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Basic Transcription Element Binding Protein (BTEB) Transactivates the Cholesterol 7α-Hydroxylase Gene (*CYP7A*)

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Cholesterol 7α-hydroxylase catalyzes the first and rate-limiting step in the conversion of cholesterol to bile acids in the liver. Previously, we have identified two bile acid response elements located in nt −74 to −54 (BARE-I) and −148 to −118 (BARE-II) regions. The nucleotide sequences in these BAREs are highly conserved and shared a novel sequence, AGTTCAAG. To identify and isolate nuclear protein factors that bind to these BAREs, we have screened a human liver cDNA expression library with oligonucleotide probes containing the sequence from nt −149 to −127. Twenty positive clones were selected and purified. Partial nucleotide sequences of these clones were determined. Nucleotide homology search of DNA databases of the sequences of these clones revealed that sequence of one clone, G13, is identical to basic transcription element binding protein (BTEB), a GC box-binding protein of Sp1 family transcription factors known to regulate many cytochrome P450 genes. Electrophoretic mobility shift assays have identified a basic transcription element (BTE) in BARE-II and a Sp1 binding site located in the nt −100/−82 region of the *CYP7A* promoter. Transient transfection assays have confirmed that BTEB was able to transactivate the *CYP7A* promoter/luciferase chimeric gene.

Cholesterol 7α-hydroxylase catalyzes the first and rate-limiting reaction in the conversion of cholesterol to bile acids in the liver (1). The gene *CYP7A* is regulated at the transcriptional level by bile acids, hormones and diurnal rhythm. We have mapped the 5′-flanking region of the gene and identified several positive and negative regulatory elements (2, 3). Several transcription factors have been shown to transactivate the *CYP7A* gene. These includes albumin D-site binding protein (DBP), CCAAT enhancer binding protein (C/EBP and LAP), hepatocyte nuclear factor 3 (HNF3), and orphan nuclear receptors such as chicken ovalbumin upstream transcription factor (COUP-TFII), retinoid receptors (RXR/RAR) and HNF4 (3–6). Hydrophobic bile acids are potent feedback inhibitors of *CYP7A* transcription and mediate their effects through *cis*-elements located in two DNase I footprinted regions in the rat *CYP7A* promoter, which we named bile acid response elements (BARE) (7, 8). Two BAREs were identified; the BARE-I is located in nt −74 to −54 and contains a direct repeat of hormone response elements (HRE) half-site separated by four nucleotides (DR4). COUP-TFII binds this DR4 and transactivates *CYP7A* gene. Recently, it has been reported that this DR4 binds LXR/RXR, which may mediate oxysterol-dependent transactivation of the *CYP7A* gene (9). The BARE-II (−149/−128) contains three HREs, which forms overlapping DR1 (−146/−134) and DR5 (139/−123) with adjacent nucleotides. We have identified that HNF4 binds DR1 and RXR/RAR binds DR5 sequence (3). This BARE-II contains a novel AGGTCAAG sequence, which is completely conserved in the *CYP7A* genes of different species. This sequence is also present in BARE-I. To characterize this response element, we have screened a human liver cDNA expression library using an oligonucleotide probe containing BARE-II to isolate transcription factors that have affinity for BARE-II sequence. Here we report the identification of a clone, which encodes BTEB, a GC-box binding protein. Several other clones were also characterized.

EXPERIMENTAL PROCEDURES

Construction of the DNA binding site probe. One hundred nanomoles of each synthetic oligomer were mixed in kinase buffer, heated at 80°C for 5 min and let cool down slowly at room temperature. A nucleotide mixture (20 mM ATP, 20 mM DTT) and T4 polynucleotide

