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# Hepatitis B Virus X Protein Interacts with a Probable Cellular DNA Repair Protein

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**The mechanism of action of hepatitis B virus (HBV) X protein in transcriptional transactivation and in tumorigenesis remains obscure. We have used the yeast two-hybrid system to identify a cellular protein that can interact with HBV X protein. This protein, designated X-associated protein 1 (XAP-1), is a human homolog of the UV-damaged DNA-binding protein (UV-DDB) recovered from a monkey cell cDNA library. UV-DDB is presumed to be involved in DNA repair. The interaction between X protein and XAP-1 protein was verified by immunoprecipitation of yeast cell lysates expressing both proteins and by in vitro mixing with X protein expressed as a glutathione *S*-transferase fusion protein and XAP-1 protein either in HeLa cell extracts or synthesized by in vitro translation. We speculate that the interaction of X protein with a DNA repair protein may recruit cellular proteins to repair the partially double-stranded HBV genome or may modify cellular transcription processes. An effect on the cellular DNA repair system may explain a cofactor role for HBV in liver cancer development.**

The hepatitis B virus (HBV) X protein plays an important regulatory role during HBV replication. It is encoded by the smallest open reading frame (ORF) of the HBV genome, with the sequence being highly conserved among different viral isolates. Even though a functional X gene appears to be unnecessary for the replication of HBV in viral DNA-transfected hepatoma cell cultures (2, 41), animal studies have indicated a requirement for X protein expression for virus multiplication in vivo (7, 42). In addition, DNA transfection approaches have clearly demonstrated that HBV X protein is a transactivator of a wide variety of viral and cellular promoters (6, 32, 36). This broad gene-regulating function suggests that X protein may not only up-regulate the expression of HBV transcripts by transactivating the HBV enhancer but may also modify the biochemical environment by transactivating cellular genes in infected cells to facilitate viral replication. Understanding the mechanism of transactivation by X protein may aid in the development of novel means to interrupt the replication cycle of HBV.

Chronic infection with HBV is a recognized risk factor in the development of human hepatocellular carcinoma (6, 36). The precise mechanism of viral involvement remains elusive, but several studies have suggested a possible role for X protein in the process of liver carcinogenesis. Identification of cellular X-interactive proteins would provide insights into the mechanism of HBV cellular effects. It is a common theme with DNA tumor viruses that the viral oncoproteins responsible for mediating cell transformation are virus-encoded replication proteins designed to modulate cell regulatory pathways to facilitate virus replication (9, 27, 28, 30).

The underlying mechanism of transactivation by X protein is currently obscure. The heterogeneity of the DNA elements responsive to X suggests an indirect mode of action, probably mediated via effects on cellular factors, rather than direct binding of a specific DNA sequence by X protein (32, 36). It has been shown that X protein interacts with factors involved in transcription, e.g., cyclic AMP-responsive element-binding protein, activating transcription factor 2, and *p53* (11, 29, 40). Association of X protein with the cellular transcriptional machinery may perturb the normal function of the latter and result in the broad transactivation of cellular genes. Alternatively, both the protein kinase C signaling pathway and mitogen-activated kinases, e.g., *raf-1*, have been proposed to be involved in X-mediated transactivation (8, 20). The interaction of X protein with cellular protein(s) may trigger a cascade of phosphorylation and dephosphorylation events which might lead to a general up-regulation of gene expression. Finally, X protein reportedly possesses the properties of a serine protease inhibitor (38). Conceivably, it might exert transactivation potential by protecting a labile protein in the transcriptional machinery from protease digestion. As the essential domains of the X protein responsible for the observed bindings or activities have usually not been characterized, it is difficult to conclude whether the described multifunctional activities are mediated through common X domains.

The most direct way to pinpoint the basis of HBV transactivational mechanisms and/or effects on cell regulatory pathways lies in identifying X-associated cellular proteins. Because the level of expression of X protein in HBV-producing cells is relatively low, it is difficult to detect and recover enough of an X-associated protein for identification and analysis by conventional microsequencing methods. A genetic approach, the yeast two-hybrid system (10, 12, 14), represents a way to identify and clone genes that interact with a protein of interest by in vivo complementation in yeast cells. Using that approach, we recovered a clone encoding an X-interactive protein (XAP-1) which is homologous to a monkey UV-damaged DNA-binding protein (UV-DDB). The interaction between the HBV X and XAP-1 proteins was verified by immunoprecipitation of yeast extracts and by in vitro binding studies.

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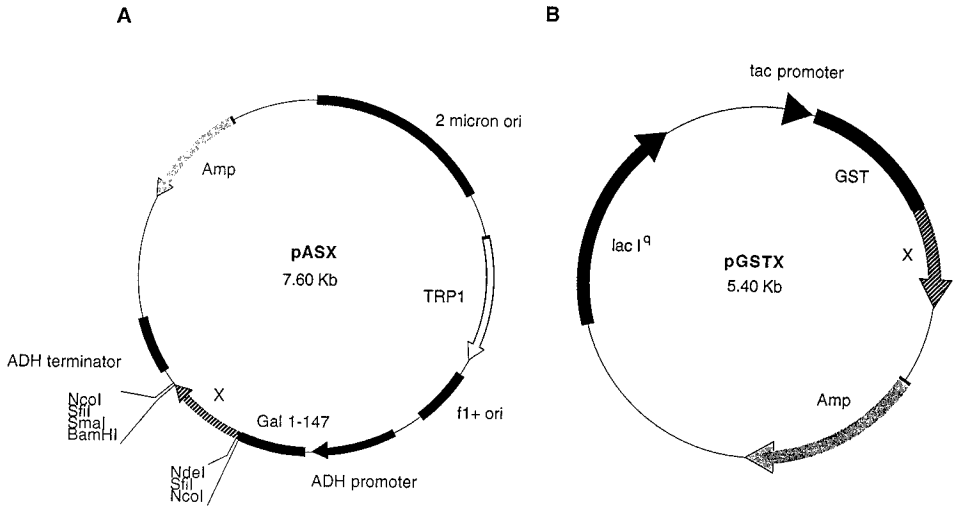


