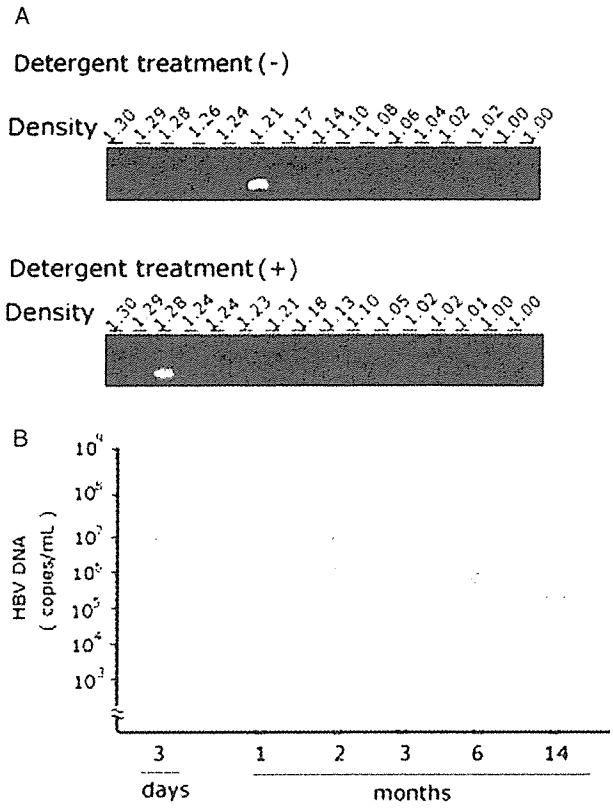

Fig. 3. Production of HBV in hydrodynamically-transfected mice. (A) Density of HBV. Sera from mice injected 3 days earlier with pHBV1.5 were treated with or without *detergent* then fractionated through sucrose density gradient. DNA was isolated from each fraction after treatment with DNase I and then the presence of the HBV DNA sequence was checked by PCR. The density (g/cm^3) of each fraction is indicated above each lane. (B) Viral titers in serum. The levels of HBV DNA were serially determined by real-time PCR.

template for HBV expression, in addition to the inoculated plasmid. To examine the presence of cccDNA in the liver, we used a PCR procedure which selectively detects cccDNA (Fig. 1). We also checked for the presence of inoculated plasmids by amplifying the ampicillin resistance gene by PCR. The authenticity of the cccDNA detection was confirmed by the detection of a specific signal from liver tissues of patients with chronic hepatitis B, but not from the serum of patients or pHBV1.5 (Fig. 5A). Viral cccDNA was clearly detected in wild-type pHBV1.5-injected livers at 3 days as well as 3 months after the injection (Fig. 5B). As expected, cccDNA was not detected in mutant pHBV1.5-injected livers. The levels of cccDNA were measured by real-time PCR (n=5 for each time point) and results were 2.4×10^7 and 6.0×10^5 copies per gram of liver tissue at 3 days and 2 months after the injection, respectively. Since the liver approximately contains 1.1×10^8 of hepatocytes, the average copy numbers of HBV cccDNA per core Ag-positive hepatocyte could be estimated to be 1 or 4. Ampicillin resistance gene was similarly amplified from both wild-type pHBV1.5- and mutant pHBV1.5-injected livers. The fact that HBV gene expression was terminated within 2 months upon injection of mutant pHBV1.5 clearly indicates that the presence of residual plasmids in the livers at later time points is not sufficient for the expression of detectable levels of HBV genes; this is consistent with a previous report [20] demonstrating that transgene expression is rapidly terminated after hydrodynamic gene delivery despite the persistence of plasmid DNA in the livers. These results support the idea that viral cccDNA is critically involved in the long-term expression and carriage of HBV in this model.

3.4. Administration of IFNα gene transiently suppressed HBV DNA replication and failed to eradicate viral template

We next sought to examine the potential usefulness of this model for the assessment of anti-viral drugs. To examine the effect of IFNα in the phase of cccDNA-dependent HBV replication, we injected either pCMV-IFNa1 or pCMV at 70 days after pHBV1.5 injection. Injection of pCMV-IFNa1 led to substantial IFNα production at day 1 (Fig. 6A), although IFNα could not be detected in the mock-injected mice (data not shown). The levels of IFNα after pCMV-IFNa1 injection rapidly declined at day 3 and could not be detected at day 28. Injection of pCMV-IFNa1 significantly suppressed viral production at day 3 but did not affect HBs production (Fig. 6B and C); this is consistent with previous findings [15,21] that IFNα suppressed HBV replication at a step of reverse transcription. In spite of the substantial suppression of HBV production at day 3, the levels of viral titers of mice injected with pCMV-IFNa1 increased to levels similar to those of pCMV-injected mice at day 14 and later. These results indicate that IFN treatment substantially suppressed viral replication, but could not eliminate the viral template from the infected host. This model should be useful for assessing anti-viral therapy aimed at eradication of the viral template.

