Prospero-related homeobox protein (Prox1) inhibits hepatitis B virus replication through repressing multiple cis regulatory elements

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Hepatitis B virus (HBV) gene transcription is controlled by viral promoters and enhancers, the activities of which are regulated by a number of cellular factors as well as virally encoded proteins. Negative regulation of HBV cis-element activities by cellular factors has been reported less widely than their activation. In this study, we report that nuclear factor Prospero-related homeobox protein (Prox1) represses HBV antigen expression and genome replication in cultured hepatocytes. By using reporter-gene analysis, three of the four HBV promoters, namely the enhancer II/core promoter (ENII/Cp), preS1 promoter (Sp1) and enhancer I/X promoter, were identified as targets for Prox1-mediated repression. Mechanistic analysis then revealed that, for ENII/Cp, Prox1 serves as a corepressor of liver receptor homologue 1 (LRH-1) and downregulates LRH-1-mediated activation of ENII/Cp, whereas for Sp1, Prox1 partially represses Sp1 activity by interacting directly with hepatocyte nuclear factor 1. Identification of Prox1 as an HBV repressor will help in the understanding of detailed interactions between viral cis elements and host cellular factors and may also form the basis for new anti-HBV intervention therapeutics.

INTRODUCTION

The species Hepatitis B virus (HBV) belongs to the family Hepadnaviridae. Approximately 350 million individuals worldwide are infected with HBV (Ocama et al., 2005); chronic HBV infection can lead to severe liver diseases such as cirrhosis and hepatocellular carcinoma (Lee, 1997). Current treatment of chronic hepatitis B does not produce satisfactory results in a considerable proportion of patients, and development of more effective therapy is still an important task.

HBV contains a 3.2 kb partially double-stranded, relaxed-circular DNA (rcDNA) genome, which is converted to covalently closed circular DNA (cccDNA) in the nuclei of infected cells. Four promoters on viral cccDNA, namely the core (Cp), preS1 (Sp1), preS2 (Sp2) and X (Xp) promoters, respectively control the production of the preC/pregenomic (3.5 kb), preS1 (2.4 kb), preS2/S (2.1 kb) and X (0.8 kb) transcripts (Fig. 1). Cp plays a critical role in HBV gene expression and replication because it controls the transcription of pregenomic RNA (pgRNA), which serves as the template for both reverse transcription, to produce progeny virus genomes, and translation of the core and polymerase proteins. Sp1 initiates transcription of the mRNA encoding the large surface protein, which is essential for virion formation and infectivity (Seeger & Mason, 2000). Sp2 directs the synthesis of the preS2/S transcript, which encodes the middle and small surface proteins. Xp is responsible for the transcription of the mRNA of the X protein, a promiscuous viral regulator (Murakami, 2001). In addition to these promoters, two enhancer elements, enhancers I (ENI) and II (ENII), which overlap Xp and Cp, respectively, also play important roles in regulating viral gene expression (Seeger & Mason, 2000).

A number of ubiquitous and liver-enriched transcription factors have been shown to regulate the activities of HBV promoters and enhancers (Moolla et al., 2002; Schaller & Fischer, 1991). The majority are activators that stimulate the activities of viral cis elements. For example, the liver-enriched liver receptor homologue 1 (LRH-1) binds to two recognition sites within ENII; such binding has been shown to be important for activation of ENII/Cp (Li et al., 1998).
On the other hand, hepatocyte nuclear factor 1 (HNF-1) binds to Sp1 and plays a critical role in regulating its basal activity (Chang et al., 1989; Raney et al., 1997). In comparison, negative regulation of HBV gene expression has been less widely reported and is less well understood. Chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) binds to both ENI and ENII and suppresses their activities (Yu & Mertz, 2003). Hepatocyte nuclear factor 4 (HNF-4) and testicular receptor 2 (TR2) exert negative effects on preC mRNA synthesis (Yu & Mertz, 1997). A negative-regulatory element that can downregulate Cp activity has also been identified upstream of ENII/Cp (Lo & Ting, 1994; Sun et al., 2001).

