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Two Adjacent C/EBP-Binding Sequences That Participate in the Cell-Specific Expression of the Mouse Serum Amyloid A3 Gene

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Serum amyloid A (SAA) is a major acute-phase protein synthesized primarily in the liver. Its expression, very low in normal animals, is increased several hundredfold following acute inflammation. To examine DNA sequences involved in liver-specific expression, 5′-flanking regions of the mouse SAA3 gene were analyzed by transient transfection, band shift, and DNase I protection assays. We found that a 56-bp fragment immediately 5′ to the TATA box spanning the region −93 to −38 relative to the transcription start site was sufficient to confer liver cell-specific transcriptional activation onto a heterologous promoter in a dose-dependent and orientation-independent manner. This DNA fragment could form DNA-protein complexes with heat-stable nuclear proteins, and the complexes formed could be specifically competed for by excess oligomers corresponding to the C/EBP- or DBP-binding sites but not by binding sites for three other liver-specific factors, HNF1, HNF3, and HNF4. Footprint analysis using Hep3B nuclear extracts revealed two adjacent footprint regions within this 56-bp fragment, the distal region having at least fivefold-greater affinity than the proximal region. Identical footprint patterns were observed when purified recombinant C/EBP protein was used. These results indicated that binding of C/EBP to this 56-bp fragment plays an important role in vivo in enhancing expression of the mouse SAA3 gene in hepatocytes.

Gene expression is regulated in part by the presence of DNA sequences that, by interacting with specific nuclear transcription factors, may greatly enhance the rate of specific initiation of mRNA transcription (39). While the more proximal sequences of a promoter, such as a TATA sequence, interact with general transcription factors, including TFIID, -E, -A, and -B (8), sequences more removed from the transcription initiation site interact with proteins that affect specialized functions of developmental, hormonal, and tissue-specific regulation of gene expression (14, 15, 28, 39, 43, 45).

Serum albumin gene expression in the liver is a well-studied system that illustrates some of the complexity of regulating transcription initiation in a development- and tissue-specific manner. A proximal 150-bp region of the albumin promoter interacts with at least six distinct nuclear proteins in addition to the general transcription factors (11, 23, 25, 34, 35, 45). Three of these DNA-binding proteins, HNF1, C/EBP, and DBP, are more abundant in hepatocytes and together are believed to be responsible not only for the exclusive expression of albumin in those cells but also for the developmental pattern of albumin expression (6, 10, 16, 17, 20, 45). Other nuclear proteins that bind to the albumin promoter, such as NF-1, are more widely distributed and found in many cell types; they contribute to the expression of many genes (29, 46). In addition, distant DNA sequences, up to 10 kbp from the transcription start site, can also enhance albumin gene expression (47).

Serum amyloid A (SAA) is a major acute-phase protein (50). Its synthesis and secretion, as in the case of serum albumin, is mainly by the liver, but its expression is readily detectable only in response to systemic inflammation (4, 5, 44). During the inflammatory response, when SAA gene expression is stimulated by early inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor (26, 48), SAA transcription rates are increased 200-fold, leading to a 1,000-fold increase in mRNA levels (37). Mouse SAA is encoded by a multigene family consisting of three transcribed genes (SAA1, -2, and -3) and one pseudogene (36). At the peak of the inflammatory response, all three SAA genes are expressed in the liver, each gene accounting for about one-third of all mRNA transcripts (37). The SAA1 and -2 mRNAs are expressed exclusively in the liver. SAA3 mRNA is expressed most abundantly in the liver, but it is also found in nearly comparable amounts in macrophages and at much lower levels in the intestine, lung, and adrenal glands (41). Although all three genes are induced by lipopolysaccharide, only SAA1 and -2 are induced by casein (41). Thus, the differential regulation of SAA1, -2, and -3, whose expression is activated by extracellular stimuli, represents an excellent model for studying the mechanisms of differential gene control.

Using transient transfection experiments to investigate the molecular mechanism of SAA3 gene regulation in cultured cells, we demonstrated that the expression is enhanced in cells of liver origin relative to non-liver-derived cells. Furthermore, we identified a transcriptional enhancer that responds to the inflammatory mediator IL-1 (26, 49). Finally, we defined and characterized sequences in the mouse SAA3 gene promoter that enhance expression of the gene in the liver and identified two adjacent sites necessary for this enhanced expression. In vitro, these two sites interact with C/EBP, a transcriptional activator most abundant in liver cells (6, 28).

