Interaction of Acute-Phase-Inducible and Liver-Enriched Nuclear Factors with the Promoter Region of the Mouse α_1-Acid Glycoprotein Gene-1†

Tawfiq Alam and John Papaconstantinou*

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, and the Shriners Burns Institute, Galveston Unit, Galveston, Texas 77550

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ABSTRACT: The synthesis and secretion of several acute-phase proteins increases markedly following physiological stress. α_1-Acid glycoprotein (AGP), a major acute-phase reactant made by the liver, is strongly induced by inflammatory agents such as lipopolysaccharide (LPS). Nuclear run-on assay showed a 17-fold increase in the rate of AGP transcription 4 h following LPS injection. DNase I footprinting assays revealed multiple protein binding domains in the mouse AGP-1 promoter region. Region B (−104 to −91) is protected by a liver-enriched transcription factor that is heat labile and in limiting quantity. An adjacent region, C (−125 to −104), is well-protected by nuclear extracts from hepatocytes. Electrophoretic mobility shift assays indicated that only one DNA–protein complex can form with an oligonucleotide corresponding to region B. However, nuclear proteins from untreated mouse liver can form three strong complexes (C1, C2, and C3) and a weak one (C4) with oligonucleotide C. An acute-phase-inducible DNA-binding protein (AP-DBP) forms complex 4. A dramatic increase (over 11-fold) in AP-DBP binding activity is seen with nuclear proteins from LPS-stimulated animals. Interestingly, AP-DBP, a heat-stable factor, can form heterodimers with the transcription factor CCAAT/enhancer binding protein (C/EBP). Furthermore, purified C/EBP also binds avidly to region C. Our studies indicate that several liver-enriched nuclear factors can interact with AGP-1 promoter and that AP-DBP binds to the AGP-1 promoter with high affinity only during the acute-phase induction.

Following a systemic injury, such as acute inflammation, bacterial infection, major surgery, metal toxicity, burn, or the development of certain forms of cancer, the liver responds with a striking increase or decrease in the synthesis of a subset of serum proteins, named the acute-phase reactants (Koj, 1974; Kushner, 1982). Currently it is thought that the acute-phase response to pathological insult primarily involves maintaining physiological homeostasis (Fey & Fuller, 1987). The changes in hepatic protein synthesis may represent an early defense mechanism.

In humans as well as other mammals, α_1-acid glycoprotein (AGP),[1] also known as orosomucoid, becomes one of the most abundant serum proteins made by the liver during the acute-phase response (Koj, 1974). AGP is a 44-kDa protein with an unusually high carbohydrate content, accounting for about 40% of its molecular weight. Recently we cloned the mouse AGP-1 gene and have shown that its expression rises dramatically following induction by agents such as turpentine, bacterial LPS, cytokines, glucocorticoids, and heavy metals (Cooper & Papaconstantinou, 1986; Copper et al., 1987; Carter et al., 1989a; Yiangou et al., 1991). The major cytokines controlling the expression of AGP in cultured hepatoma cell lines are IL-1, IL-6, and TNFα (Darlington et al., 1986; Baumann et al., 1987; Carter et al., 1989a,b). Also, there is evidence that glucocorticoids, alone or synergistically with cytokines, can increase the transcription of the AGP gene (Baumann & Maquat, 1986; Carter et al., 1989a). Transfection of L cells with a hybrid DNA construct containing the promoter region of rat AGP and chloramphenicol acetyltransferase reporter gene indicated that a cis element located between −120 and −42 is responsive to glucocorticoids (Baumann & Maquat, 1986). The glucocorticoid induction of AGP in rat hepatoma cells is diminished by inhibiting protein synthesis with cycloheximide, suggesting that the hormone may activate the synthesis of a regulatory factor that acts in trans to induce the AGP gene. Alternatively, a labile nuclear protein may act in concert with the glucocorticoid receptor on the AGP promoter (Klein et al., 1987, 1988).

We are interested in defining the interaction of nuclear factors with the AGP promoter that leads to the acute-phase-inducible and liver-specific expression of AGP. The acute-phase responsive elements (APRE) of a few other genes have been described. The angiotensinogen gene APRE binds to the cytokine-inducible NFκB protein (Brasier et al., 1990; Ron et al., 1990). A member of the C/EBP-family of nuclear proteins, IL6-DBP, has been shown to bind to the IL-6-responsive element of two acute-phase genes, hemopexin and haptoglobin (Oliviero & Cortese, 1989; Poli & Cortese, 1989; Poli et al., 1990). Another C/EBP-like protein, NF-IL6, binds to the IL-1-responsive element of the IL-6 gene; consequently, the expression of IL-6, which participates in host defense, rises rapidly during viral or bacterial infection (Akira et al., 1990; Isshiki et al., 1990).

In this study, we have conducted experiments designed to characterize the trans-acting protein factors that bind to the mouse AGP-1 promoter. We show that several liver-enriched nuclear factors can interact with the AGP promoter. Inter-

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* Corresponding author.

[1] Abbreviations: AGP, α_1-acid glycoprotein; APRE, acute-phase responsive element; AP-DBP, acute-phase-inducible DNA binding protein; IL, interleukin; IL-6DBP, interleukin-6-dependent DNA-binding protein; C/EBP, CCAAT/enhancer binding protein; NF-IL6, nuclear factor interleukin-6; NFκB, nuclear factor κB; TNF, tumor necrosis factor; GRE, glucocorticoid responsive element; EMSA, electrophoretic mobility shift assay; bp, base pairs; kb, kilobase pairs; PMSF, phenylmethanesulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LPS, lipopolysaccharide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MoCM, monocyte conditioned medium.

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estingly, a nuclear protein, capable of forming heterodimers with the transcription factor C/EBP, binds to the AGP-1 promoter with high affinity only during the acute-phase induction.

Experimental Procedures

Animals. Two-month-old male BALB/c mice were obtained from Charles River Laboratories. All animals were maintained in rooms with a 12-h light cycle and fed standard chow diets. LPS was injected intraperitoneally in pyrogen-free saline.

Nuclear Run-On Transcription. To determine the rate of transcription following LPS injection, nuclei from livers were isolated through a sucrose gradient at 4 °C (Lamers et al., 1982). The nuclear pellet was washed in cold nuclear storage buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM $MgCl_2$, and 40% glycerol). In vitro run-on transcription was carried out using 1×10^7 nuclei and 200 μCi of [^{32}P]UTP per assay at 30 °C for 45 min. The transcription reaction was stopped with DNase I and proteinase K. Following purification, a total of 1×10^7 cpm of elongated nascent RNA per assay was hybridized to a variety of denatured single-stranded cDNAs immobilized on nitrocellulose filters. The filters were washed with 2× SSC (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate) at 65 °C for 1 h and with 2× SSC and 10 μg/mL RNase A at 37 °C for 30 min.

