JOURNAL OF VIROLOGY, Aug. 1996, p. 5582–5591 Vol. 70, No. 8
0022-538X/96/$04.00+0

Proteasome Complex as a Potential Cellular Target of Hepatitis B Virus X Protein

JIAKANG HUANG,[1] JOEL KWONG,[1] EDWARD C.-Y. SUN,[1] AND T. JAKE LIANG[1,2]*

Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114,[1] and Liver Diseases Section, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892[2]

Received 18 August 1995/Accepted 9 May 1996

Although the biological importance of hepatitis B virus X protein (HBX) in the life cycle of hepatitis B virus has been well established, the cellular and molecular basis of its function remains largely undefined. Despite the association of multiple activities with HBX, none of them appear to provide a unifying hypothesis regarding the true biological function of HBX. Identification and characterization of cellular targets of HBX remain an essential goal in the elucidation of the molecular mechanisms of HBX. Using the *Saccharomyces cerevisiae* two-hybrid system, we have identified and characterized a novel subunit of the proteasome complex (XAPC7) that interacts specifically with HBX. We also showed that HBX binds specifically to XAPC7 in vitro. Mutagenesis studies have defined the domains of interaction to be critical for the function of HBX. Furthermore, overexpression of XAPC7 appeared to activate transcription by itself and antisense expression of XAPC7 was able to block transactivation by HBX. Therefore, the proteasome complex is possibly a functional target of HBX in cells.

Hepatitis B virus (HBV) has a unique fourth open reading frame coding for a protein known as hepatitis B viral X protein (HBX). HBX is well conserved among the mammalian hepadnaviruses and codes for a 16-kDa phosphoprotein, which has been detected in both the nucleus and cytoplasm (12, 47). Its message (0.7 kb) has been detected in infected liver, but the protein has not been easily detectable. However, antibodies against this protein have been detected in infected individuals, suggesting the expression of this protein in vivo (32). The HBX protein has been shown to exhibit multiple functions. First of all, the establishment of an essential role of HBX in HBV infection in vivo (7, 57) underscores the functional significance of this enigmatic protein. Second, HBX activates a variety of viral and cellular promoters in diverse cell types (9, 11, 35, 46). Although it does not bind to DNA directly, it activates transcription when it is targeted to a promoter by fusion to a heterologous DNA binding domain (46, 52). The protein has also been shown to function through AP-1 and AP-2 (46) and to interact directly with members of the CREB/ATF transcription factor family (35). Furthermore, a Kunitz domain, characteristic of Kunitz-type serine protease inhibitors, has been proposed to exist in HBX based on sequence homology (48). Although HBX has never been shown definitively to possess serine protease inhibitor activity, mutation of this putative consensus sequence inactivates the function of HBX (2). Recent evidence on the activation of the protein kinase C pathway by HBX provides a plausible hypothesis for the function of HBX (28). The pleiotropic effects of HBX mediated through the tumor promoter pathway could contribute to the long latency of cancer development.

Although this hypothesis remains attractive, it is not sufficient to explain all the findings associated with the actions of HBX. Recently, activation of the ras/raf pathway was implicated in the function of HBX (4). HBX has also been shown to interact with p53 and inhibit its function (51, 53). Furthermore, components of the basal transcription complex, such as TATA-binding protein (42) and RPB5, a common subunit of RNA polymerases (8), have been reported to be potential cellular targets of HBX. Although these findings could potentially explain the transactivation function of HBX, no functional studies have been performed to support this claim. Finally, demonstration of the oncogenic potential of the HBX gene in a transgenic mouse model suggests that HBX probably contributes to the pathogenesis of HBV-associated hepatocellular carcinoma (29). Using the *Saccharomyces cerevisiae* two-hybrid system, we have identified a putative cellular target of HBX and demonstrated that this interaction may be functionally relevant to the pleiotropic action of HBX.

(Part of this study was presented at the annual meeting on the Molecular Biology of HBV, August 1993, Washington, D.C.)

MATERIALS AND METHODS

Two-hybrid screen. The HBX gene derived from an HBV adw strain was fused to the *lexA* DNA binding domain (pEG202) through the *Nco*I site (nucleotide 1375), which contains the AUG start codon of HBX. The two-hybrid interaction cloning protocol has been described in detail previously (22, 56). Two reporter constructs were used in selection of the candidate clones: one contains the *lexA*op-Leu2 gene, which allows for growth in the absence of leucine, and the other has the *lexA*op-*lacZ* gene, which permits selection based on β-galactosidase activity (pSH18-34). For screening and our initial experiments, the pSH18-34 *lacZ* reporter which contains eight LexA binding sites was used. In subsequent experiments where the interaction is expected to be strong, such as the one between HBX and XAPC7, a less sensitive JK103, which contains two copies of LexA binding sites, was used as the *lacZ* reporter. LexA-HBX, by itself, did not activate the reporters, and functional repression assay (22, 56) showed that the fusion protein indeed binds to the LexAop sequence. The cDNA library was transfected along with the HBX expression construct into the EGY48 yeast strain harboring both reporters. A pool of cells containing 2×10^6 primary library transformants was plated onto galactose-Leu$^-$ selection plates. The Leu$^+$ clones were patch transferred to galactose-Leu$^-$ selection plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Approximately 200 clones showed detectable blue color. They were streaked out on four types of plates, Glu-Leu$^-$, Gal-Leu$^-$, Glu-Leu$^+$–X-Gal, and Gal-Leu$^+$–X-Gal. Nine clones showed definitive galactose-inducible leucine dependence and LacZ activity. Plasmids isolated from these clones were transfected into *Escherichia coli* K-12 strain KC8, which allows for selection of the Trp marker carried by the library vector JG4-5. All nine clones had cDNA inserts ranging from 0.5 to 1.5 kbp.

* Corresponding author. Mailing address: Liver Diseases Section, NIDDK/NIH, Building 10, Rm. 9B16, 10 Center Dr., Bethesda, MD 20892. Phone: (301) 496-1721. Fax: (301) 402-0491. Electronic mail address: LiangTJ@mr.gov.

Cross-hybridization demonstrated that some of them were derived from the same cDNA, resulting in a total of four groups (one group of four was of the XAPC7 clone). These clones were retransfected into EGY48 with HBX and the reporter constructs, and they again demonstrated galactose-inducible leucine dependence and LacZ activity. In addition, transfection of these cDNA clones with pEG202 that contains the LexA DNA binding domain only did not result in any transactivation of the reporter genes, indicating that specific interaction with the HBX domain of the LexA-HBX fusion protein is required. One of the cDNA clones (XAPC7) showing strong interaction was selected for further analysis. Two HBX mutants were generated for initial analysis. One contains an amino acid insertion of arginine after amino acid (aa) 68 (HBXRsr); this was constructed by digesting the HBX sequence with *Rsr*II and then filling in the 3-nucleotide overhang with Klenow enzyme. The other contains a C-terminally truncated HBX gene ending at aa 90 (HBXEn), which was generated by truncating the HBX gene at the *Eco*NI restriction site (nucleotide 1645). To switch the interaction domains, HBX was fused in frame to the B42 acid patch transactivation domain of JG4-5 and XAPC7 was fused to the *lexA* DNA binding domain of pEG202.

Plasmid construction and mutagenesis. cDNAs for human proteasome subunits LMP-2, HC2, HC3, HC5, and HC8 were obtained from Keiji Tanaka. Using PCR, the C-terminal portions of LMP-2 from aa 117, HC2 from aa 134, HC3 from aa 136, HC5 from aa 145, and HC8 from aa 138, which correspond in sequence homology alignment to the original XAPC7 clone (aa 137 to 248), were inserted into JG4-5 to form fusion proteins with the B42 activation domain. The X gene of woodchuck HBV derived from the WHV81 strain was fused to the *lexA* DNA binding domain through the *Nco*I site of woodchuck HBV (nucleotide 1501), which contains the AUG start codon of WHVX. Site-directed HBX mutations were introduced by PCR and confirmed by sequencing: C61S with Cys-to-Ser mutation at aa 61, G67A with Gly-to-Ala mutation at aa 67, P68A with Pro to Ala at aa 68, C69S with Cys to Ser at aa 69, T120R with Trp to Arg at aa 120, F132Y with Phe to Tyr at aa 132, G135A with Gly to Ala at aa 135, G136V with Gly to Val at aa 136, C137S with Cys to Ser at aa 137, R138Q with Arg to Gln at aa 138, H139D with His to Asp at aa 139, and K140Q with Lys to Gln at aa 140. An additional mutant, D2.MT, was generated by replacement of aa residues 137 to 141 (Cys-Arg-His-Lys-Leu) with Val-Met sequence. Wild-type and mutant HBX genes were cloned into the pCD.1 expression vector (Invitrogen) for transactivation studies. HBX mutants were also constructed in pEG202 for interaction studies in *S. cerevisiae.*

XAPC7 deletion mutants DM.4, DM.5, DM.6, DM.7 and DM.8 were generated using appropriate restriction sites internal to the XAPC7 cDNA and then subjected to T4 DNA polymerase treatment and ligation. DM.4 contains an in-frame deletion of aa 143 to 163 (*Avr*II to *Ban*I); DM.5 has a deletion of aa 143 to 198 (*Avr*II to *Xmn*I); DM.6 has a deletion of 143 to 220 (*Avr*II to *Dra*I); DM.7 has a deletion of aa 188 to 198 (*Bst*XI to *Xmn*I); DM.8 has a deletion of aa 188 to 220 (*Bst*XI to *Dra*I). DM.1, DM.2, DM.3, DM.9, and DM.10 were generated by PCR and contain aa 137 to 190, 137 to 207, 137 to 228, 171 to 248, and 194 to 248, respectively. All deletion mutants were confirmed by DNA sequencing analysis. The antisense constructs (pRSVRZC7.A and pRSVRZLacZ.A) were generated by cloning the cDNAs in the antisense orientation into the pRSVRZ vector, which has been shown to be a very efficient antisense vector (33). The vector contains a *cis*-acting ribozyme sequence instead of a polyadenylation signal in the 3′ region of the expression unit, resulting in nuclear retention, RNA duplex formation with sense RNA, and efficient degradation of the duplexed RNA.