FIG. 1. Construction of HBV X expression plasmids. (A) pASX. The X ORF was subcloned into the *Nco*I site of the pAS1 vector such that the Gal4 DNA-binding domain (amino acids 1 to 147) was fused in-frame with the X ORF. The recombinant plasmid was used to transform yeast cells for the expression of Gal4-X fusion protein. (B) pGSTX. The X ORF was subcloned in-frame into the *Sma*I site of the pGEX-2TK vector. The recombinant plasmid was used to transform *E. coli* for the expression of GST-X fusion protein.

## MATERIALS AND METHODS

**Plasmid construction.** The construction of plasmids pAS1 and pACT-cDNA was described in detail by Durfee et al. (10). To make the bait plasmid for this study, the complete HBV X ORF (amino acids 1 to 154) was subcloned from pGem-1-X (26) into the *Nco*I site of pAS1, creating pASX (Fig. 1A). The expressed protein has the Gal4 DNA-binding domain (amino acids 1 to 147) fused in-frame with X protein. As a negative control, a partially deleted X ORF (amino acids 1 to 67) was generated and cloned into the same position in the pAS1 plasmid. pACT-cDNA prey plasmids have the DNA sequence encoding the Gal4 DNA activation domain (amino acids 768 to 880) linked with a cDNA library made from Epstein-Barr virus-transformed human lymphocytes.

For in vitro mixing experiments, the X ORF was expressed as a glutathione *S*-transferase (GST)-X fusion protein in *Escherichia coli*. The complete X ORF was subcloned into the *Sma*I site of the pGEX-2TK plasmid vector (Pharmacia) (18) to generate plasmid pGSTX (Fig. 1B).

**Yeast two-hybrid system.** Detailed procedures for using the yeast two-hybrid system have been described previously (10, 14). Yeast strain Y153, which has *HIS3* and *LacZ* genes controlled by the *GAL1* $UAS_G$ promoter, was used as the recipient host (10). In brief, yeast strain Y153 was transformed with the pASX bait plasmid and was selected on tryptophan (Trp)-deficient synthetic complete (SC) medium. Yeast cells carrying pASX were transformed with a human cDNA library in pACT and were selected on SC medium lacking Trp, leucine (Leu), and histidine (His) and supplemented with 25 mM 3-aminotriazole (Sigma). Colonies able to grow on this medium were transferred to nitrocellulose filters for the β-galactosidase filter lift assay (β-gal assay) (4). Briefly, colonies transferred to filters were permeabilized in liquid nitrogen, were laid upon filter papers saturated with 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in Z buffer (0.1 M sodium phosphate buffer [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 40 mM 2-mercaptoethanol) per ml, and were incubated at 30°C overnight. Blue colonies were considered to be "putative positive" and were chosen for further analysis.

Putative positive clones were grown on Trp-supplemented SC medium to facilitate the loss of the resident pASX plasmid. The elimination of pASX plasmid in these colonies was confirmed by the loss of ability to grow in Trp-supplemented SC medium. These pASX-deleted clones, still carrying various pACT-cDNA plasmids, were mated with individual yeast test clones carrying the *cdk2*, cyclin D, lamin, *SNF-1*, or *p53* gene in the pAS1 vector and were grown on Trp-, Leu-, and His-deficient SC plates. Colonies that grew were tested for β-gal activity. Blue colonies were considered to be false positives and were discarded. Clones that yielded white colonies in the β-gal assay were considered to be HBV X-specific clones. pACT-cDNA plasmids were recovered from these clones and were amplified by PCR. The recovered cDNA inserts were gel purified and analyzed by DNA sequencing.

**DNA sequencing and computation.** cDNA inserts were sequenced with the Sequenase version 2.0 sequencing kit (United States Biochemical Corp.) based on the dideoxy method. Homology searches for the DNA sequences thus determined and their deduced amino acid sequences were performed at the National Center for Biotechnology Information with the BLAST network service and the Genetics Computer Group sequence analysis software package through the Molecular Biology Computation Resource, Baylor College of Medicine.

**Antiserum production.** Two hydrophilic peptide sequences of XAP-1 were chosen for the generation of anti-peptide antisera. The following peptides were used: peptide 1, REKEFNKGPWKQENVE (amino acids 198 to 213); and peptide 2, QYDDGSGMKREATA (amino acids 1113 to 1126). These peptides were chemically synthesized and were conjugated to Fmoc multiple antigenic peptide resins (Applied Biosystems) by the Advanced Technology Laboratories, Baylor College of Medicine. The peptides were mixed with adjuvant and were used to immunize rabbits intramuscularly at monthly intervals (2 mg of peptide per injection). Serum samples were collected after six immunizations. Polyclonal anti-X antiserum was prepared in rabbits against HBV X protein expressed in insect cells (25). This antiserum reacts with X protein in both immunoprecipitation and immunoblot assays (26).

**In vitro transcription and translation.** The XAP-1 ORF was amplified and subcloned into the pCRII vector (InVitrogen) for in vitro expression. The procedures for in vitro transcription with T7 RNA polymerase and in vitro translation in rabbit reticulocyte lysate (Promega) were described previously (26). Translation products were labeled with Tran$^{35}$S-label (>1,000 Ci/mmol; ICN Biomedical Inc.) at a final concentration of 0.8 mCi/ml. The synthesized protein products were analyzed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% gels (16, 23).

