

# Infection of Human Hepatocyte Chimeric Mouse With Genetically Engineered Hepatitis B Virus

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Studies of hepatitis B virus (HBV) mutants have been hampered by the lack of a small animal model with long-term infection of cloned HBV. Using a mouse model in which liver cells were highly replaced with human hepatocytes that survived over a long time with mature human hepatocyte function, we performed transmission experiments of HBV. Human serum containing HBV and the virus produced in HepG2 cell lines that transiently or stably transfected with 1.4 genome length HBV DNA were inoculated. Genetically modified e-antigen-negative mutant strain also was produced and inoculated into the mouse model. A high-level ( $\approx 10^{10}$  copies/mL) viremia was observed in mice inoculated with HBV-positive human serum samples. The level of viremia tended to be high in mice with a continuously high human hepatocyte replacement index. High levels and long-lasting viremia also were observed in mice injected with the *in vitro* generated HBV. The viremia continued up to 22 weeks until death or killing. Passage experiments showed that the serum of these mice contained infectious HBV. Genetically engineered hepatitis B e antigen-negative mutant clone also was shown to be infectious. Lamivudine effectively reduced the level of viremia in these infected mice. **In conclusion**, this mouse model of HBV infection is a useful tool for the study of HBV virology and evaluation of anti-HBV drugs. Our results indicate that HBeAg is dispensable for active viral production and transmission. (HEPATOLOGY 2005;42:1046-1054.)

**H**epatitis B virus (HBV) is a small enveloped DNA virus and causes chronic infection of the liver that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma.<sup>1-4</sup> The lack of a

practical small animal model has impeded the study of the biology of this virus and the development of effective antiviral therapies. Chimpanzee is the only natural host that allows active replication of HBV.<sup>5-7</sup> Although this animal is a valuable model for the study of hepatitis viruses,<sup>8</sup> the practical use of chimpanzees is severely limited both ethically and economically.

Several small animal models of HBV infection have been reported. The HBV transgenic mouse is a very useful model for the study of virology and evaluation of antiviral drugs.<sup>9-12</sup> However, the liver cells of this model are not permissive for HBV infection; therefore, studying virus-cell interactions such as receptor binding and entry is not possible. The HBV-trimera mouse is another useful mouse model.<sup>13</sup> In this model, *ex vivo* HBV-infected human liver fragments are implanted into lethally irradiated mice after SCID mouse bone marrow transplantation. Approximately 80% of the mice develop viremia 2 to 3 weeks after infection. However, the rate of positivity subsequently decreases to less than 20% 6 weeks after infection. The level viremia is approximately  $10^5$  copies/mL. More recently, HBV-containing human serum samples were used to infect human hepatocyte repopulated mice.<sup>14</sup> A high-level viremia ( $4.5$  and  $10 \times 10^8$  copy/mL) and HBs antigenemia are observed 8 weeks after injection. This mouse model is promising because HBV replicates in natural host cells, human hepatocytes. However,

Abbreviations: HBV, hepatitis B virus; WHV, woodchuck hepatitis virus; HSA, human serum albumin; PCR, polymerase chain reaction; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; DMEM, Dulbecco's modified Eagle's medium.

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**Table 1. Table Inoculum Used for Infection Experiments**

Inoculum	Source	Transfection	HBs Antigen	HBe Antigen	HBV DNA (LGE/mL)
Serum 1	HBV carrier 1	—	+	130	10.8
Serum 2	HBV carrier 2	—	+	150	8.7
CA59	pCAG-HB-wt	Stable	6.3 ± 2.8	15 ± 7	8.0 ± 0.2
CM3	pTRE-HB-wt	Transient	3.9 ± 1.5	105 ± 7	8.3 ± 0.4
Fresh CM3	pTRE-HB-wt	Transient	ND	ND	8.0
e-Negative	pTRE-HB (PC)	Transient	2.9 ± 0.2	0.5 ± 0.3	8.1 ± 0.3
Mouse CM3	CM3-infected mouse	—	ND	ND	ND

NOTE. Fifty microliters of each serum or cell culture supernatant was used for injection except for fresh CM3 (500–1,000  $\mu$ L) and mouse CM3 (5  $\mu$ L). Data are mean  $\pm$  SD.

Abbreviations: LGE, log genome equivalent; ND, not determined.

long-term high-level viremia has not been reported so far in this model, probably because of technical difficulties in maintaining large quantities of human hepatocytes in these mice.

Long-term HBV viremia was reported after subcutaneous transplantation of immortalized human hepatocytes in RAG-2-deficient mice after transfection of circularized full-length HBV genome.<sup>15</sup> Viremia of up to  $3 \times 10^8$  copy/mL was still observed in these mice at least 5 months after transplantation. This long-term viremia model should be useful for *in vivo* HBV studies. However, the production and selection of HBV-secreting immortalized human hepatocytes takes a long time, and the level of viremia in the transplanted animal depends on the volume of live immortalized cells in mice. The mode of viremia might be different from natural infection because the pregenome RNA is transcribed from integrated HBV. Whether the produced HBV re-infects implanted immortalized human hepatocytes has not been confirmed.

A useful woodchuck hepatitis virus (WHV) infection model was established by Petersen et al.<sup>16</sup> They showed high-level replacement of uPA/Rag-2 knockout mice liver with woodchuck hepatocytes and development of high-level ( $1 \times 10^{11}$  virion/mL) WHV viremia. Dandri et al.<sup>17</sup> transplanted *Tupaia* hepatocyte into uPA/RAG-2 mice and showed up to  $8.2 \times 10^7$  genome equivalent/mL viremia. This model is useful because viremia continued up to 29 weeks. However, probably because of different host cells, the replication levels of HBV are lower than those of woolly monkey HBV. Using SCID mouse homozygous for Alb-uPA transgene, the group of Mercer and colleagues<sup>18</sup> were the first group to report high-level replacement of mouse liver with human hepatocytes and successful infection of these mice with hepatitis C virus. Recently, we also created a human hepatocyte chimeric mouse in which the hepatocytes were highly replaced by implanted human liver cells.<sup>19</sup> The repopulation index calculated from serum human serum albumin (HSA) concentrations exceeded 70% in 32% of the transplanted mice, and these animals survived up to 80 days after transplanta-

tion with high replacement index. Using this chimeric mouse, we performed transmission experiments of HBV. Using serum samples obtained from patients with chronic HBV infection, high-level viremia (approximately  $10^{10}$  copies/mL) was observed up to 22 weeks in mice inoculated with HBV-positive human serum samples. We also performed infection study using *in vitro*-generated HBV. Infectious HBV was produced in HepG2 cell lines by transfecting with 1.4 genome length HBV DNA. Because mice injected with this *in vitro*-produced virus developed viremia, we further performed passage study. In addition, we introduced point mutations in HBV genome to create an HBe antigen-negative variant. The mice inoculated with this HBe antigen-negative variant developed viremia. Lamivudine effectively suppressed replication of HBV in mice inoculated with human serum samples and wild-type *in vitro*-created HBV. This model is a useful tool for the study of the nature of HBV mutants and development of anti-viral drugs.

## Materials and Methods

**Human Serum Samples.** Serum samples were obtained from four HBV carriers after obtaining written informed consent. Inocula for mice were obtained from two patients who tested positive for HBs and HBe antigens with slightly elevated levels of serum alanine aminotransferase and high-level viremia (Table 1). Serum samples for extraction and cloning of HBV were obtained from the remaining two patients who were positive for hepatitis B e antigen (HBeAg) and had high-level HBV DNA ( $6.9 \times 10^9$  and  $9.8 \times 10^{10}$  copies/mL by real-time polymerase chain reaction [PCR], respectively). All of these HBV belonged to genotype C.

**Analysis of HBV Markers.** Hepatitis B surface antigen (HBsAg) and HBeAg were measured by commercially available ELISA (Abbott Japan, Osaka, Japan). For quantitative analysis of HBV DNA, 100  $\mu$ L serum samples or culture supernatants were used. DNA was extracted from these samples by SMITEST (Genome

Science Laboratories, Tokyo, Japan) and was dissolved in 20  $\mu$ L H<sub>2</sub>O. One microliter DNA solution was amplified by Light Cycler (Roche Diagnostics, Japan, Tokyo) for quantitation of HBV. The primers used for amplification were 5'-TTTGGGCATGGACATTGAC-3' and 5'-GGTGAACAATGTTCCGGAGAC-3'. The amplification condition included initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 5 seconds, extension at 72°C for 6 seconds. The lower detection limit of this assay is 300 copies. Nested PCR was used to detect a small amount of HBV DNA with the outer primers X1F1 (5'-CGCGGGACGTCCTTTGTCTA-3') and X2R1 (5'-GTTACGGTGGTCTCCATGC-3') and inner primers X1F2 (5'-TACGTCCCGTTCGGCGCTGAA-3') and X2R2 (5'-CAGAGGTGAAGCGAAGTGCA-3'). The amplification condition included 35 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes after 2 minutes of initial denaturation at 94°C followed by 7 minutes of final extension using Gene Taq (Wako Pure Chemicals, Tokyo, Japan) with anti-Taq high (TOYOBO Co., Osaka, Japan) according to the instructions provided by TOYOBO.