In vitro binding and protein analysis. HBX and full-length XAPC7 were cloned into pGEM11Zf(+) vector (Promega, Madison, Wis.), and their transcripts were produced by an in vitro transcription kit (Promega). Rabbit reticulocyte lysates from Promega were used to generate [^{35}S]Met-labeled proteins, which were used immediately for binding studies. HBX (full length) and XAPC7 (aa 137 to 248) were cloned separately into the pGEX-KG vector (Pharmacia, Piscataway, N.J.) to be expressed as a fusion protein with glutathione *S*-transferase (GST). The binding reactions were performed in NETN buffer (0.5% Nonidet P-40, 20 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl) at room temperature for 1 h with constant mixing. The beads were washed extensively with the same buffer, and the bound proteins were subjected to sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis (PAGE) analysis. For immunoprecipitation and Western blot (immunoblot) analysis, cells were lysed in RIPA buffer containing protease inhibitors and subjected to standard protocols (3). The chemiluminescence kit from Amersham was used for immunoblotting detection. For analysis of transfected cells, cells were cotransfected with CD4 expression plasmid CDM8CD4 (a gift of Brian Seeds) and detached 3 days later with 0.5 mM EDTA in phosphate-buffered saline. Transfected cells (10-cm dish) were transferred to the same buffer with 5% fetal calf serum and isolated with a 50-μl suspension of Dynabeads M-450 CD4 (1.4×10^8 beads per ml), which are anti-CD4-coupled magnetic beads (Dynal, Inc., Lake Success, N.Y.), at 4°C with gentle mixing for 60 min. Cells bound to the beads were washed four times and lysed with SDS sample buffer for SDS-PAGE analysis. Half of the samples were analyzed by Western blotting with MCP34 antibody, and the other half were analyzed with antiactin antibody (Boehringer Mannheim).

Transactivation assay. Chloramphenicol acetyltransferase (CAT) reporter constructs containing either four AP-1, six AP-2, five AP-3, or three NF-κB sites in front of a minimal human metallothionein IIA promoter were described previously (2). Another reporter construct contains two SP-1 sites (5′ GGGCGGGGCAGGG 3′) cloned into the pTK81-Luc plasmid which consists of a minimal herpes simplex virus thymidine kinase (TK) promoter in front of the luciferase gene (37). The reporter construct for Rous sarcoma virus (RSV) is RSV-Luc. The minimal metallothionein IIA and TK promoter-driven reporter constructs had low activities in transfected cells and were not transactivated by cotransfection with HBX expression construct (not shown). HepG2 cells were used in all experiments. Calcium phosphate transfection was performed routinely using six-well plates. Reporter activities were typically assayed 2 days later.

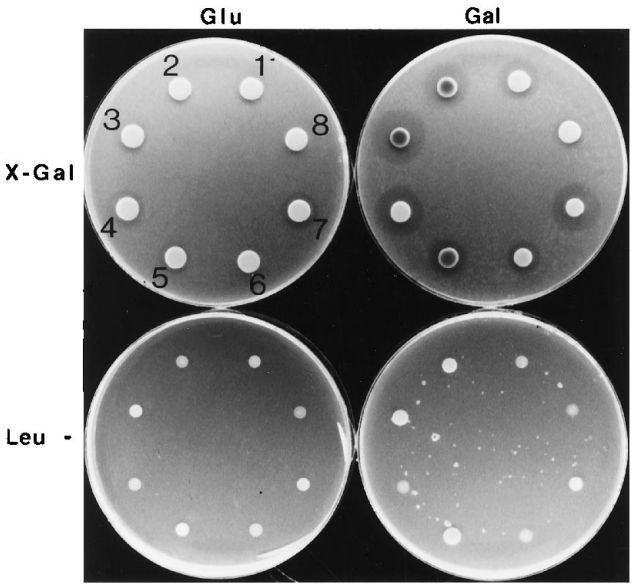

FIG. 1. Specific interaction of HBX with XAPC7 in *S. cerevisiae*. The EGY48 yeast strain containing the LexAop-Leu2 and JK103 reporters was transformed with pEG202HBX and JG4-5 (1), pEG202HBX and JG4-5XAPC7 (2), pEG202HBXRsr and JG4-5XAPC7 (3), pEG202HBXEn and JG4-5XAPC7 (4), pEG202XAPC7 and JG4-5HBX (5), pEG202- and JG4-5HBX (6), pEG202- and JG4-5XAPC7 (7), and pEG202XAPC7 and JG4-5 (8). Transformants were spotted on four types of plates as indicated at the left and top, and the plates were photographed 3 days later. The sample positions are identical on each plate. Positive interaction is scored as blue (black on this black-and-white reproduction) on X-Gal plate and as growth on Leu$^-$ plate.

RESULTS

Interaction of a proteasome subunit with HBX. In order to unravel the molecular mechanisms of HBX, we adopted the yeast two-hybrid system to identify and characterize cellular targets of HBX (13, 22). Since HBX appears to function as a transactivator in a variety of cell types including HeLa cells, a HeLa cell cDNA library containing cDNAs fused to the B42 activation domain in the yeast vector JG4-5 was used to screen for clones interacting with HBX. The result of our screening led to the identification of four independent clones. One strongly reacting clone (XAPC7) was isolated repeatedly in the two-hybrid screen and was selected for further analysis. Using a variety of controls, we demonstrated that HBX interacts specifically with XAPC7 in *S. cerevisiae* (Fig. 1). Two HBX mutants, one containing an amino acid insertion of arginine after aa 68 (HBXRsr) and the other being a truncated N-terminal HBX (aa 1 to 90) (HBXEn), were included as controls. HBXRsr appeared to interact with XAPC7, but HBXEn was totally nonreactive (Fig. 1). To demonstrate that this particular interaction between HBX and XAPC7 is not peculiar to the fusion proteins (LexA or B42), the reverse constructs were generated—HBX fused to the B42 activation domain in JG4-5 and XAPC7 fused to the *lexA* DNA binding domain. Two-

