

## Hepatitis B Virus pX Interacts with HBXAP, a PHD Finger Protein to Coactivate Transcription\*

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**Hepatitis B virus (HBV) gene expression is mainly regulated at the transcription initiation level. The viral X protein (pX) is a transcription coactivator/mediator targeting TFIIB for the recruitment of RNA polymerase II. Here we report a novel pX nuclear target designated HBXAP (hepatitis B virus X-associated protein). HBXAP is a novel cellular nuclear protein containing a PHD (plant homology domain) finger, a domain shared by many proteins that play roles in chromatin remodeling, transcription coactivation, and oncogenesis. pX physically interacts with HBXAP *in vitro* and *in vivo* via the HBXAP region containing the PHD finger. At the functional level HBXAP increases HBV transcription in a pX-dependent manner suggesting a role for this interaction in the virus life cycle. Interestingly, HBXAP collaborates with pX in coactivating the transcriptional activator NF- $\kappa$ B. Coactivation of NF- $\kappa$ B was also observed in tumor necrosis factor  $\alpha$ -treated cells suggesting that pX-HBXAP functional collaboration localized downstream to the NF- $\kappa$ B nuclear import. Collectively our data suggest that pX recruits and potentiates a novel putative transcription coactivator to regulate NF- $\kappa$ B. The implication of pX-HBXAP interaction in the development of hepatocellular carcinoma is discussed.**

Study of the interactions of the virus with the host cell is a promising approach to understand, at the molecular level, how the virus evades the defense strategies of the cells they infect. Hepatitis B virus (HBV)<sup>1</sup> is a hepatotropic virus containing a partially double-stranded circular DNA genome that causes both acute and chronic hepatic injuries. Persistent HBV infection is strongly associated with the development of hepatocellular carcinoma (1). The compact 3.2-kbp genome contains en-

hancers and multiple promoters that are regulated by the cellular transcription machinery. HBV encodes a single known regulatory polypeptide, called pX (or HBx). pX is likely to be an important regulatory protein since its sequence is conserved among the mammalian hepadnaviridae members. A debate exists regarding the role of this protein in the HBV life-cycle, however, it is evident that at least, in woodchucks, pX plays an essential role in woodchuck HBV infection (2, 3).

At the cellular level pX supports transcription and signaling (for review, see Ref. 4). pX increases HBV transcription by trans-activating the viral enhancer-I via the sequence named the E-element (5). This element binds a number of bZip cellular transcription activators including cAMP-response element-binding protein (CREB) and activating transcription factor (ATF) whose binding is potentiated by the presence of pX (6–8). In addition, pX activates transcription through NF- $\kappa$ B (5, 9–11). pX also interacts with the general transcription factors (12), consistent with its transcription coactivation function (13). Interaction of pX with TFIIB was reported by a number of groups (12, 14, 15). This interaction may be relevant not only to the role of pX in transcription but also in DNA repair. Significantly, pX interacts simultaneously with TFIIB and RNA-polymerase II possibly to facilitate polymerase recruitment to promoters (16, 17).

In addition to the transcription activators and general transcription factors a third group of proteins is needed to support transcription, collectively called coactivators or mediators. TBP-associated factors (TAFs) belong to this group of proteins. Interestingly, pX supports transcription in the absence of TAFs (12). *In vitro* studies on naked DNA templates revealed that pX functions in a TAF independent manner. *In vivo*, pX rescues the temperature-sensitive phenotype of the ts13 cell line which exhibits growth arrest at restrictive temperature due to a mutation in TAF<sub>II</sub>250. In addition, TBP mutants lacking TAF binding that poorly respond to activators exhibit wild type activity in the presence of pX, *in vivo* (18). Thus, pX can support transcription on chromatinized template in the absence of some of the transcription coactivators/mediators. While pX may be capable of alone coactivating transcription, it also may act by recruiting specific cellular coactivators. The latter possibility was addressed in this study by identifying pX-associated cellular proteins.

The two-hybrid screen is a promising experimental approach to identifying additional pX interacting proteins. The conventional screen, although very powerful, is based on a transcriptional readout, and may provide spurious data when attempting to identify proteins that interact with general transcription factors, as pX does. Recently, a two-hybrid screen which relies on a cytoplasmic signaling event was described called the Sos Recruitment System (SRS). We utilized this system and isolated two clones that specifically and repeatedly interacted with pX in our screen. One of the isolated clones is the Tat-

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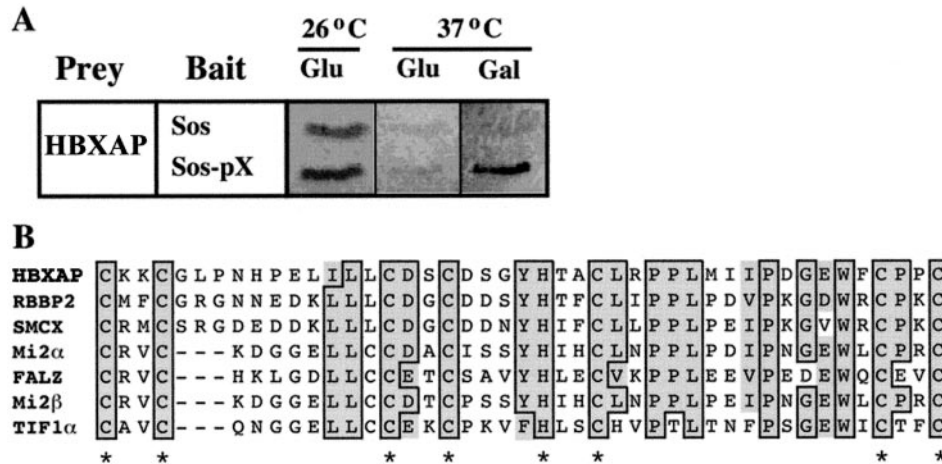
The nucleotide sequence (reported in this paper for the HBXAP mRNA has been deposited in the GenBank™/EBI Data Bank database with accession number(s) AAF61709.