**Metabolic labeling, protein extraction, immunoprecipitation, and immunoblot analysis.** Cells ($\sim 5 \times 10^6$) were metabolically labeled with 200 to 300 μCi of Tran$^{35}$S-label in methionine-free medium containing 2% serum for 2 to 3 h. Cultured cells or in vitro translated proteins were solubilized in 1% Nonidet P-40 extraction buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% Nonidet P-40, 1 mM EDTA) supplemented with protease inhibitors (1% aprotinin, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride). Lysates were clarified by centrifugation and incubated with anti-XAP-1 or anti-X antiserum, and immune complexes were adsorbed with Sepharose-protein A (Pharmacia). The precipitates were washed with extraction buffer, suspended in disruption buffer, and resolved by SDS-PAGE.

For immunoblotting, proteins in resolved gels were electrotransferred to nitrocellulose filters (35). The filters were probed with anti-XAP-1 or anti-X antiserum by following instructions for the Vectastain ABC kit (Vector Laboratories Inc.). The probed proteins on the filters were visualized by staining with 4-chloro-1-naphthol (Sigma) or the ECL system (Amersham) (17).

**In vitro mixing.** The procedures for in vitro mixing were as described by Kaelin et al. (18). Briefly, pGSTX was transformed into competent *E. coli* $DH5_2$ and expressed as a GST-X fusion protein. The transformed bacteria from a single colony were grown overnight at room temperature and were induced with IPTG (isopropyl-1-thio-β-D-galactoside) for 10 to 12 h. The IPTG-induced bacteria were harvested by low-speed centrifugation, were washed once with distilled $H_2O$, and were sonically disrupted in 0.5% Nonidet P-40 extraction buffer. Cell debris was removed by centrifugation in a microcentrifuge. Clarified cell extracts were incubated with washed GST beads (Pharmacia) at 4°C for 30 min. After several washes, the beads were mixed with cell extracts or in vitro translated XAP-1 proteins for 2 h at 4°C and were washed again. Proteins were eluted with disruption buffer and resolved by SDS-PAGE. The resolved proteins were detected by autoradiography or immunoblotting.

## RESULTS

**Screening for cellular proteins that interact with HBV X protein.** The rationale for using the yeast two-hybrid system for screening cDNAs encoding cellular proteins able to interact with a target protein of interest has been addressed in several studies (10, 12, 14). The two-hybrid system has several advantages over other commonly used methods to study protein-protein interactions: it is more sensitive than immunoprecipitation; purified proteins are not required; transient protein interactions may be stabilized by ternary interactions in transcription complexes; no preconceived notions are required about what proteins may interact; and it can be used to isolate genes encoding proteins that are normally expressed at a low level. The HBV X ORF was subcloned into plasmid pAS1 to be expressed as a fusion protein with the DNA-binding domain of the Gal4 protein (Fig. 1A). The newly constructed plasmid, pASX, was used to transform yeast strain Y153. Because the Gal4-X fusion protein would lack the activation domain of Gal4 protein, it could bind to *GAL1* promoter sequences but would not activate expression of the *HIS3* and *LacZ* genes in yeast cells. Y153 containing pASX was transformed with a human cDNA library in pACT (10). This would allow coexpression of the Gal4-X fusion protein with other fusion proteins containing the Gal4 activation domain fused to proteins encoded by human cDNAs. If a yeast cell contained a clone that expressed a human fusion protein able to associate with the X protein, the Gal4 DNA-binding domain and activation domain would colocalize and induce the expression of the reporter genes *HIS3* and *LacZ.*

About $10^6$ pASX and pACT-cDNA double transformants were screened for His-independent growth and blue-colony formation in the β-gal assay. Sixty-seven positive clones were isolated. As some pACT-cDNA-expressed proteins may interact nonspecifically with fusion proteins expressed by pAS1 hybrid plasmids, false-positive clones frequently appear in the screening procedure. To eliminate false-positive clones from further characterization, a confirmation test was used (10, 14). Plasmid pASX was eliminated from the putative positive clones by growth in Trp-supplemented SC medium. These yeast cells, which had lost pASX but still retained the pACT-cDNA plasmids, were then mated with test yeast cells containing unrelated genes inserted in pAS1 plasmids. The resulting diploids were then tested for His dependency for growth and *LacZ* expression by the β-gal assay. In this test, clones that grew in His-deficient medium or turned blue in the β-gal assay were considered to be nonspecific for X interaction. Only 1 of 67 clones survived all the genetic tests and did not interact with the other proteins expressed by the test yeast cells. It was considered a true positive and was analyzed further.

**Sequence analysis of the cDNA encoding an X-associated protein.** Plasmid pACT-cDNA was recovered from the true-positive clone and was sequenced. The cDNA was 4,072 bases long. It contained an ORF able to encode a protein of 1,140 amino acids (Fig. 2). The protein was named XAP-1 (X-associated protein 1). Sequence homology searches in GenBank revealed that XAP-1 is a human homolog of a UV-DDB gene recovered from a monkey cDNA library (39). There were 51 changes among 3,423 nucleotides of the entire ORF but only one amino acid change (99.91% identity).

**Expression of XAP-1 in vitro and in cell cultures detected with anti-peptide antisera.** The deduced amino acid sequence of XAP-1 was analyzed for hydrophilicity-hydrophobicity characters. Two oligopeptides, designated peptide 1 and peptide 2, that represent two hydrophilic regions of XAP-1 were synthesized. Peptides 1 and 2 were from the amino terminus and the carboxy terminus, respectively, of XAP-1 (see Materials and Methods). The peptides were used to raise anti-peptide antibodies in rabbits as detailed in Materials and Methods. The antisera were used to examine the expression of XAP-1 by immunoprecipitation and by immunoblotting procedures.

