Liver-specific Enhancer II Is the Target for the p53-mediated Inhibition of Hepatitis B Viral Gene Expression*

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Here, we established the inhibitory mechanism of p53 on hepatitis B viral gene expression using HepG2 cells. Our results are as follows. First, p53 down-regulated the activities of all four promoters of hepatitis B virus (HBV), suggestive of the presence of a common element mediating the p53-dependent transcriptional repression. Second, employing the 5′-deletion constructs of the pregenomic/core promoter, the liver-specific enhancer II region was localized as a target for the p53-mediated transcriptional repression. Third, in a detailed analysis of the enhancer II region, the 5′-proximal 31-base pair region was defined as a p53-repressible element. Throughout the study, p53-mediated repression was rescued upon coexpression of the X-gene product, HBx. Finally, in an electrophoretic mobility shift assay, the defined p53-repressible element did not bind purified p53 directly, but shifted three bands in HepG2 nuclear extract, two of which was supershifted upon addition of p53 monoclonal antibody. These results display a novel mechanism of p53-dependent transcriptional repression in which p53 negatively regulates the viral-specific DNA enhancer through protein to protein interaction with an enhancer-binding protein. At the same time, the results indicate that p53 plays a defensive role against HBV by transcriptionally repressing the HBV core promoter through liver-specific enhancer II and HBx is required to counteract this inhibitory function of p53.

Hepatitis B virus (HBV), a causative agent of hepatitis and hepatocellular carcinoma, contains a 3.2-kb partially double-stranded DNA genome (1). Upon infection of the virus, the viral genome is transcribed to generate a 3.6-kb pregenomic RNA used as a template for viral replication. The pregenomic/core promoter is responsible for the synthesis of 3.6-kb pregenomic RNA, and therefore the regulation of this promoter is important in the viral life cycle (1). The 3.6-kb RNA also serves as a template for the synthesis of polymerase and nucleocapsid core protein. In addition to the 3.6-kb RNA, three more transcripts are generated from the HBV genome. The large surface antigen is synthesized from 2.4-kb RNA, and the major and middle antigens are synthesized from 2.1-kb transcripts. The X-gene product (HBx) is synthesized from the smallest 0.9-kb RNA (1).

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† The abbreviations used are: HBV, hepatitis B virus; kb, kilobases(s); CAT, chloramphenicol acetyltransferase; HBx, X-gene product; PCR, polymerase chain reaction; ENII, enhancer II; CURS, core upstream regulatory sequence; TBP, TATA box-binding protein.

The transcription of these RNAs are governed by the pre-S, surface, and X promoters, respectively. The activities of these promoters are under the control of enhancer I and II. Enhancer I is located upstream from the X promoter (2, 3) and is transactivated by HBx. The mechanism of transactivation is not through DNA binding but through protein to protein interaction (4). Enhancer II is responsible for the hepatocyte-specific nature of HBV replication (5–10). HBV viral products are synthesized in a tissue-specific and differentiation state-specific manner in transgenic mice and upon transfection. The 3.6-kb transcript used as a template for the replication appears only in liver cells, indicating that liver specificity and differentiation state specificity operate at the transcription level (5–10).

p53 is a typical DNA-binding transcription factor and works either as a transcriptional activator or repressor (11). When working as an activator, p53 binds directly to a specific DNA element. However, as a repressor, p53 works through protein to protein interaction. The mechanism of transcriptional repression by p53 was suggested to be through direct association with the TATA-binding protein complex (12–15) or CCAAT-binding factor (16). The abrogation of p53 function is one of the most important steps in viral transformation. E1B of adenovirus, large T antigen of SV40, E6 of human papilloma virus, BZLF1 and EBNA-5 of Epstein-Barr virus, and HBx of HBV are examples of viral transactivator that directly bind to p53 to abolish the tumor suppressor function (11). In the HBV case, HBx to p53 interaction was suggested to be also responsible for the apoptosis observed upon expression of HBx (17–19). In addition to a function as a tumor suppressor, p53 defends host cell from the invading virus. p53 actively regulates viral replication as in the case of SV40 (20, 21) and HBV (22). In the case of SV40, p53 binds to a sequence adjacent to the replication origin of SV40 and abrogates the helicase activity of T antigen by directly binding to it (20, 21). In the case of HBV, p53 interferes with the life cycle of HBV through down-regulation of the pregenomic/core promoter as reported by us (22).

