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Hepatitis B virus transactivator protein, HBx, associates with the components of TFIIH and stimulates the DNA helicase activity of TFIIH

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ABSTRACT **Human hepatitis B virus genome encodes a protein, termed HBx, that is widely recognized as a transcriptional transactivator. While HBx does not directly bind cis-acting transcriptional control elements, it has been shown to associate with cellular proteins that bind DNA. Because HBx transactivated a large number of viral/cellular transcriptional control elements, we looked for its targets within the components of the basal transcriptional machinery. This search led to the identification of its interactions with TFIIH. Here, we show that HBx interacts with yeast and mammalian TFIIH complexes both *in vitro* and *in vivo*. These interactions between HBx and the components of TFIIH are supported by several lines of evidence including results from immunoprocedures and direct methods of measuring interactions. We have identified ERCC3 and ERCC2 DNA helicase subunits of holoenzyme TFIIH as targets of HBx interactions. Furthermore, the DNA helicase activity of purified TFIIH from rat liver and, individually, the ERCC2 component of TFIIH is stimulated in the presence of HBx. These observations suggest a role for HBx in transcription and DNA repair.**

Human hepatitis B virus (HBV) is one of the principal causative agents of chronic hepatitis. HBV infection is associated with the development of hepatocellular carcinoma (1). One of the open reading frames of the HBV genome encodes a protein termed *HBx*. It is now generally acknowledged that HBV X protein (HBx) supplied in trans can increase gene expression. This function of HBx has been assayed using both viral and cellular promoters and enhancers (for review, see ref. 2). While HBx does not bind double-stranded DNA, direct physical interaction between HBx and transcriptional factors ATF-2/CREB was demonstrated by Maguire *et al.* (3). Subsequently, the ability of HBx to associate with large variety of cellular proteins has been reported. These include basal transcriptional factor TATA box-binding protein (TBP) (4), the p53 tumor suppressor protein (5), RNA polymerase subunit RPB5 (6), and a putative DNA repair protein, UV-DDB (7). Although not shown by direct protein–protein interactions, the activities of HBx have been implicated in stimulating the cascades of signal transduction pathways (8–13). The subcellular distribution of HBx has been observed predominantly in cytoplasm, and its association with nuclear periphery and nuclear localization has been seen in transfected cells (2, 13). These findings together reinforce the notion that HBx may function by forming complexes with cellular proteins, leading to alteration of the functions associated with those proteins. However, a clear view of the mechanism(s) by which HBx influences cellular processes has remained elusive.

Initiation of eukaryotic transcription by polymerase II depends on a set of general transcription factors. These include TFIIA, TFIIB, TFIID (TBP and TBP-associated factors), TFIIE, TFIIF (RAP30 and RAP74), and TFIIH. A basal level of transcription initiation requires assembly of general transcription factors and *Pol*II onto the DNA template to form a preinitiation complex (14–17). Activation of transcription involves DNA-binding transactivators, such as, c-*fos*/c-*jun*, ATF-2/CREB, or Sp1. In addition to these cellular transcription factors, a wide variety of viral transactivators have been shown to interact directly with components of general transcriptional factors and modulate their activities (18).

Mammalian TFIIH is a multisubunit complex of approximately eight polypeptides, including p89 (ERCC3-XPB), p80 (ERCC2-XPD), p62, p50, p44 (hSSL1), p41, p38, and p34 (refs. 14 and 16 and references therein). At least three enzymatic activities are associated with TFIIH: (*i*) a DNA-dependent ATPase, (*ii*) an ATP-dependent DNA helicase, and (*iii*) a protein kinase capable of phosphorylating the C-terminal heptapeptide repeat in the largest RNA polymerase II subunit (18–28). The DNA helicase activity of TFIIH is associated with ERCC2 and ERCC3 (20–22). TFIIH has been shown to associate with TFIIE, and the complex consisting of TFIIE, TFIIH, and RNA polymerase II is involved in open complex formation and/or promoter clearance (23, 24). In yeast, TFIIH (factor b) and the associated DNA excision repair enzymes include at least 11 subunits: RAD1, RAD2, RAD3, RAD4, RAD10, RAD14, SSL1, SSL2, TFB1, TFB2, and TFB3 (21, 27).

We report here that HBx associates with components of mammalian and yeast TFIIH both *in vitro* and *in vivo*. The evidence for these associations comes from Far Western assay, immunoprecipitation, Western blot analysis, and glutathione *S*-transferase (GST)-pull down assays. Further, this study has identified the ERCC2 and ERCC3 subunits of holoenzyme TFIIH as direct targets of HBx's interactions. This interaction is directed toward a highly conserved domain of the DNA helicases associated with this complex since HBx interacts with the yeast ERCC3 and ERCC2 homologs SSL2 and RAD3 both *in vitro* and in vivo. Toward understanding the functional relevance of these interactions, evidence is presented for the stimulatory effect of HBx on the DNA helicase activity of TFIIH. These properties of HBx suggest a fuctional role of this regulatory protein in the functions associated with TFIIH.

