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XAP2, a novel hepatitis B virus X-associated protein that inhibits X transactivation

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ABSTRACT

The hepatitis B virus X protein is a promiscuous transcriptional transactivator. Transactivation by the X protein is most likely mediated through binding to different cellular factors. Using the yeast two-hybrid method, we have isolated a clone that encodes a novel X-associated cellular protein: XAP2. X and XAP2 interactions also occur *in vitro.* Antiserum raised against XAP2 recognizes a cytoplasmic protein with an apparent molecular mass of 36 kDa. The interaction between X and XAP2 requires a small region on X containing amino acids 13–26. From Northern blot analyses, XAP2 is ubiquitously expressed in both liver-derived and non-liver-derived cell lines as well as in normal non-liver tissues. In contrast, XAP2 is expressed in very low level in the normal human liver. In transfection assays, overexpression of XAP2 abolishes transactivation by the X protein. Based on these results, we suggest that XAP2 is an important cellular negative regulator of the X protein, and that X–XAP2 interaction may play a role in HBV pathology.

INTRODUCTION

The hepatitis B virus (HBV) is a small, human DNA virus that causes acute and chronic hepatitis and is strongly associated with the development of primary hepatocellular carcinoma. In addition to the genes that encode viral structural proteins, and a gene that encodes the viral polymerase, the HBV genome contains an open reading frame that encodes a protein of 154 amino acids, termed X. The X open reading frame is conserved among various HBV strains and among different hepadnaviruses. Although the X gene does not appear to be essential for virus replication in culture, it does appear to play a major role in viral replication in animals (1–4). Furthermore, in spite of the fact that there is little evidence to suggest that X is a direct agent in tumorigenesis, there is good reason to speculate that the X gene product may contribute to HBV-induced tumorigenesis. This is reinforced by findings that the X protein is capable of inducing transformation of NIH3T3 cells (5) and mouse hepatocytes (6), and causes liver tumors in some transgenic mice (7).

The X gene product is expressed during HBV infection. Two mRNAs of 0.7 and 3.9 kb that potentially encode X have been detected in mammalian cells transfected with the complete HBV genome (4,8–10). Direct evidence for the ability of the X gene to encode a protein product has been provided by its expression in both prokaryotic and eukaryotic cells (e.g., 11,12). Antibodies to the X protein have been detected in sera of HBV infected patients (e.g., 11,13–15), and antisera raised against the X protein have been used to detect cross-reacting proteins in HBV infected livers (14,16,17).

Many studies have demonstrated that the X protein is a transcriptional activator (reviewed in ref. 18). Based on the experimental results from these studies, several non-mutually exclusive models have been proposed to explain the promiscuous transactivational activity of X. In one model, X stimulates the activity of a number of different transcription factors. For example, it has been reported that the expression of X coincides with an elevated level of active TFIIIC (19). In a second model, X uses a complex signal transduction pathway for activation (20–22). A third model proposes that X is a protease inhibitor and functions by modulation of serine protease activity, giving rise to quantitative or qualitative changes of cellular transcription factors (23). Alternatively, a fourth model proposes that X acts as a coactivator and potentiates potent activation domains (24). Finally, it has been proposed that X binds to transcription factors, which in turn modulate upstream promoter elements found in X-responsive promoters (25–27).

Despite the different proposed mechanisms for X transactivation, overwhelming evidence supports the hypothesis that transactivation by the X protein is mediated by cellular factors. First, investigation of the DNA sequences susceptible to transactivation by X revealed that no single recognition sequence is present in all of the targets (reviewed in ref. 18). Second, transactivation by X is cell type but not species-specific and that only a subset of promoters are transactivated in any particular cell type (28). Finally, although X functions in the vicinity of the promoter, it does not directly bind to DNA under conditions in which other known DNA-binding proteins bind DNA efficiently (25,29–31). Instead,

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X may belong to a class of transcriptional activators believed to be brought to DNA by binding to a second protein.

Previously, several groups found that cellular transcription factors such as ATF-2, CREB, RPB5, TATA-binding protein and p53 can directly bind to X *in vivo* and *in vitro* (25,32–34). In addition, the SV40 large T antigen and a serine protease tryptase TL2 can also bind X under various conditions (23,35). Recently, using the yeast two-hybrid system, Lee *et al.* (36) identified an X-associated protein (XAP1) that is a human homolog of a monkey UV-damaged DNA-binding protein. The physiological significance of the X–XAP1 interaction, as well as other X-cellular factors interaction, remains to be determined.

As a further step toward understanding the mechanism by which X activates transcription of many different genes, we have independently used the yeast interaction trap cloning method to isolate other cellular proteins that may interact with X. Among the X-interacting clones identified, many of them were found to encode a novel 36 kDa cytoplasmic protein designated X-associated protein 2 (XAP2). XAP2 can form a complex with X *in vitro*, and overexpression of XAP2 interferes with the transcriptional activation function of X.

MATERIALS AND METHODS

Bacterial and yeast strains

Escherichia coli DH5α (F$^-$ ϕ80d*lac*ZΔM15 Δ[*lac*ZYA–*arg*F]U169 *deo*R *rec*A1 *end*ΔA1 *pho*A *hsd*R17[r_K^-, m_K^+] *sup*E44 λ$^-$ *thi*-1 *gyr*A96 *rel*A1) was the recipient for plasmid transformations and was also used for the expression of GST fusion proteins. *Escherichia coli* BNN132 (37) was used to convert λACT phage DNA to plasmid DNA. *Escherichia coli* JA226 (*hsd*R, *hsd*M, *leu*B6, *lop*11, *thi*1, *rec*BC, *str*R) was used to recover expression plasmids from yeast. *Escherichia coli* LE392 (*supE*44 *supF*58 *hsdR*514 *galK*2 *galT*222 *metB*1 *trpR*55 *lacY*1) was used to propagate the HeLa λgt10 recombinant library. *Saccharomyces cerevisiae* Y153 (38) was used to screen for HBV X-associated protein clones and to detect for protein–protein interactions.

Plasmids

pAS-X was constructed by subcloning the HBV [subtype adw2 (39)] fragment from nucleotide 1375 to 1853 (*Nco*I–*Afl*III) into the Gal4 DNA-binding domain (DBD) tagged plasmid, pAS1 (38). This plasmid expresses a fusion protein containing the Gal4DBD and the full-length wild-type X protein. pAS-Rb2, which encodes a Gal4DBD-Rb fusion protein and pAS-SNF1, which encodes a Gal4DBD-yeast SNF1 fusion protein have been described (38). pAS-E12 and pAS-NB encode Gal4DBD fused to transcription factors E12 and Zif268, respectively, and were kindly provided by B. Christy (University of Texas Health Science Center). pAS-YY1, which contains a 1.2 kb fragment of YY1 cDNA in pAS1 has previously been described (40). To construct pGST-X, the *Nco*I–*Sma*I fragment from pAS-X containing the entire HBV-X ORF was subcloned into the pGSTag vector (41) in-frame with the GST polypeptide. pGEM-X1-3 was constructed by ligating the *Bgl*II X1-3 insert fragment from pACT-X1-3 with a *Bam*HI-digested pGEM7Zf(+) vector (Promega). pGEM-CyPA was constructed by taking the human cyclophilin A cDNA, an *Eco*RI fragment from pGCyPA (42), and ligating it to the *Eco*RI site of pGEM3Z (Promega). pGEM-1-1FL was constructed by ligating the *Eco*RI 1-1FL insert fragment from the 1-1FL λgt10 clone with a *Eco*RI-digested pGEM7Zf(+) vector (Promega). Plasmid pGST-XAP2 was constructed by subcloning a *Bgl*II fragment from pACTX1-3 into the *Bam*HI site of pGEX3X (Pharmacia). pECE-X, which contains the HBV fragment from nucleotide 1355 to 1987 in the SV40-derived expression vector pECE, has been described (43). pXD1, pXD3, pXD5, pXD8, pXD6, pXD7, pXI5, pXI2, pXI3 and pXI4 were constructed by first subcloning the X coding sequence (a *Bgl*II fragment from pECE-X) into the *Bam*HI site of a modified pGEM7Zf(+) plasmid (pGEM7Zf-3X) that would provide stop codons in all three reading frames immediately after the X sequence, then using different restriction enzymes to subdivide the X coding region, and finally subcloning each individual X mutant into the *Nco*I/*Bam*HI site of pAS1. Reporter plasmids pHIVCAT, pSV2CAT and pRSVCAT have previously been described (43–45). pCMV-XAP2, which contains a full-length XAP2 cDNA under the control of the CMV promoter, was constructed by ligation of an *Eco*RI fragment from the 1-1FL λgt10 clone into the *Eco*RI site of pcDNAI/Amp (Invitrogen). pTAT-6 expresses the HIV1 transactivating protein TAT under the SV40 promoter/enhancer (46). Effector pGal4-VP16 and reporter pG5BCAT have previously been described (47,48). All plasmid constructions were verified by dideoxy sequencing.