Isolation and Analysis of RNA. Total RNA from liver, kidney, and brain was isolated by the method of Chirgwin et al. (1979). Tissues were homogenized in 7.5 M guanidine hydrochloride, 1% 2-mercaptoethanol, and 25 mM sodium citrate followed by ultracentrifugation through a 5.7 M CsCl cushion. Northern blotting was done as described previously (Carter et al., 1989b). Filters were probed with ^{32}P-labeled cDNA in hybridization buffer containing 50% formamide, 4× SSC, 0.2 mg/mL sheared salmon sperm DNA, 4× Denhardt's solution, 0.1% sodium dodecyl sulfate, and 50 mM $NaPO_4$ buffer, pH 6.5. After hybridization, filters were washed three times for 15 min at 22 °C in 2× SSC and 0.1% sodium dodecyl sulfate and then three times for 45 min at 60 °C in 0.1× SSC and 0.1% sodium dodecyl sulfate.

Plasmid Construction. A *Bam*HI/*Ban*I 613-bp fragment of the mouse AGP-1 gene extending from −595 to +18 was isolated from a Charon 28 λ genomic clone (Cooper et al., 1987), blunt-ended by treatment with the Klenow fragment of DNA polymerase I and deoxynucleotide triphosphates, and subcloned into the *Hin*cII site of pUC18. The fragment was removed by digestion with *Bam*HI and *Hin*dIII, followed by digestion with *Nhe*I, which cleaves at −229. Both fragments were subcloned individually into the *Pst*I site of the pT7/T3-19 vector (Bethesda Research Laboratories). The DNA fragments were end-labeled at the *Eco*RI or *Hin*dIII sites and gel-purified.

Preparation of Nuclear Extracts. Liver, kidney, and brain nuclear extracts from control and LPS-injected mice were prepared according to Gorski et al. (1986) and as modified by Zhang et al. (1990). Briefly, fresh tissues were homogenized at 4 °C in buffer A (0.25 M sucrose, 15 mM Tris-HCl, pH 7.9, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol, 0.4 mM PMSF, 2 mM benzamidine, and 1 μg/mL each of the protease inhibitors antipain, chymostatin, leupeptin, and pepstatin A). After homogenization, 2 volumes of buffer B (buffer A with 2.3 M sucrose) were added, mixed, and layered over a 10-mL cushion of buffer C (buffer A with 1.8 M sucrose) and centrifuged at 25 000 rpm at 4 °C in a SW27 rotor. The nuclear pellets were resuspended in buffer D (100 mM KCl, 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.4 mM PMSF, 2 mM benzamidine, and 1 μg/mL each antipain, chymostatin, leupeptin, and pepstatin A) and extracted with 0.10 volume of 4 M $(NH_4)_2SO_4$ at 4 °C. The suspension was centrifuged at 30 000 rpm for 45 min in an SW40 rotor. The nuclear protein in the supernatant was precipitated by 0.3 g/mL $(NH_4)_2SO_4$ and resuspended in buffer E (20 mM HEPES, pH 7.8, 10% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothrietol, 0.5 mM PMSF, 0.2 mM benzamidine, and 1 μg/mL each antipain, chymostatin, leupeptin, and pepstatin A) and dialyzed against buffer E at 4 °C. The dialysates were microcentrifuged to remove precipitates, and the protein concentration was determined by the method of Bradford (1976). Aliquots of the nuclear protein were frozen in liquid nitrogen and stored at −90 °C.

DNase I Footprinting. DNase I footprint assays (Lichsteiner et al., 1987; Zhang et al., 1990) were performed in a 20-μL reaction mixture containing 10 mM HEPES, pH 7.8, 30 mM KCl, 12% glycerol, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 0.2 mM PMSF. Variable amounts of nuclear proteins were incubated with 4000 ng of unlabeled poly(dI·dC) nonspecific competitor DNA and approximately 1 ng of end-labeled promoter DNA fragments for 60 min on ice. The incubation was followed by a brief digestion for 60 s at room temperature with 0.4–4 μL of freshly diluted DNase I solution (10 μg/mL). The reactions were terminated by the addition of 100 μL of stop buffer (10 mM EDTA, 0.5% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.5, 75 μg/mL sheared salmon sperm DNA, and 100 μg/mL proteinase K). The DNA was extracted with phenol–chloroform and fractionated in a 6% polyacrylamide/7 M urea sequencing gel.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays were performed with double-stranded oligonucleotides radioactively labeled with [^{32}P]ATP and T4 polynucleotide kinase (Fried & Crothers, 1981; Garner & Crothers, 1981). Approximately 1 ng of each of these DNAs was incubated for 20 min at 22 °C with various amounts of nuclear extracts or purified proteins in a 20-μL reaction containing 4000 ng of poly(dI·dC), 25 mM HEPES, pH 7.9, 60 mM KCl, 12% glycerol, 0.1 mM EDTA, 0.75 mM dithiothreitol, and 0.2 mM PMSF. The DNA–protein complexes were resolved on 5% nondenaturing polyacrylamide gels.

Cell Culture, DNA Transfection, and CAT Assays. HepG2 cells were cultured in DMEM–F12 (1:1) supplemented with 10% fetal calf serum.

The SV40 enhancer elements from pSV2CAT vector were removed to give pSV1CAT, which contains only the minimal promoter. A set of synthetic oligonucleotides corresponding to region C was cloned into pSV1CAT. The plasmid AGP-X3 APRE-CAT contained three copies of region C.

Cells were cotransfected by the calcium phosphate precipitation method (Graham & Van der Eb, 1973) with 15 μg (60-mm dishes) of plasmid DNA and 1.5 μg of the β-galactosidase reporter plasmid, pCH110, as internal control. Twenty-four hours after transfection, cells were induced by 10^{-6} M dexamethasone and 50% MoCM from LPS-stimulated peripheral blood monocytes from human donors as described (Darlington et al., 1986). The measurements of CAT and β-galactosidase activities were as described by Izban and Papaconstantinou (1989).

Results

Rate of Transcription of the AGP Gene Increases during the Acute-Phase Response. The increase in steady-state levels

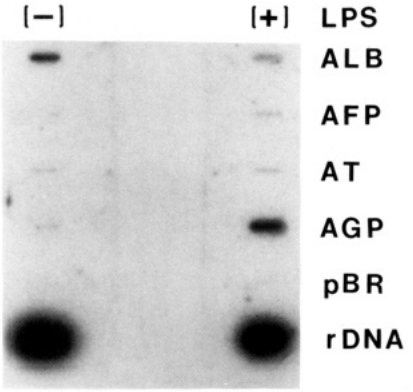

FIGURE 1: Nuclear run-on transcription assay. Two-month-old BALB/c mice (20 g) were injected with 100 μg of LPS. Transcription assays were performed on nuclei isolated from livers. The increase in AGP transcription rate seen at 4 h was estimated to be at least 17-fold. A scanning densitometer (BVI 400 biological analysis system) was used to quantitate the signals on the autoradiograph. The slight decrease in rDNA signal is due to methodology. ALB, albumin; AFP, α-fetoprotein; AT, α_1-antitrypsin; rDNA, ribosomal RNA gene.

of AGP mRNA during the acute-phase response may result from either transcriptional or posttranscriptional mechanisms (Carter et al., 1989a). Nuclear run-on transcription assays were performed to measure the relative level of AGP gene transcription in BALB/c mice. Nuclei isolated from livers were incubated with ^{32}P-labeled ribonucleotides. The elongated nascent RNA was then hybridized to cDNA probes immobilized on nitrocellulose filters. The constitutive AGP transcription is very low in control animals but increases markedly (17-fold) within 4 h following injection with LPS (Figure 1). The rate of AGP transcription in LPS-injected animals peaks at 4 h and subsequently declines rapidly (data not shown). In contrast, the rate of albumin gene transcription decreases in LPS-stimulated animals, confirming that albumin is a negative acute-phase reactant (Darlington et al., 1986).

