MOLECULAR AND CELLULAR BIOLOGY, Jan. 1993, p. 432–442
0270-7306/93/010432-11$02.00/0

Vol. 13, No. 1

Induction of Liver Alpha-1 Acid Glycoprotein Gene Expression Involves Both Positive and Negative Transcription Factors

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Received 11 September 1991/Returned for modification 9 April 1992/Accepted 13 October 1992

Expression of the alpha-1 acid glycoprotein (AGP) gene is liver specific and acute phase responsive. Within the 180-bp region of the AGP promoter, at least five *cis* elements have been found to interact with *trans*-acting factors. Four of these elements (A, C, D, and E) interacted with AGP/EBP, a liver-enriched transcription factor, as shown by footprinting analysis and by an anti-AGP/EBP antibody-induced supershift in a gel retardation assay. Modification of these sites by site-directed mutagenesis coupled with transfection analysis indicated that AGP/EBP binding to all of these sites resulted in positive regulation of the promoter. Dose-response data suggest that AGP/EBP binding to these sites results in the cooperative activation of the promoter. In contrast, functional assays showed that element B is a negative regulatory element; this element is recognized by heat-stable DNA-binding factors which are found in many cells and tissues. The regulation of these binding proteins was studied in rat liver treated with lipopolysaccharide (LPS), which induced an acute-phase reaction. We found that LPS treatment resulted in a two- to threefold increase in AGP/EBP activity and a severalfold decrease in the activity of factors that bind to element B in the liver. These results indicate that expression of the AGP gene can be regulated by both positive and negative factors and that the modulation of these factors can account for the LPS induction of the AGP gene.

In recent years, considerable progress has been made in understanding the molecular mechanisms that regulate liver gene expression (15–18, 20, 25, 32, 33, 37, 44, 63, 64). The liver not only offers a system for detailed analysis of tissue-specific gene expression but also provides a unique system for studying expression of certain kinds of genes during an acute-phase response. Alpha-1 acid glycoprotein (AGP) is an acute-phase-responsive, liver-specific plasma protein (3–5, 55). In rats, the levels of liver AGP mRNA and plasma AGP protein increase 10- to 100-fold within 24 h of experimentally induced inflammation (3, 4, 6, 9, 17, 27, 30, 39, 41, 45, 52, 53). These increases are primarily attributed to changes in AGP gene transcription (9, 41), although some evidence for additional posttranscriptional modulation has been reported (10, 61). Expression of the rat AGP gene is also modulated by humoral factors such as interleukin-1 (IL-1) (8, 54), IL-6 (6), and glucocorticoids (3, 40).

Among the most fascinating aspects of AGP gene expression are the molecular mechanisms underlying differential expression of multiple AGP genes. Multiple forms of mouse (42) and human (57) AGPs and their corresponding genes have been described (19, 42). In mice, two functional AGP genes and a pseudogene have been analyzed and characterized (14). The mouse AGP genes are located proximal (*Agp-1*) and distal (*Agp-2*) to the centromere (in chromosome 4), with the pseudogene (*Agp-3*) located between them (14). The level of mRNA for AGP-1 is higher than that for AGP-2. Both genes are induced dramatically during an acute-phase response (14).

We have previously identified and cloned a transcription factor, AGP/EBP, which plays a crucial role in regulating AGP gene transcription (13). How AGP/EBP regulates the AGP gene, however, is unknown. An AGP/EBP homolog, LAP (also known as C/EBP-beta), has been implicated in regulating rat albumin gene expression (21), while its human homolog, NF-IL-6, has been defined as an IL-6-responsive nuclear factor which regulates a number of IL-6-responsive genes (1). It has been demonstrated that an IL-1/IL-6-responsive element exists in the distal region of the rat AGP gene (−5300 to −5160) (53). We have identified a number of *cis* elements in the 5′-flanking region of the *Agp-1* gene (13, 62). In addition to the AGP/EBP-binding elements, one glucocorticoid-responsive element (24, 39) and other *trans*-acting factor-binding sites (24) have been defined. AGP/EBP, like most widely distributed and cell-specific factors, functions positively during transcription. Despite progress in understanding DNA-binding factors and their recognized *cis* elements in the AGP promoter, we know nothing about how AGP gene transcription is induced during an acute-phase response. As a general rule, the regulation of gene transcription involves both positive and negative *cis* elements and, presumably, positive and negative *trans*-acting factors.

We have made a careful analysis of the positive regulatory role of AGP/EBP and identified a negative factor in the promoter region of the AGP gene. Upon treatment with lipopolysaccharide (LPS), AGP/EBP increased while the negative factor decreased in liver; therefore, the induction of AGP gene transcription during LPS treatment can be explained by the increase of positive and the decrease of negative transcription factors.

MATERIALS AND METHODS

DNA and plasmids. Mouse *Agp-1* genomic DNA was isolated from a genomic library of BALB/c DNA and se-

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quenced (13). The promoter region containing the 190-bp fragment (*Rsa*I-*Sau*3AI) (from −180 to +10) was isolated and inserted into pCAT-basic (Promega) [referred to as pAGP(wt)CAT]. In deletion mutant p-71 CAT, the sequences upstream from −71 to +10 were deleted. Mutant pE'AGPCAT contained two nucleotide changes at site E, which were constructed by site-directed mutagenesis as described below. Expression vector pCMV-AGP/EBP was constructed by fusing the full-length cDNA of AGP/EBP (13) to the cytomegalovirus long terminal repeat vector. Recombinant AGP/EBP protein was obtained by expression of the recombinant pET-3a containing the full-length AGP/EBP cDNA (13) in *Escherichia coli* BL21-(DE3) (60). Recombinant AGP/EBP was eluted and purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Nuclear extract preparation. Liver nuclear extracts from 200- to 300-g brown Norway rats were prepared as described by Gorski et al. (32). For LPS-treated nuclear extracts, the rats were sacrificed 4 h after LPS treatment (10 μg/g of body weight). Following ammonium sulfate precipitation, protein pellets were dissolved at a concentration of 5 to 7 mg/ml in dialysis buffer (NDB; 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.8], 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol). Nuclear extracts were dialyzed and then stored in small aliquots at −135°C. Nuclear extracts from cultured cells were prepared as described by Dignam et al. (23). DNase I footprinting assays were performed according to the method of Galas and Schmitz (29). An end-labeled DNA fragment (approximately 1 ng) from polymerase chain reactions was added to a 20-μl reaction mixture containing 25 mM HEPES (pH 7.8), 60 mM KCl, 7.5% glycerol, 0.1 mM EDTA, 5 mM $MgCl_2$, and 1 μg of double-stranded poly(dI-dC) DNA. Crude nuclear extracts or heparin-agarose fractions were added last, and the binding reaction was allowed to proceed for 90 min on ice. Then 1 to 8 μl of DNase I (50 to 100 U/ml; Worthington), freshly diluted in 10 mM $MgCl_2$ and 5 mM $CaCl_2$, was added. Digestion was performed at room temperature for 2 min and halted by the addition of 80 μl of a solution containing 75 μg of sonicated *E. coli* DNA per ml, 20 mM EDTA, 0.5% SDS, and 100 μg of proteinase K per ml. The samples were then incubated for 30 min at 65°C, extracted twice with phenol-chloroform, and precipitated with ethanol at −70°C. The DNA pellets were dried, resuspended in formamide-dye (95% formamide, 1% xylene cyanol FF, 1% bromphenol blue), heated at 95°C for 3 min, and loaded onto an 8% polyacrylamide–10 M urea sequencing gel. A sequence ladder was prepared from AGP promoter plasmid DNA by a polymerase chain reaction primer. The gels were dried and autoradiographed at −70°C with a Dupont-Cronex intensifying screen. For gel retardation assays, protein-DNA complexes were formed as described for the footprint experiments but in a 10-μl total volume. Oligonucleotides used were as follows.