EXPERIMENTAL PROCEDURES

Plasmids. The construction of GST gene fusion of full-length X gene (pGST-X) and three of its deletion mutants, pGST-XN (aa 1–60), pGST-XC (aa 104–154), pGST-XSM (aa 114–143), have been described previously (4). In the plasmid pSPX, the X gene was cloned within the polylinker in pSP65

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Abbreviations: HBV, hepatitis B virus; HBx, hepatitis B virus X protein; TBP, TATA box-binding protein; GST, glutathione *S*-transferase; GST-X, GST fusion with HBx; ERCC2/3, excision repair cross complementing; ssDNA, single-stranded DNA; Ad, adenovirus; AdX, Ad expressing HBx.

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in front of the SP6 promoter for *in vitro* translation of HBx (4). pGSTERCC3 and pSGSME3 (22) were a gift of J. Hoeijmakers (Erasmus University, The Netherlands). pMD2.45 (Mal-ERCC2 fusion construct) was a gift of A. Sancar (University of North Carolina, Chapel Hill). T7 expression plasmids for all the identified subunits of yeast TFIIH (SSL1, SSL2, TFB1, TFB2, TFB3, CCL1, RAD3, and KIN28) were a kind gift of J. Feaver of R. Kornberg's laboratory (Stanford University).

***In Vitro* Transcription and Translation.** RNA and proteins were transcribed and translated *in vitro* using either T7 or SP6 polymerase with TNT-coupled *in vitro* transcription and translation kit (Promega) in the presence of [^{35}S]methionine. Plasmid pSPX, in which X gene is cloned in front of SP6 promoter, was used for *in vitro* transcription and translation of HBx.

Purification of Fusion Proteins Expressed in Bacteria. The GST fusion proteins were purified as described (30). High salt conditions (1.2 M NaCl) were used during binding and washing to eliminate nonspecific binding of bacterial proteins to GST or GST-X. A second step of purification was introduced by passing the purified GST-X fusion protein through single-stranded DNA (ssDNA) agarose column (Sigma). The protein fractions from ssDNA agarose were first eluted in 500 mM KCl, 25 mM Hepes (pH 7.9), 1 mM DTT, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride, and then dialyzed in buffer containing 25 mM KCl, 25 mM Hepes·NaOH (pH 7.9), 10% glycerol (vol/vol), 1 mM ATP, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotenin. The plasmid pMD2.45 contains the ERCC2 coding sequences downstream of the maltose binding sequences. The expression of ERCC2 was induced in BL21(Lsy$^-$) *Escherichia coli* strain by 1.0 mM isopropyl β-D-thiogalactoside and the fractions were immobilized on amylose-resin (New England Biolabs). The fusion protein was eluted in purified form with 1 mM maltose. Protein–protein interaction studies using affinity beads were performed as described previously (4).

Far Western Assay. After blocking the nitrocellulose filter with 1% nonfat dry milk, it was incubated with both 6 M and 3 M guanidine·HCl for 30 min each (two times) at room temperature. Incubation was continued for additional 30 min each (two times) in buffer A containing 100 mM KCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EDTA, 0.1 mM DTT, 1% Triton-X100, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotenin. During binding reaction, 1×10^5 cpm of labeled proteins were supplemented with 1% nonfat dry milk and 2 mM ATP. Filters were rotated in the binding mixture for 3–4 hr at 4°C and washed four times in buffer A.

Immunoprecipitation and Immunoblotting. About 100 μg of total cellular protein was used in immunoprecipitation and immunoblotting. All incubations were carried out in a buffer containing 100 mM KCl, 5 mM $MgCl_2$, 25 mM Hepes·NaOH (pH 7.9), 10% (vol/vol) glycerol, 0.1% Nonidet P-40, 1 μg/ml ethidium bromide, 1 mM ATP, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotenin with gentle shaking at 4°C for 3–4 hr. Immune complexes were collected by precipitation with protein G-Sepharose beads (Sigma) and washed six times before SDS/PAGE analysis. For immunoblotting, antiserum reactive protein bands were visualized using the alkaline phosphatase kit (Promega).

To demonstrate the formation of HBx-TFIIH complexes *in vivo*, HeLa cells were infected with adenovirus (Ad) and Ad expressing HBx (AdX) with a multiplicity of infection of 10 for 12 hr. Yeast extracts were prepared from cells transformed with pGAL-4$_{DBD}$-X according to a procedure described previously (27). GAL-4 $_{DBD}$-X construct contained 1–147 amino acids that encodes the DNA binding domain of the protein.

DNA Helicase Assay. An M13 primer oligonucleotide was labeled *in vitro* at the 5′ terminus in the presence of [γ-^{32}P]ATP by T4 polynucleotide kinase. The labeled probe was gel purified and annealed to single-stranded filamentous phage M13 mp18 DNA. The ^{32}P-labeled template-primer complex was then eluted from nondenaturing 6% polyacrylamide gel and used as a substrate for helicase assay. First, the helicase activities of purified TFIIH and Mal-ERCC2 were titrated on this substrate. Then, the minimal amount required for helicase activity was used in conjunction with increasing amounts of fusion proteins. The reaction mixture was incubated in a buffer that contained 20 mM Hepes·NaOH (pH7.9), 5 mM $MgCl_2$, 5% glycerol (vol/vol), 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotenin at 37°C for 30 min. The product of helicase assay (^{32}P-labeled 40 nucleotide, oligonucleotide) was resolved in a nondenaturing 6% polyacrylamide gel in 0.5 × TBE/0.01% SDS running buffer at 4°C. The gels were dried and exposed to x-ray film (XAR; Kodak) at −80°C with intensifying screens. *Eco*SSB was a gift of C. McHenry (University of Colorado Health Sciences Center, Denver).

