Hepatitis B virus is a causative agent of hepatocellular carcinoma, and in the course of tumorigenesis, the X-gene product (HBx) is known to play important roles. Here, we investigated the transforming potential of HBx by conventional focus formation assay in NIH3T3 cells. Cells were cotransfected with the HBx expression plasmid along with other oncogenes including Ha-ras, v-src, v-myc, v-fos, and E1a. Unexpectedly, the introduction of HBx completely abrogated the focus-forming ability of all five tested oncogenes. In addition, the cotransfection of Bcl-2, an apoptosis inhibitor, reversed the HBx-mediated inhibition of focus formation, suggesting that the observed repression of focus formation by HBx is through the induction of apoptosis. Next, to test unequivocally whether HBx induces apoptosis in liver cells, we established stable Chang liver cell lines expressing HBx under the control of a tetracycline-inducible promoter. Induction of HBx in these cells in the presence of 1% calf serum resulted in typical apoptosis phenomena such as DNA fragmentation, nuclear condensation, and fragmentation. Based on these results, we propose that HBx sensitizes liver cells to apoptosis upon hepatitis B virus infection, contributing to the development of hepatitis and the subsequent generation of hepatocellular carcinoma.

Hepatitis B virus is a small DNA virus with a 3.2-kilobase partially double-stranded DNA genome and is a causative agent of acute and chronic hepatitis (1). Through epidemiological studies, chronic hepatitis B virus infection has been linked to the high incidence of hepatocellular carcinoma (HCC) generation (2). Among the four proteins translated from the hepatitis B virus genome, the X-gene product (HBx) has drawn much attention for its role as a transacting factor for exploitation of the host cell machinery. Up to now, several significant discoveries have been made regarding the functions of HBx, and from these studies, HBx has been established as essential in viral replication, HCC, and the activation of certain signal transduction pathways (3, 4).

Although there is a controversy about the role of HBx in viral replication, the X-gene product was shown to be required for the replication of woodchuck hepatitis virus in animal studies, which are considered to reflect the in vivo phenomenon more precisely (5, 6). A similar conclusion was drawn by us in a transfection-based replication assay (7).

Generally, it is believed that HBx contributes to the generation of hepatocellular carcinoma. HBx was reported to induce transformation of NIH3T3 cells (8). Furthermore, the development of HCC was observed in HBx transgenic liver (9). A possible mechanism of HBx-mediated HCC is the disruption of p53 function as a tumor suppressor. There are several reports describing the direct association between HBx and p53 (7, 10–12). Such interaction presumably suppresses p53 function in G1 cell cycle arrest. The other possible mechanism is based on the ability of HBx to activate cellular signal transduction pathways. HBx stimulates the ras/raf/mitogen-activated protein kinase cascade, leading to the activation of AP-1-dependent transcriptional activation (13, 14). In addition, c-Jun N-terminal kinase (15) and NF-kB (16, 17) were shown to be activated by HBx. Probably, through the combined actions of the mechanisms listed above, HBx contributes to tumorigenesis. In fact, HBx has been shown to deregulate the cell cycle check point controls through the activation of ras (18).

It seems that most animal viruses have evolved strategies to block and/or induce apoptosis depending on the cellular environment (19). The representative examples of the viral transactivators inducing apoptosis include E1a of adenovirus (20, 21), Tax of human T-cell leukemia virus (22), and Tat of human immunodeficiency virus (23). On the other hand, other viral transactivators such as E1b of adenovirus (21, 24) and IE1 and IE2 of cytomegalovirus (25) were shown to have the ability to block apoptosis. As for hepatitis B virus, HBx was shown to block the apoptosis induced by the overexpression of p53 (26). Notably, as exemplified by E1a/E1b of adenovirus (20, 21, 25) or T-antigen of SV40 (27, 28), viral gene products from the same virus may either block or induce apoptosis.