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TABLE 1

Oligonucleotide Probes and Consensus Sequences

−149/−127	gatcCTATGGACTTAGTTCAAGGCCGGatc
−149/−118	gatcCTATGGACTTAGTTCAAGGCCGGGTAATGCTAgatc
−138/−118 (R138)	gatccGTTCAAGGCCGGGTAATGCTAgatc
M138	gatccGTTCccttaatttgAATGCTAgatc
H138	gatcGTTCAAGGCCAGTTACTACCAgatc
BTE	gatcGAGAAGGAGGCGTGGCCAACgatc
Sp1	ATTCGATCGGGGCGGGC
−100/−82	gatcCTAGTAGGAGGACAAATAGgatc
M100	gatcCTAGTAttcttcaAAATAGgatc
GC box consensus sequence	GG GGGC GGCG TA TAAT

kinase were added and incubated at 37°C for 2 h, inactivated at 65°C and extracted once with phenol/chloroform and once with chloroform. After ethanol precipitation, the DNA pellet was resuspended in ligase buffer. Ligation reaction was carried out overnight at 15°C with T4 DNA ligase. The mixture was phenol/chloroform extracted and precipitated. The ligation products were labeled by Klenow reaction with [α-^{32}P]dCTP, purified by a G-50 Sephadex column.

Screening of human liver cDNA library. A human liver Uni-ZAP XR cDNA expression library (Stratagene, La Jolla, CA) was plated according to manufacturer's instruction. The plates were overlaid with nitrocellulose filters, which had previously been soaked in 10 mM IPTG. The filters were immersed in BLOTTO (10 mM Tris, 50 mM NaCl, 1 mM DTT and 1 mM EDTA containing 5% non-fat dried milk) and subsequently with labeled, double-stranded DNA probe ($1–2 \times 10^6$ cpm/ml). After overnight incubation at 4° the filters were washed three times in binding buffer and exposed to X-ray films. Positive plaques were picked up, and the phages were eluted and further purified through secondary or tertiary screening. Nucleotide sequences of these clones were determined using sequencing primers of pBluescript and Sequenase (USB/Amersham, Cleveland, OH) to obtain about 200 to 300 bp sequences of both ends.

Nuclear extracts. Nuclear extracts of rat liver were isolated as described (10). HepG2 cell nuclear extracts were prepared according to Dent and Latchman (11) after transfecting cells with 20 μg of pRSVBTEB or empty vector.

Transient transfection assay. Transient transfection assay of *CYP7A*/luciferase chimeric construct in HepG2 cells was performed as previously described (2). HepG2 cells grown to confluence in 12-well tissue culture plates were transfected with *CYP7A*/luciferase chimeric construct p-376/Luc as described previously by calcium phosphate method (2). This construct contains a *CYP7A* 5′-upstream sequence from −376 to +32. To study the effect of BTEB on transcriptional activity of *CYP7A*, pRSVBTEB plasmid was cotransfected with chimeric construct in HepG2 cells. Luciferase activities were determined with the luciferase assay kit (Promega, Madison, WI) using a Lumat LB9501 luminometer (Berthold System, Inc., Pittsburgh, PA). Luciferase activities were normalized for transfection efficiencies by dividing relative light units by β-galactosidase activity expressed from cotransfected pCMVβ plasmid. The graphs were plotted and statistics analyzed using Sigma Plot software (Jandel Scientific, San Rafael, CA).

Electrophoretic mobility shift assay (EMSA). Double-stranded probes for EMSA were prepared by heating 100 pmol each of complementary synthetic oligomers to 95°C in 2× SSC (0.3 M NCl, 0.03M trisodium citrate, pH 7.0) and allowing to cool to room temperature. The resulting double-stranded oligonucleotides were labeled by incorporation of [α-^{32}P]dCTP with the Klenow fragment of DNA polymerase I and purified through 15% polyacrylamide gels. Binding reactions were initiated with the addition of protein to 100,000 cpm of oligomer dissolved in 12 mM Hepes buffer, pH 7.4, 50 mM KCl, 2 mM DTT, 15% glycerol, 2 μg single stranded unrelated oligomer and 2μg poly (dI-dC)–poly (dI-dC). After incubation for 10 min at 25°C, samples were run on 4% polyacrylamide gels, dried and the resulting images were analyzed with PhosphorImager 445Si (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Screening of a human liver cDNA library for proteins bound to BARE-II. A double stranded oligonucleotide probe −149 to −127 (Table 1) was used to screen a human liver cDNA expression library to identify protein factors that specifically bind to this sequence. A total of 1×10^6 pfu were screened as described in Experimental Procedures. Following a tertiary screening, 20 positive plaques were selected, from which the recombinant pBlueScript was rescued from the Uni-ZAP XR vector. Analysis of the 3′ end 150 to 300 bp sequences using BLAST search of genomic databases allowed the identification of many of the clones. Ten of those clones have been identified as strongly related to the albumin, α-fetoprotein and vitamin D-binding family protein. Four others clones matched, respectively, with the human mitochondrial genome in the region from 7951 to 8631 that codes for the ATP synthase subunit 6, α-fibrinogen-like protein, a surface glycoprotein, and a 14-kDa unknown protein mainly expressed in human liver. These proteins are expressed abundantly in the liver and are likely to have bound non-specifically to the DNA probe during screening. Another clone, G13, contains sequences identical to BTEB, a GC box binding protein. BTEB binds to a *cis*-acting element named basic transcription element (BTE) and is required for a high level of inducible expression of the rat cytochrome P450c (CYP1A1) by xenobiotics (12, 13). Sequences of the remaining clones have not been matched to any sequences in the databases by BLAST search. However, analysis of amino acid composition by PROPSEARCH of EMBL database has revealed that Clone C has similarity to the human COUP-transcription factor and retinoic acid receptor. Clone G23 has functional homology to the StAR pro-

