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Overexpression of Hepatitis B Virus-binding Protein, Squamous Cell Carcinoma Antigen 1, Extends Retention of Hepatitis B Virus in Mouse Liver

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Abstract How receptors mediate the entry of hepatitis B virus (HBV) into the target liver cells is poorly understood. Recently, human squamous cell carcinoma antigen 1 (SCCA1) has been found to mediate binding and internalization of HBV to liver-derived cell lines *in vitro*. In this report, we investigate if SCCA1 is able to function as an HBV receptor and mediate HBV entry into mouse liver. *SCCA1* transgene under the control of Rous sarcoma virus promoter was constructed in a minicircle DNA vector that was delivered to NOD/SCID mouse liver using the hydrodynamic technique. Subsequently, HBV-positive human serum was injected intravenously. We demonstrated that approximately 30% of the mouse liver cells expressed a high level of recombined SCCA1 protein for at least 37 d. The HBV surface antigen was found to persist in mouse liver for up to 17 d. Furthermore, HBV genome also persisted in mouse liver, as determined by polymerase chain reaction, for up to 17 d, and in mouse circulation for 7 d. These results suggest that SCAA1 might serve as an HBV receptor or co-receptor and play an important role in mediating HBV entry into hepatocytes, although its role in human HBV infection remains to be determined.

Key words HBV receptor; HBV-binding protein; minicircle DNA plasmid; hydrodynamics-based procedure

Hepatitis B virus (HBV) is a human hepadnavirus that causes acute and chronic hepatitis and hepatocellular carcinoma [1]. As with other viral diseases, HBV infection is likely initiated by specific binding of the virus to cell membrane structures through one or several viral envelope proteins. HBV has not been propagated in established cell lines, and only humans and higher apes are susceptible to infection. HBV replication and cellular injury are largely confined to the liver, and the hepatocytes are considered the primary target cells for infection, whereas the significance of extrahepatic replication of HBV is not yet well understood.

The HBV envelope consists of three distinct coterminal proteins encoded by a single *env* gene. The domains of these proteins encoded by the pre-S region of the viral genome represent potential attachment sites of HBV to the hepatocyte, as pre-S1 and pre-S2 antibodies neutralize infectivity *in vitro* [2] as well as in experimental models [3].

Although the viral structures involved in attachment to the target cell have been identified, the cellular receptors for HBV have not yet been determined and the biochemical events leading to intention remain unknown. As well as hepatocytes, many cells of nonhepatic origin, such as hematopoietic cells of the B lymphocyte lineage, peripheral blood lymphocytes, and even some simian virus 40-transformed cell lines, have receptors for the pre-S1 domain at amino acid 21–47 region [4]. The pre-S1 domain of the large envelope protein has a partial sequence homology with the Fc moiety of the α chain of immunoglobulin (Ig) A [5], therefore a common receptor for the attachment of HBV and IgA to human liver cells has been proposed [6]. Interleukin 6, containing recognition sites for the pre-S1 domain, could mediate HBV-cell interaction [7]; and the transferring receptor might also play a role in the binding of HBV to hepatocytes through the pre-S2 protein sequence, as this domain is involved in the binding of hepatitis B

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surface antigen (HBsAg) particles with T cells [8]. Another HBV binding factor (HBV-BF) was identified in normal human serum interacting with the pre-S1 and pre-S2 epitopes of the viral envelope located within the protein domain involved in the recognition of hepatocyte receptors. Monoclonal antibodies to HBV-BF recognized a membrane component of the normal human liver cells but were unreactive with the hepatocyte membrane of other species, and with that of the HepG2 cell line. The results suggested that HBV-BF represents a soluble fragment of the membrane component and might be related to the HBV receptor mediating the attachment of HBV to human liver cells [9].