During the study of the HBx-mediated transformation process, we found that HBx sensitizes/induces apoptosis in liver cells. This observation may provide a pathogenic mechanism of hepatitis B virus-related hepatitis and subsequent hepatocellular carcinoma generation.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmid of HBx and internal deletion mutants pcDNA-X, Xdel-1, and Xdel-2 were described previously (9). The plasmids employed for transformation assay, pMT13S (29), pEJ (30), pMC29 (31), pFB/JR (32), and pcDNA-src, encode the E1a 13 S gene product, Ha-ras, v- myc, v-fos, and v-src, respectively, and have been described. The human Bcl-2 expression plasmid has been described (33). The parental plasmids for the construction of the tetracycline-inducible cassette, pHUD10-3 and pHUD172-1, were developed by Gossen et al. (34). pTet-X was obtained by polymerase chain reaction using TAAATGACTCACTATAGGG as a 5′-primer and GCTCAAGAT- TCTTACTGCTAGTATCGAAACTATGATGATTGATCCGCAGAGGTTGAAAAAGTTG as a 3′-primer with pcDNA-X as a template. The underlined sequences denote the EcoRI site introduced for the facilitation of subcloning and the sequence encoding the hemagglutinin tag, respectively. After HindIII/EcoRI digestion, the polymerase chain reaction product obtained was inserted into pcDNA1 (Invitrogen), yielding the plasmid pcDNA-HBx-HA. Subsequently, a KpnI/XbaI fragment from pcDNA-HBx-HA was subcloned into pUC18, yielding the plasmid

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resulting pellets were resuspended in 50 mM NaCl and 0.5 volume of isopropyl alcohol. Following storage at
was precipitated from the supernatants by the addition of 0.1 volume of
pH 7.5, 1 mM EDTA, and 0.2% Triton X-100), vortexed, and incubated
for 30 min on ice, and permeated by 100% methanol treatment at room temperature. Subsequently, the slides were stained with 2 µg/ml Hoechst 33258 in PBS at 37 °C for 1 h, washed with PBS, and visualized under a fluorescent UV microscope.

Analysis of the p53 Status by Doxorubicin Treatment—Chang liver or HepG2 cells were maintained in DMEM supplemented with 10% calf serum or 10% fetal bovine serum, respectively. For induction of p53 and p21, the cells were treated with the chemotherapeutic agent doxorubicin at a concentration of 0.4 or 1.0 µg/ml. After the indicated times, the cells were harvested and subjected to Western analysis.

RESULTS

HBx Abrogates ras-induced Focus Formation—To investigate the transforming potential of HBx, the HBx expression plasmid pcDNA-X was transfected into NIH3T3 cells alone or together with the Ha-ras expression plasmid pEJ (Table I, part A). No foci were observed upon expression of HBx alone, whereas the expected focus formation was detected upon expression of Ha-ras. Unexpectedly, the coexpression of HBx and Ha-ras resulted in the abrogation of Ha-ras-induced focus formation, suggesting that HBx somehow interfered with the transforming potential of Ha-ras. The observed effect was specific for wild-type HBx, as two deletion mutants of HBx (Xdel-1 and Xdel-2) were not able to disrupt focus formation (Table I, part B). As a control, we cotransfected the adenvirus E1a 13 S expression plasmid (pMT13S) along with pEJ. E1a and Ha-ras acted cooperatively as expected, and an increased number of foci were observed, confirming that the abrogation of the Ha-ras-induced foci by HBx is a specific nature of HBx.

Focus Formation by All Five Tested Oncogenes Is Disrupted by HBs—Next, we examined whether the disruption of focus formation by HBx was restricted to the Ha-ras case or could be extended to other oncogenes. We cotransfected pcDNA-X along with five different expression plasmids, pMC29, pFB/J/R, pcDNAv-src, pMT13S, and pEJ, each encoding v-myc, v-fos, v-src, E1a, and Ha-ras, respectively (Fig. 1). Each of these oncogenes induced a fair number of foci, and the coexpression of

| Transfected genes | 9 days | 12 days | 16 days | 19 days |
|-------------------|--------|---------|---------|---------|
| A. neo            | 0      | 0       | 1       | 4       |
| hbx + neo         | 0      | 0       | 1       | 3       |
| Ha-ras + neo      | 4      | 4       | 20      | 30      |
| hbx + Ha-ras + neo| 0      | 0       | 3       | 7       |
| Ela + Ha-ras + neo| 9      | 30      | 47      | 51      |

Transfected genes

| Transfected genes | 11 days | 14 days |
|-------------------|---------|---------|
| B. neo            | 0       | 0       |
| Ha-ras + neo      | 19      | 25      |
| hbx + Ha-ras + neo| 1      | 3       |
| Xdel1 + Ha-ras + neo| 7     | 13      |
| Xdel2 + Ha-ras + neo| 19     | 21      |

* Days after selection with G418.

pUC18-HBX-HA, followed by the transfer of an EcoRI fragment into the pUHD10-3 plasmid.