Prospero-related homeobox (Prox1) is a nuclear protein expressed in lens, heart, liver, kidney, spleen, skeletal muscle, pancreas and the central nervous system (Zinovieva et al., 1996). Previous work has identified Prox1 as a corepressor of LRH-1 in hepatocytes. In adult hepatocytes, Prox1 is recruited to the target gene promoter by binding LRH-1, and suppresses LRH-1-mediated transcriptional activation of human cholesterol 7-α-hydroxylase and small heterodimer partner genes (Qin et al., 2004; Steffensen et al., 2004).

In the present study, we investigate the potential regulation of HBV gene transcription by Prox1. Knockdown of endogenous Prox1 expression in cultured hepatocytes by RNA interference (RNAi) resulted in a significant upregulation of HBV replication and antigen expression, whereas overexpression of Prox1 reduced HBV replication and antigen expression dramatically. Further analysis revealed that Prox1 downregulates the activities of three viral cis elements: ENII/Cp, Sp1 and ENI/Xp. Mechanistic studies then demonstrated that Prox1 functions as a corepressor for LRH-1-mediated activation of ENII/Cp and partially suppresses Sp1 activity by interacting directly with HNF-1. Suppression of viral promoter/enhancer activities by Prox1 might constitute an important mechanism for the negative regulation of HBV transcription by host cellular factors and could be a potential target for the development of novel anti-HBV therapeutics.

**METHODS**

**Plasmids.** p1.3HBV contains a terminally redundant (1.3 × copy) replication-competent HBV genome (subtype adr) (Wang et al., 2007). The HNF-1-dependent artificial promoter was constructed by placing three copies of the HNF-1 consensus binding-site sequence from the human albumin gene promoter (Ott et al., 1991) upstream of the herpes simplex virus thymidine kinase promoter. The artificial promoter and HBV regulatory elements (ENI/Xp, nt 957–1354; ENII/Cp, nt 1627–1878; Sp1, nt 2704–2823; Sp2, nt 2978–3207) were inserted into pGL2basic (Promega) to create corresponding luciferase reporter plasmids. The complete cDNA of human HNF-1 (kindly provided by Dr Moshe Yaniv, CNRS Institut Pasteur, Paris, France) with or without an upstream FLAG tag was inserted into pcDNAs (Invitrogen Life Technologies). Fragments of HNF-1 (corresponding to aa 1–100, 1–281, 1–682, 76–198, 196–281 and 263–628) were fused downstream of the herpes simplex virus thymidine kinase promoter. The artificial promoter was constructed by placing three copies of the HNF-1 consensus binding-site sequence from the human albumin gene promoter (Ott et al., 1991) upstream of the herpes simplex virus thymidine kinase promoter. The artificial promoter and HBV regulatory elements (ENI/Xp, nt 957–1354; ENII/Cp, nt 1627–1878; Sp1, nt 2704–2823; Sp2, nt 2978–3207) were inserted into pGL2basic (Promega) to create corresponding luciferase reporter plasmids.

**Cell culture and transfections.** Human embryonic kidney (HEK)293, cervical carcinoma HeLa, hepatoma HepG2 and Huh7 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Invitrogen Life Technologies) at 37 °C and 5% CO2. HeLa, HepG2 and Huh7 cells were plated in 35 mm dishes and transfected by using the calcium phosphate precipitation method. Typical transfection efficiency was about 5–15%, as measured by fluorescence microscopy using the pEGFP-N1 reporter (Clontech). In general, cells were cotransfected with 0.5 μg luciferase reporter plasmid, 0.5 μg p1830 or pSuper along with 0.3 μg β-galactosidase control plasmid pCMV-lacZ (Promega). Forty-eight hours post-transfection, cells were harvested and luciferase activities were determined by using the Luciferase Assay system (Promega) and normalized against β-galactosidase activities, measured by using a standard colorimetric method. Each transfection was performed in duplicate dishes and repeated at least three times.

**Analysis of viral RNA and DNA.** HepG2 cells grown in 60 mm dishes were cotransfected with 2 μg p1.3HBV, 2 μg Prox1 expression plasmid or the empty vector pcDNAs3 and 1 μg pCMV-lacZ, along with 2 μg p1830 or pSuper and harvested 4 days later. Total RNA was isolated by using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA (20 μg) was separated on a denaturing formaldehyde 1% agarose gel, transferred onto nylon membranes (Amerham Pharmacia Biotech) and fixed by UV irradiation. The membranes were hybridized at 65 °C with an HBV-specific 32P-radiolabelled...
proteins were expressed in induced by 0.1 mM IPTG for 4 h at 37°C according to the manufacturer’s instructions. To control for non-specific interactions, FLAG-tagged coronin 1C, a cytoskeleton protein, was used as negative control.