#### **Cloning of HBV DNA and Plasmid Construction.**

Full-length HBV DNA was amplified using these HBV DNA samples by the method of Gunther et al.<sup>20</sup> and cloned into pBluescript SK+ (Stratagene, La Jolla, CA). HBV DNA, 1.4 genome length, obtained from one of these two patients was cloned into pcDNA3 (Invitrogen, San Diego, CA) after replacement of CMV promoter with CAG to yield pCAG-HB-wt. Similarly, 1.4 genome length HBV DNA from the other patient was cloned into a plasmid vector pTRE2 (BD Biosciences, Franklin Lakes, NJ) and designated pTRE-HB-wt. A modified plasmid pTRE-HB-PC was generated by introducing a G-to-A point mutation to nucleotide 1896 to create pre-core stop codon (TTG to TAG). The substitution was introduced by a commercially available site directed mutagenesis kit (QuickChange Site-Directed Mutagenesis Kit, Stratagene). Nucleotide sequences of the HBV cloned into plasmids pCAG-HB-wt and pTRE-HB-wt were deposited into the GenBank database under accession numbers AB206817 and AB206816, respectively.

**Transfection of HepG2 Cell Lines With 1.4 Genome Length HBV DNA and Endogenous Polymerase Reaction Analysis.** HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37°C and under 5% CO<sub>2</sub>. The cells were seeded to semi-confluence in 6-well tissue culture plates. For transient transfection experiments, two plasmids; pTRE-HB-wt and pTRE-HB-PC, were used. Two micrograms of each plasmid was transfected using Fu-

gene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the instructions provided by the supplier. Three to five days after transfection, the culture supernatant was collected for infection of mice and quantitative analysis of HBV DNA by real-time PCR. Alternatively, calcium phosphate precipitation was performed to prepare fresh supernatant for large-dose administration experiments. Concentrated supernatants were prepared by using Microsep 10K spin filter, according to the instructions provided by the manufacturer (Pall Life Sciences., Ann Arbor, MI). The HBV particles produced in the supernatants were immunoprecipitated with protein A sepharose and mouse anti-HBs monoclonal antibody 2Z824Z (Institute of Immunology, Tokyo, Japan) and subjected to endogenous polymerase reaction<sup>21</sup> and Southern blot analysis after sodium dodecyl sulfate/proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was electrophoresed in a 1% agarose gel and transferred onto a nylon membrane. The transferred DNA was detected with full-length HBV DNA probe synthesized with the PCR DIG probe synthesis kit and the DIG Nucleic Acid Detection kit and CSPD, ready-to-use (Roche Diagnostics) in the Fluor-S Max MultiImager (BIO-RAD Laboratories, Hercules, CA).

For the production of stably transfected cell lines, HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO<sub>2</sub>. Cells were seeded into 90-mm-diameter culture dishes. Twenty micrograms of the plasmid pCAG-HB-wt was transfected by calcium precipitation. Twenty-four hours after transfection, the cells were split and cultured in G418 selection DMEM (1 mg/mL). One hundred fifty colonies were isolated and amplified for identification of virus-producing cell lines. Clones positive for both HBs and HBe antigens were selected and further analyzed for production of HBV particles. Finally, one of five cell lines that produced more than 10<sup>5</sup> copy/mL HBV DNA in supernatant were selected and used for further experiments. This cell line produced stable levels of HBV DNA for more than 12 months (data not shown).

#### **Analysis of HBV Produced in the Supernatant of Transfected HepG2 Cell Lines by Sucrose Density Gradient.**

Five milliliters HBV-positive serum (10<sup>8</sup> copy/mL) or 100 mL cell culture supernatant (10<sup>7</sup> copy/mL) was layered on a 20% (wt/wt) sucrose gradient, and centrifuged at 24,000 rpm for 1 hour at 4°C with a Beckman SW28 rotor Beckman Coulter, Fullerton, CA). The precipitate was resuspended with 500  $\mu$ L phosphate-buffered saline. These HBV samples were layered on a linear 20% to 50% (wt/wt) sucrose gradient. Centrifugation was carried out at 24,000 rpm for 21 hours at 4°C with a Beckman SW40 rotor. The gradients were frac-

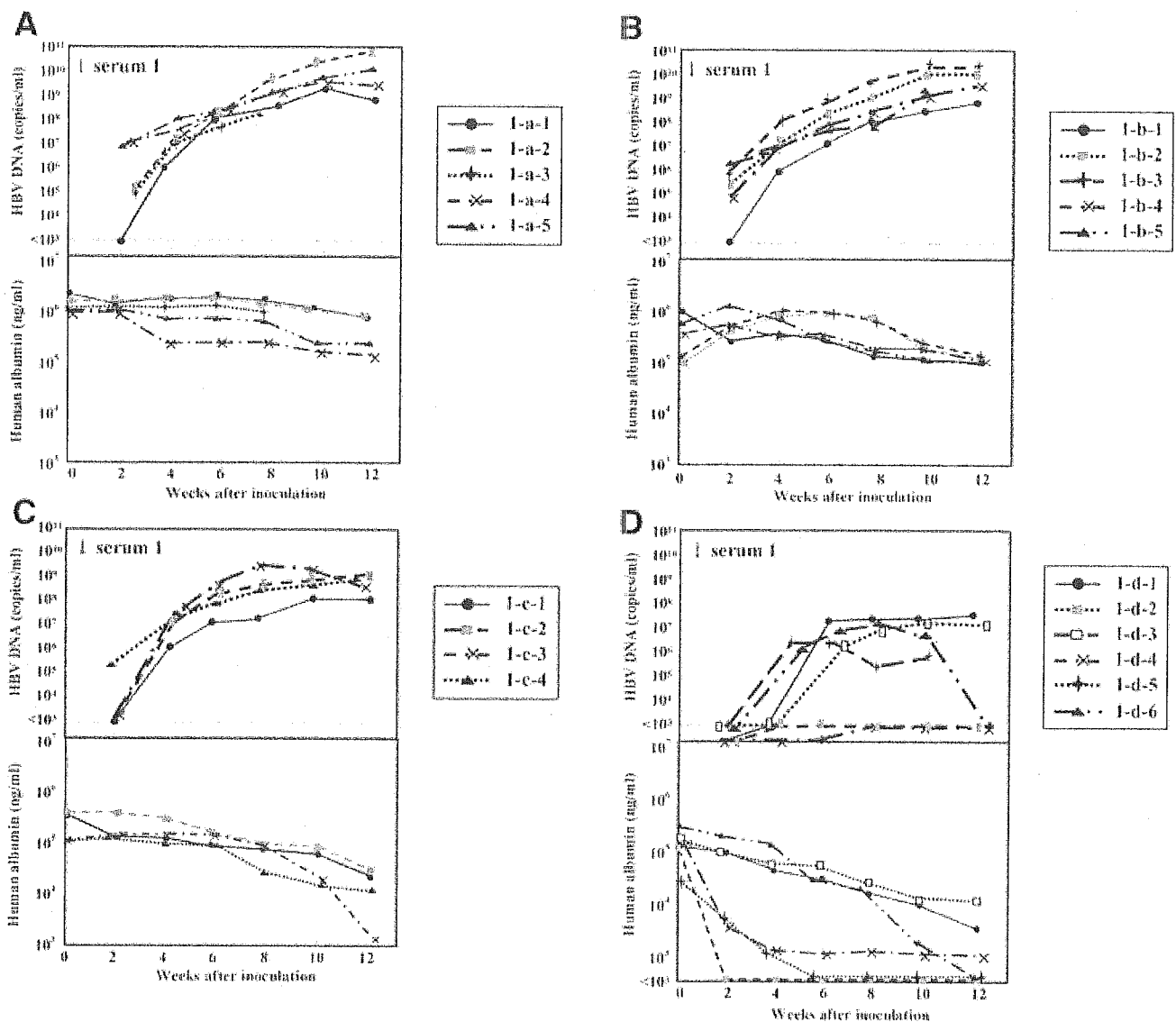


Fig. 1. Time course studies in 20 mice inoculated with human serum samples positive for hepatitis B virus (HBV). Fifty microliters serum samples (serum 1, Table 1) was intravenously injected into each mouse. The upper half of each panel shows HBV DNA, and the lower panel shows concentrations of human serum albumin (HSA). Mice were divided according to the levels of HSA; (A) initial HSA > 1,000,000 ng/mL with only slight decline ( $n = 5$ ); (B) initial HSA > 500,000 ng/mL, with slight decline ( $n = 5$ ); (C) initial HSA > 200,000 ng/mL, but declined to less than 100,000 ng/mL during observation ( $n = 4$ ); (D) initial HSA = 100,000 ng/mL, but diminished to less than 30,000 ng/mL ( $n = 6$ ).

tionated into 500- $\mu$ L 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.

**Generation of Human Hepatocyte Chimeric Mice and Analysis of Serum Samples.** Generation of the  $uPA^{+/+}/SCID^{+/+}$  mice and transplantation of human hepatocytes were performed as described previously by our group.<sup>19</sup> Animal protocols were performed in accordance with the guidelines of the local committee for animal experiments. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. HSA was measured with a Human Albumin ELISA Quantita-

tion kit (Bethyl Laboratories Inc., Montgomery, TX) according to the instructions provided by the manufacturer. Serum samples obtained from mice were aliquoted and stored in liquid nitrogen until use.

**Histochemical Analysis of Mouse Liver.** The liver specimens of infected mice were fixed with 10% buffered-paraformaldehyde and embedded in paraffin blocks for histological examination. The liver sections were stained with hematoxylin-eosin or subjected to immunohistochemical staining by using an antibody against hepatitis B core antigen (HBc-Ag) (DAKO Diagnostika, Hamburg, Germany) or HSA (Bethyl Laboratories Inc.). Endogenous peroxidase activity was blocked with 0.3%  $H_2O_2$

and methanol. Immunoreactive materials were visualized by using a streptavidin-biotin staining kit (Histofine SAB-PO kit; Nichirei, Tokyo) and diaminobenzidine.