4. Discussion

In the present study, we demonstrated that hydrodynamic injection of a plasmid encoding an overlength of HBV DNA into nude mice established long-term replication of HBV in the liver. Since hepatic damage was not observed, this model mimics the chronic carrier-like state of human HBV infections. This model reminds us of a 1988 report by Feitelson et al., [22] in which they stated that intrahepatic injection of replication competent HBV DNA led to persistent HBs antigenemia as well as chronic liver injury in nude mice. They had no evidence of HBV replication such as production of Dane particles in the circulation. In a preliminary experiment, we intrahepatically injected pHBV1.5 into nude mice and monitored viral production in the serum. DNase I-resistant HBV DNA could not be detected in most mice tested; a small number of mice

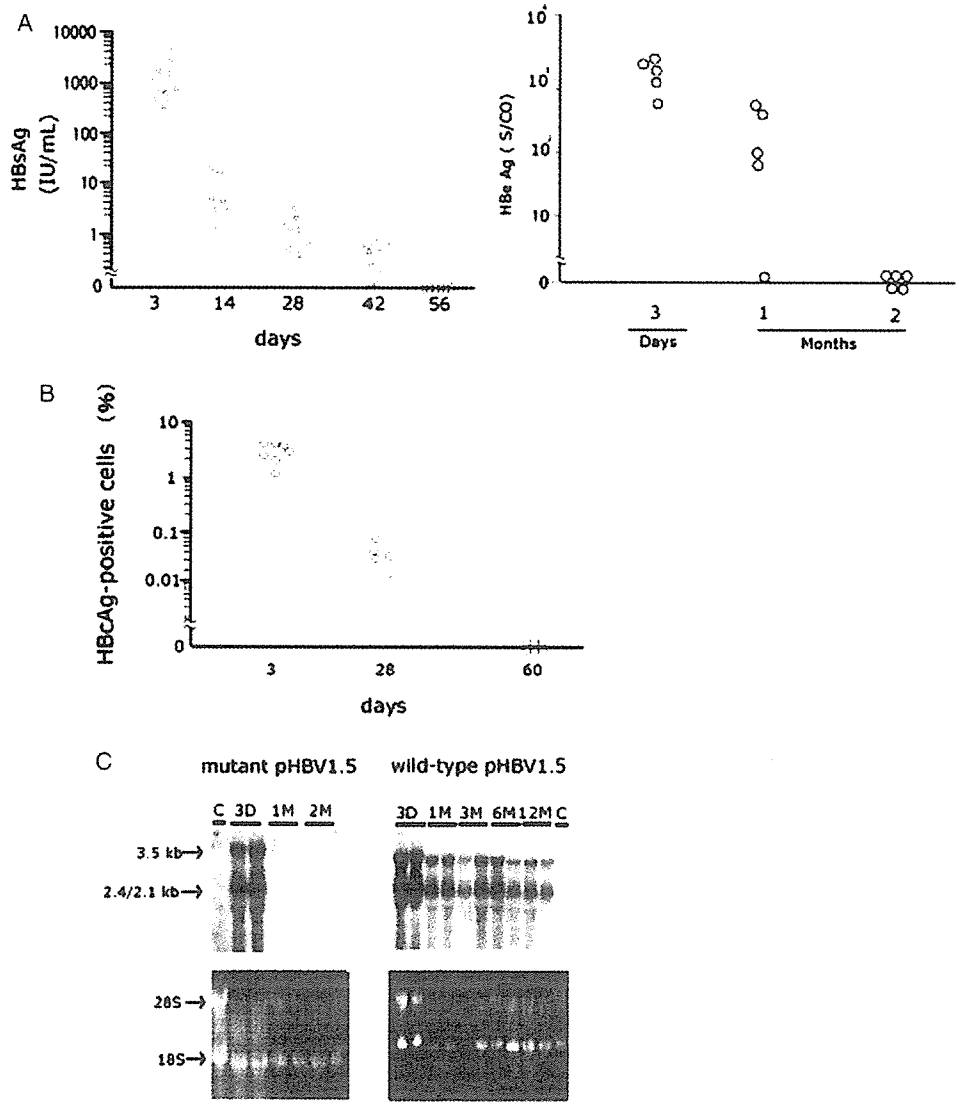

Fig. 4. Expression of HBV genes in nude mice injected with replication-incompetent pHBV1.5. (A) Serial detection of HBsAg and HBeAg in serum. (B) Frequency of HBcAg-positive hepatocytes in the livers. (C) Time-course of HBV RNA expression in the livers transfected with mutant pHBV1.5 or wild-type pHBV1.5 determined by Northern blot. C, control livers; 3D, 1M, 3M, 6M, and 12M, liver samples obtained at 3 days, 1 month, 3 , 6 , and 12 months after hydrodynamic injection, respectively.

produced low levels of virus at 3 days after injection but not at later time points (our unpublished data). Thus, the transfection efficiency of hydrodynamic injection of HBV DNA appeared to be higher than that of intrahepatic injection. Despite the difference in liver damage observed among these studies, we considered the absence of hepatitis in the present model reasonable, since the T-cell immune response towards HBV-related antigens could not occur. Furthermore, it should be noted that the antigenemia as well as HBV production achieved by the hydrodynamic procedure was very reproducible, which is critically important when applying this model to evaluate the efficacy of anti-viral drugs.