Squamous cell carcinoma antigen 1 (SCCA1), which is a member of the ovalbumin family of serine protease inhibitors, was found to play a major role in HBV infection and might be a new candidate for HBV-BF. By using tetravalent derivative chromatography from detergent-solubilized HepG2 plasma membranes, a 44 kDa HBV binding protein (HBV-BP) was found to closely correspond to human SCCA1. There are only three amino acid changes between them. Direct binding experiments confirmed the interaction of recombinant HBV-BP with the HBV pre-S1 domain. And in transfected cells, native HBV particle entry was enhanced. For example, HepG2 cells overexpressing HBV-BP after transfection with corresponding cDNA showed an increased virus binding capacity by two orders of magnitude compared with normal cells; and Chinese hamster ovary cells, which normally do not bind to HBV, acquired susceptibility to HBV binding after transfection. Both recombinant HBV-BP and antibodies to recombinant HBV-BP blocked virus binding and internalization in transfected cells as well as in primary human hepatocytes in a dose-dependent manner [10]. Although many candidates for the HBV receptor have been suggested in studies *in vitro*, none of them was proved *in vivo* that is much closer to the natural HBV infection in the human body.

In the present study, we get a special trangene mouse that expresses the HBV receptor candidate, the 44 kDa HBV-BP, in the liver cells by using a hydrodynamics-based intravenous injection procedure and a novel minicircle plasmid as vector. This novel and economical mouse model will be very useful in HBV receptor research.

Materials and Methods

Materials

Rabbit anti-SCCA1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Rabbit anti-HBsAg antibody was from ViroStat (Portland, USA). X-gal was from TaKaRa Bio. (Kyoto, Japan). The SABC kit was from Boster Biological Technology (Wuhan, China). The infectant human serum (HBV-DNA$^+$, 10^6 copies/ml) was provided by Prof. Ling LI (Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China). The HBV-DNA PCR detection kit was purchased from Da'an Gene Technology (Guangzhou, China). NOD/SCID mice were purchased from the Center of Experimental Animals (Sun Yat-Sen University). All other chemicals used were of analytical grade.

Plasmid

Minicircle-producing plasmid p2ΦC31 was a gift from Dr. M. A. KAY (Stanford University, Stanford, USA) [11]. SCCA1 cDNA was purchased from Open Biosystems (Huntsville, USA). p2ΦC31.SCCA1 was constructed by cloning *SCCA1* under the control of RSV promoter into the *Xho*I site of plasmid p2ΦC31. p2ΦC31.LacZ was constructed by placing the bacterial β-galactosidase (*LacZ*) gene under the control of CMV promoter into the *Xho*I site of plasmid p2ΦC31. Minicircles encoding CMV-LacZ (MC.LacZ) and RSV-SCCA1 (MC.SCCA1) were prepared according to the procedure described by Chen *et al.* [11]. Regular plasmid DNA was purified by using the CsCl-density gradient centrifugation method and kept in saline at –20 ºC until use. Purity of plasmid DNA was checked by absorbency at 260 and 280 nm and by 2% agarose gel electrophoresis.

X-gal staining

To examine the site of transgene expression, two mice were given 2 ml saline containing 5 μg of pMC-LacZ plasmid DNA through the tail vein, using a 2.5 ml latex-free syringe with a 0.45×16 RW LB needle (Shuang Ge, Shanghai, China). The injection rate was kept at 0.4 ml/s. The location and level of *LacZ* gene expression in mouse liver was determined by the X-gal staining method. Tissue sections (5 μm thick) were made 24 h after plasmid injection, stained with X-gal for 2 h, then counterstained in Nuclear Fast Red (Boster Biological Technology) for 30 s. The positive cells showed blue staining in the cytoplasm or nucleus and the connective tissues were stained pink.

Immunohistochemistry evaluation for SCCA1

Two mice were killed on day 3, 7, 17, 27 and 37 after injection of pMC-SCCA1, respectively. Paraffin sections of liver were analyzed for the presence of SCCA1 by

immunohistochemistry. For SCCA1 detection, rabbit polyclonal antibody SCCA1/2 (H-390) (Santa Cruz Biotechnology) was used at 1:200 dilution. Sections were incubated with primary antibody overnight at 4 ºC. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide, then the slides were heated in 10 mM sodium citrate in a microwave oven to block nonspecific protein binding in normal goat serum. Biotinylated goat anti-rabbit IgG (Boster Biological Technology) was then added at room temperature for 20 min. Samples were incubated with avidin-peroxidase and stained with a mixture of 3,3′-diamino-benzidine tetrahydrochloride and hydrogen peroxide (Boster Biological Technology).

