HBx protein is indispensable for development of viraemia in human hepatocyte chimeric mice

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The non-structural X protein, HBx, of hepatitis B virus (HBV) is assumed to play an important role in HBV replication. Woodchuck hepatitis virus X protein is indispensable for virus replication, but the duck hepatitis B virus X protein is not. In this study, we investigated whether the HBx protein is indispensable for HBV replication in vivo using human hepatocyte chimeric mice. HBx-deficient (HBx-def) HBV was generated in HepG2 cells by transfection with an overlength HBV genome. Human hepatocyte chimeric mice were infected with HBx-def HBV with or without hepatic HBx expression by hydrodynamic injection of HBx expression plasmids. Serum virus levels and HBV sequences were determined with mice sera. The generated HBx-def HBV peaked in the sucrose density gradient at points equivalent to the generated HBV wild type and the virus in a patient’s serum. HBx-def HBV-injected mice developed measurable viraemia only in continuously HBx-expressed liver. HBV DNA in the mouse serum increased up to 9 log10 copies ml−1 and the viraemia persisted for more than 2 months. Strikingly, all revertant viruses had nucleotide substitutions that enabled the virus to produce the HBx protein. It was concluded that the HBx protein is indispensable for HBV replication and could be a target for antiviral therapy.

INTRODUCTION
Chronic hepatitis B virus (HBV) infection is associated with the development of virus-related liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). HBV is a member of the family Hepadnaviridae, which consists of hepatotropic, small DNA viruses that infect their respective animal hosts (Ando et al., 1999; Ganem & Schneider, 2001; Raney & McLachlan, 1991). HBV particles contain a 3.2 kb partially double-stranded circular DNA genome encoding four open reading frames (ORFs). The preS/S, pre-core/core, polymerase/reverse transcriptase and non-structural X protein (HBx) mRNAs are transcribed from each of the four ORFs. The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the HBV genome cloned into plasmid pTRE-HB-wt is AB206817.

Although previous works have demonstrated that HBx protein is necessary for maximal HBV replication in cultured cells (Bouchard et al., 2001; Keasler et al., 2007; Leupin et al., 2005; Tang et al., 2005) and in mouse hepatocytes (Keasler et al., 2007), the precise function of HBx in the virus life cycle remains poorly defined in human hepatocytes under physiological conditions because there is no natural infection–replication system available. Accordingly, all previous work has been done using hepatocarcinoma cell lines with transfection or mouse hepatocytes with hydrodynamic injection. Analysis of HBx under physiological conditions will provide more accurate information for the function of the HBx protein.

The nucleotide and amino acid sequences of the X genes are well-conserved among all mammalian hepadnaviruses. Expression of HBx protein in hepatocytes has been reported

(Seeger & Mason, 2000; Tang et al., 2001).
both in humans (Su et al., 1998) and in woodchucks (Dandri et al., 1996; Jacob et al., 1997). Previous reports have shown that the X protein of the woodchuck hepatitis virus (WHV) is important for the virus life cycle (Chen et al., 1993; Sitterlin et al., 2000a; Zhang et al., 2001; Zoulim et al., 1994). In contrast, in non-oncogenic avian hepatitis viruses, such as duck hepatitis B virus (DHBV), the X protein (DHBx) is not necessary for virus replication in vivo (Meier et al., 2003). The HBx and WHV X proteins (WHx) localize both in the cytoplasm and in the nucleus (Dandri et al., 1998; Doria et al., 1995; Sitterlin et al., 2000b; Wang et al., 1991), and both of them have similar multi-phasic activities for transcription, DNA repair, cell growth and apoptotic cell death in tissue-culture cells (Arbuthnot et al., 2000; Murakami, 2001). HBx and WHx have also been shown to stimulate virus replication in cell lines by activating viral transcription (Colgrove et al., 1989; Melegari et al., 2005; Zhang et al., 2001) or by enhancing the reverse transcription activity of the viral polymerase (Bouchard et al., 2001; Klein et al., 1999). Although it has been shown that the WHx protein is indispensable for virus replication in vivo (Zoulim et al., 1994), which of the above functions is indispensable remains unknown. As HBV infects only humans and chimpanzees, it has been difficult to perform intensive studies in vivo.