To study protein synthesis in vitro, the XAP-1 cDNA was subcloned into the pCRII expression vector, was transcribed with T7 RNA polymerase, and then was translated with a rabbit reticulocyte lysate. A 127-kDa protein was the largest translated product (Fig. 3, lane 2); it was immunoprecipitated by anti-peptide 2 antiserum (lane 4) but not by the control normal rabbit serum (lane 3). Several smaller protein bands, which were recognized by the antiserum, may represent degradation products of full-length XAP-1 or proteins whose translations initiated from internal methionine codons, as anti-peptide 2 antiserum was directed against a carboxy-terminal epitope. Anti-peptide 1 antiserum reacted only weakly by immunoprecipitation (data not shown).

The expression of UV-DDB mRNA in human tissues and cell cultures has been characterized by Takao et al. (39). They found it expressed in all human cells and tissues examined, including liver. We further analyzed the expression of XAP-1 in human cell lines, concentrating on those of liver origin. The human hepatoma cell line HepG2 was labeled, extracted, and immunoprecipitated with antisera against XAP-1. Anti-peptide 2 antiserum immunoprecipitated a protein that migrated at about 127 kDa (Fig. 4A, lane 2) and was not recovered in the control reaction with the preimmune serum (lane 1). Immunoblot experiments were then carried out to prove that the 127-kDa protein was the product of the XAP-1 gene. Resolved gel profiles of unlabeled immunoprecipitates from HepG2 cells that were duplicates of samples shown in Fig. 4A were transferred to nitrocellulose filters and tested by immunoblotting (Fig. 4B). Normal serum did not react with the 127-kDa protein immunoprecipitated by anti-peptide 2 antiserum (lane 2), nor did other non-XAP-1-specific antisera (data not shown). However, both anti-peptide 1 and anti-peptide 2 antisera recognized the 127-kDa protein in samples immunoprecipitated by anti-peptide 2 antiserum (Fig. 4B, lanes 4 and 6). No similar protein was present in samples obtained with preimmune serum (lanes 3 and 5). It should be noted that although anti-peptide 1 antiserum immunoprecipitated XAP-1 protein only weakly from in vitro translation mixtures or from HepG2 cell lysates (data not shown), it did react well with XAP-1 by immunoblot (Fig. 4B, lane 4). This suggests that the XAP-1 amino-terminal epitope targeted by anti-peptide 1 antiserum is probably masked by conformational folding of the native protein or by binding of unknown factors. These results suggest that the 127-kDa protein recovered from the human hepatoma cells is XAP-1.

This 127-kDa XAP-1 was also expressed in other human cell lines, e.g., HeLa and PLC/PRF/5 cells (data not shown), indicating that the expression of XAP-1 (as noted for UV-DDB [39]) was not restricted to cells of skin origin. XAP-1 expressed in cell lines migrated slightly faster on an SDS-gel than did XAP-1 expressed in vitro. It is unclear whether this difference reflects posttranslational modification(s).

**Interaction between X and XAP-1 proteins in yeast cells.** Yeast experiments were performed to confirm the interaction between the X and XAP-1 proteins. Yeast cells transformed singly with plasmid pASX or plasmid pACT–XAP-1 or with both plasmids together were metabolically labeled, extracted, and immunoprecipitated with anti-X antiserum (Fig. 5). A 140-kDa protein (the predicted size of the Gal4–XAP-1 protein) was immunoprecipitated with anti-X serum from yeast cells that contained both plasmids (Fig. 5A, lane 4) but was not

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   1  TCGCGCTCGAGTCCCGACGGGCCGCTCCAAGCCTCGACATGTCGTACAACTACGTGGTAACGGCCCAGAAGCCCACCG  78
                                        M  S  Y  N  Y  V  V  T  A  Q  K  P  T  A
  79  CCGTGAACGGCTGCGTGACCGGACACTTTACTTCGGCCGAAGACTTAAACCTGTTGATTGCCAAAAACACGAGATTAG 156
      V  N  G  C  V  T  G  H  F  T  S  A  E  D  L  N  L  L  I  A  K  N  T  R  L  E
 157  AGATCTATGTGGTCACCGCCGAGGGGCTTCGGCCTGTCAAAGAGGTGGGCATGTATGGGAAGATTGCGGTCATGGAGC 234
      I  Y  V  V  T  A  E  G  L  R  P  V  K  E  V  G  M  Y  G  K  I  A  V  M  E  L
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      F  R  P  K  G  E  S  K  D  L  L  F  I  L  T  A  K  Y  N  A  C  I  L  E  Y  K
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      L  D  R  D  N  K  E  L  K  A  F  N  I  R  L  E  E  L  H  V  I  D  V  K  F  L
 547  TATATGGTTGCCAAGCACCTACTATTTGCTTTGTCTACCAGGACCCTCAGGGGCGGCACGTAAAAACCTATGAGGTGT 624
      Y  G  C  Q  A  P  T  I  C  F  V  Y  Q  D  P  Q  G  R  H  V  K  T  Y  E  V  S
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 781  TTGCCCCTCCTATCATCAAGCAAAGCACGATTGTGTGCCACAATCGAGTGGACCCTAATGGCTCAAGATACCTGCTGG 858
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      I  P  R  S  I  L  M  T  T  F  E  S  S  H  Y  L  L  C  A  L  G  D  G  A  L  F
1873  TCTACTTTGGGCTCAACATTGAGACAGGTCTGTTGAGCGACCGTAAGAAGGTGACTTTGGGCACCCAGCCCACCGTAT 1950
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1951  TGAGGACTTTTCGTTCTCTTTCTACCACCAACGTCTTTGCTTGTTCTGACCGCCCCACTGTCATCTATAGCAGCAACC 2028
      R  T  F  R  S  L  S  T  T  N  V  F  A  C  S  D  R  P  T  V  I  Y  S  S  N  H
2029  ACAAATTGGTCTTCTCAAATGTCAACCTCAAGGAAGTGAACTACATGTGTCCCCTCAATTCAGATGGCTATCCTGACA 2106
      K  L  V  F  S  N  V  N  L  K  E  V  N  Y  M  C  P  L  N  S  D  G  Y  P  D  S
2107  GCCTGGCGCTGGCCAACAATAGCACCCTCACCATTGGCACCATCGATGAGATCCAGAAGCTGCACATTCGCACAGTTC 2184
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2497  AGCTGGGCAAAGACCCCAACACTTACTTCATTGTGGGCACAGCAATGGTGTATCCTGAAGAGGCAGAGCCCAAGCAGG 2574
      L  G  K  D  P  N  T  Y  F  I  V  G  T  A  M  V  Y  P  E  E  A  E  P  K  Q  G
2575  GTCGCATTGTGGTCTTTCAGTATTCGGATGGAAAACTACAGACTGTGGCTGAAAAGGAAGTGAAAGGGGCCGTGTACT 2652
      R  I  V  V  F  Q  Y  S  D  G  K  L  Q  T  V  A  E  K  E  V  K  G  A  V  Y  S
2653  CTATGGTGGAATTTAACGGGAAGCTGTTAGCCAGCATCAATAGCACGGTGCGGCTCTATGAGTGGACAACAGAGAAGG 2730
      M  V  E  F  N  G  K  L  L  A  S  I  N  S  T  V  R  L  Y  E  W  T  T  E  K  E
2731  AGCTGCGCACTGAGTGCAACCACTACAACAACATCATGGCCCTCTACCTGAAGACCAAGGGCGACTTCATCCTGGTGG 2808
      L  R  T  E  C  N  H  Y  N  N  I  M  A  L  Y  L  K  T  K  G  D  F  I  L  V  G
2809  GCGACCTTATGCGCTCAGTGCTGCTGCTTGCCTACAAGCCCATGGAAGGAAACTTTGAAGAGATTGCTCGAGACTTTA 2886
      D  L  M  R  S  V  L  L  L  A  Y  K  P  M  E  G  N  F  E  E  I  A  R  D  F  N
```

FIG. 2. Sequence of XAP-1. The nucleic acid sequence of the XAP-1 cDNA and the deduced amino acid sequence of XAP-1 protein are shown.

