The Role of HNF1α, HNF3γ, and Cyclic AMP in Glucose-6-phosphatase Gene Activation†

Baochuan Lin,‡,§ David W. Morris,§ and Janice Yang Chou*,‡

Heritable Disorders Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, and Department of Biological Sciences, George Washington University, Washington, DC 20052

Received February 12, 1997; Revised Manuscript Received August 29, 1997⊗

ABSTRACT: The gene for glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis, is expressed in a tissue-specific manner in the liver and kidney. To understand the molecular mechanisms regulating liver-specific expression of the *G6Pase* gene, we characterized *G6Pase* promoter activity by transient expression assays. The *G6Pase* promoter is active in HepG2 hepatoma cells, but inactive in JEG3 choriocarcinoma or 3T3 cells. DNA elements essential for optimal and liver-specific expression of the *G6Pase* gene were contained within nucleotides −234 to +3. Deletion analysis revealed that the *G6Pase* promoter contained three activation elements (AEs) at nucleotides −234 to −212 (AE-I), −146 to −125 (AE-II), and −124 to −71 (AE-III). AE-I contains binding sites for hepatocyte nuclear factors (HNF) 1 and 4. Electromobility shift and cotransfection assays demonstrated that HNF1α, but not HNF4, bound to its cognate site and transactivated *G6Pase* gene expression. The *G6Pase* promoter contained five HNF3 motifs, 1 (−180/−174), 2 (−139/−133), 3 (−91/−85), 4 (−81/−75), and 5 (−72/−66), and all five sites bound HNF3γ with high affinity. Transient expression and cotransfection assays showed that HNF3 site 1 is not required for basal promoter activity, but is essential for HNF3γ-activated transcription from the *G6Pase* promoter. We further showed that HNF3 sites 3, 4, and 5 were essential for basal *G6Pase* promoter activity and transactivation by HNF3γ. AE-II contains, in addition to a HNF3 motif, a cAMP-response element (CRE) and a C/EBP half-site. The G6Pase(−146/−116) DNA containing AE-II formed multiple protein−DNA complexes with HepG2 nuclear extracts, including HNF3γ, CRE-binding protein (CREB), C/EBPα, and C/EBPβ. We showed that AE-II mediated transcription activation of the *G6Pase* gene by cAMP.

Glucose-6-phosphatase (G6Pase, EC 3.1.3.9),[1] which catalyzes the terminal step in gluconeogenesis and glycogenolysis, is the key enzyme in glucose homeostasis (Nordlie & Sukalski, 1985). In humans, deficiency of G6Pase activity causes glycogen storage disease type 1a (GSD-1a), also known as von Gierke disease (Moses, 1990; Chen & Burchell, 1995). This autosomal recessive disorder is characterized by severe hypoglycemia, growth retardation, hepatomegaly, kidney enlargement, hyperlipidemia, hyperuricemia, and lactic acidemia (Moses, 1990; Chen & Burchell, 1995). Long-term complications include gout, hepatic adenomas, osteoporosis, and renal failure. In order to understand the biology and pathophysiology of GSD-1a, we have first characterized the mouse (Shelly et al., 1993) and human (Lei et al., 1993a) *G6Pase* cDNAs and genes. These studies revealed that mammalian G6Pases are hydrophobic proteins of 357 amino acids which contain 6 putative membrane-spanning segments. Human and mouse G6Pases are encoded by structurally similar genes containing five exons, spanning approximately 12 kb (Shelly et al., 1993; Lei et al., 1993a). We have also identified a large number of mutations in the *G6Pase* gene of GSD-1a patients and have shown that these mutations inactivate or greatly reduce G6Pase activity (Lei et al., 1993a, 1994, 1995a,b), thus establishing the molecular basis of this disorder.

Expression of the *G6Pase* gene occurs primarily in the liver and kidney (Nordlie & Sukalski, 1985). It has been demonstrated that liver-specific gene expression is regulated mainly at the transcriptional level (Johnson & Mcknight, 1989; Sladek & Darnell, 1992). A large number of liver-enriched transcription factors essential for the expression of liver genes have now been identified and characterized. These include hepatocyte nuclear factor (HNF) 1 (Frain et al., 1989; Baumhueter et al., 1990; Mendel & Crabtree, 1991), HNF3 (Costa et al., 1989; Lai et al., 1991, 1993), HNF4 (Costa et al., 1989; Sladek et al., 1990), and C/EBP (Johnson et al., 1987; Cao et al., 1991; Williams et al., 1991). HNF1, which belongs to the homeodomain family of DNA-binding proteins and binds to its cognate sequence as a dimer, is the dominant liver-enriched transcription factor (Frain et al., 1989; Baumhueter et al., 1990; Mendel & Crabtree, 1991). HNF3 belongs to the *forkhead* or winged helix family of transcription factors, which are characterized by a highly conserved region of about 100 amino acids shown to be necessary for monomeric recognition of specific DNA target sites (Lai et al., 1991, 1993). Three HNF3 members, α, β, and γ, were initially identified as transcription factors essential for liver-

† B.L. is supported in part by the Weintraub Fellowship.

* To whom correspondence should be addressed at Building 10, Room 9S241, NIH, Bethesda, MD 20892. Telephone: 301-496-1094. Fax: 301-402-7784.

‡ National Institutes of Health.

§ George Washington University.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1997.

[1] Abbreviations: G6Pase, glucose-6-phosphatase; GSD-1a, glycogen storage disease type 1a; HNF, hepatocyte nuclear factor; CRE, cAMP response element; CREB, CRE-binding protein; ATF, activating transcription factor; AE, activation element; CAT, chloramphenicol acetyltransferase.

S0006-2960(97)00324-3 CCC: $14.00

specific gene expression. HNF4 is a member of the steroid hormone receptor superfamily, resides exclusively in the nucleus, and binds to its recognition site only as a dimer (Sladek et al., 1990). The C/EBP family belongs to the bZIP class of transcription factors which contain a basic DNA-binding region adjacent to a leucine zipper dimerization domain (Cao et al., 1991; Williams et al., 1991). At least four C/EBP isoforms, C/EBPα, C/EBPβ, C/EBPγ, and C/EBPδ, have been identified.

G6Pase is an inducible enzyme, and *G6Pase* mRNA expression (Chou et al., 1991) and enzymatic activity (Greengard, 1969) can be increased by cAMP. The most common mechanism by which the second-messenger cAMP stimulates eukaryotic genes is by directing the protein kinase A-dependent phosphorylation of the transcription factor, cAMP-response element binding factor (CREB) (Montminy & Bilezikjian, 1987). CREB and related family members, including the activating transcription factor (ATF) subfamily, are leucine zipper class transcription factors which activate gene transcription in the form of homo- or heterodimers (Habener, 1990; Brindle & Montminy, 1992).

To understand the control of *G6Pase* expression, we undertook studies to identify and characterize cis-acting regulatory elements in the 5′-flanking region of the human *G6Pase* gene essential for its expression in human hepatoma cells. We identified three activation elements (AEs) within the nucleotide −234 to +3 region of the *G6Pase* promoter which are essential for optimal and liver-specific expression of the gene. HNF1α and HNF3γ are liver-enriched factors that bind to the *G6Pase* promoter and transactivate *G6Pase* expression. We also showed that cAMP stimulated *G6Pase* transcription through the CRE within AE-II.

MATERIALS AND METHODS

Construction of Promoter−CAT Fusion Genes. The *G6Pase* promoter−chloramphenicol acetyltransferase (CAT) fusion gene constructs were synthesized by PCR using a human *G6Pase* genomic clone (Lei et al., 1993a) as a template. The 3′ primer for the *G6Pase* 5′ deletion mutants consisted of nucleotides −21 to +3, and the 5′ primers consisted of nucleotides −1219 to −1200, −508 to −493, −245 to −228, −234 to −219, −211 to −194, −181 to −163, −146 to −131, −124 to −107, and −70 to −54. Each primer contained an additional *Hin*dIII or *Xba*I site at the 5′ end. After digestion with *Hin*dIII and *Xba*I, the amplified fragments were inserted upstream of the bacterial CAT gene of a modified promoter-less and enhancer-less pCAT-Basic-N plasmid (Lei et al., 1993b). Some of the constructs were inserted in the modified pCAT-Enhancer-N plasmid, which contains the SV40 enhancer (Lei et al., 1993b). Mutagenesis was carried out as described by Higuchi (1990). All constructs were verified by DNA sequencing. The pSVCAT, which contains both the SV40 enhancer and promoter, and pCAT-Basic-N or pCAT-Enhancer-N plasmids were used as positive and negative controls, respectively.

