EFFECT OF HEPATITIS B VIRUS X PROTEIN ON THE EXPRESSION OF RETINOBLASTOMA GENE PRODUCT

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Received April 3, 1997

Summary. – Hepatitis B virus X protein (HBX) was studied for its capacity to form a specific complex with the retinoblastoma tumour suppressor protein (pRB), and for its effect on the expression of pRB. HBX was synthesized by *in vitro* transcription and translation in the presence of [^{35}S]methionine. The synthesized HBX was assayed for its binding to a glutathione-S-transferase (GST)-pRB fusion protein bound to Sepharose beads. The *in vivo* binding was investigated by a co-immunoprecipitation and Western blot analysis of the cell extract from a CMV-HBX-transfected hepatoblastoma cell line, Hep G2 cells. These experiments demonstrated that HBX was unable to form a detectable complex with pRB. However, the level of pRB increased considerably in Hep G2 cells transfected with CMV-HBX clone. The alteration of pRB expression by HBX could be a mechanism, contributing to the development of hepatocellular carcinoma (HCC) in human.

Key words: hepatitis B virus; X protein; retinoblastoma tumour suppressor protein; hepatocellular carcinoma

Introduction

Hepatitis B virus (HBV) infection is closely associated with the development of HCC. However, the mechanism by which HBV may play a role in the induction of HCC has not yet been identified. Several lines of evidence indicate that the HBX gene may have a major role in the pathogenesis of HBV. This gene is highly conserved among the subtypes of the human virus and the viral homologs that infect other species (Ganem and Varmus, 1987; Rossner, 1992; Henkler and Koshy, 1996). It has been shown that HBX can function as a transcriptional transactivator, positively regulating the transcription of a wide variety of viral and cellular promoters (Levrero *et al.*, 1990; Rossner, 1992; Henkler and Koshy, 1996). Moreover, HBX is reported to complex with p53 protein, and to effect a wide range of its function (Feitelson *et al.*, 1993; Wang *et al.*, 1994; Truant *et al.*, 1995). In this study, a possible interference by HBX with the normal function of a tumour suppressor gene, pRB gene or its product was examined.

pRB plays an important role in the control of cell proliferation, apparently by binding to, and regulating cellular transcription factors (Helin *et al.*, 1992; Chen *et al.*, 1995; Beijersbergen and Bernards, 1996). The disruption of pRB activities, either by mutation or by interaction with viral oncoproteins, has been implicated in the development of variety of cancers including HCC (Reissmann *et al.*, 1989; Nakamura *et al.*, 1991).

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Abbreviations: BSA = bovine serum albumin; CMV = cytomegalovirus; FBS = foetal bovine serum; GST = glutathione-S-transferase; HBV = hepatitis B virus; HBX = hepatitis B virus X protein; HCC = hepatocellular carcinoma; HPV = human papillomavirus; MEM = Eagle's Minimal Essential Medium; MoAb = monoclonal antibody; PCR = polymerase chain reaction; nt = nucleotide; ORF = open reading frame; pRB = retinoblastoma tumour suppressor protein; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate

It has been shown that pRB forms stable complexes with oncoproteins from three different classes of small DNA tumour viruses: adenovirus E1A, SV40 large T antigen and papilloma virus E7 proteins (Dyson *et al.*, 1989a,b; Kaelin *et al.*, 1990; Munger *et al.*, 1990). The transforming properties of these viruses are dependent, in part, on the ability of their oncoproteins to bind to pRB and to block its function.

Analogous to the transforming proteins of DNA tumour viruses, HBX may also target and inactivate pRB, and thus contribute to the malignant transformation of HBV-infected liver cells.