The amino acid sequence of HBXAP reported in this paper can be accessed through NCBI Protein Database under NCBI accession number NP\_057662.

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<sup>1</sup> The abbreviations used are: HBV, hepatitis B virus; TAF, TBP-associated factor; SRS, Sos recruitment system; PHD, plant homology domain; GST, glutathione S-transferase; TRITC, tetramethyl rhodamine isothiocyanate; HBXAP, hepatitis B virus X-associated protein; HDAC, histone deacetylase; HA, hemagglutinin; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .



**FIG. 1. HBXAP is a specific pX interacting protein.** *A*, complementation of yeast *cdc25-2* strain through pX and HBXAP interaction. Yeast cells were transformed with the prey construct pYes2-HBXAP, and the bait pADNS-Sos or pADNS-Sos-pX and plated on glucose minimal medium supplemented with the appropriate amino acids and nucleotides. Transformants were grown at 26 °C, replica plated onto appropriately supplemented galactose or glucose minimal plates, and grown at 37 °C. Only transformants expressing both pYes2-HBXAP and pADNS-Sos-pX, grow efficiently at 37 °C. *B*, HBXAP contains a PHD finger. Sequence comparison of a number of known PHD finger containing proteins, transcription intermediary factor 1- $\alpha$  (*hTIF1* $\alpha$ ), fetal Alzheimer antigen (*hFALZ*), chromodomain helicase DNA-binding protein-4 (*CHD-4* also named Mi2 $\beta$ ), *CHD-3* (also named Mi2 $\alpha$ ), xel69 protein (*hSMCX*), and retinoblastoma-binding protein 2 (*hRBBP-2*), to the putative PHD finger present in HBXAP. Similarity in sequence is indicated in *gray*, while the identical sequences are *boxed*. The conserved cysteine and histidine residues are indicated by *asterisks*.

binding protein 1 that is described elsewhere (19). Tat-binding protein-1 and its homologues, such as Sug1, are components of the proteasome 19S regulatory cap particle. These proteins have also been identified as transcription mediators. The second clone is a novel gene which we designated HBXAP (hepatitis B virus X-associated protein). HBXAP is a nucleoprotein of 240 kDa in size that contains a PHD/leukemia-associated protein finger, a motif shared by a variety of chromatin-associated proteins (20). We show that pX interacts with HBXAP both *in vitro* and *in vivo* and that this interaction potentiates the ability of HBXAP to coactivate NF- $\kappa$ B.

#### EXPERIMENTAL PROCEDURES

**Plasmids**—The protocol of the yeast Sos-recruitment two-hybrid system for screening of a cDNA library for X interacting proteins was reported elsewhere (19). The initial HBXAP clone isolated from this screening, designated mini-HBXAP, was used to screen a human spleen cDNA  $\lambda$ gt11 phage library (CLONTECH). Two such clones were inserted into pCDNA3 (Invitrogen) to generate pCDNA3-HBXAP-(1–814 amino acids), nucleotides 1–2444 of HBXAP open reading frame. The full-length HBXAP was generated by ligation of a reverse transcriptase-PCR product (nucleotides 2432–3567) that was cloned into *EcoRV* + *XhoI* sites in pCDNA3-HBXAP-(1–814). HA-tag was inserted in-frame of the HBXAP open reading frame by PCR to generate pCDNA3-HA-HBXAP. The pSG5-HA-HBXAP was generated by ligation of HA-HBXAP into the *Bam*HI site of the pSG5 vector (Stratagene). HA-mini-HBXAP was constructed by ligation of the *EcoRV* fragment of HBXAP into *Sma*I-digested pCGN vector. pRSET-HBXAP was generated by ligation of an *EcoRI*-*Hind*III fragment of EST clone, accession number N91426, into *EcoRI* + *Hind*III-digested pRSETb vector (Invitrogen). The HBV plasmid contains two tandem copies of HBV full-length DNA (subtype adw), ligated via the unique *EcoRI* site. The X-KO HBV mutant was constructed by generating a stop codon at position 27 of the X gene (21). The pSG5-HA-pX was generated by inserting an *EcoRI* fragment that contains the HA-tag and *NcoI* site at the *EcoRI* site of pSG5 vector, then this construct was digested with *NcoI* + *Bgl*II and ligated with *NcoI* + *Bgl*II fragment of the X open reading frame. pGFP (CLONTECH) and pGL3-control (Promega) are commercial plasmids.

**Cell Culture, DNA Transfection, and RNA Analysis**—HepG2, 293T, MCF-7, Hep3B, and Huh-7 cells were cultured and transfected as previously described (13). Approximately 8 h before transfection,  $2 \times 10^5$  and  $1.5 \times 10^6$  cells were plated per 3.5 and 10-cm dish, respectively. For reporter assays, each 3.5-cm dish was transfected with a constant amount of 1.5  $\mu$ g of DNA, consisting of 0.8  $\mu$ g of reporter plasmid, expressor vectors as indicated, and carrier (pBluescript SK). In each

case we included empty vectors with the relevant enhancers to eliminate *in vivo* competition. 48 h post-transfection, cells were harvested and assayed for luciferase activity. All the experiments were repeated at least three times. Transfection and Northern blot analysis of HBV RNA was performed as described (21).