C 5'-GATCAGATTGTGCCACAG-3'
DE 5'-GATCACATTTTGTGTAAGACATTTCCCAAGTG-3'
D 5'-GATCACATTTCCCAAGTG-3'
E 5'-GATCATTTTGTGTAAGAC-3'
B 5'-GATCTACTGTCCCTGGCTTCAGTCCCATGCCCT-3'

After incubation for 20 min at room temperature (for liver extract), 1 μl of 1% bromphenol blue was added, and the samples were directly loaded onto a 6% polyacrylamide gel (30:1 acrylamide/bisacrylamide) containing 4% glycerol. Electrophoresis was performed with 150 V at room temperature for probes C, D, and E and at 4°C for probe B.

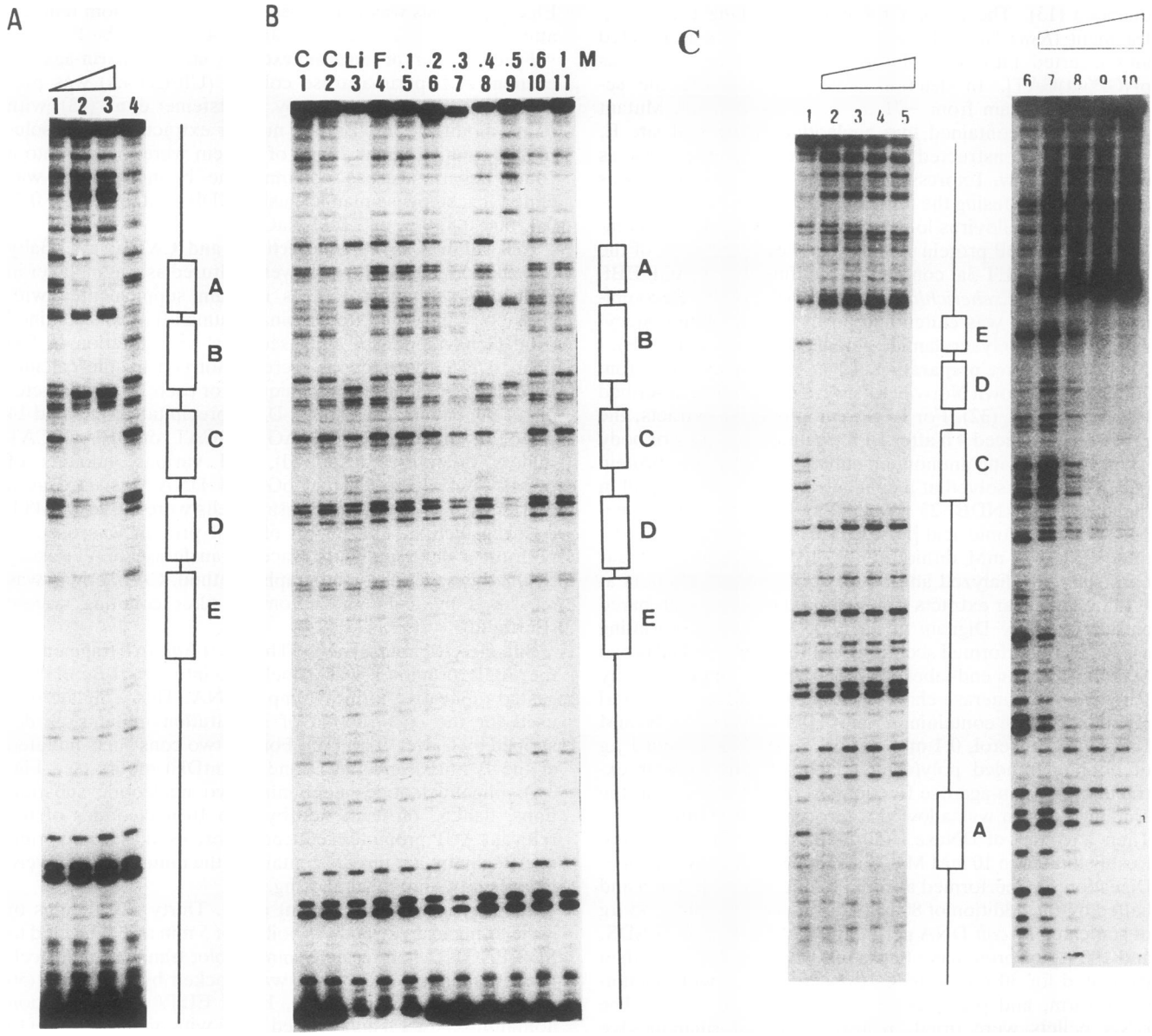
Fractionation of nuclear extracts on a heparin-agarose column. A heparin-agarose column (Ultogel-4A) was prepared and run as described by Lichtsteiner et al. (43), with minor modifications. Briefly, nuclear extracts in NDB solution containing about 25 mg of protein were loaded onto a 10-ml heparin-agarose column. The bound protein was eluted in a stepwise manner, using NDB containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, or 1.0 M NaCl.

Cell cultures, DNA transfections, and CAT assays. Baby hamster kidney (BHK) cells were cultured as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Recombinant human IL-6 was obtained from Genzyme Corp. and used at a concentration of 500 U/ml. DNA transfections were performed by the calcium phosphate precipitation technique. For each 10-cm-diameter plate, the calcium phosphate-DNA precipitate contained 14 μg of a target plasmid, pAGP(wt)CAT or pE'AGPCAT (unless otherwise described), and various amounts of pCMV-AGP/EBP. Plasmid pGem 4 DNA was used as a carrier to give 20 μg in the assay. Cells were harvested 48 h posttransfection, and 30 μg of total protein was used to perform a chloramphenicol acetyltransferase (CAT) assay by the thin-layer chromatography method. CAT activity was measured by using the automatic filter counting system (Berthold).

Site-directed mutagenesis. The *Rsa*I-*Sau*3AI fragment of the AGP promoter was subcloned into *Sma*I-*Bam*HI-digested replicative-form M13mp18 DNA. This template was used for the construction of substitution mutants as described by Carter et al. (12). For the two constructs mutated at the E (mtE) and the D and E (mtDE) motifs (see Fig. 3A), oligonucleotides containing two nucleotide substitutions, flanked on each side by 7 to 10 nucleotides of the relevant AGP promoter sequence, were used as single primers. Recombinant phages containing the mutated insert were identified by direct sequencing.

