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Direct Interaction of the Hepatitis B Virus HBx Protein with p53 Leads to Inhibition by HBx of p53 Response Element-Directed Transactivation

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Hepatitis B virus is a major risk factor in human hepatocellular carcinomas. We have used protein affinity chromatography to show that the 17-kDa hepatitis B virus gene product, HBx, binds directly to the human tumor suppressor gene product, p53. Interaction of HBx with p53 did not prevent p53 from specifically binding DNA. Instead, HBx enhanced p53's oligomerization state on a DNA oligonucleotide containing a p53 response element. Optimal binding of HBx to p53 required intact p53, but weaker binding to both the N-terminal activation domain of p53 and a protein fragment containing the C-terminal DNA-binding and oligomerization domains of p53 was observed. In transient transfection experiments with human Calu-6 cells, HBx inhibited transactivation by p53 of a reporter gene containing a p53 response element. Also, HBx inhibited p53-stimulated transcription in vitro even when added to the reaction mixture after the formation of the preinitiation complex. Interaction of HBx with p53 did not prevent the activation domain of p53 from binding two general initiation factors, the TATA-box binding protein subunit of TFIID and the p62 subunit of TFIIH. To explain these results, we propose that localization of HBx to a promoter by interaction with DNA-bound p53 enables a repression domain in HBx to directly contact the basal transcription machinery and thereby repress transcription.

Chronic infection with human hepatitis B virus (HBV) is believed to be a leading cause of human hepatocellular carcinoma (HCC) (3, 69). Tumor formation in HBV-associated HCC has a relatively long latency (7). In order to explain this latency, it has been suggested that an HBV gene product, HBx, may perturb cell growth, expanding the target for subsequent genetic events or favoring the outgrowth of populations that have already sustained genetic damage (23). Compared with the HBV surface and core antigens, HBx was found to be the most prevalent marker of HBV infection in HCC patients (73, 82). Moreover, the expression of HBx in transgenic mice results in liver cancer (31, 32). This may be due, in part, to the interaction of HBx with the tumor suppressor, p53, because HBx and p53 can be coimmunoprecipitated from extracts of HBV-induced liver tumors (16). Mutant forms of the p53 antioncogene have been implicated in the etiology of many types of human cancer, including HCC, but p53 mutations do not appear to play a significant role in HBV-associated HCC (26, 62).

With the finding that the HBx protein of HBV is in a protein complex containing p53 (16), there are now five different classes of DNA viruses that encode gene products which appear to interact with p53 in order to stimulate tumor cell growth (reviewed in references 34, 68, 74, and 81). However, HBx also stimulates the transcription of many cellular and viral genes, and this seems to involve both direct interaction with promoter-bound transcription factors (1, 40) and the regulation of cellular second-messenger systems (11, 30, 38, 46, 59).

Although the cellular functions of p53 have not yet been clearly defined, possible roles as an inducer of differentiation and/or apoptosis and as a checkpoint gene product that causes cell cycle arrest in response to DNA damage have been suggested (reviewed in reference 54). Since wild-type p53 had to be present in the cell nucleus in order to exert its antiproliferative activity (21, 61), it appeared likely that biochemical processes such as transcription, DNA replication, or RNA processing involving p53 would constitute the tumor suppression activity of p53. Consistent with this idea, wild-type p53 has been identified as a transcriptional activator of a gene encoding WAF1, a protein that blocks the activity of cyclin-dependent kinases and thus cell division (14). The abrogation of p53-dependent transcriptional activation may therefore contribute to cell transformation (78). In support of the important role of p53 as a transcription factor, the sequence-specific transcriptional activation activity of p53 has been found to be essential for tumor suppression (52). The transcriptional activation potential of p53 is thought to involve direct protein-protein interactions with the RNA polymerase II general transcription factors TFIID and TFIIH (reviewed in reference 10) via their TATA-box binding protein (TBP) (8, 37, 41, 60, 72) and p62 (77) subunits, respectively.

In this report, we present transfection data that are consistent with a role of HBx in the repression of p53 response element-directed transactivation of cellular genes. Protein affinity chromatography and gel mobility shift analysis were used to show that HBx binds directly to p53 and promotes the oligomerization of p53 on a DNA oligonucleotide containing a p53 response element. HBx also inhibits the transcription activity of a p53-transactivated promoter when added either before or after the assembly of a preinitiation complex. On the other hand, the binding of HBx to p53 does not interfere with the ability of p53 to recognize TBP or p62. We suggest that a

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repression domain in HBx (44) is presented to the RNA polymerase II general initiation complex when HBx is localized in the vicinity of the promoter via direct protein-protein interaction between HBx and p53.

MATERIALS AND METHODS

Plasmids. The adenovirus type 2 major late promoter (AdML promoter) (positions −34 to +33) 98-bp *Bgl*II-*Bam*HI fragment (positions −65 to +33) from pAL (53) cloned into the *Bgl*II site of the promoterless firefly luciferase reporter construct pXP2 (47) was used to generate our p53 response element-containing constructs. Both a 20-bp consensus binding site for p53, 5′-tcgagAG GCATGTCTAGACATGCCTc-3′, and a nonbinding mutant sequence, 5′-tc gagAGGaATtTCTAGAaATtCCTc-3′ (13), were cloned separately into the *Xho*I polylinker site located at position −77 to generate p53 response element (pluc-wtp53) and control (pluc-mutp53) promoter constructs, respectively.

