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Analysis of the promoter element of the serum amyloid A gene and its interaction with constitutive and inducible nuclear factors from rabbit liver

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In an effort to identify regulatory elements of the serum amyloid A (SAA) gene that play a major role in its expression under acute-phase conditions, we studied the expression of a set of chimeric SAA-chloramphenicol acetyltransferase (CAT) plasmids containing a progressively deleted upstream 5′ sequence of the SAA gene. Two regulatory regions (−314 to −135 and −135 to −31) capable of driving cytokine-induced transcription have been identified. Gel retardation assays revealed that the regulatory region located between positions −314 and −135 is a major site of interaction for highly inducible and constitutive nuclear proteins in acute-phase rabbit liver. DNase I footprint and competition analyses showed that this region contains two adjacent nuclear protein binding sites (between −191 and −140) with varying affinity for protein binding. Both of these binding sites are capable of driving cytokine-induced transcription of a reporter gene containing a minimal promoter. Detailed analyses of the inducible nuclear proteins that bind to this promoter element showed that they are homologues of the CCAAT/enhancer binding protein (C/EBP) family. Accumulation of the inducible nuclear factors under acute conditions, when maximal transcription activity has been reported, suggests a critical role for these proteins in the expression of the SAA gene.

In response to inflammation caused by infection, tissue injury, or malignancy, hepatic synthesis of a group of proteins, called acute-phase proteins (Kushner, 1982), increases dramatically. Serum amyloid A (SAA), one of the major acute-phase proteins, increases in concentration up to a thousandfold during periods of inflammation in the rabbit, human, and mouse (Anders et al., 1977; Mackiewicz et al., 1988; McAdam et al., 1976). As in other species, increased synthesis of SAA in rabbit corresponds to an increased level of messenger RNA for this protein in the liver (Ray and Ray, 1991a; Rygg et al., 1991; Sipe et al., 1985). Such an increase is a consequence of transcriptional induction (Lowell et al., 1986b), and several different cytokines, including IL-1, IL-6, and TNF-α, either alone or in combination, have been shown to be capable of inducing SAA gene expression (Ganapathi et al., 1991). A family of SAA genes exists in mouse, human, and possibly rabbit genomes (Lowell et al., 1986a; Ray and Ray, 1991b; Woo et al., 1987). In the mouse, individual members of this gene family respond differently to inflammatory agents (Lowell et al., 1986b), which suggests that transcriptional induction of the SAA gene is uniquely regulated. Hepatic and extra-hepatic expression of various members of the SAA gene family has recently been implicated in different functions

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of this protein, as a consequence of either systemic or local response (Meek et al., 1992).

Though in the past several years considerable knowledge has been gained about the mechanism of regulation of tissue-specific and inducible gene expression, little is known about the cis functional elements and the associated transcription factors that regulate the expression of serum amyloid A protein. Earlier, in an effort to elucidate the mechanism(s) of SAA gene regulation, we cloned and characterized the 5′-flanking region of the rabbit SAA gene (Ray and Ray, 1991b). In the present study, we analyze the cis-acting promoter elements and characterize the putative transcription factors that may be involved in SAA gene induction under acute inflammatory conditions. By using transient transfection assays, with a set of chimeric constructs harboring progressively deleted 5′ ends of the SAA gene fused to a reporter chloramphenicol acetyltransferase (CAT) gene, we have determined the boundaries of the functional promoter region that responds to cytokine-mediated transcriptional induction and defined the structural elements responsible for this induction. We also report activation of C/EBP-like DNA-binding factors in acute-phase rabbit liver nucleus and their interaction with regulatory elements of the 5′ proximal promoter region of the rabbit SAA gene.

Materials and methods

Construction of plasmids and cell lines

Plasmid pSAA-0.46 CAT was constructed by using a Sac I-Dra I fragment of a previously cloned rabbit SAA gene (Ray and Ray, 1991b). This 0.46 kbp DNA was ligated to the Pst I site (blunted) of plasmid vector pJF CAT1 (Fridovich-Keil et al., 1991), kindly provided by Dr. J. L. Fridovich-Keil. Plasmids pSAA-0.37 CAT and pSAA-0.2 CAT were constructed by ligating two separate Hpa II-Dra I fragments of 0.37 kbp and 0.2 kbp, respectively, derived from partial digestion of the 0.46 kbp Sac I-Dra I DNA of the SAA gene, into the Pst I site (blunted) of plasmid vector pJF CAT1. Plasmid pSAA-0.08 CAT was a 5′ deletion construct generated by Bal 31 exonuclease digestion of linearized pSAA-0.2 CAT and contained the SAA gene sequence from −31 to +61. Oligonucleotides corresponding to the region C_1 (−191 to −167), C_2 (−160 to −140), mutant C_1, and mutant C_2 were synthesized, annealed to make double-stranded oligonucleotides, and ligated to the pBL CAT2 (Luckow and Schutz, 1987) vector. The resulting clones were analyzed by DNA sequencing to determine authenticity and orientation. A control plasmid for transfection assays, pSV-β-galactosidase, was obtained from Promega Corporation.

BNL liver cell (BNL CL.2) used in the transfection assays (TIB 73, obtained from the American Type Culture Collection) is a normal embryonic liver cell line derived from a BALB/c mouse. It was selected to grow in medium in which ornithine was substituted for arginine and phenylalanine for tyrosine. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/L) and supplemented with 10% fetal calf serum (FCS).

Transient transfection assays

BNL cells were transfected with various plasmid constructs described above, both in the presence and in absence of a conditioned medium (CM) from a monocyte culture, which provided crude cytokines for studying inducibility of promoters under investigation. CM was prepared from rabbit peripheral monocytes treated with 20 μg/ml of E. coli lipopolysaccharide (LPS), phenol extracted (Sigma Chemical Company). CM prepared in this manner contains a wide variety of cytokines shown to mediate acute-phase response (Mackiewicz et al., 1988).