Screening of cDNA libraries

For the yeast two-hybrid screen, Y153 cells were sequentially transformed with pAS-X using the lithium acetate method, followed by transformation with a human cDNA library constructed in λ ACT using mRNA prepared from Epstein–Barr virus-transformed human peripheral lymphocytes [kindly provided by S. Elledge at the Baylor College of Medicine (38)]. Transformants were plated on 150 mm dishes containing SC medium lacking tryptophan, leucine and histidine, but containing 50 mM 3-aminotriazole, and incubated for 3 days. His$^+$ and Leu$^+$ prototroph colonies were rescreened for β-gal activity using a filter lift assay (49). Colonies corresponding to positive (blue color) in this second screen were then patched onto a master plate for further characterization. Plasmids were recovered and electroporated into *E.coli* strain JA226 (50), grown in minimal media lacking leucine but containing 50 μg/ml ampicillin. Isolated plasmids were rescreened by transforming Y153 alone or Y153 harboring pAS-X, pAS-Rb2 or pAS-SNF1. Positive clones showing specific interactions with X but not to others were subcloned into Bluescript (Stratagene) or pGEM7Zf(+), and nucleotide sequences of the subcloned cDNA were obtained using dideoxy sequencing (51). The final sequence of the XAP2 cDNA was determined from both DNA strands.

To obtain a full length XAP2 cDNA, a HeLa λgt10 cDNA library (kindly provided by B. Shan at the University of Texas Health Science Center) was screened with a ^{32}P-labeled, random-primed 200 bp probe (*Xho*I–*Msc*I fragment from clone X1-3) using standard protocols (52). Inserts from positive clones were subcloned into pGEM7Zf(+) and sequences were determined as described above.

β-galactosidase (β-gal) assays

Filter lift assays were performed essentially as described (49). Briefly, transformants were allowed to grow at 30°C for 2–4 days, transferred onto nitrocellulose filters, and freezed under liquid nitrogen. Filters were then placed on Whatman 3MM paper presoaked with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

(X gal) solution, incubated at 30°C, and checked periodically for production of blue color.

For quantitation of β-gal activity in yeast, liquid culture assays were done using *o*-nitrophenyl-β-D-galactoside (ONPG) as described (53).

In vitro transcription/translation and *in vitro* binding assays

To generate ^{35}S-labeled XAP2 and CyPA, pGEM-X1–3 (Fig. 2), pGEM-1-1FL (Fig. 4) or pGEM-CyPA were transcribed and translated with T7 RNA polymerase and [^{35}S]methionine in a transcription/translation coupled system (Promega). Bacterially-expressed GST protein and GST-X fusion protein were purified according to Frangioni *et al.* (54). Briefly, DH5α cells harboring either the pGSTag or the pGST-X plasmid were grown to log phase and induced with isopropyl-thio-β-D-galactoside (IPTG) for 4 h. After sonication in STE buffer [10 mM Tris–HCl (pH 8), 150 mM NaCl, 1 mM EDTA and 5 mM dithiothreitol] containing 1% sarcosyl (w/v, final concentration), solubilized proteins were recovered by centrifugation and incubated with glutathione–agarose beads in the presence of 3% Triton X-100 (final concentration) for 30 min at 4°C, and washed several times with ice-cold phosphate buffer saline (PBS).

For binding assays, beads were mixed with *in vitro* translated, ^{35}S-labeled proteins for 1 h at room temperature. Unbounded proteins were washed extensively with STE buffer containing 0.1% NP-40 and bound proteins were eluted from the beads by boiling in SDS loading buffer [50 mM Tris–HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol]. Final products were analyzed on a 15% SDS–polyacrylamide gel and detected by fluorography.

Antibodies

Polyclonal anti-X antibody prepared in rabbits againist HBV X protein expressed in insect cells was kindly provided by T. H. Lee and J. Butel (Baylor College of Medicine). Polyclonal antibodies to Gal4DBD and YY1 were obtained from Santa Cruz Biotechnology. Polyclonal antibody to GST-XAP2 fusion protein was raised in Balb-C mice as described by Oettinger *et al.* (55). Briefly, 100 µg of GST-XAP2 fusion protein bound to glutathione–agarose beads were injected into five animals intraperitoneally. Booster injections containing 50 µg of proteins were given at 15 day intervals. Forty-five days post-priming, mice were subjected to ocular-vein bleeding and sera were prepared. Antisera were evaluated for reactivity to bacterially-expressed XAP2 protein in immunoblot analysis using standard protocols (56).

Western blot analysis

Standard protocols were followed (56). Briefly, 3×10^6 HeLa cells (Fig. 4) were lysed by boiling for 5 min in sample buffer [62.5 mM Tris–HCl (pH 6.8), 1% SDS, 10% glycerol and 5% β-mercaptoethanol]. Fifty µl of the resulting extract were separated on a 15% SDS–polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. After blocking with nonfat dried milk, the membrane was treated with 1:1000 diluted XAP2 polyclonal antiserum followed by 1:7500 diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG. Subsequently, the blot was developed by 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

For each sample in Figure 6B, yeast transformants were grown at 30°C in 1 ml of selective SC medium containing 2% dextrose to an OD_{600} of 1–2. Cells were collected by centrifugation and lysates were prepared according to a standard protocol (52). One-tenth of each sample was resolved on a 12.5% SDS–polyacrylamide gel and subjected to Western blot analysis with 1:500 diluted anti-Gal4DBD antibody.

Intracellular localization of X and XAP2

HeLa cells were grown on acid-etched cover slips inside a 100 mm tissue culture plate for ~24 h and transfected with 25 µg of pECE-X plasmids as described below. Two days later, cells were washed with ice-cold PBS, and fixed with a 50:50 acetone:methanol mixture for 2 min and then treated with anti-XAP2 (1:600 dilution) and anti-X (1:500 dilution) antibodies. Cells were then incubated for 30 min at room temperature, followed by washing with PBS and further incubated with 1:200 diluted sheep anti-mouse IgG coupled with FITC (Sigma) and goat anti-rabbit IgG coupled with Texas-Red (Southern Biotechnology Associates). Subsequently, cells were subjected to extensive washings with PBS and cover slips were applied with 40% glycerol before analyzing under a Carl Zeiss confocal microscope.

Cellular fractionation

Briefly, cells were collected by scraping into PBS, centrifuged, and resuspended in 1 ml of ice cold RSB buffer [10 mM HEPES (pH 6.2), 10 mM NaCl, 1.5 mM $MgCl_2$ and 0.5 mM phenylmethylsulfonyl fluoride] with a Kontes Dounce homogenizer (10 strokes with a type A pestle). The resulting suspension was centrifuged for 10 min at 700 *g* at 4°C and the pellet was resuspended again in 1 ml of RSB buffer. After homogenization as before, the suspension was combined with the supernatant obtained from the previous step and centrifuged further for 10 min at 700 *g*. The final pellet (nuclear fraction) was washed with RSB buffer and resuspended in 2 ml of SDS sample buffer and the supernatant (cytoplasmic fraction) was combined with 2 ml of 2× SDS sample buffer. A small aliquot of each fraction was subjected to electrophoresis and Western blot analysis.

