

Hepatocyte Nuclear Factor-1 α Recruits the Transcriptional Co-Activator p300 on the GLUT2 Gene Promoter

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Mutations in the hepatocyte nuclear factor (HNF)-1 α gene have been linked to subtype 3 of maturity-onset diabetes of the young (MODY), a disease characterized by a primary defect in insulin secretion. Here we show that the human GLUT2 gene is closely regulated by HNF-1 α via sequences downstream of the transcriptional start site by interaction with transcriptional co-activator p300. The promoter region of the human GLUT2 gene was subcloned into luciferase expression plasmids that were transfected together with HNF-1 α expression plasmid into a pancreatic β -cell line, HIT-T15, to evaluate transcriptional activities. HNF-1 α enhanced human GLUT2 promoter activity sixfold. Site-direct mutagenesis and footprint analyses showed that the HNF-1 α binding site (+200 to +218) is critical in human GLUT2 gene expression. Furthermore, mammalian two-hybrid and immunoprecipitation studies revealed the transactivation domain of HNF-1 α (amino acids 391–540) to interact with both the NH₂-terminal region (amino acids 180–662) and the COOH-terminal region (amino acids 1,818–2,079) of p300. These findings demonstrated that HNF-1 α binds to the 5'-untranslated region of GLUT2 and that p300 acts as a transcriptional co-activator for HNF-1 α . In addition, these results provided new insight into the regulatory function of HNF-1 α by suggesting a molecular basis for human GLUT2 gene expression. *Diabetes* 51:1409–1418, 2002

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ABC, avidin-biotin complex; CBP, CREB-binding protein; CREB, DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; β -gal, β -galactosidase; GSIS, glucose-stimulated insulin secretion; GST, glutathione *S*-transferase; HAT, histone acetyltransferase; HNF, hepatocyte nuclear factor; MODY, maturity-onset diabetes of the young; P/CAF, p300/CBP-associated factor; PMSF, phenylmethylsulfonyl fluoride; SRC, steroid receptor co-activator; TBS, Tris-buffered saline; TBS-T, TBS containing 0.1% Tween-20; VP16, herpes virus acidic activation region.

Maturity-onset diabetes of the young (MODY), a monogenic form of diabetes, is characterized by autosomal-dominant inheritance, onset usually before age 25 years, and impaired glucose-stimulated insulin secretion (GSIS). MODY phenotypes have been linked so far to mutations in five genes, namely, hepatocyte nuclear factor (HNF)-4 α on chromosome 20q for MODY1 (1), glucokinase on chromosome 7p for MODY2 (2), HNF-1 α on chromosome 12q for MODY3 (3), pancreatic homeodomain transcription factor IPF-1 (also designated PDX-1, IDX-1, or STF-1) on chromosome 13q for MODY4 (4), and HNF-1 β on chromosome 17q for MODY5 (5).

Mutation in the HNF-1 α gene is a common cause of MODY in the majority of this population. Phenotypic analysis of MODY3 pedigrees revealing hyperglycemia resulting from severe defects in GSIS suggests that β -cell dysfunction plays an important pathophysiological role in the development of MODY3 (6,7).

HNF-1 α (also called LF-B1) is the homeodomain transcription factor, which is consisted with dimerization, POU, and transactivation domains. It is highly expressed in liver, but is also expressed in kidney, spleen, and intestine. HNF-1 α regulates the expression of many liver-specific genes by directly binding to the promoter and enhancer regions, including albumin, fibrinogen, and α_1 -anti-trypsin genes (consensus site: GTTAATNATTAAC, where N = A, C, G, T, or no nucleotide) (8,9). It has been reported that HNF-1 α is expressed in the insulin-producing cell-lines (10–13), HIT-T15 and INS-1. However, it is not known if HNF-1 α is expressed in pancreatic β -cells or which genes HNF-1 α regulates in pancreatic β -cells.

Extensive studies have detailed the mechanism by which glucose regulates insulin secretion. After being transported into the pancreatic β -cells through the facilitative glucose transporter GLUT2, glucose is metabolized to generate ATP, which triggers the exocytosis of insulin from the pancreatic β -cells. Because of the GSIS mechanism, the GLUT2 gene is a candidate HNF-1 α target gene in pancreatic β -cells. GLUT2 is present in the plasma membrane of pancreatic β -cells, hepatocytes, intestine, and kidney. The expression pattern of GLUT2 is quite similar to that of HNF-1 α . HNF-1 α , therefore, might play an important role in the regulation of GLUT2 transcription. The activity of various DNA-binding transcription factors

is regulated by global transcriptional co-activators such as p300 and CREB-binding protein (CBP). p300 and CBP exhibit strong sequence similarity and similar functions (14–16). p300/CBP binds not only to the activation domain of some transcription factors, such as CREB and the glucocorticoid receptor, but also to multiple components of the basal transcriptional machinery, including the TFIIB (15) and RNA polymerase II holoenzyme complex (17), suggesting that p300/CBP serves as a molecular bridge between the transcription factors and the basal transcriptional machinery. In addition, p300/CBP itself has histone acetyltransferase (HAT) activity, and p300/CBP forms a complex with multiple HATs such as p300/CBP-associated factor (P/CAF) (18) and steroid receptor coactivator (SRC)-1(19), suggesting that the p300/CBP complex contributes to transcriptional activation by disrupting the repressive chromatin structure.

In this study, we investigated a possible role of HNF-1 α during human GLUT2 gene expression using promoter deletion experiments, DNase I footprint analyses, and electrophoretic mobility shift assays (EMSAs). Prompted by the result of these studies, we went on to investigate the role of p300 in transcriptional regulation by HNF-1 α using binding and reporter gene analyses. Our results demonstrated that p300 acts as a transcriptional co-activator for HNF-1 α and that the transcriptional complex binds to the 5'-untranslated region of the human GLUT2 gene.

RESEARCH DESIGN AND METHODS

Plasmids. Plasmids for 5 \times GAL4-TATA-luciferase reporter gene and expression of p300 and CBP were kindly provided by Dr. Maurer (Oregon Health Sciences University, Portland, OR) and Dr. Goodman (Oregon Health Sciences University, Portland, OR), respectively.

The human GLUT2 promoter-luciferase reporter gene contains the human GLUT-2 promoter upstream of the minimal promoter and the coding sequence of the firefly luciferase. An expression vector for the HNF-1 α was constructed by PCR. The sequence of the cloning fragment was confirmed.

