Induction of apoptosis after switch-on of the hepatitis B virus X gene mediated by the Cre/loxP recombination system

Yoshizumi Shintani,1 Hiroshi Yotsuyanagi,1† Kyoji Moriya,1 Hajime Fujie,1 Takeya Tsutsumi,1 Yumi Kanegae,2 Satoshi Kimura,1 Izumu Saito2 and Kazuhiko Koike1

1 First Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
2 Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, Tokyo 108-0072, Japan

The HBx protein of hepatitis B virus is a multifunctional protein that is implicated in the pathogenesis of hepatocellular carcinoma by regulating gene transcription, causing cell proliferation and, as shown recently, inducing cell death. However, analysis of the effects of HBx in stable cultured cell clones has been hampered because only cell lines that adapted to the effects of HBx were selected during the establishment of cell clones. Here, we describe a system in which transcription of the X gene of hepatitis B virus is switched on by the use of the site-specific Cre recombinase. Two human liver cell lines, HLF and HepG2, were used, the former with a mutant p53 allele and the latter with wild-type p53. The stable cell clones isolated, which carried the X gene in a transcriptionally silent state, were infected with recombinant adenovirus carrying Cre recombinase. Ninety-six hours after adenovirus infection, cell clones that expressed HBx had undergone TUNEL-positive cell death with characteristics of apoptosis. Apoptosis was induced despite concomitant inactivation of the p53 protein as a result of its cytoplasmic translocation by HBx. In contrast, neither the X gene-carrying cells infected with wild-type adenovirus nor various control cells infected with Cre-expressing adenovirus exhibited apoptosis. These results indicate that the expression of HBx protein leads to liver cell apoptosis independently of the p53 pathway. The significance of HBx-induced apoptosis in natural infection is unclear, but it may contribute to the development of hepatitis and serve to spread progeny virus to neighbouring cells while evading the host immune responses.

Introduction

The X gene is one of four genes encoded by the hepatitis B virus (HBV) genome. It encodes a protein, HBx, that is composed of 154 amino acids, the sequence of which is well-conserved in other hepadnaviruses such as woodchuck hepatitis virus and ground squirrel hepatitis virus. HBx is a multifunctional protein with a number of activities that may be related to the pathogenesis of hepatitis B virus and hepatocarcinogenesis (Kim et al., 1991; Zoulim et al., 1994; Koike, 1995; Caselmann, 1995; Paterlini et al., 1995; Su et al., 1998). These functions include transactivation of other genes (Twu et al., 1993; Avantaggiati et al., 1992; Balsano et al., 1991), cell cycle progression (Koike et al., 1994b; Benn & Schneider, 1995), transformation of cultured cells (Shirakata et al., 1989; Seifer et al., 1991) and induction of liver tumours in transgenic mice (Kim et al., 1991; Koike et al., 1994a; Yu et al., 1999). These activities may be mediated by activation of the Ras–Raf mitogen-activated protein kinase (Benn & Schneider, 1994; Cross et al., 1993; Natoli et al., 1994) and protein kinase C (Kekule et al., 1993) signal transduction pathways, stimulation of transcription by RNA polymerases (Aufero & Schneider, 1990; Kwee et al., 1992) and binding to some transcription factors (Williams & Andrisani, 1995; Haviv et al., 1996; Cheong et al., 1995). In particular, deregulation of cell cycle control (Koike et al., 1994b; Benn & Schneider, 1995), induction of myc genes (Avantaggiati et al., 1992; Balsano et al., 1991) and DNA repair abnormalities (Lee et al., 1995) may play a role in the pathogenesis of HBV infection.

