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• VIRAL HEPATITIS •

Expression of hepatitis B virus X protein in transgenic mice

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Abstract

AIM: To establish a mice model harboring hepatitis B virus *x* gene (adr subtype) for studying the function of hepatitis B virus X protein, a transactivator of viral and cellular promoter/enhancer elements.

METHODS: Expression vector pcDNA3-*HBx*, containing CMV promoter and hepatitis B virus *x* gene open reading fragment, was constructed by recombination DNA technique. Hela cells were cultured in DMEM and transfected with pcDNA3-*HBx* or control pcDNA3 plasmids using FuGENE6 Transfection Reagent. Expression of pcDNA3-*HBx* vectors in the transfected Hela cells was confirmed by Western blotting. After restriction endonuclease digestion, the coding elements were microinjected into male pronuclei of mice zygotes. The pups were evaluated by multiplex polymerase chain reaction (PCR) at genomic DNA level. The *x* gene transgenic mice founders were confirmed at protein level by Western blotting, immunohistochemistry and immunogold transmission electron microscopy.

RESULTS: Expression vector pcDNA3-*HBx* was constructed by recombination DNA technique and identified right by restriction endonuclease digestion and DNA direct sequencing. With Western blotting, hepatitis X protein was detected in Hela cells transfected with pcDNA3-*HBx* plasmids, suggesting pcDNA3-*HBx* plasmids could express in eukaryotic cells. Following microinjection of coding sequence of pcDNA3-*HBx*, the embryos were transferred to oviducts of psedopregnant females. Four pups were born and survived. Two of them were verified to have the *HBx* gene integrated in their genomic DNA by multiplex PCR assay, and named C57-TgN(*HBx*)SMMU1 and C57-TgN(*HBx*)SMMU3 respectively. They expressed 17KD X protein in liver tissue by Western blotting assay. With the immunohistochemistry, X protein was detected mainly in hepatocytes cytoplasm of transgenic mice, which was furthermore confirmed by immunogold transmission electon microscopy.

CONCLUSION: We have constructed the expression vector pcDNA3-*HBx* that can be used to study the function of *HBx* gene in eukaryotic cells *in vitro*. We also established *HBx* gene (adr subtype) transgenic mice named C57-TgN (*HBx*) SMMU harboring *HBx* gene in their genome and express X protein in hepatocytes, Which might be a valuable animal system for studying the roles of *HBx* gene in hepatitis B virus life cycle and development of hepatocellular carcinoma *in vivo*.

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INTRODUCTION

Human hepatitis B virus (HBV) is the prototype for a family of viruses, referred to as *Hepadnaviridae*[1,2]. It has at least 4 subtypes, ayw, adr, ayr, and adw, among which adr is the most prevailing subtype in China. The complete genomic DNA of subtype adr has been cloned and demonstrated only 3.2kbp in length, andwhich is different from the other 3 subtypes in DNA and protein sequence[3]. HBV genome has 4 open reading frames (ORFs), including envelope genes coding region (*pre-s1*, *pre-s2* and *s* gene coding region), precore (*pc*) gene and core(*c*) gene coding region, polymerase (*p*) gene coding region, *x* gene coding region[4-6].

Chronic HBV infection is associated with a high incidence of liver disease, including hepatocellular carcinoma (HCC)[7-9]. Based on epidemiologic studies involving chronic HBV infection, it is estimated that the relative risk of developing HCC for HBV carriers may be 100- to 200-fold higher than that for non-carriers. It is proposed that the role of HBV played in HCC predisposition is modifying host gene regulation. Integration of viral DNA into the host genome can mediate host gene deregulation by a variety of mechanisms[10-15]. X protein may alter host gene expression leading to the development of HCC[16]. It has been demonstrated that X protein is a transactivator of a variety of viral and cellular promoter/enhancer elements and can mediate the activation of signal transduction pathways. Besides, it may affect DNA repair, cell cycle control, and apoptosis[17-22]. It is now clear that X-defective virus is unable to initiate infection *in vivo*. However, the physiological role of X protein during the course of an infection remains a major issue unresolved in hepadnavirus biology[23-27].

