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Research Communication

E2F1 Activates the Human *p53* Promoter and Overcomes the Repressive Effect of Hepatitis B Viral X Protein (HBx) on the *p53* Promoter

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Summary

The functional effect of the interaction of E2F1 and hepatitis B virus X protein (HBx) on the promoter of human *p53* gene was studied using chloramphenicol acetyl transferase (CAT) assay. E2F1 activated the *p53* promoter through E2F1 binding site. As previously reported, HBx repressed the *p53* promoter through E-box. When E2F1 was cotransfected with HBx, E2F1 overcame the repressive effect of HBx on the *p53* promoter through the E2F1 site. However, in the thymidine kinase (*tk*) heterologous promoter system with the E2F1 binding sites, cotransfection of E2F1 and HBx showed a strong synergistic activation. An in vitro interaction assay showed that E2F1 and HBx physically bind with each other. Analyses of the interaction domain with the GAL4 fusion protein showed that the pRb-binding domain of E2F1 was necessary for the functional interaction of these two proteins. Taken together, these results imply the functional inhibitory action of E2F1 on the HBV life cycle and HBV-mediated hepatocellular carcinogenesis (HCC). Therefore, the normal or enhanced function of E2F1 gene would be important in controlling the HBx function in HCC.

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Keywords E2F1; HBx; *p53*; promoter; transcription.

INTRODUCTION

E2F is a transcriptional regulator that plays a pivotal role in the regulation of cellular proliferation (*1*, *2*), encompassing E2F1-6 as family members and forming a heterodimer with a DP family member (DP1 or DP2) (*3*, *4*). Many E2F-responsive genes have been identified, and their products are components of either cell cycle control (e.g., *cyclin E*, *cyclin A*, and *cdc2*) or DNA synthesis machinery (e.g., dihydrofolate reductase, thymidine kinase, and DNA polymerase α). E2F acts as a transcription activator or repressor, depending on the promoter context (*5*).

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The regulation of E2F is a key target for DNA viruses, which have played an important role in the elucidation of the role of E2F in cellular proliferation and the mechanisms of its control. Binding of pRb and the other pocket proteins by E1A and other viral immediate-early (IE) proteins (e.g., SV40 large T antigen and papillomavirus E7) blocks their ability to associate with E2F and leads to an induction of E2F-dependent transcription (*6*, *7*). This activity is essential for the ability of the viral proteins to transform cells, highlighting the importance of E2F-pocket protein complexes in cell cycle and growth regulation.

E2F1 has been implicated as an oncogene from the studies in cultured cells in which E2F1 overexpression drove quiescent cells through the G1 into the S phase of the cell cycle (*8*, *9*), ultimately leading to apoptosis (*8–11*), or neoplastic transformation (*12*, *13*). Previous studies have shown that E2F1 can cooperate in inducing *p53*-dependent apoptosis (*8*, *11*, *14*), which is associated with an increase in *p53* level (*14*, *15*). Recently, it was reported that *p73*, the homologue of *p53*, is activated by E2F1 at the transcriptional level and also mediates the apoptotic signal by E2F1 (*16*). It is still not clear how E2F1 contributes in the accumulation of *p53* in the cell, and which ones of these potential downstream targets of E2F1 are biologically important in E2F1-mediated apoptosis.

It was previously reported that HBx could show oncogenic potential in a transgenic model (*17*) and activated several host genes important for cell proliferation and acute inflammatory responses, such as *c-fos*, *c-jun*, *c-myc*, *IL-8*, and *TNF-α* (*18–20*). In addition to the cellular promoters, HBx is known to transactivate many viral promoters (*21*, *22*), including HBV enhancer itself, simian virus 40 (SV40), Rous sarcoma virus, and HIV. Unable to bind to DNA directly, the activity of HBx is known to be mainly mediated through the binding sites for other transcription factors such as AP-1, NK-κB, ATF/CREB, acidic activators, and general transcription machinery (*23–25*). A recent study reported that C/EBPα also mediates the activity of HBx by direct binding (*26*).

p53 is a potent transcriptional regulator of cell growth whose induction leads either to cell-cycle arrest (*27*) or apoptosis (*28*, *29*). Loss of *p53* function correlates with cell transformation and oncogenesis (*30*), and several viral oncoproteins interact with *p53* and modulate its biological activity (*31*, *32*). Above all, in the onset of tumorigenesis, HBx and *p53* are known to control each other's level by mutually inhibiting each other at the transcriptional level (*33*, *34*) and protein-protein level (*35*). More detailed study has shown that HBx inhibits the ability of *p53* by sequestrating it to the cytoplasm and abrogating the *p53*-mediated apoptosis (*36*).

In this study, we investigated the role of E2F1 on promoter of *p53* gene. E2F1 activated *p53* promoter and overcame the negative effect of HBx. Whereas E2F1 antagonized HBx on the promoter functionally, interaction of E2F1 and HBx showed synergistic activation on the heterologous promoter system. Also, an EMSA and in vitro interaction assay showed that HBx augmented the DNA binding activity of E2F1 by direct interaction with it, probably through the pRb-binding domain of E2F1.

