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Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3

XIN WEI WANG*, KATHLEEN FORRESTER*, HEIDI YEH*, MARK A. FEITELSON†, JEN-REN GU‡, AND CURTIS C. HARRIS*§

*Laboratory of Human Carcinogenesis, Division of Cancer Etiology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; †Department of Pathology and Cell Biology, Jefferson Medical College, Philadelphia, PA 19107; and ‡Department of Molecular Biology and Biochemistry, Shanghai Cancer Institute, Shanghai, People's Republic of China.

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ABSTRACT **Chronic active hepatitis caused by infection with hepatitis B virus, a DNA virus, is a major risk factor for human hepatocellular carcinoma. Since the oncogenicity of several DNA viruses is dependent on the interaction of their viral oncoproteins with cellular tumor-suppressor gene products, we investigated the interaction between hepatitis B virus X protein (HBX) and human wild-type p53 protein. HBX complexes with the wild-type p53 protein and inhibits its sequence-specific DNA binding *in vitro*. HBX expression also inhibits p53-mediated transcriptional activation *in vivo* and the *in vitro* association of p53 and ERCC3, a general transcription factor involved in nucleotide excision repair. Therefore, HBX may affect a wide range of p53 functions and contribute to the molecular pathogenesis of human hepatocellular carcinoma.**

The p53 tumor-suppressor gene product has pleiotropic functions including control of genomic plasticity (1). p53 modulates gene transcription by binding to specific DNA sequences and to other cellular factors (such as Mdm-2, TBP, and WT1) and also may be involved in DNA replication and repair processes (2–6). Mutations in p53 are commonly found in human cancers (1, 7). Disruption of p53 activity either by mutations or by interaction with viral oncoproteins or cellular proteins has been implicated in the development of many human cancers. p53 missense mutants not only lose normal function but often gain oncogenic properties (8–10). In virally transformed cells, p53 complexes with viral oncoproteins, such as the simian virus 40 large tumor (T) antigen (11, 12), the adenovirus E1B 55-kDa protein (13), the human papillomavirus E6 protein (14), or the Epstein–Barr virus-encoded nuclear antigen EBNA-5 (15). These protein–protein interactions can inactivate p53 functions—e.g., sequence-specific DNA binding and gene transactivation—and thus are an important aspect of viral oncogenicity.

Human hepatitis B virus (HBV) is one of the major risk factors associated with primary hepatocellular carcinoma (HCC) (16). The X protein encoded by HBV (HBX) is a protein of 154 amino acids and enhances the activity of both cellular and viral promoters. While HBX does not bind DNA directly, it modulates gene expression by interacting directly with cellular transcription factors (17, 18) or indirectly via activation of the cellular protein kinase C (PKC) signal transduction pathway (19, 20). Unlike retroviral oncogenes, HBX does not appear to have a cellular homologue. HBX is capable of transforming normal rodent cells (21–23) and causes HCC in certain strains of transgenic mice, either alone (24) or in combination with *N*-nitrosodiethylamine exposure (J. Butel, personal communication). However, little is known about the role of HBX in HCC development. In this report, we present results consistent with the hypothesis that HBX binds to p53 and abrogates its normal cellular functions.

