

Establishment of transgenic mouse harboring hepatitis B virus (adr subtype) genomes

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INTRODUCTION

Hepatitis B virus (HBV) belongs to the group of hepatovirus, a major pathogen of human acute and chronic hepatitis B^[1-4], which has a very close association with human hepatocellular carcinoma (HCC)^[5-8]. For example, a statistical data from a hospital in Shanghai showed that 80% of HCC patients were positive for HBsAg (personal communication). The published data reported that people infected with HBV have a 200-fold greater risk of developing HCC than age-matched population not infected with HBV^[9-11]. So HBV infection, including its consequences, is one of the key problems for the human health in the world. Since HBV only infects human beings and a few kinds of higher primates^[12-14], the lack of culture cell and animal model impede the study of biological characteristics of HBV and human hepatitis B in the laboratory^[15,16]. In 1985, the first transgenic mouse carrying HBV genes was generated^[17], and thereafter the transgenic mouse system was applied in HBV studies^[18-26].

HBV prevailing in the world has at least 4 subtypes^[27-30], ayw, adr, ayr and adw, among which subtype adr is the most prevailing in China^[31,32]. The complete genome of subtype adr has been cloned and analyzed from HBV infected

population in China. It was identified that subtype adr was different from other 3 subtypes in DNA and protein sequence^[33]. In order to produce the animal model for exploring adr subtype of HBV associated biomedical issues, we generated transgenic mice harboring complete genome from subtype adr of HBV by microinjection method, in which the HBsAg gene and HBeAg gene could be expressed, and the genomic DNA of HBV could be replicated and packed into complete virus particles. This model will be very useful for examination of many aspects of HBV and its associated biomedical issues *in vivo*.

MATERIALS AND METHODS

HBV genome of *adr* subtype was obtained from plasmid *-padr-1-dimer*. C57 mouse strain was bought from Animal Center of Shanghai Birth Control Research Institute. PCR primers for identification were designed by ourselves according to S region of HBV genome, primer 1: 5'CCCAACCTCCAATCACTCACC3', primer 2: 5'ACGAACCACTGAACAAATGGC3', synthesized by GIBCO BRL company (USA). The thermostable DNA polymerase was bought from Sangon Co. (Canada), antigen-antibody ELISA kit of HBV was the product of Sino-American Bio-Engineering Co. (China). Primera gene Labeling System kit was bought from Promega Co. (USA). Antibodies for immunohistochemistry assay were products of Dako Co. (USA). Reagents for transmission electron microscopy were standard ones (China).

Preparation of HBV DNA and microinjection

padr-1-dimer has two copies of intact HBV (*adr* subtype) genome head to tail, cloned in BamHI site of plasmid pBR322 (Figure 1). Restriction enzymes *Sal*I and *Cla*I were used to digest *Padr-1-dimer*, followed by 7 g·L⁻¹ agarose gel electrophoresis of the digested products, the 7.0 kb band was retrieved, purified and dissolved in TE buffer for microinjection. Microinjection and embryo manipulation were performed according to the methods described in References^[34-36].

PCR and Southern-blotting of integrated transgenes

Genomic DNA from tail tissue of transgenic mice and normal mouse were prepared, and amplified with primers in S region according to the cycling program: 94°C, 30s; 60°C, 40s; 72°C, 45s; run 35

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cycles in 50 μL reaction system. Fifteen μL of PCR products were loaded on 15 $\text{g}\cdot\text{L}^{-1}$ agarose gel in the presence of 0.5 μg ethidium bromide per mL. 380 bp band was visualized by UV fluorescence. Southern blot was performed on genomic DNA by agarose electrophoresis of 30 μg restricted DNA with *Bam* HI. Nylon filtrates were hybridized with the probe including HBV genome labeled with $\alpha\text{-P}^{32}\text{-dATP}$ for further characterization of the transgenic mice.

HBV gene expression in transgenic mice

HBV gene expression in serum Venous blood in eye sockets of mice was collected, serum was isolated by centrifugation, and then used for HBsAg and HBeAg ELISA or RIA.

HBV gene expression in tissues The distribution of HBsAg and HBcAg was assessed by the labeled-avidin-biotin detection procedure. Briefly paraffin-embedded sections were treated for 10 min at 37°C with 30 $\text{mL}\cdot\text{L}^{-1}$ hydrogen peroxide and washed with PBS. Rabbit anti-HBsAg primary antiserum at 1:100 dilution, and goat anti-HBcAg primary antiserum at 1:200 dilution were applied over night at room temperature. After washing with PBS, a secondary antiserum consisting of biotin-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG was applied at 1:200 dilution for 40 min at 37°C, and then washed with PBS. Then the preparation was treated with 1:100 dilution ABC complex stained with DAB, and finally counterstained with hematoxylin.