Southwestern (DNA-protein) assay. Thirty micrograms of rat liver nuclear extract was boiled for 5 min and subjected to SDS-PAGE. After Western immunoblot transfer to nitrocellulose paper, the membrane was blocked by BLOTTO (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 5% Carnation nonfat milk) for 1 h and washed twice with binding buffer (10 mM Tris-HCl [pH 7.6], 50 mM NaCl, 1 mM EDTA) for 10 min. The membrane was then subjected to a binding reaction with probe B for 1 h in the presence of 5 μg of tRNA per ml and washed with binding buffer four times for 30 min at 4°C. After being air dried, the membrane was autoradiographed. To estimate the molecular weight and determine the sequence binding specificity of purified factor B, both the wild-type probe B (B*) and mutated probe B (mtB*) (sequences are shown in Fig. 6B) were used in a Southwestern assay.

Purification of factor B on oligonucleotide affinity columns. Partially purified factor B (i.e., the 0.4 M NaCl fraction of the heparin-agarose column) was further purified on oligonucleotide C and oligonucleotide B affinity columns. The flowthrough fraction of the oligonucleotide C column was passed through the oligonucleotide B column. Factor B was eluted at 0.5 M NaCl. Sequences of oligonucleotide B were 5'-GATCCGAAGGGGCTGTCCCTGGCTTCAGTCCCAT GCCCTC-3' (upper strand) and 5'-GAGGGCATGGGACT GAAGCCAGGGACAAG-3' (lower strand); sequences of oligonucleotide C were 5'-GATCCGAAGGGGCTGGTGAG



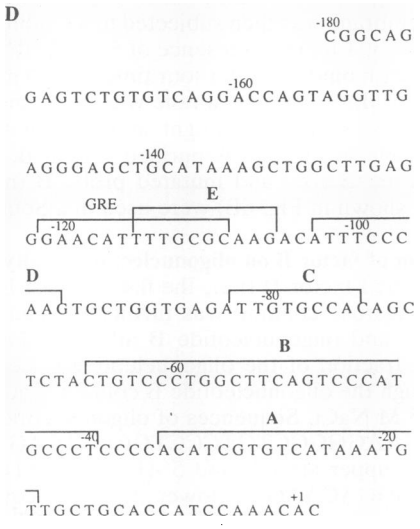


FIG. 1. DNase I footprinting analysis of the AGP promoter fragment (−180 to +10) with crude and fractionated liver nuclear extract. (A) Footprinting analysis with unfractionated liver nuclear extract. The noncoding strand was end labeled with [γ-^{32}P]ATP by polynucleotide kinase. Lanes 1 to 4 contain 12, 20, 32, and 0 μg of liver nuclear extract, respectively. (B) Footprinting analysis with heparin-agarose column-fractionated liver nuclear extract. Lane 1 and 2 show the DNase I digestion pattern observed in the absence of nuclear extract; lane 3 contains 20 μg of crude nuclear extract; lanes 4 to 11 indicate the flowthrough and the fractions eluted at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 1 M NaCl, respectively. (C) DNase I footprint analysis of the AGP promoter with recombinant AGP/EBP and crude liver nuclear extract. The coding strand was end labeled with [γ-^{32}P]ATP by polynucleotide kinase. Lanes 1 and 6 contain bovine serum albumin as a control; lanes 2 to 5 contain 0.5, 1, 2, and 4 μg of recombinant AGP/EBP, respectively; lanes 7 to 10 contain 8, 12, 20, and 32 μg of crude liver nuclear extract, respectively. Boxes A, C, D, and E indicate the protected regions that appeared in both recombinant AGP/EBP and the nuclear extract. Box B is not visualized by recombinant AGP/EBP and therefore is not shown. (D) Summary of DNase I footprints on the AGP promoter. Protein-binding sites are defined by footprinting analysis of both DNA strands. GRE, glucocorticoid-responsive element.

ATTGTGCCACAGCTCTAC-3′ (upper strand) and 5′-GTA GAGCTGTGGCACAATCCTCACCAG-3′ (lower strand).

RESULTS

Multiple nuclear proteins in rat liver interact with 5′-flanking sequences of the AGP gene. To define the *trans*-acting factors that interact with the promoter region of the AGP gene, the AGP sequence spanning from +10 to −180 (13) was labeled and used as a probe for our DNase I footprinting analysis using rat liver nuclear extracts. Five regions spanning from −120 to −27 were protected by nuclear factors from DNase I digestion in a dose-dependent manner (Fig. 1A, lanes 1 to 3). To characterize the nuclear factors involved in the binding of DNA and to precisely define the boundaries for each binding site, the liver nuclear extract was subsequently fractionated by heparin-agarose column chromatography. Figure 1B shows that the binding activities for sites A, C, D, and E are eluted predominantly in the 0.5 M NaCl fraction, while that for site B is eluted mainly in the 0.4 M fraction. This result suggests that the site B-binding protein is different from the factors binding the other regions, while the same or similar factors may bind to sites A, C, D, and E simultaneously. The DNase I footprinting pattern was further studied by using recombinant AGP/EBP and unfractionated nuclear extract with the end-labeled coding strand as a probe (Fig. 1C). The results clearly show that AGP/EBP is involved in the binding of sites A, C, D, and E. The factor that binds to site B is distinct from AGP/EBP and is readily detectable in liver nuclear extract. The boundaries of each element are summarized in Fig. 1D.

The factors that bind to the A, C, D, and E regions are similar to AGP/EBP. We previously showed (13) that recombinant AGP/EBP could bind to the C and E regions. To confirm whether recombinant AGP/EBP can bind to the A, C, D, and E regions, footprinting patterns of these regions from recombinant AGP/EBP were compared with those from liver nuclear extracts. The footprinting patterns (Fig. 1C) were essentially the same except for narrower protected regions for C and D and a hypersensitive site at −88 (G) with recombinant AGP/EBP (data not shown). To further characterize the similarity of factors that bind to these regions and AGP/EBP, we synthesized oligonucleotides containing the C, D, and E sequences and used them for gel retardation assays using partially purified liver nuclear extracts (i.e., the 0.5 M NaCl fraction of the heparin-agarose column). In this assay, all three probes had similar band shift patterns (Fig. 2). The complex could be supershifted by an anti-AGP/EBP antibody in all cases (Fig. 2, lanes 2, 4, and 6). These results suggest that AGP/EBP was the major factor in nuclear extracts that bound to the AGP promoter and that AGP/EBP could bind to multiple sites in the promoter. However, these results do not exclude the possibility that other factors, such as C/EBP or immunoglobulin (Ig)/EBP, may also participate in the binding of these elements as a heterodimer with AGP/EBP (see Discussion).