Transfection and CAT Assays. HepG2 human hepatoma cells (Aden et al., 1979), JEG3 human choriocarcinoma cells (Wada & Chou, 1993), or murine 3T3 cells were grown at 37 °C in α-modified minimal essential medium supplemented with 4% fetal bovine serum, streptomycin (100 mg/mL), and penicillin (100 units/mL). Cells in 25-cm^2 flasks were transfected with 10 μg of DNA by the calcium phosphate−DNA coprecipitate method as previously described (Lei et al., 1993b). To correct for transfection efficiency, pRSVLuc (de Wet et al., 1987) was cotransfected with the G6Pase plasmid DNA. The CAT activity was assayed by incubating total cellular protein in a buffer containing 250 mM Tris-HCl, pH 7.8, 4 mM acetylcoenzyme A, and 0.1 μCi of [^{14}C]chloramphenicol (Fordis & Howard, 1987). Routinely, the assay was run for 1 h with the amount of extract required to convert 0.5−50% of the substrate to the acetylated forms. Assays outside this range were repeated using the appropriate amount of extract. The acetylated compounds were separated from chloramphenicol by thin-layer chromatography (95% chloroform−5% methanol, v/v) on silica gel IB2 (Gilman Sciences). Spots were quantitated on an AMBIS Radio-analytic Imaging System (San Diego, CA). For luciferase assays, portions of freeze−thawed extracts were adjusted to 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, and 1% Triton X-100, and frozen at −70 °C. Luciferase activity was assayed in a buffer containing 25 mM glycyl-glycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 15 mM K_2HPO_4, pH 7.8, and 1 mM dithiothreitol (Lei et al., 1993b). The reaction was initiated by the addition of luciferin, and the light emission was monitored by a luminometer (Lumat LB9501, EG & G berthold, Natick, MA). Protein was estimated using the Micro BCA protein assay kit obtained from Pierce (Rockford, IL).

CAT activity was corrected either for protein alone or for both protein and luciferase activity. The relative CAT activities were essentially the same, regardless of the correction method. The CAT activity was determined for each construct in duplicate after transient transfection into HepG2 cells. At least three independent experiments were conducted with two preparations of each construct.

The HNF1α (Baumhueter et al., 1990) and HNF1β (Mendel et al., 1991) cDNAs were obtained from Drs. L. Hansen and G. R. Crabtree, HNF3α (Lai et al., 1991) cDNA was from Drs. W. S. Chen and J. E. Darnell, Jr., CREB cDNA (Montminy & Bilezikjian, 1987) was from Dr. M. R. Montiminy, and C/EBPα (Williams et al., 1991) and C/EBPβ (Williams et al., 1991) cDNAs were from Dr. P. F. Johnson. The HNF3γ cDNA was isolated from rat liver mRNA by RT-PCR using a First-Strand cDNA Synthesis Kit (Pharmacia Biotech) and oligonucleotide primers containing nucleotides 1−18 (5′-ATGCTGGGCTCAGTGAAG-3′) and 1065−1047 (5′-CTAGGATGCATTGAGCAG-3′) of the HNF3γ sequences (Lai et al., 1991). Each primer contained an additional *Sac*II or *Eco*RI linker at the 5′ end. The amplified fragment was subcloned into the pCR™II vector (Invitrogen, San Diego, CA) and confirmed by DNA sequencing. For cotransfection assays, the HNF3γ insert was released by *Eco*RI and subcloned into the pBJ5 expression vector (Kuo et al., 1990).

In Vitro Transcription and Translation. *In vitro* transcription−translation of HNF3α, HNF3γ, CREB, C/EBPα, and C/EBPβ cDNAs was performed using the TnT-coupled reticulocyte lysate system obtained from Promega Biotech.

Preparation of Nuclear Extracts. HepG2 nuclear extracts were prepared essentially as described by Ohlsson and Edlund (1986). Liver nuclear extracts were prepared by a modification of the method described by Lichtsteiner and Schibler (1989). Briefly, the pelleted nuclei were resuspended in a lysis buffer [10 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.6, 100

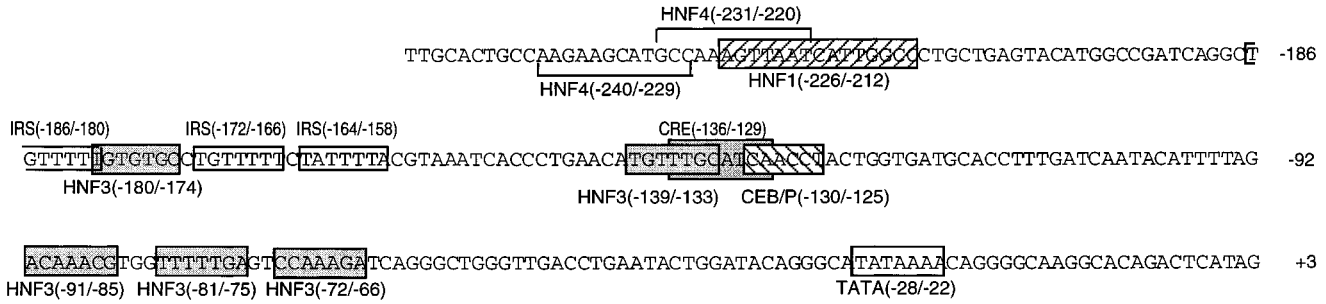

FIGURE 1: Nucleotides −250 to +3 of the 5′-flanking region of the human *G6Pase* gene. The numbers indicate the distance in nucleotides from the transcription start site (+1) of the *G6Pase* gene. The TATA box, IRS, and consensus sequences that bind to transcription factors, HNF1, HNF3, HNF4, CREB, or C/EBP are boxed and indicated.

mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 10% glycerol], and ammonium sulfate was added to a final concentration of 0.36 M. After gently stirring for 30 min at 4 °C, the homogenates were centrifuged at 100000*g* for 1 h at 4 °C. Then, ammonium sulfate was added to the supernatant to 0.25 g/mL. After 30 min at 4 °C, nuclear proteins were pelleted by centrifuging at 100000*g* for 15 min and resuspended and dialyzed against a buffer containing 25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol. The dialyzed nuclear extract was centrifuged at 10000*g* for 5 min, and aliquots of the supernatant solution were frozen and stored in a liquid N_2 freezer.

Electromobility Shift Assays. End-labeled oligonucleotide probes [0.2−1 ng; (0.5−1) × 10^5 cpm] were incubated for 20 min at room temperature in binding reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% NP-40, 1 mM EDTA, 0.5 mM DTT, and 10% glycerol) containing 1 μg of poly(dI-dC) and 3 μg of nuclear extract. Following binding, the mixture was electrophoresed through a 5% nondenaturing polyacrylamide gel in 1 × Tris−borate buffer, dried, and autoradiographed. For competition experiments, competitor DNA was incubated in the mixture prior to the addition of probe. For gel supershift or antibody inhibition assays, specific antisera were preincubated with nuclear extracts at 4 °C for 20 min before the addition of probe.

Two types of antisera, blocking and supershift, which were raised against the DNA-binding domain and non-DNA-binding domain of a transcription factor, respectively, were employed in this study. Antisera to HNF1α (supershift) were obtained from Drs. L. Hansen and G. R. Crabtree, antisera to HNF3α (supershift), HNF3β (blocking), and HNF3γ (blocking) were obtained from Drs. W. S. Chen and J. E. Darnell, Jr., antisera to HNF4 (supershift) were obtained from Dr. F. M. Sladek, and antisera to CREB (blocking), ATFs (supershift), C/EBPβα (blocking), and C/EBPβ (supershift) were obtained from Santa Cruz Biotechnology.

