activity of telaprevir (VX-950) and peginterferon alfa-2a in patients with hepatitis C. *Hepatology* **46**, 640–648.

Fried, M. W., Shiffman, M., Reddy, K. R., Smith, C., Marinos, G., Gonçales, F. L., Jr, Häussinger, D., Diago, M., Carosi, G. & other authors (2002). Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* **347**, 975–982.

Herrmann, E., Lee, J.-H., Marinos, G., Modi, M. & Zeuzem, S. (2003). Effect of ribavirin on hepatitis C viral kinetics in patients treated with pegylated interferon. *Hepatology* **37**, 1351–1358.

Hézode, C., Forestier, N., Dusheiko, G., Ferenci, P., Pol, S., Goeser, T., Bronowicki, M., Bourlière, J.-P., Gharakhanian, S. & other authors (2009). Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* **360**, 1839–1850.

Hiraga, N., Imamura, M., Tsuge, M., Noguchi, C., Takahashi, S., Iwao, E., Fujimoto, Y., Abe, H., Maekawa, T. & other authors (2007). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Lett* **581**, 1983–1987.

Katoh, M., Sawada, T., Soeno, Y., Nakajima, M., Tateno, C., Yoshizato, K. & Yokoi, T. (2007). *In vivo* drug metabolism model for human cytochrome P450 enzyme using chimeric mice with humanized liver. *J Pharm Sci* **96**, 428–437.

Katoh, M., Tateno, C., Yoshizato, K. & Yokoi, T. (2008). Chimeric mice with humanized liver. *Toxicology* **246**, 9–17.

Kimura, T., Imamura, M., Hiraga, N., Hatakeyama, T., Miki, D., Noguchi, C., Mori, N., Tsuge, M., Takahashi, S. & other authors (2008). Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice. *J Gen Virol* **89**, 2108–2113.

Kneteman, N. M., Weiner, A. J., O'Connell, J., Collett, M., Gao, T., Aukerman, L., Kovelsky, R., Ni, Z.-J., Hashash, A. & other authors (2006). Anti-HCV therapies in chimeric *scid*-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* **43**, 1346–1353.

Kneteman, N. M., Howe, A. Y. M., Gao, T., Lewis, J., Pevear, D., Lund, G., Douglas, D., Mercer, D. F., Tyrrell, D. L. J. & other authors (2009). HCV796: a selective nonstructural protein 5B polymerase inhibitor with potent anti-hepatitis C virus activity *in vitro*, in mice with chimeric human livers, and in humans infected with hepatitis C virus. *Hepatology* **49**, 745–752.

Lauer, G. M. & Walker, B. D. (2001). Hepatitis C virus infection. *N Engl J Med* **345**, 41–52.

Liang, T. J., Rehermann, B., Seeff, L. B. & Hoofnagle, J. H. (2000). Pathogenesis, natural history, treatment and prevention of hepatitis C. *Ann Intern Med* **132**, 296–305.

Lindenbach, B. D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A. J., McKeating, J. A., Lanford, R. E., Feinstone, S. M., Major, M. E. & other authors (2006). Cell culture-grown hepatitis C virus is infectious *in vivo* and can be recultured *in vitro*. *Proc Natl Acad Sci U S A* **103**, 3805–3809.

Lohmann, V., Körner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113.

Manns, M. P., McHutchison, J. G., Gordon, S. C., Rustgi, V. K., Shiffman, M., Reindollar, R., Goodman, Z. D., Koury, K., Ling, M.-H. & other authors (2001). Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* **358**, 958–965.

Manns, M. P., Foster, G. R., Rockstroh, J. K., Zeuzem, S., Zoulim, F. & Houghton, M. (2007). The way forward in HCV treatment – finding the right path. *Nat Rev Drug Discov* **6**, 991–1000.

McHutchison, J. G., Everson, G. T., Gordon, S. C., Jacobson, I. M., Sulkowski, M., Kauffman, R., McNair, L., Alam, J., Muir, A. J. & other

authors (2009). Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* **360**, 1827–1838.

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., Addison, W. R., Fischer, K. P., Churchill, T. A. & other authors (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**, 927–933.

Neumann, A. U., Lam, N. P., Dahari, H., Gretch, D. R., Wiley, T. E., Layden, T. J. & Perelson, A. S. (1998). Hepatitis C viral dynamics *in vivo* and the antiviral efficacy of interferon-α therapy. *Science* **282**, 103–107.

Pawlotsky, J.-M., Dahari, H., Neumann, A. U., Hezode, C., Germanidis, G., Lonjon, I., Castera, L. & Dhumeaux, D. (2004). Antiviral action of ribavirin in chronic hepatitis C. *Gastroenterology* **126**, 703–714.

Pereira, A. A. & Jacobson, I. M. (2009). New and experimental therapies for HCV. *Nat Rev Gastroenterol Hepatol* **6**, 403–411.

Perelson, A. S. & Ribeiro, R. M. (2008). Estimating drug efficacy and viral dynamic parameters: HIV and HCV. *Stat Med* **27**, 4647–4657.

Perni, R. B., Almquist, S. J., Byrn, R. A., Chandorkar, G., Chaturvedi, P. R., Courtney, L. F., Decker, C. J., Dinehart, K., Gates, C. A. & other authors (2006). Preclinical profile of VX-950, a potent, selective, and orally bioavailable inhibitor of hepatitis C virus NS3-4A serine protease. *Antimicrob Agents Chemother* **50**, 899–909.

Powers, K. A., Ribeiro, R. M., Patel, K., Pianko, S., Nyberg, L., Pockros, P., Conrad, A. J., McHutchison, J. & Perelson, A. S. (2006). Kinetics of hepatitis C virus reinfection after liver transplantation. *Liver Transpl* **12**, 207–216.

Poynard, T., Yuen, M.-F., Ratziu, V. & Lai, C. L. (2003). Viral hepatitis C. *Lancet* **362**, 2095–2100.

Quinkert, D., Bartenschlager, R. & Lohmann, V. (2005). Quantitative analysis of the hepatitis C virus replication complex. *J Virol* **79**, 13594–13605.

Ramratnam, B., Bonhoeffer, S., Binley, J., Hurley, A., Zhang, L., Mittler, J. E., Minarkowitz, M., Moore, J. P., Perelson, A. S. & Ho, D. D. (1999). Rapid production and clearance of HIV-1 and hepatitis C virus assessed by large volume plasma apheresis. *Lancet* **354**, 1782–1785.

Reesink, H. W., Zeuzem, S., Weegink, C. J., Forestier, N., Vliet, A., van de Wetering de Rooij,, J., McNair, L, Purdy, S., Kauffman, R. & other authors (2006). Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. *Gastroenterology* **131**, 997–1002.

Sarrazin, C., Kieffer, T. L., Bartels, D., Hanzelka, B., Möh, U., Welker, M., Wincheringer, D., Zhou, Y., Chu, H.-M. & other authors (2007). Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* **132**, 1767–1777.

Schiano, T. D., Gutierrez, J. A., Walewski, J. L., Fiel, M. I., Cheng, B., Bodenheimer, H., Jr, Thung, S. N., Chung, R. T., Schwartz, M. E. & other authors (2005). Accelerated hepatitis C virus kinetics but similar survival rates in recipients of liver grafts from living versus deceased donors. *Hepatology* **42**, 1420–1428.

Sherman, K. E., Fleischer, R., Laessig, K., Murray, J., Tauber, W. & Birnkrant, D. (2007). Development of novel agents for the treatment of chronic hepatitis C infection: summary of the FDA antiviral products advisory committee recommendations. *Hepatology* **46**, 2014–2020.

Talal, A. H., Ribeiro, R. M., Powers, K. A., Grace, M., Cullen, C., Hussain, M., Markatou, M. & Perelson, A. S. (2006). Pharmacodynamics of PEG-IFN α differentiate HIV/HCV coinfected sustained virological responders from nonresponders. *Hepatology* **43**, 943–953.

Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K. & other authors (2004). Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 165, 901–912.

Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y. & other authors (2005). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 42, 1046–1054.

Vanwolleghem, T., Meuleman, P., Libbrecht, L., Roskams, T., De Vos, R. & Leroux-Roels, G. (2007). Ultra-rapid cardiotoxicity of the hepatitis C virus protease inhibitor BILN 2061 in the urokinase-type plasminogen activator mouse. *Gastroenterology* 133, 1144–1155.

Vona, G., Tuveri, R., Delpuech, O., Vallet, A., Canioni, D., Ballardini, G., Trabut, J. B., Le Bail, B., Nalpas, B. & other authors (2004). Intrahepatic hepatitis C virus RNA quantification in microdissected hepatocytes. *J Hepatol* 40, 682–688.

Wasley, A. & Alter, M. J. (2000). Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 20, 1–16.

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

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The non-structural X protein, HBx, of hepatitis B virus (HBV) is assumed to play an important role in HBV replication. Woodchuck hepatitis virus X protein is indispensable for virus replication, but the duck hepatitis B virus X protein is not. In this study, we investigated whether the HBx protein is indispensable for HBV replication in vivo using human hepatocyte chimeric mice. HBx-deficient (HBx-def) HBV was generated in HepG2 cells by transfection with an overlength HBV genome. Human hepatocyte chimeric mice were infected with HBx-def HBV with or without hepatic HBx expression by hydrodynamic injection of HBx expression plasmids. Serum virus levels and HBV sequences were determined with mice sera. The generated HBx-def HBV peaked in the sucrose density gradient at points equivalent to the generated HBV wild type and the virus in a patient's serum. HBx-def HBV-injected mice developed measurable viraemia only in continuously HBx-expressed liver. HBV DNA in the mouse serum increased up to 9 $\log_{10}$ copies ml^{-1} and the viraemia persisted for more than 2 months. Strikingly, all revertant viruses had nucleotide substitutions that enabled the virus to produce the HBx protein. It was concluded that the HBx protein is indispensable for HBV replication and could be a target for antiviral therapy.

INTRODUCTION

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

(Seeger & Mason, 2000; Tang *et al.*, 2001). Although previous works have demonstrated that HBx protein is necessary for maximal HBV replication in cultured cells (Bouchard *et al.*, 2001; Keasler *et al.*, 2007; Leupin *et al.*, 2005; Tang *et al.*, 2005) and in mouse hepatocytes (Keasler *et al.*, 2007), the precise function of HBx in the virus life cycle remains poorly defined in human hepatocytes under physiological conditions because there is no natural infection–replication system available. Accordingly, all previous work has been done using hepatocarcinoma cell lines with transfection or mouse hepatocytes with hydrodynamic injection. Analysis of HBx under physiological conditions will provide more accurate information for the function of the HBx protein.