```
2887  ATCCCAACTGGATGAGTGCTGTGGAAATCTTGGATGATGACAATTTTCTGGGGGCTGAAAATGCCTTTAACTTGTTTG  2964
        P  N  W  M  S  A  V  E  I  L  D  D  D  N  F  L  G  A  E  N  A  F  N  L  F  V
2965  TGTGTCAAAAGGATAGCGCTGCCACCACTGACGAGGAGCGGCAGCACCTCCAGGAGGTTGGTCTTTTCCACCTGGGCG  3042
        C  Q  K  D  S  A  A  T  T  D  E  E  R  Q  H  L  Q  E  V  G  L  F  H  L  G  E
3043  AGTTTGTCAATGTCTTTTGCCACGGCTCTCTGGTAATGCAGAATCTGGGTGAGACTTCCACCCCCACACAAGGCTCGG  3120
        F  V  N  V  F  C  H  G  S  L  V  M  Q  N  L  G  E  T  S  T  P  T  Q  G  S  V
3121  TGCTCTTCGGCACGGTCAACGGCATGATAGGGCTGGTGACCTCACTGTCAGAGAGCTGGTACAACCTCCTGCTGGACA  3198
        L  F  G  T  V  N  G  M  I  G  L  V  T  S  L  S  E  S  W  Y  N  L  L  L  D  M
3199  TGCAGAATCGACTCAATAAAGTCATCAAAAGTGTGGGGAAGACGAGCACTCCTTCTGGAGATCCTTTCACACCGAGCT  3276
        Q  N  R  L  N  K  V  I  K  S  V  G  K  I  E  H  S  F  W  R  S  F  H  T  E  R
3277  GGAAGACAGAACCAGCCACAGGTTTCATCGACGGTGACTTGATTGAGAGTTTCCTGGATATTAGCCGCCCCAAGATGC  3354
        K  T  E  P  A  T  G  F  I  D  G  D  L  I  E  S  F  L  D  I  S  R  P  K  M  Q
3355  AGGAGGTGGTGGCAAACCTACAGTATGACGATGGCAGCGGTATGAAGCGAGAGGCCACTGCAGACGACCTCATCAAGG  3432
        E  V  V  A  N  L  Q  Y  D  D  G  S  G  M  K  R  E  A  T  A  D  D  L  I  K  V
3433  TTGTGGAGGAGCTAACTCGGATCCATTAGCCAAGGGCAGGGGGCCCCTTTGCTGACCCTCCCCAAAGGCTTTGCCCTG  3510
        V  E  E  L  T  R  I  H  *
3511  CTGCCCTCCCCCTCCTCTCCACCATCGTCTTCTTGGCCATGGGAGGCCTTTCCCTAAGCCAGCTGCCCCCAGAGCCAC  3588
3589  AGTTCCCCTATGTGGAAGTGGGGCGGGCTTCATAGAGACTTGGGAATGAGCTGAAGGTGAAACATTTTCTCCCTGGAT  3666
3667  TTTTACCAGTCTCACATGATTCCAGCCATCACCTTAGACCACCAAGCCTTGATTGGTGTTGCCAGTTGTCCTCTTCCG  3744
3745  GGAAGATTTGCAGTTCTTTGGCTGAAGGAAGCTGTGCGTGTGTGTGTGTGTATGTTGTGGGTATGTGTATCTCACACT  3822
3823  CATGCATTGTCCTCTTTTTATTTAGATTGGCAGTGTAGGGAGTTGTGGGTAGTGGGGAAGAGGGTTAGGAGGGTTTCA  3900
3900  TTGTCTGTGAAGTGAGACCTTCCTTTTACTTTTCTTCTATTGCCTCTGAGAGCATAGCTAGAGGCCTGACTGCCAAGC  3978
3979  CATGGGTAGCCTGGGTGTAAAACCTGGAGATGGTGGATGATCCCCACGCCACAGCCCTTTTGTCTCTGCAAACTGCCT  4056
4057  TCTTCGGAAAGAAGAA                                                                  4072
```

FIG. 2—*Continued.*

recovered from yeast cells transformed by plasmid pASX or pACT–XAP-1 alone (Fig. 5A and B, lanes 1 and 2). The Gal4–XAP-1 protein was recognized by immunoblotting with anti-peptide 2 antiserum (Fig. 5B, lane 4). We interpret these results to suggest that the 140-kDa species was the Gal4–XAP-1 fusion protein expressed by the pACT–XAP-1 plasmid and that it was coprecipitated by anti-X antibody because it was associated with the Gal4-X protein in the yeast cells.

A deletion mutant, encoding only the first 67 amino acids of the X ORF, was generated in plasmid pASX to test the specificity of the association of X with XAP-1. Cotransformation of this deletion mutant in pASX with pACT–XAP-1 did not produce colonies that activate *LacZ* expression, suggesting that amino acids 68 to 154 of X are important for the binding interaction. The protein expressed from the deletion mutant of pASX in yeast cells was immunoprecipitated by anti-X antiserum as a 24-kDa protein (Fig. 5A, lane 3). As expected from the in vivo data, this 24-kDa protein did not coprecipitate the 140-kDa Gal4–XAP-1 protein (Fig. 5A and B, lanes 3).

**Interaction between X and XAP-1 proteins by in vitro mixing.** The association between X and XAP-1 was further substantiated by in vitro binding experiments with a GST-X fusion protein. The X ORF was subcloned into the pGEX-2TK vector (Fig. 1B), and the recombinant plasmid was transformed into *E. coli* to be expressed as a GST-X fusion protein. GST-X fusion protein in bacterial lysates was purified by binding with

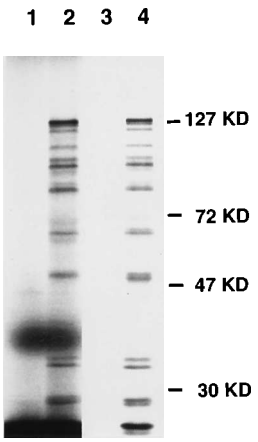


FIG. 3. Expression of XAP-1 in vitro. XAP-1 cDNA was subcloned into the pCRII vector, transcribed in vitro with T7 RNA polymerase, and translated in vitro with a rabbit reticulocyte lysate. Translated proteins were resolved by SDS-PAGE. No mRNA was added to the reticulocyte lysate as the translational control (lane 1). The largest radiolabeled XAP-1 in the lysate migrated at 127 kDa (lane 2). The same amounts of proteins translated in vitro as shown in lane 2 were immunoprecipitated with control normal rabbit serum (lane 3) or anti-peptide 2 antiserum (lane 4). XAP-1 (127 kDa) was recognized by anti-peptide 2 antiserum. Molecular mass markers are shown on the right. KD, kilodaltons.