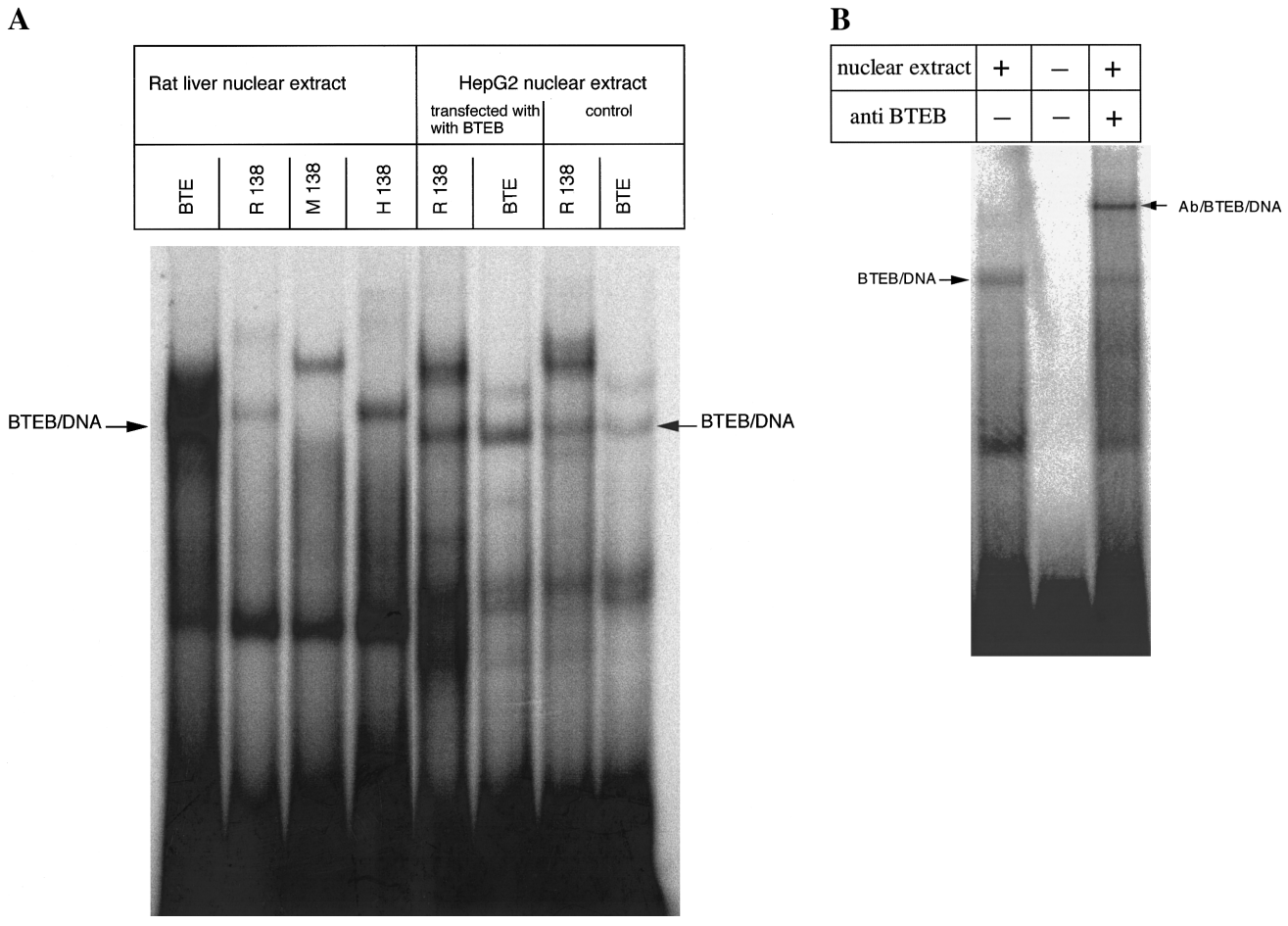

FIG. 1. Electrophoretic mobility shift assay of BTEB sequences in *CYP7A* promoter. (A) Double stranded oligonucleotide probes (sequences listed in Table 1) were labeled with ^{32}P. About 100,000 cpm of labeled probes was incubated with either rat liver nuclear extract (5 μg) or nuclear extracts of HepG2 cells transfected with pRSVBTEB or empty vector. EMSA was performed as described under Experimental Procedures. (B) Antibody super-shift assay. Antibody against BTEB (4μl) was added in EMSA of rat nuclear extract using −138/−118 probe. PhosphorImager scans of EMSA are shown.

tein, which is involved in steroid hormone synthesis. Clone H31 has similarity to nuclear hormone receptors FTZ-F1, and HNF4. These clones are potentially interesting since we have reported previously that COUP-TFII and HNF4 bind to this sequence (3, 6).

EMSA of BTEB binding to BARE-II. The isolation of BTEB as a result of our screening suggested that the GC-rich sequence GGCCGG in the probe might be the binding site for BTEB. Therefore, we attempted to verify the binding of BTEB to BARE-II by electrophoretic mobility shift assay. A double stranded oligonucleotide probe R138 (nt −138/−118 of the rat *CYP7A* gene, Table 1) shifted a faint band when mixed with rat liver nuclear extracts (Fig. 1A). This band is similar to the band shifted with a known BTE sequence of *CYP1A1* gene (12). R138 also shifted a band similar in mobility to BTEB when HepG2 nuclear extracts were used in EMSA. The slight difference in mobility of these BTEB/DNA complexes is likely due to different sizes of probes and different isoforms present in nuclear extracts of different species (Fig. 1A). Using rat liver nuclear extracts, the corresponding sequence of the human *CYP7A* (H138) showed a shifted band that was much stronger than that shifted by R138. Mutagenesis of GC rich sequences in −138/−118 (M138) completely abolished the binding of BTEB. Using either R138 or BTE as the probe, the BTEB binding activity was increased in nuclear extracts of HepG2 cells transfected with BTEB expression plasmid compared with that using nuclear extracts from HepG2 cells transfected with empty plasmid. This experiment further supports the identification of BTEB/DNA complex in EMSA. To further verify the binding of BTEB to probe R138, specific antibody against BTEB was added in reaction mixture and EMSA was performed to detect the formation of antibody/BTEB/DNA complexes. As shown in Fig. 1B, this BTEB specific antibody was able to supershift a band in rat liver nuclear extract with the R138 probe.

