# A Long-Term Hepatitis B Viremia Model Generated by Transplanting Nontumorigenic Immortalized Human Hepatocytes in Rag-2–Deficient Mice

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Development of new therapies for human hepatitis B virus infection (HBV) would be greatly facilitated by the availability of a suitable small-animal model for HBV virus production *in vivo*. To develop a murine model for HBV production, we established an immortalized, cloned liver cell line by transferring the Simian Virus 40 Large T-Antigen into primary human hepatocytes. These cells were stably transfected with a full-length HBV genome to generate a clone that expresses HBV genes and replicates HBV. The HBV–producing cells were transplanted into the livers of mice with combined immunodeficiency (Rag-2 deficient) by intrasplenic injection. Survival of the engrafted human hepatocytes was shown in several ways: fluorescent *in situ* hybridization (FISH) with a human-chromosome–specific DNA probe (human alpha satellite), dot-blot hybridization of the genomic DNA extracted from liver biopsy specimens with a human-specific Alu repetitive DNA probe, Blur-8, as well as with an HBV DNA probe, and secretion of human proteins into plasma. Histological examination of mouse liver up to 8 months following human cell transplant shows completely normal architecture. Determination of plasma HBV DNA levels indicated that engrafted cells secreted $3\times10^7$ to $3\times10^8$ virions per mL into the blood, and HBsAg was detected in plasma. This new murine model of HBV viremia should be useful for *in vivo* HBV studies. (HEPATOLOGY 2000;31:173-181.)

Hepatitis B virus (HBV) is a major cause of morbidity, that affects as many as 350 million people worldwide, resulting in an estimated 2 million deaths per year.[1,2] Currently available therapies for HBV–related hepatitis have limited success.[3] Therefore, new antiviral therapies that inhibit HBV replication or gene expression are being developed. Severe adverse reactions to nucleoside analogues in recent clinical trials[4,5] underscore the need for a small animal model to screen the safety and efficacy of new therapeutic modalities. Because of the narrow host range of HBV, existing animal models are limited to chimpanzees[6] and the recently reported tupaia,[7-10] which are expensive and in short supply. Related hepadnaviridae, duck HBV,[11] and woodchuck HBV,[12] infect their respective natural hosts; however, these viruses are of limited relevance to human HBV infection because of significant structural divergence among the viruses.[13,14] For these reasons, it would be advantageous to have a small animal model for human HBV.

In efforts to produce murine models of HBV viremia, other investigators have transferred HBV to mice as a transgene, or by ectopic transplantation of human tumor cell lines producing HBV. HBV transgenic mice produce virus from HBV DNA, which is integrated into mouse chromosomes of all mouse cells.[15] Viral replication and production of all viral antigens have been shown in this model. Disadvantages of transgenic mice include the production of HBV in mouse hepatocytes but not in human hepatocytes, the absense of covalently closed circular DNA (CCC) viral transcription templates, and vertical rather than horizontal transmission of the virus. The other available murine model is the immunodeficient mouse with ectopically transplanted human tumor cell lines containing or producing HBV.[16,17] BALB/c nude mice injected with PLC/PRF/5 tumor cells subcutaneously were shown 2 decades ago to produce tumors, secrete HBsAg, and contain HBV DNA, however their life span and usefulness are restricted by the tumor growth. HepG2.2.15 tumor cell lines subcutaneously injected in SCID mice[17] replicate HBV and secrete viral markers. This model is limited by the ectopic location of the human tumor cells and by their transformed phenotype, both of which prevent normal interactions of engrafted cells with host hepatocytes. Furthermore, human tumor growth in the host mice limits their survival to only a few weeks.[17,18]

We developed an immortalized, but nontumorigenic human hepatocyte clone by stable transfection of the HBV genome, which expresses HBV antigens and replicates virus.

Abbreviations: HBV, hepatitis B virus; FISH, fluorescent *in situ* hybridization; Rag-2, recombination activation gene 2; HIV, human immunodeficiency virus; SV40, simian virus 40; UGT, uridinediphosphoglucuronate glucuronosyltransferase; PBS, phosphate-buffered saline, PCR, polymerase chain reaction; SSC, sodium citrate buffer; SDS, sodium dodecyl sulfate; HBsAg, hepatitis B virus surface antigen; HBcAg, hepatitis B virus core antigen; NK, natural killer cells; HAAT, Human α-1 antitrypsin.

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The HBV–producing cells were transplanted into the livers of immunodeficient mice (Rag-2 knock-out[19]) by intrasplenic injection.[20] The human cells exhibited long-term survival without producing tumors, generating constant HBV viremia in the host mouse. This new human-mouse chimeric model should permit long-term studies of HBV replication *in vivo*, which have been limited, heretofore, to human subjects and primates.

## MATERIALS AND METHODS

*Animals.* Rag-2–deficient mice were obtained from Taconic Farms (Germantown, NY) and bred, under agreement, in isolation chambers, at the Central Animal Institute of the Albert Einstein College of Medicine. All animal-use protocols were approved by the institutional regulatory group of the Albert Einstein College of Medicine and conform to established NIH guidelines for animal use.

*Immortalization of Human Hepatocytes.* Immortalization of human primary hepatocytes by transduction with the SV40 T-antigen gene was performed as described previously.[21] In brief, hepatocytes were isolated by collagenase perfusion of a histologically normal segment of a liver that had been resected from a 59-year-old male with hepatic metastasis of colon cancer. The liver tissue and the patient serum were negative for HBV or HIV. The cells were transfected with a plasmid encoding the large T-antigen of the simian virus 40 (SV-40) to overcome growth arrest and selected for survival and growth in cell culture. A clone derived from this cell population was termed IHH and was used for subsequent experiments. The cells were cultured on Primaria plates (Falcon) in Chee's medium (Gibco/BRL) supplemented with 5% fetal bovine serum, 100 units/mL pennicillin, 100 mg/mL streptomycin, 2 mmol/L L-glutamine, 50 mmol/L dexamethasone, 20 mU/mL recombinant insulin (Boehringer Mannheim, Indianapolis, IN), 168mg/L arginine, and 10 mg/L thymidine. Media were replaced every other day and the cells were split 1:3 when they reached confluence (approximately in 7 days).

*Characterization of the Immortalized Human Hepatocytes.* The IHH cells were examined by phase contrast microscopy, and their doubling time was determined. To evaluate these cells for expression of hepatocyte-specific genes, immunotransblot analysis was performed as described previously.[22] Briefly, when the cells grew to confluence, they were harvested, washed, and homogenized by sonication, and the proteins were separated by electrophoresis on sodium dodecylsulfate-7.5% polyacrylamide gels. The separated proteins were electroblotted to PVDF membranes, and Western blot analysis was performed for the hepatocyte-specific proteins, albumin, bilirubin-uridinediphosphoglucuronate glucuronosyltransferase (bilirubin-UGT), and asialoglycoprotein receptor. For comparison, immunoblot analysis was performed on isolated primary hepatocytes (Clonetics, Walkersville, MD), cultured for 24 hours under similar conditions.