Focus Formation Assay—NIH3T3 cells were plated in DMEM containing 10% calf serum at a density of 3 × 10^4 cells/35-mm plate. Sixteen h later, the cells were transfected using the lipofection technique with 1 µg of each of the indicated expression plasmids along with 0.4 µg of pSV2neo. A total amount of transfected DNA was kept constant using the pUC19 plasmid DNA as a carrier. At 48 h post-transfection, the cells were split at a 2:1 ratio and cultured in the presence of 400 µg/ml G418. After the indicated days in selective medium, the cells were fixed in a PBS solution containing 0.2% glutaraldehyde and 0.5% formaldehyde for 10 min on ice and stained with 0.2% crystal violet for 10 min at room temperature, and the number of foci was counted. The experiments were performed in duplicate and at least twice.

Establishment of HBx-expressing Stable Cell Lines—Chang liver cells were obtained from American Type Culture Collection (passage 259), and all experiments were performed using cells of passage 15 passages from the original stock. Chang liver cells were cultured in DMEM containing 10% calf serum and cotransfected with pTet-X and pUC18-HBx-HA, followed by the transfer of an EcoRI fragment into the pUHD10-3 plasmid.

Antibodies and Western Analysis—For the detection of HBx protein, polyclonal rabbit antiserum was raised against a synthetic peptide (35). The sequence of the peptide was SPAPCNFFTSA, and the peptide was conjugated to keyhole limpet hemocyanin for immunization. The polyclonal anti-p53 antiserum was raised against glutathione S-transferase-p53 protein purified from Escherichia coli and has been described (7). For p21hottranscriptase-p53 protein purified from Escherichia coli was used in the present study. The specificity for wild-type HBx, as two deletion mutants of HBx (Xdel-1 and Xdel-2) were not able to disrupt focus formation (Table I, part B). As a control, we cotransfected the adenvirus E1a 13 S expression plasmid (pMT13S) along with pEJ. E1a and Ha-ras acted cooperatively as expected, and an increased number of foci were observed, confirming that the abrogation of the Ha-ras-induced foci by HBx is a specific nature of HBx.

Focus Formation by All Five Tested Oncogenes Is Disrupted by HBs—Next, we examined whether the disruption of focus formation by HBx was restricted to the Ha-ras case or could be extended to other oncogenes. We cotransfected pcDNA-X along with five different expression plasmids, pMC29, pFB/J/R, pcDNAv-src, pMT13S, and pEJ, each encoding v-myc, v-fos, v-src, E1a, and Ha-ras, respectively (Fig. 1). Each of these oncogenes induced a fair number of foci, and the coexpression of

TABLE I

Focus formation assay after transfection of NIH3T3 cells with hbx and five other oncogenes including Ha-ras, v-fos, v-src, v-myc, and E1a. The experimental methods are described in the legend to Table II.

Focus assay after cotransfection of NIH3T3 cells with hbx and five other oncogenes including Ha-ras, v-fos, v-src, v-myc, and E1a. The experimental methods are described in the legend to Table II.
HBx resulted in the abrogation of focus formation by all five tested oncogenes (Fig. 1 and Table II). Therefore, the ability of HBx to mediate disruption of focus formation seems to be a general property of HBxs regardless of the type of partner oncogenes tested.

**Bcl-2, an Apoptosis Inhibitor, Rescues the HBx-mediated Inhibition of Focus Formation**—Inasmuch as many viral transactivators were reported to induce apoptosis depending on the environment (20, 22, 23), we tested whether the observed inhibition of focus formation by HBx was mediated through the induction of apoptosis. For this purpose, we employed the Bcl-2 protein with an established anti-apoptotic property (22, 24). When Bcl-2 was coexpressed with HBx and Ha-ras, the suppression of focus formation was observed upon HBx expression alone. Therefore, more than half of the foci were rescued upon coexpression of Bcl-2. These results suggest that the HBx-induced abrogation of focus formation is probably mediated through the induction of apoptosis.