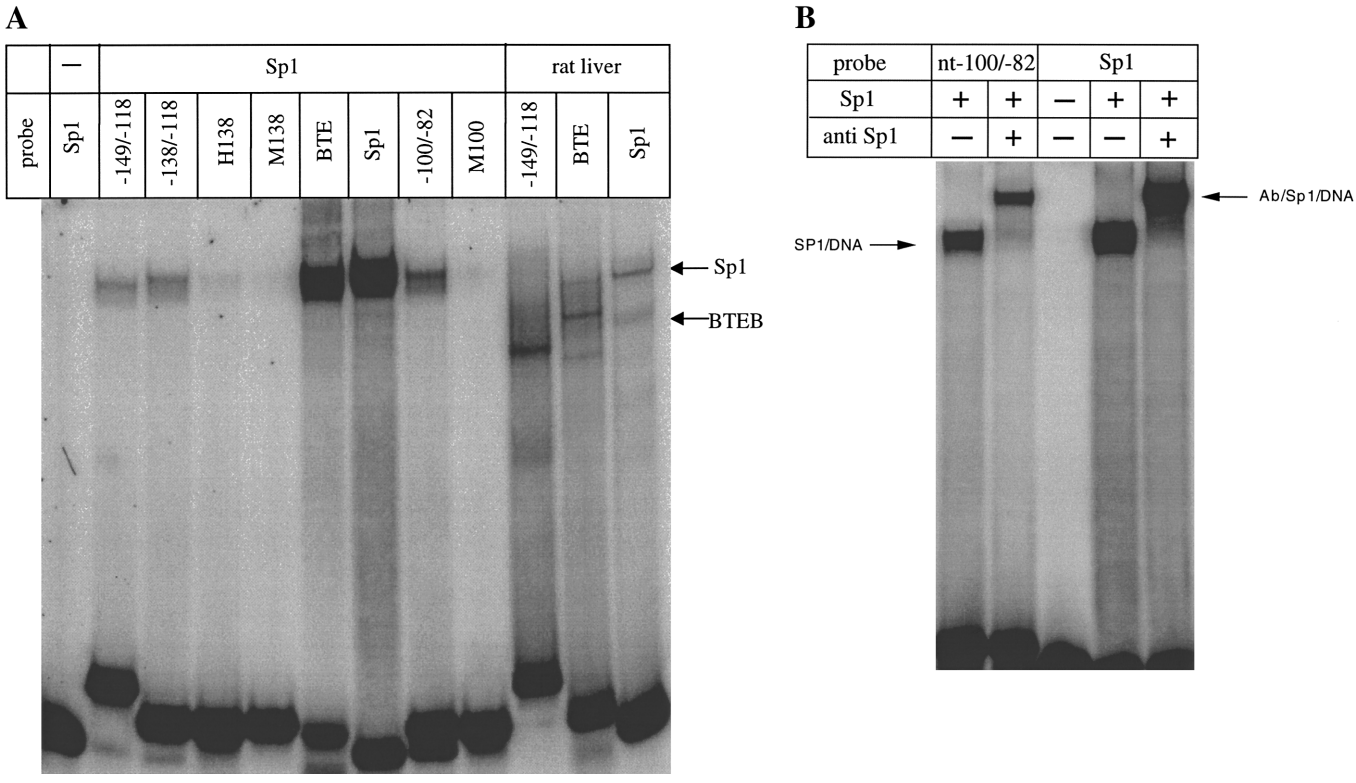

FIG. 2. Identification of Sp1 binding sites in *CYP7A* promoter. (A) Double stranded oligonucleotide probes (sequences listed in Table 1) were labeled with ^{32}P. About 100,000 cpm of labeled probes was incubated with 1 footprint unit of purified Sp1 (Promega) or rat liver nuclear extract (5 μg). (B) Double-stranded oligonucleotide containing nt −100/−82 or Sp1 sequence was used as a probe in EMSA of purified Sp1 protein. Antibody against Sp1 (1 μl) was added to interact with the DNA-protein complexes. EMSA was performed as described. PhosphorImager scans of EMSA are shown.

Identification of a SP1 binding site. The rat sequence −149/−118 contains overlapping DR1 and DR5 motifs and showed a major binding activity which was different from those using BTE or Sp1 as a probe and incubated with rat liver nuclear extract (Fig. 2A). This binding activity is due to binding of HNF4 to DR1 motif (3). The shorter sequence −138/−118 (R138) does not bind to HNF4 and contains a DR5 motif, which is GC rich. It was previously suggested th-rich sequence in −100/−82 of the *CYP7A* gene (Table 1, Ref. 14) might be a potential BTEB binding site. Since BTEB and Sp1 share similar binding specificity (15), we want to study the binding of purified Sp1 protein to these sequences by EMSA. As shown in Fig. 2A, Sp1, BTE and −100/−82 probes all shifted a strong band of similar mobility with Sp1 protein. On the other hand, −100/−82 probe did not bind to BTEB (data not shown). Both −149/−118 and −138/−118 bound weakly to Sp1. Interesting, the human −138/−118 probe (h138), which bound strongly to BTEB, did not bind to Sp1 protein. However, Sp1 protein bound much stronger to the rat sequence −100/−82 than to −149/−118 (or −138/−118) (Fig. 2A). Mutation of GC sequences in either −100/−82 (M100) or −138/−118 (M138) completely abolished Sp1 binding. To further confirm the binding of Sp1 protein to −100/−82 probe, antibodies against Sp1 was used to interact with DNA-protein complexes in EMSA. Antibody against Sp1 was able to supershift purified Sp1 with either −100/−82 or Sp1 cognate sequence as a probe (Fig. 2B). We conclude that there are two GC-rich sequences in the proximal promoter; BTEB preferentially binds to −138/−118 sequence, whereas Sp1 binds to the −100/−82 sequence.

