

HBV X Protein Targets HIV Tat-Binding Protein 1

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The HBV X protein (HBx) is implicated in infection and development of hepatocellular carcinoma. HBx has a pleiotropic effect on cells, suggesting multiple targets in the virus–host cell interaction. We employed the cytoplasmic-based two-hybrid screen and identified the HIV Tat-binding protein 1 (Tbp1) as a novel HBx interacting protein. Tbp1 interacts *in vivo* with HBx both in yeast and in animal cells. This interaction maps to the functionally important ATP-binding motif of Tbp1. Furthermore, HBx and Tbp1 interaction is functionally significant and regulates HBV transcription. Tbp1 homologues, such as Sug1, are known members of the proteasome 19S regulatory cap particle and have also been implicated in transcription coactivation. Remarkably, Tbp1 and Sug1 interact with multiple viral effector proteins including HIV Tat, SV40 large T antigen, and adenovirus E1A, establishing these proteins as important targets of the viral oncogenes. © 2001 Academic Press

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INTRODUCTION

The Hepatitis B X protein (HBx) has been implicated in HBV infection and development of hepatocellular carcinoma (Cromlish, 1996). At the cellular level, one major role attributed to HBx is that of a coactivator of transcription. In the presence of a potent transcription activator, HBx increases transcription by many folds (Haviv *et al.*, 1995). Moreover, HBx is able to abrogate transcriptional squelching, whereby overexpression of activators inhibit transcription. Biochemical interaction assays were very helpful in elucidating the mechanism by which HBx coactivates transcription. These studies revealed that HBx binds to several components of the basal transcription complex including TFIIB (Lin *et al.*, 1997; Haviv *et al.*, 1998b), the RPB5 subunit of RNA polymerase II (Cheong *et al.*, 1995), and TFIIF (Wang *et al.*, 1994; Haviv *et al.*, 1996; Qadri *et al.*, 1996). HBx binds transcription activators such as CREB and other basic region/leucine zipper-containing factors (Maguire *et al.*, 1991; Williams and Andrisani, 1995; Perini *et al.*, 1999) and Vp16 (Haviv *et al.*, 1996) as well. It has been proposed that HBx minimizes the requirements of the general transcription factors and enables transcription in the absence of essential factors including, TAFII250 and TFIIA (Haviv *et al.*, 1996, 1998a). The ability to activate a minimal transcription complex would prove very advantageous to a virus dependent on the cellular transcriptional machinery for replication.

The cytoplasm also represents a significant site to where a number of HBx interacting proteins have been localized. This suggests a role for HBx outside of the nucleus as a possible activator of signaling pathways or in proteasomal regulation. For example, it has been reported that HBx binds to I κ -B (Weil *et al.*, 1999), a component of NF κ -B signaling. Other signaling pathways such as the MAP kinase pathway have also been suggested as targets of HBx (Klein and Schneider, 1997). Recently, studies have shown a component of the 20S core of the proteasome to be a physical target of (Huang *et al.*, 1996) and regulated by (Hu *et al.*, 1999) HBx.

In an attempt to clarify the role of or to identify new roles for HBx, the two-hybrid screen has been utilized for the investigation of HBx-associated proteins (XAPs). To date, the interactors identified include a DNA repair protein (Lee *et al.*, 1995), a component of the arylhydrocarbon receptor complex (Kuzhandaivelu *et al.*, 1996), a PKC binding protein (Cong *et al.*, 1997), and a component of the 20S core of the proteasome (Huang *et al.*, 1996). The relevance of these interactors to HBx function is still under investigation.

In this study we attempt to discover new interacting proteins using a novel type of two-hybrid-like screen called the Sos recruitment system (SRS) (Aronheim *et al.*, 1997). The SRS is based on the rescue of a temperature-sensitive yeast strain with a mutation in *cdc25* by membrane localization of Sos, the human homologue of *cdc25*, thus reconstituting the ras signaling pathway. This membrane localization is dependent on the interaction of a Sos–bait fusion protein and a membrane-localized prey protein expressed from a cDNA library (Fig. 1). SRS is different from the classic two-hybrid (Fields and

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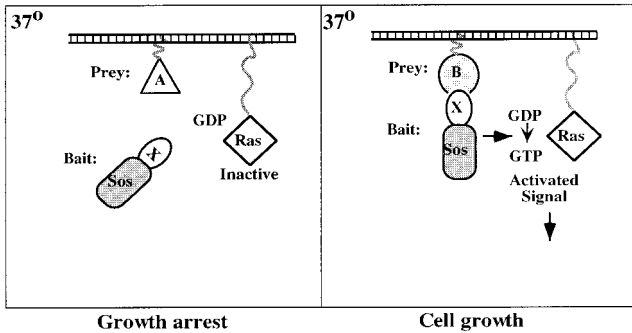


FIG. 1. Schematic representation of the Sos recruitment system. The SRS employs the temperature-sensitive *cdc25-2 S. cerevisiae* strain containing a point mutation in the *cdc25* Guanine nucleotide exchange factor (GEF). Yeast transformed with a bait chimera consisting of a bait protein fused to Sos, the human homologue of *cdc25*. Yeast cells were also transformed with the prey cDNA library fused to a membrane localization signal. A lack of interaction between bait and prey (left) fails to localize the Sos–bait construct to the plasma membrane, leading to cell growth arrest at the restrictive temperature. A stable interaction between the Sos–bait and prey (right) results in localization of bait with Sos to membrane, thus reconstituting Ras signaling and conferring cell growth upon these colonies at the restrictive temperature.

Song, 1989) in two respects that may reduce false-positives. First, it is cytoplasmic rather than nuclear, so a primarily nuclear bait brought out to the cytoplasm, a foreign ionic environment, would select for only the strongest of interactions and would also reduce the bridging phenomenon that may occur via endogenous yeast proteins. Second, it relies on a temperature-resistant survival readout rather than a white–blue colony transcription-based readout. The use of a transcriptional coactivator such as HBx in a transcriptionally driven readout may increase false-positives as well as fall subject to interference by endogenous factors.