**Analysis of secreted HBV antigens.** HepG2 or HuH7 cells grown in 35 mm dishes were cotransfected with 1 μg pHBV1.3, 1 μg Prox1 expression plasmid or pcDNA3 and 0.5 μg pSEAP2-Control expressing secreted alkaline phosphatase (SEAP), along with 2 μg pI830 or pSuper. SEAP was used for transfection-efficiency normalization instead of β-galactosidase because secreted proteins were analysed in this assay. Seventy-two hours post-transfection, culture media were collected and levels of HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) were measured by standard ELISA (Kehua Biotech). SEAP activity was measured as reported previously (Lin et al., 2001).

**GST pull-down assay.** Prox1 proteins were produced in vitro by using the TNT Quick Coupled Transcription/Translation system (Promega) and labelled with [35S]methionine (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. GST fusion proteins were expressed in *Escherichia coli* BL21(pLys) (Novagen) induced by 0.1 mM IPTG for 4 h at 37°C. Pull-down assays were performed by using 1 μg GST fusion protein and 10 μl [35S]-labelled protein. Purification of GST fusion proteins and GST pull-down assays were carried out essentially as described previously (Qin et al., 2004). To control for non-specific interactions, GST-fused coronin 1C, a cytoskeleton protein, was used as negative control.

**Co-immunoprecipitation.** HEK293 cells plated in 60 mm dishes were cotransfected with the expression plasmids of FLAG-tagged HNF-1 and Prox1 or HA-tagged Prox1 by using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer’s protocol (3 μg per plasmid per dish). Typical transfection efficiency was about 40–60%, as measured by fluorescence microscopy using the pEGFP-N1 reporter. Forty-eight hours post-transfection, cells were rinsed once with ice-cold PBS and lysed in 0.5 ml modified RIPA buffer [50 mM Tris/HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 0.03% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF and protease inhibitor cocktail (Roche)] at 4°C for 15 min. After centrifugation at 10,000 r.p.m. for 10 min, the supernatant was cleared with preimmune mouse serum (Santa Cruz) for 1 h at 4°C. Protein (1 mg) was incubated overnight with 5 μg mouse anti-FLAG monoclonal antibody (mAb) M2 (Sigma) or 5 μg anti-HA mAb (Santa Cruz) and 25 μl protein A-Sepharose (Amersham Pharmacia Biotech) for 4 h at 4°C. After washing four times with 0.5 ml wash buffer [8% glycerol, 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF and protease inhibitor cocktail (Roche)] the gel matrix was boiled in SDS loading buffer for 5 min and separated on a SDS/8% polyacrylamide gel and then transferred onto a nitrocellulose membrane (Schleicher & Schuell). Immunoblotting of the precipitated and 1% input proteins was performed by using anti-HA mAb (1:2000 dilution) and anti-FLAG mAb M2 (1:3000 dilution), respectively. Rabbit anti-mouse IgG-horseradish peroxidase (1:1000 dilution; DAKO) was used as secondary antibody and signals were developed by using Western blot LuminoL reagent (Santa Cruz) according to the manufacturer’s instructions. To control for non-specific interactions, FLAG-tagged interferon-regulating factor 3 (IRF-3) and HA-tagged coronin 1C were used as negative controls.

**Western blot.** HepG2 cells were cotransfected with 2 μg expression plasmid of Prox1 or pcDNA3, 2 μg pI830 or pSuper and 0.3 μg pCMV-lacZ. Forty-eight hours post-transfection, enhanced green fluorescent protein-expressing cells were enriched by using fluorescence-activated cell sorting. Ten per cent of the cells were removed for measurement of β-galactosidase activity to ensure equal loading, whilst the remaining cells were lysed. Cell lysates were separated by SDS-PAGE (8% gel) and transferred onto a nitrocellulose membrane. Prox1 and β-actin were detected by using mouse anti-Prox1 mAb (1:2500 dilution; Upstate Biotechnology) and anti-actin mAb (1:1500 dilution; Sigma) as primary antibody, respectively.

