JOURNAL OF VIROLOGY, Aug. 1997, p. 6194–6199 Vol. 71, No. 8
0022-538X/97/$04.00+0


# Interaction of the UV-Damaged DNA-Binding Protein with Hepatitis B Virus X Protein Is Conserved among Mammalian Hepadnaviruses and Restricted to Transactivation-Proficient X-Insertion Mutants

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Received 9 January 1997/Accepted 8 May 1997

**We carried out a comparative analysis of several proposed host protein partners of the human hepatitis B virus X protein (HBx) using both the GAL4- and the LexA-based yeast two-hybrid system. We showed that the interaction of HBx with the UV-damaged DNA-binding protein (UVDDB) is positive in both yeast systems, detectable in cotransfected human cells, conserved by rodent hepadnavirus X proteins (known to transactivate in human cells), and tightly correlated with the transactivation proficiency of X-insertion mutants. Taken together, our results strongly suggest that UVDDB is involved in X-mediated transactivation.**

Despite the existence of efficient vaccines, the human hepatitis B virus (HBV) is responsible for a major health problem: worldwide, more than 200 million people are persistently infected with HBV, and their risk of developing liver cancer is 200 times higher than that of noninfected individuals (2). HBV is the prototype of the hepadnavirus family, which includes rodent viruses, such as the woodchuck hepatitis virus (WHV) and the ground squirrel hepatitis virus (GSHV), as well as the more distantly related duck hepatitis B virus. The unique replication mode of hepadnaviruses, which involves reverse transcription of encapsidated viral RNA leading to synthesis of the circular and partially double-stranded DNA genome, has been unraveled during the past years (reviewed in reference 28). Our knowledge is much less complete regarding other aspects of the biology of hepatitis B viruses. Thus, the precise function in the virus life cycle of the X gene found in the mammalian hepadnaviruses, as well as the specific effects mediated by the corresponding protein on the properties of the host cell, remains to be elucidated.

Engineered X-deficient HBV genomes lead to virion synthesis when they are transfected into hepatocytic cell lines (4, 37). Therefore, the X-encoded protein (referred to as HBx, WHx, or GSHx, depending on its viral species of origin) has been considered an accessory molecule, in keeping with the absence of the corresponding gene in duck hepatitis B virus. However, other studies conducted in vivo with the woodchuck model have challenged this view: X mutant WHV strains have been found to be unable to establish infection (6, 39).

It is also noteworthy that the property shared by mammalian hepadnaviruses of increasing the risk of host liver cancer (5) best correlates with presence of the X gene in their genomes. To date, however, only circumstantial evidence has been obtained for a role of HBx in carcinogenesis (reviewed in reference 38).

A number of studies indicate that HBx affects multiple cellular processes, including transcription (reviewed in references 30 and 38) and signal transduction (3, 9, 10, 23), although any direct relationship with an essential function of HBx in the virus life cycle remains to be established. To date, the best documented HBx activity is indirect transcriptional transactivation of a large number of cellular and viral enhancers-promoters (reviewed in reference 30). Because HBx shows no specific DNA-binding activity, its activity is thought to be mediated via protein-protein interactions. Several approaches have been used to identify primary host targets of HBx. Where a functional interaction was suspected, such as for the transcription factor CREB (27, 36) or the tumor suppressor p53 (12, 33, 35), the protein was assayed directly for physical interaction with HBx. In an alternative strategy, no prediction was made regarding the identities of possible targets and putative candidates for interaction with HBx were isolated from screens of a large number of cellular proteins. The RNA polymerase subunit RPB5 (7), the proteasome subunit HU28K (14, 20), and the UV-damaged DNA-binding protein UVDDB or XAP-1 (25) have been identified in screens with λ gt11, a LexA-based two-hybrid system, and a GAL4-based two-hybrid system, respectively. Each of the potential partners has been studied individually by different investigators, and no common criteria have been applied to address the biological significance of interaction.

In this study, we first used two distinct interaction assays for a comparative analysis of several proposed HBx partners, in addition to other candidates isolated in a two-hybrid screen of human liver proteins. This led us to focus on the UVDDB-HBx interaction, as it behaved like the two well-characterized interactions used as positive controls in the assays. We show that rodent hepadnavirus X proteins also bind to UVDDB and that, among a panel of HBx insertion mutants, efficient interaction with UVDDB is restricted to transactivation-competent species.