MATERIALS AND METHODS

Plasmid constructs. pSAA3/CAT (−306) was constructed by inserting the 5′-flanking and exon 1 sequence (positions −306 to +45) of the mouse SAA3 gene into the *Bam*HI site

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of the pCATβ′ vector (49). pSAA3/CAT (−93) and pSAA3/CAT (−63) are the 5′ deletion mutants of pSAA3/CAT (−306) generated by BAL 31 exonuclease digestion. A 56-bp oligonucleotide (PR) corresponding to positions −93 to −38 of the SAA3 promoter as well as two other oligonucleotides (PR-I, positions −58 to −37; PR-II, positions −79 to −59) were synthesized and cloned into the *Bam*HI site of the pBLCAT vector (32). The resulting clones were analyzed by restriction enzyme digestion and DNA sequencing to determine copy number and orientation of each insert.

Cell lines and transient transfection assay. Both Hep3B and HeLa cells were cultured in modified Eagle medium and Waymouth MAB (3:1, vol/vol) plus 10% fetal calf serum (19) and passaged at confluence by trypsinization about once a week.

DNA transfections were performed by the Polybrene precipitation procedure (31) as described by Huang et al. (26). The cell extracts were assayed for protein content by the method of Bradford (7), and chloramphenicol acetyltransferase (CAT) activity was determined as described by Gorman et al. (22), with modifications described by Cato et al. (9). The CAT assays were quantitated by counting in a liquid scintillation counter the [^{14}C]chloramphenicol spots corresponding to acetylated and nonacetylated forms.

Nuclear extracts. Nuclear extracts from Hep3B and HeLa cells were prepared as described by Shapiro et al. (54). 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-ethanesulfonic acid [HEPES, pH 7.9], 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM dithiothreitol [DTT], and 25% glycerol). The resulting suspension was mixed by gentle rocking for 30 min at 4°C, after which the chromatin was sedimented at 150,000 × *g* for 90 min at 2°C. The supernatant was collected, and proteins were precipitated by the addition of ammonium sulfate (0.33 g/ml), followed by centrifugation at 85,000 × *g* 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, 0.7 mM EGTA, 2 mM DTT) per 10^9 nuclei and dialyzed for 4 h against 300 ml of nuclear dialysis buffer, with one change of buffer. The dialysate was then clarified by centrifugation at 10,000 × *g* for 10 min, and aliquots were frozen in dry ice and stored at −80°C.

Electrophoresis mobility shift assay. Reactions for the electrophoretic mobility shift assay 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), 1 to 2 μg of Hep3B nuclear protein, and 10^4 cpm (0.25 to 1 ng) of ^{32}P-labeled DNA fragment 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 20 min more at room temperature. Samples were loaded onto a low-ionic-strength, 6% acrylamide gel (19:1 cross-linking ratio) containing 0.25× TBE (1× TBE is 89 mM Tris [pH 7.8], 89 mM boric acid, and 1 mM EDTA) and subjected to electrophoresis at 200 V for 2 h. The gel was then dried and autoradiographed.

DNase I footprint analysis. Reactions for the DNase I footprint assays were done in a final volume of 20 μl containing 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), 3 to 6 μg of Hep3B nuclear protein, and 1 to 2 ng of end-labeled DNA fragment. The nuclear extract was preincubated with 4 μg of poly(dI-dC) carrier DNA for 5 min at room temperature, after which the end-labeled fragment was added and the mixture was incubated for another 20 min at room temperature. After freshly diluted DNase I was added, digestion was allowed to proceed for 30 s at room temperature. The final DNase I concentration in the reaction mixture varied between 4 and 8 μg/ml. Reactions were stopped by the addition of 100 μl of proteinase K (60 μg/ml) in 12 mM EDTA–0.12% sodium dodecyl sulfate–16μg of pBR322 DNA per ml. The reaction mixtures were incubated for 45 min at 37°C. Nucleic acids were extracted with 1 volume of phenol-chloroform (1:1), ethanol precipitated, dissolved in 99% formamide, heated at 90°C for 1 min, and loaded onto an 8% polyacrylamide-urea gel.

Oligonucleotides. The oligonucleotides and their complementary sequences used as competitors in band shift experiments were sequences for the C/EBP-binding site (5′-CACA CTTGGAATTAGCAATAGATGCAATTTGGGACTTG-3′) from the human albumin promoter (J. H. Kelley, D. R. Wilson, K. M. Huber, M. Wilde, and G. J. Darlington, J. Biol. Chem., in press), the DPB-binding site sequence (5′-CGATTTTGTAATG-3′) from the mouse albumin promoter (11, 45), and the HNF1-binding sequence (5′-TGAGGTTAA TAATTTTCCA-3′) from the *Xenopus* albumin promoter (52). Binding sites for HNF3 (5′-TGACTAAGTCAATAATC AGAATCAGCA-3′) and HNF4 (5′-CCCTAGGCAAGGTT CATATGGCC-3′) are derived from the mouse transthyretin promoter (13), and the consensus NFκB-binding sequence (5′-CGGGGACTTTCCG-3′) is derived from the mouse immunoglobulin κ gene promoter (53). For annealing, equal amounts of complementary strands of oligonucleotides were heated to 95°C for 2 min in a buffer containing 50 mM Tris (pH 7.4), 60 mM NaCl, and 1 mM EDTA and allowed to cool to room temperature in about 6h.