Transfection and chloramphenicol acetyltransferase (CAT) assay

HepG2 or CV1 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum. Transfections were done using the calcium phosphate method (57) which included a 1 min glycerol shock 4 h after the addition of DNA precipitate. Forty-eight hours after transfection, cells were harvested and CAT activity was determined (45) in 1 h reactions. All transfections were normalized to equal amounts of DNA with pGEM7Zf(+) or pcDNAI/Amp plasmids.

Purification of mRNA and Northern blots

Using the micro-fast track mRNA isolation kit (Invitrogen), Poly(A)$^+$ RNA was purified from normal frozen human liver tissues (obtained from the Liver Tissue Procurement and Distribution System at the University of Minnesota) and from different cell lines. Two µg of each RNA sample were then separated on a 1% agarose/formaldehyde gel and transferred onto a Hybond-N$^+$ nylon membrane using standard protocols (52). XAP2 cDNA probe was prepared with [α-^{32}P]dCTP using a

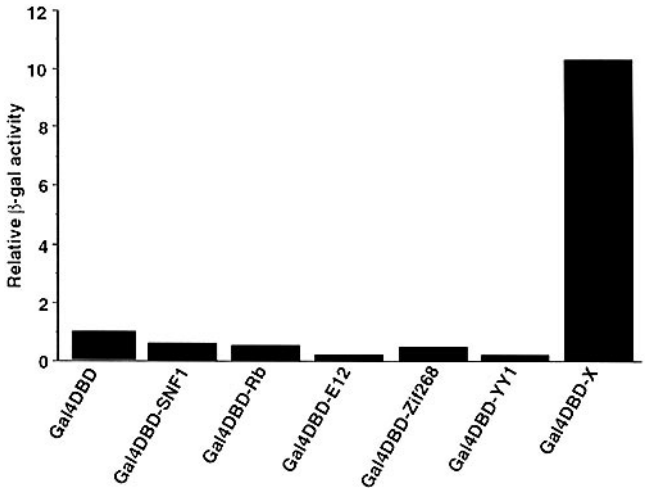

Figure 1. Specific interaction between X and XAP2 as determined by quantitative liquid β-gal assays. The numbers represent the average fold of activation above background in three independent experiments with < 5% standard deviations. Background is arbitrarily set at 1 and refers to the amount of β-gal activity observed with cotransformation of bait plasmid with Gal4DBD alone (pAS1) and the plasmid with the Gal4AD fused to XAP2 (clone X1-3). The relative amount of β-gal activity observed with cotransformation of Gal4DBD-X (pAS-X) and the plasmid with the Gal4AD in the absence of XAP2 insert is 0.73. Similar results were obtained using a full length XAP2 (clone 1-1FL fused to Gal4AD).

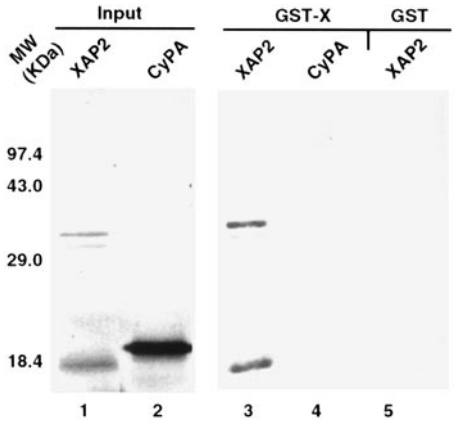

Figure 2. Representative autoradiogram of *in vitro* translated XAP2 protein captured by GST-X fusion proteins. Input lanes were loaded with one-quarter the amount of proteins used in the binding reactions. Bound proteins were eluted and analyzed by SDS–polyacrylamide gel as described in Materials and Methods. Three independent experiments yielded consistent results. The sizes of molecular weight markers are indicated to the left.

random prime kit (Stratagene). Pre-hybridization, hybridization and washing were performed under high stringency conditions before exposure to X-ray films (52). Multiple human tissue Northern blots (Clontech) were treated identically. To control for the relative amount of RNA in each lane, after hybridization with XAP2, the blots were stripped by incubation in 0.5% SDS at 95°C, and reprobed with the human β-actin cDNA.

Accession number

The nucleotide sequence data reported in this paper will appear in GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number U31913.

RESULTS

Cloning of cDNAs encoding X-associated proteins

To identify human cDNAs encoding proteins that interact with X, we used the *in vivo* two-hybrid system developed by Fields and colleagues (58,59) as modified by Durfee *et al.* (38). A gene encoding the X protein was fused to the DBD of Gal4 that did not activate transcription by itself in yeast. The Gal4DBD-X fusion gene was introduced into Y153 cells together with a human lymphoma cDNA library fused to the activation domain of Gal4. *HIS*3 expression was directly selected by plating transformants on media lacking histidine. To eliminate false positive clones, a second screen was performed with the *Lac*Z reporter gene, for which *Lac*Z transcription was monitored by growing cells in the presence of X-gal. Further, all positive clones were tested against two unrelated proteins [the retinoblastoma protein (Rb) and the yeast SNF1 protein] fused to the DBD to eliminate those that did not show specificity for the X protein.

Of 5.6×10^6 colonies screened, 51 grew in the absence of His. Out of these 51 His^+ prototrophs, 23 scored positive for β-gal activity. Though none of the plasmids recovered from the positive clones produced β-gal activity when singly transformed into yeast, 11 of the clones were active when cotransformed with plasmids encoding Gal4DBD fused either to Rb or the yeast SNF1 protein. This suggested that these 11 clones did not depend on the presence of X for activity. The remaining 12 clones were active only when cotransformed with plasmid encoding Gal4DBD fused to X, but not to Rb or SNF1.

Each of the 12 potential X-binding protein cDNAs were subcloned into a Bluescript or pGEM7Zf(+) plasmid and sequenced by the dideoxy method. Six clones, X1-3, X1-3A, X2, X3, X6 and X10, are overlapping clones of cDNA encoding the same gene [we will refer to this gene as X-Associate Protein-2 (XAP2)]. Sequence comparison of XAP2 with GenBank and EMBL databases did not reveal any homologous protein, although a small part of the cDNA resembles several partial human cDNA fragments deposited in GenBank.

X binds XAP2 specifically *in vivo* and *in vitro*

To confirm the specificity of X and XAP2 interaction, clone X1-3 which expresses the Gal4 activation domain (Gal4AD) fused to XAP2, was retransformed into Y153 cells with plasmids expressing the Gal4DBD, Gal4DBD-X or with Gal4DBD fused to five different unrelated proteins. β-Gal activity (blue color) was observed by the colony filter lift method only in the presence of a Gal4DBD-X fusion protein but not with other unrelated fusions. Also, the β-gal activity produced in the presence of Gal4DBD-X, as measured by an ONPG assay was almost 10-fold higher compared with Gal4DBD alone or compared with Gal4DBD fused to other unrelated proteins (Fig. 1).