Our SRS screen resulted in the isolation of two specific HBx interacting clones. The first was a novel gene that we call XAP8, a putative transcription coactivator, which will be described in a future publication. The second clone isolate contained a complete ORF identical to the previously isolated Tat-binding protein 1 (Tbp1). Tbp1 was isolated in a screen for proteins interacting with the HIV Tat protein (Nelbock *et al.*, 1990). It was originally described as a transcription factor and was later characterized as a member of the 19S regulatory cap associated with the 26S proteasome (Schnall *et al.*, 1994). While they are associated with the proteasome, their role may not be strictly proteasomal. The 19S cap proteins including Tbp1 and Sug1 have been implicated in transcription (Kim *et al.*, 1994; Rubin *et al.*, 1996; vom Baur *et al.*, 1996; Makino *et al.*, 1997, 1999; Russell *et al.*, 1999a) independent of their role in the proteasome. Furthermore, Tbp1 was recently described as a tumor suppressor-like protein causing a reversion in the transformant phenotype (Park *et al.*, 1999). Finally, together Sug1 and Tbp1 are targets of multiple viral effector proteins,

including HIV Tat (Nelbock *et al.*, 1990), SV40 large T, and adenovirus E1A (Grand *et al.*, 1999), establishing these proteins as some of the few cellular components targeted by multiple viruses.

We show in this study that Tbp1 is indeed a specific target of HBx in both the yeast and *in vivo* coimmunoprecipitation experiments. Using functionally deficient mutants we were able to map the interacting regions between the two proteins. Furthermore, via Northern blot analysis of HBV genomic transcripts, we show a functional interaction of wild-type Tbp1 with HBx, resulting in an increase in HBx-specific transcription, which may prove critical to the virus life cycle. By incorporating the mutant Tbp1 constructs into the functional experiments, we show that the non-HBx-binding Tbp1 mutants have a negligible effect on HBx-mediated transcription. Together, these data support the role of Tbp1 as a novel target of HBx in the HBV virus–host cell interaction, with possible implications for HBx-mediated transcription and cellular transformation.

RESULTS

HBx–Sos chimera expression in the *cdc25-2* yeast strain

The HBx ORF was cloned into the bait vector in two orientations, either 5′ or 3′ to the Sos fusion ORF (Fig. 2A). Since establishment of a properly expressed fusion gene is primarily empirical, we assayed expression of each of the bait by phenotypic rescue with a Sos-dependent prey and by Western analysis of the bait-containing yeast lysates. For phenotypic rescue, we employed the galactose-induced pYes2-M#7, a Sos-dependent Ras clone that is localized to the plasma membrane via a hydrophobic myristyl group and requires Sos exchange activity to rescue *cdc25-2* mutation (Aronheim, 1997). The *cdc25-2* yeast strain was cotransformed with pYes2-M#7 along with the two bait clones having HBx in both orientations with respect to Sos (Fig. 2B, top). At 37°C, we obtained rescue of the temperature-sensitive phenotype in a galactose-expressed pYes2-M#7-dependent manner with both baits. Repression of pYes2-M#7 expression by growth on glucose abrogates the rescue.

We then prepared total cell lysate from yeast transformed with either one of the HBx fusion baits or a Sos-alone bait to perform Western analysis of bait expression. Equal amounts of cell lysate were fractionated by SDS–PAGE and immunoblotted using a monoclonal α -HBx antibody (Fig. 2B, bottom). Comparison of the two HBx bait constructs with respect to the negative control, Sos alone, revealed that HBx fused 3′ to the Sos ORF shows much higher expression than HBx in the 5′ orientation to Sos. We concluded that, while expression levels of both constructs were sufficient for rescue by a highly specific Sos-binding prey clone, actual levels of expression from each construct differed greatly. In this

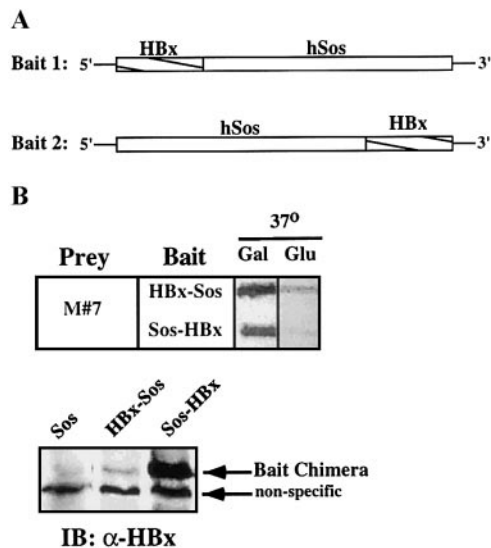


FIG. 2. Schematic and expression of chimeric Sos/ HBx constructs in *cdc25-2 S. cerevisiae*. (A) Schematic representation of the two baits constructed for screening. Bait 1 (HBx-Sos) contained the complete ORF for HBx cloned 5' to hSos (human homologue of *cdc25*) while bait 2 (Sos-HBx) contained the HBx ORF 3' to hSos. (B) Yeast transformed with either HBx-Sos or Sos-HBx constructs assayed for expression. Expression from both plasmids is sufficient for rescue in the context of a Sos-specific prey, M#7, allowing rescue at the restrictive temperature in a galactose-dependent manner (B, top). Western blot of yeast lysates indicates detectable expression of Sos-HBx, but not HBx-Sos or Sos alone (B, bottom). The blot was probed with monoclonal anti-HBx antibodies (Haviv *et al.*, 1998b).

study, the bait with HBx fused to the 3' end of Sos was employed for the subsequent cDNA library screen.

Isolation and identification of two putative HBx targets

A HeLa cell cDNA library was screened for HBx interacting genes. In total, 400,000 transformants were scanned. Briefly, *cdc25-2* yeast were serially transformed first with the Sos-HBx bait followed by the HeLa cDNA library and plated at room temperature on glucose containing selective media. Colonies were then replicate plated at the restrictive temperature of 37°C onto either glucose or galactose containing media. Colonies growing on galactose but not glucose were selected and cDNA plasmids were isolated from the yeast and shuttled to bacteria. Upon retransformation into yeast, these two clones exhibited growth in a galactose-dependent manner at 37°C in the context of the Sos-HBx bait (Fig. 3). Moreover, they did not grow in the context of the Sos-alone bait, indicating that the interaction was specific to HBx and not Sos. Interestingly, the isolated clones failed to interact and hence to rescue the Sos bait containing the HBx at the N-terminus. This might be due to the poor HBx-Sos production (Fig. 2) or, alternatively, to the specific interaction of the HBx C-terminus that is sterically hindered in HBx-Sos. In addition to these pu-

tative HBx interacting proteins, we also isolated a clone that exhibited bait-independent growth (data not shown). The sequence of this clone revealed a small G-protein, rit, a known false-positive of the SRS screen. These data satisfy the screening requirements for a putative HBx interacting protein for both clones 1 and 2.