In each case of immunohistochemistry experiment, sections incubated with the appropriate non-immune IgG were used as the negative control. For antibody specificity confirmation, human skin specimens for SCCA1 were used as the positive control and the liver samples from a normal mouse were used as the negative control.

In each case, the semiquantitative immunoreactivity of SCCA1 was independently evaluated by two pathologists. In all immunohistochemical analyses, necrotic areas and edges of tissue sections were not included in the counting to avoid possible immunohistochemical false positivity.

In vivo transfection

Animals were divided into four groups.

Group 1 (negative control 1, two mice): the mice did not receive injection.

Group 2 (negative control 2, two mice): the mice were injected through the tail vein with 5 μg pMC-SCCA1 in 2 ml saline, and killed 3 d later. Group 3 (negative control 3, 10 mice): the mice were injected through the tail vein with 2 ml saline. Twenty-four hours later the mice were injected through the tail vein with 0.3 ml of HBV-DNA$^+$ serum, and 1 d later the same quantity of serum was given to each mouse through the celiac artery. Two mice were killed on day 3, 7, 17, 27 and 37, respectively.

Group 4 (experimental group, 10 mice): the mice were injected through the tail vein with 5 μg pMC-SCCA1 in 2 ml saline and 24 h later the mice were injected through the tail vein with 0.3 ml of HBV-DNA$^+$ serum. One day later the same quantity of serum was given to the mice through the celiac artery. Two mice were killed on day 3, 7, 17, 27 and 37, respectively.

For each group, the liver and serum samples were collected at appropriate times.

Immunohistochemistry detection for HBsAg

Liver sections from the four groups above were analyzed for the presence of HBsAg by immunohistochemistry. For HBsAg detection, rabbit anti-HBsAg antibody (ViroStat) was used as the primary antibody at 1:200 dilution. The protocols were the same as for SCCA1 detection.

Enzyme-linked immunosorbent assay (ELISA) for HBsAg in mouse serum

The collected mouse sera were tested for HBsAg using an ELISA kit and the AxSYM system (Abbott Diagnostics, Chicago, USA) according to the manufacturer's protocols.

PCR detection of HBV-DNA in mouse serum and liver

The collected mouse sera were tested for HBV-DNA using the HBV-DNA detection kit (Da'an) according to the protocols.

The genomic DNA was extracted from 200–300 mg mouse liver tissue. Five hundred microliters of 1×lysis buffer (40 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA) and 20 μl of RNase solution (10 mg/ml) was added to each pre-cooled tube. The live tissue was homogenized while keeping the tube in the pre-cooled heating-block and transferred to a 15 ml F2097 tube, then 3500 μl of 1×lysis buffer, 300 μl of 0.10% sodium dodecylsulfate and 1000 μl of proteinase K solution (2.0 mg/ml) were added. The mixture was incubated at 55 ºC for 2 h and at 37 ºC overnight with shaking. Ten microliters of 10 mg/ml RNase was added, mixed and incubated at 55 ºC for 2 h with shaking. Two milliliters of saturated NaCl solution was added, mixed by pipetting up and down five times using a 5 ml pipetter, then centrifuged at 3500 *g* for 30 min using a clinical centrifuge. The supernatant was removed to a fresh tube, 1 ml of saturated NaCl solution was added, and the mixture was centrifuged for 30 min as above. The supernatant was removed to a fresh tube and two volumes of 100% ethanol was added. DNA will show up after mixing the solution by reversing the tube several times. The DNA was taken to a 1.5 ml Eppendorf tube, washed twice with 75% ethanol and centrifuged at 6000 *g* to remove as much ethanol as possible. The DNA was air dried at room temperature for 5 min, then 600 μl of TE buffer (1 mM EDTA, pH 8.0, 10 mM Tris-HCl) was added to dissolve it. Two microliters of the DNA solution was used as the template of following PCR. The mouse liver samples were tested for HBV-DNA using the HBV-DNA detection kit (Da'an) according to the protocols. Twelve microliters of samples was analyzed by 2.0% agarose gel electrophoresis. One day later the negative control PCR reaction was carried out with liver samples from a mouse injected with pMC-SCCA1 but not with infectant human serum. The positive control PCR

reaction was carried out with 2 μl of positive DNA template solution provided with the kit.