To explore the function of *HBx* gene *in vivo*, we generated transgenic mice harboring *HBx* gene from subtype adr by microinjection method, in which *HBx* gene could be expressed. This model might be valuable for the study of *HBx* biology and its associated biomedical issues *in vivo*.

MATERIALS AND METHODS

Reagents, antibodies, cells and animals

Restriction Endonucleases and T_4 DNA ligase were obtained from Promega Co. (USA). The mouse monoclonal antibody against X protein was purchased from DAKO (USA). Sheep anti mouse IgG-HRP was obtained from CALBIOCHEM (Germany). Gel extraction kit was purchase from QIAGEN. Hela cells were preserved in our laboratory. C57BL/6 mice were maintained in our Transgenic Animal Laboratory (SPF level).

Plasmid constructions

Plasmids pBR322-HBV (containing two tandem copies of the HBV genome of adr subtype) and pcDNA3 (containing CMV promoter) were preserved in our laboratory. Expression plasmid pcDNA3.1 (containing CMV promoter) was generously provided by Dr. Yu Hong-Yu. An 0.894-Kilobase pair DNA fragment containing *HBx* gene was isolated by gel extraction from plasmid pBR322-HBV after *Hind*III and *Bgl*II restriction digest. The fragment was then subcloned into plasmid pcDNA3.1 that has been digested by *Hind*III and *BamH*I to yield intermediate plasmid pcDNA3.1-*HBx*, which was employed as a template for polymerase chain reaction (PCR) amplification of the *HBx* coding fragments. The primers (A: 5' -ACACA AGCTT CATAT GGCTG CTCGG G-3' , B: 5' -CATGA ATTCT AGATG ATTAG GCAGA GGTG-3') were synthesized by Sangon Co. (Shanghai). Thirty five cycles of amplification were done in a total volume of 50 μl with an annealing temperature of 58 ℃. PCR product and pcDNA3 were isolated after *Hind*III and *Xba*Ⅰ digestion. After ligation, the plasmid of pcDNA3-*HBx* was confirmed by restriction endonucleases digestion and direct DNA sequencing.

Cell culture and DNA transfection

Hela cells were cultured in DMEM (Gibco) supplemented with 10 % FCS (Gibco) to confluence. Cells at 50 % confluency were transfected with pcDNA3-*HBx* or contol pcDNA3 plasmids using FuGENE6 Transfection Reagent (Roche) with a total of 1 ug of DNA per 3.5-cm plate of cells. Selection in medium containing geneticin (G418; Gibco) at a concentration of 500 μg/ml was started 48 hours later. After 2 weeks selection, positive clones that were named Hela-*HBx* were isolated and further expanded.

Assay pcDNA3-HBx expression in hela cells

Hela-*HBx* cells cultured in 10-cm dishes were rinsed with phosphate-buffered saline (pH7.4) three times and collected in a microcentrifig tube by trypsinzation. Cells were lysed with lysis buffer[18]. Supernatants were then diluted 5 times with phosphate-buffered saline (pH7.4) to assay the expression of the transfected pcDNA3-*HBx* vectors in Hela cells by Western blotting.

Microinjection and production of HBx transgenic mice

The pcDNA3-*HBx* plasmid was digested by *Sal* I and purified by gel extraction (Qiagen gel extraction kit). Purified coding fragment containing CMV promoter and *HBx* ORF were dissolved in TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH7.5) at a final concentration of 2 ug/L (-4 000 copies/pl) and microinjected into zygotes. Microinjection and embryo manipulation were performed according to standard protocols.