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

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

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

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

RESULTS

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

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

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

Infectivity of HBx-def HBV particles

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

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

We then investigated the effect of *trans*-complementation of the HBx protein both *in vitro* and *in vivo*. Since the C-terminal two-thirds (aa 51–154) domain of HBx has been reported to contain a transactivation domain (Tang *et al.*, 2005), we constructed three plasmids (full length and residues 1–50 and 51–154), as shown in Fig. 3(a). To analyse the effect of co-transfection of these three plasmids on intracellular replication of HBV, the cells transfected using *Trans*IT-LT1 reagent were harvested 24 h after transfection and analysed by Southern blotting. As shown in Fig. 3(b), *trans*-complementation of HBx enhanced the
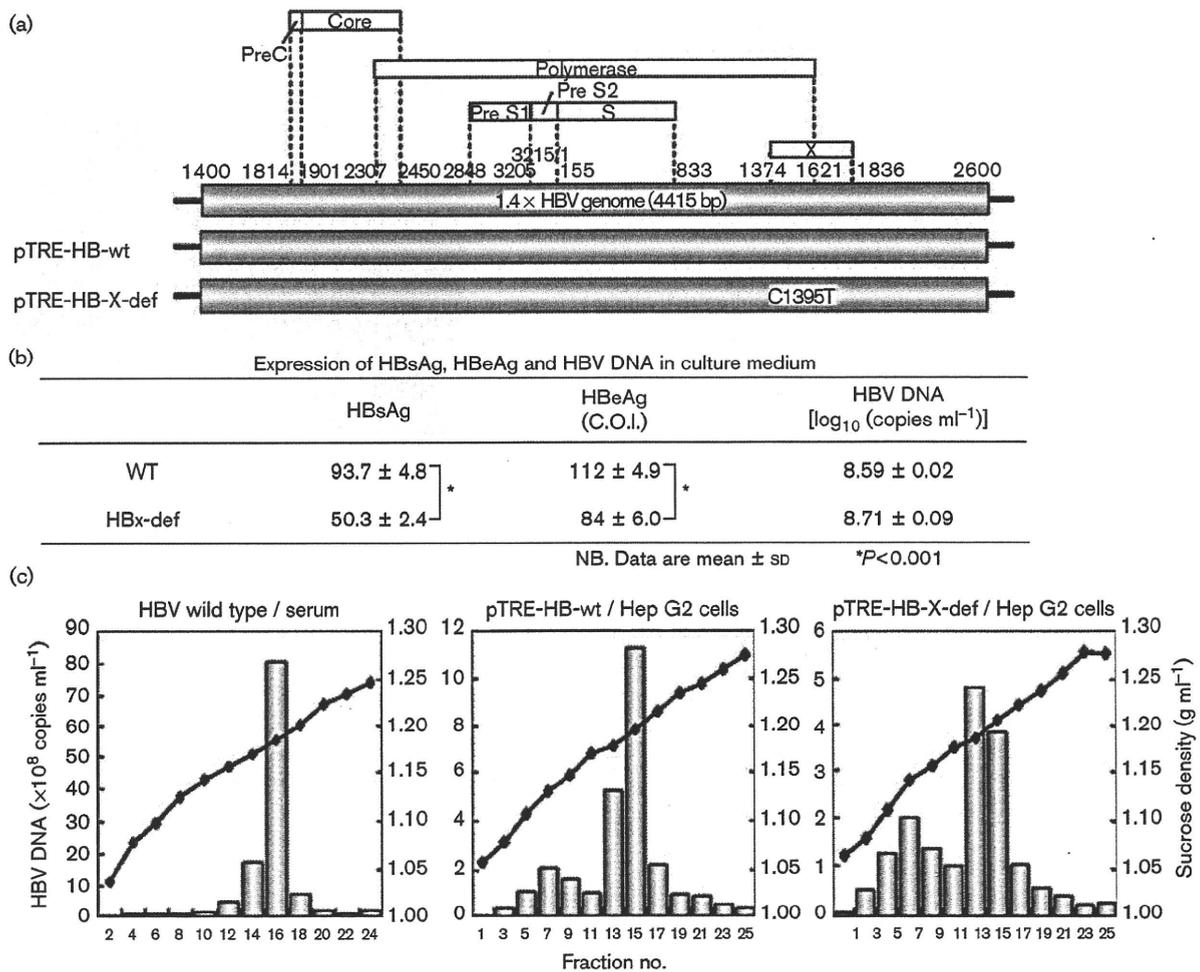
Fig. 1. Construction of HBV expression plasmids. (a) Wild type (WT) 1.4× genome length HBV was cloned into the pTRE2hyg vector (pTRE-HB-wt) and a nucleotide substitution, C1395T, was introduced to create the HBx-def mutant pTRE-HB-X-def. (b) Comparison of expression of HBsAg, HBeAg and HBV DNA in culture medium between WT and HBx-def. (c) Sucrose density gradient analysis of HBV particles (◆) and HBV DNA copies (bars) obtained from a serum sample (left) and supernatants from a cell culture transfected with WT HBV (pTRE-HB-wt, middle) and HBx-def pTRE-HB-X-def. C.O.I., cut-off index.

replication of HBV to the WT level. The effects of HBx protein were also evident on the expression of HBsAg (Fig. 3c) and HBeAg (Fig. 3d). As reported previously, the effect of the C-terminal two-thirds (aa 51–154) of the HBx protein was stronger than that of the entire protein and the N-terminal one-third (aa 1–50) (Tang *et al.*, 2005). The production of replication intermediates was increased similarly by co-transfection of the X proteins (Fig. 3e). To further study the effect of HBx expression, we analysed the levels of intracellular core protein expression. As shown in Fig. 4(a), the expression levels of the core protein were upregulated with the expression of the entire (WT) and C-terminal two-thirds (aa 51–154) of the HBx protein. Immunocytochemical analysis showed that only the cells with strong HBx protein expression were stained with the

core protein (Fig. 4b). The core and HBx proteins in these cells were stained mainly in the cytoplasm.

Expression of HBx protein in mouse liver by hydrodynamic injection

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

(a)

(b)

No. analysed mice

Weeks		2	4	6	8	10	12	14	16
HBV	(−/+)	1	3	3	0	1	0	0	0
DNA	(−)	15	10	6	9	6	5	5	3

(c)
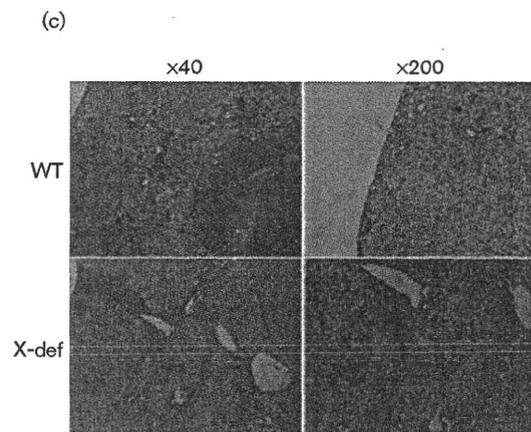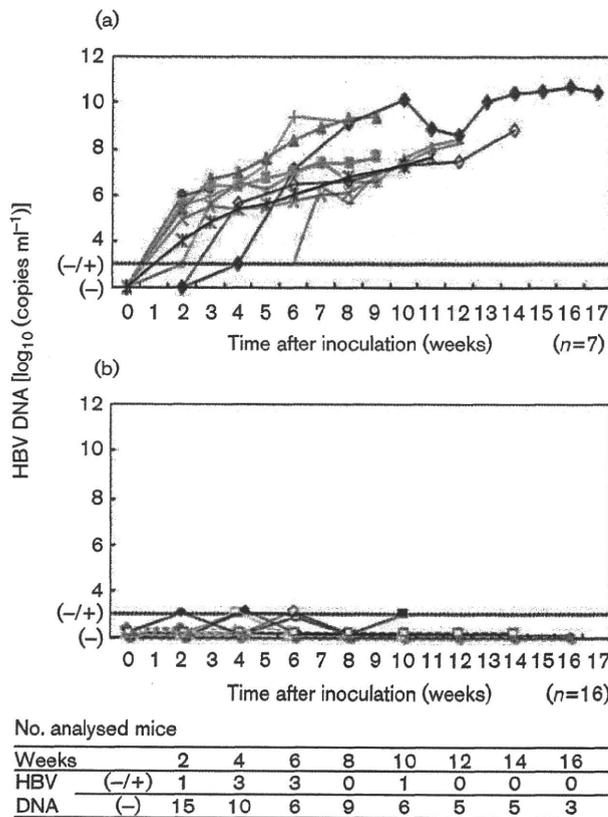
Fig. 2. Infection of human hepatocyte chimeric mice with cell-culture-derived HBV. (a, b) WT (a) and HBx-def (b) HBV particles generated from HepG2 cell lines by transfection of overlength HBV plasmid injected into chimeric mice. (c) Immunohistochemical analysis of mouse liver infected with WT (upper panel) and HBx-def (lower panel) HBV using anti-HBc antibody. HBV-infected hepatocytes were stained brown.

liver. Notably, the HBx protein staining was strong around the central vein (Fig. 5b).

Infection of HBx-def HBV particles with intrahepatic expression of the HBx protein

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

Sequence analysis of inocula and the infected mouse sera

We analysed nucleotide sequences of the virus recovered from all six infected mice and compared them with those of inoculated HBx-def HBV. As shown in Fig. 7(a), direct sequencing analyses of the amplified HBV DNA products showed that all revertant viruses had T1395C (mouse

MHX#1, 3, 5–7) or T1395A (mouse MHX#2) point mutations, which reverted the introduced stop codon to amino acids. We further analysed nucleotide sequences of HBV by cloning and sequencing using serum samples obtained from two mice (MHX#1, 33 clones; MHX#2, 38 clones) (Fig. 7b). Only one of 33 clones obtained from MHX#1 and none of the 38 clones from MHX#2 had the stop codon mutation that was introduced into the transfected plasmid.