Phenotypic rescue is dependent on HBx and not other nonspecific transcription factors

To ensure the specificity of the interaction between HBx and clone 2 within the SRS screen, the *cdc25-2* strain was cotransformed with a number of baits and clone 2. We employed HBx-Sos and Sos alone as well as a number of other genes fused to Sos, including Jun, Jun activation domain, Creb, JDP2, and GHF1 as nonspecific (Aronheim *et al.*, 1997). These baits were selected for their roles as transcription factors, a known function of HBx (Haviv *et al.*, 1996, 1998a,b). While colonies from all of the cotransformed yeast grew on both glucose and galactose at room temperature, indicating that these constructs did not alone inhibit growth (Fig. 4A, top panels), only the HBx-expressing colony rescued the temperature-sensitive phenotype in a galactose-specific manner (Fig. 4A, bottom panels). We concluded that by repeatedly and specifically rescuing the temperature-sensitive phenotype, clone 2 and Sos-HBx interact within the *cdc25-2* yeast strain.

Upon sequencing of the putative HBx interacting protein, clone 2, which was isolated twice in the screen, was found to contain a complete ORF of about 1.3 kb, identical to a previously isolated gene, Tat binding protein 1 (Nelbock *et al.*, 1990). Tbp1 was previously isolated in a phage expression screen for proteins interacting with recombinant HIV Tat (Nelbock *et al.*, 1990). It was found to be a nuclear protein with functional and structural roles in transcription (Nelbock *et al.*, 1990; Ohana *et al.*, 1993) and the 19S cap of the proteasome (Schnall *et al.*,

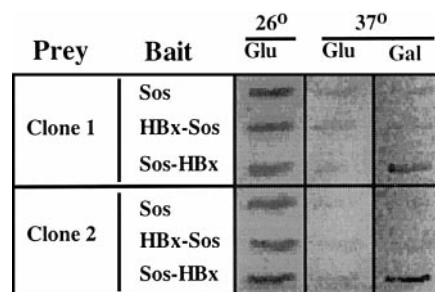


FIG. 3. Rescue of the temperature-sensitive phenotype by the clones isolated from HBx-binding genetic screen. Plasmids from positive colonies were purified and retransformed into naive *cdc25-2* yeast with Sos, HBx-Sos, or Sos-HBx as bait. Yeast were then replica plated onto glucose and galactose and grown at the restrictive temperature. Clone 1 and clone 2 rescued the temperature-sensitive phenotype of *cdc25-2* strain in a Sos-HBx-specific manner, but failed to rescue HBx-Sos or Sos alone baits. This rescue is in a galactose-specific manner.

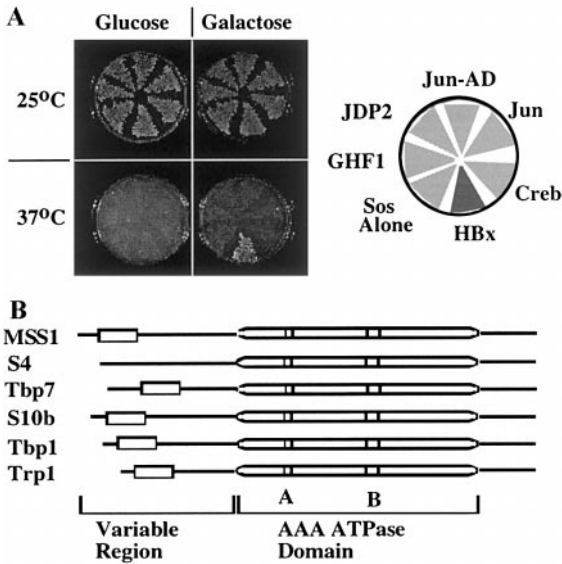


FIG. 4. Clone 2 interacts specifically with HBx, but not other transcription factors. (A) Naive *cdc25-2* yeast were cotransformed with the isolated clone 2 and various chimeric bait constructs including HBx, Jun, Jun activation domain, CREB, JDP2, GHF1, and Sos alone. The employed baits had been shown previously to express in yeast (data not shown). Transformants were replica plated onto glucose and galactose at 25°C and 37°C. The only bait that confers temperature resistance upon the yeast on galactose at the restrictive temperature, 37°C, is HBx. This indicates a specific interaction between clone 2 and HBx, but not other nonspecific bait constructs. (B) A schematic of the AAA motif containing components of the 19S cap of which Tbp1, the putative HBx interacting protein, is a member. Box A contains the ATPase domain and box B the DExD helicase domain.

1994). Tbp1 was shown to be one of the six components of the 19S cap belonging to the AAA gene family (Fig. 4B) (Beyer, 1997).

Flag-HBx and HA-Tbp1 interact in cell extracts

Having established a genetic interaction in the yeast between HBx and Tbp1, we then assayed for interaction between the two proteins in a mammalian system. To this end we cloned Tbp1 into the pSG5 mammalian expression vector. We included an N-terminal hemagglutinin (HA) epitope tag in order to detect expression of Tbp1. We cotransfected this Tbp1 construct along with an epitope-tagged Flag-HBx into the 293T human kidney cell line. Following 60 h incubation, cells were collected, fractionated into nuclear and cytoplasmic fractions, and incubated with a monoclonal antibody specific to the flag epitope tag. After stringent washing, the samples were fractionated by SDS-PAGE and immunoblotted with a monoclonal antibody specific for the HA epitope on Tbp1.

The monoclonal anti-HA antibody proved very specific for detecting in the samples transfected with HA-Tbp1 only a 45-kDa band in the nuclear fractions, but in the cytoplasmic fraction detected some nonspecific bands as well (Fig. 5). A distinct band at around 45 kDa correlating with the size of Tbp1 was coimmunoprecipitated in the nuclear but not cytoplasmic fraction (Fig. 5, lane 8). These results indicate specific HA-Tbp1 interaction with

