Employing the glutathione S-transferase column retention method and far Western analysis, we found a physical association between tumor suppressor p53 and the hepatitis B virus X-gene product, which led us to study the function of observed interaction in relation to viral propagation. In the cell culture-based in vitro replication system, expression of p53 resulted in a dramatic inhibition of viral replication, and this inhibition was relieved by the coexpression of the X-gene product in a dose-dependent manner. Furthermore, the activity of pregenomic/core promoter, responsible for the synthesis of pregenomic RNA, was almost completely inhibited upon expression of p53, and as in the replication assay, the inhibition was rescued by the coexpression of the X-gene product in a dose-dependent manner. Based on these results, we propose that the ratio of X-gene product to p53 is an important factor determining the fate of viral replication through modulation of the pregenomic/core promoter.

The hepatitis B virus (HBV), the genome of which consists of a partially double-stranded 3.2-kb circular DNA, is a causative agent of acute and chronic hepatitis. Upon infection of the hepatocytes, a partially double-stranded genome is converted to a complete double-stranded circular, supercoiled DNA. Employing this as a template, 3.6 kb pregenomic RNA, which is under the control of pregenomic/core promoter, is transcribed. The pregenome is packaged into a nucleocapsid and is reverse-transcribed using viral polymerase as an initiation primer (1, 2) to generate single-stranded minus DNA. The polymerization of the second strand follows until approximately half of the genome is synthesized, resulting in the generation of a partially double-stranded circular genome, which is coated and then secreted from the infected cells (3, 4).

Among the proteins synthesized from HBV, the X-gene product (HBV-X) attracts much attention for its various roles in the regulation of host cell machinery. The most well-known function of HBV-X is its role as a regulator of transcriptional activation. HBV-X does not bind to DNA but modifies the function of basic transcriptional machinery. Interaction with RPB5, a subunit of eukaryotic RNA polymerase (8), as well as a possible role as a coactivator of acidic transactivators, was reported (9). On the other hand, to explain the promiscuity of HBV-X in transcriptional transactivation, Kekule et al. (10) have suggested that the X-gene product acts through the protein kinase C pathway (10, 11), while others reported the activation of the Ras pathway (12–14).

It is generally believed that HBV-X contributes to the generation of hepatocellular carcinoma. HBV-X was reported as a weak oncogene (15) and induced hepatocellular carcinoma in transgenic liver (16). One possible mechanism of HBV-X-induced malignancy is the disruption of p53 function by direct association. The interaction between these two molecules was reported previously (17–20), especially in tumor samples of transgenic liver expressing the X-gene product (20). In addition, other viral oncoproteins were reported to induce malignancy through dysregulation of wild-type p53 function (21, 22). Examples include E6 of the human papilloma virus (23–25), E1B 55k of the adenovirus (26, 27), and SV40 large T antigen (26, 28).

Furthermore, p53 has been reported to interfere with the replication of several viruses. In the case of SV40, wild-type p53 binds to SV40 large T antigen and blocks the function of large T antigen in mediating viral replication (29, 30). In addition, p53 seems to interfere with the replication of the Herpesvirus (31), the latency of the Epstein-Barr virus (32), and human immunodeficiency virus (33). Because the X-gene product is indispensable for the replication of the woodchuck hepatitis virus in the woodchuck model that is believed to represent the in vivo situation (34, 35), we hypothesized that p53 might inhibit the replication of hepatitis B virus and that the role of HBV-X is to overcome the negative effect of p53 by direct interaction. As a first step, we studied whether p53 binds to HBV-X (during our study, others (17–20) have reported the direct association of p53 and HBV-X) and then extended our study to the role of p53 and HBV-X in the viral life cycle. We found that p53 inhibited the replication of HBV as well as the activity of pregenomic/core promoter and that HBV-X rescued these inhibitory functions of p53.

