MOLECULAR AND CELLULAR BIOLOGY, Dec. 1990, p. 6642–6653
0270-7306/90/126642-12$02.00/0

Vol. 10, No. 12

Molecular Cloning of a Transcription Factor, AGP/EBP, That Belongs to Members of the C/EBP Family

CHING-JIN CHANG,[1] TUNG-TI CHEN,[1] HUAN-YAO LEI,[2] DING-SHINN CHEN,[3] AND SHENG-CHUNG LEE[1,3]*

Institute of Biological Chemistry, Academia Sinica,[1] and Department of Microbiology, School of Medicine, National Cheng Kung University,[2] Tainan, and Graduate Institute of Clinical Medicine, School of Medicine, National Taiwan University, Taipei,[3] Taiwan

Received 21 May 1990/Accepted 24 September 1990

A C/EBP-like transcription factor, AGP/EBP, that binds to three distinct motifs in the 5′-flanking region of α_1-acid glycoprotein gene (AGP) has been identified. Here we report the cloning and properties of cDNA corresponding to mouse AGP/EBP. AGP/EBP and C/EBP share 87% amino acid sequence homology in the "leucine zipper" and its associated DNA-binding domains, while their sequences outside these domains and the sizes of their mRNAs are different. Unlike the limited expression of C/EBP in tissues and cells, AGP/EBP appears to be ubiquitously expressed in tissues like lung, spleen, kidney, heart, testis, and liver and cell lines like p388D1, 129P (hepatoma cell line of C3H/HeJ), FO (mouse myeloma), and L929. Antibody against cloned and expressed AGP/EBP which was raised in rabbits could recognize AGP/EBP from nuclear extract of a number of cells and tissues. On the basis of our findings about the structural relationship and the similarity of motif recognition, we propose that a family of C/EBP-like transcription factors exists.

α_1-Acid glycoprotein (AGP) is a liver-derived plasma glycoprotein which increases severalfold during acute-phase reaction (3–5, 37). AGP is transcriptionally regulated in rat, mouse, and human hepatocytes by interleukin-1 (IL-1) (31, 34, 36), hepatocyte-stimulating factor (beta interferon subtype 2 and IL-6) (6, 13, 16, 24), and glucocorticoids (3, 4, 7, 22, 23, 40). In the rat AGP gene, the hormonal stimulation by IL-1 and IL-6 is mediated by the distal regulatory element located at −5300 to −5150 (34), and the glucocorticoid-responsive element has been limited to positions −120 to −42 (7). The corresponding enhancer elements in the human and mouse AGP genes have not yet been defined.

Unlike many glucocorticoid-responsive genes, such as those encoding the mouse mammary tumor virus (38, 40), human metallothionein (21), and tyrosine aminotransferase (33), the induction of AGP RNA by glucocorticoids is diminished in cells treated with protein synthesis inhibitor (e.g., cycloheximide) (3, 22, 23). It has been demonstrated that the rat AGP 5′-flanking region contains a DNA sequence (−121 to −107), exhibiting a high degree of homology to the glucocorticoid-responsive element consensus sequence 5′-$^{GG}_{TT}$TAC$^{A}_{C}$N$_3$TGT$^{T}_{C}$CT-3′, which serves to specifically bind purified rat glucocorticoid receptor in vitro (22). A 15-bp oligonucleotide representing the rat AGP glucocorticoid-responsive element (5′-GGAACATTTTGTGCA-3′) confers glucocorticoid responsiveness on a heterologous promoter; such regulation is not diminished by concurrent inhibition of protein synthesis. However, inclusion of the AGP sequences immediately downstream of the AGP glucocorticoid-responsive element (−106 to −42) renders the hormonal induction sensitive to inhibition of protein synthesis (22). DNase I footprinting with nuclear extracts prepared from HTC hepatoma cells indicates the presence of DNA-protein interactions spanning the region from −110 to −68 of the rat AGP gene (22).

Three functional AGP genes (Agp-1, Agp-2, and Agp-3) have been isolated from the mouse genomic library (9, 27). The nucleotide sequences corresponding to the 5′-flanking regions of these AGP genes have been determined (9) and shown to be highly homologous to the rat AGP sequence. We have identified a number of *cis* elements in the 5′-flanking region of the Agp-1 gene. Among them, three motifs (−121 to −70) could be bound with C/EBP or C/EBP-like factors. The identification and characterization of the factors that interact in this region (−121 to −70) could probably shed some light on the responsiveness of the AGP gene to glucocorticoid induction as previously identified in the rat system (22). Therefore, we initiated a project to clone and identify the gene corresponding to the C/EBP-like (i.e., AGP/EBP) factor. Here we report the cloning and properties of cDNA corresponding to AGP/EBP.

MATERIALS AND METHODS

Isolation of recombinant clones encoding AGP/EBP. Poly $(A)^+$ RNA was isolated from BALB/c mouse livers. One microgram of poly$(A)^+$ RNA was used to synthesize cDNA with a cDNA synthesis kit (Amersham). A cDNA library of 2×10^5 plaques was obtained. The library was not amplified before use. Lambda gt11 expression was screened with an end-labeled synthetic oligonucleotide probe as previously described (39, 41); the sequence of this probe is as follows:

```
5′-GATCGAACATTTTGCGCAAGACATTTCCCAAGT-3′
3′-     CTTGTAAAACGCGTTCTGTAAAGGGTTCACTAG-5′
```

Briefly, culture plates were grown for 3 h at 42°C, overlaid with isopropyl-β-D-thiogalactopyranoside-impregnated nitrocellulose filters (BA85; Schleicher & Schuell), and incubated for an additional 6 h at 37°C. After removal from culture plates, nitrocellulose filters were treated with BLOTTO for 60 min at room temperature. The nitrocellulose filters were washed with binding buffer (25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.9) and incubated with rat liver nuclear extracts in binding buffer. The filters were washed with binding buffer and incubated with DNA probe (10^6 cpm/ml in binding buffer) with gentle shaking. Filters were washed with binding buffer

* Corresponding author.

```
C/EBP       5'-AATTCAATTGGGCAATCAGG-3'
                3'-GTTAACCCGTTAGTCCTTAA-5'

Alb         5'-GATCTGGTATGATTTTGTAATGGGGTAGGA-3'
                3'-ACCATACTAAAACATTACCCCATCCTCTAG-5'

α1-AT       5'-GATCGCTTTGCTTAAGACTCCATTGATTTAGGAG-3'
                3'-CGAAACGAATTCTGAGGTAACTAAATCCTCCTAG-5'

HBV         5'-GATCACAATGTGGATATCCTG-3'
                3'-TGTTACACCTATAGGACCTAG-5'


Oligonucleotide I
                5'-GATCGCTGGTGAGATTGTGCCACAGCT-3'
(-92 to -70)        3'-CGACCACTCTAACACGGTGTCGACTAG-5'


Oligonucleotide II
  (Agp-wt)      5'-GATCGAACATTTTGCGCAAGACATTTCCCAAGT-3'
(-121 to -93)       3'-CTTGTAAAAAGCGTTCTGTAAAGGGTTCA-5'
```

FIG. 1. Double-stranded oligonucleotides used in binding experiments. These oligonucleotides were synthesized according to the sequences described in the following references: Landschulz et al. (25) (C/EBP), Johnson et al. (20) (hepatitis B virus [HBV]), Costa et al. (10) (α1-AT), and Maire et al. (28) (Alb).

and exposed to Kodak X-Omat AR film. A single positive clone, termed 1e, was identified from a screen of roughly 170,000 plaques.

Preparation of bacterial extracts containing AGP/EBP. The preparation of bacterial extracts containing AGP/EBP was carried out as described by Landschulz et al. (25). λgt11 recombinant (1e)-infected Y1089 cells were induced by isopropyl-β-D-thiogalactopyranoside and then harvested by centrifugation. The cells were resuspended in TBS (10 mM Tris, pH 7.5, 150 mM NaCl) supplemented with 0.05% Triton X-100, 1 mM benzamidine, 1 mM EDTA, and 2.5 M urea. Resuspended cells were lysed with sonication or freezing and thawing. The extracts were partially purified with a DE-52 (Whatman) column. Flowthrough fractions were dialyzed against TGEDK (10 mM Tris, pH 7.9, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 100 mM KCl). Aliquots of this partially purified AGP/EBP were kept frozen at −70°C. A fusion protein of T7 gene 10 protein and AGP/EBP was prepared by cloning the DNA fragment derived from 1e-7 into pGEMEX.1 (Promega) and was expressed in *Escherichia coli* JM109(DE). The bacterial extracts containing the fusion protein were processed according to the procedures of Landschulz et al. (25).

