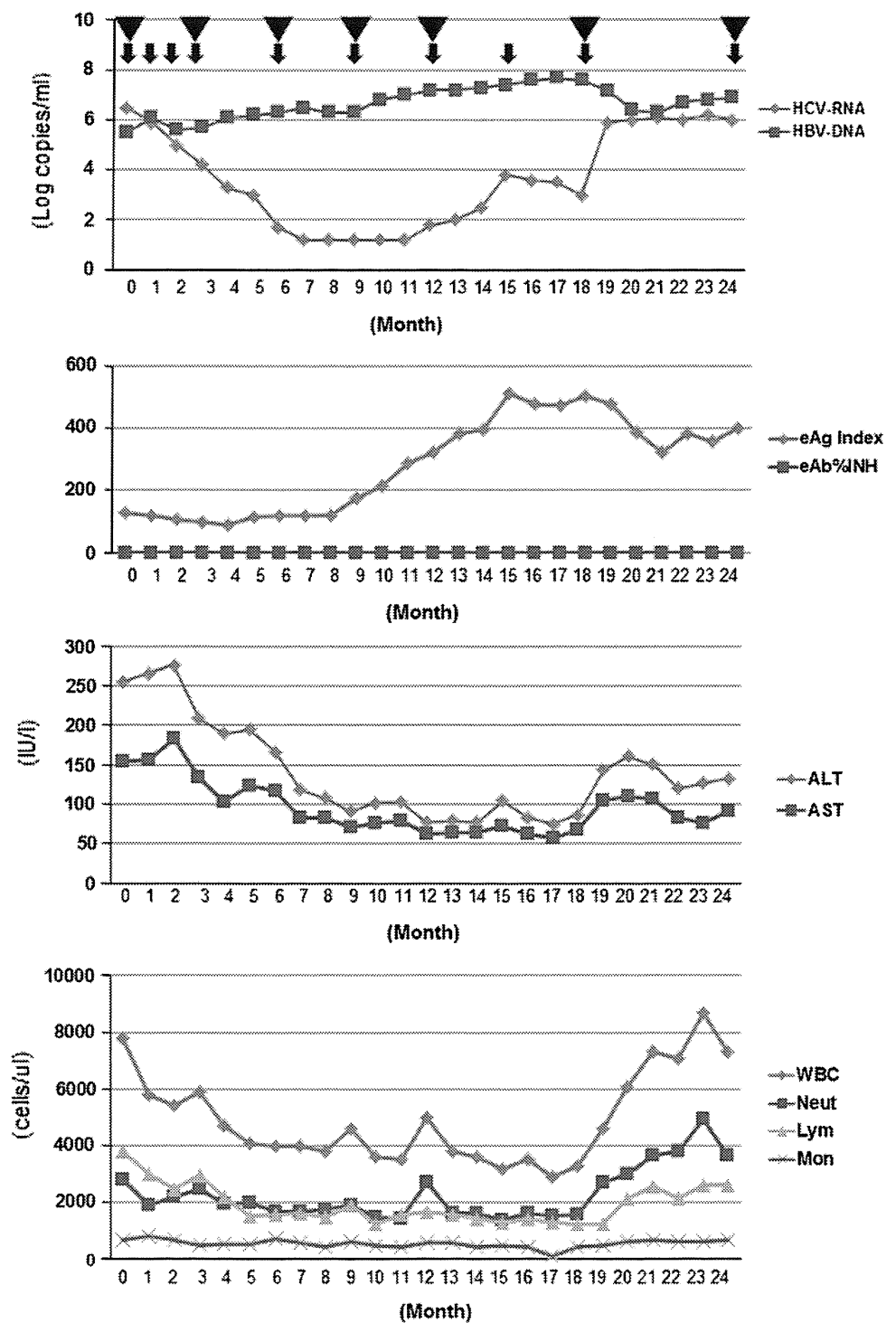


**Fig. 3** Sequential biochemical data analysis during Peg-IFN/RBV therapy. The titers of HBV-DNA and HCV-RNA; the amounts of envelope antigen (*eAg*) and envelope antibody (*eAb*), and alanine aminotransferase (*ALT*) and aspartate aminotransferase (*AST*); and the numbers of WBCs, neutrophils (*Neut*), lymphocytes (*Lym*), and monocytes (*Mon*) in patients A (a) and B (b) are shown in these graphs. *Arrows* indicate the sampling points of FACS analysis. *Triangles* indicate the sampling points of the ELISPOT assay. *INH* inhibition

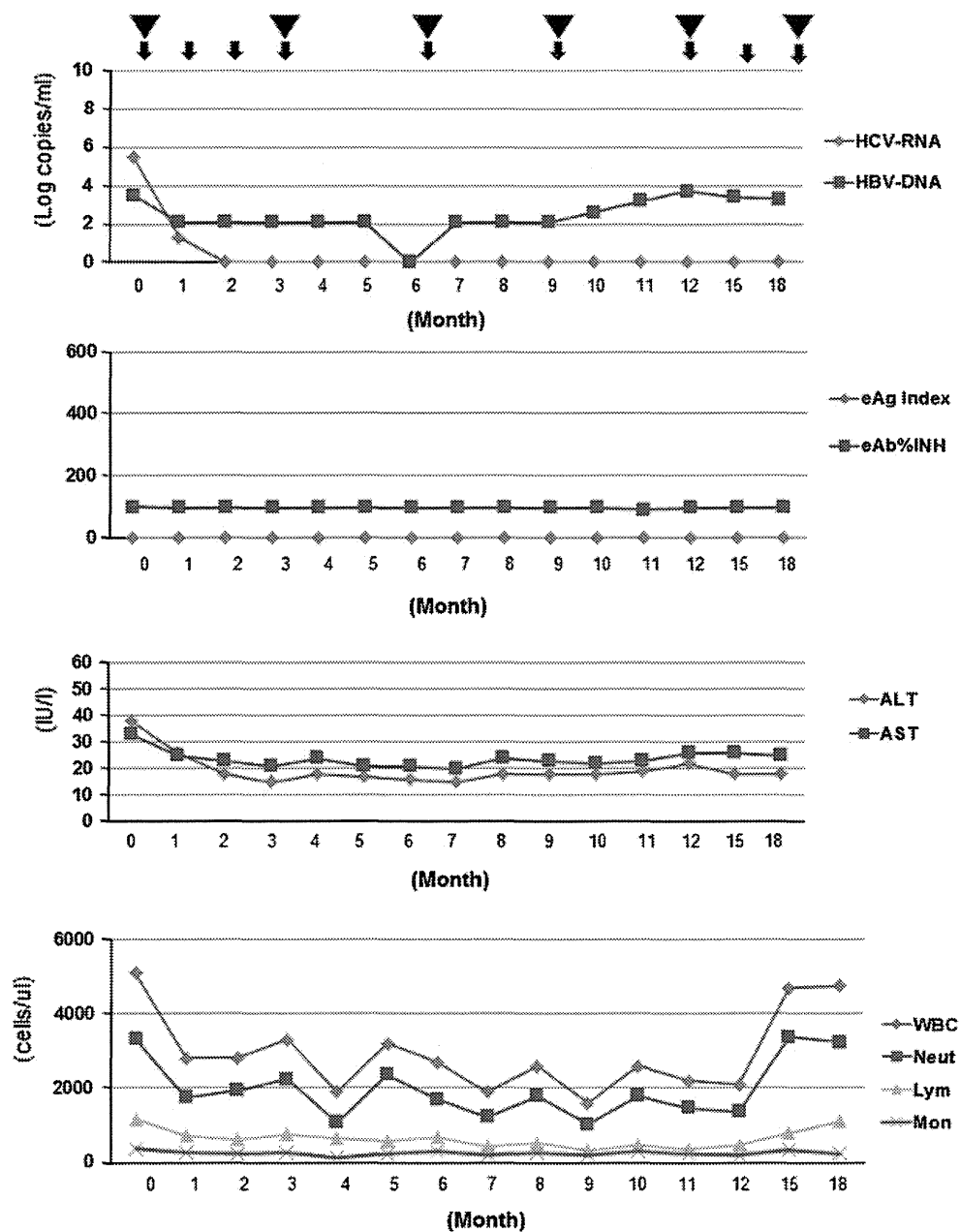
**A HCV High/HBV High [Sequential Biochemical Data During PEG-IFN+RBV Therapy]**



HBV-genotype C-monoinfected patients who were HBeAg-positive. However, HBeAg-specific IL-10 secretion in patient A was stronger than that in HBV-genotype C monoinfected patients who were HBeAg-positive. These data indicated that the presence of HCV might also suppress the HBV-specific immune response in regard to certain host

factors (e.g., in the presence of IL-28B polymorphism, and depending on the body mass index [BMI] and  $\gamma$ -guanosine triphosphate [ $\gamma$ -GTP] level), because the presence of HCV did not suppress the HBV-specific immune response either in patient B or in the patients with dual HCV-dominant infection. Otherwise, we could deny the possibility indicating that

