ABSTRACT

The hepatitis B virus (HBV) X protein (HBx) is a transcriptional transactivator that has been implicated in the development of HBV-related hepatocellular carcinoma. Mutations in the HBx open reading frame have been reported, but their general impact on the biological function of HBx remains unknown. To address this issue, we comparatively analyzed the structures and biological functions of HBx sequences isolated from sera and from tumor and nontumor tissues of patients with a HBV-related hepatocellular carcinoma. In addition to the HBx sequences derived from free HBV genomes, HBx from HBV integrants was also obtained from the tumor tissues by use of a HBs-Alu PCR-based approach. Sequence analysis showed that the HBx sequences derived from tumor tissues (6 of 7) were more precisely map the functional domains located at the COOH-terminal region. Interestingly, most of the COOH-terminally truncated HBx sequences obtained from tumor tissues, in contrast to the full-length HBx isolated from the sera and nontumor tissues, lost their transcriptional activity and their inhibitory effects on cell proliferation and transformation. Importantly, although full-length HBx suppressed the focus formation induced by the cooperation of ras and myc oncoproteins in primary rat embryo fibroblasts, COOH-terminally truncated HBx enhanced the transforming ability of ras and myc. Finally, by analyzing the artificial mutants, we were able to more precisely map the functional domains located at the COOH-terminal end of HBx. Taken together, our results suggest a key role for the HBx COOH-terminal end in controlling cell proliferation, viability, and transformation. This study further supports the hypothesis that natural HBx mutants might be selected in tumor tissues and play a role in hepatocarcinogenesis by modifying the biological functions of HBx.

INTRODUCTION

HCC is one of the most common human cancers on a worldwide basis. Among the different risk factors involved in liver carcinogenesis, HBV chronic infection has been shown to play a major role (for a review, see Ref. 1). HBx, a small regulatory protein that is required for the establishment of viral infection, has been claimed to contribute to the development of HCC. However, the molecular mechanisms involved in HBx-mediated hepatocarcinogenesis remain obscure.

HBx is a promiscuous transactivator. It can activate a variety of viral and cellular promoters and enhancers (for a review, see Ref. 2). HBx does not bind directly to DNA. Its transcriptional activity is therefore mediated via a protein-protein interaction. The transactivation function of HBx may be exerted both in the cytoplasm, via signaling pathways, and in the nucleus, via DNA-binding proteins. The transcriptional activation triggered by HBx was reported to be essential for viral replication (3). It may also be implicated in regulating the expression of certain cellular genes that are crucial to hepatocyte proliferation, viability, and transformation.

HBx has been reported to either induce or block apoptosis. Initially, HBx was shown to abrogate p53-induced apoptosis by direct association with p53 (4). HBx has also been reported to inhibit tumor necrosis factor-α, anti-Fas antibody, and transforming growth factor-β-induced apoptosis (5–7). However, a growing body of evidence, including results from our group, has shown that HBx can induce apoptosis or sensitize cells to apoptotic death induced by proapoptotic stimuli (8–18). It is important to point out that in the livers of transgenic mice and in primary hepatocyte cultures, HBx has demonstrated a proapoptotic effect (10, 16). The apoptotic effect of HBx could be partially relieved by Bcl-2 (11, 17), E1B (17), or mitogenic growth factor insulin-like growth factor II (13) and could be suppressed by activated Ha-ras through activation of the phosphatidylinositol-3 kinase and Akt pathway (18). The sensitization of cells by HBx to tumor necrosis factor-α-induced apoptosis may involve activation of mitogen-activated-protein kinase kinase 1 and Myc protein (12, 13). The association of HBx with mitochondria, which causes an abnormal aggregation of mitochondrial structures in the cell, suggests one of the possible mechanisms underlying HBx-related apoptotic cell death (15, 19), although there is no evidence for cytochrome c release in HBx-expressing cells (20). Interestingly, in three independent studies, the proapoptotic domains on HBx were found to overlap with transactivation domains (8, 9, 17).