Functional roles of sites A, C, D, and E. Having characterized AGP/EBP biochemically, we tested the functional role of the *cis* elements, A, C, D, and E, by a CAT assay with site-directed and deletion mutants (Fig. 3A). Both wild-type and mutant promoter sequences were linked to the CAT reporter gene. To determine the *trans*-acting role of AGP/EBP, the expression vector pCMV-AGP/EBP was used for cotransfection analysis. The CAT assay was performed in BHK cells, in which the endogenous AGP/EBP level was low (unpublished data). mtE responded to pCMV-AGP/EBP at a lower level than did the wild type (Fig. 3B). Mutation of both D and E elements (mtDE) further impaired the AGP/EBP responsiveness (data not shown). The poorest response was observed when sites C, D, and E were deleted (con-

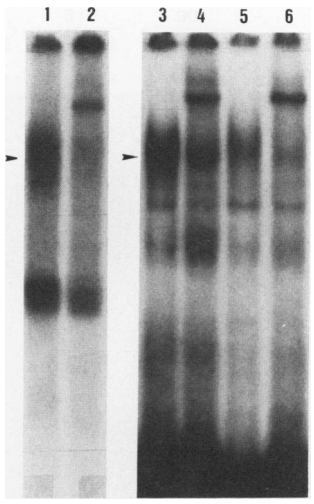


FIG. 2. Similarity of the factors that bind to sites C, D, and E. A gel retardation assay was performed on the 0.5 M fraction from the heparin-agarose column. Oligonucleotides E (lanes 1 and 2), D (lanes 3 and 4), and C (lanes 5 and 6) were labeled as probes. Anti-AGP/EBP antibody was added to lanes 2, 4, and 6, while preimmune serum was added to lanes 1, 3, and 5 after the binding reaction. Arrowheads indicate the major DNA-protein complexes that were supershifted.

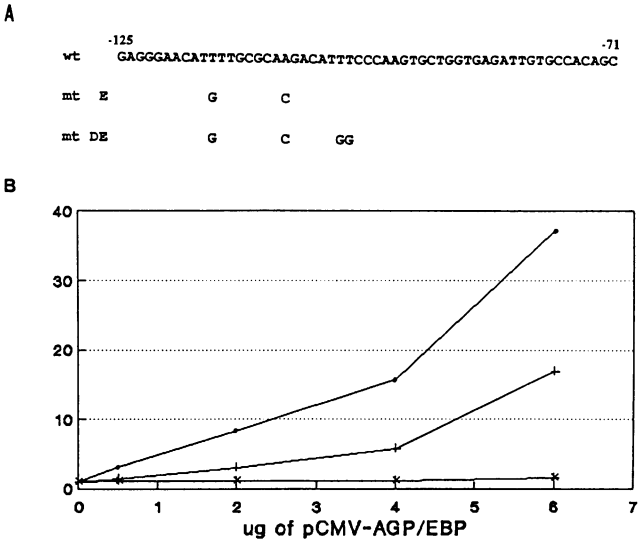


FIG. 3. Functional analysis of the A, C, D, and E motifs. (A) Partial sequences of the wild-type (wt) AGP promoter [pAGP(wt) CAT] and mutants. Only mutated nucleotides are shown for mutants. mtE contains two nucleotide changes at site E. Mutants of site B contain triple nucleotide changes at various positions. Deletion mutant CDE contains nucleotides −71 to +10 of the AGP promoter. (B) Cotransfection assay (in BHK cells) of the wild type and mutants with AGP/EBP expression vector pCMV-AGP/EBP. The *x* axis indicates the amounts of pCMV-AGP/EBP used, while the *y* axis indicates the percentage of chloramphenicol conversion. AGP/CAT (●) responds to AGP/EBP much better than do mtE/CAT (+) and CDE/CAT (×).

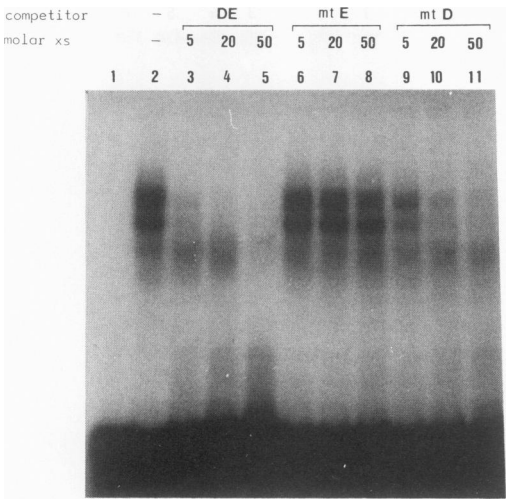


FIG. 4. Relative efficiency of competition of factors that bind to regions D and E by DE, E, and D sequences. End-labeled oligonucleotide DE (5′-GATCGAACATTTTGCGCAAGACATTTCCCAA GTGC-3′) was incubated with no protein (lane 1) and with 4 μg of the 0.5 M NaCl fraction from the heparin-agarose column in the presence of no competitor (lane 2) and of oligonucleotides DE, mtE (5′-GATCGAACATTTTGCGCCCGACATTTCCCAAGTGC-3′), and mtD (5′-GATCGAACATTTTGCGCAAGACACCCCCCAAGT GC-3′) (molar excess is indicated) in a gel retardation assay. It is evident that factors bind to the D and E motifs cooperatively.

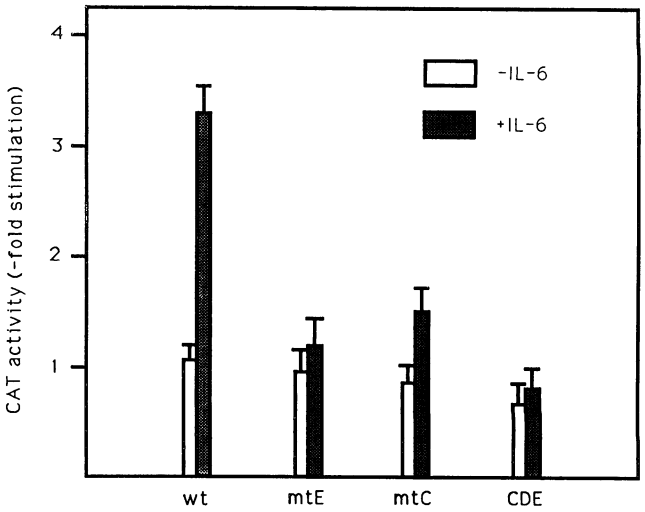


FIG. 5. Functional roles of the C, D, and E motifs in the acute-phase induction of the AGP gene. The AGP promoters of the wild-type (wt), mtE, mtC, and CDE constructs were ligated to the CAT reporter gene. These plasmids were transfected into HepG2 cells. At 24 h posttransfection, 500 U of human IL-6 per ml was added. The cells were harvested 48 h posttransfection, and CAT activity was determined. IL-6 stimulation is shown.

struct CDE). However, because it retained the A element, CDE still showed low levels of response. This finding suggests that sites A, C, D, and E in the AGP promoter region function positively. Furthermore, these motifs respond positively to the overexpression of AGP/EBP. However, other AGP/EBP-like factors (e.g., C/EBP) may also be involved in transactivation of the AGP gene through binding to these sites (see Discussion).