RESULTS

The 5′-Flanking Sequences Required for Liver-Specific Expression of the Human G6Pase Gene. The *G6Pase* gene is primarily expressed in the liver and kidney, and liver G6Pase plays a major role in glucose homeostasis (Nordlie & Sukalski, 1985). To delineate the DNA sequences responsible for liver-specific expression of the *G6Pase* gene, we examined *G6Pase* promoter activity by transient expression assays. We have sequenced nucleotides −1219 to −1 upstream of the transcription start site (+1) of the human *G6Pase* gene; the sequence is identical to that reported by

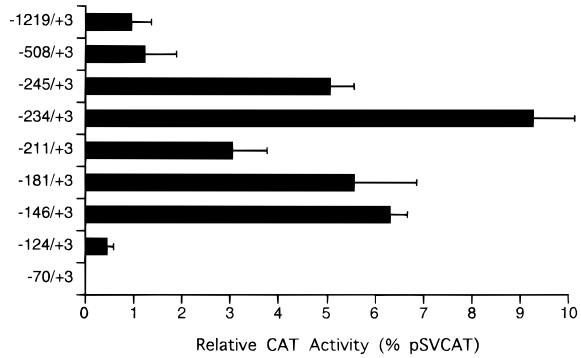

FIGURE 2: Promoter activity of the *G6Pase* 5′-flanking region. Nucleotides −1219/+3, −508/+3, -245/+3, −234/+3, −211/+3, −181/+3, −146/+3, −124/+3, or −70/+3 region 5′ of the transcription initiation site of the *G6Pase* gene were inserted upstream of a promoter-less and enhancer-less CAT gene (pCAT-Basic-N) (Lei et al., 1993b). The pSVCAT plasmid, which contains both the SV40 enhancer and promoter, and the pCAT-Basic-N plasmid were used as positive and negative controls, respectively. Specific CAT activities directed by the pSVCAT and pCAT-Basic-N plasmids were 10.6 and 0.01 nmol min^{-1} (mg of protein)$^{-1}$, respectively. At least three independent experiments were conducted with two preparations of each construct.

Schmoll et al. (1996). Sequence analysis predicts the presence of multiple transcription factor binding sites at the *G6Pase* 5′-flanking region with a putative TATA box located at nucleotides −28 to −22 (Figure 1). Motifs for liver-enriched transcription factors were clustered between nucleotides −250 and −1 (Figure 1). These included binding sites for HNF1 at nucleotides −226 to −212; for HNF3 at nucleotides −180 to −174 (site 1), −139 to −133 (site 2), −91 to −85 (site 3), −81 to −75 (site 4), and −72 to −66 (site 5); for HNF4 at nucleotides −240 to −229 and −231 to −220; and for CRE at nucleotides −136 to −129 (Figure 1). The HNF1 site overlapped the downstream HNF4 site, and HNF3 site 2 and the CRE site overlapped a C/EBP half-site at nucleotides −130 to 125 (Figure 1).

We generated *G6Pase* promoter−CAT fusion genes containing sequential 5′ deletions and examined their promoter activities after transient transfection into HepG2 human hepatoma cells as well as nonhepatic human JEG3 choriocarcinoma and mouse 3T3 cells. The *G6Pase* promoter−CAT constructs directed high levels of CAT activity in HepG2 cells (Figure 2). On the other hand, none of these *G6Pase* promoter−CAT constructs was able to direct CAT expression in choriocarcinoma or 3T3 cells. Although pSVCAT directed only low CAT expression in 3T3 cells [0.14 nmol min^{-1} (mg of protein)$^{-1}$], high specific CAT activity directed by pSVCAT was obtained in HepG2 [10.6 nmol min^{-1} (mg of protein)$^{-1}$] and JEG3 [6.5 nmol min^{-1}

(mg of protein)$^{-1}$] cells. This suggests that the inactivity of the *G6Pase* promoter in nonhepatic cells was not due to a difference in transfection efficiency.

In HepG2 cells, the G6Pase(−1219/+3)CAT construct exhibited a relatively low level of CAT activity, and CAT expression increased with *G6Pase* promoter−CAT fusion genes containing sequential 5′-deletions (Figure 2). Maximal CAT activity was achieved with a construct containing the −234 to +3 bp region of the *G6Pase* promoter. Our data suggested that the −508 to −246 and −245 to −235 bp regions contained negative regulatory elements that suppress *G6Pase* expression. Further deletion of nucleotides −234 to −212 markedly reduced CAT activity, indicating that this DNA region (−234 to −212, AE-I) contained cis-acting elements that positively regulate *G6Pase* expression (Figure 2). Indeed, binding sites for HNF1 (−226/−212) and HNF4 (−231/−220) were located within this region (Figure 1). Deletion of nucleotides −211 to −182 [G6Pase(−181/+3)-CAT] increased CAT expression, suggesting the presence of negative regulatory elements at this region (−211/−182). The G6Pase(−181/+3)CAT and G6Pase(−146/+3)CAT constructs expressed similar high promoter activity (Figure 2), suggesting that nucleotides −181 to −147 containing the HNF3 site 1 are not required for basal promoter activity. Further deletion of nucleotides −146 to −125 [G6Pase-(−124/+3)CAT] greatly reduced CAT expression, and the G6Pase(−70/+3)CAT construct directed little or no CAT expression (Figure 2). This analysis located two additional activation elements at nucleotides −146 to −125 (AE-II) and −124 to −71 (AE-III). AE-II contains a HNF3 (−139/−133, site 2), a CRE (−136/−126), and a half-C/EBP (−130/−125) site that overlap, and AE-III contains HNF3 site 3 (−91/−85), site 4 (−81/−75), and part of site 5 (−72/−66). Our data suggest that nucleotides −124 to +3 constitute a minimal *G6Pase* promoter. As controls, we constructed *G6Pase* promoter−CAT constructs in the reverse orientation, G6Pase(+3/−508)CAT and G6Pase(+3/−234)-CAT, and showed that these two plasmids directed little or no CAT expression (data not shown). An increase in CAT activity to 54.5% of the activity expressed by the G6Pase-(−234/+3)CAT was achieved after fusing a SV40 enhancer downstream of G6Pase(−70/+3)CAT, indicating the presence of basal promoter elements at the −70 to +3 bp region.

Characterization of Nuclear Proteins Binding to the G6Pase Promoter Elements. To characterize individual protein-binding sites within the *G6Pase* promoter, electromobility shift assays were performed using HepG2 or rat liver nuclear extracts and double-stranded synthetic oligos corresponding to the transcription activation regions. Specific protein−DNA interactions were identified by the ability to block complex formation by homologous oligos but not by unrelated ones. *G6Pase* AE-I (nucleotides −234 to −212) contains binding sites for HNF1 (−226/−212) and HNF4 (−231/−220), and two protein−DNA complexes (CIa and CIIa, Figure 3A, lane 2) were detected with G6Pase-(−234/−194) oligo and HepG2 nuclear extracts. The formation of complexes was efficiently blocked by an excess of unlabeled target DNA (lane 3), but not by an unrelated Oct1 oligo (lane 5). An oligo containing the binding site for HNF1 effectively blocked the formation of complex CIa (lane 4). Moreover, an antiserum to HNF1α caused a shift in mobility of complex CIa (lane 6), indicating that the protein factor in complex CIa is HNF1α. On the other hand, an oligo containing the binding site for HNF4 failed to compete for complex formation (lane 7), and an anti-HNF4 serum did not alter the mobility of either complex (lane 8). However, a protein−DNA complex (Figure 3B, lane 2) formed between an HNF4 oligo and HepG2 nuclear extracts was supershifed by the antiserum to HNF4 (lane 4), demonstrating that AE-I of the *G6Pase* promoter had little or no affinity for HNF4.

To demonstrate that HepG2 cells mimic liver tissue, we performed electromobility shift assays using nuclear extracts prepared from rat liver. As shown in Figure 3C, lanes 1−7, HNF1α was the protein factor in the liver that bound to AE-I in the *G6Pase* promoter.