To determine whether XAP2 binds X *in vitro*, we expressed the X protein as a fusion to glutathione *S*-transferase (GST) and used it to test for its ability to bind specifically to *in vitro* ^{35}S-labeled XAP2. As shown in Figure 2, XAP2 binds to GST-X but not GST (lanes 3 and 5). An unrelated protein, cyclophilin A (CyPA), was

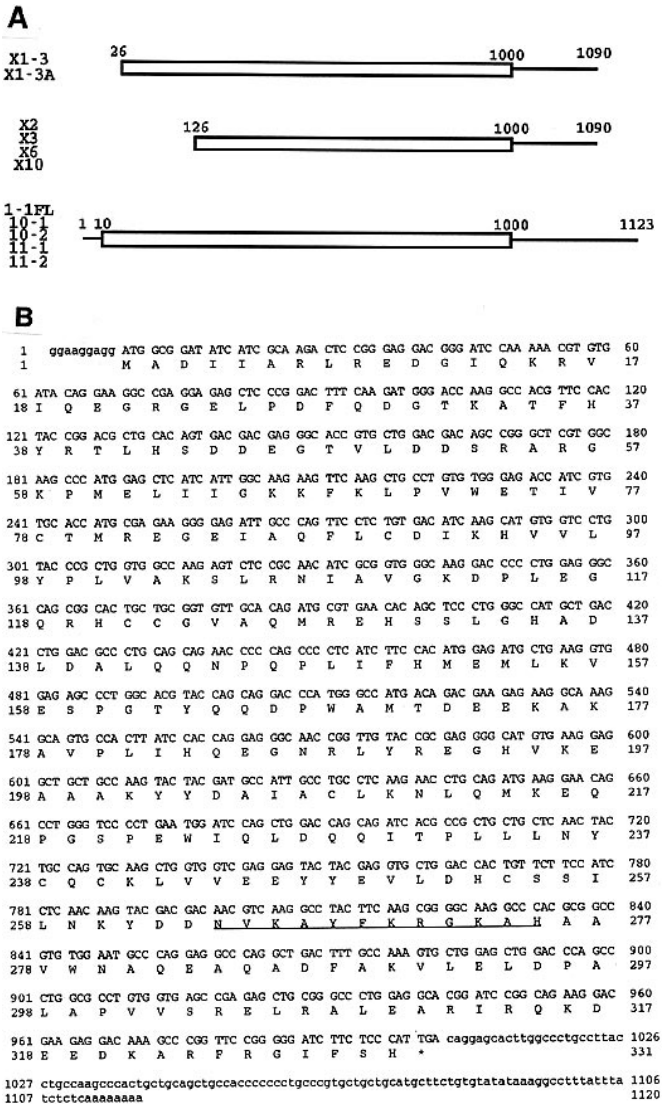

Figure 3. DNA and amino acid sequence of XAP2. (**A**) Schematic representation of the 11 XAP2 cDNA clones. The boxed region denotes the predicted open reading frame. (**B**) The entire nucleotide sequence and the deduced amino acid sequence of the XAP2 cDNA. The amino acid sequences underlined indicate the region that shares similarity with the X protein. (*) The deduced stop codon for translation. (**C**) Sequence similarity between X and XAP2. Similar amino acids are indicated with a semi colon and identical amino acids are indicated with a vertical line.

used as an additional negative control; and, as expected, CyPA did not bind GST-X in this assay (lane 4). Taken together, this suggests that X and XAP2 interact both *in vivo* and *in vitro* and the interaction is highly specific.

Isolation and analysis of full-length XAP2 cDNA

The six cDNA clones (X1-3, X1-3A, X2, X3, X6 and X10) isolated from the two-hybrid screen were divided into two

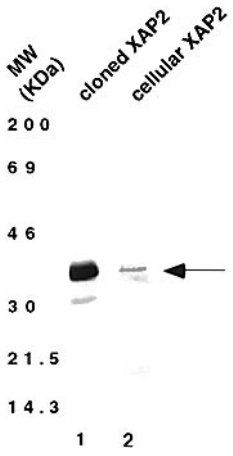

Figure 4. *In vitro* transcription/translation of XAP2 cDNA (clone 1-1FL) and Western blot analysis of native XAP2. Lane 1, vector construct containing XAP2 cDNA (pGEM-1-1FL) was used as template for coupled *in vitro* transcription/translation. The [^{35}S]methionine-labeled protein product was separated by electrophoresis and autoradiographed. Lane 2, antibody to XAP2 specifically recognizes a single 36 kDa protein that corresponds identically to the *in vitro* translated product. HeLa cell extract was separated by electrophoresis along side the *in vitro* translated product and Western blot was performed as described in Materials and Methods. The position of XAP2 in the gel and in the blot is indicated by an arrow.

independent isolates according to DNA sequences of the recombinant junctions between the cDNA and the vector (illustrated in Fig. 3A). The predicted amino acid sequence was determined by theoretical translation of the cDNA clone open reading frame (Fig. 3B). A consensus sequence for initiation of translation (60) was not found in the 5′ end of the two longest cDNAs (X1-3 and X1-3A), and the reading frame remains open at the 5′ side, suggesting that these six clones represent only a partial XAP2 coding sequence.

To obtain a full-length XAP2 cDNA, a λgt10 HeLa cDNA library was screened with a radiolabeled probe corresponding to the 5′ end of clone X1-3. Five identical cDNA clones (1-1FL, 10-1, 10-2, 11-1 and 11-2) were isolated from 10^6 phage plaques (Fig. 3A). The DNA sequence of clones is illustrated in Figure 3B. Analysis of the predicted amino acid sequence of XAP2 reveals an open reading frame of 1 kb, beginning with a Kozak consensus at nucleotide 10 and ending with a stop codon at nucleotide 1000. As shown in Figure 3C, it is noteworthy that residues 264–275 of XAP2 are similar to a region of the X protein (residues 107–118) that has previously been shown to interact with cellular proteins and is necessary for transactivation by X (61). cRNA synthesized from clone 1-1FL and translated in a rabbit reticulocyte lysate produced a protein that migrates in SDS–polyacrylamide gels as an ~36 kDa polypeptide (Fig. 4, lane 1), which is consistent with the size of HeLa XAP2 from Western blot analysis with a polyclonal anti-XAP2 antibody prepared against GST-XAP2 fusion protein expressed in bacteria (lane 2). Thus, the five newly isolated cDNAs appear to contain the entire coding region of XAP2.

X and XAP2 colocalize in mammalian cells

Although we have shown that X and XAP2 interact both in yeast cells and *in vitro*, we reasoned that if the consequence of X and

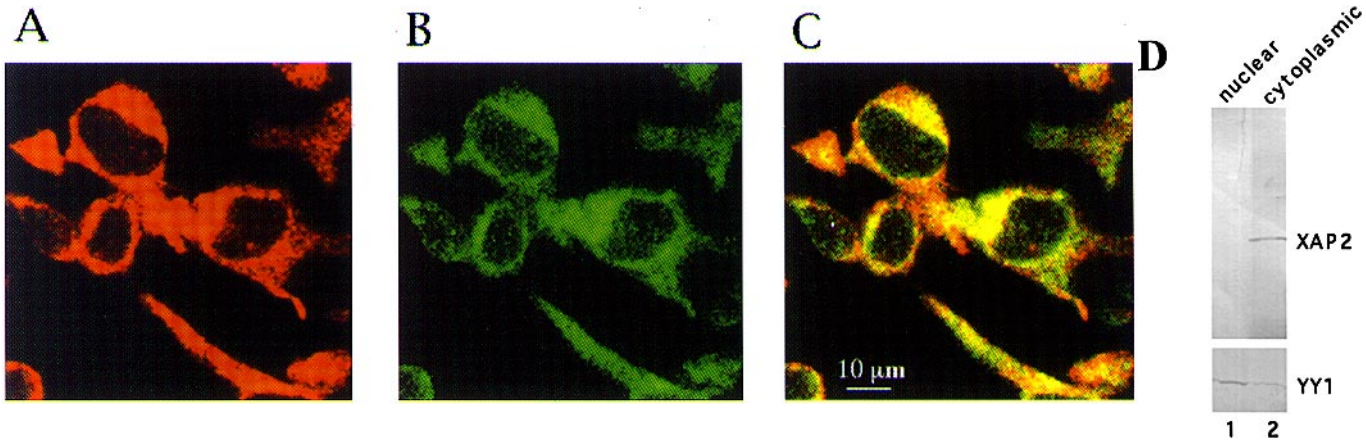

Figure 5. Colocalization of X and XAP2. Indirect immunofluorescence was used to detect X and XAP2 proteins in cultured HeLa cells. (**A**) Representative staining pattern for X protein (red) obtained with rabbit polyclonal antibody to X. No staining was seen with mock transfected cells (data not shown). (**B**) Representative staining pattern for XAP2 protein (green) in the same cells with mouse polyclonal antibody specific for XAP2. No staining was seen with preimmune sera (data not shown). (**C**) Double-staining for X and XAP2 displayed by superimposing the images shown in (A) and (B). Three independent experiments yielded consistent results, and in general ~50–75% of the cells are stained with the anti-X antibody in a given image. (**D**) Western blot analysis of XAP2 protein distribution by subcellular fractionation. Immunoblotting with an anti-YY1 antibody is shown as a control.