The pECE53 p53 expression vector under the control of the simian virus 40 (SV40) promoter and enhancer (42) was obtained from S. Benchimol. A wild-type p53 expression vector under the control of the Ad E1A enhancer, major late promoter, tripartite leader 5′ noncoding region, and polyadenylation signal in pNL3c (12) was obtained by adding *Sal*I linkers to the 1,430-bp *Sma*I fragment containing the human p53 cDNA in pECE53 (42) and cloning the fragment into the *Sal*I site of plasmid pNL3c (12). The HBV Ad wild-type X gene expression vector construct (pAdwtXO) under the control of the major late promoter and E1A enhancer present in pNL3c was obtained from R. Schneider (33). The construction of the T7 Flag-HMK 17-kDa HBx pET-3b expression plasmid has been previously described (1). To construct the GAL4(1-96):p53(1-73) T7 plasmid, PCR was used to amplify the GAL4 cDNA in pJL6 with 5′ *Nde*I and 3′ *Bam*HI restriction sites flanking the cDNA encoding amino acids 1 to 96 of *Saccharomyces cerevisiae* GAL4 [GAL4(1-96)]. This fragment was cloned, along with a *Bam*HI-*Eco*RI fragment containing the cDNA encoding p53(1-73) from plasmid pGST-p53(1-73) (72), into the *Nde*I- and *Eco*RI-cut plasmid pET5a (Novogen), resulting in a GAL4(1-96):p53(1-73) fusion protein under T7 RNA polymerase promoter control. To construct the plasmid pHBx, which encodes the HBx cDNA under bacteriophage T7 RNA polymerase promoter control, PCR was used to amplify the HBx cDNA in the T7 Flag-HMK 17-kDa HBx pET-3b plasmid (1) with 5′ *Nde*I and 3′ *Eco*RI restriction sites flanking the entire coding sequence for HBx. This fragment was blunt ligated into the *Sma*I site of plasmid pBluescript KS+ (Stratagene). Plasmid pHT-HBx, which encodes an N-terminal 10X histidine-tagged version of HBx, was created by the subsequent cloning of an *Nde*I-*Eco*RI fragment of DNA from pHBx into *Nde*I- and *Eco*RI-cut pET16b (Novogen). Plasmid pREP4 was obtained from Qiagen. The chloramphenicol acetyltransferase (CAT) gene under the control of the AdML promoter (ML-CAT) was obtained from J. Orlowski.

Factor preparation. Flag-HMK 17-kDa HBx expressed in BL21(DE3) and a control fraction prepared from BL21 were prepared by preparative isoelectric focusing and reverse-phase high-performance liquid chromatography (HPLC), as previously described (1). Human wild-type p53 was prepared from recombinant baculovirus-infected sf21 insect cells by immunoaffinity procedures (20). GAL4(1-96):p53(1-73), expressed in BL21(DE3) (65), was extracted from the insoluble 10,000 × *g* pellet prepared from a sonicated resuspended bacterial pellet (resuspended pellet from 1 liter of bacteria in 80 ml of 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.5–0.2 M KCl) by a modification of the procedure described by Carey et al. (6). Briefly, 20 ml of solubilization buffer (50 mM Tris [pH 7.4], 2 M urea, 0.2 M KCl, 0.1% Triton X-100, and 50 mM dithiothreitol [DTT]) was added to the insoluble pellet and the mixture was gently agitated for 2 h at 4°C. After centrifugation at 10,000 × *g* for 10 min, the resulting supernatant was applied to a 3-ml native DNA cellulose column (Pharmacia) equilibrated with solubilization buffer. The column was step eluted with 10 ml of solubilization buffer containing 1.0 M KCl, and the eluate was dialyzed twice against 2 liters of 25 mM HEPES [pH 7.9]–50 mM KCl–20% glycerol–10 μM $ZnCl_2$. The integrity of the purified GAL4(1-96):p53(1-73) preparation was verified by Western blot (immunoblot) analysis, following electrophoresis on a 12% polyacrylamide–urea–sodium dodecyl sulfate (SDS) gel (67), by using the appropriate alkaline phosphatase-coupled second antibody (either goat anti-mouse or goat anti-rabbit immunoglobulin G at a 1:7,500 dilution). GAL4(1-96) was detected with rabbit polyclonal serum 181 (1:2,000 dilution) raised against GAL4 residues 1 to 147 (Brendon Bell, University of British Colombia), and p53(1-73) was detected with mouse monoclonal antibody Ab1801 (1:2,000 dilution, Ab-2; Oncogene Science). The yeast TBP (yTBP) expressed in bacteria was purified as previously described (72). All factors were stored as aliquots at −85°C.

Histidine-tagged HBx (HT-HBx) used for affinity chromatography was produced by coexpression of the plasmids pHT-HBx and pREP4 (Qiagen) in *Escherichia coli* BL21(DE3) (65). A 1-liter overnight culture grown at 25°C was used to inoculate 14 liters of Luria-Bertani (LB) medium and grown at 25°C to an A_{600} of 1.5. The culture was subsequently induced for 1 h with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside), and the cells were pelleted at 3,000 × *g* (Sorvall SS34 rotor) for 15 min. The cell pellet was resuspended in sonication buffer (20 mM HEPES [pH 7.5], 0.5 M KCl, 10% glycerol), sonicated for three 1-min intervals on salt ice, and then centrifuged at 30,000 × *g* (Sorvall SS34 rotor) for 30 min. About 0.05% of the total HT-HBx produced in the cells was present in the soluble fraction. This material was purified under nondenaturing conditions on a His-Bind resin column as described by the manufacturer (Novogen) with a supplement of 20 mM NaH_2PO_4 to all buffers. The purified protein was dialyzed against 0.1 M ACB (72) and stored at −80°C.

[^{35}S]methionine (NEN Dupont)-labeled HBx, human TBP, p62, and wild-type p53 for affinity chromatography were prepared with T7 or SP6 RNA polymerase in a coupled transcription-translation rabbit reticulocyte lysate reaction (50-μl reaction volume) (Promega TNT system) by using plasmids pHBx, pET27 (51), pET11a-BTF2 (generously provided by R. Roy and J. M. Egly), and pSpProp53 (42), respectively.

Glutathione *S*-transferase (GST) and GST-p53 (wild type) proteins were prepared exactly as described elsewhere (72).

Affinity chromatography. ^{35}S-labeled HBx (5 μl of a 50-μl reaction volume) was chromatographed through 20-μl microaffinity columns containing immobilized (Affi-gel 10; Bio-Rad) GST, GST-p53(1-393), GST-p53(1-73), and GST-VP16 (36), all at a 2-mg/ml protein concentration in ACB loading buffer (10 mM HEPES [pH 7.5], 0.1 M NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 2 mg of bovine serum albumin and 0.1% Nonidet P-40, as previously described (72). Affinity columns were eluted sequentially with 300 μl of ACB buffer containing 0.5 M NaCl and 50 μl of ACB buffer containing 1% SDS. The eluates were electrophoresed on SDS–15% polyacrylamide gels at 40 mA by using the Laemmli buffer system (9), and the binding of HBx was detected by fluorography (Dupont).

^{35}S-labeled p53 (5 μl of a 50-μl reaction volume) was chromatographed through 20-μl microaffinity columns containing HT-HBx immobilized on His-Bind resin (Novogen) at a concentration of 1 mg/ml. The sample was loaded and elution was carried out as described above, except that no DTT or EDTA was present in any of the buffers (ACB*). Eluates were electrophoresed on SDS–10% polyacrylamide gels at 40 mA by using the buffer system described above, and the binding of p53 was also detected by fluorography.

