trans-Activation of the α1-Acid Glycoprotein Gene Acute Phase Responsive Element by Multiple Isoforms of C/EBP and Glucocorticoid Receptor*

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α1-Acid glycoprotein (AGP) is a major acute phase protein synthesized primarily by the liver. The AGP gene is transcriptionally activated in hepatocytes during the acute phase response to bacterial lipopolysaccharide. In this study, we analyzed an acute phase responsive element (APRE) located between nucleotide residues −127 to −104 relative to the transcription initiation site of the mouse AGP gene. Binding studies show that several trans-acting factors interact with the APRE. Using monospecific antibodies we demonstrate that these isoforms of the CCAAT/enhancer-binding protein (C/EBP) family, namely C/EBPα, C/EBPβ, and C/EBPγ, bind to the APRE. Furthermore, with liver nuclear protein from control animals, C/EBPα is the predominant form that binds to the APRE, whereas with nuclear proteins from acute phase-induced animals, C/EBPα is replaced by C/EBPβ. The mechanism of activation of the AGP gene during the acute phase response appears to involve an exchange of C/EBPα by C/EBPβ. C/EBPβ does not play a role in this reaction. Interestingly, the C/EBP binding site of the APRE partially overlaps a functional glucocorticoid responsive element. We present evidence that both purified C/EBPα and glucocorticoid receptor bind strongly to the APRE. By site-specific mutation, we have identified the C/EBP and glucocorticoid receptor binding sites in the APRE. These mutants were used in expression vectors to demonstrate that both C/EBP and glucocorticoid receptor are essential for maximal response to interleukin-6 and dexamethasone. These results demonstrate that the APRE is a composite binding site for multiple factors that are responsible for the transcriptional control of the mouse AGP. Finally, functional analyses indicate that C/EBPα, C/EBPβ, and C/EBPγ are strong transcriptional trans-activators of the AGP APRE in hepatoma cells. These data suggest that the regulatory activity of the C/EBP with the APRE in the liver may require interactions with adjacent proteins.

An informative model system to study the interactions of trans-acting factors with cis-acting elements during gene regulation is the hepatic acute phase response to inflammation (Fey and Fuller, 1987). The strong and rapid changes in the expression of acute phase reactant genes provide an excellent system to study the mechanism of gene regulation by transcription factors. In particular, we are interested in the interaction of the CCAAT/enhancer-binding proteins (C/EBPs) with the acute phase responsive element (APRE) of mouse α1-acid glycoprotein (AGP) promoter.

In humans as well as other mammals, AGP, also known as orosomucoid, becomes one of the most abundant proteins made by the liver during the acute phase response. The expression of AGP rises dramatically following induction by agents such as bacterial LPS, turpentine, cytokines, glucocorticoids, and heavy metals (Cooper and Papaconstantinou, 1986; Carter et al., 1989; Yiangou et al., 1991). Recent biochemical and clinical investigations have suggested that AGP has a pleiotropic role. Due to its resemblance to sialomembrane proteins of the cell surface, AGP has been proposed to function as a nonspecific anti-infectious agent (Friedman, 1983). AGP also has the ability to bind and transport a wide variety of drugs, lipids, and proteins in circulation (Baumann, 1989). There are some indications that AGP may function in regulating the immune system, in particular suppressing the immune response; purified AGP has been shown to reduce antibody formation by spleen cells in vitro (Bennet and Schmid, 1980). We, as well as others, have cloned the mouse, rat, and human AGP genes (Reinke and Feigelson, 1985; Cooper et al., 1987; Dente et al., 1987). The rat genome is known to contain just one AGP gene. In contrast, the mouse and human genomes contain three AGP genes clustered in one locus. Studies with transgenic animals have shown that sufficient information for tissue-specific and acute phase-regulated expression is contained within the 1.2-kilobase pair 5'-flanking DNA and coding region of the human AGP gene (Dente et al., 1988).

C/EBP belongs to a class of transcription factors, termed bZIP proteins, characterized by a basic domain linked to leucine repeats (Landschulz et al., 1988b, 1989). The leucine zipper region is required for the dimerization of C/EBP. C/EBP is primarily expressed in fully differentiated cells (Birkenmeier et al., 1989). Moreover, C/EBP can trans-activate the genes for serum albumin (Friedman et al., 1989; Friedman and McKnight, 1990), stearoyl acyl-CoA desaturase-1 and 422/aP2 protein (Christy et al., 1993).

The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein(s); AGP, α1-acid glycoprotein; APRE, acute phase responsive element; IL, interleukin; GR, glucocorticoid receptor; hGR, human glucocorticoid receptor; GRE, glucocorticoid responsive element; EMSA, electrophoretic mobility shift assay; bp, base pair(s); LPS, lipopolysaccharide; PMSF, phenylmethylsulfonyl fluoride; CAT, chloramphenicol acetyltransferase; NF, nuclear factor; HNF, hepatocyte nuclear factor.