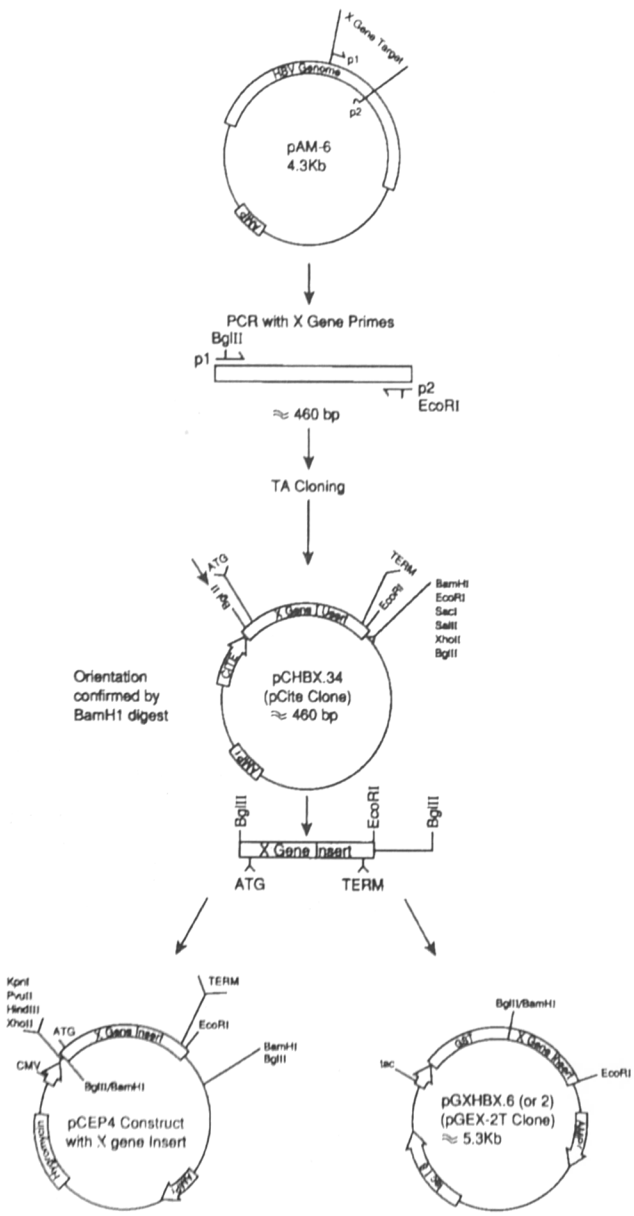

Fig. 1
Construction of plasmids CMV-HBX and GST-HBX
CMV-HBX: expression plasmid containing HBX gene under control of the CMV promoter. GST-HBX: expression plasmid containing HBX gene ORF fused to GST gene.

Materials and Methods

Cell lines. Hep G2 cell line was derived from a hepatoblastoma of 15-year-old white male from Argentina (American Type Culture Collection [ATCC], Rockville, MD). Cell line 2.2.15 was derived by transfection of Hep G2 cells with a plasmid containing multiple copies of the HBV genome. It was kindly provided by Dr. G. Acs, Mount Sinai School of Medicine, New York, NY. HCC cell line, Hep3B, and a human bladder carcinoma cell line, 5637 (ATCC), both known to be negative for the expression of pRB, were used as negative controls. PLC/PRF/5, a human HCC cell line (ATCC), known to be positive for the expression of pRB, was used as a positive control. All cell lines used in this study were grown as a monolayer in Eagle's Minimal Essential Medium (MEM) with 10% foetal bovine serum (FBS).

Plasmid constructions. All HBV-related plasmids were constructed using HBV DNA from plasmid pAM6 (ATCC). The pGEM-HBX, used for *in vitro* translation of HBX, contains HBV DNA nt 1250-1710, including the entire ORF of HBX gene. The gene was amplified by polymerase chain reaction (PCR) and inserted into the *Eco*RI site of pGEM-3Z under the control of the T7 promoter (Promega). The plasmids CMV-HBX and GST-HBX were constructed as shown in Fig. 1. Briefly, the PCR-amplified HBX ORF was inserted into the *Eco*RV (T) site of pCITE-2a vector to obtain a pCHBX clone; pCHBX was then used to construct CMV-HBX and GST-HBX plasmids. The plasmids GST-RB and pGEM-RB (containing the binding domain of pRB to SV40 T antigen, adenovirus E1A and papilloma virus E7 proteins) were kindly provided by Dr. F. Kashanchi, NIH, Bethesda, MD.