The HNF3 site 1 (−180/−174) is located between AE-I and AE-II, and a protein−DNA complex (CIb) was formed between a G6Pase(−198/−165) oligo and HepG2 nuclear extracts (Figure 4A, lane 2). The complex formation was efficiently blocked by an excess of unlabeled target DNA (lane 3) and a consensus HNF3 oligo (lane 4), but not by a G6Pase(−189/−166-M) DNA with a mutated HNF3 motif (TGTG to ATAT at nucleotides −178 to −175) (Figure 4A, lane 5), suggesting that the protein factor that binds to G6Pase(−198/−165) is HNF3-like. CIb complex formation was not affected by an antiserum to HNF3α (lane 6) or HNF3β (lane 7), but was efficiently blocked by an antiserum to HNF3γ (lane 8), suggesting HNF3γ is the factor binding to the *G6Pase* promoter.

The *G6Pase* AE-II (nucleotides −146 to −125) contains overlapping binding sites for HNF3 (−139/−133), CREB (−136/−129), and C/EBP (−130/−125, a half-site) (Figure 1). When G6Pase(−146/−116) was used as a target, more than one band was observed using HepG2 nuclear extracts (Figure 4C, lane 2; 4D, lane 4; 4E, lane 5), indicating that several proteins in hepatocytes bound to AE-II. The formation of these complexes was efficiently blocked by an excess of unlabeled target DNA (4C, lanes 3−5; 4D, lane 5; 4E, lane 6), CRE (4C, lanes 9−11), and HNF3 (4C, lanes 12−14) oligos, was partially blocked by a C/EBP (4C, lanes 6−8) oligo, and was unaffected by an HNF4 oligo (4D, lane 6; 4E, lane 7). The G6Pase(−146/−116) DNA also bound recombinant HNF3α (4D, lane 2), HNF3γ (4D, lane 3), CREB (4E, lane 2), C/EBPα (4E, lane 3), and C/EBPβ (4E, lane 4).

An antiserum to HNF3γ (4D, lane 9), but not HNF3α (4D, lane 7) or HNF3β (4D, lane 8), inhibited complex formation, indicating that the HNF3 family member that binds to G6Pase(−146/−116) DNA is HNF3γ. This is supported by the fact that HNF3α, with a molecular mass of 50 kDa (Lai et al., 1991), formed a complex (4D, lane 2) that migrated slower than the complexes formed with HepG2 extracts. Moreover, a protein−DNA complex (Figure 4B, lane 2) formed between an HNF3β oligo (Costa et al., 1989) and HepG2 extracts was efficiently blocked by the antiserum to HNF3β (lane 4).

Antisera to ATF (4E, lane 9) had no effects on complex formation. However, complexes formed between G6Pase-(−146/−116) and HepG2 extracts were completely blocked by an antiserum to CREB (4E, lane 8) and partially inhibited by an antiserum to C/EBPα (4E, lane 10), and one of the protein complexes was supershifted by an antiserum to C/EBPβ (4E, lane 11, indicated by an arrow). This again indicates AE II has a high affinity for CREB and that C/EBPα and C/EBPβ also bind to this element in the *G6Pase*

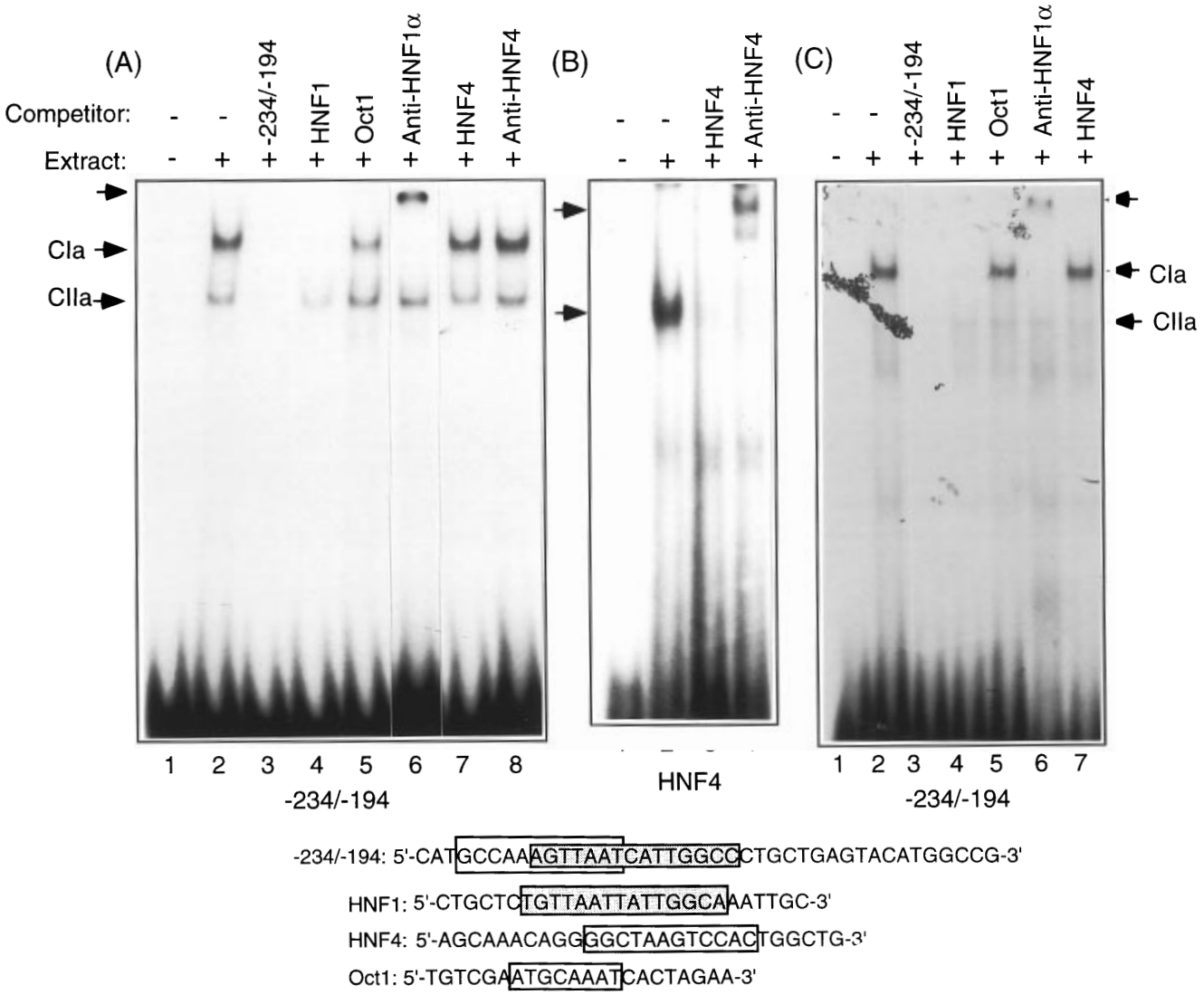

FIGURE 3: Binding of nuclear proteins to G6Pase(−234/−194) in the *G6Pase* promoter. The G6Pase(−234/−194) fragment encompassing HNF1 (−226/−212) and HNF4 (−231/−220) motifs within AE-I was labeled, and used in electromobility shift assays with nuclear extracts prepared either from HepG2 human hepatoma cells (A) or from rat livers (C). As a control, electromobility shift assay was also performed with HepG2 extracts and an HNF4 oligo (B). Reaction mixtures were preincubated with a competitor oligo, an antiserum to HNF1α (supershift), or an antiserum to HNF4 (supershift). The competitor oligonucletides were present at a 100-fold excess over the amount of the target DNA. The reaction mixture was analyzed on a 5% nondenaturing polyacrylamide gel. Consensus sequences that bind to transcription factors are boxed.

promoter. It has been shown that C/EBPα and C/EBPβ bind to the phosphoenolpyruvate carboxykinase (*PEPCK*) promoter through a CRE (Park et al., 1993), and this may explain the affinity of C/EBPα and C/EBPβ to the C/EBP half-site in the *G6Pase* promoter. The complexes formed between G6Pase(−146/−116) and recombinant HNF3γ, CREB, C/EBPα, and C/EBPβ migrated faster than the complexes formed between G6Pase(−146/−116) and HepG2 extracts, suggesting that the latter represent multimeric DNA−protein complexes, which include HNF3γ, CREB, C/EBPα, and C/EBPβ.