RESULTS

Identification of TFIIH Subunits That Interact with HBx. Using a highly purified rat liver preparation of TFIIH (34), specific interactions between HBx and subunits of TFIIH were demonstrated by direct protein–protein interactions (Far Western assay). Five identical aliquots of this preparation were electrophoresed through a SDS/12% gel. One lane was characterized by silver-staining after SDS/PAGE, to show the composition of TFIIH preparation (Fig. 1, lane 1). A similar pattern has been previously reported (34). Four lanes of TFIIH were transferred onto a nitrocellulose filter. One strip was incubated with ^{32}P-labeled GST (lane 2) and the second was incubated with ^{32}P-labeled GST-XC (lane 4). ^{32}P-labeled GST did not show any binding with the purified TFIIH (lane 2), whereas ^{32}P-labeled GST-XC showed binding to two discrete higher M_r bands, indicating a direct protein–protein interaction between HBx and TFIIH (lane 4). ^{32}P-labeled GST-XC showed no binding to the BSA that was as a control (lane 3). Because the approximate electrophoretic mobilities of these protein bands corresponded to the p89 (ERCC2) and p80 (ERCC3) subunits of TFIIH, two separate strips from the same gel were immunoblotted with antisera against ERCC2 and ERCC3. The results of the Western blot analysis unambiguously identified the ERCC3 and ERCC2 subunits of mammalian TFIIH as targets of HBx interaction.

HBx Directly Binds to the ERCC3 and ERCC2 Subunits of TFIIH. To demonstrate direct interactions between HBx and the ERCC2/ERCC3 components of TFIIH, *in vitro* translated HBx ([^{35}S]methionine-labeled) was allowed to bind either Mal-ERCC2 immobilized on amylose resin or GST-ERCC3 immobilized on gluthathione affinity beads. After extensive washing, the bound HBx was resolved by SDS/PAGE. Bacterial lysates immobilized on either amylose resin or GST affinity beads were used as a control. The results of this experiment show that HBx bound specifically to ERCC2 and ERCC3 (Fig. 2*A*, lanes 2 and 4). In reciprocal approach, ERCC3 was translated *in vitro*, labeled in the presence of [^{35}S]methionine, and allowed to bind with GST, GST-X, and three deletion mutants of HBx [GST-XN (aa 1–64), GST-XC (aa 104–154), and GST-XSM (aa 110–143)] that were immobilized on glutathione affinity beads (Fig. 2*B*). After extensive washing, the bound fractions were fractionated by SDS/PAGE. The results described in Fig. 2*B* show that while ERCC3 interacted with GST-X (lane 2), GST-XC (lane 4), and GST-XSM (lane 5), it failed to interact with GST (lane 1) and GST-XN (lane 3).

We next investigated the possible interactions between HBx and the components of yeast TFIIH, RAD3, and SSL2, the homologs of human ERCC2 and ERCC3. These proteins were translated *in vitro* in the presence of [^{35}S]methionine and allowed to interact with GST, GST-XC, and GST-X. The results of this analysis show that both SSL2 and RAD3 bound specifically to

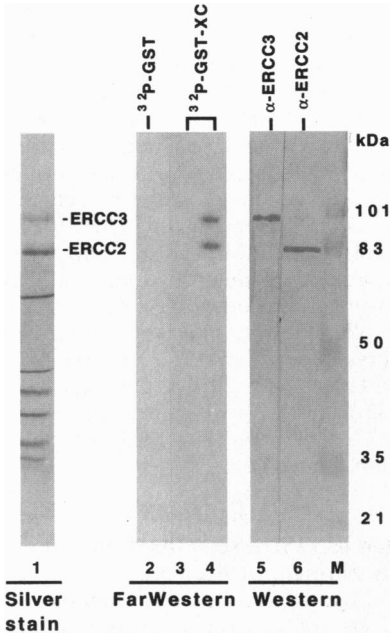

FIG. 1. Identification of the interactions between HBx and the components of TFIIH. Rat liver TFIIH was purified according to a previously described procedure (34). Five identical aliquots of 50 ng of purified rat liver TFIIH preparation were subjected to SDS/PAGE. One was characterized by silver staining (lane 1), and four lanes were transfered onto a nitrocellulose filter. Two strips were subjected to Far Western assay using ^{32}P-labeled GST (lane 2) and ^{32}P-labeled GST-XC (lane 4). Lane 3 was also assayed by Far Western method but contains 200 ng of BSA that was used for analysis using ^{32}P-labeled GST-XC as a probe. Approximately 1×10^5 cpm of the ^{32}P-labeled GST and ^{32}P-labeled GST-XC protein probes were used in this assay. Two additional strips containing purified rat liver TFIIH were immunoblotted with anti-ERCC3 (lane 5) and anti-ERCC2 (lanes 6), respectively. M, M_r standard.