Southwestern (DNA-protein) blot analysis. Partially purified AGP/EBP and proteins from the parental λgt11-infected Y1089 cells were electrophoresed on sodium dodecyl sulfate (SDS) gels and transferred to nitrocellulose membranes. Nitrocellulose filters were probed for sequence-specific DNA-binding activity by the method of Miskimins et al. (29). The probes used were the same as those used for screening the cDNA library.

DNase I footprinting. DNase I footprint analyses were performed according to the method of Galas and Schmitz (15). A filled-in, labeled DNA fragment (5′ protruding) (1 to 2 ng) was added to a 50-μl reaction mixture containing 25 mM Tris hydrochloride (pH 7.9), 60 mM KCl, 6.2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol, and 1 μg of double-stranded poly(dI-dC). Extract was added last, and the binding reaction was allowed to proceed for 30 min at room temperature. Fifty microliters of 5 mM $CaCl_2$ and 10 mM $MgCl_2$ was added to the reactions. One microliter of DNase (10 ng/ml), freshly diluted in 10 mM Tris hydrochloride (pH 7.5) and 10% glycerol, was added to the reactions. Digestion of DNA-protein complexes was allowed to proceed for 1 min at room temperature. The digestions were stopped by the addition of 100 μl of a solution containing 200 mM NaCl, 20 mM EDTA, 1% SDS, 100 μg of tRNA per ml, and 75 μg of proteinase K per ml. The samples were incubated for 30 min at 45°C and extracted once with chloropane, and the nucleic acids were precipitated with 2.5 volumes of ethanol. The DNA pellets were dried, resuspended in formamide-dye (80% formamide, 1% xylene cyanol, 1% bromophenol blue), heated at 90°C for 3 min, and loaded on an 8% polyacrylamide–10 M urea sequencing gel. The gels were dried and autoradiographed at −70°C with a Dupont-Cronex intensifying screen.

Antibody to AGP/EBP. A fusion protein of β-galactosidase and AGP/EBP (from recombinant λgt11 clone 1e) was isolated by SDS-polyacrylamide gel electrophoresis (PAGE). The protein band was visualized by soaking in 3 M KCl. The gel which contained the fusion protein was excised and crushed in phosphate-buffered saline. Crushed gel containing about 200 to 300 μg of fusion protein was mixed with Freund complete adjuvant and sonicated briefly (Ultrasonic 2000 Microprobe; ARTEK). The emulsion was then injected into a rabbit subcutaneously. For the first two injections (3 weeks apart), Freund complete adjuvant was included. For the next four injections (once every 3 weeks), Freund incomplete adjuvant was added to the fusion protein-crushed gel mixture. One week after a boost with about 200 μg of SDS-PAGE-purified fusion protein (eluted from the gel), the rabbit was bled and tested for anti-AGP/EBP. Before starting immunization, the same rabbit was bled and the serum served as a preimmune serum control. All serum samples were treated at 56°C for 30 min and stored in aliquots at −20°C.

In vitro transcription and translation. The insert of a 1e-7 clone containing AGP/EBP of nucleotides 1 to 1416 was cloned into the *Eco*RI site of pGEM4 vector. This plasmid (CL-1) was linearized at the *Bam*HI site. In vitro transcription was performed on 4 μg of linearized template in a 50-μl reaction mixture with a kit from Promega. After 1 h at 38°C, DNase I was added and incubated for an additional 15 min. The mixture was extracted once with chloropane, and the

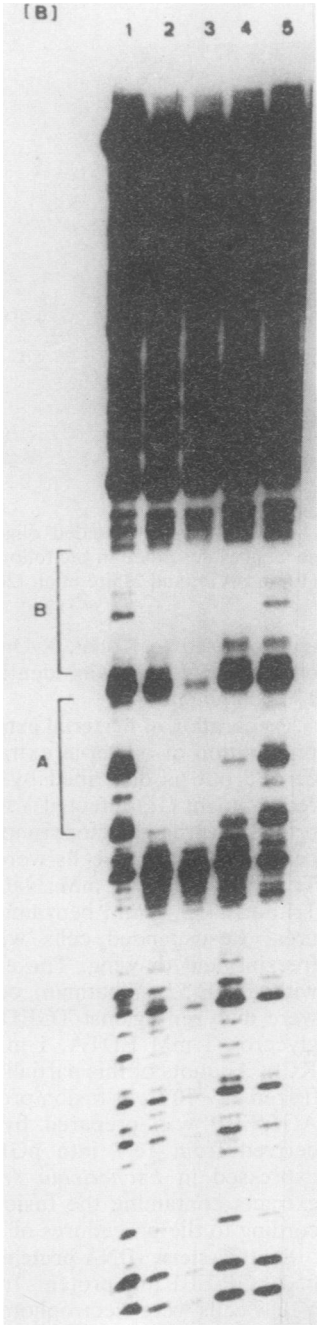

FIG. 2. Nucleotide sequence and DNase I footprint analysis of AGP-1 promoter region. (A) Nucleotide sequence of AGP-1 promoter region. The TATA sequence is boxed. The transcription initiation site is indicated (+1 arrow). Regions A and B (underlined) are the two regions protected from DNase I digestion. Oligonucleotides corresponding to −97 to −70 (I) and −121 to −93 (II) were used for competition inhibition in footprinting analysis. (B) DNase I footprinting analysis of AGP-1 promoter. A 470-bp fragment of the AGP-1 5′-flanking sequence was used for footprinting (sense strand is shown). Lane 1, Bovine serum albumin control; lanes 2 and 3, 7 and 14 μg of rat liver nuclear extract; lanes 4 and 5, 14 μg of rat liver nuclear extract and a 100 M excess of oligonucleotides I (lane 4) and II (lane 5) as competitors. (C) Determination of the relative binding affinities of AGP/EBP to motifs recognized by C/EBP and oligonucleotide II (Agp-wt) from AGP promoter by gel mobility shift assay. Oligonucleotide II was used as a probe; 20, 50, and 100 M excesses of competitors were used. Lanes: 1, in the absence of rat liver nuclear extract; 2, in the presence of rat liver nuclear extract; 3, 4, and 5, same as lane 2, except in the presence of 20, 50, and 100 M excesses, respectively, of the indicated competitors (Fig. 1). HBV, Hepatitis B virus.

RNA was precipitated with ethanol. The RNA was suspended in 20 μl of distilled H_2O. Approximately 2 μl was translated in a 50-μl reaction mixture containing 20 mM amino acid mix (minus methionine), 1 mCi of [^{35}S]methionine per ml, and 35 μl of rabbit reticulocyte lysate (Promega) for 1 h at 30°C. The translation product was analyzed by SDS-PAGE and by gel mobility shift assay.

DNA-binding assays. For mobility shift DNA-binding assays, in vitro translation products or rat liver nuclear extracts were incubated in a 20-μl reaction mixture containing 20 mM Tris (pH 7.6), 10% glycerol, 40 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 1 μg of poly(dI-dC), and 0.5 ng (10,000 to 20,000 cpm) of labeled synthetic oligonucleotide II (Fig. 1). Free DNA and DNA-protein complexes were resolved on a 4% polyacrylamide gel in 25 mM Tris–190 mM glycine buffer, pH 8.3. For the antibody-binding assay, antibody or preimmune serum was added after each component was mixed. For competition experiments, 2 to 8 ng of unlabeled oligonucleotide was added prior to the addition of in vitro translation products.

Northern (RNA) blot analysis. The AGP/EBP probe corresponding to the insert of clone 1e was prepared by the random hexamer priming method (14). For Northern blot analysis, 1 to 2 μg of poly(A)$^+$ RNA or 20 μg of total RNA from each tissue or cell line was size fractionated on a 1% agarose–2.2 M formaldehyde gel, transferred to a nitrocellulose membrane, and hybridized with ^{32}P-labeled AGP/EBP probe overnight. The quantity and integrity of the RNA loaded were subsequently probed with the housekeeping gene probe GAPDH.