The dose-response curve of CAT activity to pCMV-AGP/EBP in the cotransfection assay suggested that these sites functioned cooperatively. The highest response (roughly 75- to 78-fold above the basal level for the wild-type sequence) was achieved with approximately 6 μg of pCMV-AGP/EBP. When the level of pCMV-AGP/EBP was increased to 8 μg, CAT activity decreased (data not shown). To confirm the cooperative effect, oligonucleotides mtE and mtD were synthesized (see legend to Fig. 4) and used for competition in a gel retardation assay (Fig. 4). Nearly complete competition by a 20-fold molar excess of oligonucleotide DE was observed (Fig. 4, lane 5). In contrast, a 50-fold molar excess of oligonucleotide mtE as a competitor (lane 11) and a greater than 50-fold molar excess of oligonucleotide mtE (lane 8) were required for a comparable degree of competition. These results demonstrate that significant decreases in binding affinities are produced by each motif, thus supporting our theory that AGP/EBP binds cooperatively to these elements. To further address the functional roles of the C, D, and E motifs in acute-phase induction of the AGP gene, wild-type, mtE, mtC, and CDE AGP promoter sequences were linked to the CAT reporter gene and used in a transfection assay in the absence and presence of IL-6. As shown in Fig. 5, the wild-type AGP promoter responds to IL-6 stimulation quite well, while the mutants respond poorly or not at all. These results also show that site E is more important than site C in the response to IL-6.

Negative factors interact with the B region. To study the functional activity of the B region, oligomeric oligonucleotide B (10 copies of oligonucleotide B) and an AGP promoter sequence containing mutants of the B region (created by site-directed mutagenesis and by deletion) were linked to the CAT reporter gene. As shown in Fig. 6A, CAT activity of the pCAT-promoter-containing oligomeric B was much lower than that of the control (pCAT-promoter) in HepG2, Chinese hamster lung, and BHK cells, suggesting that B is a negative element. This conclusion is further strengthened by the finding that mutant B had much higher CAT activity (7- to 15-fold) than did the wild-type construct (Fig. 6B and C).

The results of DNase I footprinting analysis indicated that some nuclear factors should bind to the B region. We have further characterized the B factor(s). The sequence-binding specificity, tissue or cell distribution, heat stability, and chromatographic properties of the nuclear factor(s) derived from rat liver that bind to the B element were studied in a gel retardation assay (Fig. 7 and 8). Figure 7A shows that the liver nuclear factor binds the B sequence in a dose-dependent manner. The binding is specific for the B element, as shown by competition by oligonucleotide B but not by oligonucleotide GCF (39). However, oligonucleotide Sp-1 competes partially (Fig. 7A, lanes 5 and 6). The nuclear factors that bind to the B element appear to exist in three complexes, a, b, and c (Fig. 7B, lanes 1, 2, 4, and 6; Fig. 8A, lanes 1 and 5). Although the gel shift patterns of HeLa (Fig. 7B, lane 3) and BHK (Fig. 7B, lane 5) cells appear to be different from liver and kidney patterns, they do have some common bands; the nature of these differences and similarities remains to be investigated. The factors that bind to the B element can be separated from AGP/EBP with a heparin-agarose column (Fig. 8A). Band a appeared in the 0.3 M NaCl fraction, while bands b and c appeared in the 0.4 M fraction (Fig. 8A, lanes 3 and 4). Moreover, the nuclear factor eluted in the 0.3 M NaCl fraction is not specific for the B element, as demonstrated by its competition for binding by oligonucleotides Sp-1, GCF, and B (Fig. 8B). In contrast, the

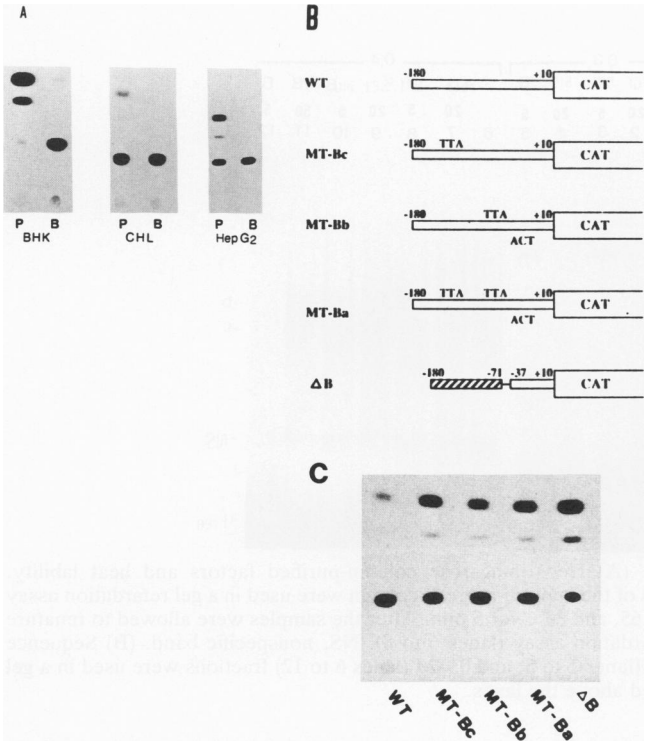


FIG. 6. Functional analysis of the B element. (A) Effect of oligomerized B elements on the simian virus 40 promoter. CAT activities in BHK, Chinese hamster lung (CHL), and HepG2 cells are shown. P, pCAT-promoter (Promega) control; B, B10-pCAT-promoter, which contains 10 copies of synthetic oligonucleotide B in front of the simian virus 40 promoter in pCAT-promoter. (B) Mutants created by site-directed mutagenesis. Detailed sequences of these constructs (from −70 to −32) are as follows: wild type (WT), 5′-CTCTACTGTCCCTGGCTTCAGTCCCATGCCCTCCCC ACAT-3′; MT-Ba, 5′-CTCTACTTTACCTGGCTTCATTACCAT ACTCTCCCCACAT-3′; MT-Bb, 5′-CTCTACTGTCCCTGGCTT CATTACCATACTCTCCCCACAT3′; and MT-Bc, 5′CTCTACTTT ACCTGGCTTCAGTCCCATGCCCTCCCCACAT-3′. The deletion mutant has an internal deletion from −70 to −38. (C) CAT activity of each construct.