The duration of hydrodynamics-based gene expression varies among reports from days to months [19,23,24]. The plasmid-based gene expression of our model terminated within 2 months, as demonstrated by the injection of replication-incompetent HBV DNA (mutant pHBV1.5). Replication-competent HBV DNA (wild-type pHBV1.5)-injected mice displayed a rapid decline of HBsAg production followed by relatively stable antigenemia for more than 1 year (Fig. 2B). Although the rapid decline observed in the first 2 weeks may reflect the plasmid-based gene expression, stable expression of HBsAg at later time points did not depend on residual plasmids in the livers, but required intracellular reproduction of HBV DNA. These results indicate that HBV replication in addition to immunological tolerance is critically important for long-term HBV expression in this system. Previous research on in vivo gene transfer [8] and transgenic mice [5] has indicated that HBV cccDNA, the template of HBV replication in natural infection, could not be detected in

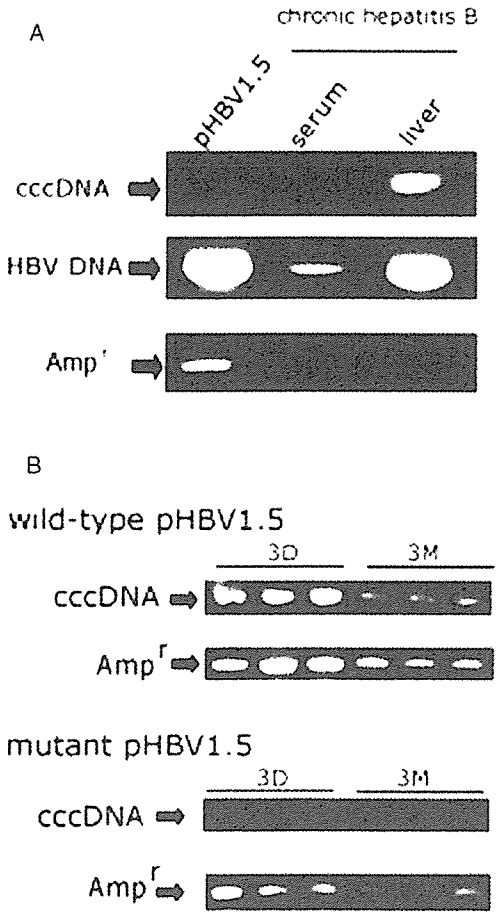

Fig. 5. Detection of HBV cccDNA and ampicilin resistance gene by PCR. (A) Specificity of HBV cccDNA detection by PCR. DNA were isolated from the following samples and amplified for the detection of cccDNA, ampicilin resistance gene, and HBV DNA sequence by corresponding PCRs. pHBV1.5, sample containing pHBV1.5; serum, serum from a patient with chronic hepatitis B; liver, a liver specimen from the same patient. (B) DNA was isolated from the liver samples 3 days (3D) or 3 months (3 M) after hydrodynamic injection of either wild-type pHBV1.5 or mutant pHBV1.5 ($N=3$ for each group) and examined the presence of cccDNA and ampicilin resistance gene by PCRs.

murine livers by Southern blot analysis. In the present study, we applied a highly sensitive PCR procedure and detected HBV cccDNA in pHBV1.5-injected livers. What is important is that the estimated numbers of HBV cccDNA per hepatocyte were 1 or 4, which should be sufficient for HBV gene expression. Taken together, the present study is the first demonstration of the production of viral cccDNA and its contribution to HBV replication in mice. Thus, the species restriction on the production of HBV cccDNA may not be as strict as has previously been believed.