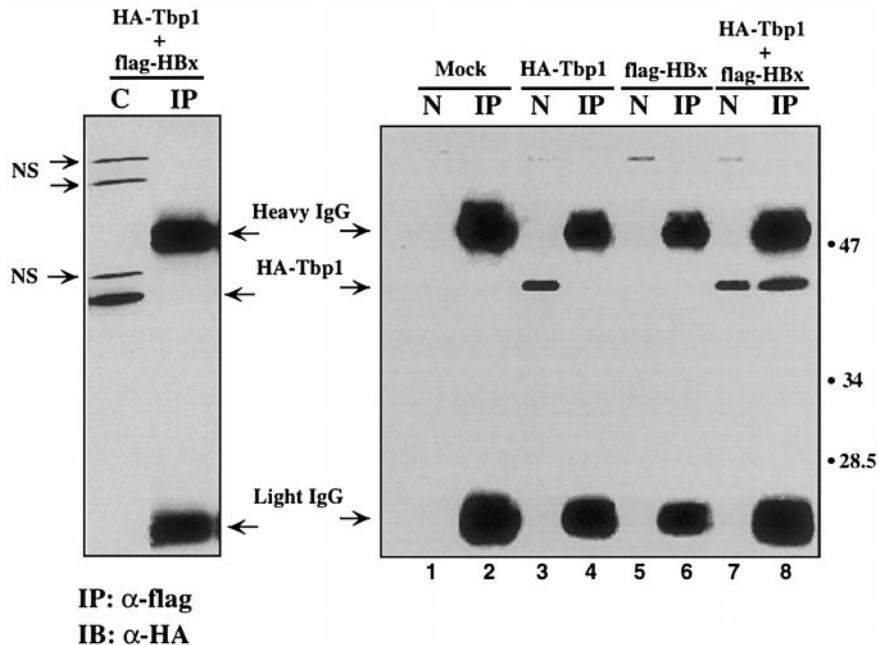


FIG. 5. HA-Tbp1 coimmunoprecipitates with Flag-HBx. 293T cells were transfected with various combinations of HA-Tbp1 and Flag-HBx. Lysates (2 mg) were then subjected to immunoprecipitation with the monoclonal M2 anti-flag beads (Kodak). Cytoplasmic (C), nuclear (N) lysates (100 μ g of each), and immunoprecipitates (IP) were then analyzed by Western blot probed with a monoclonal anti-HA antibody. HA-Tbp1 alone was not immunoprecipitated by the M2 beads (lane 4). However, cotransfection of HA-Tbp1 with Flag-HBx resulted in successful coimmunoprecipitation of HA-Tbp1 (lane 8). Negative controls include mock transfection (lanes 1 and 2) and Flag-HBx alone (lanes 5 and 6). The positions of the antibody chains used for immunoprecipitation are indicated. NS, nonspecific reactive bands.

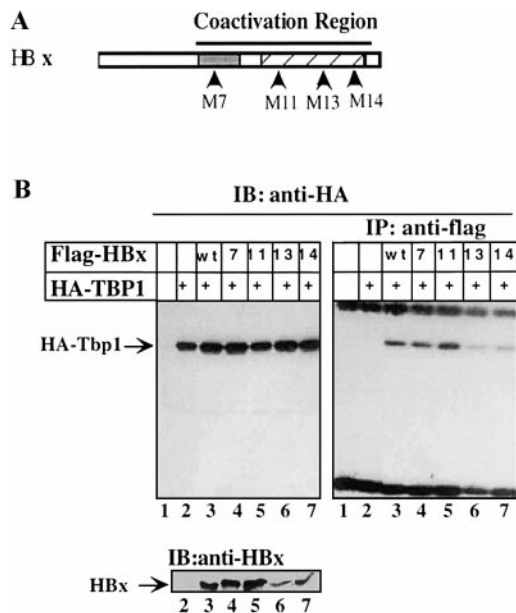


FIG. 6. Mapping the Tbp1 interacting domain of Flag-HBx. Wild-type HA-Tbp1 was cotransfected with wild-type Flag-HBx. (A) Schematic of the various mutants within the coactivation domain of HBx. The M7 mutation is specifically in the pol II binding region (gray box), while M11, M13, and M14 mutants are within the TFIIIB binding region (hatched region). (B) 293T cells were transfected, HA-Tbp1 and HBx production was monitored (left panels), and extracts were coimmunoprecipitated as described in the legend to Fig. 5. HA-Tbp1 was coimmunoprecipitated with Flag-HBx as well as the M7 and M11 mutants of HBx (right panel, lanes 3, 4, and 5). The M13 and M14 mutants pull down HA-Tbp1 much less efficiently (right panel, lanes 6 and 7). However, since these mutants accumulate to lower levels the possibility that the HBx C-terminus region mediates Tbp 1 interaction has to be further substantiated.

Flag-HBx in nuclear extracts and supports a role for Tbp1 as a putative physical target of the HBx *in vivo*.

Having demonstrated coimmunoprecipitation of Tbp1 by HBx, we attempted to delineate the interacting region of each protein. The domains of HBx have been elucidated by a number of groups with respect to target proteins and function (Takada and Koike, 1994; Cheong *et al.*, 1995; Haviv *et al.*, 1998b). It was shown previously that the N-terminus of HBx serves as a negative regulator of transcriptional activation (Takada and Koike, 1994), and the C-terminus is both necessary and sufficient for this activity. Therefore, we chose a series of four double-codon mutants in the transcriptionally active C-terminus of HBx for our interaction mapping. The four double-codon insertion mutants, coined M7, M11, M13, and M14 (Runkel *et al.*, 1993), were previously shown not only to abrogate transcriptional coactivation of HBx but also to actually inhibit transcriptional activity by as much as 10-fold less than basal (Haviv *et al.*, 1998b). The M7 mutant is in the region implicated in Pol-II binding, while M11, M13, and M14 are all in the TFIIIB binding region (Fig. 6A). These mutants are all expressed well in transient transfection (Fig. 6B, lower panel).

For the mapping, the four Flag-HBx mutants, as well as wild-type (wt) HBx, were cotransfected with HA-Tbp1 (Fig. 6B). Expression of HA-Tbp1 was apparent in the immunoblot of the total extract (Fig. 6B, left panel). As expected, HA-Tbp1 was coimmunoprecipitated only in the presence of Flag-HBx (Fig. 6B, lanes 2 and 3). While the M7 and M11 Flag-HBx mutants brought down HA-wtTbp1 (lanes 4 and 5), the ability of the M13 and M14 mutants to coimmunoprecipitate HA-Tbp1 was greatly reduced (Fig. 6B, right panel, lanes 6 and 7). Since these mutants were expressed to a lower level, the possibility that the Tbp1-interacting site in HBx is located in the C-terminus should be further investigated.

HBx binds to the ATPase domain of Tbp1

Tbp1 is a component of the 19S cap which, along with the 20S core unit, forms the 26S proteasome (Glickman *et al.*, 1998). As mentioned previously, it is a member of a large family of proteins called the AAA ATPase gene family (Beyer, 1997), with its closest relatives being five other members of the 19S cap of the proteasome, including Mss1, S4, Tbp7, S10b, and Trp1 (Fig. 4B). Characteristic of the AAA family of proteins is a highly conserved 230-amino-acid region containing ATPase and DEXD domains called boxes A and B, respectively. These regions were found to be essential to the function of Tbp1 (Ohana *et al.*, 1993). For our mapping studies, we employed two variants of Tbp1, one with a deletion of the ATPase domain (box A) called Δ ATP and one with a point mutation in the helicase domain (box B) called D285A (Fig. 7A).