RESULTS

Sequences between −93 and −63 in the mouse SAA3 gene promoter direct liver cell-specific expression. In a previous study, we had observed that a 138-bp fragment from the mouse SAA3 promoter containing only 93 bp of sequence 5′ to the start of transcription and that 45 bp of exon 1 sequence was sufficient to direct expression of a reporter gene in liver-derived cells (26), whereas expression was not detectable in HeLa cells (Fig. 1A) or other nonliver cells (26). Further deletion of the 5′-flanking sequence to −63 resulted in complete loss of promoter activity in Hep3B cells (Fig. 1A). These results suggest that the sequences conferring liver cell-specific expression reside around positions −93 and −63 relative to the start of SAA3 transcription.

To examine further the functional domains in this 93-bp 5′-flanking fragment and to assay for regions that may confer liver cell-specific expression to a heterologous promoter, a 56-bp oligonucleotide corresponding to this proximal region (PR) of the SAA3 promoter (position −93 to −38) was synthesized (Fig. 1B) and cloned into the unique *Bam*HI site of the pBLCAT vector containing the thymidine kinase (TK) promoter (32). Resulting constructs containing a single copy of PR sequence in the same orientation [pPR(1+)/TK] with respect to the direction of transcription or in the opposite orientation [pPR(1−)/TK], two copies [pPR(2)/TK], or four copies [pPR(4)/TK] were assayed for promoter activities by transient transfection into Hep3B and HeLa cells. When transfected into Hep3B cells, pPR(1+)/TK gave a 39-fold increase in CAT activity over the parental pBLCAT vector,