GAL4-HNF-1 α and GAL4-p300 expression plasmids containing the DNA-binding domain of GAL4 and the transcriptional activation domain of HNF-1 α or p300 were constructed as follows. The control pM vector (Clontech, Palo Alto, CA) was generated by introducing the coding sequence for the GAL4 DNA-binding domain (amino acids 1–147) from pGBT9 downstream of the SV40 early promoter in plasmid. The NH₂-terminal region of the human HNF-1 α (amino acids 281–631) and the human p300 (amino acids 1–2,414) were then fused in frame to the 3' end of the GAL4 DNA-binding domain in plasmid pM. To construct the herpes virus acidic activation region (VP16) fusion constructs, HNF-1 α cDNA sequences were fused in frame to VP16 (Clontech), which has SV40 large T-antigen nuclear localization signal. The sequences of the cloning fragments were confirmed. The plasmid to express the FLAG-tagged full length of HNF-1 α was created via PCR amplification of the human HNF-1 α , and the product was subcloned into the pFLAG-CMV-2 expression vector (Sigma, St. Louis, MO). We used the pRSV- β -gal plasmid (β -galactosidase expression vector) for internal control in transient transfection.

Cell culture and transfections. HIT-T15 cells, a hamster insulin-producing cell line, were maintained at 37°C in RPMI 1640 medium supplemented with 10% FCS. HepG2, MIN6, and COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS.

Plasmid DNAs were transfected into HIT-T15 and HepG2 cells by the standard calcium phosphate precipitation procedure, and were transfected into COS-7 cells by the lipofection method using Lipofect AMINE reagent (Gibco-BRL, Santa Clara, CA). These cells were harvested 48 h after transfection, and luciferase activity was determined according to the manufacturer's manual (Nippon Gene, Tokyo, Japan). Luciferase activity values were normalized to β -gal activity.

Preparation of glutathione S-transferase (GST)–HNF-1 α fusion protein and DNase I footprinting. A full-length cDNA clone for HNF-1 α was subcloned into the pGEX-4T vector (Amersham Pharmacia Biotech), and the resulting clone was verified by sequencing. *E. coli* BL21 was transformed, and

the fusion proteins were purified according to the standard protocol, using glutathione sepharose 4B beads (Amersham Pharmacia Biotech). For DNase I footprinting, the probe A (nucleotide –46 to +125), B (nucleotide +29 to +216), or C (nucleotide +126 to +308) was Cy5-labeled at the 5' end of the upper strand. DNA-binding reaction and DNase I digestion were done in 60 μ l with 1–4 ng of the fragment and 30 μ g of the bacterially expressed HNF-1 α , as previously described (20).

Electrophoretic mobility shift assays. MIN6 and HepG2 cell nuclear extracts were prepared according to the method of Dignam et al. (21). The sequences of the oligonucleotides used in the gel retardation assay were the following: BS1 (sense), 5'-AAGCACTTATTGATTAGATCCCATCAATA-3'; mutated BS1 (sense), 5'-AAGCACTCTGTTGCCAGATTCATCAATA-3'; BS2 (sense), 5'-CCTCTGCAATGCATAGCCTAGGCA-3'; the mutated BS2 (sense), 5'-CCTCTGCAGCGCATGGCTAGGCCTAGGCA-3'. Complementary sense and antisense oligonucleotides were hybridized. The double-stranded oligonucleotide probes were end-labeled with T₄ polynucleotide kinase and [γ -³²P]ATP. Nuclear extracts (5 μ g) were preincubated for 20 min at room temperature with 1.5 μ g of poly(dI-dC) in a buffer containing 10 mmol/l Tris (pH 7.4), 50 mmol/l NaCl, 1 mmol/l dithiothreitol, 1 mmol/l EDTA, 1 mg of bovine serum albumin per ml, and 10% glycerol, in a final volume of 20 μ l. After the addition of the probe (3 \times 10⁴ cpm), the samples were incubated for 30 min at room temperature, and then electrophoresis was performed on 5% nondenaturing polyacrylamide gels in 0.5 \times TBE (Tris-borate-EDTA) buffer for 120 min at 150 V at 4°C. In the competition studies, a 1-, 10-, 50-, or 100-fold molar excess of unlabeled oligonucleotide competitor was added with the probe. The gels were fixed in a solution of 10% acetic acid and 30% methanol, dried, and exposed to Kodak X-Omat film (Kodak, Rochester, NY).

Immunoprecipitation. Then 48 h after transfection, COS-7 cells were washed with ice-cold PBS and then extracted with lysis buffer-1 containing 10 mmol/l Tris-HCl (pH 7.5), 1% wt/vol Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 50 mmol/l NaF, 1 mmol/l β -glycerophosphate, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 2 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin A. Lysates were centrifuged at 4°C for 20 min at 12,000g, and the anti-GAL4 DNA binding domain antibody (Santa Cruz, CA) was incubated with 300 μ g of cell-free extracts for 3 h at 4°C. The immune complexes were coupled to protein A–sepharose beads (Amersham Pharmacia Biotech) and washed four times with the lysis buffer-1. The beads-bound proteins were solubilized by addition of SDS-containing sample buffer. Immunoblot analysis was done using the monoclonal anti-FLAG antibody (Sigma).

Immunoblotting. After 48 h, transfected COS-7 cells were washed with ice-cold PBS and extracted by lysis buffer-2 containing 50 mmol/l Tris-HCl (pH 7.5), 1% wt/vol Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l sodium orthovanadate, 1 mmol/l PMSF, and 2 μ g/ml aprotinin. The debris was pelleted at 4°C for 20 min at 12,000g. Samples (supernatant) normalized for total protein content were resolved by electrophoresis through NuPAGE Bis-Tris gels (10% MOPS buffer; Novex) and transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membranes were blocked in 5% low-fat dried milk dissolved in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T; pH 8.0) overnight at 4°C. After extensive washing in TBS-T, the membranes were incubated for 1 h at 37°C with the primary antibody. The primary antibody, mouse anti-FLAG monoclonal antibody, was used at a 1:5,000 dilution with TBS-T. Detection was performed using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and required the secondary horseradish peroxidase-conjugated antibody (diluted to 1:10,000).

Immunohistochemistry. The pancreases of 8-week-old Wistar rats were removed under pentobarbital anesthesia (40 mg/kg body wt) and fixed in Bouin's solution. Pancreatic specimens were embedded in paraffin and divided into 3.5- μ m sections. The avidin-biotin complex (ABC) method with alkaline phosphatase was used, as previously described (22), with a slight modification. After deparaffinization, normal goat serum (diluted to 1:75; DAKO, Kyoto, Japan) for the inhibition of nonspecific binding of secondary antibody, rabbit anti-p300 polyclonal antibody (diluted to 1:500; Santa Cruz), or rabbit anti-insulin polyclonal antibody (diluted to 1:500; DAKO); the biotin-labeled anti-rabbit IgG serum (diluted to 1:300; DAKO); and the avidin-biotin-alkaline phosphatase complex (diluted to 1:100; Vector Laboratories, Burlingame, CA) were sequentially applied on consecutive sections, followed by hematoxylin nuclear counterstaining (insulin). Staining was visualized in black (p300) and red (insulin) by alkaline phosphatase substrate (Vector Laboratories).