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al., 1989, 1991), and the insulin responsive glucose transporter (Kaeestner et al., 1990). Recent evidence suggests that C/EBP may play an important role in cell growth and differentiation (Umek et al., 1991). Indeed a strong correlation exists between C/EBP expression and terminal differentiation of preadipocytes (Christy et al., 1989). It has also been proposed that C/EBP may play a central role in energy metabolism in liver and adipose tissues (McKinney et al., 1989).

More recently, evidence has emerged indicating the presence of isoforms of C/EBP, bearing sequence homology in the bZIP region. A characteristic feature of these isoforms is the ability to form heterodimers. The cytokine-inducible NF-IL6r, the first C/EBP-related protein to be identified, binds to the IL-1-responsive element of the IL-6 gene; consequently, the expression of IL-6, which participates in host defense processes, rises rapidly during viral and bacterial infection (Akira et al., 1991; Ishihara et al., 1990). Subsequently, the mouse and rat homologs of NF-IL6 were independently identified by several investigators (Descombes et al., 1990; Poli et al., 1990; Chang et al., 1990). To avoid confusion, from hereon these proteins will be collectively called C/EBPB, whereas the first C/EBP-related protein to be identified, binds to the APRE and is now called C/EBPa. A new family member, C/EBPδ, is distinct from all other known isoforms of C/EBP (Cao et al., 1991).

Given that multiple isoforms of C/EBP exist, it was of interest to investigate how these transcription factors interact with the APRE during the LFS-mediated activation of the mouse AGP gene. In this paper, we also describe functional elements of pSV2-CAT (Gorman et al., 1982) were excised from the plasmid DNA and purified recombinant of C/EBP (600 units/mg) from Boehringer Mannheim. After a further 24 h, cells were harvested, and CAT and β-galactosidase activities were measured as described previously by Izban and Papaconstantinou (1989).

Preparation of Nuclear Extracts—Liver and kidney 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 from a pool of 10 mice were homogenized at 4°C in buffer A (0.25 M sucrose, 15 mM Tris-Cl, 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 protease inhibitors, antipain, chymostatin, leupeptin, and pepstatin A). After homogenization, 2 volumes of buffer B (buffer A with 2.5 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 35,000 rpm at 4°C in a SW 27 rotor for 1 h. The nuclear pellets were resuspended in buffer D (100 mM KCl, 10 mM Tris-Cl, pH 8.0, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.4 mM PMSF, 2 mM benzamidine, 1 μg/ml each of antipain, chymostatin, leupeptin, and pepstatin A) and extracted with 0.10 volume of 4 M (NH₄)₂SO₄ at 4°C overnight. The suspension was centrifuged at 35,000 rpm for 45 min in an SW 40 rotor. The nuclear protein was precipitated adjusting the supernatant to 0.3 g/ml NH₄SO₄ and resuspended in buffer E (20 mM HEPES pH 7.8, 10% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 0.2 mM benzamidine, 1 μg/ml each of 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 proteins were frozen in liquid nitrogen and stored at -90°C.

Electrophoretic Mobility Shift Assays—Electrophoretic mobility shift assays were performed with double-stranded oligonucleotides radioactively labeled with [³²P]ATP and T4 polynucleotide kinase (Fried and Crothers, 1981; Garner and Rezvani, 1981). Approximately 1 ng of these DNAs was incubated for 20 min at 22°C with various nuclear extracts, purified C/EBP (provided by Dr. S. L. McKnight, Carnegie Institute of Washington, Baltimore, MD), a polypeptide corresponding to the DNA binding domain of GR (provided by Dr. K. R. Yamamoto, University of California, San Francisco, CA), or baculovirus-expressed human GR (provided by Dr. E. B. Thompson, University of Texas Medical Branch, Galveston, TX). The C/EBP and GR proteins also exhibited strong binding to oligonucleotide probes containing previously published high affinity GRE and C/EBP consensus binding sites. The DNA-protein complexes were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels. Oligonucleotides—The following oligonucleotides and their complementary strands were used as probes or competitors in electrophoretic mobility shift assays.