*Evaluation of Tumorigenicity of Immortalized Human Hepatocytes.* Soft agar colony growth was assessed for immortalized human hepatocytes, by plating cells in soft agar and counting colonies surviving at various time points.

*Stable Transfection With Genome Length HBV DNA.* IHH cells were stably transfected with circular HBV DNA prepared by *Eco*RI–linearization of a cloned full-length HBV genome, purification of linear monomers, and recircularization by ligation. This procedure generates replication-competent HBV circular genomes free of plasmid sequence.[23] The circularized HBV DNA was cotransfected into the IHH cells, using Lipofectamine (Gibco/BRL) according to manufacturer's recommendations, along with pRC/CMVneo (Invitrogen, SanDiego, CA), which confers resistance to the antibiotic, G418. Stable transfectants were selected initially by resistance to G418 at 300 mg/mL (Gibco/BRL) and then screened in multiwell plates by immunological analysis of the media for HBsAg (HBV AUS Ab ELISA, Abbott). HBsAg–expressing clones were subcloned by limiting dilution and selection in cloning rings and rescreened for viral gene expression. Clone IHBV6.7, expressing high levels of HBsAg, was used for further experiments.

*Evaluation of IHBV6.7 Cells for Tumorigenicity in Rag-2 Mouse Liver,* In Vivo. Following transplantation into the liver of Rag-2–deficient mice, the recipients were observed for up to 8 months. After this period, the livers were harvested, examined for evidence of tumors, and sections were examined histologically by hematoxylin-eosin staining for any alterations in liver structure.

*Subcutaneous Injection of IHBV6.7 Cells In SCID (Rag-2) Mice to Assay Tumorigenicity.* Recombination activation gene-2 (Rag-2M) mice were injected with IHBV6.7 cells, or as a positive control for tumor growth, HuH-7 cells, at 1 million cells per 0.1 mL (in sterile PBS) under the skin of the scapular region. Mice were observed weekly for the following 6 months for the appearance of tumors at the injection site.

*Evaluation of HBV Production by IHBV6.7 Cells.* DNA was extracted from nuclear and cytoplasmic fractions of IHBV6.7 cells, washed twice in ice-cold PBS, and resuspended in 10mmol/L Tris HCl pH7.5, 150mmol/L NaCl, 10mmol/L disodium EDTA (TNE). Cells were pelleted and resuspended in TNE containing 0.1% Triton-X100 to solubilize cell membranes. Lysates were centrifuged at 2,000 *g* for 5 minutes to separate the nuclei from the cytoplasmic fraction. DNA was extracted from both the nuclear and the cytoplasmic fraction by incubation in TNE containing 0.1% SDS and 100 mg/mL proteinase K, at 55°C for 2 hours, followed by 2 phenol/chloroform extractions and ethanol precipitation. The DNA fractions were analyzed by restriction-enzyme digestion, gel electrophoresis, and Southern blot using a full-length HBV DNA probe, labeled by random priming with $^{32}$P-dCTP (Amersham).

*Transplantation of IHBV6.7 Cells Into the Livers of Rag-2–deficient Mice.* Six- to eight-week-old homozygous Rag-2–deficient mice housed under sterile conditions in a barrier facility were anaesthetized by intraperitoneal injection of a sterile 2% solution of 2-2-2-tribromoethanol solution (Aldrich Chemicals, Milwaukee, WI) in PBS (80 mg/100 g body weight). Hepatocyte transplantation was performed essentially as described.[20] Briefly, under aseptic conditions, in a laminar flow hood, the lower pole of the spleen was exposed by a small left subcostal incision. The spleen was stabilized by holding the fat pad of the splenic mesentery with an iris forceps. One million IHBV6.7 cells for each transplantation were harvested from culture plates, washed in PBS, resuspended in 0.1 mL of PBS, and injected into the splenic pulp over 30 seconds using a 27-gauge needle. The spleen was returned to the body cavity, and the incision was closed in 2 layers with 4.0 chromic gut. Untreated sibling mice were kept as controls in separate cages.

*Analysis of HBV Secretion Into the Blood.* After transplantation of the IHBV6.7 cells, blood samples were collected from the mouse tail vein at various time intervals under sterile conditions. For extracting DNA, 50 mL of the plasma was added to 0.4 mL of 50 mmol/L Tris-HCl, pH 8.0/ 100 mmol/L EDTA, and 0.5% SDS/100 mg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN). Following overnight incubation at 55°C, the DNA was purified by phenol extraction, phenol/chloroform extraction, and ethanol precipitation. The DNA was then resuspended in 30 mL 10 mmol/L Tris, 1mmol/L EDTA, pH7.5. The conserved sequences within the HBV core region were amplified by polymerase chain reaction (PCR), using specific amplimers (HB01 and HB02, Enzo Diagnostics, Farmingdale, NY) and the following thermocycling program: 94°C for 8 minutes, 95°C for 2 minutes, 55°C for 1 minute, 74°C for 2 minutes, followed by 8 cycles of 95°C for 55 seconds, 58°C for 50 seconds, and a final cycle of 74°C for 8 minutes. Generation of the specific amplicon was evaluated using the HBV Microplate Hybridization Assay kit (Enzo Diagnostics, Farmingdale, NY). For dot-blot hybridization analysis, the DNA was blotted to Biodyne B nylon membranes (Gibco/BRL) as described[24] and hybridized to a $^{32}$P-dCTP–labeled full-length HBV DNA probe. Blots were washed under stringent conditions: 0.1 × SSC and 0.1 % SDS at 60°C for 30 minutes.

*Identification of Human Genomic DNA in Transplant Recipient Mouse Liver.* Under general anesthesia with 2-2-2-tribromoethanol, tissues were removed from transplant recipient and control untransplanted

mice, which were promptly euthanized by anesthesia overdose. The presence of human genomic DNA in the liver, spleen, lung, and kidney was evaluated by dot-blot hybridization of extracted DNA and fluorescent *in situ* hybridization of tissue sections.

*Dot-Blot Hybridization.* Total genomic DNA was extracted by standard methods from human hepatocytes, or Rag-2M mice, or Rag-2 mice, which received human hepatocyte transplants intrasplenically.[25] The DNA specimens were applied as dot-blots (5 mg/blot) and hybridized to a $^{32}$P-labeled, purified DNA insert, derived from a cloned human-specific Alu repetitive DNA element (Blur8, ATCC, Rockville, MD). Serial dilutions of human DNA were prepared using mouse DNA as the diluent. These were the standards used to quantitate the level of human DNA present in the chimeric mouse human livers created by the hepatocyte transplantation. Levels of Alu hybridization of the serially diluted human controls were compared with levels of hybridization of the experimental mice that received human hepatocyte transplants.