Fig. 3 continued

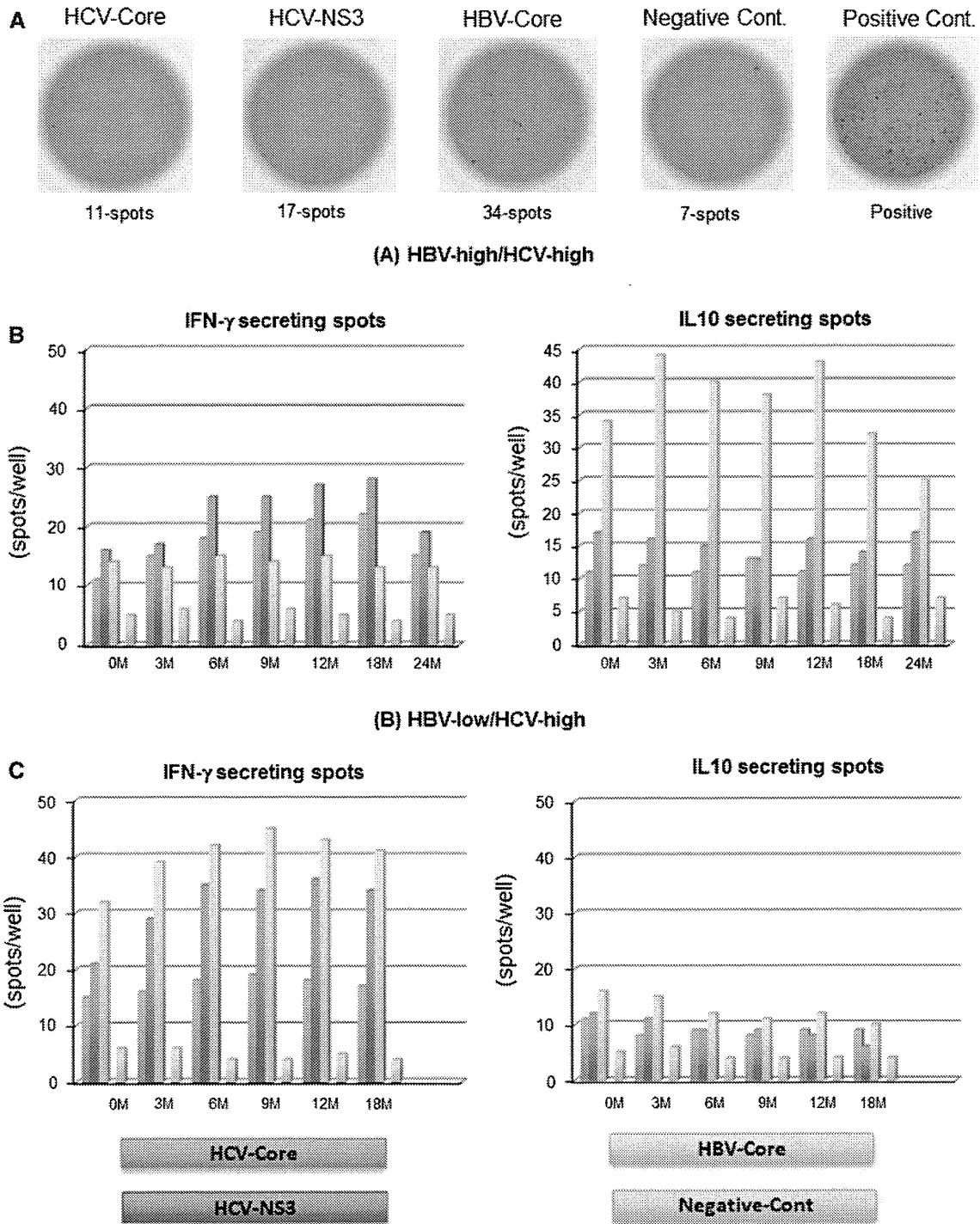
**B HCV High/HBV Low [Sequential Biochemical Data During PEG-IFN $\alpha$ +RBV Therapy]**

the certain background of host factors could allow the existence of dual virus actively. These data indicated that HBV-specific IL-10-secreting cells and/or certain kinds of host factors had an important role in HBV- and HCV-specific immune suppression in patient A, but not in patient B.

#### In vitro analysis of HBV/HCV dual infection

We carried out in vitro analysis of HBV/HCV infection using Huh-7 cells that were susceptible to the HCV-JFH-1 strain

and HBV expression plasmids. The amount of the JFH-1 strain did not change with the various kinds of HBV expression plasmids (Fig. 5a). Moreover, the amounts of the various HBV strains did not change in the presence of JFH-1 infection. These data indicated that no direct effect of HBV and HCV could be detected in Huh 7 cells. We carried out experiments to analyze the effect of IFN- $\alpha$  treatment on HCV Huh-7 cells with various kinds of HBV expression (Fig. 5b). In our systems, it appeared that HBV expression could not significantly affect the suppressive effect of IFN- $\alpha$ .



**Fig. 4** The sequential analysis of HBV/HCV-specific immune reactions during Peg-IFN/RBV therapy. Representative spots of the ELISPOT assay are shown (a). The sequential data of IFN- $\gamma$ - and interleukin-10 (*IL-10*)- secreting spots in patient A are shown (b). The sequential data of IFN- $\gamma$ - and IL-10-secreting spots in patient B are shown (c). Comparison of IFN- $\gamma$ - and IL-10- secreting spots in patient A before starting therapy, patient B before starting therapy, dual HCV-dominant patients, HCV-monoinfected patients, HBV-Bj

(HBeAb<sup>+</sup>) monoinfected patients, HBV-Bj (HBeAg<sup>+</sup>) monoinfected patients, HBV-C (HBeAb<sup>+</sup>) monoinfected patients, and HBV-C (HBeAg<sup>+</sup>) monoinfected patients (d). In these bar graphs, the blue bars indicate HCV-core specific reaction. The red bars indicate HCV-NS3 specific reaction. The green bars indicate HBV-core specific reaction. The aqua blue bars indicate the negative control (Cont.). Error bars indicate standard deviations (color figure online)

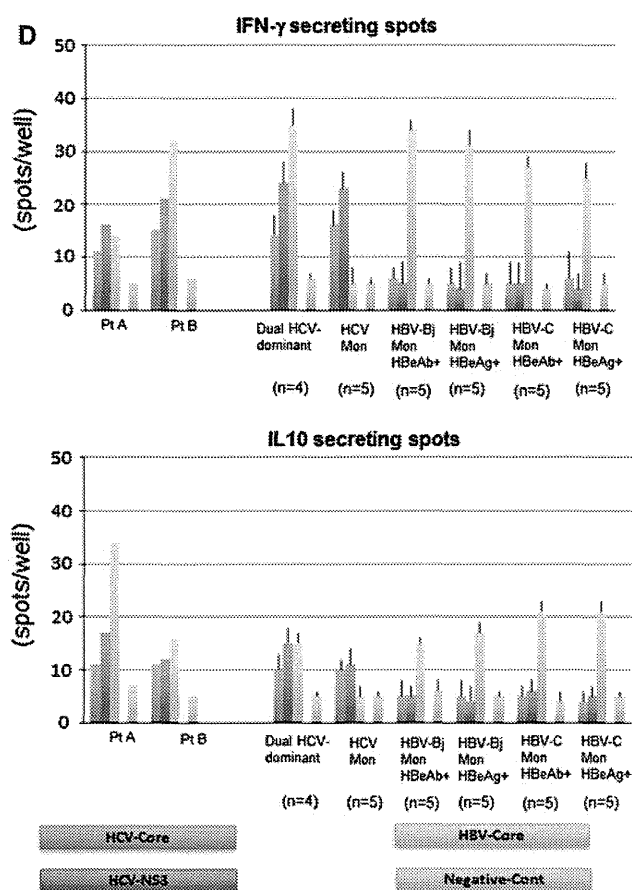


Fig. 4 continued

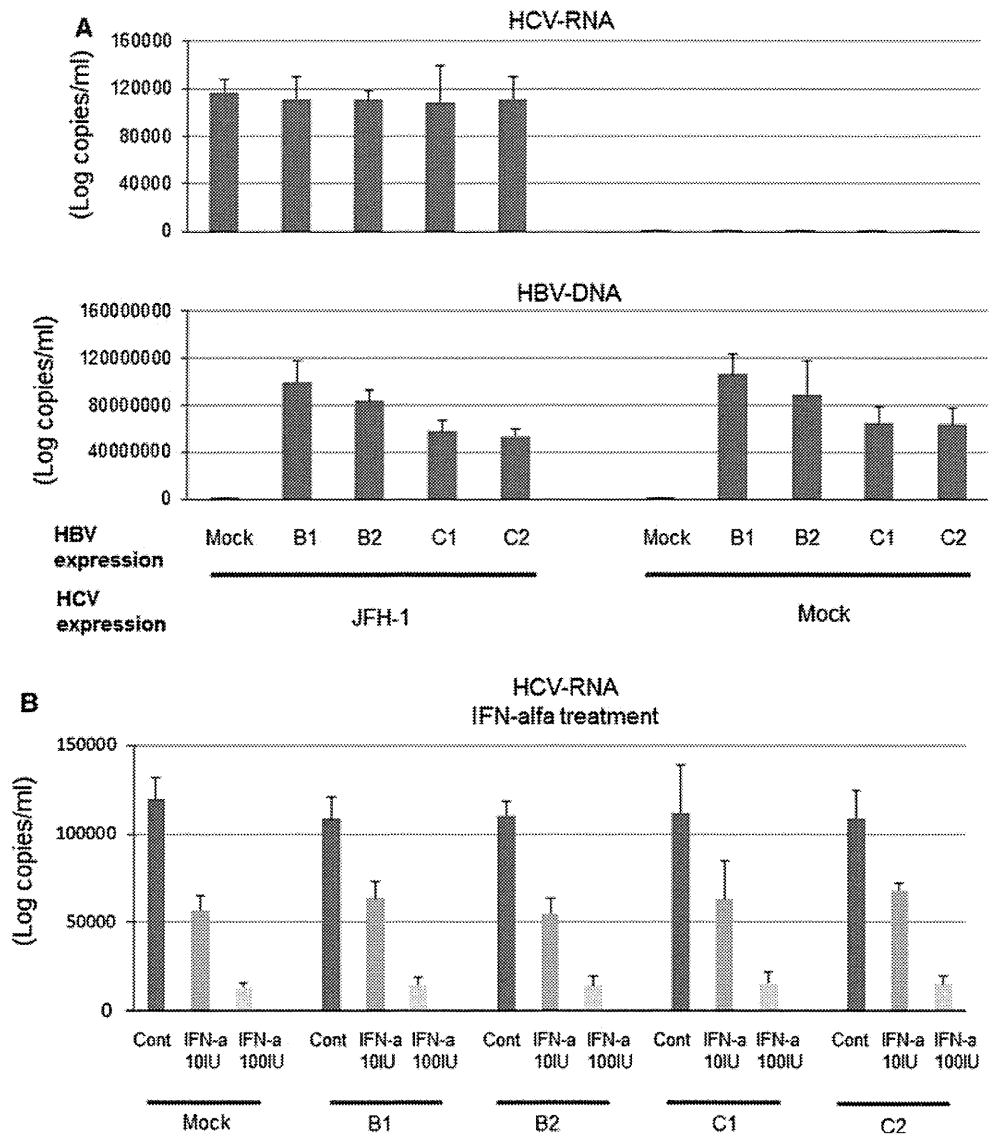
## Discussion

The immunopathogenesis of dual hepatitis B and C infection is not clear, given the complexity of viral and host factors [19, 21, 48–50]. However, detailed understanding of specific patients with dual hepatitis B and C infection could contribute to improving the treatment and follow up of these patients. Therefore, we focused on two representative patients with HBV/HCV dual infection who received Peg-IFN/RBV therapy.