GST-XC (Fig. 2*C*, lanes 2 and 5) and to GST-X (lanes 3 and 6) but not to GST (lanes 1 and 2). Other components of yeast TFIIH (TFB1, TFB2, TFB3, SSL1, CCL1, and KIN28) failed to interact with GST-X (data not shown). The amount of input that binds to affinity-retained proteins is uniformly above 10%. In summary, the ERCC3 and ERCC2 subunits of mammalian TFIIH complex and their yeast homologs, SSL2 and RAD3, appear to be the targets of HBx interaction.

HBx Associates with TFIIH in Cell Lysates. To test for the interactions between HBx and TFIIH in the cell lysates, liquid immunoprecipitation, Western blot analysis, and direct retention of these complexes on GST-X immobilized glutathione beads were used. To investigate the HBx's ability to bind mammalian TFIIH, cell lysates were prepared from a human liver-derived Huh-7 cell line. The lysates were incubated with either GST or GST-XC labeled with [γ-^{32}P]ATP by an *in vitro* phoshorylation reaction. Since TFIIH is a multisubunit complex of eight or more tightly associated polypeptides, an antibody against any of its subunits should precipitate the entire complex. Based on this rationale, after incubation complexes were immunoprecipitated with antibody directed against p62, one of the TFIIH subunits (Fig. 3*A*, lanes 1 and 2). While ^{32}P-labeled GST is not immunoprecipitated by anti-p62 (lane 1), ^{32}P-labeled GST-XC is immunoprecipitated specifically (lane 2). To determine if p62 is also immunoprecipitated in this experiment, the same gel was immunoblotted with anti-p62 serum. In all cases, p62 was present (data not shown). Immunoprecipitation using full-length GST-X also gave similar results (data not presented). To determine whether p62 is a direct target of HBx, the cloned p62 (a gift of J.-M. Egly) was translated *in vitro* ([^{35}S]methionine-labeled) and used in the binding studies with GST-X immobilized on glutathione affinity beads. No binding was detected (data not shown). The data demonstrate that HBx can associate with holoenzyme TFIIH in the cell lysates.

Next, we investigated if HBx could interact with yeast TFIIH. This analysis was carried out using an antiserum directed against the SSL2 subunit of yeast TFIIH, a DNA helicase, and a homolog of human ERCC3 (28). ^{32}P-labeled GST and ^{32}P-labeled GST-XC were mixed with unlabeled yeast lysates (Fig. 3*B*). Anti-SSL2 serum specifically precipitated ^{32}P-labeled GST-XC (lane 2), but not ^{32}P-labeled GST (lane 1).