EXPERIMENTAL PROCEDURES

Plasmid Construction

The reporter plasmids for the *p53* promoter, p53p1CAT, *p53–416*, *p53–320*, *p53–67*, p53mEbox, and pBLCAT2Δ were described previously (*33*). From the p53p1CAT, *p53* promoter fragment for p53mE2F1 with 5′ *Pst*I and 3′ *Xba*I ends were prepared by PCR and were cloned into pBLCAT2Δ digested with *Pst*I and *Xba*I. The E2F1 heterologous promoter pE2F1tkCAT was constructed by inserting three copies of the E2F1 binding sites in the p53p1CAT (−28 to −8) into pBLCAT2, which has a minimal promoter (−150 to +50) of herpes simplex viral (HSV) thymidine kinase (*tk*) gene, respectively. The sequences of the wild-type and mutant E2F1-binding sites are as follows: p53E2F1wt, 5′-GATCCTCAAGACTGGCGCTAAAAG-3′; p53E2F1mt, 5′-GATCCTCAAGACTGGC*AT*TAGGAG-3′. Sequences homologous to the consensus E2F1 site are underlined, and mutated sequences in mutant E2F1-binding sites are described in italic. The reporter plasmid pG5SVCAT was made by inserting five copies of the consensus yeast GAL4 binding sites into the pCAT-promoter (Promega Co.), which contains the minimal SV40 early promoter. The eukaryotic expression vector for the human E2F1, pSG5E2F1, was kindly provided by Dr. William G. Kaelin, Jr. The expression vector for HBx, pMGX, was described previously (*26*). The GAL4-E2F1 fusion protein expression vector was constructed by the digestion of pSG5E2F1 by *Bgl*II and ligated into the *Bam*H1 cut GAL4 expression vector, pSG424. The pRb-binding domain deletion clone GAL4-E2F1ΔRb was made by the complete digestion of pGAL4-E2F1 by *Xba*I and the partial digestion of *Xho*I.

Cell Culture, Transient Transfection, and CAT Assay

HepG2 and HeLa cells were grown in Dulbecco's modified Eagle's medium/10% fetal bovine serum/penicillin G sodium at 100 units/ml/streptomycin sulfate at 100 μg/ml/amphotericin B at 250 ng/ml. Cells were seeded into 60-mm dishes (30–50% confluence) for 24 h prior to transfection. These cell lines were transfected with reporter and activator plasmids using the calcium phosphate coprecipitation method with Bes as described previously (*37*). Typically 2 μg each of reporter, 0.5 μg of pSG5E2F1, and 1 μg of pMGX were used. One microgram of pCMV-β-gal plasmid was introduced in all experiments to correct the variations of transfection efficiency. The total amount of transfected DNA was adjusted to 5 μg with pBluCAT. The CAT assay was described previously (*26*) and normalized by β-galactosidase assay. CAT activity was quantified by measuring the conversion of [^{14}C]chloramphenicol to its acetylated forms using a Fuji BAS bioimaging analyzer.

Preparation of Recombinant Proteins and Electophoretic Mobility Shift Assay (EMSA)

Dr. William G. Kaelin, Jr., kindly provided the *E. coli* expression plasmid of GST-fused E2F1. The GST and GST-E2F1 were affinity-purified, and the amount of purified proteins was determined by a Bradford assay (Bio-Rad). For DNA binding reactions, 100 ng of purified GST-E2F1 was mixed (where appropriate) with 100 ng of the DNA competitors and binding buffer. The mixture was incubated for 10 min on ice; about 1 ng of end-labeled probe was added, then the incubation was continued for another 15 min on ice. Antibody against human E2F1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mixtures were then electrophoresed in 5% acrylamide gels in 0.5 × TBE. After electrophoresis, the gels were dried and exposed to X-ray film.

In Vitro Interaction Assay and Western Blotting

One hundred nanograms of purified GST-E2F1 were incubated with 100 μl of amylose resin bound to MBP or MBP-fused HBx proteins for 1 h at 4 °C. After extensive washing with buffer A, bound proteins were eluted with 100 μl buffer A containing 10 mM maltose. Western blotting to detect bound proteins was carried out as described previously (*26*) with minor modifications. Anti-E2F1 antibody (1:10,000) was used for the primary antibody, and a horseradish peroxidase-linked, anti-mouse antibody (1:10,000) was used for the secondary antibody. Protein-antibody complexes were visualized by the ECL Western blotting detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

RESULTS

E2F1 Activates the Human p53 *Promoter through the E2F1 Site*

To investigate the role of E2F1 on the expression of *p53* gene, we transfected an increasing amount of E2F1 expression vector (pSG5E2F1) with the human *p53* full-length promoter (p53p1CAT) into HeLa and HepG2. As shown in Fig. 1B, E2F1 activated the *p53* promoter activity up to 10-fold in HepG2 cells.