Concerning the virological results, patient A had genotype 1b, HCV-Core 70 wild-type and low mutation of ISDR HCV and genotype C HBV. It has been reported that genotype 1b HCV is common in Japan and is usually difficult to treat in comparison to genotypes 2a and 2b [51]. Among genotype 1b HCV strains, HCV-Core 70 wild-type HCV is easily decreased by Peg-IFN/RBV therapy [51]. On the other hand, it has been reported that in genotype 1b HCV low mutation of ISDR is difficult to treat [52]. Patient B had almost the same background of HCV–genotype 1b, HCV-Core 70 wild-type, and low mutation of ISDR—as patient A. However, the background of host factors that could affect the responsiveness of IFN-based therapy was

different between patients A and B. For example, patient A had a hetero allele of the IL-28B polymorphism, advanced fibrosis, and fatty changes of the liver. On the other hand, patient B had the major allele of the IL-28B polymorphism and mild fibrosis. Moreover, the background of HBV in patient B was completely different from that in patient A. It has been reported that HBV genotype Bj is usually more susceptible to IFN-based therapy than genotype C [45, 53]. Therefore, not only the HBV factors but also the combination of host factors and HBV factors might affect the responsiveness to IFN-based therapy. In patient A, the responsiveness of HCV during Peg-IFN/RBV therapy was relatively poor. However, the viral titers of HCV were lower than 1.2 log copies/ml at 7 months after the start of therapy. During the reduction of the HCV viral titers, the titers of HBV and HBeAg specific IL-10-secreting cells were gradually increased. Although patient A had received Peg-IFN/RBV therapy for up to 18 months, HCV-RNA increased again 12 months after the start of the therapy. The sustained Th1 immune suppression might have contributed to the relapse of HCV. Not only weak up-regulation of HCV-specific Th1 immune reaction but also strong up-regulation of HBV-specific IL-10-secreting activity was detected during Peg-IFN/RBV therapy in patient A [26, 35]. Moreover, increased HBeAg could be detected 9 months after the start of the therapy. Fluctuations of activated CD4 cells, CD8 cells, NK cells, and NK-T cells could be seen in patient A. On the other hand, in patient B, the responsiveness of HBV and HCV during Peg-IFN/RBV therapy was good. Moreover, the immune response of patient B was almost comparable to the responses in the patients with HCV mono-infection and those with HBV-genotype Bj mono-infection. Previously, it has been reported that Peg-IFN/RBV therapy could achieve almost the same SVR rates in patients with HCV/HBV dual infection and those with HCV mono-infection [54–56]. We assume that the results in these studies were obtained from patients similar to our patient B, because the number of patients with HCV-dominant infection is much higher than the number of those with HBV/HCV dual active infection such as our patient A. Patients with HBV/HCV dual active infection such as patient A are relatively rare in Japan. However, it is necessary to understand the immunopathogenesis of these patients, because Peg-IFN/RBV therapy might not be sufficient to eradicate or control HBV/HCV in these difficult-to-treat patients. One of the candidate therapies for such patients might be Entecavir (ETV)/Peg-IFN/RBV sequential therapy. The effect of HBV specific regulatory T cells might contribute to the immunosuppression of not only HBV but also HCV [35]. In some previous studies, including ours, it has been reported that HBV replication might contribute to immune suppression [19, 29].

**Fig. 5** In vitro analysis of HBV/HCV dual infection. The titers of HCV-RNA and HBV-DNA are shown. *B1* indicates genotype Bj35 clone. *B2* indicates genotype Bj56 clone. *C1* indicates genotype C-AT clone. *C2* indicates genotype C-22 clone (a). The titers of HCV-RNA after the IFN- $\alpha$  treatment are shown (b)



In the present study, we employed an in vitro coinfection system to analyze the direct interaction between HBV and HCV. In our system, we used several different HBV clones, because it is necessary to consider the effects of different genotypes. Although we could not detect the direct interaction of HBV/HCV in our system, we could not exclude the possibility of indirect interaction between cytokines and chemokines produced from virus-infected hepatocytes. We are now analyzing the chemokines produced from hepatoma cells with different HBV genotype clones (ongoing study).

In conclusion, we analyzed data from representative patients with HBV/HCV dual infection sequentially and precisely. Because many different kinds of backgrounds might affect immunoreactions, we focused on representative patients and analyzed the immunological responses extensively. There might be a group of patients with very

difficult-to-treat dual infections. We need to understand the immunopathogenesis of such patients to develop the appropriate therapy.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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## 2'-Fluoro-6'-methylene-carbocyclic adenosine phosphoramidate (FMCAP) prodrug: In vitro anti-HBV activity against the lamivudine–entecavir resistant triple mutant and its mechanism of action

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### ABSTRACT

Novel 2'-fluoro-6'-methylene-carbocyclic adenosine (FMCA) monophosphate prodrug (FMCAP) was synthesized and evaluated for its in vitro anti-HBV potency against a lamivudine–entecavir resistant clone (L180M + M204V + S202G). FMCA demonstrated significant antiviral activity against wild-type as well as lamivudine–entecavir resistant triple mutant (L180M + M204V + S202G). The monophosphate prodrug (FMCAP) demonstrated greater than 12-fold (12×) increase in anti-HBV activity without increased cellular toxicity. Mitochondrial and cellular toxicity studies of FMCA indicated that there is no significant toxicity up to 100 μM. Mode of action studies by molecular modeling indicate that the 2'-fluoro moiety by hydrogen bond as well as the Van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug-resistant mutants.

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The chronic HBV infection is strongly associated with liver diseases like chronic hepatic insufficiency, cirrhosis and hepatocellular carcinoma (HCC).<sup>1</sup> According to the World Health Organization (WHO), currently about 2 billion people world-wide have been infected with HBV and more than 350 million live with chronic infection. Acute or chronic outcomes of HBV infection are estimated to cause the deaths of 600,000 people worldwide every year.<sup>2</sup>

Currently, there are several nucleos(t)ide analogues available to treat chronic hepatitis B virus infection.<sup>3–6</sup> The major target of these drugs is to inhibit the viral reverse transcriptase (RT)/DNA polymerase, which is responsible for the synthesis of the minus-strand DNA. Although the currently used agents are well tolerated and effective in suppressing the viral replication for extended periods, the significant rate of virological relapse caused by drug resistance remains a critical issue.

Lamivudine (LVD) was first introduced as the orally active anti-HBV agent in 1998. Lamivudine profoundly suppresses HBV replication in patients with chronic hepatitis B infection; however, lamivudine-resistant HBV (LVD<sub>r</sub>) was isolated from a significant numbers of patients during the treatment with lamivudine.

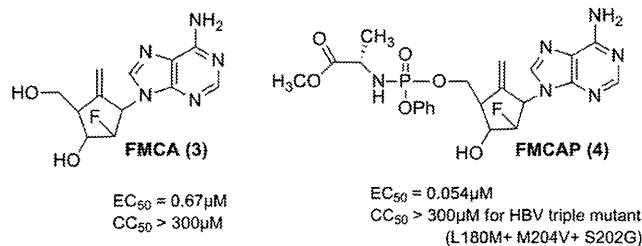
Currently, there are several antiviral options exist for these patients viz., to use adefovir or high dose (1.0 mg/day) of entecavir, or more recently tenofovir. However, this resulted in also the development of resistance mutants during the long term therapy. At present, entecavir is the most prescribed drug, and is recommended for patients with the wild-type as well as for those harboring adefovir and lamivudine-resistant strains. However, recent clinical studies by Tanaka and his co-workers suggested that the entecavir mutant in the lamivudine-resistant patients (L180M + M204V + S202G) causes a viral breakthrough: 4.9% of patients at baseline increases to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.<sup>7</sup> Therefore, it is of great interest to discover novel anti-HBV agent, which is effective against lamivudine- and entecavir-resistant triple mutants (L180M + M204V + S202G).

The potency of a nucleos(t)ide analogue is determined by its ability to serve as a competitive inhibitor of the HBV polymerase relative to that of the natural substrate, the nucleotide triphosphate.<sup>8</sup> However, host cellular kinases limit the pharmacological potency of nucleoside analogues by phosphorylation to their corresponding triphosphates. Particularly, the initial kinase action on the nucleoside to the monophosphate is the rate-limiting step. However, many synthetic nucleosides are not phosphorylated or the rate of phosphorylation is very slow due to the structural requirement of the kinases, resulting in only generating a low quantity of the triphosphate. To overcome this phosphorylation issue, nucleoside phosphoramidate prodrugs have been introduced,<sup>8,9</sup> which

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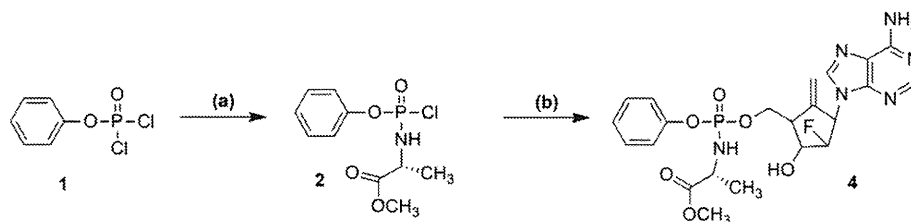
**Figure 1.** Structures of 2'-fluoro-6'-methylene-carbocyclic adenosine (FMCA; **3**) and its prodrug (FMCAP; **4**).

can bypass the rate-limiting first step of monophosphorylation. Phosphoramidate prodrugs have demonstrated to enhance the nucleoside potency in cell culture as well as in patients.<sup>10,11</sup> This methodology greatly increases the lipophilicity of the nucleotide to increase the cell penetration as well as to target the liver cells in vivo.

In this communication, we present that a FMCA phosphoramidate prodrug is such an agent, which can potentially be used for the treatment of patients who experience viral breakthrough due to the triple mutants caused by the use of lamivudine and entecavir.

In our previous report, we have demonstrated that the novel carbocyclic adenosine analog **3** (FMCA Fig. 1) exhibits significant anti-HBV activity against wild type as well as adefovir/lamivudine resistant strains.<sup>12</sup> The present study describes the synthesis and antiviral evaluation of a phosphoramidate of FMCA (FMCAP), which demonstrated the significantly improved in vitro potency. Additionally, we studied its mechanism of action how FMCA-TP can effectively bind to the HBV polymerase by molecular modeling and still exerts the antiviral activity against the lamivudine-entecavir triple mutant (L180M + M204V + S202G).