| Oligonucleotide | Description |
|----------------|-------------|
| 5'-GAAATTTGCGCAAGAATTTCCCAGAG-3' | Oligonucleotide APRE (wt) |
| 5'-GAAATTTGCGCAAGAATTTCCCAGAG-3' | Oligonucleotide APRE (mt-1) |
| 5'-ATAATTGTTTTTGCAGAAGATTTTTCCCAGAG-3' | Oligonucleotide APRE (mt-2) |
| 5'-AGTTGCTGAAAGCCATATG-3' | Oligonucleotide NF-1 |
| 5'-GAACCTTTGCGCAAGAATTTCCCAGAG-3' | Oligonucleotide APRE (wt) |
| 5'-GAACCTTTGCGCAAGAATTTCCCAGAG-3' | Oligonucleotide APRE (mt-1) |
| 5'-AAATAATTGTTTTTGCAGAAGATTTTTCCCAGAG-3' | Oligonucleotide APRE (mt-2) |
| 5'-ATTGCTGAAAGCCATATG-3' | Oligonucleotide NF-1 |

Equa amounts of the complementary strands of the oligonucleotides were heated at 100°C for 3 min in STE buffer (10 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0) and allowed to anneal by slowly cooling to room temperature.
RESULTS

**Definition of an APRE on the Mouse AGP-1 Promoter**—One of the three mouse AGP genes, AGP-1, has been cloned, and its 5'-flanking region was identified and sequenced (Cooper et al., 1987). Transgenic animal studies with the human AGP gene indicated that sufficient information for tissue-specific and acute phase-regulated expression is contained within 1.2 kilobase pairs of the 5'-flanking DNA (Dente et al., 1988). Also, we have demonstrated that a CAT expression vector containing -595 bp of the 5'-flanking region of the mouse AGP-1 gene is responsive to inflammatory agents in HepG2 cells (Yangou et al., 1991). These observations prompted us to examine the interaction of trans-acting factors with the 5'-flanking sequence of the mouse AGP-1 gene. Using DNase I footprint assays, we have previously shown the presence of multiple protein binding domains in the 5'-flanking region of mouse AGP-1 gene (Alam and Papadimitrionou, 1991). Footprint assays with nuclear protein from mouse liver revealed a very strongly protected segment of DNA between nucleotide residues -127 to -104 (region C). This region showed a high degree of homology to the human and rat AGP genes (Fig. 1). Moreover, region C contains the consensus binding site (T(T/G)NNGAAA(G/T)) for C/EBPβ (NF-IL6), an acute phase-inducible transcription factor (Akira et al., 1990; Alam et al., 1992). The C/EBP binding site overlaps a putative GRE. Thus, we have named the nucleotide residues from -127 to -104 as the AGP APRE.

**Acute Phase-inducible Nuclear Proteins Bind to the APRE**—To analyze the trans-acting factors that recognize the APRE, we performed electrophoretic mobility shift assays (EMSA) with oligonucleotides corresponding to this region. Liver nuclear extracts from control and LPS-stimulated animals formed four complexes (C1, C2, C3, and C4) with the APRE (Fig. 2A). The formation of C3 and C4 increased reproducibly and have been repeated with several groups of control and induced animals. As control, the same nuclear extracts were used to show that the binding of nuclear factor-1 (NF-1) and hepatocyte nuclear factor-1 (HNF-1) to their cognate site was not significantly changed (Fig. 2A).

To identify the precise location of the binding site(s) of the four complexes, oligonucleotides with site-specific mutations were used. When the potential C/EBP binding site, TTGCGAATAAGGTTTGCAGGCAGCATTTCC

HUMAN:  g G g A g g T T T G 1 G C g A G g C A T T T C

RAT:  g G A C A T T T T G i G C A A G A C A T T T C

MOUSE:  A A A C A T T T T G i G C A G A C A T C A T T C

C/EBP:  Y T C T G G T A A A C C g C G T

GRE:  T C T G G T a a C A T G T

**FIG. 1. Sequence comparison of the AGP APRE.** The APRE sequence corresponds to region C (124 to 104 of the coding strand) that is protected in a DNase I footprint assay with nuclear extract from mouse liver. Since the GRE extends to -127, we have termed the nucleotide residues between -127 and -104, relative to the transcription initiation site, as the AGP APRE. Rat (Reinke and Feigelson, 1985) and human (Dente et al., 1987) nucleotides that diverge from the mouse are indicated by lower case letters. The putative C/EBP and GR binding sites of the APRE are boxed. The stronger half-site of the GRE is shaded. For comparison, the GRE of the tyrosine aminotransferase gene is shown at the bottom.

GCAA G, was mutated to TCTGCAGAG, the formation of all four complexes was completely abrogated (Fig. 2B). Moreover, the formation of the four complexes was sequence-specific since binding was totally abolished by a 100-fold molar excess of the wild-type APRE (wt) but not with the APRE (mt-I) in which the potential C/EBP binding site has been mutated (mutated sequence is shown under "Materials and Methods"). Competition assays were performed with 100× molar excess of unlabeled wild-type oligonucleotide (100×wt) or the mutated form (100×mt-I).