**Pull-down Assays**—Recombinant GST-X and GST proteins were produced in *Escherichia coli* and purified on glutathione-Sepharose beads, as described previously (22). *In vitro* transcription/translation kit (Promega) was used to generate [<sup>35</sup>S]Met-labeled HBXAP protein. Recombinant HBXAP was produced from BL21 cells transformed with pRSET-HBXAP. Bacteria were lysed by sonication (in buffer containing 50 mM HEPES pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2  $\mu$ g/ml pepstatin A) followed by a 30-min centrifugation at 15,000 rpm. The supernatant was either used in pull down assays or purified on nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrix (Qiagen). The purified protein was used to generate polyclonal antibodies. Cell extracts containing HA-mini-HBXAP were prepared by transfection of pCGN-HA-mini-HBXAP into 293T cells. Forty-eight h post-transfection cells were lysed with detergent-based lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 0.2% Nonidet P-40, 0.025% deoxycholate, 10% glycerol, and protease inhibitor mixture (Sigma)). Lysis proceeded for 10 min on ice. Lysates were then centrifuged for 10 min at 13,000 rpm. Supernatants were collected and stored for further analysis. Binding reactions were performed in WASH buffer (20 mM Hepes-KOH pH 7.9, 150 mM KCl, 25% glycerol, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 2 mM dithiothreitol, and protease inhibitor mixture (Sigma)) at room temperature for 1 h. Beads were washed extensively with the same buffer, and bound proteins were separated on SDS-PAGE. In the case of *in vitro* translated HBXAP, higher concentrations of salt were tested, and HBXAP was pulled down at 350 mM NaCl.

**Immunoprecipitation and Western Blot Analysis**—For the coimmunoprecipitation experiments, 10-cm plates (four for each sample) of 293T cells, were transfected with 2  $\mu$ g of pGFP-pX or pGFP and 5  $\mu$ g of pCDNA3-HA-HBXAP expression vectors, and 8  $\mu$ g of carrier DNA, as described (13). Cells were harvested, and nuclear extracts were prepared. Anti-HA monoclonal antibody 12CA5 was bound to Affi-Gel 10 (Bio-Rad) column and incubated for 4 h at 4 °C with the nuclear extracts. Beads were washed 5 times with WASH buffer and proteins were eluted and separated on SDS-PAGE, electroblotted, and incubated with specific monoclonal antibodies, anti-GFP (BAbCO) or anti-HA 12CA5 (Pharmingen), followed by a peroxidase-conjugated goat anti-mouse IgG secondary antibody, and visualized using an ECL detection kit (Pierce). HBXAP was detected by polyclonal anti-HBXAP antibody (described above), followed by peroxidase-conjugated protein-A (ICN).

**Immunofluorescence Microscopy**—COS-1 cells were seeded in 8-well chamber slides, and were transfected with 125 ng of DNA. Cells were fixed 40 h post-transfection using 4% paraformaldehyde and treated

with 0.5% Triton X-100 for 30 min. Blocking was carried out with 6% skim milk, 3% bovine serum albumin, and 0.2% Tween 20 in 100% fetal calf serum. Cells were then incubated for 16 h at 4 °C with mouse monoclonal anti-HA antibodies (BAbCO), and goat polyclonal Affinipure anti-lamin B (Santa Cruz Biotechnology). Reacting antibodies were visualized with fluorescein isothiocyanate-conjugated Affinipure donkey anti-mouse and rhodamine (TRITC)-conjugated Affinipure donkey anti-goat (Jackson ImmunoResearch Laboratories).

## RESULTS

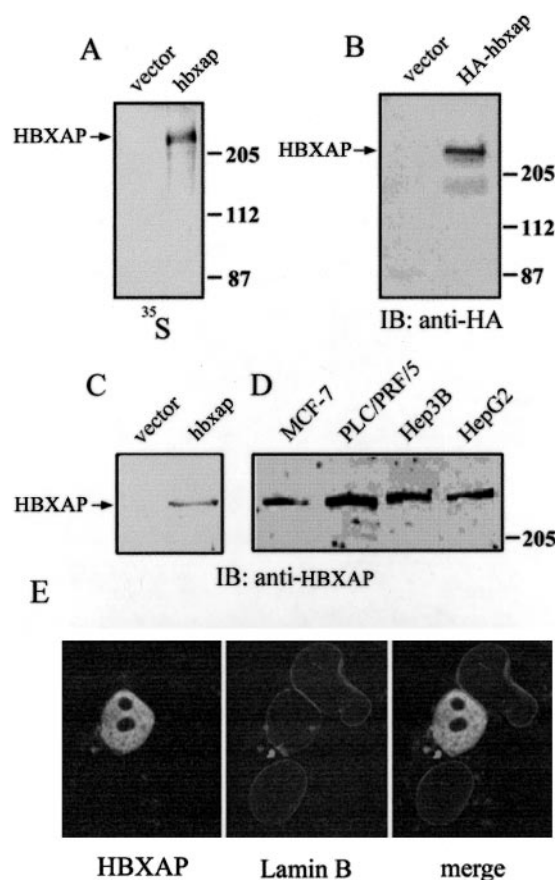
**Isolation of a Novel pX-binding Protein**—Fourteen cDNA positive clones were isolated by SRS two-hybrid screening for pX interacting proteins (19). Eleven were similar and one of them was reintroduced back into the *cdc25-2* cells and, as expected, it rescued the cells in the presence of Sos-X and in a galactose inducible manner (Fig. 1A), suggesting a specific Sos-X interaction. This clone was used to isolate the full-length 5-kbp cDNA from a human spleen cDNA library. Inspection of the nucleotide sequence revealed that it contains a novel 1189-amino acid long open reading frame that we designated HBXAP. After HBXAP sequence was deposited to the GenBank™ data base (accession number AAF61709), three predicted human proteins with significant relatedness were deposited (accession numbers: XP\_006161, BAA91591, and AAG43114).