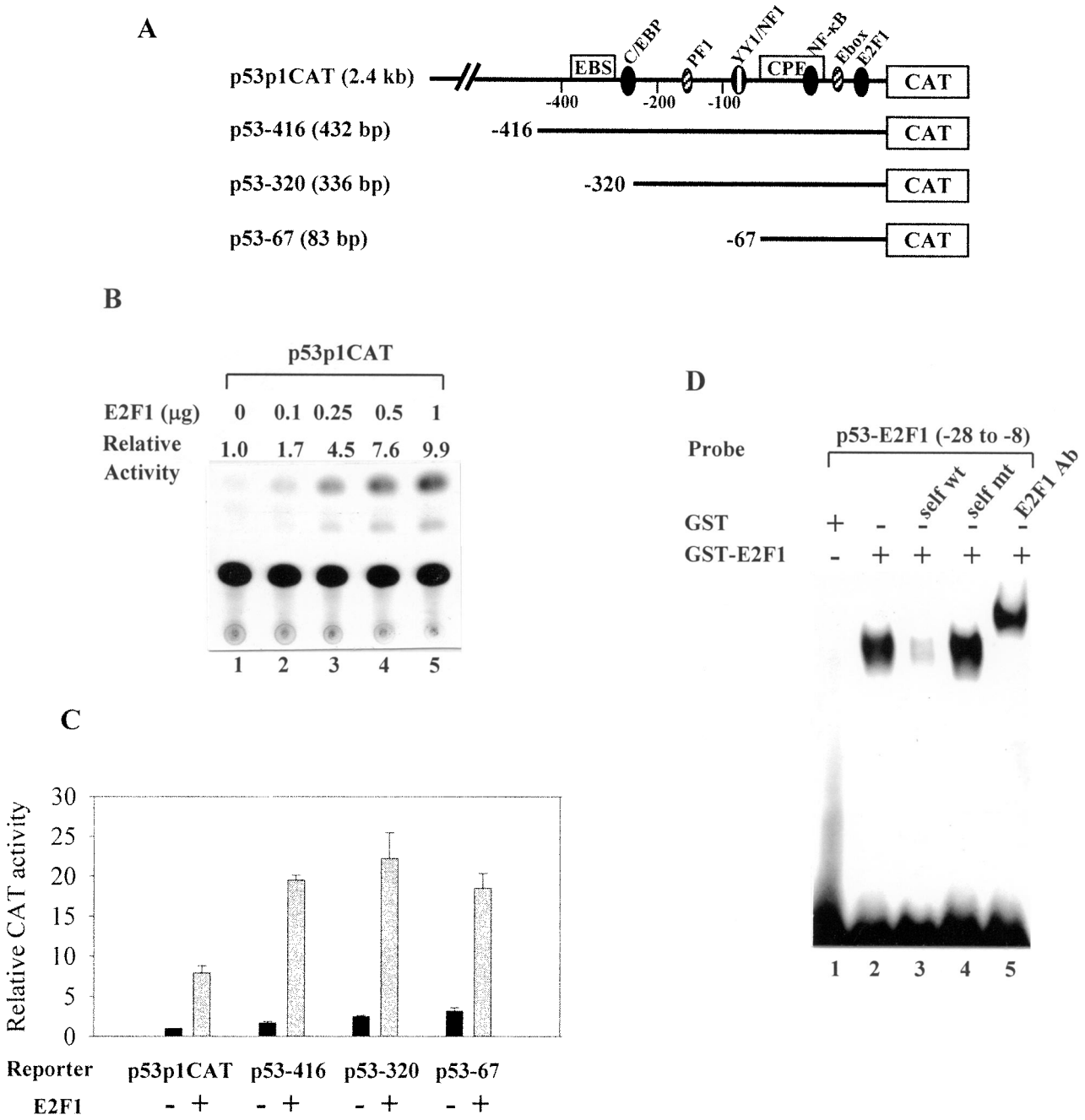

Figure 1. E2F1 activates the *p53* promoter through the E2F1 binding site located from nt −28 to −8. (A) Schematic presentation of transcription factor binding sites and approximate location in the *p53* promoter and its serial deletion mutant clones. EBS, ETS-binding site; C/EBP, CCAAT/enhancer binding protein site; CPE, *p53* core promoter element; arrow, major transcription start site. (B) Dose-dependent activation of *p53* promoter by E2F1. Two micrograms of p53p1CAT and increasing amount of E2F1 expression vector (pSG5E2F1) were transfected into HepG2 cells. (C) Localization of E2F1 binding sites in the *p53* promoter by serial deletion of the *p53* promoter. HepG2 cells were cotransfected with the same amount of each reporter and 0.5 μg of E2F1 expression vector as indicated. (D) Identification of E2F1-binding site in *p53* promoter. EMSA was performed using bacterially expressed GST and GST-E2F1 protein and the E2F1 probe corresponding to the nt −28 to −8 region of the *p53* promoter. All experiments were repeated at least three times. All the transient transfection experiments were performed in HepG2 and HeLa cell lines and obtained the same results except Fig. 3D. A representative autoradiography is shown, and standard deviations were <12% in all cases for the CAT assay data.

We also obtained the same result in HeLa cells (data not shown). To localize the active E2F1 site in the promoter, we used the serial deletion mutant clones of the *p53* promoters (Fig. 1C). The activation effect of E2F1 was maintained in the clone *p53–67*, which contains the putative binding site for E2F1 in the region nt −28 to −8, which has 91% homology with the consensus E2F1 element.

To confirm the interaction of E2F1 with this region, we performed EMSA with oligonucleotides synthesized based on the region nt −28 to −8. Bacterially expressed and purified GST-E2F1 bound this region (Fig. 1D) and competed out completely when excess wild-type cold probe was added, whereas mutant probe did not (Fig. 1D, lanes 3 and 4). Addition of anti-E2F1 antibody supershifted the band (lane 5). Also, site-directed mutagenesis of the E2F1-binding site of *p53–67* clone (p53mE2F1) disrupted the E2F1 activity completely when compared to the wild-type clone (Fig. 2C, compare lanes 2 and 6). E2F1 binding activity of this region was also tested with the nuclear extracts of HepG2 cell, and got the similar data (data not shown). These results imply that E2F1 activates *p53* promoter through its binding site.