FMCAP (**4**, Scheme 1)<sup>13</sup> was synthesized using a known method in the literature,<sup>14,15</sup> in which the phosphorylation of phenol with phosphorus oxychloride generates phenyl dichlorophosphate **1**, which was coupled with L-alanine methyl ester in the presence of tri-ethyl amine in dichloromethane to give chlorophosphoramidate reagent **2**, which, in turn, was coupled with FMCA **3** in the presence of 1-methyl imidazole in THF to furnish the phosphoramidate **4** in good yield.

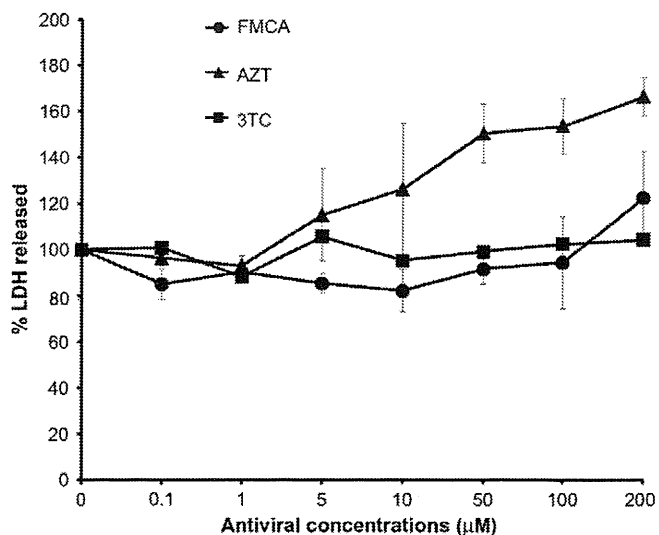


**Scheme 1.** Reagent and conditions: (a) L-alanine methyl ester hydrochloride, Et<sub>3</sub>N, CH<sub>2</sub>Cl<sub>2</sub>; (b) FMCA (**3**), NMI, THF, rt overnight.

**Table 1**

In vitro anti-HBV activity of FMCA **3**, FMCAP **4**, lamivudine and entecavir against wild-type and entecavir drug-resistant mutant (L180M + M204V + S202G) in Huh7 cells

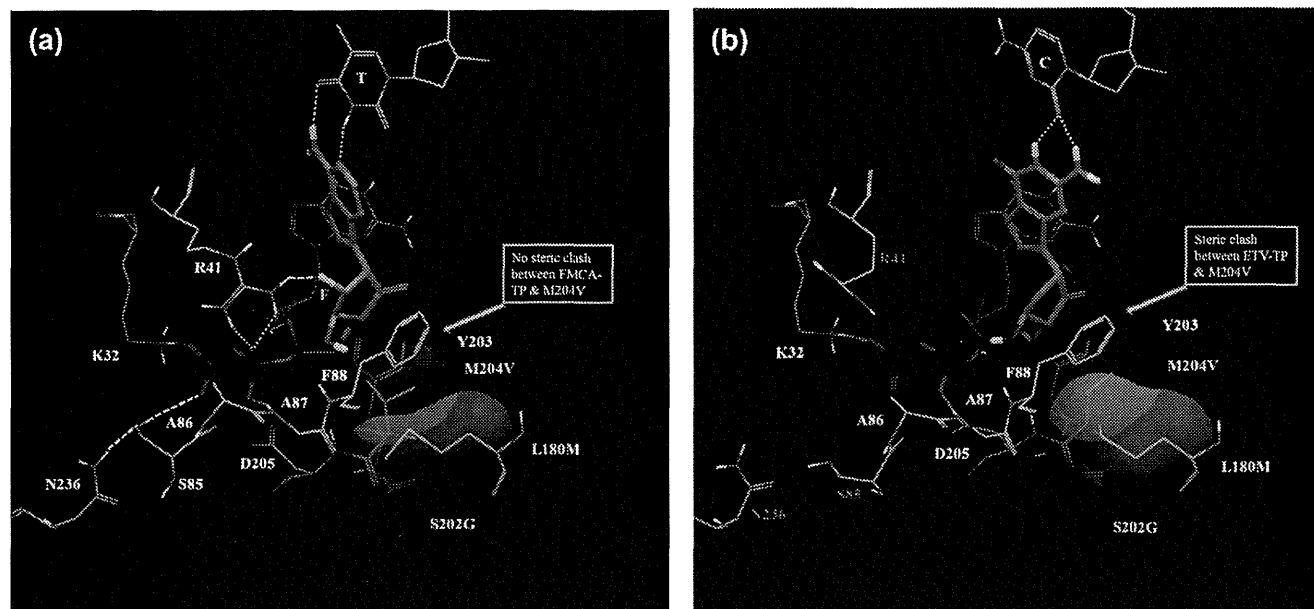
Compounds	HBV Strains			L180M + M204V + S202G EC <sub>50</sub> (μM)
	EC <sub>50</sub> (μM)	Wild-type EC <sub>90</sub> (μM)	CC <sub>50</sub> (μM)	
FMCA <b>3</b>	0.548 ± 0.056	6.0 ± 0.400	>300	0.67
FMCAP <b>4</b>	0.062 ± 0.011	0.46 ± 0.060	>300	0.054
Lamivudine	0.056 ± 0.003	0.142 ± 0.008	>300	>500 <sup>17</sup>
Entecavir	0.008	0.033	28	1.20 <sup>16</sup>



**Figure 2.** Mitochondrial toxicity of FMCA **3**, AZT and 3TC through lactate dehydrogenase release (LDH) assay.

FMCA **3** and FMCAP **4** were evaluated in vitro against the wild-type as well as the lamivudine-entecavir resistant clone (L180M + S202I + M202V). The FMCA **3** and FMCAP **4** demonstrated significant anti-HBV activity ( $EC_{50}$  0.548 ± 0.056 & 0.062 ± 0.011 μM, respectively) against the wild-type virus, while lamivudine and entecavir also demonstrated potent anti-HBV activity ( $EC_{50}$  0.056 ± 0.003 & 0.008 μM, respectively) (Table 1). It is noteworthy to mention that the anti-HBV potency of FMCAP (**4**) was increased to eight-fold (8×) in comparison to that of FMCA **3**, which indicates the importance of the initial phosphorylation of the nucleoside.

FMCA **3** and FMCAP **4** were further evaluated for their in-vitro antiviral potency against a lamivudine-entecavir resistant clone (L180M + M204V + S202G). It was observed that the anti-HBV potency of both FMCA **3** and FMCAP **4** ( $EC_{50}$  0.67 & 0.054 μM, respectively) were maintained against the resistant clone, and furthermore, the anti-HBV activity of FMCAP **4** was enhanced a 12-fold (12×) with respect to that of FMCA without significant enhancement of cellular toxicity. It was also noteworthy to mention that the anti-HBV potency of entecavir against the mutant



**Figure 3.** (a) FMCA-TP binding mode in ETVr (L180M + M204V + S202G); and (b) ETV-TP binding mode in ETVr (L180M + M204V + S202G) and there is a steric hindrance. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å). The Van der Waals surface of L180M is colored yellow. The Van der Waals surface of M204V is shown in spring green. The Van der Waals surface of S202G is colored orange. The exocyclic double bond is shown blue color.

**Table 2**

MBAE (multi-ligand bimolecular association with energetics) calculation of FMCA-TP and ETV-TP after Glide XP docking<sup>21</sup> and energy minimization<sup>22</sup>

Strains	Compounds	Energy difference results ( $\Delta E$ , kcal/mol)		
		Total energy	VdW <sup>a</sup>	Electrostatic
Wild-type	FMCA-TP	-588.05	375.78	-6341.08
	ETV-TP	-597.25	350.35	-6009.65
ETVr (L180M + M204V + S202G)	FMCA-TP	-591.54	359.91	-6245.68
	ETV-TP	-320.28	248.82	-4831.12

<sup>a</sup> Van der Waals interaction.

was reduced by 150-fold ( $EC_{50}$  1.2  $\mu$ M) in comparison to wild type.<sup>16</sup>

In the preliminary mitochondrial toxicity studies in HepG2 cells by measuring the lactic dehydrogenase release,<sup>18</sup> FMCA 3 did not exhibit any significant toxicity up to 100  $\mu$ M like lamivudine (3TC), while azidothymidine (AZT) shows the increase of toxicity (Fig. 2).

In our previous report, we described molecular modeling studies for favorable anti-HBV activity of FMCA-TP in wild-type as well as in N236T adefovir resistant (ADVr) mutant.<sup>12</sup> In the current studies, it was of interest to know how the FMCA and its prodrug maintain the anti-HBV activity against ETVr triple mutant (L180M + M204V + S202G) in comparison to entecavir. Therefore, molecular modeling studies were conducted to obtain the insight of the molecular mechanism of FMCA-TP by using the Schrodinger Suite modules.<sup>19</sup> A previously described homology model was used to further explore the impact of the ETVr to the HBV-RT.<sup>12</sup> The homology model of HBV-RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD).<sup>20</sup>

The binding mode of FMCA-TP and ETV-TP in ETVr (L180M + M204V + S202G) HBV-RT are depicted in Figure 3a and b, respectively. Their MBAE (multi-ligand biomolecular association with energetics)<sup>22</sup> calculations of FMCA-TP (total energy, wt -588.05 & ETVr -591.54 kcal/mol) and ETV-TP (total energy, wt -597.25 & ETVr -320.28 kcal/mol) after glide XP (extra precision) docking<sup>21</sup> and energy minimization in ETVr HBV-RT are shown in

Table 2. The triphosphate of FMCA-TP forms all the network of hydrogen bonds with the active site residues (Fig. 3a), K32, R41, S85 & A87 in the similar manner as in wild-type,<sup>12</sup> whereas ETV-TP lose the hydrogen bonding with R41 & S85. The  $\gamma$ -phosphate of FMCA-TP maintains a critical H-bonding with the OH of S85 with connection of hydrogen bonds between S85 and N236 in ETVr HBV-RT also. However,  $\gamma$ -phosphate ETV-TP does not maintain this critical H-bonding with S85 and N236 (Fig. 3b).

The carbocyclic ring with an exocyclic double bond of FMCA-TP and ETV-TP makes the favorable Van der Waals interaction with F88 in ETVr HBV-RT (Fig. 3a and b). There is no steric clash in between exocyclic double bond of FMCA-TP and M204V residue, whereas ETV-TP exocyclic double bond has steric clash with M204V residue in ETVr HBV-RT. The 2'-fluorine substituent in the carbocyclic ring of FMCA-TP appears to promote an additional binding with the NH of R41 guanidino group as shown in Figure 3a, which is in agreement with the antiviral activity of FMCA-TP shown in Table 1. Overall, the modeling studies can qualitatively explain the favorable anti-HBV activity of FMCA-TP against ETVr mutant (L180M + M204V + S202G) in comparison to entecavir as shown in Table 1.