HBXAP is a novel protein containing a PHD/leukemia-associated protein finger motif (640–692 amino acid). Interestingly, the original DNA fragment that was isolated by SRS two-hybrid, encodes only a short region encompassing the PHD finger (amino acids 517–742) and will be referred to as mini-HBXAP. The PHD finger is a Cys<sup>4</sup>-His-Cys<sup>3</sup> zinc finger found primarily in a wide variety of chromatin-associated proteins (20) including Trithorax, Polycomb-like, ALL-1, CBP, p300, MOZ, Tip60, and HAT3.1, a plant homeobox gene (Fig. 1B). Although the exact function of the PHD finger is not known it was reported that in the Mi2β corepressor, the two PHD fingers are essential but not sufficient for interaction with histone deacetylase 1 (HDAC1) (23). In addition, HBXAP also contains two putative nuclear localization signals (amino acids 832–838 and 951–957). The C terminus (731–1189 amino acids) of the protein is highly acidic, a characteristic feature of potent transcription activation domains.

**HBXAP Is a Ubiquitously Expressed 240-kDa Protein**—To analyze the HBXAP polypeptide it was *in vitro* synthesized by a transcription/translation kit and separated by SDS-PAGE. Unexpectedly, the [<sup>35</sup>S]methionine-labeled HBXAP migrates as a 240-kDa protein (Fig. 2A) far larger than the predicted 135 kDa. The slow migration of HBXAP was also confirmed when HA-tagged HBXAP was overexpressed in 293T cells (Fig. 2B). It is possible that highly negative charged acidic stretches at the HBXAP C terminus are responsible for the slow migration, although we cannot exclude other possibilities.

To determine the expression pattern of HBXAP, we probed a CLONTECH Human RNA Master-blot composed of poly(A)-purified mRNA from 50 different tissues, and found that HBXAP is ubiquitously expressed. However, we found higher levels of expression in adrenal and pituitary glands, a lower level in skeletal muscle and average levels in both fetal and adult liver (data not shown). As HBV is a hepatotropic virus, it was reassuring to our investigation that HBXAP was indeed expressed in this tissue. This point was confirmed by the presence of HBXAP content in a set of three liver-derived cell lines by immunoblotting with anti-HBXAP-specific antibodies (Fig. 2D).

**HBXAP Is a Nuclear Protein**—To test the cellular localization of HBXAP COS-1 cells were transfected with an HA-tagged HBXAP expression vector. After 48 h cells were fixed and reacted with an anti-HA antibody to detect HA-HBXAP,

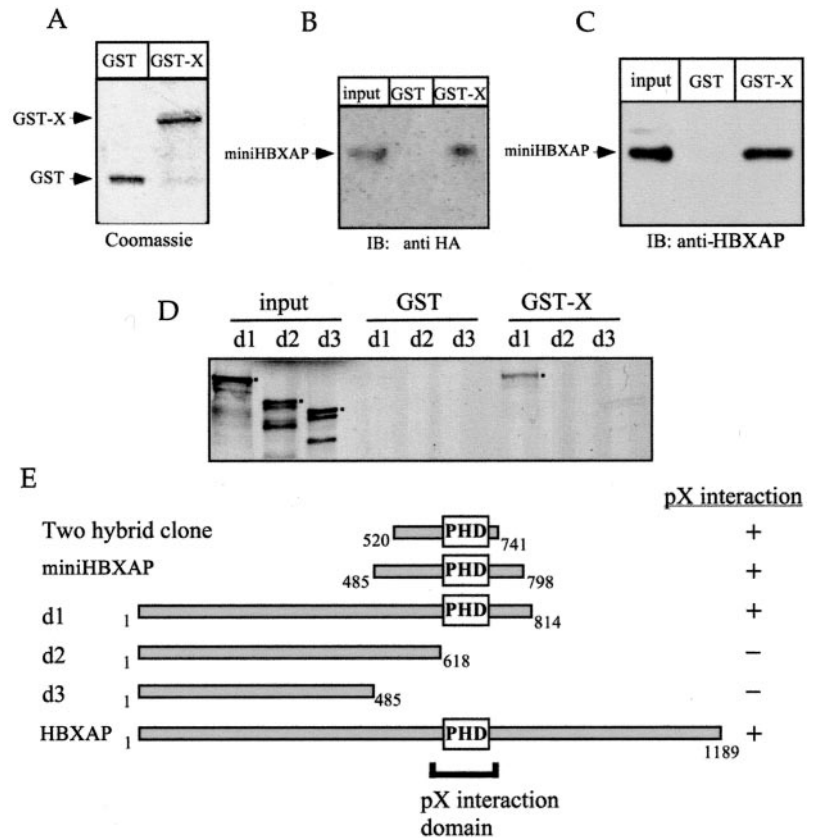


**FIG. 2. Expression of HBXAP.** A, [<sup>35</sup>S]methionine-labeled HBXAP was synthesized *in vitro* using a transcription/translation kit and was fractionated on SDS-PAGE and exposed to an x-ray film. B, HA-HBXAP was expressed in 293T cells, the cellular extract was fractionated on SDS-PAGE and immunoblotted with an anti-HA antibody. C, extracts as in B were immunoblotted with anti-HBXAP antibodies. D, four indicated cell lines were analyzed by immunoblotting for their HBXAP content by employing anti-HBXAP polyclonal antibodies. E, HBXAP is a nuclear protein. COS-1 cells were transfected with HA-HBXAP and 40 h later cells were fixed and reacted with mouse monoclonal anti-HA antibody and goat anti-lamin-B antibody that were visualized with fluorescein isothiocyanate-conjugated anti-mouse antibody and rhodamine (TRITC)-conjugated donkey anti-goat.