E2F1 Overcomes the Negative Effect of HBx on the p53 *Promoter*

As reported previously concerning the *p53* promoter (*33*), HBx alone repressed the *p53* promoter at the transcriptional level through an E-box region located about nt −25 to −40 (Fig. 2B, lane 3). Interestingly, E2F1 successfully abolished the repressive effect of HBx (Fig. 2B, lane 4) and activated the *p53* promoter from 5.8 fold by E2F1 alone (Fig. 2B, lane 2) to 8.4-fold by E2F1 and HBx together (Fig. 2B, lane 4). This result implies that E2F1 overcame the repressive effect of HBx on the *p53* promoter by the synergistic activation of the *p53* promoter.

To further analyze the effect of E2F1 and HBx on the *p53* promoter, the site-directed mutant clones of *p53–67* were cotransfected with these two factors (Fig. 2A). The wild-type clone *p53–67* showed similar behavior as a full-length promoter (p53p1CAT) (compare to Fig. 2B). When we focus on E2F1, HBx repressed the reporter as the wild-type clone (Fig. 2C, lane 7), because the E-box element was intact (lane 7), but the mutation of the E2F1 binding site (p53mE2F1) inhibited the effect of these two factors from 7.1 to 2.6 (Fig. 2C, compare

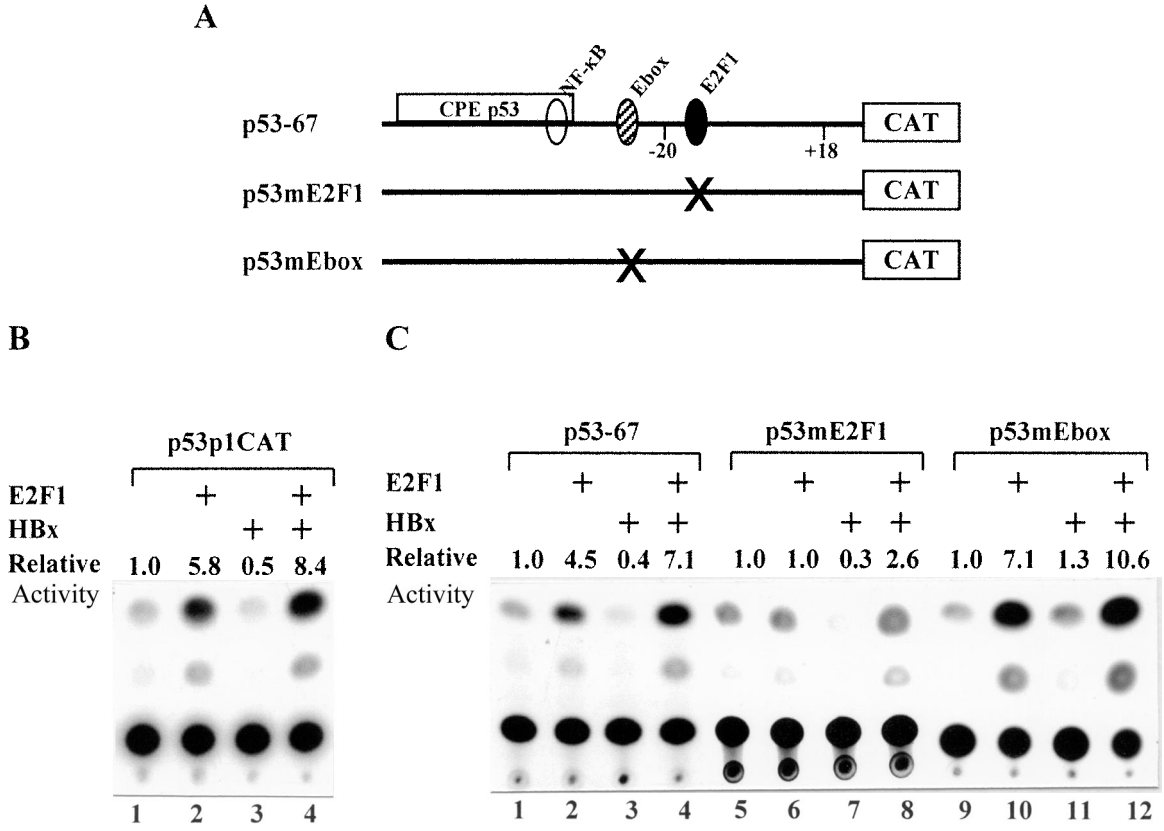

Figure 2. E2F1 overcomes the repressive effect of HBx on the *p53* promoter through E2F1 site. (A) Schematic presentation of the *p53–67* promoter and its point mutant derivative clones, p53mE2F1 and p53mEbox. (B) Cotransfection of E2F1 and HBx on the *p53* promoter. A half microgram of E2F1 and 1 μg of HBx expression vector (pMGX) was transfected with p53p1CAT into HepG2 cells. Note that E2F1 and HBx show the synergistic activation on the *p53* promoter (lane 4). (C) Comparison of effect of E2F1 and HBx on the mutant clones and the wild-type promoter *p53–67*. Note that the synergistic effect was abolished in p53mE2F1, while this effect was maintained in p53mEbox.