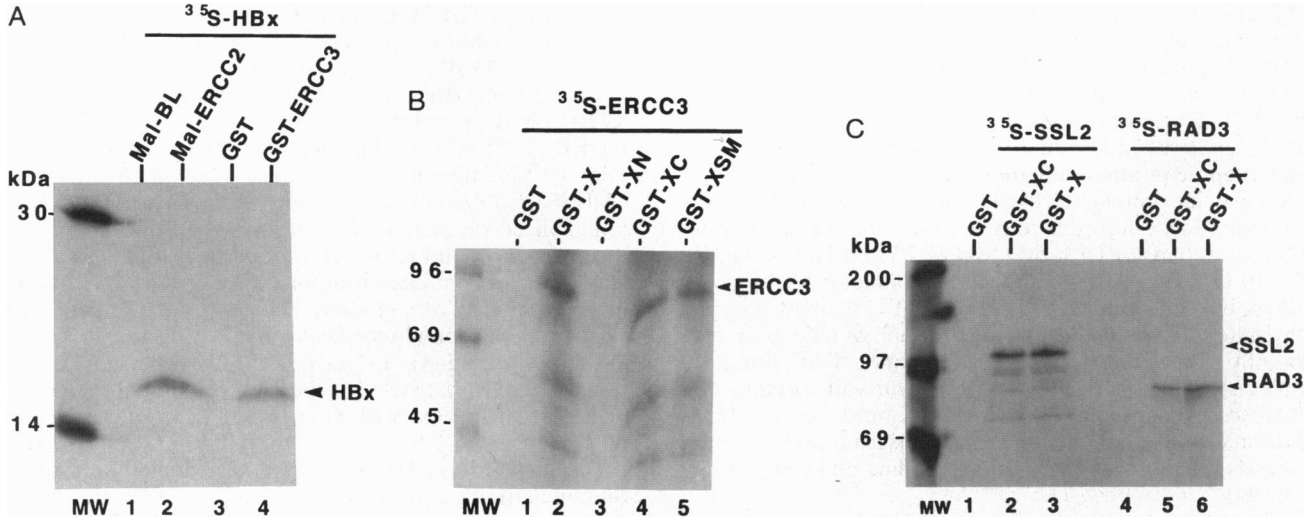

FIG. 2. Direct interactions between HBx and the components of mammalian and yeast TFIIH. (*A*) HBx directly interacts with ERCC2 or ERCC3. *In vitro* translated, [^{35}S]methionine-labeled HBx was added to the amylose-resin column with immobilized bacterial lysate (lane 1) or Mal-ERCC2 (lane 2). The expression of ERCC2 was induced in BL21(Lsy$^-$) *E. coli* strain by 1.0 mM isopropyl β-D-thiogalactoside and the fractions were immobilized on amylose-resin by a maltose tag. After extensive washing of the column, the ^{35}S-labeled HBx was added and washed again before SDS/PAGE analysis. ^{35}S-labeled HBx was added to the glutathione beads immobilized with GST (lane 3) or GST-ERCC3 (lane 4), respectively. (*B*) Direct interaction between ^{35}S-labeled ERCC3 and full-length HBx and its deletion mutants. ERCC3 was translated *in vitro* in the presence of [^{35}S]methionine. Binding of ^{35}S-labeled ERCC3 to GST (lane 1), GXT-X (lane 2), GST-XN (lane 3), GST-XC (lane 4), and GST-XSM (lane 5) immobilized on glutathione affinity beads is shown. M, M_r standard. (*C*) Direct interactions between ^{35}S-labeled SSL2 or -RAD3 and GST-X. *In vitro* translated ^{35}S-labeled SSL2 and ^{35}S-labeled RAD3 were added to glutathione affinity beads with immobilized GST, GST-XC, and GST-X (lanes 1–3 and 4–6), respectively.

In an alternative experimental approach, the yeast lysates were immobilized on glutathione affinity beads containing GST or GST-XC followed by Western blot analysis using anti-SSL2 serum (Fig. 3*C*). Anti-SSL2 serum did not show any cross-reactivity with either GST or GST-X (lanes 1 and 2). A protein band of 110 kDa is observed only in the reaction mixture containing GST-X affinity beads. The 110-kDa band is consistent with the M_r of SSL2 (lane 4).

In summary, these studies provide indirect evidence for a probable interaction between HBx and the component(s) of both yeast and mammalian TFIIH. Because HBx binds single-stranded nucleic acids as well (33), a possibility that the observed protein–protein interactions are mediated by nucleic acids may exist. To rule out this possibility, the bacterial extracts and cellular lysates were treated uniformly with DNase and RNase before incubation in the presence of ethidium bromide.

HBx Associates With TFIIH Subunits *in Vivo*. To document interaction of HBx with TFIIH *in vivo*, two approaches were undertaken. In the first approach, HeLa cells were infected with an Ad containing the X coding sequences (AdX). Wild-type Ad was included as a control. Cell lysates were immunoprecipitated with anti-ERCC2 and anti-ERCC3 sera. As a control, AdX-infected lysates were immunoprecipitated with normal serum. The immunoprecipitates were subjected to SDS/PAGE and then immunoblotted with anti-HBx serum. Results described in Fig. 4*A* show HBx with an expected M_r of 16.7 that was brought down by both anti-ERCC2 and ERCC3 sera, respectively (lanes 3 and 5), but not by normal rabbit serum (lanes 1 and 2) or G Sepharose beads alone (lanes 6 and 7). This result suggests that HBx and TFIIH (subunits) form complexes *in vivo*.

In the second approach, yeast cells transformed with GAL-4_{DBD}-X vector were immunoprecipitated with anti-HBX (Fig. 4*B*) and -anti-SSL2 (Fig. 4*C*) sera and fractionated by SDS/PAGE. The SDS gels were immunoblotted with anti-SSL2 (Fig. 4*B*) and anti-HBx sera (Fig. 4*C*), respectively. Fig. 4*B* (lane 2), shows the SSL2 protein band with a M_r of 110. Similarly, the GAL-4_{DBD}-X protein band with an expected M_r of 29 is observed (Fig. 4*C*, lanes 2 and 3). Treatment of the lysates with normal serum did not bring down either SSL2 or HBx (Fig. 4 *B* and *C*, lane 1). Anti-HBx serum did not cross-react with GAL-4_{DBD} moiety of the fusion protein (data not shown). The results of this analysis indicate that HBx could associate with yeast TFIIH *in vivo* and that this interaction is mediated by SSL2 and RAD3 subunits of the complex. In summary, the studies described above confirm the association of HBx with components of both mammalian and yeast TFIIH *in vivo*.

HBx Stimulates the DNA Helicase Activity of TFIIH. To address the possible functional relevance of these interactions, we investigated the effect of HBx on the DNA helicase activity of TFIIH, since the subunits of TFIIH that associate with HBx contain intrinsic helicase activities (14, 16). The DNA helicase activity of TFIIH is attributed to ERCC2 and ERCC3 (18, 20). To assay DNA helicase activity, a substrate consisting of single-stranded M13 bacteriophage DNA was annealed to a 40-nucleotide-long M13 primer that had been previously labeled with ^{32}P by polynucleotide kinase reaction as shown in Fig. 5*A*. This substrate was first incubated with various amounts of TFIIH in a helicase reaction. The minimal amount of TFIIH required to yield a barely detectable ^{32}P primer was used along with increasing concentrations of GST-X fusion protein in the subsequent reactions. The results of this analysis show proportionally intense bands corresponding to released ^{32}P primer as a function of GST-X concentration (Fig. 5*A*, lanes 5–8). GST, GST-X, or an unrelated fusion protein GST-HNF4 alone were unable to stimulate helicase activity (lanes 2, 3, and 13, respectively). In the presence of ATPγS, AMP-PNP (ATP analogs), and CCCP (an inhibitor of ATPase), no stimulation was observed (lanes 9–11, respectively). This is consistent with previous results that indicate that the DNA helicase activity of TFIIH requires ATP hydrolysis (34). A residual level of activity still seen in lanes 9–11 may be due to the partial presence of functional ATP bound to HBx. To confirm that stimulation is indeed due to HBx, anti-HBx serum was used in the helicase reaction mixture. In the presence of anti-HBx serum, the stimulatory effect of HBx was abrogated (lane 12).