BNL cells were grown in plates (6 cm in diameter) by seeding at a density of 1×10^4 cells per plate. Twenty-four hours later, the cells were transfected with 5 to 10 μg of plasmid DNA by the calcium phosphate precipitation method (Graham and van der Eb, 1973). The DNA-calcium phosphate precipitate was allowed to settle on cells overnight, and the next day the cells were shocked with 15% glycerol in HEPES-buffered saline for 60 seconds and washed three times with HEPES-buffered saline. Medium (DMEM + 4.5 g/L glucose + 10% FCS) containing 20% CM was placed on cells for induction. Forty-eight hours after washing out the DNA-calcium precipitate, cells were harvested and lysed by 3 freeze-thaw cycles. CAT enzymatic activity was assayed on cellular extracts after heating the extracts at 60°C for 10 minutes to inactivate the endogenous acetylase enzymes.

We performed β-galactosidase assays as de-

scribed (Sambrook et al., 1989) using o-nitrophenol β-D-galactopyranoside, and a CAT assay using β-galactosidase-equivalent amounts of cell extracts incubated in a mixture containing 250 mM Tris, pH 8.0, 4 mM acetyl CoA, and 0.1 μCi [^{14}C]chloramphenicol (NEN) in a total volume of 100 μl. The reaction products (chloramphenicol and its acetyl derivatives) were extracted with ethyl acetate, dried, resuspended in 15 μl of ethyl acetate, and spotted on Silica Gel 60 plastic TLC plates (VWR Scientific). The TLC plate was developed in $CHCl_3$:CH_3OH (95:5, v:v) solvent system. Autoradiographs were obtained after exposing the dry TLC plates to Kodak XAR film.

Preparation of nuclear extracts

Nuclear extracts were prepared from normal and 24 hour acute-phase rabbit liver. The acute-phase condition in rabbit (New Zealand strain, male) was developed by a single injection of concentrated turpentine oil (1 ml/kg body weight) in the dorsal lumbar region. The animal was sacrificed 24 hours later, and liver tissue was collected. Nuclei were prepared essentially as described (Dignam et al., 1983). Fresh livers were minced and suspended in sterile phosphate-buffered saline (PBS). The suspension was filtered through cheesecloth and spun at 1,000 rpm in an IEC centrifuge for 10 minutes to pellet the cells. The cells were suspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.5 μg/ml each of leupeptin, antipain, pepstatin, 0.1 μg/ml chymostatin, 0.3 TIU/ml aprotinin, 0.5 mg/ml benzamidine) and broken by using a Dounce homogenizer with pestle B. Nuclei were isolated by centrifugation at 1,200 rpm for 10 minutes in the IEC centrifuge. The nuclear pellet was resuspended in buffer A and recentrifuged to remove any remaining postnuclear fractions. The nuclei were resuspended in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% [v/v] glycerol, 0.5 mM PMSF, 0.5 μg/ml each of leupeptin, antipain, pepstatin, 0.1 μg/ml chymostatin, 0.3 TIU/ml aprotinin, 0.5 mg/ml benzamidine), incubated on ice for 30 minutes with occasional shaking, dounced using pestle A, and centrifuged at 20,000 × g for 30 minutes. The supernatant was dialyzed for 5 hours in buffer D (20 mM HEPES, pH 7.9, 0.1 MKCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, 0.5 mM PMSF, 0.5 mg/ml benzamidine). Following dialysis, the nuclear extract was spun in a microcentrifuge for 15 minutes, and the clear supernatant was stored at −70°C in small aliquots and used as the source of nuclear factors in the binding assays.

Binding assays

DNA fragments were labeled at their 5′ ends with γ[^{32}P]ATP and T_4 polynucleotide kinase. Nuclear extracts (2–10 μg protein) were preincubated for 10 minutes at 25°C in a binding mixture containing 10 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM $ZnCl_2$, 0.05% (v/v) NP40, 10% (v/v) glycerol, and 50 μg/ml poly(dI-dC). The labeled DNA probe was added with or without 100-fold molar excess of competitor DNA, and incubation in a total volume of 10 μl was continued for an additional 30 minutes at 25°C. DNA–protein complexes were fractionated in a 4% nondenatured polyacrylamide gel with recirculation of running buffer (7 mM Tris-HCl, pH 7.5, 3 mM Na-acetate, 1 mM EDTA). Gels were dried and autoradiographed at −70°C with an intensifying screen. For antibody interaction studies, a combination of antibodies raised against three C/EBP isoforms, α, β, and δ (a gift of Dr. S. L. McKnight), was added to the reaction mixture during the preincubation period.

Oligonucleotides

The oligonucleotide used as competitor for the C/EBP binding site consisted of the self-complementary, dyad-symmetric sequence 5′-TGCAGATTGCGCAATCTGCA-3′ (Cao et al., 1991). The sequence of mutant C/EBP oligonucleotide used in this study was 5′-TACAGACTACGAACTCTGCA-3′, the sequence of wild-type C_1 oligonucleotide was from −191 to −167, and wild-type C_2 oligonucleotide was from −160 to −140 (Fig. 2B). The sequence of the mutant C_1 oligonucleotide (C_1′) was 5′-CCT TCATAGACTACACAACTAGGCA-3′, and the mutant C_2 oligonucleotide (C_2′) was 5′-CTG CGCATCACGCAACCCTGT-3′. In each case the underlined nucleotides represent the mutated bases. For annealing, equal amounts of complementary strands of oligonucleotides were heated to 95°C for 2 minutes in 50 mM Tris, pH 7.4, 60 mM NaCl, 1 mM EDTA and allowed to cool to room temperature slowly in about 5–6 hours. Other DNA fragments used as competitors in gel retardation assays were gener-

ated from the cloned SAA gene fragment using several restriction endonucleases. For example, DNA fragments A, B, and C of sizes 89, 34, and 60 bp, respectively, were obtained by digesting a Hpa II fragment of the SAA gene (−314 to −136) with Hae III and fractionating the products on a preparative polyacrylamide gel. The isolated fragments were eluted from gel and purified by passing through a Sephadex G-25 (coarse) minicolumn.