*Fluorescent* In Situ *Hybridization (FISH).* Liver sections were analyzed for presence of human DNA using negative control Rag-2M mice, or IHBV transplant recipient Rag-2M mice, or as positive controls, HuH-7 human hepatoma tumors grown subcutaneously in Rag-2 mice. Formalin-fixed, paraffin embedded tissue sections of liver were deparaffinized by baking at 60°C for 2 hours, rinsed twice in xylene, and then hydrated in progressively decreasing concentrations of ethanol. After incubation with proteinase K (0.4 mg/mL in PBS) at 37°C for 10 minutes, the tissue sections were rinsed in cold PBS, fixed in buffered 10% formalin for 1 minute and washed again with PBS. The sections were dehydrated in progressively increasing concentrations of ethanol and air-dried for 10 minutes. Hybridization was carried out using 20 ng of fluorescein-labeled DNA probe for human chromosomes, alpha satellite (Boehringer Mannheim), in 20 mL of hybridization solution (Gibco/BRL) per slide. DNA was denatured by a brief exposure to 100°C on a heating block, following which the slides were kept at 42°C overnight. Slides were protected from light. After washing twice in 2 × SSC for 15 minutes, 2 sections from each mouse liver were viewed and selected fields photographed using filters for fluorescein.

*Human α-1 Antitrypsin Detection in Transplant Recipient Mouse Plasma.* Human-specific rabbit antihuman human α-1 antitrypsin (HAAT) antibody (Boehringer Mannheim) was used at 1:500 dilution for ELISA, with a secondary antibody of goat antirabbit (BioRad) at 1:3000 dilution. The presence and concentration of HAAT in mouse plasma from transplant recipients was determined by comparison to dilutions of normal human plasma samples and negative control mouse plasma samples in the same plate assay as experimental samples.

## RESULTS

Our experimental design was to transplant nontumorigenic human hepatocytes to the liver of mice that are unable to mount an immunological rejection of xenografts. The engrafted human cells within the mouse liver were identified using human genome-specific DNA probes. In addition, the survival and function of the transplanted cells were detected by the secretion of human proteins, HBV DNA, and viral antigens in plasma.

*Characteristics of the Immortalized Human Hepatocytes.* The immortalized cells doubled every 72 hours, but the proliferation was inhibited when the cells became confluent. Immunotransblot experiments showed that the immortalized hepatocytes contained several hepatocyte-specific proteins. Densitometry of the immunoreactive bands indicated that the immortalized hepatocytes contained human serum albumin, apolipoprotein B100, bilirubin-UGT, and asialoglycoprotein receptor at 80%, 40%, 20%, and 18% of the levels observed in primary human hepatocytes cultured for 24 hours. A large fraction of the cells became polarized in culture, forming bile canaliculi-like structures.[21] The cells do not form 3-dimensional colonies when cultured on soft agar and do not form tumors when transplanted into the livers of mice with severe combined immunodeficiency (SCID) (unpublished observation, 1997).

*Generation of HBV DNA by IHBV6.7 Cells.* IHBV6.7 cells were produced by DNA transfection of cloned recircularized HBV DNA, as shown in Fig. 1. The restriction map expected from single or tandem integrations of HBV into human chromosomal DNA is shown. HBV viral content of cloned populations of IHBV6.7 cells was determined by analysis of HBV DNA in nuclear and cytoplasmic DNA fractions. The HBV virion genome consists of a partially double-stranded, circular 3.2-kb DNA, open-circular DNA (OC DNA). The incomplete second DNA strand is completed during viral replication, whereby the HBV DNA molecule becomes fully double-stranded, covalently closed circular DNA (CCC DNA). The CCC DNA becomes supercoiled and, therefore, migrates ahead of linear or OC HBV DNA on agarose gel electrophoresis. Both circular HBV genomes are linearized by *Eco*RI digestion. In IHBV6.7 cells, Southern blot analysis of the DNA extracted from the cytoplasmic fraction showed HBV DNA forms migrate as circular episomes at linear marker sizes of 4 kb and 2 kb, characteristic of replicating virus open-circular and CCC (Fig. 1). Linearization of these eposiomal virus DNA's by *Eco*RI digestion resulted in a single species of 3.2 kb, which is the size of the complete linear HBV genome. Although it is possible that a small percentage of the DNA isolated by this method was genomic DNA, the gross majority of the HBV hybridization in the undigested cytoplasmic DNA preparation is found at the expected HBV circular form size. The resolution of the HBV hybridizing episomes to a single size class of 3.2 kb following *Eco*RI digestion, strongly indicates that the low molecular weight HBV fraction contains viral circular forms, including CCC DNA.

High molecular weight, genomic DNA isolated from nuclear fraction of IHBV6.7 cells hybridized to HBV indicating that the HBV DNA had integrated into the chromosomal DNA. Restriction enzyme digestion of the integrated HBV DNA resolved to 3.2-kb fragments following *Eco*RI digestion, 2.4 kb following *Bgl*2 digestion, and 1.8 kb and 1.4 kb after *Bam*HI digestion (Fig. 1). These are the sizes of HBV DNA expected from full-length, unrearranged HBV genomes, integrated in tandem in the human chromosomes, as shown in the retriction map in Fig. 1.

*Expression of Viral Genes by IHBV6.7 Cells.* The IHBV6.7 cell lysates contained HBV endogenous polymerase activity (not shown). IHBV6.7 cells secrete HBsAg into the culture medium at a concentration of 121 ng/mL in confluent cultures of 5 to 6 million cells per 10-cm plate in stationary phase of growth, for up to 20 passages in culture (Table 1). The level of HBsAg in cell culture supernatants produced the first day after passage was 45 ng/mL and reached 121 ng/mL at 7 to 12 days after passage (*i.e.*, at confluence, when cells have ceased dividing). This level compares favorably with that found in existing human hepatoma cells lines that generate HBV (Table 1). The secreted viral marker E antigen was not detected in cell culture supernatants using standard clinical assays. However, the IHBV6.7 cell nuclei were positive for the viral core protein marker, HBcAg, by standard clinical immunocytochemical staining (Enzo Diagnostics, not shown).