While overwhelming numbers of clinical and epidemiological studies indicate a relationship between HBV and hepat-
cellular carcinoma (HCC) (for reviews see Ganem & Varmus, 1987; Tiollais et al., 1985) and the X gene has been implicated in the pathogenesis of HCC, several questions remain regarding the pathogenesis of HCC associated with chronic HBV infection. One of these relates to the long latency before the development of HCC; in general, it takes 40–50 years from infection to the development of HCC (Okuda, 1986). If the X gene of HBV is involved in hepatocarcinogenesis through the mechanisms mentioned above, why is such a long incubation period necessary? A possible explanation involves apoptosis. Most animal viruses possess strategies to block and/or induce apoptosis depending on the intracellular conditions or external environment (for a review see O'Brien, 1998). For example, E1A of adenovirus, Tax of human T-cell leukaemia virus type I and T-antigen of simian virus 40 (SV40) have been shown to have the ability to induce apoptosis, in addition to their ability to induce cell growth. In an animal model for HBV hepatocarcinogenesis, increased apoptosis of hepatocytes in the precancerous liver was noted (Koike et al., 1998; Terradillos et al., 1998). Alternatively, apoptosis in HBV infection may be associated with the pathogenesis of hepatitis or may lead to impaired cell-mediated immunity that could allow the virus to evade host inflammatory responses. Therefore, the possibility that HBX can induce apoptosis must be explored.

One of the problems in studying apoptosis in cultured cells lies in the difficulty in establishing cell lines with a sufficient level of apoptosis-inducing product, since a high level of such a protein may result in cell death during the selection process for cell establishment. In fact, we had previously attempted to establish stable cell lines expressing high levels of HBx, but were unable, in spite of repeated trials, to obtain such a cell line: the only cell lines that were obtained had very low levels of HBx. To overcome this problem, we employed the Cre/loxP system in combination with a recombinant adenovirus vector expressing Cre (Kanegae et al., 1995). By using this system, one can introduce a potentially toxic gene, in a completely silent state, permanently into cell lines. In order to make the X gene active in our study, the stuffer DNA was excised by a sequence-specific recombinase, Cre, upon infection with recombinant adenovirus. Driven by a strong promoter, the established cells now produced a high level of HBx, allowing us to carry out biological and biochemical analyses.

**Methods**

- **Gene construction.** pCALNLX is a plasmid that contains the expression-switching transgene unit CALNLX, which is similar in design to CALNLZ (Kanegae et al., 1996). CALNLX consists of the CAG promoter (Miyazaki et al., 1989), a loxP sequence, the neo neomycin-resistance gene, the SV40 poly(A) signal, a second loxP sequence, the X gene open reading frame (Koike et al., 1988) and the globin poly(A) signal, in this order (Fig. 1A). For construction, the X gene open reading frame was excised from plasmid pUCHBx (Koike et al., 1994b) by digestion with Hinfl and MfeI followed by Klenow treatment and ligated into the SpeI site of pCALNLw (Kanegae et al., 1996). Three plasmids, pCALNLZ, pxCALNLNZ and pCALNLLE, were used as controls. pCALNLZ has a lacZ gene, pxCALNLNZ has a lacZ gene tagged with a nuclear localization signal (Kalderon et al., 1984) and pCALNLLE has the envelope genes of hepatitis C virus (HCV) in place of the X gene of plasmid pCALNLX. The p53 expression plasmid, pCAGp53, was constructed by inserting the human wild-type p53 gene into a pCAGGS vector (Miyazaki et al., 1989).

- **Cell culture, DNA transfection and establishment of stable cell lines.** Human hepatoma cell lines, HLF and HepG2, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. HLF has been shown to have a mutant p53 gene, while HepG2 has the wild-type counterpart (Hsu et al., 1993). We confirmed the mutation in the p53 gene in the HLF cell line by using PCR–single-strand conformation polymorphism analysis and direct