DNase I protection assay

Single 5′ end ^{32}P-labeled SAA DNA fragments (−314 to +61) were incubated with nuclear extracts from both normal and acute-phase rabbit livers. In one incubation reaction, purified C/EBP was used. Incubation conditions were the same as those of the binding assays, except that reaction volumes were 20 µl. After incubation, the samples were treated with 0.2 unit of DNase I (Promega Corporation) for 30 seconds at room temperature, and the reaction was stopped by adding 100 µl DNase I stop buffer (100 mM Tris-HCl, pH 7.6, 100 mM NaCl, 15 mM EDTA, 0.3% [w/v] SDS, 50 µg/ml sonicated salmon sperm DNA) and 10 µg proteinase K (Bethesda Research Laboratory). The mixture was incubated at 50°C for 30 minutes. The DNA was extracted with phenol, precipitated with ethanol, and dried. Samples were resuspended in formamide buffer containing tracking dye, and electrophoresis was performed in a 6% polyacrylamide-urea gel. Following electrophoresis, the gel was dried and autoradiographed. To determine precisely the DNA sequences protected from DNase I digestion, Maxam-Gilbert G reactions (Maxam and Gilbert, 1980) were also performed on the ^{32}P-labeled SAA DNA and electrophoresed together with the DNase I-treated samples.

Results

The 5′-flanking region of the rabbit SAA protein gene contains sufficient information for acute-phase inducibility

We employed a transient expression assay to determine whether the upstream region of the rabbit SAA gene contains a functional, inducible promoter. A 462 bp DNA fragment of the SAA gene extending from a Sac I (−401) site to a Dra I (+61) site in the first intron (Fig. 1A) was ligated in front of the bacterial CAT gene using plasmid vector pJF CAT 1. This vector carries a trimer cassette of the simian virus 40 major late polyadenylation site to block plasmid-initiated readthrough expression of the CAT gene. Thus it is very useful for analyzing the effect of even weak promoters, whose activity may otherwise be masked by background CAT activity. The resulting construct, pSAA-0.46 CAT, was used in transfection assays. Transient transfections were performed in duplicate, and one set of plates was treated with 20% CM, a source of active cytokines obtained from LPS-induced monocyte cells. Forty-eight hours after transfection, cells were harvested, and CAT activity was measured. Each test plasmid was co-transfected with a second plasmid pSV-β galactosidase, which directs expression of E. coli *lacZ* gene under control of SV40 promoter. The latter activity served as an internal measure for transfection efficiency. Results presented in Figure 1B show that pSAA-0.46 CAT is expressed at a very low level in absence of CM but is highly induced in presence of CM, which contains various cytokines. These data suggest that the 462 bases of the 5′ region of the SAA gene contain sequences necessary for induction of transcription and reflect the acute-phase response in vivo. IL-1 and IL-6 produced a similar kind of induction, which indicates that all of these agents (CM, IL-1, and IL-6) act through the same promoter element. This is consistent with the previous finding, that IL-1 and IL-6 are involved in the induction of several acute-phase proteins (Ganapathi et al., 1991).

In order to determine precisely the region of upstream sequence that responds to CM-mediated induction, a series of deletion constructs were made (Fig. 1A). These plasmids were transiently transfected with or without CM, and CAT activity was measured. pSAA-0.37 CAT contains DNA from the Hpa II (−314) site to the Dra I (+61) site; pSAA-0.2 CAT contains DNA from the Hpa II (−135) site to the Dra I (+61) site; and pSAA-0.08 CAT contains DNA from position −31 to the Dra I (+61) site. As shown in Figure 1B, deletion of sequences from −401 to −314 has no effect on CAT activity. Deletion of sequences from −314 to −135 retains the responsiveness to CM but results in more than 50% reduction of CAT activity. Deletion of sequences from −135 to −31 abolishes the remaining level of expression and inducibility. Thus, two distinct regions of DNA sequences capable of regulating CAT expression are evi-