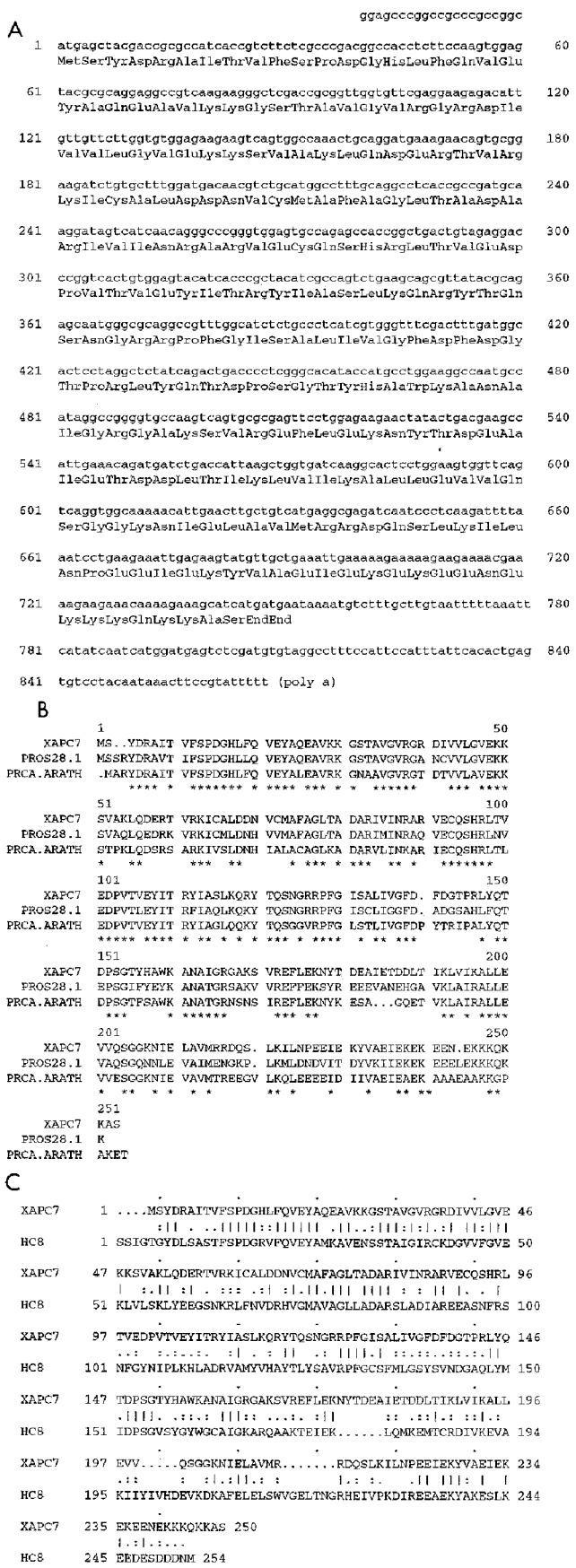

FIG. 2. XAPC7 is a new human proteasome subunit. (A) A full-length XAPC7 cDNA clone was isolated from a λGT11 HeLa cDNA library, and its complete sequence and open reading frame are shown. (B) Comparison of protein sequences between XAPC7 and PROS28.1 proteasome subunit of *D. melanogaster* and the α proteasome subunit of *A. thaliana* is shown. Sequence identities among the three proteins are marked beneath the amino acid residues with asterisks. (C) XAPC7 is then aligned with its most homologous human proteasome subunit, HC8.

hybrid analysis showed that this interaction between HBX and XAPC7 is specific regardless of the backbone of the fusion protein (Fig. 1).

The full-length cDNA of XAPC7 was subsequently obtained from a λGT11 HeLa cDNA library (Fig. 2). The initiation AUG codon was identified at the 5′ end with appropriate Kozak consensus sequence. The entire open reading frame codes for 248 aa with a M_r of 27,896. Sequencing analysis of the XAPC7 clone (Fig. 2A) reveals that it encodes a polypeptide with high sequence homology to the PROS-28.1 subunit of proteasome (multicatalytic proteinase complex) of *Drosophila melanogaster* (23) and the α proteasome subunit of *Arabidopsis thaliana* (16) (Fig. 2B). It shows weaker but significant sequence homology to the other members of the proteasome family (50). Sequence alignment between XAPC7 and its closest human relative, HC8, is also shown (33% identity and 57% similarity) (Fig. 2C). The fact that XAPC7 represents a highly conserved proteasome subunit with >65% amino acid identity among members of both animal and plant kingdoms supports the functional importance of this subunit. The domain interacting with HBX resides in the C terminus of the XAPC7 protein (aa 137 to 248). The full-length XAPC7 was also shown to interact with HBX in *S. cerevisiae* (data not shown). Northern (RNA) blot analysis of several human cell lines revealed a 1.0-kb transcript (Fig. 3). A similar-size RNA species was also identified in RNA from a mouse lymphoma cell line.

Specific interaction of HBX with XAPC7 but not other proteasome subunits. Since XAPC7 belongs to a multigene family, we proceeded to examine whether other subunits of the proteasome family also interact with HBX. cDNAs of five other human proteasome subunits (LMP-2, HC2, HC3, HC5, and HC8) (5, 18, 49) were cloned into the yeast JG4-5 vector for interaction studies in the yeast (Fig. 4) (20). No interaction was observed between HBX and these subunits. Since woodchuck HBV also encodes an X protein (WHVX) with transactivation function and significant homology to HBX, WHVX was ana-

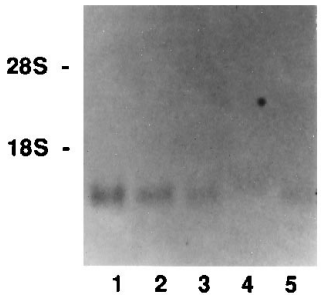

FIG. 3. The XAPC7 gene codes for a 1-kb mRNA. Using the cDNA as hybridization probe and washing under stringent condition, Northern blot analysis of RNAs from several human and mouse cell lines and human placenta (Clonetech) was performed: lane 1, human hepatoma cell line HepG2; lane 2, human hepatoma cell line HuH-7; lane 3, COS7; lane 4, mouse lymphoma cell line EL4; lane 5, human placenta RNA. The positions of 28S and 18S are indicated.

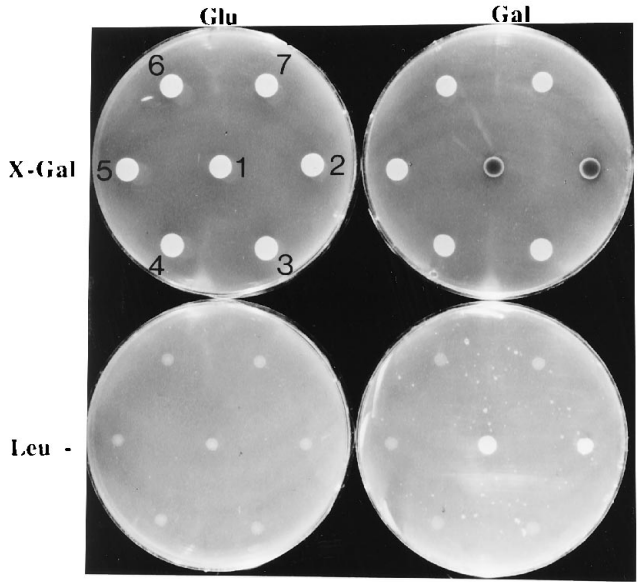

FIG. 4. Specific interaction of HBX with XAPC7 proteasome subunit but not other subunits. The carboxy-terminal portion of each cDNA for LMP-2, HC2, HC3, HC5, and HC8 proteasome subunits was inserted into JG4-5 to form fusion protein with the B42 activation domain. The resulting constructs by themselves did not activate the reporters (not shown). They were then tested with pEG202HBX as in Fig. 1. Sample 1, pEG202HBX and JG4-5XAPC7; sample 3, pEG202HBX and JG4-5LMP-2; sample 4, pEG202HBX and JG4-5HC2; sample 5, pEG202HBX and JG4-5HC3; sample 6, pEG202HBX and JG4-5HC5; sample 7, pEG202HBX and JG4-5HC8. Interaction between pEG202WHVX and JG4-5XAPC7 was shown in sample 2.

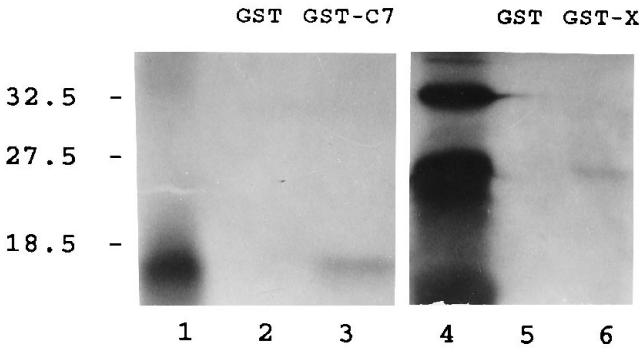

FIG. 5. In vitro binding of HBX and XAPC7. In vitro-translated [^{35}S]Met-labeled HBX and XAPC7 proteins were incubated with GST-XAPC7 and GST-HBX bound to the glutathione beads, respectively. Binding reactions with GST only were performed as controls in each binding assay. The in vitro-translated protein products are shown in lane 1 as HBX and in lane 4 as XAPC7. The other lanes are as indicated. The background labeling of a 35-kDa protein is often seen in this preparation of rabbit reticulocyte lysates (Promega). This irrelevant protein product represents an internal control for binding to the GST fusion protein-containing beads.

lyzed for its interaction with XAPC7 in *S. cerevisiae*. Figure 4 shows that WHVX also interacts specifically with XAPC7.

In vitro binding of XAPC7 and HBX. In order to demonstrate interaction of HBX and XAPC7 proteasome subunits in vitro, we constructed two GST-fusion expression plasmids, one with HBX and the other with the XAPC7 protein. The GST-HBX and -XAPC7 fusion proteins expressed in bacteria were purified with glutathione beads and then incubated with in vitro-translated, [^{35}S]Met-labeled full-length XAPC7 and HBX polypeptides, respectively. The beads were then washed, and the bound polypeptides were subjected to SDS-PAGE analysis. The results are shown in Fig. 5. HBX bound specifically to GST-XAPC7 and not to GST alone; similar binding was also seen between C7 and GST-HBX. The fact that the binding is not particularly strong could be a result of several factors. First, this in vitro binding system may not reflect the in vivo environment in which the two proteins interact. Second, this may reflect the intrinsic difference between the in vitro binding assay and the two-hybrid system, which is more sensitive in detecting weaker interaction. Third, endogenous rabbit XAPC7 present in the reticulocyte lysate may compete with binding to HBX.