Localization of Multiple Sites in the Regulatory Region of the AGP-1 Gene Which Bind Liver Nuclear Proteins. DNase I footprint analyses were performed to map the DNA–protein complexes in the 5′-flanking region of the mouse AGP-1 gene. Incubation of two DNA fragments, extending from −229 to +18 and −595 to −229, with liver nuclear extracts from a pool of 10 2-month-old BALB/c mice results in the formation of multiple DNA–protein complexes (Figure 2A). We focused our investigation on regions B and C because these sequences showed the strongest binding with nuclear proteins

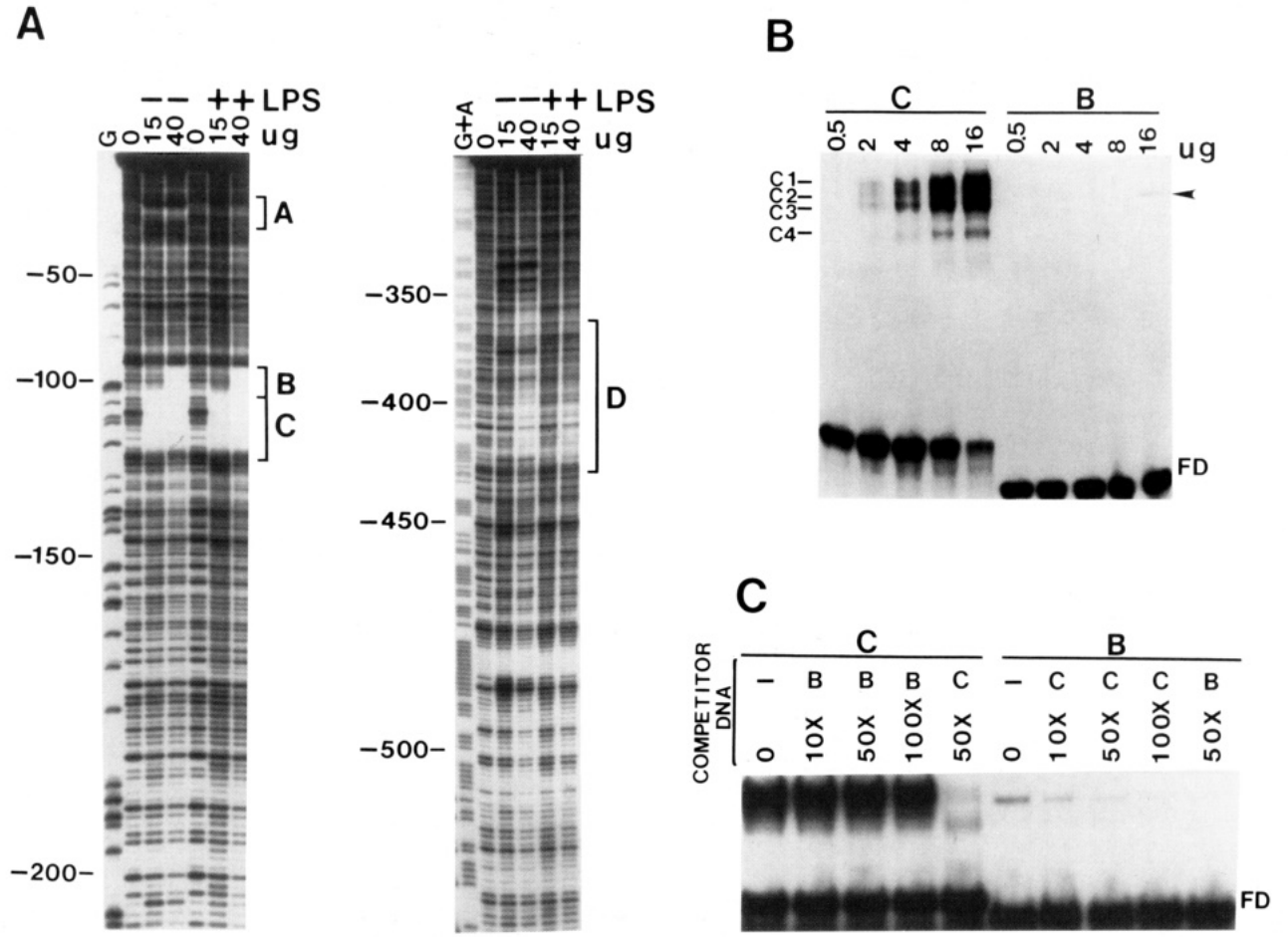

FIGURE 2: DNA-binding assays demonstrating the interaction of liver nuclear proteins with AGP-1 promoter. (Panel A) DNase I footprinting of the AGP promoter was performed using liver nuclear proteins from control or LPS-treated mice. The two fragments of DNA used range from −230 to +18 and from −595 to −230. The noncoding strands are shown. The coding strand showed a similar protection pattern. Labeled DNA fragments were incubated with mouse liver nuclear protein in the presence of 4000 ng of poly(dI·dC). Lanes G and G+A indicate Maxam–Gilbert chemical cleavage reactions of the probe DNA. A, B, C, and D refer to the protected regions. Numbers on the left side indicate nucleotides upstream (−) of the site of transcription initiation. (Panel B) Various concentrations of liver nuclear proteins from LPS-stimulated mice (24 h postinjection) were analyzed by EMSA on native gels. Each reaction contained 4000 ng of nonspecific poly(dI·dC). B and C indicate oligonucleotide probes corresponding to regions B and C, respectively, of the DNase I protection assay. The four DNA–protein complexes are indicated as C1, C2, C3, and C4. The arrowhead indicates the position of the single complex seen with oligonucleotide B. FD, unbound or free DNA. (Panel C) Competition EMSA was performed in the presence of 0, 10×, 50×, or 100× molar excess of unlabeled oligonucleotides B or C. The four bands are not well separated because the gel was run for a much shorter time; moreover, to show the relatively weak band with oligonucleotide B, the autoradiograph was overexposed. All lanes contained 16 μg of liver nuclear protein.