![Fig. 1. (A) Structure of the Cre-mediated activation unit of the HBV X gene outlining activation of the X gene in the CALNLX unit by Cre recombinase. The Cre-mediated excision removes both the neo coding region and the poly(A) sequence, consequently generating a functional X gene expression unit. CAG, CAG promoter; SpA, SV40 early poly(A) site; GpA, rabbit ß-globin poly(A) site. (B) Southern blot analysis of a representative cell line. Genomic DNA extracted from Fx19 cells either before (–) or after (+) infection with recombinant adenovirus AcxCANCre was digested with HindIII. A 6.5 kb band in the (–) lane became a 5.2 kb band in the (+) lane due to the excision of "stuffer" DNA as described in (A). Numbers on the left indicate the positions of the DNA size markers (sizes in kb).](image)
sequencing: Ala is substituted for Gly in codon 244. DNA transfection to cells was done by the calcium phosphate method and selection in medium sequencing; Ala is substituted for Gly in codon 244. DNA transfection to cells was done by the calcium phosphate method and selection in medium containing geneticin (G418) at a concentration of 500 μg/ml was started 24 h later as described previously (Koike et al., 1994b). After the transfection of the plasmids to HLF cells, we established cell lines that contained pCALNLX (designated Fx), pCALNLZ (Fz), pxCALNLNZ (Fnz) and pCALNLLE (FE). We also established cell lines from HepG2 that contained pCALNLX (designated Gx) and pCALNLZ (Gz). Each cell line had between two and 10 copies of the intact transgenes as determined by Southern blotting.

For transient transfection of pCAGGp53 plasmid by the calcium phosphate method, HepG2 cells were incubated with DNA precipitates for 6 h at 37 °C, followed by AxCANCre infection (see below).

■ Generation and administration of recombinant adenovirus. AxCANCre virus, which expresses Cre DNA recombinase, was generated as described previously (Kanegae et al., 1995). As a control for adenovirus infection, Adex1w1, not containing any exogenous genes, was used. A concentrated and purified virus stock was prepared and the titre of the purified stock was determined as described previously (Kanegae et al., 1994). Infection of adenovirus into cultured cells was carried out at the m.o.i. indicated (Kanegae et al., 1996).

■ Infection with recombinant adenovirus. We determined the optimal concentration of recombinant adenovirus needed to infect a maximal number of cells with minimal cytopathic effect (Yeh & Perricaudet, 1997). For this purpose, recombinant adenovirus, AxCANCre, was infected into HLF-derived Fz and HepG2-derived Gz cell lines, both of which possessed the lacZ gene, at m.o.i. of 0, 0.5, 2.0, 5.0, 30 and 100. A representative experiment is shown in Fig. 2 for Fz3 cells. By infection with AxCANCre at an m.o.i. of 0.5 to 5.0, nearly 100% of the cells were stained with X-Gal. However, a cytopathic effect was observed to a mild degree with infection at an m.o.i. of 2.0, to a moderate degree at an m.o.i. of 5.0 and to a severe degree at an m.o.i. of 30. From these results, we decided to use an m.o.i. of 0.5 for the remaining experiments. It is not surprising that nearly 100% of the cells were infected at a m.o.i. < 1.0, as an m.o.i. of 1.0 does not mean one virus particle per cell on the basis of the titration method (Kanegae et al., 1996).

■ Detection of lacZ gene expression. To detect β-galactosidase activity by staining, 3 days after infection, cells were washed twice with PBS, fixed with 0.25% glutaraldehyde and stained with 0.1% X-Gal.

■ Southern hybridization of genomic DNA. Ten micrograms of genomic DNA was digested with the appropriate restriction enzymes, separated through a 0.8% agarose gel and transferred to a nylon membrane (Schleicher & Schuell). The membrane was then hybridized with a digoxigenin-labelled (Boehringer Mannheim), 1.2 kb linearized pUCHBx probe.

■ Antibody and indirect immunofluorescence. HLF or HepG2 cells were grown on Lab-Tek cell culture chambers (Nunc) and infected with adenovirus as described above. Cells were washed with PBS after an appropriate interval after the infection and then fixed with fresh acetone–methanol (70:30) at −20 °C for 7 min. The cells were then reacted with rabbit anti-HBx antibody (Koike et al., 1988), washed and incubated with FITC-conjugated secondary antibody (Organon Teknika). For the detection of p53 protein, mouse monoclonal antibody PAB 1801 (Oncogene Science) was used as the first antibody and rhodamine-conjugated goat anti-mouse IgG (Organon Teknika) was used as the second antibody. Stained cells were examined and photographed by using a fluorescent UV microscope.

■ Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) and nuclear condensation assays. We applied the TUNEL assay (Gavrieli et al., 1992) by using a TACS2 TdT in vitro apoptosis detection kit ( Trevigen). Fixed cells were subjected to the TUNEL assay according to the manufacturer’s protocol. To detect nuclear condensation, cells were fixed and stained with Hoechst 33258 (Wako) in PBS at 37 °C for 1 h. After washing with PBS, the cells were visualized under a fluorescent UV microscope.

■ Flow cytometry. After the indicated interval following infection with adenovirus, cells were harvested and resuspended in DMSO–sucrose buffer. After being stained with propidium iodide, the cells were analysed by FACSscan (Becton Dickinson) with the CellFIT program.

■ Statistical analysis. Fisher’s exact test was used for statistical evaluation.

Results

By selection with geneticin, we obtained 27 Fx cell clones derived from HLF and 15 Gx cell clones derived from HepG2, all of which possessed the HBV X gene in the inactive state. As control cell clones, we established 15 Fz, 17 Fnz and 11 FE clones from HLF cells, which possessed the lacZ gene, nucleus-localized lacZ gene and HCV envelope genes, respectively. In addition, 10 Gz cell clones, which possessed the lacZ gene, were established from HepG2 cells. The following experiments were performed using randomly chosen cell clones.

Cre-mediated recombination and switch-on of the X gene

A series of Fx and Gx cell lines was infected with AxCANCre (m.o.i. of 0.5). After incubation at 37 °C for 60 min, the cells were cultured for an additional 2 days and
harvested for the determination of gene recombination and HBx protein expression. Firstly, genomic DNA was prepared from the cells and analysed by Southern blot analysis. Recombination of the introduced plasmid, pCALNLX, had occurred in all the cell lines analysed. The result of a representative experiment on the Fx19 cell line is shown in Fig. 1(B). The size of the band detected after HindIII digestion confirmed the expected HBx-on structure, as shown by the shift of the specific band from 6–5 to 5–2 kb. Immunofluorescence staining demonstrated the expression of HBx upon infection with AxCANCre (Fig. 3 B), while no expression

Fig. 3. Expression of the HBx protein after switch-on by AxCANCre. HBx protein was detected 2 days after infection with adenoviruses. HBx protein was chiefly expressed in the cytoplasm and perinuclear region of Fx cells following infection with AxCANCre (B), while no HBx protein was detectable in cells of the same line that were infected with a control adenovirus, Adex1w1 (A). Cells were stained with rabbit anti-HBx antibody, washed and incubated with FITC-conjugated secondary antibody.

Fig. 4. Induction of apoptosis by the HBx protein as detected by the TUNEL method. (A)–(B) Fx cell lines, which had the X gene in a silent state, were infected with recombinant adenoviruses and analysed by TUNEL at 4 days after infection. Cells from a representative cell line, Fx2, infected with control Adex1w1 showed only slight TUNEL positivity (A), while those infected with AxCANCre showed a marked increase in the number of TUNEL-positive cells (B). (C) Summary of the results of the TUNEL assay on Fx cell lines and control Fz cell lines (means of triplicate experiments; error bars indicate SD). 1w1, Control adenovirus Adex1w1; Cre, recombinant Cre-expressing adenovirus AxCANCre. ***, P < 0.005 compared with 1w1 control.

Fig. 5. Analysis of HBx-induced apoptosis by flow cytometry. Cells were harvested 2 or 4 days after adenovirus infection and subjected to flow cytometric analysis; a representative result is shown. Numbers above the graphs indicate the percentage of cells in the hypoploid fractions. (A) Fx19 cells were infected with either AxCANCre (Cre) or Adex1w1 (1w1) and harvested at day 2 or day 4 after infection. Apoptosis was induced at 4 days after infection with AxCANCre (33% of total nuclei were in the hypoploid fraction, indicating apoptosis), while only 5% of cells were apoptotic on day 2. Fx19 cells infected with control adenovirus showed no increase in the hypoploid fraction either on day 2 or day 4. (B) Four days after infection, 40% of Fx23 cells exhibited apoptosis following AxCANCre infection while only 7% exhibited apoptosis following control Adex1w1 infection. The control cell line Fz5 did not show any increase in the hypoploid fraction.
Induction of apoptosis by HBx

To examine the consequences of HBx protein expression, we infected a series of Fx and Gx cell lines, as well as the control cell lines, Fz, Fnz, FE and Gz, with AxCANCre or Adex1w1. After incubation with the recombinant adenovirus for 60 min, the cells were incubated in DMEM with 10% FCS. After 4 days, both floating and attached cells were collected for analysis.