and anti-lamin B antibodies to detect the nuclear membrane. HA-HBXAP staining suggested localization to the nucleus with notable absence of staining in the cytoplasm and nucleolar structures (Fig. 2E). A similar result was obtained with the endogenous HBXAP protein that was visualized by polyclonal anti-HBXAP specific antibodies (not shown). HBXAP was predicted to localize to the cell nucleus as it contains two putative NLS sequences. All known PHD finger containing proteins are localized to the nucleus, although there are reports of cytoplasmic and tight junction localization in addition to nuclear. Within the nucleus, PHD-finger containing proteins are either distributed throughout or localized to nuclear speckles (24). We could not detect HBXAP in nuclear speckles.

**pX Directly Interacts with the PHD Region of HBXAP**—To further characterize the HBXAP and pX interaction, recombinant GST-X (Fig. 3A) was employed to conduct pull down experiments. To this end, the mini-HBXAP protein containing the sequence isolated in the SRS two-hybrid screen was HA tagged and expressed in 293T cells. Cell extracts were prepared and loaded on either fusion GST-X or naive GST bound columns as a control. Following extensive washes the retained proteins were eluted, separated on SDS-PAGE, and immunoblotted with an anti-HA antibody. The mini-HA-HBXAP protein bound specifi-

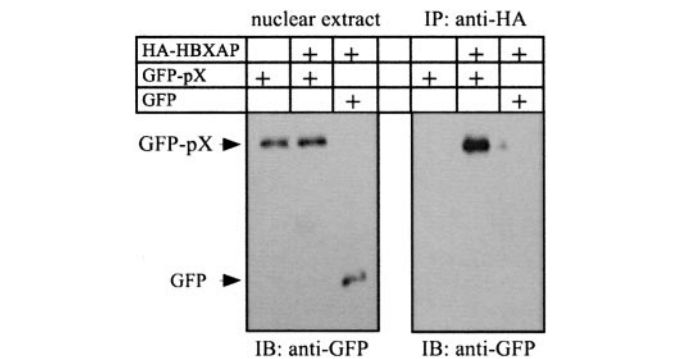
**FIG. 3. HBXAP interacts with pX *in vitro*.** *A*, a Coomassie Blue gel staining showing the recombinant GST-X and GST proteins. *B*, extracts from 293T cells transfected with a plasmid encoding the mini-HA-HBXAP protein, were used in GST pull-down assays. The eluted proteins were separated by SDS-PAGE, and immunoblotted with an anti-HA antibody. *C*, the experiment in *B* was repeated using the bacterial recombinant mini-HBXAP proteins instead of an extract from transfected 293T cells. The eluted proteins were subjected to SDS-PAGE, and immunoblotted with anti-HBXAP antibodies. *D*, specific interaction of GST-X with *in vitro* synthesized HBXAP truncations. The [<sup>35</sup>S]methionine *in vitro* synthesized HBXAP truncation mutant (*d1-d3*) proteins, were used in GST pull-down assays. The eluted proteins were separated by SDS-PAGE, and exposed to an x-ray film. *E*, schematic presentation of HBXAP and the various deletion constructs that were analyzed in binding pX.



cally to GST-X (Fig. 3B). To rule out the possibility of an indirect interaction via other unknown proteins the experiment was repeated with the recombinant mini-HBXAP protein. Here again the mini-HBXAP protein was specifically retained on the GST-X but not naive GST column (Fig. 3C).

**The HBXAP PHD Finger Is Necessary but Not Sufficient for pX Interaction**—As only the mini-protein was isolated in the SRS screen, it was important to show that the capacity of pX to bind HBXAP was not restricted to this small fragment. To this end we produced a set of constructs including the full-length HBXAP along with a number of deletion mutants. These constructs were *in vitro* translated and incubated with the GST-X protein and subjected to pull down experiments. Here again we recapitulated the interaction with recombinant pX using HBXAP (1–814 amino acids) (Fig. 3D). However, fragments deleted for the region isolated in the SRS screen failed to be retained in the eluate by GST-X. Collectively, these data suggest that the region contained in the SRS isolate was necessary for the pX interaction (Fig. 3E). To investigate the possibility that the PHD finger *per se* mediates this interaction, a point mutant of the mini-protein, at the conserved His residue of the PHD finger was analyzed for interaction with pX in the SRS screen. We found that this mutation did not affect the pX interaction (not shown), suggesting that the interaction is not mediated by the PHD finger most conserved residues.