lanes 4 and 8), which means that the E2F1-binding site in the region −28 to −8 is important for the synergistic activation of E2F1 and HBx. When tested on the E-box mutant clone of *p53–67* (p53mEbox), the negative effect of HBx was abolished completely as previously reported (Fig. 2C, lane 11) (*33*). For this clone, the E2F1 activity was stronger than for the wild-type clone (Fig. 2C, compare lanes 2 and 10), which may be as a result of the easy binding of E2F1 due to the inaccessibility of bHLH proteins to E-box. The synergistic effect of E2F1 and HBx was also observed in the p53mEbox as shown in the wild-type clone (Fig. 2C, lane 12), which implies that E-box is not important for the synergistic activation of E2F1 and HBx. These results suggested that the synergistic effect of E2F1 and HBx was dependent on the E2F1-binding site, in which the E2F1 binds directly on the DNA and HBx behaves as a coactivator.

E2F1 and HBx Show a Synergistic Effect on the Heterologous Promoter

To study the functional relevance of E2F1 and HBx in detail, we used the minimal *tk* promoter of the HSV with or without E2F1 binding sites of the *p53* promoter (Fig. 3A), and transfected into the HeLa and HepG2 cell lines. As shown in Fig. 3C, E2F1 and HBx showed strong synergistic activity in HepG2 cells when cotransfected (lanes 1–4). This strong synergistic activity was not observed in the control reporter plasmid pBLCAT2 (Fig. 3B) and the mutant E2F1 reporter plasmid pmE2F1tkCAT (Fig. 3C,