AE-III at nucleotides −124 to −71 contains HNF3 site 3 (−91/−85), site 4 (−81/−75), and part of site 5 (−72/−66) (Figure 1). Electromobility shift assays were performed using G6Pase(−105/−82) (Figure 5A) or G6Pase(−84/−64) (Figure 5B) as a target. A protein−DNA complex (CIc) (Figure 5A, lane 2) was detected with G6Pase(−105/−82) and HepG2 cell extracts. The formation of this complex was efficiently blocked by an excess of unlabeled target DNA (lanes 3−6), G6Pase(−84/−64) DNA (lanes 7−10), and an HNF3 oligo (lanes 11−14). Mutation of the HNF3 site 3 (AA to GG conversion at nucleotides −88 and −87) decreased the ability of the mutant oligo, G6Pase(−105/−82-M), to compete for complex formation (lanes 15−18), and antiserum to HNF3γ inhibited complex CIc formation (lane 19). However, antisera to HNF3α or HNF3β had no effects on complex CIc formation (data not shown). Electromobility shift assays using G6Pase(−105/−82) and a recombinant HNF3γ (Figure 5A, lanes 20−26) provided further support that HNF3γ is the transcription factor that binds to its cognate site (−91/−85) within AE-III.

When G6Pase(−84/−64) was used as a target, a protein−DNA complex (CId, Figure 5B, lane 2) was formed with HepG2 cell extracts. The formation of this complex was efficiently blocked by an excess of unlabeled target DNA (lanes 3−5), G6Pase(−105/−82) DNA (lanes 6−8), and an HNF3 oligo (lanes 9−11). Mutation of either HNF3 site 4 [G6Pase(−84/−64-M1); TTTG to CCCT conversion at nucleotides −79 to −76] (lanes 12−14) or site 5]G6Pase-(−84/−64-M2); AA to GG conversion at nucleotides −69

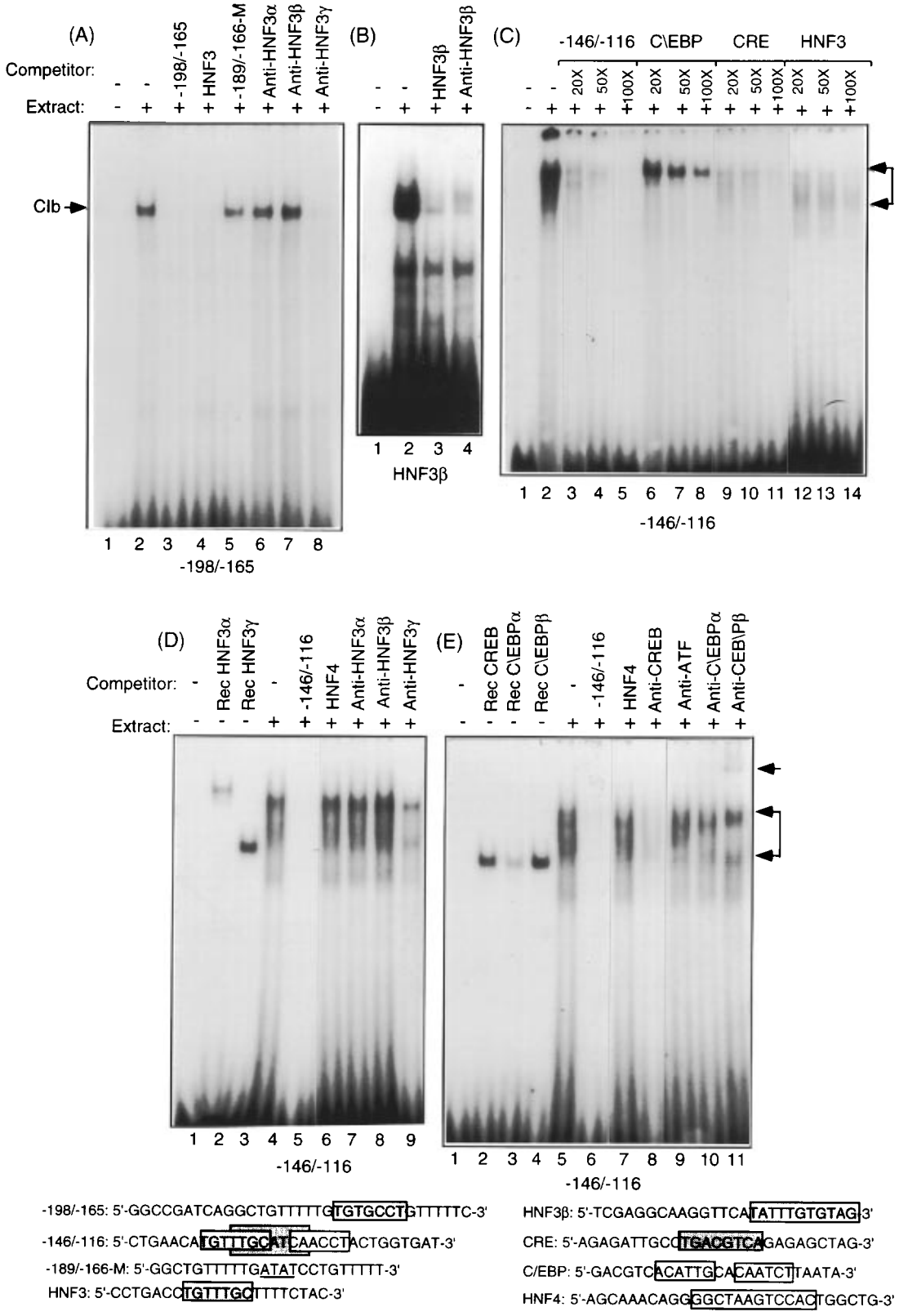

FIGURE 4: Binding of HepG2 nuclear proteins to G6Pase(−198/−165) and G6Pase(−146/−116) in the *G6Pase* promoter. The G6Pase-(−198/−165) fragment (A) encompassing HNF3 site 1 (−180/−174) or the G6Pase(−146/−116) fragment (C−E) encompassing HNF3 site 2 (−139/−133), CRE (−136/−129), and C/EBP half-site (−130/−125) within AE-II was labeled and used in electromobility shift assays with nuclear extracts prepared from HepG2 cells or with a recombinant protein synthesized by *in vitro* transcription−translation. As a control, electromobility shift assay was also performed with HepG2 extracts and an HNF3β oligo (B). Reaction mixtures were preincubated either with a competitor oligo or with antisera to HNF3α (supershift), HNF3β (blocking), HNF3γ (blocking), C/EBPα (blocking), C/EBPβ (supershift), CREB (blocking), or ATF (supershift). Unless specified, the competitor oligonucleotides were present at a 100-fold excess over the amount of target DNA. The reaction mixture was analyzed on a 5% nondenaturing polyacrylamide gel. Consensus sequences that bind to transcription factors are boxed.

