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Direct binding of hepatitis B virus X protein and retinoid X receptor contributes to phosphoenolpyruvate carboxykinase gene transactivation

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Abstract The X gene product of the human hepatitis B virus (HBx), a major factor responsible for hepatitis and hepatocellular carcinoma, modulates transactivation by a variety of transcription factors. Herein, expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene was found to be regulated transcriptionally by HBx through two distinct promoter regions. The cAMP response element (CRE)-1 site within the proximal promoter region mediated the HBx-induced transactivation of the PEPCK gene through C/EBPα and ATF-2. A retinoid X receptor (RXR) response element within the distal promoter region also contributed to the HBx-induced transactivation. Consistent with these results, HBx directly interacted with RXR, and the interaction interfaces were localized to the transactivation domain of HBx and the ligand binding domain of RXR.

Key words: Hepatitis B virus X protein; Phosphoenolpyruvate carboxykinase; Retinoid X receptor; ATF-2; Transactivation

1. Introduction

Hepatitis B virus (HBV) mainly infects liver tissue and is associated with hepatitis and hepatocellular carcinomas [1,2]. Several significant findings suggest that HBV-X protein (HBx) could be a causative factor in the progression of these diseases [3]. HBx is known to transcriptionally regulate a variety of cellular and viral genes. The finding that HBx itself does not bind to double-stranded DNA and that genes stimulated by HBx lack any obvious consensus sequences suggest that HBx stimulates transcription presumably by interacting with cellular proteins and/or components of signal transduction pathways. Consistent with this hypothesis, HBx has been shown to directly interact with transcriptional factors such as RPB5 of RNA polymerase [4], TATA binding protein [5] and bZIP proteins [6], and to activate signal transduction pathways, including Ras/Raf/MAP kinase, protein kinase C, Jak1-STAT and NF-κB [7,8].

The phosphoenolpyruvate carboxykinase (PEPCK) promoter is a well-defined model for metabolic regulation of gene expression [9]. PEPCK, which catalyzes a regulatory step in gluconeogenesis, is expressed primarily in the liver, kidney, small intestine and adipose tissue, where its synthesis is regulated at the level of transcriptional initiation. The PEPCK promoter integrates cues arising from diverse signaling pathways. PEPCK mRNA is induced by glucocorticoids, thyroid hormone or glucagon [10], whereas insulin results in a repression of the promoter activities in a dominant manner [11]. The PEPCK promoter fragment encompassing −460 to +73 was demonstrated to be sufficient for hormonal regulation in liver, and many of the transcription factors that bind elements in this region have been identified [12,13]. Proteins demonstrated to bind and impact regulation of the PEPCK promoter include CREB, C/EBPα, C/EBPβ, ATF-2, NF1, HNF3, glucocorticoid receptor (GR), thyroid hormone receptor (TR), retinoic acid receptor (RAR) and retinoid X receptor (RXR). The energy balance state can effect signals for the PEPCK gene regulation through activating CREB, C/EBPα and C/EBPβ [14], whereas ATF-2 mediates the stress response signals [15].

The overlapping expression profile of HBV and PEPCK led us to determine whether HBV infection impacts transcription of the PEPCK gene. Notably, adenovirus infection was previously shown to regulate the PEPCK gene transcription through E1A expression [16]. In this report, we show that HBx indeed regulates the transcription of the PEPCK gene through direct interaction with both RXR and CRE-1 binding proteins.

2. Materials and methods

2.1. Plasmids

pSG5-HBx, -D1 and -D5 constructs for mammalian transient transfection were kindly provided by Dr. Seishi Murakami (Kanazawa University, Japan) [17]. HBx, D1 and D5 fragments amplified by polymerase chain reaction (PCR) were cloned into the *Bam*HI and *Eco*RI restriction sites of pCMV-GAL4 plasmid (pCMX1) for the mammalian two-hybrid assays. The reporter plasmids, PEPCK-275 and PEPCK-543, were constructed by using PCR amplified fragments of rat genomic DNA, which encompasses positions −275/−543 through +73 of the PEPCK promoter [18].

2.2. Cell culture and transient transfection

HepG2 cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal

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Abbreviations: HBV, hepatitis B virus; PEPCK, phosphoenolpyruvate carboxykinase; HBx, hepatitis B virus X protein; CRE, cAMP response element; RAR, retinoic acid receptor; RXR, retinoid X receptor; GR, glucocorticoid receptor; TR, thyroid hormone receptor; LBD, ligand binding domain; GST, glutathione *S*-transferase

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bovine serum (FBS) (Gibco BRL) and 1% antibiotics. The cells were seeded in 24-well plates with growth medium supplemented with 10% charcoal-stripped serum and co-transfected with pRSV/β-gal vector and expression vectors for HBx, D1, D5, C/EBPα, ATF-2 and RXR. Total amounts of expression vectors were kept constant by adding pcDNA3.1/His C, along with the reporter constructs containing the PEPCK gene promoter sequences. The relative luciferase and β-galactosidase activities were determined as described [19]. All the transfection results represent the mean of three independent experiments.