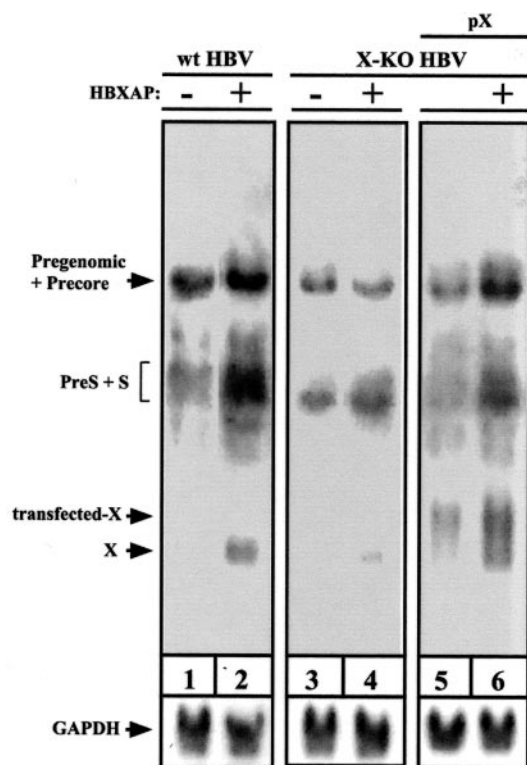
**HBXAP Interacts with pX in Extracts of Transfected Cells**—To examine the interaction between HBXAP and pX *in vivo* coimmunoprecipitation experiments were performed. For this purpose we employed a GFP-pX chimeric construct. pX activity, as assayed by transcription coactivation, was not compromised when fused to GFP (not shown). 293T cells were transfected with a plasmid that directed the synthesis of the HA-HBXAP protein together with either GFP-pX or the control GFP expressor plasmids. Cell extracts were prepared and level of the expression of the employed GFP proteins was examined



**FIG. 4. HBXAP interacts with pX in extracts of transfected cells.** 293T cells were transfected with a plasmid directing the synthesis of the HA-HBXAP protein together with either a GFP-pX or the control GFP expressor plasmids. Cell lysates were prepared and analyzed for either GFP-pX or GFP production. The same lysates were subjected to immunoprecipitation with an anti-HA antibody and the immunoprecipitates were analyzed by Western blotting using an anti-GFP antibody.

by immunoblotting with an anti-GFP specific antibody (Fig. 4). The same extracts were immunoprecipitated with an anti-HA antibody and the precipitated fractions were analyzed by Western blotting using anti-GFP antibody. Significantly, GFP-pX was coimmunoprecipitated only in the presence of HA-HBXAP. Furthermore, this interaction was not seen when cells were transfected with the naive GFP, indicating a specific association between pX and HBXAP *in vivo*.

**pX Collaborates with HBXAP to Support HBV Transcription**—To look into the functional significance of pX-HBXAP interaction, the effect of HBXAP on the transcription from the whole HBV genome was investigated. HepG2 cells were transfected with a head to tail dimer of genomic HBV DNA, and either with or without the HBXAP expressor plasmid. The

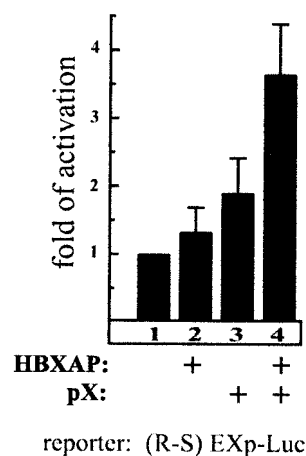


**FIG. 5. pX collaborates with HBXAP to coactivate HBV transcription.** HepG2 cells were transfected with plasmids containing two tandem HBV full-length DNA, either wt (lanes 1 and 2) or a mutant carrying a stop codon at position 27 of the X open reading frame (X-KO HBV, lanes 3–6). Cells were co-transfected with increasing amounts of the pCDNA3-HA-HBXAP and pSG5-HA-X plasmids (lanes 5 and 6). A  $^{32}\text{P}$ -labeled HBV DNA probe was used to detect the known viral transcripts, and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to quantify the amount of RNA per lane. Efficiency of transfection was monitored by GFP as previously reported (46).

levels of the different HBV RNA species were quantified by Northern blot analysis. Interestingly, the level of the viral transcripts, in particular the 0.9-kb short X-RNA, was dramatically increased in the presence of HBXAP (Fig. 5, lanes 1 and 2). However, when an HBV mutant (X-KO HBV) that does not express pX (21) was employed, the effect of HBXAP on HBV transcription was much lower (Fig. 5, lanes 3 and 4), suggesting a collaborative action between pX and HBXAP in supporting HBV transcription. This possibility was further demonstrated by complementation experiments whereby the X-gene expressed from an SV40 vector was provided in *trans*. Under these conditions the viral RNA level was significantly increased (Fig. 5, lanes 5 and 6), suggesting that pX supplementation in *trans* was sufficient to restore the HBXAP transcription coactivation activity on HBV transcription.

To demonstrate that the effect of pX-HBXAP is mediated via coactivation of HBV enhancer we employed the reporter assay. HBXAP only slightly affected the activity of the luciferase reporter gene under HBV enhancer-I and pX promoter (25), however, in the presence of pX the reporter activity was significantly increased (Fig. 6). Thus, HBXAP and pX collaborate to potentiate HBV enhancer-I by increasing the transcription rate.