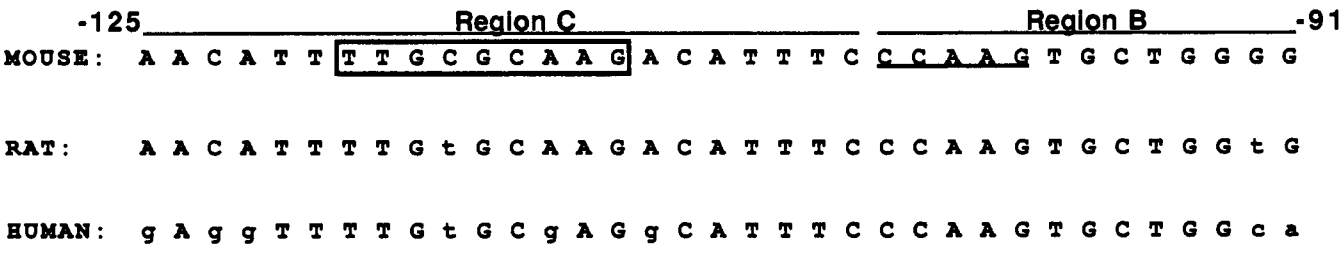

FIGURE 3: Sequence comparison of the 5′-flanking regions of mouse, rat, and human AGP genes. The lines above the sequences indicate regions B and C. The 9-nucleotide sequence identical to the NF-IL6 consensus binding site is enclosed in a box. A putative CCAAT box is underlined. Rat (Reinke & Feigelson, 1985) and human (Dente et al., 1987) nucleotides that diverge from the mouse sequence are denoted by lowercase letters.

and revealed a high degree of homology to the human and rat AGP genes (Figure 3). Additional weak protections from nucleolytic attack are also seen in regions A and D; however, further studies are required to determine if these represent specific protein binding domains. Region B (−104 to −91), containing a CCAAT motif at its 5′-end, is protected only when the protein concentration is increased to 40 μg, suggesting that this factor is in limiting quantity. The juxtaposed region C (−125 to −104) is well-protected even at low protein concentrations. The protection patterns for both coding and noncoding strands were similar. Using a heterologous system, Chang et al. (1990) have shown that rat liver nuclear extract can protect two regions (−85 to −70 and −121 to −94) of the mouse AGP promoter. These differences in the footprint pattern may reflect the properties of species-specific transcription factors.

Liver nuclear extracts were also prepared from 10 BALB/c mice 4 h following injection with LPS. Although the rate of transcription peaks at 4 h, no large changes in footprinting patterns were observed between liver nuclear extracts from control and LPS-injected animals (Figure 2A).

To analyze the protein binding domains of regions B and C in more detail, electrophoretic mobility shift assays (EMSA) were performed with synthetic oligonucleotides corresponding to these two sequences (Figure 3). Liver nuclear extracts produced a single gel-shifted band with labeled oligonucleotide B (Figure 2B). The weak intensity of this band suggests that this protein is in limiting quantity or binds with low affinity; alternatively, the protein binding to region B is an accessory factor, which is dependent on the factors binding to region C. In contrast, three prominent gel-shifted bands (C1, C2, and C3) were formed with labeled oligonucleotide C even when low amounts of nuclear extracts from LPS-treated mice (24 h postinjection) were used. A faster-migrating band (C4) can also be observed. The shifting of four bands with oligonucleotide C may indicate the presence of multiple C-region binding proteins or the possibility of protein–protein interactions. In a similar experiment, Brasier et al. (1990) have shown that several different proteins can bind to a single cis element, the APRE of the angiotensinogen gene.

To determine whether the proteins binding to regions B and C are related, we carried out competition experiments. Increasing amounts of unlabeled oligonucleotide B did not inhibit the four-complex formation with oligonucleotide C; however, cold oligonucleotide C abrogated the formation of the four specific complexes (Figure 2C). In the reciprocal experiment a slight decrease in protein binding to oligonucleotide B is seen when increasing amounts of oligonucleotide C are used as competitor. This minor change may represent nonspecific competition, especially since 50× molar excess of unlabeled oligonucleotide B and 100× molar excess of oligonucleotide C had an identical effect. However, it is possible that C-like molecules bind to oligonucleotide B with low affinity.

Liver-Enriched Transcription Factors Bind to the AGP-1 Promoter. Studies with transgenic animals have shown that sufficient information for tissue-specific and acute-phase regulated expression is contained within the 1.2-kb 5′-flanking DNA and coding region of the human AGP gene (Dente et al., 1988). To examine the tissue-specific binding sites within the context of the AGP-1 promoter sequence, we performed DNase I protection assays with nuclear extracts from liver, where AGP is normally expressed, and two other differentiated tissues (kidney and brain). Region B is protected when liver nuclear protein concentration is increased to 50 μg (Figure 4A). Increasing the protein concentration of nuclear extract from brain has no effect on region B; however, 50 μg of kidney nuclear extract causes a slight increase in the protection of this region. Region C is well-protected with only 15 μg of liver nuclear extract; however, 50 μg of kidney nuclear protein is needed to protect this region. Fifty micrograms of brain nuclear protein also protects region C, albeit with much less efficiency. These observations suggest that the transcription factors binding to region C are liver-enriched.

Since some protection of regions B and C is seen with kidney nuclear protein, we wanted to examine if ectopic expression of AGP is possible in this differentiated tissue. Northern analysis revealed that indeed a low level (25% of control liver) of AGP mRNA is present in the kidney 24 h following injection with LPS (Figure 4B). However, no AGP mRNA was detectable in the brain even after prolonged exposure of the autoradiographs.

Constitutive and Acute-Phase-Inducible Nuclear Factors Bind to Region C. In view of the demonstrated lack of differential footprinting between control and LPS-stimulated nuclear extracts, we asked whether the same proteins bind to region C during the acute-phase response. We performed EMSA with liver nuclear extracts from untreated and LPS-stimulated animals. A dramatic increase (11-fold over control level) in C4 complex is seen with nuclear extracts prepared from mice 4 h following injection with LPS (Figure 5). The protein(s) responsible for complex 4 formation has been named acute-phase-inducible DNA-binding protein (AP-DBP). The level of AP-DBP is extremely low in control animals even when 40 μg of protein was used in the EMSA. On the other hand, the formation of C1 is significantly reduced in LPS-stimulated animals as compared to untreated mice. The data suggest that quantitative differences in binding may exist between the two groups: there is a 5-fold increase in binding to labeled oligonucleotide C by nuclear proteins from the LPS-injected group (Figure 5B). Nuclear run-on data indicated that the peak of transcription is at 4 h postinjection, followed by a decline. A correlative decrease in AP-DBP complex formation is seen in nuclear protein from 12 h post-LPS-treated mice (Figure 5A). At this time point the formation of C3 complex rises significantly. When compared to control protein, the level of AP-DBP binding increases significantly in nuclear extract