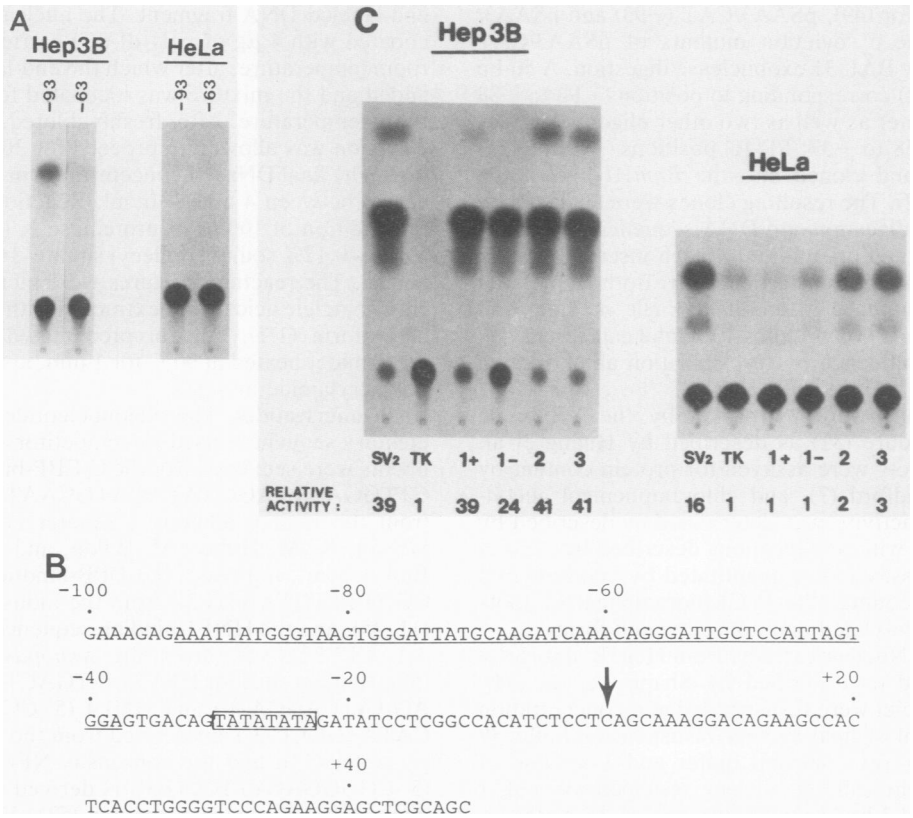

FIG. 1. Sequences in the proximal promoter region responsible for liver cell-specific expression of the mouse SAA3 gene. (A) CAT assay. Two constructs, pSAA3/CAT (−93) and pSAA/CAT (−63), which contain 93 and 63 bp, respectively, of the mouse SAA3 5′-flanking promoter sequences fused immediately 5′ of the CAT reporter gene (26), were transfected into Hep3B and HeLa cells. Cells were harvested 24 h later; cell extracts were prepared, and CAT assay were performed as described in Materials and Methods. (B) Nucleotide sequence of the immediate 5′-flanking region and part of exon 1 of the mouse SAA3 gene (37). Arrow denotes the transcription start site (+1), and the TATA region is boxed. The underlined region is the 56-bp PR sequence that was synthesized. (C) CAT assay. Synthetic oligomers corresponding to the PR sequence shown in panel B were inserted in either orientation and in multiple copies into the *Bam*HI site of the pBLCAT vector containing the TK promoter (32). The number of copies of oligomer in each construct is indicated. pSV_2CAT (SV_2) and pBLCAT (TK) DNAs were used as controls. Similar results were obtained from three independent experiments, only one of which is shown. CAT activities are expressed relative to level of CAT activity for pBLCAT vector in each cell line, which was assigned a value of 1.0.

whereas basal CAT activity was observed in HeLa cells (Fig. 1C). The increased activity observed in Hep3B cells was independent of PR sequence orientation, since the pPR(1−)/TK construct had similarly enhanced expression in liver cells. Moreover, constructs pPR(2)/TK and pPR(4)/TK, containing multiple copies of the PR sequence, resulted in slightly higher CAT activity in both Hep3B and HeLa cells than did constructs containing only a single copy (Fig. 1C). These results demonstrated that the 56-bp PR sequence contributes to the enhanced basal expression observed with the pSAA3/CAT (−93) construct in liver cells. Furthermore, this PR element alone was sufficient to confer liver cell-enhanced activation onto a heterologous promoter in an orientation-independent manner, suggesting that this fragment plays an important role in the tissue-specific expression of the mouse SAA3 gene.

Interaction of a C/EBP-like nuclear factor with the PR element. To identify the proteins interacting with the PR sequence, we prepared nuclear extracts from Hep3B and HeLa cells and assayed for specific protein-DNA interactions. Two discrete DNA-protein complexes were formed with 1 μg of Hep3B nuclear extract, while the same amount of protein from HeLa nuclear extract resulted in a similar gel shift pattern but with an intensity approximately 10-fold lower (Fig. 2A). This finding suggested that the PR sequence either interacts with at least one nuclear protein present at higher levels in liver cells than in HeLa cells or binds with much higher affinity. Since liver-specific *trans*-acting factors constitute a limited number of proteins that recognize similar binding sites in many genes, and a number of them have already been described (6, 13, 20, 45), we were interested in determining whether the nuclear protein or proteins binding to the PR sequence are among these known factors. Five oligonucleotides corresponding to the binding sites of C/EBP, DBP, HNF1, HNF3, and HNF4 were synthesized and used in competition experiments. Only C/EBP and DBP oligomers could efficiently and specifically compete for binding of protein to the PR sequence, while HNF1, HNF3, and HNF4 oligonucleotides, as well as the nonspecific competitor control NFκB, could not compete for the protein-PR sequence complexes even at a 100-fold molar excess of competitor DNA (Fig. 2B). These results indicated that the protein factor binding to the PR element is a C/EBP- or DBP-like nuclear protein that is abundant in liver cells.

Because heat stability is a characteristic of C/EBP (27, 33), we tested whether binding to the 56-bp PR sequence is also resistant to heat treatment. Nuclear extracts heated to 90°C for 15 min still formed the specific doublet DNA-protein

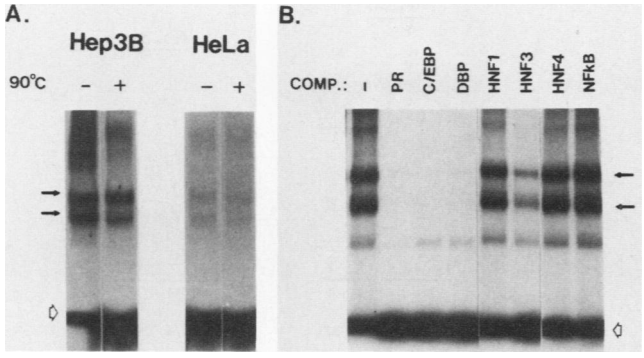

FIG. 2. Detection of nuclear factors from Hep3B cells that interact with the PR element of SAA3 by gel retardation assay. (A) Heat stability of the nuclear factors. Hep3B and HeLa nuclear extracts were not heated (−) or were heated at 90°C for 15 min (+) before incubation with the PR probe. DNA-protein complexes were then resolved in a native 6.5% polyacrylamide gel. (B) Competition of factor binding to the PR sequence. The 56-bp PR sequence was used in binding reactions with Hep3B nuclear extracts. Competitor oligonucleotides corresponding to the binding site of liver-specific nuclear factors C/EBP, DBP, HNF1, HNF3, and HNF4 were used at a 100-fold molar excess with respect to the PR probe. The NFκB oligomer was used as a nonspecific competitor. Positions of specific complexes formed are indicated by filled arrows; open arrows indicate positions of free probe.