## Results

**Human Hepatocyte Chimeric Mice Develop High-Level and Long-Term Viremia After Inoculation of Serum Samples Obtained From Carriers.** Twenty chimeric mice were inoculated with 50  $\mu$ L serum 1 (Table 1). We used mice that had relatively low-level HSA because we had previously found that mice with low-level replacement are susceptible to HBV (Chayama K and Tateno C, unpublished results). The HSA of these mice was 300,000 ng/mL (median, range, 40,000-3,090,000, Fig. 1A). All 20 mice tested positive for HBV DNA by nested PCR 2 to 4 weeks after inoculation. Eighteen of 20 mice developed quantitatively measurable viremia, but two mice showed very low-level viremia that was detectable only by nested PCR. Mice with persistently high-level HSA tended to show high virus titer (Fig. 1A-C). The maximum level of viremia was  $9.5 \times 10^{10}$  copy/mL. The viremia reached a plateau 4 to 6 weeks after infection. In contrast, mice with a rapid decrease in HSA or persistently low-level HSA showed low virus titer (Fig. 1D). We also performed infection experiments using serum 2 (Table 1). Of the five mice inoculated with this serum, all developed quantitatively measurable viremia 2 to 4 weeks after inoculation (Fig. 2). The level of viremia reached  $1 \times 10^7$  to  $1 \times 10^9$  copies/mL. The level of viremia also tended to be high in mice with high HSA levels.

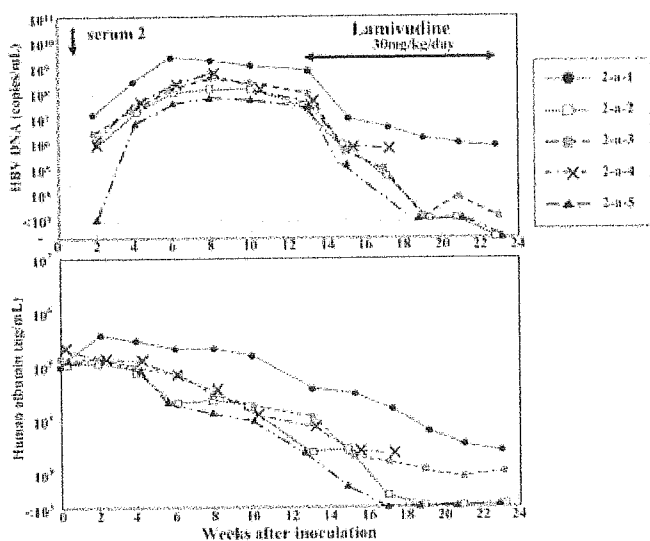


Fig. 2. Time course studies in five mice inoculated with human serum samples positive for hepatitis B virus (HBV). Fifty microliters serum samples (serum 2, Table 1) was intravenously injected into each mouse. The upper panel shows HBV DNA, and the lower panel shows concentrations of human serum albumin. The effects of lamivudine are shown in the upper panels.

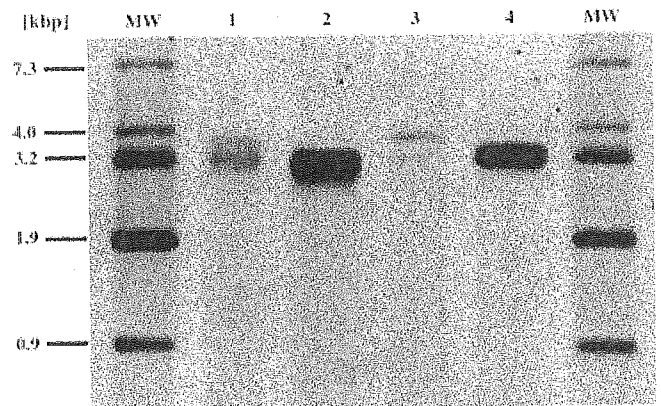


Fig. 3. Formation of fully repaired, relaxed circular hepatitis B virus (HBV) DNA after endogenous polymerase reaction. HBV particles produced into supernatant were immunoprecipitated with a monoclonal antibody against HBs antigen and subjected to Southern blot analysis before (lanes 1 and 2) and after (lanes 3 and 4) endogenous polymerase reaction. The undigested (lanes 1 and 3) and unique *Sma*I-digested DNA was electrophoresed in a 1% agarose gel and detected by Southern blot hybridization.

**HBV Generated in HepG2 Cell Lines Are Infectious to Human Hepatocyte Chimeric Mice.** HBV markers and endogenous polymerase experiments with Southern blot analysis of HBV produced by transiently or stably transfected HepG2 cell lines are shown in Table 1 and Fig. 3. The results indicated that these cell lines produced the expected HBV antigens and HBV DNA into the supernatant. Using virus particles produced by transient transfection of plasmid pTRE-HB-wt, we performed endogenous polymerase chain reaction experiments. Formation of fully double-stranded, relaxed circular DNA was observed after the reaction (Fig. 3). Sucrose density gradient analysis of HBV produced by stably transfected cell line (CA59, Table 1) showed that the produced viruses were sedimented to similar fractions of HBV obtained from the serum of the HBV carrier (Fig. 4), suggesting that HBV particles similar to those in serum are produced in these cell lines.

In the next step, we inoculated each chimeric mouse with 50  $\mu$ L of the supernatants produced by transiently or stably transfected cell lines (Table 1). Three mice were inoculated with CM3 (Fig. 5A). Four weeks later, one of these three mice developed measurable viremia. It reached a high level ( $7.3 \times 10^8$  copy/mL) at week 14. A serum sample obtained from this mouse at week 6 was stored in liquid nitrogen and used in the subsequent passage experiments. The other two mice developed viremia, but its level was so low that HBV was only detectable by nested PCR. At week 13, these two mice (5-a-2, 5-a-3, Fig. 5A) were inoculated with serum 1, which induced high-level viremia in mice with high HSA levels (Fig. 1). These mice did not develop measurable viremia, suggesting that the

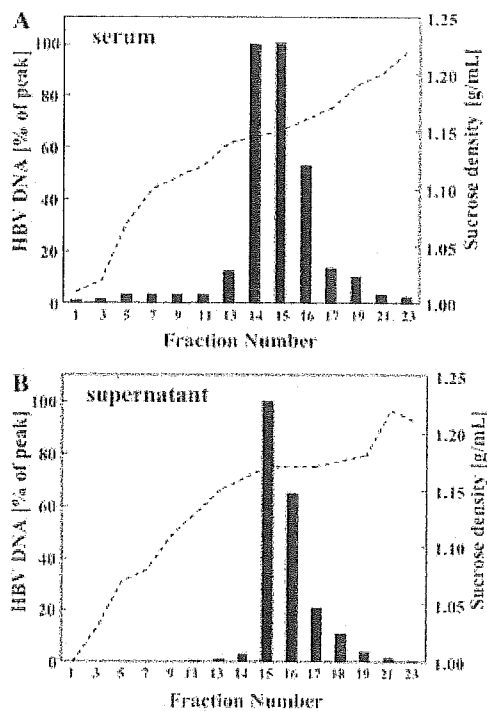


Fig. 4. Sucrose density gradient analysis of hepatitis B virus (HBV) produced into the supernatant of the transfected HepG2 cell line (CA59). Sucrose density is indicated by the dotted line. The amount of HBV DNA in each fraction was measured by real-time PCR.

low-level viremia in the latter two mice was due to low-levels of human hepatocyte replacement. Similarly, six mice were inoculated with supernatant CA59. One of these six mice developed quantitatively measurable viremia (peak,  $2.6 \times 10^9$  copies/mL) (Fig. 5B). Two of the six mice developed viremia only detectable by nested PCR. For a more efficient reverse genetics infection procedure, we used mice with higher human albumin concentrations and inoculated each with 500 to 1,000  $\mu$ L freshly prepared high-titer virus particles. This resulted in infection of all 10 mice (Fig. 5C). Thus, we established a highly effective infection procedure of reverse genetics of HBV.

**Infection of Genetically Engineered Mutant Viruses.** Four mice were inoculated with the supernatant of genetically engineered e-antigen-negative HBV generated in a pTRE-HBV-PC-transfected HepG2 cell line (e-negative, Table 1). Three of these four mice developed quantitatively measurable, but relatively low-level (less than  $10^7$  copies/mL) viremia, 2 to 6 weeks after inoculation (Fig. 6). Nucleotide sequence analysis of the precore region showed that the sequence obtained from the infected mice was completely in agreement with the transfected plasmid with precore stop codon at 1896.

**Passage Experiment of HBV From a Mouse Infected by In Vitro Generated HBV to Naïve Chimeric Mice.** Each of four naïve mice was injected with 5  $\mu$ L serum

samples obtained from a mouse that developed HBV viremia after inoculation of *in vitro* generated virus (CM3, Table 1). All four mice developed viremia at 2 to 6 weeks after inoculation (Fig. 7). One of the four mice that developed measurable viremia died at week 5 (7-a-3, Fig. 7). Another mouse (7-a-2) was weak and was sacrificed at week 13. The high-level viremia in the third mouse (7-a-1) increased further to  $8.5 \times 10^9$  copies/mL. The remaining mice developed viremia detectable only by nested PCR (7-a-4).

**Histochemical Analysis of the Liver of Mice Infected With HBV.** Liver specimens from mice that became positive for HBV DNA after the inoculation of the described passage experiment were subjected to histological and immunohistochemical analyses. Multiple foci of replaced human hepatocytes were noted in hematoxylin-eosin-stained sections (Fig. 8A) that were positive for HSA (Fig. 8B). Such positive human hepatocytes were also positive for the HBV core antigen in serial sections (Fig. 8C).