Protein encoded by XAPC7 is a subunit of 20S proteasome complex. In order to demonstrate that the XAPC7 cDNA indeed codes for a proteasome subunit, we obtained a panel of monoclonal antibodies specific for various subunits of human proteasome from Keiji Tanaka and Klaus Hendil (24, 30). One antibody (MCP34) specific for a 28-kDa subunit of human proteasome (HC6) recognizes the recombinant XAPC7 polypeptide. It detects a 28-kDa protein in several cell lines (Fig. 6A), which is consistent with the estimated molecular mass of the XAPC7 open reading frame. Comparison of the encoded protein sequence of XAPC7 cDNA with the partial protein sequence of purified HC6 subunit (49a) showed a 100% match. In order to demonstrate that the XAPC7 subunit is part of the proteasome complex, we treated lysates from several cell lines with monoclonal antibody 2-17 or MCP21, which has been shown to specifically precipitate the whole proteasome complex (24, 30), and performed Western blot analysis on the immunoprecipitates using the MCP34 (anti-XAPC7) antibody (Fig. 6B). These results indicate that the XAPC7 protein is a member of the proteasome complex.

Mutational analysis of HBX and XAPC7 interaction. Functional mapping of HBX has defined two structural domains that are crucial for the transactivation function of HBX (2, 45, 48). These two domains appear to overlap with the putative Kunitz-type domain of protease inhibitor that is present in both HBX and WHVX. We mutated several key residues in these two domains and studied the effects of these mutations on transactivation function of HBX and interaction between HBX and XAPC7 in the yeast two-hybrid system (Fig. 7). Since glycine residues are important structural determinants of pro-

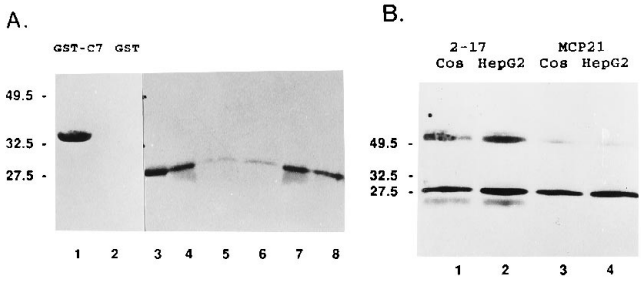

FIG. 6. (A) Purified GST-XAPC7 (lane 1) and GST (lane 2) (0.5 μg each) and 50 μg of cell lysates from HepG2 (lane 3), HuH-7 (lane 4), Hep3B (lane 5), focus hepatoma (lane 6), COS-7 (lane 7), and HeLa (lane 8) cells were electrophoresed through a 14% gel and subjected to Western blot analysis using MCP34 antibody. XAPC7 subunit has the apparent molecular mass of 28 kDa on SDS-PAGE. (B) Proteasome complex was immunoprecipitated from 50 μg of cell lysates from COS and HepG2 cell lines using either 2-17 or MCP21 antibody. Western blot analysis was performed on the immunoprecipitated proteasome using MCP34 antibody. Immunoglobulin heavy (50 kDa) and light (25 kDa) chains are also detected on the blot. Control immunoblotting with mouse immunoglobulin was performed in parallel and confirmed the specificity of the 28-kDa band as the XAPC7 protein (not shown).

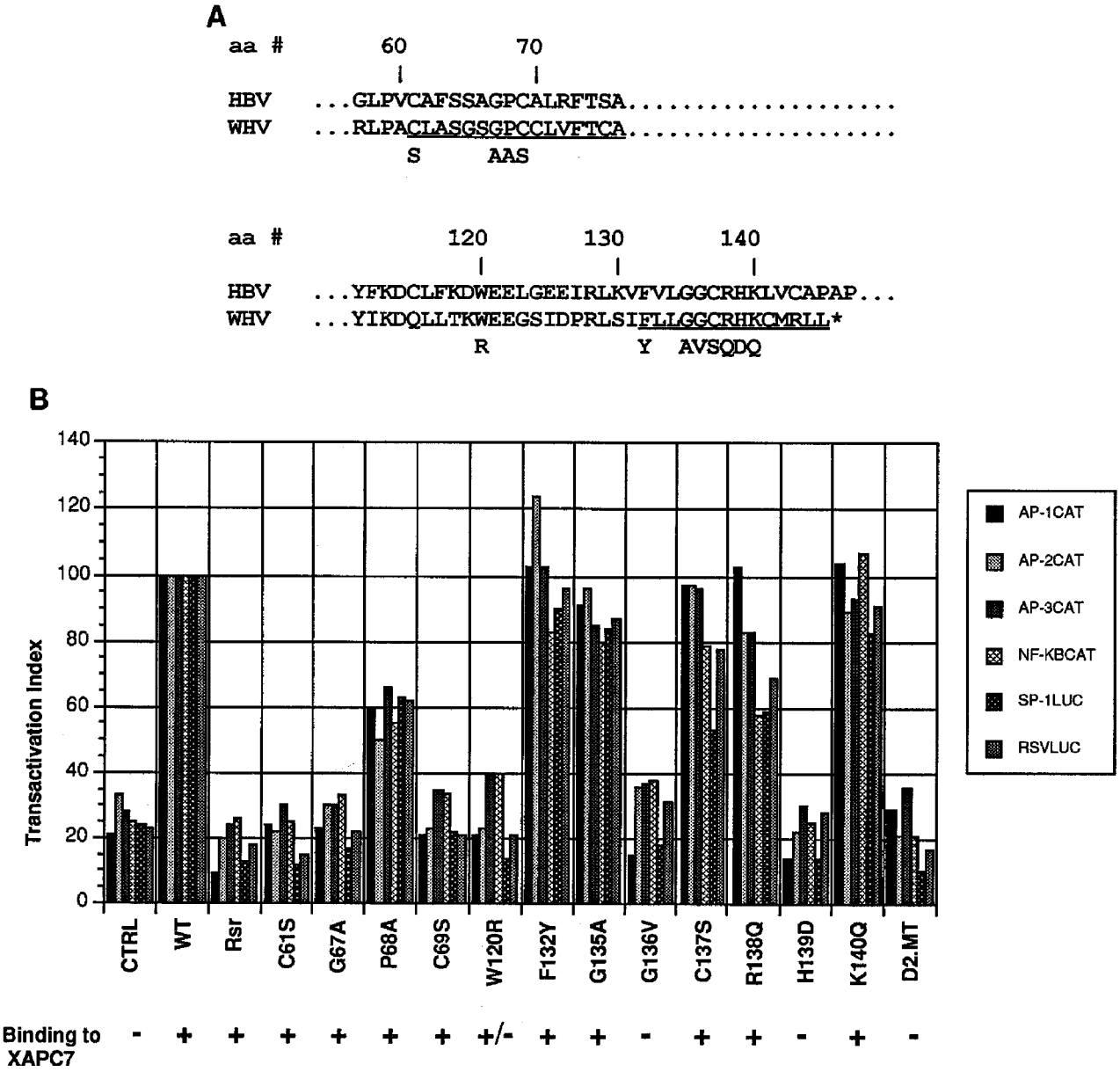

FIG. 7. Mutagenesis studies of HBX and XAPC7 interaction. (A) Protein sequences of HBX and WHVX around the putative Kunitz domains (underlined) are shown. Amino acid numbers of the HBX protein are shown at the top. Site-directed HBX mutations (shown at the bottom of the amino acid residues) were introduced by PCR as described in Materials and Methods. The HBXRsr and D2.MT mutant has been described previously. (B) Transactivation and XAPC7 interaction of HBX mutants. Calcium phosphate cotransfection of HBX expression constructs and reporter plasmids was performed at a ratio of 1 to 5 with a total DNA of 0.6 μg per well in a six-well plate. Reporter activities were assayed 2 days later. Data presented are the mean values of transfections done in triplicate, and the results are representative of three separate experiments. The reporter activity of each mutant was shown as percentage of activity of wild-type HBX (labeled as transactivation index). Interactions of HBX mutants and XAPC7 are shown below the transactivation activity of each mutant.

tein (10) and cysteine residues may form disulfide bonds, we mutated the glycine and cysteine residues in both domains (Fig. 7A). Several other conserved residues were also mutated. Western immunoblots of cell lysates transfected with these mutants have shown that these mutations have minimal effect on the level of the protein (not shown). To obtain a structure-function correlation of this interaction, the mutants were also tested for their transactivation activities. Since HBX has been shown to activate transcription through AP-1, AP-2, AP-3, NF-κB, and SP-1 factors (28, 35, 46), we tested the effects of these mutants on five reporter constructs, each of which contains a *cis*-acting sequence responsive to each of the five factors. RSV long terminal repeat, which has been shown to be transactivated by HBX, was also tested. The results are shown in Fig. 7B. The mutations tested here affected the transactivation of all the reporters equally. This observation was also corroborated by transfection studies using different concentrations of HBX as well as in CV-1 cells (data not shown). The glycine (G136V) and histidine (H139D) mutations in the second domain eliminated both the transactivation and binding properties. D2.MT with a partial deletion of the second domain also had no activities in both transactivation and binding. The cysteine mutation (C137S) did not affect the binding or transactivation. Other conserved residues in the second do-

main, such as phenylalanine (aa 132), glycine (aa 135), arginine (aa 138), and lysine (aa 140), appeared not to be essential for XAPC7 binding or function of HBX. Mutagenesis analysis in the second domain demonstrated a close association between the ability of the mutants to interact with XAPC7 and the transactivation activity of the mutants. This structure-function correlation suggests that the interaction between the XAPC7 proteasome subunit and the second domain of HBX may play an important role in the function of HBX.