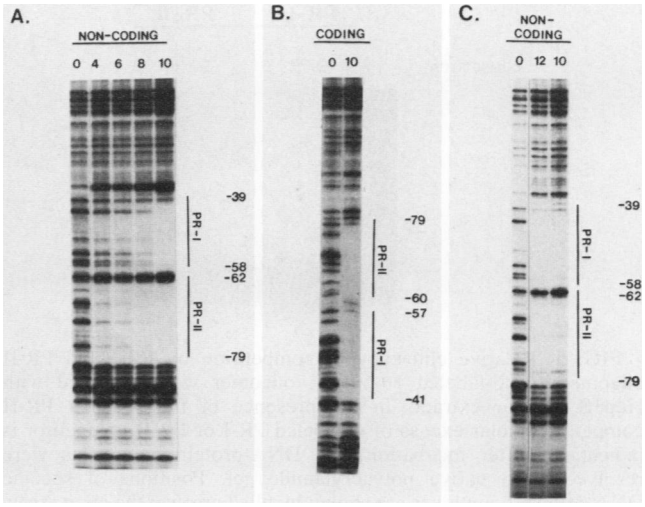

FIG. 3. Determination of binding sites for Hep3B nuclear factor and recombinant C/EBP by DNase I footprint assay. (A) A restriction fragment containing the 56-bp PR sequence was end labeled on the noncoding strand and incubated with 0, 4, 6, 8, or 10 μl of Hep3B nuclear extract (0.5 μg/μl) under conditions used for gel retardation assays. DNA-protein complexes were incubated with DNase I as described in the text, and the DNA was extracted, denatured, and electrophoresed on a sequencing gel. The extent of protection from DNase I cleavage is shown by the solid bar. (B) DNase I footprint analysis is identical to that in panel A except that the single end label was on the coding strand. (C) End-labeled fragment as in panel A was incubated with 0 or 12 μl of Hep3B nuclear extract or with 10 μl of a 1:10 dilution of purified recombinant rat C/EBP.

complexes with little or no loss in binding activity (Fig. 2A). This result provided further evidence that C/EBP or a similar nuclear protein binds to the PR sequence of the mouse SAA3 gene.

PR sequence contains two adjacent C/EBP-binding sites. To localize the binding site of the C/EBP factor more closely within the PR sequence, DNase I protection assays were performed by using Hep3B nuclear extracts. End-labeled restriction fragments from plasmids pPR(1+)/TK and pPR(1−)/TK were used as substrates to footprint the noncoding and coding strands, respectively. With increasing concentrations of Hep3B nuclear extract, two distinct DNase I-protected regions became apparent on the noncoding strand (Fig. 3A) Locations of the protected regions relative to the SAA3 gene transcription start site are indicated. The PR sequence from −58 to −39 was designated PR-I, and the more distal region from −79 to −62 was designated PR-II (Fig. 3A). Overlapping regions were protected on the coding strand, one extending from −41 to −57 (PR-I) and the other extending from −60 to −79 (PR-II) (Fig. 3B). These footprint results indicated that there are actually two adjacent protein-binding sites within the 56-bp PR sequence, each binding site spanning approximately 20 bp. To further establish that the footprint regions were the result of C/EBP binding, we performed footprint analyses with the purified recombinant C/EBP protein. An identical footprint pattern was obtained with the recombinant C/EBP (Fig. 3C). These results are consistent with the existence of C/EBP-binding sites within the PR sequence and demonstrate conclusively that the PR sequence contains two C/EBP-binding sites.

The PR-II region is a stronger C/EBP-binding site. Results from the DNase I protection experiments revealed that complete protection of PR-II requires two- to threefold less Hep3B nuclear extract than is required to fully protect PR-I, which suggested that C/EBP has a stronger affinity for the PR-II-binding site. To resolve these questions, we synthesized two oligonucleotides corresponding to PR-I and PR-II and used them in gel shift and oligonucleotide competition assays. Both PR-I and PR-II oligonucleotides formed a doublet of DNA-protein complexes with Hep3B nuclear extract (Fig. 4), similar to the complexes formed with the entire PR element. As expected, synthetic oligonucleotides containing the C/EBP- and DBP-binding sites and the 56-bp PR sequence competed efficiently and specifically for this binding, whereas a nonspecific oligonucleotide containing

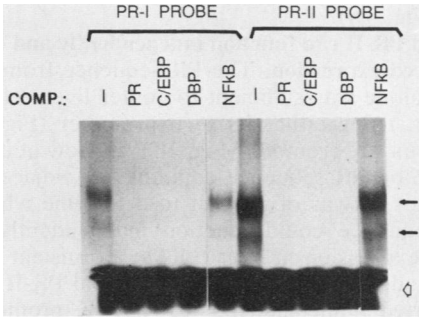

FIG. 4. Gel retardation assays using oligomers corresponding to PR-I and PR-II. PR-I oligomer (−58 to −37) and PR-II oligomer (−79 to −59) corresponding to the two DNase I-protected regions were synthesized as double-stranded oligomers and used in gel retardation assays. The oligomers were end labeled and incubated with nuclear extracts from Hep3B cells. Filled arrows indicate the positions of specific DNA-protein complexes; the open arrow denotes free oligomers. The specificity of factor binding was determined by competition with a 100-fold molar excess of unlabeled oligonucleotide competitors PR, C/EBP, and DBP. Nonspecific competitor NFκB oligomer was used as a control.