Primer extension analysis. An oligonucleotide (5′-CAGCA GGCGGTGCATGA-3′) complementary to the mRNA of AGP/EBP was end labeled by [^{32}P]rATP and T4 polynucleotide kinase. The labeled primer was annealed to 2 μg of poly(A)$^+$ RNA from mouse liver or p388D1 cells in 20 μl of buffer containing 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), 1 mM EDTA, 400 mM NaCl, and 60% formamide. The reaction mixtures were covered

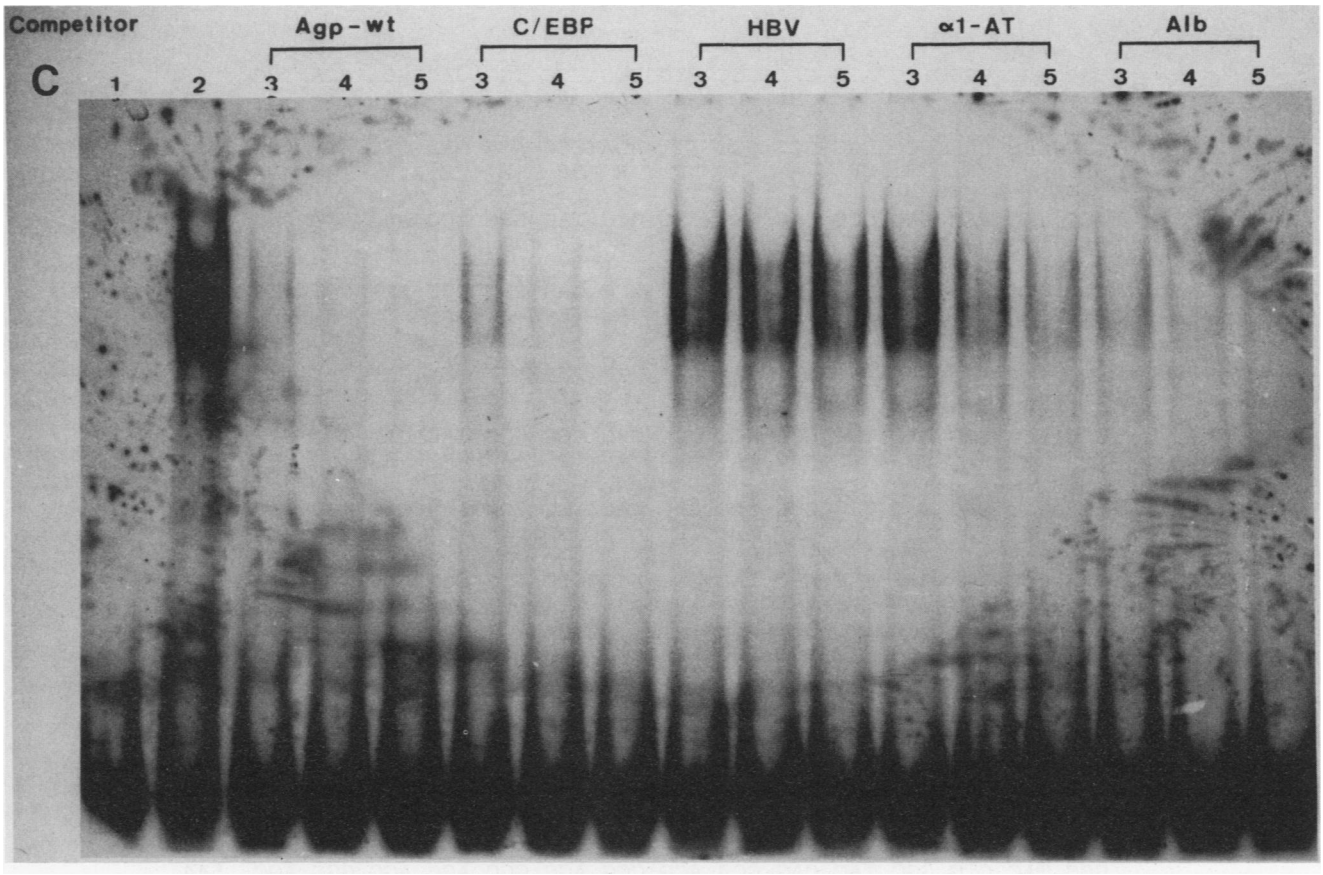

FIG. 2—*Continued.*

with 20 μl of mineral oil, heated to 85°C for 10 min, and then incubated at 42°C for 12 h. The mixtures were precipitated with ethanol, and the pellet was dried. The pellet was then suspended in a reaction mixture of 20 μl containing 4 μl of 5× reverse transcription buffer, 4 μl of 0.1 M dithiothreitol, 1.5 μl (2 μg/μl) of dactinomycin, 4 μl of 2.5 mM dNTPs, and 40 U of mouse mammary tumor virus reverse transcriptase. The mixtures were incubated at 42°C for 1 h and then precipitated with ethanol. The pellet was suspended in a sequencing loading buffer and analyzed with an 8% sequencing gel.

RESULTS

Identification of factor-binding sites in AGP that are related to the C/EBP recognition sequences. We investigated the AGP (Fig. 2A)-binding activities present in rat liver nuclear extract by using DNase I footprinting assays. The sequences spanning from −121 to −70 were the most prominent region protected from nucleolytic attack (Fig. 2B). By using the preparation of crude nuclear extract, the precise boundaries of individual binding sites could not be determined. The analysis of nuclear factors interacting in the region of −121 to −70 was chosen as our primary goal. At least two factors interacted with the sequence −121 to −70. Two oligonucleotides (I and II) corresponding to the regions of −97 to −70 and −121 to −93 were synthesized (Fig. 1) and used for a protection competition experiment. Oligonucleotide I could compete for the binding of factor A, while oligonucleotide II could block the binding of both factors A and B (Fig. 2B). Oligonucleotide II competed much more effectively than oligonucleotide I for the factor A (Fig. 2B). Substantial sequence homology between the core sequences of oligonucleotides I and II has been noted. By examination of the sequence of oligonucleotide II, we found that it contained an apparent dyad symmetric sequence, 5′-TTTGCGCAAG-3′, and a closely related motif, 5′-ATTTCCCAAG-3′, similar to the one recognized by C/EBP (1), while oligonucleotide I contained the sequence 5′-ATTGTGCCACAGCT-3′.

It has been reported (10, 11, 18) that a liver-specific DNA-binding protein recognizes multiple nucleotide sites in regulatory regions of transthyretin, α_1-antitrypsin, albumin, and simian virus 40 genes. To identify the core motif recognized by AGP/EBP-containing nuclear factors from rat liver and p388D1 cells, synthetic oligonucleotides from different genes (Fig. 1) were used for competition in mobility shift assays. For the labeled probe derived from synthetic oligonucleotide II (Agp-wt), the unlabeled Agp-wt competed most effectively, followed by those derived from C/EBP and Alb. α1AT and hepatitis B virus competed relatively poorly (Fig. 2C). These results suggest that C/EBP or C/EBP-like factors could bind to two regions (−85 to −70 and −121 to −93) of the AGP gene. The relative affinities for these three motifs included in the two regions were different. The motif 5′-TTTGCGCAAG-3′ (−115 to −106) is closer to the dyad-symmetric sequence (5′-ATTGCGCAAT-3′) and thus has higher binding affinity for the factor than the motif 5′-ATTGTGCCAC-3′ (−83 to −74) does. This and the results