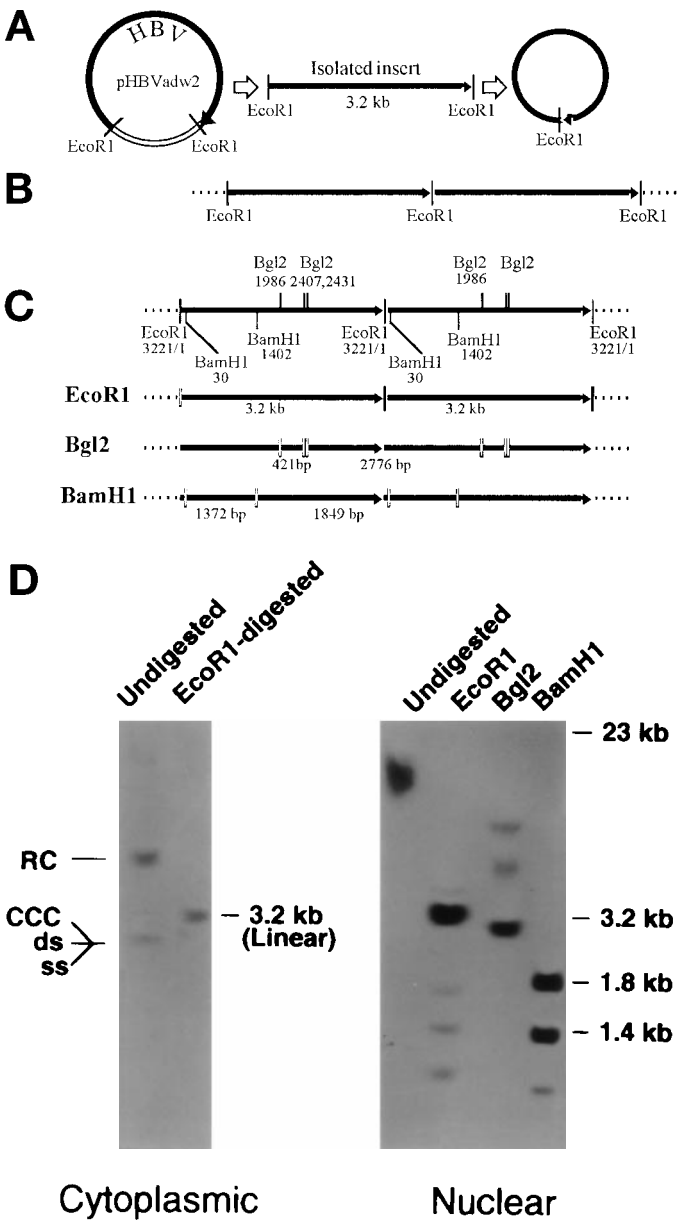


FIG. 1. Southern blot analysis of DNA extracted from cell fractions of IHBV6.7 cells. (A) HBV transfection strategy. Full-length, cloned HBV DNA was excised from a plasmid by *Eco*RI digestion, the HBV insert was purified from vector sequences by gel electrophoresis (*first arrow*), then self ligated (*second arrow*). Ligation products include circular HBV genomes and tandem linear repeats, which may also circularize. Both products of ligation are replication competent in transfections. (B) HBV restriction map. The restriction sites in the HBV adw2 DNA sequence for the restriction enzymes *Eco*RI, *Bgl*2, and *Bam*HI are shown as they would appear if integrated as head to tail dimers. (C) Restriction fragments expected from chromosomally integrated HBV genomes. Sizes of the expected restriction fragments generated by *Eco*RI, *Bgl*2, and *Bam*HI digestion of full-length tandem multimers of HBV DNA are shown. Southern blot of cytoplasmic IHBV6.7 DNA fractions hybridized to HBV. 100 ng of undigested cytoplasmic IHBV6.7DNA, in the first lane, and *Eco*RI–digested cytoplasmic DNA in the second lane were hybridized to an HBV probe. The undigested cytoplasmic HBV migrates at 2 and 4 kb, as expected for circular forms CCC and OC, whereas the *Eco*R1 digest migrates at 3.2 kb linear as complete linear HBV viral genomes do. (D) Southern blot of nuclear IHBV6.7 DNA fractions. Ten micrograms of undigested genomic DNA extracted from nuclear IHBV6.7 fractions show HBV DNA integrated in high molecular weight genomic DNA (*first lane*). Digestion of 10 μg of the nuclear DNA with *Eco*R1 in the *second lane*, generated 3.2-kb fragments, and *Bgl*2 digestion (*third lane*) generated 2.4-kb fragments, whereas *Bam*HI (*fourth lane*) generated 1.8-kb and 1.4-kb bands, as expected from unrearranged HBV genomes, integrated in tandem into the host chromosomes (see B).

*IHBV6.7 Cells Retain the Nontumorigenic Phenotype of Their Progenitors, IHH.* IHBV6.7 cells were characterized for potential tumorigenicity in the Rag-2 mouse host. Following subcutaneous injection of 1 million IHBV6.7 cells into each of 9 Rag-2M mice, 8 of the mice remained tumor free up to and including the experimental observation period endpoint, 6 months. One mouse produced a single tumor detected at 56 days. This is in sharp contrast to the results of tumorigenic hepatoma cell injections into Rag-2M mice, in which we observed all mice, 4 of 4, developed tumors at 21 to 28 days, and HepG2.2.15 hepatoma cell injections into mice, which all developed tumors by 4 weeks.[17] Therefore IHBV6.7 cells have a low tumorigenicity in this mouse host and a long latency period when compared with hepatoma tumor cell lines.

*Long-Term Survival and Proliferation of the Transplanted IHBV6.7 Cells in Mouse Liver.* At various time points after transplantation of IHBV6.7, the presence of human-specific markers in the livers of the recipient Rag-2–deficient mice was evaluated by dot-blot hybridization of liver DNA and by FISH–labeling of human chromosomes in liver tissue sections. Dot-blot analysis of genomic DNA extracted from the livers of the transplant recipients with a human-specific, highly repetitive DNA Alu element probe, Blur-8, showed the persistence of human genomic DNA in the livers of transplant recipients throughout the 8-month period of observation (Fig. 2). The relative amount of human cell DNA contributing to total genomic DNA of the transplanted mouse liver was determined by comparing Blur-8 hybridization signal intensity of liver DNA of the transplanted animals to serially diluted human DNA control DNA samples on the same blot. The ratio of human DNA to mouse DNA detected in 12 of these transplanted mice ranged from 0.2% to 0.5%. The dot-blot assay for human DNA also gave detectable but low signal from lung and kidney corresponding to approximately 0.02%, which may indicate spill over of isolated human hepatocytes into these organs during the transplantation to spleen. Intrasplenic transplantation is known to result in migration of most of the injected hepatocytes to liver parenchyma where they can integrate and, if syngeneic, survive indefinitely, but small percentages of cells can migrate to lung, or less commonly, kidney. One example of this analysis is shown in Fig. 2, in which the relative concentration of human DNA was approximately 0.5% at 8 months following transplantation. In genomic DNA from livers of 6 control untransplanted mice, hybridization to Blur-8 was undetectable, showing the specificity of the probe for human DNA.

FISH labeling of human chromosomes with fluorescein-tagged human satellite DNA identified the nuclei of human cells transplanted to mice. As positive controls, the HuH-7

TABLE 1. HBsAg Levels Secreted by IHBV6.7 and HBV-Producing Human Hepatoma Cell Lines

| Cell Line | Origin | HBsAg Concentration In Media (ng/mL) |
|---|---|---|
| IHBV6.7 | normal liver, HBV transfected | 121 |
| PLC/PRF/5(25) | tumor, HBV+ | 18 |
| HepG2.2.15(24) | tumor, HBV transfected | 94 |
| **Cell Line Transplanted to Mice** | | **HbsAg Concentration in Plasma** |
| IHBV6.7 | intrasplenic transplantation | 7000 |