EXPERIMENTAL PROCEDURES

Plasmids—p53 or HBV-X was expressed as a fusion protein to glutathione S-transferase (GST). pGST-p53 was constructed by subcloning the XbaI partial digestion product of human p53 cDNA (36) into pGEX-KG (37). pGST-X contains a phosphorylation site for cAMP-dependent protein kinase A, -RRPSY-, upstream from the HBV-X open reading frame for in vitro labeling. pGST-X was generated by PCR, using TACGGATCCCAGGAGGGCTTCTATAGCTGCTCAGG-GTGGC as a 5′-primer and TACGGATCCGATCTCCTAGGCGAGGTT-GAAAAA as a 3′-primer. The underlined sequence codes for RRPSY.

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The obtained PCR product was subcloned into pGEX-2T (Pharmacia Biotech Inc.) after digestion with BamHI. adv R9 containing the dimer of HBV DNA was a kind gift from Dr. Blum (38). Expression plasmid pCDNA-p53 was created by subcloning a 2.55-kb BamHI fragment of p53 cDNA (36) to the BamHI site of pcDNA.1 (Invitrogen). Mutant p53 expression plasmids G154V and R273L were created by site-directed mutagenesis using the Kunkel method (39). pcDNA-X encoding the HBV-X open reading frame was obtained by PCR. The PCR template was adv 4 type HBV DNA (40). The sequence of the primers contains BamHI sites for subcloning, TACCAGTCATCATGCTGTCGTTGTC for a 5′-primer and TACCAGTCATCATGCTGTCGTTGTC for a 3′-primer. After BamHI digestion, the obtained PCR product was subsequently inserted into pcDNA.1 to generate pCDNA-X. Xdel-1 and Xdel-2 were constructed by site-directed mutagenesis (39). CpLUC, which was a kind gift from Dr. Raney (41), was the source for CpCAT. From CpLUC, 3.2-kilobase pair HindII fragment covering the HBV genome was isolated and subcloned into the HindIII site of pGEM.1CAT (Genbank accession number U17116), which has the chloramphenicol acetyltransferase gene subcloned into pGEM.1CAT (Promega). CEP-CAT was generated by insertion of the 2.2-kb NsiI digestion product of CpCAT into pGEM.1CAT. Plasmid PERCAT2 (42) was kindly provided by Dr. Merlino.

Purification of Recombinant Proteins from Escherichia coli—E. coli strain XL-1 BLUE was used to express GST, GST-X, and GST-p53. Proteins were purified using a glutathione-Sepharose 4B column. The fusion proteins were cleaved with thrombin when necessary. For in vitro labeling, purified GST-X was incubated with [γ-32P]ATP and the catalytic subunit of protein kinase A (Sigma). The details of purification and labeling will be described elsewhere.

Transfection—Transfections were carried out following the established protocols (43). HepG2 cells were transfected by the DEAE-dextran method. One day before transfection, approximately 2 × 106 cells were seeded on a 60-mm diameter plate. On the day of transfection, the cells were washed twice with phosphate-buffered saline and treated with 0.4 mg/ml of DEAE-dextran and an appropriate amount of DNA mixture made in phosphate-buffered saline. The cells were treated with 0.1 mM chloroquine diphosphate at 37 °C in a 70% humidified CO2 incubator. Subsequently, shock with 10% dimethylsulfoxide was given. Transfected cells were then incubated for additional 40 h with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum unless stated otherwise. Hep3B cells were transfected by the calcium phosphate coprecipitation method according to Sambrook et al. (43) except that cells were incubated with the coprecipitate for 24 h followed by 15% glycerol shock. Transfected cells were incubated with Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum.

CAT Assay—The method for CAT assay was basically the same as the established protocol (43). Transfection of cells was performed in duplicate, and the results from at least three independent experiments were analyzed.

Assay of Tissue Culture Medium for HBV Antigens—Experiments were carried out after Blum et al. (38). From the transfected cells, 1 ml of the culture medium was taken daily for assay and replaced by the same amount of fresh media. The surface antigen (HBsAg) released into the culture medium was measured using an enzyme-linked immunoassay (E.I.A. Genedia-S. M., Korea Green Cross Co.). Core-related antigens (HBcAg and/or HBeAg) were detected using the HBc/eAg R.I.A. (Abbott HBe diagnosis kit). These results suggest that HBV-X binds to HBcAg and HBeAg. The assays were performed in the linear range of the kits by diluting the media with phosphate-buffered saline.