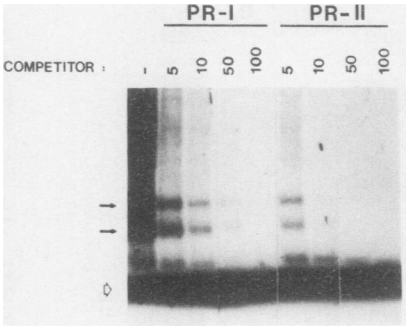

FIG. 5. Relative efficiency of competition by PR-I and PR-II oligomers. End-labeled 56-bp PR oligomer was incubated with Hep3B nuclear extracts in the presence of the PR-I or PR-II competitor (molar excess of unlabeled PR-I or PR-II competitor is indicated). After incubation, the DNA-protein complexes were resolved on a native polyacrylamide gel. Positions of specific DNA-protein complexes are shown by filled arrows; the open arrow indicates free DNA.

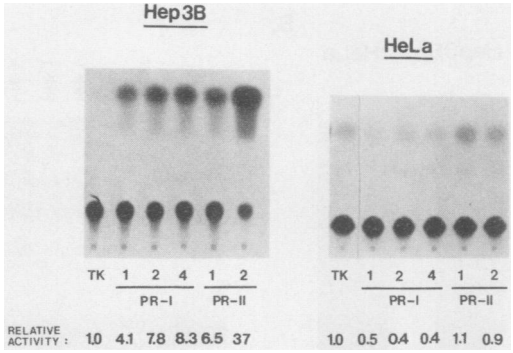

FIG. 6. Demonstration that PR-I and PR-II sequences functioned independently and conferred liver cell-enhanced expression. Synthetic oligomers corresponding to PR-I (−58 to −37) and PR-II (−79 to −59) were inserted in one or multiple copies into the *Bam*HI site of the pBLCAT vector (number of copies of oligomer in each construct is indicated at the bottom). pBLCAT (TK) DNA was used as a control. CAT activities are expressed relative to level of CAT activity for pBLCAT vector in each cell line, which was assigned a value of 1.0.

the NFκB-binding site did not compete. Under identical experimental conditions using equal amounts of probe and nuclear extract, the intensity of the DNA-protein complexes formed with PR-II probe was always much greater than that obtained with the PR-I probe (Fig. 4). Consistent with the footprint results, this finding indicates that the PR-II region is a higher-affinity binding site for C/EBP than is PR-I. To assess the relative binding affinities of PR-I and PR-II, they were used as specific competitors in gel shift experiments in which the 56-bp PR sequence was used as a probe. Both PR-I and PR-II oligomers efficiently competed with the labeled PR fragment, although the PR-II oligonucleotide competed much better than did the PR-I oligonucleotide (Fig. 5). A 10-fold excess of unlabeled PR-II oligomer was sufficient to eliminate the C/EBP-PR complexes completely, whereas a 50-fold excess of PR-I oligomer was needed to eliminate these complexes. As these competition experiments indicate, the PR-II-binding site had at least a three- to fivefold-higher affinity for C/EBP protein than did the PR-I-binding site, a result consistent with the DNase I protection analysis (Fig. 3A).

PR-I and PR-II can function independently and confer liver cell-enhanced expression. The PR sequence from the SAA3 promoter alone was sufficient to confer liver cell-enhanced expression, using either its own promoter (Fig. 1A) or a heterologous TK promoter (Fig. 1C). In view of our findings that the 56-bp PR sequence contains two adjacent C/EBP-binding sites, it was of interest to determine whether each binding sequence could function independently to direct enhanced expression in liver cells in a transient expression assay. To address this question, PR-I and PR-II sequences were inserted immediately 5′ to the TK promoter in the vector pBLCAT, and the chimeric DNAs were transfected into Hep3B and HeLa cells. When transfected into Hep3B cells, both constructs containing either the PR-I or PR-II sequence gave significantly higher CAT activity than did the parental vector (Fig. 6), those containing two copies of PR-II yielding the highest activity. This finding agreed with results of our gel shift and competition experiments in which PR-II bound C/EBP with higher affinity than did PR-I. When transfected into HeLa cells, the same constructs showed no enhancement compared with the parental pBLCAT vector.