Assay of serum antibody of transgenic mice

Fifty μL of the serum from transgenic mouse was assayed for anti-HBsAg, anti-HBeAg and anti-HBcAg by ELISA according to the Kit protocol.

Pathological findings analysis

Serum ALT and AST Serum ALT and AST were tested with auto-biochemical analyzer.

Histopathological study The specimens of liver, kidney, heart, spleen from transgenic mice were fixed in formalin, paraffin embedded, sectioned and stained with HE.

Examination of HBV Dane's particles in liver tissue from transgenic mice under TEM

Several immunohistochemically HBV-positive mouse liver specimens were fixed over night at 4°C in 40 $\text{mL}\cdot\text{L}^{-1}$ paraformaldehyde and 1 $\text{g}\cdot\text{L}^{-1}$ glutaraldehyde in PBS. They were then postfixed in 10 $\text{g}\cdot\text{L}^{-1}$ OsO_4 in cacodylate buffer for 1hr at room temperature, dehydrated in gradient ethanol, and embedded in epoxy resin. This sections were cut on an LKB Ultratome III, mounted on copper grids, stained in uranyl acetate and lead citrate, and examined with electron microscope.

RESULTS

Microinjection and embryo manipulation

Hundreds of molecules of target fragment were microinjected into male pronuclei of fertilized eggs. Of 355 fertilized eggs microinjected, 262 of manipulated eggs survived; the survival rate was 73.8%. Thirteen of female recipient mice were transplanted with microinjected eggs through one of the ovarioles, and 2 of the transplanted mice were pregnant; the pregnant rate was 15.4%. Six small mice were born and all of them survived; the survival rate was 100%.

Founder mice and the identification of HBV DNA integration in transgenic mice

PCR and Southern-blotting results indicated that 4 mice were positive. So four founders were obtained. The founder mice were named HB *adr* dimer-*x*. The positive rate was 66%. Then the founders were mated with the normal mice of the same line. 27 F_1 offspring of the founder mice were born. In 14 of them PCR showed positive genomic DNA PCR. The offspring of F_1 were 56 mice, 28 of them were positive, and the positive rate was 50%. The exogenous genes can be transmitted through the germ line (Figure 2).

HBV genes expression in transgenic mice

HBV gene expression in serum The HBsAg and HBeAg were detected in the serum with ELISA and RIA. And the expression level of HBsAg was higher than that of HBeAg, and the gene expression was different in different founders (Table 1). The concentration of HBsAg in the serum was about 3000 $\text{IU}\cdot\text{L}^{-1}$.

Table 1 The ELISA result of founder mice and normal mice (A450)

Antigen	Control		Transgenic mice			
	Negative	Positive	HB-2	HB-3	HB-4	HB-5
HBsAg	0.01	1.42	0.17	0.06	0.26	0.23
HBeAg	0.01	1.56	0.06	0.06	0.08	0.09

Distribution of HBsAg and HBcAg in transgenic mice

Immunohistochemical study of liver and kidney tissues from transgenic mice indicated that the distribution of positive hepatocytes was uneven, HBsAg-positive particles were located mainly in cytoplasm of hepatocytes. HBsAg expression level was weaker in kidney than in liver; the products of immune reactions of HBcAg-positive were not very certain, and mainly emerged in a few nuclei of hepatocytes from the individuals that were invariably HBsAg-positive (Figure 3). No obvious product of immune reaction from HBsAg and HBcAg positive mice was found in spleen, skin, testis and other detected tissues.

Serum antibody in transgenic mice The ELISA test indicated that there was no significant positive result of HBV antibodies in HBV transgenic mouse serum.

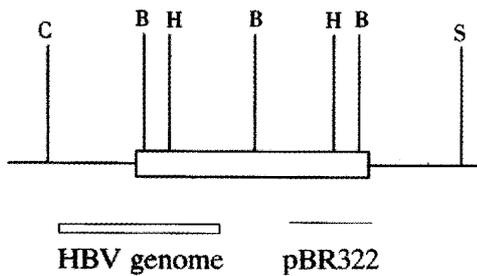


Figure 1 Restriction map and microinfection fragment of padr-1-dimer.

C: *Cla* I; B: *Bam* HI; H: *Hind* III; S: *Sall*

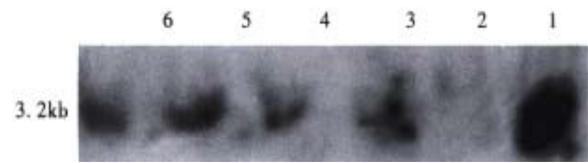


Figure 2 Southern-blot analysis of the HBV transgenic mice.