HBx has been shown to induce HCC in vivo in certain lines of transgenic mice (16, 21, 22) and in vitro to have a weak oncogenic potential in several immortalized cell lines. In the NIH3T3 cell line, an injection in nude mice of cells expressing HBx could induce tumors, although transfected HBx alone in the cells did not form foci (23). Similar results were obtained with FMH202 cells, a murine immortalized hepatocyte cell line (24, 25), and AML12, a differentiated murine hepatocyte cell line (26, 27). Recently, HBx was shown to transform REV-2 rat fibroblasts by itself (28) and could transform REF52 cells (18) when cotransfected with activated ras oncogene. Although the data from transgenic mice and in vitro studies have provided evidences implicating HBx in carcinogenesis, the impact of these data on human HCC still remains highly controversial. Indeed, to date, only three reports have indicated that HBx can induce hepatocarcinoma in vivo in transgenic mice (16, 21, 22). In most other studies, HBx transgenic mice developed tumors only after the administration of chemical carcinogens or the coexpression of other oncogenes (29–32). In the context of in vitro experiments, certain groups have recently reported that HBx may act as a tumor suppressor gene. Transfection of HBx into NIH3T3 cells completely abrogated the focus formation ability of all tested oncogenes, including Ha-ras, v-src, v-myc, v-fox, and E1a (11). In another study performed in primary REFs, HBx also exhibited a strong suppressive effect on focus formation induced by cooperating oncogenes (17).
studies, the inhibitory effect on cell transformation was related to the proapoptotic activity of HBx.

In humans, HBx has been shown to be expressed in HCC tissues and is the most preferential sequence retained in the integrated form of HBV (9, 33–35). Several mutants have been identified from sera and/or liver tissues from patients with HCC. HBx sequences isolated from integrated HBV genomes in tumor tissues have frequently been reported to have a deletion at the 3’ end (36–43). Recently, a Ser31Ala point mutation (44) and a linked-point mutation, Lys130Met and Val131Ile (45–47), in HBx were shown to be prevalent in HCC patients. However, the biological functions of these mutants have not been explored to date.

During a previous study based on a small number of HBsAg-negative patients with HCC, we showed that the HBx mutants isolated from tumor tissues abrogated both the transactivation and antiproliferative effects of wild-type HBx (8). A major difficulty when studying HBx genetic variability in HBsAg-positive HCC is the mixture of both free and integrated HBV genomes. In this work, we have taken the advantage of a HBx-Alu PCR-based approach, which allowed us to separately isolate uninterrupted HBx sequences, presumably referring to replicative genomes in HCC tumor tissues, from those interrupted by integration of the HBV genomes into the cellular DNAs. By comparatively analyzing the structures and biological functions of HBx sequences isolated from the sera and from nontumor and tumor tissues of these patients, we are able to report here for the first time that the HBx sequences isolated from tumor tissues differ considerably from wild-type HBx in all of the functions tested. Together with the results generated from the artificial mutants, we provide evidence for the role of such mutations in the development of HCC.

MATERIALS AND METHODS

Patients and Samples. We studied four HBsAg-positive patients with HCC, two [patients 1 and 2 (P1 and P2)] from France, and two [patients 3 and 4 (P3 and P4)] from South Africa. All of the patients were anti-HBV core protein-positive and anti-HBs-negative. HCC was developing in a cirrhotic liver in patients 1, 2, and 4. Tumor tissues and paired distal normal liver tissues (referred as nontumor) were obtained at surgery and defined by microscopic examination.

Amplification and Sequencing of HBx from Sera and Liver Tissues. DNA was extracted from sera and liver tissues by a previously reported method (33). The uninterrupted HBx sequences were amplified from sera and from tumor and nontumor tissues by regular PCR using primer 37 (5’-TGCCAAGTGTGTTTGCGACC-3’; HBV 1176–1195; located upstream to the X gene) and primer 25 (5’-AGGGAAGAAGTGACGAGG-3’; HBV 1978–1996; located downstream to the X gene). To amplify the integrated HBx sequences from tumor tissues, we used an HBx-Alu PCR-based approach (48). PCR and Alu PCR products were purified and analyzed by PCR direct sequencing. At least two independent DNA extractions and PCR reactions were performed for each sample, and four sequencing reactions (two for each strand) were carried out to confirm that the reported sequence reflected the most prevalent HBx in a specific sample.