Results

We used *LacZ* gene as the reporter gene to identify the site of transgene expression. Twenty four hours after infusion of 5 μg of pMC-LacZ plasmid DNA, the animals were killed and *LacZ* gene expression was assessed in the liver tissue. *LacZ* gene expression in the liver tissue seems restricted within certain areas as X-gal-positive cells are clustered around the central vein [**Fig. 1(A)**].

The pattern of *SCCA1* transgene expression and the cell types that express SCCA1 protein were evaluated by immunohistochemical analysis [**Fig. 1(B,C)**]. Compared with the negative control, a number of cells in the liver tissue were stained positively by the SCCA1 antibody after injection of 5 μg pMC-SCCA1 plasmid into mice. The cells expressing SCCA1 are located around the central vein.

Positive cell counting was used to evaluate the persistence of *SCCA1* gene expression. **Table 1** shows that *SCCA1* gene expression was persistent at a stable level for at least 37 d after transfection with the positive cell ratio being approximately 30%.

It is evident that no cell was detected to be positively immunoreactive for HBsAg in the liver of the mouse injected with infectant human serum only (**Fig. 2**). The cells around the central vein and hepatic sinusoid, including the Kupffer cells, sinusoidal endothelial cells and some hepatocytes, were found to be positively immunoreactive up to day 17 of pMC-SCCA1 group. No positive cells were detected in the mice of day 27 and 37 of pMC-SCCA1 group.

Table 1 Ratio of positive cells in livers of mice injected with pMC-squamous cell carcinoma antigen 1

Mouse	Percentage at different time points				
	day 3	day 7	day 17	day 27	day 37
Mouse 1	33%	30%	33%	28%	30%
Mouse 2	30%	27%	30%	33%	26%

Cells were counted under a microscope. Shown is the average percentage of three random views.

The HBsAg qualitative ELISA analysis showed that the mouse serum on day 3 was positive and the others (day 7, 17, 27 and 37) were negative in the control group. In the experimental group, the mouse sera of day 3 and 7 were positive and the others (day 17, 27 and 37) were negative (**Table 2**).

Data in **Fig. 3** show that HBV DNA was detected only in the mouse serum and liver tissue of day 3 of control group injected with infectant human serum and not injected with pMC-SCCA1 in advance. However, HBV DNA was found to be positive in the liver of day 3, 7 and 17 and in the mouse serum of day 3 and 7 injected with pMC-SCCA1 24 h before injection with infectant human serum. No HBV DNA was detected in the liver or serum of day 27 or 37.

