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The cloning of 3'-truncated preS/S gene from HBV genomic DNA and its expression in transgenic mice

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INTRODUCTION

Hepatitis B virus (HBV) is regarded as one of the main etiologic factors involved in the development of human hepatocellular carcinoma (HCC)[1-20]. The open reading frame (orf) of *X* gene of HBV encoded a transactivating factor is the evidence that strongly supported the notion that the *X* gene of HBV DNA integrated in HCC genomic DNA could contribute to the carcinogenesis of liver cells by activation of some related cellular genes *in trans*[8,9]. But it was found that the functional orf of *X* gene was absent in some HCCs harbouring HBV genomic DNA[6-14]. However, the 3'-truncated preS/ S sequence of HBV DNA, which also encodes a transcriptional transactivation factor, was found in all analyzed HCCs harbouring HBV genomic DNA[20-27]. These findings indicate that transactivation of some cellular genes by the expression product of 3'-truncated preS/S sequence of HBV integrated in the genomic DNA of liver cells is a possible mecha nism for HBV-associated oncogenesis[11]. The transcriptional transactivity also can be produced in the cultured cells transfected with an artificial 3'-truncated preS/S gene of HBV genomic DNA[1]. To explore the *in vivo* function of 3'- truncated preS/S region of HBV, we cloned the 3'-truncated preS/S region from wild-type HBV genomic DNA and constructed its expression vector for using in transgenic mice. Then, by using pronuclear microinjection method, we obtained two transgenic mouse lines expressing 3'-truncated preS/S region from 15 new born mice. These transgenic mouse lines are helpful to identify the function of the expression product of 3 '-truncated preS/S *in vivo* and the relationship between 3 '-truncated preS/S and HBV-associated oncogenesis.

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MATERIALS AND METHODS

Materials

Plasmids Vectors pBR322HBV carrying wild-type HBV genomic DNA and pBluscript were preserved in our laboratory. Expression vector pcDNA3.1, containing MCV promoter was provided by Dr. Yu Hong-Yu.

Cells *E. coli* DH5α was preserved in our laboratory.

Animals C57BL/6 and BALB/ c mice were preserved by our transgenic animal laboratory (SPF level). All mice were maintained on a 14:10 light-dark schedule (lights off at 10 pm, on at 8 am.).

Main reagents Restriction endonucleases, T_4 DNA ligase and DNA large fragment (klenow) ploymerase were purchased from Promega company. QIA quick gene gel kit and plasmid extraction kit were from QIA gene. Anti-HBV preS1 kit was purchased from α- company.

PCR primers design and synthesis Primers were synthesized by Sangon. Positive primer: 5' GGCCAGA- GGCAAATCAGGTAGGAGG 3', Negative primer: 5' TGGGTGAGGCAGTAGTCGG- AACAGG 3'. The primers are from 1607 to 1934bp of HBVadr genomic DNA sequence, containing 327bp. We also used the T_7 primer, upstream the positive primer.

Methods

Plasmid construction A 2.0kb fragment, containing 3'-truncat ed preS/S of HBV genome, was cut out of pBR322HBV digested with *Xba*-I and was subcloned into pBluescript, which was named pBluescript - *Xba* 2.0. The 3'-truncated preS/S region was obtained from pBluescript - *Xba* 2.0 digested with *Bst* E II and *xba* I. Its 3'-end was filled with klenow fragment and dNTPs, and inserted into *Bam* HI site of expression vector pcDNA3.1 which also filled, named pcDNA3.1 PreS/S. Restriction endonucleases digesting and sequencing were used to identify the construction.

Transgenic mice The pcDNA3.1-PreS/S DNA was purified and dissolved in TE buffer (10mM Tris-HCl, 0.2mM EDTA, pH 7.5) at a final concentration of 1mg/L (-2000 copies/pl). After pronuclear microinjection, the eggs were implanted into oviducts of pseudopregnant recipients to enable further development before term.

DNA isolation To isolate tail fragments from 10-day-old mice, approximately one third of the tail was cut and place into a screw-capped 1.5mL microcentrifuge tube containing 500μL of TB buffer. The tubes containing the tail fragments were incubated overnight at 55℃. They were extracted once with 500μL of 1:1 (v/v) equilibrated phenl-chloroform, and precipitated with 2 volumes of ethanol. After centrifugation, precipitates were resupended in 500μL water.

DNA analysis The PCR amplification conditions were used with Taq DNA ploymerase. For a 50μL reaction, mix the following components: 1μg template DNA, 0.5μL dNTP 10mm, 10u-Taq, 5μL PCR Buffer (10×), 41.5μL deionized water. We use the following cycling parameters: initial denaturation at 94℃ for 5min; followed by 35 cycles at 94℃ for 30s; 58℃ for 30s; and 72℃ for 1min; and then final extension at 72℃ for 7min. The products were run on a 2% agarose gel.