The coimmunoprecipitation experiments were again performed employing wild-type HBx with wild-type HA-Tbp1 and the two mutants, D285A and Δ ATP. The two are expressed as well as the wild-type Tbp1 (Fig. 7B, left). Again, the controls exhibited HBx-specific coimmunoprecipitation (Fig. 7, lanes 2 and 3, right). Interestingly, while D285A was successfully pulled down (Fig. 7, lane 4, right), the Δ ATP mutant of HA-Tbp1 completely abrogated the interaction between HBx and Tbp1 (Fig. 7, lane 5, right). Collectively, these data suggest that HBx interacts specifically with the ATPase domain of Tbp1.

Tbp1 supports HBV transcription in a HBx-dependent manner

Finally, we investigated the ability of Tbp1 and the two mutants to influence HBV expression. We employed a construct containing two tandem repeats of the HBV genome (Ori *et al.*, 1998). The genomic plasmid was transfected with and without Tbp1, D285A, or Δ ATP into the Huh-7 hepatoma cell line. Following transfection, RNA was prepared for Northern analysis probing for HBV RNA. A GAPDH control was employed to ensure equal amounts of RNA being loaded into each lane (Fig. 8). Compared to wild-type HBV DNA alone, cotransfection of

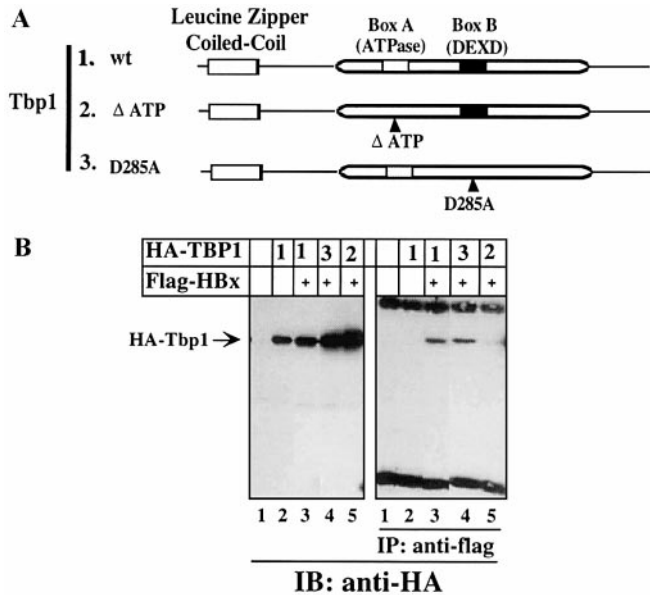


FIG. 7. The HBx interacting domain of HA-Tbp1 maps to the ATPase domain. Wild-type (wt) Flag-HBx was cotransfected with wild-type HA-Tbp1 and mutations in the two critical regions, box A and box B. (A) The box A mutant is the Δ ATP clone while the box B mutant is the D285A clone. (B) 293T cells were transfected, production of wt and mutant HA-Tbp1 was monitored (left panel), and extracts were coimmunoprecipitated as described in the legend to Fig. 5. Flag-HBx pulls down the HA-Tbp1 as well as the D285A mutant (lanes 3 and 4, right), whereas the Δ ATP mutation abrogates the interaction (lane 5, right). These data suggest that box A is the HBx interacting domain.

Tbp1 resulted in a significant increase in the levels of HBV transcripts (Fig. 8, left panel, lane 2). The D285A mutant, which does not bind HBx, decreased the level of HBV transcripts (Fig. 8, left panel, lane 3). The Δ ATP mutant had no significant effect on the HBV RNA levels (Fig. 8, left panel, lane 4). We performed the same experiment but with a genomic HBV construct containing a stop codon mutation in the HBx ORF abrogating production of HBx protein (Ori *et al.*, 1998; Doitsh and Shaul, 1999). Cotransfection of the HBx knockout HBV genome with the wild-type Tbp1, D285A, and Δ ATP constructs did not significantly affect expression of HBV (Fig. 8, right panel). Together, these data suggest that Tbp1 may increase transcription from HBV genomic DNA in an HBx-dependent manner. This is further supported by the observation that with regards to the wild-type HBV, D285A, which binds HBx, exhibited a dominant negative effect, while the Δ ATP lacking the ability to bind HBx does not influence genomic expression.

DISCUSSION

To elucidate the function of HBx we chose a genetic screen to identify HBx interacting proteins, which would gain us additional insight into its role in the virus-host cell interaction. To this end we employed the SRS (Aronheim *et al.*, 1994), a cytoplasmic yeast two-hybrid-like,

but not the conventional, screen, since we assumed that the SRS is more likely to result with reliable candidates while also dealing with nuclear transcription factors. We isolated two clones that specifically and repeatedly interacted with HBx in our screen. We confirmed the specificity and reproducibility of this interaction by employing a number of target proteins in the *Saccharomyces cerevisiae* system. Clone 1, named XAP8 (X-associated protein 8), proved to be a novel gene with the functional and structural characteristics of a transcription coactivator (unpublished observation). In this study we investigated clone 2 (Tbp1) as a genuine HBx nuclear target.

Coimmunoprecipitation studies from nuclear extracts of transfected 293T cells exhibited a specific and reproducible interaction between Tbp1 and HBx. Mutations in the functionally important ATPase domain (box A) of Tbp1 abrogated coimmunoprecipitation of HA-Tbp1 by Flag-HBx. However, we could not show specific Tbp1 interaction with glutathione-S-transferase (GST)-HBx in pull-down *in vitro* assays since Tbp1 binds GST alone (data not shown and Cong *et al.*, 1997). Thus, we cannot exclude the possibility of the involvement of a third partner in HBx and Tbp1 interaction in both yeast and mammalian cells. This option is very unlikely in yeast since the Tbp1 homologue is exclusively nuclear (Russell *et al.*, 1999b), and, therefore, excludes the likelihood of any endogenous Tbp1 and HBx interacting protein in the cytoplasm where the genetic interaction is necessary.