Transactivation of CYP7A promoter by BTEB. We then studied the effect of BTEB on transcriptional activity of the *CYP7A*/luciferase chimeric construct using transient transfection assay assays in HepG2 cells. Previously we have shown that *CYP7A* promoter in p-376/Luc chimeric reporter gene could drive a high level of expression of luciferase activity when transiently transfected into HepG2 cells. Co-transfection of BTEB expression plasmid pRSV-BTEB together with p-376/Luc plasmid in HepG2 cells stimulated promoter activity in a dose-dependent manner with a maximal stimulation of about fourfold (Fig. 3). Thus, BTEB functions as a positive transcription factor on *CYP7A* promoter.

DISCUSSION

Screening of expression library with *cis*-acting element for DNA-binding proteins has been used to iso-

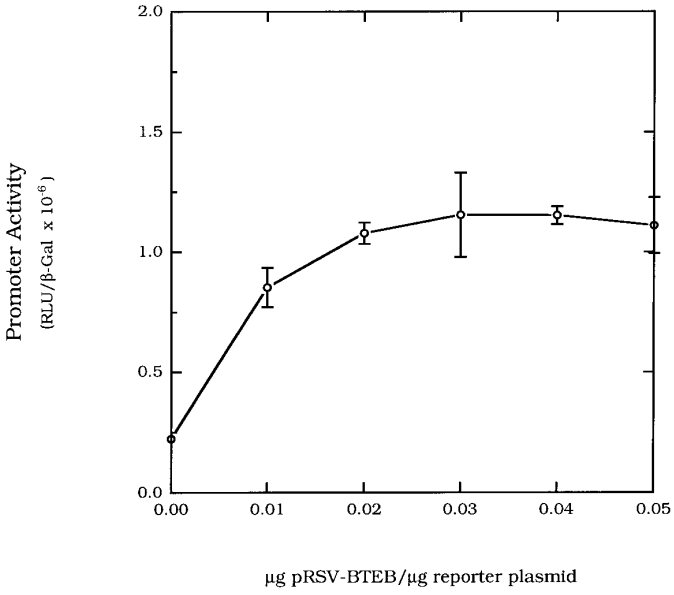

FIG. 3. Effect of BTEB on *CYP7A* transcription. Increasing amounts of pRSVBTEB plasmid were cotransfected with p-376/Luc chimeric construct in HepG2 cells. Luciferase activities expressed in HepG2 cells were measured as random light units (RLU) divided by β-galactosidase activity, which served as an internal standard for normalization of transfection efficiency. Results represent the mean $\pm$standard deviations of triplicate samples.

late potential transcription factors interacting with the bile acid response elements we identified previously. Although many clones isolated by DNA-protein interactions are apparently non-specific, this method directly isolates cDNA clones, the identity of which can be readily verified by searching expressed sequence tags (EST) in databases. The identification of cDNA clones encoding proteins with functional similarity to COUP-TF and HNF4 is consistent to our recent finding that the sequence from -149 to -127 (footprint B) binds these two nuclear hormone receptors (3, 6). One clone has functional homology to the StAR protein, which has been reported to play a role in regulation of steroid 27-hydroxylase, a mitochondrial enzyme in bile acid biosynthesis pathway (16).