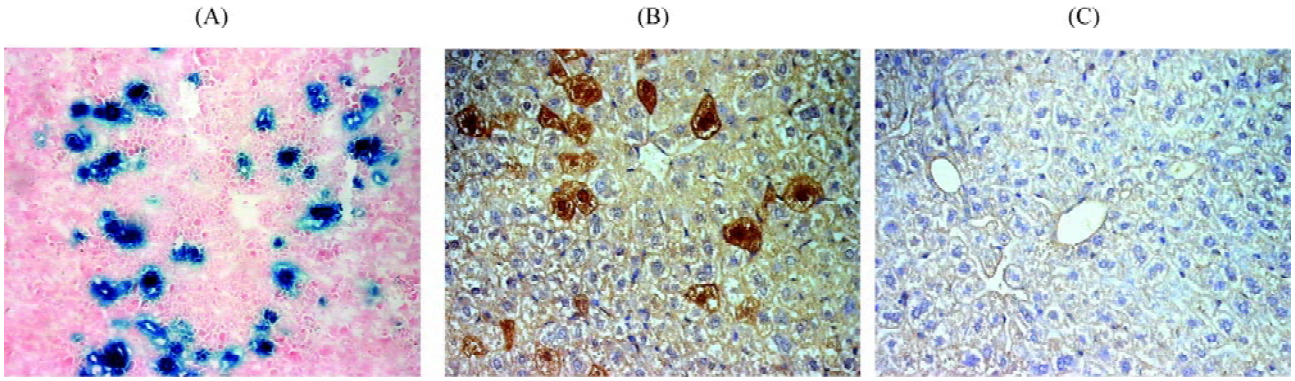

Fig. 1 SCCA1 transgene expression in mouse liver

(A) The mouse was injected with 2 ml of saline containing 5 μg of pMC-LacZ plasmid DNA. The animal was killed and the liver section was taken 24 h after injection. Sections were stained with X-gal solution at 37 ºC overnight followed by counterstaining with Nuclear Fast Red for 30 s. (B) Squamous cell carcinoma antigen 1 (SCCA1) transgene expression in mouse liver determined by immunohistochemistry. (C) Negative control for SCCA1 expression. The mouse was injected with 2 ml of saline. Magnification, 400×.