**FIG. 2. Acute phase-inducible nuclear proteins bind to the APRE.** Panel A, determination of relative binding of liver nuclear extracts from control and LPS-treated mice. End-labeled oligonucleotides corresponding to AGP APRE, NF-1, and HNF-1 were incubated with hepatic nuclear extracts (10 µg) from control (+) or 12 h post-LPS-injected (+) mice. The four complexes seen with AGP APRE have been termed C1, C2, C3, and C4. Panel B, localization of the C/EBP binding site in AGP APRE. EMSA was performed with liver nuclear protein. The four complexes can be seen with wild-type AGP APRE (wt) but not with the APRE (mt-I) in which the potential C/EBP binding site has been mutated (mutated sequence is shown under "Materials and Methods"). Competition assays were performed with 100× molar excess of unlabeled wild-type oligonucleotide (100×wt) or the mutated form (100×mt-I).

**Interaction of Three Isoforms of C/EBP with the APRE**—To demonstrate that the four DNA-protein complexes were formed by members of the C/EBP family of transcription factors, we used specific antibodies raised against synthetic peptides unique to individual isoforms of C/EBP as described previously (Landschulz et al., 1988a; Cao et al., 1991). The antisera against C/EBPβ and C/EBPδ recognized unique carboxyl-terminal sequences, and the antiserum against C/EBPα was raised against a 14-amino acid sequence at the NH2 terminus. The inclusion of antibody to C/EBPα with hepatic nuclear extract converted both C1 and C2 to more slowly migrating forms (supershift); antibody to C/EBPβ, however, recognized mainly C4 (Fig. 3A). This is more clearly seen with nuclear extract from LPS-treated animals where C4 is the major complex formed. The C3 complex is recognized by both C/EBPα and C/EBPβ antibodies, suggesting that it may be an αβ-heterodimer. The complexity of the recognition pattern by the monospecific antibodies is most likely due to various combinations of homo- and heterodimer formation; however, post-translational modification or the generation of bands due to proteolytic cleavage cannot be excluded. Nevertheless it is interesting to note that the acute phase inducible...
pressed in the kidney, and our experiments showed that ever, the data shown in Fig. 3 suggest that either the concentration or binding activity of CIEBPP in the nuclei was increased and that the binding affinity of C/EBPa to the APRE was lower than that of C/EBPβ. Indeed, Cao et al. (1991) have shown that a hierarchy of binding affinities exists among the various C/EBP isoforms (C/EBPβ > C/EBPa > C/EBPδ) when an oligonucleotide containing the motif, TTGGCGCAAT, similar to the AGP APRE, was used in Scatchard analysis.

Both Glucocorticoid Receptor and C/EBP Bind to the APRE Template in Vitro—Examination of the APRE sequence revealed that the C/EBP binding site is interdigitated with a potential GRE (Fig. 1). The stronger half-site (TGTCTT) of the GRE is located at the 5’ end of the APRE, whereas the weaker half-site (TGCGCA) is overlapped by the C/EBP binding site. By using purified recombinant C/EBPa and GR, the binding characteristics of these two sites were assessed. Purified C/EBPa bound avidly to the wild-type APRE, but binding was completely abolished when its cognate site was mutated (Fig. 4, lane 2). A small 10-kDa polypeptide encompassing the DNA binding domain of the glucocorticoid receptor (amino acids 440–525) bound to both the APRE (wt) and APRE (mt-1) probes, implying that the GR binding site is located outside the C/EBP binding site (Fig. 4). Further confirmation of the specificity of GR binding was obtained by using human GR (hGR), which was expressed in a baculovirus system (Srinivasan and Thompson, 1990; Tsai et al., 1990). A similar GR binding site in the rat AGP gene has also been reported (Klein et al., 1988). The binding of GR and hGR to mt-1 was relatively stronger than binding to the wild-type APRE; it is possible that the mutation of the C/EBP site strengthened the weaker half-site of the GRE. When the stronger half-site of the GRE was mutated, APRE (mt-2), the binding of hGR was abolished; however, the binding of C/EBPa was not significantly affected. Next we wanted to determine if simultaneous binding of hGR and C/EBPa was possible. Inclusion of both hGR and C/EBPa together in the EMSA did not produce additional intermediate or slower migrating bands, arguing against the simultaneous binding of these transcription factors. Titrations were performed with hGR and C/EBPa as well as with recombinant hGR and GR, and the results were essentially the same as those described under “Materials and Methods.”
factors bind to the APRE in a mutually exclusive manner (data not shown).