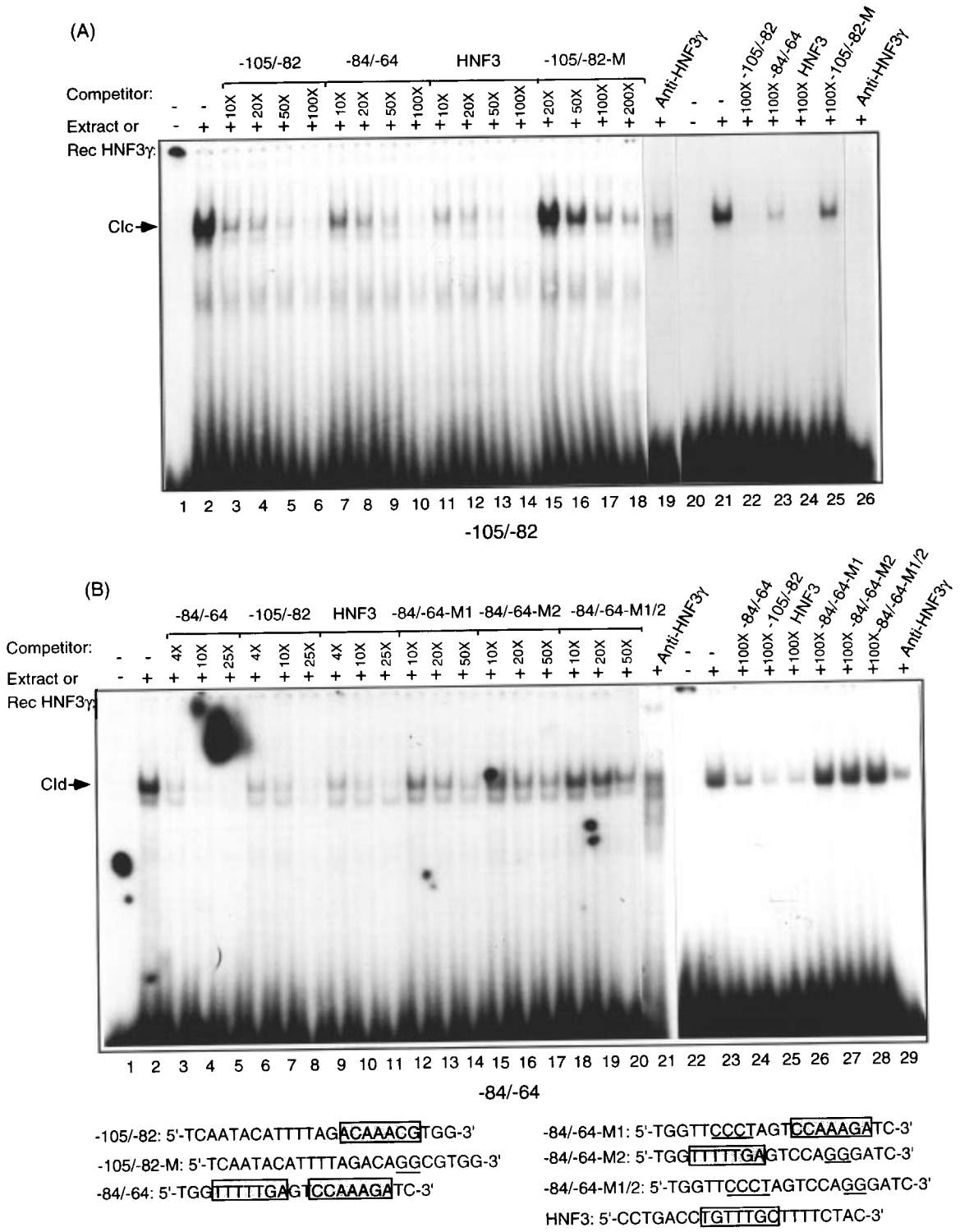

FIGURE 5: Binding of HepG2 proteins to G6Pase(−105/−82) and G6Pase(−84/−64) in the *G6Pase* promoter. The G6Pase(−105/−82) fragment (A) encompassing HNF3 site 3 (−91/−85) or the G6Pase(−84/−64) fragment (B) encompassing HNF3 sites 4 (−81/−75) and 5 (−72/−66) was labeled and used in electromobility shift assays with whole cell extracts prepared from HepG2 cells or with a recombinant HNF3γ synthesized by *in vitro* transcription−translation. Reaction mixtures were preincubated with a competitor oligo or an antiserum to HNF3γ (blocking). The reaction mixture was analyzed on a 5% nondenaturing polyacrylamide gel. Consensus sequences that bind to transcription factors are boxed.

and −68] (lanes 15−17) decreased its efficiency as a competitor (Figure 5), and G6Pase(−84/−64-M1/2) containing mutations in both HNF3 sites was essentially ineffective as a competitor (lanes 18−20). Again, an antiserum to HNF3γ inhibited the formation of complex CId (lane 21), and antisera to HNF3α and HNF3β had no effects on complex CId formation (data not shown). Moreover, assays using G6Pase(−84/−64) oligo and a recombinant HNF3γ (Figure 5B, lanes 22−29) demonstrated that HNF3γ bound to nucleotides −84 to −64 of the *G6Pase* promoter.

Effects of HNF1α*, HNF3*γ*, and cAMP on G6Pase Gene Expression.* HNF1, a member of the homeodomain family of DNA-binding proteins, exists in two forms, HNF1α and HNF1β (variant HNF1) (Frain et al., 1989; Baumhueter et al., 1990; Mendel et al., 1991; Rey-Campos et al., 1991). The two proteins share 58% overall amino acid sequence

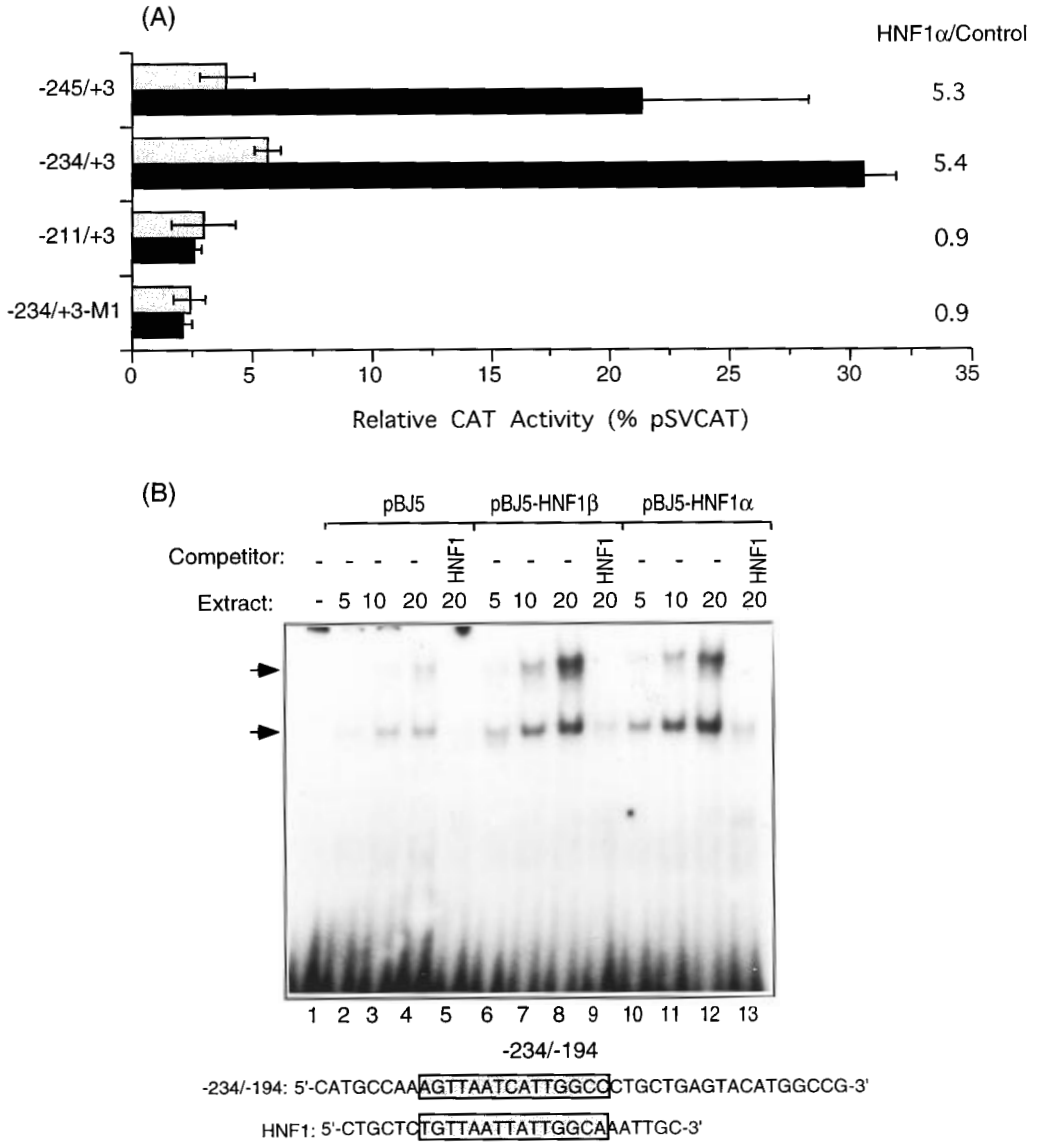

FIGURE 6: (A) Stimulation of CAT expression directed by *G6Pase* promoter−CAT constructs (5 μg) were transfected into HepG2 cells in the presence of 1 μg each of pBJ5 (open bar) or pBJ5-HNF1α (filled bar). The four constructs were G6Pase(−245/+3)CAT and G6Pase(−234/+3)CAT, containing the HNF1 motif, G6Pase(−211/+3)CAT, lacking the HNF1 site, and G6Pase(−234/+3-M1)CAT, containing a mutated HNF1 site. The pSVCAT plasmid which contains both the SV40 enhancer and promoter and the pCAT-Basic-N were used as positive and negative controls, respectively. At least three independent experiments were conducted with two preparations of each construct. (B) Synthesis of HNF1α and HNF1β in transfected HepG2 cells. The G6Pase(−234/−194) fragment was used in electromobility shift assays with whole cell extracts (μg) prepared from HepG2 cells cotransfected with G6Pase(−245/+3)-CAT and pBJ5, pBJ5-HNF1α, or pBJ5-HNF1β. The competitor HNF1 was present at a 100-fold excess over the amount of target DNA, and the reaction mixture was analyzed on a 5% nondenaturing polyacrylamide gel. Consensus sequences that bind to transcription factors are boxed.