DNA Analysis from Cell Extract and Culture Media—DNA preparation was performed as described (44) with slight modification. For analysis of DNA in cell lysates, cells were collected 3 days after transfection and suspended in hypotonic buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). After homogenization, 10 mM of MgCl2 and 23 units/ml of DNase I (final concentration) were added and incubated at 37 °C for 3 h, followed by centrifugation at 5,000 rpm for 1 h. The supernatant was layered over 30% sucrose solution (in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) and centrifuged at 35,000 rpm for 12 h at 4 °C in SW41 (Beckman) for 24 h. The RNA pellet was resuspended in RNAase-free water and ethanol-precipitated with 0.3 M sodium acetate, pH 5.0. 30 μg of the total RNA was fractionated on a 2.2 M formaldehyde-denaturing agarose gel and transferred onto Nylon membrane. Hybridization was carried out following the established protocol (43).

RESULTS

Hepatitis B Virus X-gene Product Is Associated with Tumor Suppressor p53 in Vitro—First, we investigated whether the X-gene product is associated with p53 through protein-protein interaction. HBV-X was expressed in E. coli as a fusion protein to GST (GST-X). GST-X contains a phosphorylation site (-RRPSY-) for labeling by protein kinase A. [γ-32P]-labeled GST-X was specifically retained by GST-p53 but not by GST bound to glutathione-Sepharose beads (Fig. 1A). Furthermore, in far Western analysis, γ-32P-labeled GST-X specifically recognized GST-p53 on the blot (Fig. 1B). These results suggest that HBV-X binds to p53 in vitro.

Wild-type but Not Mutant p53 Inhibits the Replication of Hepatitis B Virus—The interaction between wild-type p53 and HBV-X led us to hypothesize that p53 might interfere with the replication of the hepatitis B virus and that the role of HBV-X is to overcome the negative effect of p53. As a first step to test this hypothesis, we adopted an established HBV replication system (44). adv R9 (38), the plasmid containing a head-to-tail dimer of HBV DNA, was transiently transfected into HepG2 cells along with the p53 expression plasmids (Fig. 2, A and B). The effect of p53 on HBV replication was assayed by determining the levels of surface antigen (HBsAg) and the core protein (HBeAg) secreted into the culture medium. Experiments were carried out in duplicate and were repeated at least 4 times. The data presented in Fig. 2 are from a single representative experiment. In the adv R9-transfected set, starting from day 2, continuously increasing amounts of HBsAg were secreted into the culture medium with a continuously increasing amount of HBsAg secreted into the culture medium.
In contrast, cotransfection of wild-type p53 expression plasmid resulted in dramatically reduced levels of HBsAg (95% reduction on day 3 post-transfection). This observation was specific for wild-type p53, since cotransfection of the plasmids encoding mutant p53 resulted in only a modest degree of repression in HBsAg production, i.e. the level of reduction was 40 and 10%, respectively, for R273L and G154V on day 3 post-transfection. The time-dependent alteration in the level of HBc/eAg displayed a similar pattern to that of HBsAg (Fig. 2B). Cotransfection of the wild-type p53 expression plasmid resulted in a marked reduction in the HBc/eAg level, an 80% reduction on day 3 post-transfection, whereas the R273L and G154V mutants affected the HBc/eAg level only slightly, a 30% reduction for R273L and 10% reduction for G154V on day 3 post-transfection. In addition, the authenticity of secreted particles was analyzed by CsCl density gradient centrifugation of the spent culture media as reported by others (38, 44). The major peaks of HBsAg or HBc/eAg were observed at a buoyant density of 1.15 g/ml and 1.3 g/ml, respectively (data not shown). These values are identical to those reported previously, showing that the secreted particles are indistinguishable from wild-type virion.