**Establishment of Cell Lines Expressing HBx in a Tetracycline-inducible Manner**—Next, to confirm the possibility that HBx possesses an ability to induce apoptosis, we established cell lines expressing HBx in a tetracycline-inducible manner. For this purpose, we constructed an HBx expression plasmid named pTet-X employing the system developed by Gossen et al. (34) (Fig. 2A). In pTet-X, the expression of HBx was under the control of the tetracycline-inducible promoter, tetO, tetracycline operator; TATA, cytomegalovirus immediate early minimal promoter; HA, hemagglutinin; SV40-polyA, poly(A)+ additional signal of SV40 DNA virus. Western analysis of HBx expression in stable Chang liver cell lines. Two HBx-expressing stable cell lines (CLX1 and CLX2) are compared with the parental Chang liver cells (CL). The cell lysates were prepared and subjected to SDS-polyacrylamide gel electrophoresis and subsequent Western analysis using polyclonal antiserum raised against a synthetic peptide corresponding to residues 144–154 of HBx. As a control (C) for Western analysis, E. coli cell-derived purified HBx (7) was employed. Dox, doxycycline. The numbers represent the concentration of doxycycline in μg/ml.

**Expression of HBx Induces Typical Apoptosis Phenomena**—Such as DNA Fragmentation and Nuclear Condensation—To test whether HBx really induces apoptosis, DNA fragmentation and nuclear condensation were assayed upon HBx expression. To assay for DNA fragmentation, the parental Chang liver cells and two HBx-expressing stable clones were employed. In DMEM supplemented with 1% calf serum, 0, 2, or 5 μg/ml doxycycline was added to induce HBx expression. Seventy-two h later, DNA was extracted from the harvested cells and run on an agarose gel (Fig. 3). No sign of DNA fragmentation was observed with control Chang liver cells, whereas typical DNA ladders were detected with CLX1 and CLX2 when 2 and 5 μg/ml doxycycline were added to the medium. The amount of fragmented DNA increased in a doxycycline dose-dependent manner.

**TABLE II**

| Transfected gene selection | Number of foci |
|---------------------------|---------------|
|                           | 11 days*      | 13 days |
| neo                       | 0             | 0       |
| v-fos + neo               | 16            | 32      |
| hbx + v-fos + neo         | 0             | 4       |
| v-src + neo               | 7             | 12      |
| hbx + v-src + neo         | 0             | 2       |
| Ha-ras + neo              | 26            | 36      |
| hbx + Ha-ras + neo        | 0             | 1       |
| E1a + neo                 | 9             | 18      |
| hbx + E1a + neo           | 1             | 4       |
| v-src + v-myc + neo       | 4             | 8       |
| hbx + v-myc + neo         | 0             | 2       |
| E1a + Ha-ras + neo        | 36            | 63      |

* Days after selection with G418.

**TABLE III**

| Transfected genes                        | Number of foci |
|------------------------------------------|----------------|
|                                          | 11 days*       | 13 days |
| neo                                      | 0             | 0       |
| Ha-ras + neo                            | 18            | 22      |
| bel-2 + neo                             | 1             | 1       |
| hbx + Ha-ras + neo                      | 2             | 3       |
| hbx + Ha-ras + bel-2 + neo              | 5             | 12      |
| E1a + Ha-ras + neo                      | 21            | 32      |

* Days after selection with G418.

**FIG. 2. Establishment of Chang liver cell lines stably expressing HBx in a tetracycline-inducible manner.** A, schematic representation of the HBx expression vector pTet-X. In this construct, the expression of HBx is under the control of the tetracycline-inducible promoter. tetO, tetracycline operator; TATA, cytomegalovirus immediate early minimal promoter; HA, hemagglutinin; SV40-polyA, poly(A)+ additional signal of SV40 DNA virus. B, Western analysis of HBx expression in stable Chang liver cell lines. Two HBx-expressing stable cell lines (CLX1 and CLX2) are compared with the parental Chang liver cells (CL). The cell lysates were prepared and subjected to SDS-polyacrylamide gel electrophoresis and subsequent Western analysis using polyclonal antiserum raised against a synthetic peptide corresponding to residues 144–154 of HBx. As a control (C) for Western analysis, E. coli cell-derived purified HBx (7) was employed. Dox, doxycycline. The numbers represent the concentration of doxycycline in μg/ml.
manner. Next, the effects of HBx on nuclear condensation were assayed. Forty-eight h after doxycycline treatment, the cells were fixed and stained with Hoechst 33258 (Fig. 4). Again, no signs of nuclear condensation were observed with control Chang liver cells at any concentration of doxycycline, whereas with CLX1, nuclear condensation was evident at 2 μg/ml doxycycline. The same result was observed with CLX2 (data not shown). From these results, we conclude that HBx possesses the ability to induce apoptosis.