identity while the DNA-binding homeodomain is 92% identical. To demonstrate that binding of HNF1α to its cognate site in the *G6Pase* promoter transactivates *G6Pase* gene expression, we examined CAT expression after cotransfecting *G6Pase* promoter−CAT fusion genes with pBJ5, pBJ5-HNF1α, or pBJ5-HNF1β. HNF1α increased CAT activity directed by G6Pase(−245/+3)CAT and G6Pase(−234/+3)CAT, both of which contain the HNF1 motif (−226 to −212), by 5.3- and 5.4-fold, respectively (Figure 6A). In contrast, CAT expression directed by the G6Pase(−211/+3)-CAT, which lacks the HNF1 site, and G6Pase(−234/+3-M1)CAT, which contains a mutated HNF1 site (AGTTAA to GGCCGC conversion at nucleotides −226 to −221), was unaffected by HNF1α (Figure 6A). Moreover, CAT activity directed by G6Pase(−234/+3-M1)CAT was 2.4-fold lower than the activity directed by the parental plasmid, G6Pase-(−234/+3)CAT, indicating that the HNF1 motif within AE-I is essential for basal *G6Pase* promoter activity. Although HNF1β (Figure 6B, lanes 6−8) and HNF1α (lanes 10−12) concentrations in HepG2 cells transfected with either plasmid were markedly higher than HepG2 cells transfected with pBJ5 (lanes 2−4), HNF1β had no effect on CAT expression directed by G6Pase(−245/+3)CAT or G6Pase(−234/+3)-CAT (data not shown). Therefore, HNF1α is the transcription factor that binds to the *G6Pase* promoter at −226 to −212 and transactivates *G6Pase* expression.

The electromobility shift assays presented earlier showed that HNF3γ is the HNF3 family member that bound to the *G6Pase* promoter. Liver expresses at least three HNF3 members, HNF3α, HNF3β, and HNF3γ (Costa et al., 1989; Lai et al., 1991, 1993). To demonstrate that the binding of HNF3γ transactivates the *G6Pase* gene, we examined CAT

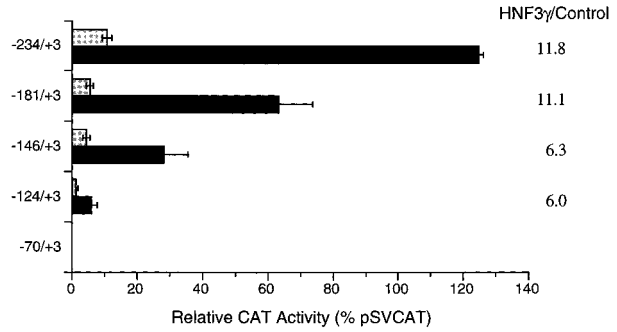

FIGURE 7: Stimulation of CAT expression directed by *G6Pase* promoter constructs by HNF3γ. Five *G6Pase* promoter−CAT constructs (5 μg) were transfected into HepG2 cells in the presence of 1.25 μg each of pBJ5 (open bar) or pBJ5-HNF3γ (filled bar). The five constructs were G6Pase(−234/+3)CAT and G6Pase(−181/+3)CAT, containing HNF3 sites 1−5, G6Pase(−146/+3)CAT, containing HNF3 sites 2−5, G6Pase(−124/+3)CAT, containing HNF3 sites 3−5, and G6Pase(−70/+3)CAT, containing no HNF3 sites. The pSVCAT plasmid which contains both the SV40 enhancer and promoter and the pCAT-Basic-N were used as positive and negative controls, respectively. At least three independent experiments were conducted with two preparations of each construct.

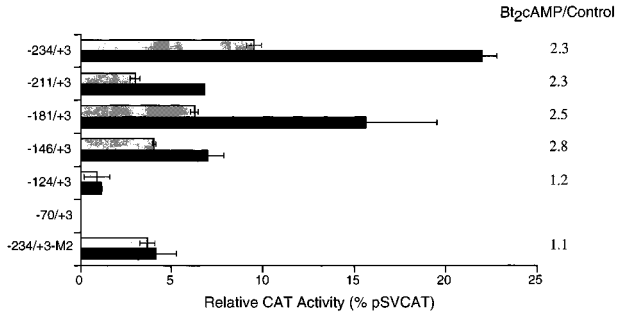

FIGURE 8: Stimulation of CAT expression directed by *G6Pase* promoter constructs by Bt_2cAMP. Six *G6Pase* promoter−CAT constructs were transfected into HepG2 cells in the absence (open bar) or presence (filled bar) of 0.5 mM Bt_2cAMP. The six constructs were G6Pase(−234/+3)CAT, G6Pase(−181/+3)CAT, and G6Pase(−146/+3)CAT, which contain the CRE (−136/−129), G6Pase(−124/+3)CAT and G6Pase(−70/+3)CAT, which lack this CRE, and G6Pase(−234/+3)M2CAT, which contains a mutated CRE. The pSVCAT plasmid which contains both the SV40 enhancer and promoter and the pCAT-Basic-N were used as positive and negative controls, respectively. At least three independent experiments were conducted with two preparations of each construct.

expression directed by *G6Pase* promoter−CAT fusion genes in the presence of pBJ5 or pBJ5-HNF3γ (Figure 7). HNF3γ stimulated CAT expression directed by G6Pase(−245/+3)-CAT and G6Pase(−181/+3)CAT, both of which contain all five HNF3 sites, by 11.8- and 11.1-fold, respectively (Figure 7). Deletion studies (Figure 2) indicated that HNF3 site 1 is not required for basal *G6Pase* promoter activity. However, HNF3γ increased CAT activity directed by G6Pase(−146/+3)CAT, containing HNF3 sites 2−5, and G6Pase(−124/+3)CAT, containing HNF3 sites 3−5, by only 6-fold (Figure 7), suggesting that the HNF3 site 1 (−180/−174) is essential for HNF3γ-activated transcription from the *G6Pase* promoter. CAT activity directed by G6Pase(−70/+3)CAT, lacking all HNF3 motifs, was undetectable either in the presence or in the absence of HNF3γ, demonstrating that HNF3 sites 3, 4, and 5 are essential for basal and HNF3γ-activated transcription.

The expression of the *G6Pase* gene is stimulated by cAMP (Chou et al., 1991), and AE-II contains a CRE (−136/−129) which binds to CREB. We therefore examined CAT expression directed by *G6Pase* promoter fusion genes in the presence of a cAMP analog, dibutyryl-cAMP (Bt_2cAMP) (Figure 8). CAT activity directed by G6Pase(−234/+3)-CAT, G6Pase(−211/+3)CAT, G6Pase(−181/+3)CAT, and G6Pase(−146/+3)CAT, all of which contain the CRE, was stimulated by Bt_2cAMP, 2.3−2.8-fold. In contrast, CAT activity directed by G6Pase(−124/+3)CAT and G6Pase(−70/+3)CAT, which lack this CRE, was unaltered by this analog (Figure 8). CAT activity directed by G6Pase(−234/+3)M2CAT, which contains a mutated CRE (TTG to ATC conversion at nucleotides −136 to −134), was 2.6-fold lower than the activity directed by the parental plasmid, G6Pase(−234/+3)CAT (Figure 8). Moreover, cAMP did not affect CAT activity directed by the G6Pase(−234/+3)M2CAT. Our data indicated that the CRE motif within AE-II is essential for basal *G6Pase* promoter activity and transactivation by cAMP.