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MATERIALS AND METHODS

Plasmids. Plasmid constructs encoding GST–p53-WT, containing glutathione *S*-transferase (GST) fused to human wild-type p53, and GST–p53-135Y, containing GST fused to the mutant p53 containing a His → Tyr mutation at codon 135, were provided by Jon Huibregtse (National Cancer Institute) (25). The remaining GST–p53 constructs were made by PCR amplification of the p53 open reading frame encoding missense mutations at codon 248 (Arg → Trp), 249 (Arg → Ser), or 273 (Arg → His) from plasmids provided by Bert Vogelstein (Johns Hopkins University) and insertion in frame into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia LKB). pSPX46, used for *in vitro* translation of HBX protein, contains HBV nucleotides (nt) 1248–1718, including the entire open reading frame of the HBX gene amplified by PCR and inserted into the *Bgl* II site of pSP72 under the control of the T7 promoter (Promega). pSHDX42 was constructed by inserting the *Bgl* II fragment of pSPX46 into the *Bam*HI site of pLXSHD, a retroviral expression vector provided by Andrea Pfeifer (Nestec, Lausanne, Switzerland). pCMV-X1 was constructed by inserting the *Bgl* II fragment of pSPX46 into the *Bam*HI site of pCMVneo (Bert Vogelstein). The plasmid encoding GST–X was constructed by inserting a PCR-amplified fragment corresponding to the HBX open reading frame from pSPX46 into the *Bam*HI site of pGEX-2T. PG_{13}-CAT, containing the chloramphenicol acetyltransferase (CAT) reporter gene adjacent to the p53 DNA binding site (26), and pC53-SN, encoding human wild-type p53 under the control of the cytomegalovirus (CMV) IE promoter, were provided by Bert Vogelstein. pSelectp53 was constructed by inserting the 1.8-kb *Bam*HI fragment of pC53-SN into the *Bam*HI site of pALTER-1 (Promega). pSelectp53, constructed by Emanuela Felley-Bosco (Université de Lausanne, Lausanne, Switzerland), was used for *in vitro* translation of p53 protein. pZAP10, used for *in vitro* translation of ERCC3 protein, was provided by Jan Hoeijimakers and Geert Weeda (Erasmus University, Rotterdam, The Netherlands).

Expression and Purification of Recombinant Proteins. GST fusion proteins were produced in *Escherichia coli* and purified on glutathione-Sepharose 4B beads (Pharmacia LKB) as

Abbreviations: CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; HBV, hepatitis B virus; HBX, hepatitis B virus X protein; HCC, hepatocellular carcinoma; PKC, protein kinase C; T antigen, simian virus 40-encoded large tumor antigen.

§To whom reprint requests should be addressed at: Laboratory of Human Carcinogenesis, Building 37, Room 2C01, National Cancer Institute, Bethesda, MD 20892.

described (27). The fusion proteins immobilized on the surface of the Sepharose beads were stored at 4°C in phosphate-buffered saline (pH 7.4), containing 1% (vol/vol) Triton X-100 for up to 2 months. The protein concentrations were determined by Coomassie blue staining of SDS/polyacrylamide gels and comparison with molecular weight standards run on the same gel (Bio-Rad). The purified baculovirus-produced p53-WT and p53-273^{His} proteins were provided by Carol Prives (Columbia University). The purified baculovirus-produced simian virus 40 T antigen was provided by Daniel Simmons (University of Delaware). For generation of *in vitro* translated ^{35}S-labeled proteins, the plasmids encoding the corresponding genes driven either by T7 or SP6 promoters were subjected to a one-step *in vitro* transcription and translation system (TNT System; Promega) at room temperature for 90 min in the presence of [^{35}S]cysteine (DuPont/NEN). Each *in vitro* translated protein was freshly prepared.

***In Vitro* Protein Binding and Analysis of Protein Complexes.** Binding assays were carried out in 500 μl of immunoprecipitation (IP) buffer (50 mM Tris·HCl, pH 8.0/120 mM NaCl/0.5% Nonidet P-40) containing 2–15 μl of the ^{35}S-labeled *in vitro* translated proteins and Sepharose beads loaded with GST fusion proteins either at room temperature or at 4°C for 60 min. The beads were washed five times with IP buffer. The bound proteins were eluted by boiling the beads in the presence of Laemmli buffer for 5 min, separated by SDS/PAGE, and visualized by fluorography. An aliquot of *in vitro* translated HBX or ERCC3, immunoprecipitated by anti-HBX polyclonal antibody (28) or anti-ERCC3 polyclonal antibody (29), respectively, was included as a reference (see Fig. 1, lane 3, and Fig. 5, lane 2). For detection of unlabeled baculovirus-produced p53 following *in vitro* binding to GST-X (see Fig. 2), the ECL Western blot detection system (Amersham) was used with polyclonal anti-p53 antibody (CM-1) (Signet Laboratory, Dedham, MA).