DISCUSSION

We sought a molecular explanation for the high level of SAA3 expression in hepatocytes by assaying the promoter for sequences responsible for this phenotype. Our previous studies have shown that constructs containing 93 bp of the mouse SAA3 proximal promoter region can direct basal expression of a reporter gene in a liver cell-specific manner (26). Further deletion to position −63 resulted in complete loss of promoter activity, suggesting that sequences around positions −93 and −63 in the mouse SAA3 gene are important for its enhanced expression in liver cells. In this study, we showed that a 56-bp sequence spanning positions −93 to −38 relative to the transcription start site could, when ligated to a heterologous promoter, enhance expression in cells derived from liver, yet this sequence had no comparable ability to enhance expression in HeLa cells. We also demonstrated, by both in vitro binding and DNase I protection assays, that this 56-bp region is capable of being recognized by the transcription factor C/EBP, which is much more abundant in liver cells than in other cells (6). Moreover, DNase I protection analysis of this region of the SAA3 promoter revealed two adjacent C/EBP-binding sites. Purified recombinant C/EBP gave a pattern of DNase I protection identical to that seen with crude Hep3B nuclear extracts. Heat treatment of the nuclear extracts in which binding activity was evident did not affect the pattern of binding, a result consistent with the known heat-stable characteristics of C/EBP (27, 33). These results suggested strongly that C/EBP, a transcription activator abundant in the liver, is one nuclear factor in hepatoma cells that recognizes the SAA3 promoter and confers the enhanced transcriptional activity of the mouse SAA3 gene in hepatocytes.

C/EBP is a heat-stable DNA-binding protein that is highly abundant in the liver though not found exclusively there. Several liver-specific gene promoters, such as the albumin, α_1-antitrypsin, and transthyretin promoters, all contain C/EBP-binding sites (14). Deletion or loss of C/EBP-binding sites lead to loss of transcription regulation of these liver-specific genes. In fact, natural mutations that disrupt a C/EBP-binding site in the factor IX promoter have been shown to associate with an inherited bleeding disorder,

hemophilia B (18). Furthermore, when cotransfected into cultured cells, C/EBP can *trans*-activate the transcription of promoters containing the C/EBP-binding sites (21). That C/EBP functions as a transcriptional activator in the expression of liver-specific genes is consistent with our results in which the two adjacent C/EBP-binding sites of the mouse SAA3 gene conferred liver cell-specific transcriptional activation onto a heterologous promoter. Although both PR-I and PR-II sites interact with C/EBP, binding of C/EBP to PR-II is about five times stronger than binding to PR-I. Deletion of the PR-II region completely abolished the SAA3 promoter function. It is thus apparent that the binding of C/EBP also plays an important role in liver-specific transcription driven by the SAA3 promoter.

The optimal C/EBP-binding site consists of the dyad half-site GCAAT, but it can also recognize sequences significantly divergent from the optimum (24, 55). Compared with an expanded C/EBP consensus sequence T(T/G)NNG(A/C/G)AA(T/G) (51), both PR-I and PR-II, containing the sequences ATTGCTCCA and TTATGCAAG, respectively, are consistent with this consensus sequence, yet C/EBP binds these two sites with quite different binding affinities, as demonstrated by the DNase I protection and oligomer competition assays. Because of its broad sequence specificity, C/EBP may bind sequences that are more specific for other C/EBP-like DNA-binding proteins. This is the case for the D region of the rat albumin promoter, which can interact with both C/EBP and a protein designated DBP (38, 45). DBP binds only the D site, whereas C/EBP binds the D site as well as the A, C, and F sites in the albumin promoter (45). These observations suggest, therefore, that DBP binds with a higher degree of sequence specificity to its recognition site. We believe, however, that C/EBP rather than DBP is the factor recognizing this region of the SAA3 promoter. Several lines of evidence support this conclusion. First, the PR-I and PR-II sites bear no obvious sequence similarity to the D region (CGATTTTGTAATG) of the albumin promoter. This lack of sequence homology suggests that a protein other than DBP recognizes the SAA3 promoter. Second, oligonucleotides corresponding to the binding sites of both C/EBP and DBP can compete effectively for the binding activity with the PR-I and PR-II oligonucleotides. Since DBP has a higher degree of sequence specificity than C/EBP, then binding with DBP as the PR-sequence-binding protein should not be competed for by the C/EBP oligomers. Moreover, the protein binding to PR-I and PR-II is heat stable, a characteristic property that distinguishes C/EBP from DBP. Third, electrophoretic mobility shift experiments with the PR sequence and Hep3B nuclear extracts yielded gel shift patterns identical to those of the albumin C/EBP-binding site but different from those done with the D region of the albumin promoter (unpublished data).