DISCUSSION

In previous studies, HBx has been reported to be a multi-functional protein affecting cell growth and proliferation and activating transcription of mRNA (Arbuthnot *et al.*, 2000; Bouchard *et al.*, 2001; Klein *et al.*, 1999; Murakami, 2001) and virus replication in HCC cell lines (Bouchard *et al.*, 2001; Keasler *et al.*, 2007; Leupin *et al.*, 2005; Tang *et al.*, 2005) and mouse hepatocytes (Keasler *et al.*, 2007; Xu *et al.*, 2002). However, these results were obtained by introduction of HBV genomes into cells using artificial methods such as transfection, gene transfer and hydro-dynamic injection. Recently, we established an *in vivo* HBV infection system using human hepatocyte chimeric mice (Tsuge *et al.*, 2005). The system enabled us to perform infection experiments using HBV-containing patient sera and cell-culture medium. Using this system,
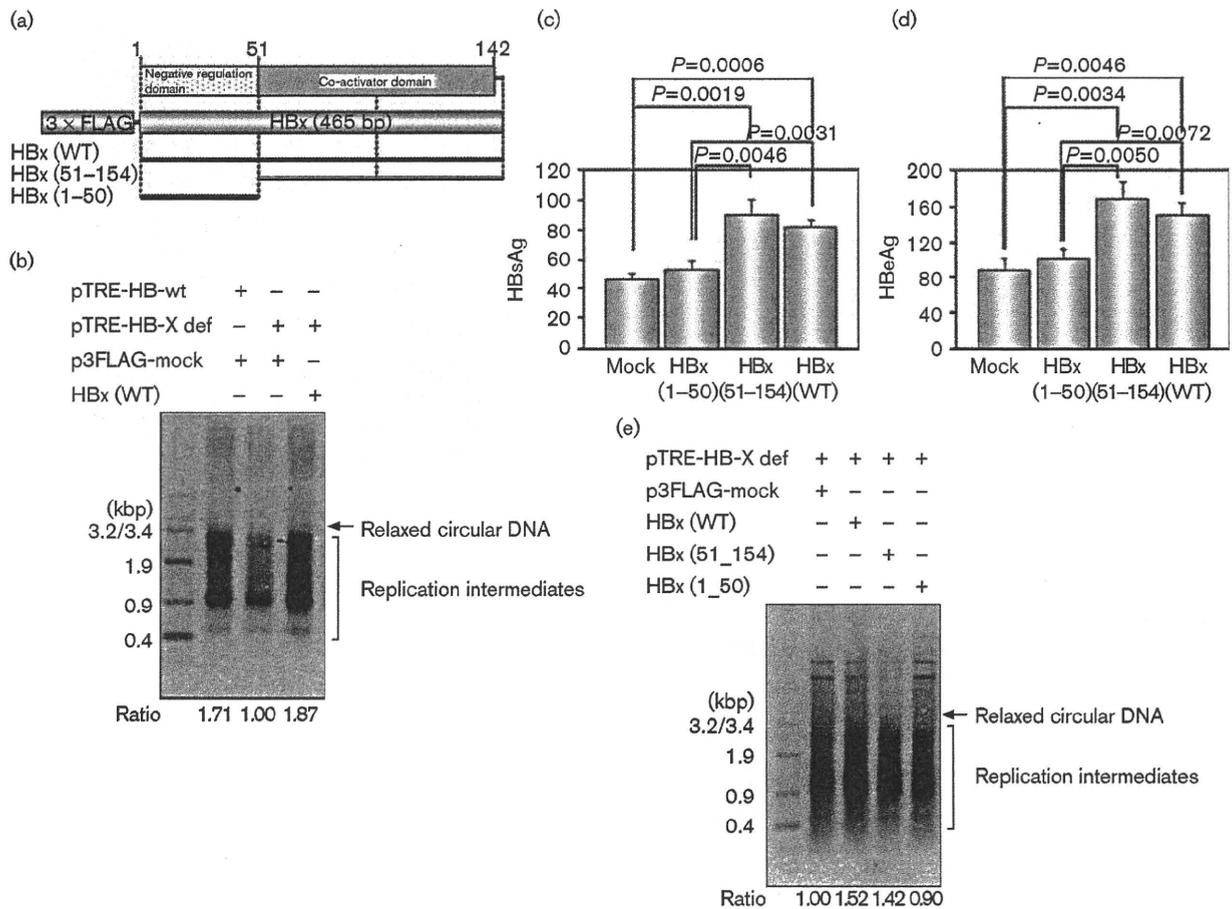
Fig. 3. Recovery of reduced formation of replication intermediate and HB antigens from HBx-def HBV by *trans*-complementation of HBx. (a) Construction of HBx expression plasmids. Full-length and deletion mutants of HBx gene were cloned into the p3FLAG-CMV10 or pcDNA3 or pcDNA3.1-3HA vector. Examples of three FLAG-tagged plasmids are shown. (b) *Trans*-complementation of HBx protein restored the reduced formation of replication intermediates of HBx-def HBV. The core-associated HBV replication intermediates were collected from HepG2 cells and detected with Southern blot hybridization with a full-length HBV probe. (c–e) Recovery of reduced production of HBsAg (c) and HBeAg (d) in culture medium and replication intermediates (e) of HBx-def HBV with *trans*-complementation of HBx expression plasmids. HBx (WT) and HBx(51–154), but not HBx(1–50), effectively enhanced the formation of HBV products. (b, e) The levels of core-associated HBV DNA are shown at the bottom of each lane. Data in (c) and (d) are mean ± SD of three experiments.

we showed previously that HBeAg is dispensable for HBV infection and active replication *in vivo* (Tsuge *et al.*, 2005). Virus replication following infection of HBV particles is quite similar to natural infection. We thus applied the system to study the function of HBx protein in this study. We also utilized hydrodynamic injection of HBx expression plasmid to *trans*-complement the defective HBx. As shown by Western blot analysis (Fig. 4a), HBx protein of the expected size was produced without development of antibody in this SCID-mouse-based model system.

This natural infection mode is quite different from previous animal studies. Virus titres of HBx-def HBV were approx. 50–99 % compared with WT HBV *in vitro*

(Bouchard *et al.*, 2001; Keasler *et al.*, 2007; Leupin *et al.*, 2005; Tang *et al.*, 2005) and *in vivo* (Keasler *et al.*, 2007; Xu *et al.*, 2002). High-level HBx-def virus production seen in these experiments may be the result of expression of HBV proteins other than HBx following forced introduction of plasmids into mouse liver cells by hydrodynamic injection or transgenes. Such introduction probably resulted in virus production that is similar to *in vitro* transfection experiments using cultured cells.

In vitro experiments in this study showed that normal-density HBV particles (Fig. 1c) were produced in the absence of HBx. Curiously, the amount of HBV DNA released from the cells into the supernatant was not different between WT and HBx-def HBV, even though the
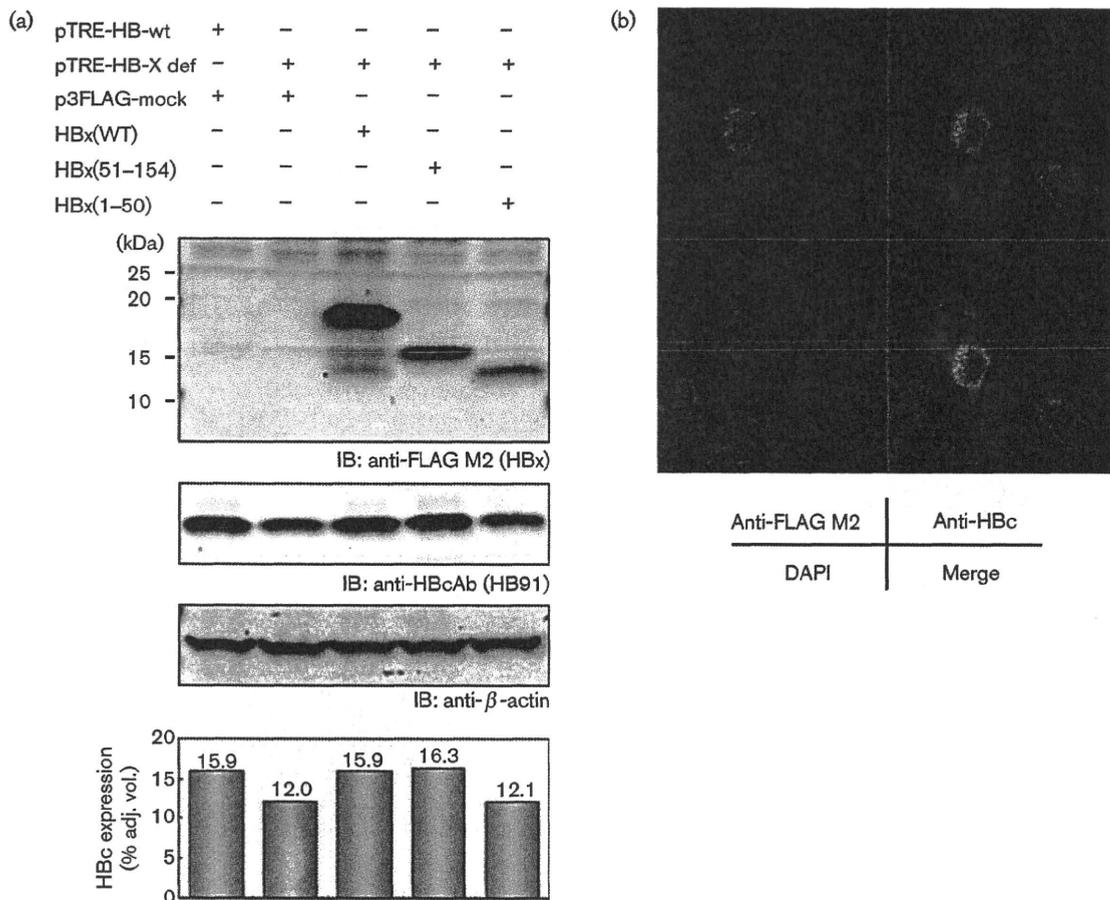
Fig. 4. Upregulation of intracellular core protein formation by *trans*-complementation of the HBx proteins. (a) Western blot analysis of intracellular proteins. Expression of the HBx proteins (percentage adjusted volume) is shown by staining the fused FLAG tag (upper panel). The membrane was also stained by the anti-HBc (middle) and anti-β-actin (lower) antibodies. Values obtained by scanning via densitometer are shown at the bottom of each lane. (b) Immunohistochemical analysis of HepG2 cells co-transfected with pTRE2-HB-X-def and p3FLAG-HBx plasmids. The expression of HBx and HBc proteins was detected by anti-FLAG (upper left) and anti-HBc (upper right) antibody, respectively. The merged image is shown in the lower right and nuclei are shown in the lower left panel. Note that only cells positive for HBx are also positive for HBc protein.

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

Similarly, in the absence of HBx protein *in vitro*, the formation of the replication intermediates (Fig. 3) and production of intracellular core protein (Fig. 4) continued, although their amounts were much lower. It is thus difficult to explain the inability of HBx-def HBV to infect *in vivo* simply from its transcription-activating ability,

although our results confirmed that HBx has *trans*-activation ability, as reported previously (Keasler *et al.*, 2007; Tang *et al.*, 2005; Xu *et al.*, 2002). A different mode of introduction of viral nucleic acid might explain the difference seen in *in vitro* and *in vivo* experiments. In the transfection experiments, a relatively large amount of HBV DNA is introduced by transfection. In contrast, only successfully attached virus particles can introduce viral DNA into liver cells.