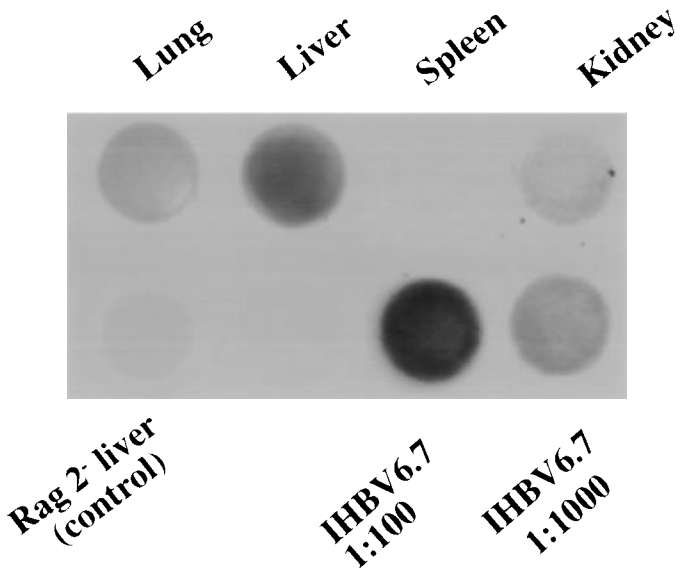


FIG. 2. Human cell detection in mouse by Alu DNA probe. Dot-blot of DNA from tissues of mice transplanted with human hepatocytes 8 months previously, hybridized to the Human BLUR-8 probe detects human DNA in the liver. Control and experimental mouse liver, spleen, lung, or kidney tissue DNA's are compared with dilutions of human IHBV6.7 DNA (*second row*). Mouse DNA, which does not hybridized to BLUR-8, was used as diluent for the concentration controls.

human hepatoma cell line was subcutaneously injected into Rag-2 mice and produced tumors. These human tumor tissues embedded in mouse were excised and labeled by FISH (Fig. 1A). FISH labeling was not detected in the 2 Rag-2M negative control mice assayed, as shown in Fig. 3B. Two transplant recipient mice were analyzed 3 months after transplantation of the IHBV6.7 cells. The IHB6.7 cells are identified in the liver sections by the fluorescent dots within their nuclei (Fig. 3C). Clusters of human cells were observed in the liver lobules of the transplant recipient mice. These human cell clusters contain 24 to 112 cells in the 5 μm section and were distributed randomly throughout the liver lobe, interspersed with mouse hepatocytes. This indicates human hepatocyte groups comprised 88 to 500 cells each, and were patchily distributed throughout the liver. The human cells were fully integrated into the normal architecture of the mouse liver. We did not detect human cells in lung or kidney by this analysis, but cannot rule out that rare, isolated hepatocytes may have migrated to and survived in these organs after intrasplenic transplantation.

*HAAT Detection in IHBV–Transplanted Mice.* Human-specific, secreted protein HAAT is a normal component of human plasma and a biochemical product of human hepatocytes. Human HAAT is not detected in plasma of Rag-2 mice. However, in Rag-2 mice, which received IHBV6.7 cell transplants, HAAT is detected at levels that are 11% to 14% of the level found in normal human plasma samples. The detection of this biochemical marker is an indicator of survival of the transplanted human cells.

*Histological Examination of the Liver and Absence of Tumorigenicity.* In long-term studies of IHBV6.7–transplanted mice, no tumors were detected in the liver of any of 12 transplant recipients at autopsies performed at various time points up to 8 months after transplantation. Histological examination of hematoxylin-eosin-stained liver sections from the same transplant recipient mice used for the FISH analysis showed normal architecture of the liver (Fig. 4) at 3 months following transplantation. The spleen, kidney, and lung of these mice were also histologically normal (not shown). The long-term survival of the mice hosting human cell transplantation allowed analysis of the HBV production in these mice.

*IHBV6.7 Human Cells Produce HBsAg and Chronic HBV Viremia in Transplanted Mice.* The presence of HBV DNA in plasma is the most sensitive indicator of active HBV replication *in vivo*. To determine whether the human hepatocytes of the IHBV6.7 clone, when transplanted to Rag-2 knockout mice, continue to support the replication of HBV, we transplanted IHBV6.7 into 12 mice by intrasplenic injection and then assayed HBV DNA in the mouse plasma. Plasma virus load was analyzed by DNA dot-blot, with a lower limit of detection of 20 pg HBV DNA per mL plasma. Three and five mice were tested at 8 to 11 days and 5 to 12 weeks after transplantation, respectively. Four additional mice were tested at a long-term time point, 5 months after transplantation, 12 mice in total. Untreated sibling mice were examined as negative controls. At all time points from day 11 onward, samples of 50 μL each of the sera of IHBV6.7–transplanted mice contained detectable levels of HBV DNA (Fig. 5), corresponding to from 100

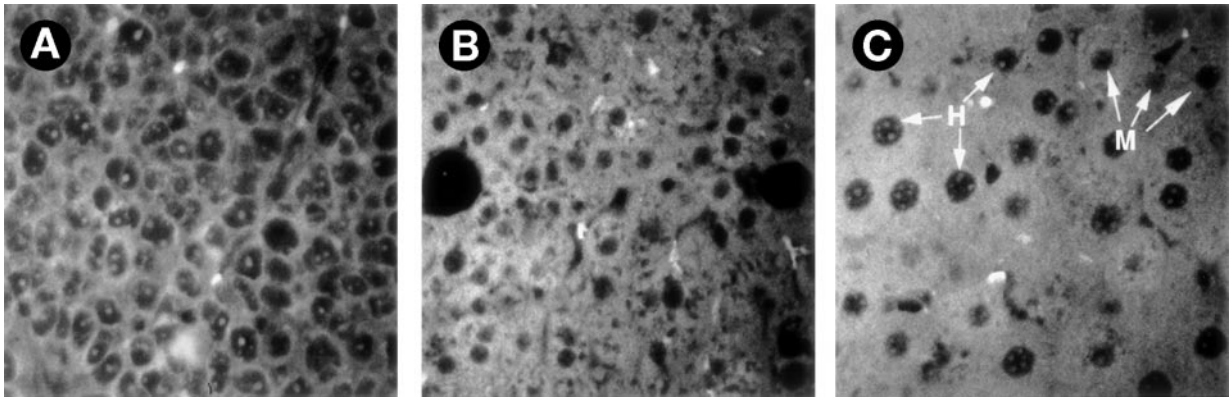


FIG. 3. FISH (fluorescence *in situ* hybridization) of IHBV transplanted liver tissue sections with Human-specific alfa satellite probe. (A) Positive control HuH-7 human hepatoma cells implanted in mice subcutaneously, (B) Rag-2 mouse negative control tissue is shown, and (C)IHBV transplanted mouse liver tissue 3 months post transplant. Human cells are detected in this method by their bright nuclear fluorescence, seen in the postive control A and the experimental transplant C, marked with *arrows* labeled "h". Mouse cells in the section are indicated by *arrows* labeled "m".