**Effect of Lamivudine Treatment in Mice Infected With HBV.** Five mice that became positive for HBV DNA by inoculation with serum 2 (Fig. 2) were fed lamivudine (30 mg/kg/day)-containing food. A rapid reduction of HBV DNA level was observed in all 5 mice. Although two of five mice showed graft failure reflected by a decrease in HSA levels to the lower limits of the assay (2-a-2 and 2-a-5), the reduction of HBV DNA levels appeared before the decrease in HSA in these mice, suggesting that the decrease in HBV DNA was due to both the effect of lamivudine and the loss of virus replicating human hepatocytes (Fig. 2). Similarly, 1 mouse with high-level viremia as described in the above passage experiment (7-a-1, Fig. 7) showed a marked reduction of HBV DNA.

## Discussion

The major finding of the current study was the successful establishment of a model of HBV infection with long-term and high-level HBV viremia in the human hepatocyte chimeric mouse. The level of viremia correlated with the degree of human hepatocyte replacement indicated by HSA levels. We also showed that HBV created *in vitro* using HepG2 cell lines are infectious to this mouse model. Thus, a combination of chimeric mouse and molecularly cloned virus enabled us to prepare a practical model for the study of HBV virology. Chimpanzee is also a useful model for the study of HBV virology. Injection of molecularly cloned HBV into the liver of chimpanzee induced HBV infection and hepatitis.<sup>22</sup> However, there might be some difference between hepatocytes of human and chimpanzee that could affect the nature of infection and the replication of this narrow host virus.

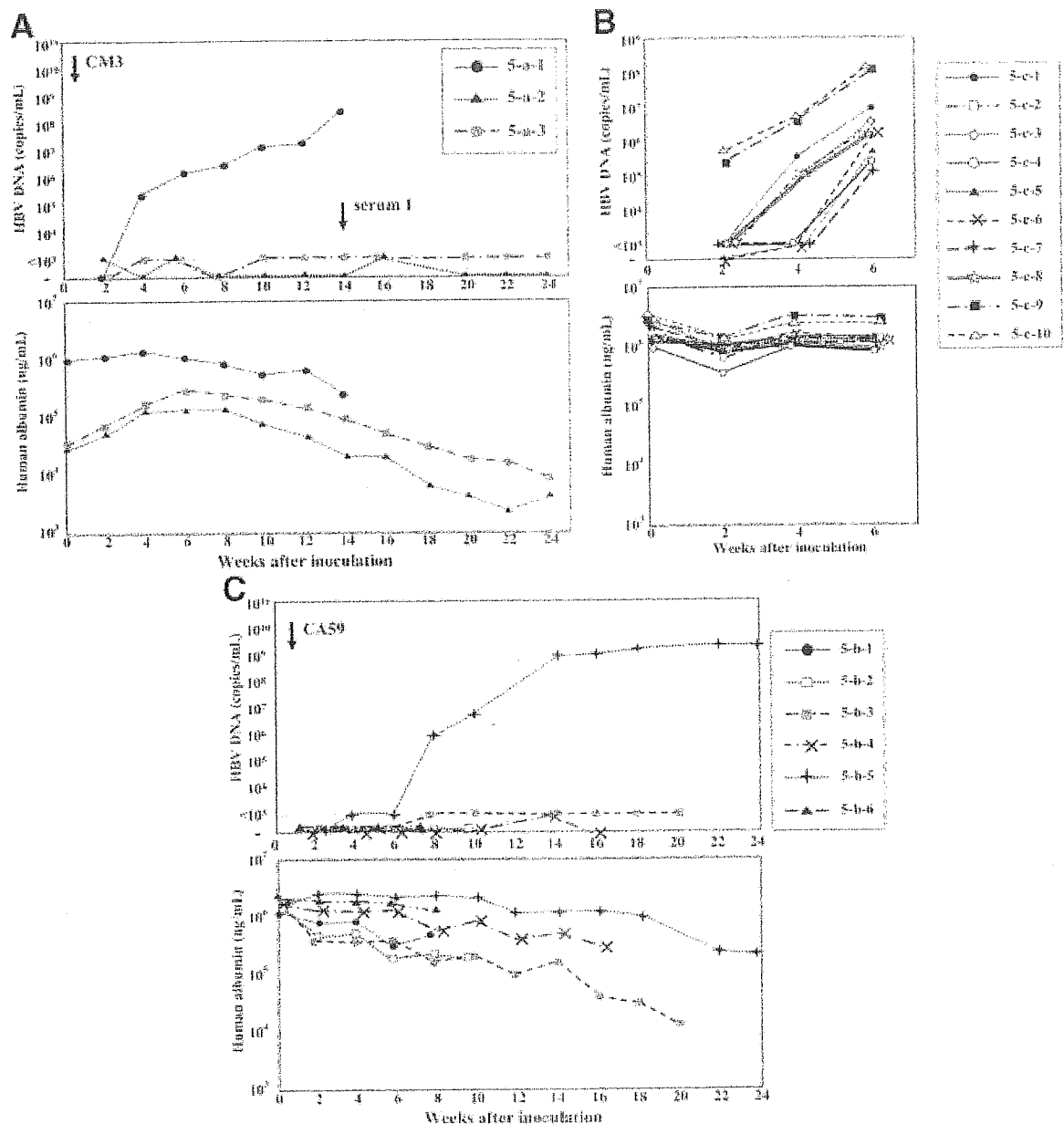


Fig. 5. (A) Time course studies in three mice inoculated with supernatants of HepG2 cell lines transfected with hepatitis B virus (HBV) DNA. Fifty microliters culture supernatant (CM3, Table 1) was inoculated intravenously into each mouse. Upper panel: HBV DNA; lower panel: concentrations of human serum albumin. (B) Time course studies in 6 mice inoculated with supernatants of HepG2 cell lines transfected with HBV DNA. Fifty microliters culture supernatant (CA59, Table 1) was inoculated intravenously into each mouse. (C) Time course studies in 10 mice inoculated with freshly prepared supernatants of HepG2 cell lines transfected with HBV DNA. Five hundred (mice 5-c-1 to 5-c-4) and 1,000  $\mu$ L culture supernatants (5-c-5 to 5-c-8) and 500  $\mu$ L concentrated (from  $1.1 \times 10^9$  copies/mL to  $1.3 \times 10^{10}$  copy/mL by ultrafiltration) supernatant (5-c-9 and 5-c-10) were inoculated intravenously into each mouse.

A critical difference between the chimeric mouse model reported here and chimpanzee is that there is no immune system active for HBV in the mouse model. Although the chimpanzee model is known to cause hepatitis and is suitable for the study of HBV-induced hepatitis,<sup>23</sup> the mouse model is expected to be free from inflammation because these mice are SCID and do not have any human cytotoxic T lymphocytes. Actually, we observed no lymphocyte infiltration or focal necrosis of human hepatocytes in our

mouse model. Recently, similar morphological changes in a similar model were reported by Meuleman et al.<sup>24</sup>; they also observed no alteration of liver architecture by HBV and hepatitis C virus infections. Interestingly, however, we observed a poor increase in the viral titer during the early phase of infection in some mice (Figs. 5A, 6). This might represent some innate anti-viral defense mechanism of liver cells themselves against viral infection. Further investigation is necessary to explore this issue.

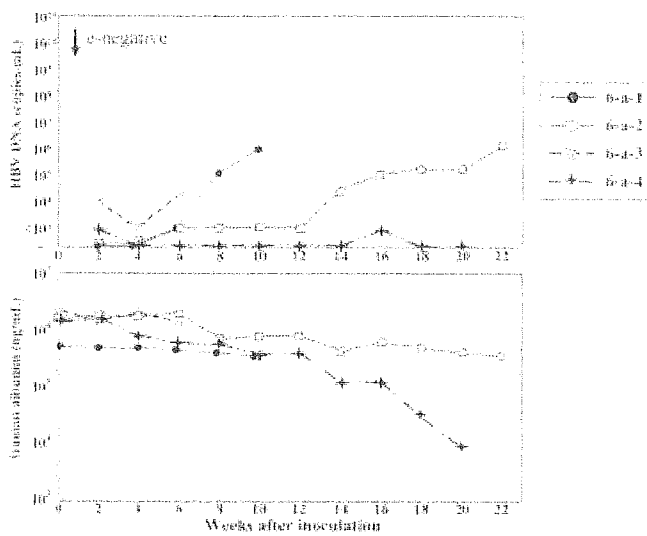


Fig. 6. Time course studies in four mice inoculated with supernatants of HepG2 cell lines transfected with e-antigen-negative (G1896A) hepatitis B virus (HBV) DNA. Fifty microliters culture supernatant (e-negative, Table 1) was inoculated intravenously into each mouse. The upper panel shows HBV DNA, and the lower panel shows the concentrations of human serum albumin.

A mouse model without any inflammation is an advantageous phenotype because it allows the study of HBV replication without any influence of immunological reaction. The model is also beneficial for studying the effects of drugs without any influence of fluctuation of the virus by immunological reaction. The HBV-infected mouse described here opens the way to create a long desired practical small animal model that overcomes economical

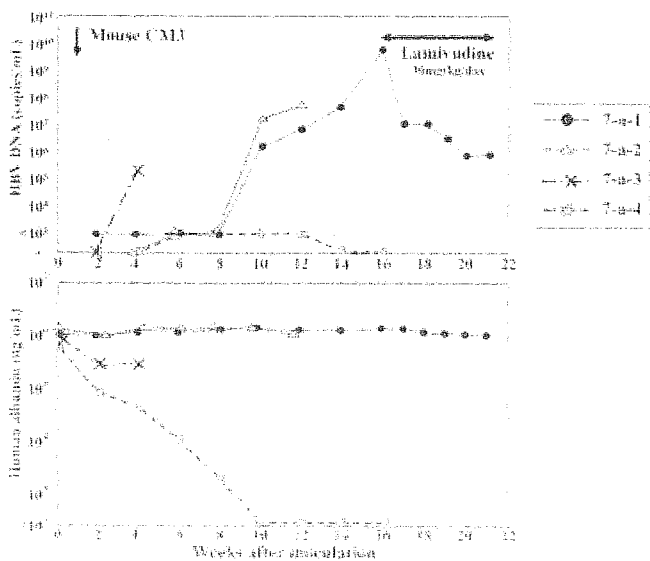


Fig. 7. Passage experiments in four mice. Five microliters mouse serum (mouse CM3, Table 1) was intravenously inoculated into each of four naïve mice. Upper panel: HBV DNA, lower panel: concentrations of human serum albumin.