Strikingly, all but one (70 of 71 clones) revertant viruses had nucleotide substitutions that reversed the introduced stop codon to a coding amino acid. This is in contrast to the fact that HBV replicates in the HBx-def form in cultured cells, even though the efficacy is lower than in WT. We assumed that complemented HBx protein stimulated the replication of HBx-def HBV and increased the chance of nucleotide sequence substitutions in the HBx
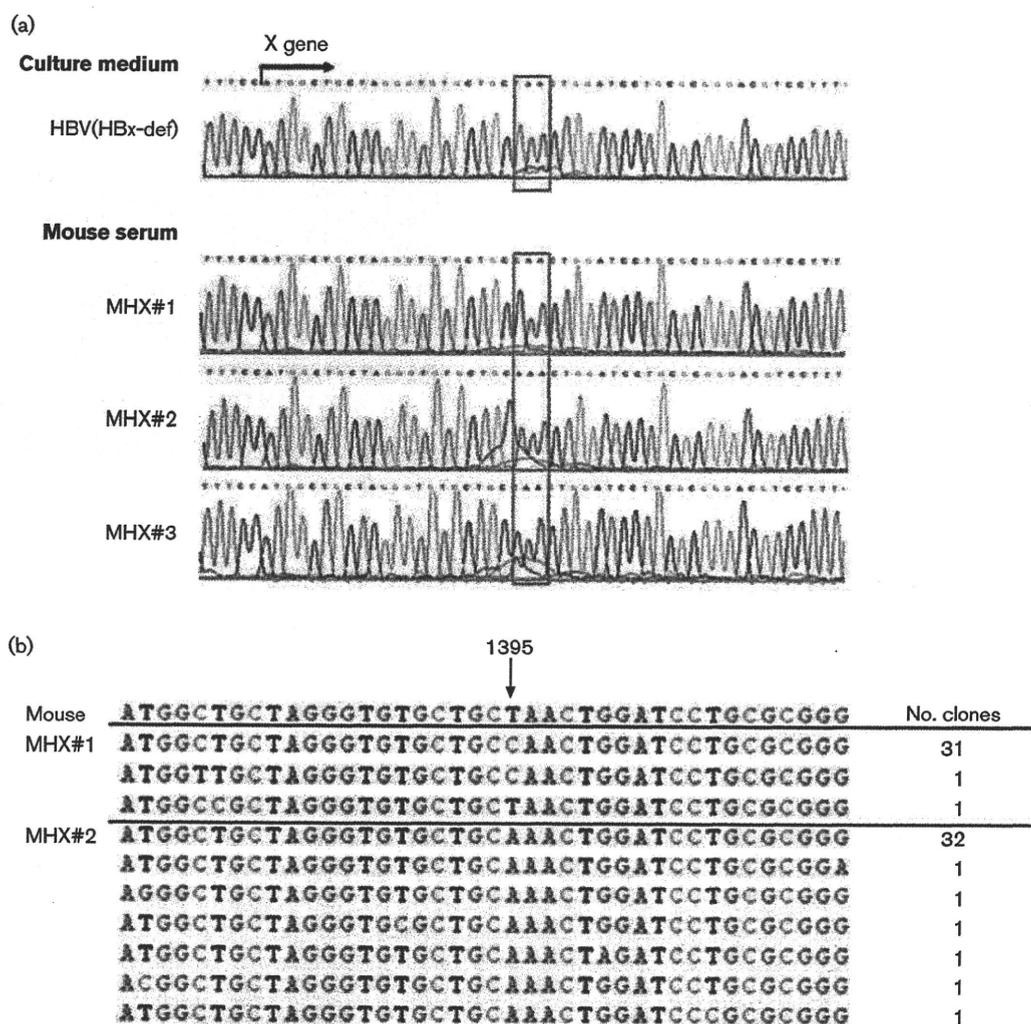
— 940 —
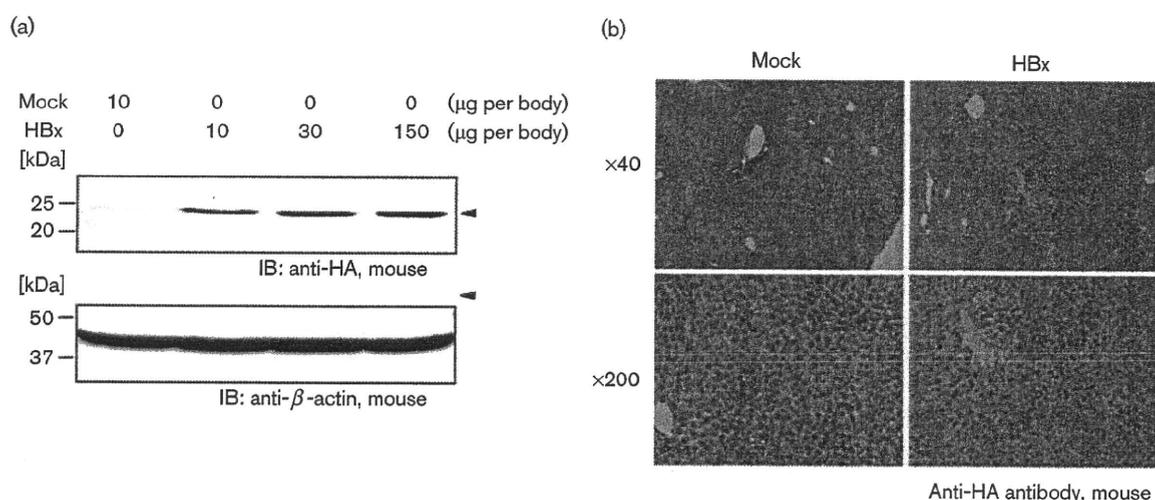
Fig. 5. Expression of HBx protein by hydrodynamic injection of HBx plasmid. (a) Liver-expressed HA-tagged HBx proteins were detected by Western blot analysis using anti-HA antibody (HA tag was used to avoid non-specific binding of anti-FLAG tag to mouse liver proteins). Dose-dependent expression of the protein was observed with different doses of the injected plasmid. (b) Immunohistochemical analysis of mouse liver using anti-HA antibody revealing expression of HBx protein. The protein was mainly expressed around the central vein.

gene, and that only revertant HBV variants predominantly increased, due to their rapid replication ability through the infection–replication cycle that only exists in the *in vivo* model. One might consider the possibility that the HBx protein works as a mutagen. However, we did not observe clear differences in the incidence of nucleotide sequence substitutions between the presence and absence of HBx (Fig. 7b and data not shown).
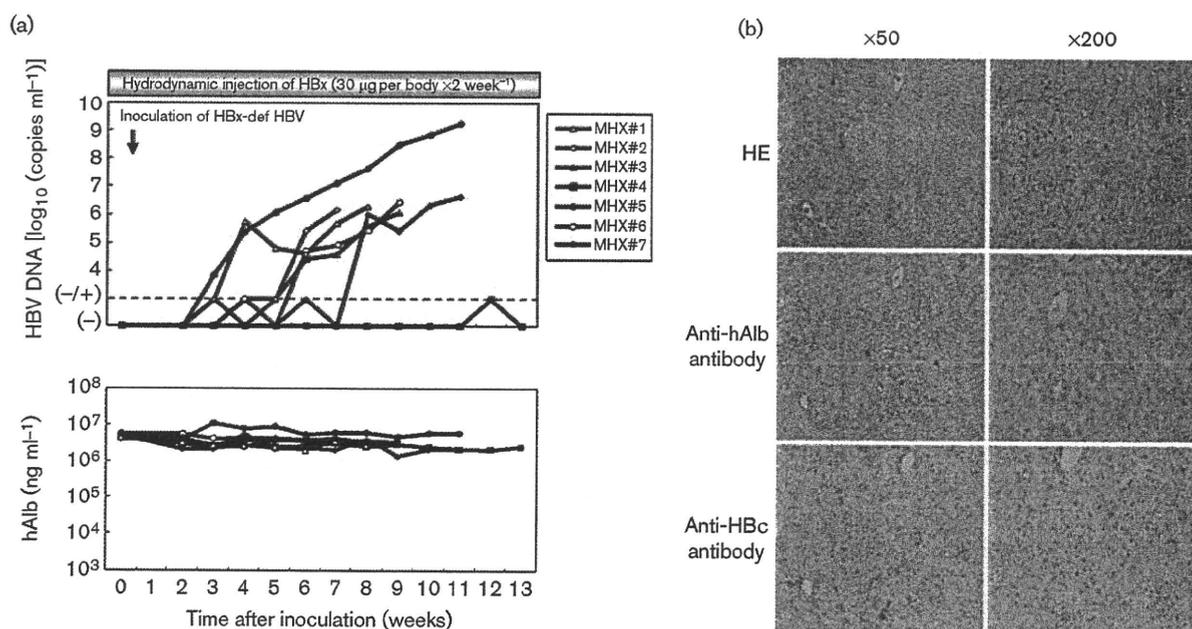
Fig. 6. Infection of HBx-def HBV particles after hydrodynamic injection of HBx expression plasmid. (a) Full-length HBx protein expression plasmid was hydrodynamically injected twice a week into human hepatocyte chimeric mice. Two weeks after the beginning of the injections, cell-culture-derived HBx-def HBV particles were injected through the tail vein. HBV DNA (upper panel) and hAlb (lower panel) were measured. (b) Immunohistochemical analysis of the infected mouse. The liver was stained with haematoxylin and eosin (HE) (upper), antibody against hAlb (middle) and anti-HBc antibody (lower).

(a)

Culture medium

X gene

HBV(HBx-def)

Mouse serum

MHX#1

MHX#2

MHX#3

(b)

1395

		No. clones
Mouse	ATGGCTGCTAGGGTGTGCTGCTAACTGGATCCTGCGCGGG	
MHX#1	ATGGCTGCTAGGGTGTGCTGCCAACTGGATCCTGCGCGGG	31
	ATGGTTGCTAGGGTGTGCTGCCAACTGGATCCTGCGCGGG	1
	ATGGCCGCTAGGGTGTGCTGCTAACTGGATCCTGCGCGGG	1
MHX#2	ATGGCTGCTAGGGTGTGCTGCAAACTGGATCCTGCGCGGG	32
	ATGGCTGCTAGGGTGTGCTGCAAACTGGATCCTGCGCGGA	1
	AGGGCTGCTAGGGTGTGCTGCAAACTGGATCCTGCGCGGG	1
	ATGGCTGCTAGGGTGCGCTGCAAACTGGATCCTGCGCGGG	1
	ATGGCTGCTAGGGTGTGCTGCAAACTAGATCCTGCGCGGG	1
	ACGGCTGCTAGGGTGTGCTGCAAACTGGATCCTGCGCGGG	1
	ATGGCTGCTAGGGTGTGCTGCAAACTGGATCCCGCGCGGG	1

Fig. 7. Nucleotide sequence analysis of HBV recovered from HBx-def HBV-injected mice. (a) Nucleotide sequences of the HBx region of HBV determined by direct sequencing of PCR products using serum samples obtained from three mice (#1, #2 and #3 in Fig. 6a). The sequences were compared with that of inoculated HBV. Note that one of the three mice (#2) had a unique sequence different from the original sequence before introduction of the stop codon (C1395T). (b) Nucleotide sequences of the HBx gene determined by cloning and sequencing of PCR-amplified DNA from mice #1 and #2. Note that only one of 63 clones showed the introduced stop codon mutation. As we used a large amount of HBV plasmids, special care was taken to avoid contamination of DNA. Water was used as a negative control for all experiments and we observed no inappropriate amplification in these experiments.