Recently, Mercer et al. (2001) reported that transplanted human hepatocytes in SCID mice homozygous for the Alb-uPA transgene resulted in replacement of the mouse liver. They also reported that the highly replaced mice are susceptible to hepatitis C virus (Mercer et al., 2001). Tateno et al. (2004) also created human hepatocyte chimeric mice with an improved replacement rate. Using this chimeric mouse model and the cell-culture-created HBV, we showed previously that hepatitis B e antigen (HBeAg) is dispensable for virus infection and replication (Tsuge et al., 2005).

In this study, we tested whether the cell-culture-generated HBx-defective (HBx-def) HBV infects and replicates in the chimeric mice. As HBx-def HBV did not develop measurable viraemia, we expressed the HBx protein in the chimeric mouse liver by hydrodynamically injecting HBx-expression plasmid. It was noted that this trans-complementation of HBx helped the replication of HBx-def virus in the chimeric mice, and revertant viruses showed nucleotide substitutions that reversed the introduced stop codon [CAA to TAA created by a C-to-T point mutation at nt 1395 (aa 7) in the HBx gene; Fig. 1a] and restored expression. The HBx protein is thus indispensable for infection and proliferation of HBV. The protein thus might be a target for therapy development against HBV.

RESULTS

Production of HBV particles and antigens in cell culture and effect of HBx ablation

We initially examined nucleotide sequences of the cell-line-produced HBV by direct sequencing of the PCR products using cell-culture supernatants. As expected, HBV DNA was released from HepG2 cells transfected with the HBx-def plasmid with an introduced stop codon mutation by calcium phosphate precipitation (data not shown). We then analysed hepatitis B surface antigen (HBsAg), HBeAg and HBV DNA in the supernatants 3 days after transfection. While HBV DNA titres were not significantly different between the wild-type (WT)- and HBx-def HBV-transfected cultures, the HBsAg and the HBeAg levels were significantly lower in HBx-def HBV- than in WT-transfected cultures (Fig. 1b).

To examine the particle formation in the transfection experiments, we analysed the density of generated HBV by sucrose density gradient sedimentation analysis. The density of the cell-culture-produced HBx-def HBV was compared with those of WT HBV and HBV obtained from human serum. As shown in Fig. 1(c), each of the three preparations of HBV sedimented at sucrose density 1.18 g ml⁻¹, suggesting that cell-culture-produced HBV particles were similar to those obtained from human serum.

Infectivity of HBx-def HBV particles

To analyse the infectivity of HBx-def HBV, we inoculated cell-culture-produced recombinant HBV (WT HBV or HBx-def HBV) into chimeric mice. All seven mice injected with cell-culture-generated WT HBV developed measurable viraemia 2–7 weeks after inoculation. The virus titre reached 6–10 log₁₀ copies ml⁻¹ and the viraemia persisted for more than 4 months (Fig. 2a). In contrast, we did not observe any measurable viraemia in HBx-def HBV-injected mice within a period of 16 weeks after inoculation (Fig. 2b). Only five of 16 HBx-def HBV-inoculated mice became occasionally positive for HBV DNA by nested PCR assay. We then examined the mouse livers 14 weeks after inoculation by immunohistochemical staining with anti-HB core (HBC) antibody. As shown in Fig. 2(c), human hepatocytes of WT-injected mice were positive for HBV core antigen (HBCAg). In contrast, the staining was negative in mouse liver injected with HBx-def HBV.

Effect of trans-complementation of entire and partial HBx protein on replication of HBx-def HBV

We then investigated the effect of trans-complementation of the HBx protein both in vitro and in vivo. Since the C-terminal two-thirds (aa 51–154) domain of HBx has been reported to contain a transactivation domain (Tang et al., 2005), we constructed three plasmids (full length and residues 1–50 and 51–154), as shown in Fig. 3(a). To analyse the effect of co-transfection of these three plasmids on intracellular replication of HBV, the cells transfected using TransIT-LT1 reagent were harvested 24 h after transfection and analysed by Southern blotting. As shown in Fig. 3(b), trans-complementation of HBx enhanced the

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replication of HBV to the WT level. The effects of HBx protein were also evident on the expression of HBsAg (Fig. 3c) and HBeAg (Fig. 3d). As reported previously, the effect of the C-terminal two-thirds (aa 51–154) of the HBx protein was stronger than that of the entire protein and the N-terminal one-third (aa 1–50) (Tang et al., 2005). The production of replication intermediates was increased similarly by co-transfection of the X proteins (Fig. 3e).