Mutation of HBV DNA occurring during therapy with various nucleotide analogues leads to drug resistance and limits the success of these drugs for controlling HBV replication in humans [1,2]. Thus far, except for the in vitro recombinant HBV baculovirus system [25], there has been no useful model supporting reproduction of the HBV viral

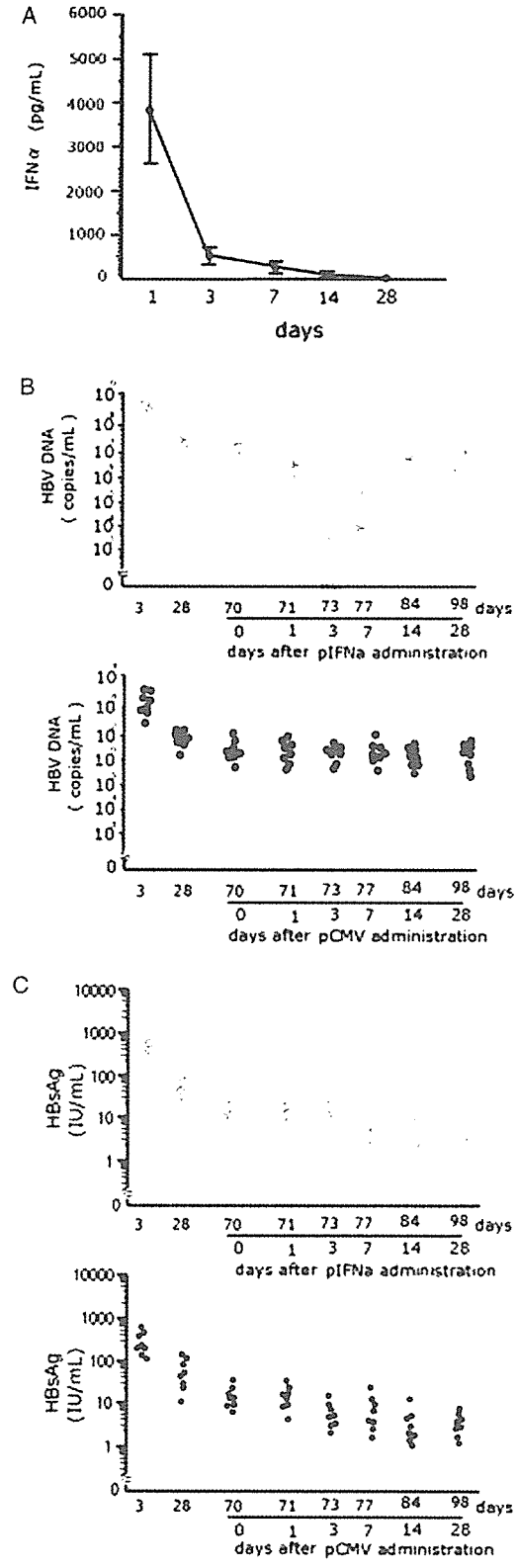

Fig. 6. Transient suppression of HBV production by IFNα gene therapy. (A) Serial determination of serum IFNα levels of nude mice after injection of pCMV-IFNa1. Horizontal bars indicate SD. (B and C) Nude mice were transfected with pHBV1.5 and, at 70 days later, transfected with either pCMV-IFNa1 (open circles) or pCMV (closed circles). Sera were serially obtained from the retro-orbital plexus, with HBV DNA (B) and HBsAg (C) titers being determined.

template as is the case of hepatitis C virus replicon systems [26,27]. Although HBV could not 'infect' murine hepatocytes, intracellular 'reinfection', namely recycling of HBV DNA occurs and leads to chronic viral production in the present model. Therefore, this model may provide a unique opportunity for analyzing possible mutations induced by long-term usage of various nucleotide analogs. Further study is needed to examine this possibility. Finally, intentional mutation could be easily introduced in inoculated DNA and a wide variety of mice with different genetic backgrounds can be used. The model presented here should enable analysis of viral as well as host factors that may regulate HBV replication.