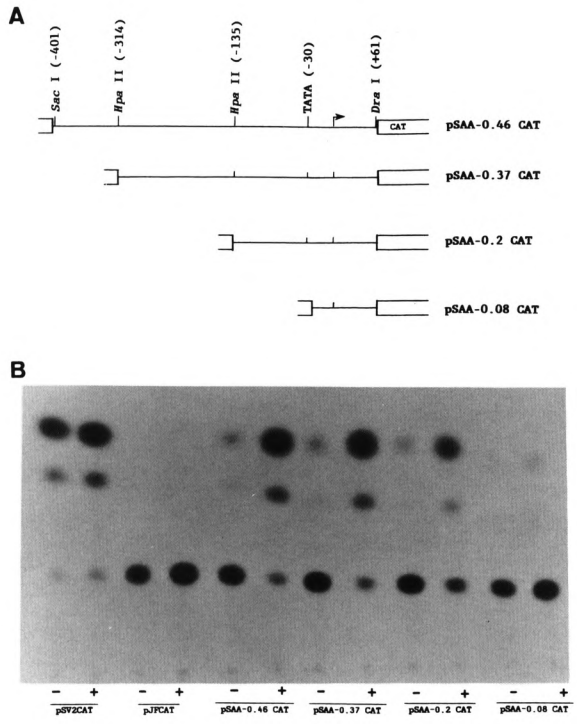

Figure 1. Analysis of rabbit SAA proximal promoter function. **A.** Map of the plasmid vector pJF CAT containing the Sac I-Dra I fragment of rabbit SAA gene (pSAA-0.46 CAT) and three deletion constructs, pSAA-0.37 CAT, pSAA-0.2 CAT, and pSAA-0.08 CAT, containing progressively shorter SAA gene fragments. These fragments were inserted into pJF CAT as described in Materials and Methods. The position of the TATA box is identified, and the transcription start site and direction of transcription are indicated by an arrow. **B.** Result of a CAT assay using cell extracts obtained from cells transfected with the chimeric SAA-CAT constructs in the absence (−) and presence (+) of conditioned medium (CM). Various active cytokines present in the CM can induce transcription of the reporter gene CAT, covalently linked to the SAA promoter. These results are representative of three separate transfection experiments.

dent. This finding suggests that two cis-acting elements necessary for the regulation of the rabbit SAA gene by cytokines are present in this 5′ proximal promoter region of the gene and are located at positions between −314 to −135 and −135 to −31.

Identification of constitutive and inducible DNA-binding proteins in rabbit liver nuclear extract

Since the acute-phase condition results in the induction of SAA gene transcription, we planned to examine whether any DNA-binding protein(s) capable of interacting with the promoter region of the SAA gene are present specially in acute liver nuclear extract. End-labeled DNA fragments from −314 to −136 and −135 to +61 were used as probes in gel retardation assays. Nuclear extracts were prepared from both normal and acute-phase induced (turpentine-injected) rabbit livers. Using the DNA fragment from −314 to −136, we detected only one DNA–protein complex (complex 3) when the probe was incubated with normal liver nuclear extract (Fig. 2A, lane 2). This complex appeared as a doublet and may therefore indicate involvement of more than one factor. However, the same probe, when incubated with the same amount (protein) of acute liver nuclear extract, yielded three DNA–protein complexes, designated complexes 1, 2, and 3 (Fig. 2A, lane 6). We also noticed that the intensity of complex 3 increased several-fold when acute liver nuclear extract was used, suggesting that the nuclear factor involved in the formation of complex 3 was either induced under an acute condition, or an additional protein present in acute nuclear extract could form a DNA–protein complex that co-migrated with complex 3. Appearance of these DNA-binding proteins in acute-phase rabbit liver nuclear extracts and their interaction with the SAA promoter are of considerable interest, since they correlate with increased transcription of the SAA gene under an acute-phase condition (Ray and Ray, 1991a; Rygg et al., 1991; Sipe et al., 1985).

To define more closely the binding sites involved in the formation of complexes 1, 2, and 3, we allowed them to form in presence of unlabeled competing DNA fragments containing different regions of this probe (−314 to −136). The DNA fragment from −314 to −136 was cleaved with Hae III restriction enzyme, which yielded three smaller fragments spanning −314 to −228 (A), −227 to −194 (B), and −193 to −136 (C). These DNA fragments (Fig. 2B) were used as competitors in a gel retardation assay. Results presented in Figure 2A indicate that DNA sequences in fragments A and B failed to compete, whereas that in fragment C efficiently blocked the formation of all DNA–protein complexes by both normal and acute liver nuclear extracts. These data suggest that the nuclear factor binding site is present in DNA fragment C between positions −193 and −136.

Since an earlier transfection assay (Fig. 1B) indicated that the −135 to +61 region of the SAA gene might contain an additional promoter

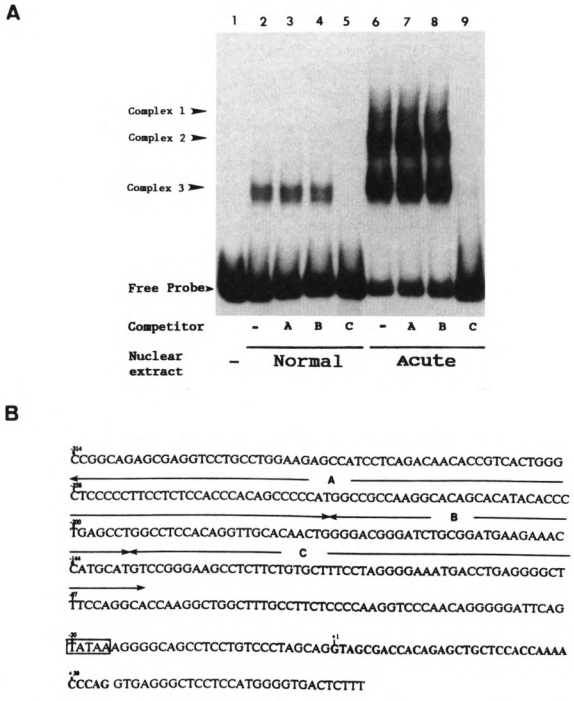

Figure 2. Detection of DNA–protein interaction by gel retardation assay. **A.** The assay was performed in the absence (−) or presence of double-stranded DNA fragments (A, B, and C) as competitors. Nuclear extracts (10 μg protein) prepared from normal (lanes 2–5) or turpentine-induced acute-phase (lanes 6–9) rabbit liver were incubated as described in Materials and Methods with a ^{32}P-labeled DNA fragment containing the SAA gene sequence from −314 to −136. The products were fractionated in a 4% nondenaturing polyacrylamide gel. Lane 1 contains the labeled probe only. Position of migration of the free (unbound) probe and three DNA–protein complexes, designated 1, 2, and 3, are shown. Competitor DNA fragment A contains the sequence from −314 to −228 (lanes 3 and 7), B contains the sequence from −227 to −194 (lanes 4 and 8), and C contains the sequence from −193 to −136 (lanes 5 and 9). **B.** DNA sequence of the rabbit SAA 5′ proximal promoter region. The TATA element is boxed, and the transcription start site is designated +1. Different regions of this DNA used as competitors are underlined and labeled A, B, and C.