The isolation of a clone identical to BTEB suggests that Sp1 family proteins are important in regulating *CYP7A* gene transcription. Sp1 family proteins have been shown to be involved in transcription of many eukaryotic genes by binding to GC-rich sequences in the promoters (17). BTEB and Sp1 activate the expression of genes with tandem repeats of GC box sequence in promoters, while BTEB represses the activity of a promoter containing a single GC box sequence BTE (13). In this study, we identified two GC box sequences in the proximal promoter of the rat *CYP7A* gene. These two GC box sequences apparently are functional to explain the stimulation of *CYP7A* promoter by overexpressed BTEB in transfection assay. Recently, Sp1 proteins have been reported to play an essential role in transactivation of LDL receptor and fatty acid synthase genes by SREBP, a steroid response element binding protein (18). The close proximity of BTE to HNF4 and RXR/RAR binding sites, and of the downstream Sp1 site to LXR/RXR binding sites in the *CYP7A* promoter suggest that Sp1 family proteins may also play important roles in transcriptional regulation of *CYP7A* gene.

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REFERENCES

1. Chiang, J. Y. L. (1998) *Front Biosci.* **3,** D176–D193.
2. Crestani, M., Stroup, D., and Chiang, J. Y. L. (1995) *J. Lipid Res.* **36,** 2419–2432.
3. Crestani, M., Sadeghpour, A., Stroup, D., Gali, G., and Chiang, J. Y. L. (1998) *J. Lipid Res.* **39,** 2192–2200.
4. Lavery, D. J., and Schibler, U. (1993) *Genes Dev.* **7,** 1871–1884.
5. Lee, Y.-H., Alberta, J. A., Gonzalez, F. J., and Waxman, D. J. (1994) *J. Biol. Chem.* **269,** 14681–14689.
6. Stroup, D., Crestani, M., and Chiang, J. Y. L. (1997) *J. Biol. Chem.* **272,** 9833–9839.
7. Stroup, D., Crestani, M., and Chiang, J. Y. L. (1997) *Am. J. Physiol.* **273,** G508–G517.
8. Chiang, J. Y. L., and Stroup, D. (1994) *J. Biol. Chem.* **269,** 17502–17507.
9. Lehmann, J. M., Kliewer, S. A., Moore, L. B., Smith-Oliver, T. A., Oliver, B. B., Su, J.-L., Sundseth, S. S., Winegar, D. A., Blanchard, D. E., Spencer, T. A., and Wilson, T. M. (1997) *J. Biol. Chem.* **272,** 3137–3140.
10. Sierra, F., Tian, J.-M., and Schibler, U. (1993) *in* Gene Transcription: A Practical Approach (Hames, B. D., and Higgins, S. J., Eds.), pp. 125–152, IRL Press, Oxford.
11. Dent, C. D., and Latchman, D. S. (1993) *in* Gene Transcription: A Practical Approach (Hames, B. D., and Higgins, S. J., Eds.), pp. 1–26, IRS Press, Oxford.
12. Yanagida, A., Sogawa, K., Yasumoto, K.-I., and Fuji-Kuriyama, Y. (1990) *Mol. Cell. Biol.* **10,** 1470–1475.
13. Imataka, H., Sogawa, K., Yasumoto, K.-i., Kikuchi, Y., Sasano, K., Kobayashi, A., Hayami, M., and Fujii-Kuriyama, Y. (1992) *EMBO J.* **11,** 3663–3671.
14. Cohen, J. C., Cali, J. J., Jelinek, D. F., Mehrabian, M., Sparkes, R. S., Lusis, A. J., Russell, D. W., and Hobbs, H. H. (1992) *Genomics* **14,** 153–161.
15. Sogawa, K., Kikuchi, Y., Imataka, H., and Fujii-Kuriyama, Y. (1993) *J. Biochem.* **114,** 605–609.
16. Sugawara, T., Lin, D., Holt, J. A., Martin, K. O., Javitt, N. B., Miller, W. L., and Strauss, J. F. I. (1995) *Biochemistry* **34,** 12506–12512.
17. Lania, L., Majello, B., and De Luca, P. (1997) *Int. J. Biochem. Cell Biol.* **29,** 1313–1323.
18. Yieh, L., Sanchez, H. B., and Osborne, T. F. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6102–6106.