Expression of hepatitis B virus X protein in transgenic mice

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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 3.2kb in length, and which 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[4,5].

Chronic HBV infection is associated with a high incidence of liver disease, including hepatocellular carcinoma (HCC)[6-9]. 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[10-12]. X protein may alter host gene expression leading to the development of HCC[13]. 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 hepatadnavirus 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 T4 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 HindIII and BglII restriction digest. The fragment was then subcloned into plasmid pcDNA3.1 that has been digested by HindIII and BamHI 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 °C. PCR product and pcDNA3 were isolated after HindIII and XbaI 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 control 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 genetin (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 microcentrifug tube by trypsinization. Cells were lysed with lysis buffer⁴¹. 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. PCR reaction was performed using 1 µl of dissolved DNA, 0.2 µM HBx gene specific primers (C: 5'-GGACG TCCTC TTGTCT AGGTG CGTC-3', D: 5'- CCTAA TACTC TCCCC CAACC CCTCTC-3'), synthesized by Sangon Co. (Shanghai), and 0.1 µM IL3 gene specific primers (E: 5'-GGGAC TCCCA GCTTC ATTAG CCAGA-3', F: 5'-TGAG GAGAG AGAAA CCGAA-3'), synthesized by Sangon Co. (Shanghai) in a total volume of 50 µl according to the cycling program: 94 °C, 40 s; 61 °C, 40 s; 72 °C, 60 s; 35 cycles.

**Analysis of HBx gene expression in transgenic mice**

**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 microcentrifuge tube and lysed in lysis buffer (0.5 % Nonidet P-40, 10 mM Tris (pH7.4), 150 mM NaCl, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluoride). 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 nitrocellulose filter. After blocked with 50 g/L defatted milk, the filter was incubated with X protein mouse monoclonal antibody for 40 min at 37 °C, then washed with TBS (three times, 15 min each time) and incubated with HRP-conjugated sheep anti-mouse IgG for 30 min at 37 °C. 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 (H₂O₂) for 10 min at 37 °C and washed with PBS. Subsequently, the sections were incubated in the X protein mouse monoclonal antibody (diluted 1:100) for 2hr at 37 °C. After washing with PBS, the sections were incubated in horseradish peroxidase-labeled sheep anti mouse IgG (diluted 1:50) for 40 min at 37 °C. Washed with PBS three times, the sections were subjected to color reaction with 0.02 % 3, 3-diaminobenzidine tetrahydrochloride containing 0.005 % H₂O₂ 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 in 2 % paraformaldehyde and 0.5 % glutaraldehyde mixture buffer for 2hr at 4 °C, washed three times with PBS, acted upon by 0.25 % Triton X-100 for 10 min. After being blocked with blocking buffer, the pieces were incubated with X protein mouse monoclonal antibody over night at 4 °C, washed with TBS and incubated with avidin-gold (15 nm) for 2hr at room temperature; then postfixed in 1 % osmium 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 microscope.

**RESULTS**

**pcDNA3-HBx vector construction and expression in Hela cells**

A 0.46kb HBx gene was amplified 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 before⁴¹, 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

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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 HMU1 and C57-TgN(HBx)SMMU3 (Figure 2).

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 hepatocytes was determined by immunohistochemistry and immunogold electron microscopy, which revealed that X protein was mainly distributed in hepatocytic cytoplasm, little on plasm membrane and in nucleus (Figure 3B-3D).

**DISCUSSION**
Transgenic mice are the valuable 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 mechanism of transactivation in vitro. We also generated two founders of transgenic mice with HBx gene(adr subtype) by microinjections,
named C57-Tg(NHBx)SMMU1 and C57-Tg(NHBx)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 hepatocytes cytoplasm by Western blotting, immunohistochemistry 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 amplify target genes, but also shows its amplification efficiency.

In conclusion, we have established HBx mice, which can not only amplify target genes, but also indicate that X protein was probably involved in some phases of cell cycle checkpoint controls. The transgenic mice could be an important tool in studying the function of HBx gene in vivo. And the multiplex PCR is a rapidly and accurately method to detect the transgenic mice with HBx gene.

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