**RESULTS**

**Prox1 inhibits HBV replication and antigen expression in cultured hepatocytes**

The nuclear receptor LRH-1 has been shown to be important for HBV ENII/Cp activity (Li et al., 1998) and previous work by ourselves (Qin et al., 2004) and others (Steffensen et al., 2004) has identified Prox1 as a corepressor for LRH-1 in regulating transcription of a couple of liver-specific genes. We therefore deduced that Prox1 might also have a negative effect on HBV transcription and replication in hepatocytes. To test this hypothesis, cultured HepG2 cells were transfected with p1.3HBV plasmid, which contains a terminally redundant (1.3 × copy) replication-competent HBV genome, and HBV transcription, replication and antigen expression were analysed in the context of overexpressed or knocked-down Prox1 (Fig. 2e).

As shown in Fig. 2(a), in the presence of Prox1 overexpression, both of the major HBV transcripts, namely the 3.5 kb preC/pgRNA mRNAs and the 2.4/2.1 kb preS1 + preS2/S mRNAs, displayed a significant reduction in signal strength, whereas when Prox1 was knocked down by using RNAi, the levels of both these transcripts increased. When HBV rcDNA contained in intracellular core particles was analysed as a measure of HBV replication, a similar pattern was observed: rcDNA was reduced significantly in the presence of Prox1 overexpression, but increased when Prox1 was knocked down (Fig. 2b). A reverse correlation between Prox1 dose and HBV activity was further confirmed by analysing secretion of HBsAg and HBeAg by transfected HepG2 cells. As shown in Fig. 2(c, d), levels of both HBsAg and HBeAg in the culture media were reduced significantly when Prox1 was overexpressed, but increased when endogenous Prox1 was knocked down by RNAi. Similar results were also obtained by using HuH7 cells (see Supplementary Fig. S1, available in...
These data demonstrate that Prox1 indeed has a negative effect on HBV transcription and replication in cultured hepatocytes.

Prox1 suppresses multiple cis elements on the HBV genome

As the observed effects of Prox1 on HBV were in agreement with our deduction based on LRH-1-mediated activation of ENII/Cp and Prox1-mediated corepression of LRH-1, we went on to confirm whether Prox1 represses ENII/Cp activity by using reporter analysis in HepG2 cells. The three other HBV enhancer/promoter sequences, Sp1, Sp2 and ENI/Xp, were also analysed in parallel. As shown in Fig. 3, not only was ENII/Cp activity repressed significantly by Prox1 overexpression as expected, but both Sp1 and ENI/Xp were also markedly repressed in the presence of Prox1 overexpression. Sp2 activity was apparently not altered in the presence or absence of Prox1 overexpression. Correspondingly, when Prox1 was knocked down by using RNAi, the activity of ENII/Cp, Sp1 and ENI/Xp was obviously increased, whereas Sp2 activity displayed no marked change. Such a pattern was also reproduced in Huh7 cells (see Supplementary Fig. S2, available in JGV Online). These results suggest that the observed repressive effects of Prox1 on HBV gene expression and replication are the cumulative result of its effects on multiple HBV cis elements.

Prox1 suppresses ENII/Cp by acting as corepressor for LRH-1

As reporter analysis confirmed repression of ENII/Cp by Prox1, and LRH-1 is a known activator of ENII/Cp (Li et al., 1998), the possibility that such repression involves the established mechanism of Prox1 acting as corepressor of
LRH-1 was explored by performing reporter assays in HeLa cells, which express neither Prox1 nor LRH-1. As shown in Fig. 4, the activity of ENII/Cp was stimulated nearly 3-fold by LRH-1 coexpression (compare bars 2 and 3). However, when wild-type Prox1 was co-expressed with LRH-1, such stimulation was almost completely abolished (Fig. 4, bar 5). Corepression of LRH-1 by Prox1 requires physical interaction between the two factors. Prox1-DM is a Prox1 mutant that loses most of the ability to bind LRH-1 (Qin et al., 2004; Steffensen et al., 2004). When Prox1-DM was coexpressed with LRH-1, LRH-1-mediated ENII/Cp activation was only marginally affected (Fig. 4, bar 7), possibly due to residual interaction between Prox1-DM and LRH-1 (Qin et al., 2004). In the absence of LRH-1 expression, the activity of ENII/Cp was not affected by either wild-type Prox1 or Prox1-DM (Fig. 4, bars 4 and 6). These results indicate that Prox1 suppresses LRH-1-mediated activation of HBV ENII/Cp by binding to and corepressing LRH-1.