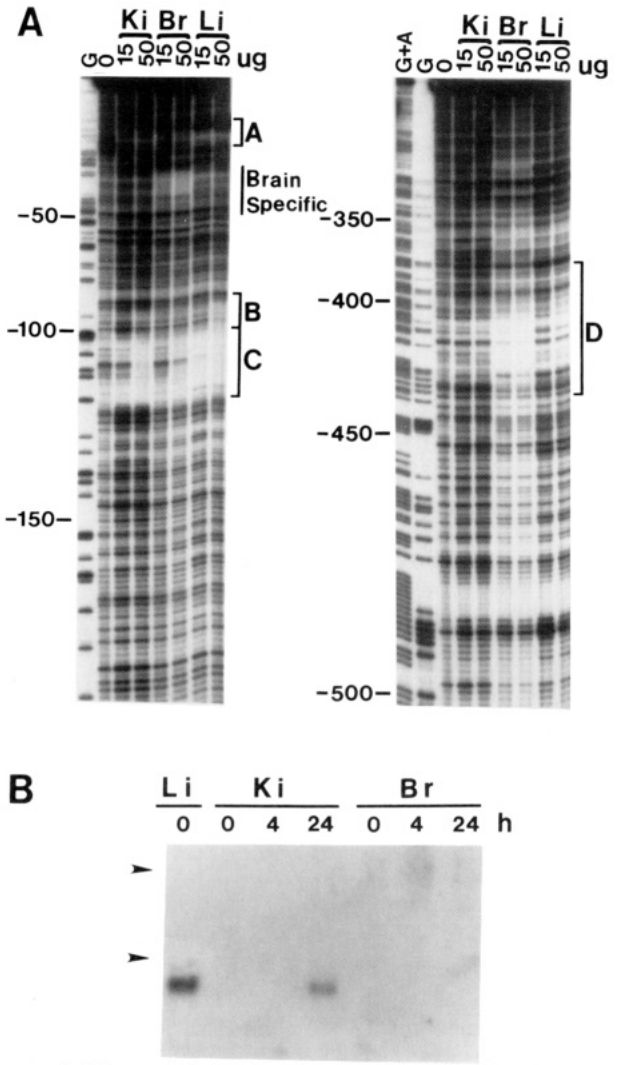

FIGURE 4: Liver-enriched nuclear factors bind to the AGP-1 promoter. (Panel A) DNase I footprints with nuclear extracts from kidney (Ki), brain (Br), and liver (Li). Nuclear protein binding to region B is liver-enriched. Only 15 μg of liver nuclear protein protects region C, whereas with 50 μg, protection is seen with other tissues. A unique region of protection is seen only with brain nuclear protein. All the other labels are the same as in Figure 2. (Panel B) Northern blot analysis of total RNA (20 μg/lane) from liver (Li), kidney (Ki), and brain (Br). AGP mRNA can be detected in the kidney 24 h following LPS injection; however, no AGP expression was detectable in the brain even after longer exposure of the autoradiographs. The arrowheads denote the positions of 28S and 18S rRNA.

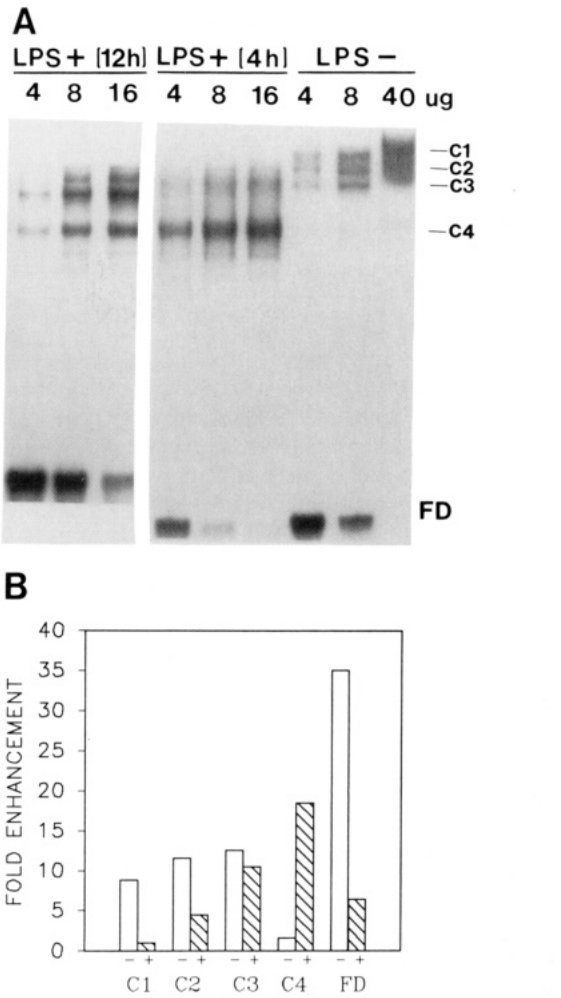

FIGURE 5: Induction of AP-DBP binding during the acute-phase response. (Panel A) Nuclear proteins from control [LPS(−)] and LPS-stimulated (4 h and 12 h postinjection) livers were analyzed by EMSA. Each reaction contained 4000 ng of poly(dI·dC). A sharp increase in AP-DBP (C4) binding is seen 4 h following LPS stimulation. The binding of C3 increases at 12 h postinjection. FD, free DNA. (Panel B) Relative region C binding activities of nuclear factors forming complexes C1, C2, C3, and C4 (AP-DBP). FD indicates free or unbound DNA. The DNA–protein complexes seen with nuclear protein from untreated (−) and 4 h post-LPS-treated (+) mouse livers were quantitated by video densitometric scanning of the autoradiographs with BVI 4000 biological analysis system.

from LPS-stimulated kidney but not brain (data not shown). Thus, AP-DBP represents an acute-phase-inducible complex. Interestingly, region C contains a palindromic motif (TTGCGCAAG) that is identical to the consensus binding site for NF-IL6 ($T^T/_GNNGNAA^G/_T$), a nuclear factor recently cloned from a cDNA library of LPS-activated human monocytes (Akira et al., 1990). Similar to the C4 complex, the constitutive level of NF-IL6, a member of the C/EBP family, is extremely low in the liver and kidney; however, 4 h following stimulation with LPS, IL-1, or IL-6, a rapid and drastic increase in NF-IL6 level was observed (Akira et al., 1990). NF-IL6 and C/EBP recognize the same nucleotide sequences. A putative homologue of human NF-IL6, called IL6-DBP, has been cloned from rat cDNA library. This C/EBP-like protein is also inducible by IL-6 and is known to bind to hemopexin gene IL-6-responsive element, which contains a motif (TTACGAAAT) similar to the NF-IL6 consensus sequence (Poli et al., 1990).

Purified C/EBP and Heat-Stable C/EBP-like Proteins Bind to Region C. To determine if region C is capable of binding to the C/EBP family proteins, EMSA was performed with purified C/EBP (a gift from Dr. Steven L. McKnight). Purified C/EBP binds avidly to the oligonucleotide C probe (Figure 6A). This single band comigrated closely with the C1 complex but had substantially slower mobility than the AP-DBP complex. Moreover, the absence of multiple bands when C/EBP interacts with oligonucleotide C suggests that region C contains a single consensus site for the binding of C/EBP-like transcription factors (Figure 6A). It is possible that the C4 complex is formed due to protein–protein interaction; however, this is highly unlikely because such a complex would migrate more slowly than the C/EBP band.