p53 Inhibits the Synthesis of Hepatitis B Viral DNA and RNA—The role of p53 in viral replication was further assessed by quantitating the HBV DNA and RNA levels after cotransfection of adw R9 and p53 expression plasmid. On day 3, viral
DNA levels in cellular extracts and culture media were analyzed by Southern hybridization, using 32P-labeled adw R9 DNA as a probe (Fig. 2C). The levels of HBV DNA in both cell extract (CE) and media (M) were reduced by more than 90% when p53 expression plasmid was cotransfected. Next, we analyzed the levels of HBV RNAs by Northern blot hybridization with 32P-labeled adw R9 DNA as a probe. Total RNAs were isolated from HepG2 cells transfected with adw R9 ± p53 expression plasmid and analyzed. The probe detected the 3.6-kb pregenomic RNA as well as the 2.4- and 2.1-kb RNA species. Cotransfection of p53 resulted in a more than 95% reduction of all the viral transcript levels even though the staining of the gel displayed that the level of total RNA on each lane was apparently equal (Fig. 2D). The reduction of the 3.6-kb mRNA level is especially important in light of the fact that this species of RNA is utilized as a pregenome in HBV replication (46). Based on these results, we propose that wild-type p53 is a negative regulator of hepatitis B viral replication. The possible p53-mediated alteration in the transfection efficiency or the viability of the cell was ruled out by cotransfection of the β-galactosidase expression plasmid under the control of either the Rous sarcoma virus promoter or the epidermal growth factor receptor promoter along with the adw R9 ± p53 expression plasmid. Upon in situ staining on day 3 post-transfection, the number of stained cells was identical, indicating that the expression of p53 did not affect the number of viable, transcriptionally competent, transfected cells under the employed experimental conditions (data not shown). Furthermore, microscopic observation of the p53-transfected cells showed no difference in morphology when compared with the control cells (data not shown).

p53 Inhibits the Activity of Pregenomic/ Core Promoter—To explain the molecular mechanism of the p53-mediated repression of HBV replication, we investigated the possibility of p53 regulating the pregenomic/core promoter, which is responsible for the synthesis of pregenomic RNA. Initially, HepG2 cells were employed for transfection experiments. Insomuch as HepG2 is believed to retain the wild-type p53 (49), the effects of overexpression of exogenous p53 over endogenous p53 were investigated in this experimental system by cotransfection of p53 expression plasmids (Fig. 3).

When CpCAT, containing a complete copy of the HBV genome, was employed as a reporter, cotransfection of wild-type p53 expression plasmid led to the dramatic repression of core promoter activity, down to 3% of the control. On the other hand, cotransfection of two p53 mutants, R273L and G154V, resulted in CAT activity, down to 16 and 83% of the control value, respectively, indicating requirement of the wild-type p53 action for the complete shutdown of the HBV core promoter (Fig. 3B). The observed behavior of two p53 mutants in transcriptional regulation of the core promoter is similar to the results published by others studying p53-dependent transcriptional activation using other systems; i.e. R273L retains a part of wild-type p53 function, while G154V behaves as a null mutant (50, 51). In the same transfection experiments, we included a control reporter, pERCAT2, in which CAT activity is driven by the promoter of the epidermal growth factor receptor (42). Previously, the activity of this promoter was shown to be up-regulated by p53 (47). In contrast to the down-regulation of core promoter activity by p53, the activity of a control reporter, pERCAT2, was enhanced more than 18-fold by the overexpression of p53 (Fig. 3B). These results suggest that the observed down-regulation of core promoter by p53 is not caused by the general inhibition of transcriptional machinery.

Next, we tested whether a short version of the core promoter, CEP-CAT, was also under the same inhibitory regulation by p53 (Fig. 3C). In CEP-CAT, a 740-base pair segment of the core promoter, including liver-specific enhancers I and II, is placed upstream from the CAT gene. CEP-CAT was regulated in an almost identical fashion by effectors plasmids as was CpCAT. Wild-type p53 repressed CAT activity down to 6% of the control, whereas the R273L and G154V mutants reduced the activity down to 10 and 40% of the control, respectively. Therefore, the sequence responsible for the down-regulation of the core promoter is contained in the 740-base pair region of the HBV genome between positions 1060 and 1804. As already stated, HepG2 cells retain endogenous p53. To assay the p53 effect without interference by endogenous p53, we performed an identical transfection experiment in a p53-deficient cell line, Hep38, employing CpCAT as a reporter (Fig. 3D). Again, an almost identical regulation pattern was observed; the CAT activities obtained upon transfections of the wild-type p53, R273L, and G154V were 5, 40, and 86% of the control, respectively.

As a control experiment, expression levels of wild-type and mutant p53s were investigated. Cells were harvested 2 days after transfection, and the lysates were subjected to Western analysis employing rabbit serum raised against GST-p53. The expression levels of R273L and G154V were rather higher than wild-type p53, indicating that the failure of mutant p53s to repress the core promoter activity or viral replication was not due to reduced expression of mutant p53s (Fig. 3E). Taking all of these results together, we conclude that the activity of pregenomic/core promoter of HBV is repressed by wild-type p53.

The Inhibitory Function of p53 on Viral Replication and the Core Promoter Activity Is Relieved by Overexpression of the X-gene Product—Insomuch as p53 and HBV-X associate in vitro, we tested whether the inhibitory function of p53 on viral replication and transcription is relieved by the coexpression of HBV-X. First, we tested whether HBV-X overcomes the p53-mediated negative regulation of viral replication. We cotransfected pcDNA-X for expression of HBV-X, since 0.9-kb HBV-X mRNA was not readily detectable upon transfection of adw R9 (Fig. 2D) despite the presence of an intact transcription unit of HBV-X in adw R9. The low level expression of HBV-X from adw R9 may explain the discrepancy in the requirement of the X-gene product in viral replication assayd in a cell culture system (38) or in an animal model (34, 35). Coexpression of HBV-X recovered the p53-mediated inhibition of viral replication in a dose-dependent manner (Fig. 4, A and B). When 10 μg of pcDNA-X was cotransfected, we observed almost complete reversal of the p53-mediated down-regulation of HBsAg or HBc/eAg levels. Next, we tested the effect of the X-gene product on p53-mediated inhibition of the pregenomic/core promoter. The expression plasmid of the X-gene product was cotransfected along with the CpcAT and p53 expression plasmid. Again, overexpression of p53 repressed the core promoter function, which was relieved by the cotransfection of the HBV-X expression plasmid in a dose-dependent manner (Fig. 4C). When 10 μg of HBV-X expression plasmid was cotransfected, we observed the complete recovery of core promoter activity.

Next, as controls for the effect of wild-type HBV-X, two mutants of HBV-X, Xdel-1 and Xdel-2, were employed for the experiment identical to that shown in Fig. 4. In Xdel-1 and Xdel-2, amino acids 75–100 or 101–125, respectively, of wild-type HBV-X were deleted. The deleted regions were selected based on the report (52) that these regions are important for the functions of wild-type HBV-X. In contrast to the effect of wild-type HBV-X, the mutants barely rescued the p53-mediated down-regulation of the HBSAg and HBc/eAg levels (Fig. 5, A and B) as well as core promoter-driven CAT activity (Fig. 5C), despite the approximately equal level of expression (Fig. 5D).
FIG. 3. Effect of p53 on the activity of HBV pregenomic/core promoter. A, maps of the reporter plasmids are described. Arrows indicate the position and direction of transcription from preS1 (PS), major surface antigen (S), X-gene (X), and the core (C) promoters, respectively. In addition, the locations of liver-specific enhancer I (ENI) and enhancer II (ENII) are indicated. B, HepG2 cells were cotransfected with 5 μg of a reporter plasmid and 3 μg of an effector plasmid. The numbers in the bottom row represent the relative CAT activity. C, identical to B except that the short version of the core promoter, CEP-CAT was employed. D, identical to B except that p53-deficient cell line Hep3B was employed. E, analysis of wild-type and mutant p53 levels after transient transfection. HepG2 cells grown on a 60-mm plate were transfected with 3 μg of pcDNA1 (lanes 1 and 2), pcDNA-p53 (lanes 3 and 4), R273L (lanes 5 and 6), or G154V (lanes 7 and 8). One-fiftieth of the prepared lysates were used for Western analysis by the Immune Lite kit (Bio-Rad). A 1:1000 dilution of antisera raised against GST-p53 was employed as a primary antibody.
Taking these results together, we propose that X-gene product is a functional antagonist of p53 and recovers the p53-mediated inhibition of HBV replication by preventing the down-regulation of the pregenomic/core promoter.