a

```
                    1
CCGCGGGCCCGCGTTC ATG CAC CGC CTG CTG GCC TGG GAC GCA GCA TGC   49
                 M   H   R   L   L   A   W   D   A   A   C   11

CTC CCG CCG CCG CCC GCC GCC TTT AGA CCC ATG GAA GTG GCC AAC   94
 L   P   P   P   P   A   A   F   R   P   M   E   V   A   N   26

TTC TAC TAC GAG CCC GAC TGC CTG GCC TAC GGG GCC AAG GCG GCC  139
 F   Y   Y   E   P   D   C   L   A   Y   G   A   K   A   A   41

CGC GCC GCG CCG CGC GCC CCC GCC GCC GAG CCG GCC ATT GGC GAG  184
 R   A   A   P   R   A   P   A   A   E   P   A   I   G   E   56

CAC GAG CGC GCC ATC GAC TTC AGC CCC TAC CTG GAG CCG CTC GCG  229
 H   E   R   A   I   D   F   S   P   Y   L   E   P   L   A   71

CCC GCC GCG GAC TTC GCC GCG CCC GCG CCC GCG CAC CAC GAC TTC  276
 P   A   A   D   F   A   A   P   A   P   A   H   H   D   F   86

CTC TCC GAC CTC TTC GCC GAC GAC TAC GGC GCC AAG CCG AGC AAG  319
 L   S   D   L   F   A   D   D   Y   G   A   K   P   S   K  101

AAG CCG GCC GAC TAC GGT TAC GTG AGC CTC GGC CGC GCG GGC GCC  364
 K   P   A   D   Y   G   Y   V   S   L   G   R   A   G   A  116

AAG GCC GCG CCG CCC GCC TGC TTT CCG CCG CCG CCT CCC GCC GCG  409
 K   A   A   P   P   A   C   F   P   P   P   P   P   A   A  131

CTC AAG GCG GAG CCG GGC TTC GAA CCC GCG GAC TGC AAG CGC GCG  454
 L   K   A   E   P   G   F   E   P   A   D   C   K   R   A  146

GAC GAC GCG CCC GCC ATG GCG GCC GGT TTC CCG TTC GCC CTG CGC  499
 D   D   A   P   A   M   A   A   G   F   P   F   A   L   R  161

GCC TAC CTG GGC TAC CAG GCG ACG CCG AGC GGC AGC AGC GGC AGC  544
 A   Y   L   G   Y   Q   A   T   P   S   G   S   S   G   S  176

CTG TCC ACG TCG TCG TCG TCC AGC CCG CCC GGC ACG CCG AGC CCC  589
 L   S   T   S   S   S   S   S   P   P   G   T   P   S   P  191

GCC GAC GCC AAG GCC GCG CCC GCC GCC TGC TTC GCG GGG CCG CCG  634
 A   D   A   K   A   A   P   A   A   C   F   A   G   P   P  206

GCC GCG CCC GCC AAG GCC AAG GCC AAG AAG ACG GTG GAC AAG CTG  679
 A   A   P   A   K   A   K   A   K   K   T   V   D   K   L  221

AGC GAC GAG TAC AAG ATG CGG CGC GAG CGC AAC AAC ATC GCG GTG  724
 S   D   E   Y   K   M   R   R   E   R   N   N   I   A   V  236

CGC AAG AGC CGC GAC AAG GCC AAG ATG CGC AAC CTG GAG ACG CAG  769
 R   K   S   R   D   K   A   K   M   R   N   L   E   T   Q  251

CAC AAG GTG CTG GAG CTG ACG GCG GAG AAC GAG CGG CTG CAG AAG  814
 H   K   V   L   E   L   T   A   E   N   E   R   L   Q   K  266

AAG GTG GAG CAG CTG TCG CGA GAG CTC AGC ACC CTG CGG AAC TTG  859
 K   V   E   Q   L   S   R   E   L   S   T   L   R   N   L  281

TTC AAG CAG CTG CCC GAG CCG CTG CTG GCC TCG GCG GGC CAC TGC  904
 F   K   Q   L   P   E   P   L   L   A   S   A   G   H   C  296

TAGCGCGGCGCGGTGGCGTGGGGGGCGCCGCGGCCACCGTGCGCCCTGCCCCGCGCGCT  963
CCGGCCCCGCGCGCGCGCCCGGACCACCGTGCGTGCCCTGCGCGCACCTGCACCTGCAC 1022
CGAGGGGACACCGCGGGCACACCGCGGGCACGCGCGGCGCACGCACCTGCACAGCGCAC 1081
CGGGTTTCGGGACTTGATGCAATCCGGATCAAACGTGGCTGAGCGCGTGTGGACACGGG 1140
ACTACGCAACACACGTGTAACTGTCTAGCCGGGCCCTGAGTAATCACCTTAAAGATGTT 1199
CCTGCGGGGTTGTTGATGTTTTTGGTTTTGTTTTTGTTTTTTGTTTTGTTTTGTTTTTT 1258
TTTTTGGTCTTATTATTTTTTTTTGTATTATATAAAAAAGTTCTATTTCTATGAGAAAAG 1317
AGGCGTATGTATATTTTGAGAACCTTTTCCGTTTCGAGCATTAAAGTGAAGACATTTTA 1376
ATAAACTTTTTTGGAGAATGTTTAAAAGCCAAAAAAAAAA                     1416
```

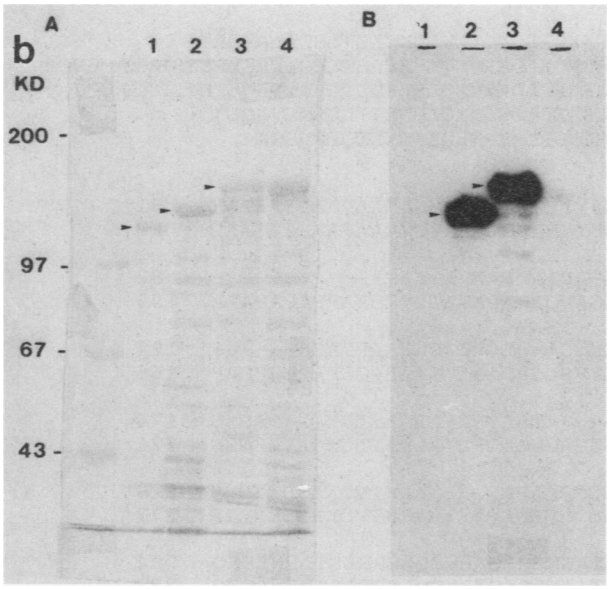

FIG. 3. (a) Nucleotide sequence of mouse AGP/EBP and deduced amino acid sequence of AGP/EBP protein. The DNA and deduced amino acid sequences of the AGP/EBP derived from the λgt11-1e clone (arrowhead) and the λgt11-1e-7 clone. The numbering system at the right of the sequence begins for both nucleotide and peptide sequences at the putative initiation codon ATG (labeled 1 over the underlined Met). The position of consensus polyadenylation hexamer AATAAA is underlined. (b) SDS-PAGE and Southwestern blot analysis of the β-galactosidase fusion protein specified by λgt-11 recombinant 1e, 1e-7, and *Pst*I-truncated 1e-7. (A) Coomassie blue staining patterns of SDS gel-separated proteins that were extracted from *E. coli* cells infected with λgt11 (lane 1), 1e (lane 2), 1e-7 (lane 3), or *Pst*I-truncated 1e-7 (lane 4) recombinant. KD, Kilodaltons. (B) Results of Southwestern blot of the same proteins that were visualized in panel A. Protein derived from the *Pst*I-truncated 1e-7 bound to the probe extremely poorly.

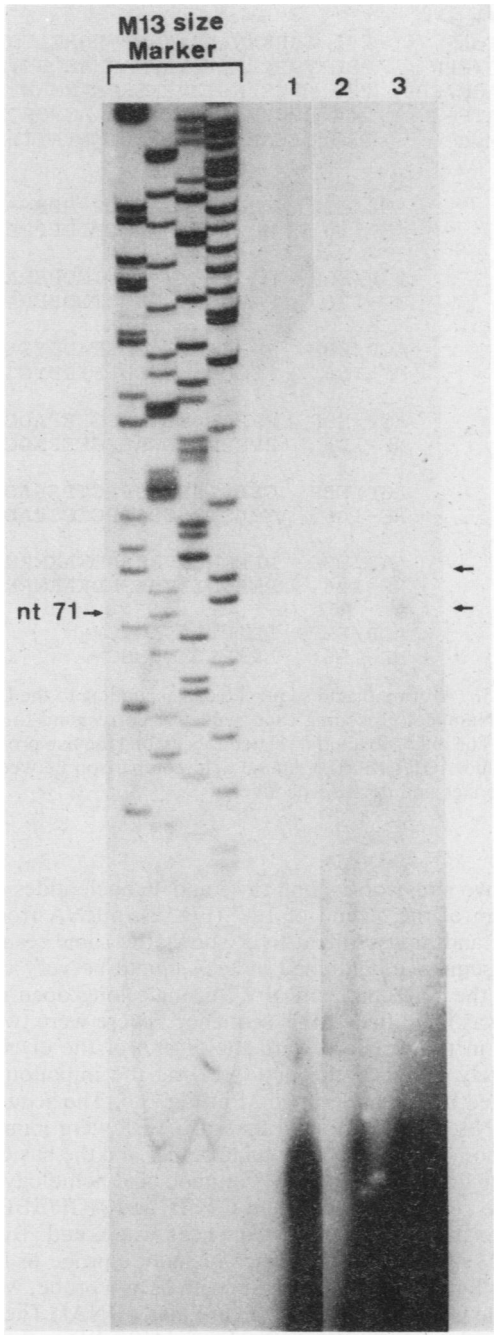

FIG. 4. Determination of transcription initiation site of AGP/EBP. Primer extension was conducted with ^{32}P-labeled primer (30 ng) and 2 μg of poly(A)$^+$ RNA from either mouse liver (lane 2) or p388D1 (lane 3) cells. The control consisted of the reaction mixture without exogenous RNA added (lane 1). An M13mp18 sequencing ladder was used as the size marker. The arrows indicate two transcription initiation sites located 43 and 46 nucleotides from the 5′ end of 1e-7. nt, Nucleotides.

from the footprinting competition indicate that factors A and B are related to each other in terms of sequence specificity.

Molecular cloning of the gene encoding AGP/EBP. By direct screening of a λgt11 library with a specific DNA sequence as a ligand, cDNA clones encoding Oct-2 and C/EBP have been obtained (39, 41). By employing a similar strategy, it should be possible to isolate clones encoding factors that interact with the promoter region of the AGP gene. Initial screening assays were carried out with synthetic oligonucleotide II probe. Rat liver nuclear extract was incubated with the membrane prior to incubation with the probe. We reasoned that if any protein-protein interactions increased the binding activity of the probe, it should be easier to pick up the signal by this treatment. A clone (1e) that expressed the DNA-binding domain of the factor binding to the B region was obtained.