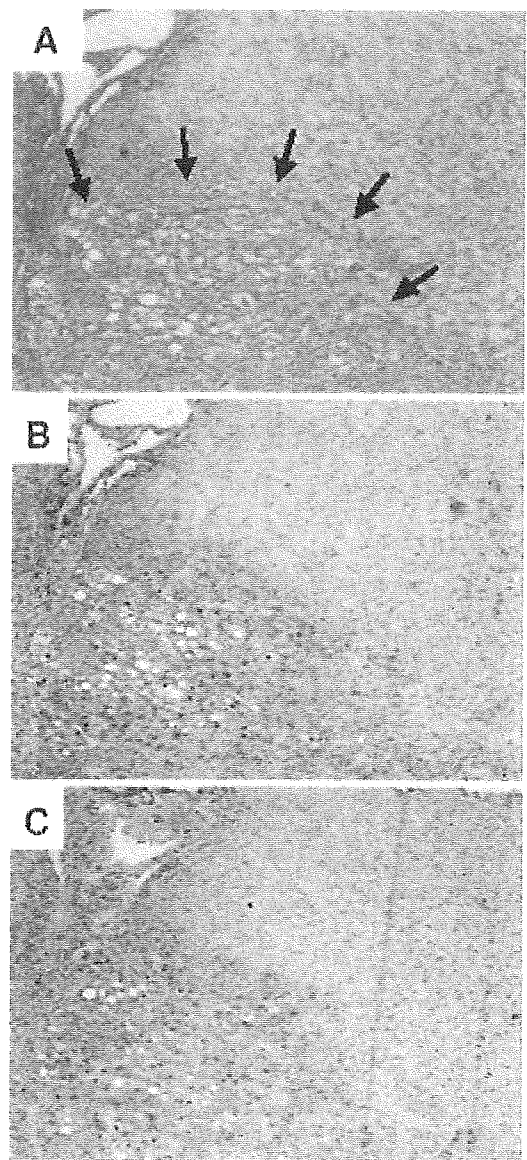


Fig. 8. Histochemical analysis of liver samples obtained from mice infected with hepatitis B virus generated in the 1.4 genome transfected HepG2 cell line. (A) Hematoxylin-eosin staining. Human hepatocytes are indicated by arrows. Immunohistochemical staining with anti-human serum albumin antibody (B) and anti-HBc-Ag antibody (C) (original magnification,  $\times 40$ ).

and ethical problems associated with the chimpanzee model.

We showed in this study that reverse genetics of HBV can be achieved highly efficiently by using mice with high human albumin levels and inoculating mice with large amounts of freshly prepared virus particles (Fig. 5C). We further showed in this study that e-antigen is completely dispensable for infection and replication. The e-antigen-negative HBV-containing serum was previously used in chimpanzee and is known to induce more severe hepatitis.<sup>25</sup> However, it is difficult to exclude the possible presence of a small amount of e-antigen-producing virus that might help infection and



replication of HBe antigen-negative HBV strain. Our results clearly demonstrate that HBV can infect and replicate in the complete absence of e-antigen-producing species. However, the level of viremia was relatively low (less than  $1 \times 10^7$  copies/mL) in these mice. Whether this is due to lack of e-antigen should be further confirmed in a larger number of mice with high replacement index.

Because the mice treated with lamivudine showed a reduction of viremia (Figs. 2, 7), our infected mouse is suitable for the study of new drugs. Lamivudine is a potent anti-HBV drug that reduces the virus and induces clinical remission and histological improvement.<sup>26-28</sup> Emergence of drug-resistant HBV mutants against this drug as well as other anti-viral drugs is a serious problem in the treatment of HBV,<sup>29-31</sup> as has been seen in the therapy of human immunodeficiency virus infection. Our model is especially useful for the study of the biology and drug susceptibility of such mutants, because almost all such drug resistances are based on only one or two point mutation(s).<sup>29,31</sup>

In conclusion, the mouse model presented in this study is very useful for the study of HBV biology and evaluating of anti-HBV drugs. Furthermore, many applications of this model are expected because we can easily create, manipulate, and modify the model compared with other models.

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## References

1. Wright TL, Lau JY. Clinical aspects of hepatitis B virus infection. *Lancet* 1993;27:342:1340-1344.
2. Bruix J, Llover JM. Hepatitis B virus and hepatocellular carcinoma. *J Hepatol* 2003;39 Suppl 1:S59-S63.
3. Raimondo G, Pollicino T, Squadrito G. Clinical virology of hepatitis B virus infection. *J Hepatol* 2003;39(Suppl 1):S26-S30.
4. Ganem D, Prince A. Hepatitis B virus infection: natural history and clinical consequences. *N Engl J Med* 2004;350:1118-1129.
5. Hong HJ, Ryu CJ, Hur H, Kim S, Oh HK, Oh MS, et al. *In vivo* neutralization of hepatitis B virus infection by an anti-preS1 humanized antibody in chimpanzees. *Virology* 2004;318:134-141.
6. Gagneux P, Muchmore EA. The chimpanzee model: contributions and considerations for studies of hepatitis B virus. *Methods Mol Med* 2004;96:289-318.
7. Muchmore EA. Chimpanzee models for human disease and immunobiology. *Immunol Rev* 2001;183:86-93.
8. Bukh J. A critical role for the chimpanzee model in the study of hepatitis B. *HEPATOLOGY* 2004;39:1469-1475.
9. Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus replication in transgenic mice. *J Virol* 1995;69:6158-6169.
10. Uprichard SL, Wieland SF, Althage A, Chisari FV. Transcriptional and posttranscriptional control of hepatitis B virus gene expression. *Proc Natl Acad Sci U S A* 2003;100:1310-1315.
11. Nakamoto Y, Suda T, Momoi T, Kaneko S. Different procarcinogenic potentials of lymphocyte subsets in a transgenic mouse model of chronic hepatitis B. *Cancer Res* 2004;64:3326-3333.
12. Iyer RP, Roland A, Jin Y, Mounir S, Korba B, Julander JG, et al. Anti-hepatitis B virus activity of ORI-9020, a novel phosphorothioate dinucleotide, in a transgenic mouse model. *Antimicrob Agents Chemother* 2004;48:2318-2320.
13. Ilan E, Burakova T, Dagan S, Nussbaum O, Ido L, Eren R, et al. The hepatitis B virus-trimer mouse: a model for human HBV infection and evaluation of anti-HBV therapeutic agents. *HEPATOLOGY* 1999;29:553-562.
14. Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, Sommer G, et al. Repopulation of mouse liver with human hepatocytes and *in vivo* infection with hepatitis B virus. *HEPATOLOGY* 2001;33:981-988.
15. Brown JJ, Parashar B, Moshage H, Tanaka KE, Engelhardt D, Rabbani E, et al. A long-term hepatitis B viremia model generated by transplanting nontumorigenic immortalized human hepatocytes in Rag-2-deficient mice. *HEPATOLOGY* 2000;31:173-181.
16. Petersen J, Dandri M, Gupta S, Rogler CE. Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepatitis B virus infection and clonal growth of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1998;95:310-315.
17. Dandri M, Burda MR, Zuckerman DM, Wursthorn K, Matschl U, Pollok JM, et al. Chronic infection with hepatitis B viruses and antiviral drug evaluation in uPA mice after liver repopulation with tupaia hepatocytes. *J Hepatol* 2005;42:54-60.
18. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfort A, et al. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;7:927-933.
19. Tatenno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004;165:901-912.
20. Gunther S, Sommer G, Von Breunig F, Iwanska A, Kalinina T, Sterneck M, et al. Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: frequency and functional consequences of PCR-introduced mutations. *J Clin Microbiol* 1998;36:531-538.
21. Kim HY, Park GS, Kim EG, Kang SH, Shin HJ, Park S, et al. Oligomer synthesis by priming deficient polymerase in hepatitis B virus core particle. *Virology* 2004;322:22-30.
22. Will H, Cattaneo R, Koch HG, Darai G, Schaller H, Schellekens H, et al. Cloned HBV DNA causes hepatitis in chimpanzees. *Nature* 1982;299:740-742.
23. Gagneux P, Muchmore EA. The chimpanzee model: contributions and considerations for studies of hepatitis B virus. *Methods Mol Med* 2004;96:289-318.
24. Meuleman P, Libbrecht L, De Vos R, de Hemptinne B, Gevaert K, Vandekerckhove J, et al. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *HEPATOLOGY* 2005;41:847-856.
25. Ogata N, Miller RH, Ishak KG, Purcell RH. The complete nucleotide sequence of a pre-core mutant of hepatitis B virus implicated in fulminant hepatitis and its biological characterization in chimpanzees. *Virology* 1993;194:263-276.
26. Honkoop P, de Man RA, Zondervan PE, Schalm SW. Histological improvement in patients with chronic hepatitis B virus infection treated with lamivudine. *Liver* 1997;2:103-106.
27. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 1995;25:1657-1661.
28. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *HEPATOLOGY* 1996;24:711-713.
29. Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine *in vivo*. *HEPATOLOGY* 1996;3:714-717.
30. Honkoop P, Niesters HG, de Man RA, Osterhaus AD, Schalm SW. Lamivudine resistance in immunocompetent chronic hepatitis B. *J Hepatol* 1997;26:1393-1395.
31. Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, et al. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. *HEPATOLOGY* 1998;27:1711-1716.