2.3. Gel shift analysis

Full-length C/EBPα and ATF-2 proteins were prepared by in vitro transcription-coupled translation (TNT, Promega, Madison, WI, USA) under conditions described by the manufacturer. A set of double-stranded oligonucleotides encoding the PEPCK CRE-1 sequence (promoter positions 99 to 76) was used as a probe: 5′ CCGGCCCCTTACGTCAGAGGCG. Binding reactions were assembled without the labeled probe and held 5 min on ice followed by 5 min at room temperature. The labeled probe was added and further incubated for 30 min. Samples were separated in 4% acrylamide–0.5× TBE (0.045 M Tris, 0.045 M boric acid, 1.0 mM EDTA, pH 8.0) gels run at constant voltage (200 V).

2.4. Glutathione S-transferase (GST)-pull down assay

GST-fusion proteins were purified as described previously [19]. Equal amounts (approximately 1 μg) of GST, GST-HBx, GST-D1 or GST-D5 immobilized on 20 μl of glutathione Sepharose beads were incubated with in vitro translated ^{35}S-RXR, RXR-ABC, RXR-ligand binding domain (LBD), and RAR in the reaction buffer (25 mM HEPES, pH 7.6, 20% glycerol, 120 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.1% bovine serum albumin, 0.1% Triton X-100) in the absence or presence of 100 nM of 9-*cis*-RA for 4 h at 4°C. After washing three times with phosphate buffered saline, the bound proteins were eluted with reduced glutathione and boiled with an equal volume of 2× Laemmli sample buffer at 100°C for 3 min prior to electrophoresis. After electrophoresis, the gel was dried and analyzed with the Molecular Imager Fx (Bio-Rad).

2.5. Mammalian two-hybrid assay

Cells were seeded with growth medium supplemented with 10% FBS and 1% antibiotics, and co-transfected with expression vectors encoding Gal4-DNA binding domain fusions (pCMX/Gal4N-D1 or pCMX/Gal4N-D5) and VP16-activation domain fusions (pCMX/VP16/-, pCMX/VP16-RXR-LBD or pCMX/VP16-RAR-LBD) as well as the previously described Gal4-tk-luc reporter plasmid. After 48 h, cells were harvested and the luciferase activity was normalized to the β-galactosidase expression. All the results represent the average of at least three independent experiments.

3. Results

3.1. HBx stimulates transcription of the PEPCK promoter

To examine the effect of HBV on the regulation of PEPCK promoter activity, we transfected an expression vector encoding HBx, a transactivator protein of HBV, into HepG2 cells. For these experiments, we used two different PEPCK promoter/reporter plasmids, PEPCK-275 and PEPCK-543 (Fig. 1A). Transfection with HBx expression plasmid resulted in increased luciferase activity of both PEPCK reporters (Fig. 1B). HBx stimulated the promoter activity of PEPCK-275 construct approximately four-fold. The HBx-D1 fragment containing the HBx transactivation domain resulted in approximately six-fold activation, whereas the N-terminal domain D5 led to marginal transactivation (Fig. 1B). Thus, HBx appeared to positively regulate the PEPCK promoter activity in a manner specifically dependent on the transactivation domain of HBx. Interestingly, the transcriptional activity of the PEPCK-543 reporter was more strongly induced by HBx than that of the PEPCK-275 reporter. For instance,