**Comparative analysis of potential HBx partners in two distinct two-hybrid interaction assays.** In order to analyze several of the proposed HBx partners in the same interaction assay, we performed quantitative analysis of *lacZ* induction in a yeast GAL4 two-hybrid system (13). Briefly, the test proteins were coexpressed with HBx (ayw subtype) from yeast two-hybrid vectors encoding the GAL4 activation domain (GAL4AD) or

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TABLE 1. UVDDB-HBx interactions are detectable in two distinct yeast two-hybrid assays

| Coexpressed test protein | β-Galactosidase activity of interaction with indicated protein[a] in the | | | | | |
|---|---|---|---|---|---|---|
| | GAL4-based assay with: | | | LexA-based assay with: | | |
| | HBx | PRP21 | PRP9 | HBx | PRP21 | PRP9 |
| UVDDB | 0.7 ± 0.1[d] | ≤0.1 | ND[c] | 1,360 ± 146[d] | ≤0.1 | ND |
| Clone 77 | 6.0 ± 1.4 | ≤0.1 | ND | 1.6 ± 0.6 | ≤0.1 | ND |
| HU28K | ≤0.1 | ≤0.1 | ND | 1 ± 0.3 | ≤0.1 | ND |
| RPB5 | ≤0.1 | ≤0.1 | ND | ≤0.1 | ≤0.1 | ND |
| CREB | 0.2 ± 0.05 | ≤0.1 | ND | ND | ND | ND |
| p53[b] | ≤0.1 | ≤0.1 | ND | ND | ND | ND |
| PRP9 | 0.2 ± 0.05 | 29 ± 7[d] | 51 ± 5[d] | 1.5 ± 0.3 | 249± 6[d] | 258 ± 0.7[d] |
| PRP21 | ≤0.1 | 0.5 ± 0.2 | 82 ± 14[d] | ≤0.1 | 0.5 ± 0.2 | 1,284 ± 18[d] |

[a] For each potential interaction, two independent yeast cotransformants were assayed in duplicate for β-galactosidase activity with *o*-nitrophenyl–β-galactoside as the substrate. Results are expressed in mean β-galactosidase units ± standard deviations.
[b] P53 self-interaction measured in the same assay gave rise to 4 β-galactosidase units.
[c] ND, not determined.
[d] Values corresponding to interactions detected with increased sensitivity in the LexA versus the GAL4-based assay.

the GAL4 DNA-binding domain (GAL4BD) in the yeast strain Y190 (17). β-Galactosidase activity was quantitated as described previously (15). Except for those with UVDDB, which was tested as the full-length protein, the assays were performed with protein domains previously reported to interact with HBx: RPB5 (amino acids 47 to 210) (7), CREB (amino acids 219 to 327), which contains the basic and leucine zipper motifs (36), murine p53 (amino acids 72 to 390), which includes the oligomerization motif of the protein (33), and HU28K (amino acids 168 to 248), which is the protein domain encoded by the original clone identified in the screening experiment (14). UVDDB has been isolated in a GAL4-based two-hybrid screen of a lymphoblastoid cell cDNA library (25). In a similar two-hybrid screening experiment of a liver cDNA library, we isolated several classes of positive clones (data not shown). One such class, represented by several independent clones that gave strong signals in qualitative *lacZ* assays, corresponded to partial cDNAs of the metallothionein gene. One representative of this class, clone 77, was included in the quantitative assay. Two yeast splicing factors, PRP9 and PRP21, were used both as irrelevant proteins and as positive controls. The existence of functional PRP9-PRP9 and PRP9-PRP21 dimers has been demonstrated by both genetic and biochemical methods (26).

The results of this analysis are illustrated in Table 1. As expected, PRP9-PRP9 and PRP9-PRP21 dimers gave rise to significant and specific signals. In contrast, an HBx interaction with HU28K, CREB, RPB5, or p53 could not be detected in this assay, whereas p53 self-interaction was easily detectable. UVDDB gave rise to a significant interaction signal with HBx ayw, although it was twofold lower in intensity than that with the HBx adw subtype that was used in its identification. In this assay, the strongest interaction signal with HBx was obtained with clone 77. Therefore, the GAL4 two-hybrid assay failed to reveal any of the interactions previously detected in other assays, raising the question of its sensitivity for an analysis of HBx partners. However, identification of Cdi1 (also called CIP2) as a CDK2 partner has been independently obtained by two groups using LexA- and GAL4-based two-hybrid screens (16, 17), suggesting that proteins scoring positive in the two systems are attractive candidates for authentic protein-protein interactions.