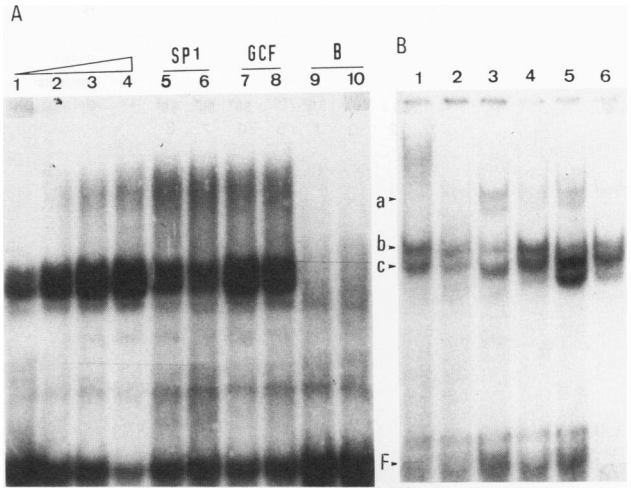


FIG. 7. Biochemical analysis of the factor that bind to B elements. (A) Sequence specificity of the factor that binds to the B element determined by a gel retardation assay. Oligonucleotide B was labeled as the probe. Lanes 1 to 3 contain 0.5, 1, and 2 μg of liver nuclear extract; lanes 4 to 10 contain 4 μg of liver nuclear extract; lanes 5, 7, and 9 contain 20 ng of the indicated competitors; lanes 6, 8, and 10 contain 50 ng of the indicated competitors. (B) Tissue distribution of B factors. Three micrograms of nuclear extracts from different tissues was applied to each lane. Lanes: 1, liver; 2, kidney; 3, HeLa cells; 4, p388D1; 5, BHK cells; 6, 129 P (mouse hepatoma cells). b and c indicate the major complexes. F, free DNA.

nuclear factor eluted in the 0.4 M NaCl fraction appears to be specific for the B element. Figure 8B shows that GCF, Sp-1, and mtB compete for the factor (0.4 M NaCl fraction) much more inefficiently than does the wild-type B element. We conclude that factor B eluted in the 0.4 M fraction is specific for the B element and is distinct from GCF and Sp-1. Furthermore, the B factor is relatively heat stable at 80°C for 7 min and retains some activity after treatment at 80°C (Fig. 8A, lane 8). Heat stability was also demonstrated by footprinting assays using crude or partially purified B factor (data not shown).

The heparin-agarose column-purified B factor (i.e., 0.4 M fraction) was further purified by using oligonucleotide affinity columns (see Materials and Methods for the B sequence). To exclude the AGP/EBP coeluted in the 0.4 M fraction of the heparin-agarose column, the sample was passed through the oligonucleotide C (see Materials and Methods) affinity column. The flowthrough fraction was then chromatographed on an oligonucleotide B affinity column. In a DNase I footprinting assay (Fig. 9A), the binding activity of the affinity column-purified B factor was shown to be similar to that of the factor from a heparin-agarose column. Furthermore, the B factors from the crude nuclear extract, heparin-agarose fraction, and oligonucleotide affinity column fraction were analyzed by Southwestern assay. The molecular size of the B factor was estimated to be 50 kDa (Fig. 9B). To further demonstrate the specificity of the 50-kDa protein, oligonucleotides B* and mtB* were used as probes for Southwestern assays. As shown in Fig. 10, mtB* could probe a polypeptide of approximately 60 kDa in crude and partially purified proteins, while B* could probe a 50-kDa polypeptide in the affinity column-purified protein. These results clearly show that only B* could bind specifically to the 50-kDa polypeptide.

Having addressed sequence specificity, chromatographic properties, heat stability, and molecular size, we examined the relative binding activity of the B factor derived from rat liver before and during an acute-phase reaction. Four hours after LPS treatment, binding of the B factor in rat liver nuclear extracts to the B element was studied in a gel retardation assay. The binding activity of AGP/EBP in these nuclear extracts was used as a positive control and was demonstrated by Southwestern blotting (Fig. 11A). As expected, AGP/EBP binding activity increased by severalfold during an acute-phase reaction (Fig. 11A). To demonstrate that these results were not due to the variations of protein concentration, Coomassie blue staining of the nuclear extract protein from normal liver, LPS-treated liver, and kidney was also performed (Fig. 11C). In contrast to the increased binding activity of AGP/EBP during an acute-phase reaction, binding of the B factor to its cognate sequence was decreased by severalfold (Fig. 11B). In our previous studies, the increase of the AGP/EBP protein during an acute-phase reaction was also demonstrated by Western blotting and immunohistochemical staining (data not shown). Taken together, the data show that the binding

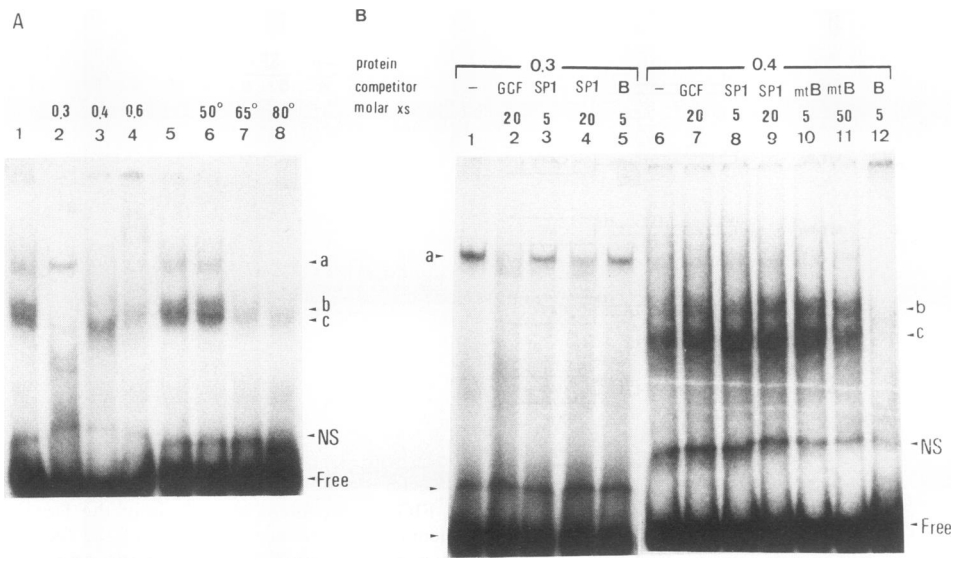


FIG. 8. Characterization of the B factor by gel retardation assay. (A) Heparin-agarose column-purified factors and heat lability. Four-microgram amounts of protein from the 0.3, 0.4, and 0.6 M fractions of the heparin-agarose column were used in a gel retardation assay (lanes 2 to 4). Heat sensitivity was assayed by heating the samples at 50, 65, and 80°C for 5 min. After the samples were allowed to renature at room temperature for 30 min, 4 μg of protein was used in a gel retardation assay (lanes 6 to 8). NS, nonspecific band. (B) Sequence specificity of the 0.3 and 0.4 M fractions. Proteins (4 μg) from the 0.3 M (lanes 1 to 5) and 0.4 M (lanes 6 to 12) fractions were used in a gel retardation assay. Competitors and the molar excesses used are indicated above the lanes.

activity of the B factor decreases substantially during an acute-phase reaction, in direct contrast to the increase of the positive factor, AGP/EBP.

The core sequence of the B element is highly conserved among a number of genes encoding acute-phase reactants, such as haptoglobin (50), C-reactive protein (CRP) (2), and angiotensinogen (49), as well as the highly regulated renin gene (11, 48). These sequences are summarized in Fig. 12. The negative effect of those elements has been confirmed for the haptoglobin (50) and renin (48) promoters by deletion analysis, which suggests that these elements are important for the regulation of genes during an acute-phase reaction.