In the next experiment, we tested the effect of three deletion mutants of HBx on the stimulatory effect on the DNA helicase activity. In these mutants the X coding sequences are as follows: GST-XN (aa 1–64), GST-XC (aa 104–154), and GST-XSM (aa 110–143) (4). In a recent analysis of the ssDNA binding properties of HBx, we used these mutants and found that none of these mutants interact with the ssDNA (I.Q. and A.S., unpublished results). In its ability to interact with ssDNA, HBx may prevent the subsequent reannealing of the separated strands. Surprisingly, in the current analysis GST-XC and GST-XSM retained the stimulatory activity (Fig. 5*B*, lanes 7 and 8). In contrast, GST-XN showed reduced stimulatory effect (lane 6). These results indicate that the ability of HBx to stimulate the DNA helicase activity may not be exclusively due to its ability to bind ssDNA. In its capacity to interact simultaneously with DNA helicases and ssDNA, HBx seems to play a role in stimulating the DNA helicase activity by perhaps stabilizing the enzymes involved in the unwinding reaction. To

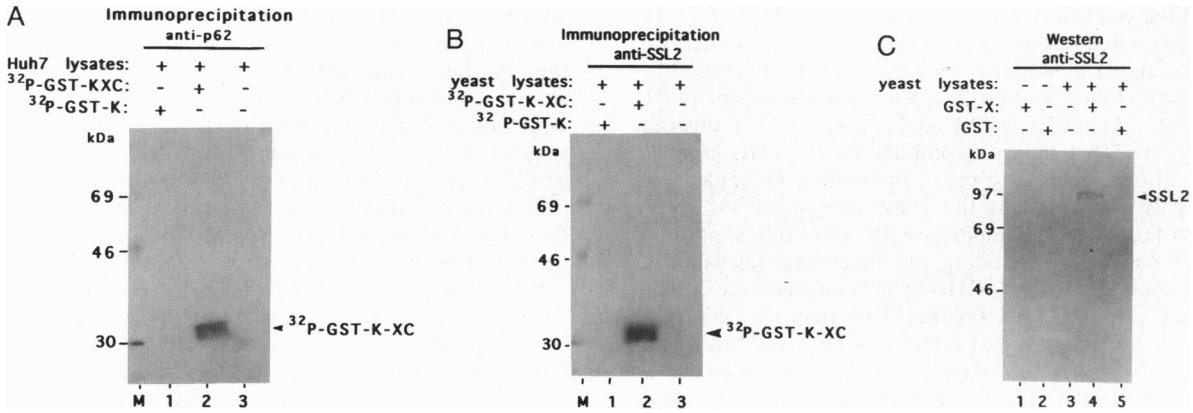

FIG. 3. Association of HBx with components of TFIIH in cell lysates. (*A*) Immunoprecipitation of Huh 7 cell lysates mixed with either ^{32}P-labeled GST (lane 1) or ^{32}P-labeled GST-XC (lane 2) with anti-p62 serum (Santa Cruz Biotechnology). Lane 3, represent the cell lysates without any exogenous protein. Immunoprecipitates were resolved by SDS/12% polyacrylamide gel and autoradiographed. (*B*) Immunoprecipitation of unlabeled yeast cell lysates mixed with either *in vitro* ^{32}P-labeled GST (lane 1) or ^{32}P-labeled GST-XC (lane 2) with anti-SSL2. Lane 3 represents the yeast lysates without any exogenous protein. (*C*) Immunoblotting of yeast cell lysates bound to GST or GST-X immobilized on glutathione affinity beads, using anti-SSL2. Lanes: 1, GST; 2, GST-X; 3, lysates alone; 4, lysates bound to GSTX; 5, lysates bound to GST.