As a positive control for binding to p53, the ^{35}S-labeled transcription factors TBP and p62 (5 μl of a 50-μl reaction volume each) were mixed and chromatographed on 20-μl microaffinity columns containing either GST or GST-p53(1-393) immobilized on glutathione-Sepharose beads (GS4B; Pharmacia) at a 1-mg/ml concentration. Columns were eluted sequentially with 200 μl of 0.1 M ACB and 50 μl of 20 mM glutathione-ACB. A mixture of ^{35}S-labeled TBP and p62 was also chromatographed through columns of HT-HBx immobilized on nickel-agarose beads (Novogen) at a 1-mg/ml concentration, or on HT-HBx immobilized at a 1-mg/ml concentration over which 20 μg of free GST-p53(1-393) in 0.1 M NaCl-ACB had been loaded. HT-HBx columns were eluted sequentially with 200 μl of 0.1 M NaCl-ACB* and 50 μl of 100 mM EDTA-ACB*. All eluates were electrophoresed on SDS–10% polyacrylamide gels (9) as described above. TBP and p62 binding was also detected by fluorography.

Gel EMSA. Electrophoretic mobility shift analysis (EMSA) was performed with human p53 consensus site DNA-binding and nonbinding mutant site DNA oligonucleotides. The oligonucleotides were radiolabeled with [α-^{32}P]dATP by 3′-end filling with the Klenow fragment of DNA polymerase. The sequences of the wild-type and mutant 26-bp DNA oligonucleotides were the same as those used for plasmid constructions as described above. Binding reaction mixtures contained 20 mM HEPES (pH 7.9), 4% (wt/vol) Ficoll (type 400), 50 mM KCl, 0.5 mM EDTA, 100 μg of poly(dI-dC), 50 ng of pBluescript SK− DNA (Stratagene), 50,000 cpm of p53 binding site DNA, and 450 ng of baculovirus-expressed human p53 protein.

Reaction mixtures (20-μl volume) were incubated for 20 min at 30°C. Interacting proteins HBx (38 ng) or yTBP (100 ng) or corresponding control fractions were added to the reaction mixture, which was then incubated for a further 20 min at 30°C. All samples were run on a 3.5% polyacrylamide gel (acrylamide-bisacrylamide, 30:0.8) at 120 V at room temperature in 0.5× TBE (Tris-borate-EDTA) buffer.

GAL4 EMSAs were performed with a 33-bp oligonucleotide, 5′-tc gagAAAGTCGGAGGACTGTCCTCCGTCAAAc-3′, which was labeled as described above. This DNA sequence was previously shown to bind a dimer of GAL4 (76). Binding reaction mixtures (25 μl) contained 20 mM HEPES (pH 7.9), 4% (wt/vol) Ficoll (type 400), 60 mM KCl, 0.5 mM EDTA, 2.5 μg of poly(dI-dC) · poly(dI-dC), 50 ng of pBluescript SK− DNA, 10,000 cpm of GAL4 oligonucleotide, and 5 μl of dialyzed DNA cellulose fraction of purified GAL4(1-96):p53(1-73) protein. The addition of the interacting proteins HBx and yTBP and the monoclonal anti-p53 antibody, Ab-2 (5 μl), and the running of the gel were as described above, except that a 5% polyacrylamide gel was used.

In vitro transcription assays. Transcription reaction mixtures (25 μl) contained 380 ng of a luciferase reporter gene containing either a wild-type consensus p53 response element or a mutant site; 11 μl of HeLa nuclear extract prepared by the Dignam procedure (Promega); ATP, GTP, UTP, and CTP (0.6 mM each); and buffer consisting of 20 mM HEPES (pH 7.7), 5 mM $MgCl_2$, 0.5 mM DTT, and a KCl concentration adjusted to 45 mM. Reaction mixtures contained baculovirus-expressed human p53 (90, 180, or 270 ng) and Flag-HMK 17-kDa HBx (29 ng) or its corresponding control fraction, as indicated. All reactions were initiated by the addition of nucleoside triphosphates, with HBx being added either after (condition 1) or before (condition 2) preinitiation complex formation. The reactions were terminated after a 60-min incubation at 30°C by the addition of stop mix (0.1 M sodium acetate [pH 5.5] containing 0.5%

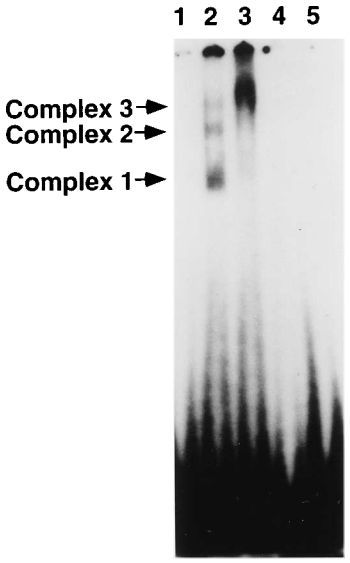

FIG. 1. Interaction of HBx with DNA-bound p53. A fluorograph of an EMSA performed with human p53 and either a consensus p53 binding site (lanes 1 to 4) or a non-DNA-binding mutant site (lane 5) is shown. The p53-containing complexes 1, 2, and 3 are indicated by arrows. Lane 2, p53-DNA complexes on a p53 consensus binding site; lane 1, p53-DNA complexes inhibited by competition by an excess of nonlabeled DNA; lane 3, HBx protein added to preformed p53-DNA complexes; lane 4, HBx alone added to p53 binding site-containing DNA; lane 5, p53 added to a mutant nonbinding p53 site oligonucleotide.

SDS, 10 mM EDTA, and 1.0 mg of yeast tRNA per ml). Transcription reactions were monitored by primer extension at 50°C as previously described (4) by using 5′-CTTATGCAGTTGCTCTC-3′, which is located at positions +85 to +68 in the luciferase gene, as the primer. This results in a 152-nucleotide product when transcriptional initiation occurs from the AdML promoter. Dried samples were suspended in loading buffer (50% deionized formamide containing 0.1% xylene cyanole and 0.1% bromophenol blue) and run at 230 V on an 8% polyacrylamide (acrylamide-bisacrylamide, 30:0.8) gel containing 7 M urea and 1× TBE. When the AdML promoter was used as a template, it contained the short G-free cassette in pML(C_2 AT)Δ-127 sh (57, 66). Reaction conditions were as described above, except for a different nucleotide mix consisting of 0.6 mM each ATP and CTP, 0.025 mM [α-^{32}P]UTP, 0.1 mM 3′-*O*-methyl GTP, and 15 U of RNase T1. The 350-nucleotide reaction product was resolved on a 4% polyacrylamide gel containing 7 M urea. Autoradiography was performed to localize transcripts. Scintillation counting was used when quantitation was required.