Analysis of HBx gene integration

Genomic DNA was extracted from tail tissue of pups mice or normal mouse and dissolved in TE buffer. It was used for PCR assays to identify founders of transgenic mice with *HBx* gene. In order to set an internal control of the efficiency of PCR amplification, we developed a multiplex PCR, using two sets of primers to amplify the *HBx* gene and the autosomal *IL3* gene in the same reaction tube[28,29]. PCR reaction was performed using 1 μl of dissolved DNA, 0.2 μm *HBx* gene specific primers (C: 5' -GGACG TCCTT TGTCT ACGTC CCGTC-3' , D: 5' -CCTAA TCTCC TCCCC CAACT CCTCC-3' , synthesized by Sangon Co. /Shanghai), and 0.1 μm *IL3* gene specific primers (E: 5' -GGGAC TCCAA GCTTC AATCA-3' , F: 5' -TGGAG GAGGA AGAAA AGCAA-3' , synthesized by Sangon Co. / Shanghai) in a total volume of 50 μl according to the cycling program: 94 ℃, 40 s; 61 ℃, 40 s; 72 ℃, 60 s; 35 cycles.

Analysis of HBx gene expression in transgenic mices

Western blotting Liver samples were obtained from the transgenic mice with *HBx* gene and normal C57BL/6 mice. Specimens (approximately 100 mg) were homogenized in a screw-capped 1.5 ml microcentrifage tube and lyzed in lysis buffer (0.5 % Nonidet P-40, 10 mM Tris (pH7.4), 150 mM NaCl, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluorid). 100 mg lysate was separated via 15 % SDS-polyacrylamide gel electrophoresis with Tris-Glycine buffer (pH8.3). One electrophoresis gel was stained with commassie brilliant blue R-250, and another was blotted to nitorcellulose filter. After blocked with 50 g/L defatted milk, the filter was incubated with X protein mouse monoclonal antibody for 40 min at 37 ℃, then washed with TBS (three times, 15 min each time) and incubated with HRP-conjugated sheep anti-mouse IgG for 30 min at 37 ℃. Finally, the filter was incubated with peroxidase substrate solution Diaminobenzidine (DAB) for 5 min to visualize the positive bands.

Immunohistochemistry analysis Hepatic tissue samples were fixed in 10 % neutral buffered formalin, paraffin-embedded and sectioned. Briefly paraffin-embedded sections were blocked with 3 % hydrogen peroxide (H_2O_2) for 10 min at 37 ℃ and washed with PBS. Subsequently, the sections were incubated in the X protein mouse monoclonal antibody (diluted 1:100) for 2hr at 37 ℃. After washing with PBS, the sections were incubated in horseradish peroxidase-labeled sheep anti mouse IgG (diluted 1:50) for 40 min at 37 ℃. Washed with PBS three times, the sections were subjected to color reaction with 0.02 % 3, 3-diaminobenzidine tetrahydrochloride containing 0.005 % H_2O_2 in PBS and counterstained with hematoxylin lightly.

Immunogold transmission electron microscopy The immunohistochemical X protein-positive mouse liver tissue was selected to be cut into small pieces (0.1 cm in diameter) and fixed first in 2 % paraformaldehyde and 0.5 % glularaldehyde mixture buffer for 2hr at 4 ℃, washed three times with PBS, acted upon by 0.25 % Triton X-100 for 10 min. After being blocked with blocking buffter, the pieces were incubated with X protien mouse monoclonal antibody over night at 4 ℃, washed with TBS and incubated with avidin-gold (15 nm) for 2hr at room temperature; then postfixed in 1 % osmiam tetroxide for 1hr at room temperature, dehydrated in gradient ethanol and embedded in epoxy resin. The sections were cut on an LKB Ultralome III, mounted on copper grids, stained with uranyl acetate and led citrate, and examined by transmission electron mocroscopy.

RESULTS

pcDNA3-HBx vector construction and expression in Hela cells

A 0.465kb *HBx* gene was amplificated from HBV genomic DNA and subcloned into the expression vector pcDNA3, with which the pcDNA3-*HBx* was constructed. The sequence of *HBx* gene in the plasmid was coincident with that reported befoer[3], as identified by restriction endonucleases digestion and confined by DNA direct sequencing. After purification by gel extraction, pcDNA3-*HBx* plasmids were transfected into Hela cells. Positive clones, Hela-*HBx* cells were isolated by G418 selection. With Western blotting, hepatitis X protein was detected in Hela cells, suggesting pcDNA3-*HBx* plasmids expressed in eukaryotic cells (Figure 1).