XAP2 interaction is biologically significant the two proteins should also colocalize in mammalian cells. The subcellular location of the X protein is controversial, ranging from cytoplasmic (16,17,62,63), nuclear (6,64), to nuclear periphery (12,65–67), or more generalized nuclear and cytoplasmic (12,66,68,69). To independently assess the subcellular location of X, HeLa cells were transiently transfected with a plasmid expressing the X protein, fixed with methanol/acetone, and immunostained with a polyclonal anti-X antibody. As shown in Figure 5A, images obtained with a confocal laser scanning system indicated that, under the present conditions, X is localized almost exclusively in the cytoplasm. Like the X protein, XAP2 was regionally dispersed throughout the cytoplasm (Fig. 5B). Interestingly, there were some distinct regions where the distribution of X and XAP2 overlapped (Fig. 5C), consistent with the observation that the two proteins physically interacted *in vivo* and *in vitro*.

Since immunostaining is sometimes prone to artifacts and different fixation methods may in many cases lead to different staining results, we decided to confirm the localization of XAP2 by subcellular fractionation. As demonstrated by Western blot (Fig. 5D), consistent with the immunostaining results, XAP2 was recovered almost entirely in the cytoplasmic fraction. To ensure the quality of the fractionation procedure, another Western blot was performed using an antibody to the transcription factor YY1. Unlike, XAP2, most of the YY1 protein was in the nuclear fraction.

Amino acids 13–26 of X are required for binding XAP2

The X protein sequences required for binding XAP2 were examined to determine whether they coincided with previously defined activation or regulatory domains. A series of C-terminal X deletion mutants, as well as internal deletion mutants, were generated and subcloned into pAS1 and tested for their abilities to interact with XAP2 in the two-hybrid system. As shown in Figure 6A and Table 1, deletion of the X C-terminal from amino acids 27 to 154 had no effect on β-gal activities, as measured by filter lift and liquid assays. However, deletions of amino acids 13–154 or 10–27 (pXD7 and pXI5) eliminated β-gal activities.

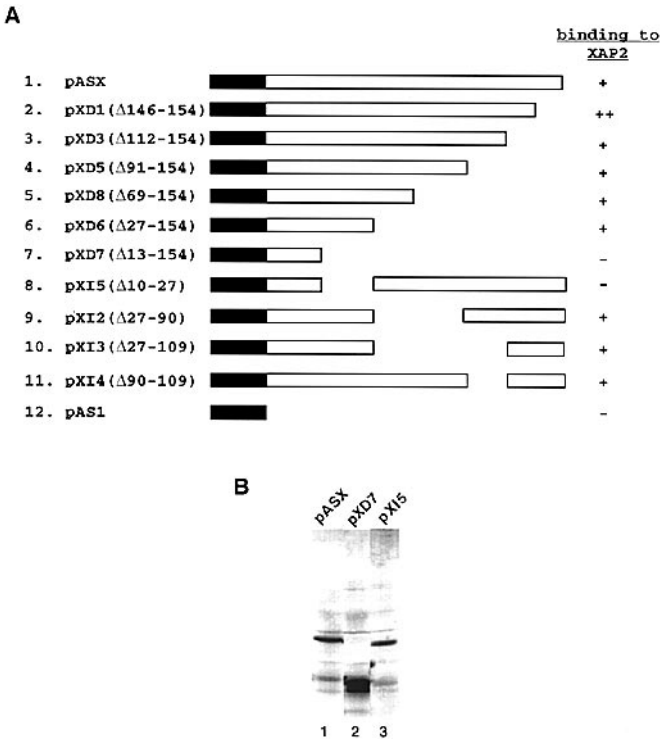

Figure 6. XAP2-binding domain in the X protein. (**A**) Schematic drawing of Gal4DBD–X fusions used to determine binding domains. The Gal4DBD (black box) is fused to various X mutants. Ability to bind XAP2 in the filter lift assay is indicated (+). (**B**) Two mutant Gal4DBD–X fusions that did not bind XAP2 were expressed as determined by Western blotting.

Three internal deletions outside of amino acids 13–27 (pXI2, pXI3 and pXI4) had no effect on β-gal activities. Western blot analysis indicated that the two mutants that did not bind XAP2 were expressed in yeast cells (Fig. 6B, lanes 2 and 3). Taken together, our data suggest that amino acids 13–26 of X are important

for binding XAP2. Interestingly, these 14 amino acids lie within a previously identified regulatory domain that represses transactivation of the X protein (70).

Table 1. Protein–protein interactions detected by the yeast two-hybrid liquid assay

Bait/prey	Gal4AD–XAP2
Gal4DBD (pAS1)	1
Gal4DBD-X (pASX)	10.9 ± 1.3
pXD1	21.3 ± 2.5
pXD3	11.3 ± 2.1
pXD5	9.1 ± 1.4
pXD8	13.6 ± 2.9
pXD6	11.9 ± 1.7
pXD7	1.2 ± 0.4
pXI5	3.0 ± 0.8
pXI2	12.4 ± 1.6
pXI3	23.4 ± 2.5
pXI4	12.3 ± 1.5

Quantitative liquid β-gal assays were performed on at least three independent experiments for each combination. The numbers represent the fold activation above background (amount of β-gal activity observed with cotransformation of the Gal4DBD vector and the prey plasmid).

Specific inhibition of X transactivation by XAP2

To determine the functional significance of X and XAP2 interaction, we tested the ability of XAP2 to influence transcription directed by an X-responsive promoter in the presence of the X protein. We constructed a plasmid that expresses XAP2 under the control of the human cytomegalovirus (CMV) immediate early promoter, cotransfected it into CV1 (Fig. 7A) or HepG2 (Fig. 7B) cells together with a plasmid that expresses the X protein and a reporter. Consistent with earlier studies (19,28,30,31,71–75), the X protein activated the human immunodeficiency virus type 1 (HIV1), simian virus 40 (SV40) and Rous sarcoma virus (RSV) promoters in CV1 (Fig. 7A, lanes 2, 6 and 10) and HepG2 cells (Fig. 7B, lanes 2 and 6). However, when XAP2 expression plasmid was added to the transfections, transcriptional activation by X was repressed (Fig. 7A, lanes 3, 7 and 11; Fig. 7B, lanes 3 and 7).

To be certain that the inhibition of transactivation by the X protein is not due to a general inhibitory effect of XAP2 overexpression, we transfected CV1 cells with pTAT6, pCMV-XAP2 and pHIVCAT. As shown, overexpression of XAP2 did not affect transcriptional activation of the HIV1 promoter by the TAT protein (Fig. 7C, compare lanes 2 and 3). Furthermore, overexpression of XAP2 had no inhibitory effect on the activation of pG5BCAT by Gal4-VP16 (compare lanes 6 and 7). Taken together, our data suggests that XAP2 is not a general cytotoxic protein but is a specific cellular inhibitor of the X protein but not other viral transactivators.