To further study the effect of HBx expression, we analysed the levels of intracellular core protein expression. As shown in Fig. 4(a), the expression levels of the core protein were upregulated with the expression of the entire (WT) and C-terminal two-thirds (aa 51–154) of the HBx protein. Immunocytochemical analysis showed that only the cells with strong HBx protein expression were stained with the core protein (Fig. 4b). The core and HBx proteins in these cells were stained mainly in the cytoplasm.

**Expression of HBx protein in mouse liver by hydrodynamic injection**

Next, we expressed the HBx protein in the chimeric mouse liver with hydrodynamic injection. As shown in Fig. 5(a), a dose-dependent expression of the HBx protein with a haemagglutinin (HA) tag was confirmed by Western blot analysis. Although Henkler et al. (2001) showed an aggregation of HBx under the control of the human cytomegalovirus (CMV) promoter, we were able to observe expression of properly sized HBx. Immunohistochemical analysis also revealed HBx protein expression in the mouse liver by hydrodynamic injection.

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**Fig. 1.** Construction of HBV expression plasmids. (a) Wild type (WT) 1.4× genome length HBV was cloned into the pTRE2hyg vector (pTRE-HB-wt) and a nucleotide substitution, C1395T, was introduced to create the HBx-def mutant pTRE-HB-X-def. (b) Comparison of expression of HBsAg, HBeAg and HBV DNA in culture medium between WT and HBx-def. (c) Sucrose density gradient analysis of HBV particles (●) and HBV DNA copies (bars) obtained from a serum sample (left) and supernatants from a cell culture transfected with WT HBV (pTRE-HB-wt, middle) and HBx-def pTRE-HB-X-def. C.O.I., cut-off index.
intrahepatic expression of the HBx protein

As the infection experiments with HBx-def HBV failed to result in measurable viraemia (Fig. 2b), we then tried to infect HBx-def HBV after expression of HBx protein by hydrodynamic injection. As shown in Fig. 6(a), six of seven mice developed measurable viraemia 2–8 weeks after inoculation. The incidence of measurable viraemia was significantly higher in mice that received hydrodynamic injection than in those without (Fig. 2b versus Fig. 6a, P < 0.0001). Immunohistochemical analysis of the infected mice showed simultaneous staining for human serum albumin (hAlb) and HBcAg in the same portion of the liver (Fig. 6b).

Sequence analysis of inocula and the infected mouse sera

We analysed nucleotide sequences of the virus recovered from all six infected mice and compared them with those of inoculated HBx-def HBV. As shown in Fig. 7(a), direct sequencing analyses of the amplified HBV DNA products showed that all revertant viruses had T1395C (mouse MHX#1, 3, 5–7) or T1395A (mouse MHX#2) point mutations, which reverted the introduced stop codon to amino acids. We further analysed nucleotide sequences of HBV by cloning and sequencing using serum samples obtained from two mice (MHX#1, 33 clones; MHX#2, 38 clones) (Fig. 7b). Only one of 33 clones obtained from MHX#1 and none of the 38 clones from MHX#2 had the stop codon mutation that was introduced into the transfected plasmid.

DISCUSSION

In previous studies, HBx has been reported to be a multifunctional protein affecting cell growth and proliferation and activating transcription of mRNA (Arbuthnot et al., 2000; Bouchard et al., 2001; Klein et al., 1999; Murakami, 2001) and virus replication in HCC cell lines (Bouchard et al., 2001; Keasler et al., 2007; Leupin et al., 2005) and mouse hepatocytes (Keasler et al., 2007; Xu et al., 2002). However, these results were obtained by introduction of HBV genomes into cells using artificial methods such as transfection, gene transfer and hydrodynamic injection. Recently, we established an in vivo HBV infection system using human hepatocyte chimeric mice (Tsuge et al., 2005). The system enabled us to perform infection experiments using HBV-containing patient sera and cell-culture medium. Using this system,
we showed previously that HBeAg is dispensable for HBV infection and active replication in vivo (Tsuge et al., 2005). Virus replication following infection of HBV particles is quite similar to natural infection. We thus applied the system to study the function of HBx protein in this study. We also utilized hydrodynamic injection of HBx expression plasmid to trans-complement the defective HBx. As shown by Western blot analysis (Fig. 4a), HBx protein of the expected size was produced without development of antibody in this SCID-mouse-based model system.