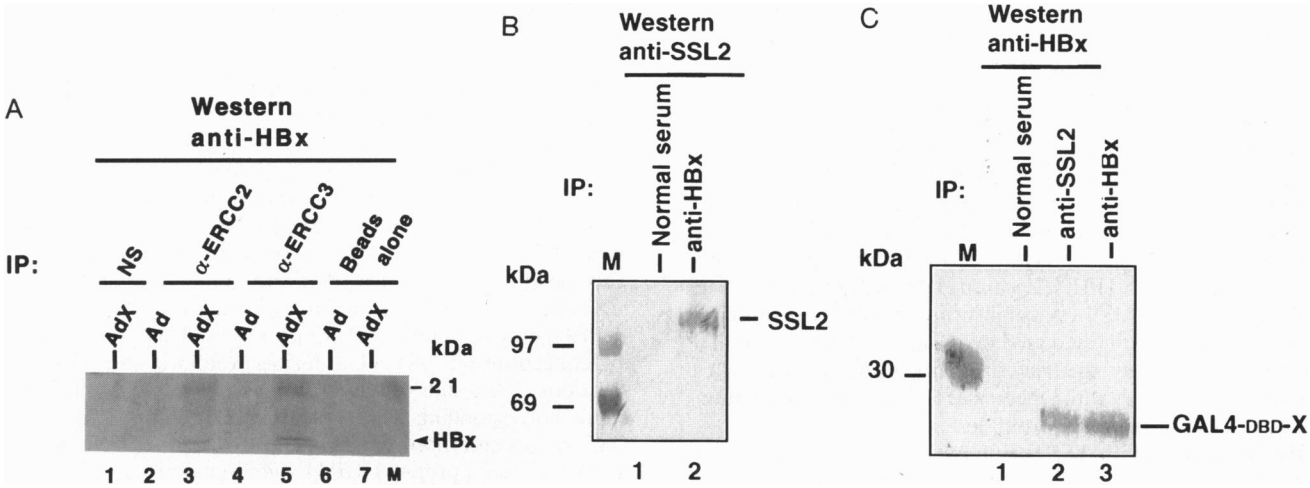

FIG. 4. HBx interacts with the components of mammalian and yeast TFIIH *in vivo*. (*A*) Cultured HeLa cells were infected with AdX and wild-type Ad. At 12 hr postinfection, the cells were lysed and immunoprecipitated with a normal serum (lane 1), anti-ERCC2 serum (lanes 2 and 3), anti-ERCC3 serum (lanes 4 and 5), and no antibodies (lanes 6 and 7). After immunoprecipitation, the samples were subjected to SDS/PAGE, followed by immunoblotting using the anti-HBx serum. NS, normal serum. (*B* and *C*) Yeast extracts were prepared from cells transformed with a GAL-4_{DBD}-HBx vector DNA. The lysates were immunoprecipitated with anti-HBx (*B*), and anti-SSL2 (*C*), and subjected to SDS/PAGE followed by immunoblotting using anti-SSL2 (*B*) and anti-HBx (*C*), respectively.

determine whether other ssDNA binding proteins had an effect on the DNA helicase activity, increasing concentrations of an unrelated *E. coli* ssDNA binding protein (*Eco*SSB) were tested in the DNA helicase assay. The results show that *Eco*SSB also stimulated the DNA helicase activity of TFIIH (lanes 10–13). This stimulation may be due to the fact that *Eco*SSB, as a ssDNA binding protein, may prevent the reannealing of the separated strand.

The stimulatory effect of HBx was also demonstrable with an individual component ERCC2 of TFIIH (Fig. 5*C*, lanes 5–9). HBx was able to stimulate ERCC2 helicase activity, however the levels of stimulation are not comparable to those observed with holoenzyme TFIIH (Fig. 5 *A* and *B*). This may be the result of a cumulative effect of the helicase activities of ERCC2 and ERCC3 in the TFIIH complex.

DISCUSSION

The mechanism(s) by which HBx transactivates gene expression is a subject of intense investigation. It is well-documented now that one possible mechanism of action by HBx is via protein–protein interactions. Physical interactions between HBx and several nuclear proteins have been demonstrated (3, 4, 6, 7). First, we showed that it interacts with TBP of TFIID complex (4). The present study, in which other components of basal transcriptional machinery were investigated as targets of HBx, revealed interactions with at least two subunits of TFIIH (i.e., ERCC2 and ERCC3). ERCC2 and ERCC3 together constitute the DNA helicase components of TFIIH complex (18). The affinity of HBx for the component of TFIIH appears to be highly conserved as the yeast homologs, SSL2, and RAD3 protein formed complexes with HBx both *in vitro* and *in vivo*. Toward understanding the functional relevance of HBx's associations with TFIIH, we present evidence for the stimulatory effect of HBx on the DNA helicase activity of TFIIH. Our mutant data has tentatively localized this stimulatory activity within the 34 amino acid residues in the C-terminal half of HBx. These sequences have been previously shown to encode the transactivation domain (for review, see ref. 2). The functional significance of these observations in the context of transcription initiation process remains to be investigated.

We have recently observed that HBx can bind single-stranded nucleic acids (33). Based on these observations, it is tempting to speculate a possible functional relevance of HBx's interactions with single-strand nucleic acids and components of TFIIH. In the latter case, its binding affinity appears to be directed toward DNA helicase activity of that complex. In that role, as a part of complex, HBx may gain access to the melted template catalyzed by TFIIH during transcription initiation, where it can bind separated DNA strands. The ability of HBx to bind ssDNA (33) may prevent reannealing of melted strands, thus leading to the stabilization of TFIIH activity. The ssDNA binding activity of HBx may also have an impact on other important cellular functions such as DNA repair. Support for HBx's role in DNA repair comes from a recent report, in which HBx was shown to interact with UV damaged DNA repair enzyme (7). ERCC3 has also been shown to be involved in human DNA repair disorders xeroderma pigmentosum and Cockayne syndrome (22, 29). Thus, the ability of HBx to interact with ERCC2/ERCC3 may implicate this viral protein in both DNA excision repair and promoter clearance.