DNA transfections and CAT and luciferase analysis. Calu-6 cells were seeded at 5×10^5 cells per 60-mm-diameter dish and grown overnight in Dulbecco's modified Eagle (DME) medium containing 10% calf serum. On the following day, cells were transfected by Ca_2PO_4 coprecipitation (MBS mammalian transfection kit; Stratagene). Transfections employed luciferase reporter constructs containing wild-type consensus or mutant p53 binding sites (5 μg) and ML-CAT control plasmid (5 μg). A p53-expressing plasmid under the control of the AdML promoter or the SV40 promoter, along with the HBx expression plasmid pAdwtXO or the control plasmid pNL3c, was cotransfected as indicated in the legend to Fig. 5. Salmon sperm DNA was added to give a total of 20 μg of DNA per plate. Cells were incubated with DNA precipitates for 3 h, washed, and harvested 30 h later. Cell lysates were prepared by using reporter lysis buffer (Promega) (400 μl per plate), and luciferase and CAT assays were performed with extracts that had been frozen as aliquots at −85°C, as described by the manufacturer of the assay kit (Promega).

RESULTS

Interaction of HBx with wild-type p53 bound to a p53 response element promotes or stabilizes the oligomerization of p53. We initially used EMSA to detect an interaction between p53 and HBx. By EMSA, multiple complexes are formed between wild-type human p53 produced in a baculovirus system (Fig. 1, lane 2; complexes 1, 2, and 3) and a DNA oligonucleotide containing a consensus binding site for p53 (13). The complexes that were observed were specific, since they were not formed in the presence of cold competitor DNA (Fig. 1, lane 1) or when the binding site contained a mutation (lane 5). Complex 1 may contain a tetramer of p53, as previously suggested (2), since nondenaturing polyacrylamide gel electrophoresis (PAGE) has revealed the existence of p53 oligomers that represent tetramers and multiples thereof (64), and since a predominant tetrameric form of p53 in solution was detected by cross-linking, gel filtration chromatography, and zonal velocity gradient centrifugation (19). This is controversial (24, 25), however, since complex 1 could also represent a dimeric p53 complex in which the quaternary status of p53 regulates DNA binding (24, 71). This is because the p53 generated by deletion of the C-terminal oligomerization domain forms a stable DNA complex only as a dimer (50). The addition of bacterially expressed Flag-HMK 17-kDa HBx (17 ng) to preformed p53 response element complexes (450 ng of p53) resulted in quantitative conversion to a complex with slightly less mobility than that of complex 3 and the disappearance of complexes 1 and 2 (Fig. 1, lane 3). The decreased mobility of complex 3 in the presence of HBx likely resulted from the incorporation of HBx into a p53 oligomeric complex, because the p53-HBx complex could be supershifted with an anti-Flag antibody against the HBx (data not shown). Although all of the p53 bound to DNA interacted with HBx under our assay conditions (450 ng of p53, 17 ng of HBx), we cannot comment on the stoichiometry since we do not know if HBx bound equally well to the substantial amount of p53 that was not bound to DNA. Note that HBx did not, by itself, interact directly with the DNA probe (Fig. 1, lane 4), and that an HPLC control fraction from a BL21 control strain of the kind that was used to make HBx had no effect on p53 complex formation (data not shown). Therefore, our data indicated that HBx could directly interact with and stabilize a highly oligomerized form of DNA-bound p53. The inclusion of yTBP (100 ng) in the p53 EMSA did not result in the detection of a mobility-shifted complex by our oligonucleotide probe, as observed previously under similar conditions for human TBP (19), nor did the presence of yTBP interfere with the observed HBx-dependent oligomerization of p53 (data not shown).

HBx binds directly to human p53. Protein affinity chromatography was used to test whether human p53 could directly and selectively bind HBx in the absence of DNA. First, HT-HBx was produced in bacteria, purified, and then immobilized on agarose beads. Full-length, ^{35}S-labeled, wild-type human p53, produced by transcription and translation in vitro (Fig. 2A, lane 2, indicated by the arrow), bound to an HBx column (lane 4) and not to a control column containing no immobilized protein (lane 3). This binding was selective and specific for the full-length translation product of the p53 cDNA: fragments of p53 visible in Fig. 2A, lane 2, and other labeled background proteins visible in a control translation reaction programmed with a plasmid lacking the p53 cDNA (lane 1) did not bind.

To confirm this direct HBx-p53 interaction in a different way, we also chromatographed HBx on p53 columns (Fig. 2B). Production of ^{35}S-labeled HBx by transcription and translation in vitro resulted mainly in the expected 17-kDa HBx polypeptide (Fig. 2B, lane 2). This protein was not produced in a control reaction programmed with a plasmid lacking the HBx cDNA (lane 1). HBx bound specifically and with about 50% efficiency to a column containing immobilized full-length human p53 (amino acids 1 to 393) produced in *E. coli* as a GST-p53 fusion protein (72) (Fig. 2B, lane 5), and not to a control GST column (lane 4). Binding of ^{35}S-labeled protein to GST-p53 was observed only if the transcription-translation reaction mixture contained HBx cDNA (Fig. 2B, lane 5) and not if it contained a control plasmid (lane 3). HBx bound to both