Mutagenesis studies of the first domain of HBX demonstrated that this domain is not important for interaction with XAPC7 but is critical for HBX transactivation function (Fig. 7B). The glycine (G67A) and two cysteine (C61S and C69S) mutations in the first domain appeared to abrogate the transactivation function of HBX without affecting binding to XAPC7. The mutant with a proline-to-alanine substitution (P68A) in the first domain seemed to retain 50 to 60% of the transactivation activities of wild-type HBX but bound to XAPC7 as wild-type HBX did. These data are consistent with the phenotype of the HBXRsr and HBXEn mutants described previously. This observation suggests that HBX likely interacts with another cellular factor through the first domain and that this interaction is equally critical for the function of HBX. It is possible that one of the other clones identified during the initial interaction cloning may represent such a factor. This possibility awaits further studies. Finally, because of the highly conserved tryptophan residue at aa 120, an additional mutation was introduced at this position (W120R). Transactivation and interaction analyses showed that this HBX mutant does not transactivate but retains a weak binding activity to XAPC7. This conserved tryptophan residue, therefore, may contribute to the binding of the second domain of HBX to XAPC7.

Structural mapping of XAPC7 domain that interacts with HBX. In order to define the XAPC7 domain interacting with HBX, we performed deletion analyses to map this domain. Since we already knew that aa 137 to 248 of XAPC7 contain the interacting domain with HBX, we generated deletion constructs from this region of XAPC7 and tested their binding to HBX in *S. cerevisiae* (Fig. 8). Although we could not rule out the possibility that truncated proteins might not fold properly or that noncontiguous regions of XAPC7 might be involved in HBX interaction, our data suggested that the interacting domain probably resides in two domains: the middle and the very C-terminal region of C7 (Fig. 8). First, the C-terminal 20 aa appear to be essential for interaction between XAPC7 and HBX. Second, DM.4 (deletion of 143 to 163) interacted positively with HBX whereas DM.5 (aa 171 to 248) was only weakly reactive. A small deletion of aa 188 to 198 in DM.7 appeared to eliminate interaction with HBX completely. These data suggest that amino acid sequences in the middle of C7 (aa 163 to 198) are also important for interaction with HBX.

Overexpression of XAPC7 leads to transactivation. The data we have presented so far relate to physical interaction and structure-function correlation of these two proteins. In order to assess the functional significance of this interaction, we needed to establish some correlation between XAPC7 and the function of HBX. Since the only well-established and testable function of HBX is its transactivation of a variety of promoters, we first tested the possible transactivation effect of XAPC7. Although XAPC7 is expressed in HepG2 cells, it is possible that overexpression of the protein can achieve additional effect. A construct expressing full-length XAPC7 (pCDXAPC7) was tested on transcriptional activation of RSV-Luc and AP-1-CAT reporters containing complex and single enhancer elements, respectively, or pTK81-Luc, a minimal promoter construct (Fig. 9). As shown in Fig. 8, the C-terminal cluster of

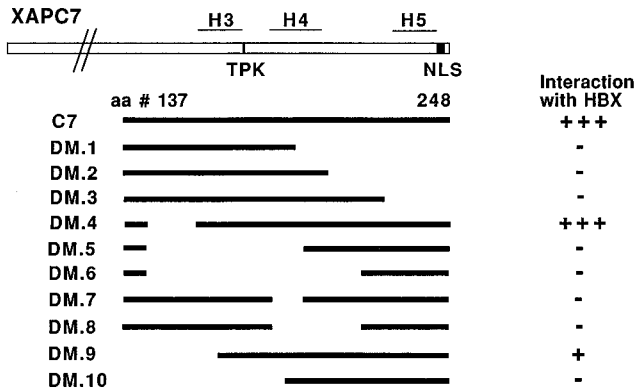

FIG. 8. Deletion analysis of HBX-XAPC7 interaction. The top illustrates regions of XAPC7 with homology to consensus sequences of nuclear localization signal (NLS) and tyrosine phosphorylation site (TPK). The H3 to -5 domains represent structurally adjacent α helices of proteasome α subunits (see text). Deletion mutants were generated as described in Materials and Methods. DM.1, aa 137 to 190; DM.2, aa 137 to 207; DM.3, aa 137 to 228; DM.4, aa 137 to 248 (deletion of 143 to 163); DM.5, aa 137 to 248 (deletion of 143 to 198); DM.6, aa 137 to 248 (deletion of 143 to 220); DM.7, aa 137 to 248 (deletion of 188 to 198); DM.8, aa 137 to 248 (deletion of 188 to 220); DM.9, aa 171 to 248; DM.10, aa 194 to 248. The C7 construct contains the original interacting sequence of XAPC7 (aa 137 to 248). The deletion mutants were constructed in JG4-5 and analyzed for interaction with pEG202HBX in *S. cerevisiae.*

highly charged amino acids of XAPC7 appears to be critical for HBX binding. Therefore, we generated a construct expressing full-length XAPC7 with this deletion of 20 aa at the C terminus (pCDXAPC7DM.3) and tested its transactivation activity. The results demonstrated that XAPC7, by itself, activated the reporter activities by approximately three- to fourfold but that the deletion mutant appeared to have no effect. Although the absence of transactivation effect could be due to misfolding of the mutant protein, it is unlikely for the following reason. The C-terminal sequences of the proteasome α subunits are highly variable; some, such as XAPC7, contain additional highly charged amino acid clusters as putative nuclear localization signal (34, 50). Despite these variabilities, the α subunits have similar tertiary structures. Therefore, it is unlikely that mutations involving this region would affect the overall folding of the protein. The other proteasome subunits, HC5 and HC8, had little or no transactivation effect (Fig. 9A). This observation was consistently reproducible in multiple experiments and was also demonstrated with reporter constructs containing other specific enhancer elements as described in Fig. 7 (data not shown). Furthermore, cotransfection of XAPC7 and HBX appeared to exhibit a much higher transactivation activity than either of the constructs alone (Fig. 9C). Expression of the XAPC7DM.3 mutant also had no effect on HBX transactivation (not shown). In Fig. 9D, a minimal TK promoter containing only the TATA box element (37) was used to study the effect of HBX and XAPC7 expression constructs on enhancerless basal promoter. Similar to the transactivation effect of HBX, XAPC7 appeared to have little or no effect on minimal TK promoter.

Antisense XAPC7 inhibits transactivation by HBX. If XAPC7 is indeed the functional target of HBX, one would expect to inhibit the function of HBX by blocking the expression of XAPC7. We constructed an XAPC7 antisense expression construct (pRSVRZXAPC7.A) using an efficient antisense vector and tested its effect on the transactivation activity of HBX (Fig. 10). The results demonstrated that the antisense XAPC7 construct was able to block the transactivation func-

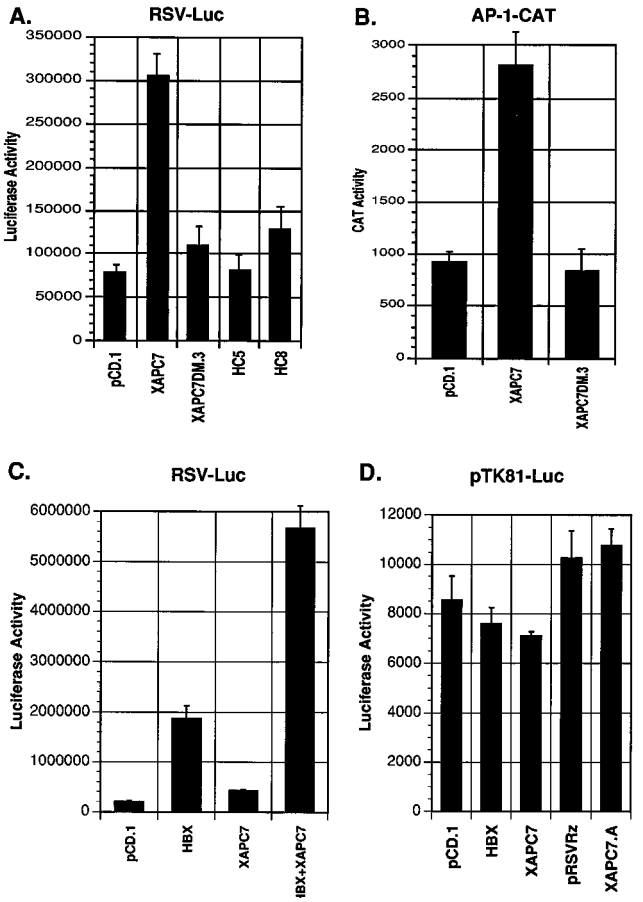

FIG. 9. Transactivation function of XAPC7. (A and B) Full-length cDNAs for HC5, HC8, XAPC7, and XAPC7DM.3 with deletion of the C-terminal 20 aa were cloned into expression vector pCD.1 and cotransfected (0.9 μg each) into HepG2 cells in triplicate with RSV-Luc (0.1 μg) as reporter in panel A and AP-1-CAT (0.1 μg) in panel B. (C) HBX (0.1 μg) and XAPC7 (0.4 μg) were transfected either together or separately with RSV-Luc reporter into HepG2 cells. (D) pCDXAPC7, pCDHBX, and pRSVRZXAPC7.A constructs (0.3 μg each) were cotransfected with minimal promoter construct pTK81-Luc (0.1 μg). Control cotransfections were done with both pCD.1 and pRSVRZ (0.3 μg each). In all experiments, pCD.1 plasmid was added to make the total amount of transfected DNA 1 μg per well. Two days posttransfection, cells were lysed and luciferase activities were measured. The results were expressed as means of luciferase activities ± standard deviations.