It is thus still uncertain why the HBx protein is indispensable for virus replication *in vivo*. However, the fact that HBV cannot replicate in the absence of HBx protein may allow development of therapeutic medicine by disturbing the unknown action of HBx. To this end, it is interesting to identify a substance that binds to HBx.

The indispensability of the X protein for virus replication is a common feature shared by HBV and WHV (Chen *et al.*, 1993; Zoulim *et al.*, 1994). Both of them cause chronic infection, inflammation, fibrosis and cancer. In contrast, DHBV, which can replicate without DHBx expression, does not cause such a pathological situation (Meier *et al.*,

2003). Further analysis of the X protein may pave the way to clarify the mechanism of cancer development caused by HBV infection.

METHODS

Human hepatocyte chimeric mice experiments. Care of uPA^{+/+}/SCID^{+/+} mice and transplantation of human hepatocytes were performed as described previously (Tateno *et al.*, 2004). The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University. Infection, extraction of serum samples and sacrifice were performed under ether anaesthesia as described previously (Tateno *et al.*, 2004).

hAlb in mouse serum was measured with a Human Albumin ELISA Quantification kit (Bethyl Laboratories Inc.) according to the instructions provided by the manufacturer. Serum samples obtained from mice were aliquotted and stored in liquid nitrogen until use.

Analysis of HBV markers. HBsAg and HBeAg were measured using a commercially available ELISA kit (Abbott). For quantitative analysis of HBV DNA, 10 µl mouse serum sample or 100 µl of culture supernatant was used. DNA was extracted from these samples using the SMITEST R&D (Genome Science Laboratories) and dissolved in 20 µl H_2O, and HBV DNA was quantified by real-time PCR using the 7300 Real-Time PCR System (Applied Biosystems). Amplification was performed as described previously (Tsuge et al., 2005). The lower detection limit of this assay is 300 copies. For detection of small amounts of HBV DNA, we also performed nested PCR. The amplification conditions were as described previously (Tsuge et al., 2005).

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

Transfection of HepG2 cell lines with HBV expression plasmids. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum at 37 °C and under 5 % CO_2. For functional analysis of the HBx protein in vitro, the HBV or HBx-def HBV expression plasmid was transfected with/without HBx expression plasmid using TransIT-LT1 (Mirus) reagent according to the instructions provided by the supplier. Three to five days after transfection, core-associated HBV DNA was extracted from cells for HBV DNA quantification (Noguchi et al., 2005). For analysing the infectivity of recombinant HBV particles, HBV expression plasmids were transiently transfected into HepG2 cells. The cells were seeded to semi-confluence in 90 mm dishes. WT HBV particles were generated from cells transfected with 20 µg pTRE2-HB-wt by calcium phosphate precipitation. HBx-def HBV particles were also generated from cells co-transfected with 10 µg pTRE2-HB-X-def and 10 µg pcDNA-HBx. Three days after transfection, the culture medium was collected and stored in liquid nitrogen until use.

Analysis of cell-culture-produced HBV by sucrose density gradient sedimentation. Five millilitres of HBV-positive human serum (8 log_{10} copies ml^{-1}) or 50 ml cell culture supernatant (8 log_{10} copies ml^{-1}) was layered on a 20 % (w/w) sucrose cushion, and centrifuged at 24 000 r.p.m. (maximum 103 864 g) for 12 h at 4 °C with a Beckman SW28 rotor (Beckman Coulter). The precipitate was resuspended in 500 µl PBS. These HBV samples were layered on a linear 20–50 % (w/w) sucrose gradient. Centrifugation was carried out at 24 000 r.p.m. (maximum 102 445 g) for 21 h at 4 °C with a Beckman SW40 rotor. The gradients were fractionated into 500 µl

samples, and the density of each fraction was calculated from the weight and volume. Each fraction was diluted 10-fold and tested for HBV DNA by real-time PCR.

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

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

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

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

Immunohistochemical analysis of mouse liver. The liver specimens of HBV-infected mice were fixed with 10 % buffered paraformaldehyde and embedded in paraffin blocks for histological

examination. The liver sections were stained with haematoxylin–eosin or subjected to immunohistochemical staining using an antibody against HBcAg (DAKO Diagnostika), anti-HA antibody or HSA (Bethyl Laboratories Inc.). Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 and methanol. Immunoreactive materials were visualized by using a streptavidin–biotin staining kit (Histofine SAB-PO kit; Nichirei) and diaminobenzidine.

Sequence analysis of the HBV genome. Genome-length HBV DNA was amplified by PCR as described by Günther *et al.* (1995). HBV genome-length PCR products were subjected to 1 % agarose gel electrophoresis and the 3.2 kbp band was extracted using a QiaEx II Gel Extraction kit (Qiagen). Direct sequencing, cloning and sequencing (Ohishi *et al.*, 2004) were performed in an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) with a Big Dye Terminator version 3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems).

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

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REFERENCES

Ando, T., Sugiyama, K., Goto, K., Miyake, Y., Li, R., Kawabe, Y. & Wada, Y. (1999). Age at time of hepatitis Be antibody seroconversion in childhood chronic hepatitis B infection and mutant viral strain detection rates. *J Pediatr Gastroenterol Nutr* 29, 583–587.

Arbuthnot, P., Capovilla, A. & Kew, M. (2000). Putative role of hepatitis B virus X protein in hepatocarcinogenesis: effects on apoptosis, DNA repair, mitogen-activated protein kinase and JAK/STAT pathways. *J Gastroenterol Hepatol* 15, 357–368.

Bouchard, M. J., Wang, L. H. & Schneider, R. J. (2001). Calcium signaling by HBx protein in hepatitis B virus DNA replication. *Science* 294, 2376–2378.

Chen, H. S., Kaneko, S., Girones, R., Anderson, R. W., Hornbuckle, W. E., Tennant, B. C., Cote, P. J., Gerin, J. L., Purcell, R. H. & Miller, R. H. (1993). The woodchuck hepatitis virus X gene is important for establishment of virus infection in woodchucks. *J Virol* 67, 1218–1226.

Colgrove, R., Simon, G. & Ganem, D. (1989). Transcriptional activation of homologous and heterologous genes by the hepatitis B virus X gene product in cells permissive for viral replication. *J Virol* 63, 4019–4026.

Dandri, M., Schirmacher, P. & Rogler, C. E. (1996). Woodchuck hepatitis virus X protein is present in chronically infected woodchuck liver and woodchuck hepatocellular carcinomas which are permissive for viral replication. *J Virol* 70, 5246–5254.

Dandri, M., Petersen, J., Stockert, R. J., Harris, T. M. & Rogler, C. E. (1998). Metabolic labeling of woodchuck hepatitis B virus X protein

in naturally infected hepatocytes reveals a bimodal half-life and association with the nuclear framework. *J Virol* 72, 9359–9364.

Doria, M., Klein, N., Lucito, R. & Schneider, R. J. (1995). The hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of Ras and nuclear activator of transcription factors. *EMBO J* 14, 4747–4757.

Ganem, D. & Schneider, R. J. (2001). *Hepadnaviridae*: the viruses and their replication. In *Fields Virology*, 4th edn, pp. 2923–2969. Edited by D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman & S. E. Straus. Philadelphia, PA: Lippincott Williams & Wilkins.

Günther, S., Li, B. C., Miska, S., Kruger, D. H., Meisel, H. & Will, H. (1995). A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 69, 5437–5444.

Henkler, F., Hoare, J., Waseem, N., Goldin, R. D., McGarvey, M. J., Koshy, R. & King, I. A. (2001). Intracellular localization of the hepatitis B virus HBx protein. *J Gen Virol* 82, 871–882.

Jacob, J. R., Ascenzi, M. A., Roneker, C. A., Toshkov, I. A., Cote, P. J., Gerin, J. L. & Tennant, B. C. (1997). Hepatic expression of the woodchuck hepatitis virus X-antigen during acute and chronic infection and detection of a woodchuck hepatitis virus X-antigen antibody response. *Hepatology* 26, 1607–1615.

Keasler, V. V., Hodgson, A. J., Madden, C. R. & Slagle, B. L. (2007). Enhancement of hepatitis B virus replication by the regulatory X protein *in vitro* and *in vivo*. *J Virol* 81, 2656–2662.

Klein, N. P., Bouchard, M. J., Wang, L. H., Kobarg, C. & Schneider, R. J. (1999). Src kinases involved in hepatitis B virus replication. *EMBO J* 18, 5019–5027.

Leupin, O., Bontron, S., Schaeffer, C. & Strubin, M. (2005). Hepatitis B virus X protein stimulates viral genome replication via a DDB1-dependent pathway distinct from that leading to cell death. *J Virol* 79, 4238–4245.

Meier, P., Scougall, C. A., Will, H., Burrell, C. J. & Jilbert, A. R. (2003). A duck hepatitis B virus strain with a knockout mutation in the putative X ORF shows similar infectivity and *in vivo* growth characteristics to wild-type virus. *Virology* 317, 291–298.

Melegari, M., Wolf, S. K. & Schneider, R. J. (2005). Hepatitis B virus DNA replication is coordinated by core protein serine phosphorylation and HBx expression. *J Virol* 79, 9810–9820.

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., Addison, W. R., Fischer, K. P., Churchill, T. A. & other authors (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 7, 927–933.

Murakami, S. (2001). Hepatitis B virus X protein: a multifunctional viral regulator. *J Gastroenterol* 36, 651–660.

Noguchi, C., Ishino, H., Tsuge, M., Fujimoto, Y., Imamura, M., Takahashi, S. & Chayama, K. (2005). G to A hypermutation of hepatitis B virus. *Hepatology* 41, 626–633.

Ohishi, W., Shirakawa, H., Kawakami, Y., Kimura, S., Kamiyasu, M., Tazuma, S., Nakanishi, T. & Chayama, K. (2004). Identification of rare polymerase variants of hepatitis B virus using a two-stage PCR with peptide nucleic acid clamping. *J Med Virol* 72, 558–565.

Raney, A. K. & McLachlan, A. (1991). The biology of hepatitis B virus. In *Molecular Biology of the Hepatitis B Virus*, pp. 1–37. Edited by A. McLachlan. Boca Raton, FL: CRC Press.

Seeger, C. & Mason, W. S. (2000). Hepatitis B virus biology. *Microbiol Mol Biol Rev* 64, 51–68.

Sitterlin, D., Bergametti, F., Tiollais, P., Tennant, B. C. & Transy, C. (2000a). Correct binding of viral X protein to UVDDB-p127 cellular protein is critical for efficient infection by hepatitis B viruses. *Oncogene* 19, 4427–4431.