DISCUSSION

AGP/EBP is involved in regulating expression of the AGP gene. The expression of AGP is limited to liver cells and some hepatoma cells (7) stimulated with IL-1, while the expression of AGP/EBP is constitutive in those cells and other nonhepatocyte cells. Clearly, AGP/EBP is not the only factor responsible for AGP expression. AGP/EBP binding sites appear four times in the AGP promoter (within the 180 bp in the 5′-flanking region). This tandem array of binding sites has been reported for C/EBP-like factors in multiple-drug resistance genes (35) and other genes (65), as well as for factors such as Ap-1 and Sp-1 in many other genes. Although in most cases the role of the multiplicity of elements involved in transcription is not understood, these elements may contribute to the inducibility of highly regulated genes (e.g., acute-phase-responsive genes). During an acute-phase response, the levels of C/EBP (36) and DBP (46) are down-regulated, while that of AGP/EBP (and the rat homolog LAP) is up-regulated; hence, it is likely that AGP/EBP plays a more critical role in AGP expression than does C/EBP or DBP. Whether the same is true under normal physiological conditions remains to be determined. We can speculate that C/EBP or C/EBP-like factors in conjunction with AGP/EBP play a more important role under these conditions. Both AGP/EBP and C/EBP or C/EBP-like homodimers and their heterodimers may be functional in regulating AGP gene expression. These four motifs may be recognized by different homodimers or heterodimers. This issue remains to be resolved. Upon LPS stimulation, the levels of AGP/EBP were elevated two- to threefold in liver, but the levels of C/EBP decreased (36). As a result, C/EBP may not contribute to the LPS induction of AGP. However, cotransfection experiments establish that C/EBP could transactivate the AGP promoter. Therefore, C/EBP may regulate AGP expression under normal physiological conditions.

Footprinting data obtained for recombinant AGP/EBP and rat liver nuclear AGP/EBP suggest that qualitative differences exist. The G residue at position −88 (AGP promoter) was exposed to nucleophilic attack by recombinant AGP/EBP but not by nuclear AGP/EBP; this finding may reflect binding by the homodimer of AGP/EBP (recombinant AGP/EBP) versus the heterodimer, which may exist in nuclear AGP/EBP. It may also be the result of binding by factors other than AGP/EBP (e.g., C/EBP or C/EBP-like factors), although some posttranslational modification of AGP/EBP may also be responsible. These theories can be resolved by future studies.

Recently, it was found that both the transcriptional activator protein LAP and the transcriptional inhibitory protein LIP are translated from the same mRNA (22). A moderate increase in the LAP/LIP ratio results in a significantly high transcriptional activation of the appropriate gene. We have found that the AGP/EBP protein in mouse liver is regulated in the same manner as is the LAP protein in rat liver. However, upon LPS induction, it is very clear that only the level of AGP/EBP increased (14a). LIP could not be detected in other tissues (e.g., kidney) regardless of the physiological conditions (LPS stimulated or untreated). Although there is only a moderate increase in the ratio of the AGP/EBP to LIP in liver during an acute-phase reaction, the resulting increase

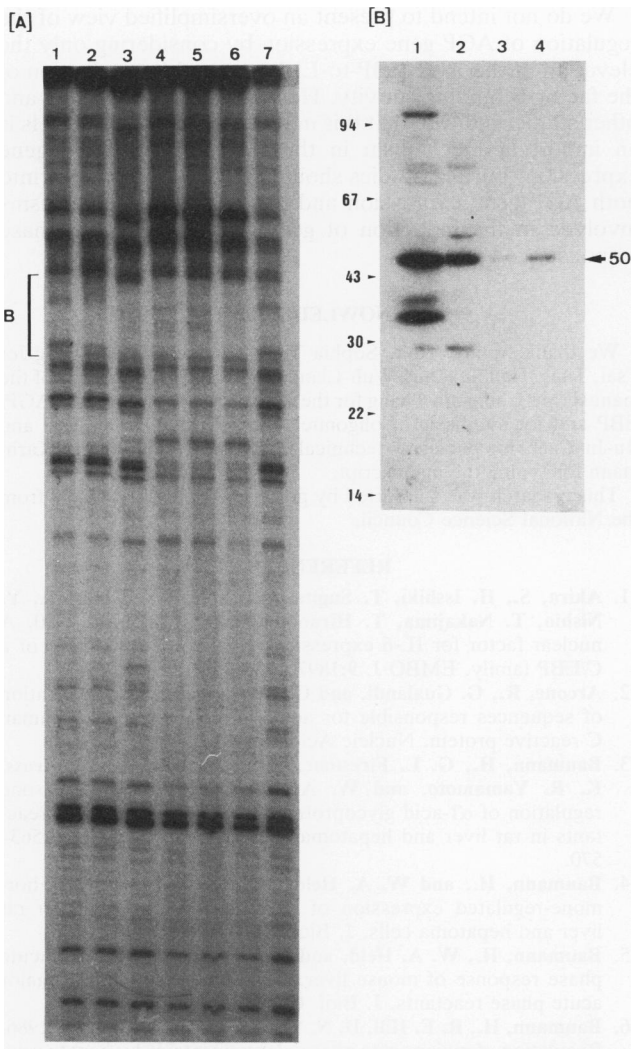


FIG. 9. Characterization of oligonucleotide affinity column-purified factor B. (A) DNase I footprinting analysis of purified factor B. An AGP probe (−180 to +10 fragment labeled at the 5′ end) was used for footprinting analysis. Lanes: 1 and 7, no protein factor; 2, 10 μg of crude liver nuclear extract; 3, 10 μg of the 0.4 M NaCl fraction from the heparin-agarose column; 4 to 6, 10 μl of the oligonucleotide affinity column fractions. The factor B protected region is indicated by a bracket. (B) Estimation of the molecular size of affinity column-purified B factor by Southwestern blot analysis. Oligonucleotide B (see Materials and Methods) was labeled and used as a probe. Lanes: 1, 30 μg of rat liver nuclear extract; 2, 3 μg of the 0.4 M NaCl fraction from the heparin-agarose column; 3 and 4, 10 and 20 μl, respectively, of affinity column-purified B factor. Molecular sizes are indicated in kilodaltons.

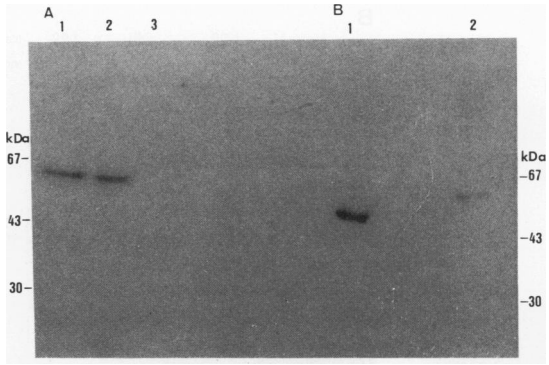


FIG. 10. (A) Southwestern blot analysis of the crude nuclear extract (lane 1), the 0.4 M NaCl fraction from heparin-agarose column (lane 2), and affinity column-purified proteins (lane 3). Mutated oligonucleotide B was used as a probe. A protein of approximately 60 kDa was detected. (B) Southwestern blot analysis of affinity column-purified factor B. Oligonucleotides B* (lane 1) and mtB* (lane 2) were used as probes. A protein of 50 kDa was detected by the B* probe, while a band corresponding to 60 kDa was detected by the mtB* probe.

in AGP gene transcription is quite dramatic. Furthermore, during postnatal development in the liver, the LAP/LIP protein ratio increases significantly from 3 at birth to 15 in the adult; thus, the production of LIP is a dynamic and developmentally regulated process (22). How the ratio of LAP to LIP (AGP/EBP to LIP) is up-regulated during an acute-phase response remains to be investigated.