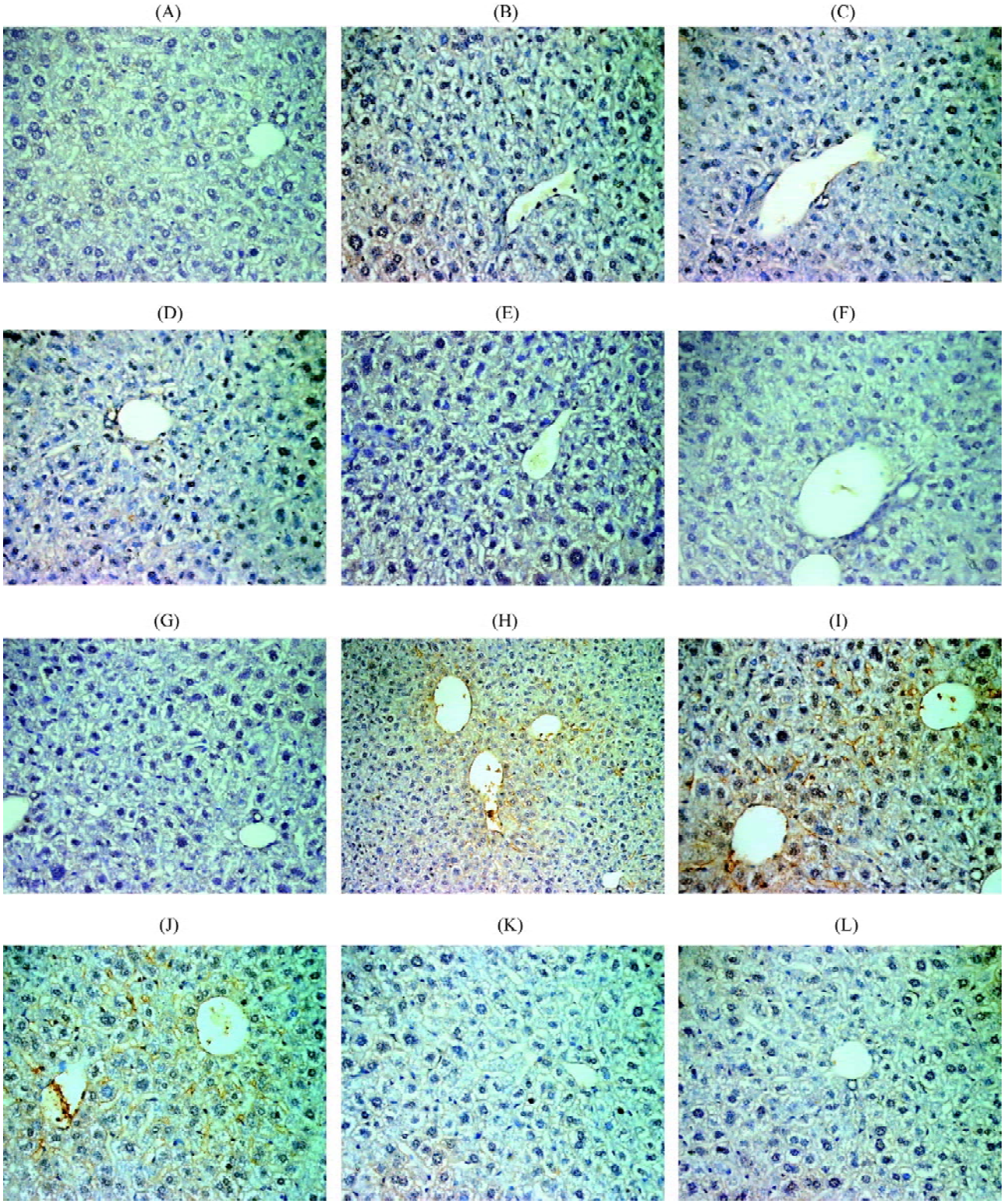

Fig. 2 Hepatitis B surface antigen in mouse liver determined by immunohistochemistry

(A) Negative control 1, normal mouse. (B–F) Negative control 3. Mice were injected with infectant human serum only and killed on day 3, 7, 17, 27 and 37, respectively. (G) Negative control 2. The mouse was injected with pMC-SCCA1 only. (H–L) Mice were injected with infectant human serum after pMC-SCCA1 injection, and killed on day 3, 7, 17, 27 and 37, respectively. The sinusoidal endothelial and Kupffer cells, and the hepatocytes near the central vein and the hepatic sinusoid were found to be positive (H–J) in the immunohistochemical test. Magnification, 400×.

Table 2 Hepatitis B surface antigen (HBsAg) qualitative enzyme-linked immunosorbent assay (ELISA) analysis of mouse serum

Group	S/N at different time points				
	day 3	day 7	day 17	day 27	day 37
Control group	+ (2.86)	– (1.28)	– (1.16)	– (1.20)	– (1.00)
	+ (2.93)	– (1.26)	– (1.16)	– (1.20)	– (1.20)
Experimental group	+ (10.80)	+ (3.08)	– (1.90)	– (1.26)	– (1.20)
	+ (10.00)	+ (3.90)	– (1.92)	– (1.26)	– (1.22)

In the control group, mice were injected with infectant human serum only. In the experimental group, mice were injected with pMC-SCCA1 24 h before injection with infectant human serum. The mouse serum was tested for HBsAg using an ELISA kit and an AxSYM system according to the manufacturers' protocols. S/N, the ratio of specimen to negative control. +, HBsAg positive (S/N≥2.10); –, HBsAg negative (S/N<2.10).

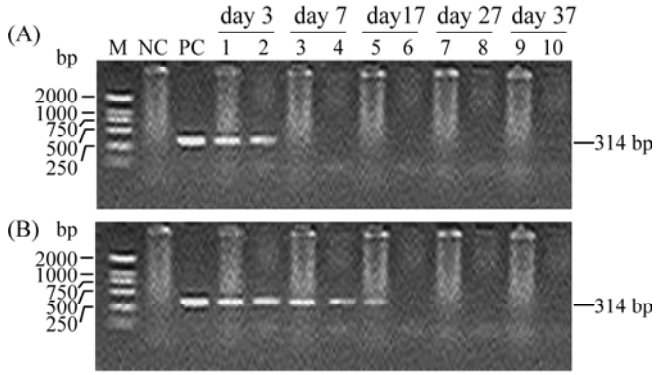

Fig. 3 Electrophoresis analysis of the polymerase chain reaction products for hepatitis B virus (HBV) DNA in mouse liver and serum samples

(A) The mouse was injected with infectant human serum and not injected with pMC-squamous cell carcinoma antigen 1 (pMC-SCCA1) in advance. (B) The mouse was injected with infectant human serum 24 h after injection with pMC-SCCA1. M, marker; NC, negative control; PC, positive control, provided by the HBV-DNA detection kit; 1, 3, 5, 7 and 9, liver tissue samples; 2, 4, 6, 8 and 10, serum samples.