element, we used this region as a probe in the gel retardation assay to evaluate whether any nuclear factor present in either normal or turpentine-induced acute-phase rabbit liver could interact with this region. No DNA–protein complex was formed by either of these two extracts (data not shown), suggesting that a transcription factor capable of binding to this region was not present in the nucleus of these liver tissues. However, recent studies have revealed that LPS-mediated acute-phase induction activates transcription factor NF-κB in the liver, which then binds to a potential NF-κB element present in this region (unpublished data). In turpentine-mediated acute-phase response, this element apparently remains inactive due to the absence of an active NF-κB transcription factor.

The DNA–protein complexes are formed by C/EBP-like nuclear factors

As liver tissue is a rich source of C/EBP transcription factors known to be involved in liver-specific gene regulation (Alam and Papaconstantinou, 1992; Baumann et al., 1992; Birkenmeier et al., 1989; Johnson et al., 1987; Landschulz et al., 1988; Poli et al., 1990; Ray et al., 1993), we were interested in examining whether the DNA-binding nuclear factors responsible for the formation of complexes 1, 2, and 3 with the SAA promoter described above were homologous to C/EBP. A gel retardation assay was performed using a ^{32}P-labeled SAA probe (−314 to −136) to characterize the involved transcription factors. As seen in Figure 3, addition of excess unlabeled C/EBP-specific oligonucleotide efficiently competed and abolished the formation of all three complexes, whereas the oligonucleotide containing a mutated C/EBP binding site failed to do so. Also, addition of a combination of C/EBP antibodies raised against three isoforms (α, β, and δ) in the incubation mixture prevented any DNA–protein complex formation (Fig. 3, lanes 5 and 9). These results indicate that either C/EBP or C/EBP-like nuclear proteins are present in both normal and acute-phase rabbit liver and bind to region C of the SAA gene. At least one such protein is constitutively present, while others are highly induced during the acute-phase condition.

High-resolution mapping of the binding sites

The competition experiments described in Figure 2A indicated that the nuclear factor binding site might be located within fragment C, from −193 to −136. To map it with greater precision, we performed a DNase I protection assay using a single end-labeled probe (−314 to +61) and acute liver nuclear extract (Fig. 4). Two protected regions, from −191 to −167 (C_1) and −160 to −140 (C_2), were detected. This result indicates that there are two adjacent C/EBP-like protein binding sites within region C. When purified recombinant C/EBP was used in the

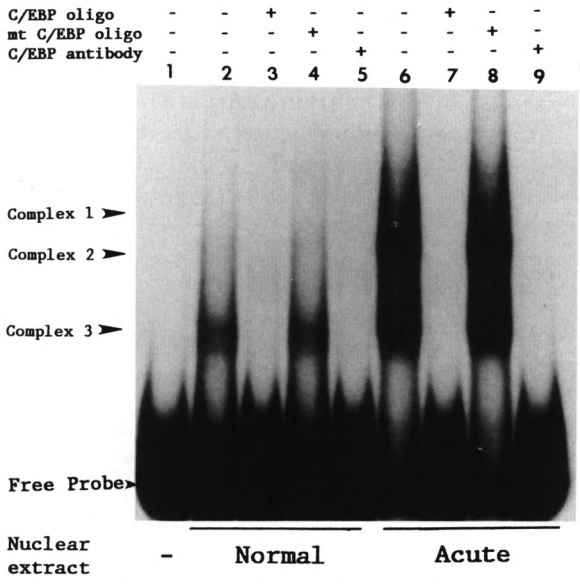

Figure 3. Characterization of the nuclear factors that form DNA–protein complexes. A ^{32}P-labeled DNA fragment (bases −314 to −136) was incubated with nuclear extracts (10 μg protein) prepared from normal (lanes 2–5) or turpentine-induced acute-phase (lanes 6–9) rabbit liver. In some binding reactions, 100-fold molar excess of unlabeled oligonucleotide containing the C/EBP binding sequence (lanes 3 and 7) or mutant C/EBP binding sequence (lanes 4 and 8) was added. In lanes 5 and 9, a combination of antibodies raised against three C/EBP isoforms (α, β, and δ) was added 10 minutes prior to the addition of labeled probe in the reaction mixture.

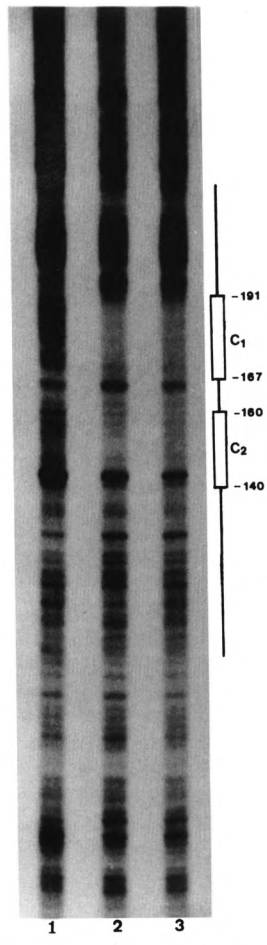

Figure 4. DNase I footprinting analysis of the SAA promoter region. A single 5′ end-labeled DNA fragment (−314 to +61) was incubated with acute liver nuclear extract (lane 2) and purified rat C/EBP (lane 3) under the conditions used for gel retardation assays. DNA–protein complexes were incubated with DNase I, as described in Materials and Methods, and analyzed on a sequencing gel containing 8 M urea. In lane 1, the probe was incubated without any added nuclear extract.