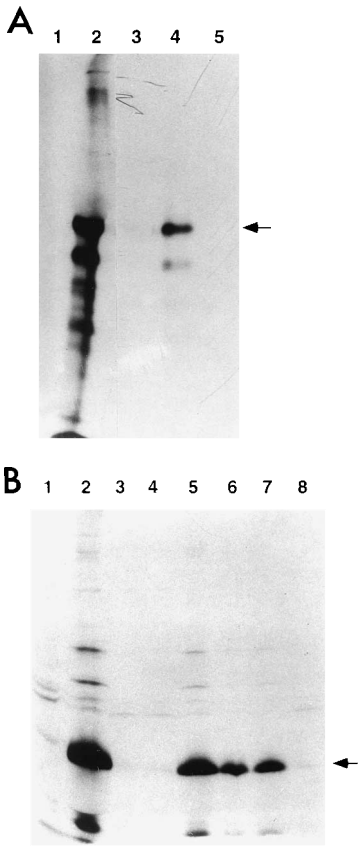

FIG. 2. Direct interaction between HBx and p53. (A) Chromatography of ^{35}S-labeled wild-type p53 on an HT-HBx column. Each column was loaded with 5 μl of an in vitro transcription-translation reaction mixture. The 1% SDS eluates from various columns were subjected to SDS-PAGE and fluorography. Lane 1, 2.5 μl of a ^{35}S-labeled transcription-translation reaction mixture programmed with a control plasmid containing no inserted cDNA; lane 2, 2.5 μl of a ^{35}S-labeled transcription-translation reaction mixture programmed with p53 wild-type cDNA; lane 3, 25 μl of eluate from an HT-HBx column loaded with the control translation reaction mixture shown in lane 1; lane 4, 25 μl of eluate from an HT-HBx column loaded with the p53 translation reaction mixture shown in lane 2; lane 5, 25 μl of eluate from a control column containing no immobilized protein and loaded with the p53 translation reaction mixture shown in lane 2. The migration of full-length ^{35}S-labeled p53 protein at approximately 53 kDa is indicated by the arrow. (B) Chromatography of ^{35}S-labeled HBx on p53 columns. Each column was loaded with 5 μl of an in vitro transcription-translation reaction mixture. The 1% SDS eluates (50 μl) of the various columns were analyzed by SDS-PAGE and fluorography. In vitro transcription-translation reaction mixtures were programmed with a plasmid containing HBx cDNA (lane 2) or a control plasmid containing no inserted cDNA (lane 1). A 2.5-μl volume of the in vitro transcription-translation reaction mixtures is loaded in each lane. Lane 3 contains 25 μl of eluate from a GST control column loaded with the HBx translation reaction mixture shown in lane 2; lane 4 contains 25 μl of eluate from a GST-p53(1-393) column loaded with the control translation reaction mixture shown in lane 1; lane 5 contains 25 μl of eluate from a GST-p53(1-393) column loaded with the HBx translation reaction mixture shown in lane 2; lane 6 contains 25 μl of eluate of a GST-p53(1-73) column over which HBx was loaded; lane 7 contains 25 μl of eluate from a GST-p53(141-393) column loaded with ^{35}S-labeled HBx; and lane 8 contains 25 μl of eluate from a GST-VP16 column loaded with ^{35}S-labeled HBx. The migration of ^{35}S-labeled HBx at approximately 17 kDa is indicated by the arrow.

GST-p53(1-73), which contains the N-terminal activation domain of p53 (17) (Fig. 2B, lane 6), and to GST-p53(141-393), which contains the DNA-binding (63) and dimerization (27) domains of p53 (lane 7), but binding in these cases was only 25% as efficient as it was to full-length p53 (lane 5). This suggests either that there are two binding sites in p53 for HBx or that the binding site in p53 for a single molecule of HBx is

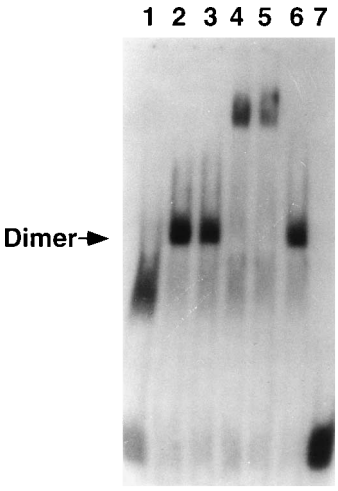

FIG. 3. Interaction between HBx and the p53 acidic activation domain is not detected by EMSA. Nondenaturing PAGE (EMSA) of the GAL4(1-96):p53(1-73) protein bound to a radiolabeled GAL4 binding site-containing DNA oligonucleotide is shown in lane 2. In the other lanes, the reagents were added as follows: lane 1, GAL4(1-96) rather than the GAL4(1-96):p53(1-73) fusion protein; lane 3, HBx protein; lanes 4 and 5, anti-p53 monoclonal antibody, Ab-2, with (lane 4) and without (lane 5) HBx protein; lane 6, yTBP and HBx proteins; lane 7, HBx, yTBP, and Ab-2 anti-p53 monoclonal antibody in the absence of GAL4:p53 protein. The migration of the GAL4:p53 protein DNA complex is indicated by the arrow.

bipartite. The MDM2 oncoprotein has previously been shown to interact with p53(76-78). Although we have not determined a dissociation constant for the p53-HBx interaction, we found that HBx made in vitro bound about 10 times as efficiently as MDM2 made in vitro to p53 columns under our binding conditions (data not shown).

Although the N-terminal activation domain of p53 is very acidic, HBx did not bind to a column containing immobilized GST-VP16 (Fig. 2B, lane 8), even though the VP16 activation domain on this column is similar to that of p53 in size, amino acid composition, net charge, and activation potential (17). Moreover, all of the interactions between HBx and p53 were insensitive to salt, although they were disrupted by 6 M guanidine-HCl or 1% SDS, indicating the predominant involvement of nonionic interactions (data not shown). Nevertheless, the interaction between HBx and the p53 activation domain may be quite weak, since we could not detect it by EMSA (Fig. 3). For this experiment, a fusion protein containing the DNA-binding and dimerization domains of *S. cerevisiae* GAL4 (amino acids 1 to 96), linked to the p53 activation domain (amino acids 1 to 73), was produced in *E. coli* and purified. This GAL4(1-96):p53(1-73) fusion protein bound to an oligonucleotide containing a GAL4 binding site (Fig. 3, lane 2), presumably as a dimer (6), but its position was not shifted by inclusion of HBx in the binding reaction mixture (lane 3). TBP, which also binds to the p53 activation domain (72), also did not produce a supershift in these circumstances (Fig. 3, lane 6), presumably because its binding to p53 is also too weak to be detected in this way. Only a monoclonal antibody produced a supershift in this analysis (Fig. 3, lanes 4 and 5). Failure to observe binding of HBx to the activation domain of p53 in this gel shift assay, in contrast to the result of the affinity chromatography assay shown in Fig. 2B, may be due to the different fusion proteins used in the two assays [GST versus GAL4(1-96)] or the use of EMSA conditions which were optimized for DNA binding to the GAL4 oligonucleotide.