This natural infection mode is quite different from previous animal studies. Virus titres of HBx-def HBV were approx. 50–99% compared with WT HBV in vitro (Bouchard et al., 2001; Keasler et al., 2007; Leupin et al., 2005; Tang et al., 2005) and in vivo (Keasler et al., 2007; Xu et al., 2002). High-level HBx-def virus production seen in these experiments may be the result of expression of HBV proteins other than HBx following forced introduction of plasmids into mouse liver cells by hydrodynamic injection or transgenes. Such introduction probably resulted in virus production that is similar to in vitro transfection experiments using cultured cells.

In vitro experiments in this study showed that normal-density HBV particles (Fig. 1c) were produced in the absence of HBx. Curiously, the amount of HBV DNA released from the cells into the supernatant was not different between WT and HBx-def HBV, even though the
amounts of HBsAg and HBeAg as well as the amount of HBV DNA in cells were significantly greater in WT (Fig. 1b). Efficacy of release of the virus from the cells might be different between WT and HBx-def HBV. Alternatively, production of defective virus, which appeared as the second peak of HBV DNA in the sucrose gradient experiment (Fig. 1c, right panel), might be enriched in HBV DNA in the supernatant of HBx-def HBV. The reason for this discrepancy is unknown. Previous papers did not mention such production of HBV into the supernatant.

Similarly, in the absence of HBx protein in vitro, the formation of the replication intermediates (Fig. 3) and production of intracellular core protein (Fig. 4) continued, although their amounts were much lower. It is thus difficult to explain the inability of HBx-def HBV to infect in vivo simply from its transcription-activating ability, although our results confirmed that HBx has trans-activation ability, as reported previously (Kessler et al., 2007; Tang et al., 2005; Xu et al., 2002). A different mode of introduction of viral nucleic acid might explain the difference seen in in vitro and in vivo experiments. In the transfection experiments, a relatively large amount of HBV DNA is introduced by transfection. In contrast, only successfully attached virus particles can introduce viral DNA into liver cells. Strikingly, all but one (70 of 71 clones) revertant viruses had nucleotide substitutions that reversed the introduced stop codon to a coding amino acid. This is in contrast to the fact that HBV replicates in the HBx-def form in cultured cells, even though the efficacy is lower than in WT. We assumed that complemented HBx protein stimulated the replication of HBx-def HBV and increased the chance of nucleotide sequence substitutions in the HBx.
gene, and that only revertant HBV variants predominantly increased, due to their rapid replication ability through the infection–replication cycle that only exists in the in vivo model. One might consider the possibility that the HBx protein works as a mutagen. However, we did not observe clear differences in the incidence of nucleotide sequence substitutions between the presence and absence of HBx (Fig. 7b and data not shown).

**Fig. 5.** Expression of HBx protein by hydrodynamic injection of HBx plasmid. (a) Liver-expressed HA-tagged HBx proteins were detected by Western blot analysis using anti-HA antibody (HA tag was used to avoid non-specific binding of anti-FLAG tag to mouse liver proteins). Dose-dependent expression of the protein was observed with different doses of the injected plasmid. (b) Immunohistochemical analysis of mouse liver using anti-HA antibody revealing expression of HBx protein. The protein was mainly expressed around the central vein.

**Fig. 6.** Infection of HBx-def HBV particles after hydrodynamic injection of HBx expression plasmid. (a) Full-length HBx protein expression plasmid was hydrodynamically injected twice a week into human hepatocyte chimeric mice. Two weeks after the beginning of the injections, cell-culture-derived HBx-def HBV particles were injected through the tail vein. HBV DNA (upper panel) and hAlb (lower panel) were measured. (b) Immunohistochemical analysis of the infected mouse. The liver was stained with haematoxylin and eosin (HE) (upper), antibody against hAlb (middle) and anti-HBc antibody (lower).
It is thus still uncertain why the HBx protein is indispensable for virus replication in vivo. However, the fact that HBV cannot replicate in the absence of HBx protein may allow development of therapeutic medicine by disturbing the unknown action of HBx. To this end, it is interesting to identify a substance that binds to HBx. The indispensability of the X protein for virus replication is a common feature shared by HBV and WHV (Chen et al., 1993; Zoulim et al., 1994). Both of them cause chronic infection, inflammation, fibrosis and cancer. In contrast, DHBV, which can replicate without DHBx expression, does not cause such a pathological situation (Meier et al., 2003). Further analysis of the X protein may pave the way to clarify the mechanism of cancer development caused by HBV infection.