Statistical analysis. Statistical analyses were performed by an unpaired *t* test.

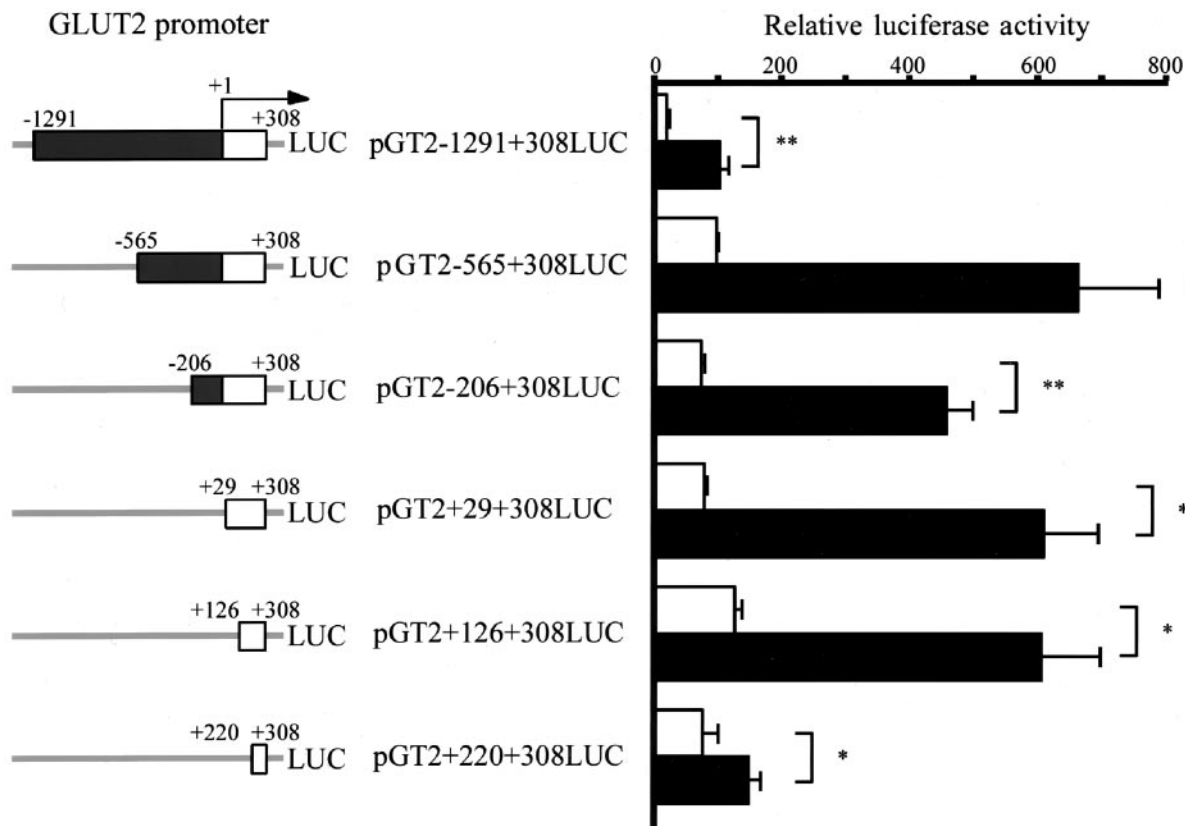


FIG. 1. HNF-1 α responsive region within the GLUT2 promoter. Each restriction fragment of human GLUT2 was placed upstream of a minimal promoter and luciferase cassette. HIT-T15 cells were co-transfected with 4 μ g of the indicated reporter plasmids plus 2.5 μ g of HNF-1 α expression construct or a control plasmid. Luciferase activities in the cells transfected with HNF-1 α (■) relative to that in cells transfected with the control plasmid (□) are shown. Luciferase activities were normalized on the basis of β -galactosidase activity. Results are given as means \pm SE of five independent experiments in triplicate. * P < 0.05; ** P < 0.01.

RESULTS

Transcriptional activation of the GLUT2 gene by HNF-1 α . A 1.6-kb genomic fragment encompassing the human GLUT2 promoter (−1,291 to +308) was inserted upstream of the luciferase reporter gene. This GLUT2 promoter region conferred significant transcriptional activity to the basal reporter gene transfected into the hamster insulinoma cell line HIT-T15, and this activity was significantly enhanced by HNF-1 α . Nested deletions of the promoter were used to map the HNF-1 α responsive region to a fragment containing nucleotides +126 to +308 relative to the TATA box at the human GLUT2 transcription start site (Fig. 1).

Multiple HNF-1 α –binding sites in the human GLUT2 gene. To identify the HNF-1 α –binding sites within the human GLUT2 gene, a DNase I footprint analysis using the bacterially synthesized HNF-1 α was performed. Two sites, BS1 and BS2, were protected from DNase I digestion in the presence of HNF-1 α . BS1 is located between +95 and +117 and BS2 is located between +200 and +218 (Fig. 2A). Furthermore, we performed EMSA analysis using nuclear extracts of the pancreatic β -cell line, MIN6, or hepatoblastoma cell line, HepG2, to confirm that HNF-1 α binds to the regions in these cells. Both binding sites can be interacted with the endogenous HNF-1 α protein (Fig. 2B). Within the core of each protected region, we found a DNA sequence similar to the HNF-1 α binding consensus sequence (5′-GTTAATNATTAAC-3′) (Fig. 2C).

To determine if the HNF-1 α responsiveness of the

human GLUT2 gene requires the two binding sites, we constructed mutant GLUT2 promoter-luciferase plasmids containing mutated HNF-1 α binding sites. Luciferase activities expressed in HIT-T15 cells by the wild-type and mutant plasmids are shown in Fig. 2D. The level of luciferase activity expressed from the control plasmid pGT2–206 + 308LUC was increased by about sixfold with HNF-1 α . When the BS1 was disrupted (pGT2–206 + 308LUC-m1), the degree of induction by HNF-1 α was similar to the wild-type promoter. The introduction of mutations into BS2 (pGT2–206 + 308LUC-m2) reduced the HNF-1 α transcriptional increment to one-third. Disruption of both BS1 and BS2 (pGT2–206 + 308LUC-m1,2) also reduced the HNF-1 α transcriptional inducibility to one-third. These results indicate that BS2 within the 5′ untranslated region contributes significantly to the HNF-1 α responsiveness of the GLUT2 promoter.