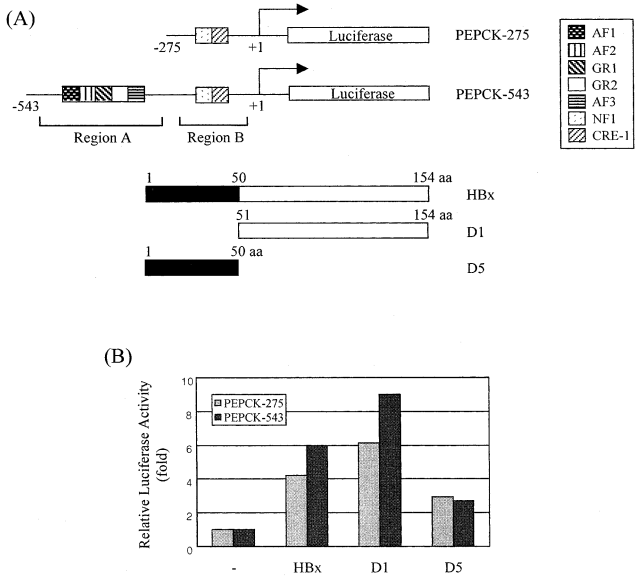

Fig. 1. HBx enhances the PEPCK transactivation. A: Schematic representation of two luciferase reporter constructs as well as various HBx constructs are shown. The region A contains the glucocorticoid response unit (GRU) composed of two glucocorticoid regulatory elements, three accessory factor binding sites and a CRE. This region provides several factor binding sites for RAR, RXR, GR, TR, C/EBP, and HNF-3 [22]. The region B contains CRE-1 (−91 to −84) and is immediately adjacent to a nuclear factor 1 (NF1) binding site [22]. The HBx fragments containing two different functional domains, the regulatory domain (D5) and the transactivation domain (D1), are as shown [17]. B: The hepatoma cell line HepG2 was transfected with 20 ng of reporter plasmid alone, or along with 50 ng of the indicated mammalian expression vector. All the transfection results were normalized to β-galactosidase activity, and the presented results represent the average of three independent experiments, with fold induction over the level observed with the reporter alone.

coexpression of D1 resulted in approximately nine-fold potentiation (Fig. 1B). Thus, not only the region B but also the region A of the PEPCK promoter (Fig. 1A) should contain HBx-responsive elements that mediate the HBx-dependent transactivation of the PEPCK promoter constructs.

3.2. C/EBPα and ATF-2 mediate the HBx-dependent transactivation of PEPCK promoter through the PEPCK CRE-1 site

We next wanted to determine the role of the CRE-1 site within the PEPCK-275 construct in the HBx-mediated transactivation. Previous reports have shown that both CREB/ATF and C/EBP proteins bind this site [15]. Thus, we examined whether overexpression of C/EBPα or ATF-2 can enhance the HBx-induced transactivation activity of the PEPCK promoter. Cotransfection of HBx with C/EBPα exerted a synergistic effect on the transcription activities of the PEPCK-275 construct (Fig. 2A). Similar results were also obtained with ATF-2. Given the presence of the additional CRE sites in the PEPCK-543 construct, one might expect stronger transactivation of the PEPCK-543 construct relative to the PEPCK-275. However, overexpression of C/EBPα or ATF-2 did not increase the transcription activity of the PEPCK-543 construct beyond that of the PEPCK-275 reporter (Fig. 2A). These results suggest that C/EBPα or ATF-2 mediates the HBx-induced transactivation of the PEPCK-275 promoter construct,

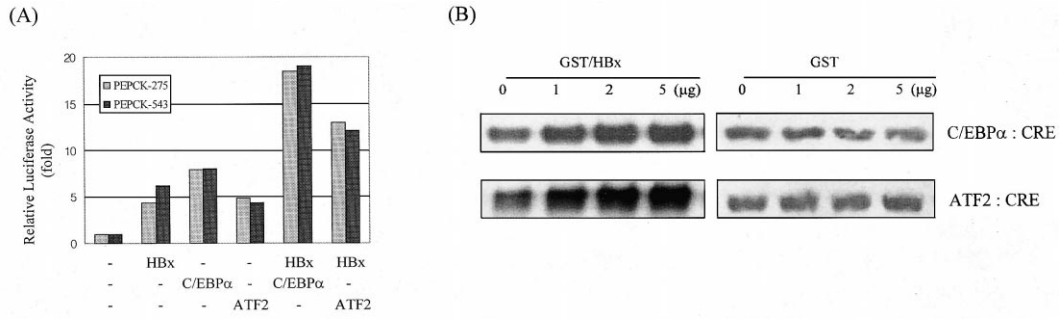

Fig. 2. C/EBPα and ATF-2 mediate the HBx-induced transactivation of the PEPCK gene expression. A: Expression vectors for C/EBPα and ATF-2 were transfected into HepG2 cells along with reporter constructs PEPCK-275 or PEPCK-543, either in the presence or absence of HBx-expression plasmid. All the transfection results were normalized to β-galactosidase activity, and the presented results represent the average of three independent experiments, with fold induction over the level observed with the reporter alone. B: A double-stranded oligonucleotide probe containing the CRE-1 site of the PEPCK promoter was used in electrophoretic mobility shift analysis. 2 μl of in vitro transcription coupled translation reactions was added in a binding reaction either in the absence or presence of increasing amount of GST protein or GST-HBx protein.

whereas yet other factors are involved in the HBx-mediated transactivation through the region A of the PEPCK promoter (i.e. the PEPCK promoter sequences −543 to −276). Since HBx stimulates in vitro DNA binding of a variety of cellular proteins that contain bZIP DNA binding domains [6], we examined whether HBx increases the DNA binding activity of those factors at the PEPCK CRE-1 site. Interestingly, HBx significantly enhanced the DNA binding activity of C/EBPα and ATF-2 in a dose-dependent manner (Fig. 2B). Thus, the observed HBx-dependent transactivation of the PEPCK promoter constructs may involve the HBx-dependent increase in the DNA binding activity of C/EBPα and ATF-2, although HBx may also affect other aspects of C/EBPα and ATF-2.