***In Vitro* DNA-Binding Assay.** The p53 sequence-specific DNA-binding assay was performed essentially as described (30). A ^{32}P-labeled DNA fragment (PG259) containing 13 copies of the p53 binding sequence (26) was incubated with 100 ng of either baculovirus-produced human wild-type p53 or mutant p53-273^{His} followed by immunoprecipitation with anti-p53 antibody (DO-1; Oncogene Science). p53-bound PG259 was released by digestion with proteinase K and analyzed by electrophoresis in a nondenaturing 5% polyacrylamide gel.

Cell Culture, Transfection, and CAT Assay. THLE-5b cells were originally established in this laboratory (National Cancer Institute) as a T-antigen-immortalized normal human hepatocyte line and were grown in fibronectin-coated flasks containing LCM medium essentially as described (31). The culture conditions for SK-Hep-1 cells, Calu 6 cells, and Hep3B cells are described elsewhere (32).

For transfection, cultured cells in a 100-mm-diameter tissue culture plate (Corning) at about 50% confluence were transiently transfected by Lipofectin (GIBCO/BRL) with 5 μg of p53-responsive reporter construct (PG_{13}-CAT) and 5 μg of pC53-SN in the presence or absence of various amounts of HBX expression vectors for 5 hr. All plates were transfected with 30 μg of total DNA by addition of pLXSHD or pBluescript SKII(+) vector (Stratagene) as a carrier. After 16 hr, cell extracts were prepared and subjected to analysis of CAT activity (Promega). CAT activity, quantified by counting in an AMBIS β-scanner, is expressed as the fold activation by p53 relative to vector alone. Data shown are representative of at least five experiments.

RNase Protection Assay. RNase protection assay of endogenous and exogenous p53 mRNA levels was essentially as described (10). Briefly, an antisense RNA probe was synthesized from the pBluescript SKII(+) vector containing nt 1750–2138 of the 3′ untranslated region of the human p53 gene. Endogenous p53 mRNA protected this entire sequence, whereas exogenous p53 mRNA protected a fragment of 167 nt. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe vector was constructed by subcloning a 129-bp PCR-generated *Hin*dIII–*Pst* I fragment corresponding to human GAPDH gene positions 256–385 into pBluescript SKII(+). The ^{32}P-labeled *in vitro* transcribed p53 and GAPDH riboprobes were gel purified and hybridized to 5 μg of total cellular RNA, prepared by acid/guanidinium extraction, for 16 hr at 58°C in the presence of 25 μl of hybridization buffer (85% formamide/0.4 M NaCl/1 mM EDTA/40 mM Pipes, pH 6.7). Hybridized RNA was digested for 1 hr at 30°C with 300 μl of digestion buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA/0.3 M NaCl) containing RNase A (type IIIA; Sigma) at 35 μg/ml. The reaction was terminated by adding 15 μl of proteinase K (10 mg/ml) and 15 μl of 10% SDS, with incubation for 15 min at 37°C followed by phenol/chloroform extraction and ethanol precipitation. Fragments were separated by electrophoresis in a denaturing 5% polyacrylamide gel. Levels of endogenous and exogenous p53 and GAPDH mRNA were determined by densitometry.

RESULTS AND DISCUSSION

Interaction Between p53 and HBX Proteins. The p53–HBX interaction *in vitro* was studied by fusing the entire HBX or p53 open reading frame to the GST gene. The GST fusion proteins were produced in *E. coli* and purified on glutathione-Sepharose beads (27). The loaded beads were used as "bait" in incubations with *in vitro* translated ^{35}S-labeled HBX or p53 protein. *In vitro* transcription and translation of a vector encoding the HBX gene under the control of the T7 promoter (pSPX46) resulted in three major ^{35}S-labeled bands, one of which appeared to be an authentic HBX protein, since it had a molecular mass of 17 kDa and was immunoprecipitated by rabbit anti-HBX antibody (Fig. 1, lane 3) but not by normal rabbit serum (data not shown). The other two proteins, 34 and 28 kDa, appeared to be nonspecific products associated with this vector, since they were not recognized by the anti-HBX antibody and were evident when a different gene was trans-