## EDITORIAL

# Integration of hepatitis B virus DNA and hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common malignant human tumors worldwide.<sup>1</sup> Hepatitis B virus (HBV) and the hepatitis C virus (HCV) infections are etiologically linked to this cancer. HCC often emerges after the development of cirrhosis in patients with chronic HCV infection. In contrast, in patients with the HBV infection, HCC sometimes develop in non-cirrhotic patients, although the incidence is much lower than in patients with cirrhosis.<sup>2</sup> The HBV is a DNA virus that integrates into the chromosome of the host. In contrast, the HCV is a RNA virus that does not integrate into the host genome. It seems that the integration of the HBV into host chromosome(s) plays an important role in the development of HCC, especially in non-cirrhotic patients with HBV infection.

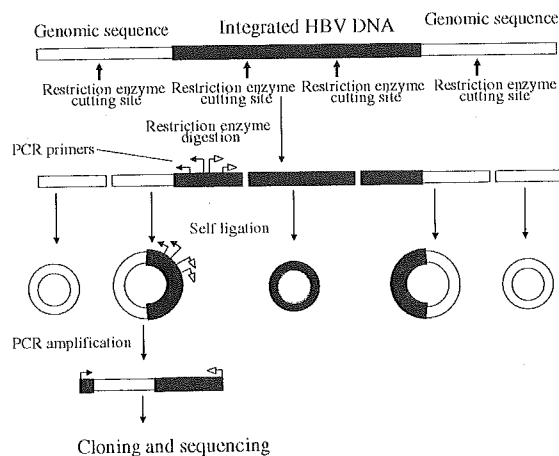
There are two major possible carcinogenic effects of HBV on the development of HCC. One is the direct effect of viral proteins such as HBx. In fact, this protein has been proven to be a transactivator that activates a variety of viral and cellular promoters. It has also been reported that HBx binds to p53 and inhibits p53-mediated cellular processes, including DNA binding, transcriptional transactivation and apoptosis. The viral proteins that might be produced as a result of integration and truncation of preS2/S and hepatitis B spliced proteins have also been reported to function as transcriptional modulators.<sup>3</sup> These proteins have been shown to modulate the transcriptional activation of cellular growth-regulating genes, to modify apoptosis and to inhibit nucleotide excision and the repair of damaged cellular DNA. The other possible carcinogenic effect of HBV is the integration of HBV-DNA into a host chromosome. This might interrupt cellular tumor suppressor genes, or cause alterations in the expression of cellular growth factors and/or apoptosis regulating

factors. Although frequent activation of the N-myc oncogene has been reported in woodchuck hepatitis virus integration,<sup>4</sup> no apparent favored locus of human oncogene/tumor suppressor gene has been found. However, there are many reports that describe the alteration of cellular proliferation pathways (important in the control of cell signaling, proliferation and viability) as a result of the insertion of HBV-DNA. Recent reports have shown that in at least some hepatocellular carcinomas, the human telomerase reverse transcriptase (hTERT) gene is a non-random integration site of the HBV genome, which activates the hTERT transcription *in cis*.<sup>5</sup>

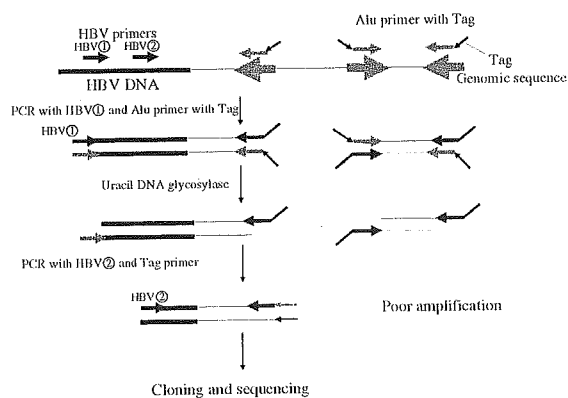
In this issue of the Journal, Huang *et al.*<sup>6</sup> reported a high incidence of HBV-DNA integration in tumor and non-tumor tissues of children with chronic HBV infection. They detected a higher incidence of the HBV integration using a sensitive inverse polymerase chain reaction (IPCR). This method was developed to amplify unknown sequences that flank a region of a known sequence (Fig. 1)<sup>7,8</sup>. This technique is very sensitive because a nested polymerase chain reaction is used for amplification of DNA. However, in some instances, it fails to detect integrated HBV-DNA. For example, integrants that do not contain a full four site of primer escape detection. Alternatively, the flanking region that does not contain an employed restriction enzyme digestion site near the integration portion fails to be amplified. The latter possibility is less likely because the employed restriction enzymes are four base recognizing cutters, the restriction site often appearing in common nucleotide sequences. However, it sometimes the restriction enzyme site is too near to the integration site and consequently, the amplified flanking sequences are too short to analyze. This could be avoided if one were to use several different pairs of enzymes in order to analyze the samples. It is, thus, highly likely that the incidence of integration of HBV-DNA in a host chromosome is higher than that detected.

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**Figure 1** Alu-polymerase chain reaction to detect the integrated hepatitis B virus DNA in host chromosomes.



**Figure 2** Cellular DNA was amplified using primer HBV1 and Alu specific primer with 5'-Tag sequence. Primers were synthesized using dUTP. The amplified products were then digested with uracil DNA glycosylase. Primers in the amplified DNA were digested with this enzyme leaving complementary sequences. The second round PCR was then carried out using HBV2 and a Tag primer. Only sequences that contain HBV-DNA and Alu sequence were effectively amplified.

An alternative method used to detect integration of HBV-DNA is Alu PCR (Fig. 2).<sup>9</sup> A recent study showed that this method is very sensitive, detecting more than 70% of HCC related to HBV containing integrated HBV-DNA.<sup>10</sup> However, this approach might fail to detect integrated HBV-DNA if the site of integration is too far from the Alu sequences. Also, it is possible that the flanking sequence contains a PCR resistant secondary structure or guanine-cytosine rich regions. Furthermore, similarly to IPCR, if the integrated sequence does not contain a sequence for primers to anneal, the examined integration rate might be underestimated.

The high integration rate (related or unrelated to carcinogenesis) and the fact that the related hepadna virus, the duck hepatitis B virus (DHBV), integrates into a host genome very early after infection, show us that

almost all infected cells contain integrated HBV-DNA.<sup>11</sup> However, previous studies have shown that a greater proportion of HBV-DNA integrations are not related to cancer development. Although it is obvious that chronic inflammation and fibrosis are main factors for accelerated cell proliferation and transformation, there are many steps to the development of HCC. Although it remains unclear if the integration of HBV plays an important role in hepatocarcinogenesis, it might open up new avenues of research for hepatocarcinogenesis, for example, to improve the detection of HBV-DNA integration (see Huang *et al.*<sup>6</sup>), and to continue the analysis of the effect of integrated HBV-DNA to intracellular biological phenomenon. Tissues obtained from HCC in children might be good candidates for such analysis because they might cause a serious alteration in chromosomal genetic functions.

## REFERENCES

- Zhu AX. Hepatocellular carcinoma. Are we making progress? *Cancer Invest.* 2003; 21: 418-28.
- Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000; 118: 554-9.
- Anzola M. Hepatocellular carcinoma: role of hepatitis B virus and hepatitis C virus proteins in hepatocarcinogenesis. *J. Viral Hepat.* 2004; 11: 383-93.
- Bruni R, Dugo E, Giuseppetti R, Argentini C, Rapicetta M. Activation of the N-myc2 oncogene by woodchuck hepatitis virus integration in the linked downstream b3n locus in woodchuck hepatocellular carcinoma. *Virology* 1999; 257: 483-90.
- Horikawa I, Barrett JC. Transcriptional regulation of the telomerase hTERT gene as a target for cellular and viral oncogenic mechanism. *Carcinogenesis* 2003; 24: 1167-76.
- Huang HP, Tsuei DJ, Wang KJ *et al.* Differential integration rates of hepatitis B virus DNA in the liver of children with chronic hepatitis V virus infection and with hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* 2005; 20: 1206-1214.
- Huang SH, Hu YY, Wu CH, Holcenberg J. A simple method for direct cloning cDNA sequence that flanks a region of known sequence from total RNA by applying the inverse polymerase chain reaction. *Nucleic Acids Res.* 1990; 18: 1922.
- Ochman H, Gerber AS, Hartl DL. Genetic applications of an inverse polymerase chain reaction. *Genetics* 1988; 120: 621-3.
- Minami M, Poussin K, Bressshot C, Paterlini P. A novel PCR technique using Alu-specific primers to identify unknown flanking sequences from the human genome. *Genomics* 1995; 29: 403-8.
- Brechot PP, Saigo K, Murakami Y *et al.* Hepatitis B virus-related insertional mutagenesis occurs frequently in human liver cancers and recurrently targets human telomerase gene. *Oncogene* 2003; 22: 3911-16.
- Cullen JM, Marion PL, Sherman GJ, Hong X, Newbold JE. Hepatic neoplasms in aflatoxin B1-treated, congenital duck hepatitis B virus-infected, and virus-free pekin ducks. *Cancer Res.* 1990; 50: 4072-80.

## Clinical Studies

## Liver International

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# Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.