3.3. 9-*cis*-RA potentiates the HBx-mediated transactivation of the PEPCK promoter

From the results presented in Fig. 1, an additional element(s) in the promoter region A (Fig. 1A) may mediate the HBx-dependent transactivation of the PEPCK promoter. This region of the promoter has binding sites for nuclear receptors [20], including RAR, RXR, GR and TR. Since TR and RAR mediate the basal repression and GR is cytoplasmic in the absence of their cognate ligand, we reasoned that RXR is a good candidate factor responsible for the HBx-mediated transactivation of the PEPCK promoter region A. Confirming this prediction, coexpression of RXR alone enhanced the HBx-mediated transactivation of the PEPCK-543 promoter, which was further stimulated by 9-*cis*-RA (Fig. 3A). This increased transcriptional activity by RXR and HBx was not observed with the PEPCK-275 promoter (results not shown). Interestingly, coexpression of the HBx-D1 fragment alone stimulated the PEPCK–RXR transactivation at a level similar to that observed with the full length HBx. In contrast, RAR did not enhance the HBx-induced transactivation of the PEPCK-543 construct (results not shown). These results indicate that RXR, either in the absence or presence of 9-*cis*-RA, renders HBx to stimulate the PEPCK transactivation through the PEPCK promoter region A. Consistent with these results, 9-*cis*-RA increased the HBx-dependent transactivation of the PEPCK-543 reporter construct, whereas all-*trans*-RA, dexamethasone and T3 were without any significant effect, although treatment of each ligand marginally increased the PEPCK-543 transactivation in the absence of HBx (approximately two-fold) (Fig. 3B).

3.4. HBx can directly interact with RXR

Given the combined results of HBx and RXR (Fig. 3), we

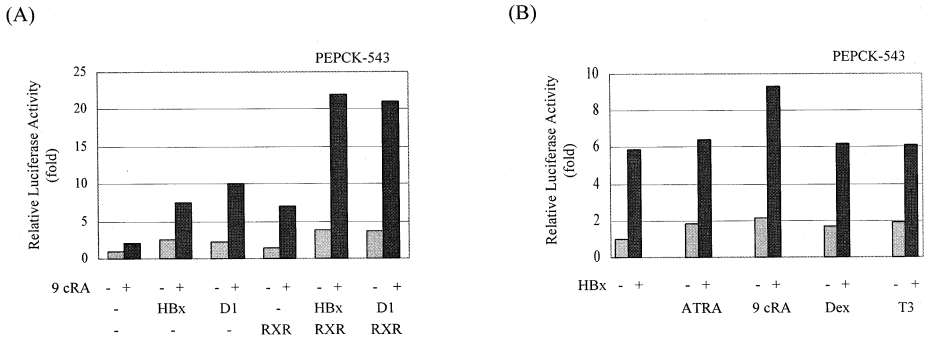

Fig. 3. HBx and RXR synergize in the PEPCK transactivation. HepG2 cells were transfected with the indicated expression plasmids and assayed for reporter activity with the PEPCK-543 reporter construct. 20 ng of reporter vector was transfected into HepG2 cells along with 50 ng of the indicated expression plasmid, either in the absence or presence of 100 nM of 9-*cis*-RA (A, B), all-*trans*-RA, dexamethasone or triiodothyronine (B). 48 h after transfection, cells were harvested for luciferase activities. All the transfection results were normalized to β-galactosidase activity, and the presented results represented the average of four independent experiments, with fold induction over the level observed with the reporter alone.