Plasmids. To express HBx mutants in the mammalian cells, the DNA fragment encompassing precisely the whole HBx or HBx with the downstream cellular flanking sequence before the first stop codon were cloned into the vector pcDNA3.1 (Invitrogen Life Technologies, Inc., Cergy Pontoise, France) by HindIII and XhoI sites. Positive clones were sequenced to confirm their compliance with the original results. Artificial mutants used in this study were generated by a PCR cloning method using HBx ayw as the template. The CRE-Luc reporter construct was a kind gift from Dr. C. Desdoguet (INSERM V370, Paris, France). It contains two copies of the cyclin A CRE in the sense orientation upstream to the thymidine kinase promoter of the TK-Luc reporter construct (49). C-Ha-ras and c-myc constructs have been described previously in detail (50).

Cells and Cell Culture. Human hepatocarcinoma cell lines Huh7 and HepG2, the human liver cell line CCL13 (Chang), the murine liver cell line α-ML, and REFs were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS. REFs were prepared from Fischer rats (IFFA-CREDO, Lyon, France) at day 14 of gestation, according to the protocol described by Land (51).

Luciferase Test. HBx (1 μg) and reporter CRE-Luc (0.5 μg) plasmids were cotransfected into the α-ML cells in 6-well plates, using Exgen reagent (Euromedex, Soufflewysersheim, France). Cells were transfected at 48 h posttransfection and lysed in Chris buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.5% NP-40, 0.1 mM EDTA, 10% glycerol]. After protein normalization by the Bradford assay (Bio-Rad Laboratories, Hercules, CA), equal amounts of protein (150 μg) were used for the luciferase assay. The data presented are the means of three independent transfections.

 Colony Formation Assay. We transfected Chang, Huh7, and HepG2 cells with 10 μg of HBx constructs or empty vector per 10-cm plate of cells, using a standard calcium phosphate precipitation method. Two days after transfection, the cells were subcultured at a ratio of 1:6 for Chang and 1:4 for Huh7 and HepG2. Two plates were retained for each transfection, and the cells were cultured in G418 (Invitrogen Life Technologies) selective medium for 2 weeks (800 μg/ml G418 for Chang, 700 μg/ml for Huh7, and 1000 μg/ml for HepG2). Drug-resistant colonies were fixed with cold methanol, stained with Giemsa, and then scored.

Focus Formation Assay. We transfected second-passage REF cells (5 × 10⁵) with 20 μg of DNA for each 10-cm plate of cells, using a calcium phosphate precipitation method (51). Two days after transfection, the cells were split 1:20 for subculture in normal medium and 1:6 for subculture in G418 (500 μg/ml) selective medium. The cell culture medium was changed every 3 or 4 days. Foci and colonies formed in G418 selective medium were scored at day 10 after selection, whereas foci developed in normal medium were scored at week 4 posttransfection. Statistical analysis was performed using a paired t test.

Growth in Soft Agar. Soft agar dishes were prepared with an underlayer of 0.5% agar (Sigma-Aldrich, Saint Quentin Fallavier, France) in DMEM supplemented with 20% FCS. Five hundred cells from each focus-derived clonal cell line were plated in the same medium containing 0.375% agar. The dishes were examined microscopically for colony formation after a 2-week incubation period.

Immunoblot Analysis. Approximately 2.5 × 10⁵ cells were pelleted and lysed in Laemmli buffer. Cell lysates were separated by 12% SDS-PAGE, transferred onto Immobilon membranes (Millipore, Molsheim, France), and revealed by monoclonal anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated antimouse immunoglobulin and the Western blotting detection kit (ECL Plus) were obtained from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