Sitterlin, D., Bergametti, F. & Transy, C. (2000b). UVDDB p127-binding modulates activities and intracellular distribution of hepatitis B virus X protein. *Oncogene* **19**, 4417–4426.

Su, Q., Schroder, C. H., Hofmann, W. J., Otto, G., Pichlmayr, R. & Bannasch, P. (1998). Expression of hepatitis B virus X protein in HBV-infected human livers and hepatocellular carcinomas. *Hepatology* **27**, 1109–1120.

Tang, H., Banks, K. E., Anderson, A. L. & McLachlan, A. (2001). Hepatitis B virus transcription and replication. *Drug News Perspect* **14**, 325–334.

Tang, H., Delgermaa, L., Huang, F., Oishi, N., Liu, L., He, F., Zhao, L & Murakami, S. (2005). The transcriptional transactivation function of HBx protein is important for its augmentation role in hepatitis B virus replication. *J Virol* **79**, 5548–5556.

Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K. & other authors (2004). Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165**, 901–912.

Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y. & other authors (2005). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* **42**, 1046–1054.

Wang, W. L., London, W. T. & Feitelson, M. A. (1991). Hepatitis B X antigen in hepatitis B virus carrier patients with liver cancer. *Cancer Res* **51**, 4971–4977.

Xu, Z., Yen, T. S., Wu, L., Madden, C. R., Tan, W., Slagle, B. L. & Ou, J. H. (2002). Enhancement of hepatitis B virus replication by its X protein in transgenic mice. *J Virol* **76**, 2579–2584.

Yang, P. L., Althage, A., Chung, J. & Chisari, F. V. (2002). Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc Natl Acad Sci U S A* **99**, 13825–13830.

Zhang, Z., Torii, N., Hu, Z., Jacob, J. & Liang, T. J. (2001). X-deficient woodchuck hepatitis virus mutants behave like attenuated viruses and induce protective immunity *in vivo*. *J Clin Invest* **108**, 1523–1531.

Zoulim, F., Saputelli, J. & Seeger, C. (1994). Woodchuck hepatitis virus X protein is required for viral infection *in vivo*. *J Virol* **68**, 2026–2030.

Original article

Differential effects of interferon and lamivudine on serum HBV RNA inhibition in patients with chronic hepatitis B

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Background: Lamivudine and interferon have been widely used for the treatment of patients with chronic HBV infection. Serum HBV RNA is detected during lamivudine therapy as a consequence of interrupted reverse transcription and because RNA replicative intermediates are unaffected by the drug. In this study, we aimed to determine the detectability of serum HBV RNA during sequential combination therapy of interferon and lamivudine.
Methods: HBV DNA and RNA in serum samples were quantified by reverse transcription of HBV nucleic acid extract and real-time PCR. Samples were analysed every 2 weeks to 3 months from three groups of patients: 10 male patients treated with nucleoside analogue monotherapy for 44–48 weeks (5 with lamivudine and 5 with entecavir), 6 males on sequential interferon and lamivudine combination therapy, and 3 males on lamivudine monotherapy for 20–24 weeks.

Results: HBV RNA was not detectable in any patients before treatment, but became detectable in 15 during antiviral treatment. Among the three groups, pretreatment HBV DNA (8.1 ±2.4 versus 7.7 ±1.4 versus 5.1 ±0.3 $\log_{10}$ copies/ml; P=0.06), treatment and follow-up durations (45.5 ±2.0 versus 49.7 ±5.6 versus 48.7 ±6.4 weeks; P=0.32) were comparable. HBV RNA was detectable at the end of treatment or follow-up in all patients with monotherapy, but in none of those with sequential combination therapy (100% versus 0%; P<0.001).
Conclusions: Compared with lamivudine therapy with detectable serum HBV RNA in patients with chronic HBV infection, interferon treatment might reduce HBV DNA replication through the inhibition of HBV RNA replicative intermediates, resulting in the loss of serum HBV RNA.

Introduction

Although effective HBV vaccines have been available for more than two decades, HBV infection remains a global health problem. It is estimated that more than 350 million people are chronic carriers of HBV worldwide [1,2]. In the US, 1.2 million individuals have chronic HBV infection [3]. HBV infection causes a wide spectrum of clinical manifestations, ranging from acute or fulminant hepatitis to various forms of chronic liver disease, including inactive carrier state, chronic hepatitis, cirrhosis and even hepatocellular carcinoma [2,4].

HBV is a unique DNA virus that replicates via pre-genomic RNA. There are several key steps in HBV replication. Firstly, in the nucleus of infected hepatocytes, the asymmetric DNA in virions converts to

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covalently closed circular DNA (cccDNA); the cccDNA is then transcribed to pre-genomic RNA. Next, the minus strand of viral DNA is synthesized by reverse transcriptase. Finally, there is synthesis of the plus strand to form mature genomic DNA [5]. Both interferon and nucleos(t)ide analogues have been approved for the treatment of chronic hepatitis B (CHB). All of these agents have viral suppression effects, whereas interferon has additional immunomodulatory properties [6]. Lamivudine is the first approved nucleoside analogue for the treatment of CHB; however, it does not affect the integrated HBV DNAs or their transcripts, the RNA replicative intermediates [7]. Thus, lamivudine, as well as other nucleos(t)ide analogues, needs indefinite duration of therapy for continued viral suppression. By contrast, interferon has a finite duration of therapy and a higher rate of hepatitis B surface antigen (HBsAg) seroclearance than nucleos(t)ide analogues [8]. Our study and others showed that serum HBV RNA could be detected during lamivudine therapy, as the consequence of unaffected RNA replicative intermediates as well as interrupted reverse transcription [9,10]. In addition, serum HBV RNA might serve as a predictor of early emergence of viral mutation during lamivudine therapy [10].

Previous clinical trials indicated that simultaneous combination therapy of interferon-α plus lamivudine leads to greater on-treatment viral suppression and higher sustained response rates than lamivudine monotherapy [8]. However, the detectability and patterns of serum HBV RNA in patients receiving sequential combination therapy of interferon and lamivudine compared with those on lamivudine monotherapy remain largely unknown. Thus, we explored the differential effects of interferon and lamivudine on serum HBV RNA inhibition in CHB patients with various treatment regimens.

Methods

Patients

We enrolled 19 CHB patients treated with nucleoside analogue alone or sequential combination therapy of conventional interferon and lamivudine. These patients were divided into three groups on the basis of treatment regimen. Group I consisted of 10 male patients (mean age 44.2 years; range 30–74) treated with nucleoside analogue monotherapy for 44–48 weeks (5 patients with lamivudine and 5 with entecavir). Group II consisted of six male patients (mean age 47.3 years; range 39–56). Five of these patients were treated with lamivudine for 34–52 weeks and then shifted to conventional interferon for 24–36 weeks; there was an overlap of the two drugs for 4–20 weeks. The remaining patient in this group was treated with conventional interferon for 36 weeks and then shifted to lamivudine for 32 weeks; there was an overlap of the two drugs for

12 weeks. Group III consisted of three male patients (mean age 51.3 years; range 41–64) who were treated with lamivudine for 20–24 weeks and then followed for 22–36 weeks. Serum samples from enrolled patients were obtained just before the initiation of therapy and every 2 weeks to 3 months until the end of treatment or follow-up. These samples were stored at -80°C until use. Informed consent was obtained from each patient.

Extraction of HBV nucleic acid and reverse transcription

Extraction of HBV nucleic acid and reverse transcription with subsequent quantification were performed as previously described [10]. Nucleic acid was extracted from 100 µl serum using the SMI TEST EX-R&D kit (Genome Science Laboratories, Tokyo, Japan) and dissolved in 18 µl of ribonuclease-free water. The extract was then divided into two aliquots of equal size, termed solutions I and II. Solution I was mixed with an equivalent amount of water for DNA quantification. Solution II underwent reverse transcription using a random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) with subsequent DNA plus cDNA quantification. The steps in reverse transcription were as follows: 25 pM random primer was added and the sample heated to 65°C for 5 min; the sample was then put on ice for 5 min; 5× reverse transcription buffer (4 µl), 10 mM dNTPs (2 µl), 0.1 M dithiothreitol (2 µl), 8 units of ribonuclease inhibitor and 100 units of M-MLV reverse transcriptase were added; the sample was incubated at 30°C for 10 min and 42°C for 60 min; and inactivation was carried out at 99°C for 5 min.

Quantification of HBV DNA and cDNA by real-time PCR

HBV DNA and cDNA quantification were performed as previously described [10]. A 1 µl aliquot of solution I and solution II were each amplified by real-time PCR with an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Amplification was performed in a 25 µl reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystems), 200 nM forward primer (5'-TTTGGGGCATGGACATTGAC-3', nucleotides 1893–1912), 200 nM reverse primer (5'-GGTGAACAATGGTCCGGAGAC-3', nucleotides 2029–2049) and 1 µl of solution I or solution II. The steps in real-time PCR were as follows: incubation at 50°C for 2 min, denaturation at 95°C for 10 min, and PCR cycling comprising 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. The lower detection limit of this assay was 10^3 copies/ml. The HBV RNA quantity is obtained by subtracting the quantification result of solution I from solution II (that is, HBV nucleic acid determined by real-time PCR after reverse transcription reaction minus HBV DNA determined by real-time PCR).

Serological assays

Serum hepatitis B e antigen (HBeAg) and levels of antibodies against HBV e antigen (anti-HBe) were tested by chemiluminescent immunoassay (Architect HBeAg and Architect HBeAb, Abbott Japan, Tokyo, Japan).

Statistical analysis

Baseline characteristics including age, gender, serum alanine aminotransferase (ALT) level, HBeAg and HBV DNA level were compared among study groups. Continuous variables were expressed as mean ± SD and evaluated by Student's t-test for comparison between two groups and by Kruskal–Wallis test for comparison among three groups. Categorical variables were expressed as frequencies with proportions and compared using Pearson's χ^2 test; Fisher's exact test was applied when at least one cell of the table has an expected frequency <5. All of the tests were two-tailed and a P-value <0.05 was considered statistically significant.

Results

Demographic profiles of patients

Baseline characteristics of CHB patients treated with nucleoside analogues and/or conventional interferon are shown in Table 1. There was no significant difference in terms of age, gender ratio, ALT level, HBeAg status or HBV DNA level among the three groups.

Serum HBV RNA levels after nucleoside analogue therapy

The detectability of serum HBV RNA before, after and at the end of nucleoside analogue therapy or follow-up is shown in Table 2. Serum HBV RNA was undetectable in all patients before the initiation of nucleoside analogue therapy; however, it became detectable in 15 patients (79%) after therapy. Of 14 patients treated with lamivudine, serum HBV RNA was detectable in 10 (71%). By contrast, of five patients treated with entecavir, all (100%) had detectable serum HBV RNA. The peak serum HBV RNA level ranged from 4.2 to 7.0 $\log_{10}$ copies/ml in lamivudine-treated patients and from 7.2 to 9.6 $\log_{10}$ copies/ml in entecavir-treated patients.