Heat stability is an important property of the C/EBP family proteins (Landschulz et al., 1988a; Isshiki et al., 1990). Thermal denaturation experiments revealed that the nuclear factor(s) binding to region C are extremely heat stable (Figure 6B). Also, EMSA indicated that treatment of nuclear extracts from control and LPS-stimulated animals up to 100 °C did not significantly reduce the formation of C1, C2, C3, or C4

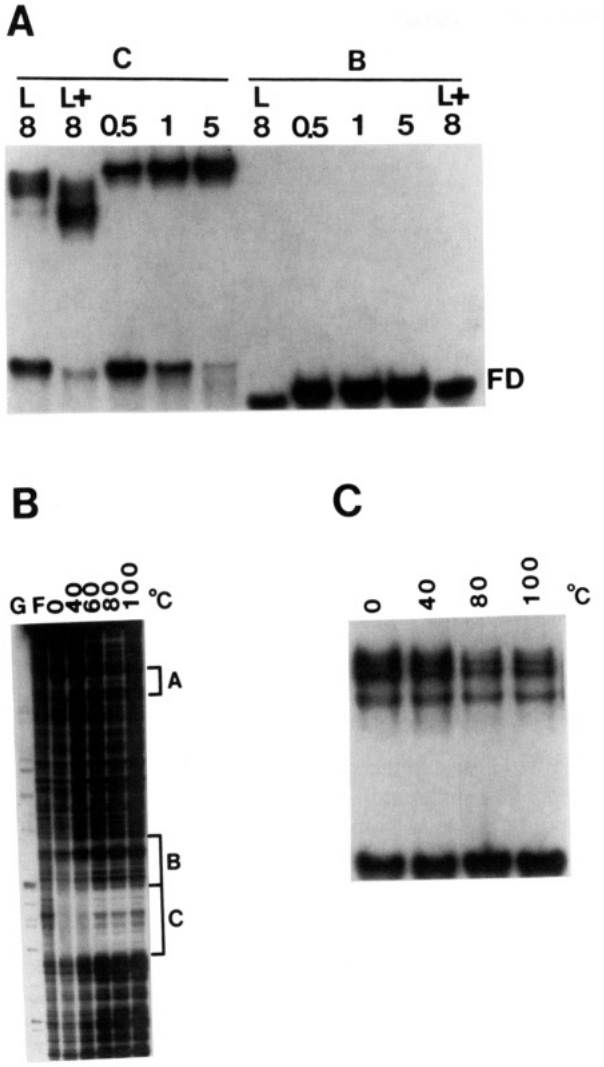

FIGURE 6: Purified C/EBP and heat-stable nuclear factors bind to region C. (Panel A) EMSA showing the binding of full-length 42-kDa C/EBP to oligonucleotide C but not B. The binding reactions were performed in the presence of 0.5, 1, and 5 μL of C/EBP (Landschulz et al., 1988a) or 8 μg of nuclear proteins from control (L) or LPS-stimulated (L+) liver. C, oligonucleotide C; B, oligonucleotide B; FD, free DNA. (Panel B) Effect of temperature on the DNA-binding activities of nuclear proteins from LPS-stimulated liver. After heating for 5 min at the temperatures indicated, the precipitate of the denatured protein was pelleted and the supernatant assayed by DNase I footprinting. Lane F is the DNase I digested DNA probe in the absence of any nuclear protein. All other lanes contained 50 μg of nuclear extract. All the other labels are the same as in Figure 2. (Panel C) Effect of temperature on the formation of C1, C2, C3, and C4 complexes. EMSA was performed with nuclear protein treated at different temperatures.

complexes (Figure 6C). In contrast, the nuclear factor binding to region B is very heat labile since exposure to temperatures above 40 °C completely abolishes its binding activity (Figure 6B). Our data indicate that the proteins interacting with regions C and B are not similar: the protein binding to region B is heat labile and purified C/EBP binds to region C but not B. Thus we have named the protein binding to region B as hepatocyte-enriched labile factor (HLF).

Down-Regulation of C/EBP Expression during the Acute-Phase Response. Because of the demonstrated similarity between C/EBP and the nuclear factors binding to region C, we asked whether C/EBP expression is altered during the acute-phase reaction. Interestingly, Northern analysis revealed that the level of C/EBP mRNA declined to a level 4.5-fold that of control within 4 h following injection

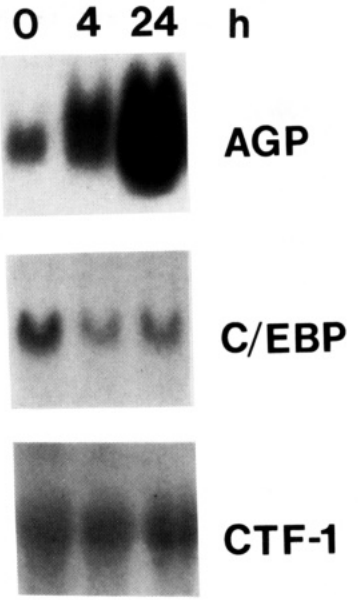

FIGURE 7: Decrease in C/EBP expression during the acute-phase response. Northern blot analysis was performed on total RNA (20 μg) from control and LPS-stimulated (4 h and 24 h postinjection) livers. Identical blots were probed with labeled cDNAs for AGP-1, C/EBP, and CTF-1 (CCAAT-binding transcription factor).

with LPS (Figure 7). This result is consistent with the low levels of the constitutive C1 complex that comigrates with C/EBP, seen with nuclear extract from LPS-stimulated animals (Figure 5A).

C/EBP and AP-DBP Compete for Binding to Region C and Can Form Heterodimers. The presence of a leucine zipper motif allows C/EBP to form homodimers which can bind to DNA. If AP-DBP belongs to the C/EBP family, it may form heterodimers with C/EBP. To test this hypothesis, EMSA was performed with mixtures of purified C/EBP and LPS-stimulated nuclear protein. While holding the concentration of one protein constant, a progressively larger amount of the competitor protein was added. A new band of intermediate mobility was observed in addition to the complexes for C/EBP and AP-DBP only when both proteins were present, indicating that the two proteins are capable of forming heterodimers (Figure 8A). In the competition assay the diminution of C/EBP complex as the AP-DBP complex increases also suggests that these two proteins can displace each other from the cognate site. The preferential formation of AP-DBP complex is also seen when control and LPS-stimulated nuclear fractions are allowed to compete for the oligonucleotide C probe (Figure 8B). It has been shown that the subunits of C/EBP and IL6-DBP are exchangeable, leading to the formation of a heterodimer of intermediate mobility (Poli et al., 1990). A similar heterodimer complex between C/EBP and immunoglobulin/EBP-1 was observed when crude nuclear extract from plasmacytoma P3X was allowed to interact with C/EBP (Roman et al., 1990).