**Prox1 suppresses HNF-1-mediated activation of Sp1**

As HNF-1 has been shown to be critical for Sp1 activity (Chang et al., 1989; Raney et al., 1991), we wondered whether suppression of Sp1 by Prox1 might involve this key activator. A luciferase reporter under the control of Sp1 was cotransfected, with expression plasmids of HNF-1 and Prox1, into HepG2 cells. As shown in Fig. 5, in the absence of Prox1 overexpression, the activity of Sp1 was stimulated nearly 2-fold by HNF-1 overexpression (compare black bars 1 and 2). When Prox1 was also overexpressed, however, the effects of HNF-1 overexpression on Sp1 activity were abolished (Fig. 5, compare grey bars 1 and 2). In order to demonstrate further the involvement of HNF-1 in Prox1-mediated repression of Sp1, the HNF-1-binding site in Sp1 was mutated so that it was no longer recognized by HNF-1 (data not shown). In HepG2 cells, the activity of mutated Sp1 was not affected significantly by Prox1 overexpression (Fig. 5, bar 3), which is in distinct contrast to wild-type Sp1. These results indicate that HNF-1 is indeed involved in Prox1-mediated repression of Sp1.

**Prox1 interacts directly with HNF-1**

Dependence of Prox1 repression of Sp1 on the presence of an HNF-1-binding site suggested the possibility of Prox1 acting as a corepressor of HNF-1. To probe such a possibility, HNF-1–Prox1 interaction was first tested by using GST pull-down and co-immunoprecipitation assays. As shown in Fig. 6(a), in vitro-translated Prox1 could be pulled down specifically by HNF-1 (lane 3). In HEK293
cells, which lack endogenous Prox1 expression (Shan et al., 2008), coexpressed FLAG–HNF-1 and HA–Prox1 were co-immunoprecipitated using anti-FLAG antibody, and coexpressed FLAG–HNF-1 and HA–Prox1 were co-immunoprecipitated by using anti-HA antibody (Fig. 6b). These results indicate that Prox1 interacts directly with HNF-1 in vitro and in cultured cells.

The domains of Prox1 and HNF-1 that mediate their interaction were then mapped by using deletion mutants in GST pull-down assays. As shown in Fig. 7(b), HNF-1 interacts with Prox1 mainly through its DNA-binding domain (DBD) (lane 3), which encompasses both the Pit-Oct-Unc (POU) domain and homeodomain. Although sharing no apparent sequence similarity, the POU domain and homeodomain displayed similar binding to Prox1 (Fig. 7b, lanes 8 and 9), indicating that they both contribute to DBD-mediated interaction with Prox1. On the other hand, Prox1 seems to bind HNF-1 through its N-terminal 120 residues, and the integrity of this domain is required for such binding, as manifested by the failure of non-full-length translation products of Prox1-N to pull down any HNF-1 (Fig. 7d, compare lanes 1 and 2). The nuclear receptor (NR) boxes that are critical for the interaction of Prox1 with the LRH-1 ligand-binding domain (Qin et al., 2004) were apparently not required for the interaction with HNF-1, as Prox1-DM was fully capable of interacting with HNF-1 (Fig. 7e, compare lanes 2 and 5).