We next analyzed several of the HBx target candidates, as well as the control interactions, in the LexA-based system developed by Votjek (34) (Table 1). Note that in this system, the same GAL4AD constructs can be used but that the other test protein is fused to the bacterial LexA protein. The hybrid proteins were analyzed for interaction in the appropriate yeast reporter strain L40 (19) by a quantitative assay of *lacZ* induction (Table 1). Remarkably, the control interactions, PRP9-PRP9, PRP9-PRP21, and PRP21-PRP9, were not only detectable in the LexA-based system but also showed stronger signals (between 5- and 16-fold higher) than in the previous GAL4-based assay. Interestingly, the UVDDB-HBx interaction behaved similarly, with an even more dramatic increase in signal intensity (>1,700-fold). In contrast, a decreased signal was obtained with the clone 77-HBx interaction (Table 1). All other clones selected in the screen of human liver proteins were negative by the LexA-based two-hybrid assay (not shown). Finally, an RPB5-HBx interaction was not detectable in the LexA system whereas the HU28K-HBx interaction gave rise to a weak but significant signal. We note that the LexA-based system developed by Gyruis and colleagues (16), which led to an independent identification of HU28K from a HeLa cell cDNA library by two groups of researchers, requires expression vectors and a yeast reporter strain different from those used in our study.

Taken together, our results indicate that among the combinations analyzed, only the UVDDB-HBx interaction followed

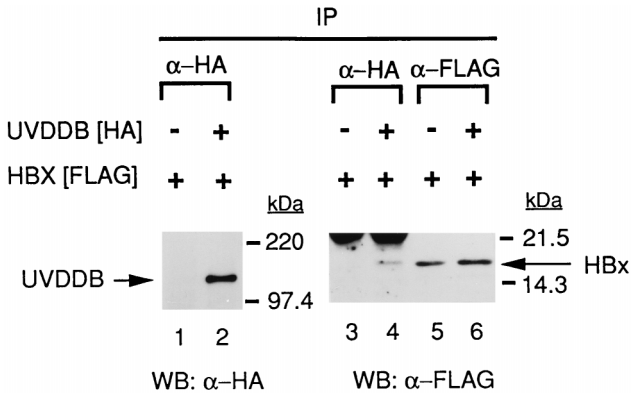


FIG. 1. HBx interacts with UVDDB in mammalian cells. HBx-FLAG was transiently expressed in Chang liver cells either alone or in combination with UVDDB-HA. The cell extracts were immunoprecipitated with anti-HA (lanes 1 to 4) or anti-FLAG (lanes 5 and 6) antibodies. HBx-FLAG and UVDDB-HA contents of the immunoprecipitates (IP) were separated in sodium dodecyl sulfate–15% and–8% polyacrylamide gels, respectively, and subjected to Western blotting (WB) with the indicated antibodies. Exposure times were 10 and 30 min for the anti-HA and anti-FLAG Western blots, respectively.