G6Pase AE-II also bound to transcription factors C/EBPα and C/EBPβ. However, cotransfection of *G6Pase* promoter fusion genes containing AE-II with either a C/EBPα or a C/EBPβ expression plasmid failed to stimulate CAT expression (data not shown).

DISCUSSION

The liver is the predominant site of gluconeogenesis where hepatic G6Pase plays a key role in glucose homeostasis. In this study, we have demonstrated that the cis-acting DNA elements essential for optimal and liver-specific expression of the *G6Pase* gene *in vitro* are contained in the 5′-flanking gene region at nucleotides −234 to +3. Within this region, the *G6Pase* promoter contains three activation elements at nucleotides −234/−212 (AE-I), −146/−125 (AE-II), and −124/−71 (AE-III), which bind to transcription factors in hepatocytes and transactivate *G6Pase* gene expression. We further showed that HNF1α, HNF3γ, and CREB play important roles in activating the *G6Pase* gene.

AE-I contains both HNF1 and HNF4 motifs. We demonstrated that HNF1α is the transcription factor in the liver that transactivated *G6Pase* expression and bound to its cognate site within AE-I; in contrast, AE-I had little or no affinity for HNF4. Cotransfection and site-directed mutagenesis studies demonstrated that HNF1α activated *G6Pase* expression only with fusion genes containing this HNF1 motif. Deleting or mutating this HNF1 site decreased basal as well as HNF1α-activated transcription from the *G6Pase* promoter. HNF1α is a liver-enriched transcription factor (Frain et al., 1989; Baumhueter et al., 1990; Mendel & Crabtree, 1991) required for the expression of many liver genes, including albumin (Lichtsteiner & Schibler, 1989), α1-antitrypsin (Monaci et al., 1988), *PEPCK* (Roseler et al., 1989), and fibrinogen (Courtois et al., 1987). It is expressed in hepatocytes and kidney proximal tubules (Pontoglio et al., 1996) where the *G6Pase* gene is expressed at high levels (Nordlie & Sukalski, 1985). Moveover, mice lacking HNF1α are characterized by failing to thrive, hepatomegaly, and renal proximal tubular dysfunction (Pontoglio et al., 1996), all of which are clinical presentations seen in GSD-1a patients (Moses, 1990; Chen & Burchell, 1995). The vital role of HNF1α in activating the G6Pase gene is suggested by some of the manifestations suffered by HNF1α knockout

mice (Pontoglio et al., 1996), where the absence of HNF1α may cause a reduced expression of the *G6Pase* gene.

AE-II contains overlapping CRE, HNF3, and C/EBP motifs, and we showed that CREB, HNF3*γ*, C/EBPα, and C/EBP*β* bind to this activator sequence. However, cotransfection assays showed that C/EBPα or C/EBP*β* could not enhance G6Pase transcription. In addition, HNF3*γ* increased CAT activity 6-fold with either G6Pase(−146/+3)CAT, containing HNF3 site 2, or G6Pase(−124/+3)CAT, which lacked this site. Thus, our data lead us to conclude that AE-II plays a minor role in mediating C/EBPα, C/EBP*β*, and HNF3*γ* action in the *G6Pase* promoter. On the other hand, the finding that cAMP stimulated *G6Pase* promoter activity with fusion genes containing AE-II strongly indicates that binding of CREB to the CRE within AE-II is necessary to transactivate *G6Pase* expression.

We showed that HNF3*γ* did play a major role in transactivating *G6Pase* promoter which contains five HNF3 sites: 1 (−180/−174), 2 (−139/−133), 3 (−91/−85), 4 (−81/−75), and 5 (−72/−66). Electromobility shift assays identified HNF3*γ* as the factor in HepG2 extracts that bound to all five HNF3 sites. This is supported by the observations that the characteristics of the DNA−protein complexes formed between AE oligos encompassing HNF3 motifs and HepG2 extracts were indistinguishable from complexes formed with a recombinant HNF3*γ*. However, transient transfection assays demonstrated that HNF3 site 1 is required only for HNF3*γ*-activated transcription from the *G6Pase* promoter, whereas HNF3 sites 3, 4, and 5 are essential for both basal promoter activity and transactivation by HNF3*γ*. This was demonstrated when CAT expression, directed by G6Pase(−181/+3)CAT containing the HNF3 site 1, was stimulated 11-fold by HNF3*γ*, whereas CAT activity directed by G6Pase(−146/+3)CAT lacking site 1 was increased only 6-fold by HNF3*γ*. However, in the absence of HNF3*γ*, similar CAT activity was directed by G6Pase(−181/+3)-CAT and G6Pase(−146/+3)CAT. Moreover, G6Pase(−124/+3)CAT, containing HNF3 sites 3, 4, and 5, constituted a minimal *G6Pase* promoter which expressed significant CAT activity in HepG2 cells. In contrast, G6Pase(−70/+3)CAT, containing no HNF3 motifs, directed no CAT expression. Additionally, CAT expression directed by G6Pase(−124/+3)CAT was stimulated 6-fold by HNF3*γ* which had no effect on CAT activity directed by G6Pase(−70/+3)CAT.

HNF3 belongs to the *forkhead* or winged helix family of transcription factors that binds DNA as a monomer (Costa et al., 1989; Lai et al., 1991, 1993), and HFN3α, HNF3*β*, and HNF3*γ* were originally discovered to be required for the hepatocyte-specific expression of transthyretin and antitrypsin genes (Costa et al., 1989). In the liver, HNF3*β* and HNF3*γ* mRNAs are more abundant than HNF3α mRNA (Kaestner et al., 1994). HNF3*γ* is expressed at low levels in the small intestine and testis, but absent in brain, spleen, and kidney. The important role of HNF3*γ* in liver-specific expression of the *G6Pase* gene raised the possibility that kidney *G6Pase* expression is regulated by a different mechanism. A well documented example of differential regulation of a single gene in liver and kidney is the *PEPCK* gene (Shoshani et al., 1991).

The promoter of G6Pase appears to be complex, allowing it to maintain tight regulation of glucose homeostasis in response to various stimuli. It has been shown that administration of insulin to rats down-regulates *G6Pase* expression (Liu et al., 1994; Lange et al., 1994; Argaud et al., 1996) and inhibited G6Pase enzymatic activity (Spagnoli et al., 1983; Argaud et al., 1996). Similarly, in the liver, insulin also down-regulates the *PEPCK* and insulin-like growth factor-binding protein 1 (*IGFBP-1*) genes (Unterman et al., 1994; O'Brien et al., 1995). O'Brien et al. (1995) showed that the insulin response elements (IREs) in the *PEPCK* and *IGFBP-1* promoters bind HNF3 and suggested that insulin may elicit its negative effect by inhibiting HNF3 action. The G6Pase(−234/+3) region contains multiple IREs at nucleotides −186 to −180, −172 to −166, and −164 to −158 (Figure 1). The IREs are clustered at and around the HNF3 site 1 at nucleotides −180 to −174. It is possible that insulin down-regulation of *G6Pase* gene expression is mediated by a similar mechanism.

In summary, we have identified cis-acting DNA elements and transcription factors essential for liver-specific expression of the *G6Pase* gene. However, these *in vitro* findings need to be considered in the context of the whole cell, where genomic DNA is complexed with proteins to form a highly compact and organized structure (Wolffe, 1992). The chromosomal context of the gene is essential for correct regulation *in vivo* (Fascher et al., 1990; Straka & Horz, 1991). Future studies will focus on regulation of the expression of the G6Pase gene *in vivo*.

ACKNOWLEDGMENT

We thank Drs. L. Hansen and G. R. Crabtree for gifts of expression vector for HNF1α, HNF1*β*, and an antiserum to HNF1α; Drs. W. S. Chen and J. E. Darnell, Jr. for gifts of cDNA for HNF3α and antisera to HNF3α, HNF3*β*, and HNF3*γ*; Dr. M. R. Montminy for gifts of cDNA for CREB; Dr. F. M. Sladek for an antiserum to HNF4, and Dr. M. Chamberlin for critical reading of the manuscript.

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BI9703249