Potential of HNF-1 α –dependent transactivation by p300/CBP. To determine if p300 is involved in transcriptional activation of the GLUT2 gene by HNF-1 α , we used a reporter gene containing sequences from −206 to +308 of the GLUT2 gene. When co-expressed transiently in HIT-T15 cells, HNF-1 α stimulated reporter gene expression in a dosage-dependent manner (Fig. 3A). p300 or CBP enhanced the luciferase activities from −206 to +308 in the GLUT2 gene. In contrast, the BS2-mutated GLUT2 promoter (pGT2–206 + 308LUC-m2) was not activated by p300 or CBP. We next used the fusion protein consisting of the DNA-binding domain of GAL4 and the transactivation

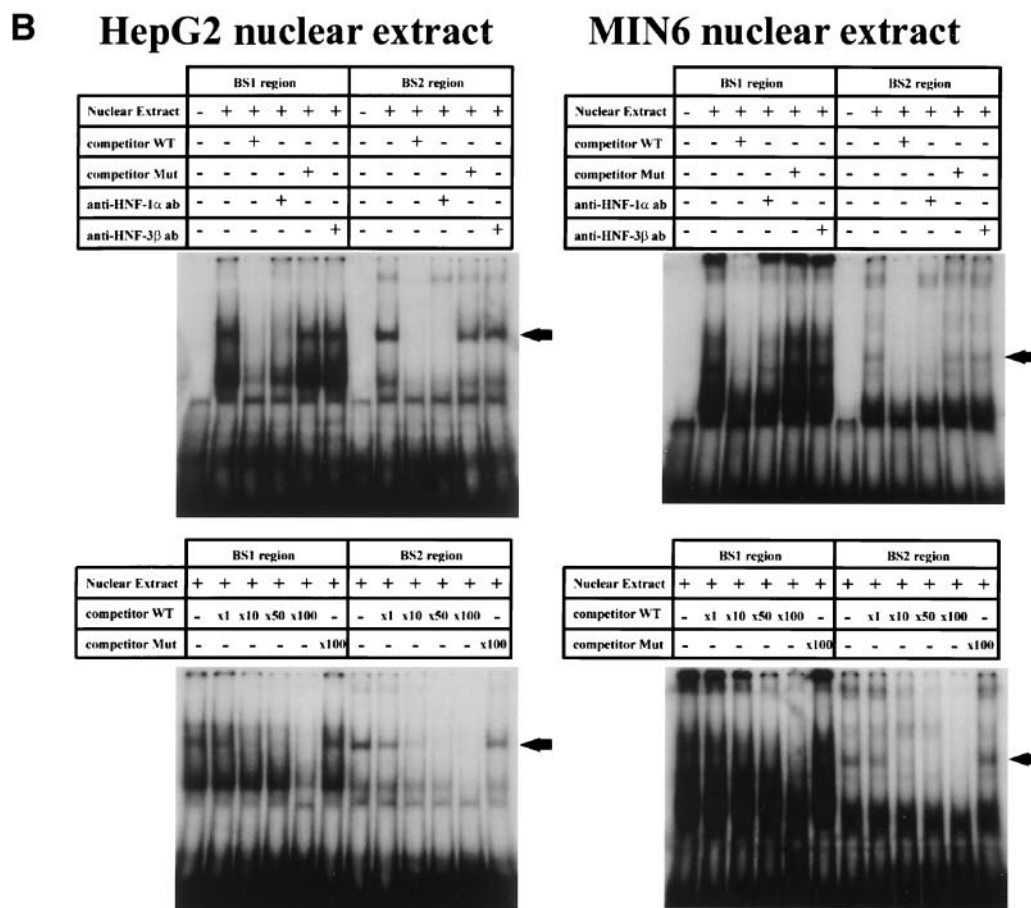
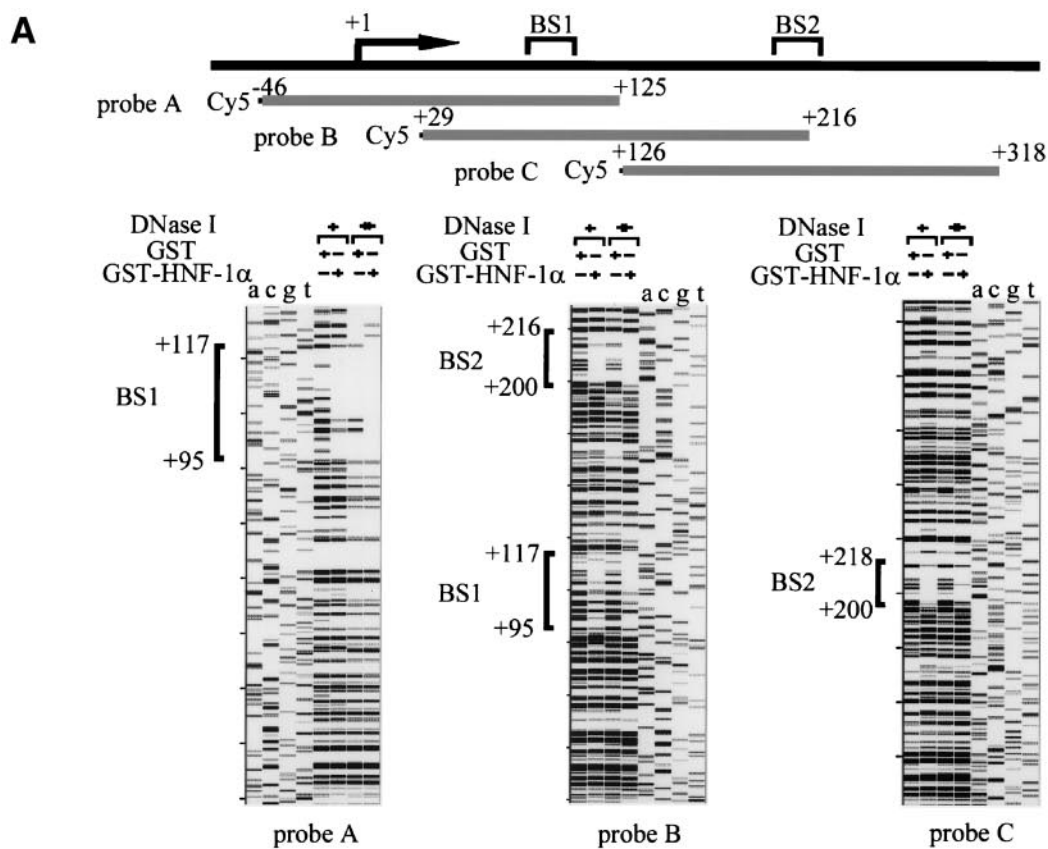


FIG. 2

C BS1

	+95	+117
GLUT2 sequence	aagcACTTATTGATTAGATTCCCATCAatattcag	
consensus	GTTAATNATTAAC	
mutant (m1)	aagcACTCGTTGGCCAGATTCCCATCAatattcag	

BS2

	+200	+218
GLUT2 sequence	ctccTGCAATGCATAACTAGGCCta	
consensus	GTTAATNATTAAC	
mutant (m2)	ctccTGCAGCGCATGGCTAGGCCta	