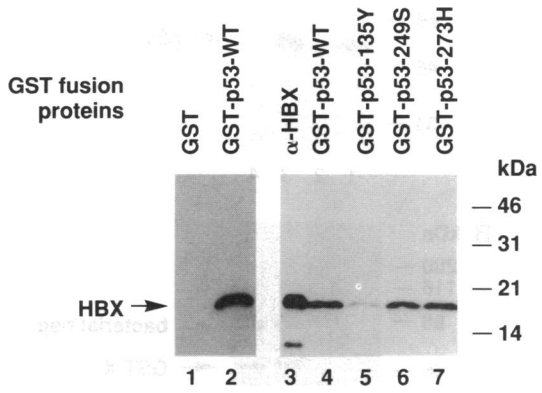

FIG. 1. Physical association between GST–p53 and *in vitro* translated HBX. HBX and human p53 proteins were obtained from *in vitro* translation or *E. coli*-produced GST-fusion systems. Fifteen microliters of ^{35}S-labeled HBX was mixed with glutathione-Sepharose beads loaded with either 4 μg of GST (lane 1) or 2 μg of GST–p53-WT (lanes 2 and 4), GST–p53-135Y (lane 5), GST–p53-249S (lane 6), or GST–p53-273H (lane 7). Beads were washed with IP buffer and proteins which remained bound were analyzed by SDS/PAGE as described in *Materials and Methods*. An equal amount of *in vitro* translated HBX, immunoprecipitated by anti-HBX (α-HBX) polyclonal antibody, was used as a reference (lane 3). Lanes 2 and 4 represent two independent experiments. GST–p53-WT, wild-type p53; GST–p53-135Y, p53 mutant at codon 135 (His → Tyr); GST–p53-249S, p53 mutant at codon 249 (Arg → Ser); GST–p53-273H, p53 mutant at codon 273 (Arg → His).

lated from this vector (data not shown). When these *in vitro* translated proteins were used for binding studies, only the beads loaded with GST–p53-WT (Fig. 1, lanes 2 and 4), but not with GST (Fig. 1, lane 1), bound the *in vitro* translated HBX (10–50% of input). No binding was observed between GST–p53-WT and the 34- or 28-kDa nonspecific proteins. Fig. 2 shows the reciprocal experiment in which the beads loaded with the GST or GST–X fusion proteins were used as a "bait." When beads loaded with these proteins were incubated with a purified baculovirus-produced p53-WT protein, only GST–X bound p53-WT (20% of input) (Fig. 2, lane 3). GST–X also bound the endogenous wild-type p53 from a hepatoblastoma cell line (HepG2) (data not shown). The specificity of the binding was demonstrated by the lack of binding of the 34- and 28-kDa proteins, as well as GST, to either p53 or HBX. These results are consistent with those recently reported (28).

HBX Exhibits Different Binding Abilities with Various p53 Mutants. Human p53 mutants exhibit diversity in complex formation with T antigen (33). To examine the binding of HBX to certain p53 mutants, we used GST–p53-135Y, GST–p53-249S, and GST–p53-273H, mutants found in human cancers (7). Equal amounts of the GST–p53 mutant proteins as determined by Coomassie blue staining of SDS/polyacrylamide gels were used for binding with *in vitro* translated proteins. To ensure equal loading in the binding reaction, GST–p53 mutant proteins were tested for their abilities to form heterodimers with p53-WT. *In vitro* translated p53-WT bound to each of the GST–p53 mutants as well as to GST–p53-WT (data not shown). In contrast, *in vitro* translated HBX bound to GST–p53-249S and GST–p53-273H but bound only minimally to GST–p53-135Y (Fig. 1, lanes 5–7). Interestingly, the p53-135Y mutant has diminished binding to the E6 protein of human papillomavirus via the E6-AP linker protein (25, 34). In addition, some mutants, such as 175H, 248Q, and 249S, retain their ability to bind T antigen (33). These data are consistent with previous findings and support the hypothesis that different p53 mutants vary in their biochemical and biological activities (35, 36). It is intriguing that p53-249S retains its ability to bind to both HBX and T antigen, since this mutation is commonly found in cases of HCC from areas of China and Africa with high dietary aflatoxin and HBV exposure (37, 38). Further analysis of this interaction may help define the pathobiological nature of the p53-249S mutation.