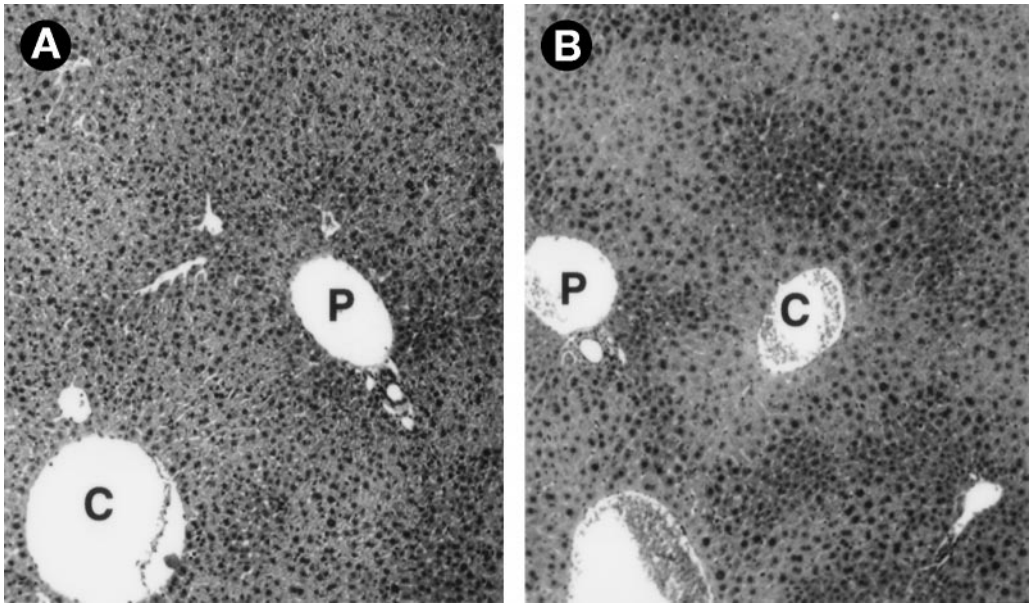


FIG. 4. Histology of IHBV transplanted liver. Hematoxylin and eosin stained sections of liver tissue serial to sections used in the FISH analysis 3 months post-transplant show normal liver structure and cell morphology. Sections shown are (A) mouse control and (B) 3 months post-transplantation.

pg to 1 ng HBV DNA per mL mouse plasma, a viral load of up to $3 \times 10^8$/mL. Plasma HBV DNA was evaluated by PCR as well, which showed HBV DNA sequences in the plasma of transplant recipient mice by 4 days following transplantation, absent in untransplanted control mice. This constitutes a chronic, HBV viremia among transplanted Rag-2M mice. Similarly, the secreted form of the HBV envelope protein, which is produced by IHBV6.7 cells in culture, is also detected in plasma of IHBV6.7 transplanted mice, at levels of up to 7 μg/mL (Table 1B).

## DISCUSSION

The requirement for specific interactions between HBV virions and host cell membranes has limited the natural HBV infection to humans and a few other primate species.[26] Because the higher primates are expensive, endangered, and

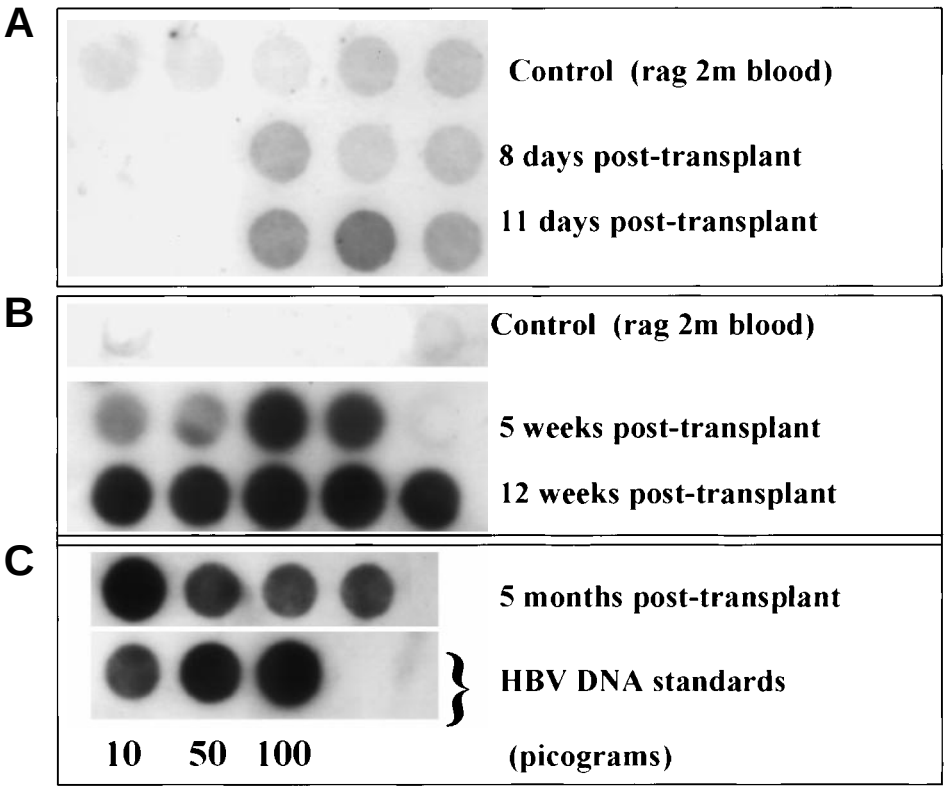


FIG. 5. Human HBV detection in blood of transplanted mice. Dot-blot of DNA prepared from 50 μL sera of control mice or mice transplanted with HBV+ human cells at time points following transplantation are hybridized to an HBV probe. (A) Five negative control mice (*first row*). Three IHBV transplanted mice at 8 days and 11 days post-transplant (*rows 2 and 3)*. HBV DNA is detected in sera of 1 mouse at day 11. (B) Five negative control mice (*first row*). Five IHBV transplanted mice at 5 weeks and 12 weeks post-transplant (*rows 2 and 3*). HBV DNA is detected in 2 of the mice at 5 weeks and all of the mice at 12 weeks. (C) Four IHBV–transplanted mice at 5 months post-transplant (*first row*). HBV DNA detected in all mice. Control HBV DNA standards shown are dilutions of HBV DNA in mouse DNA to quantitate the viral load.

in short supply, we have transplanted HBV–producing human hepatocytes into the livers of immune-deficient Rag-2 knockout mice to generate a new murine model. The Rag-2 deficient mice have a combined immunodeficient phenotype[15] in which B and T cell lineages are absent, although NK cells, macrophages, and granulocytes are present. Because of their lack of the humoral and cellular immune response, these mice may accept xenografts as the Swiss athymic nude nu/nu mice are known to,[27] without exogenous immunosuppression.

IHBV6.7, the human cell line used in this study, produces HBsAg at a level comparable with those produced by commonly used HBV–expressing human hepatoma tumor cell lines such as HepG2 2.2.15[28] and Alexander cells PLC/PRF/5.[29] HBV core protein is detected in IHBV6.7 nuclei, and also episomal viral replication intermediates are found in the cytoplasm, apparently including CCC DNA. As in human infection, but in contrast to transgenic mouse models, the immortalized human hepatocytes are the natural host for HBV infection. Following transplantation, the human cells are engrafted in the mouse liver, secrete human specific products HAAT and HBsAg into the mouse plasma, and appear in normal anatomical relationship with the host cells. Most importantly, unlike the HBV–producing human hepatoma cell lines, the IHBV6.7 cells do not generate tumors in the host liver. This allows long-term survival of mice after transplantation of IHBV.7 cells to the liver. Therefore, the transplanted cells support chronic HBV viremia, without limiting the longevity and health of the recipient mice.