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**Abstract:** *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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## Prediction of lamivudine resistance

Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

### Patients and methods

#### Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retro-

spectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

#### Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 µL serum was mixed with 50 µL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 µL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 µL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

#### Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

#### Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance		<i>P</i>
	Negative ( <i>n</i> = 54)	Positive ( <i>n</i> = 27)	
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	> 0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	> 0.2‡
HBV genotype (A/B/C)	2/2/50	0/1/26	> 0.2‡
HBe antigen (positive %)	59%	70%	> 0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	> 0.2‡
At 6 months	27 (11–115)	30 (15–92)	> 0.2‡
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	> 0.2‡
At 6 months	< 2.6 (< 2.6–4.8)	3.3 (< 2.6–6.6)	< 0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (< 3.0–8.8)	7.3 (4.4–9.1)	0.073‡
At 6 months	5.2 (< 3.0–6.7)	5.8 (4.7–8.4)	< 0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. \*Data are expressed as median (range). †Mann–Whitney *U* test. ‡ $\chi^2$ -test.

antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it (*P* > 0.2).

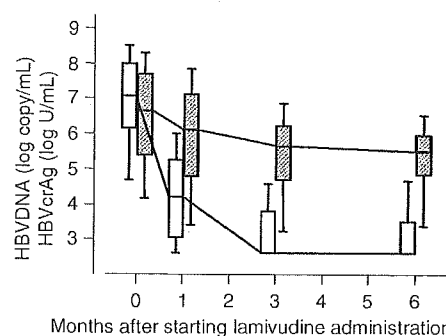


Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, *P* < 0.001), 3 (3.60 log copy/ml vs. 0.83 log U/ml, *P* < 0.001) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, *P* < 0.001) after the initiation of lamivudine administration.

### Prediction of lamivudine resistance

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test,  $P < 0.001$  at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine

resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

### Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

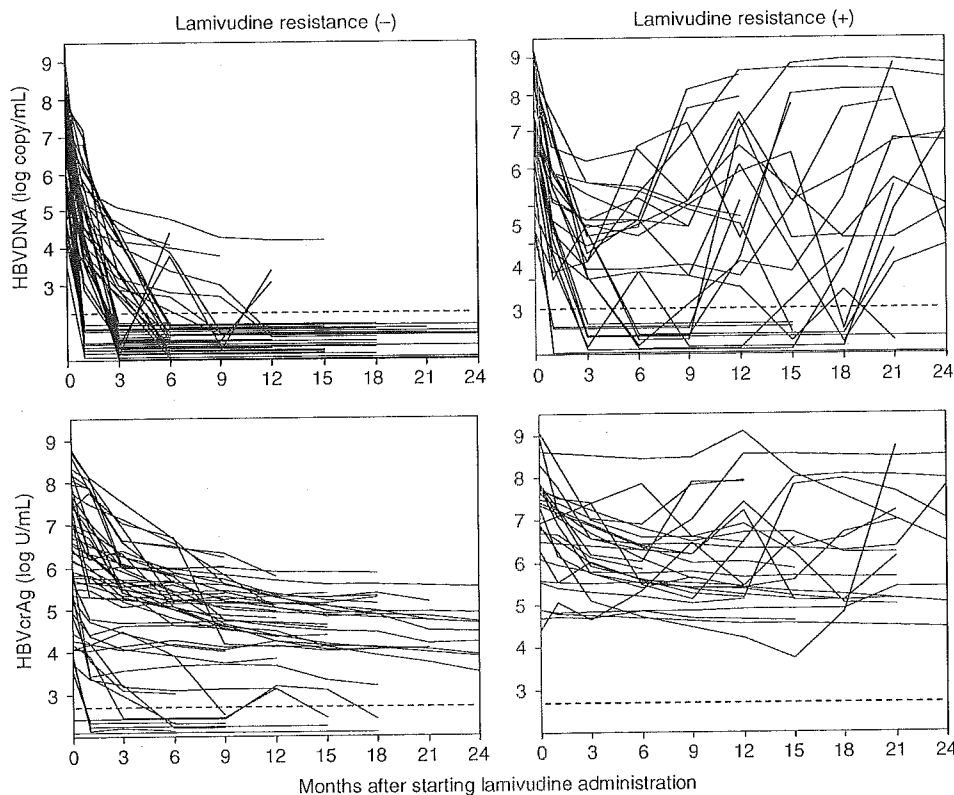


Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

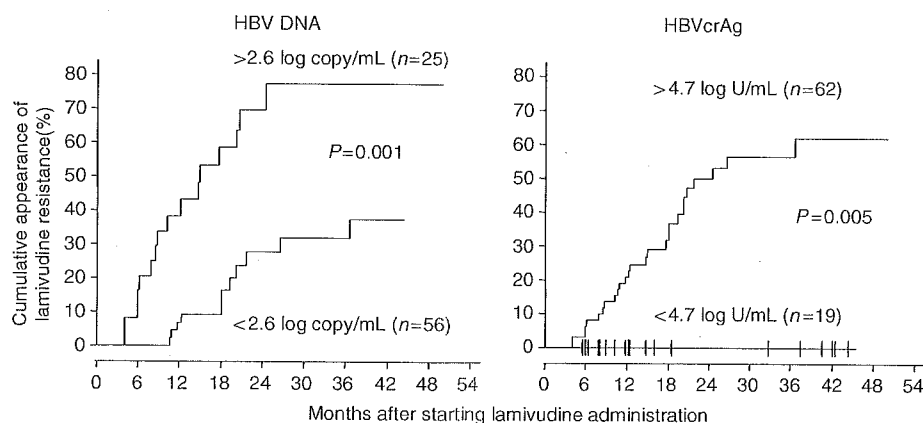


Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than

HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine



## Prediction of lamivudine resistance

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

### Acknowledgements

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### References

1. DOONG S L, TSAI C H, SCHINAZI R F, LIOTTA D C, CHENG Y C. Inhibition of the replication of hepatitis B virus in vitro by 2', 3'-dideoxy-3'-thiacytidine and related analogues. *Proc Natl Acad Sci USA* 1991; 88: 8495-9.
2. BENHAMOU Y, DOHIN E, LUNEL-FABIANI F, POYNARD T, HURAOX J M, KATLAMA C, et al. Efficacy of lamivudine on replication of hepatitis B virus in HIV-infected patients. *Lancet* 1995; 345: 396-7.
3. DIENSTAG J L, GOLDIN R D, HEATHCOTE E J, HANN H W, WOESSNER M, STEPHENSON S L, et al. Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; 124: 105-17.
4. DIENSTAG J L, PERRILLO R P, SCHIFF E R, BARTHOLOMEW M, VICARY C, RUBIN M. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 1995; 333: 1657-61.
5. LAI C L, CHIEN R N, LEUNG N W, CHANG T T, GUAN R, TAI D I, et al. A one-year trial of lamivudine for chronic hepatitis B. *Asia Hepatitis Lamivudine Study Group. N Engl J Med* 1998; 339: 61-8.
6. LIAW Y F, SUNG J J, CHOW W C, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; 351: 1521-31.
7. MATSUMOTO A, TANAKA E, ROKUHARA A, et al. Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2,795 patients. *Hepatology* 2005; 42: 173-84.
8. LING R, MUTIMER D, AHMED M, et al. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* 1996; 24: 711-3.
9. LOK A S, LAI C L, LEUNG N, YAO G B, CUI Z Y, SCHIFF E R, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003; 125: 1714-22.
10. TIPPLES G A, MA M M, FISCHER K P, BAIN V G, KNETEMAN N M, TYRRELL D L. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. *Hepatology* 1996; 24: 714-7.
11. LIAW Y F, CHIEN R N, YEH C T, TSAI S L, CHU C M. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. *Hepatology* 1999; 30: 567-72.
12. SUZUKI F, TSUBOTA A, ARASE Y, SUZUKI Y S, AKUTA N, HOSAKA T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. *Intervirology* 2003; 46: 182-9.
13. ZOLLNER B, SCHAFER P, FEUCHT H H, SCHROTER M, PETERSEN J, LAUFS R. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. *J Med Virol* 2001; 65: 659-63.
14. KIMURA T, ROKUHARA A, SAKAMOTO Y, YAGI S, TANAKA E, KLYOSAWA K, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; 40: 439-45.
15. ROKUHARA A, TANAKA E, MATSUMOTO A, KIMURA T, YAMAURA T, ORII K, et al. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepatol* 2003; 10: 324-30.
16. BRUSS V, GERLICH W H. Formation of transmembraneous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. *Virology* 1988; 163: 268-75.
17. GARCIA P D, OU J H, RUTTER W J, WALTER P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. *J Cell Biol* 1988; 106: 1093-104.
18. LEE W M. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733-45.
19. MIZOKAMI M, NAKANO T, ORITO E, TANAKA Y, SAKUGAWA H, MUKAIDE M, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; 450: 66-71.
20. KOBAYASHI S, SHIMADA K, SUZUKI H, TANIKAWA K, SATA M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). *Hepatology* 2000; 31: 31-42.
21. MASON W S, HALPERN M S, ENGLAND J M, SEAL G, EGAN J, COATES L, et al. Experimental transmission of duck hepatitis B virus. *Virology* 1983; 131: 375-84.
22. SUMMERS J, SMITH P M, HORWICH A L. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol* 1990; 64: 2819-24.
23. TUTTLEMAN J S, POURCEL C, SUMMERS J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986; 47: 451-60.