Extending our previous study (22), we investigated the mechanism of the inhibitory function of p53 on the HBV life cycle. We report here a novel mechanism of p53-dependent transcriptional repression in which p53 inhibits hepatitis B viral gene expression by repressing the virus-specific enhancer, not the basal promoter, and HBx rescues viral gene expression. Our finding displays that the function of p53 extends to protection against viral infection by down-regulating the virus-specific DNA enhancer, which is tissue-specific and differentiation state-specific.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmids of wild type p53, pcDNA-p53, and of two p53 mutants, R273L and G154V, have been described previously (22). HBx expression plasmid, pcDNA-X and reporter plasmids, CpCAT, and CEP-CAT have also been described previously (22). SpCAT, S1pCAT, and XpCAT were derived from SpLuc, S1pLuc, and XpLuc kindly provided by Dr. McLachlan (23). In these constructs, a
3.2-kb HindIII fragment containing whole HBV DNA was linked to the CAT gene such that the expression of the CAT gene was governed by the surface, pre-S, and X gene promoters, respectively. The parental pGEMCAT vector has been described previously (22). Serial deletion constructs of CpCAT were generated by restriction digestion or PCR. CEP-del1 and CEP-del2 were derived from digestion of CpCAT with SphI or AvaI, respectively, and subsequent self-ligation. CEP-del3 and CEP-del4 were generated by PCR and subsequent ligation to the SmaI and HindIII sites of pGEMCAT. The 5’ PCR primers used were CCAAAATATGCGGCAAGGTCTT for CEP-del3 and AAAGACTGTGTTTGGTATAGCAC for CEP-del4. The underlined sequence represents the additional HindIII site introduced for subcloning. All deletion series have the same 3’ sequence as depicted in Fig. 2A. pINr-CAT was kindly provided by Dr. Laimins (13). EN2-InrCAT was generated by subcloning the PCR product of enhancer II core to the SmaI and NsiI site in the multi-cloning site of pINr-CAT. The 5’ primer was TCGAGTGCACATGGCGCAAGGTCTTTAC for 5’ and TGCATG- CATCCCAAATCTCCTCC for 3’. The underlined sequence represents an additional Sall or NsiI site introduced for the convenience of subcloning. EN2-A, EN2-B, and EN2-C were generated by subcloning the annealed oligonucleotides to the multi-cloning site of pINr-CAT at the XbaI and NsiI site. The sequences of the corresponding oligonucleotides are shown in Fig. 4, except that either side of the synthetic oligonucleotides have an XbaI and NsiI hanger for the convenience of subcloning.

**Transfection and CAT Assay**—The transfection and CAT assays were performed as described (22). The liver cell line HepG2 cells were used throughout this study. Where indicated, non-liver cell lines 293 and C33A were also employed.

**Electrophoresis Mobility Shift Assay**—Probes were labeled with [γ-32P]ATP and T4 polynucleotide kinase. Three ng of labeled oligonucleotides were used for each reaction. The total 40 μl of reaction mixture consists of 8 μl of 5× electrophoresis mobility shift assay buffer (100 mM HEPES, pH 7.9, 125 mM KCl, 0.5 mM EDTA, 50% glycerol, 10 mM MgCl2), 2 μl of 40 mM spermidine, 2 μl of 10 mM dithiothreitol, 2 μl of 0.5% Nonident P-40, 2 μl of 60 μg/ml poly(dl-dc), and 4 μl of 1 mg/ml bovine serum albumin. Sixty ng of purified p53 or 10 μg of HepG2 nuclear extract was employed for each reaction, and 0.1 μg of mononucleotides was included when necessary. Anti-p53 monoclonal antibody Do-1 and pAb1801 and polyclonal anti-TBP antibody (SI-1) were from Santa Cruz Biotechnology. When necessary, 104-fold cold oligonucleotides were employed as competitor. The sequences of competitor oligonucleotides were as follows: E2F, 5’-ATTATTAGTTCCGC-GCCCTTTTCTCA-3’; Sis-inducible element, 5’-GTCAGATTCCCTCCGTA-AATCTTGTCTCA-3’; acute phase response element, 5’-GATCTCTTGGAGATCCTGATC-3’. All the reaction mixtures were incubated for 60 min at room temperature and were run on a 5% polyacrylamide gel with 0.5× TBE as a running buffer.

**RESULTS**

p53 Represses the Activity of All Four Promoters of HBV—In the previous paper (22), we reported that tumor suppressor p53 negatively regulates the HBV life cycle through down-regulation of the 3.6-kb pregenomic/core RNA. In addition to the down-regulation of 3.6-kb RNA, we have also observed that the levels of other HBV transcripts are repressed by p53, which led us to test whether other HBV promoters are also influenced by p53. CpCAT, SpCAT, XpCAT, and S(1)pCAT, each containing a 3.2-kb full context of the viral genome in the configuration that the CAT genes are under the control of pregenomic/core, surface, pre-S, or X promoters, respectively, were employed as reporters (Fig. 1). In the transient transfection assay in HepG2 cells, the activities of all four promoters were down-regulated by the cotransfection of pcDNA-p53. The observed p53-mediated down-regulation of HBV promoters was the specific nature of wild type p53 insomuch as two p53 mutants, R273L and G154V, were found to be deficient in this activity. In addition, the coexpression of HBx resulted in the full or partial rescue of the CAT activity depending on the promoter used. The p53-mediated inhibition of CpCAT was fully rescued upon cotransfection of 5 μg of pcDNA-X, whereas the activities of SpCAT, XpCAT, and S(1)pCAT were rescued partially (Fig. 1). The HBx-mediated rescue was specific on the p53-mediated transcriptional repression, as the expression of HBx did not elevate but rather reduced the activity of all 4 promoters (Fig. 1). These results suggest the possibility that the effect of p53 was mediated through a common element in the HBV genome.

**Liver-specific Enhancer II of HBV Is the Target Site for p53-mediated Transcriptional Repression**—To identify the p53-responsive element in the HBV genome, we employed five 5’-deletion series of core promoter-CAT constructs shown in Fig. 2A. These deletion constructs were designed mainly to test the effects of liver-specific enhancers I and II, inasmuch as these two enhancers in combination are responsible for the liver-specific expression of HBV genes (6, 8, 9). The regulation pattern of CEP-CAT, CEP-del1, CEP-del2, and CEP-del3 in

**Purification of p53 Expressed in the Baculovirus System**—Human p53 baculovirus was a kind gift of Dr. Y. C. Sung. The purification was after Bargiotti et al. (20).

**Preparation of HepG2 Nuclear Extract**—The preparation of HepG2 nuclear extract was basically after Dignam et al. (24).
HepG2 cells was basically identical in that the CAT activity was repressed by p53 and was rescued by the coexpression of HBx. Further deletion of enhancer II in CEP-del4 resulted in the complete loss of the effects of p53 and HBx expression in HepG2 cells. These results suggest that the liver-specific enhancer II region contains the target site for p53-mediated transcriptional repression.

Next, to confirm that the enhancer II region is responsible for the p53-mediated transcriptional repression, we transferred enhancer II to the heterologous promoter, pInr-CAT (13, 22), and obtained ENII-Inr-CAT (Fig. 3A). As reported by Mack et al. (13), pInr-CAT was insensitive to p53 in HepG2 cells, whereas the activity of ENII-Inr-CAT was repressed by p53 and was recovered by the coexpression of HBx (Fig. 3B). To further confirm that liver-specific enhancer II is the target for the p53-mediated repression, we employed non-liver cell lines 293 and C33A (Fig. 3C). The activity of ENII-Inr-CAT was not repressed at all by p53 in these non-liver cells, indicating that the basal activity of the Inr promoter is insensitive to p53 and that the observed p53-mediated repression of enhancer II activity in HepG2 cells is a liver-specific phenomena. Again, the expression of HBx only did not elevate the activity of the reporter. From these results, we conclude that the liver-specific enhancer II is the target site for p53 to inhibit viral gene expression.

Fine Mapping of the p53-repressible Element in the Enhancer II Region—For detailed mapping of the element mediating p53-dependent transcriptional repression, an 81-base pair enhancer II core sequence was divided into three domains and oligonucleotides were synthesized with a five-base pair overlap (Fig. 4A). The synthetic oligonucleotides were inserted into pInr-CAT, and ENII-A, -B, and -C were generated. In transient transfection assays in HepG2 cells, the CAT activity of ENII-A was repressed upon expression of p53 and rescued by the coexpression of HBx (Fig. 4B). Again, the coexpression of HBx did not elevate the reporter activities, indicating that the rescue by HBx is specific to p53-mediated repression. Therefore, the 31-base pair ENII-A region, corresponding to nucleotide positions 1637–1667, was defined as a p53-repressible element. Previously, this region was reported to be protected in footprinting experiments with liver nuclear extracts (7, 9, 10, 26).

The Mechanism of p53-dependent Repression Assayed by Gel Shift—Inasmuch as the sequence of ENII-A shows no homology to the previously reported p53-responsive element (27), we were interested in whether p53 binds directly to the element or not. As a first step, we tested whether p53 binds directly to the ENII-A sequence by electrophoresis mobility shift assay using purified p53 from the baculovirus expression system (Fig. 5A). The purified p53 readily shifted the previously characterized
p53-binding sequence, RGC-W (5'TCGAGTTGCCTG- 
GACTTGCGCTTGCCCTTTTCC3'), and the band was super- 
shifted by the addition of p53 monoclonal antibody DO-1 or 
pAb1801. However, no shifted band was observed with ENII-A 
as a probe, eliminating the possibility that p53 directly binds to 
this element (Fig. 5A).

As a second step, we tested the possibility that p53 associates 
with an enhancer binding protein through protein to protein 
interaction. Employing HepG2 nuclear extract and ENII-A as a 
probe, we observed three shifted bands marked as A, B, and C 
(Fig. 5B, lane 2) and specifically competed (Fig. 5B, lanes 6–11). 
Upon addition of the p53 monoclonal antibody DO-1 (Fig. 5B, 
lane 4), the intensity of band B and C was reduced and the 
newly appearing supershifted band (SS) was observed indicat- 
ing that p53 is present in complexes B and C. No or weak 
supershifted band was detected with pAb1801 perhaps because
FIG. 5. Electrophoresis mobility shift assay with ENII-A oligonucleotides. A, gel shift assay with purified human p53 from the baculovirus expression system. For lanes 1–6, a p53-responsive element was used as a probe, and for lanes 7 and 8, ENII-A oligonucleotide was employed as a probe. 60 ng of purified p53 was used for lanes 2–6 and 8. Bands a and b represent the shifted p53 protein and supershifted p53 protein, respectively. Lanes 1 and 7, free probe. Lanes 2 and 8, purified p53. Lane 3, addition of p53 monoclonal antibody pAb1801. Lane 4, addition of p53 monoclonal antibody DO-1. Lane 5, competition with cold p53-responsive element oligonucleotide. Lane 6, competition with E,F-binding oligonucleotide. B, Gel shift assay with HepG2 nuclear extract and ENII-A probe. Three shifted bands are marked as A, B, and C and the supershifted band is marked as ss. Lane 1, free probe. Lane 2, HepG2 nuclear extract. Lane 3, addition of rabbit polyclonal anti-TBP (SI-1). Lane 4, addition of p53 monoclonal antibody DO-1. Lane 5, addition of p53 monoclonal antibody pAb1801. Lane 6, competition with 104-fold cold ENII-A oligonucleotide. Lanes 7–11, competition with 105-fold cold E,F (lane 7), Sis-inducible element (lane 8), acute phase response element (lane 9), ENII-B (lane 10), and ENII-C (lane 11) oligonucleotides, respectively. The pAb1801-binding site is shielded when p53 is complexed to an enhancer-binding protein. Addition of anti-TBP antibody used as a control did not cause any alteration of the gel shift pattern (Fig. 5B, lane 3). These results indicate that p53 is a part of the complex formed on an ENII-A and negatively regulates HBV gene expression via protein to protein interaction with one of the ENII-A binding proteins.

**DISCUSSION**

Previously, we have shown that p53 negatively regulates HBV replication through down-regulation of the pregenomic/core promoter, and that HBx is required to counteract this p53-mediated inhibition (22). In this report, we have localized the liver-specific enhancer II region as the target for the p53-mediated transcriptional repression of the pregenomic/core promoter. The enhancer II region is essential for the activities of all four promoters and is regarded as liver cell- and differentiation state-specific (5–10). For the pregenomic/core promoter, the enhancer II region stimulates basic core promoter activity more than 100-fold and works in a position- and orientation-dependent manner, and therefore was previously referred to as the core upstream regulatory sequence (CURS) (9). The EN-IIA region corresponds to “box α” within CURS, which was reported to be essential for full core promoter activity (9). Inasmuch as CURS was shown to be essential for the transcription of 3.6-kb pregenomic RNA and the production of 42-nm virions from transiently transfected hepatoma cells (9), the observed p53-mediated repression of CURS activity through ENII-A provides the mechanism for the antagonistic role of p53 against HBV infection or propagation. At the same time, the observation suggests that the p53-mediated inhibition of HBV replication probably operates under a physiological setting. As suggested by Lowe and Ruley (29), upon viral infection, the levels of p53 may rise, which represents the protection mechanism of p53 from the invading viruses. Upon initial infection or in the process of fluctuation of replication observed in chronic hepatitis patients, the ratio of p53 to HBx will determine the activity of ENII-A, which is important in viral replication and transcription.

For the pre-S, surface, and X promoters, enhancer II operates in a position- and orientation-independent manner (5–10). Previously, the EN-IIA region has also been shown to be essential for the activity of enhancer II (8, 10). Even though we tested only the deletion constructs of the core promoter to map the p53-repressible element, the observed down-regulation of the other three promoter activities by p53 and the fact that the employed promoter constructs all contain the full context of the HBV genome suggests that p53 probably down-regulates the other promoters of HBV through the same ENIIA element. Previous studies of HBV promoters have shown that each promoter possesses different characteristics: Cp, initiator/TATA-like; Sp, SV40-like; Xp, initiator-like; S(1)p, TATA-like (30–34). Considering these various characteristics of HBV promoters, it is not likely that p53 acts on each basal promoter element to mediate the common effect. Recently, Takada et al. (34) reported that p53 repressed the activity of the basal X-gene promoter. However, they employed constructs containing the basal promoter only and did not test the effect through enhancer II. Taken together, by acting on the EN-IIA sequence, p53 is expected to disrupt the activity of enhancer II as well as the activity of CURS, thereby leading to the repression of all four promoters and viral replication.

The ENII-A region was reported to be protected in a footprinting experiment with rat liver nuclear extract (26) or HepG2 nuclear extract (7, 9, 10). Although CCAAT/enhancer-binding protein has been proposed as a binding factor for this region (26), it is probably not the case since only a trace amount of CCAAT/enhancer-binding protein is present in HepG2 cells and the sequence preference and heat sensitivity are different (10).

According to the reports to date, p53-dependent transcriptional repression was suggested to be mediated through the
protein to protein interaction with basic transcription factors such as transcription factor IID and the CAAT box-binding factor (12–16). However, the sequence of enhancer II-A, a p53-repressible element, has no homology to the “TATA” or “CAAT” box sequence. Furthermore, the anti-TBP antibody did not cause any alteration of the gel shifted bands formed on ENII-A, eliminating the possibility that p53 down-regulated enhancer II activity through interaction with these basal transcription factors. In addition, Ori et al. (35) recently reported the p53-mediated repression of HBV enhancer I through the mechanism different from our finding. In their study, p53 bound to a defined region in the enhancer I in a sequence-specific manner and, with the help of an adjacent enhancer element, acted as a transrepressor. As far as we know, the finding in this report provides the previously unidentified mechanism in that p53 represses the activity of a enhancer-binding factor through protein to protein interaction, not the activity of a general transcription factor like transcription factor IID or CCAAT-binding factor.

Throughout the study, HBx antagonized the p53-mediated inhibition of transcriptional repression. In particular, the rescue of ENII-A activity reveals that HBx rescues viral gene expression and replication by acting on this element. Since HBx is not a DNA-binding protein, HBx may work by forming a complex with p53 (36–39) to antagonize the negative effect of p53. Alternatively, HBx may regulate certain signal transduction pathways (40–42), leading to the posttranslational modifications of p53. Regardless of the mechanism, these results suggest that p53 acts as a defense mechanism against HBV propagation, and that HBx is required to counteract this inhibitory function of p53 for the survival of HBV. Dysregulation of p53 function by viral oncoproteins is indispensable in tumorigenesis (43), and one of the proposed mechanisms of HBV-induced tumorigenesis is the disruption of p53 function by HBx (39). From the viral point of view, transformation can be caused by the previously unidentified mechanism in that p53 acts as a defense mechanism against HBV replication, and that HBx is required to counteract this inhibition pathways (40–42), leading to the posttranslational modification systems of p53.

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