The AGP APRE Sequence Confers Cytokine and Glucocorticoid Responsiveness to a Heterologous Promoter in Hepatoma Cells—To demonstrate that the highly conserved APRE sequence is functionally important, we performed an enhancer-substitution experiment. We deleted the 72-bp enhancer elements from pSV2CAT and cloned in monomer or multimerized copies of the synthetic AGP APRE DNA templates, generating pX1-APRE-CAT and pX3-APRE-CAT (Fig. 5A). As controls we used the enhancerless pSV1-CAT containing the minimal promoter or the original pSV2-CAT. The ability of these constructs to respond to combined dexamethasone plus IL-6 treatment was analyzed by transient expression analysis in the human hepatoma cell line, HepG2, which is responsive to mediators of the acute phase response. Transfected cells were harvested after 24 h of stimulation with IL-6 and dexamethasone (Fig. 5B). The cell extracts were assayed for CAT activity, which was normalized to cotransfected β-galactosidase activity. The data indicate that pX1-APRE-CAT and pX3-APRE-CAT are stimulated by dexamethasone plus IL-6 and that the APRE is a functional promoter element.

\[
\text{AGP-APRE-CAT} \quad \text{C} \quad \text{CAT}
\]
\[
\text{AGP-X3 APRE-CAT} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{CAT}
\]
\[
\text{pSV1-CAT} \quad \text{72} \quad \text{72}
\]
\[
\text{pSV2-CAT}
\]

**A**

**FIG. 5.** The AGP APRE sequence confers IL-6 and glucocorticoid responsiveness to heterologous promoters. Panel A, schematic representation of the reporter gene (CAT) chimeric constructs. APRE-CAT contains one copy of the APRE (corresponding to region C of the footprint), and X3 APRE-CAT contains three copies. The SV40 72-bp enhancer elements are indicated in pSV2CAT. The pSV1-CAT vector contains only the enhancerless SV40 minimal promoter. Panel B, transient expression of CAT chimeric constructs in HepG2 cells. Expression vectors X1-APRE-CAT and X3-APRE-CAT were cotransfected with a GRE expression plasmid (RShGR) and β-galactosidase reporter plasmid (pCH110). Twenty-four hours after transfection, cells were treated for 20 h with dexamethasone (DEX, 10^{-6} M) and IL-6 (800 units/ml) and prepared for analysis of CAT and β-galactosidase activity. The results represent an average of three separate experiments. CAT activities were expressed as nm/mg/h and normalized to β-galactosidase activity.

Since HepG2 cells are relatively deficient in functional glucocorticoid receptors (Ron et al., 1990) this provided an opportunity to assess the role of GR in the APRE by selective cotransfection of a hGR-expressing plasmid. Cells cotransfected with pX3-APRE-CAT and a GR expression vector (RShGR) displayed a 2.3-fold dexamethasone inducibility and a 2.10-fold IL-6 inducibility, whereas a 13-fold increase in CAT activity was observed when dexamethasone was used with IL-6 (Fig. 6). Therefore, ligand-activated GR may mediate the increased CAT expression by directly binding to the APRE. Alternatively, dexamethasone may increase the synthesis of the APRE-binding proteins or IL-6 receptor, which is involved in signal transduction. Recent studies indicate that dexamethasone increases the expression of the IL-6 receptor in HepG2 cells (Snyers et al., 1990). Our results clearly indicate the functional importance of the APRE in controlling the acute phase-specific expression of the mouse AGP gene.

To identify the specific sites within the APRE that respond to dexamethasone and IL-6, we constructed pX3-APRE-CAT plasmids using APRE mutant-1 and APRE mutant-2 sequences. The gel-shift analyses shown in Fig. 4 clearly show that mutant-1 sequences do not bind C/EBP and that mutant-2 sequences do not bind GR. The data in Fig. 6 demonstrate that with wild-type pX3-APRE-CAT there is a 2-fold response to dexamethasone or IL-6 alone whereas the treatment with dexamethasone plus IL-6 gives a 10-15-fold stimulation. On the other hand, the mutant-1 expression vector, which does not bind C/EBP, does not respond to any of the treatments, while the mutant-2 expression vector, which does not bind GR, shows no response to either dexamethasone or IL-6 alone but shows a 4-5-fold response with both factors. These data clearly indicate that the AGP-1 APRE is a functional element that mediates a minimal response to dexamethasone and IL-6 alone and that it mediates a synergistic response with both factors. The fact that mutant-2 can mediate a partial response to dexamethasone plus IL-6 but not to IL-6 alone suggests that dexamethasone may play another role in addition to its direct interaction with the APRE. For example, it may inhibit C/EBPα and stimulate C/EBPβ expression or it may stimulate expression of the IL-6R (Snyers et al., 1990). Overexpression of C/EBP Isoforms Mediates trans-Activat-