Gene transfer and Western blot analysis. Hep G2 cell cultures at about 50% confluence were transiently transfected by the calcium phosphate method with 20 µg of CMV-HBX plasmid for 4 hrs. In a parallel experiment, Hep G2 cells were transfected with the CMV expression vector as a control. Cells were harvested 24 hrs after transfection, rinsed twice in PBS, and total protein was extracted in a lysis buffer (100 mmol/l Tris-HCl pH 8.0, 100 mmol/l NaCl, 0.1% aprotinin, 1 mmol/l phenylmethylsulfonyl fluoride, and 0.5% Nonidet P-40). Aliquots containing 100 µg protein from each sample were separated by 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), electroblotted onto a nitrocellulose membrane and probed with monoclonal antibody (MoAb) to human pRB (PMG3-245) (PharMingen, San Diego, CA) as previously described (Farshid *et al.*, 1994). A protein extract from human bladder carcinoma cell line 5637, known to be negative for the expression of pRB, was used as a negative control.

Expression and purification of recombinant proteins. GST is a plasmid expression vector that was constructed to direct the synthesis of foreign polypeptides in *E. coli* fused with the C-terminus of GST, coded by *Schistosoma japonicum* (Smith and Johnson, 1988). GST-HBX was expressed and purified from *E. coli* as described (Smith and Johnson, 1988). After binding to GST-Sepharose, the protein was washed and eluted with reduced glu-

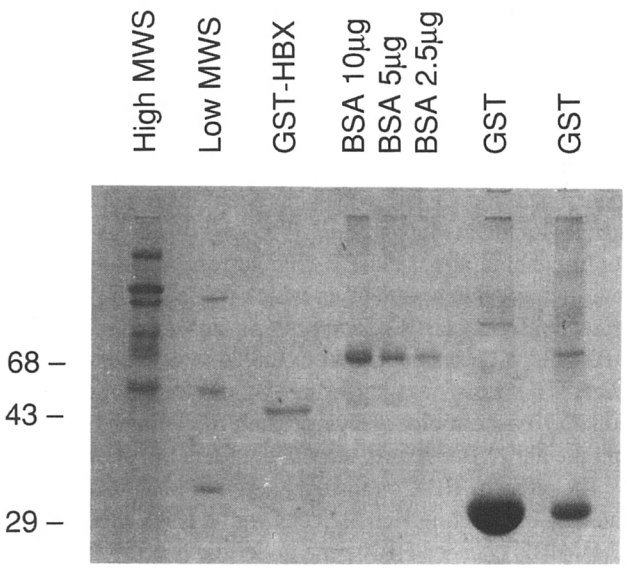

Fig. 2
Expression and purification of GST-HBX fusion protein
SDS-PAGE followed by Coomasie Blue staining. Purified GST, BSA and molecular size markers (low MWS and high MWS) are also shown. The numbers at the left of the figure indicate M_r (K) of the molecular size markers.

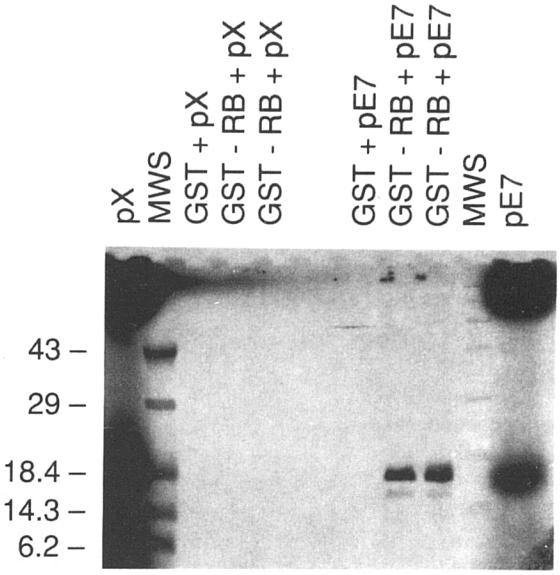

Fig. 3
Results of *in vitro* assay of binding between GST-RB fusion protein and ^{35}S-HBX
SDS-PAGE followed by autoradiography. pX = ^{35}S-HBX. GST-RB = GST-RB fusion protein (two GST-RB preparations, originating from two slightly different plasmid constructs, were used). pE7 = ^{35}S-HPV E7 protein (positive control). MWS = molecular size markers. The numbers at the left of the figure indicate M_r (K) of the molecular size markers.

tathione. The purity and size of the eluted proteins were then evaluated by SDS-PAGE and Coomassie Blue staining (Fig. 2). GST-HBX was immunoprecipitated with a rabbit anti-HBX serum but not with normal rabbit serum. The anti-HBX serum was raised in a rabbit using three synthetic peptides containing amino acid residues 6 – 85, 82 – 100 and 116 – 134 of HBX as described (Osamu *et al.*, 1987).

