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The Synergistic Transactivation of the Hepatitis B Viral (HBV) Pregenomic Promoter by the E6 Protein of Human Papillomavirus Type 16 (HPV-16 E6) with HBV X Protein Was Mediated through the AP1 Site of E Element in the Enhancer I (EnI) in Human Liver Cell

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Infection by HBV of a cell already infected with other viral species or vice versa has been suggested as being involved in hepatocellular carcinoma. Using the CAT assay method, we investigated the interactive roles of HBx and potentially oncogenic and transactivating viral early proteins such as Ad5 E1A, HPV-16 E6, and SV40 T ag. In the presence of HBx, only HPV-16 E6 showed significant synergistic transactivation of EnI. We further investigated the function of the HPV-16 E6 using deletion, heterologous promoter, and mutation analyses on the EnI promoter. The results showed that the synergistic effect was mediated through the AP1 site of the E element in EnI by the direct activation of AP1 and support the idea that the infection by HBV of the cell with other viral species such as HPV-16 could increase the transcription activity of the HBV and other oncogenes containing an AP1 site in the promoter.

Key Words: **HBx; HPV-16 E6; AP1; HBV EnI; synergism.**

Hepatitis B virus (HBV), a 3.2 kb, partially double-stranded DNA virus, replicates through the reverse transcription of pregenomic RNA (1) and, despite its small size, the HBV genome contains 4 open reading frames (ORF), and four promoters regulated by two enhancers, enhancer I (EnI) and enhancer II (EnII) (2, 3). EnI has been shown to contain overlapping binding sites for multiple liver-enriched and ubiquitous transcription factors including NF1, C/EBP (4), AP-1, CREB, and ATF (5), HNF4, EF-C, RXR, HNF-3, and RFX-1 (6, 7). EnII located upstream of the core/pregenomic promoter is highly liver specific (8, 9), and its activity is regulated according to the differentiation state of the hepatoma cell lines and includes binding sites for transcription factor Sp1, C/EBP, HNF3 (10, 11), HNF4, RXR, and PPAR (3).

In relationship between HBV and other viruses, adenovirus type 5 (Ad5) E1A repressed HBV EnII activity, but its responsive element was not identified in the viral promoters (12). Simian virus 40 (SV40) T antigen induces the nuclear sequestration of HBx, and blocks stimulation of the Ras-Raf-MAP kinase cascade by HBx. Human papillomavirus type 16 (HPV-16) E6 with 2 hypothetical zinc fingers, has been shown to be responsible for HPV-induced cervical cancer (13). The E6 has the ability to bind the tumor suppressor p53, resulting in abrogation of p53-mediated cell cycle control (14).

Previous studies on the coinfection of HBV with other oncogenic viruses (HCV, HDV and HIV) have been focused on a wide range of clinical manifestations, from hepatitis to the development of HCC (15–18). In this case, several oncogenes may interactively accumulate biochemical and genetic alterations to disrupt a number of cellular barriers on the way to HCC. In particular, Scinicariello *et al.* (19) reported that they detected human papillomaviral DNA in human primary hepatocellular carcinoma, and suggested that oncogenic HPV might contribute a cofactor acting synergistically with HBV in the development of the HCC. In this study, we investigated differential regulations of HBV gene expression by HBx and potent transforming/transactivating viral proteins such as Ad5 E1A, HPV-16 E6, and SV40 T antigen. Using a series of

Abbreviations used: HBV, hepatitis B virus; CAT, chloramphenicol acetyltransferase; HBx, hepatitis B viral X prtotein; Ad5, adenovirus type 5; HPV-16, human papillomavirus type 16; SV40, simian virus 40; EnI, hepatitis B virus enhancer I.

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functional assays, we here report that cotransfection of HBx with E6 stimulated the activity of EnI region of a pregenomic promoter.

MATERIALS AND METHODS

Plasmids. The plasmids, pCENCAT, pEIXp, and pCpBm were described previously (5). The expression vector of HBx (pCMV-X) was constructed by placing its cDNA (nt 1371-1833) under the control of the cytomegalovirus enhancer and early promoter of pcDNA3. The expression vectors of c-Jun and c-Fos contain RSV LTR in front of their cDNAs, and the ATF2 expression vector was described previously (20). The heterologous reporter, pEtkCAT, has 5 copies of E element (5′-CTGCCAAGTATTTGCTGACGCAACCCCCA-3′) in front of the tk promoter and its mutant plasmid, pEMtkCAT, has mutations in the E element (5′-CTGCCAAGTATTTGCTAATT-CAACCCCCA-3′). The underlined sequences in the pEMtkCAT represent mutated bases, which inhibit binding of both AP1 and ATF2. A heterologous reporter, pAP1tkCAT which has 3 copies of consensus AP1 site (5′-CGCTTGATGAGTCAGCCGGAA-3′) in front of the tk promoter. The control plasmid pBLCAT2 has a minimal promoter (−150 to +50) of the HSV tk gene (20).

Transfection and CAT assay. Transfections of plasmid DNAs into HepG2 cells were carried out using the calcium phosphate coprecipitation method (21). After forty-eight hours, the cells were harvested. Subsequently, CAT assay was performed as described previously (20) and the activities of reporters were quantitated using a Fuji BAS bio-imaging analyzer.

RESULTS

Effects of DNA Tumor Viral Early Proteins on the HBV Pregenomic Promoter

To study the effects of other transiently expressed viral proteins (Ad5 E1A, HPV-16 E6, and SV40 T antigen) on the HBV gene expression, cotransfection assays were performed with a reporter, pCENCAT, and each plasmid encoding viral proteins in HepG2 cells. Ad5 E1A repressed the CAT activity of pCENCAT (Fig. 1A, lane 2), whereas other viral proteins slightly activated transcription (Fig. 1A). However, the E6 increased the CAT value 2- to 6-fold in the presence of the transactivator HBx (Fig. 1B). In particular, there was a synergistic transactivation between HBx and the oncoprotein E6 of HPV-16 (Fig. 1B, lane 3).

The E6 of HPV-16 Transactivated Synergistically the HBV EnI/Xp in the Presence of HBx

The plasmid pEIXp containing HBV EnI/Xp in front of the CAT reporter gene was cotransfected with each expression vector of three viral proteins in the absence (Fig. 2B) and in the presence of HBx expression plasmid (Fig. 2B). In the presence of HBx, the E6 of HPV-16 synergistically enhanced the transcription activity more than 6-fold (Fig. 2B, lane 8) as compared with the control value (Fig. 2B, lane 3). Ad5 E1A alone and even with HBx repressed the activity of EnI (Fig. 2B, lanes 2 and 7). This repression was not consistent with the data of Chen *et al.* (12), in which this discrepancy might have been due to the different promoter and cell lines used.

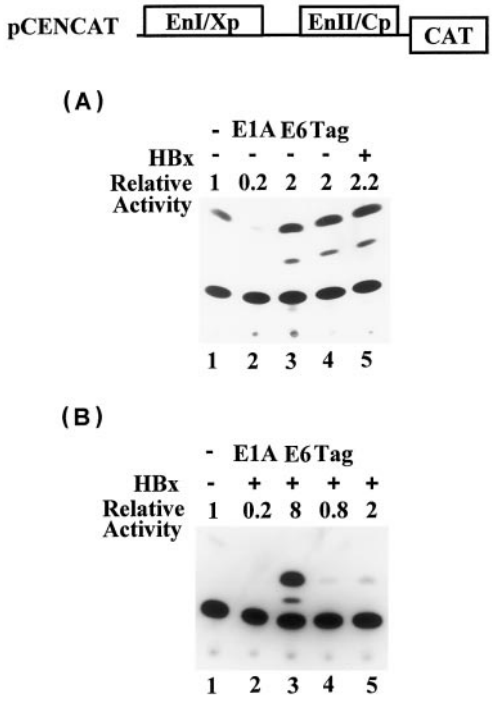

FIG. 1. Effects of four viral proteins on the HBV pregenomic promoter (pCENCAT). A map of the full-length HBV pregenomic promoter region (nt 247–1873) including EnI and EnII is shown at the top. (A) Two micrograms each of pCENCAT, and the expression plasmids of the four viral proteins were cotransfected into HepG2 cells. (B) The effects of other viral proteins on the HBV pregenomic promoter in the presence of HBx. The amount of each cotransfected plasmid was 2 μg each. Relative CAT activities are average values of three independent experiments.

To investigate whether EnII can mediate the cooperative effects of HBx with viral proteins, transient cotransfection assays were performed with a reporter pCpBm containing EnII/Cp and each expression vector of viral proteins without or with the HBx expression vector pCMV-X (Fig. 2D). Unlike the results of pEIXp, the E6 of HPV-16 stimulated the CAT activity somewhat about 1.9-fold in the presence of HBx (Fig. 2D, lane 8) as compared with the control (Fig. 2D, lane 3). Results so far indicate that the E6 of HPV-16 shows major synergistic effect with HBx on EnI.

We then examined which site in the EnI was responsible for the synergistic activation of HBx with the E6. Previous reports described that the EnI contains five well-characterized distinct factor binding sites, namely 2C, GB, EP, E, and NF-1 sites (20). Preliminary observations implied that the responsive elements in EnI for those two proteins (HBx and E6) were in the E element where the AP1 and ATF2/CREB overlapped. Therefore, it is most likely that the E element was responsible for the synergism of E6 with HBx. CAT assays were performed directly with the heterologous system, pEtkCAT contain-

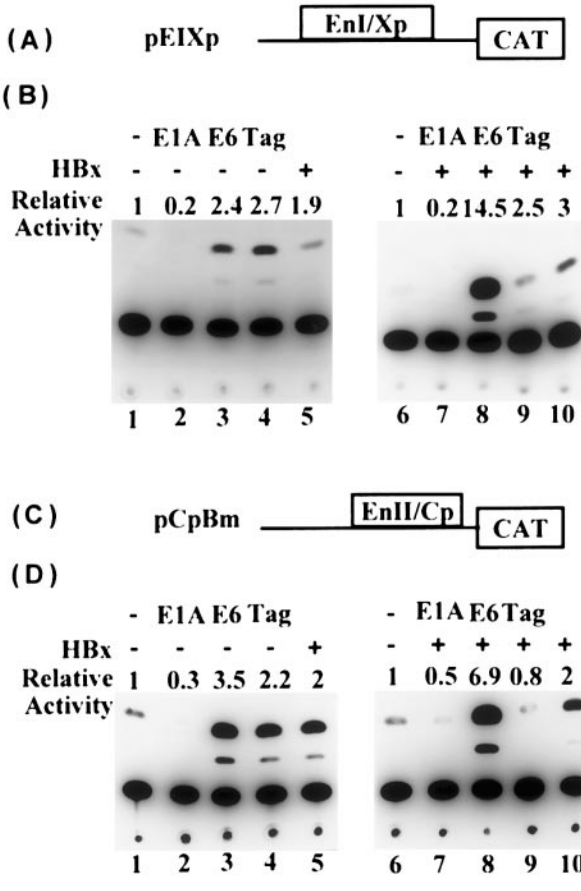

FIG. 2. Effects of four viral proteins and HBx on the HBV EnI/X promoter (pEIXp) and the HBV EnII/core promoter (pCpBm) in the absence and the presence of HBx (B, D). Schematic diagrams of pEIXp and pCpBm are shown (A, C). HepG2 cells were cotransfected with 2 μg of each reporter and the expression plasmids of other viral proteins with or without HBx expression plasmid (2 μg). Relative CAT activities are average values of three independent experiments.

ing the E element in front of the herpes simplex virus thymidine kinase (tk) gene promoter. The results showed that the E element mediated the E6-HBx synergism in EnI (Fig. 3B). A mutant reporter, pEMtkCAT having mutations in both AP1 and ATF2 binding sequences exhibited no synergistic effect of E6-HBx (Fig. 3B, lane 8). This observation suggests that the E element is responsible for the E6-HBx synergism.

Synergistic Transactivation by the Interaction of HBx and E6 through the AP1 Binding Site in the E Element of EnI

To determine which transcription factor binding site in the E element mediated the synergistic activation of E6 and HBx, cotransfection assay was carried out with pEtkCAT and pAP1CAT as reporters, and expression vectors of c-Jun, c-Fos, ATF2, HBx, and E6 in various combinations in HepG2 cells (Fig. 4). The AP1 alone transactivated CAT activity about 4-fold (Fig. 4A, lane 5), and higher levels of activation were observed following the addition of E6 and HBx (Fig. 4A, lanes 8 and 9). Furthermore, consistent with these observations, we observed that the transcription was synergistically activated when all three proteins are present (Fig. 4A, lanes 12 and 13). The presence of ATF2 exhibited rather a marginal repressive effect on the synergistic activation of E6-HBx (Fig. 4A, lanes 10–13). This implies that the repressive effect of ATF2 on the E element, which we previously reported (20) was abolished in the presence of E6 and HBx.

To confirm the AP1 site mediated E6-HBx synergism, CAT assays were performed with the heterologous reporter, pAP1tkCAT. The E6 alone activated transcription slightly (Fig. 4B, lane 2), and HBx about 8-fold (Fig. 4B, lane 3). When both proteins were present, the transcription was synergistically increased about 34-fold (Fig. 4B, lane 4) as expected. This result supports the view that the E6 significantly activated the effect of HBx on the AP1 site. Taken together, the results demonstrated that the synergistic transactivation occurred through the AP1 binding site in the E element by the coexpression of E6 and HBx.

DISCUSSION

Previously, HBx interactions with cellular proteins such as C/EBP, ATF2, p53, TFIIB and TBP, have been

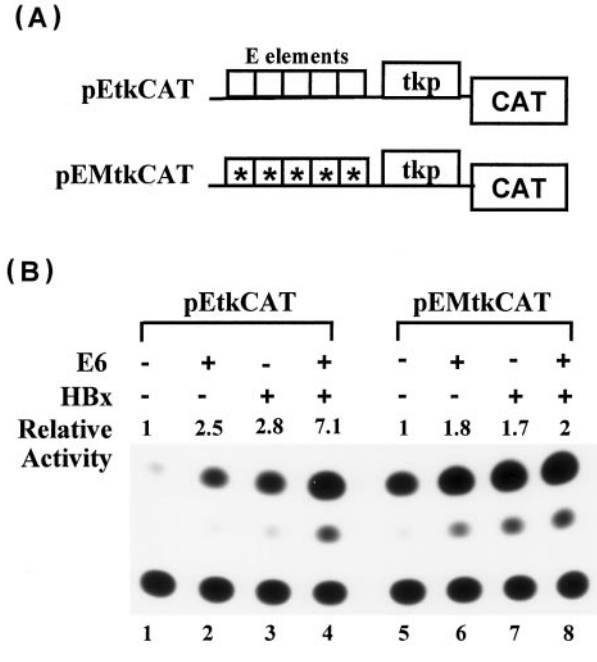

FIG. 3. Effects of HPV-16 E6 and HBx on the E element of HBV EnI. (A) Schematic diagrams of pEtkCAT and pEMtkCAT are shown. (B) Two micrograms of each heterologous reporter plasmid were cotransfected into HepG2 cells with pHPV16E6 and pCMV-X (2 μg each). The relative CAT activities are the average values of three independent experiments.

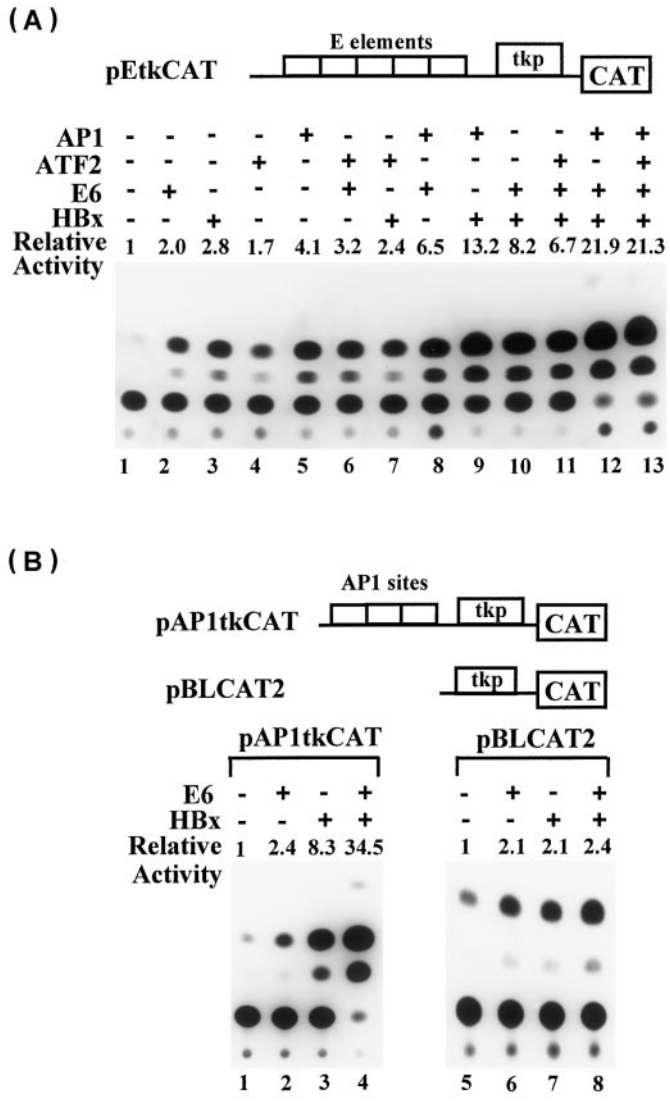

FIG. 4. AP1 mediates the synergism of HBx and HPV-16 E6. (A) Two micrograms each of the plasmids that express the HPV-16 E6, HBx, c-Jun, c-Fos, and ATF2 proteins were cotransfected with the reporter, pEtkCAT (2 μg) into HepG2 cells. Note that the combination of AP1, E6 and HBx showed the highest CAT activity suggesting that the AP1 binding site is responsible. (B) The synergistic effect of HBx and E6 on a heterologous reporter, pAP1tkCAT which has 3 copies of consensus AP1 site in front of the tk promoter. The control plasmid pBLCAT2 has a minimal promoter (−150 to +50) of the HSV tk gene (20). The relative CAT activities represent the mean values of three independent experiments.

reported (5, 22, 23). However, the effects of HBx with other viral transactivators on the transcription of HBV have not been studied much. Here, we showed that the coexpression of HBx with the HPV-16 viral transactivator E6 synergistically stimulated the activities of HBV EnI and EnII whereas E7 activated EnI activity only.

It has been reported that HBx not only transcriptionally transactivates the c-Jun and c-Fos promoter in transient transfection assays (24, 25), but also increases the DNA binding activity of AP1 (c-Jun/c-Fos heterodimer) in HepG2 cells (26). It was suggested that the activity of AP1 is modulated at least at three levels: the regulation of the nuclear translocation and transcriptional activation of c-Fos, the regulation of the DNA binding activity of the AP1, and the modulation of the transactivating potential of c-Jun (26, 27). Although we could not clearly elucidate the synergistic mechanism among the three proteins AP1, E6 and HBx, two possible explanations, not mutually exclusive, suggest themselves. First, the highest activation in the presence of AP1, E6 and HBx (Fig. 4A, lane 12) can be explained by the fact that the E6 significantly stimulated the activity of HBx directly and/or that another protein(s) in the signal pathway involved in the AP1 activation did. Early study (28) supporting the presence of another protein demonstrated that HPV-16 E6 was associated with at least seven cellular proteins, and this complex manifests kinase activity. Moreover, it has been reported that c-Jun seemed to be the primary target of HPV-16 E6 and c-Fos is also transcriptionally activated by the E6 (29, 30). As previously known in the case of HBx, another possibility would be that the E6 acts as a coactivator that can mediate crosstalk between activator and elements of the basal transcription complex (31).

In conclusion, these results strongly imply that the infection by HBV of the cell already infected with other viral species such as HPV or vice versa could increase the transcription activity of the HBV and other oncogenes containing an AP1 site in the promoter.

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