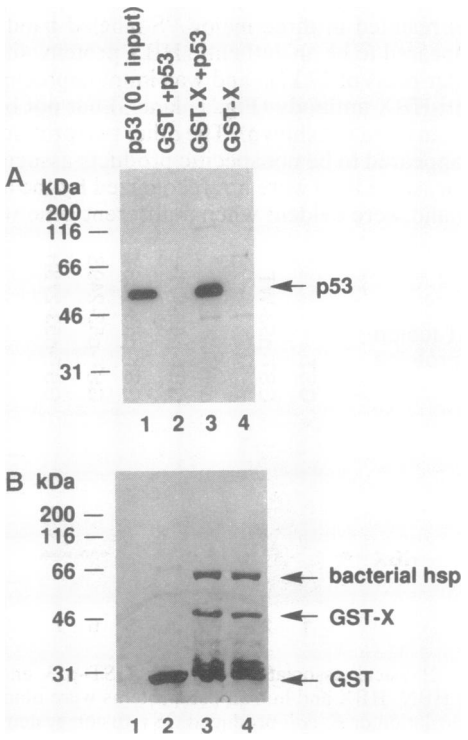

FIG. 2. Physical association between GST–X and baculovirus-produced p53-WT protein. Fifty nanograms of purified baculovirus-produced p53-WT (lanes 2 and 3) was incubated with Sepharose beads loaded with either 4 μg of GST (lane 2) or 2 μg of GST–X (lanes 3 and 4) in the presence of 10 μl of rabbit reticulocyte lysate to block nonspecific binding sites. Sepharose-bound proteins were analyzed by SDS/PAGE and immunoblotted with anti-p53 polyclonal antibody (CM-1, 1:1,000 dilution) (*A*). Five nanograms of purified p53 was loaded in parallel as a reference (lane 1). A duplicate experiment was analyzed by Coomassie blue staining (*B*). Bacterial heat shock protein (hsp) often coprecipitated with GST fusion protein in this system.

HBX Inhibits p53 Sequence-Specific DNA-Binding Activity. The tumor-suppressive activity of human wild-type p53 may be the result of its interaction with specific DNA sequence adjacent to functionally important cellular genes such as WAF1/Cip1 (26, 39, 40). We hypothesized that HBX may block the tumor-suppressive activity of p53 by preventing its sequence-specific DNA binding. To test this hypothesis, we used a previously described assay (30) and found, consistent with previous findings (26), that baculovirus-produced p53-WT bound specifically to the p53 consensus sequence (PG259), whereas baculovirus-produced p53-273H bound with very low affinity (Fig. 3). When *in vitro* translated HBX was added to the binding reaction mixture at conditions known to bind p53 protein effectively (Fig. 1), p53-WT binding activity was diminished nearly 90% (Fig. 3, lane 3). Similar results were obtained when GST–p53 fusion proteins were used for binding studies (data not shown). These results strongly suggest that the HBX–p53 complex may influence wild-type p53 activity by blocking sequence-specific DNA binding of p53. The complex between HBX and p53 may alter the conformation of p53 in a way analogous to p53 missense mutants found in human cancers. Interestingly, complexes between HBX and other cellular transcription factors—e.g., CREB and ATF-2—enhance the DNA-binding specificity of these proteins (41). These contrasting results indicate that the functional consequence of HBX on gene expression may depend on its interaction with specific cellular transcription factors.

HBX Expression Inhibits p53-Mediated Transcriptional Activation *in Vivo*. Binding of p53 to its DNA consensus sequence is necessary for transactivation of genes adjacent to these binding sites (30). We cotransfected p53 and HBX expression vectors to determine whether HBX modulated the transactivation ability of p53-WT. Since HBV replicates in

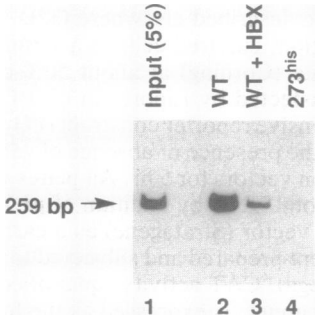

FIG. 3. Inhibition of p53 DNA-binding properties by HBX protein. ^{32}P-labeled PG259 DNA fragments were incubated with 100 ng of baculovirus-produced human wild-type (WT) p53 (lane 2) or mutant p53-273^{His} (lane 4). Fifteen microliters of *in vitro* translated unlabeled HBX was used for competition (lane 3). The p53-bound DNA was analyzed by PAGE (see *Materials and Methods*). Five percent of original DNA was loaded in parallel as a reference (lane 1).