For the generation of *in vitro* translated ^{35}S-labelled proteins, the plasmids encoding the corresponding genes, driven by the T7 promoter, were subjected to an *in vitro* transcription and translation (TNT System; Promega) at 30°C for 90 mins in the presence of [^{35}S]methionine (Amersham).

In vitro protein binding and analysis of protein complexes. The binding assay was performed in 200 µl of binding buffer (50 mmol/l Tris-HCl pH 8.0, 120 mmol/l NaCl and 0.5% Nonidet P-40) containing 2 – 15 µl of the *in vitro* translated, ^{35}S-labelled HBX and Sepharose beads loaded with GST-RB fusion protein at 4°C for 2 hrs. The beads were washed five times with binding buffer. The bound proteins were eluted by boiling the beads in the presence of the Laemmli buffer for 5 mins, separated by SDS-PAGE, and visualized by autoradiography. An aliquot of the *in vitro* translated HBX was included as a reference. A labelled E7 protein of human papilloma virus (HPV), which has a high affinity for pRB, was used as a positive control. The specificity of the binding was shown by the lack of binding of the E7 protein to a GST vector. In a reciprocal experiment, ^{35}S-labelled pRB was mixed with GST-HBX fusion protein in a similar assay as described above.

Results and Discussion

The exact role of HBX in the disease pathogenesis and in the development of HCC has not yet been determined. However, several studies, using transient transfection experiments with cultured cells, have revealed a transcriptional transactivator function of HBX on a wide variety of cellular and viral promoters (Rossner, 1992; Henkler and Koshy, 1996). The function of HBX as a transcriptional activator has often been linked to its potential role in carcinogenesis. In addition to its function as a transcriptional transactivator, there is growing evidence that HBX shares functional similarities with oncoproteins of some transforming DNA viruses. HBX has been shown to bind *in vitro* to the tumour suppressor gene product p53 and affect a wide range of its functions with a potential for neoplastic development (Feitelson *et al.*, 1993; Wang *et al.*, 1994; Truant *et al.*, 1995). Similarly to p53, pRB plays an important role in the control of cell proliferation, apparently by binding to, and regulating cellular transcription factors (Helin *et al.*, 1995; Beijersbergen and Bernards, 1996). It has been shown that pRB is frequently targeted by different classes of small DNA tumour viruses (Dyson *et al.*, 1989; Kaelin *et al.*, 1990; Munger *et al.*, 1990). The transforming properties of these viruses are dependent, in

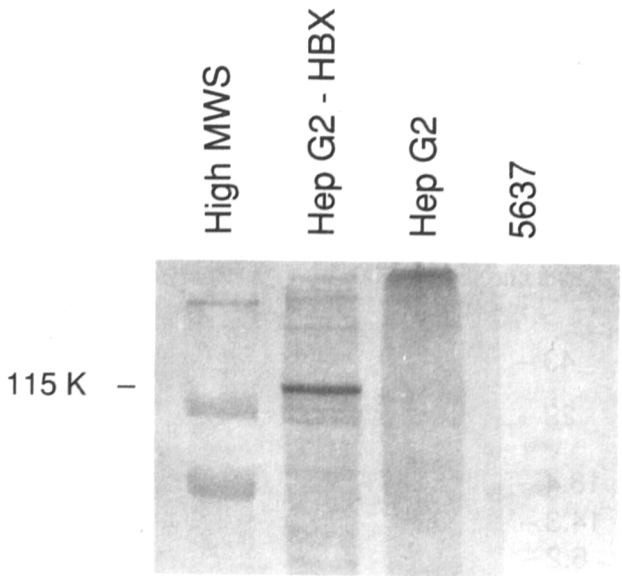

Fig. 4
Western blot analysis of pRβ in CMV-HBX-transfected Hep G2 cells
Hep G2-HBX = Hep G2 cells transfected with CMV-HBX vector. Hep G2 = Hep G2 cells transfected with CMV vector alone. 5637 = human bladder carcinoma cell line known to be negative for pRB. High MWS = molecular size markers with high M_r. The M_r of pRB is indicated at the left of the figure.