The roles of Tbp1 in cellular processes are wide ranging. It is known to be a member of the AAA gene family of ATPases characterized by a 220- to 250-amino-acid

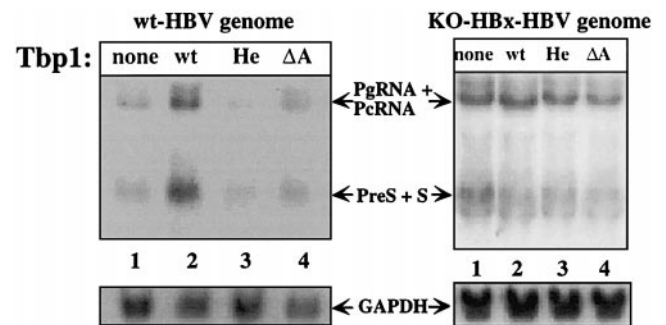


FIG. 8. Effects of wtTbp1 and mutants on the HBV genome. (Left) Huh-7 cells were transfected with 20 μ g plasmids containing two tandem full-length HBV genomic sequences, as has been reported (Ori *et al.*, 1998). In the indicated lanes, 1 μ g of Tbp1 and derivative mutant plasmids was cotransfected. Cells were collected, and total RNA was separated by gel electrophoresis and analyzed by Northern blot. The HBV genome alone is in lane 1. Tbp1 (wt) alone increases expression of all transcripts from the genome (lane 2). The D285A (He) mutant decreases the amount of HBV transcripts (lane 3), while the Δ ATP (Δ A) mutant has no effect (lane 4). Probing for GAPDH indicates similar amounts of RNA loaded into each lane. (Right) Cells were transfected with the genomic construct with a stop codon in the HBx ORF leading to no HBx protein (Ori *et al.*, 1998). Under this condition none of the constructs show any effect on HBV transcripts, indicating that the Tbp1 effect is HBx dependent.

region containing two highly conserved domains, one resembling an ATP-binding site and the other a motif with helicase activity (Beyer, 1997). Tbp1, along with the other closely related ATPases, was shown to be in a complex called the 19S regulatory particle or PA700. This complex has been implicated in regulation and associated with the 20S core to form the 26S proteasome (DeMartino *et al.*, 1994). Thus, the Tbp1 interacting proteins, Tat and HBx, might regulate proteasome activity. Indeed, HIV Tat has been shown to inhibit the peptidase activity of the 26S proteasome by blocking formation of the 19S–20S complex (Seeger *et al.*, 1997). HBx has been reported to interact with the components of the 20S core (Hu *et al.*, 1999; Huang *et al.*, 1996) and 19S proteasome (Zhang *et al.*, 2000). Subcellular colocalization of HBx with the proteasome using immunofluorescence also suggests the proteasome as an important target (Sirma *et al.*, 1998).

Tbp1 and the other 19S cap ATPases were originally credited with a role in transcription. Tbp1's interaction with and repression of the HIV1 Tat protein, the HIV effector of transcription (Nelbock *et al.*, 1990), suggested a designation of transcription factor. In addition, Tbp1 is localized to the nucleus and activates transcription in the absence of HIV1 Tat when fused to a Gal4 DNA-binding domain (Ohana *et al.*, 1993). Tbp1 is not the only 19S ATPase protein that has been implicated in transcription regulation. Sug1, another member, has been isolated in a genetic screen for suppressors of Gal4 proteins with a deleted acidic activation domain and is able to rescue mutants harboring a defective transcription factor (Swaffield *et al.*, 1992). Furthermore, Sug1 has been found to be a purified mediator of the holo-RNA polymerase II complex (Kim *et al.*, 1994) and has been coeluted from fractionation of the yeast lysates with transcription factors (Swaffield *et al.*, 1995), as well as RNA polymerase II and yeast Tbp1 (Fraser *et al.*, 1997). In addition, Sug1 interacts with a subunit of the basal transcription factor TFIIF (Weeda *et al.*, 1997). Interestingly, mouse Sug1 homologue plays a role in transcription as well and interacts with a number of nuclear receptors to regulate their activity (vom Baur *et al.*, 1996). Collectively, the documented data attribute a role of transcription mediator/coactivator to some of the members of this protein family.

The possible crosstalk between the transcription machinery and protein degradation apparatus also became apparent from the analysis of the transcription activators, which showed that many of them are unstable proteins degraded by an ubiquitin-dependent pathway. In fact, it has been proposed that these activators may be destroyed because of their ability to activate transcription (Salghetti *et al.*, 2000). This trend characterizes, in particular, the potent acidic activation domains, the preferred target of HBx (Haviv *et al.*, 1995). Thus, HBx interaction with Tbp1 might enhance transcription also by increasing the activator's stability.

The documented data support the possibility that the viral effectors that target Tbp1 are likely to regulate transcription. A functional assay allowed us to further establish this possibility. We relied on Northern analysis of RNA transcripts from genomic wild-type HBV and HBV knockout HBV (Ori *et al.*, 1998) under the influence of cotransfected wild-type Tbp1 or mutants. The Northern analysis of the effect of Tbp1 and mutants on HBV genomic transcripts supported a functional role for the interaction between HBx and Tbp1. We found that wild-type Tbp1 resulted in a significant increase in expression from the wt HBV genome. The D285A mutant, which binds HBx but is known to have a functional deficit (Ohana *et al.*, 1993), resulted in a decrease in levels of HBV RNA transcripts, while the Δ ATP mutant deficient in HBx binding had no effect. This suggests that the D285A mutation in the critical box B of Tbp1 with HBx binding capacity might act as a dominant negative and hence abrogate HBx activity. Interestingly, the HBx knockout HBV genome, unable to produce HBx, failed to show any response to the Tbp1 constructs. This would suggest that the effect of Tbp1 on HBV transcription is HBx specific and dependent.

The functional interaction between HBx and Tbp1 can be attributed to any of the cited functions of both proteins. In fact, there is some resemblance between the activities of these proteins. Both were shown to interact with transcription activators and with general transcription factors. However, each targets a different member. For example, HBx was shown to interact with CREB (Maguire *et al.*, 1991; Williams and Andrisani, 1995; Perini *et al.*, 1999), while Tbp1 failed to do so (Fig. 4). Tbp1 and HBx were both shown to be associated with the proteasome. They were also implicated in the DNA nucleotide excision repair (NER) machinery (Russell *et al.*, 1999a; Jia *et al.*, 1999). Furthermore, HBx, just as Tbp1, has been shown to have ATPase activity (De-Medina *et al.*, 1994). This striking functional similarity between a viral effector and a cellular protein and the fact that one targets the other must be of functional significance. The finding that HBx interacts with Tbp1 via its ATPase domain is intriguing, considering the fact that HBx itself has the same activity. An attractive possibility would be that via this interaction a complex is generated that has a more potent and constitutive ATPase domain.