Recently, Akira et al. (1) described yet another DNA-binding factor, NF-IL-6, that also interacts with C/EBP-binding consensus, T(T/G)NN GNAA(T/G). In contrast to C/EBP and DBP, NF-IL-6 is normally expressed at very low levels in the liver but can be induced to high levels by IL-1 and binds to the IL-1 response element in the IL-6 gene promoter. Furthermore, the expression of NF-IL-6 is not restricted to liver. Therefore, instead of being a liver tissue-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. Coincidentally, IL-1 is a known regulator of SAA3 expression (26). This raises an interesting possibility that the two C/EBP-binding sites described herein might in fact be sites for NF-IL-6 binding in vivo. Two lines of evidence suggest that this is not the case. First, construct pSAA/CAT (−93), which retains the two C/EBP-binding sites, PR-I and PR-II, has lost the ability to respond to IL-1 (26, 50). We have mapped IL-1 responsiveness to a region 5′ of PR-I and PR-II (26). Indeed, a potential NF-IL-6-binding site in the IL-1 response element of the mouse SAA3 promoter is present. Second, pPR/TK constructs in cells stimulated with IL-1 or conditioned media prepared from activated mixed lymphocytes do not show an increase in CAT activity (data not shown). The fact that the SAA3 promoter is both IL-1 responsive and able to bind C/EBP suggest that NF-IL-6 and C/EBP can discriminate between highly similar if not identical binding sites.

Although C/EBP protein is abundant in the liver, it is also found in high concentrations in adipose tissues and at significantly lower levels in the lung, small intestine, and adrenal glands (6). Interestingly, SAA3 expression is also highest in the liver after acute inflammation, but it is also expressed in the lung, adrenal gland, and intestine, albeit at much lower levels (41). To account for the widespread distribution of C/EBP protein, McKnight et al. (40) proposed that it may be a central regulator for genes encoding proteins involved in energy metabolism, particularly those participating in the synthesis and catabolism of lipids. This hypothesis is based on the coincidence of expression of C/EBP and enzymes involved in regulating intermediary energy metabolism (6, 40), the *trans* activation of some promoters by C/EBP of genes encoding proteins belonging to this class (12, 21, 30), and the reduced level of C/EBP mRNA in livers of mice homozygous for deletions near the albino locus, the physiological consequence of which is neonatal liver dysfunction and death (40, 42). Consistent with this hypothesis is the finding that SAA proteins have been shown to associate with the high-density lipoprotein fraction of serum lipids and are therefore apolipoproteins (2). Association of high-density lipoprotein particles with SAA during the acute-phase response greatly reduces the half-life of high-density lipoprotein particles in serum, which led to the proposal that SAA functions by facilitating the redistribution of lipids during inflammation (3). Many genes involved in energy or lipid metabolism, such as those encoding glucokinase, tyrosine aminotransferase, and phosphoenopyruvate carboxykinase, are also hormonally regulated. In this respect, SAA3 is similarly regulated by circulating inflammatory mediators (26, 48). Since SAA3 expression in liver is low and only detectable on induction, it is possible that the coexpression of C/EBP and SAA mRNAs in various tissues represents coregulation of SAA3 by C/EBP, along with the necessary transcription factor or factors activated by inducing signals that interact with the IL-1 response element of the SAA3 promoter. The activity of these transcription factors that follows IL-1 induction helps to initiate SAA3 gene transcription. Cooperation among all of these nuclear proteins might initiate gene expression during inflammation. Accordingly, the function of C/EBP in SAA3 expression might be to enable the SAA3 promoter to increase transcription rates in the liver in response to inducing signals. The diminished SAA3 expression in nonliver cells such as HeLa cells may be due to the absence or low levels of C/EBP protein in those cells. The regulation of mouse SAA3 gene expression, however, is further complicated by the finding of comparable amounts of SAA3 mRNA in macrophages and in liver cells (41). Whether C/EBP is as abundant in macrophages as in liver cells is not known. It will be of interest to determine whether the regulation of SAA3 gene expression is depen-

dent on the same *cis*-acting sequences and *trans*-acting factors in macrophages as it is in liver cells.

We demonstrated that the SAA3 promoter binds purified recombinant C/EBP protein in a pattern similar to that seen in a crude nuclear extract made from cells in which the SAA3 gene is expressed. Tissue distribution of SAA3 is correlated with the expression of C/EBP, levels of both being highest in the liver. Whether this correlation directly reflects the role C/EBP in regulating SAA3 expression is not clear. The implication that C/EBP participates in SAA3 expression in the liver broadens the role of C/EBP to include genes of the acute-phase class. The in vivo effects of C/EBP on SAA3 expression and the mechanism of cooperation between C/EBP and the proteins that bind to the SAA3 IL-1-responsive element are being investigated.

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