Negative factors bind to the B element of the AGP gene. Among the numerous negative control factors that have been identified are beta interferon (31), c-Fos (56), c-Myc (54), α-fetoprotein (47), lysozyme (59), Igκ and IgH (51), the adeno-associated virus P5 promoter (58), the long terminal repeat of Molony murine leukemia virus (MuLV) (28), and the GC-rich sequences present in the epidermal growth factor receptor (EGFR) and the beta-actin and calcium-dependent protease (CANP) promoters (34). The factors that bind to some of these sequences have been identified, and their genes have been cloned (51). The factor (GCF) that binds to the consensus sequence, 5′-NNGCGGGGCN-3′, in EGFR, CANP, and the beta-actin promoter can repress the corresponding gene expression both in vitro and in vivo (58). In contrast to GCF, Sp-1 is a well-characterized transcription factor that binds to GC boxes and stimulates transcription from promoters that contain those sites (26). An activator, ETF, also binds to GC-rich sequences, including GC boxes (38). It is likely that various factors with different functions interact with the same or similar sequences to control gene expression in a flexible manner.

The long terminal repeat of MuLV contains the upstream conserved region (UCR). The UCR core sequence, CGC CATTTT, binds a ubiquitous nuclear factor and mediates negative regulation of MuLV promoter activity. The cDNA of the UCR-binding protein has been cloned and has been shown to be identical to NF-E1 (51) and YY-1 (58). These results indicate that the UCR-binding protein, NF-E1, and YY-1 have unusually diverse DNA-binding specificities and are likely to regulate the expression of many different genes.

The consensus sequence, 5′-NTGYCCCNN-3′, recognized by factor B appeared to be different from the sequences recognized by other known DNA-binding factors. The sequence is also found in the promoters of the haptoglobin (50), CRP (2), and angiotensinogen (49) genes and in the promoter of the highly regulated renin gene (11) (Fig. 12). It has been shown that the sequence (or a similar sequence) recognized by factor B is a negative element in AGP, CRP, and renin. A negative control element (i.e., sequence similar to that of the factor B-binding element) may be responsible for modulating the basal levels of renin gene expression (11, 18). The mechanism of this negative regulation remains to be studied.

One of the most important observations in this study is that the binding activity of factor B is reduced severalfold in the liver following LPS treatment. Therefore, we infer that

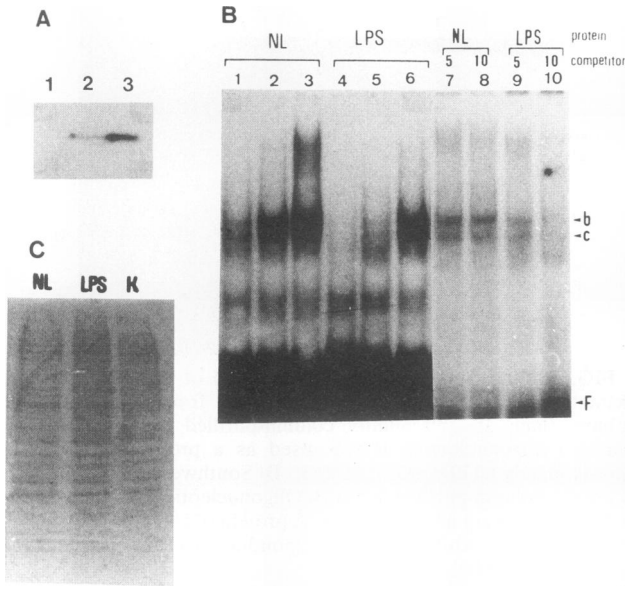


FIG. 11. Binding activity of factor B in nuclear extract derived from normal liver and LPS-treated rat liver. (A) Southwestern blot analysis shows the increase of AGP/EBP binding activity in the LPS-treated liver nuclear extract. The blot was probed with probe C. Lanes 1, 2, and 3 contain kidney, normal liver, and LPS-treated liver nuclear extracts, respectively. (B) Gel retardation assay. Nuclear extracts from normal liver (NL; lanes 1 to 3, 7, and 8) and LPS-treated liver (LPS; lanes 4 to 6, 9, and 10) were used in a gel retardation assay. Oligonucleotide B was labeled as a probe. Lanes 1 to 3 and 5 to 6 contain 1, 2, and 4 μg of nuclear extract; lanes 7 to 10 contain 2 μg of nuclear extract. The amounts of competitor B are indicated above lanes 7 to 10. Lanes 7 to 10 were exposed for 6 h at −70°C with an intensifying screen; lanes 1 to 6 were exposed overnight. (C) SDS-PAGE of nuclear proteins from normal liver (NL), LPS-treated liver (LPS), and kidney (K). A 30-μg sample of each extract was electrophoresed on an SDS–12.5% gel and stained with Coomassie blue. The similar patterns of major nuclear proteins suggest that the three extracts used are of similar overall quality and quantity.

factor B is one of the key factors in regulating AGP gene expression during an acute-phase response. It also serves as a key homeostatic factor under normal physiological conditions.

AGP	-66	C T G T C C C T G	-58
	-46	A T G C C C C T C	-38
	-53	C A G T C C C A T	-45
Hp/Hpr	-12	A T G C C C C A C	-4
	+2	C T G C T C T T C	+10
CRP	-94	C T G C C C C A A	-86
Renin	-320	T T G T C C C A G	-312
	-291	C T G T C C T T C	-283
	-171	C T G C T C C C C	-164

T8 C5 C7 C7
C6 G T4 C T2 T2
A1

FIG. 12. Presence of similar B-motif sequences in different genes. Hp/Hpr, haptoglobin.

We do not intend to present an oversimplified view of the regulation of AGP gene expression by considering only the elevation of the AGP/EBP-to-LIP ratio and the reduction of the factor B-binding activity. However, the data of this and other studies indicate that this modulation of protein levels is an important mechanism in the regulation of AGP gene expression. Further studies should provide more insight into both AGP gene expression and the molecular mechanisms involved in the induction of genes during an acute-phase response.

ACKNOWLEDGMENTS

We thank Sunny Yum, Sophia Tsai, George Bolton, Ming-Jer Tsai, Tung-Tien Sun, and Wuh-Liang Hwu for critical reading of the manuscript, Ching-Jin Chang for the expression vector pCMV-AGP/EBP and for synthesis of oligonucleotides, Miaw-Sheue Tsai and Ru-Ju Chen for excellent technical assistance, and Joanne Karhmann for typing the manuscript.

This research was supported by grant NSC80-0412-B002-09 from the National Science Council.

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