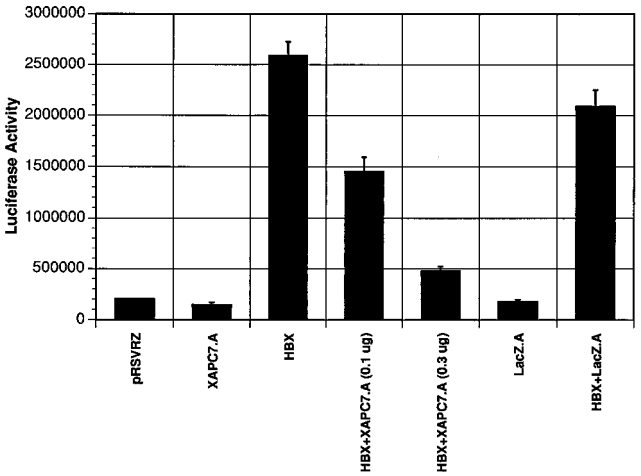

FIG. 10. Expression of antisense XAPC7 blocks transactivation by HBX. Antisense construct (pRSVRZXAPC7.A, 0.1 and 0.3 μg, or pRSVRZLacZ, 0.3 μg each) was cotransfected with or without pCDHBX (0.1 μg) and RSV-Luc reporter (0.1 μg) into HepG2 cells in triplicate. pRSVRZ vector was also cotransfected as control. pCD.1 plasmid was added to make the total amount of transfected DNA 0.5 μg per well. Three days posttransfection, cells were lysed and luciferase activities were measured. The results were expressed as means of luciferase activities ± standard deviations.

tion of HBX on RSV-Luc in a dose-dependent manner. A control antisense expression construct of the *lacZ* gene (pRSVRZLacZ.A) and the pRSVRZ vector had no effect on the reporter activities. pRSVRZXAPC7.A had minimal, if any, inhibitory effect on baseline expression of the RSV-Luc reporter; in this experiment, we noted ~25% inhibition (baseline luciferase activities of 202,734 ± 2,609 versus 148,613 ± 26,017), but in many other experiments, we did not see any inhibitory effect. These observations suggest that XAPC7 is important for transactivation activity of HBX. Since XAPC7 is a subunit of proteasome which is important for a variety of cellular functions, antisense expression of XAPC7 may be toxic to cells, causing a general depression of cellular function including transcription. This possibility could explain our findings. However, this is unlikely for several reasons. First, antisense expression of XAPC7 had no effect on minimal TK promoter (Fig. 9C) and little, if any, inhibitory effect on the highly active RSV promoter, whereas its inhibitory effect on HBX transactivation is dramatic (~6-fold inhibition to almost the baseline; luciferase activities of 2,592,607 ± 135,456 versus 458,009 ± 43,621 with the baseline being 202,734 ± 2,609). Second, cells cotransfected with XAPC7 antisense construct and a LacZ expression construct (pJ3β-Gal) visualized by X-Gal staining had normal appearance and showed no signs of cytotoxicity. Third, antisense expression of HC5 and HC8 subunits' cDNAs did not result in inhibition of reporter activities (not shown). Although this effect could still be a nonspecific effect of antisense expression of XAPC7, we think that it is unlikely based on our reasoning above.

Finally, to demonstrate that antisense expression was able to reduce the endogenous level of XAPC7 protein, CDM8CD4 (a CD4 expression plasmid) was cotransfected with pRSVRZ XAPC7.A or pRSVRZ vector into HepG2 cells. The transfected populations of cells were isolated using anti-CD4-coupled magnetic beads. Counting of cells bound to the beads was similar in all samples. The isolated cells were lysed and analyzed for XAPC7 expression by Western immunoblot analysis; actin levels were analyzed in parallel as controls (Fig. 11). The results showed that XAPC7 expression was reduced significantly in the antisense-treated cells (by four- to fivefold as measured by densitometry: control, 1,774 and 1,643 arbitrary optical density units; antisense, 337 and 420); the differences were even more pronounced when they were corrected for actin levels. In a parallel experiment, pJ3β-Gal was cotransfected with CDM8CD4 and cells were subjected to the same isolation procedure. X-Gal staining showed that >95% of cells isolated and bound to the beads were positive, confirming the efficiency of this procedure (Dynal, Inc.). This finding further supports the argument that antisense expression of XAPC7 exerts a specific effect on XAPC7 synthesis.

DISCUSSION

Several recent studies have reported identification of possible cellular target(s) of HBX (8, 31). Using recombinant HBX as probe in an in vitro binding assay, Cheong et al. identified RPB5, a common subunit of RNA polymerases, as a putative target of HBX (8). Another study reported the specific inter-

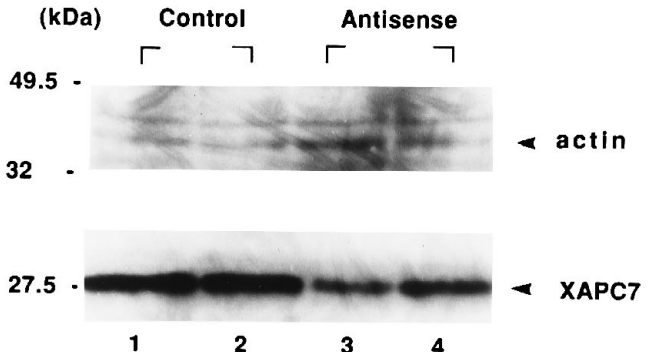

FIG. 11. Antisense expression of XAPC7 specifically reduces XAPC7 protein level. HepG2 cells were cotransfected with CDM8CD4 and pRSVRZ (control) or pRSVRZXAPC7.A (antisense) in duplicate. Transfected cells were isolated using anti-CD4 coupled magnetic beads and lysed for SDS-PAGE analysis. Half of the samples were subjected to Western blot analysis with anti-XAPC7 antibody MCP34 (bottom panel), and the other half were analyzed with antiactin antibody (top panel). Lanes are as indicated. XAPC7 (28 kDa) and actin (42 kDa), marked by arrowheads, were specifically recognized by the respective antibodies since parallel immunoblotting with irrelevant antibodies was negative for these bands (not shown).

action of a probable DNA repair protein with HBX using the yeast two-hybrid system (31). Although both of these findings are interesting and could potentially explain the action of HBX, there were few functional data to support the biological significance of these interactions. Using a similar yeast two-hybrid system, our studies demonstrate that HBX interacts specifically with a novel subunit of the proteasome family. This subunit, XAPC7, is highly conserved across species and belongs to the regulatory α subunit of the proteasome complex. We have confirmed the importance of the putative Kunitz domain of serine protease inhibitor in the function of HBX. We have mapped the HBX domain required for interaction with the XAPC7 subunit to the second domain of the Kunitz domain. A structure-function correlation based on mutagenesis analyses has been established for the HBX-XAPC7 interaction in the second domain of HBX. We also showed that HBX binds specifically, although not strongly, to XAPC7 in vitro. So far, we have not been able to demonstrate in vivo association of HBX and XAPC7 by coimmunoprecipitation. One reason is that HBX protein has been particularly difficult to detect in vivo and that good antibodies against HBX have also been difficult to generate. Another possible explanation is that the interaction of HBX and XAPC7 is not sufficiently strong to be detected by the coimmunoprecipitation method.