Interaction of HBx with p53 does not block recognition by

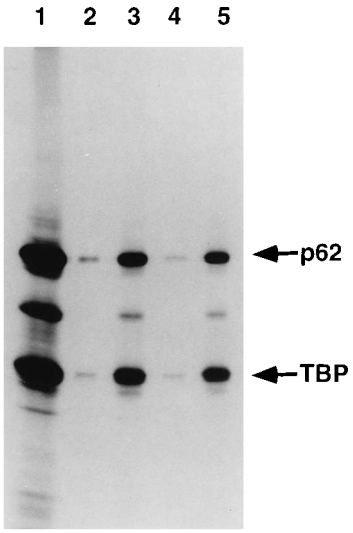

FIG. 4. HBx-bound p53 is able to complex with transcription factor proteins TBP and p62. A mixture of ^{35}S-labeled TBP and p62 made by translation in vitro (lane 1, 5 μl) was chromatographed on various columns. The bound proteins were eluted and analyzed by SDS-PAGE and fluorography (lanes 2 to 5). Lane 2, 25 μl of the 50-μl 20 mM glutathione eluate from a control column containing GST immobilized on glutathione-Sepharose; lane 3, 25 μl of the 50-μl 20 mM glutathione eluate from a column containing GST-p53(1-393) immobilized on glutathione-Sepharose; lane 4, 50 μl of the 50-μl 100 mM EDTA eluate from a nickel chelate column containing immobilized HT-HBx; lane 5, 50 μl of the 50-μl 100 mM EDTA eluate from a nickel chelate column containing immobilized HT-HBx that was preloaded with free GST-p53(1-393).

p53 of TBP or p62. The ability of HBx to bind p53 and inhibit transactivation by p53 suggested that HBx might accomplish this by blocking the interaction between p53 and either the TBP subunit of TFIID (8, 37, 41, 60, 72) or the p62 subunit of TFIIH (77). However, in initial experiments we observed no effect of HT-HBx produced in *E. coli* on the binding of TBP or p62 to a GST-p53 column (data not shown). This suggested that p53 could simultaneously bind both HBx and either p62 or TBP. To test this idea, we chromatographed ^{35}S-labeled TBP and p62 made by transcription and translation in vitro (Fig. 4, lane 1) on an HBx column that had been preloaded with recombinant GST-p53. As expected (72, 77), TBP and p62 bound to a GST-p53 column (Fig. 4, lane 3) but not to a GST control column (lane 2). More importantly, TBP and p62 both also bound to an HBx column that had been preloaded with GST-p53 (Fig. 4, lane 5) but not to an HBx column in the absence of GST-p53 (lane 4). Therefore, p53 which is bound to HBx is still able to bind TBP and p62.

HBx inhibits the transactivation of a p53-responsive reporter plasmid by p53 in Calu-6 cells. Calu-6 cells, a human lung carcinoma cell line (18), were chosen for use in transient transfection analysis because they do not express p53 mRNA and therefore lack p53 (5). The reporter plasmid pluc-wtp53 contained a firefly luciferase gene under the control of the AdML basal promoter (positions −34 to +33) and a p53 response element. Cotransfection with p53 expression constructs in the 0- to 0.5-μg range, driven either by the AdML promoter and enhancer or by the SV40 promoter and enhancer, specifically increased luciferase production by 2.5- and 4-fold, respectively (Fig. 5A), but only if the luciferase reporter had a wild-type p53 response element and not if it had a mutant element (data not shown). The reason for the quantitative difference with the AdML and SV40 expression constructs is not clear, since cotransfection with 1.0 μg or more of either

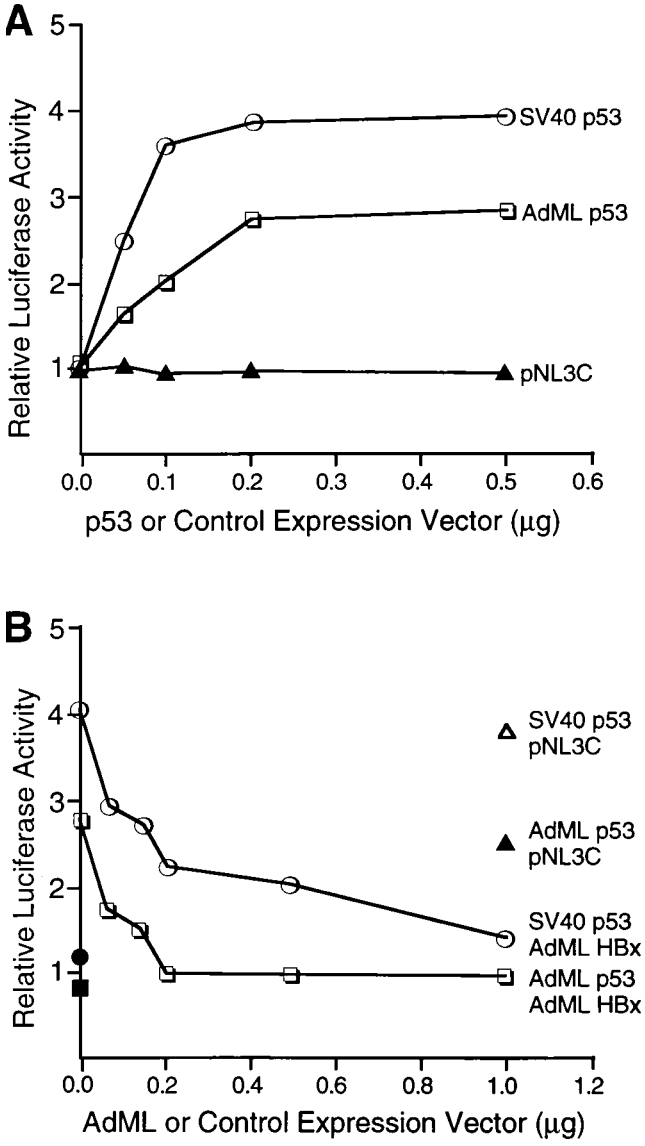

FIG. 5. HBx inhibits activation by p53 in vivo. (A) Activation by p53. Calu-6 cells were cotransfected with 5 μg of a luciferase reporter construct containing a p53 binding site and 0 to 0.5 μg of either the control vector, pNL3C, or a p53 expression vector under the control of the AdML promoter or the SV40 promoter-enhancer. Luciferase activity was measured relative to the CAT activity produced by a cotransfected ML-CAT control plasmid. (B) Inhibition of p53-activated transcription by HBV. Calu-6 cells were cotransfected with a mixture containing 5 μg of a luciferase reporter construct (pluc-wtp53) with a consensus p53 binding site; 0.2 μg of either the AdML-p53 or the SV40-p53 expression vector; and 0 to 1.0 μg of AdML promoter-driven HBx expression vector, pAdwtXO. Control cells were cotransfected with 1.0 μg of the control plasmid, pNL3C, rather than pAdwtXO, as indicated. The relative luciferase activities produced by a control luciferase construct, pluc-mutp53, containing a mutant p53 binding site, in cells that were cotransfected with p53 expression vectors driven by either the AdML (■) or SV40 (●) promoter are also shown.