DNase I protection assay (Fig. 4, lane 3), an identical footprint pattern was detected. This result also suggests that the DNA-binding nuclear factors in acute-phase rabbit liver are functionally similar to C/EBP, which has been implicated in liver-specific gene expression. Normal liver nuclear extract produced a similar DNase I protection pattern, albeit with a significantly reduced level of protection (data not shown). This is consistent with the observed binding pattern of the normal and acute liver nuclear extracts in gel retardation assays and indicates that both constitutive and inducible nuclear factors bind to common elements of the SAA promoter.

Some of the inducible C/EBP-like proteins are heat labile

One characteristic of C/EBP protein is its heat stability (Johnson et al., 1987). Since the gel retardation and DNase I footprint assays, described above, indicated a functional relationship between the nuclear factors that bind to the 5′ proximal promoter region and C/EBP, we investigated whether these DNA-binding factors are thermostable. We heated both normal and acute liver nuclear extracts to different temperatures and tested their DNA-binding ability in a gel retardation assay (Fig. 5). Interestingly, inducible nuclear protein present in acute liver extract, which formed complex 1, was found to be heat labile and lose its ability for complex formation when heated to 65°C for 8 minutes (Fig. 5, lane 5). The inducible nuclear protein that formed complex 2 was partially heat stable (Fig. 5, lane 7), while the protein that formed

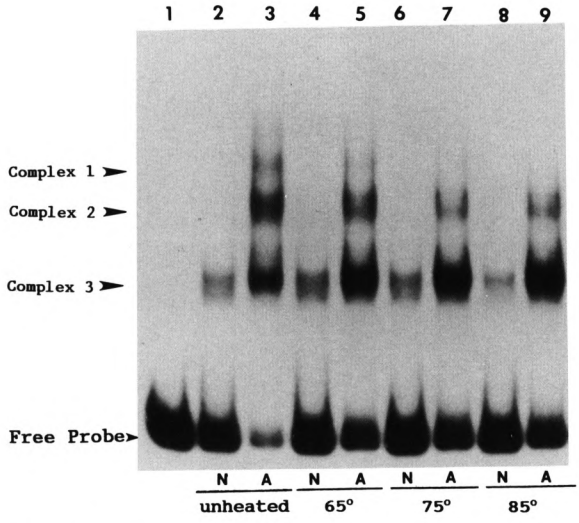

Figure 5. Effect of heat treatment of nuclear extracts on DNA–protein complex formation. The 5′ end-labeled SAA probe (−314 to −136) was incubated with normal (N) and acute-phase (A) rabbit liver nuclear extracts (10 μg protein). The products were fractionated as described in Figure 2. Prior to incubation with a ^{32}P-labeled probe, the nuclear extracts were heated, wherever mentioned, to 65°C, 75°C, and 85°C for 8 minutes. Slight stimulation of binding of preheated nuclear extracts might be the result of thermal inactivation of accompanying nucleases in the nuclear extracts.

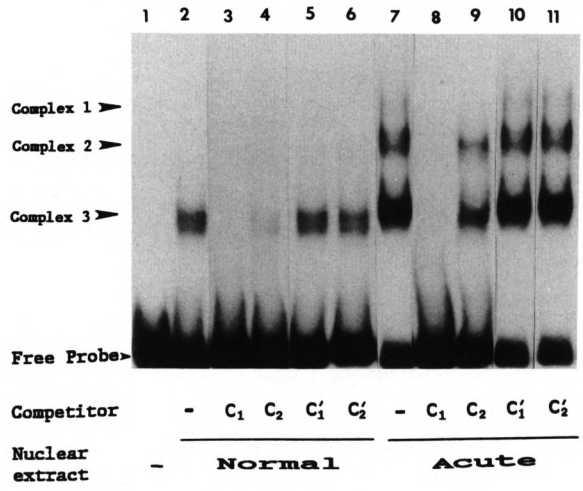

Figure 6. Competition analysis with double-stranded oligonucleotides using a ^{32}P-labeled DNA fragment (−314 to −136) as a probe and nuclear extracts (10 μg protein) prepared from normal (lanes 2–6) or acute-phase induced (lanes 7–11) rabbit liver. Incubations were carried out without any nuclear extract (lane 1), in the absence of any competitor DNA (lanes 2 and 7), in the presence of 100-fold molar excess of oligonucleotide C_1 (lanes 3 and 8), oligonucleotide C_2 (lanes 4 and 9), oligonucleotide C_1' (lanes 5 and 10), and oligonucleotide C_2' (lanes 6 and 11). Oligonucleotide C_1 contains the sequence from −191 to −167, C_2 contains the sequence from −160 to −140, and oligonucleotide C_1' and C_2' contain mutated sequences of C_1 and C_2 respectively (see Materials and Methods).

complex 3 was highly heat stable in nature. This result indicates that functionally related C/EBP-like factors that interact with the 5′ proximal promoter element of the SAA gene are structurally distinct, and—unlike C/EBP—some of the factors in acute-phase rabbit liver nuclear extract are heat labile.