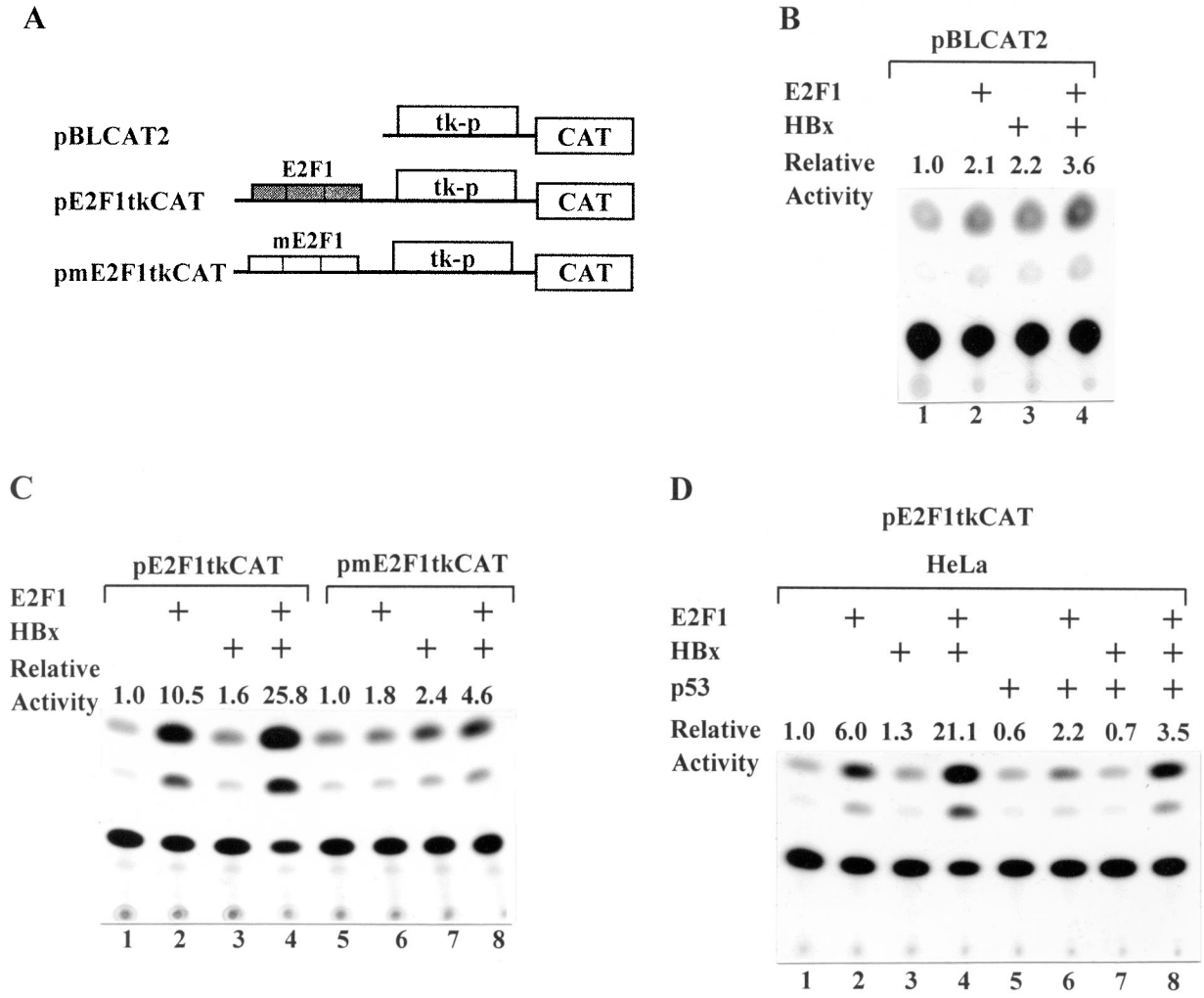

Figure 3. E2F1 and HBx show the synergistic effect on the heterologous promoter system in two cell lines, HeLa and HepG2. The same amount of reporter and expression vectors were used as indicated. (A) Schematic presentation of pBLCAT2-derived heterologous promoters. pBLCAT2 has a CAT expression reporter plasmid under the influence of a minimal promoter of HSV *tk* gene. pE2F1tkCAT and pmE2F1tkCAT have three repeats of E2F1 wild-type and mutant-type binding site, respectively. (B) Effect of E2F1 and HBx in the control reporter plasmid pBLCAT2. (C) The synergistic effect of E2F1 and HBx on pE2F1tkCAT was observed in HepG2 cells, while this effect disappeared in pmE2F1tkCAT. (D) The synergistic effect was relieved when 1 μg of *p53* expression vector was transfected with E2F1 and HBx in HeLa cells, where the endogenous *p53* is inactivated.

lanes 5–8). We observed the same result in HeLa cells (data not shown). These results showed that these two factors work synergistically, with dependence on the E2F1 binding site. We also constructed the minimal *tk* promoter with E2F1 binding sites of the HBx promoter and got the same results with those of *p53* promoter (data not shown).

It was previously reported that *p53* mutually inhibits the activities of E2F1 and HBx at the protein-protein level (*35*, *38*). We tested the effect of *p53* on the synergistic activation of E2F1 and HBx in HeLa cells, where endogenous *p53* protein is deactivated by human papilloma virus (Fig. 3D) (*39*). Addition of *p53* to E2F1 and HBx repressed the activity of these factors as reported previously (compare lanes 2, 3 and lanes 6, 7) (*35*, *38*). When *p53* was cotransfected with E2F1 and HBx, *p53* was also able to repress the activity of these two factors (compare lanes 4 and 8). These results suggest that there is a new role of *p53* in regulating the activity of HBx by abolishing the synergistic activation with E2F1.

The Physical Interaction Between E2F1 and HBx

To confirm the activation domain of E2F1 in the synergistic effect of E2F1 and HBx, we used a yeast GAL4 fusion protein of E2F1 and tested on the reporter plasmid pG5SVCAT, which has a minimal SV40 early promoter with five copies of consensus GAL4 binding sites (Fig. 4A). The transactivation domain (amino acids 238–437 of E2F1) encompassing a pRb-binding domain of E2F1 was excised from the E2F1 and fused into the DNA binding domain of the GAL4 protein (amino acids 1–147 of GAL4) [Fig. 4A, GAL4-E2F1 (283–437)]. As expected, the GAL4-E2F1 (283–437) and HBx showed strong synergistic activity in HepG2 cells (Fig. 4B, lane 6), whereas the same amount of GAL4 DNA binding domain expression vector (GAL4) failed to show this activation effect (lane 4). With an assumption that the pRb-binding domain of E2F1 would play some role in the interaction with HBx, we constructed the GAL4-fusion version of the pRb-binding domain deletion clone of E2F1 (pGAL4-E2F1ΔRb, amino acids 238–413 of E2F1) (Fig. 4A). The transactivation ability of the GAL4-E2F1ΔRb decreased about twofold compared to the GAL4-E2F1 when transfected alone (compare lanes 5 and 7). Interestingly, when cotransfected with HBx, GAL4-E2F1ΔRb also did not show any more synergistic activation (compare lanes 7 and 8), which implies that the synergistic activation of these two factors are dependent on the pRb-binding domain of E2F1. The same result was observed in HeLa cells (data not shown).

The effect of HBx on the DNA binding affinity of E2F1 was examined by EMSA of the E2F1-binding site in the *p53* promoter. As shown in Fig. 5A, MBP-X increased the binding of GST-E2F1 to the E2F1-binding site (Fig. 5A, lanes 4 and 5), whereas MBP did not (Fig. 5A, lane 3). We then analyzed the direct interaction of E2F1 and HBx using an in vitro interaction assay with MBP-X protein-coupled amylose resin (Fig. 5B). Results showed by pull-down experiment with amylose that GST-E2F1 directly bound to MBP-X, (Fig. 5B, lane 3) but not to