The duality of functions ascribed to TFIIH make this multiprotein complex a prime regulatory target for a wide variety of cellular and viral proteins. Indeed, several viral regulatory proteins have been shown to interact with TFIIH, including herpes virus VP16 (31), Epstein–Barr virus nuclear antigen EBNA2 (32), HIV Tat (ref. 16 and references therein), and, now, the HBx protein. These viral proteins may have acquired the potential to modulate the activities of TFIIH, thereby significantly influencing transcription initiation process and possibly DNA excision repair. Interestingly, these viral factors also interact with TFIID (TBP), suggesting a dual regulatory control mechanism both at the level of preinitiation complex assembly and promoter clearance during transcription initiation. Sharing these common cellular targets implies that HBx, EBNA2, and Tat may exploit similar mechanism(s) in regulating gene expression. It is interesting to note that DNA genomes of several animal viruses such as papilloma viruses, herpes viruses, simian virus 40, insect viruses, and bacteriophages encode a helicase activity. HBV, being one of the smallest virus (3.2 kb), does not encode a helicase activity but one of its encoded proteins (HBx) harbors an affinity for cellular helicases, suggesting the requirement for helicase activity for the establishment of infectious process. HBx is indispensable for viral lifecycle (35).

The data presented here invoke a fundamental role of HBx in cellular processes including transcription and repair. Indeed, our preliminary studies with yeast expressing HBx displayed to in-

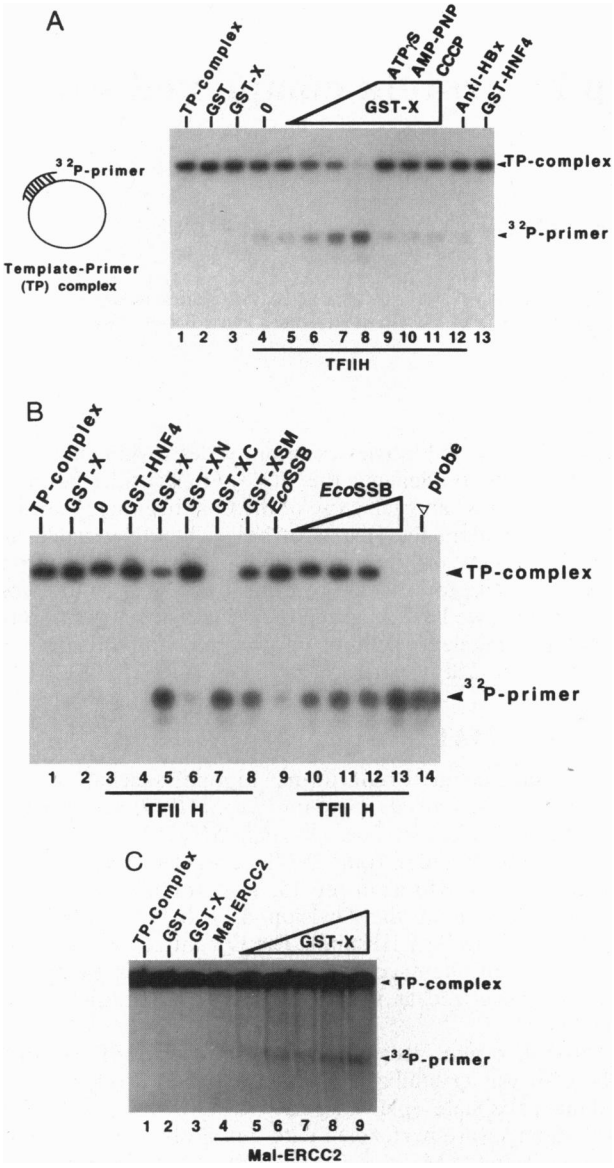

FIG. 5. Stimulation of DNA helicase activity of TFIIH in the presence of HBx. (*A*) Purified rat liver TFIIH preparation (34) was assayed for DNA helicase activity using a M13 (ssDNA)-^{32}P-primer DNA as a substrate. Lanes 2 and 3 contain 100 ng of GST and GST-X alone, respectively. Lanes 4–12 contain 5 ng of purified TFIIH. Lanes 5–8 contain increasing concentrations (10, 25, 50, and 100 ng, respectively) of GST-X. Lanes 9–11 contain 100 ng of GST-X and ATPγS, AMP-PNP, and CCCP (1.0 mM in each lane). Lane 12, anti-HBx serum. Lane 13, 100 ng of GST-HNF4 (without TFIIH). (*B*) Effect of deletion mutants of HBx and *Eco*SSB on the stimulatory activity of TFIIH. Lanes 3–8 and 10–13 contain constant amount (5 ng) of purified TFIIH. GST-X (100 ng; lane 5) and its deletion mutants (lanes 6–8) were added. Lane 9 contains 50 ng of purified *Eco*SSB alone. Lanes 10–13 contain 1, 10, 25, and 50 ng of *Eco*SSB respectively and constant amount (5 ng) of purified TFIIH. Lane 14 contains a heat-denatured substrate. (*C*) Effect of HBx on the helicase activity of bacterially expressed ERCC2. Lanes 4–9 contain constant amount (5 ng) of purified Mal-ERCC2 fusion protein expressed in bacteria. Lanes 5–9 show an increasing concentration of 1, 10, 25, 50, and 100 ng of added GST-X.

creased UV hypersensitivity, implying a functional role of HBx in DNA excision repair. These properties of HBx further lend support to its suspected role in the development of hepatocellular carcinoma by directly regulating cellular gene expression in infected hepatocytes.

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