expression vector resulted in substantially lower levels of luciferase production, perhaps due to the "squelching" phenomenon that has been observed previously (49) (data not shown). Cotransfection with 0 to 1.0 μg of an HBx expression vector under the control of an AdML promoter (pAdwtXO) (33), along with 0.2 μg of the AdML promoter- or SV40 promoter-driven p53 expression construct, resulted in inhibition of the p53-activated luciferase expression to a level close to the basal

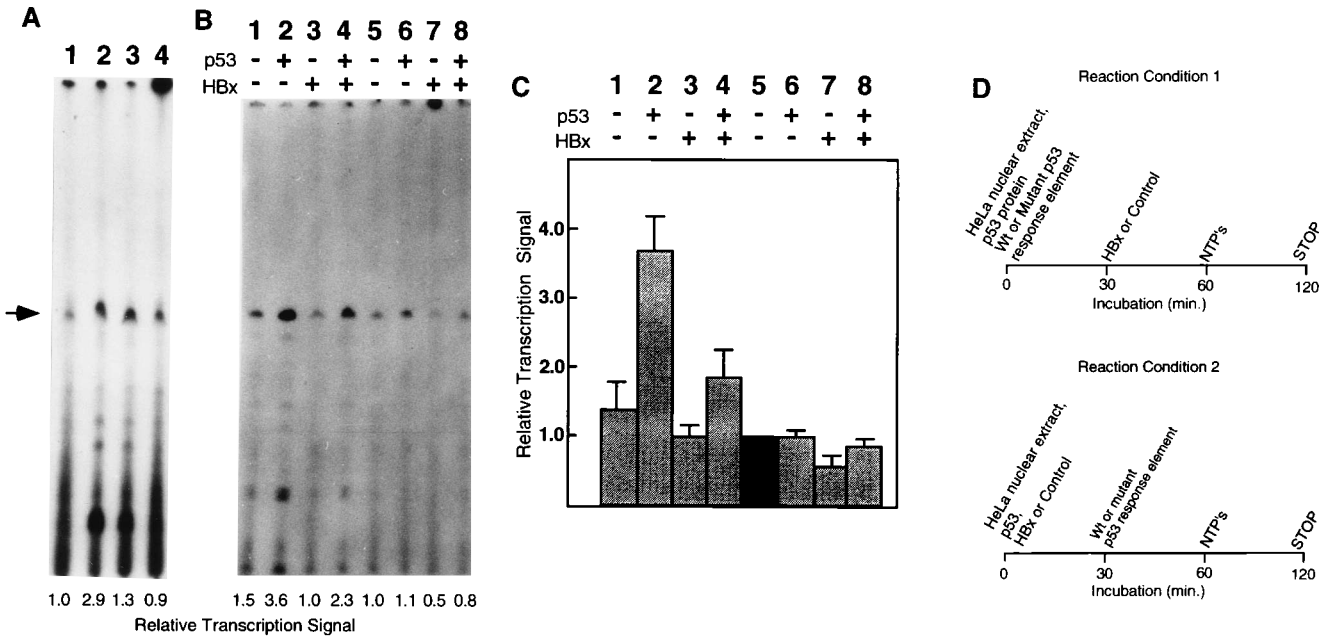

FIG. 6. HBx inhibits p53-activated transcription in vitro. (A) Stimulation by baculovirus-produced p53 of transcription in vitro from an AdML promoter containing a consensus p53-binding site. Reaction mixtures contained 0 (lane 1), 90 (lane 2), 180 (lane 3), and 270 (lane 4) ng of p53. The relative amounts of the 152-nucleotide promoter-specific primer extension product (indicated by the arrow) are shown at the bottom. (B) Inhibition by HBx of transcription from preassembled transcription complexes (see panel D, condition 1) formed on DNA templates containing a consensus p53 binding site (lanes 1 to 4) or a mutant p53 binding site (lanes 5 to 8). As indicated by the plus and minus signs above the lanes, 90 ng of p53 and 29 ng of HBx protein were added to the reaction mixtures. (C) Bar graph showing relative transcription signals and standard deviations from four sets of reactions performed as shown in panel B, two under reaction condition 1 and two under reaction condition 2 (see panel D). The reaction mixtures contained templates with either a consensus p53 binding site (lanes 1 to 4) or a mutant p53 binding site (lanes 5 to 8). The transcription signals were normalized relative to the mutant template in the absence of p53 or HBx, as indicated by the darker bar in column 5. (D) Schematic representations of reaction conditions 1 (HBx addition after preinitiation complex formation) and 2 (HBx addition before preinitiation complex formation). NTPs, nucleoside triphosphates.

activity that was observed with a luciferase construct containing a mutant p53 site (Fig. 5B). This inhibition was specific for HBx, because it was not observed upon cotransfection with the control vector pNL3c that was used to make the HBx expression construct. Our results are in apparent contrast to those of a recent study in which HBx was found to enhance transactivation by p53 in Calu-6 cells, but this appeared to result from transactivation by HBx of the cytomegalovirus promoter-driven p53 expression vector used in these experiments (75).

HBx inhibits p53-transactivated transcription from preassembled initiation complexes in vitro. To further characterize the mechanism by which HBx inhibits activation by p53, we examined the effect of HBx on transactivation by p53 in vitro using reaction mixtures containing HeLa nuclear extracts. The DNA templates used in these reactions contained a luciferase gene driven by the AdML promoter and either a wild-type or mutant p53 consensus binding site (13). The addition to the reaction mixture of 90 ng of human baculovirus-produced p53 led to a 2.9-fold increase in the level of reporter transcript, as measured by primer extension analysis (Fig. 6A, lane 2), as has been observed previously with a different reporter construct (15). The addition of more p53 (180 or 270 ng) to the reaction mixture led to decreased production of the transcript, as has also been previously observed by others (39, 55). This repressing effect of p53 in vitro may be explained by several models (reviewed in reference 35), including the squelching model (22). Note that in contrast to the approximately threefold activation observed with a promoter containing a consensus p53 binding site, there was little or no activation with a promoter containing a mutant p53 binding site (Fig. 6B and C). The addition of HBx (29 ng) before or after the assembly of the preinitiation complex (see the protocols in Fig. 6D) led to a twofold reduction in transcription (Fig. 6C), suggesting that HBx can contact or disrupt the formation of preformed preinitiation complexes. Under our transcription conditions, which involved a larger amount of template DNA than was previously used (375 ng compared with the 25 ng used in the study described in reference 15), we observed basal p53-independent transcription (Fig. 6A through C). HBx sometimes had small effects on basal transcription as well, but these effects were not statistically significant.