Region C Acts as a Functional Acute-Phase Responsive Element (APRE) in Hepatoma Cells. In order to analyze the functional role of region C identified by EMSA and footprinting assays, CAT constructs were generated in which the SV40 enhancers of pSV2CAT (Gorman et al., 1982) were replaced with multimerized copies of region C. HepG2 cells were transiently transfected with the plasmid AGP-X3 APRE-CAT, containing three copies of region C. Dexamethasone alone was not significantly effective in increasing the CAT activity (Figure 9). This confirms earlier reports which showed that dexamethasone alone does not significantly

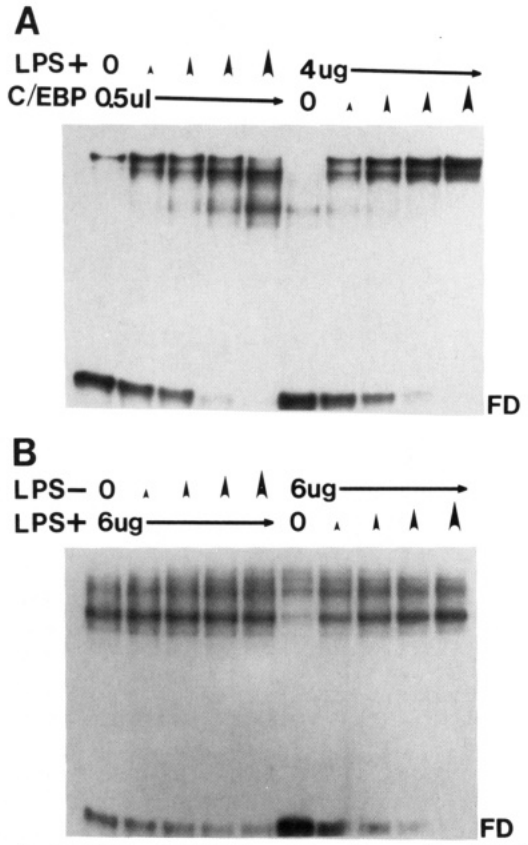

FIGURE 8: AP-DBP and C/EBP form heterodimers. (Panel A) EMSA of oligonucleotide shifting patterns produced by addition of increasing concentrations of LPS-stimulated liver nuclear protein (0, 2, 4, 8, and 16 μg) to a constant amount (0.5 μL) of full-length C/EBP (first five lanes) in the presence of 4000 ng of poly(dI·dC). Conversely, increasing amounts of C/EBP (0, 0.25, 0.5, 1, and 5 μL) were added to a constant amount (4 μg) of LPS-stimulated nuclear protein (last five lanes). FD, free DNA. (Panel B) EMSA showing titration of LPS-induced nuclear protein into control nuclear extract. Increasing concentrations of control or LPS-stimulated nuclear proteins (0, 2, 4, 6, and 8 μg) were added to a constant amount (6 μg) of the opposing extract. FD, free DNA.

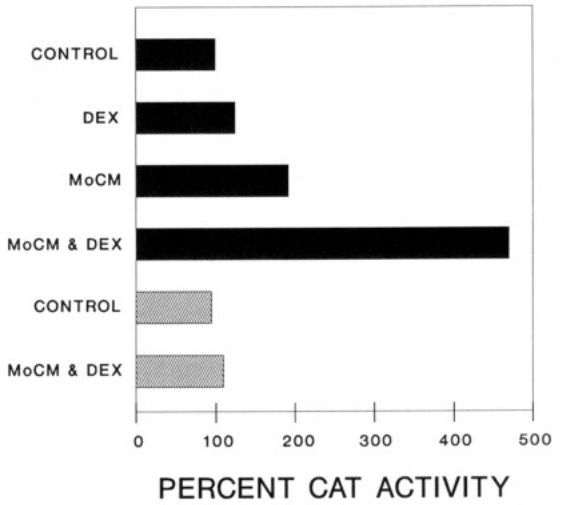

FIGURE 9: Region C DNA sequence confers acute-phase responsiveness to a CAT chimeric construct. Three copies of region C were cloned upstream of pSV1CAT, containing only the SV40 minimal promoter. Twenty-four hours after transfection, HepG2 cells were stimulated as indicated. After another 20 h, CAT activity was assayed and normalized by β-galactosidase activity. Solid bars represent transfection with AGP-X3 APRE-CAT, and hatched bars indicate transfection with the control plasmid, pSV1CAT. CAT activity is presented relative to untreated cells (control), which is set at 100%.

enhance the transcription of the AGP gene (Baumann et al., 1990). Our recent studies indicate that HepG2 cells are relatively deficient in functional glucocorticoid receptor; indeed, a 23-fold dexamethasone inducibility can be observed when a functional glucocorticoid receptor expression vector was cotransfected (T. Alam, M. R. An, and J. Papaconstantinou, manuscript submitted). Increased CAT activity was observed when cells were induced with LPS-stimulated monocyte conditioned medium; furthermore, a synergistic induction of CAT activity was detected when cells were treated with MoCM and dexamethasone together (Figure 9). This synergistic response suggests that glucocorticoids may activate the synthesis of region C binding proteins; alternatively, the glucocorticoid receptor acts in concert with these trans-acting factors. These results indicate that the DNA sequence contained in region C is a functional acute-phase responsive element.

Discussion

We have localized the protein binding domains of the mouse AGP-1 promoter. Regions B (−104 to −91) and C (−125 to −104) showed the strongest protection in DNase I footprint assays with hepatic nuclear extracts; moreover, these two regions are highly homologous to the corresponding sequences of the rat and human AGP promoters (Reinke & Feigelson, 1985; Dente et al., 1987). Region B binds with low affinity to a very heat-labile protein. In contrast, region C binds strongly to liver-enriched nuclear factors that are heat stable. We have demonstrated significant differences in the region C binding proteins from control and acute-phase animals.

Our studies indicate that multiple protein interactions occur with region C in constitutive and LPS-stimulated animals. Electrophoretic mobility shift assays show that nuclear protein from hepatocytes can form four complexes with oligonucleotide C. Three complexes (C1, C2, and C3) are formed with hepatic nuclear extract from unstimulated animals. With the control extract, only a trace amount of the faster migrating complex 4 is seen. Interestingly, formation of complex 4 (AP-DBP) shows a dramatic increase, as indicated by binding activity, following stimulation with LPS. This may be due to either de novo synthesis of AP-DBP or modification of a precursor protein. The kinetics of induction of AP-DBP activity are consistent with the transcriptional activation of the AGP gene: the rate of transcription peaks at 4 h, followed by a decline at 12 h. Also, an increase in the AP-DBP binding complex is seen in extracts from LPS-stimulated kidney but not brain. A correlative induction of AGP mRNA is observed in the kidney during the acute-phase response.