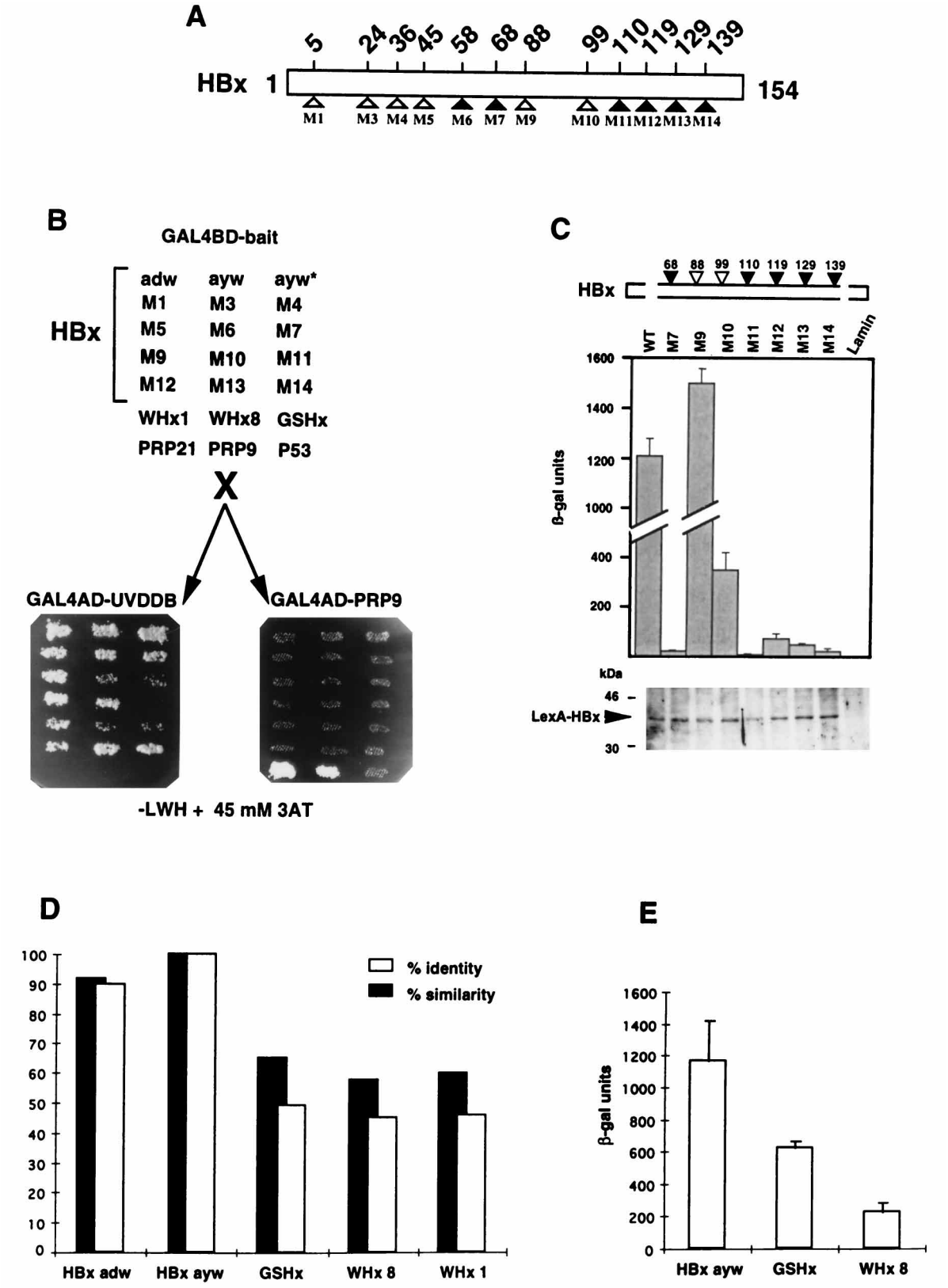


FIG. 2. Transactivation competence of various HBx species correlates with UVDDB binding activity. (A) Locations of insertional mutations and reported transactivation phenotype of HBx mutants (31) used in two-hybrid assays. The 154-amino-acid-long HBx protein (ayw subtype) is represented as a bar. The amino acid positions preceeding Arg-Pro insertions are indicated at the top. Mutants strongly affected in transactivation efficiency are indicated by filled triangles, and mutants conserving more than 60% of wild-type transactivation activity are indicated by open triangles below the bar. (B) Qualitative analysis of the interactions of different X proteins with UVDDB in a GAL4-based two-hybrid assay. The indicated diploid cells ($Trp^+$, $Leu^+$) were replica plated on medium lacking leucine, tryptophan, and histidine (−LWH) to assay induction of the GAL4-driven *HIS3* reporter gene. 3AT, triaminotriazole. The asterisk refers to the weak GAL4DB

the profile of the control interactions. UVDDB was also the only protein yielding a strong signal in an assay distinct from the one that led to its identification as an HBx-interacting protein. However, we do not wish to imply from this analysis that other proposed HBx partners are not relevant, because the yeast two-hybrid system may fail to detect interactions under some circumstances for a variety of reasons. For example, constitutive phosphorylation of E2F in yeast has been shown to impair its interaction with Rb (11). Conversely, phosphorylation of E2F is a prerequisite for binding to adenovirus E4 protein, and this phosphorylation prevents detection in in vitro assays.