Production of transgenic mice

The pcDNA3-*HBx* plasmid was digested by *Sal* I and target fragments containing CMV promoter and *HBx* ORF were purified by gel extraction. Target fragments then were microinjected into male pronuclei of zygotes from C57BL/6

mice. 45 zygotes were microopreated. 41 microinjected eggs were implanted into oviducts of 3 pseudopregnant recipient mice, and 4 pups were born and survived. The born rate was 11 %. By multiplex PCR screening, two of the pups were identified to harbour *HBx* gene in their genomic DNA, named C57-TgN *H*MU1 and C57-TgN(*HBx*)SMMU3 (Figure 2).

Figure 1 Detected hepatitis X protein expression in transfected Hela cells. M_r17 000 X protein was detected in Hela-*HBx* cells. Lane1: control cells; Lane 2: Hela-*HBx* cells.

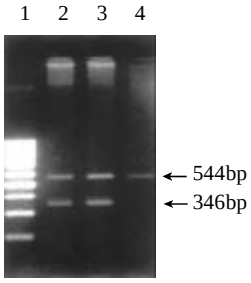

Figure 2 Analysis of *HBx* gene integration in transgenic mice by PCR. 544 bp *IL3* gene fragment and 346 bp fragment were amplified with positive and negative primers. Lane 1: 100 bp ladder Marker; Lane 2: C57-TgN (*HBx*) SMMU1 transgenic mice; Lane3: C57-TgN(*HBx*)SMMU3 transgenic mice; Lane 4: normal C57BL/6 mice.

Expression of HBx gene in transgenic mouse

To detect the expression of hepatitis X protein in transgenic mice, liver samples were obtained from C57-TgN(*HBx*) SMMU1 mice and normal C57BL/6 mice. Specimens were homogenized and lyzed in lysis buffer. 100 mg lysate was used to assay HBX protein. A component of relative molecular mass 17 000 befitting the X protein was specifically detected with anti X protein monoantibody by Western blotting (Figure 3A), suggesting the transgenic mice with *HBx* gene could express X protein in the liver tissue. The distribution of X protein in hepacytes was determined by immunohistochemisty and immunogold electron microscopy, which revealed that X protein was mainly distributed in hepatocytic cytoplasma, little on plasm membrane and in nucleus (Figure 3B-3D).