RESULTS

Comparison of HBx Sequences Isolated from Sera and from Nontumor and Tumor Tissues of HBV-related HCC Patients. The HBx in the sera and tumor of nontumor liver tissues of four HBV-related HCC patients were amplified by PCR using primers encompassing the entire HBx region (40). We previously reported that free HBV genome was identified as a major form in the liver of patients whose serum scored positive for HBsAg, although viral load was low in such subjects with HCC (2). In this context, free HBV genome will be preferentially amplified with a regular PCR assay. We therefore considered that the PCR products obtained from tumor and nontumor tissues corresponded to the HBx from free HBV genome. To study the status of the HBx sequences from integrated HBV DNA in tumor tissues, we used an Alu PCR-based technique (48). PCR and Alu PCR products were then analyzed by direct sequencing, and nucleotide variations were considered only when consistent in at least two independent experiments. Thus, the sequences reported here represent the most prevalent sequence in each sample. We failed to amplify the HBx sequence from the free HBV genome in the tumor tissue of patient 2. This may have been attributable to the low copy of virus in the tumor tissue or to a viral genetic variation in the primer regions. Fig. 1 summarizes the aa sequences of HBx isolated from the free
HBx sequences derived from patient 1 were close to the HBV adr subtype (genotype C), whereas HBx sequences derived from the other three patients were close to the HBV adw subtype (genotype A). In a given patient, there was only a small difference between the HBx sequences isolated from serum and from nontumor tissue. In fact, in patients 1 and 4, the HBx from nontumor tissue was identical to that obtained from the serum, whereas 1- and 5-aa substitutions were observed in patients 2 and 3, respectively. HBx sequences derived from free and integrated HBV genomes in tumor tissues diverged more from each other, particularly in the distal COOH-terminal part. As for the seven HBx sequences isolated from tumor tissues, six exhibited deletions (two of three and four of four for HBx sequences derived from free and integrated HBV genomes in tumor tissues, respectively) at the COOH terminus, with sizes ranging from 3 to 74 aa. For patient 1, the COOH-terminal deletion of HBx sequence derived from free HBV genome in tumor tissue was the result of an 8-nucleotide deletion (nt 396–403), which introduced a stop codon (codon 135). No point mutation through the HBx region was found to be associated with tumor phenotype. Therefore, the deletion of amino acids at the COOH-terminal end is a major feature of HBx identified in tumor tissue.

The serum and liver tissue of patient 4 exhibited a particular free form HBx sequence. At the nucleic acid level, the 3' part of the sequence showed only 45% homology with the HBV X gene (data not shown). A similar HBV integrated sequence has previously been described in the PLC/PRF/5 cell line (52, 53). The deduced amino acid sequence showed that the first 79 aa shared high homology with the HBx adw subtype, but because of a frame shift, the 42 aa in COOH-terminal part showed no similarity with HBx.

**Analysis of Transactivation Activity of HBx Natural Mutants.**

To investigate the biological properties of different HBx sequences, HBx PCR products were cloned into mammalian expression vector. After sequencing analysis, clones with the sequence reflecting the major form of HBx in the population, as depicted in Fig. 1, were selected for further study. The level of expression of HBx natural mutants was determined by Western blot and immunostaining analyses. In all HBx full-length sequences (P1/S/NT, P2/S, P2/NT, P3/S, P3/NT, and P3/T), a similar level of HBx expression was observed when compared with HBx ayw (data not shown). A lower level of HBx expression was observed in almost all truncated HBx (P1/T, P1/TI, P2/TI, P3/TI, and P4/TI) by Western blot (Fig. 2B). The expression of P4/S/NT/T was below the sensitivity threshold of the Western blot. However, the immunofluorescence results showed very

Fig. 1. Amino acid sequence of naturally occurring HBx mutants. HBx sequences from sera (S), nontumor (NT), and tumor (T) liver tissues of four HBV-related HCC patients (P1, P2, P3, and P4) were obtained using PCR (for the free form of HBV) and Alu PCR (for the integrated form of HBV). TI, integrated HBx in tumor. The amino acid sequences of HBV adr and adw subtypes are shown at the top. Identical amino acid residues are represented by dots. The underlined amino acids were deduced from cellular flanking sequences.

Fig. 2. Transactivation activity of HBx mutants. A, α-ML cells were cotransfected with the HBx expression plasmids indicated and the CRE-Luc reporter construct. Cells were harvested for luciferase assay 48 h after transfection. Relative luciferase activities indicate the mean of three independent transfections. B, expression of wild-type HBx and COOH-terminally deleted mutants in α-ML 48 h after transfection. S, serum; NT, nontumor; T, tumor; TI, integrated HBx in tumor.

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formed. Murine hepatocyte cells (α-luciferase assay using a reporter construct of CRE-Luc was performed. Murine hepatocyte cells (α-luciferase assay using a reporter construct of CRE-Luc was performed. Murine hepatocyte cells (α-luciferase assay using a reporter construct of CRE-Luc was performed. Murine hepatocyte cells (α-luciferase assay using a reporter construct of CRE-Luc was performed. Murine hepatocyte cells (α-luciferase assay using a reporter construct of CRE-Luc was performed.