XAP2 tissue distribution

Using Northern blot analysis, we explored the tissue expression pattern of XAP2 in hope of further understanding the biological

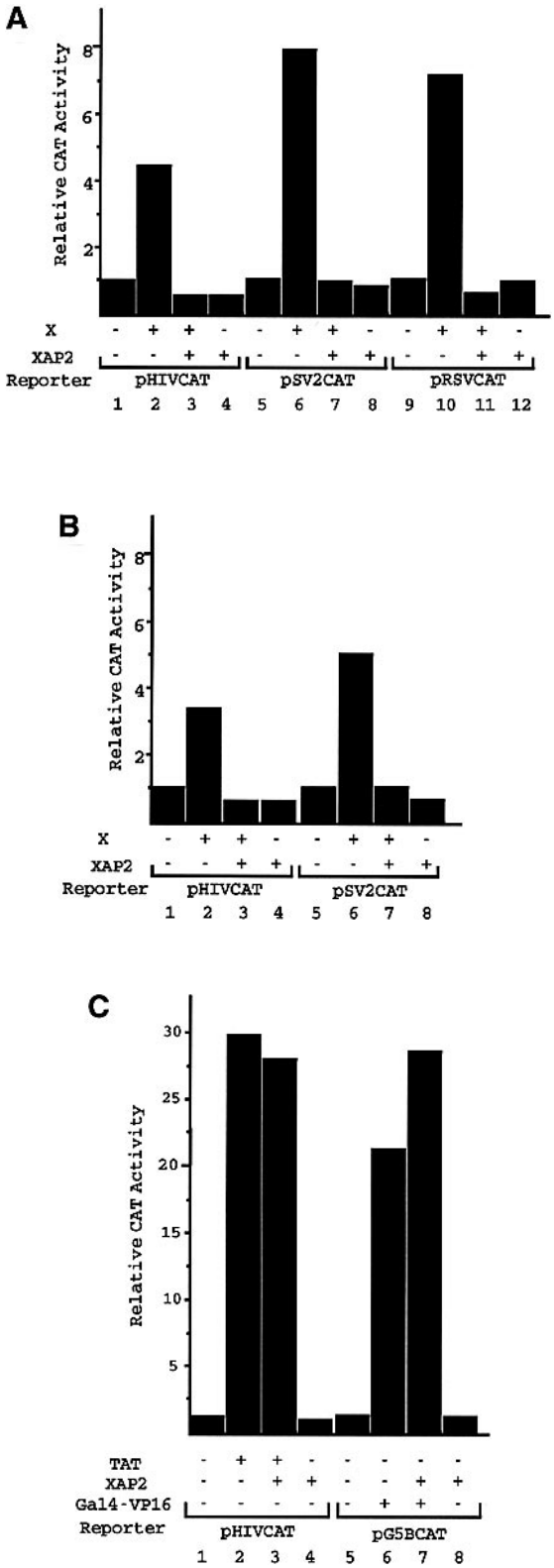

Figure 7. Repression of X transactivation by XAP2. Assay results with different CAT reporter plasmids (5 µg each) transfected into CV1 (**A** and **C**) or HepG2 (**B**) cells in the presence or absence of a plasmid encoding X (5 µg), XAP2 (10 µg), TAT (1 µg) or Gal4-VP16 (5 µg). The extent of acetylation in various reactions was determined relative to that for the CAT reporter, and results are presented as the mean of three independent transfections with <8% standard deviations.

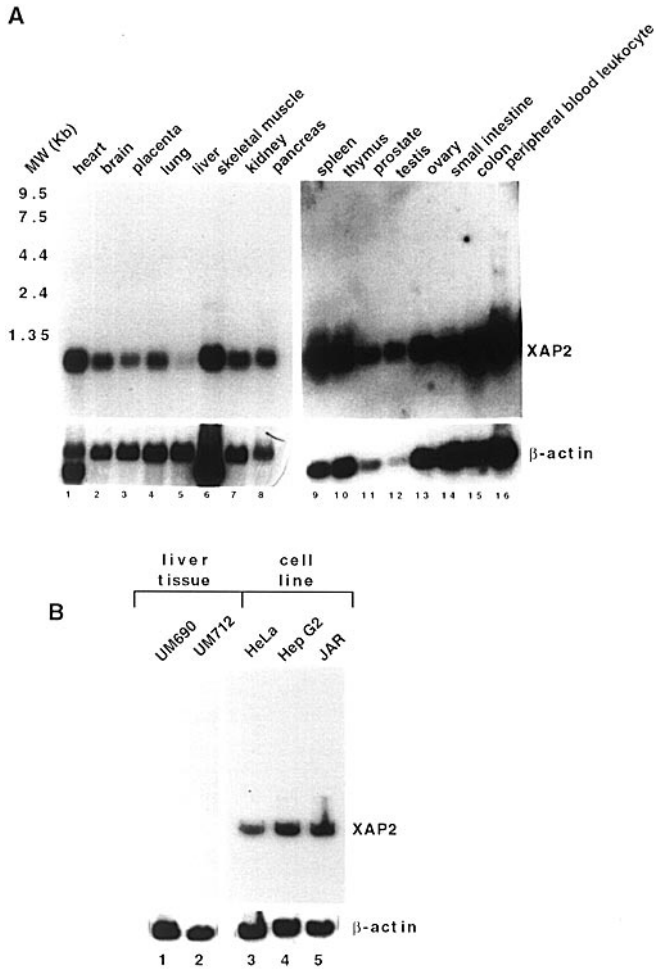

Figure 8. Northern blot analyses of poly $(A)^+$ RNA from human tissues and cell lines. Northern blot analyses of XAP2 mRNA were performed on (**A**) multiple normal human tissues; and on (**B**) normal human liver tissues (lanes 1 and 2), HeLa human cervical carcinoma cells (lane 3), Hep G2 human hepatocellular carcinoma cells (lane 4), and JAR human choriocarcinoma cells (lane 5). Hybridization to a human β-actin probe is shown as a control.

significance of X and XAP2 interaction. Interestingly, we found that a message of ~1.25 kb was present in every tissue we examined, with the exception of the liver (Fig. 8A; Fig. 8B, lanes 1 and 2). In addition, XAP2 was found to be abundantly expressed in three different cell lines including one derived from human hepatocellular carcinoma.

DISCUSSION

It has been postulated that a key mechanism by which many viral activators regulate transcription is by forming complexes with cellular factors. The HBV X protein is a promiscuous transcription activator. In order to understand its mechanism we used the HBV X gene as the bait in a yeast two-hybrid screen to identify proteins that are capable of forming heterologous complexes with X. Six clones isolated in this screen encode an identical protein that we named XAP2. Sequence comparison of XAP2 with protein sequences in the GenBank and EMBL databases indicate it is a novel protein.

Three lines of evidence confirmed that XAP2 is a genuine X-associated protein. First, we shown that XAP2 interacts only with X but not a number of other unrelated proteins in the yeast two-hybrid system. Second, using a GST-X fusion protein we obtained biochemical evidence that X and XAP2 interact *in vitro*. Finally, we used immunocytochemical methods to demonstrate similar subcellular distributions of X and XAP2 in mammalian cells.

Analysis of X mutants indicated that the interaction with XAP2 probably occurred through X residues 13–26. This N-terminal region is highly conserved among all mammalian hepadnaviruses (reviewed in ref. 18). The importance of this region with respect to transactivation is unclear. It has been reported that deletion of the N-terminal rendered the X protein incapable of transactivation of the HBV enhancer (76) but did not affect its ability to transactivate the SV40 early promoter/enhancer, the RSV long terminal repeat (30), or a hybrid SV40/HBV promoter/enhancer (70). Similarly, a finer deletion of amino acids 4–20 indicated that this region is not important in the transactivation of the SV40 early promoter/enhancer (77). However, there is evidence that the N-terminal one-third of the X protein can bind the X protein itself and repress the X transactivation function (70,78). In addition, this identical region is sensitive to transrepression as well (70). Our finding that XAP2 interacts with this particular domain raises the possibility that the X–X interaction and X transrepression function described previously is mediated by association of X and XAP2. One can imagine that XAP2 functions as a bridging protein that brings two X molecules together. Alternatively, XAP2 may compete for binding to X protein, preventing X–X dimer formation, and hence repress X transactivation.