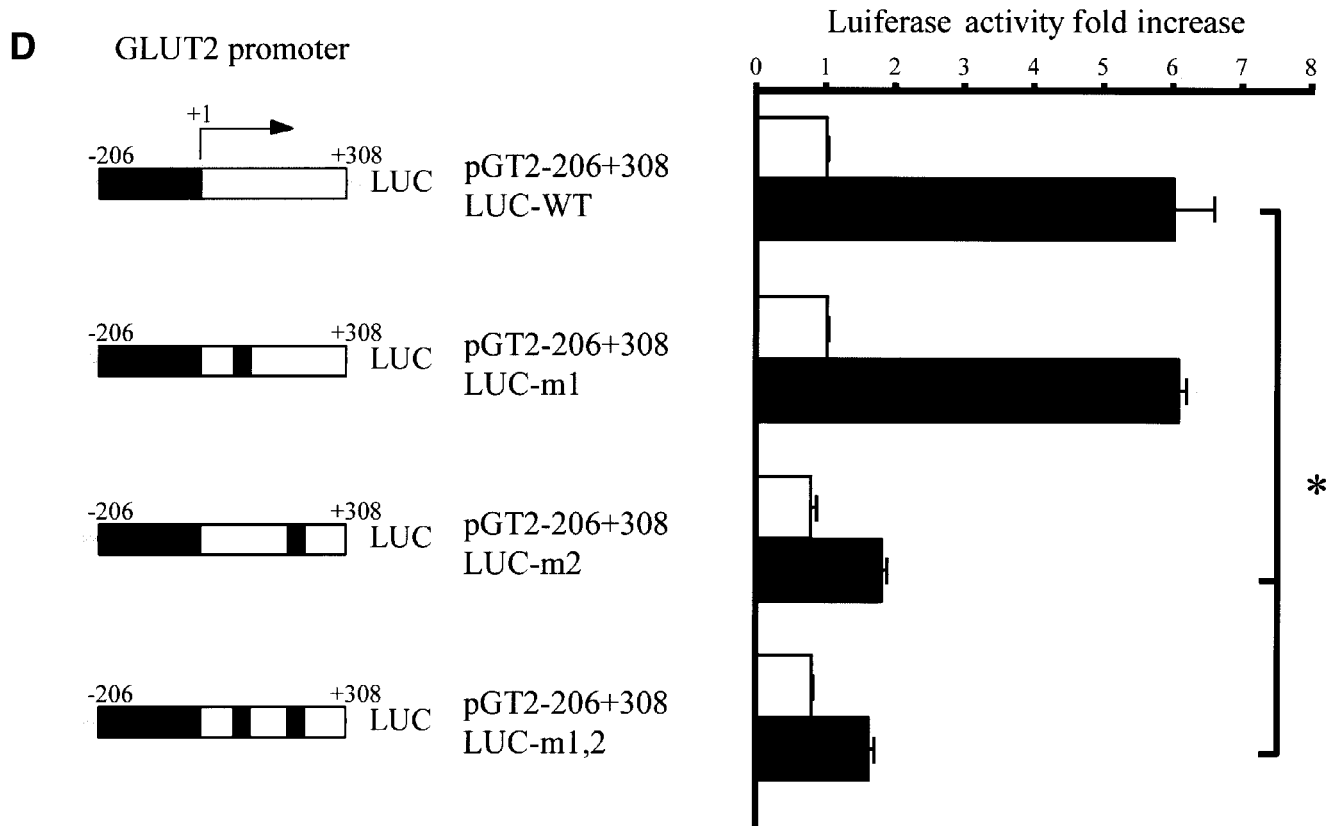


FIG. 2. HNF-1 α binding sites in the GLUT2 promoter region. **A:** Binding of HNF-1 α to the 5' flanking region of human GLUT2 gene. The purified GST-HNF-1 α was bacterially generated. Three probes were end-labeled with Cy5, incubated with 30 μ g of GST-HNF-1 α or GST protein, and digested with DNase I. The protected regions are depicted on the left. **B:** EMSAs were performed with HepG2 or MIN6 cell nuclear extracts and a labeled double-strand oligonucleotide probe representing the binding site BS1 or BS2. Wild-type or mutant probes were introduced as unlabeled competitors. Lengths of the competitors were the same as that of the binding site (30mer). Each competitor was added at a 100-fold molar excess. Similar results were obtained in five independent experiments. **C:** Nucleotide sequences of the HNF-1 α binding sites in the GLUT2 promoter region. Nucleotide sequences of two sites protected by HNF-1 α , HNF-1 α consensus binding site, and introduced mutations are shown. **D:** Effects of mutation of the HNF-1 α -binding sites on GLUT2 promoter activity. Schematic representation of the luciferase plasmids containing the mutated binding sites is shown in the left. Luciferase activities have been normalized on the basis of β -galactosidase activity. Results are given as means \pm SE of five independent experiments in triplicate. \square , control; \blacksquare , +HNF-1 α . * P < 0.01.

domain (amino acids 281–631) of HNF-1 α , and the 5 \times GAL4-TATA-luciferase reporter gene when co-expressed transiently in HepG2 cells. Under these conditions, the GAL4-HNF-1 α fusion protein activated luciferase expres-

sion from this reporter plasmid (Fig. 3B). Furthermore, co-transfection of the p300 or CBP expression plasmid further potentiated HNF-1 α -dependent transcriptional activity by about 5.5- and 3-fold, respectively. These re-