DISCUSSION

Several mechanisms have been proposed to explain why the HBx protein of HBV is oncogenic (31): direct inactivation of p53 (16); transactivation of cellular genes by HBx acting as a coactivator that interacts with cellular DNA-binding transcription factors (1, 40; reviewed in reference 56); stimulation of cellular second-messenger systems (11, 30, 38, 45, 46); and direct inactivation of certain cellular serine proteases, such as tryptase TL2 (70). We have shown here that HBx binds directly to human p53 and represses transactivation by p53 in vivo in transfection experiments. This is consistent with the observation that transcriptional activation by p53 is essential for its ability to act as a growth suppressor (52). In this way, the activity of HBx is similar to that of the cellular oncoprotein MDM2, which also transforms cells by interacting with p53 and repressing its ability to activate transcription (43, 48, 80). Our observations are also consistent with the finding that HBx is in a protein complex that contains wild-type p53 in liver tumor tissues from HBV-infected HCC patients (16, 26, 62). Evi-

dence that p53 can directly bind HBx was also recently reported by Wang et al. (75).

It is striking that HBx can be either an activator or a repressor of transcription. Since HBx has no known DNA-binding activity, its ability to activate transcription seems to depend on interactions with cellular DNA-binding proteins like CREB, ATF-2, and Oct-1 (1, 40). Stimulation of transcription by HBx probably involves an activation domain in its C-terminal 104 amino acids (44). On the other hand, interaction of HBx with p53 represses activation by p53, and high concentrations of HBx lead to dimerization of HBx and repression of the X gene promoter in HBV DNA (44). Therefore, whether HBx will behave as an activator or repressor of transcription seems to depend on the manner in which it is presented to a particular promoter.

Although HBx represses activation by p53, we found that HBx did not prevent DNA binding by p53. Instead, HBx promoted or stabilized the oligomerization of p53 on its DNA site. Our results are different from those of Wang et al. (75), who used HBx made in a reticulocyte lysate, rather than purified recombinant HBx, and found that HBx apparently prevented DNA binding by p53 in an in vitro DNA-binding immunoprecipitation assay. Differences in the assay conditions (e.g., the DNA-binding assay technology or the presence or absence of reticulocyte lysate proteins) may explain this discrepancy.

Transcriptional activation by p53 appears to depend not only on its ability to bind DNA (71) but also on interactions of its N-terminal acidic activation domain with the general initiation factors TFIID (8, 37, 41, 60, 72) and TFIIH (77). Nevertheless, we found that p53 can simultaneously bind both HBx and either the TBP subunit of TFIID or the p62 subunit of TFIIH. It is possible that in the context of a complete initiation complex, which contains the other subunits of TFIID and TFIIH, as well as RNA polymerase II, TFIIB, TFIIE, and TFIIF, HBx can, nevertheless, sterically block the interaction of p53 with TFIID or TFIIH. However, for two reasons, we favor the idea that HBx that is bound to p53 can act as a direct repressor of the transcriptional machinery, as illustrated in Fig. 7. Firstly, there is evidence that the N-terminal 50 amino acids of HBx constitute a repression domain, as well as a dimerization motif (44). Secondly, we found that HBx can inhibit p53-activated transcription in vitro even when it is added to the reaction mixture after the formation of the preinitiation complex. This observation is most easily explained by an interaction between a repressing region of HBx and a basal transcription factor.

Many DNA tumor viruses are known to produce oncogenic proteins that inactivate p53. For example, SV40 T antigen prevents DNA binding by p53 (28) while the E6 proteins of the oncogenic human papillomaviruses target p53 to the ubiquitin-dependent proteolytic pathway (58). The Ad E1B 55-kDa protein may, however, provide the best model for understanding the activity of HBx. The 55-kDa protein of both HBx and E1B can interact with p53 that is bound to its specific DNA recognition element (79). Moreover, E1B interacts with the activation domain of p53 (29) and thereby tethers a transcriptional repression domain to p53 (79). In a similar way, direct interaction of HBx with p53 may tether HBx's repression domain (44) in the vicinity of a promoter that is normally activated by p53. Identification of the general initiation factor that is targeted by the repression domain of HBx will require further investigation.

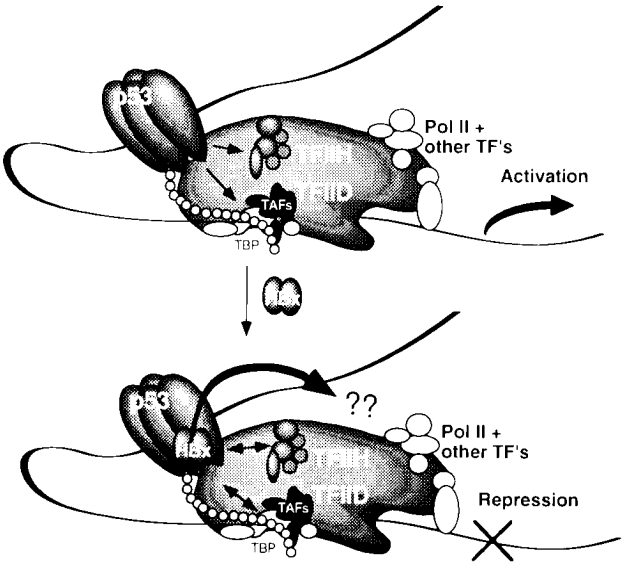

FIG. 7. Model for inhibition by HBx of p53-activated transcription. Activation and repression in the absence (top) and presence (bottom) of HBx are shown. The smaller arrows denote interactions between p53 and either the TBP subunit of TFIID or the p62 subunit of TFIIH. The large arrow suggests that HBx inhibits transcription by interacting with one or more of the general initiation factors. TFs, transcription factors; TAFs, TATA-binding protein-associated factors.

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The first two authors (R.T. and J.A.) contributed equally to this work.

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