**FIG. 6.** The role of dexamethasone (DEX) and IL-6 in the induction of pX3-APRE-CAT. The pX3-APRE-CAT vectors were cotransfected with RShGR and β-galactosidase reporter plasmid (pCH110). Twenty-four hours after transfection, HepG2 cells were treated for 20 h with dexamethasone, IL-6, or dexamethasone plus IL-6 and then prepared for analysis of CAT and β-galactosidase activity. The results represent an average of 3–4 separate experiments. Open bars represent CAT expression by pX3-APRE-CAT (wt); hatched bars represent expression by pX3-APRE-CAT (mt-1); and solid bars represent expression by pX3-APRE-CAT (mt-2).
tion of the AGP-1 APRE—Since all three C/EBP isoforms bind to the APRE in vitro we asked whether these proteins can trans-activate the APRE in hepatoma cells. Expression plasmids encoding the C/EBP isoforms driven by the murine sarcoma virus long terminal repeat sequence (Cao et al., 1991) and pX3-APRE-CAT were cotransfected into HepG2 cells. Overexpression of each of the C/EBP isoforms markedly increased CAT reporter activity clearly demonstrating that, in addition to GR, C/EBP also trans-activates the AGP APRE (Fig. 7). We also transfected various combinations of C/EBP expression vectors to get a general idea of how these isoforms function together. During the acute phase response, in vivo, C/EBPα mRNA levels are reduced, and C/EBPβ and C/EBPδ mRNA levels rise dramatically. However, when the C/EBPs are transfected singly or in combination there does not appear to be any difference in the level of expression of the reporter activity, with the possible exception of the C/EBPα-C/EBPβ cotransfection. Thus, although these studies clearly demonstrate that the trans-activation potential of the three isoforms, singly and in combination, do not vary significantly with an isolated cis-acting element (APRE), they also suggest that in the liver nuclear environment, factors such as other proteins that bind to cis-elements adjacent to the APRE, C/EBP mRNA, and protein pool levels, and posttranslational modification may differentially influence the trans-activation potential of the C/EBP isoforms.

DISCUSSION

The acute phase response represents the body’s well orchestrated reaction to permit survival immediately following pathological insult, such as inflammation, bacterial and viral infection, myocardial infarction, surgery, burns, or neoplastic disease (Fey and Fuller, 1987; Kushner et al., 1989). Mediators of the acute phase response, in particular IL-6 and glucocorticoid, through their receptors evoke a signaling cascade that ultimately activates or represses gene expression. The mouse AGP gene is induced dramatically during acute inflammation. In this study, we identified and characterized a functionally defined acute phase responsive element in the promoter of the mouse AGP-1 gene.

We present evidence that the mouse liver nuclear extract contains several proteins that bind strongly to the APRE to form four distinct complexes. Site-specific mutation of the APRE indicates that these proteins bind to a common site. Moreover, the formation of complex 4 shows a dramatic increase following stimulation with LPS, whereas complexes 1 and 2, which are more pronounced with nuclear proteins from control liver nuclei, are decreased by LPS treatment. Several studies have demonstrated that multiple proteins can interact with a common DNA sequence. For instance, when EMSA was performed with the minimal element of the hemopexin gene promoter, at least six DNA-protein complexes were formed with nuclear extracts from Hep3B cells (Polli and Cortese, 1989). Similarly, multiple bands were observed with the haptoglobin gene promoter (Oliviero and Cortese, 1989). Mobility shift assay also revealed that two nuclear factors, one constitutive and the other IL-1 inducible, bind to a 14-bp palindromic sequence of the IL-6 gene (Ishikii 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 increased formation of one DNA-protein complex, containing NF-κB (Brasier et al., 1990).

A direct demonstration of the identity of the proteins forming the four complexes with APRE was made by using monospecific antibodies against C/EBP isoforms. The existence of several C/EBP-like transcription factors has been reported from several laboratories. Similar to C/EBPα, a liver-enriched transcriptional activator that binds to protein binding site D of the albumin gene (Mueller et al., 1990). Furthermore, it has been shown that region D binding protein also contains a leucine zipper dimerization domain (see Mueller et al. (1991)) and that it is therefore a member of the leucine zipper family of trans-acting factors. Additionally, several C/EBP isoforms have been identified; a characteristic feature of these isoforms is the ability to form heterodimers with C/EBPα and bind DNA with similar specificity (Descombes et al., 1990; Polli et al., 1990; Roman et al., 1990). For instance, Ig/EBP-1 (C/EBPγ), a protein capable of cross-dimerization with C/EBPα, binds to the E site of the immuno globulin heavy chain gene enhancer (Roman et al., 1990). The human, rat, and mouse homologs of C/EBPδ have also been cloned, and the protein products of these genes have been variously termed as NF-IL6, LAP, AGP-EBP, and IL-6DBP (Akira et al., 1990; Descombes et al., 1990; Chang et al., 1990; Polli et al., 1990; Cao et al., 1991). NF-IL6, which binds to the IL-1-responsive element of the IL-6 gene, is inducible by LPS and interleukins. In the present study, we showed that in control nuclear extract the binding activity of C/EBPα with AGP-APRE is significantly stronger than C/EBPβ and that this pattern of binding activity is reversed during the acute phase, i.e. binding activity of the acute phase-inducible C/EBPβ is significantly stronger than that of C/EBPα, with the same AGP-APRE. Our studies show, therefore, that C/EBPα occupies the APRE site in control liver and that C/EBPβ replaces C/EBPα in the LPS-induced liver. These observations suggest that C/EBPδ may have a higher affinity for this site and that this may be an important factor in this C/EBPα-C/EBPβ exchange process. Cao et al. (1991) have demonstrated a hierarchy of binding affinities among the C/EBP isoforms, i.e. C/EBPβ > C/EBPα > C/EBPδ. Thus, our observations are in agreement with their Scatchard analysis, which demonstrated the hierarchy of affinities for synthetic oligonucleotides.