Long-term survival of nontumorigenic human hepatocytes in a nonhuman host is a novel aspect of this work. Two different markers, specific for human genomic DNA, were used to evaluate the survival of the transplanted cells. Dot-blot hybridization of the genomic DNA extracted from the liver of recipient mice with a human-specific Alu sequence probe (Blur8) unequivocally showed the long-term survival up to 8 months of the transplanted human cells in 12 mice. This method revealed that 0.2% to 0.5% of the liver mass of the recipient mice had been replaced by human hepatocytes. The human cells were also readily visible in histological sections of transplanted mouse liver when stained by FISH for human chromosomes using an alpha satellite probe. Groups of 24 to 112 human cells in a planar section, representing 88 to 500 cell clusters, were found integrated normally within the architecture of mouse liver lobule at 3 months following transplantation. The number of the transplanted IHB6.7 cells initially represented only 1% of the hepatocyte mass of the recipient. Because previous experiments indicate that 10% to 20% of injected syngeneic cells[30] engraft, our findings indicate amplification of our xenogeneic transplanted cells in some of the mice. Immediately following transplantation, single transplanted cells are typically found in the liver. Cell clusters are therefore formed only on proliferation of the transplanted cells. Our data indicate that, although the transplanted human immortalized hepatocytes are not tumorigenic, they retain proliferative capacity, approximately 5 to 9 divisions within the liver of the recipient mice.

Human-specific secreted protein HAAT is an abundant serum protease inhibitor and a biochemical product of human hepatocytes. Human HAAT is detected in plasma of mice, which received IHBV6.7 cell transplants at 12% of the level found in normal human plasma samples. The detection of this biochemical marker indicates that the transplanted human cells continue their natural hepatocyte functions in the mouse liver environment.

Characteristics of the IHBV6.7–transplanted Rag-2 mice as HBV animal models are summarized in Table 2. When compared with the previously published HBV transgenic models or ectopic HepG2.2.15 mouse model, the IHBV-Rag-2 model has the unique features of using human cells as hosts of HBV, and long-term *in vivo* survival. The levels of HBV DNA sustained in the mouse plasma and the levels of HBsAg detected in plasma are high relative to the human tumor cell subcutaneous transplant model, perhaps because of the location of the human hepatocytes in liver, which is their native organ location.

Long-term, continuous secretion of HBV into the transplant recipient mouse plasma is a feature of our model. HBV virion DNA was detected in the peripheral circulation of recipient mice throughout the duration of the study (5 months). The presence of HBV DNA in blood is a reliable indicator of HBV viral replication in human liver.[1] HBV virion DNA present in human HBV carrier plasma samples is normally cleared from Rag-2M mouse plasma within 2 hours of intraperitoneal injection (J.J. Brown, unpublished observation, 1998). Because HBV does not infect mouse liver cells, our finding of HBV DNA in blood by PCR and dot-blot analysis supports the conclusion that HBV is continuously replicating for up to 5 months in the transplanted cells. This viremia, combined with evidence of human cell survival in

TABLE 2. Comparison of Results With Cell Culture Systems and Mouse Models Regarding HBV Expression

| | Model System | | | | |
|---|---|---|---|---|---|
| | Cells (*In Vitro*)[28] | | Mice (*In Vivo*)[17] | | Transgenic Mice[15] |
| Markers | IHBV 6 | HepG2215 | IHBV 6 | HepG2215 | |
| DNA | | | | | |
| Integrated transgenes | + | + | + | + | + |
| Replicative intermediate | + | + | + | + | + |
| ccc-DNA | + | + | NT | | − |
| Secreted DNA (pg/mL) | − | + | 100-1000 | 0.5-2 | + |
| Antigens | | | | | |
| HBs-Ag secreted (ng/ml) | 121 | 94 | 7000 | NT | +/− |
| e-antigen secreted (ng/ml) | − | + | NT | NT | + |
| c-antigen (nuclear) | + | + | + | NT | + |
| Host cell type | Human | Human | Human | Human | Mouse |
| Duration of HBV marker detection | >20 passages | ∝ | 5 months | 4 weeks | ∝ |

the mouse liver, indicates that it is unlikely that HBV DNA is derived from degradation of the injected HBV–positive human hepatocytes, which are relatively few in number. Rather, we believe the high levels of human serum proteins, and of HBV DNA and HBsAg present in transplanted mouse blood indicate ongoing differentiated function of the human hepatocytes engrafted in the mouse liver. The observed viral load, up to 300 million viral particles per mL of mouse plasma, parallels that found in some cases of chronic active HBV viral hepatitis.[31] Similarly, the secreted envelope protein HBsAg is detected in IHBV6.7 transplant recipient mouse plasma, whereas the normal half-life of HBsAg in mice is relatively short (less than 6 hours).[32] The viremia in the IHB6.7–transplanted immune-deficient mice, which lack T-cell and B-cell differentiated functions, may mimic the viral replication seen in those human subjects whose immune systems are unable to eliminate the virus.[33]

In summary, the mouse model described in this article has several important novel features. First, we demonstrated long-term survival of mice transplanted with nontumorigenic human hepatocytes in the liver. This is an advance over transplantation of human hepatoma cells, in which tumors rapidly kill the hosts.[17,18] Previously, woodchuck liver cells have been transplanted into immune deficient mice as hosts of woodchuck hepatitis virus, but similar to human hepatoma cell lines, they develop hepatocellular carcinoma in the host.[34] Second, in contrast to previous studies in which HBV–producing human cell lines were transplanted ectopically, we transplanted the IHBV6.7 cells into the liver, permitting their exposure to portal venous blood and their natural interactions with the parenchymal and nonparenchymal cells in the host mouse liver. This may contribute to their long-term survival and function. Development of this model overcomes the constraints of previous models and presents opportunities to perform pathophysiological and pharmacological studies of human hepatocytes in an *in vivo* setting. It is also likely that the human hepatocyte-mouse chimera will support other human specific infectious agents, such as hepatitis virus C and malaria parasites[35] and will, therefore, have an even broader application.