Firstly, Fx cell lines Fx2, Fx19 and Fx23, which had the X gene in the silent state, and Fz cell lines Fz3 and Fz5, which had a silent lacZ gene, were infected with recombinant adenoviruses and analysed by TUNEL at 4 days after infection. While the cells infected with the control Adex1w1 showed only slight TUNEL positivity (Fig. 4A), those infected with AxCANCre showed a marked increase in the number of TUNEL-positive cells (Fig. 4B). The results together with those of the control cell lines are summarized in Fig. 4(C). The Fx cell line contained 29–38% TUNEL-positive cells after induction of the X gene, while only 5–8% TUNEL-positive cells were observed following infection with control Adex1w1. Also, the control cell lines showed only 7–9% positive cells, irrespective of the expression of the lacZ gene. Thus, the HBx protein induced cell death, which was not due either to the effect of adenovirus or to the Cre recombinase itself, in hepatoma-derived cells. Importantly, even after more than 15 passages, Fx or Gx cells could still be induced to express the same levels of HBx protein after infection with AxCANCre recombinant adenovirus, suggesting that the potentially detrimental HBx protein is completely silent unless the stuffer is removed.

We next analysed the cell lines by flow cytometry. In this analysis, the cell clones Fx7, Fx19, Fx23 and Fz5 were subjected to flow cytometry 2 and 4 days after infection with adenoviruses. A representative result is shown in Fig. 5. Two days after infection, only a small proportion of the cells were apoptotic, as shown by the hypoploid fraction, while 33% of the cells became apoptotic at 4 days after infection (upper panel of Fig. 5A). Because HBx was detectable 2 days after the adenovirus infection, at least 1 day of exposure of the cells to HBx may be necessary for the induction of apoptosis. Infection of Fx19 cells with control Adex1w1 (lower panel of Fig. 5A) or infection of a control Fz5 cell line with AxCANCre (Fig. 5B) did not increase the apoptotic fraction even at 4 days after infection. Overall, these FACS analysis results agree well with the results of the TUNEL analysis.

To characterize the cell death induced by HBx, a nuclear condensation assay was carried out. Four days after infection with the adenoviruses, cells were fixed and stained with Hoechst 33258. No signs of nuclear condensation were observed following infection with control adenovirus, Adex1w1, whereas after AxCANCre infection, nuclear condensation was evident at 4 days after infection of Fx or Gx cells (Fig. 6A, B). A summary of the nuclear condensation assay results for a series of cell lines is shown in Fig. 6(C). Gx cell lines, which had the X gene in a silent state in HepG2-derived cells, also exhibited changes of apoptosis upon induction of
HBx. Thus, HBx induced apoptosis irrespective of whether the p53 gene was wild-type or mutant in these two hepatoma cell lines. Control cell lines, FE, Fnz and G2, did not show any significant increase in the fraction of apoptotic cells upon induction of the HCV envelope proteins, nucleus-directed lacZ or cytoplasmic lacZ, respectively.