hepatocytes and HBX-transgenic mice develop only HCC (24), we used a T-antigen-immortalized normal human hepatocyte line (THLE-5b) in which the endogenous p53 is inactivated by T antigen. Cotransfection of pC53-SN and PG_{13}-CAT into THLE-5b cells resulted in a 16-fold activation of CAT gene expression (Fig. 4*A*, lane 2). Addition of HBX expression vectors under the control of either the Moloney murine leukemia virus long terminal repeat promoter (LTR–X) or the CMV promoter (CMV–X) resulted in about a 90% reduction of p53-mediated transactivation (Fig. 4*A*). We measured exogenous p53 mRNA levels by RNase protection (Fig. 4*B*) and total p53 levels by immunoprecipitation (data not shown) and observed no significant differences in cells transfected with the HBX gene. Therefore, HBX did not inhibit p53-mediated transactivation by down-regulation of p53 expression in THLE-5b cells.

HBX modulation of p53 transactivation activity in this transient transcription assay is dependent on cellular context. Although inhibition of p53-mediated transactivation by HBX was observed in THLE-5b cells (Fig. 4) and in a HCC cell line without T antigen (SK-Hep-1) (data not shown), an apparent enhancement of p53-mediated transactivation by HBX was observed in another HCC cell line (Hep3B) which contains an integrated partial HBV genome (32) and a lung adenocarcinoma cell line (Calu 6) (data not shown). The cell-type specificity observed in these experiments appears to contradict our *in vitro* results. However, since HBX can transactivate many cellular and viral promoters in a variety of cell lines through activation of the PKC signal transduction pathway (19, 20), we hypothesized that inhibition of p53-mediated transactivation by HBX could be masked by HBX-mediated activation of the CMV promoter through the PKC pathway to elevate the levels of p53 in the Hep3B and Calu 6 cell lines. To test this hypothesis, we cotransfected the HBX expression vector with a CMV–CAT reporter construct and found that HBX transactivated the CMV promoter in the Hep3B and Calu 6 cell lines, but not in the THLE-5b and SK-Hep-1 cell lines (X.W.W. and C.C.H., unpublished work). Furthermore, transactivation of the CMV promoter by HBX in the Hep3B cell line was blocked by potent PKC inhibitors (X.W.W. and C.C.H., unpublished work), suggesting activation of PKC by HBX in this cell line. Further analysis of the pathways mediated by HBX may be important in understanding its oncogenic activity and the divergent effects observed in various cell types (42, 43). In addition, the effects of HBX on expression of WAF1/Cip1 (39, 40), a downstream effector of p53, will be interesting to investigate.

HBX Interferes with Binding of p53 to ERCC3. p53 is postulated to modulate gene transcription by interacting with the basic transcriptional machinery. TFIID, a part of the transcription initiation complex, has been shown to be a p53 target, resulting in down regulation of TFIID-dependent genes (2, 44). Wild-type p53 also forms complexes with ERCC3 (Fig. 5, lane 4; unpublished data), a basic transcription factor that is involved in transcription-coupled repair (29). Furthermore, GST–p53 mutants 135Y, 249S, and 273H bound ERCC3 more strongly than wild-type p53 (Fig. 5, lanes 6–9). Although HBX did not bind to ERCC3 itself (data not shown), it almost completely inhibited binding by p53 (Fig. 5, lane 5).