The proteasome is involved in both ubiquitin-dependent and -independent proteolytic pathways (20, 43, 50). Interaction of HBX with a polypeptide involved in the degradation of protein is consistent with the report that a putative Kunitz domain, characteristic of Kunitz-type serine protease inhibitors, is present in HBX (48). Many growth-related gene products or transcription activators, such as c-Fos, c-Myc, p53, and the cyclin family, are tightly regulated at the protein degradation level (17, 25). Targeted degradation of c-Fos, but not v-Fos, has also been shown to be regulated by intracellular signal transduction (40). Furthermore, a recent study demonstrated that activation of NF-κB depends on the inducible degradation of I-κB through the proteasome complex (39). A parallel situation exists in yeast MATα2 repressor, selective degradation of which via the proteasome pathway results in activation of transcription (26). Proteolytic modification, therefore, may be a general pathway for the activation of cellular machinery in growth and malignant transformation, and HBX may exert its function through the regulation of such a process.

Recently, the crystal structure of the 20S proteasome from the archaebacterium *Thermoplasma acidophilum* has been elucidated (34). XAPC7 belongs to the α subunit of the 20S proteasome, which is located on the outer ring of the proteasome complex. We have mapped the sequences of XAPC7 interacting with HBX to two domains, the middle and the C-terminal regions of C7. It is interesting to note that the two domains—one contains two contiguous α helices (H3 and H4) and the other contains α helix H5 at the C terminus—are structurally adjacent to one another (34). This region is apparently exposed to the entrance of the channel where proteolysis occurs and likely represents a structural point where the 19S cap structure of 26S interacts with the core 20S proteasome (19, 27, 54). This observation together with the notion that the α subunit of proteasome regulates the activity of the complex (20, 41, 50) suggests that HBX may modulate the function of proteasome by interacting with XAPC7. Furthermore, the C terminus of XAPC7 and its homologs in other species contain a putative nuclear localization signal (20, 23, 50). Interaction of HBX with this domain may affect the nuclear-cytoplasmic distribution of the proteasome complex and alter the function of proteasome in the nucleus. Interference with proper nuclear localization of proteasome may lead to altered activities of transcription factors in the nucleus.

Finally, we have functional data that overexpression of XAPC7 appeared to activate transcription by itself and that antisense expression of XAPC7 was able to block specifically transactivation by HBX. The observation that HBX and XAPC7 effects were at least additive, if not synergistic, is consistent with the notion that overexpression of XAPC7 could provide additional substrate for HBX to act on, leading to a further increase in transactivation activity. We also showed that the transactivation effect is unique to this XAPC7 proteasome subunit and not to other subunits; expression of two other proteasome subunits, HC5 and HC8, did not result in transactivation. Although this experiment demonstrates that XAPC7 can transactivate a variety of promoters, it does not directly support the contention that HBX functions through interaction with XAPC7. It is possible that HBX and XAPC7 transactivate through two entirely independent pathways. Overexpression of XAPC7 may alter the activity of proteasome complex in regulating the level of a transcriptional factor which, in turn, is tightly linked to general transcriptional activities. The effect of XAPC7 overexpression could either up- or down-regulate the level of this putative transcriptional factor, which could either positively or negatively modulate transcription. The observation that antisense expression of XAPC7 blocked the transactivation activities of HBX appears to lend further support to the notion that the XAPC7 is involved in transcriptional activation by HBX. However, an independent transcriptional factor as described above could again be responsible for the antisense effect of XAPC7. Since antisense expression of XAPC7 blocked specifically transactivation by HBX, this putative factor would somehow have to act in concert with HBX to activate transcription of promoters with enhancer elements. At present, we have no direct proof that such a scenario does not exist. If such a factor indeed exists, further studies of the effect of HBX on function of XAPC7 should allow us to identify this factor.

The interesting question remains as to how the overexpression of a specific proteasome subunit results in transactivation. We can only speculate that the composition of the subunits within the proteasome complex may change, leading to functional alteration of proteasome, which is important for regu-

lation of a variety of transcriptional factors. Recently, composition of proteasome subunits has been shown to determine specific proteolytic activities of the proteasome. Increased incorporation of the LMP-2 subunit into the proteasome complex results in enhanced peptidase activity after basic residues and reduced hydrolysis after acidic residues without affecting hydrophobic activity; LMP-7 overexpression leads to increased basic and hydrophobic peptidase activity but does not affect hydrolysis after acidic residues (14, 15). At present, we do not have any data on the functional effect of HBX on XAPC7 and proteasome. We favor the idea that HBX, by interacting with XAPC7 subunit, alters the activities of proteasome complex. Alternatively, XAPC7 may have an entirely different function other than being a subunit of proteasome. It may interact directly with transcriptional machinery to affect gene expression. Both of these possibilities await further studies.

In addition to the diverse roles of proteasome in cell metabolism and growth, the most intriguing function of proteasome is its involvement in cellular antigen presentation. Recent evidence has pointed to a critical role of this complex in the pathway of protein degradation and delivery of peptides to major histocompatibility complex class I molecules (20, 36, 44). One of the major sequelae of HBV infection is a persistent infectious state with progression to chronicity and end-stage liver disease. HBV infection appears to persist despite the presence of ample neutralizing antibodies and active cytotoxic T-lymphocyte responses (21). Many viruses have evolved various novel mechanisms to evade host immune response and achieve persistent infection (38). In light of our findings on the interaction of HBX and proteasome as well as observations by others that HBX is essential for establishment of infection in vivo, it is tempting to speculate that the expression of HBX, by interfering with proteasome functions, prevents viral antigen presentation in infected cells and leads to a state of persistent infection. This functional effect may underlie the biological importance of HBX in the life cycle of HBV. Such a notion is not without precedence; herpes simplex virus encodes a protein, ICP47, which was recently shown to interfere with major histocompatibility complex class I antigen presentation to $CD8^+$ lymphocytes (55), and adenovirus 19-kDa glycoprotein (E3-gp19K) binds to class I molecules and prevents egress of the complexes from the endoplasmic reticulum (1, 6). This intriguing possibility regarding the function of HBX awaits further studies.

ACKNOWLEDGMENTS

We thank Hucheng Bei for excellent technical assistance; Keiji Tanaka, Klaus Hendil, T. S. Ben Yen, and Robert Schneider for providing plasmids and reagents for this study; Roger Brent for helpful advice on the interaction trap system; and Margaret Koziel, Bruce Walker, and Alfred Goldberg for critical discussions.

This work was supported by grants CA-54524 and DK-01952 from the National Institutes of Health. Oligonucleotides were provided through the core support of NIH Center Grant P30DK-43351.

ADDENDUM IN PROOF

After the manuscript was accepted, we learned that Fischer et al. also identified interaction between HBX and the same proteasome subunit in the two-hybrid system (M. Fischer et al., Annual Meeting on the Molecular Biology of Hepatitis B Virus, San Diego, Calif., 1995).