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## REFERENCES

1. Hess G, Reuschling M. Toward routine diagnosis of hepatitis B virus DNA. Clinical Biochemistry 1993;26:289-293.
2. London W and Evans A. The epidemiology of hepatitis viruses B, C and D. Clinics in Laboratory Medicine 1996;16:251-271.
3. Rothstein KD, Munoz SJ. Interferon and other therapies for Hepatitis B virus and Hepatitis C virus infections. Clinics in Laboratory Medicine 1996;16:465-491.
4. McKenzie R, Fried MW, Sallie R, Conjeevaram H, DiBisceglie AM, Park Y, Savarese B, et al. Hepatic failure and lactic acidosis due to fialuridine (FIAU), and investigational nucleoside analoque for chronic hepatitis B. N Engl J Med 1995;333:1099-1105.
5. Rensen P, Van Dijk M, Havenaar E, Busterbosch M, Kruijt J, Van Berkel T. Selective liver targetting of antivirals by recombinant chylomicrons. Nature Medicine 1995;1:221-225.
6. Shouval D, Chakraborty P, Ruiz-Opazo N, Baum S, Spigland I, Muchmore E, Gerber MA, et al. Chronic hepatitis in chimpanzee carriers of hepatitis B virus;morphologic, immunologic and viral DNA studies. Proc Natl Acad Sci U S A 1980;77:6147-6151.
7. Walter E, Keist R, Niederost B, Pult I, Blum HE. Hepatitis B virus infection of Tupaia hepatocytes in vitro and in vivo. HEPATOLOGY 1996;24:1-5.
8. Yan RQ, Su JJ, Huang DR, Gan YC, Yang C, Huang GH. Human hepatitis B virus and hepatocellular carcinoma 1. Experimental infection of tree shrews with hepatitis B virus. J Cancer Res Clin Oncol 1996;122:283-288.
9. Yan RQ, Su JJ, Huang DR, Gan YC, Yang C, Huang GH. Human hepatitis B virus and hepatocellular carcinoma II. Experimental induction of hepatocellular carcinoma in tree shrews exposed to hepatitis B virus and aflatoxin B1. J Cancer Res Clin Oncol 1996;122:289-295.
10. Brown JJ, Parashar B, Kadakol A, Rabbani E, Engelhardt D, Roy-Chowdhury J. Serological and pathomorphological characterization of human hepatitis B virus in a small primate, *Tupaia belangeri*. HEPATOLOGY1998;28(Suppl):927S.
11. Offensperger WB, Blum HE, Gerok W. Molecular therapeutic strategies in hepatitis B virus infection. Clinical Investigator 1994;72:737-741.
12. Korba BA, Xie H, Wright KN, Hornbickly WE, Gerin JL, Tennant BC, Hostetler KL. Liver-targetted antiviral nucleosides: enhanced antiviral activity of phosphatidyl-dideoxyguanosine versus ddG in woodchuck hepatitis virus infeciton in vivo. HEPATOLOGY 23:958-963.
13. Mandart E, Kay A, Galibert F. Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human HBV sequences. J. Virol 1984;49:782-792.
14. Seeger C, Ganem D, Varmus HE. Biochemical and Genetic Evidence for the Hepatitis B virus replication strategy. Science 1986;232:477-484.
15. Chisari F. Hepatitis B Virus Transgenic Mice: models of Viral Immunobiology and Pathogenesis. Current Topics in Microbiology and Immunology 1996;206:149-173.
16. Shouval D, Reid LM, Chakraborty PR, Ruiz-Opazo M, Morecki R, Gerber MA, Thung SM, Shafritz DA. Tumourigenicity in nude mice of a human hepatoma cell line containing hepatitis B virus DNA. Cancer Res 1981;41:1342-1350.
17. Condreay LD, Jansen RW, Powdrill RF, Johnson LC, Selleseth DW, Paff MT, Daluge SM, et al. Evaluation of the potent anti-hepatitis B virus agent (-)cis-5-fluoro-1-(2-hydroxymethly)-1,3-oxathiolan 5-yo)cytosine in a novel in vivo model. Antimicrobial Agents and Chemotherapy 1994;38:616-619.
18. Shouval D, Schuger L, Levij IS, Reid LM, Neeman Z, Shafritz DA. Comparative morphology and tumorigenicity of human hepatocellular carcinoma cell lines in athymic rats and mice. Virchows Arch A Pathol Anat Histopathol 1988;412:595-606.
19. Shinkai U, Rathbun G, Lam KP, Oltz EM, Stewart V, Mendelsohn M, Charron J, et al. Rag-2-deficient mice lack mature lymphocytes owing to an inability to initiate V (D) J rearrangement. Cell 1992;68:855-867.
20. Gupta S, Chowdhury NR, Jagtiani R, Gustin K, Aragona E, Shafritz DA, Chowdhury JR, et al. A novel system for transplantation of isolated hepatocytes utilizing HBsAg producing transgenic donor cells. Transplantation 1990;50:472-475.
21. Schippers IJ, Moshage H, Roelofsen H, Muller M, Heymans HB, Ruiters M, Kuipers F. Immortalized human hepatocytes as a tool for the study of hepatocytic (de)differentiation. Cell Biol Toxicol 1997;13:375-386.
22. Fox IJ, Roy Chowdhury N, Gupta S, Kondapalli R, Schilsky ML, Stockert RJ, Roy Chowdhury J. Conditional Immortalization of Gunn rat hepatocytes: an *ex vivo* model for evaluating methods for bilirubin-UDP-glucuronosyltransferase gene transfer. HEPATOLOGY 1995;21:837-846.
23. Galle PR, Schlicht HJ, Fischer M, Schaller H. Production of Infectious Duck Hepatitis B virus in a human hepatoma cell line. J Virol 1988;62:1736-1740.
24. Scotto J, Hadchouel M, Hery C, Yvart J, Tiollais P, Brechot C. Detection of hepatitis B virus DNA in serum by a simple spot hybridization technique. HEPATOLOGY 1983;3:279-284.
25. Maniatis T, Fritsch E F, Sambrook J. Molecular Cloning, a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1982.
26. Medhi H, Yang X, Peeples M. An altered form of apolipoprotein H binds Hepatitis B virus surface antigen most efficiently. Virology 1996;217: 58-66.
27. Rhim JA, Sandgren EP, Palmiter RD, Brinster RL. Complete reconstitution of mouse liver with xenogeneic hepatocytes. Proc Natl Acad Sci U S A 1995;92:4942-4946.
28. Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci U S A 1987;84:1005-1009.

29. Alexander J J, McNab G, Saunders R. Studies on in vitro production of hepatitis B surface antigen by a human hepatoma cell line. Perspect. Virol 1978;10:103-117.
30. Gupta S, Vemuru RP, Lee CD, Yerneni PR, Aragona E, Burk RD. Hepatocytes exhibit superior transgene expression after transplantation into liver and spleen compared with peritoneal cavity or dorsal fat pad: implications for hepatic gene therapy. Human Gene Therapy 1994;5:959-967.
31. Omata M, Yokosuka O, Imazeki F, Ito Y, Mori J, Uchiumi K, Okuda K. Correlation of Hepatitis B virus DNA and antigens in the liver. Gastroenterology 1987;92:192-196.
32. Guidotti LG, Ando K, Hobbs MV, Ishikawa T, Runkel L, Schriber RD, Chisari FV. Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice. Proc Natl Acad Sci 1994;91:3764-3768.
33. Nowak MA, Bonhoeffer S, Hill AM, Boehme R, Thomas HC, McDade H. Viral Dynamics in hepatits B virus infection. Proc Natl Acad Sci U S A 1996;93:4398-4402.
34. Petersen J, Dandri M, Gupta S, Rogler CE. Liver repopulation with xenogeneic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. Proc Natl Acad Sci U S A 1998;95:310-315.
35. Sacci JB, Schriefer ME, Resau JH, Wirtz RA, Detolla LJ, Markham RB, Azad AF. Mouse model for exoerythrocytic stages of plasmodium falciparum malaria parasite. Proc Natl Acad Sci U S A 1992;89:3701-3705.