Expression analysis The 100μL of blood was extracted from the mouse developed from a microinjected eggs. After centrifuged in microfuge for 5min, the supernatants were isolated and analyzed by ELISA.

RESULTS

Clone of 3'-truncated preS/S and construction of its expression vector

The approximate 2.0kb fragment containing the preS/S was cloned from HBV genomic DNA, from which 3'-truncated preS/ S region was cut out and subcloned into the expression vector pcDNA3.1, and then was identified by the restrictive enzyme and sequence analysis. The results showed that the structure was identical with our design (Figures 1 and 2).

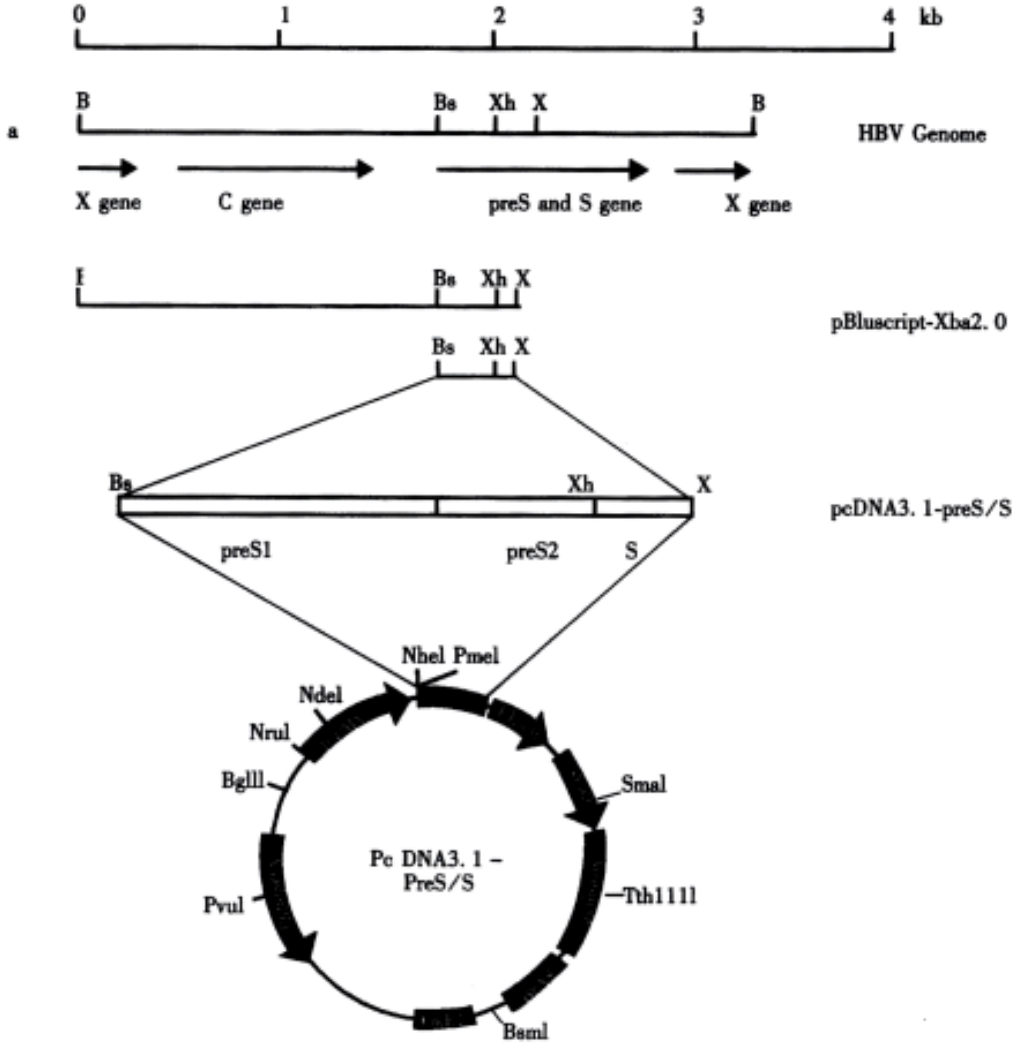
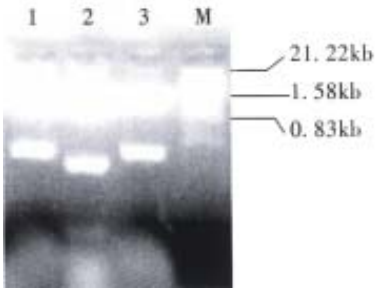
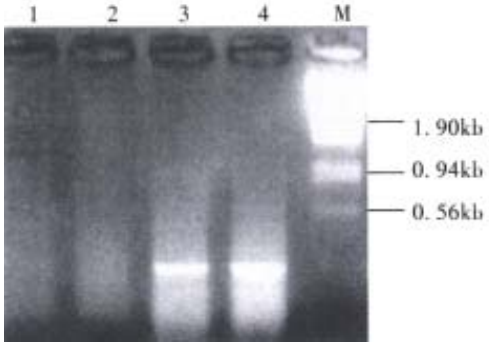