Repression of HBV Sp1 by Prox1 involves Prox1–HNF-1 interaction

The role played by interactions between HNF-1 and Prox1 in repression of Sp1 by Prox1 was then investigated by performing reporter analysis in HeLa cells, which lack both endogenous Prox1 and HNF-1 expression. As shown in Fig. 8, HNF-1 expression stimulated Sp1 activity by about 100 % (compare lanes 2 and 3). When wild-type Prox1 was coexpressed with HNF-1, however, such stimulation was reduced dramatically to about 20 % (Fig. 8, lane 5). When Prox1 deletion mutant aa 108–737, which loses the ability to bind HNF-1 (Fig. 7; data not shown), was coexpressed with HNF-1, stimulation of Sp1 activity was only reduced to nearly 60 % (Fig. 8, lane 7). Interestingly, when wild-type Prox1 and aa 108–737 fragment were expressed in the absence of HNF-1 expression, slight but obvious repression of Sp1 activity was also observed (Fig. 8, lanes 4 and 6), indicating that other repression mechanisms not involving HNF-1 are at work. Taken together, these results suggest that the interaction between Prox1 and HNF1 is involved in the repression of Sp1 activity by Prox1.
Fig. 6. Prox1 interacts directly with HNF-1. (a) GST pull-down of in vitro-translated and $^{35}$S-methionine-labelled Prox1. The lanes show 10% of labelled Prox1 input, GST-fused coronin 1C as negative-control bait protein and GST-fused full-length HNF-1 as bait protein. The lower panel shows 5% of each bait protein analysed by SDS-PAGE. (b) Co-immunoprecipitation of Prox1 and HNF-1. Transfection of HEK293 cells and co-immunoprecipitation were performed as described in Methods. Co-precipitated Prox1 (left) and HNF-1 (right) in the top panels are indicated. FLAG-tagged HNF-1 or IRF-3 and HA-tagged Prox1 or coronin 1C in total extracts (1% input) were analysed by Western blotting using respective antibodies to ensure equal loading. IP, Immunoprecipitation; IB, immunoblotting.

Fig. 7. Mapping of regions mediating interactions between Prox1 and HNF-1. (a) Domain organization of HNF-1. DD, Dimerization domain; Homeo, homeodomain; DBD, DNA-binding domain; AD, activation domain. GST-fused HNF-1 fragments used in pull-down assays are depicted underneath. (b) Mapping HNF-1 regions required for the interaction with Prox1. Left and middle panels, in vitro-translated and $^{35}$S-methionine-labelled Prox1 were pulled down by using the indicated GST-fused HNF-1 fragments. Lanes 1 and 5 show 10% of input Prox1; lanes 2 and 6 show GST as negative-control bait protein. The right-hand panel shows 10% of each bait protein analysed by SDS-PAGE. (c) Domain organization of Prox1. Fragments used for pull-down are depicted underneath. (d) Mapping of Prox1 regions required for interaction with HNF-1. Indicated Prox1 fragments were in vitro-translated and $^{35}$S-methionine-labelled, and pulled down by using GST-fused full-length HNF-1 (lanes 2, 5, 8 and 11) or GST (lanes 3, 6, 9 and 12). Lanes 1, 4, 7 and 10 show 10% of the input labelled Prox1; lanes 2 and 6 show GST as negative-control bait protein. The right-hand panel shows 10% of each bait protein analysed by SDS-PAGE. (e) NR boxes are not required for interaction with HNF-1. Prox1 and Prox1-DM were in vitro-translated and $^{35}$S-methionine-labelled, and pulled down by using GST-fused full-length HNF-1 (lanes 2 and 4) or GST (lanes 3 and 6). Lanes 1 and 4 show 10% of input Prox1 proteins.
Prox1 suppresses HBV gene transcription and replication

Fig. 8. Repression of HBV Sp1 by Prox1 involves Prox1–HNF-1 interaction. HeLa cells were transfected with the indicated reporter and expression plasmids as described in Methods. Relative luciferase activities were calculated, taking pcDNA3 transfection as 1. Transfections were performed in duplicate dishes and repeated three times. Means ± SD are presented.

DISCUSSION

Understanding the mechanisms of negative regulation of HBV transcription is not only one of the key tasks of molecular HBV research, but may also provide new information and candidate targets for the development of novel anti-HBV intervention therapy. In this study, we identified the nuclear protein Prox1, an important player in liver lipid-metabolism regulation (Qin et al., 2004), as a potent negative regulator of HBV transcription, antigen expression and replication (Fig. 2).

Reporter analysis revealed that three of the four HBV enhancer/promoter sequences, namely ENII/Cp, Sp1 and ENI/Xp, were repressed by Prox1 (Fig. 3). The existence of multiple targets of repression by Prox1 clearly contributes to its strong potency as an HBV repressor (Fig. 2). It should be noted, however, that it is possible that other responsive elements for Prox1 exist on the HBV genome that are not encompassed by the four cis elements tested in this work.