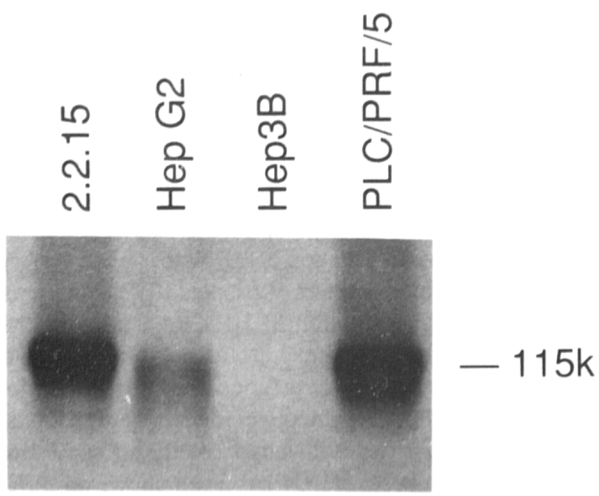

Fig. 5
Western blot analysis of pRB in Hep G2 and 2.2.15 cells
The cell line 2.2.15 is derived from Hep G2 cells and contains full HBV genome. PLC/PRF/5 and Hep3B are cell lines known to be positive and negative for pRB, respectively. The number at the right of the figure indicates M_r of pRB.

part, on the ability of their oncoproteins to bind to pRB and to block its function.

In this study, the effect of HBX on the expression of pRB, and a possible association between these two proteins was studied. The interaction between HBX and pRB was investigated by *in vitro* and *in vivo* binding assays. The results of the *in vitro* binding assay indicate that HBX did not co-precipitate with GST-RB. This was determined by the absence of the ^{35}S-labelled X protein in the gel, following incubation with GST-RB (Fig. 3). The labelled E7 protein of human papilloma virus, which has been shown to have high affinity for pRB (Munger *et al.*, 1990), was used as a positive control in binding to GST-RB. Similar results were obtained in a reciprocal experiment, in which ^{35}S-labelled pRB was mixed with GST-HBX fusion protein. No complex between HBX and pRB was detected in the *in vivo* binding assay either. This assay was performed by co-immunoprecipitation and Western blot analysis, using the anti-pRB MoAb and the anti-HBX serum, alternately (data not shown).

The effect of HBX on the expression of pRB was investigated by transfecting Hep G2 cells with a CMV-HBX clone. CMV-HBX encodes HBX gene under the control of the cytomegalovirus (CMV) promoter. After transfection, the cells were lysed, and the Western blot analysis was performed on the extracted protein using the anti-pRB MoAb. The results indicate that in the presence of HBX, the level of pRB increased considerably in Hep G2-HBX cells, compared to the pRB level in Hep G2 cells, transfected with CMV vector alone (Fig. 4). The 2.2.15 cells (Hep G2 cells transfected with complete HBV genome) demonstrated similar increases in the level of pRB expression (Fig. 5), as we have previously reported (Farshid *et al.*, 1994). It should be noted that the higher expression of pRB in the 2.2.15 cells could be a result of transactivation by a HBV gene(s) other than, or in addition to, the HBX gene.

The molecular mechanism of pRB activation by HBX has not been determined yet. HBX may alter the level of pRB through an indirect mechanism(s), mediated by transcriptional factors, and/or by acting on the components of cellular transduction pathways (Rossner, 1992; Kekule *et al.*, 1993). Interestingly, pRB detected in Hep G2-HBX and 2.2.15 cells was exclusively in a phosphorylated form, as indicated by its size (115 K) in Western blot analysis (Figs. 4 and 5). The phosphorylated pRB has been shown to be its inactive form (Mihara *et al.*, 1989). Thus, HBX may inactivate pRB by inducing its phosphorylation and/or by stabilizing its phosphorylated form, thereby contributing to the malignant transformation of HBV-infected liver cells.

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