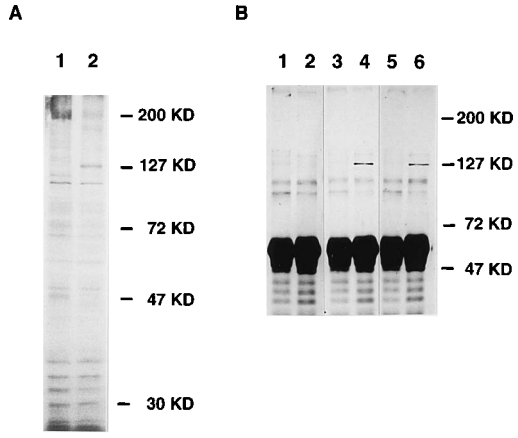


FIG. 4. Expression of XAP-1 in human hepatoma HepG2 cells. (A) Immunoprecipitation of XAP-1. HepG2 cells were radiolabeled with Tran$^{35}$S-label and detergent extracted; extracts were immunoprecipitated with preimmune serum (lane 1) or anti-peptide 2 antiserum (lane 2), and the precipitated proteins were resolved by SDS-PAGE. A 127-kDa protein was immunoprecipitated by anti-peptide 2 antiserum but not by control normal rabbit serum. (B) Immunoblot of XAP-1. Unlabeled duplicates of immunoprecipitates from panel A were transferred onto a nitrocellulose filter; separated proteins were reacted with control normal rabbit serum (lanes 1 and 2), anti-peptide 1 antiserum (lanes 3 and 4), or anti-peptide 2 antiserum (lanes 5 and 6) against XAP-1; and bound antibodies were visualized by staining with 4-chloro-1-naphthol. XAP-1 protein (127 kDa) was detected by both anti-peptide 1 and anti-peptide 2 antisera. Nonspecific bands shown at the bottom of the filters are the heavy chain of immunoglobulin G. Molecular mass markers are shown on the right. KD, kilodaltons.

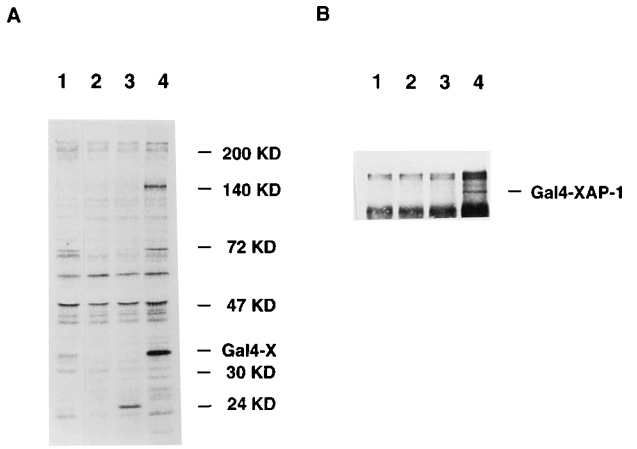


FIG. 5. Association of X and XAP-1 proteins in yeast cells. (A) Immunoprecipitation of X–XAP-1 complexes from yeast cells with anti-X antiserum. Yeast cells harboring pASX alone (encoding full-length X [amino acids 1 to 154]) (lane 1), pACT–XAP-1 alone (lane 2), a deletion mutant of X ORF (amino acids 1 to 67) in pAS1 together with pACT–XAP-1 (lane 3), or both pASX and pACT–XAP-1 (lane 4) were metabolically labeled and extracted. The lysates were immunoprecipitated with anti-X antiserum, and the proteins were resolved by SDS-PAGE. The 140-kDa protein that was coprecipitated by anti-X antibody from yeast cells coexpressing intact Gal4-X and Gal4–XAP-1 (lane 4). Molecular mass markers are shown on the right. (B) Immunoblot detection of XAP-1 coimmunoprecipitated by anti-X antiserum. Duplicates of immunoprecipitates shown in panel A were transferred to nitrocellulose and reacted with anti-peptide 2 antiserum against XAP-1, and bound antibodies were visualized with an ECL kit (see Materials and Methods). The 140-kDa protein that coprecipitated with intact X was XAP-1 (lane 4). KD, kilodaltons.

glutathione beads and then was mixed with in vitro translated, radiolabeled XAP-1. The 127-kDa XAP-1 protein, as well as several partial XAP-1 proteins synthesized in vitro (Fig. 6, lane 1), was recovered by the GST-X fusion protein (lane 3). XAP-1 was not recovered by GST alone (lane 2), indicating that XAP-1 interacts specifically with the HBV X portion of the GST-X fusion protein. Although XAP-1 protein bound to GST-X represented <10% of the total input, the binding was reproducible in multiple experiments. This relatively low percentage of binding could be due to imperfect binding conditions, an absence of necessary cofactors, or the failure of translated XAP-1 to achieve an authentic native conformation or modification.

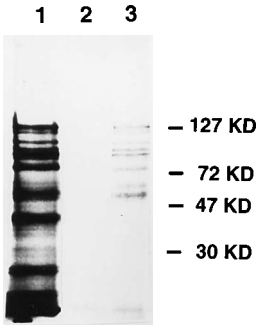


FIG. 6. Association of GST-X and in vitro translated XAP-1 by in vitro mixing. XAP-1 was synthesized and radiolabeled by in vitro translation (lane 1). The same amount of translation mixture containing labeled XAP-1 shown in lane 1 was applied to glutathione-Sepharose beads containing immobilized GST control protein (lane 2) or GST-X fusion protein (lane 3). Bound protein was eluted and analyzed by SDS-PAGE. The 127-kDa XAP-1 was recovered by using the GST-X fusion protein. Molecular mass markers are shown on the right. KD, kilodaltons.

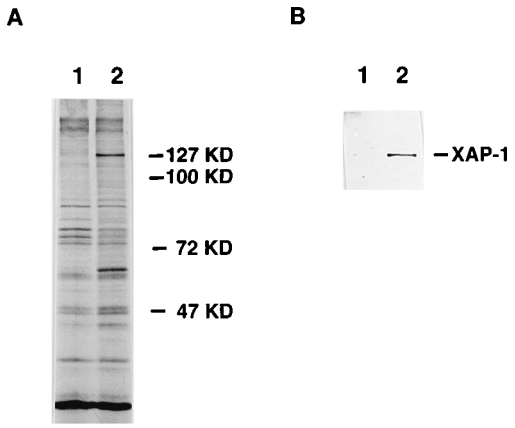


FIG. 7. Association of GST-X and authentic XAP-1 from HeLa cell extracts by in vitro mixing. (A) Radiolabeled HeLa cell extracts prepared as described in Materials and Methods were mixed with GST control (lane 1) or GST-X (lane 2) immobilized on glutathione-Sepharose beads. Bound proteins were eluted and were resolved by SDS-PAGE. A 127-kDa protein was recovered by GST-X but not by the GST control. Molecular mass markers are shown on the right. (B) Immunoblot detection of XAP-1 recovered from GST-X-bound proteins. Duplicates of eluted proteins shown in panel A were transferred to nitrocellulose and reacted with anti-peptide 2 antiserum against XAP-1, and bound antibodies were visualized with an ECL kit. The 127-kDa protein recovered by using GST-X was XAP-1.