Intracellular localization of p53

HBx has been shown to inactivate p53 by translocating it from the nucleus to the cytoplasm (Ueda et al., 1995; Takada et al., 1997). Therefore, it would be of great interest to determine whether or not p53 was inactivated in our system in order to study the p53-dependency of HBx-induced apoptosis. We determined the intracellular localization of the p53 protein under conditions of HBx induction or its absence in our system using the immunofluorescent double-staining technique. Since the level of p53 in HLF cells was about 20 times higher than that in HepG2 cells (data not shown), we first determined the localization of endogenous p53 in HLF-derived Fx clones. In two independent Fx clones, Fx2 and Fx19, endogenous p53 was found to be located in the nucleus in the absence of induction of HBx. However, induction of HBx by infection with AxCANCre changed the localization of the endogenous p53 protein from the nucleus to the cytoplasm in all the cells analysed (52/52 in Fx2 and 49/49 in Fx19). In the representative case shown in Fig. 7, dispersed staining of endogenous p53 protein was detected consistently in the cytoplasm of HBx-expressing cells 2 days after infection with AxCANCre. Thus, HBx inactivates endogenous p53 by translocating it from the nucleus to the cytoplasm in cells in which HBx induces apoptosis, suggesting that HBx-induced apoptosis is independent of the p53 pathway. Because the endogenous level of p53 in HepG2 cells was low, its localization was analysed by transfecting HepG2-derived Gx3 and Gx7 cells with the wild-type p53 gene. The nuclear localization of transiently expressed p53 protein was changed to cytoplasmic localization by HBx co-expression (data not shown), as described previously by Takada et al. (1997).

Discussion

Recent advances in molecular biological techniques have enabled analysis of the function of genes of interest by making possible the production of stable cell lines with constitutive expression of these genes. However, in cases in which an introduced gene has a deleterious effect on cell viability or cell proliferation, the stable cell lines obtained may first be genetically altered to tolerate or modulate the effects of the products of the introduced genes. In particular, for assessment of the effect of any given protein on commitment of cells to cell-suicide pathways, it is ideal to analyse the fate of cells in which expression of the protein is strictly regulated by an inducible transcriptional control machinery: i.e. expression is completely silent until induction of the gene. A transient expression system using DNA transfection may be an alternative, but its application is limited because, in general, it only allows a low rate of transduction.

To overcome the aforementioned problems, several inducible expression systems have been developed and used to analyse the functions of proteins. These include (i) hormone- or metal ion-inducible expression vectors such as those containing the mouse mammary tumour virus (MMTV) long terminal repeat (LTR) or the regulatory region of metallothionein gene, (ii) the tetracycline (Tc)-inducible expression system and (iii) the Cre/loxP switch-on system. To date, only the first two
systems have been applied to the assessment of the function of HBx. In our earlier study, we utilized an MMTV LTR-driven HBV X gene construct and demonstrated the promotion of cell cycle progression by HBx in NIH3T3 cells (Koike et al., 1994b). Recently, Chirillo et al. (1997) employed the same system and showed that HBx induced apoptosis in NIH3T3 cells in addition to its effect of promoting cell cycle progression. A potential problem in this system lies in the leaky character of the MMTV promoter: i.e. HBx was expressed at a basal level even before induction with glucocorticoids (Koike et al., 1994b). Therefore, it is possible that specific cells such as those tolerant of or resistant to death signals are selected during the process of establishment of stable cell lines. This may explain the failure of HBx to induce apoptosis in our previous study (Koike et al., 1994b) despite the experimental conditions being the same as those used in the study of Chirillo et al. (1997).

The second system, the Tc-inducible gene, has been established rather recently. This system is a very useful one, in which gene transcription is regulated by the use of a Tc-inducible promoter and a mutated Tc repressor. Recently, Kim et al. (1998) have shown the induction of apoptosis by HBx by the use of this system. By obtaining cell lines that were initially silent for the X gene, they showed directly that HBx induced apoptosis in Chang liver cells, although they used no controls to exclude the possibility that proteins other than HBx may also induce apoptosis when expressed in that system. In addition, as they described in their paper, the Tc-inducible promoter was leaky and allowed a low level of expression of the X gene even without induction; after multiple passages, the expression level of the X gene in their cell lines decreased rapidly to an undetectable level (Kim et al., 1998).

These problems may be circumvented by the use of the Cre/loxP-mediated conditional expression system, which has already been successfully applied to cultured cells and transgenic animals. We employed a replication-deficient recombinant adenovirus system harbouring Cre recombinase tagged with a nuclear localization signal (Kanegae et al., 1995, 1996). The advantages of this system are that (i) almost 100% of the culture cells are infected, (ii) the silent transgene is efficiently switched on in infected cells and (iii) the transgene is completely silent before infection with Cre recombinase-expressing adenovirus (Kanegae et al., 1995, 1996). However, this system suffers from the disadvantage of the cytotoxic effect of adenovirus (Yeh & Perricaudet, 1997). To minimize this effect, we carried out the titration of recombinant adenovirus using lacZ gene-carrying cells and used adenovirus for the X gene experiment at the lowest m.o.i. that gave 100% efficiency of switching on of the gene. We also utilized controls for the adenoviruses and the cell lines: ‘wild-type’ adenovirus Adex1w1 and a number of cell lines that carried the lacZ gene, the lacZ gene tagged with a nuclear localization signal and the HCV envelope genes.

Our data demonstrate that the HBx protein specifically induced apoptosis in human liver-derived cells when X gene expression was turned on. Selection of cell lines that had a completely silent objective gene (Kanegae et al. 1995) by the use of the Cre/loxP system avoided the possibility that cell lines were selected that were more resistant or susceptible to signals from HBx. Our observation that X gene expression was activated reproducibly even after more than 15 passages by the use of the Cre/loxP system is concordant with this notion. We could not observe the cell cycle progression induced by the HBx protein in the current study that we demonstrated previously by using the MMTV inducible system and NIH3T3 cells (Koike et al., 1994b). Both of the cell lines used in the present study, HLF and HepG2, are hepatoma-derived and do not become quiescent upon the depletion of serum from the culture medium. This may be the reason why the effect of the HBx protein of promoting cell cycle progression was not detectable in the present study.

HBx-induced apoptosis described in previous studies was reported to be either p53-dependent (Chirillo et al., 1997) or p53-independent (Terradillos et al., 1998). Our data indicate that HBx-induced apoptosis occurs via the p53-independent pathway, since apoptosis occurred in cell lines with both mutant and wild-type p53 genes, in which the p53 protein was inactivated by HBx. The mutant p53 in HLF, which contained a mutation from Ala to Gly at codon 244 in the DNA-binding domain, retained its binding capability for HBx and was inactivated by HBx, as was also shown for HuH7 cells by Takada et al. (1997). Nonetheless, apoptosis occurred in HLF-derived clones and in HepG2-derived clones with the wild-type p53. Thus, it is considered that HBx inactivated p53 and induced apoptosis concurrently in our system.

Our results show a discrepancy from the previously reported results of Wang et al. (1995), in which HBx was shown to block apoptosis induced by the overexpression of p53 in primary fibroblasts. It is known that p53 modulates the apoptotic response in various ways. It may exert a protective effect by the induction of cell cycle arrest or induce apoptosis through a transcription-dependent pathway (Amundson et al., 1998; Evan & Littlewood, 1998). Thus, differences in cell type and culture conditions may account for the differences. However, it should be noted that Wang et al. (1995) could not detect HBx in their system: in contrast, in our system, HBx protein was easily detectable after induction.

The precise role of HBx-induced apoptosis in the virus life cycle is not clear. There are some examples of viral or cellular oncoproteins involved in both cell death and oncogenesis. Adenovirus E1A, human T-cell leukaemia virus type I Tax and human papillomavirus E7 stimulate cell proliferation and apoptosis in both p53-dependent and p53-independent manners (Debbas & White, 1993; Pan & Grieben, 1994; Yamada et al., 1994). Having the ability to promote both cell proliferation and cell death, HBx may give the virus an advantage in persistence by boosting both virus and host DNA replication and thereafter destroying host cells in order to release virions. Also, these two apparently opposing functions of HBx,
inducing both cell death and proliferation, might explain the long incubation period of 40–50 years from HBV infection to the development of HCC in humans, given that HBx plays a central role in hepatocarcinogenesis (Paterlini et al., 1995; Koike, 1995; Koike & Takada, 1995; Caselmann, 1995; Feitelson & Duan, 1997).

We thank Ms C. Ariga for her technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

References


Induction of apoptosis by HBx


Received 19 July 1999; Accepted 3 August 1999