Figure 1 a: The HBV genomic sequence, cloned in pBR322. Positions of restriction site (B, *Bam* HI; Bs, *Bst* EII; X, *Xba* I; Xh, *Xho* I) b: 0.65kb fragment, containing the 3'-truncated preS / S. c: Construction of the vector (pcDNA3.1-preS / S) for expressing 3' -truncated preS / S.



Figures 2 The pcDNA3.1 digested with *Nhe* I, *Pme* I, *Xba* I and *Xho*I.
1: *Nhe* + *Xba* I; 2: *Xho* I + *Nhe* I; 3: *Pme* I + *Xba* I; M: λ/*EcoR* I+ *Hin*d-III marker



Figures 3 The DNA analysis of founder mice by PCR. 324bp fragment was amplified with positive and negative primers.
1: blank control; 2: PCR for genomic DNA of normal mice; 3: PCR for genomic DNA of transgenic mice (No.1); 4: PCR for genomic DNA of transgenic mice (No.3); M: λ/*Eco* RI + *Hind* III marker

Table 1 The ELISA results of the surme of transgenic mice expressing the 3'-truncated preS/ S at different times

Serial times (week)	Positive control	Negative control	Normal mice	Transgenic mice	
				No.1	No.3
15	0.21	0.01	0.00	0.14[a]	0.09[a]
19	0.34	0.04	0.00	0.11[a]	0.11[a]
23	0.31	0.04	0.01	0.10[a]	0.26[a]

[a]preS1 Ag-positive was defined as ⩾negative control ×2.1

Production of transgenic mice

The recombinant construct containing CMV promoter sequences fused to 3'-truncated preS/S region, which encodes a transcriptional transactivation factor, was microinjected into fertilizted eggs from C57BL/6 mice. Of 243 microinje cted eggs implanted into oviducts of 20 pseudopregnant recipient mice 15 developed to term and gave rise pups. However, only 7 of them survived and the others died within several hours after birth. DNA from the 7 mice were isolated and analyzed by PCR. It was found that 2 of them were positive for the injected tran sgene (Figure 3). Besides, we also noticed that the embryos from the microinjected eggs had a high miscarriage and mortality rate during the course of their development and growth in comparison with the experience in our laboratory.

Expression of 3'-truncated preS/ S gene in Transgenic mice

The serum samples were collected from the 2 mice harbouring the 3'-truncated preS/S region under the control of CMV promoter and the expression product of the recombinant gene in transgenic mice was analyzed by ELISA, in which the antibody against HBV preS1 was used as the first antibody. The results showed that 2 of them were positive for PreS1 (Table 1). Following the founders conformed, a series of expression analysis was carried out at different time points during the development. It was found that the 3'-truncated preS/S region could been stably expressed in the transgenic mice.

DISCUSSION

The full-length preS/S sequence integrated in nearly all HCCs can't show any trans-activity. However, one copy of the preS/ S sequence with 3'-truncation could show a definite trans-activity[1]. We constructed the expression vector of 3'-truncated preS/S gene, which can be expressed in cultured mammalian cells. So this vector should be very useful for exploring the biological function of expression product of 3'-truncated preS/S gene and for identifying whether 3'-truncated preS/S gene in HCCs is a causative factor of HBV-associated oncogenesis.

During the process of generating transgenic mice, the miscarriage rate and mortality rate seemed to be much higher than that in the producing transgenic mice harbouring other genes[28-30]. This phenomenon indicates that bes ide the some common reasons for the death of transgenic mice there might be some other factors. It is possible that there are some effects of the trans-activation of the expression product of the 3'-truncated preS/S like those of the 3'-truncated preS/S intergrated in HCCs of human being on the develop ment of mouse embroys and its early growing of the pups after birth.

The 2 transgenic mouse founders could express 3'-truncated preS/S sequences stably. These results indicate that the 3'-truncated preS/S is integrated in their genomic DNA, which is similar to those existing in the HCCs of human being. So we believe that the 2 transgenic mouse lines can be employed as the model for exploring the *in vivo* function of the expression product of 3'-truncated preS/S and relationship between 3'-truncated preS/S and HBV-associated oncogenesis.

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