In conclusion, 2'-fluoro-6'-methylene-carbocyclic adenosine phosphoramidate prodrug (FMCAP) was synthesized, which demonstrated the significantly increased anti-HBV potency relative to the parent compound, FMCA in vitro. Molecular modeling studies delineated the mechanism of FMCA-TP and how it can effectively bind to the lamivudine-entecavir resistant triple mutant resulting

in maintaining the anti-HBV activity against the mutant. Furthermore, FMCA has been studied for the release of lactic dehydrogenase for potential mitochondrial toxicity and found that no significant increase of toxicity of FMCA compared with other commonly used anti-HIV nucleoside drugs. Very recently, a preliminary in vivo study in chimeric mice harboring the triple mutant, FMCAP was found to reduce HBV viral load while entecavir did not (data not shown). In view of these promising anti-HBV activities and non-toxicity of FMCAP as well as the interesting mechanism of antiviral activity, the chiral synthesis of FMCAP and its mitochondrial toxicity studies for preclinical investigation are warranted.

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13. **Compound 4**:  $^1\text{H NMR}$  (500 Mz,  $\text{CDCl}_3$ )  $\delta$  8.35 (s, 1H), 7.86 (d,  $J = 3.0$  Hz, 1H), 7.34–7.15 (m, 5H), 5.95 (m, 3H), 5.26 (d,  $J = 8.0$  Hz, 1H), 5.01–4.90 (m, 1H), 4.83 (s, 1H), 4.50–4.41 (m, 2H), 4.25–4.04 (m, 3H), 3.71 (s, 3H), 3.07 (s, 1H), 1.40 (d,  $J = 6.5$  Hz, 3 H);  $^{19}\text{F NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$  -192.86 (m, 1F);  $^{13}\text{C NMR}$  (125 MHz,  $\text{CDCl}_3$ )  $\delta$  171, 159.0, 156.5, 152.5, 150.4, 142.9, 130.1, 121.2, 120.3, 106.7, 102.4, 72.2, 71.1, 62.3, 51.9, 46.3, 43.9, 19.1;  $^{31}\text{P NMR}$  (202 MHz,  $\text{CDCl}_3$ ):  $\delta$  2.67, 2.99. Anal. Calcd For  $\text{C}_{22}\text{H}_{26}\text{FN}_6\text{O}_6\text{P}\cdot 0.5\text{H}_2\text{O}$ : C, 49.91; H, 5.14; N, 15.87; Found C, 49.84; H, 5.06; N, 15.22.
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# Mechanism of the dependence of hepatitis B virus genotype G on co-infection with other genotypes for viral replication

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**SUMMARY.** Hepatitis B virus (HBV) is classified into several genotypes. Genotype G (HBV/G) is characterised by world-wide dispersion, low intragenotypic diversity and a peculiar sequence of the precore and core region (stop codon and 36-nucleotide insertion). As a rule, HBV/G is detected in co-infection with another genotype, most frequently HBV/A2. In a previous *in vivo* study, viral replication of HBV/G was significantly enhanced by co-infection with HBV/A2. However, the mechanism by which co-infection with HBV/A2 enhances HBV/G replication is not fully understood. In this study, we employed 1.24-fold HBV/A2 clones that selectively expressed each viral protein and revealed that the core protein expressing construct significantly enhanced the replication of HBV/G in Huh7 cells. The introduction of the HBV/A2 core promoter or core protein or both genomic regions into the HBV/G genome showed

that both the core promoter and core protein are required for efficient HBV/G replication. The effect of genotype on the interaction between foreign core protein and HBV/G showed that HBV/A2 was the strongest enhancer of HBV/G replication. Furthermore, Western blot analysis of Dane particles isolated from cultures of Huh7 cells co-transfected by HBV/G and a cytomegalovirus (CMV) promoter-driven HBV/A2 core protein expression construct indicated that HBV/G employed HBV/A2 core protein during particle assembly. In conclusion, HBV/G could take advantage of core proteins from other genotypes during co-infection to replicate efficiently and to effectively package HBV DNA into virions.

**Keywords:** co-transfection, core protein, genotype A, genotype G, hepatitis B virus, replication.

## INTRODUCTION

Hepatitis B virus (HBV) infection affects more than 350 million people and is one of the major causes of acute and chronic liver disease. Acute HBV infection in adults is usually self-limiting, while chronic HBV infection can cause chronic hepatitis, liver cirrhosis or hepatocellular carcinoma [1]. As the clinical course in infected individuals depends on a complex interplay among various factors including viral, host and environmental factors, molecular characteristics of HBV including the genotype could become increasingly important in our understanding of HBV clinical implications [2].

Abbreviations: CMV, cytomegalovirus; CP, core promoter; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SEAP, secreted alkaline phosphatase.

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Eight major HBV genotypes (A–H) have been identified by a sequence divergence >8% in the entire HBV genome [3,4] and have a relatively distinct geographical distribution, which may be associated with anthropological history [5]. Hepatitis B virus genotype G (HBV/G) was first described in 2000 by studies carried out in France [6]. It is usually detected during co-infection with other genotypes, most frequently with HBV/A2 [7,8]. Co-infection with HBV/C and H has also been reported [9–11]. One of the features distinguishing HBV/G from other genotypes is the 36-nucleotide (nt) insertion in its core gene [6,12]. Recent studies indicated that the 36-nt insertion increased core protein translation without enhancing mRNA abundance [13], and insertion of the 36-nt in the core region of genotypes A and D impaired genome replication, despite upregulation of core protein expression, indicating that the 36-nt insertion could alter core protein expression without altering the mRNA expression [14]. The other feature of the HBV/G genome that is unique is the possession of two stop codons in the precore region that prevents the expression of hepatitis B e antigen (HBeAg) [6,12]. Nevertheless, some HBV/G carriers are

HBeAg positive, which is explained by co-infection with an HBeAg-expressing HBV/A strain [7].

As previously reported, HBV/G monoinfection in uPA/SCID mice that had been transplanted with human hepatocytes (hereafter referred to as chimeric mice) resulted in very low level viral replication, but HBV replication increased markedly when the animals were co-infected with HBV/A2, C or H [11,15]. Furthermore, the co-infection induced more pronounced fibrosis, which concurs with findings from studies of immunosuppressed patients [16]. However, as it is still unclear how the interaction between HBV/G and other genotypes enhances the replication of HBV/G and affects the virological and clinical manifestation within an individual, we conducted *in vitro* studies using 1.24-fold HBV clones to elucidate the mechanism of HBV/G replication during co-infection.

## MATERIALS AND METHODS

### *Plasmid constructs of HBV DNA and sequencing*

Hepatitis B virus DNA was extracted from 100  $\mu$ L of serum using the QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments (A and B) covering the entire HBV/G genome. PCR with nested primers was performed using TaKaRa LA Taq polymerase (Takara Biochemicals, Kyoto, Japan) for 35 cycles (30 s at 95° C, 30 s at 60° C and 2 min at 72° C). The primer pairs and protocols for plasmid construction are outlined in the Supporting Information. As reported previously [17], these fragments were added to the pUC19 vector, which had been deprived of promoters (Invitrogen Corp., Carlsbad, CA, USA), by digestion with *Hind*III and *Eco*RI, resulting in the 1.24-fold HBV genome – required to transcribe the oversized pregenome and precore messenger RNA. Cloned HBV DNA sequences were confirmed with Prism BigDye (Applied Biosystems, Foster City, CA, USA) using the ABI 3100 automated sequencer.

### *HBV DNA mutagenesis and construct design*

HBV/A2 and HBV/G clones containing the 1.24-fold HBV genome were constructed using isolates obtained from a co-infected Caucasian patient from the San Francisco cohort described in our previous study (patient #1) [7]. The study design conformed to the 1975 Declaration of Helsinki and was approved by our institutional ethics committee. Written informed consent was obtained from the patient. The HBV/A2 clones isolated from the patient's blood specimen did not possess any precore or core promoter mutations that are known to affect HBeAg expression. To study the interaction between the different genotype isolates, the following viral protein expression constructs were prepared (outlined in Fig. 1) in HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X and were each able to

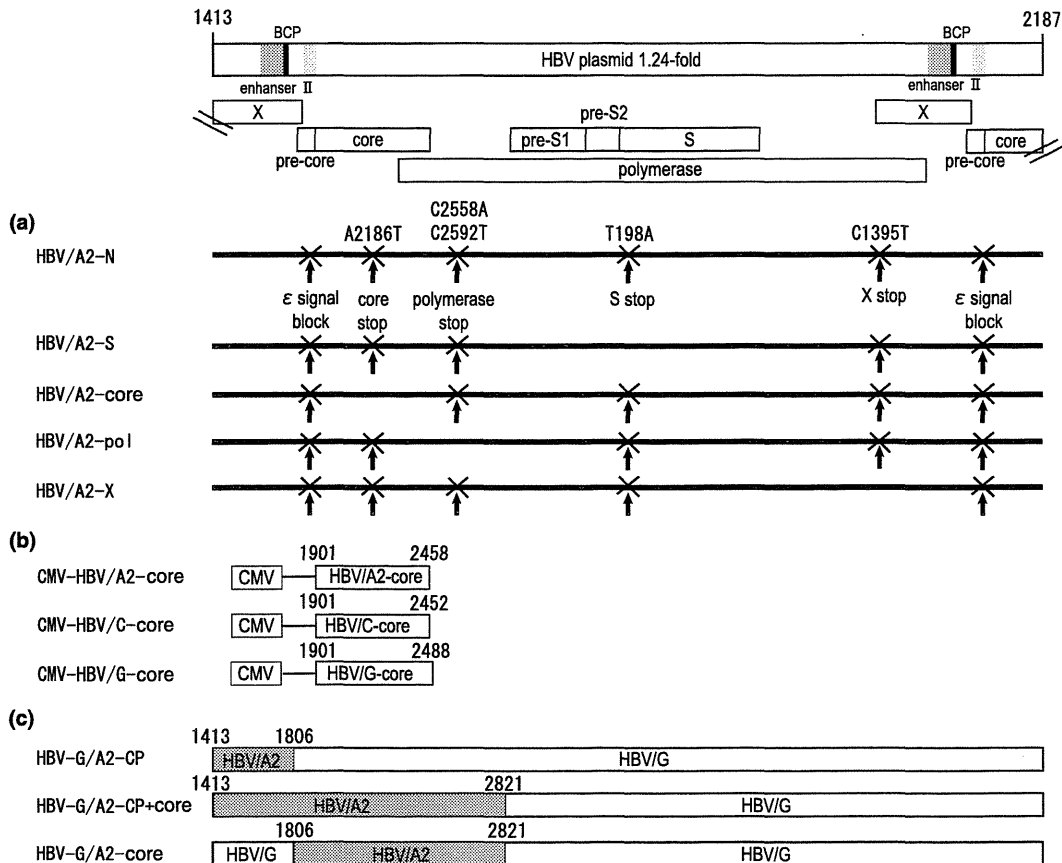
selectively translate one of the four viral proteins (the large surface, precore/core, polymerase and X proteins, respectively), whereas translation of the other three was prevented by the introduction of point mutations that produced corresponding stop codons (Fig. 1a). The following stop codons were used: (i) for surface protein: change from TTA to TAG in the 15th codon of the S gene (T198A) [18], (ii) for core protein: change from AAG to TAG in the 96th codon of the core gene (A2186T), (iii) for polymerase: change from CA-ACAA to TAATAA in the 283rd and 284th codons of the pol gene (C2558T/C2592T) and (iv) for X protein: change from CAA to TAA in the 7th codon of the HBx gene (C1395T) [19]. All of the above HBV/A2 recombinant plasmids possessed a TCTG motif after nucleotide position 1876, which abolished genome replication by altering the  $\epsilon$  loop (CTGT to TCTG, nt 1877–1880) [20]. The 'HBV/A2-N' clone contained all six mutations and was used as an experimental negative control. All of the mutations in this study (substitutions, insertions and deletions) were created by overlapping PCR extension followed by the exchange of endonuclease enzyme-restricted fragments, as described previously [13,21].