Another isoform studied in this report, termed C/EBPδ, is distinct from all other C/EBP-related proteins. The expression of C/EBPδ is also highly inducible in a variety of tissues.

Fig. 7. Overexpression of C/EBP isoforms mediates the trans-activation of AGP APRE. A constant amount of APRE-CAT or pSV1-CAT was cotransfected with β-galactosidase plasmid as internal control. The transfections included an equal amount of MSV-C/EBPα, -β, and -δ expression plasmids as indicated. When combinations of C/EBP expression vectors were used, a 1:1 ratio was maintained. Thus the total amount of C/EBP expression plasmids, whether alone or in combination, was always the same. These experiments were repeated three times, and similar trends were observed. The bar graph shows the level of induction of CAT activity due to overexpression of C/EBP isoforms. Solid bars represent transfections that contained pX3-APRE-CAT, and open bars indicate transfections that contained the control plasmid, pSV1-CAT. The CAT activity was normalized to β-galactosidase activity. CAT activity is presented relative to control transfection (-), which contained no C/EBP expression plasmid.
during the acute phase response (Alam et al., 1992). There are at present no genes known to utilize C/EBPβ as a trans-acting factor even though its ability to trans-activate an APRE-containing expression vector is well documented by us and others (Baumann et al., 1992).

There is also considerable evidence demonstrating a hierarchy of inducibility among various acute phase genes, ranging from 2-fold to 1000-fold increases in mRNA levels (Fey and Fuller, 1987). We propose that the APRE sequences of other genes may be sufficiently different and that C/EBPβ may bind to these sites with greater affinity. Furthermore, since these proteins cross-dimerize in vitro, it is also possible that combinatorial diversity in activation and binding potential can be achieved by the formation of heterodimers in vivo (Lamb and McKnight, 1991).

Our data suggest that the C/EBP transcription factors play an important functional role in the expression of the mouse AGP gene. Binding activity of the C/EBPβ isoform, for example, increased significantly in the liver during the acute phase response while the activity of C/EBPα decreased. Given that C/EBPβ replaces C/EBPα at the APRE site, this is the isoform that is likely to play a key role in the acute phasespecific induction of the mouse AGP gene expression. On the other hand, C/EBPα occupies the APRE site in the unstimulated animal and since the AGP gene exhibits a low level of expression a possible role for C/EBPα may be to attenuate the activity of AGP in the control animal. Recently, repressor and attenuator domains have been identified within the trans-activation domains of C/EBPα (Pei and Shih, 1990, 1991).

A mechanism on the negative regulation of the mouse AGP gene has been presented in which nuclear proteins from rat liver that bind to the mouse AGP promoter (element B) function as negative regulators (Lee et al., 1993). In this mechanism LPS-mediated induction of AGP is associated with a decrease in binding activity of the factor(s) that bind to this promoter region. Mutation of this region resulted in increased expression of a transient expression vector, thus supporting the possibility that this is a repressor binding site. Our DNase I protection analyses using liver nuclear proteins from control and LPS-treated mice consistently fail to detect protein(s) that bind to this region even though the sequences used in both of these studies are the same (Alam and Papaconstantinou, 1991). Although the basis for these differences is not clear, these studies indicate that there are significant differences between the nuclear proteins of the mouse versus those of the rat and that the mechanism of negative regulation of the mouse AGP gene may differ with respect to the proteins that interact with the promoter.