Many cellular proteins have the ability to bind and inhibit the transactivation function of transcription activators. For examples, the MDM-2 protein can suppress p53 transactivation (79,80), the Id family proteins bind to and inhibit transcription activation by the family of basic helix–loop–helix transcription factors (81–85), p107 can suppress c-Myc transactivation (86), and a cellular protein BS69 inhibits E1A transactivation (87). Perhaps the best characterized case of suppression of transactivation is the interaction between the Rb protein, transcription factor E2F, and the adenovirus E1A protein. Studies of transcription control mediated by E1A identified a cellular DNA-binding protein, termed E2F, that recognizes key regulatory elements in many promoters (88). In the last several years, it became clear that when E2F is complexed with cellular Rb, its ability to activate transcription is prevented. Several laboratories have demonstrated that E1A can release E2F from the Rb complex, making it available to bind other proteins and hence activate transcription (89–91). Although a number of cellular proteins have been shown to bind the X protein, XAP2 is the first cellular protein identified that appears to directly inhibit X transactivation. It is possible that XAP2 has a similar function to Rb in that it forms complexes with certain transcription factors. In this case, X could then release these transcription factors from XAP2, making them available to activate transcription. In contrast, when XAP2 is abundant it would prevent the X protein from releasing transcription factors for activation. Understanding the normal function of XAP2 and its binding proteins will help resolve whether this is a novel mechanism for X transactivation.

Another possible, though not mutually exclusive, significance of the X and XAP2 interaction is that XAP2 may function as a cytoplasmically located inhibitor. Our finding that X and XAP2 colocalize in the cytoplasm of HeLa cells suggests that by

association with X, XAP2 may prevent X from entering the nucleus to activate transcription. This would be similar to the transcription factor NF-κB whose ability to activate transcription is regulated by association and dissociation with the cytoplasmically located inhibitor IκB (92,93). Experiments involving comparisons of X subcellular localization to its ability to transactivate, similar to those described by Doria *et al.* (68), as well as studies to compare XAP2 expression with X subcellular localization are now in progress.

Although computer searches of the database failed to detect any proteins with sequence identity to XAP2, a closer inspection of the XAP2 sequence revealed a region (amino acids 264–275) that is similar to a region of the X protein (amino acids 107–118). Interestingly, amino acids 105–115 of the X protein, coincidewith a site termed X1 that has been shown to interact with cellular proteins and is necessary for X transactivation (61). This observation, together with the finding that excess XAP2 can prevent X transactivation, suggests that XAP2 may inhibit X transactivation simply by sequestering cellular proteins important for X transactivation. Elucidation of the exact mechanism by which XAP2 inhibits X activity will provide insight into the molecular basis of X transactivation.

The chief biologic features of HBV infection are species specificity and relative hepatotropism (reviewed in ref. 94). In productive infections, viral antigens and DNA are found primarily within liver cells (although viral DNA sequences have been detected in low copy numbers in cells other than hepatocytes). Furthermore, successful infection of permanent cell lines by HBV has not yet been documented. Propagation of HBV in human hepatoma cell lines has been achieved with modest success only after transfection with large amounts of HBV DNA (4,95–97). Although there are probably multiple factors that contribute to HBVs hepatotropism, our finding that XAP2 is abundantly expressed in every human tissue we examined, with the exception of the liver, is intriguing. If XAP2 is complexed with X and prevents the X protein's normal function; and since XAP2 is not expressed (or expressed in a very low level) in the human liver, this may partially allow for X to contribute to the virus' hepatotropism. Second, our finding that XAP2 is present in different cell lines (including one that is derived from human hepatocellular carcinoma, Hep G2), may also partially explain why X is not essential for the viral life cycle *in vitro* but is important for viral infection *in vivo* (1–4), and why attempts to propagate HBV *in vitro* have not been successful.

While most previous studies of the X protein focused on the transcriptional activation potential of X, clearly X may also play a role in the viral life cycle (1,2) and the development of liver cancer (6,7). Our findings that XAP2 inhibits X transactivation and is expressed in low level in normal human liver, point to the possibility that XAP2 may also interfere with HBV replication and transformation *in vivo*. The cloning of XAP2 allows us now to systematically characterize the protein, and determine its role not only in X-mediated transcription activation, but also in HBV infection and transformation. Ultimately, it may be possible to target XAP2 to inactivate X as an antiviral agent for treatment of patients with chronic hepatitis B.

Note

Current sequence comparisons with the BLAST algorithm show that partial DNA sequences for XAP2 have been isolated randomly (accession numbers T17260, R50134, Z41802, F02680, F03063, R46618, T32201, H17133, Z46173 and R50188). No function for these partial cDNAs has been described.

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REFERENCES

1 Chen, H. S., Kaneko, S., Girones, R., Anderson, R. W., Hornbuckle, W. E., Tennant, B. C., Cote, P. J., Gerin, J. L., Purcell, R. H., and Miller, R. H. (1993) *J. Virol.* **67**, 1218–1226.
2 Zoulim, F., Saputelli, J., and Seeger, C. (1994) *J. Virol.* **68**, 2026–2030.
3 Blum, H. E., Zhang, Z.-S., Galun, E., von Weizsacker, F., Garner, B., Liang, T. J., and Wands, J. R. (1992) *J. Virol.* **66**, 1223–1227.
4 Yaginuma, K., Shirakata, Y., Kobayashi, M., and Koike, K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2678–2682.
5 Shirakata, Y., Kawada, M., Fujiki, Y., Sano, H., Oda, M., Yaginuma, K., Kobayashi, M., and Koike, K. (1989) *Jpn J. Cancer Res.* **80**, 617–621.
6 Hshne, M., Schaefer, S., Seifer, M., Feitelson, M. A., Paul, D., and Gerlich, W. H. (1990) *EMBO J.* **9**, 1137–1145.
7 Kim, C. M., Koike, K., Saito, I., Miyamura, T., and Jay, G. (1991) *Nature* **351**, 317–320.
8 Gough, N. M. (1983) *J. Mol. Biol.* **165**, 683–699.
9 Guo, W., Wang, J., Tam, G., Yen, T. S. B., and Ou, J. (1991) *Virology* **181**, 630–636.
10 Zelent, A., Sells, M. A., Price, P. M., Mohamad, A., Acs, G., and Christman, J. K. (1987) *J. Virol.* **61**, 1108–1115.
11 Kay, A., Mandart, E., Trepo, C., and Galibert, F. (1985) *EMBO J.* **5,** 1287–1292.
12 Levrero, M., Jean-Jean, O., Balsano, C., Will, H., and Perricaudet, M. (1990) *Virology* **174**, 299–304.
13 Feitelson, M. A., and Clayton, M. M. (1990) *Gastroenterology* **99**, 500–507.
14 Moriarty, A. M., Alexander, H., Lerner, R., and Thornton, G. B. (1985) *Science* **227**, 429–433.
15 Vitvitski-Trepo, L., Kay, A., Pichoud, C., Chevallier, P., De-Dinechin, S., Shamoon, B. M., Mandart, E., Trepo, C., and Galibert, F. (1990) *Hepatology* **12**, 1278–1283.
16 Katayama, K., Hayashi, N., Sasaki, Y., Kasahara, A., Ueda, K., Fusamoto, H., Sato, N., Chisaka, O., Matsubara, K., and Takenobu, K. (1989) *Gastroenterology* **97**, 990–998.
17 Vitvitski, L., Meyers, M. L., Sninsky, J. J., Berthillon, P., Chevalier, P., Sells, M. A., Acs, G., and Trepo, C. (1988) In Zuckerman, A. J. (ed.), *Viral Hepatitis and Liver Disease.* Alan R. Liss, New York, pp 341–344.
18 Rossner, M. T. (1992) *J. Med. Virol.* **36**, 101–117.
19 Aufiero, B., and Schneider, R. J. (1990) *EMBO J.* **9**, 497–504.
20 Cross, J. C., Wen, P., and Rutter, W. J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8078–8082.
21 Kekul, A. S., Lauer, U., Weiss, L., Luber, B., and Hofschneider, P. H. (1993) *Nature* **361**, 742–745.
22 Lucito, R., and Schneider, R. J. (1992) *J. Virol.* **66**, 983–991.
23 Takada, S., Kido, H., Fukutomi, A., Mori, T., and Koike, K. (1994) *Oncogene* **9**, 341–348.
24 Haviv, I., Vaizel, D., and Shaul, Y. (1995) *Mol. Cell. Biol.* **15**, 1079–1085.
25 Maguire, H. F., Hoeffler, J. P., and Siddiqui, A. (1991) *Science* **252**, 842–844.
26 Seto, E., Mitchell, P. J., and Yen, T. S. B. (1990) *Nature* **344**, 72–74.
27 Unger, T., and Shaul, Y. (1990) *EMBO J.* **9**, 1889–1895.
28 Seto, E., Zhou, D. X., Peterlin, B. M., and Yen, T. S. B. (1989) *Virology* **173**, 764–766.
29 Jameel, S., Siddiqui, A., Maguire, H. F., and Rao, K. V. S. (1990) *J. Virol.* **64**, 3963–3966.