Analysis of the p53 Status in Chang Liver Cells—We studied the p53 status in Chang liver cells insomuch as viral transactivators may induce apoptosis in either a p53-dependent or p53-independent manner. Upon doxorubicin treatment, wild-type p53 is known to accumulate and participate in the cellular response to DNA damage (36). As a control, the HepG2 cell line was employed since p53 in this cell line was reported as a wild type (36). As shown in Fig. 5A, the basal p53 level was low, and upon treatment with doxorubicin, p53 began to accumulate and reached the maximal level after 24 h in both cell lines. Accompanying the accumulation of p53, the induction of p21waf1/cip1 expression in Chang liver and HepG2 cells when exposed to doxorubicin (0.4 μg/ml). After the indicated hours, the cells were harvested for Western blot analysis using a monoclonal anti-p21 antibody (Upstate Biotechnology, Inc.). The numbers represent hours after treatment with doxorubicin.

DISCUSSION

In this report, we have shown that HBx possesses an ability to induce apoptosis using two different criteria. First, HBx disrupted focus formation by other oncogenes in NIH3T3 cells, which is blocked by the anti-apoptotic protein Bcl-2. Second, the induction of HBx in Chang liver cells in the presence of 1% serum resulted in typical apoptosis phenomena such as DNA fragmentation and nuclear condensation.

The experimental results reported here differ from the previous ones in two aspects. The first point of discrepancy is observed in the ability of HBx to transform NIH3T3 cells. Previously, Shirakata et al. (8) reported that the expression of HBx led to the stimulation of growth of drug-resistant colonies and tumor formation in nude mice. In our case, even though the assay was different, HBx alone did not induce any focus formation, but rather abrogated focus formation by other oncogenes. The reason for the difference is not clear at this stage. Perhaps, the expression of HBx alone is sufficient for the stimulation of cell growth, but is not a strong enough stimulus for the formation of foci, and the cell cycle perturbation caused by
the coexpression of other oncoproteins revealed the otherwise latent apoptosis-inducing ability of HBx. At any rate, disruption of focus formation is a unique property of HBx in that the coexpression of E1a with Ha-ras resulted in cooperative focus formation in the same experiment. With regard to the second point of discrepancy, in contrast to our finding, Wang et al. (26) showed that the microinjection of an HBx expression plasmid blocks the apoptosis induced by the overexpression of p53 in primary fibroblasts. Possibly, the discrepancy originated from a different cellular environment in which the apoptosis was assayed since the induction of apoptosis is determined by the combined effects of external stimulus and the physiology of the cell. Recent reports suggest that the normal physiological level of p53 displays an anti-apoptotic activity, whereas a high dose of p53 induces apoptosis (37, 38). Considering that HBx binds to p53 and blocks the p53 function, HBx may have induced apoptosis by antagonizing the anti-apoptotic function of the normal level of p53 in Chang liver cells as observed in our case, but, on the other hand, HBx may have an ability to block apoptosis when the apoptosis-inducing stimulus is the overexpression of p53, as reported previously (26).

There are several other known functions of HBx that may also serve as the mechanism of apoptosis. The HBx-mediated activation of the mitogen-activated protein kinase/Jun N-terminal kinase pathway (15) may be responsible since the abnormal regulation of this pathway was shown to lead to apoptosis (39). Alternatively, HBx may work through the induction of transforming growth factor β insomuch as HBx induces transforming growth factor β expression, which is a well-established apoptosis stimulus for liver cells (40), and HBx is colocalized with transforming growth factor β in transgenic liver (41). In addition, HBx was shown to bind damaged DNA and to sensitize liver cells to cell death by ultraviolet irradiation (42). Further study is required to clarify which of the mechanisms listed above is responsible for HBx-mediated apoptosis.

At this stage, the precise role of HBx-mediated apoptosis during natural infection is not clear, but there are reported correlations of apoptosis with hepatitis or HCC. For example, apoptosis in the liver has been observed during hepatitis B viral infection (43, 44). Apoptotic bodies and "piecemeal necrosis" are frequently detected in viral hepatitis and are likely to occur from apoptosis of individual infected cells rather than focal lysis of hepatocytes, as they are often observed in the absence of adjacent inflammation and features of necrosis. Furthermore, in hepatocarcinogenesis, preneoplastic and neoplastic cell populations show an increased rate of apoptosis as well as enhanced cell proliferation (45). The possible involvement of HBx in these pathological phenomena needs further investigation.

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