Lane 1: positive control; Lane 2: negative control;
Lane3-6: transgenic mouse

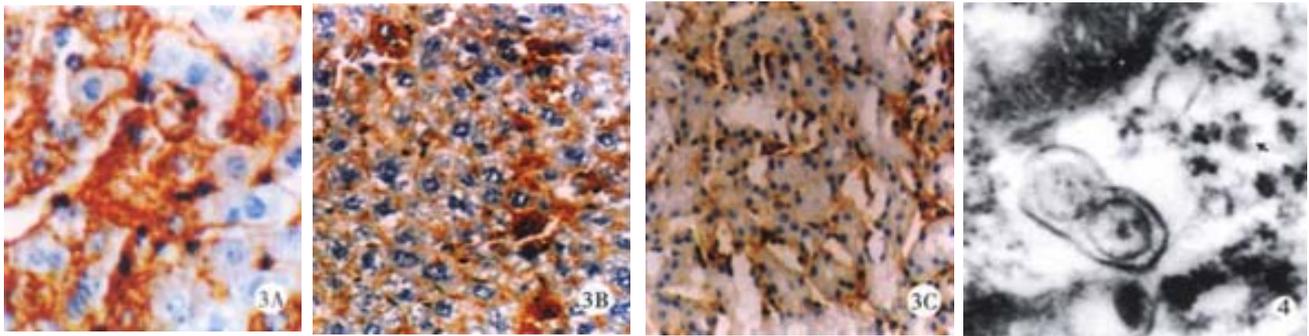


Figure 3 Immunohistochemical analysis of HBV antigens in transgenic mouse tissues.

A. HBsAg in the liver; B. HBcAg in the liver; C. HBsAg in the kidney

Figure 4 Dane's particles under electron microscope.

HBV Dane's particles in liver in transgenic mice under TEM HBsAg particles (spherical and filamental) were found in the endoplasmic reticulum of some hepatocytes, a few of in renal tubule-epithalia, and the cytoplasm of a few Kupffer cells (Figure 4).

Histopathological findings in transgenic mice

Histopathologic observation in transgenic mice tissues Mice have been monitored histologically for over 1 year without evidence of pathological changes in any organ, especially the liver and kidney. The serum ALT and AST assays indicated that there was no significant difference between the transgenic mice and the normal ones. ($P > 0.05$) (Table 2).

Table 2 Aminotransferase in the transgenic mice ($n = 12, \bar{x} \pm s$)

Mice	ALT(nkat·L ⁻¹)	AST(nkat·L ⁻¹)
Normal	698.5 ± 471.8	3597.4 ± 7669.9
Transgenic	708.5 ± 491.8	4349.2 ± 7248.1

DISCUSSION

The HBV transgenic mice show replication of the HB virus in the hepatocytes, but have no evidence of cytopathology of the liver and other organs or tissues.

In theory, HBV genes microinjected are complete in structure, and can be expressed and replicated in their bodies^[37,38], because we have purposefully prepared the fragment which contains

two copies of intact HBV (*adr* subtype) genomes head-to-tail to make it sure that all of four open reading frames of HBV genome are complete in structure. Four of the founders all showed serum HBsAg-positive, but the expression level is different. We think this is due to the different integrated site of HBV DNA. In general, the exogenous gene will be integrated into multi-sites in transgenic mice. This is similar with the other reports^[39,40].

Immunohistochemical assays of several tissues from transgenic mice revealed that HBV gene tissue-dependent expressions were tissue-dependent, and the genes were mainly expressed in the liver and kidney, but the expression level was different; this is similar to HBV-infection in nature. Examination of liver and kidney tissue from transgenic mice under transmission electron microscope revealed the existence of Dane's particles and HBsAg particles in hepatocytes, which confirmed the ability of HBV genome replication in the transgenic mice.

Serum anti-HBs, anti-HBc and anti-HBe detected by ELISA were negative in all of the transgenic mice, and suggested that these mice were tolerant to HBsAg, HBcAg and HBeAg; this result was consistent with that of other author's reports^[41]. Now, we obtain the F₃ generation positive mice. The HBV DNAs were able to be transmitted through the germ line of the mice from one generation to another.

The results of our experiments shows that HBV genome introduced by microinjection is integrated into the mouse genome, and the HBV genes can be expressed, replicated and packaged. So these lines of HBV transgenic mice may be used as the animal model for the study on HBV and its associated biomedical issue. And it can also be applied in the selection of anti-HBV drugs and vaccine^[42,43]. It is also useful for exploring the mechanism of HBV infection.

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