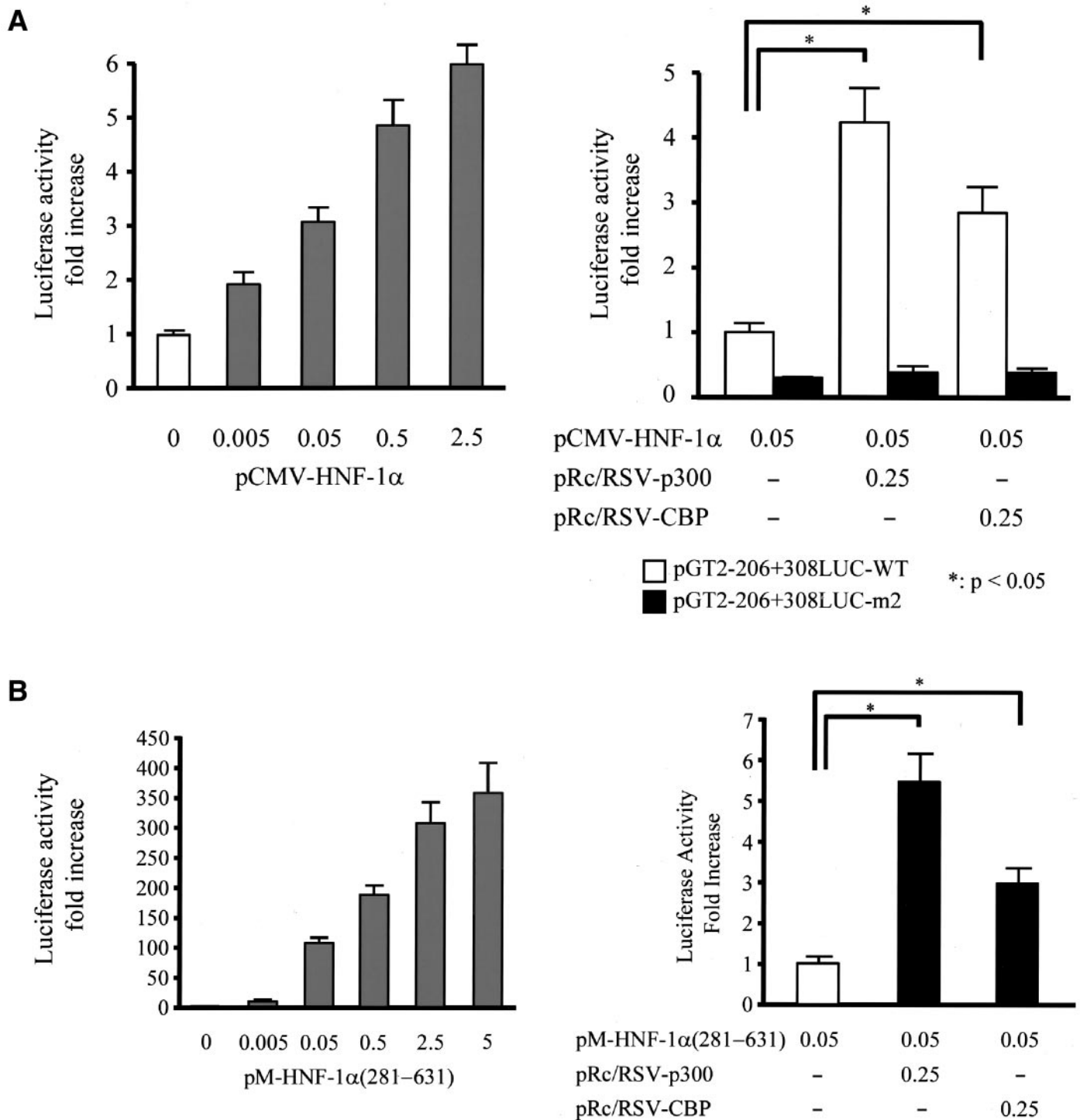


FIG. 3. Potentiation of HNF-1 α -activated gene expression by p300. **A:** p300/CBP as a transcriptional co-activator for HNF-1 α . An increasing amount of HNF-1 α expression plasmid was co-transfected with 4 μ g of pGT2-206 + 308 wild-type luciferase reporter gene into HIT-T15 cells by the calcium phosphate precipitation method. Relative luciferase activities compared without the HNF-1 α expression plasmid were determined 48 h after transfection (left). HIT-T15 cells were co-transfected with 4 μ g of pGT2-206 + 308 wild-type (\square) or m2 (\blacksquare) luciferase reporter gene, 0.05 μ g of expression plasmid for HNF-1 α , and 0.25 μ g of expression plasmid for CBP or p300 by the calcium phosphate precipitation method. Luciferase activity was determined 48 h after transfection (right). Results are given as means \pm SE of five independent experiments in triplicate. * $P < 0.05$. Luciferase activities were normalized on the basis of β -galactosidase activity. **B:** p300/CBP cooperates with the HNF-1 α transactivation domain. An increasing amount of the expression plasmid for GAL4-HNF-1 α was co-transfected with 4 μ g of 5 \times GAL4-TATA-luciferase reporter gene into HepG2 cells by the calcium phosphate precipitation method. Relative luciferase activities compared without the GAL4-HNF-1 α expression plasmid were determined 48 h after transfection (left). HepG2 cells were co-transfected with 4 μ g of 5 \times GAL4-TATA-luciferase reporter gene, 0.05 μ g of GAL4-HNF-1 α , and 0.25 μ g of expression plasmid for CBP or p300 by the calcium phosphate precipitation method. Luciferase activity was determined 48 h after transfection (right). Results are given as means \pm SE of five independent experiments in triplicate. * $P < 0.05$. Luciferase activities were normalized on the basis of β -galactosidase activity.