Similar mixing experiments were performed with radiolabeled HeLa cell extracts in place of in vitro translated XAP-1. A 127-kDa protein was recovered with GST-X (Fig. 7A, lane 2) but not with the GST control (lane 1). This 127-kDa HeLa cell protein was proven to be XAP-1 by immunoblotting. A 127-kDa protein that reacted with the anti-peptide 2 antiserum was present in the GST-X sample (Fig. 7B, lane 2) but not in the sample recovered with GST alone (lane 1). These results indicated that the 127-kDa XAP-1 from HeLa cells can associate with HBV X protein. Other lower-molecular-weight proteins also bound to the GST proteins. Most reflected nonspecific binding, as similar bands appeared with both GST and GST-X (Fig. 7A). Other bands that may be specific for interaction with GST-X did not react with the XAP-1 antiserum and have not been identified.

## DISCUSSION

The identification of the HBV X-associated cellular protein(s) represents a major goal in order to define the function of X protein in HBV replication and liver carcinogenesis. In this study, we have identified an X-associated protein, designated XAP-1, by using the yeast two-hybrid system. Sequence analysis revealed that XAP-1 is the human homolog of a UV-damaged DNA-binding protein, UV-DDB, recovered from a monkey cell cDNA library (39).

The association of HBV X protein with XAP-1 was established in several ways. His-independent growth and blue-colony formation in the β-gal assay by yeast cells harboring both pASX and pACT–XAP-1 recombinant plasmids and the behavior of the cells in false-positive elimination tests suggested that the binding of XAP-1 was X specific. The interaction between X and XAP-1 was confirmed, first by immunoprecipitation with XAP-1-specific peptide antisera of yeast cells carrying both plasmids and second by in vitro mixing of the GST-X fusion protein with XAP-1 from in vitro translation or in HeLa cell extracts. The amount of XAP-1 which interacted with X represented only a small percentage of total input protein in the in vitro mixing experiments. This is probably

because X and XAP-1 expressed in bacterial cultures or in rabbit reticulocyte lysates may not be in optimal physiological conformations or because necessary cofactors may be lacking. Even in the yeast lysates containing both X and XAP-1, not all the X and XAP-1 proteins were complexed. It is not clear if this is due to imperfect interaction conditions, innate weak interaction properties, or a requirement for other cellular facilitative factors.

The deletion mutant of X, containing only amino acids 1 to 67, did not interact with XAP-1 in the yeast transformation and immunoprecipitation studies. These results suggest the importance of the carboxyl terminus of the X protein in the target protein interaction. It has been reported that a region of the X protein, located at amino acid sequences 132 to 139, was one of the essential regions responsible for transactivation (1, 33). We are currently generating a series of deletion mutants to determine if the essential domains of X required for XAP-1 interaction are located at those same regions. Other than binding to XAP-1, HBV X protein has been shown to be a cyclic AMP-responsive element-binding protein and activating transcription factor 2-binding protein (29), a protease inhibitor (38), and a *p53*-binding protein (11, 40). The X protein may exert these functions through different domains, or it may have a common mechanism for mediating these seemingly diverse functions. Determination of the domain(s) of X required for each function will indicate if they are related mechanistically.