Three cytomegalovirus (CMV) promoter-driven expression clones were constructed containing the whole core genes (not including the precore section) of HBV/G (nt 1901–2488), HBV/A2 (nt 1901–2458) and HBV/C (nt 1901–2452): CMV-HBV/G/core, CMV-HBV/A2/core and CMV-HBV/C/core, respectively (Fig. 1b).

Three replicating recombinant constructs were created by recombination of different genomic sections of HBV/G and HBV/A2 (Fig. 1c). The 'HBV-G/A2-CP' clone was a HBV/G-based construct in which the leading fragment containing the core promoter (CP) region (nt 1413–1806) was replaced with that of HBV/A2. The 'HBV-G/A2-CP+core' clone was also an HBV/G-based construct, in which the leading fragment containing the core promoter (CP), precore and core region (nt 1413–2821) of HBV/G was replaced with those of HBV/A2. The 'HBV-G/A2-core' clone was an HBV/G-based construct in which the fragment of the precore and core region (nt 1806–2821) was replaced with those of HBV/A2.

### *Cell culture and transfection*

After 16 h of culture, Huh7 cells were transfected with 5  $\mu$ g of DNA construct per 10-cm diameter dish using the Eugene 6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol and harvested 3 days later. Transfection efficiency was measured by co-transfection with 0.5  $\mu$ g of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and normalised with subsequent SEAP measurement from culture supernatant using a SEAP reporter assay kit (Toyobo, Osaka, Japan) [17]. Three experiments were conducted for each clone.



**Fig. 1** HBV constructs (1.24-fold) and CMV-driven HBV core protein expression constructs used for the present study. CP, core promoter; BCP, basal core promoter; CMV, cytomegalovirus promoter. Stop codons for the corresponding HBV protein are indicated by crosses and arrows. All HBV/A2 recombinant plasmids consisted of the packaging-negative mutation ( $\epsilon$  signal block). In three recombinant constructs between HBV/A2 and HBV/G, the corresponding recombinant genomic parts are shown by the grey bar. CMV-core constructs produce core protein without generating HBeAg in the absence of the preceding  $\epsilon$  signal.

#### Determination of HBV markers

The expression levels of hepatitis B surface antigen (HBsAg) and HBeAg were determined by chemiluminescent enzyme immunoassay using commercial assay kits (Fujirebio Inc., Tokyo, Japan). The detection limit of the HBsAg assay is 0.05 IU/mL. HBV core-related antigen (HBcrAg) was measured in serum using a previously described chemiluminescent enzyme immunoassay [22]. The detection limit of the HBcrAg assay is 1.0 kU/mL.

#### Southern blot hybridisation

Southern blot hybridisation was performed with full-length probes for each genotype/subgenotype according to previously described methods [23]. In brief, cells were harvested and lysed in 1.5 mL of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA and 1% NP-40. Half of the cell lysate was treated with 100  $\mu$ g/mL of RNase A and 200  $\mu$ g/mL of DNase I for 2 h at 37°C, in

the presence of 6 mM Mg acetate. Then, HBV DNA was released by proteinase K digestion, extracted with phenol and precipitated with ethanol after the addition of 20  $\mu$ g of glycogen. DNA was separated on a 1.2% agarose gel, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) and hybridised with an alkaline phosphatase-labelled full-length HBV/G or HBV/A2 fragment generated with a Gene Images AlkPhos direct labelling module (GE Healthcare, Hertfordshire, UK). The detection was performed with CDP-Star, ready-to use (Roche Diagnostics GmbH). The signals were analysed by using a LAS-3000 image analyzer (Fuji Photo Film, Tokyo, Japan).

#### Western blot analysis

Serum or culture medium samples were subjected to SDS-PAGE under 15–25% polyacrylamide gel electrophoresis conditions. The proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA) at 15 V for 45 min. The

membrane was then blocked and probed using alkaline phosphatase-conjugated HB50 (for HBcAg) or HB91 (for HBcrAg) monoclonal antibody [22] at room temperature for 1 h, before being washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (KPL, Gaithersburg, MD, USA) for 15 min (for HBcrAg) or 90 min (for HBcAg).

#### Sucrose density gradient ultracentrifugation

Aliquots (1.7 mL) of 10%, 20%, 30%, 40%, 50% or 60% (w/w) sucrose in 10 mM Tris-HCl, 150 mM NaCl and 1 mM EDTA (pH 7.5) were carefully layered in a 12-mL ultracentrifuge tube and left at room temperature for 6 h. The culture supernatant of Huh7 cells that had been co-transfected with the 1.24-fold HBV genome construct (HBV/G or HBV/A) and/or the CMV-HBV/A2-core plasmid was layered onto this sucrose gradient, and ultracentrifugation was performed at  $200\,000 \times g$  for 15 h at 4° C in a Beckman Sw40Ti rotor (Beckman Coulter, Chaska, MN, USA). Fractions were collected from the top to the bottom of the gradient. The density of each fraction was calculated from its weight and volume. Each fraction was diluted 10-fold and tested for HBcrAg, HBsAg, HBeAg and HBV DNA.

#### Immunoprecipitation

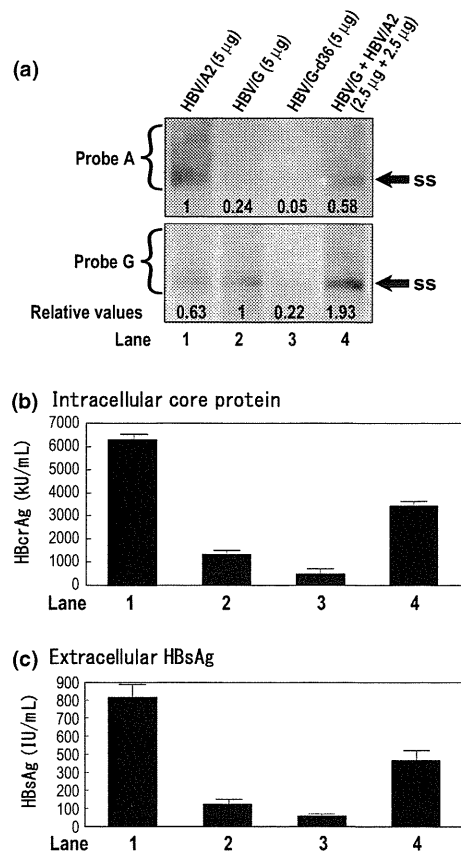
Immunoprecipitation was carried out using magnetic beads coated with monoclonal anti-HBs from the 'MagneSphere™ MS300/Caboxyl' kit (JSR Corp., Tokyo, Japan) [24]. A 100- $\mu$ L aliquot of sample was mixed with 100  $\mu$ L of a magnetic bead suspension. The mixture was then incubated for 1 h at room temperature under gentle agitation and then magnetically separated. The core protein in the precipitate was analysed by Western blotting.

## RESULTS

#### The replication of HBV/G is enhanced by HBV/A2 in co-transfection experiments

In this study, HBV/G and HBV/A2 genome clones (1.24-fold) were constructed from the serum of a HBV carrier that had been co-infected with HBV/G and HBV/A2. The HBV/G-d36 clone is a HBV/G genome-based construct in which the genotype-specific 36-nt insertion was deleted. We performed co-transfection with HBV/A2 and HBV/G clones and assessed virological features. Because of an over 12% sequence divergence between genotype A and G at the nucleotide level [12], the blot was hybridised successively with genotype-specific probes to DNA of each genotype. However, due to the unbiased binding of each probe at lower efficiency in Southern blot analysis [although the replication of HBV/A2 was higher than that of HBV/G, relative value of HBV/A2 with probe G became lower

(0.63), as well as the detection of HBV/G with probe A was very weak (0.24)], each probe of genotype G or A was used for hybridisation with the HBV/A2 and HBV/G clones (Fig. 2a). The density of single-strand HBV DNA detected by the genotype-specific probes in Southern blot analysis revealed that co-transfection with HBV/A2 resulted in increased replication of the wild-type HBV/G



**Fig. 2** (a) Southern blot analysis for replicative activity among HBV/G monotransfection, HBV/G-d36 monotransfection, HBV/A2 monotransfection and co-transfection with HBV/A2 and HBV/G (3 days after transfection). HBV/G-d36 clone was a deletion mutant lacking the 36-nt unique insertion in the core gene of the wild-type HBV/G clone. Hybridisation of the blot with genotype-specific probes of genotype A2 (upper) and G (lower). The density values shown at the bottom were measured to the probe-specific DNA sample. Single-stranded (SS) DNA is indicated by arrows. (b) Intracellular expression of core protein was estimated by detecting HBV-core-related antigen (HBcrAg) [22] as measured by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation,  $n = 3$ ). (c) HBsAg levels in the supernatant as detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation,  $n = 3$ ). All experiments were tested at least three times.