A 0.9-kb cDNA insert was isolated from the 1e/λgt11 recombinant. From the nucleotide sequence analysis (Fig. 3a, started at nucleotide 539, as indicated by arrow) and the predicted amino acid sequence, a typical "leucine zipper" and the adjacent basic amino acid-rich domains were identified. The 3′-untranslated region of this cDNA consists of 502 nucleotides.

Using the insert of 1e as a hybridization probe, we obtained several additional clones from mouse liver and p388D1 cDNA libraries constructed in λgt10. The insert of one of these clones, termed 1e-7, was 1.4 kb in length and terminated with a poly(A) tail. DNA sequence analysis indicated that 1e-7 shares an identical sequence with 1e except that 1e-7 has an additional 538 bp of the 5′ sequence (Fig. 3a). Northern blot analysis (presented below) showed the AGP/EBP mRNA to be 1.6 kb in length. Two transcription initiation sites have been detected by primer extension.

FIG. 5. (A) Amino acid sequence corresponding to the DNA-binding and leucine zipper domains of v-Fos, C/EBP, AGP/EBP, ATF, and c-Myc. Numbers preceding each sequence correspond to the distance, in amino acid residues, from the NH_2 terminus of the respective protein. The only substantial relatedness among the five proteins is a heptad repeat of leucine spanning the region that is most similar between C/EBP and AGP/EBP. (B) Amino acid comparison between AGP/EBP and NF-IL6. Identical and conserved amino acids are marked by shaded boxes and dots, respectively.

These two sites correspond to 43 and 46 nucleotides (Fig. 4) upstream of the 5′ end of 1e-7 (Fig. 2). mRNA from both p388D1 and mouse or rat liver shows the same result. The cDNA sequence contained in 1e-7 should be very close to that of the full-length mRNA. A single long open reading frame was identified in this sequence. There were two ATG codons in this frame toward the 5′ end of the cDNA. We tentatively assigned the first ATG as the initiation codon (indicated by the underlined M in Fig. 3a). The sequence of this cDNA (AGP/EBP) and that of C/EBP were found to be highly homologous in the leucine zipper and the basic amino acid-rich domains, sharing 87% amino acid homology. However, no relationship between C/EBP and AGP/EBP in the 3′-untranslated and 5′-end sequences was noted. By direct binding screening with an oligonucleotide probe or by subsequent hybridization screening with 1e as a probe, we failed to isolate C/EBP or other closely related cDNAs. The spatial distribution of basic amino acids of AGP/EBP in the region that encompasses its DNA-binding domain and part of the leucine zipper domain was very similar to that of C/EBP.

Infection of Y1089 bacterial cells with 1e and 1e-7 resulted in the synthesis of a β-galactosidase fusion protein that migrated more slowly in SDS-PAGE than the form specified by the parental λgt11. The fusion polypeptides specified by 1e and 1e-7 were estimated to exceed the mass of its λgt11 counterpart by 13 and 33 kDa (Fig. 3b). Proteins from infected Y1089 were partially purified, sized by SDS-PAGE, and transferred to nitrocellulose membranes. The filters were exposed to end-labeled oligonucleotide II (Fig. 3b). When 1e-7 was truncated with *Pst*I and the resulting fragment (3′ deleted and thus lacking the leucine zipper domain) was ligated to λgt11, the expressed protein (Fig. 3b) could not bind to the oligonucleotide probe in a Southwestern blot (Fig. 3b). Thus, the leucine zipper domain is critical for DNA binding.

Sequence comparison. To investigate the conservation of amino acid sequences in other leucine-zipper-containing genes, we compared the sequences of c-Myc, v-Fos, C/EBP, AGP/EBP, and ATF (12, 19, 42). The results are shown in Fig. 5A. AGP/EBP and C/EBP are more closely related to each other than c-Myc and v-Fos are. However, when we compared the protein sequences of AGP/EBP and NF-IL6 (2), it was clear that these two proteins have identical sequences in the basic region and the leucine zipper domain (Fig. 5B). The sequences outside these domains showed some variations. Notably, three stretches of proline-rich region and a stretch of glycine-rich region in NF-IL6 were missing in AGP/EBP. The AGP/EBP gene may be a mouse homolog of the NF-IL6 gene, or these two genes may be more closely related than other members (e.g., C/EBP) of the C/EBP family. We will address this point in the Discussion.

Characterization of clone 1e-7. The partially purified proteins were tested for AGP promoter-binding activity by DNase I footprinting assays. Proteins from 1e-infected Y1089 cells contained an activity that bound to regions of AGP promoter at both −121 to −93 and −85 to −70 (Fig. 6A). Side-by-side comparisons showed no qualitative differences between the footprint produced by rat liver nuclear extract and that specified by the 1e phage in the regions −121 to −93 and −85 to −70 (Fig. 6A).

Nuclear extracts (14 μg) were treated by heating at 80°C. Untreated samples were used as controls. Both oligonucleotides I and II (Fig. 1) were used as probes for a gel mobility shift assay. AGP/EBP was stable upon heating at 80°C. Both oligonucleotides I and II could be bound by AGP/EBP.

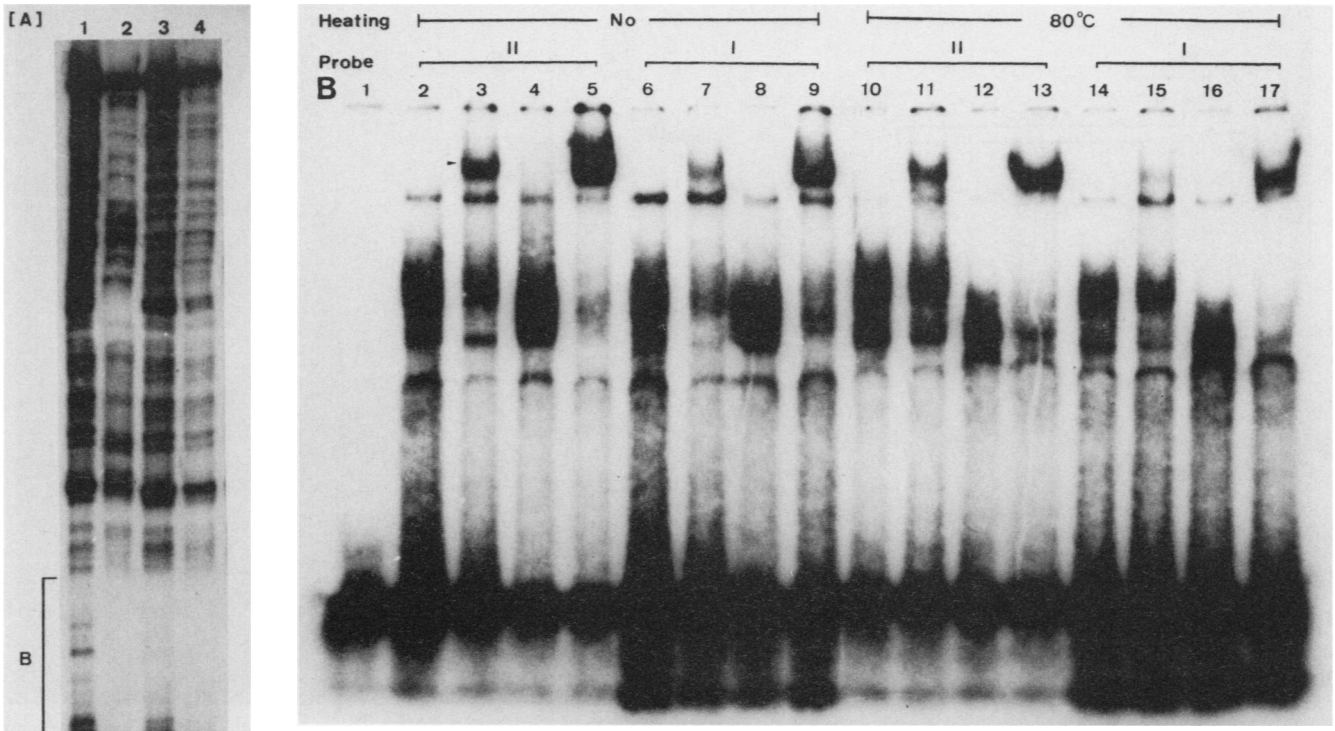

FIG. 6. A. DNase I footprint analysis of β-galactosidase fusion protein specified by λgt11 recombinant 1e. DNase I digestion patterns generated on a fragment (470 bp) from the AGP-1 promoter in the absence (lane 1) or presence of 7 and 14 μg (lanes 3 and 4) of rat liver nuclear extract or β-galactosidase fusion protein from 1e (lane 2). Regions A and B correspond to positions −70 to −85 and −93 to −121 of the AGP-1 promoter, respectively. (B) Heat stability and antigenic cross-reactivity of AGP/EBP from rat liver and p388D1 cells. Nuclear extracts from liver and p388D1 cells were heated at 80°C for 7 min, and the supernatant was saved while the pellets were reextracted with nuclear dialysis buffer. The combined supernatant (adjusted to the original volume of the nuclear extract) was assayed in a gel mobility shift assay with oligonucleotide I (lanes 6 to 9 and 14 to 17) and oligonucleotide II (lanes 2 to 5 and 10 to 13) probes. Lanes 2 to 9, Unheated controls; lanes 10 to 17, heated (80°C) samples; lanes 2, 4, 6, 8, 10, 12, 14, and 16, preimmune serum controls; lanes 3, 5, 7, 9, 11, 13, 15, and 17, anti-AGP/EBP-treated samples; lane 1, no nuclear extract; lanes 2, 3, 6, 7, 10, 11, 14, and 15, liver nuclear extracts; lanes 4, 5, 8, 9, 12, 13, 16, and 17, p388D1 nuclear extracts.