Fraser *et al.* (1997) noted a DNA helicase activity in Sug1 supporting a functional role for the putative DEXD helicase domain in the Sug1 ORF. The same activity is likely to be presented in Tbp1. Interestingly, a Tbp1 mutant in the DEXD helicase box represses transcription in the presence of HBx, suggesting that this activity is essential for the proper function of Tbp1 in transcription. Furthermore, as this gain of function of the Tbp1 helicase mutant is HBx dependent, we can assume their *in vivo* association.

Finally, HBx is implicated in the progression of chronic

HBV infection to neoplastic transformation of hepatocytes and the ultimate diagnosis of hepatocellular carcinoma (Cromlish, 1996). Tbp1 has also been studied for its role in transformation of cells. The genomic sequence was localized to chromosome 11p12–13 (Hoyle *et al.*, 1997), a region noted to be deleted in a variety of cancers (Bepler and Garcia-Blanco, 1994; Shipman *et al.*, 1993). Most recently, Park *et al.* (1999) isolated Tbp1 in a screen for genes implicated in the reversion of a transformed phenotype in a number of different cell lines, suggesting a tumor suppressor-like role. The transforming capacity of HBx might be Tbp1 dependent. Although a likely mechanism would be modulation of transcription, alternative possibilities must be considered. For example, the 19S cap was implicated in the DNA NER machinery independent of the 20S core of the proteasome (Russell *et al.*, 1999a). Inhibition of NER was a recently described function of HBx (Jia *et al.*, 1999). Sug1, the close relative of Tbp1, was reported to be a target of multiple viral transcriptional regulators, including E1A of adenovirus and the large T antigen of SV40 (Grand *et al.*, 1999). Thus, our model may not only explain transformation by HBx, but also transformation mediated by E1A and SV40.

MATERIALS AND METHODS

Plasmids

The HBx–Sos bait chimera for the SRS screen was constructed as follows: An *NcoI*–*BglII* insert was removed from the HBV Adw2 genome containing the HBx ORF and ligated into an *NcoI*–*BamHI*-digested pGL3 basic vector (Promega). A *HinDIII*–*FspI* insert was excised from this construct and ligated into a *HinDIII*–*SmaI* digest of the pADNS-p110–Sos (Aronheim *et al.*, 1997) bait vector. The Sos–HBx chimeric bait was constructed by removing a *BglII*-digested insert from a pECE–Flag–HBx expression plasmid containing the HBx ORF and ligating it into a *BamHI*-digested pYes2–Sos yeast expression vector. Subsequent cloning of this chimera into the pADNS bait vector required ligating a *HinDIII*–*NotI* insert from the pYes2–Sos–HBx clone into a *HinDIII*–*NotI*-digested pADNS.

The Tbp-1 expressing vectors were constructed by a PCR reaction amplifying the Tbp-1 gene from the full-length positive clones isolated in the SRS screen. The sense primer, 5'GCTCTAGAATGTCCACGGAGGAGATCAT3', includes a *XbaI* site at the 5' end of the PCR product, and the antisense primer, 5'GCAGATCTTAGCGTAGTATTGTAGGTT-3', includes a *BglII* site at the 3' end of the PCR product. The product was then digested with *XbaI*–*BglII* and ligated into an *NheI*–*BamHI*-digested pSG5–HA expression vector. The Tbp-1 helicase and Δ ATP mutants have been described previously (Ohana *et al.*, 1993).

Construction of the flag epitope-tagged HBx in the pECE expression vector is as described elsewhere

(Haviv *et al.*, 1996). Flag epitope-tagged HBx mutants were constructed using PCR amplification of mutants constructed previously, using the following sense and antisense primers, respectively: 5'GGAGATCTTCATGCTGCTAGGCTGTACTGCC3' and 5'TCACTCGAGTTAGGCAGAGGTGAAAAAGTTGC3'. The PCR product was subsequently digested with *BglII*–*XhoI* and ligated into a *BamHI*–*SacI*-digested pECE–Flag expression vector.

Yeast transformation

The *cdc25-2* yeast strain was grown to 0.5 OD₆₀₀ units on 200 ml yeast extract/peptone/dextrose (YPD). Cells were centrifuged at 2500 rpm for 5 min and washed twice in 20 ml of LISORB (100 mM LiAcetate, 1 M Sorbitol, 1 mM EDTA, 10 mM Tris, pH 8.0). The pellet was then resuspended in 2 ml of LISORB. Cells were rotated for 30 min at room temperature followed by plasmid addition (2 μ g of plasmid DNA and 20 μ g of boiled sheared salmon sperm) for transformation to the 0.2 ml of cells. To the yeast and DNA, 1.2 ml of LIPEG (40% PEG-3350 in 100 mM LiAcetate in TE) was added and incubated for 30 min at room temperature with gentle mixing. The cells were then supplemented with 0.1 ml of DMSO and incubated at 42°C for 10 min. The cells were centrifuged at 5000 rpm for 1 min and resuspended in 0.15 ml of TE. Cells were plated under appropriate amino acid selection for transformants at room temperature.

Library screening

cdc25-2 yeast cells previously cotransformed with pADNS (leucine) bait construct and Yes2–GAP (tryptophan) plasmid were grown on selective media (+glucose –Leu –Trp) in 10 ml until saturation. Cells were then diluted in YPD and transformed according to protocol with 3 μ g per plate of HeLa (TNF- α and IFN- γ treated for 30 min) cDNA library fused to v-Src myristoylation sequence in pYes2 (uracil-derived expression plasmid). Transformants were plated on selective medium (+glucose –Leu –Ura –Trp) at room temperature. Following 4 days of growth, colonies were replica plated using velvet squares to galactose plates with the same amino acid and base selection and placed at 37°C. Colonies growing at 37°C were then replica plated to assay for galactose-dependent growth. Clones found to be galactose-positive but glucose-negative at 37°C were subjected to plasmid DNA purification and retransformation into DH5a bacteria for further characterization.

Yeast protein extracts

Yeast with expression plasmid was grown in 50 ml of medium to 0.5 OD₆₀₀. Cells were then centrifuged for 3 min at 2500 rpm, and the pellet was washed in 1 ml of TE, followed by resuspension in 0.3 ml of 1% SDS in 1 \times PBS, and transferred to a 0.5-ml tube. Glass beads (400 μ m; Sigma) were added and the mixture was vortexed at 4°C

for 20 min. Cells were inspected with a light microscope for spheroplast formation. The yeast extract was centrifuged at 13,000 rpm for 10 min at 4°C followed by boiling for 2 min.