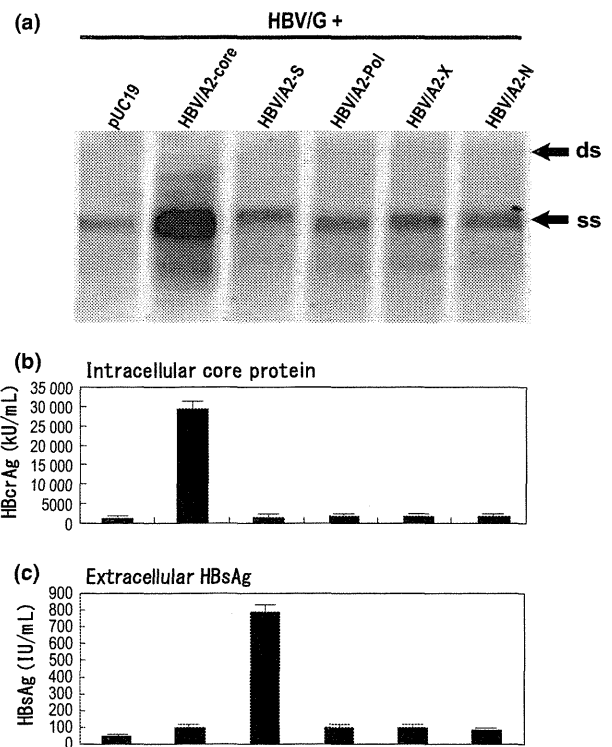
clone, compared with monotransfection of the wild-type HBV/G (Fig. 2a). The intracellular expression of core protein in the cell lysates and the expression of HBsAg in the culture supernatant were also enhanced by the co-transfection with both HBV/A2 and HBV/G clones (Fig. 2b and 2c). Removing the 36-nt insertion from the wild-type HBV/G genome resulted in a significant reduction in viral replication and core protein expression compared with the wild-type HBV/G clone. These results are in agreement with the observations of a previous study [13].

#### *The core protein of HBV/A2 is essential for efficient replication of HBV/G*

To determine how HBV/A2 rescues HBV/G replication during co-transfection, we constructed four HBV/A2 recombinant plasmids that selectively expressed one of the four viral proteins, whereas translation of the other three was prevented by the introduction of stop codons (Fig. 1a). All of these plasmids were prevented from coding for the viral pregenomic RNA containing the 'packaging-negative' mutation in the  $\epsilon$  signal loop to abrogate encapsidation (see Materials and methods). Huh7 cells were co-transfected with the wild-type HBV/G clone and one of the four plasmids expressing a single viral protein. According to Southern blot analysis, the expression of intracellular HBV DNA was greatly increased when HBV/G was co-transfected with HBV/A2-core compared with the other three expression plasmids or the experimental control (pUC19 or HBV/A2-N) (Fig. 3a). The intracellular expression of core protein in the cell lysates was also the highest when HBV/G was co-transfected with HBV/A2-core (Fig. 3b). The expression of HBsAg in the culture supernatant was only increased when HBV/G was co-transfected with the HBV/A2-S plasmid (Fig. 3c). These results indicated that the core protein translated from the HBV/A2 recombinant plasmid can enhance HBV/G replication.

#### *The core protein of HBV/A2 is more effective than those of HBV/C and HBV/G at promoting HBV/G replication*

To compare the effects of genotype on the ability of the core protein to increase HBV/G replication in co-transfection experiments, we generated three genotype-specific core protein expression constructs (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding  $\epsilon$  signal (Fig. 1c). Huh7 cells were co-transfected with HBV/G and one of the three core protein expression vectors. Southern blot analysis showed that the level of intracellular HBV DNA was highest during co-transfection with CMV-HBV/A2/core, followed by CMV-HBV/G/core, and was lowest for CMV-HBV/C/core (Fig. 4a), although the expression of core protein in the cell lysates was the highest during co-transfection with CMV-HBV/C/core, followed by CMV-HBV/G/core, and CMV-HBV/A2/core (Fig. 4b). As



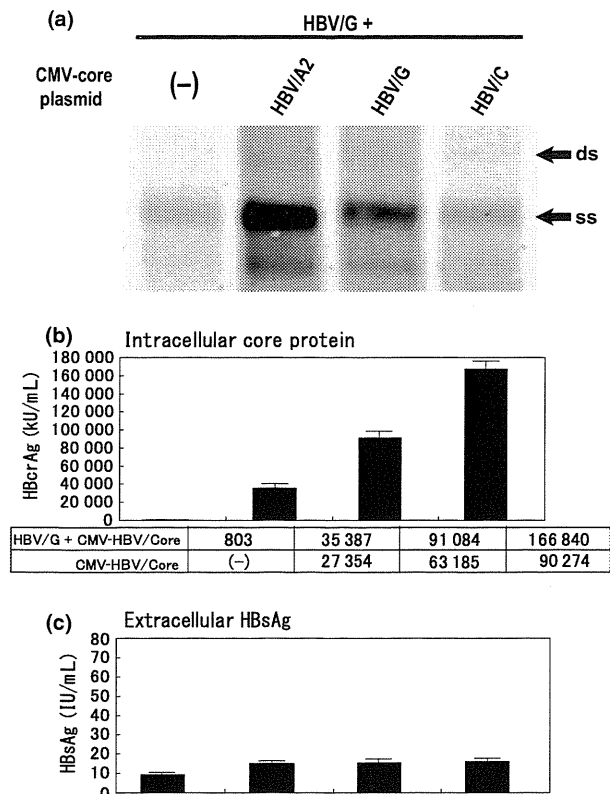
**Fig. 3** (a) Southern blot analysis for replication competence of HBV/G clones co-transfected with each of the four HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X selectively expressing only one of the four viral proteins (large surface, precore/core, polymerase or X protein, respectively). The 'HBV/A2-N' contained all the six mutations to be used as an experimental control. All of the above HBV/A2 recombinant plasmids had the 'packaging-negative' mutation in the  $\epsilon$  signal to abrogate encapsidation. (b) Intracellular expression of core protein was measured as described in Fig. 2b. (c) The expression of HBsAg in the culture supernatant was detected as described in Fig. 2c.

anticipated, there was no difference in the expression levels of HBsAg in any co-transfection experiment (Fig. 4c).

#### *A comparison of viral replication among HBV/G and recombinant HBV/G clones*

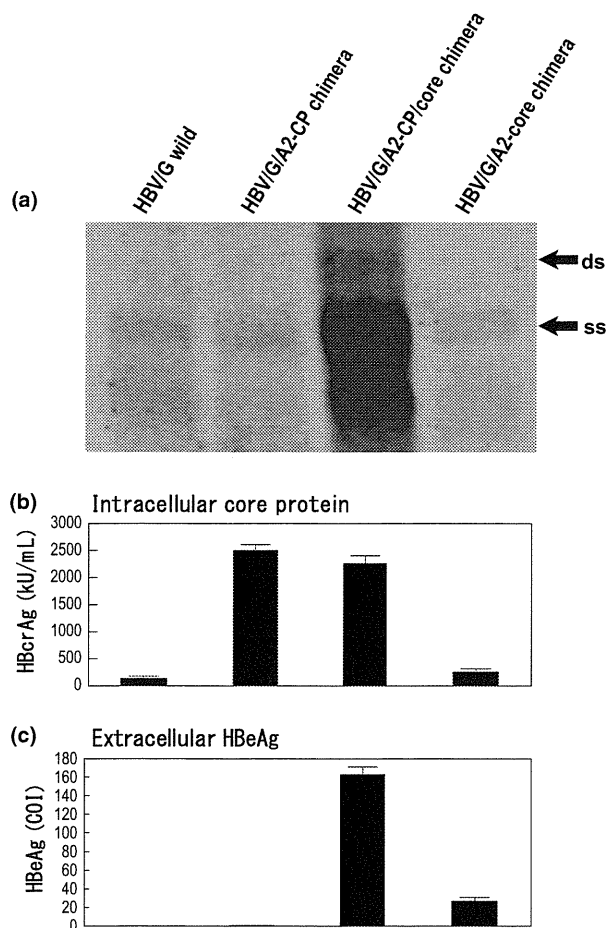
To examine the effects of genetic recombination and the roles of the core promoter, precore and core genomic regions in the interaction of HBV/G and HBV/A2 during co-transfection, we employed three HBV/G and HBV/A2 chimaeric replicating constructs (see Materials and methods), which are shown in Fig. 1c. After the transfection experiment, Southern blot analysis of cell lysates indicated an abundant level of DNA expression in HBV/G/A2-CP/core-transfected cells compared with those in the cells transfected with HBV/G-wild type, HBV/G/A2-CP and HBV/G/A2-core (Fig. 5a). As shown in Fig. 5b, the highest





**Fig. 4** (a) Southern blot analysis for the expression of intracellular HBV/G DNA during co-transfection with the three core protein expression constructs for each genotype (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding  $\epsilon$  signal. (b) Intracellular expression of core protein. (c) The expression of HBsAg in the culture supernatant.

levels of core protein (HBcrAg) expression were observed for the HBV/G/A2-CP and HBV/G/A2-CP/core-transfected cultures, which was in sharp contrast with the low levels observed in the HBV/G/A2-core and the wild-type HBV/G cultures. The discrepancy between viral replication and core production of the HBV/G/A2-CP clone might indicate insufficient virion assembly. Figure 5c shows the HBeAg levels measured in culture supernatants. The expression of HBeAg was the highest in the HBV/G/A2-CP/core culture distantly followed by that in the HBV/G/A2-core culture. The HBV/G/A2-CP and wild-type HBV/G clones expressed HBeAg protein at levels close to or below the level of detection. Nevertheless, a high HBcrAg titre was detected in the cell lysate of the HBV/G/A2-CP clone, although its DNA level was as low as that of the wild-type HBV/G clone (Fig. 5a). These results indicated that low replication of HBV/G might be explained by low synthesis of HBV/G core protein due to weak core promoter activity or dysfunction, as well as insufficient virion assembly due to the larger core protein of HBV/G (12-aa unique insertion).