Furthermore, rabbit antibodies produced from the purified fusion protein of β-galactosidase and AGP/EBP (from 1e) could recognize AGP/EBP in rat liver and p388D1 nuclear extracts (Fig. 6B). These data suggested that the cDNA is corresponding to the mRNA encoding AGP/EBP.

AGP/EBP produced by in vitro transcription and translation behaved similarly to that from liver nuclear extract. To further test the authenticity of the cloned cDNA in AGP/EBP, the cDNA of 1e-7 was subcloned into pGEM4 and the resultant plasmid (CL-1) (Fig. 7A, top) was transcribed in vitro with SP6 RNA polymerase. The in vitro-transcribed RNA was then used for in vitro translation with rabbit reticulocyte lysate. The in vitro-translated AGP/EBP had a molecular mass of approximately 35 kDa (Fig. 7A).

Furthermore, the in vitro-translated AGP/EBP could bind to the oligonucleotide probe which was also recognized by AGP/EBP from nuclear extract. AGP/EBP produced by in vitro translation could result in a retarded band in the presence of anti-AGP/EBP antibodies but not of preimmune sera in the gel mobility shift assay (Fig. 7B). The binding of in vitro-translated AGP/EBP could specifically be inhibited with unlabeled oligonucleotide. Mutated oligonucleotide competed very poorly (Fig. 7B).

Expression of AGP/EBP mRNA in cells and tissues. The expression of AGP/EBP in different cells and tissues was investigated by Northern blot analysis with a 0.9-kb cDNA fragment (derived from 1e) as a probe. Total RNA was isolated from various cells and tissues. mRNA of 1.6 kb was observed in every sample. By comparing the same amount of total RNA (10 μg) from various tissues and cells, we found that testis tissue had the lowest level of expression among

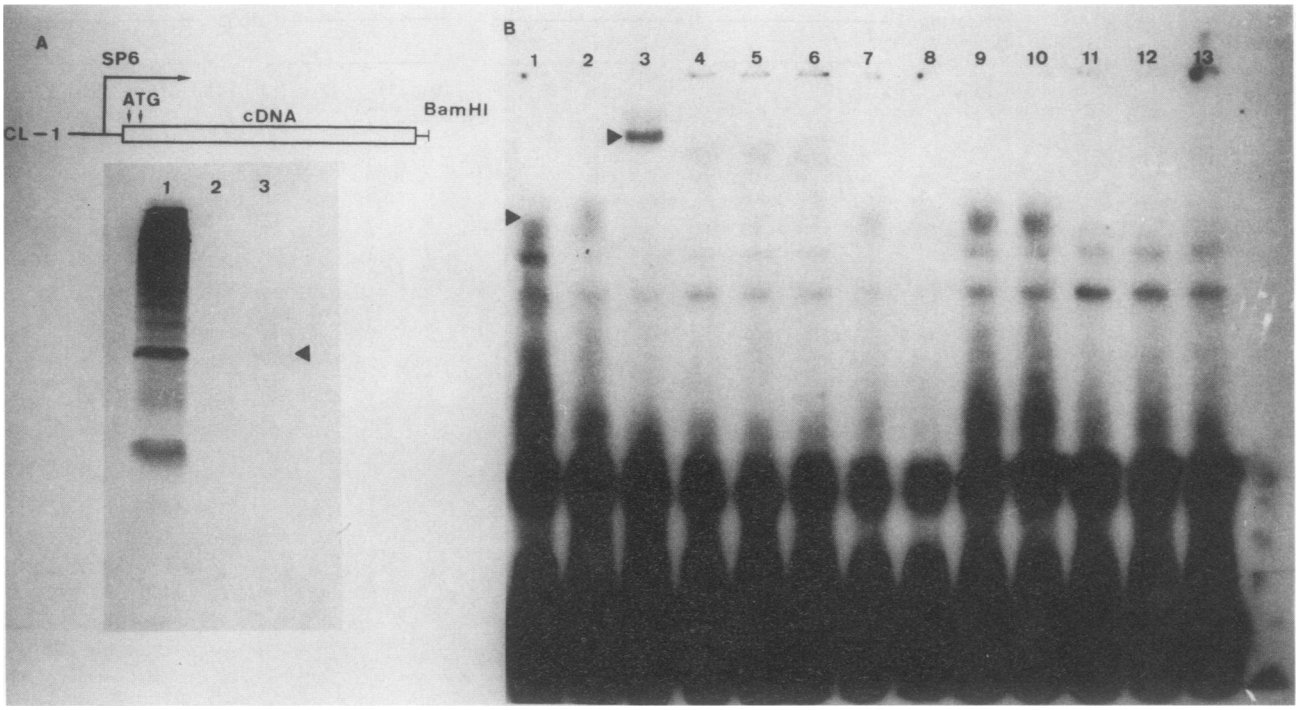

FIG. 7. In vitro expression of AGP/EBP cDNA. (A) Analysis of 1e-7 cDNA in vitro translation product by SDS-PAGE. A vector (CL-1) containing the AGP/EBP cDNA insert (1 to 1416) into pGEM4 under the control of the SP-6 promoter was constructed (top). In vitro [^{35}S] methionine-labeled translation products generated with rabbit reticulocyte lysate with SP-6 RNA polymerase-transcribed capped AGP/EBP transcripts, with CL-1 as a template (lane 3), were separated on a 15% polyacrylamide-SDS gel. The autoradiograph exposure was for 15 h. Brome mosaic virus RNA was translated in a separate reaction and used as a positive control for in vitro translation (lane 1). In vitro translation in the absence of any exogenous RNA was used a negative control (lane 2). (B) Gel mobility shift assay with an oligonucleotide II probe (Fig. 1). CL-1 in vitro translated-protein (lanes 1 to 3 and 7 to 10) was assayed for binding to 0.5 to 1.0 ng (10,000 to 20,000 cpm) of oligonucleotide II probe in the presence of preimmune serum (lane 2), anti-AGP/EBP antibody (lane 3), and 20 and 50 M excesses of unlabeled oligonucleotide II (lanes 7 and 8, respectively) (Fig. 1) and the mutated oligonucleotide II (lanes 9 and 10). Lanes contained in vitro-translated products containing no exogenous RNA (in the absence [lane 11] or presence [lane 12] of preimmune serum or anti-AGP/EBP antibody [lane 13]) or Brome mosaic virus RNA (in the absence [lane 4] or presence [lane 5] of preimmune serum or anti-AGP/EBP antibody [lane 6]).

six tissues checked (Fig. 8A) while 129P and FO cells had lower levels of AGP/EBP mRNA than L929 and p388D1 cells did (Fig. 8B).

Furthermore, AGP/EBP expression from different rat tissues and other cells was compared in a gel mobility shift assay with anti-AGP/EBP antibodies. Nuclear extracts from rat liver, kidney, lung, and brain cells, HeLa cells, and p388D1 cells were incubated with probe (detailed in the legend to Fig. 8 and Materials and Methods) and then reacted with preimmune serum (1 μl) or specific antiserum (1 μl) (Fig. 8C).

Taken together, these data indicate that AGP/EBP is expressed in tissues and cell lines that we have checked.