Discussion

Due to its role in synthesizing many proteins and its involvement in numerous genetic and acquired diseases, the liver is an important target organ for gene transfer. A variety of vectors have been used for introducing genes into the liver, including recombinant retrovirus [12], adenovirus [13], adeno-associated virus [14], and nonvirus vectors such as liposome [15], cationic polymer [16], and reconstituted chylomicron remnant [17]. Although significant progress has been made in the successful delivery of genes to the liver, many problems are associated with each of these methods. Retrovirus vector can generate long-term transgene expression, but partial hepatectomy or liver damage is usually required to stimulate cell division [18]. Adenoviral vector allows for high transferring efficiency, but gene expression lasts for a short period due to the host's immune response against viral protein [19]. Adeno-associated viral vectors produce long-term gene expression, but they can not deliver genes of a size more than 5.2 kb [20]. The current nonviral vectors suffer from the major limitation of low transfection efficiency. To overcome these problems, Zhang *et al*. [21] explored the hydrodynamics-based procedure. Using this method, liver cells can be transfected with a foreign gene by rapid injection of a large volume of plasmid DNA solution. It was demonstrated that the liver was the only organ that expressed the transferred gene and the expression persisted for at least 6 months [22]. In our present study, by using this method we transferred the candidate HBV receptor gene *SCCA1* into mouse liver, the target organ of HBV.

The loss of transgene expression has been a major obstacle to the development of nonviral vectors for the treatment of human diseases. Chen *et al*. [11] previously demonstrated that bacterial DNA linked to a mammalian expression cassette resulted in transcriptional silencing of the transgene *in vivo*. To develop a means to produce a robust DNA vector that is not silenced *in vivo*, they developed a phage ΦC31 integrase-mediated intramolecular recombination technology to prepare minicircle vector DNA devoid of the bacterial backbone. The authors reported that minicircle DNAs devoid of bacterial sequences expressed 45- and 560-fold more serum human factor IX and α1-antitrypsin, respectively, compared to standard plasmid DNAs transfected into mouse liver. Their data suggest that minicircles are capable of expressing high and persistent levels of therapeutic products *in vivo* and have a great potential to serve as episomal vectors for the treatment of a wide variety of diseases. The present data showed that mouse liver expressed a high level of

recombined SCCA1 protein at a detectable level under our experimental conditions for at least 37 d after injection. This HBV-BP transgene mouse would be a very useful animal model for the study of the function of SCCA1 involved in HBV binding and internalization *in vivo*.

Our data of the HBV study in this mouse model suggested that HBV can stay longer in the liver by intravenous injection of pMC-SCCA1 using a hydrodynamics-based procedure. Although the liver tissues of the 3, 7 and 17 d groups showed positive in the immunohistochemical examination and HBV-DNA PCR test, there was no HBV-DNA detected in the 27 or 37 d groups [**Fig. 2(G–L)** and **Fig. 3**]. These findings suggested that this protein *in vivo* might help HBV binding to the mouse liver cells that express this protein, and that SCCA1 might serve as an HBV receptor or co-receptor and play an important role in mediating HBV entry to hepatocytes. However, its role in human HBV infection remains to be further determined.

The sinusoidal endothelial and Kupffer cells near the hepatic sinusoid were found to be strongly positive in the immunohistochemical test [**Fig. 2(H–J)**]. This suggests that the cells might play an important role in HBV clearance as the first defence against HBV infection, and this function deficiency might give HBV more access to hepatocytes.

In summary, our data in this report show that the long-term expression of the suspected HBV receptor gene can be achieved in a mouse model by simple tail vein injection of the minicircle plasmid using a hydrodynamics-based procedure. This method gives access to the HBV receptor research, and our studies proved *in vivo* that this HBV-BP can extend its retention of HBV in mouse liver. However, there are still other factors involved in HBV infection and further quantitive research on this animal model is needed.

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