Yeast plasmid preparation

Yeast cells were grown in 3 ml of selective liquid medium for the desired plasmid until saturated. Cells were washed once in water and resuspended in 0.1 ml of STET (8% sucrose, 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 5% Triton X-100) followed by addition of 0.2 g of 400- μ m glass beads (Sigma). The mixture was vortexed for 5 min. An additional 0.1 ml of STET was added with a brief vortex. The mixture was boiled for 3 min, cooled on ice, and centrifuged at 13,000 rpm for 10 min at 4°C. A quantity of 0.1 ml of this mixture was added to 50 ml of 8 M ammonium acetate and incubated for 1 h at -20°C followed by centrifugation at 13,000 rpm for 10 min at 4°C. A quantity of 0.1 ml of the supernatant was transferred to 0.2 ml of ice-cold ethanol and centrifuged at 13,000 rpm for 10 min at 4°C. After a final wash with 70% ethanol, the pellet was resuspended in 20 μ l of water for electroporation to bacteria.

Cell culture and transfections

HepG2 and 293T cells were maintained in Dulbecco's modified Eagle minimal medium (Sigma) containing penicillin (100 IU/ml) and streptomycin (100 μ g/ml) and supplemented with 8% fetal calf serum. At the time of transfection, cells were seeded to be 40 to 60% confluent. All transfections were performed in 10-cm plates with 20 μ g of total DNA. The cells were transfected with the calcium phosphate precipitation techniques. Transfection efficiency was assayed by cotransfection with 100 ng of EGFP reporter plasmid (Clontech).

Subcellular extraction

Sixty hours following transfection, 293T cells were washed once with dialysis buffer (20 mM HEPES-KOH, pH 7.6, 20% glycerol, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 50 mM KCl, 5 mM MgAcetate) and collected in 1 ml of dialysis buffer using a rubber policeman. Cells were then centrifuged for 1 min at 4000 rpm at 4°C. The supernatant was discarded and cells were lysed in 0.5 ml of hypotonic buffer (25 mM Tris, pH 7.5, 0.7 M sucrose, 5 mM KCl, 1.5 mM MgCl₂, 12 mM EGTA, 0.25% Triton X-100, 2 mM DTT, 1:100 of Sigma protease inhibitor cocktail) using a Dounce homogenizer. Following 10 min on ice, the extract was centrifuged for 2 min at 13,000 rpm at 4°C. The cytoplasmic supernatant was set aside for further analysis. CI buffer (50 ml per plate of cells) (20 mM HEPES-KOH, pH 7.9, 0.2 mM EDTA, 2 mM DTT, 25% glycerol, 140 mM KCl, and protease inhibitors) was added and homogenized. Two volumes of CII (20 mM HEPES-KOH, pH 7.9, 0.2 mM EDTA, 2 mM DTT, 25%

glycerol, 700 mM KCl, 0.025% sarcosyl, 0.025% DOC) were added and the extract was incubated for 30 min on ice followed by centrifugation for 20 min at 13,000 rpm at 4°C. The supernatant containing nuclear material was kept for further analysis.

Coimmunoprecipitation

One volume of dilution buffer (25 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 2 mM DTT, and 0.25 mg/ml bovine serum albumin) was added to the nuclear extract and centrifuged at 13,000 rpm for 15 min at 4°C to remove precipitated extract. Of the total extract, 1/10 vol was set aside and 0.1 ml of M2 beads (Kodak) in wash buffer (25 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 0.25% Triton X-100, 10% glycerol, 150 mM NaCl, 2 mM DTT, and 0.25 mg/ml bovine serum albumin) was added. The immunoprecipitation reaction was incubated at 4°C with gentle shaking. After the incubation, the reaction was washed three times in wash buffer by centrifugation for 3 min at 4000 rpm at 4°C to bring down the beads. SDS sample buffer (50 μ l) was added and fractionated by SDS-PAGE.

SDS-PAGE and transfer

Cell extracts in sample buffer were boiled for 5 min and fractionated on 10% or 15% polyacrylamide gels (30:0.8 acrylamide-bis-acrylamide) depending on the protein to be visualized. Running buffer contains 25 mM Tris-HCl, 192 mM glycine, and 0.1% w/v SDS. Gels were electroblotted for 2 h at 200 mA to nitrocellulose membranes in cold blotting buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol).

Western blot analysis

Membranes posttransfer were incubated in blocking solution (5% nonfat milk, 1 \times PBS, and 0.05% Tween 20) at 4°C. Following blocking, primary antibody at the desired concentration was added to the blocking solution (1:1000 for α -HA mouse monoclonal antibodies, and 1:2000 for α -HBx mouse monoclonal antibodies). One hour later, the membrane was washed three times in blocking buffer minus milk. The membrane was incubated in a 1:10,000 dilution of HRP-conjugated goat anti-mouse (The Jackson Laboratories) in blocking solution for 1 h. This was followed by three washes with blocking solution minus milk. Proteins were visualized using the ECL detection system (Amersham).

Northern blot analysis

Huh-7 or HepG2 cells were transfected with a head-to-tail genomic HBV construct (Ori *et al.*, 1998) together with various Tbp1 constructs. RNA was extracted with TRI reagent (Molecular Research Center) and DNaseI treated. RNA (20 μ g) was raised in 20 μ l of sample buffer

(160 μ l formaldehyde; 100 μ l of 10 \times MOPS buffer: 0.2 M MOPS, 0.05 M NaCl, 0.01 M EDTA, pH 7.0; 50 μ l glycerol, 90 μ l water, 0.6 ml formamide, bromophenol blue) and incubated at 65°C for 10 min. Samples were electrophoresed onto a 1% agarose gel (1 gm agarose, 1.8 ml formaldehyde, 10 ml 10 \times MOPS, 90 ml water) with RNA running buffer (100 ml 10 \times MOPS, 18 ml formaldehyde, 880 ml water) at 120V. Following electrophoresis, the gel was soaked in 10 \times SSC and set overnight for capillary transfer to a Hybond-N membrane (Amersham) in 10 \times SSC. Following capillary transfer, RNA was UV cross-linked to the membrane and hybridized overnight with 1 million cpm/ml of HBV DNA or GAPDH probe. The washed membrane was exposed to photographic film.

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