**Detection of UVDDB-HBx complex formation in mammalian cells.** Because yeast cells may be considered an artificial expression system, we wished to examine whether a UVDDB-HBx interaction also occurs in mammalian cells. For this reason, we performed coimmunoprecipitation experiments of epitope-tagged proteins from transiently transfected human cells. Chang liver cells ($10^6$) were cotransfected with equal amounts (10 μg) of an expression vector, pcDNA3, which carries the HBx open reading frame fused to the FLAG epitope sequence at its 3′ end (HBx-FLAG), and either empty pcDNA3 or pcDNA3 expressing UVDDB fused to the hemagglutinin (HA) epitope at its carboxy terminus (UVDDB-HA). Equal amounts of cell lysates prepared in SD buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40) were immunoprecipitated with rabbit polyclonal antibodies directed against the HA epitope (Babco) or against the FLAG epitope (Santa Cruz). The presence of the epitope-tagged proteins in the immunoprecipitates was detected by Western blotting on Hybond C extra membranes (Amersham) with the murine monoclonal antibodies anti-HA (12CA5) and anti-FLAG (M2; Kodak). Proteins were detected with an alkaline phosphatase-CDP star-based system (Tropix). As expected, UVDDB-HA was specifically detected in the lysate from cells transfected with pcDNA3-UVDDB (Fig. 1, compare lane 2 to lane 1). Assuming that anti-HA and anti-FLAG antibodies have similar affinities for their respective epitope targets, UVDDB-HA appeared to accumulate in larger amounts than HBx-FLAG (compare lane 2 to lanes 5 and 6). In the anti-FLAG immunoprecipitates, comparable amounts of HBx-FLAG were revealed (compare lanes 5 and 6), whereas in the anti-HA immunoprecipitates, the protein was detected only from the cell lysate containing UVDDB-HA (compare lanes 4 and 3). As analysis of anti-HA and anti-FLAG immunoprecipitates for HBx content was performed with equivalent amounts of cell lysates, we conclude that a significant proportion of HBx was stably bound to UVDDB, confirming the validity of the yeast two-hybrid method in the study of this particular interaction.

**Correlation between the transactivation potential of various X protein species and their ability to interact with UVDDB.** We next examined whether the UVDDB-HBx interaction is relevant to the transactivation activity of the viral protein. If so, the interaction would be expected both to be affected by mutations known to inactivate the transactivation potential of HBx and to be retained by rodent hepadnavirus X proteins, previously shown to transactivate in human cells (8).

A panel of X insertional mutants previously characterized for transactivation activity (31) (a generous gift of H. Schaller) as well as the X genes from WHV and GSHV were subcloned in the GAL4BD yeast two-hybrid vector pAS2 (17) and used to transform the Y187 yeast strain (17) (Fig. 2A and B). The corresponding $Trp^+$ Y187 transformants were mated to $Leu^+$ Y190 transformants expressing either GAL4AD-UVDDB or GAL4AD-PRP9 from the pACT2 vector (Clontech). The resulting diploid cells were then analyzed for induction of the *HIS3* reporter gene by a growth assay in medium lacking histidine. Triaminotriazole was included in the medium to increase the threshold of *HIS3* activity required for growth (17). When they were coexpressed with GAL4AD-PRP9, none of the GAL4BD-X fusions supported growth in selective medium (Fig. 2). In contrast, the control interactions PRP9-PRP9 and PRP9-PRP21 were readily detectable. As expected from our previous results, both HBx ayw and HBx adw subtypes interacted with UVDDB. In addition, UVDDB showed an interaction with WHx from subtypes 1 and 8, as well as with GSHX, whereas no interaction was observed with three irrelevant proteins (PRP9, PRP21, and p53). Importantly, a UVDDB interaction was demonstrated only with the HBx insertion mutants that have retained significant transactivation activity (i.e., mutants M1 to M5 and M9 to M10 [31]). Qualitative analysis of *lacZ* induction confirmed these results (not shown). Note that in the same assay, two substitution mutants at positions known to be critical for transactivation (C61→L and C69→L) (1, 24) were also negative for interaction with UVDDB (data not shown).

In order to confirm these results on a more quantitative basis, we performed a similar analysis in the LexA-based two-hybrid system using the *lacZ* reporter gene. All HBx mutants affected in their transactivation potential showed a strongly reduced signal with UVDDB (Fig. 2C). Interestingly, mutant M11, previously shown to be completely inactive in transactivation (31), was also the most dramatically affected in the interaction (500-fold signal reduction compared to the level of the signal produced by the wild type). Conversely, M9 and M10, which have conserved significant transactivation activity, also retained the ability to interact efficiently with UVDDB (Fig. 2C). The possibility that these results merely reflected differential levels of stability of mutant proteins was excluded by Western blotting analysis of yeast extracts with polyclonal anti-HBx antibodies. Similar amounts of different X mutant proteins were accumulated in the yeast transformants (Fig. 2C). We note that a similar analysis of the Hu28K-HBx interaction identified only M13 as a mutant affected in both transactivation and interaction (14). Finally, as rodent and human hepadnavirus X proteins are quite distantly related (Fig. 2D), the possible interaction of these proteins with UVDDB was also examined by the LexA-based method. Both WHx and GSHx showed efficient interaction with UVDDB, although