The molecular mechanisms of Prox1-mediated repression of ENII/Cp and Sp1 were investigated. In the case of ENII/Cp, we demonstrate that Prox1 suppresses LRH-1-mediated activation of ENII/Cp (Fig. 4) in a fashion similar to Prox1 corepressing LRH-1-mediated activation of cyp7a1 (Qin et al., 2004). Although LRH-1 has been shown to be important for ENII/Cp activity, and suppression of ENII/Cp by Prox1 is therefore probably achieved mainly through corepressing LRH-1, other mechanisms may also exist. In preliminary studies, we have observed that ENII/Cp containing mutated LRH-1-binding sites was still repressed by Prox1, albeit to a lesser degree (data not shown), suggesting the involvement of factors other than LRH-1. These factors will hopefully be identified in our future work.

By using coexpression and binding-site mutation, it was demonstrated that Prox1 exerts its repressive effects on HBV Sp1 through HNF-1 (Fig. 5), a regulator of many cellular genes important for development, differentiation and metabolism (Arrese & Karpen, 2002; Davies & Fisher, 2002; Dukes et al., 1998; Ellard, 2000; Pontoglio et al., 1996, 2000) that is also a critical activator of Sp1 basal activity (Raney et al., 1994). Further investigation revealed a direct interaction between HNF-1 and Prox1 both in vitro and in vivo (Figs 6 and 7). Moreover, a Prox1 mutant lacking the HNF-1-interacting domain displayed reduced repression of Sp1 (Fig. 8). It is clear that HNF-1-mediated activation of Sp1 is downregulated by Prox1–HNF-1 interaction. However, as abolition of Prox1–HNF-1 interaction only partially relieved Prox1-mediated repression of Sp1, and Prox1 is capable of repressing Sp1 in the absence of HNF-1 (Fig. 8, lanes 4 and 6), it is evident that additional repression mechanisms exist that do not involve HNF-1.

Suppression of HNF-1-mediated transcriptional activation by Prox1 is probably not restricted to HBV Sp1. For instance, Prox1 represses the activity of an HNF-1-dependent artificial promoter in reporter assays (data not shown). As Prox1 and HNF-1 are both expressed in tissues such as liver and pancreas (Burke & Oliver, 2002; Courtois et al., 1987; Oliver et al., 1993; Zinovieva et al., 1996), downregulation of HNF-1-mediated transcriptional activation by Prox1 could also occur to other HNF-1 target genes.

The mechanism of Prox1-mediated suppression of ENI/Xp is unknown. The ENI/Xp region contains at least five different binding sites for transcription factors: 2C, GB, EP, E and NF1 sites (Ben-Levy et al., 1989; Chen et al., 1994; Dikstein et al., 1990; Garcia et al., 1993; Guo et al., 1991), which are recognized by a variety of transcription factors, including HNF-3 (Chen et al., 1994; Dikstein et al., 1990; Guo et al., 1991), HNF-4, COUP-TF1 and RXRα (Garcia et al., 1993), EF-C and NF1 (Ostapchuk et al., 1989), as well as ATF-2/CREB and AF-1 (Choi et al., 1997; Gustin et al., 1993; Ori et al., 1994). We are currently investigating whether any of these are involved in Prox1-mediated repression of ENI/Xp.

Suppression of multiple HBV cis elements by Prox1 results in significant inhibition of viral gene expression and replication in cultured hepatocytes, suggesting that Prox1 is a potential potent negative regulator during natural HBV infection. Due to the lack of inexpensive and reliable cell or animal infection models, the regulatory role of Prox1 in the HBV life cycle, like those of many other putative regulators, cannot be readily confirmed in vivo. However, recent
progress in the generation of novel cell and animal models for HBV infection, as well as improved infection models of HBV-related animal hepatadnaviruses (woodchuck hepatitis virus, etc.), may eventually pave the way for the investigation of Prox1 functions in natural HBV infection. Alternatively, study of HBV-infected patients might reveal a possible correlation between Prox1 expression levels in liver and HBV antigen expression, genome replication and/or disease status, thus providing evidence of Prox1 acting as an HBV repressor in vivo. Nevertheless, Prox1-mediated suppression of HBV certainly offers the possibility of designing new anti-HBV therapeutics targeting such mechanisms.

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