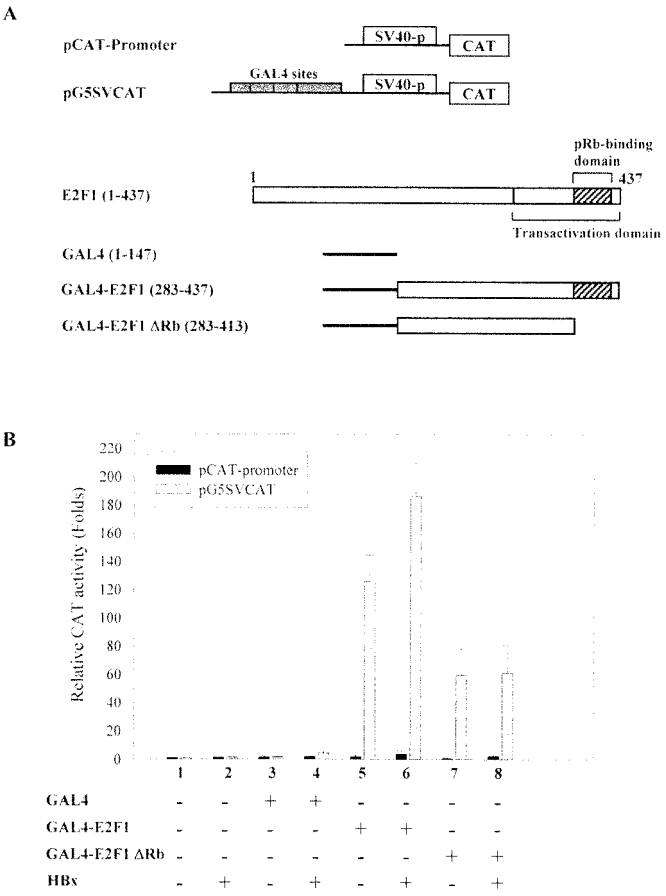

Figure 4. The synergistic effect of E2F1 and HBx by GAL4-E2F1 fusion protein is dependent on the pRb-binding domain of E2F1. (A) Schematic presentation of plasmids for the GAL4-heterologous promoter reporters and GAL4-E2F1 fusion protein expression vectors, GAL4-E2F1 and GAL4-E2F1ΔRb, which were made by fusing the DNA-binding domain of GAL4 protein (amino acids 1–147) with amino acids 283–437 and 283–413 of E2F1, respectively. (B) 0.25 μg of GAL4, GAL4-E2F1, and GAL4-E2F1ΔRb were transfected into HepG2 cells without (−) or with (+) 1 μg of HBx expression vector as indicated. Error bars indicate standard errors.

MBP alone (Fig. 5B, lane 2). Purified GST-E2F1 was used as a marker (lane 1). Also, the amino acid 47–133 region of HBx is found out to be important for the interaction with E2F1 (*40*). These results demonstrate that the Rb-binding domain of E2F1 and the amino acid 47–133 region of HBx are responsible for the functional interaction with each other. In addition, by direct binding with E2F1, HBx enhances the DNA binding activity of E2F1, which induces a synergistic activation dependent on the promoter contexts.

DISCUSSION

In this report, we showed that E2F1 activated the *p53* promoter and successfully blocked the effect of HBx, that is, E2F1

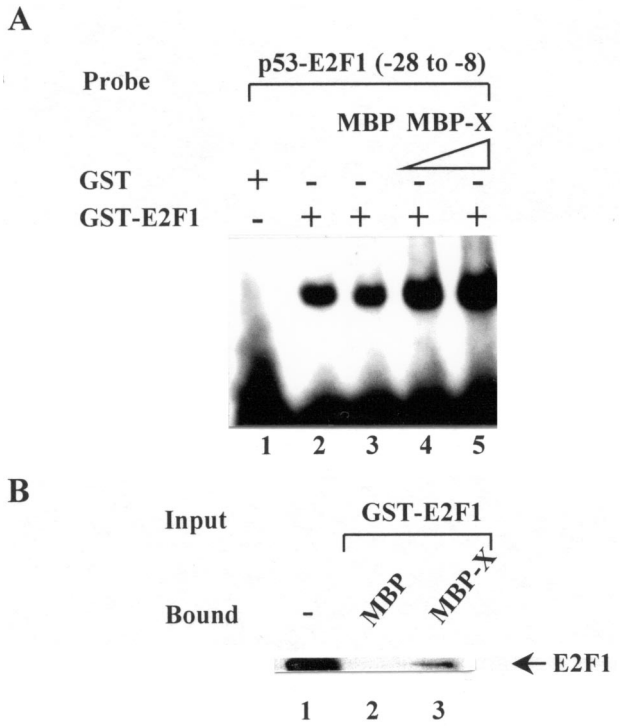

Figure 5. HBx directly interacts with E2F1 and enhances its binding to the E2F1-binding site. (A) HBx enhanced binding of E2F1 to the E2F1-binding site in the *p53* promoter. Constant amount (100 ng) of GST or GST-E2F1 was incubated with 500 ng of MBP or increasing amounts of MBP-X (250 ng, lane 4; 500 ng, lane 5) in the presence of ^{32}P-labeled E2F1-binding site oligonucleotide. (B) In vitro interaction assay of the direct interaction of E2F1 and HBx. MBP or MBP-X protein was used as bait for full-length E2F1. Eluted proteins were subjected to Western blotting using anti-E2F1 antibody. Purified GST-E2F1 was used as a marker (lane 1).

overcame the negative effect of HBx on the *p53* promoter. On the other hand, E2F1 and HBx together showed a synergistic effect on the heterologous systems, which suggests the possibility that these two factors may also show a synergistic effect on other target promoters of E2F1. In addition, by demonstrating the direct binding of E2F1 and HBx in vitro, we proposed a mechanism for this synergistic effect and the role of this complex in the process of carcinogenesis. In short, new target gene of E2F1 has been identified, and E2F1 and HBx showed various functional interactions on the gene expression of target genes depending on promoter contexts, while HBx may disrupt the E2F1-Rb complex by binding with E2F1.