expression vector pGBT9 (Clontech). The HBx mutants are the same as those shown in panel A. WHx1 and WHx8 are X proteins from WHV subtypes 1 and 8, respectively. GSHx is the X protein from GSHV. (C) Quantitative analysis of UVDDB interactions with HBx insertion mutants in a LexA-based two-hybrid system. Two independent L40 yeast transformants expressing GAL4AD-UVDDB together with the indicated LexA-HBx fusions were assayed in duplicate for β-galactosidase (β-gal) activity. LexA fused to lamin was used as a negative control. The bottom panel shows immunodetection of LexA-HBx fusion proteins from yeast transformants. WT, wild type. (D) Comparisons of the amino acids of X proteins from different viral species. Each of the indicated X proteins was compared to HBx of subtype ayw, which was chosen as the reference protein, with the GAP program from Genetics Computer Group software. (E) Quantitative analysis of UVDDB interaction with rodent hepadnaviruses X proteins. The GAL4AD-UVDDB fusion was coexpressed with the indicated LexA-X fusions, and *lacZ* reporter gene induction was monitored as described above for panel C. Parallel assays, conducted with WHx and GSHx in combination with the irrelevant protein PRP21, gave background levels of <0.04 β-galactosidase unit.

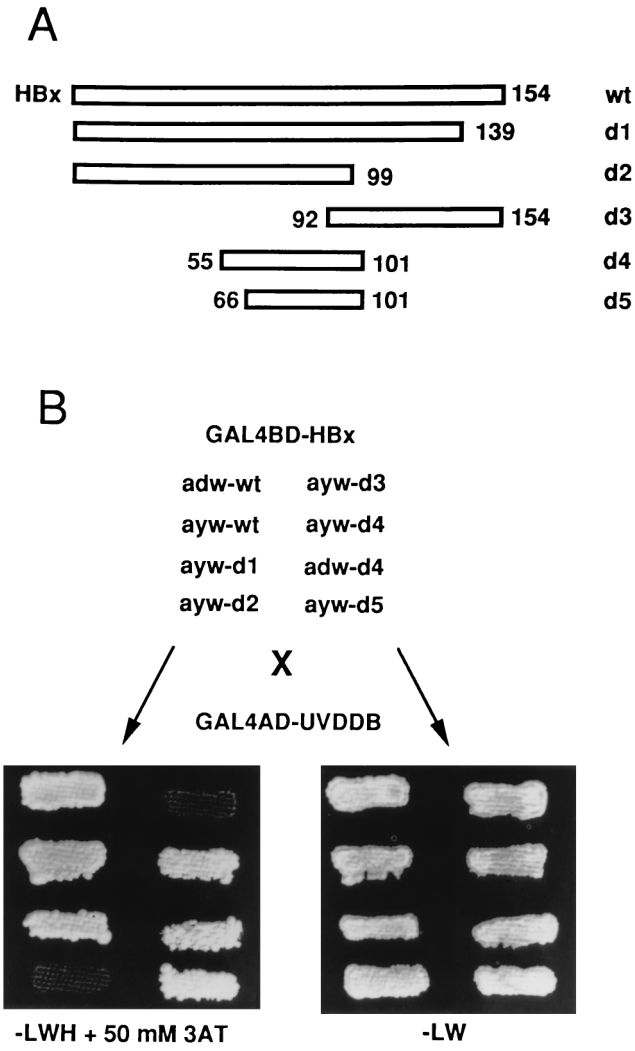


FIG. 3. The HBx domain (amino acids 65 to 101) is sufficient to confer binding to UVDDB. (A) Schematic representation of HBx truncation mutants. The positions of the first and last amino acids of the truncated HBx molecules are indicated. (B) Qualitative analysis of interactions with UVDDB in a GAL4-based two-hybrid assay with the *HIS3* reporter gene. Diploid yeast cells were obtained and analyzed for *HIS3* induction as described in the legend to Fig. 2B. wt, wild type; −LWH, medium lacking leucine, tryptophan, and histidine; −LW, medium lacking leucine and tryptophan; 3AT, triaminotriazole.

with reduced levels of *lacZ* induction compared to that of HBx (Fig. 2E).