**DISCUSSION**

In this report, we have shown that HBV-X relieves the inhibitory effect of p53 on HBV replication and pregenomic/core promoter activity. The requirement of HBV-X in the viral life cycle has been controversial. In a cell culture-based system, HBV-X does not appear to be essential (38), whereas in the woodchuck model, the X-gene product is indispensable (34, 35). Inasmuch as an adequate animal model for HBV is not available yet, we consider the information from woodchucks as reflecting the *in vivo* phenomenon more precisely. The results of this study provide a model explaining the requirement of the X-gene product in HBV replication. Both the viral replication and the activity of the core promoter were repressed by p53, and these inhibitions were relieved by the coexpression of HBV-X in a dose-dependent manner. Based on the observed correlation between viral replication and core promoter activity, we propose that the balance between HBV-X and p53 is one of the important factors determining the extent of viral replication during the course of HBV-mediated hepatitis.

The requirement of the X-gene product in HBV replication observed in this study is in good agreement with the reports that HBV-X is expressed during certain stages of viral infection (53, 54) and that the prevalence of anti-X is unusually higher in anti-HBeAg positive serum, which is a marker for viral replication (53). It can be postulated that, depending on the ratio of HBV-X to p53, HBV replication may fluctuate as observed in some chronic hepatitis patients (55). Otherwise, the HBV-X/p53 ratio may play a role in the initial phase of HBV infection, reflecting the presence of an incubation period of 6 weeks to 6 months (56).

From this study, we have not definitely proven that direct interaction between HBV-X and p53 is responsible for the HBV-X-mediated reversal of the inhibitory effect of p53 on viral replication or core promoter activity. However, in vitro interaction observed by us (Fig. 1) and others (17–20) suggests that the most likely mechanism involves a protein-protein interaction between two molecules. Previously, HBV-X was shown to reverse p53-mediated transcriptional activation through the p53 response element (18, 19). In addition, in vivo interaction between the two has been reported in a hepatocellular carci-
Our study does not deal with the known functions of p53 in cell cycle arrest or apoptosis. In fact, it is not likely that p53 inhibits general growth control of the established cell line harboring wild-type p53, especially in a transient transfection system. However, in an in vivo situation, we cannot rule out the possibility of p53 inducing cell cycle arrest or apoptosis as reported for other viruses (57). In this case, by relieving the negative effect of p53, HBV-X may antagonize the effect of p53, causing growth arrest or apoptosis, which may lead to tumorigenesis.

Currently, we do not know the sequence of the core promoter responsible for p53-mediated transcriptional repression. Recent reports suggest that p53 acts directly on the TATA box by binding to TFIIID (58–61) and represses the activity of various promoters (62), including several viral promoters (48). Since the TATA-box is not found in the HBV core promoter, it is unlikely that this mechanism applies to p53-mediated repression of the core promoter, even though other AT-rich elements may functionally interact with TFIIID as reported earlier for other promoters (63, 64). Another possibility is that p53 acts through enhancer I or enhancer II, since they are known to be responsible for the high transcription efficiency of the viral promoters in the liver cell (65). The specific cis-acting element on which p53 acts to repress the pregenomic/core promoter function is under investigation.

HBV is not the only virus whose replication is inhibited by p53. Previously, p53 has been shown to inhibit the replication of SV40 by binding to replication origin (29) and to large T antigen (30). Although the inhibitory mechanisms may not be the same for all viruses, the finding in this report raises the possibility that p53-mediated inhibition of viral replication can be a generalized phenomena. The presence of other viral transactivating factors like E1B, E6, and EBNA5 interacting with p53 raises the possibility that p53 may inhibit the replication of these viruses as observed in HBV and that the role of these viral transactivating factors is to overcome the inhibitory effect of p53 for survival.

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