The C_1 region is a stronger C/EBP-like protein binding site

Since DNase I footprint studies indicated two adjacent nuclear factor binding sites, we were interested in defining the role of each site in DNA–protein complex formation. As seen in Figure 6, both of these regions appear to be involved in complex formation, as evidenced by their ability to compete effectively for binding (Fig. 6, lanes 3, 4, 8, and 9). However, comparison of lanes 3 to 4 and 8 to 9 shows that region C_2 has lower affinity for binding with nuclear proteins. We also used mutated sequences of the C_1 and C_2 regions (see Materials and Methods) as competitors (designated C_1' and C_2'; Fig. 6, lanes 5, 6, 10, and 11), and these failed to compete in the binding reactions.

Because this result suggested that there might be a difference in the affinity for nuclear protein binding to the DNA elements in C_1 and C_2, we performed a gel retardation assay in which a constant amount of nuclear extract was titrated with increasing amounts of unlabeled C_1 and C_2 DNA, respectively. As shown in Figure 7, much more C_2 DNA was needed to eliminate the DNA–protein complex formations than C_1. It is estimated that the C_1 region has at least 5- to 8-fold higher affinity for nuclear factors than the C_2 region. Examination of the DNA sequence within the C_1 region (−191 to −167) reveals that it contains a dyad-symmetric structure (located at −182 to −173), which may be why it displays higher affinity. Existence of a similar structure has been noted in bZIP protein binding, in which the basic regions of the paired subunits are believed to interact with each half of a dyad-symmetric DNA-binding site, forming a "scissors grip" around the DNA substrate (Cao et al., 1991; Williams et al., 1991). Interestingly,

comparison of this sequence with an optimal recognition site for C/EBP (ATTGCGCAAT) (Vinson et al., 1989) reveals a close match. Absence of such a closely matched sequence in the C_2 region (−160 to −140) may explain its apparent low-affinity binding profile. Ability of both C_1 and C_2 to prevent formation of all three complexes (albeit with varying affinities) suggests that each element contains binding sites for all of the involved factors.

The two C/EBP binding domains are transcriptionally active

Since the DNA sequences in the C_1 and C_2 elements were found to be involved in DNA–protein complex formation, we were interested in seeing whether these protein-binding elements were functional, and whether they could confer cytokine responsiveness to a reporter gene. Wild-type and mutated sequences of the C_1 and C_2 regions were ligated in front of a thymidine kinase promoter (containing a minimal promoter region) linked to a reporter CAT gene using the vector pBLCAT2. The chimeric constructs were used in transient transfection assays performed in the presence and absence of CM, and CAT activity was measured 48 hours after the transfection. As shown in Figure 8, the plasmid constructs pSAA-C_1 (wt) and pSAA-C_2 (wt) give rise to several-fold induction of CAT activity in the presence of CM. No induction or very little induction is seen for the constructs carrying mutant sequences of C_1 and C_2 sites. These results indicate that both nuclear factor binding sites are functionally active and can confer cytokine responsiveness to a reporter gene.

Discussion

Increased biosynthesis of serum amyloid A protein in liver under an acute-phase condition has been found to correlate with increased production of the corresponding messenger RNA. Thus a transcriptional regulatory mechanism controlling the expression of this gene is evident. In the present study, we have identified and characterized a DNA fragment of about 375 bp at the 5′ proximal promoter region of the rabbit SAA gene that is capable of conferring cytokine-mediated transcriptional induction to a reporter gene. Responsiveness of this DNA to stimulatory signals provided by the CM indicates that cis-acting DNA elements are present in two major segments (see Fig. 1). One of

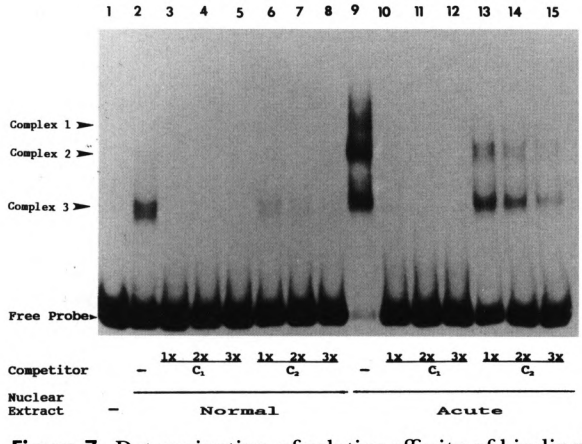

Figure 7. Determination of relative affinity of binding sites for nuclear factors. ^{32}P-labeled DNA (−314 to −136) was incubated in the presence of nuclear extracts (10 μg protein) prepared from normal (lanes 2–8) or acute-phase induced (lanes 9–15) rabbit liver. Increasing concentrations (1×: 50-fold molar excess) of double-stranded oligonucleotide C_1 (lanes 3–5 and lanes 10–12) and oligonucleotide C_2 (lanes 6–8 and lanes 13–15) were added in some binding reactions. Lane 1 contained the probe only, and lanes 2 and 9 contained no competitor DNA.

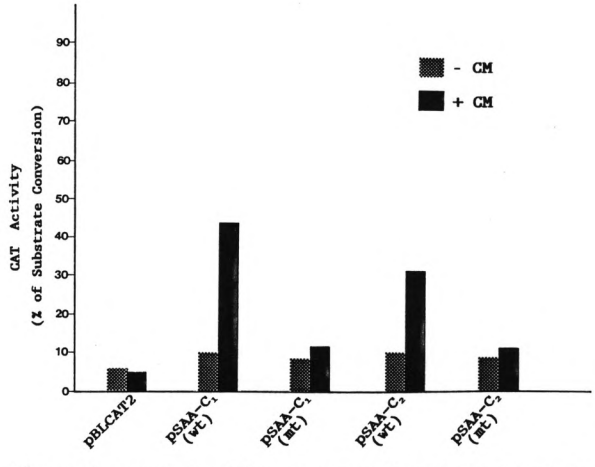

Figure 8. Analysis of the promoter function of the two binding sites, C_1 and C_2. Synthetic oligonucleotides corresponding to the wild-type (wt) and mutant (mt) sequences of C_1 (−191 to −167) and C_2 (−160 to −140) regions were ligated in front of a reporter CAT gene using the vector pBLCAT2. The resulting CAT constructs were used to transiently transfect the BNL liver cells in the presence or absence of conditioned medium (CM). pBLCAT2 DNA was used as a control, and CAT activity was measured as described in Materials and Methods.

these is located in a Hpa II–Hpa II fragment of about 180 bp, while the other element is present within the Hpa II (at −135) and TATA box (−30) sites. Our study indicated that the latter promoter element, which contains a potential NF-κB transcription factor binding site, is not involved in SAA gene induction under all inflammatory conditions but participates only under certain circumstances. An active NF-κB-like transcription factor is detected in LPS-induced but not in turpentine-induced acute liver extract, suggesting that this is an alternative pathway for SAA gene induction. This may explain how the SAA gene in different species, some containing an NF-κB protein-binding element (human, Edbrooke et al., 1989; and rabbit, Ray and Ray, 1991b) and some lacking it (mouse, Lowell et al., 1986a), can respond to various inflammatory agents. The major promoter elements responsive to both turpentine- and LPS-mediated SAA gene induction are present in region C (between −193 and −136 bp), described here. This represents the first functional delineation of cytokine-responsive promoter elements and binding factors for the rabbit SAA gene.

A gel retardation assay (Fig. 2) has demonstrated for the first time that a group of nuclear factors are activated under the turpentine-induced acute-phase condition in liver tissue of a treated animal. All of the factors, including the constitutively expressing proteins seen in normal rabbit liver and the inducible proteins found in acute-phase rabbit liver, are similar in their DNA-binding ability, which is inhibited by an oligonucleotide containing a C/EBP binding site. Such binding of the acute-phase inducible nuclear factors to this element suggests a possible relationship between these factors and C/EBP, which is known to bind this oligonucleotide. This possibility is strengthened by the observation that the antibodies raised against three individual C/EBP isoforms prevent binding of these nuclear factors to the promoter element (see Fig. 3), and by the fact that both C/EBP and inducible nuclear factors in rabbit liver bind to the same regulatory element of the SAA gene (see Fig. 4). Existence of C/EBP-like proteins in rat liver (Landschulz et al., 1988) and several other inducible forms—IL-6DBP (Poli et al., 1990), LAP (Descombes et al., 1990), NF-IL6 (Akira et al., 1990), and NF-IL6β (Kinoshita et al., 1992)—have been reported. AGP/EBP, a mouse homologue of rat protein LAP, was found to bind to the promoter region of the AGP gene (Chang et al., 1990). More recently, the role of C/EBP has been documented in mouse AGP gene expression (Alam and Papaconstantinou, 1992). The role of C/EBP in tissue-specific gene expression has been proposed by Li et al. (1990), who reported two adjacent C/EBP binding sites in the mouse SAA_3 gene. Appearance of multiple proteins in acute-phase liver, which we have demonstrated here, correlates with enhanced transcription of the SAA gene and suggests that these inducible proteins might be involved in SAA gene activation. Interestingly, both constitutive and inducible factors recognize the same DNA element and bind to it. It will be interesting to find out how inducible forms of the nuclear factors form an effective complex for transcription induction.

Another interesting feature of this study is the variable affinities of two adjacent binding sites for the nuclear factors, both the constitutive and the inducible forms (Fig. 4). The high-affinity binding site present in the promoter region designated C_1 is favored by all of the binding factors. The same factors can also bind DNA sequences within the promoter region designated C_2, albeit with a much lower affinity. The significance of this phenomenon in the expression of the SAA gene is not clear. However, such characteristics of binding sites have been found for other nuclear factors. For instance, variable affinities of binding of NF-κB subunits p50 and p65 for different κB motifs have been shown to be responsible for transcriptional activation (Fujita et al., 1992). The ability of different subunits to form homo- and heterodimers at different binding sites has been found to be responsible for distinct transcriptional activation. C/EBP is a member of the bZIP family of proteins, which have a basic amino acid domain for DNA binding and a hydrophobic domain with a leucine zipper structure capable of forming dimers and are known to form both homo- and heterodimers at the DNA-binding site (Cao et al., 1991; Williams et al., 1991). Homologues of C/EBP, NF-IL6, and NF-IL6β are similarly capable of forming homo- and heterodimers (Kinoshita et al., 1992). It is therefore conceivable that the multiple complex formed during the DNA–protein interaction of the rabbit SAA promoter with the nuclear factors from acute-phase rabbit liver is a consequence of several homo- and heterodimer

formations by the different isoforms of C/EBP-like factors that are activated under this condition. Variable interactions of these isoforms of both constitutive and inducible nuclear factors at two adjacent sites in the SAA 5′ proximal promoter region may be necessary for the transcriptional induction of SAA under the acute condition. Involvement of the inducible form of C/EBP has been implicated in developmentally regulated gene expression in adipocytes (Cao et al., 1991; Cheneval et al., 1991) and in the expression of the human alcohol dehydrogenase gene family during liver development (van Ooij et al., 1992). Recently we detected involvement of such nuclear factors in the expression of the AGP gene in rabbit liver (Ray et al., 1993). The current study shows that the involved transcription factors are homologues of C/EBP. The role of the individual members of this family (α, β, and δ) in the formation of the complexes seen here (Fig. 2) will be an interesting mechanism to investigate. We are currently studying this phenomenon.

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