Acknowledgements

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References

[1] Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. N Engl J Med 2004;350:1118–1129.
[2] Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. Lancet 2003;362:2089–2094.
[3] Feitelson MA, Larkin JD. New animal models of hepatitis B and C. ILAR J 2001;42:127–138.
[4] Araki K, Miyazaki J, Hino O, Tomita N, Chisaka O, Matsubara K, et al. Expression and replication of hepatitis B virus genome in transgenic mice. Proc Natl Acad Sci USA 1989;86:207–211.
[5] Guidotti LG, Matzke B, Schaller H, Chisari FV. High level hepatitis B virus replication in transgenic mice. J Virol 1995;69:6158–6169.
[6] Takahashi H, Fujimoto J, Hanada S, Isselbacher KJ. Acute hepatitis in rats expressing human hepatitis B virus transgenes. Proc Natl Acad Sci USA 1995;92:1470–1474.
[7] Suzuki T, Takehara T, Ohkawa K, Ishida H, Jinushi M, Miyagi T, et al. Intravenous injection of naked plasmid DNA encoding hepatitis B virus (HBV) produces HBV and induces humoral immune response in mice. Biochem Biophys Res Commun 2003;300:784–788.
[8] Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. Proc Natl Acad Sci USA 2002;99:13825–13830.
[9] McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, et al. Inhibition of hepatitis B virus in mice by RNA interference. Nat Biotechnol 2003;21:639–644.
[10] Klein C, Bock CT, Wedemeyer H, Wustefeld T, Locarnini S, Dienes HP, et al. Inhibition of hepatitis B virus replication in vivo by nucleoside analogues and siRNA. Gastroenterology 2003;125:9–18.
[11] Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E. Small interfering RNA inhibits hepatitis B virus replication in mice. Mol Ther 2003;8:769–776.
[12] Harle P, Noisakran S, Carr DJ. The application of a plasmid DNA encoding IFN-alpha 1 postinfection enhances cumulative survival of herpes simplex virus type 2 vaginally infected mice. J Immunol 2001; 166:1803–1812.
[13] Liu F, Song YK, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther 1999;6: 1258–1266.
[14] Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injection of naked plasmid DNA. Hum Gene Ther 1999;10:1735–1737.
[15] Kawanishi Y, Hayashi N, Katayama K, Ueda K, Takehara T, Miyoshi E, et al. Tumor necrosis factor-α and interleukin-γ inhibit synergistically viral replication in hepatitis B virus-replicating cells. J Med Virol 1995;47:272–277.
[16] Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantification of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;37:2899–2903.
[17] Jun-Bin S, Zhi C, Wei-Quin N, Jun F. A quantitative method to detect HBV cccDNA by chimeric primer and real-time polymerase chain reaction. J Virol Methods 2003;112:45–52.
[18] Kaplan PM, Ford EC, Purcell RH, Gerin JL. Demonstration of subpopulation of Dane particles. J Virol 1976;17:885–893.
[19] Seeger C, Mason WS. Hepatitis B virus biology. Microbiol Mol Biol Rev 2000;64:51–68.
[20] Harweijer H, Zhang G, Subbotin VM, Budker V, Williams P, Wolff JA. Time course of gene expression after plasmid DNA gene transfer to the liver. J Gene Med 2001;3:280–291.
[21] Ueda K, Tsurimoto T, Nagahata T, Chisaka O, Matsubara K. An in vitro system for screening anti-hepatitis B virus drugs. Virology 1989;169:213–216.
[22] Feitelson MA, DeTolla LJ, Zhou XD. A chronic carrierlike state is established in nude mice injected with cloned hepatitis B virus DNA. J Virol 1988;62:1408–1415.
[23] Jiang J, Yamato E, Miyazaki J. Intravenous delivery of naked plasmid DNA for in vivo cytokine expression. Biochem Biophys Res Commun 2001;289:1088–1092.
[24] Yang J, Chen S, Huang L, Michalopoulos GK, Liu Y. Sustained expression of naked plasmid DNA encoding hepatocyte growth factor in mice promotes liver and overall body growth. Hepatology 2001;33: 848–859.
[25] Delaney IV WE, Isom HC. Hepatitis B virus replication in human HepG2 cells mediated by hepatitis B virus recombinant baculovirus. Hepatology 1998;28:1134–1146.
[26] Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–113.
[27] Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972–1974.

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主にアジアに蔓延するウイルス性肝疾患の制御に資する為の
日米合作的肝炎ウイルス基礎研究

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