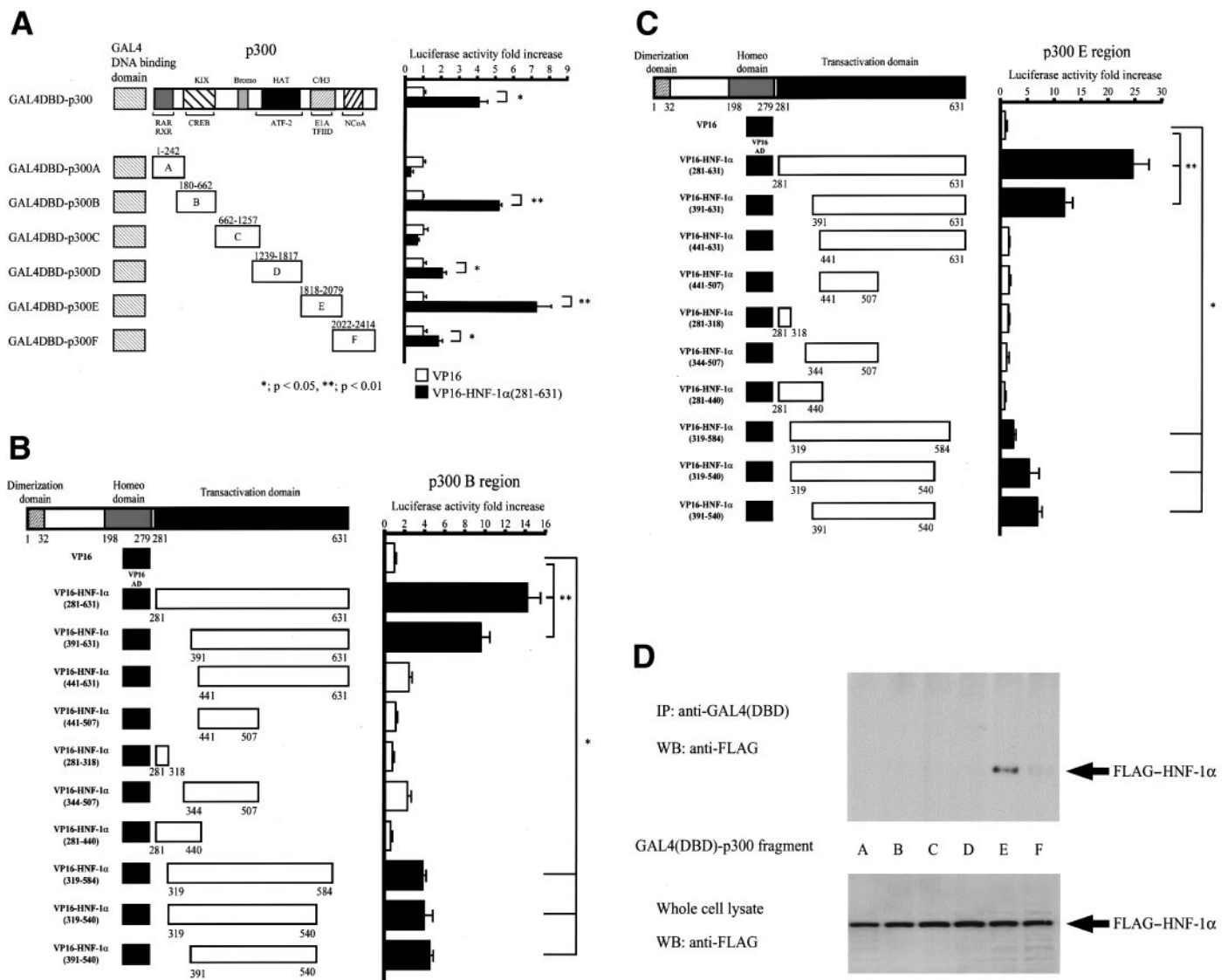


FIG. 4. Interaction between HNF-1 α and p300. **A:** In vivo interaction of HNF-1 α to the various fragments of p300. The structures of the GAL4-p300 fusion proteins used for mammalian two-hybrid assay are indicated. HepG2 cells were co-transfected with 4 μ g of 5 \times GAL4-TATA-luciferase reporter gene, 4 μ g of GAL4-p300 or one of the p300 deletion vectors, and 4 μ g of VP16 alone (□) or VP16-HNF-1 α expression plasmids (VP16-HNF-1 α [281-631]; ■), by the calcium phosphate precipitation method. Luciferase activity was determined 48 h after transfection. Results are given as means \pm SE. * $P < 0.05$; ** $P < 0.01$. Luciferase activities were normalized on the basis of β -galactosidase activity. **B** and **C:** In vivo interaction of the p300 B (**B**) and p300 E (**C**) regions with various fragments of HNF-1 α . The structures of the VP16-HNF-1 α fusion proteins used for mammalian two-hybrid assay are indicated. HepG2 cells were co-transfected with 4 μ g of 5 \times GAL4-TATA-luciferase reporter gene, 4 μ g of VP16-HNF-1 α (amino acids 281-631) or one of the HNF-1 α deletion vectors, and 4 μ g of GAL4-p300B (amino acids 180-662) or p300E (amino acids 1,818-2,079) expression plasmids by the calcium phosphate precipitation method. Luciferase activity was determined 48 h after transfection. Results are given as means \pm SE * $P < 0.05$; ** $P < 0.01$. Luciferase activities were normalized on the basis of β -galactosidase activity. **D:** In vitro binding of HNF-1 α to the p300 region. A mixture of the GAL4-p300 expression plasmid and the plasmid to express the FLAG-tagged full-length HNF-1 α were transfected into COS-7 cells. Whole-cell lysates were prepared from the transfected cells and immunoprecipitated with the anti-GAL4 DNA binding domain antibody. The immune complexes were analyzed by 10% SDS-PAGE, followed by Western blotting using the anti-FLAG antibody (upper panel). The expression levels of transfected FLAG-tagged HNF-1 α were analyzed by 10% SDS-PAGE, followed by Western blotting using the anti-FLAG antibody (lower panel). Similar results were obtained in three independent experiments.

sults indicated that p300 specifically activates HNF-1 α -dependent transcription by interaction with the HNF-1 α activation domain.

The p300 co-activator interacts with sequences in HNF-1 α essential for activation. To confirm in vivo interaction between HNF-1 α and p300, we performed two-hybrid assays in mammalian cells. Chimeric proteins were created by fusing the p300 fragments in frame to the DNA-binding domain of GAL4 and fusing the COOH-terminal regions of HNF-1 α and the transcriptional activation domain of VP16. Transcriptional activation was then

examined in HepG2 cells transfected with a combination of these constructs. The basal activity is the luciferase activity obtained by a combination of expression plasmids of the GAL4 DNA-binding domain and the VP16 activation domain. The VP16 protein fused to the COOH-terminal region of HNF-1 α (281-631) stimulated GAL4-p300B (amino acids 180-662) activity sixfold and GAL4-p300E (amino acids 1,818-2,079) activity fivefold (Fig. 4A). The regions of HNF-1 α involved in interaction with the p300B or p300E region were then determined. NH₂-terminal or COOH-terminal truncation of HNF-1 α revealed that the p300B and

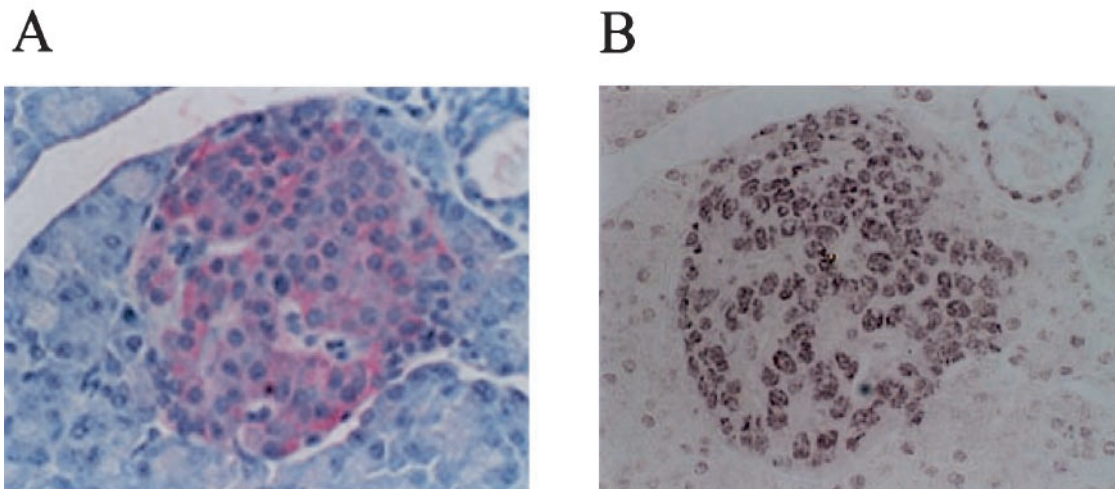


FIG. 5. Expression of insulin and p300 in pancreatic islets of 8-week-old rats was examined on consecutive 3.5 μ m sections. **A:** Immunohistochemical analysis of insulin (red). The pancreatic β -cells were confirmed by staining the cytoplasm with anti-insulin antibody. Hematoxylin nuclear counterstaining; magnification $\times 400$. **B:** Immunohistochemical analysis of p300. Immunohistochemical localization of the p300 protein in pancreatic islets was shown using anti-p300 antibody (black). Intense nuclear staining for p300 was observed in pancreatic β -cells; magnification $\times 400$.

p300E regions interacted with the transactivation domain of HNF-1 α containing the amino acids 391–540 structure (Fig. 4B and C).