In vitro binding assays alone cannot predict if a transcriptional factor functions as an authentic trans-activator. We assayed the enhancer activity of the APRE in hepatoma cells in the absence or presence of expression vectors for the various C/EBP isoforms. Overexpression of each of the C/EBP isoforms in these cells markedly increased reporter activity driven by APRE multimers. However, our studies show that C/EBPα overexpression in HepG2 cells can trans-activate pX3-APRE-CAT. Thus, although these experiments demonstrate the ability of C/EBPα to trans-activate an APRE-containing expression vector in the liver of control, untreated animals, C/EBPα occupies the APRE site and the AGP gene is only expressed at a very low level. These data suggest that the function of C/EBPα and C/EBPβ may depend upon adjacent promoter-binding proteins. In recent experiments we have shown that overexpression of C/EBPα in AGP expressing HepG2 cells results in reduced endogenous AGP expression while activating the expression vector.2

Our CAT expression vectors contain cis-elements (Sp1 binding sites) of the SV40 promoter adjacent to the APRE, which might explain the trans-activation caused by overexpression of C/EBPβ. In vivo proteins binding to the sites adjacent to the APRE may influence the activation and binding potential of C/EBPα as well as the activity of the AGP gene. For example, in vivo C/EBPα occupies the APRE site when the AGP gene is repressed, whereas the overexpression of C/EBPβ in HepG2 cells transactivates the pX3-APRE-CAT expression vector. It is possible, therefore, that the proteins binding to adjacent elements in vivo may prevent trans-activation by C/EBPα and stimulate trans-activation by C/EBPβ. Studies are now in progress to determine if C/EBPα plays an attenuator role by regulating the low level of AGP expression.

Our studies demonstrate that purified C/EBPα and GR bind to the APRE. The binding sites of these two transcription factors are interdigitated, and binding studies suggest that GR and C/EBP do not bind to the APRE simultaneously. Furthermore, dexamethasone and IL-6 directly induce CAT reporter activity driven by the APRE in HepG2 cells that produce pGR. Dexamethasone may also have a synergistic effect by inducing the genes for some of the C/EBP isoforms and the IL-6 receptor (Snyers et al., 1990; Cao et al., 1991). Alternatively, recent evidence suggests that the steroid receptors can recruit or stabilize transcription factors, thereby augmenting the initiation of transcription. (Klein-Hitpass et al., 1990). Schule et al. (1988a, 1988b) have shown that progesterone or glucocorticoid receptor binding site can be tightly clustered with the binding sequences of other transcription factors. These authors conclude that a CACCC box binding factor immediately upstream of the GRE facilitates the steroid induction of the tryptophan oxygenase gene; the observed cooperativity is mediated by protein-protein interactions. Similar to C/EBP, the glucocorticoid receptor contains a well conserved region of heptad repeat of hydrophobic residues (Fawell et al., 1990). Any possible protein-protein relationship between GR and C/EBP remains to be determined.

Recent studies indicate that inducible transcriptional factors can compete for a common DNA motif. For instance, NFκB can displace constitutive proteins from an enhancer element of the angiotensinogen gene (Brasier et al., 1990). The vitamin A and D3 steroid hormone receptors can compete with Jun/Fos for a vitamin D-responsive element (Schule et al., 1990), whereas NF-1 competes with GR for binding to mouse mammary tumor virus promoter (Bruggemeier et al., 1990). Thus, the relative abundance and/or increase in binding activity of various transcription factors that bind to a common DNA sequence may be important in the dynamic regulation of gene expression.

Our studies with the wild-type and mutant pX3-APRE-CAT expression vectors strongly suggest a role for both GR and C/EBPβ in the activation of the AGP genes. For example, mutation of the C/EBP binding site (mt-1) completely abolished both the IL-6- and GR-mediated induction of the gene. These data suggest that C/EBPβ plays a dominant role in the regulation of AGP. On the other hand, mutation of the GRE binding site (mt-2) reduced by ~65% but did not abolish the IL-6-mediated induction of the gene, suggesting the need for GR to mediate maximal expression of the gene. Thus, a possible role for GR may be to mediate a change in chromatin structure that would facilitate a C/EBPα-β/C/EBPβ exchange as well as to facilitate the maximal expression of AGP via synergistic action with C/EBPβ. Studies by Rigaud et al. 2

T. Alam, M. R. An, R. C. Mifflin, C.-C. Hsieh, X. Ge, and J. Papaconstantinou, unpublished data.
promoter APRE binding site,

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activities. Thus, we propose in our model that activation of the

decrease in binding activity of a "C/EBP-like" protein and an

increase in the binding activity of AGP-EBP. These obser-

vations are in agreement with our studies in which we show that activation of the mouse AGP gene involves a decrease in C/EBPα and an increase in C/EBPβ (NF-IL6) binding activities. Thus, we propose in our model that activation of the mouse AGP gene involves an exchange of these factors at the promoter APRE binding site, i.e. C/EBPα is replaced by C/EBPβ and furthermore that during this activation the GR also interacts with this composite binding site.

Our report establishes a basis for understanding the mechanism of transcriptional regulation of the mouse AGP gene. These studies should also contribute to the analysis of the regulatory mechanisms of other acute phase genes.

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Translocation of the AGP APRE