METHODS

Human hepatocyte chimeric mice experiments. Care of uPA<sup>+/−</sup>/SCID<sup>+/−</sup> mice and transplantation of human hepatocytes were performed as described previously (Tateno et al., 2004). The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University. Infection, extraction of serum samples and sacrifice were performed under ether anaesthesia as described previously (Tateno et al., 2004).
hAlb in mouse serum was measured with a Human Albumin ELISA Quantification kit (Bethyl Laboratories Inc.) according to the instructions provided by the manufacturer. Serum samples obtained from mice were aliquotted and stored in liquid nitrogen until use.

**Analysis of HBV markers.** HBsAg and HBeAg were measured using a commercially available ELISA kit (Abbott). For quantitative analysis of HBV DNA, 10 μl mouse serum sample or 100 μl of culture supernatant was used. DNA was extracted from these samples using the SMITEST R&D (Genome Science Laboratories) and dissolved in 20 μl H2O, and HBV DNA was quantified by real-time PCR using the 7300 Real-Time PCR System (Applied Biosystems). Amplification was performed as previously (Tsuge et al., 2005). The lower detection limit of this assay is 300 copies. For detection of small amounts of HBV DNA, we also performed nested PCR. The amplification conditions were as described previously (Tsuge et al., 2005).

**Plasmid construction.** The construction of wild-type (WT) HBV 1.4 genome length, pTRE-HB-wt, was described previously (Tsuge et al., 2005). We used pTRE2 vector without pTet-off vector and doxycycline because a sufficient amount of HBV transcripts was produced from internal HBV promoters, and transcription from the pTRE2 promoter is negligible under these conditions. The nucleotide sequence of the HBV genome that we cloned into plasmid pTRE-HB-wt was deposited in GenBank under accession number AB206817. A modified plasmid, pTRE-HB-X-def, was generated by introducing a C-to-T point mutation at nt 1395 (aa 7) to create a stop codon (CAA to TAA) in the HBx gene (Fig. 1a). The substitution was introduced by using a QuikChange Site-Directed Mutagenesis kit (Stratagene). For the construction of the HBx gene expression plasmid, the HBx gene was amplified from pTRE-HB-wt and cloned into pcDNA3, pcDNA3-3×HA, p3×FLAG-CMV10 vectors and designated pcDNA-HBx, pcDNA3-3×HA-HBx, p3FLAG-HBx, respectively. Partially truncated HBx plasmids, with a deletion of the N-terminal 50 aa [HBx(51–154)] and the C-terminal 50 aa [HBx(1–50)], were also cloned into pcDNA3 or p3FLAG-CMV10 vectors.

**Transfection of HepG2 cell lines with HBV expression plasmids.** HepG2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum at 37°C and under 5% CO2. For functional analysis of the HBx protein in vitro, the HBV or HBx-def HBV expression plasmid was transfected with/without HBx expression plasmid using TransIT-LT1 reagent (Mirus) according to the instructions provided by the supplier. Three to five days after transfection, core-associated HBV DNA was extracted from cells for HBV DNA quantification (Noguchi et al., 2005). For analysing the infectivity of recombinant HBV particles, HBV expression plasmids were transiently transfected into HepG2 cells. The cells were seeded to semi-confluence in 90 mm dishes. WT HBV particles were generated from cells transfected with 20 μg pTRE2-HB-wt by calcium phosphate precipitation. HBx-def HBV particles were also generated from cells co-transfected with 10 μg pTRE2-HB-X-def and 10 μg pcDNA-HBx. Three days after transfection, the culture medium was collected and stored in liquid nitrogen until use.

**Analysis of cell-culture-produced HBV by sucrose density gradient sedimentation.** Five millilitres of HBV-positive human serum (8 log10 copies ml-1) or 50 ml cell culture supernatant (8 log10 copies ml-1) was layered on a 20% (w/w) sucrose cushion, and centrifuged at 24 000 r.p.m. (maximum 103 864 g) for 12 h at 4°C with a Beckman SW28 rotor (Beckman Coulter). The precipitate was resuspended in 500 μl PBS. These HBV samples were layered on a linear 20–50% (w/w) sucrose gradient. Centrifugation was carried out at 24 000 r.p.m. (maximum 102 445 g) for 21 h at 4°C with a Beckman SW40 rotor. The gradients were fractionated into 500 μl samples, and the density of each fraction was calculated from the weight and volume. Each fraction was diluted 10-fold and tested for HBV DNA by real-time PCR.