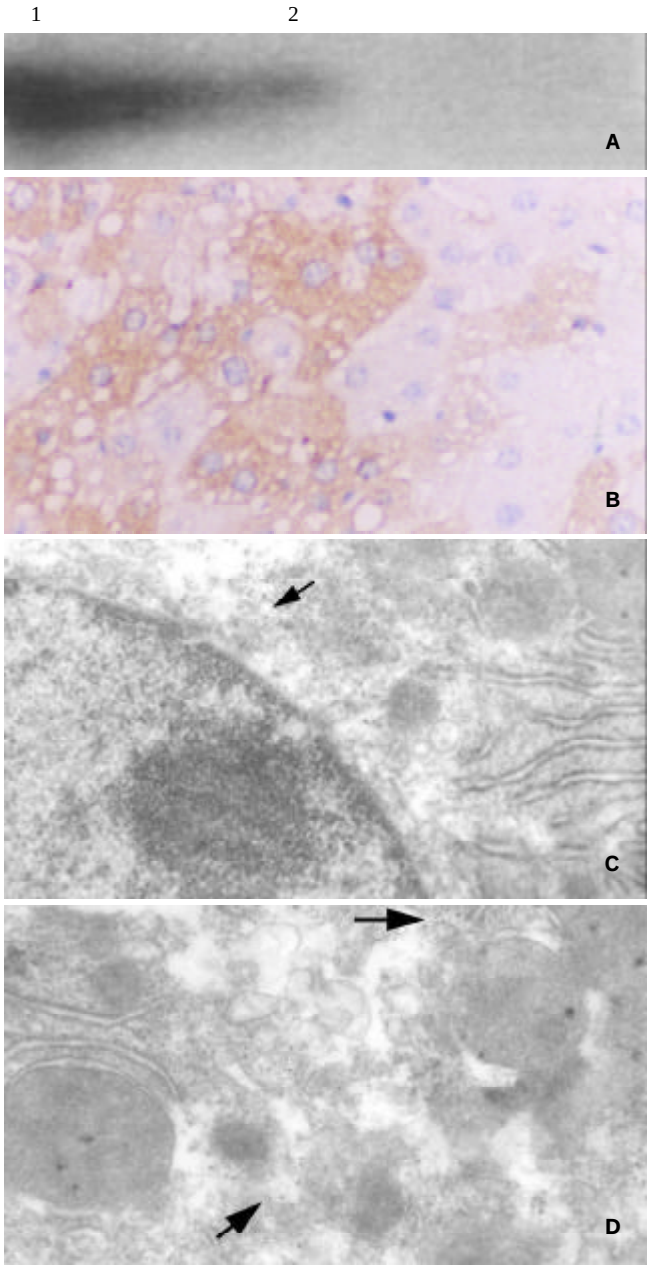

Figure 3 Expression of *HBx* gene in transgenic mice. (A) Western blot analysis of *HBx* gene expression in liver tissue from transgenic mouse. M_r 17 000 X protein was detected with X protein mouse monoclonal antibody; Lane 1: C57-TgN(*HBx*) SMMU1 transgenic mice; Lane 2: normal C57BL/6 mice; (B): *HBx* gene expression mainly in hepatocytes cytoplasm of C57-TgN(*HBx*)SMMU1 transgenic mice demonstrated by immunohistochemistry (×132). (C, D) *HBx* gene Expression in hepaticyte cytoplasm of C57-TgN (*HBx*) SMMU1 transgenic mice detected by immunogold electron microscopy (arrows) 3C: ×20 000; 3D: ×40 000.

DISCUSSION

Transgenic mice are the valueble animal models to study the functions of genes[30]. Although transgenic mice containing different HBV genes, including the entire viral genome, have been established and analysed before, there is little evidence to suggest that the virus plays a direct role in inducing hepatocellular carcinoma[31-41]. Hepatitis X protein is essential for HBV genes expression and replication[42,43]. *In vitro*, X protein exhibits a plethora of activities. From cell culture studies, it is believed that X protein can activate the transcription of host genes, including the major histocompatibility complex and c-myc, as well as viral genes. Aside from the transactivation of many promoters, the other activities linked to X protein include stimulation of signal transduction and binding, to various degrees, to well-known protein targets such as p53, proteasome subunit, and UV-damaged DNA binding protein[44-58].

However, the role of *HBx* gene in the course of HBV infection and in inducing HCC is unknown. In the present study, we constructed an *HBx* gene (adr subtype) expression vector pcDNA3-*HBx* containing CMV promoter and *HBx* gene ORF. By Western blotting, we found that it could express X protein in eukaryotic cells. pcDNA3-*HBx* may be a useful vector to study the role of X protein and explore the machansim of transactivation *in vitro*. We also generated two founders of transgenic mice with *HBx* gene(adr subtype) by microinjections,

named C57-TgN(*HBx*)SMMU1 and C57-TgN(*HBx*)SMMU3, which harboured *HBx* gene in their genomic DNA. The birth rate of the pups was lower than that of other transgenic mice, including entire hepatitis viral genome transgenic mice. This indicated that X protein was probably involved in some phases of development. The hepatitis X protein was expressed in the liver tissue of transgenic mice and distributed mainly in hepacytes cytoplasm by Western blotting, immunohistochemisty and immunogold electron microscopy, which suggested that the transgenic mice could be an important tool in studying the function of *HBx* gene *in vivo*. Besides, we also developed a multiplex PCR to rapidly and accurately screen the transgenic mice with *HBx* gene. This method, using an optimized ratio of primer pairs, allows for the detection of *HBx* gene in transgenic mice, which can not only amplificate target genes, but also show its amplification efficiency.

In conclusion, we have established *HBx* (adr subtype) transgenic mice as a model systerm for defining the function of *HBx* gene (adr subtype) and the role of X protein in the virus life cycle and HCC. And the multiplex PCR is a rapidly and accurately method to detect the transgenic mice with *HBx* gene.

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