Serum HBV RNA levels after interferon treatment in patients with prior lamivudine therapy

In patients with detectable serum HBV RNA after nucleoside analogue monotherapy, HBV RNA persisted until the end of therapy (group I; Table 2). Similarly, serum HBV RNA was persistently detectable after short-term lamivudine therapy (group III; Table 2). By contrast, those with sequential lamivudine and interferon therapy experienced undetectable

Table 1. Baseline characteristics of chronic hepatitis B patients treated with nucleoside analogues and/or conventional interferon

| Characteristic	Group			P-value
	I[a]	II[b]	III[c]	
Patients (3TC/ETV), n	10 (5/5)	6	3	–
Mean age, years ±SD	44.2 ±13.7	47.3 ±6.5	51.3 ±11.7	0.31[d]
Gender (male/female), n	10/0	6/0	3/0	1.0[e]
Mean ALT, U/l ±SD	164.4 ±105.5	180.3 ±121.2	154 ±142	0.88[d]
HBeAg, % (+/–)	70 (7/3)	17 (1/5)	66.7 (2/1)	0.13[e]
Mean HBV DNA, $\log_{10}$ copies/ml ±SD	8.1 ±2.4	7.7 ±1.4	5.1 ±0.3	0.06[d]

[a]Group I, lamivudine (3TC) or entecavir (ETV) monotherapy for 44–48 weeks. [b]Group II, sequential 3TC for 34–52 weeks and conventional interferon for 24–36 weeks; there was overlap of the two drugs for 4–20 weeks. [c]Group III, 3TC therapy for 20–24 weeks, then follow-up for 22–36 weeks. [d]Kruskal–Wallis test; [e]Fisher's exact test. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.

Table 2. Serum HBV RNA in chronic hepatitis B patients treated with nucleoside analogues and/or conventional interferon

	Group			P-value
	I[a]	II[b]	III[c]	
Patients (3TC/ETV), n	10 (5/5)	6	3	–
Detectability before treatment, %	0	0	0	1.0[e]
Detectability after treatment, n (%)	8/2 (80.0)	5/1 (83.0)	2/1 (66.7)	1.0[e]
Mean peak level, $\log_{10}$ copies/ml ±SD	7.4 ±1.9	6.1 ±0.9	4.6 ±0.1	0.08[d]
Mean total duration (treatment plus follow-up), weeks ±SD	45.5 ±2.0	49.7 ±5.6	48.7 ±6.4	0.32[d]
Detectability at end of treatment plus follow-up, n (%)	8/0 (100)	0/5 (0)	2/0 (100)	<0.001[e]

[a]Group I, lamivudine (3TC) or entecavir (ETV) monotherapy for 44–48 weeks. [b]Group II, sequential 3TC for 34–52 weeks and conventional interferon for 24–36 weeks; there was overlap of the two drugs for 4–20 weeks. [c]Group III, 3TC therapy for 20–24 weeks, then follow-up for 22–36 weeks. [d]Kruskal–Wallis test; [e]Fisher's exact test.

Figure 1. Sequential changes of serum HBV RNA, DNA and ALT levels in patients treated with lamivudine or entecavir monotherapy for 44–48 weeks with detectable serum HBV RNA (group I)
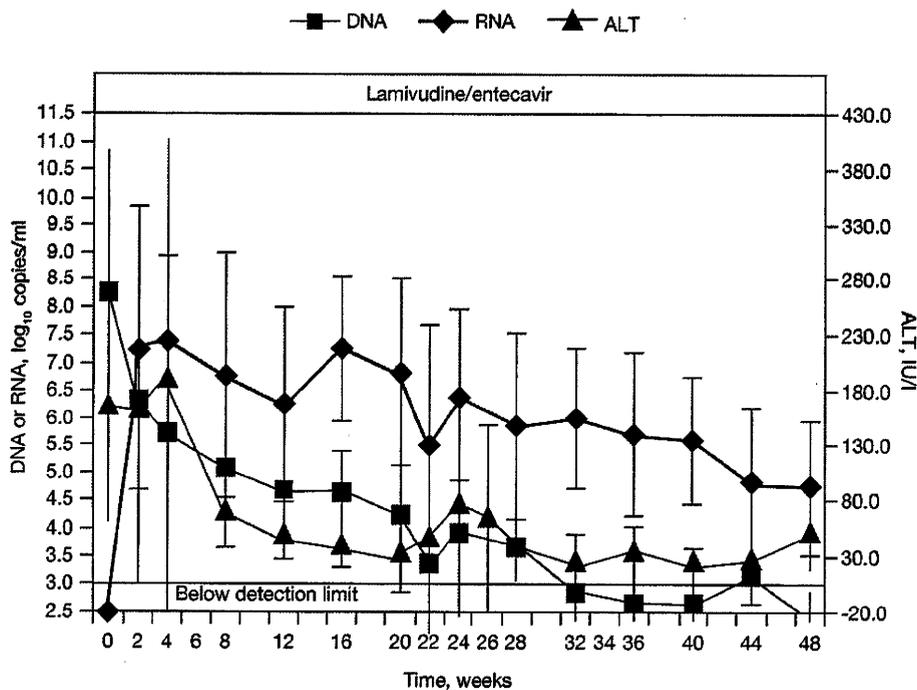
Serum HBV RNA was detectable until the end of treatment. ALT, alanine aminotransferase.

serum HBV RNA at the end of treatment (group II; Table 2).

Sequential changes of serum HBV RNA levels in patients with various treatments

The sequential changes of serum HBV RNA in patients with detectable HBV RNA in group I, II and III are shown in Figure 1, Figure 2 and Figure 3, respectively. As early as 2–4 weeks after starting nucleoside analogue therapy, serum HBV RNA could be detected in 13 patients (87%) and reached the peak level in 11 (73%).

Discussion

The specific presence of serum HBV RNA in patients with CHB infection was confirmed in our previous study using ribonuclease digestion [10]. Ribonuclease treatment reduced the amount of HBV DNA detected by real-time PCR after reverse transcription to about 1% of that originally detected [10]. In this study, detectable serum HBV RNA persisted during nucleoside analogue therapy including lamivudine and entecavir (group I), whereas it was inhibited under sequential lamivudine

and interferon therapy (group II). The difference between these two groups is significant even in a small number of patients, demonstrating the substantial HBV RNA inhibitory effect of interferon. The decline in serum HBV RNA level was not simply the result of discontinuation of lamivudine, because serum HBV RNA was persistently detectable even after discontinuation of short-term lamivudine therapy in group III patients. The inhibition of serum HBV RNA was found in patients treated with lamivudine and then shifted to conventional interferon and in those on conventional interferon treatment and shifted to lamivudine. In the latter patients, the inhibition of serum HBV RNA might be due to the delayed therapeutic effect of interferon.

Several previous studies have proven that sequential combination therapy of lamivudine and interferon has a better efficacy than lamivudine monotherapy. For example, sequential lamivudine and interferon therapy increased HBeAg seroconversion rate [11] and had a higher response rate in terms of sustained HBeAg seroconversion, ALT normalization, HBV DNA loss and reduced rates of relapse after stopping therapy [12]. In addition, the initial use of lamivudine before interferon

Figure 2. Sequential changes of serum HBV RNA, DNA and ALT levels in patients treated with lamivudine for 34–52 weeks with detectable serum HBV RNA (group II)
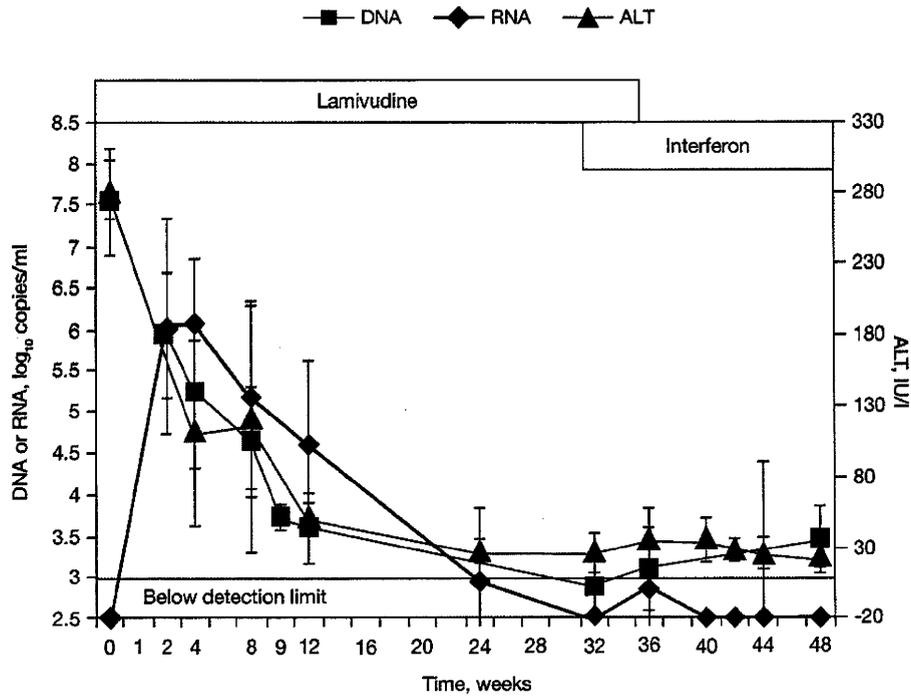

Patients were treated with lamivudine for 34–52 weeks and then shifted to conventional interferon therapy for 24–26 weeks; there was overlap of the two drugs for 4–20 weeks. Serum HBV RNA was undetectable at the end of treatment. ALT, alanine aminotransferase.

led to an improved sustained virological response compared with the use of interferon alone from the start [13]. Nevertheless, the underlying mechanisms of these convincing findings remain unclear and deserve further investigation. In this study, the inhibitory effect of interferon on serum HBV RNA in lamivudine-treated patients might explain why these patients have a higher sustained response rate than those treated with lamivudine monotherapy.

The weak point of this study is the small number of patients in groups II and III. Because this is a pilot study on the differential effects of various treatment regimens on serum HBV RNA, and because the treatment regimens in group II and group III patients are not the current standard of care, we did not intend to include additional patients treated with these regimens. In line with this study, however, our unpublished data on three patients also indicate that *de novo* combination therapy of pegylated interferon plus lamivudine therapy could inhibit serum HBV RNA levels. In these three patients, serum HBV RNA levels started to rise at 12–24 weeks of combination therapy and became undetectable at 48–72 weeks of therapy (YWH, JHK, *et al.*, unpublished data).