This may be attributable to a reduction in the HBx stability or length of HBx might therefore affect the level of protein expression. Weak expression of P4/S/NT/T in the cells (data not shown). The loss of HBx in the cells (Fig. 3A) may be attributable to a decrease in the HBx stability or length of HBx might therefore affect the level of protein expression. Weak expression of P4/S/NT/T in the cells (data not shown). The loss of HBx sequences obtained from tumor tissues, a 3-fold transactivation was observed only in full-length HBx (P3/T) or in HBx with the deletion of 3 aa (P2/TI). In the other HBx sequences from tumors (P1/TI/TI, P3/TI, and P4/TI), with a COOH-terminal deletion of >18 aa, an abrogation of HBx transactivation was observed. The loss of transactivation activity was not attributable to the decreased expression of truncated HBx because HBx ayw can maintain transactivation activity even when the expression level is lower than that of the most truncated HBx (Fig. 2B, Lanes P1/T, P1/TI, P2/TI, and P4/TI).

Our results thus revealed that the COOH-terminal region of HBx is crucial to the transcriptional function and that in tumor tissues, most HBx natural mutants had lost their transcriptional activity.

Characterization of Growth-suppressive Effect of HBx Natural Mutants. Because HBx can induce cell apoptosis (5, 24–33), we next investigated the proapoptotic ability of different HBx sequences, using a colony formation assay. Chang cells were transfected with the different HBx expression plasmids, and 2 weeks after G418 selection, the number of colonies was scored (Fig. 3A). As expected, the expression of HBx ayw caused a substantial reduction (5-fold) in the number of colonies compared with the control cells transfected with the empty vector pcDNA3.1. A degree of colony formation inhibition similar to that of HBx ayw was observed in HBx sequences from sera and nontumor tissues of patients 1, 2, and 3. P4/S/NT/TI failed to suppress colony formation. Strikingly, in tumor tissue, all four HBx sequences from integrated HBV, and two of three of the HBx sequences from free HBV exhibited abrogation of the growth-suppressive effect. The detection of HBx in the pool of drug-resistant clones at the end of the experiments indicated that the loss of suppressive effect on colony formation was a result of the absence or lower expression of HBx in the cells (Fig. 3C). It should be noted that the inhibitory effect of HBx on colony formation was associated with its transactivation activity in most, but not all cases. For example, P2/TI, which retained transactivation activity despite a 3-aa deletion at the COOH-terminal end, lost its growth-suppressive effect on cells.

The above results were confirmed in Huh7 and HepG2 cells (Fig. 3B). It may therefore be concluded that the abrogation of HBx proapoptotic and antiproliferative functions is a common feature of HBx isolated from tumor tissues. In addition, the growth-suppressive effect is often, but not always, associated with transactivation activity.

Effect of HBx Natural Mutants on Focus Formation Induced by Cooperating Oncogenes. Using linker scanning mutants, a previous study had demonstrated in REF cells that the suppressive effect of HBx on focus formation induced by two oncogenes (ras and myc) is dependent on its transactivation activity (17). Thus, to test the effect of HBx natural mutants identified in this study on cell transformation, REF cells were transfected with HBx ayw or COOH-terminally truncated HBx (P3/TI), either alone or complemented with ras and myc oncogenes. After the transfection, REF cells were split and cultured in G418 selective medium or normal medium. As shown in Fig. 4B, when a single gene (HBx ayw, P3/TI, or ras) was introduced into REF cells, none of the genes could transform the cells after G418 selection. Although cooperating ras and myc oncogenes converted REFs into a morphologically transformed phenotype (Fig. 4A), neither HBx ayw nor truncated HBx could cooperate with ras to exert this effect. In line with the findings of a previous report (17), HBx ayw had in fact a strong suppressive effect on focus formation induced by the cooperation of ras and myc. However, truncated HBx (P3/TI) did not have any effect on focus formation by ras and myc (Fig. 4B). Similar results were obtained from the culture without G418 selection. The tumorigenicity of the transformed colony was tested in soft agar. Among the 14 clonal cell lines derived from foci, all were able to grow in soft agar (Fig. 4A). Under our experimental conditions, HBx ayw only slightly inhibited colony formation in REFs. We previously observed an apparent growth-suppressive effect of HBx ayw in REF cells (8). This discrepancy is probably related to the different batch of REFs used in the experiments.

To investigate the role of other HBx mutants on focus formation, we performed triple transfections with ras, myc, and HBx constructs in REF cells. With the exception of P2/TI, there was a correlation between transactivation activity and the suppression of
focus formation (compare Table 1 and Fig. 2A). Interestingly, except for P4/TI, the loss of suppressive effect on focus formation by HBx was associated with a modest but reproducible increase in the number of foci induced by ras and myc. Statistic analysis confirmed this enhancement of focus formation ($P < 0.05$; Table 1).

**Dissection of Multifunction Domains in the Distal COOH-Terminal Region of HBx.** The impact of the natural COOH-terminal deletion of HBx on cell proliferation, viability, and transformation is summarized in Table 2. To gain a clear understanding of the amino acid sequence essential to the transactivation, proapoptotic, and transformation functions of HBx, we constructed a series of COOH-terminal deletion mutants based on the HBx ayw sequence. The level of expression of artificial mutants in α-ML, Huh7, and REF transfected cells is shown in Fig. 5A. Whereas deletion of 1-4 aa was enough to abrogate the suppressive effects of HBx on cell growth (Fig. 5D) and cell transformation (Fig. 5, C and E), an additional 2-aa deletion was required to abolish its transactivation function (Fig. 5C). The transcriptional activity of HBx artificial mutants has also been tested by a nuclear factor-κB-luciferase reporter construct, and the results confirmed our findings (data not shown). In HBxΔ16 and HBxΔ18, the loss of HBx suppression of focus formation was associated, consistent with the above observation, with an increase in the ras and myc transforming capacity. Our results thus confirm the observation in HBx natural mutants of the importance of the COOH-terminal region to HBx function. They also indicate that the transactivation function of HBx is closely associated, but does not completely overlap with, the proapoptotic function.

**DISCUSSION**

To investigate the biological implications of HBx genetic variability on liver carcinogenesis, we studied patients with HBV-related HCC. We analyzed the effects of natural HBx mutants selected from the sera and from nontumor and tumor tissues of HCC patients on cell proliferation, viability, and transformation. We demonstrated that the HBx COOH-terminal part plays a key role in regulating its transcriptional activity and controlling cell viability and proliferation. In addition, we showed that HBx mutants with a COOH-terminal deletion enhanced transforming ability of ras and myc.

To gain an overview of HBx genetic variability, we characterized the HBx sequence present not only in serum but also in liver tissue. More importantly, an HBx-Alu PCR-based method allowed us to amplify the HBx sequence from the integrated HBV DNA in tumor tissue. Our sequence data demonstrated that, in a given patient, HBx sequences could be divergent in the liver and the serum. However, variations occurred more frequently in tumor tissues, where the HBx COOH-terminal deletion was found in all HBx derived from integrated HBV DNA, and in two of three of HBx sequences derived from free HBV DNA. In the case of HBx derived from sera and nontumor tissues, HBx deletion was not observed, except in patient 4. Hence, in our cases, deletions in the distal COOH-terminal region may be characterized as a common feature of HBx in tumor tissue, particular for the integrated HBx sequence. This conclusion is reinforced by a previous report from our laboratory that demonstrated that a 3′-end deletion in the HBx gene was found in five of nine tumor tissues, but in none of the eight nontumor tissues in a study of nine HCC patients (40). In fact, a body of evidence has shown that most, if not all, HBx

**Fig. 4.** Focus formation assay in REFs. Morphology of REF cells transfected with empty vector pcdNA3.1 (A, left panel) or triple transfected with ras, myc, and HBx mutant P3/TI (A, middle panel) and cultured in G418 selective medium for 10 days (original magnification, ×100). A (right panel), growth of transformed REF cells in soft agar 2 weeks after plating (original magnification, ×100). B, focus formation ability of transfected REFs. Second-passage REFs were transfected with the plasmids indicated. Two days after transfection, the cells were split and subcultured in normal medium for 4 weeks or in G418 selective medium for 10 days. Results show the mean of focus numbers in duplicated plates. TI, integrated HBx in tumor; ND, not determined.

Table 1 Effects of HBx natural mutants on focus formation induced by ras and myc in REFs

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Colonies with morphologically transformed cells</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ras + myc + pcdNA3.1</td>
<td>22</td>
<td>25</td>
<td>21</td>
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</tr>
<tr>
<td>ras + myc + P1/S/NT</td>
<td>2</td>
<td>1</td>
<td>ND</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P1/T</td>
<td>32</td>
<td>38</td>
<td>32</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P1/TI</td>
<td>38</td>
<td>34</td>
<td>32</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P2/S</td>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P2/TI</td>
<td>2</td>
<td>2</td>
<td>ND</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P2/NT</td>
<td>34</td>
<td>38</td>
<td>33</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P2/TI</td>
<td>1</td>
<td>2</td>
<td>ND</td>
<td>0.05</td>
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</tr>
<tr>
<td>ras + myc + P3/S</td>
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<td>2</td>
<td>ND</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P3/NT</td>
<td>0</td>
<td>3</td>
<td>ND</td>
<td>0.05</td>
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<tr>
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<td>38</td>
<td>30</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P4/S/NT</td>
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<td>40</td>
<td>35</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>ras + myc + P4/T</td>
<td>40</td>
<td>30</td>
<td>24</td>
<td>0.21</td>
<td></td>
</tr>
</tbody>
</table>

* Mean of the foci number in one 10-cm plate after G418 selection for 10 days.
* Exp, experiment; S, serum; T, tumor; ND, not determined; TI, integrated HBx in tumor.
* Statistical analysis was performed using the paired $t$ test between the results of ras + myc + pcdNA3.1 and the indicated plasmids.

Table 2 Summary of the biological impact of HBx natural mutants

<table>
<thead>
<tr>
<th>Serum</th>
<th>Nontumor</th>
<th>Tumor</th>
<th>Integrated HBx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P1</td>
<td>P2</td>
<td>P3</td>
</tr>
<tr>
<td>COOH-terminal deletion (aa)</td>
<td>0</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Transactivation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth inhibition</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Transforming inhibition</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* ND, not determined.
isolated from integrated HBV DNA in patients with HCC was in a truncated form (36, 39, 41–43). HBx truncation may also be found in chronic hepatitis B patients, but at a lower incidence than that seen in HCC (54), suggesting that HBx truncation might play a role at an early stage of liver carcinogenesis and be selected during the development of HCC.

HBx can regulate numerous signal transduction pathways. However, there is no indisputable evidence showing that the transactivation activity of HBx contributes to liver oncogenesis. It has previously been reported that an integrated form of HBx isolated from HCC tissues or from hepatoma cell lines conserved its transactivation activity (36, 39, 41–43). Our present results challenge this issue because during our investigations three of four integrated HBx abrogated the transactivation function (Fig. 2A). This apparent discrepancy could be interpreted by the size of the deletion at the COOH terminus of HBx. Indeed, we have noticed that in most publications in the literature, transcriptionally active HBx from HBV integrants exhibited the deletion within 14 aa. According to the functional domain characterized by the present study (Fig. 5C) and others (55, 56), the last 14 aa at the COOH terminus of HBx are not essential to transactivation activity. It is reasonable to suppose that with a small fragment truncation, HBx might retain its transactivation activity, as was shown in our P2/T1 sample. Deletions at the COOH terminus of HBx >16 aa in length had previously been reported in some integrated forms of HBV DNA, although the functional data regarding these mutants were missing (57, 58). In addition, in a survey carried out by our group, 33% (7 of 21) of integrated HBx sequences derived from HCC tissues showed a deletion longer than 16 aa at their COOH-terminal ends.4 Along the same lines, we note that in a report of 12 integrated HBV DNA clones from HCC, only 6 contained a functional HBx transactivator (41). Therefore, the transactivation activity of HBx appears to be abrogated frequently during HCC development.

The apoptotic effect of HBx is a subject of considerable controversy. However, increasing evidence suggests that HBx may exert a proapoptotic effect on hepatocytes and in the liver of HBx transgenic mice (8–18). During our previous study, we provided evidence that HBx inhibits cell growth by inducing apoptosis in a p53-independent manner and that late G1 cell cycle arrest induced by HBx may be related to cell demise (8). Interestingly, the transcriptional activation and apoptotic ability of HBx were shown to be tightly linked (9, 17). From our present data, based on natural and artificial mutants, it can be noted that the transactivation function of HBx is generally, but not always, linked to the proapoptotic function. Indeed, in two samples, P2/T1 and HBxΔ14, transactivation and proapoptotic function were two separable functions of HBx. In both cases, HBx retained the transactivation function but lost its proapoptotic function. This indicates that the proapoptotic function is more sensitive to amino acid changes in the COOH terminus of HBx. Strikingly, all four integrated HBx sequences had abrogated the growth-suppressive effect (Fig. 3). This suggests that abrogation of the proapoptotic effect may endow a growth advantage on cells containing these particular HBx mutants.


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**Fig. 5.** Impact of COOH-terminally truncated HBx on transactivation activity, cell proliferation, and transformation. A, expression of HBx and artificial mutants in α-ML, Huh7, and REF cells 48 h after transfection. B, focus formation of REFs cotransfected with ras and myc plus the indicated HBx artificial mutant or empty vector pcDNA3.1. Transactivation activity (C), growth-suppressive effect (D), and oncogenic potential (E) of HBx artificial mutants were determined as described in “Materials and Methods.” The results are the mean of at least two independent transfections.
Contradictory data have been reported concerning the ability of HBx to transform cells in vitro. Whereas some studies have shown that HBX has a weak oncogenic potential in several immortalized cell lines (18, 23–28), others have observed contrasting results in which HBX inhibits focus formation induced by oncogenes (11, 17). The inhibitory effect of HBx on cell transformation induced by the cooperation of ras and myc oncogenes was observed during this study. Furthermore, we provide evidence that COOH-terminally truncated HBX enhances the focus formation induced by the cooperation of ras and myc, thus suggesting that HBX COOH-terminal truncation not only induces an abrogation of HBX-related proapoptosis but may also participate in promoting cell transformation. A main implication of this observation would be that mutations in the HBX sequence would act not only by abrogating HBX biological activities but also by triggering some other biological effects of HBX on cellular pathways. With the exception of P2/TI and HBXΔ14, our results agreed with recent data supporting a correlation between the ability of HBX to suppress focus formation in REF cells and its transcriptional activity (32). Thus, the transactivation activity of HBX seems to play a negative regulatory role on cell transformation. Indeed, in an in vitro study performed in REV-2 cells, the transcriptional activation domains in HBX were neither required nor sufficient for cell transformation, and the transformation domain of HBX was mapped to the first 50 aa at the NH2 terminus (28). Therefore, a deletion at the COOH-terminal end may alter the balance of HBX functional domains in regulating cell proliferation, viability, and transformation. Thus, COOH-terminally truncated and full-length HBX may play different roles in HBX-related liver pathogenesis. In this view, it should also be noted that we do not exclude the coexistence within the same tumor cells of full-length and COOH-terminally truncated HBX sequences encoded by free or integrated HBV genome. The biological implication of the coexistence of full-length and COOH-terminally truncated HBX sequences needs further investigation.

It should be mentioned that despite the COOH-terminal region deletion, certain point mutations may also be involved in modifying the functions of HBX. Recently, a linked-point mutation (Lys130Met and Val131Ile) was reported to be prevalent in HCC patients (45). Although this linked-point mutation alone (P1/S/NT) did not show functional alterations in our study, an additional amino acid substitution introduced at aa 129 of HBX (Leu3–Val131Ile) was reported to be prevalent in HCC patients (45). It should be mentioned that despite the COOH-terminal region deletion, certain point mutations may also be involved in modifying the functions of HBX. Recently, a linked-point mutation (Lys130Met and Val131Ile) was reported to be prevalent in HCC patients (45). Although this linked-point mutation alone (P1/S/NT) did not show functional alterations in our study, an additional amino acid substitution introduced at aa 129 of HBX (Leu3–Val131Ile) was reported to be prevalent in HCC patients (45). It should be mentioned that P2/TI has a 130Lys131Val sequence. The true impact of point mutations on the regulation of the biological function of HBX needs to be further evaluated.

REFERENCES


Biological Impact of Natural COOH-Terminal Deletions of Hepatitis B Virus X Protein in Hepatocellular Carcinoma Tissues

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