E2F1 activated the expression of *p53* promoter through its binding site (Fig. 1). Although E2F1 and *p53* reciprocally antagonized each other's functions (*38*), it was observed that *p53* accumulates in response to the stably or transiently transfected E2F1 by western blotting (*14*, *15*). However, it was unclear whether the increase in *p53* levels was caused by the proposed physical interaction between E2F1 with MDM2 (*41*), or between DP-1 and *p53* (*38*), or by another mechanism. Bates et al. showed that the tumor-suppressor protein p14^{ARF} links deregulated E2F1 activity and stabilization of *p53* (*42*). We here report the other mechanism for this; direct activation of *p53* transcriptional level by E2F1, which may cause the protection of cells from oncogenic changes that result in abnormal proliferation in the cell. Also, when overexpressed in the cell, E2F1 induces apoptosis by *p53*-dependent and *p53*-independent pathways, while other members of the E2F family do not (*15*). Knock-out mice with disrupted E2F1 showed spontaneous tumors (*43*, *44*), which clearly demonstrates the role of E2F1 as a tumor-suppressor (*45*). The result in this report is consistent with this recent view that E2F1 may function in an antioncogenic role as well as an oncogenic role. More detailed studies, which will show that direct activation of *p53* expression by E2F1 causes the cell to induce *p53*-dependent apoptosis and block the malignant transformation, are needed.

HBx, the potential viral oncogene of HBV, is a versatile factor with roles as a transcription factor as well as a signal transducer. We have previously reported that HBx represses *p53* promoter through an E-box element (*33*). Also, other groups reported that *p53* represses the HBx promoter in the transcriptional level (*34*), and these two proteins are known to bind and mutually inhibit each other's function at the protein-protein level (*35*). So, the effect of E2F1 on the mutual inhibitory balance of *p53* and HBx on *p53* promoter needed to be tested. Interestingly, E2F1 succeeded in abolishing the repressive activity of HBx on the *p53* promoter, and further activated it (Fig. 2B). To examine the synergistic activation of E2F1 and HBx on the *p53* promoter more precisely, we used the site-directed mutants of the wild-type clone. Fig. 2C shows that the synergistic effect of these two factors is dependent on the E2F1 site located from −28 to −8, whereas the E-box element seems to be less important for this synergistic activation effect. This effect is probably due to the different characteristics of these two factors. HBx has no DNA-binding activity and works as a coactivator in vivo (*24*, *46*), which bridges between the transcription factor and general transcription machinery, whereas E2F1 mainly works by direct binding to DNA. So this result also implies that HBx may bridge E2F1 with general transcription machinery on the p53mEbox by direct binding with E2F1 and enhance the binding of same. Also, this result reemphasizes the importance of the E-box element in mediating the repressive effect of HBx, for the disruption of the E-box element caused enhanced activity of *p53* promoter when HBx was transfected with (Fig. 2C, compare lanes 4 and 12). The phenomenon that E2F1 as well as HBx can interact with the basal transcription machinery to activate the target promoter (*24*, *46*, *47*) is shown in Fig. 2C, lane 8, with slight activation in E2F1-binding site mutant clone. It also appears in Fig. 3B, lane 4 and Fig. 3C, lane 8.

When the heterologous promoters were tested for their ability to mediate the E2F1 effect with the E2F1 binding sites from the *p53* promoter, E2F1 activated it (Fig. 3C). Addition of HBx

showed a strong synergistic effect on the promoter, which implies a general functional interaction between E2F1 and HBx. In addition, *p53* abolished the synergistic activation effect of E2F1 and HBx complex (Fig. 3D), which implies a new role for *p53* in blocking the activity of HBx using E2F1 as a cellular transcriptional activation target.

GAL4-fusion protein analyses revealed that the pRb-binding domain in E2F1 proves to be important in the interaction with HBx (Fig. 4B). In another study of our group, the amino acid 47–133 region of HBx was responsible for the interaction with E2F1 (*40*). The result of the physical interaction of E2F1 and HBx (Fig. 5) and the result of the GAL4-fusion assay (Fig. 4) strongly suggest that HBx interacts with E2F1 through a pRb-binding domain. These results further imply that HBx has the potential to disrupt the pRb-E2F complex in inducing tumorigenesis like other DNA tumor viral oncoproteins such as SV40 Tag, Adenovirus E1A, and HSV E7. In addition, the new functional target of HBx was identified, and by direct binding with E2F1, HBx exerts its transcriptional influence through E2F1 by enhancing the DNA binding activity of E2F1 like other previously identified factors, such as AP-1, NF-κB, and ATF/CREB (Fig. 5A).

Both E2F1 and HBx work as critical factors in determining the fate of the cell in malignant transformation. When these two factors were transiently expressed in the cell lines, they showed various effects ranging from synergistic activation to mutual inhibition. In the *p53* promoter, the effect resulted in the successful blocking of HBx activity by E2F1. Yet in the heterologous promoter system, the synergistic effect can be explained by the effective utilization of E2F1 by HBx. The effects of these two factors on the individual promoters are differentially dependent on the promoter contexts, based on the different characteristics of E2F1, HBx, and promoters, whereas the net cellular effect of these two factors should be studied in detail. However, there have been numerous reports that *p53* mutation is responsible for the hepatocellular carcinoma (HCC) (for a review, see reference *48*), and it is also proposed that the balance of the reciprocal inhibitions between *p53* and HBx may play a decisive role in the development of HBV-related malignances (*33–35*). In this respect, the normal function of E2F1 gene would be important in controlling the HBx function in hepatocellular carcinogenesis.

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