DISCUSSION

Comparison of AGP/EBP, C/EBP, DBP, NF-IL6, and other related transcription factors. We have isolated the cDNA for a ubiquitously existing transcription factor that binds to three sites of the AGP promoter. One of the motifs, −115 to −106, was shown to be the same as the high-affinity binding motif for C/EBP (1). The binding specificities of AGP/EBP, C/EBP, DBP (32), and NF-IL6 (2) are very similar. In view of the common DNA-binding specificity of AGP/EBP, C/EBP, NF-IL6, and DBP, it was interesting to compare the primary structures of these four proteins. In AGP/EBP, C/EBP, and NF-IL6, two distinct adjacent domains, a basic region and a leucine zipper, are located at the C-terminal region of these proteins. Both domains are required for DNA binding. While the leucine zipper mediates protein dimerization, the basic region has been proposed to contact DNA directly (26). The primary structures of AGP/EBP and NF-IL6 are identical in the DNA-binding and basic region domains while highly homologous outside of these domains (Fig. 5B). The predicted amino acid sequences of NF-IL6 and AGP/EBP contain 345 and 296 amino acids, respectively. Several blocks of amino acid deletions and substitutions were observed when the NF-IL6 and AGP/EBP sequences were compared. We suspect that AGP/EBP is the mouse homolog of NF-IL6. We will return to this point later in this discussion. In contrast to the identity in the basic region and leucine zipper domain of AGP/EBP and NF-IL6, the homology between AGP/EBP and C/EBP is about 87% within these domains. However, the sequences outside these domains are completely different. Therefore, it is likely that AGP/EBP and NF-IL6 are more closely related to each other than AGP/EBP and C/EBP are. These proteins belong to the C/EBP family. The combination of a basic region and a leucine zipper domain appears to be conserved among a variety of other regulatory proteins, including ATF, c-Myc, and v-Fos (12, 19, 42). The basic region of DBP exhibits

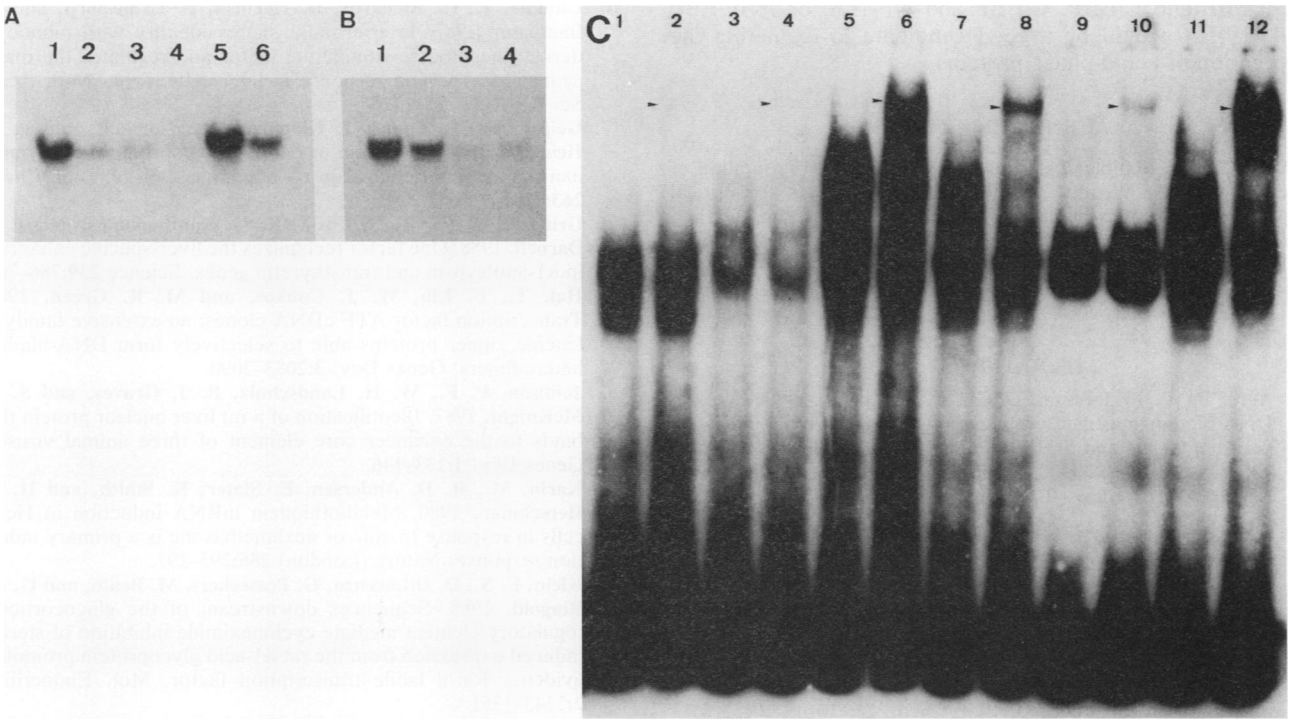

FIG. 8. (A) Northern blot analysis of RNA from mouse tissues. A 0.9-kb cDNA insert from 1e was used as a probe. Total RNA (10 μg) from mouse liver (lane 1), kidney (lane 2), spleen (lane 3), testis (lane 4), lung (lane 5), and heart (lane 6) was separated on 1% formaldehyde gel and blotted onto a nitrocellulose membrane. (B) Northern blot of 10 μg of total RNA derived from p388D1 (lane 1), L929 (lane 2), 129P (lane 3), and FO (lane 4) cells. (C) Tissue distribution of AGP/EBP. Specific antibody to AGP/EBP was used to detect the presence of AGP/EBP in different tissues and cells with oligonucleotide II probe (Fig. 1) in gel mobility shift assays. Lanes: 1 and 2, rat kidney (14 μg); 3 and 4, rat brain (6 μg); 5 and 6, p388D1 (14 μg); 7 and 8, rat lung (6 μg); 9 and 10, HeLa cells (14 μg); 11 and 12, rat liver (14 μg). Lanes 1, 3, 5, 7, 9, and 11, preimmune-serum controls; lanes 2, 4, 6, 8, 10, and 12, anti-AGP/EBP-treated samples.

significant sequence similarity with the analogous regions of these proteins (32). However, DBP is devoid of the leucine zipper present in these proteins (32).

Tissue-specific expression of AGP/EBP. Significant levels of a putative 1.6-kb AGP/EBP mRNA have been detected in mouse tissues and cell lines that we have checked. The protein of AGP/EBP has also been detected in these tissues and cells. This distinguishes AGP/EBP from C/EBP in that the level of expression of AGP/EBP in cell lines and tissues (e.g., lung tissue) is much higher than that of C/EBP. The expression of C/EBP gene, which was studied by a combination of Northern blot and antibody-staining techniques, revealed that C/EBP mRNA was detected in liver and fat cells but not found in spleen or brain cells (8, 43). Thus, AGP/EBP and other C/EBP-like factors (e.g., NF-IL6) may be involved in regulation of some other genes (i.e., nonliver genes) in other tissues. Unlike NF-IL6, which is not expressed in adult hepatocytes but is induced after lipopolysaccharide, IL-1, or IL-6 stimulation, AGP/EBP is expressed in significant levels in normal liver cells. AGP/EBP expression in mouse lung, liver, heart, kidney, and spleen but not in mouse testis is significantly elevated upon lipopolysaccharide treatment (S. C. Lee et al., unpublished results). The expression of AGP/EBP and C/EBP is similar in normal liver tissue but different in other tissues. In contrast, the level of expression of AGP/EBP and NF-IL6 is different in normal tissues but similar by induction with lipopolysaccharides. AGP/EBP and NF-IL6 may be more closely related than other members of the C/EBP family.

AGP/EBP may be involved in the expression of AGP and other acute-phase proteins. The expression of AGP is limited to liver cells or some hepatoma cells (17) stimulated with IL-1, while the expression of AGP/EBP is constitutive in these cells and other nonhepatocyte cells. Clearly, AGP/EBP is not the only factor responsible for AGP expression. The existence of three motifs that can be recognized by AGP/EBP and other C/EBP-like factors in the 5′-flanking region of the AGP gene may play some essential role in regulating the expression of AGP. The significance of the combinatorial effects of control elements was apparent in several examples in previous studies (28, 30, 35). In the case of three AGP/EBP-binding sites in the regulatory region of AGP gene, several possibilities for the interaction between protein factors and these sites emerged. (i) Homodimers of AGP/EBP are binding. (ii) Heterodimers of AGP/EBP and other C/EBP-like factors are binding. (iii) Some sites are occupied by homodimers, while others are occupied by heterodimers. (iv) Different sites are occupied by different sets of heterodimers. At least C/EBP and NF-IL6 have potential to form heterodimers with AGP/EBP. The diverged N-terminal sequences among AGP/EBP, C/EBP, and NF-IL6 suggest that once the putative heterodimers formed, they could play regulatory functions different from those of the homodimers, thus increasing the scope and effectiveness of the subtle regulatory mechanisms. The possibility of heterodimer formation between AGP/EBP or other C/EBP-like factors and DBP is uncertain. But since DBP is not a leucine zipper protein, it would be unlikely that heterodimer could be formed between DBP and C/EBP or DBP and AGP/EBP. Thus, the putative heterodimers formed between

AGP/EBP and C/EBP, C/EBP and NF-IL6, or AGP/EBP and NF-IL6 would be a good candidate in regulating the expression of acute-phase proteins.

ACKNOWLEDGMENTS

We thank U. Schibler for a detailed protocol for preparation of nuclear extracts. We are grateful to Mei-Shang Ho for critical reading of the manuscript and to Shu-Yang Yeh, Tso-Pang Yao, and Ru-Ju Chen for their excellent technical assistance.