At present, the relationships of the variably sized proteins interacting with region C are not clear. It is possible that the same protein is differentially modified or different proteins have a common region C binding specificity. However, as has been explained by Poli and Cortese (1989), we believe it is highly unlikely that artifactual proteolytic cleavage of a single DNA-binding protein contributes to the multiple complexes because the pattern of the gel mobility shift is very reproducible. We have seen the exact mobility shift pattern with nuclear extracts from several different groups of normal and stimulated animals. Extensive precautions were taken by adding various protease inhibitors to every step of nuclear protein extraction from fresh tissues. Also, no protein degradation is observed when the same extracts are used for binding to other DNA probes. Another reason why it is unlikely that AP-DBP is a proteolytic fragment of C/EBP is because a high level of C4 complex is formed with the kidney nuclear extract from LPS-stimulated animals, yet C/EBP mRNA and protein are present in very low amounts in this

tissue (Birkenmeier et al., 1989; Liu et al., 1991). Moreover, there is considerable recent evidence in the literature describing observations similar to ours. For instance, when EMSA was performed with the minimal element of the hemopexin gene promoter, at least six DNA–protein complexes are formed with nuclear extracts from Hep3B cells; the formation of complex 4 is highly inducible by IL-6 (Poli & Cortese, 1989). Similarly, multiple bands were observed with the haptoglobin gene promoter (Oliviero & Cortese, 1989). Mobility shift assay also revealed that two nuclear factors, one constitutive and the other IL-1 inducible, can bind to a 14-bp palindromic sequence of the IL-6 gene (Isshiki et al., 1990). Furthermore, multiple proteins can bind to a 29-bp APRE of the angiotensinogen gene; nuclear extract from LPS-stimulated rat liver showed that the formation of only one DNA–protein complex is induced (Brasier et al., 1990).

We have also presented evidence that region C interacts with the C/EBP family of transcription factors. Purified C/EBP binds avidly to region C. The C1 complex may represent C/EBP since they comigrate in the gel. During the acute-phase response the C/EBP mRNA is reduced, as is the C1 complex. Additionally, C1 is absent in brain and kidney, where C/EBP expression is known to be very low (Birkenmeier et al., 1989; Liu et al., 1991). All four complexes are heat-stable, a characteristic feature of the C/EBP family proteins. Another feature of C/EBP is its obligate dimerization, which is achieved through hydrophobic interactions between heptad leucine repeats located in the C-terminal α-helical domain (Landschulz et al., 1988b, 1989). AP-DBP forms heterodimers with C/EBP, which strongly suggests that this acute-phase-inducible protein is a member of the C/EBP family. The intermediate bands seen with hepatocyte nuclear extract may represent similar heterodimers. It is interesting to note that two other members of the family, IL6-DBP and immunoglobulin/EBP-1, can also form heterodimers of intermediate mobility with C/EBP (Poli et al., 1990; Roman et al., 1990).

Brasier et al. (1990) have shown that C/EBP-like proteins (BPcs) bind constitutively to the angiotensinogen APRE. Extracts from LPS-injected rat liver contain an inducible protein (BPi) that undergoes nuclear translocation and effectively competes for the APRE. NFκB, an LPS-inducible DNA-binding protein identified by Sen and Baltimore (1986), is indistinguishable from BPi (Brasier et al., 1990). Although 9 bp of the underlined angiotensinogen APRE (AGTTGGGATTTCCCAACC) resembles the 3′-end of region C, we do not think BPi is similar to AP-DBP: NFκB or BPi is heat-labile (Brasier et al., 1990; Zabel et al., 1991) and is a much larger protein (50 kDa) than AP-DBP, which is considerably smaller than the 42-kDa C/EBP. Recently two C/EBP-like factors, IL6-DBP and AGP/EBP, have been cloned independently from unstimulated rat and mouse liver cDNA libraries, respectively (Chang et al., 1990; Poli et al., 1990). Comparison of cDNA sequences revealed that these two genes are identical. The IL6-DBP has been shown to bind to the IL-6-responsive elements present in some acute-phase genes, such as haptoglobin and hemopexin (Oliviero & Cortese, 1989; Poli & Cortese, 1989). Another smaller (36 kDa) member of the family, NF-IL6, which is somewhat homologous to the above two genes, has been cloned from an LPS-stimulated human monocyte library (Akira et al., 1990). This nuclear factor binds to the IL-1-responsive element of the IL-6 gene. A crucial difference between IL6-DBP and NF-IL6 is that the latter is not expressed in the adult mouse hepatocytes unless the animals are induced with LPS, IL-1, or IL-6 (Akira et al., 1990; Chang et al., 1990; Poli et al., 1990). In contrast,

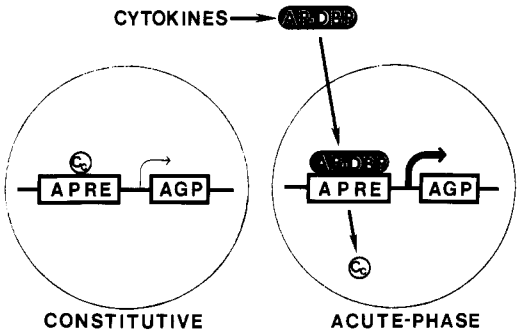

FIGURE 10: Model depicting the induction of the AGP-1 gene during the acute-phase response. In the uninduced hepatocytes, the constitutive complexes, represented as Cc, bind to the APRE, thus allowing only a minimal level of transcription. During the acute-phase response, cytokine-mediated activation of AP-DBP occurs. This potent trans-activator displaces the other proteins, resulting in a dramatic increase in transcription. A similar model has been proposed for the angiotensinogen APRE (Brasier et al., 1990).

very high levels of IL6-DBP mRNA and protein are found in the uninduced rat liver. Even when a large amount of nuclear protein (40 μg) from unstimulated hepatocytes is used in our assays, only a trace level of AP-DBP is detected. Therefore, we believe that AP-DBP is a homologue of the human NF-IL6. Within 4 h following stimulation with LPS, the binding activity of AP-DBP and the expression of NF-IL6 increase markedly in mouse liver and kidney but not brain. It is conceivable that IL6-DBP represents the constitutive intermediate complex seen in our EMSA.

Using specific antisera against synthetic peptides unique to individual isoforms of mouse C/EBP, we have recently shown that C1 is indeed formed by C/EBP, whereas the transcription factors forming C3 and C4 complexes have immunological identity with a novel acute-phase-inducible C/EBP isoform (T. Alam, M. R. An, and J. Papaconstantinou, manuscript submitted). Furthermore, Northern analysis with a cloned cDNA revealed a dramatic increase in the mRNA level of this C/EBP isoform during the acute-phase response.

On the basis of our studies, we propose a simple model as shown in Figure 10. The constitutive complexes, possibly acting as minimal activators, occupy region C in the uninduced hepatocytes; hence, very little transcription from the gene is possible. During the acute-phase response, stimulation by LPS results in a signaling cascade that releases cytokines, such as IL-1, IL-6, and TNFα (Gauldie et al., 1987; Kushner et al., 1988). Although the second messenger pathway is unknown, activation of a protein kinase may be involved because preliminary results indicate that AP-DBP is phosphorylated. In a similar example, NFκB undergoes activation and translocates into the nucleus, where it is thought to substitute for C/EBP-like factors on the angiotensinogen APRE (Brasier et al., 1990). Here we postulate that increased expression of AP-DBP during the acute-phase response allows it to displace the constitutive complexes from the AGP APRE. Furthermore, the situation favors AP-DBP since the expression of constitutive factors, such as C/EBP, is reduced during the acute-phase response. AP-DBP in concert with HLF acts as a potent trans-activator; thus, a drastic increase in AGP transcription occurs in the LPS-induced hepatocytes. A direct demonstration of the role of AP-DBP must await the isolation of this transcription factor. Further studies are in progress to discern the nature of the dynamic interplay of various nuclear factors with region C.

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