Proteins important in cellular DNA repair, including the human version of UV-DDB (XAP-1), have been studied by using cells from persons with inherited genetic defects. Individuals with the autosomal recessive genetic disease xeroderma pigmentosum (XP) have a defect in the DNA repair system. Such individuals are sensitive to sunlight and have a marked predisposition toward skin cancer. Somatic-cell fusion experiments with cells derived from XP patients have defined seven complementation groups (A to G), suggesting that a multienzyme complex is involved in efficient DNA repair (3).

Two groups have described a protein that binds to damaged DNA and that may represent the defective protein in XP group E patients (15, 39). The monkey gene, UV-DDB, was cloned by Takao et al. (39); the human cognate of the UV-DDB gene was mapped to chromosome 11. Hwang and Chu (15) purified the protein (XPE-binding factor) from human placenta. The proteins are about the same size (125 to 127 kDa) and have a high binding affinity for damaged DNA. Both groups concluded that their isolate was probably involved in the recognition step of the excision repair pathway. Injection of purified human DDB protein into XP-E cells corrected the DNA repair defect, establishing a functional role for the DDB protein in nucleotide excision repair (19). Additional functions of UV-DDB, other than binding to damaged DNA, are not known. As the expression of UV-DDB/XAP-1 is not restricted to skin cells, retention of its expression in hepatocytes and other tissues suggests involvement of UV-DDB/XAP-1 in an essential function(s) other than solely the repair of UV-damaged DNA.

It is possible to envision a role for the cellular DNA repair process during HBV replication. Following entry into susceptible cells, the HBV component is released from the virion and the partially double-stranded DNA genome is converted to a covalently closed circular form. The covalently closed circular DNA then serves as template for the production of HBV mRNAs. This repair process, including removal of HBV genome-linked polymerase protein and a small stretch of mRNA, synthesis of new DNA, and ligation of the nick left in the repaired strand, is achieved by cellular rather than by viral DNA-encoded functions (22). It remains to be determined if XAP-1 is a factor in this repair process. XAP-1 may be responsible for binding to imperfect double-helix regions of the HBV genome so that other factors in the repair complex can fix those defects into covalently closed circular DNA. We postulate that the X protein is required for the recruitment or functioning of the cellular repair proteins to repair the HBV genome. The involvement of the X protein may be to stabilize the interaction of the multiple-enzyme complex with HBV DNA, to displace or inhibit the enzymatic process(es) that is part of the normal DNA repair process and might be deleterious to HBV DNA, or to alter the function of the repair process in some other way.

On the other hand, several proteins involved in DNA repair have recently been found to be components of the cellular transcriptional machinery; e.g., the *XPBC* gene product is a component of the TFIIH transcriptional factor (34). XAP-1 may also be involved in transcription. Association with X protein may stabilize the transcriptional complex so that more HBV transcripts can be synthesized during HBV infection. Through the same type of mechanism, X may also activate or stabilize the transcription of some cellular genes, which may explain the ability of X to be a transactivator.

The functional role of X protein in causing hepatocellular carcinoma is still unclear. Even in transgenic animal models (21, 26), it is still controversial whether X protein by itself can induce hepatoma formation or whether cofactors are required (37). If X protein were one of the factors involved, in addition to just transactivating cellular genes to perturb their normal functions, X may have effects on the DNA repair process, resulting in the accumulation of mutations and genetic instability and leading to cancer development. It is noteworthy that mutated DNA repair genes have recently been identified as causative in the development of hereditary nonpolyposis colon cancer (5, 13, 24, 31). Even though the colon cancer genes are different from XAP-1 identified here, they serve to emphasize the important role of DNA repair in susceptibility to cancer development.

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