REFERENCES

1. **Andersson, M., S. Paabo, T. Nilsson, and P. A. Peterson.** 1985. Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance. Cell **43:**215–222.
2. **Arii, M., S. Takada, and K. Koike.** 1992. Identification of three essential regions of hepatitis B virus X protein for transactivation function. Oncogene **7:**397–403.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1991. Current protocols in molecular biology. John Wiley & Sons, New York.
4. **Benn, J., and R. J. Schneider.** 1994. HBV HBx protein establishes a Ras, Raf, MAP kinase signalling cascade and stimulates cell proliferation through activation of p34cdc2 kinase-cyclin B complexes. Proc. Natl. Acad. Sci. USA **91:**10350–10354.
5. **Brown, M. G., J. Driscoll, and J. J. Monaco.** 1991. Structural and serological similarity of MHC-linked LMP and proteasome (multicatalytic proteinase) complexes. Nature (London) **353:**355–357.
6. **Burgert, H.-G., and S. Kvist.** 1985. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. Cell **41:**987–997.
7. **Chen, H.-S., S. Kaneko, R. Girones, R. W. Anderson, W. E. Hornbuckle, B. C. Tennant, P. J. Cote, J. L. Gerin, R. H. Purcell, and R. H. Miller.** 1993. The woodchuck hepatitis X gene is important for establishment of virus infection in woodchucks. J. Virol. **67:**1218–1226.
8. **Cheong, J., M. Yi, Y. Lin, and S. Murakami.** 1995. Human RPB5, a subunit shared by eukaryotic nuclear RNA polymerases, binds human hepatitis B virus X protein and may play a role in X transactivation. EMBO J. **14:**143–150.
9. **Colgrove, R., G. Simon, and D. Ganem.** 1989. Transcriptional activation of homologous and heterologous genes by the hepatitis B virus X gene product in cells permissive for viral replication. J. Virol. **63:**4019–4026.
10. **Creighton, T. E.** 1984. Proteins, structures and molecular principles. Freeman, New York.
11. **Cross, J. C., P. Wen, and W. Rutter.** 1993. Transactivation by hepatitis B virus X protein is promiscuous and dependent on mitogen-activated cellular serine/threonine kinases. Proc. Natl. Acad. Sci. USA **90:**8078–8082.
12. **Doria, M., N. Klein, R. Lucito, and R. J. Schneider.** 1995. The hepatitis B virus HBX protein is a dual specificity cytoplasmic activator of Ras and nuclear activator of transcription factors. EMBO J. **14:**4747–4757.
13. **Fields, S., and O.-K. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
14. **Gaczynska, M., K. Rock, and A. Goldberg.** 1993. γ-Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. Nature (London) **365:**264–266.
15. **Gaczynska, M., K. L. Rock, T. Spies, and A. L. Goldberg.** 1994. Peptidase activities of proteasomes are differently regulated by the major histocompatibility complex-encoded genes for LMP2 and LMP7. Proc. Natl. Acad. Sci. USA **91:**9213–9217.
16. **Genschik, P., G. Philipps, C. Gigot, and J. Fleck.** 1992. Cloning and sequence analysis of a cDNA clone from Arabidopsis thaliana homologous to a proteasome a subunit from Drosophila. FEBS Lett. **309:**311–315.
17. **Glotzer, M., A. W. Murray, and M. W. Kirschner.** 1991. Cyclin is degraded by the ubiquitin pathway. Nature (London) **349:**132–138.
18. **Glynne, R., S. H. Powis, S. Beck, A. Kelly, L.-A. Kerr, and J. Trowsdale.** 1991. A proteasome-related gene between the two ABC transporter loci in the class II region of the human MHC. Nature (London) **353:**357–360.
19. **Goldberg, A. L.** 1995. Functions of the proteasome: the lysis at the end of the tunnel. Science **268:**522–523.
20. **Goldberg, A. L., and K. L. Rock.** 1992. Proteolysis, proteasomes and antigen presentation. Nature (London) **357:**375–379.
21. **Guilhot, S., P. Fowler, G. Portillo, R. Margolskee, A. Ferrari, A. Bertoletti, and F. Chisari.** 1992. Hepatitis B virus-specific cytotoxic T-cell response in humans: production of target cells by stable expression of HBV-encoded proteins in immortalized human B-cell lines. J. Virol. **66:**2670–2678.
22. **Gyuris, J., E. Golemis, H. Chertkov, and R. Brent.** 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell **75:**791–803.
23. **Haass, C., B. Pesold-Hurt, G. Multhaup, K. Beyreuther, and P.-M. Kloetzel.** 1990. The Drosophila PROS-28.1 gene is a member of the proteasome gene family. Gene **90:**235–241.
24. **Hendil, K. B., and W. Uerkvitz.** 1991. The human multicatalytic proteinase: affinity purification using a monoclonal antibody. J. Biochem. Biophys. Methods **22:**159–165.
25. **Hershko, A., and A. Ciechanover.** 1992. The ubiquitin system for protein degradation. Annu. Rev. Biochem. **61:**761–807.
26. **Hochstrasser, M., and A. Varshavsky.** 1990. In vivo degradation of transcriptional regulator: the yeast α2 repressor. Cell **61:**697–708.
27. **Jentsch, S., and S. Schienker.** 1995. Selective protein degradation: a journey's end within the proteasome. Cell **82:**881–884.
28. **Kekule, A. S., U. Lauer, L. Weiss, B. Luber, and P. H. Hofschneider.** 1993. Hepatitis B virus transactivator HBX uses a tumor promoter signalling pathway. Nature (London) **361:**742–745.
29. **Kim, C. M., K. Koike, I. Saito, T. Miyamura, and G. Jay.** 1991. HBX gene of hepatitis B virus induces liver cancer in transgenic mice. Nature (London) **315:**317–320.
30. **Kumatori, A., K. Tanaka, N. Inamura, S. Sone, T. Ogura, T. Matsumoto, T. Tachikawa, S. Shin, and A. Ichihara.** 1990. Abnormally high expression of

proteasomes in human leukemic cells. Proc. Natl. Acad. Sci. USA **87:**7071–7075.
31. **Lee, T. H., S. J. Elledge, and J. S. Butel.** 1995. Hepatitis B virus X protein interacts with a probable cellular DNA repair protein. J. Virol. **69:**1107–1114.
32. **Levero, M., M. Stemler, C. Pasquinelli, A. Alberti, O. Jean-Jean, A. Franco, C. Balsano, D. Diop, C. Brechot, M. Melegari, E. Villa, V. Barnaba, M. Perricaudet, and H. Will.** 1991. Significance of anti-HBs antibodies in hepatitis B virus infection. Hepatology **13:**131–149.
33. **Liu, Z. L., D. B. Batt, and G. G. Carmichael.** 1994. Targeted nuclear antisense RNA mimics natural antisense-induced degradation of polyoma virus early RNA. Proc. Natl. Acad. Sci. USA **91:**4258–4262.
34. **Lowe, J., D. Stock, B. Jap, P. Zwicki, W. Baumeister, and R. Huber.** 1995. Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. Science **268:**533–539.
35. **Maguire, H. F., J. P. Hoeffler, and A. Siddiqui.** 1991. HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein-protein interactions. Science **252:**842–844.
36. **Neefjes, J. J., and F. Momburg.** 1993. Cell biology of antigen presentation. Curr. Opin. Immunol. **5:**27–34.
37. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–457.
38. **Oldstone, M. B.** 1991. Molecular anatomy of viral persistence. J. Virol. **65:**6381–6386.
39. **Palombella, V. J., O. Rando, A. L. Goldberg, and T. Maniatis.** 1994. Processing of the NF-kB1 (p105) precursor protein and the degradation of IkB-a require the proteasome. Cell **78:**773–785.
40. **Papavassiliou, A. G., M. Treier, C. Chavrier, and D. Bohmann.** 1992. Targeted degradation of c-fos, but not v-fos, by a phosphorylation-dependent signal on c-JUN. Science **258:**1941–1944.
41. **Puhler, G., S. Weinkauf, L. Bachmann, S. Muller, A. Engel, R. Hegeri, and W. Baumeister.** 1992. Subunit stoichiometry and three dimensional arrangement in proteasome from thermoplasma acidophilum. EMBO J. **11:**1607–1616.
42. **Qadri, I., H. F. Maguire, and A. Siddiqui.** 1995. Hepatitis B virus transactivator protein X interacts with TATA-binding protein. Proc. Natl. Acad. Sci. USA **92:**1003–1007.
43. **Rechsteiner, M., L. Hoffman, and W. Dubiel.** 1993. The multicatalytic and 26S protease. J. Biol. Chem. **268:**6065–6068.
44. **Rock, K. L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A. L. Goldberg.** 1994. Inhibitors of the proteasome block the degradation of most cell protein and generation of peptides presented on MHC class I molecules. Cell **78:**761–771.
45. **Runkel, L., M. Fischer, and H. Shaller.** 1993. Two-codon insertion mutations of the HBX define two separate regions necessary for its trans-activation function. Virology **197:**529–536.
46. **Seto, E., P. J. Mitchell, and T. S. B. Yen.** 1990. Transactivation by the hepatitis B virus X protein depends on AP-2 and other transcription factors. Nature (London) **344:**72–74.
47. **Siddiqui, A., S. Jameel, and J. Mapoles.** 1987. Expression of the hepatitis B virus X gene in mammalian cells. Proc. Natl. Acad. Sci. USA **84:**2513–2517.
48. **Takada, S., and K. Koike.** 1990. X protein of hepatitis B virus resembles a serine protease inhibitor. Jpn. J. Cancer Res. **81:**1191–1194.
49. **Tamura, T., D. H. Lee, F. Osaka, T. Fujiwara, S. Shin, C. H. Chung, K. Tanaka, and A. Ichihara.** 1991. Molecular cloning and sequence analysis of cDNAs for five major subunits of human proteasomes (multi-catalytic proteinase complex). Biochim. Biophys. Acta **1089:**95–102.
49a. **Tanaka, K.** Personal communication.
50. **Tanaka, K., T. Tamura, T. Yoshimura, and A. Ichihara.** 1992. Proteasomes: protein and gene structures. New Biol. **4:**173–187.
51. **Truant, R., J. Antunovic, J. Greenblatt, C. Prives, and J. A. Gromlish.** 1995. Direct interaction of the hepatitis B virus HBX protein with p53 leads to inhibition by HBX of p53 response element-directed transactivation. J. Virol. **69:**1851–1859.
52. **Unger, T., and Y. Shaul.** 1990. The X protein of the hepatitis B virus acts as a transcription factor when targeted to its responsive element. EMBO J. **9:**1889–1895.
53. **Wang, X. W., K. Forrester, H. Yeh, M. A. Feitelson, J.-R. Gu, and C. C. Harris.** 1994. Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proc. Natl. Acad. Sci. USA **91:**2230–2234.
54. **Weissman, J. S., P. B. Sigler, and A. L. Horwich.** 1995. From the cradle to the cave: ring complexes in the life of a protein. Science **268:**523–524.
55. **York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Grahm, and D. C. Johnson.** 1994. A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell **77:**525–535.
56. **Zervos, A. S., J. Gyuris, and R. Brent.** 1993. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223–232.
57. **Zoulim, F., J. Saputelli, and C. Seeger.** 1994. Woodchuck hepatitis virus X protein is required for viral infection in vivo. J. Virol. **68:**2026–2030.