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24. MORALEDA G, SAPUTELLI J, ALDRICH C E, AVERETT D, CONDREAY L, MASON W S. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. *J Virol* 1997; 71: 9392-9.
25. WERLE-LAPOSTOLLE B, BOWDEN S, LOCARNINI S, WORS-THORN K, PETERSEN J, LAU G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004; 126: 1750-8.
26. ZHU Y, YAMAMOTO T, CULLEN J, et al. Kinetics of hepadnavirus loss from the liver during inhibition of viral DNA synthesis. *J Virol* 2001; 75: 311-22.
27. LAU D T, KHOKHAR M F, DOO E, GHANY M G, HERION D, PARK Y, et al. Long-term therapy of chronic hepatitis B with lamivudine. *Hepatology* 2000; 32: 828-34.
28. YUEN M F, SABLON E, HUI C K, YUAN H J, DE-CRAEMER H, LAI C L. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. *Hepatology* 2001; 34: 785-91.
29. MIHM U, SARRAZIN C, HERRMANN E, TEUBER G, VON WAGNER M, KRONEA BERGER B, et al. Response predictors and results of a long-term treatment with lamivudine in patients with chronic hepatitis B. *Z Gastroenterol* 2003; 41: 249-54.

# Hepatitis C Virus Core Protein Modulates Fatty Acid Metabolism and Thereby Causes Lipid Accumulation in the Liver

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We studied the roles of hepatitis C virus (HCV) core protein in hepatic steatosis and changes in hepatic lipid metabolism. HCV core protein expression plasmid was transfected in HepG2. Triacylglyceride (TG) and mRNA level associated with lipid metabolism were measured. Male C57BL/6 mice were infected with HCV core recombinant adenovirus and used for lipids and mRNA studies. In HCV core protein-expressing cells, peroxisome proliferator-activated receptor (PPAR) $\alpha$ , multidrug resistance protein (MDR) 3, and microsomal triglyceride transfer protein (MTP) were down-regulated 48 hr after transfection. In HCV core protein-expressing mice, hepatic TG content and hepatic thio-barbituric acid-reactive substances increased. PPAR $\alpha$ , MDR2, acyl-CoA oxidase (AOX), and carnitine palmitoyl transferase-1 (CPT-1) were down-regulated. HCV core protein down-regulated lipid metabolism-associated gene expression, Mdr2, CPT, and AOX, accompanied by down-regulation of PPAR $\alpha$ . These findings may contribute to the understanding of HCV-related steatosis, induction of reactive oxygen species, and carcinogenesis.

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**KEY WORDS:** HCV core protein; steatosis; nuclear receptor; ABC transporter.

Chronic hepatitis C virus (HCV) infection results in necroinflammatory liver disease that is characterized by the insidious progression of hepatic fibrosis and the loss of functioning hepatocytes (1–3). Little is known about the molecular mechanisms underlying liver injury due to infection with this virus, but a cell-mediated immune response associated with prominent lymphocytic infiltration of hepatic tissues is thought to play a major role (4, 5). In addition, various observations have suggested that nonimmune mechanisms may also play an important role. These

findings include the frequent presence of hepatic steatosis in patients with chronic hepatitis C, an abnormality that is not often observed in other inflammatory conditions such as autoimmune hepatitis and chronic hepatitis B (6–9). Also, a considerable number of *in vitro* studies have suggested that expression of various HCV proteins may lead to alterations of lipid metabolism and transport, cell cycle dysregulation, increased or decreased susceptibility to apoptosis, and cellular transformation (10–17). In particular, HCV core protein has been suggested to contribute to hepatic steatosis (18–20), induction of reactive oxygen species (ROS) (19–21), and hepatic carcinogenesis (22).

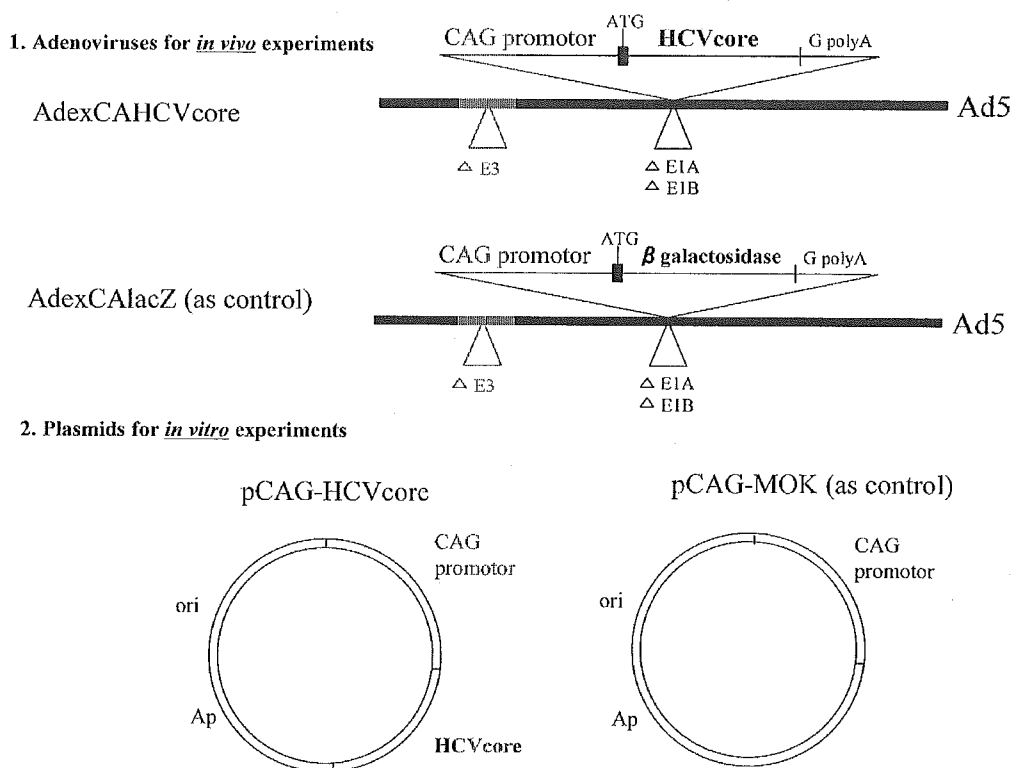
Regarding HCV core protein-induced steatosis, the following findings have been reported: (a) HCV core protein interacts with apoA2, a major component of high-density lipoprotein (10, 23), (b) HCV core protein interferes with the assembly of very low-density lipoprotein (VLDL) by reducing the level of microsomal triglyceride transfer

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**Fig 1.** Constructs of recombinant adenoviruses and plasmids employed in this study. See Materials and Methods. ATG, start codon; G poly(A), rabbit  $\beta$ -globin poly(A); CAG promoter; cytomegalovirus enhancer, chicken  $\beta$ -actin promoter, and rabbit  $\beta$ -globin poly(A); Ad5, adenovirus type 5 genome lacking E1A, E1B, and E3.

protein (24), and (c) HCV core protein causes steatosis due to mitochondrial toxicity and production of ROS (19, 20). However, the details of the interaction between HCV and lipid metabolism remain unclear. Hepatocytes represent the crossroads of various metabolic pathways, so HCV may interfere with lipid metabolism via one or several pathways. To investigate the role of HCV core protein in steatosis and the accompanying changes in hepatic lipid metabolism, we focused on fatty acid metabolism-associated proteins, including those involved in fatty acid oxidation and lipid transport into blood and bile, as well as nuclear receptors.

## MATERIALS AND METHODS

**Plasmid and Recombinant Adenovirus.** The complementary DNA clone of the full-length HCV core protein (amino acids [aa] 1–191) was derived from the serum of a patient with HCV 1b by reverse transcription and nested polymerase chain reaction. First-strand primers were 5'-CTGCTAGCCGAGTAGTGTG-3' and 5'-CATTGAGGACCACCAGGTTCT-3', while second-strand primers were 5'-CGGGAATTCTCGTAGACCGTGACCATGAGC-3' and 5'-GTTGGGATCCTCCTAAGCGGAAGCTGGAT-3'. The gene was inserted into pBluescript (Stratagene,

La Jolla, CA, USA) and cloned. Then it was made to correspond with HCV 132996 (GenBank) using a QuikChange Site-Directed Mutagenesis kit (Stratagene). The HCV core protein expression plasmid (pCAG-HCVcore), a control plasmid (MOK), and a  $\beta$ -galactosidase expression plasmid (pCAG-LacZ) were prepared using an adenovirus expression vector kit (Takara Biotechnology, Tokyo) (25, 26). The HCV core gene was inserted into the *Swa*I site in cosmid vector pAxCawt, which is a 44.741-kilobase cosmid containing a 31-kilobase adenovirus type 5 genome lacking the E1A, E1B, and E3 genes, but including the cytomegalovirus enhancer, chicken  $\beta$ -actin promoter, and rabbit  $\beta$ -globin poly(A) signal (pAxCaiHCVcore). The cosmid vector pAxCaiLacZ, with the  $\beta$ -galactosidase gene inserted into pAxCawt, was included in the adenovirus expression vector kit. These three vectors (pAxCawt, pAxCaiHCVcore, and pAxCaiLacZ) were digested at the *Sal*I site and ligated, yielding the pCAG-MOK, pCAG-HCVcore, and pCAG-LacZ expression plasmids for cell transfection experiments. The cosmid pAxCaiHCVcore or pAxCaiLacZ was cotransfected into 293 cells with adenovirus DNA by calcium phosphate precipitation. Incorporation of the expression cassette was confirmed by digestion with *Clal*. Recombinant adenovirus (AdexCAHCVcore or AdexCALacZ) was propagated in 293 cells and the viral titer was determined as the 50% tissue culture infectious dose using 293 cells. These viruses were used for animal experiments (Figure 1).

**Cell Culture.** HepG2 cells were seeded into 56-cm<sup>2</sup> tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM)