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The X Gene of Hepatitis B Virus Induced Growth Stimulation and Tumorigenic Transformation of Mouse NIH3T3 Cells

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To examine the transforming potential of the X gene product of hepatitis B virus (HBV), the X-gene-containing region (referred to as the HBx region) was introduced into mouse NIH3T3 cells. Each transformed cell line expressed X-coding mRNA at a different level. A positive correlation was found between the level of X-coding mRNA and the saturation density of the cells. The HBx-transformed cell lines exhibited X protein production and tumor formation in nude mice. The function of HBV in oncogenesis may involve the continuous expression of the X-gene-coded product in the HBV DNA-integrated cells.

Key words: Hepatitis B virus — X gene — Cell saturation density — *Trans*-activation — Oncogenesis

Hepatitis B virus (HBV) causes acute and chronic hepatitis and is strongly associated with the development of primary hepatocellular carcinoma (HCC). Four open reading frames (ORFs) in the plus strand of HBV DNA: S/preS, C/preC, P, and X were revealed,[1] and its integration into the chromosomal DNA of HCC has been demonstrated.[2,3] But the roles of HBV in pathogenesis and oncogenesis still remain unclear.

By using antibodies against synthetic peptides corresponding to the X open reading frame (ORF), a polypeptide was detected in an HBV-infected liver.[4] Antibodies containing anti-X reactivity were also detected in HBV carriers.[5,6] Our previous findings indicated the transient production of HBV particles by the transfected HBV DNA not to be strongly inhibited by a mutation in the X ORF.[7] The X gene product may thus be related to the host-factor-dependent regulation of virus replication *in vivo*.

To observe the early stage of tumor development, integrated forms of HBV DNA in HCC or chronic active hepatitis of children were characterized and compared with those from the HCC of adult patients. In many of the observed integrated forms of HBV DNA, one of the virus-chromosomal DNA junctions was noted to be close to the 5′ end of the minus-strand DNA, and a region containing the major part of X ORF and enhancer sequence was well conserved.[8] Recently, the X gene product of HBV has been shown to be a *trans*-activator of the homologous and heterologous transcriptional enhancers.[9] It thus appears reasonable to consider the expression of cellular gene(s) to be *trans*-activated through a continuous expression of the X gene-coded product at the stage of chronic infection. In this study, an attempt was made to clarify to some extent the transforming potential of the X gene through the expression of X gene-containing plasmid DNA transfected into mouse NIH3T3 cells. A preliminary examination was previously made of the expression of X-coding mRNA in HBV DNA-transfected NIH3T3 cells.[10]

The HBx DNA, 0.87 kb *StuI*/*Bgl*II fragment of HBV DNA[11] contains the X gene and a 0.26 kb upstream sequence containing an intrinsic enhancer. Using linker ligation, this fragment was inserted into the *Bgl*II site of pKSV10 (Pharmacia), which possesses the SV40 early promoter sequence and polyadenylation signal (Fig. 1A). This plasmid was designated as pKSV-HBx. All plasmid DNAs were constructed by standard recombinant DNA techniques.[12]

Several DNA transfection experiments were conducted independently with pKSV-HBx DNA (Fig. 1A). One day before DNA transfection, 1×10^6 NIH3T3 cells were plated and maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Irvine) and 60 μg/ml kanamycin in a 100 mm dish followed by cotransfecting 20 μg of pKSV-HBx DNA or pKSV10 DNA with 1 μg of pSV2-neo-SVgpt DNA into NIH3T3 cells using calcium phosphate precipitation.[13] Two days later, G418 sulfate (Geneticin) was added to the culture medium (400 μg/ml final concentration) for the selection. Drug-resistant colonies developed within 2 to 3 weeks following cotransfection and single colonies

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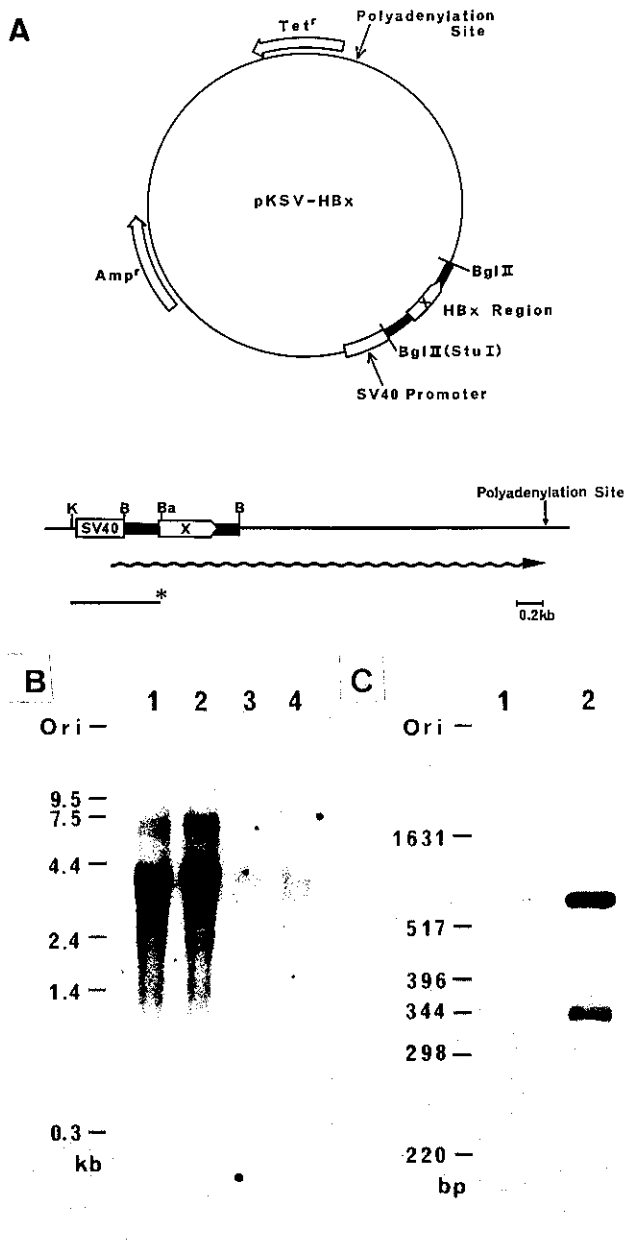

Fig. 1. A. Structure of plasmid pKSV-HBx DNA. The HBV DNA used in this study was derived from the adr subtype and its nucleotide sequences have been described.[11] The thick line represents the 0.87 kb *StuI/BglII* fragment of HBV DNA containing X ORF, shown as an open box. The thin line represents the vector pKSV10 DNA. K, *KpnI*; Ba, *BamHI*; B, *BglII*. B. Northern blot hybridization of X-coding mRNA. Ten μg of the total RNA extracted from each G418-resistant cell line was analyzed. Lane 1, NHBx-1; lane 2, NHBx-32; lane 3, NHBx-22; lane 4, NHBx-33. C. Mapping of the 5′ end of X-coding mRNA by S1 nuclease. Lane 1, NIH3T3; lane 2, NHBx-32.

of resistant cells were randomly isolated. The G418-resistant cell lines obtained were designated as a series of NHBx. The cell line transfected with pKSV10 was named NpKSV.

To examine the expression of the HBx region in these cell lines, the total RNA was extracted from these G418-resistant cell lines at the confluent stage by the guanidium/cesium chloride method.[14] After electrophoresis in a formamide/agarose gel, RNA was transferred to a nitrocellulose filter[15] and hybridized with the HBV DNA probe. A ^{32}P-labeled HBV DNA probe was made by nick translation.[16] Four G418-resistant cell lines (NHBx-1, NHBx-2, NHBx-21 and NHBx-32) exhibited significantly high expression of X-coding mRNA and three cell lines (NHBx-22, NHBx-31 and NHBx-33) that exhibited very low expression were selected and utilized for subsequent experiments. These two groups of cell lines are designated as high-expression and low-expression cell lines, respectively, for convenience. Data on X-coding mRNA from high-expression (NHBx-1, NHBx-32) and low-expression (NHBx-22 and NHBx-33) cell lines are shown in Fig. 1B. The major band at 3.4 kb in the Northern blot corresponded to the transcript started at the SV40 promoter and terminated at the SV40 polyadenylation site.

To confirm the location of the 3.4 kb RNA start site, an S1 nuclease mapping experiment was conducted. A *BamHI* fragment prepared from the pKSV-HBx DNA (Fig. 1A), was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase and then digested with *KpnI* to obtain the 5′ end -labeled probe. About 50 μg of the total cytoplasmic RNA of NHBx-32 cells, for instance, mixed with 100 μg of *Escherichia coli* tRNA was hybridized with the probe DNA at 50°C overnight and then digested with 150 units of S1 nuclease for 30 min at 37°C. Analysis of the protected fragments was carried out by 7 *M* urea/6% polyacrylamide gel electrophoresis.[12] The S1 nuclease-protected band was about 350 nucleotides long (Fig. 1C) and was thus located in the SV40 promoter region upstream from the *BglII* site (Fig. 1A, B). One minor band was observed at 5.4 kb (Fig. 1B), presumably corresponding to a read-through transcript which started at the SV40 promoter but was not terminated at the SV40 polyadenylation site.

In these cell lines, the integrated plasmid DNA was examined by Southern blot hybridization using pKSV10 DNA or HBV DNA as the probe (data not shown). High-expression cells (NHBx-1, NHBx-2, NHBx-21 and NHBx-32) contained 7–15 plasmid copies, while low-expression cells (NHBx-22, NHBx-31 and NHBx-33) contained 1–2 copies. The expression level of the X-coding mRNA may possibly depend on the number of copies of integrated pKSV-HBx DNA. The NpKSV cell line contained about 10 copies of integrated pKSV10 DNA.

High-expression cells (NHBx-1, NHBx-2, NHBx-21 and NHBx-32) did not exhibit contact inhibition and grew into several layers. However, none of the cell lines showed spindle-shaped morphology and focus formation, as did *ras*-transformed NIH3T3 cells.[17] Abnormally shaped cells were frequently observed, such as those with enlarged nuclei and cytoplasm, or multinuclear morphology (unpublished data). The low-expression cells (NHBx-22, NHBx-31 and NHBx-33) formed a monolayer of ordinary appearance like that of the cell line NpKSV or the control NIH3T3 cells at the confluent stage. These observations prompted us to examine in detail their growth and saturation density.

In Fig. 2, the high-expression cells exhibited high cell density, while low-expression cells remained indistinguishable in saturation density from the control NIH3T3 and NpKSV cells. Based on the present results, the expression level of the X-coding mRNA and the cell saturation density are correlated positively.

Although G418-resistant cell lines were randomly taken, there is a possibility that cells were selected with a bias to high saturation density. Therefore, we examined the colony-forming activity of G418-resistant cells transfected with pKSV-HBx or pKSV10 DNA in soft agar without colony isolation. After addition of G418, all G418-resistant cells transfected with each relevant

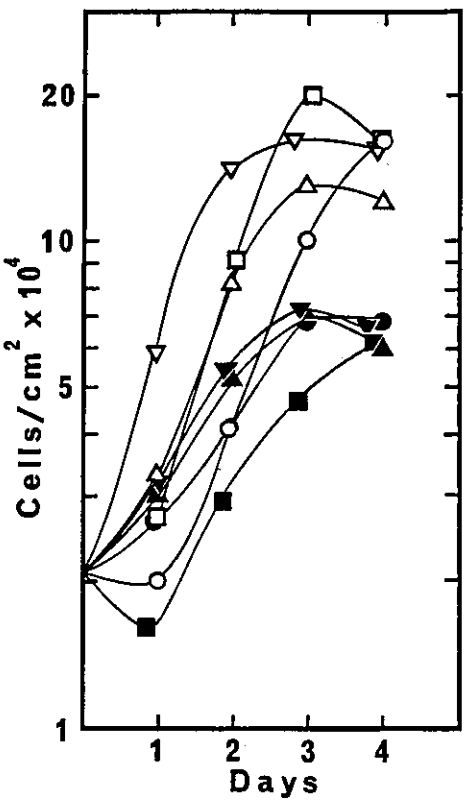

Fig. 2. Growth curve of G418-resistant cell lines. Cells (2×10^5) were plated in a 35 mm dish in DMEM supplemented with 5% calf serum, and viable cells were counted for 5 days without medium change. Each point is the average cell number in two or three dishes counted by trypan blue staining. Open symbols are cell lines NHBx-1 (○), NHBx-2 (□), NHBx-21 (△), NHBx-32 (▽). Closed symbols indicate cell lines NHBx-22 (▲), NHBx-31 (■), NHBx-33 (▼) and the control cell line NIH3T3 (●).

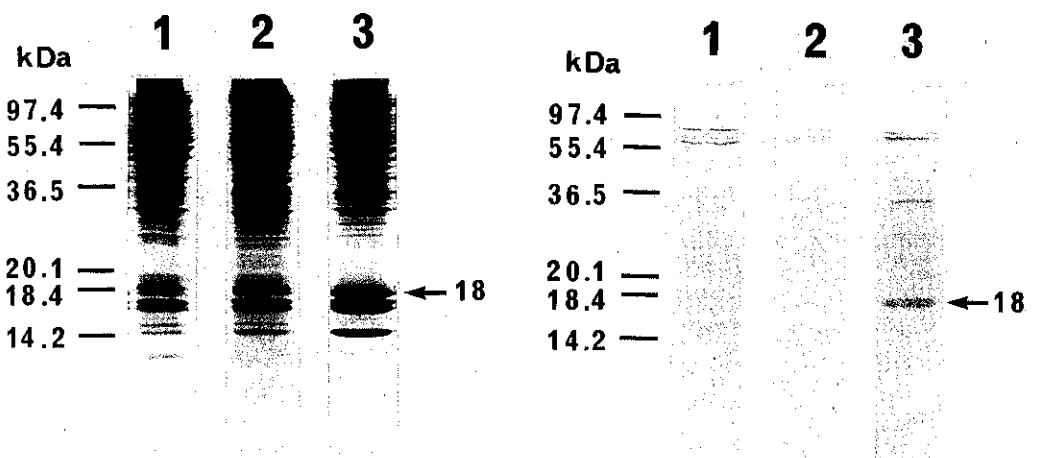

Fig. 3. SDS-polyacrylamide gel of the cell extract. Cell extract was prepared and loaded into each sample well of a 15% SDS-polyacrylamide gel.[18] In the left panel, the gel was stained with Coomassie blue. In the right panel, the gel was immunoblotted and developed with a rabbit antibody to synthetic X peptides. Lane 1, NIH3T3; lane 2, NpKSV; lane 3, NHBx-1.

Table I. Tumorigenicity in Nude Mice

Cell line	Frequency			
	3 weeks	4 weeks	6 weeks	8 weeks
NHBx-1	0/5	3/5	5/5	5/5
NHBx-32	1/5	5/5	5/5	5/5
NHBx-22	0/5	0/5	0/5	1/5
NpKSV	0/5	0/5	0/5	0/5
NIH3T3	0/5	0/2	0/5	0/5

Each of five nude mice was injected subcutaneously with 2×10^6 HBx-transformed cells. Data indicate the frequency of tumor-carrying nude mice.

plasmid DNA were collected without colony isolation. A total of 2×10^4 cells were suspended in 3 ml of DMEM containing 0.3% agar and 10% calf serum, and incubated for 2 to 3 weeks. About 10% of the suspended cells transfected with pKSV-HBx DNA formed colonies, while only 1–3% of those transfected with pKSV10 DNA did so (unpublished data). The data strongly support the idea that the continuous expression of the X gene is positively correlated to stimulation of cell growth.

To demonstrate the production of X protein in the HBx-transformed cells, the cell lysate was subjected to Western blot analysis to assay for the presence of X protein using antisera generated in rabbits for synthetic X peptides.[4] Immunoblots showed that 18 kDa protein reacted with antisera to synthetic X peptides was clearly detected in the high-expression cells (NHBx-1) when compared to the control NIH3T3 or NpKSV cells (Fig. 3). The 18 kDa protein could be the 154-amino-acid protein corresponding to the X ORF. A similar result was obtained with other high-expression cells, NHBx-32 (unpublished data). Details will be published elsewhere.

We then examined the tumorigenicity of HBx-transformed cell lines in nude mice (Table I). The high-expression cells (NHBx-1 and NHBx-32), the low-expression cells (NHBx-22), the NpKSV cells and the control NIH3T3 cells were inoculated into nude mice. The high-expression cells induced tumors generally having longer periods of latency than the *ras*-induced tumor, which usually becomes apparent 2 weeks following inoculation.[19] One nude mouse inoculated with low-expression cells (NHBx-22) exhibited tumor formation after 8 weeks. The NpKSV cells and control NIH3T3 cells failed to induce a tumor even after 8 weeks.

The X gene is located at the 3′ end of the pregenome RNA of HBV, as in the case of retroviral oncogenes.[20] The codon usage of the X coding region is probably similar to that of the genes of eukaryotic cells, whereas other HBV genes are similar to viral genes in origin.[21] In the present study, tumorigenicity in nude mice was observed in these transformed cell lines by introducing the HBx region with many copies. However, there is no evidence that the HBx region alone is directly oncogenic in action under the conditions used.

Two functional classes of oncogenes are known.[22] The first class includes the *ras* oncogene family and the second class the *myc* family, p53 and polyoma virus large T antigen. *Ras*-transformed NIH3T3 cells show unique spindle-shaped and highly refractive morphology. However, the HBx-transformed cells did not undergo such morphological change. In these transformed cells, change in gene expression may occur only in the second class of oncogenes. The present results suggest that the expression of the HBx region in HBx DNA-integrated NIH3T3 cells possibly stimulates the cell growth in cooperation with the expression of an oncogene(s) of the second class.

Recently, a cloned integrated HBx DNA sequence was shown to *trans*-activate the SV40 early promoter and enhancer.[23] When the HBx DNA is integrated and persistently expresses X protein to function as the transcriptional *trans*-activator for the cellular oncogene(s), this X gene-coded product may induce tumorigenic transformation. It is possible to speculate that once the product from the integrated X gene *trans*-activates the cellular oncogene(s) in chronic hepatitis and induces tumorigenic transformation, expression on the X gene product itself may not be needed any more in HCC and the gene could be deleted from the integrated HBV DNA in certain cases. Transforming DNA sequences of HCC have been reported recently.[24] So far, we do not know whether the X gene product directly *trans*-activates a cellular oncogene(s) or not.

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REFERENCES

1) Tiollais, P., Pourcel, C. and Dejean, A. The hepatitis B virus. *Nature*, **317**, 489–495 (1985)
2) Dejean, A., Sonigo, P., Wain-Hobson, S. and Tiollais, P. Sepecific hepatitis B virus integration in hepatocellular carcinoma DNA through a viral 11-base-pair direct repeat. *Proc. Natl. Acad. Sci. USA*, **81**, 5350–5354 (1984).

3) Mizusawa, H., Taira, M., Yaginuma, K., Kobayashi, M., Yoshida, E. and Koike, K. Inversely repeating integrated hepatitis B virus DNA and cellular flanking sequences in the human hepatoma derived cell line huSP. *Proc. Natl. Acad. Sci. USA,* **82,** 208–212 (1985).
4) Moriarty, A. M., Alexander, H., Lerner, R. A. and Thornton, G. B. Antibodies to peptides detect new hepatitis B antigens: serological correlation with hepatocellular carcinoma. *Science,* **227,** 429–433 (1985).
5) Kay, A., Mandart, E., Trepo, C. and Galibert, F. The HBV HBx gene expressed in *E. coli* is recognised by sera from hepatitis patients. *EMBO J.,* **4,** 1287–1292 (1985).
6) Meyers, M. L., Trepo, L. V., Nath, N. and Sninsky, J. J. Hepatitis B polypeptide X: expression in *Escherichia coli* and identification of specific antibodies in sera from hepatitis B virus-infected humans. *J. Virol.,* **57,** 101–109 (1986).
7) Yaginuma, K., Shirakata, Y., Kobayashi, M. and Koike, K. Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA. *Proc. Natl. Acad. Sci. USA,* **84,** 2678–2682 (1987).
8) Yaginuma, K., Kobayashi, H., Kobayashi, M., Morishima, T., Matsuyama, K. and Koike, K. Multiple integration site of hepatitis B virus DNA in hepatocellular carcinoma and chronic active hepatitis tissues from children. *J. Virol.,* **61,** 1808–1813 (1987).
9) Spandau, D. F. and Lee, C. H. *Trans*-activation of viral enhancers by the hepatitis B virus X protein. *J. Virol.,* **62,** 427–434 (1988).
10) Koike, K., Kobayashi, M., Yaginuma, K. and Shirakata, Y. Structure and function of integrated HBV DNA. *In* "Hepadna Viruses," ed. W. Robinson, K. Koike and H. Will, pp. 267–286 (1987). Alan R. Liss, Inc., New York.
11) Kobayashi, M. and Koike, K. Complete nucleotide sequence of hepatitis B virus DNA of subtype adr and its conserved gene organization. *Gene,* **30,** 227–232 (1984).
12) Maniatis, T., Fritsch, E. F. and Sambrook, J. "Molecular Cloning: A Laboratory Manual" (1982). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13) Graham, F. L. and Van der Eb, A. J. A new technique for the assay of infectivity of human adenovirus 5 DNA. *J. Virol.,* **52,** 456–467 (1973).
14) Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry,* **18,** 5294–5299 (1979).
15) Thomas, P. S. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA,* **77,** 5201–5205 (1980).
16) Rigby, P. W. J., Dieckmann, M. A., Rhodes, C. and Berg, P. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase. *J. Mol. Biol.,* **113,** 237–251 (1977).
17) Takada, S. and Koike, K. Activated N-*ras* gene was found in human hepatoma tissue but only in a small fraction of the tumor cells. *Oncogene,* **4,** 189–193 (1989).
18) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680–685 (1970).
19) Gebhardt, A., Bell, J. C. and Foulkes, J. G. Abelson transformed fibroblasts lacking the EGF receptor are not tumorigenic in nude mice. *EMBO J.,* **5,** 2191–2195 (1986).
20) Molnar-Kimber, K. L., Summers, J. W. and Mason, W. S. Mapping of the cohesive overlap of duck hepatitis B virus DNA and of the site of initiation of reverse transcription. *J. Virol.,* **51,** 181–191 (1984).
21) Miller, R. H. and Robinson, W. S. Common evolutionary origin of hepatitis B virus and retroviruses. *Proc. Natl. Acad. Sci. USA,* **83,** 2531–2535 (1986).
22) Land, H., Parada, L. F. and Weinberg, R. A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature,* **304,** 596–602 (1983).
23) Wollersheim, M., Debelka, U. and Hofschneider, P. H. A transactivating function encoded in the hepatitis B virus X gene is conserved in the integrated state. *Oncogene,* **3,** 545–552 (1988).
24) Yang, S. S., Modali, R., Parks, J-B. and Taub, J. V. Transforming DNA sequences of human hepatocellular carcinomas, their distribution and relationship with hepatitis B virus sequence in human hepatomas. *Leukemia,* **2,** 102S–113S (1988).