This research was supported by grants NSC78-0412-B002-04 and NSC79-0412-B002-10 from the National Science Council, Republic of China.

LITERATURE CITED

1. **Agre, P., P. F. Johnson, and S. L. McKnight.** 1989. Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. Science **246**:922–926.
2. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9**:1897–1906.
3. **Baumann, H., G. L. Firestone, T. L. Burgess, K. W. Gross, K. R. Yamamoto, and W. A. Held.** 1983. Dexamethasone regulation of α1-acid glycoprotein and other acute phase reactants in rat liver and hepatoma cells. J. Biol. Chem. **258**:563–570.
4. **Baumann, H., and W. A. Held.** 1981. Biosynthesis and hormone-regulated expression of secretory glycoproteins in rat liver and hepatoma cells. J. Biol. Chem. **256**:10145–10155.
5. **Baumann, H., W. A. Held, and F. G. Berger.** 1984. The acute phase response of mouse liver. Genetic analysis of the major acute phase reactants. J. Biol. Chem. **259**:566–573.
6. **Baumann, H., R. E. Hill, D. N. Sauder, and G. P. Jahreis.** 1986. Regulation of major acute phase plasma proteins by hepatocyte-stimulating factors of human squamous carcinoma cells. J. Cell. Biol. **102**:370–383.
7. **Baumann, H., and L. E. Maquat.** 1986. Localization of DNA sequences involved in dexamethasone-dependent expression of the rat α_1-acid glycoprotein gene. Mol. Cell. Biol. **6**:2551–2561.
8. **Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKnight.** 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev. **3**:1146–1156.
9. **Chang, C. J., J. H. Lin, T. P. Yao, and S. C. Lee.** 1990. Regulation of expression of mouse α1-acid glycoprotein (AGP) genes, p. 273–284. *In* F. Y. H. Wu and C. W. Wu (ed.), Structure and function of nucleic acids and proteins. Raven Press, New York.
10. **Costa, R. H., D. R. Grayson, K. G. Xanthopoulos, and J. E. Darnell.** 1988. A liver-specific DNA-binding protein recognizes multiple nucleotide sites in regulatory regions of transthyretin, α1-antitrypsin, albumin, and simian virus 40 genes. Proc. Natl. Acad. Sci. USA **85**:3840–3844.
11. **Courtois, G., J. G. Morgan, L. A. Campbell, G. Fourel, and G. R. Crabtree.** 1987. Interaction of a liver-specific nuclear factor with the fibrinogen and α1-antitrypsin promoters. Science **238**:688–692.
12. **Curran, T., G. Peters, C. Van Beveren, N. Teich, and I. Verma.** 1982. FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. J. Virol. **44**:674–682.
13. **Darlington, G. J., D. R. Wilson, and L. B. Lachman.** 1986. Monocyte-conditioned medium, interleukin-1, and tumor necrosis factor stimulate the acute phase response in human hepatoma cells in vitro. J. Cell. Biol. **103**:787–793.
14. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
15. **Galas, D., and A. Schmitz.** 1978. DNAase footprinting: a simple method for the detection of protein-DNA-binding specificity. Nucleic Acids Res. **5**:3157–3170.
16. **Gauldie, J., C. Richards, D. Harnish, P. Landsdorp, and H. Baumann.** 1987. Interferon-β2 shares identity with monocyte derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc. Natl. Acad. Sci. USA **84**:7251–7255.
17. **Geiger, T., T. Andus, J. Klapproth, H. Northoff, and P. C. Heinrich.** 1988. Induction of α1-acid glycoprotein by recombinant human interleukin-1 in rat hepatoma cells. J. Biol. Chem. **263**:7141–7146.
18. **Grayson, D. R., R. H. Costa, K. G. Xanthopoulos, and J. E. Darnell.** 1988. One factor recognizes the liver-specific enhancers in α1-antitrypsin and transthyretin genes. Science **239**:786–788.
19. **Hai, T., F. Liu, W. J. Coukos, and M. R. Green.** 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. **3**:2083–2090.
20. **Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight.** 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1**:133–146.
21. **Karin, M., R. D. Andersen, E. Slater, K. Smith, and H. R. Herschman.** 1980. Metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction response. Nature (London) **286**:295–297.
22. **Klein, E. S., D. DiLorenzo, G. Posseckert, M. Beato, and G. M. Ringold.** 1988. Sequences downstream of the glucocorticoid regulatory element mediate cycloheximide inhibition of steroid induced expression from the rat α1-acid glycoprotein promoter: evidence for a labile transcription factor. Mol. Endocrinol. **2**:1343–1351.
23. **Klein, E. S., R. Reinke, P. Feigelson, and G. M. Ringold.** 1987. Glucocorticoid-regulated expression from the 5′-flanking region of the rat α1-acid glycoprotein gene. J. Biol. Chem. **262**:520–523.
24. **Kulkarni, A. B., R. Reinke, and P. Feigelson.** 1985. Acute phase mediators and glucocorticoids elevate α1-acid glycoprotein gene transcription. J. Biol. Chem. **260**:15386–15389.
25. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2**:786–800.
26. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1989. The binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243**:1681–1688.
27. **Lee, S. C., C. J. Chang, Y. M. Lee, H. Y. Lei, M. Y. Lai, and D. S. Chen.** 1989. Molecular cloning of cDNAs corresponding to two genes of α1-acid glycoprotein and characterization of two alleles of AGP-1 in the mouse. DNA **8**:245–251.
28. **Maire, P., J. Wuarin, and U. Schibler.** 1989. The role of cis-acting promoter elements in tissue-specific albumin gene expression. Science **244**:343–345.
29. **Miskimins, W. K., M. P. Roberts, A. McClelland, and F. H. Ruddle.** 1985. Use of a protein-blotting procedure and a specific DNA probe to identify nuclear proteins that recognize the promoter region of the transferrin receptor. Proc. Natl. Acad. Sci. USA **82**:6741–6744.
30. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
31. **Mortensen, R. F., J. Shapiro, B. F. Lin, S. Douches, and R. Neta.** 1988. Interaction of recombinant IL-1 and recombinant tumor necrosis factor in the induction of mouse acute phase proteins. J. Immunol. **140**:2260–2266.
32. **Mueller, C. R., P. Maire, and U. Schibler.** 1990. DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. Cell **61**:279–291.
33. **Peterkofsky, B., and G. M. Tomkins.** 1968. Evidence for the steroid induced accumulation of tyrosine amino transferase mRNA in the absence of protein synthesis. Proc. Natl. Acad. Sci. USA **60**:222–228.
34. **Prowse, K. R., and H. Baumann.** 1988. Hepatocyte-stimulating factor, β_2 interferon, and interleukin-1 enhance expression of the rat α_1-acid glycoprotein gene via a distal upstream regula-

tory region. Mol. Cell. Biol. **8**:42–51.
35. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. Nature (London) **335**:683–689.
36. **Ramadori, G., J. D. Sipe, C. A. Dinarello, S. B. Mizel, and H. R. Colten.** 1985. Pretranslational modulation of acute phase hepatic protein synthesis by murine recombinant interleukin 1 (IL-1) and purified human IL-1. J. Exp. Med. **162**:930–942.
37. **Ricca, G. A., R. W. Hamilton, J. W. McLean, A. Conn, J. E. Kallinyak, and J. M. Taylor.** 1981. Rat α1-acid glycoprotein mRNA. J. Biol. Chem. **256**:10362–10368.
38. **Ringold, G. M., K. R. Yamamoto, G. M. Tomkins, J. M. Bishop, and H. E. Varmus.** 1975. Dexamethasone-mediated induction of mouse mammary tumor virus RNA: a system for studying glucocorticoid action. Cell **6**:299–305.
39. **Singh, H., J. H. LeBowitz, A. S. Baldwin, and P. A. Sharp.** 1988. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. Cell **52**:415–423.
40. **Vannice, J. L., G. M. Ringold, J. W. McLean, and J. M. Taylor.** 1983. Induction of the acute phase reactant α1-acid glycoprotein by glucocorticoids in rat hepatoma cells. DNA **2**:205–212.
41. **Vinson, C. R., K. L. LaMarco, P. F. Johnson, W. H. Landschulz, and S. L. McKnight.** 1989. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. Genes Dev. **2**:801–806.
42. **Vinson, C. R., P. B. Sigler, and S. L. McKnight.** 1989. Scissor-grip model for DNA recognition by a family of leucine zipper proteins. Science **246**:911–916.
43. **Xanthopoulos, K., J. Mirkovitch, T. Decker, C. F. Kuo, and J. Darnell.** 1989. Cell-specific transcriptional control of the mouse DNA-binding protein mC/EBP. Proc. Natl. Acad. Sci. USA **86**:4117–4121.