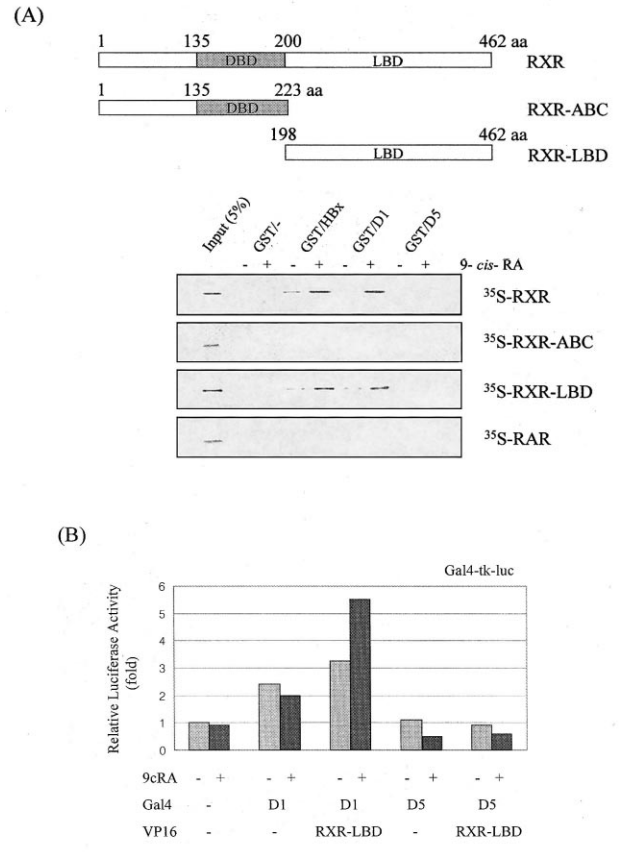

Fig. 4. HBx can directly interact with RXR. A: The full-length RXR as well as its deletion mutants are schematically shown. GST fusions to the full-length HBx, D1 and D5 were purified from *Escherichia coli*. In vitro translated proteins in the presence of ^{35}S-methionine (RXR full-length, RXR-ABC, RXR-LBD and RAR) were incubated with glutathione-resin immobilized GST, GST-HBx, GST-D1 or GST-D5 in the absence or presence of 100 nM of 9-*cis*-RA. The bound proteins were eluted with reduced glutathione and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. B: The mammalian expression plasmids encoding VP16-RXR-LBD and GAL4-HBx were transfected into HepG2 cells, as indicated. Forty-eight h after transfection, cells were harvested for luciferase activities. All of the transfection results were normalized to β-galactosidase activity, and the presented results represent the average of three independent experiments, with fold induction over the level observed with the reporter alone.

tested whether HBx protein directly interacts with RXR. GST fusion proteins encoding the full-length HBx as well as D1 and D5 were produced in bacteria, immobilized on glutathione Sepharose beads, and incubated with in vitro-translated ^{35}S-labeled RXR, RXR-ABC, RXR-LBD and RAR. Consistent with the transfection results, the full-length HBx protein and D1 interacted with RXR in a 9-*cis*-RA-enhanced manner (Fig. 4A). However, D5 encoding the regulatory domain of HBx failed to associate with RXR. In addition, this interaction was mapped to the LBD of RXR. RAR as well as the ABC domains of RXR did not interact with HBx. From these results, the transactivation domain of HBx was suggested to specifically associate with the LBD of RXR in a 9-*cis*-RA-enhanced manner. To confirm the association of HBx with RXR in vivo, we utilized the mammalian two-hybrid assays. Coexpression of Gal4-D1 and VP16-RXR-LBD enhanced the Gal4-tk-luc-dependent transactivation in a ligand-dependent manner, whereas Gal4-D5 was inert. These results indicate that the transactivation domain of HBx interacts with RXR in vivo in a ligand-dependent manner. As suspected, HBx did not interact with RAR in the mammalian two-hybrid assay (results not shown).

4. Discussion

In conclusion, we have shown that the PEPCK promoter activity is up-regulated by HBx, which involved the functional interactions of HBx with both bZIP proteins and RXR. HBx is an important regulator of HBV genome expression, infectiousness and proliferation of liver cells. Chronic liver infection by HBV leads to profound changes in hepatocyte physiology including the expression of various proteins that are not normally expressed or under-expressed in uninfected hepatocytes, including genes involved with gluconeogenesis. Thus, this report adds the PEPCK promoter to the list of gluconeogenesis genes up-regulated by HBx.

Finally, it is interesting to note that the PEPCK promoter is a typical enhancersome, comprised of a series of *cis*-elements, including binding sites for bZIP proteins and nuclear receptors. Our findings that HBx functionally interact with RXR and bZIP proteins may shed some light into how the PEPCK enhancersome is regulated upon HBV infection. HBx can stabilize the assembly of PEPCK enhancersome through association with bZIP proteins and RXR. In addition, HBx may enhance the function of PEPCK enhancersome by juxtaposing components of the transcriptional machinery in a more favorable orientation [21], and may also play a role in recruiting transcriptional coactivators.

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