**Analysis of replication intermediate of HBV.** The cells were harvested 5 days after transfection and lysed with 250 μl lysis buffer [10 mM Tris/HCl (pH 7.4), 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15 000 g. The core-associated HBV genome was immunoprecipitated by mouse anti-HBV core monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after SDS/proteinate K digestion, followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR with SYBR Green using the 7300 Real-Time PCR System and the amounts of the replication intermediates were compared. The HBV-specific primers used for amplification were 5’-TTTGGGCAATGGCATTGAC-3’ and 5’-GGTGAACATGGTCCCGGAC-3’. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

**Immunocytochemistry of HepG2 cells transfected with pTRE2-HB-X-def and p3FLAG-HBx plasmids.** HepG2 cells were seeded to semi-confluence in two-well chamber plates. Each 1 μg pTRE2-HB-X-def and p3FLAG-HBx plasmids was co-transfected using TransIT-LT1 reagent (Mirus) according to the instructions provided by the supplier. The cells were harvested 24 h after transfection and then washed with PBS and fixed with 4% (v/v) paraformaldehyde. After fixation, the cells were stained with mouse monoclonal antibody directed to FLAG (Sigma) or rabbit polyclonal antibody against hepatitis B core antigen (HBcAg, DAKO Diagnostika) as the primary antibody. The bound antibodies were detected with an Alexa Fluor 488-conjugated antibody against rabbit IgG or Alexa Fluor 568-conjugated antibody against mouse IgG, respectively (Molecular Probes). Nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

**Hydrodynamic injection of HBx expression plasmids.** Hydrodynamic injection was performed as reported previously (Yang et al., 2002) with slight modifications. As the human hepatocyte chimeric mice were quite small (12–15 g) and weak for the rapid injection and the stress, we reduced the amount of DNA solution and injection speed: 1 ml PBS containing 30 μg HBx expression plasmids was injected rapidly through the mouse tail vein within 30 s. For analysis of infectivity of HBx-def HBV particles, the plasmids were injected twice a week.

**Western blot analysis.** Mouse liver tissues or transfected HepG2 cells were cooled on ice and treated with RIPA-like buffer [50 mM Tris/HCl (pH 8.0), 0.1% SDS, 1% NP-40, 150 mM sodium chloride and 0.5% sodium deoxycholate] containing protease inhibitor cocktail (Sigma). Cell lysates were separated on SDS-polyacrylamide gels [5–20% (w/v)] (Bio-Rad) and then transferred onto nitrocellulose membranes (GE Healthcare) by electroblotting. The membranes were incubated with anti-haemagglutinin fusion epitope (anti-HA) monoclonal antibody (Roche) or with anti β-actin monoclonal antibody (Sigma) followed by incubation with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (GE Healthcare). Proteins were visualized via the ChemiDoc XRS system (Bio-Rad). Expression of HBc protein was quantified from the densities of the immunoblot signals by Quantity One software (Bio-Rad).

**Immunohistochemical analysis of mouse liver.** The liver specimens of HBV-infected mice were fixed with 10% buffered paraformaldehyde and embedded in paraffin blocks for histological
examination. The liver sections were stained with haematoxylin–eosin or subjected to immunohistochemical staining using an antibody against HBcAg (DAKO Diagnostika), anti-HA antibody or HSA (Bethyl Laboratories Inc.). Endogenous peroxidase activity was blocked with 0.3 % H$_2$O$_2$ and methanol. Immunoreactive materials were visualized by using a streptavidin–biotin staining kit (Histofine SAB-PO kit; Nichirei) and diaminobenzidine.

**Sequence analysis of the HBV genome.** Genome-length HBV DNA was amplified by PCR as described by Günther et al. (1995). HBV genome-length PCR products were subjected to 1 % agarose gel electrophoresis and the 3.2 kbp band was extracted using a QiaEx II Gel Extraction kit (Qiagen). Direct sequencing, cloning and electrophoresis and the 3.2 kbp band was extracted using a QiaEx II Biosystems).

**Statistical analysis.** All data are expressed as mean ± SD. Differences between groups were examined for statistical significance by using Student’s t-test. A P value <0.05 denoted the presence of a statistically significant difference.

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