**pX Is Required for HBXAP in Coactivating NF- $\kappa$ B**—An additional and well characterized target of pX is NF- $\kappa$ B. Therefore, we investigated the collaborative pX and HBXAP activity in the context of NF- $\kappa$ B. An NF- $\kappa$ B-specific reporter plasmid was co-transfected with the HBXAP encoding plasmid either in the presence or absence of pX. HBXAP alone marginally affected transcription from the NF- $\kappa$ B reporter plasmid (Fig. 7,



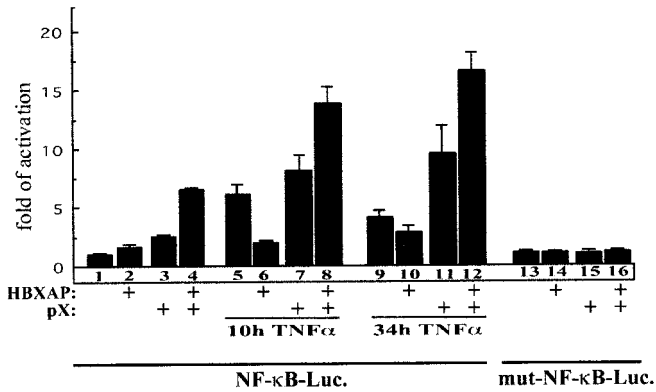
**FIG. 6. Discriminative HBXAP collaboration with pX to potentiate transcription.** A reporter luciferase plasmid (0.8  $\mu\text{g}$ ) under the control of the HBV enhancer-I and the X gene promoter ((R-S)EXp-luc (25)) was transfected into Huh7 cells. pSG5-HA-pX (70 ng/35-mm plate) and pCDNA3-HA-HBXAP (80 ng/plate), were co-transfected as indicated. In all cases the experiments were performed in triplicates and the average values with S.D. are indicated. Fold of activation was calculated by considering lanes 1 as 100%.

lanes 1 and 2). In the presence of pX, HBXAP significantly increased NF- $\kappa$ B transcription (Fig. 7, lanes 3 and 4). This enhancement of transcription is NF- $\kappa$ B dependent, as it was not obtained in the context of a reporter plasmid that carries mutations in the NF- $\kappa$ B-binding site (Fig. 7, lanes 13–16).

NF- $\kappa$ B is sequestered in the cytoplasm by the I $\kappa$ B protein. Upon TNF- $\alpha$  treatment, NF- $\kappa$ B undergoes nuclear translocation to activate target genes. We wanted to determine whether the effect of the pX and HBXAP in increasing transcription from the NF- $\kappa$ B reporter was via nuclear translocation of the cytoplasmic NF- $\kappa$ B or coactivation of the nuclear NF- $\kappa$ B. We found that pX potentiated NF- $\kappa$ B reporter activity in the presence of TNF- $\alpha$  induction at both 10 and 34 h (Fig. 7, lanes 7 and 11). Interestingly, under these conditions HBXAP represses NF- $\kappa$ B transcription activation, and blocks TNF- $\alpha$  effect. Remarkably, in the presence of pX, not only HBXAP lost the capacity to inhibit transcription, but becomes a super coactivator. These data strongly suggest the pX-HBXAP induced NF- $\kappa$ B reporter expression occurs at the level of the nuclear NF- $\kappa$ B rather than as an inducer of NF- $\kappa$ B translocation. This supports the notion that these proteins coactivate DNA bound NF- $\kappa$ B transcriptional activator.

#### DISCUSSION

Both viral and cellular regulatory proteins participate in multiple protein-protein interactions. In contrast to other viruses, such as HIV, which encode many regulatory proteins, only a few proteins control the HBV life cycle. Yet, it has an outstanding capacity to infect and propagate with an efficiency that far exceeds that of HIV as calculated by particle number per ml of serum. Although many factors might be responsible for this efficacy, we may assume that it is in part due to pX, the HBV regulatory protein, which has an extraordinary capacity to perform multiple functions. In this report we provide evidence for a physical and functional interaction between pX and HBXAP, a novel cellular nuclear protein with the attributes of a transcription coactivator. pX, like many other viral regulators, interacts with cellular proteins to recruit the machinery needed to support viral propagation and to counteract the cellular defense systems. A favorable approach to investigate the molecular mechanisms of HBV-host cell interaction is the characterization of pX target cellular proteins. Previous biochemical experiments have revealed a number of



**FIG. 7. HBXAP collaboration with pX to coactivate NF- $\kappa$ B.** Luciferase reporter gene under the control of either wild-type (NF- $\kappa$ B-Luc; lanes 1–12) or mutant (mutNF- $\kappa$ B-Luc; lanes 13–16) NF- $\kappa$ B response elements were transfected into HepG2 cells. Cells were co-transfected with pSG5-pX (30 ng) and pSG5-HBXAP (200 ng) as indicated. Cells were treated with 10 ng/ml TNF- $\alpha$  for the indicated time points. In each case the luciferase activity observed in the TNF- $\alpha$ -untreated cells (basal activity) was taken as 100% to calculate folds of activation. In all cases the experiments were performed in triplicates and the average values with S.D. are indicated.

pX-interacting cellular proteins that are components of the transcription machinery. However, none of the pX-interacting proteins isolated by the two-hybrid screen (26–30), are known components of this machinery. We hypothesized that the conventional two-hybrid screen that is based on a transcriptional readout may not be suitable for identifying transcriptional activators and effectors because the system may be compromised by pX activity. Given this rationale we utilized the SRS two-hybrid screen (31) that acts outside the nucleus at the plasma membrane. Two proteins were isolated, both are nuclear and have the characteristics of transcription coactivators, one is the Tat-binding protein 1 (TBP-1) that is described elsewhere (19) and the other HBXAP. Notably, recently we have identified three HBXAP isoforms designated  $\alpha$ ,  $\beta$ , and  $\gamma$ . In this study we used the HBXAP $\gamma$  isoform that in comparison to  $\alpha$  is N-terminal truncated (32). Although the number of interactions in which pX is engaged is unexpectedly large, these multiple interactions appear to be a hallmark of viral regulatory proteins.