Figure 3. Sequential changes of serum HBV RNA, DNA and ALT levels in patients treated with short-term lamivudine for 20–24 weeks with detectable serum HBV RNA (group III)
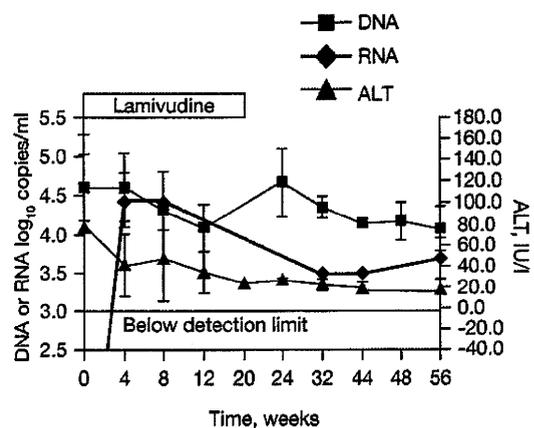
Serum HBV RNA was detectable until the end of follow-up at 44–56 weeks. ALT, alanine aminotransferase.

The presence of serum HBV RNA in patients treated with nucleoside analogues could be explained by *in vitro* data obtained in HBV-transfected HepG2.2.15 cell lines. Doong *et al.* [7] demonstrated that HBV-specific RNAs in cell lysate were not reduced after lamivudine and other nucleoside analogue treatments. Our unpublished data also showed persistent detectable HBV RNA in supernatant from day 4 to 17 of nucleoside analogue treatment when all the cells died (YWH, JHK, *et al.*, unpublished data). Lamivudine and other nucleoside analogues do not affect the integrated HBV DNAs from which HBV RNAs are transcribed [7]. Further studies are needed to evaluate the effect of long-term treatment of nucleoside analogues on serum HBV RNA.

The inhibition of serum HBV RNA by interferon-α might be supported by previous studies on transgenic mice. Intrahepatic HBV replicative intermediates were cleared by a single injection of the interferon-α/β inducer polyinosinic-polycytidylic acid [14]. It was postulated that the mechanism of action of interferon involves the post-transcriptional steps of the HBV life cycle, as the intrahepatic HBV replicative intermediates were cleared while the steady-state content of HBV RNA was unaffected [14]. This same group of researchers further demonstrated that the inhibitory effect of interferon-α/β is at the level of the capsids containing pre-genomic RNA, acting either to accelerate their degradation or to prevent their assembly [15]. Interferon might directly inhibit HBV synthesis or could act through the cellular immune response against HBV-infected hepatocytes [16]. HBV inhibition in immortalized hepatocyte cell lines from HBV transgenic mice by interferon-β and interferon-γ confirms the non-cytolytic inhibition pathway [17]. This inhibition might act through the 2',5'-oligoadenyl synthetase/RNAse L pathway [18]. Interferon can induce this multienzyme pathway that includes 2',5'-oligoadenyl synthetase, endoribonuclease RNAse L and 2',5'-oligoadenyl phosphodiesterase. Among these enzymes, RNAse L theoretically inhibits all viral replication that uses an RNA intermediate step [16]. Furthermore, activation of this ribonuclease has been proposed as the major driver by which interferon inhibits viral replication [18].

In the current study, we performed frequent detection of serum HBV DNA and RNA, in some cases as often as every 2 weeks, to determine the sequential change of serum HBV DNA and RNA during monotherapy or combination therapy. Our data showed that the peak serum HBV RNA level with entecavir treatment was significantly higher than that with lamivudine treatment (8.6 ± 1.0 versus 5.6 ± 1.0; $P<0.001$). There was also a trend towards higher detectability of serum HBV RNA in patients treated with entecavir in comparison with those treated with lamivudine (100% versus 71%; $P=0.48$). These findings suggest that the serum

HBV RNA level might reflect the antiviral potency of nucleoside analogues [19]. Further studies are needed to clarify this interesting and important issue.

Although Rokuhara *et al.* [20] showed that HBV RNA was detectable before lamivudine therapy in serum samples of 24 patients, the detection rate was not specified. Their results of sucrose density gradient fractionation studies indicated that viral particles containing HBV DNA were dominant at the start of treatment, whereas those containing HBV RNA became more prevalent after 1 and 2 months of treatment. They also suggested that under untreated conditions, viral particles containing HBV RNA accounted for only about 1% of total HBV virions. However, these specific particles became the major component under lamivudine treatment [9]. They concluded, therefore, that HBV RNA particles seemed to exist in <1% of the HBV virions among patients without lamivudine treatment [21]. By contrast, the undetectable pre-treatment HBV RNA data in this study was consistent with our previous report, showing that serum HBV RNA levels increased soon after the administration of nucleoside analogues [10]. Furthermore, our data was supported by the *in vitro* data obtained in HBV-transfected HepG2.2.15 cell lines (YWH, JHK, *et al.*, unpublished data), suggesting that HBV RNA was undetectable in the supernatant before nucleoside analogue treatment but became detectable after administration of these agents. In addition, Rokuhara and colleagues [20] reported a more significant decline of the serum HBV DNA level than HBV RNA level during lamivudine therapy, which confirmed our findings.

Serum HBV RNA was persistently detectable even after discontinuation of short-term lamivudine therapy in group III patients. This finding suggested that, although new viral particles containing HBV RNA were no longer produced after discontinuation of lamivudine, the existing viral particles containing HBV RNA during lamivudine administration were not quickly degraded. The study by Rokuhara *et al.* [20] showed a more significant decline in serum HBV DNA than RNA during lamivudine therapy, also confirming our findings on the poor immediate inhibition of serum viral particles containing HBV RNA by nucleoside analogues. Nevertheless, further studies are needed to demonstrate how long the viral particles containing HBV RNA persist in serum.

Following sequential combination therapy of lamivudine and conventional interferon, the serum HBV DNA level declined but was still detectable in all patients until the end of treatment. By contrast, serum HBV RNA was inhibited and undetectable at the end of treatment. The persistent presence of serum HBV DNA was due to the discontinuation of nucleoside analogue treatment and, thus, the lack of

continuous inhibition. The shift to interferon led to the inhibition of serum HBV RNA, but the inhibitory effect of interferon on HBV DNA was not as efficient as that of the nucleoside analogue [8]. Although northern and Southern hybridization data of intracellular RNA and DNA were not available, our study and others have confirmed the possibility of detecting serum HBV RNA. We reported the discrepant measurement of HBV nucleic acid by the transcription-mediated amplification and hybridization protection assay (TMA-HPA) and the Amplicor HBV Monitor test [10]. Because TMA-HPA uses RNA transcription and amplification of transcripts by T7 RNA polymerase [22], we assumed that the discrepancy was a result of the persistence of serum HBV RNA in nucleoside-analogue-treated patients. Zhang et al. [9] reported the presence of serum HBV RNA in a patient treated with lamivudine. The study mainly analysed truncated HBV RNA, which was assumed to be transcribed from the integrated HBV genome; the authors showed a marked difference between truncated HBV RNA and HBV DNA. In this study, HBV DNA and HBV nucleic acid were assayed by real-time PCR and real-time reverse transcriptase PCR, and <1 $\log_{10}$ difference was shown; this observation suggests that the effect of truncated serum HBV RNA was minimal. In addition, Rokuhara et al. [20] investigated the incorporation of HBV RNA into virus particles using sucrose gradient analyses: HBV RNA made a single peak in one fraction, whereas both HBV DNA and HBV core-related antigen made single peaks at three different time points during lamivudine treatment.

In conclusion, interferon can inhibit serum HBV RNA induced by lamivudine therapy. The persistence of serum HBV RNA as a consequence of unaffected HBV RNA replicative intermediates might lead to indefinite nucleoside analogue therapy. By contrast, the inhibitory effect of interferon on HBV RNA replicative intermediates might potentiate the suppression of HBV replication.

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Disclosure statement

References

1. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337:1733–1745.

2. Kao JH, Chen DS. Overview of hepatitis B and C viruses. In Goedert JJ (Editor). *Infectious Causes of Cancer: Targets for Intervention.* Totowa: Humana Press 2000; pp. 313–330.

3. McQuilland GM, Townsend TR, Fields HA, Carrol M, Leahy M, Polk BF. Seroepidemiology of hepatitis B virus infection in the United States 1976 to 1980. *Am J Med* 1989; 87 Suppl 1:S5–S10.

4. Chen DS. From hepatitis to hepatoma: lessons from type B viral hepatitis. *Science* 1993; 262:369–370.

5. Ganem D, Varmus HE. The molecular biology of the hepatitis B viruses. *Annu Rev Biochem* 1987; 56:651–693.

6. Lok ASF, McMahon BJ. AASLD practice guidelines. Chronic hepatitis B. *Hepatology* 2007; 45:507–539.

7. Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus *in vitro* by 2′,3′-dideoxy-3′-thiacytidine and related analogues. *Proc Natl Acad Sci U S A* 1991; 88:8495–8499.

8. Dienstag JL. Hepatitis B virus infection. *N Engl J Med* 2008; 359:1486–1500.

9. Zhang W, Hacker HJ, Tokus M, Bock T, Schroder CH. Patterns of circulating hepatitis B virus serum nucleic acids during lamivudine therapy. *J Med Virol* 2003; 71:24–30

10. Hatakeyama T, Noguchi C, Hiraga N, *et al.* Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. *Hepatology* 2007; 45:1179–1186.

11. Schalm SW, Heathcote J, Cianciara J, *et al.* Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomized trial. *Gut* 2000; 46:562–568.

12. Sarin SK, Kumar M, Kumar R, *et al.* Higher efficacy of sequential therapy with interferon-alpha and lamivudine combination compared to lamivudine monotherapy in HBeAg positive chronic hepatitis B patients. *Am J Gastroenterol* 2005; 100:2463–2471.

13. Sarin SK, Sood A, Kumar M, *et al.* Effect of lowering HBV DNA levels by initial antiviral therapy before adding immunomodulator on treatment of chronic hepatitis B. *Am J Gastroenterol* 2007; 102:96–104.

14. McClary H, Koch R, Chisari FV, Guidotti LG. Relative sensitivity of hepatitis B virus and other hepatotropic viruses to the antiviral effects of cytokines. *J Virol* 2000; 74:2255–2264.

15. Wieland SF, Guidotti LG, Chisari FV. Intrahepatic induction of alpha/beta interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. *J Virol* 2000; 74:4165–4173.

16. Thomas H, Foster G, Platis D. Mechanisms of action of interferon and nucleoside analogues. *J Hepatol* 2003; 39:S93–S98.

17. Pasquetto V, Wieland SF, Uprichard SL, Tripodi M, Chisari FV. Cytokine-sensitive replication of hepatitis B virus in immortalized mouse hepatocyte cultures. *J Virol* 2002; 76:5646–5653.

18. Lengyel P. Mechanisms of interferon action: the 2′5′ A synthetase-RNase L pathway. In Gresser I (Editor). *Interferon 3.* New York: Academic Press 1981; pp. 77–99.

19. Huang YW, Chayama K, Tsuge M, *et al.* Serum HBV RNA level reflects the potency of nucleos(t)ide analogue. *Hepatol Int* 2009; 3:110.