30 Ritter, S. E., Whitten, T. M., Quets, A. T., and Schloemer, R. H. (1991) *Virology* **182**, 841–845.
31 Twu, J .S., Wu, J. Y., and Robinson, W. S. (1990) *Virology* **177**, 406–410.
32 Cheong, J., Yi, M., Lin, Y., and Murakami, S. (1995) *EMBO J.* **14**, 143–150.
33 Feitelson, M. A., Zhu, M., Duan, L. X., and London, W. T. (1993) *Oncogene* **8**, 1109–1117.
34 Qadri, I., Maguire, H. F., and Siddiqui, A. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 1003–1007.
35 Seto, E., and Yen, T. S. B. (1991) *J. Virol.* **65**, 2351–2356.
36 Lee, T. H., Elledge, S. J., and Butel, J. S. (1995) *J. Virol.* **69**, 1107–1114.
37 Elledge, S. J., Mulligan, J. T., Ramer, S. W., Spottswood, M., and Davis, R. W. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1731–1735.
38 Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., and Elledge, S. J. (1993) *Genes Dev.* **7**, 555–569.
39 Valenzuela, P., Quiroga, M., Zaldivar, J., Gray, W., and Rutter, W. J. (1980) In Fields, B. N., Jaenisch, R., and Fox, C. F. (eds), *Animal Virus Genetics.* Academic Press, New York, pp 57–70.
40 Inouye, C. J., and Seto, E. (1994) *J. Biol. Chem.* **269**, 6506–6510.
41 Ron, D., and Dressler, H. (1992) *Biotechniques* **13**, 866–869.
42 Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993) *Cell* **73,** 1067–1078.
43 Seto, E., Yen, T. S. B., Peterlin, B. M., and Ou, J. H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8286–8290.
44 Gorman, C. M., Merlino, G. T., Willingham, M. C., Pastan, I., and Howard, B. H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6777–6781.
45 Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
46 Peterlin, B. M., Luciw, P. A., Barr, P. J., and Walker, M. D. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9734–9738.
47 Lillie, J., and Green, M. R. (1989) *Nature* **338**, 39–44.
48 Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988) *Nature* **335**, 563–564.
49 Breeden, L., and Nasmyth, K. (1985) *Cold Spring Harbor Symp. Quant. Biol.* **50**, 643–650.
50 Becker, D. M., and Guarente, L. (1991) *Methods Enzymol.* **194**, 182–187.
51 Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
52 Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
53 Guarente, L. (1983) *Methods Enzymol.* **101**, 181–191.
54 Frangioni, J. V., and Neel, B. G. (1993) *Anal. Biochem.* **210**, 179–187.
55 Oettinger, H. F., Pasqualini, R., and Bernfield, M. (1992) *BioTechniques* **12**, 544–549.
56 Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
57 Graham, F., and van der Eb, A. (1973) *Virology* **52**, 456–457.
58 Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9578–9582.
59 Fields, S., and Song, O. (1989) *Nature* **340**, 245–246.
60 Kozak, M. (1984) *Nucleic Acids Res.* **12**, 857–872.
61 Takada, S., and Koike, K. (1994) *Virology* **205**, 503–510.
62 Lee, T. H., Finegold, M. J., Shen, R. F., DeMayo, J. L., Woo, S. L. C., and Butel, J. S. (1990) *J. Virol.* **64**, 5939–5947.
63 Zheng, Y. W., Riegler, J., Wu, J., and Yen, T. S. B. (1994) *J. Biol. Chem.* **269**, 22593–22598.
64 Seifer, M., Heermann, K. H., and Gerlich, W. H. (1990) *Virology* **179**, 287–299.
65 Chisaka, O., Araki, K., Ochiya, T., Tsurimoto, T., Hiranyawasitte-Attatip-paholkun, W., Yanaihara, N., and Matsubara, K. (1987) *Gene* **60**, 183–189.
66 Schek, N., Bartenschlager, R., Kuhn, C., and Schaller, H. (1991) *Oncogene* **6**, 1735–1744.
67 Siddiqui, A., Jameel, S., and Mapoles, J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2513–2517.
68 Doria, M., Klein, N., Lucito, R., and Schneider, R. J. (1995) *EMBO J.* **14**, 4747–4757.
69 Hu, K. Q., Vierling, J. M., and Siddiqui, A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7140–7144.
70 Murakami, S., Cheong, J., and Kaneko, S. (1994) *J. Biol. Chem.* **269**, 15118–15123.
71 Siddiqui, A., Gaynor, R., Srinivasan, A., Mapoles, J., and Farr, R. W. (1989) *Virology* **169**, 479–484.
72 Spandau, D. F., and Lee, C. H. (1988) *J. Virol.* **62**, 427–434.
73 Takada, S., and Koike, K. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5628–5632.
74 Twu, J. S., and Robinson, W. S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2046–2050.
75 Zahm, P., Hofschneider, P. H., and Koshy, R. (1988) *Oncogene* **3**, 169–177.
76 Faktor, O., and Shaul, Y. (1990) *Oncogene* **5**, 867–872.
77 Arii, M., Takada, S., and Koike, K. (1992) *Oncogene* **7**, 397–403.
78 Lin, M. H., and Lo, S. J. (1989) *Biochem. Biophys. Res. Comunm.* **164**, 14–21.
79 Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992) *Cell* **69**, 1237–1245.
80 Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993) *Nature* **362**, 857–860.
81 Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) *Cell* **61**, 49–59.
82 Cordle, S. R., Henderson, E., Masuoka, H., Weil, P. A., and Stein, R. (1991) *Mol. Cell. Biol.* **11**, 1734–1738.
83 Jen, Y., Weintraub, H., and Benezra, R. (1992) *Genes Dev.* **6**, 1466–1479.
84 Kreider, B., Benezra, R., Roverea, G., and Kadesch, T. (1992) *Science* **255**, 1700–1702.
85 Wilson, R. B., Kledjian, M., Shen, C. P., Benezra, R., Zwollo, P., Dymecki, S. M., Desiderio, S. V., and Kadesch, T. (1991) *Mol. Cell. Biol.* **11**, 6185–6191.
86 Beijersbergen, R. L., Hijmans, E. M., Zhu, L., and Bernards, R. (1994) *EMBO J.* **13**, 4080–4086.
87 Hateboer, G., Gennissen, A., Ramos, Y. F. M., Kerkhoven, R. M., Sonntag-Buck, V., Stunnenberg, H. G., and Bernards, R. (1995) *EMBO J.* **14**, 3159–3169.
88 Kovesdi, I., Reichel, R., and Nevins, J. R. (1986) *Cell* **45**, 219–228.
89 Bagchi, S., Weinmann, R., and Raychaudhuri, P. (1991) *Cell* **65**, 1063–1072.
90 Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991) *Cell* **65,** 1053–1061.
91 Chittenden, T., Livingston, D. M., and Kaelin, W. G. (1991) *Cell* **65**, 1073–1082.
92 Baeuerle, P. A., and Baltimore, D. (1988) *Cell* **53**, 211–217.
93 Baeuerle, P. A., and Baltimore, D. (1988) *Science* **242**, 540–546.
94 Ganem, D., and Varmus, H. E. (1987) *Annu. Rev. Biochem.* **56**, 651–693.
95 Acs, G., Sells, M. A., Purcell, R. H., Price, P., Engle, R., Shapiro, M., and Popper, H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4641–4644.
96 Sureau, C., Romet-Lemonne, J. L., Mullins, J. I., and Essex, M. (1987) *Cell* **47**, 37–47.
97 Tsurimoto, T., Fujiyama, A., and Matsubara, K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 444–448.