**Delineation of an HBx domain sufficient for binding to UVDDB.** In order to delineate the HBx domain involved in UVDDB interaction, we used PCR to construct HBx deletion mutants in the pAS2 vector (Fig. 3A). The truncated mutants were assayed by the GAL4 two-hybrid method with the mating strategy described above. All transformants exhibited similar levels of growth efficiency in nonselective medium (Fig. 3B, right photo). By contrast, growth efficiency in medium lacking histidine clearly discriminated among truncated mutants with respect to UVDDB binding (Fig. 3B, left photo). For example, the truncated mutant d4 could bind UVDDB whereas d3 could not. Taken together, the results illustrated in Fig. 3 indicate that the region of HBx containing amino acids 66 to 101 is sufficient for stable interaction with UVDDB. This observation deserves two comments. First, the insertional mutations M11 to M14, found to strongly diminish interaction with UVDDB, are located outside of the mapped HBx-binding region. One likely reason for this location is that the corresponding insertions of Arg and Pro amino acids lead to incorrect folding of the more N-terminal HBx-binding domain. Second, the HBx-binding domain for UVDDB is included within, but is shorter than, a minimal HBx domain (amino acids 58 to 140), which was found to conserve transactivation activity (24). This suggests that, in addition to an HBx-UVVDB interaction, transactivation activity of the viral protein may involve other functional elements that may lie between amino acids 102 and 140. One hypothesis is that the more C-terminal region is endowed with direct effector function. The recently reported single-stranded DNA-binding activity of HBx may represent such a function (29). However, the corresponding functional domain has not yet been mapped and no relationship with transcriptional transactivation activity is apparent. Alternatively, the UVDDB-HBx complex may recruit other molecules via the carboxy-terminal domain of HBx. For example, the binding of HU28K or TFIIB to HBx that involves the region of the viral protein from amino acids 120 to 140 (18, 20) and the UVDDB-HBx interaction are not expected to be mutually exclusive.

Mapping of the UVDDB domain involved in HBx interaction has not been achieved, as large fragments containing the N- or C-terminal regions of the protein failed to show binding in yeast (data not shown). We suspect that the N-terminal region of UVDDB is somehow required for interaction to occur. Supporting this view, a single cDNA clone (>4 kb in size) encoding the full-length protein was isolated (25) despite the relatively high abundance of UVDDB mRNA in cells. That UVDDB has not been isolated in other screening experiments of proteins interacting with HBx may be due to underrepresentation of 5′ regions of long mRNAs in oligo(dT)-primed cDNA libraries.

**Concluding remarks.** In this study, we demonstrated the existence of an HBx-UVDDB complex in vivo in mammalian cells and showed a strong correlation between interaction with UVDDB and retention of transactivation activity among well-characterized X-insertion mutants. This strongly suggests that the UVDDB-HBx interaction is relevant to the transactivation activity of the viral protein. The only known activity of UVDDB is specific binding to damaged DNA (21, 32), and to date, the exact role of UVDDB in the DNA repair process remains elusive (22). Therefore, the molecular mechanism and functional significance of the UVDDB-HBx interaction in the transcription process have yet to be elucidated.

An important clue substantiating the biological relevance of UVDDB as an HBx target is the conservation of that interaction by rodent hepadnaviruses X proteins, which we demonstrated here. To the best of our knowledge, this property has not been examined for other proposed cellular protein partners of the viral protein. The phenotype of WHx random mutants selected for loss of UVDDB interaction can now be analyzed with respect to both transactivation ability and infectivities of the mutant viruses. This will offer the possibility of addressing whether UVDDB is also involved in some important contribution of the viral protein to the virus life cycle.

We thank H. Schaller for providing X-insertion mutants and Hu28K and RPB5 clones and S. Fields for providing the p53 two-hybrid construct. We are grateful to A. Kay for providing anti-HBx antisera and to R. Schneider for advice in immunodetection of epitope-tagged HBx. We thank C. Legout for technical assistance in the preparation of yeast media.

D.S. was supported by a fellowship from the French Ministère de

l'Education Nationale et de la Recherche. The work was supported in part by a grant (CA54557) from the National Institutes of Health.

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