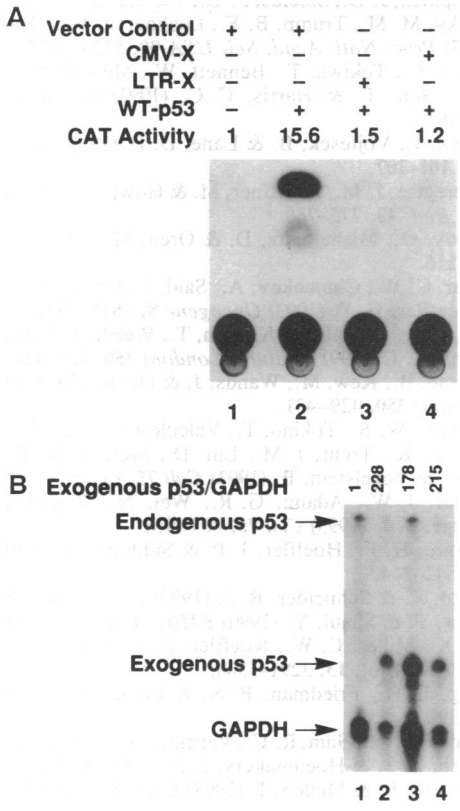

FIG. 4. Inhibition of p53 transactivation of the PG_{13}-CAT reporter by HBX. THLE-5b cells were transfected with PG_{13}-CAT alone (lane 1) or cotransfected with pC53-SN [wild-type (WT) p53] in the absence (lane 2) or presence of pSHDX42 (LTR–X) (lane 3) or pCMV-X1 (CMV–X) (lane 4). Cells were subjected for analysis of CAT activity (*A*) or for analysis by RNase protection (*B*). (*A*) CAT activity is expressed as the fold activation of PG_{13}-CAT expression by p53 relative to vector alone. (*B*) The levels of exogenous p53 displayed above lanes 1–4 are normalized to the endogenous GAPDH signal and expressed as the ratio of exogenous p53 divided by GAPDH. The p53/GAPDH ratios are as follows: lane 1, 1 (control); lane 2, 228; lane 3, 178; and lane 4, 215.

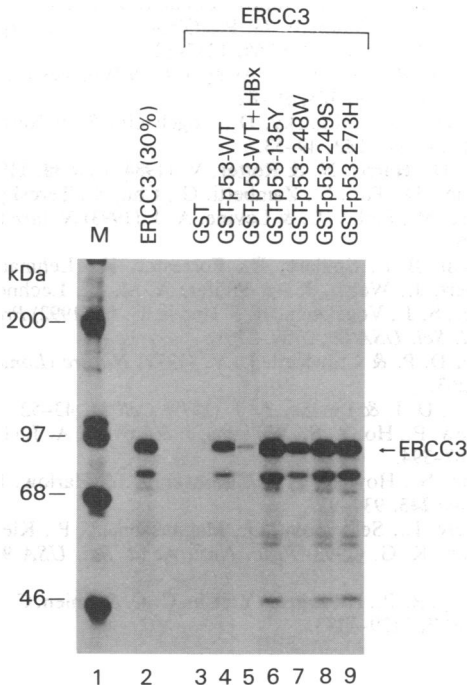

FIG. 5. Association of p53 with ERCC3. The protein binding reactions were performed as described in the Fig. 1 legend except that the GST–p53 fusion proteins were incubated with a ^{35}S-labeled *in vitro* translated ERCC3. Thirty percent of *in vitro* translated ERCC3 protein, immunoprecipitated by anti-ERCC3 polyclonal antibody, was included as a positive control (lane 2). Twenty-five microliters of unlabeled *in vitro* translated HBX was used in a competition assay (lane 5). Arrow indicates the authentic 89-kDa ERCC3 protein.

The finding that p53 and ERCC3 associate *in vitro* could be significant, since p53 plays a role both in transcription and in DNA repair processes (2, 3, 44). p53 suppresses the initiation of transcription, presumably by interacting with TFIID (2, 44), and inhibits the activity of DNA helicases (45). The ERCC3 protein harbors seven putative helicase motifs (46) and contains an intrinsic helicase activity (29). These data indicate a model for p53 being a transcription/repair modulating factor in which p53 contributes to the maintenance of genomic stability by modulating transcription and DNA nucleotide-excision repair processes. When DNA is damaged, p53 could stall transcription either at the initiation or at the elongation stage of transcription by interacting with TFIID or ERCC3, respectively, and modulate preferential repair of DNA damage within actively transcribed genes (47, 48). Inactivation of p53 by mutations or interaction with viral oncoproteins, including HBX, could increase the mutation frequency of important cellular genes and increase the probability of neoplastic transformation of human hepatocytes.

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