Experiments were conducted to show that HBXAP is a genuine pX target. HBXAP physical interaction with pX was demonstrated *in vitro* and *in vivo*. We found that recombinant HBXAP is preferentially retained on the GST-pX column in a series of pull down experiments, suggesting that these proteins directly interact. The observed interaction must be specific and with relatively high affinity as HBXAP that is expressed in transfected cells is selectively retained by a GST-pX column. Furthermore, these proteins, when expressed in cells by co-transfection experiments are coimmunoprecipitated. HA-tagged HBXAP that is immunoprecipitated by anti-HA is associated with GFP-pX. Collectively, these data suggest that pX and HBXAP are in physical and direct contact.

We provide evidence in support of the possibility that HBXAP and pX physical interaction has a functional significance. We show that HBXAP collaborates with HBV genome-encoded pX, to support HBV transcription. Co-transfection of HBV DNA with HBXAP expression vector resulted in an elevation in the level of the HBV transcripts. As this effect was not observed in the context of an HBV mutant that does not express pX, we assumed that pX is required for this process. This possibility was further supported by the fact that HBXAP activity was recapitulated by a co-transfected pX expressor plasmid. To gain mechanistic insight on the functional interaction

between these proteins we employed reporter assays. Interestingly, functional collaboration between HBXAP and pX was observed in the context of HBV and NF- $\kappa$ B-derived enhancers.

To date several mechanisms were reported to explain the pX role in activating NF- $\kappa$ B (33–35). Here we provide evidence for the nuclear pX role in NF- $\kappa$ B coactivation. Previously it has been reported that at least a fraction of pX is localized in the nucleus (17) and that pX directly interacts with I $\kappa$ B $\alpha$ , which is able to transport it to the nucleus by a piggyback mechanism (33). We took this event a step further to show that in the nucleus coactivation of NF- $\kappa$ B by pX is modulated by a novel transcription coactivator, HBXAP. We have performed a set of experiments to localize pX function along the NF- $\kappa$ B activation pathway. Given the fact that pX-HBXAP stimulate NF- $\kappa$ B activity in the presence of TNF- $\alpha$ , where the majority of NF- $\kappa$ B is nuclear, it is reasonable to localize the effect of this complex downstream to nuclear translocation of NF- $\kappa$ B. Thus, pX-HBXAP effect is restricted to the DNA-bound NF- $\kappa$ B, and therefore displays the attributes of transcription coactivators.

The finding that HBXAP blocks TNF- $\alpha$ -induced NF- $\kappa$ B activation is rather interesting given the fact that some of the well described members of the PHD family, including Trithorax, ALL-1, TIF1, CBP, and p300, are transcription coactivators. However, a number of studies indicate that the PHD finger might be involved in transcription repression. The PHD and bromo-domain of KAP-1 form a cooperative unit with silencing activity. In that case KAP-1, mediated silencing requires association with NuRD and HDAC activity (36). Furthermore, the PHD finger in context with the bromo-domain of KAP-1 are sufficient to represses transcription when artificially recruited to DNA via GAL4-DBD (36). Mi-2 $\beta$ , a PHD finger protein was also reported to interact with HDAC1, and its two PHD fingers are essential but not sufficient for the interaction (23). In addition, it was reported that the PHD-like motif in the DNA methyltransferase Dnmt3a, represses transcription via the recruitment of HDAC-1 (37). Collectively, it appears that a number of PHD proteins are active in transcription silencing and that the PHD finger via interaction with HDAC is at least partially responsible for this function. Likewise, when we recruited HBXAP to DNA via GAL4-DBD it represses transcription. Furthermore, the PHD finger of HBXAP as an independent structural unit has very strong repression activity (32). The fact that pX interacts with HBXAP in a region containing this domain might be significant. pX by binding the PHD finger may displace the associated HDAC corepressor to permit transcription activation, a switching that we have observed in the context of TNF- $\alpha$ -induced NF- $\kappa$ B activation.

PHD containing proteins are often associated with cancer. Their chromosomal locations are amplified and rearranged in many tumors, including MOZ in leukemia (38, 39), CBP in acute myelogenous leukemia (40), and ALL-1 in acute lymphocytic leukemia (41). At the moment it is too early to conclude whether HBXAP is associated with cancer, but there is evidence in support of this possibility. The region 11q13.4–14.1, where the *hbzap* gene is located was reported to be amplified in 7–10% of breast cancer (42), amplified, and rearranged in multiple endocrine neoplasia type I syndrome (MEN1) (43), and B-cells malignancies. We have found the amplification of the *hbzap* gene in a few cases of breast cancers that we have analyzed (data not shown). However, since pX is the putative HBV oncogene, the behavior of this genomic locus in hepatocellular carcinomas is of particular interest. Therefore, we found it rather remarkable that 11q13 is amplified in 15% of HBV-positive tumors, but only a rare event in HCV-positive cases (44). It has been suggested that this amplification is related to the progression of HBV-infected HCCs. Interest-

ingly, we have identified in one of the HCC cell lines an HBV integrant containing a functional enhancer at this chromosomal locus (45). The possibility that the *hbzap* gene is indeed amplified in these HCCs or activated by the integrated HBV sequences is of highest interest and may shed new light on the mechanism of oncogenic function of HBV.

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