The interaction between p300 and HNF-1 α in mammalian cells was also examined by co-immunoprecipitation procedures. The expression plasmids for the FLAG-tagged HNF-1 α and the fusion protein of partial fragments of p300 with the GAL4-DNA binding domain were transfected into COS-7 cells, and the cell lysates were immunoprecipitated with anti-GAL4-DNA binding domain antibody. Precipitates and lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibody. The results indicated that a fragment (amino acids 1,818–2,079) of p300 binds to HNF-1 α (Fig. 4D).

Expression of transcriptional co-activator p300 in pancreatic β -cells. To determine if p300 is expressed in pancreatic β -cells, immunohistochemical analyses were performed in pancreatic islets of 8-week-old rats using anti-p300 antibody and anti-insulin antibody. Pancreatic β -cells were detected by cytoplasmic staining with the anti-insulin antibody (Fig. 5A). In all, >90% of pancreatic β -cells showed intense nuclear staining by anti-p300 antibody (Fig. 5B).

DISCUSSION

Subjects with mutant HNF-1 α genes show impaired glucose-stimulated insulin secretion, even before they present with clinical manifestations of diabetes. We have shown that HNF-1 α is expressed in pancreatic β -cells. It is therefore important to determine which genes are regulated by HNF-1 α in pancreatic β -cells. Because glucose metabolism is essential in glucose-stimulated insulin secretion, GLUT2 and the glucokinase gene are candidates for HNF-1 α target genes. Glucokinase is the rate-limiting enzyme of the glycolytic pathway. In the pancreas of HNF-1 α -deficient mice, glucokinase is expressed at levels similar to those of control mice. This suggests that glucokinase is not regulated by HNF-1 α in pancreatic β -cells. GLUT2 is a facilitative glucose transporter, and its expres-

sion is strongly reduced in glucose-unresponsive islets in various animal models of diabetes (23,24).

Consensus binding sites for HNF-1 α have been identified in the promoter (–1,046 to –1,030, +87 to +116) of the human GLUT2 gene (25,26). In this study, however, we demonstrated that HNF-1 α stimulates GLUT2 gene expression through binding to its 5'-untranslated region. A series of sequential 5'-deletions of the GLUT2 gene were generated and fused to a luciferase reporter gene. An HNF-1 α response element was located in nucleotide +126 to +308 of the human GLUT2 gene, and that region was further examined by DNase I footprint analysis. Two sites were protected with the recombinant HNF-1 α protein, designated as BS1 (+95 to +117) and BS2 (+200 to +218). These two sites have sequences similar to the HNF-1 α consensus sequence. Mutation of the sequence BS2 (+200 to +218) within the human GLUT2 gene promoter markedly decreased both basal activity and HNF-1 α responsiveness, although mutation of the BS1 site did not affect basal or HNF-1 α -induced activity of the human GLUT2 promoter. These results indicated that the BS2 site is more important than the BS1 site in HNF-1 α -induced GLUT2 transcriptional activation. Recently, Cha et al. (26), using DNase I footprint analysis with liver cell extracts, showed that the regions corresponding to BS1 (Site-C) and BS2 (Site-B) in the human GLUT2 gene promoter were also protected. Those investigators focused on the BS1 (Site-C) region as the binding site of HNF-1 α , because the BS1 site was determined by the computer-based analysis to the candidate with the HNF-1 α binding consensus sequences. However, they did not analyze the BS2 region in the GLUT2 gene promoter.

Different classes of transcription factors recruit complexes with different configurations of the specific co-activator components, leading to differential requirements for their HAT activities. Soutoglou et al. (27) reported that the mechanism of HNF-1 α -mediated target gene expression *in vivo* might involve HNF-1 α -dependent remodeling of chromatin. They also reported that HNF-1 α interacts

with CBP, P/CAF, SRC-1, and RAC3. CBP and P/CAF can independently interact with the NH₂-terminal (DNA-binding domain) and COOH-terminal (transactivation domain) of HNF-1 α , respectively. However, Soutoglou et al. did not analyze p300. Our results suggest that p300 increased HNF-1 α -induced GLUT2 promoter activity more efficiently than did CBP. p300/CBP modulates the activity of key activators, including those involved in regulating cellular proliferation and differentiation. p300/CBP appears to function as a transcriptional co-activator by bridging, through direct interactions, the activator to the basal transcriptional machinery. In addition, these proteins have intrinsic HAT activity, which could modify chromatin structure by promoting a locally open and transcriptionally active configuration.

Here we have showed that p300/CBP interacts with HNF-1 α to potentiate human GLUT2 promoter activities. Results of our co-transfection experiments, using the promoter sequences from -206 to +308 in the GLUT2 gene as a reporter gene, indicate that p300/CBP additionally stimulates transcriptional activation by HNF-1 α (Fig. 3A). The BS2 site-mutated reporter gene was not activated by p300/CBP, however, indicating that HNF-1 α binding is critical in p300-induced GLUT2 gene expression. Further, p300/CBP stimulated transcriptional activation by the GAL4-HNF-1 α (amino acids 281-631) fusion protein. This suggests that p300/CBP can cooperate with the transactivation domain of HNF-1 α to induce GLUT2 gene expression.

We determined the regions of p300 interaction with HNF-1 α by mammalian two-hybrid and immunoprecipitation analyses. In the mammalian two-hybrid analysis, interactions were observed between the HNF-1 α transactivation domain and the p300B (amino acids 180-662) and p300E (amino acids 1,818-2,079) regions. In the immunoprecipitation analysis, interactions were observed between HNF-1 α and only the p300E region (amino acids 1,818-2,079). This discrepancy between immunoprecipitation and mammalian two-hybrid results has two possible explanations. First, it is possible that the association of the p300B region with HNF-1 α is not strong enough for detection by immunoprecipitation. Second, the association of the p300B region and HNF-1 α might be prevented by molecules that are present in the HepG2 cells, but not in the COS-7 cells.

In summary, the results of our study indicated that p300 is a potential co-activator of HNF-1 α -induced transcription. The transactivation domain of HNF-1 α has the ability to interact with p300. This suggests that HNF-1 α recruits p300 to regulate expression of the human GLUT2 gene, providing insight into the regulatory function of HNF-1 α and indicating a molecular basis for GLUT2 gene expression.

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