**Fig. 5** (a) Southern blot analysis for HBV replication among HBV/G and three chimeric replicating constructs created by recombination of different genomic sections of HBV/G and HBV/A2 (see Materials and methods). The 'HBV/G/A2-CP' clone was a HBV/G-based construct in which the fragment containing the core promoter (CP) region but not the precore or core was replaced by the corresponding sequence from HBV/A2. The 'HBV/G/A2-Core' clone was an HBV/G-based construct in which the section of the precore and core region was replaced with that of HBV/A2. For the 'HBV/G/A2-CP/core' clone, the CP, precore and core region of HBV/G were replaced with that of HBV/A2. (b) Intracellular expression of core protein. (c) Extracellular expression of HBeAg levels detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation,  $n = 3$ ).

*Dane particles produced by HBV/G during co-transfection with HBV/A2 were packed in HBV/A2 protein*

To investigate the effects of HBV/A2 core protein during HBV/G viral assembly, we tried to define whether the Dane particles in B Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid

(source of the Fig. 4 lane 2) contained HBV/A2 or HBV/G core protein. To extract the Dane particles, we employed ultracentrifugation of the culture media through a 10–60% (w/w) sucrose density gradient followed by immunoprecipitation using anti-HBs-coated magnetic beads. We thereby extracted Dane particles, which were then analysed using Western blotting. The obtained fractions were tested for HBcrAg, HBsAg and HBV DNA (Fig. 6a). HBcrAg appeared in the high-density fractions, and its levels peaked in the same fraction (fraction 22) as HBV DNA. As reported previously, the fraction in which the levels of HBV DNA and HBcrAg peaked contained Dane particles [22]. To eliminate contamination of the Dane particles with 'naked' core particles or core protein, they were specifically retrieved from sucrose high-density fraction 22 by means of immunoprecipitation using anti-HBs-coated magnetic beads. The media supernatant obtained from the culture of cells that had been subjected to CMV-HBV/A2/core clone monotransfection was also subjected to sucrose gradient ultracentrifugation using the same protocol. Sucrose high-density fraction 22, in which the HBcrAg concentration peaked, presumably contained 'naked' core particles or core protein (Fig. 6b). This fraction was collected and processed in the same manner via anti-HBs-coated magnetic bead separation and was used as negative control for this procedure (Fig. 6c, lane 4). To discriminate between HBV/G and HBV/A2 core proteins on Western blot analysis probed with anti-HBc antibody, we employed cell lysates produced from cells that had been transfected with the wild-type HBV/G clone and those produced with the HBV/A2 clone as controls. As can be seen on the Western blotting image (Fig. 6c), HBV/G-transfected cells (lane 1) produced larger proteins than the HBV/A2-transfected cells (lane 2), which can be explained by the 12-aa insertion in the core protein of HBV/G coded by its 36-nt unique insertion. Interestingly, the most saturated band associated with the Dane particles produced by HBV/G that had been co-transfected with CMV-HBV/A2/core (lane 3) was the same size as that for HBV/A2, suggesting that HBV/G competitively produces Dane particles consisting of HBV/A2 core protein during virion assembly.

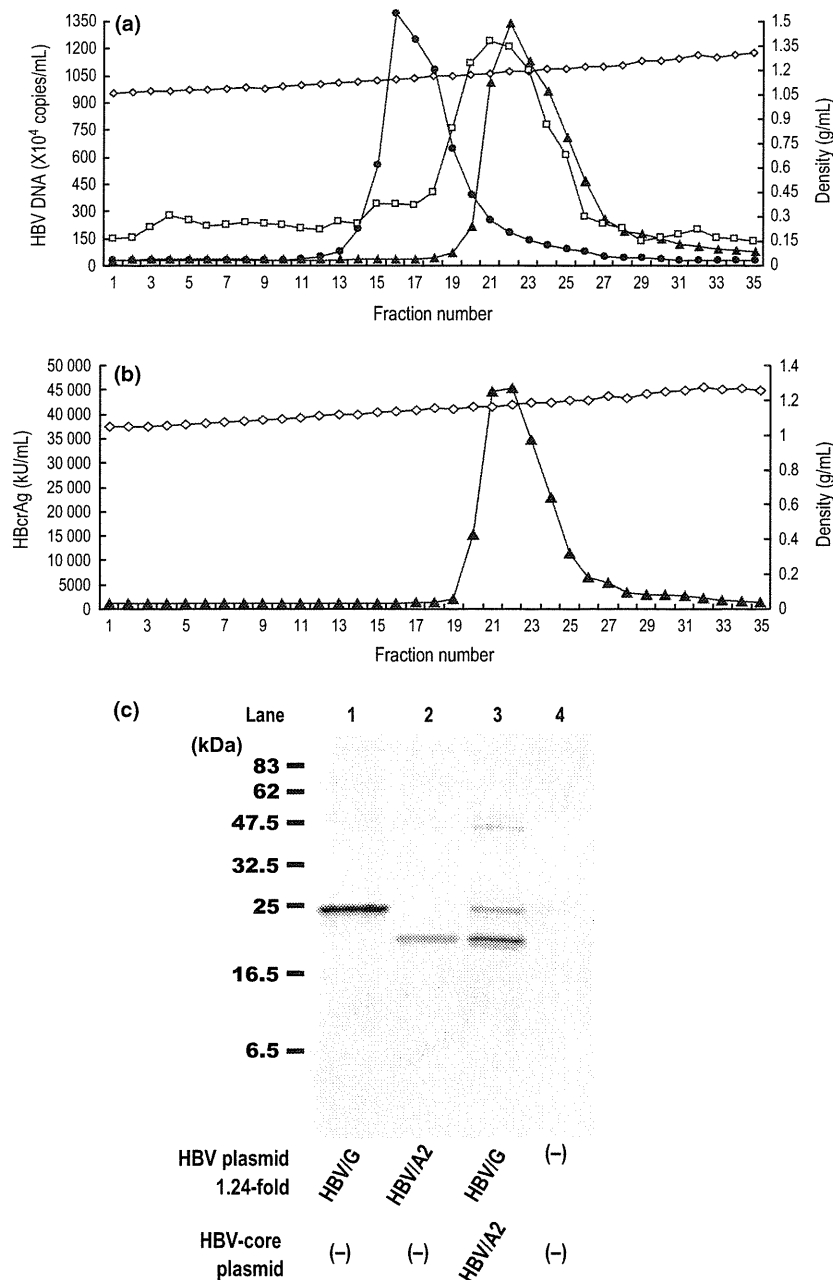
## DISCUSSION

HBV/G was first isolated in 2000 in France and the USA and was later found in Thailand, Japan and Mexico, indicating its global dissemination and association with specific risk groups, such as injection drug users (IDU) and men who had sex with men (MSM) [25]. Studies have also demonstrated that throughout the world, HBV/G strains possess unprecedented genetic homology and are mainly detected during co-infection with another genotype that is endemic in the area. Further studies have suggested that genotype G represents a 'replication-defective' variant of HBV that requires co-infection with another genotype to

establish a persistent infection. We and others have reported *in vitro* and *in vivo* experimental evidence of this HBV/G dependence [13–15]. The unique 36-nt insertion within core coding region increases core protein level and genome replication in genotype G but impairs replication, not core protein expression, in other genotypes [14]. These results strongly suggest the 36-nt/12-aa insertion has pleiotropic effects on core protein expression, genome replication and virion secretion [14]. To obtain clues about the mechanism by which genotype G works in combination with genotype A to effect its replication, we performed co-transfection experiments using Huh7 cells.

Using HBV/A2 viral proteins expressing plasmids, we determined that a HBV/A2 plasmid that selectively expressed core protein was capable of increasing the replication of the wild-type HBV/G (Fig. 3a). The replication of HBV/G during co-transfection was not affected by other viral elements of HBV/A2 because of the presence of the 'packaging-negative mutation' in the epsilon-coding region and stop codons preventing the translation of the other three viral proteins (the polymerase, surface and X proteins). The specific role of the core protein was further confirmed in experiments with CMV promoter-driven core expressing constructs, in which the core protein alone enhanced HBV/G replication in the absence of HBV pre-genomic RNA. Interestingly, co-transfection of HBV/G with the CMV-HBV/A2/core expression construct produced the highest levels of intracellular DNA, even though this combination produced the lowest intracellular core protein level, compared with the CMV-core constructs of the other two genotypes (HBV/G and HBV/C) (Figs 4a,b). The replication of HBV/G was the highest during co-transfection with the CMV-HBV/A2/core expression construct, which agreed with the results of experiments using other genotype (HBV/D, HBV/B1) CMV-core constructs (data not shown). Thus, the core protein of HBV/A2 was confirmed to play an important role in upregulating HBV/G replication and performed this task more efficiently than the other genotypes. These experimental results might explain why HBV/A is the genotype that is most frequently found in co-infections with HBV/G [12,26].

Moreover, HBV/G core protein overexpression achieved by the co-transfection of HBV/G with CMV-HBV/G/core did not enhance replication, suggesting that HBV/G core protein is functionally defective; that is, results in insufficient viral packaging. To investigate the functional defect in the HBV/G core protein, we exchanged the core gene of the wild-type HBV/G for the corresponding gene of HBV/A2 (HBV/G/A2-core); the introduction of the HBV/A2 core promoter together with core coding region into the HBV/G genome (HBV/G/A2-CP/core) significantly enhanced replication. However, the replication of the recombinant construct (HBV/A2 core coding region; HBV/G/A2-core) did not differ from that of the wild-type HBV/G, suggesting that the replacement of HBV/G/A2-core alone was not



**Fig. 6** (a) Sucrose gradient analysis of the culture media of Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid. It was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient. Density of each fraction is shown as a line with diamond symbols. Fractions were diluted 10-fold and tested for HBsAg (●) (IU/mL), HBcrAg (▲) (KU/mL) and HBV DNA (□) ( $10^4$  copies/mL). (b) Sucrose gradient analysis of culture supernatant obtained from the cells that were subjected to CMV-HBV/A2/core monotransfection using the same protocol. (c) Western blot analysis for HBV core protein was probed by anti-HBc antibody. HBV/G/core and HBV/A2/core were obtained from cell lysates that were transfected with the wild-type HBV/G clone and the wild-type HBV/A2 clone, respectively. The ‘HBV/G + CMV-HBV/A2/core’ was obtained from sucrose high-density fraction 22 (Fig. 6a) that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid by means of immunoprecipitation using anti-HBs-coated magnetic beads.

enough for viral replication because the core promoter of HBV/G was not capable of generating sufficient amounts of core protein to enhance HBV replication. As well, an HBV/

G/A2-CP construct containing the HBV/A core promoter region in the context of the wild-type HBV/G genome did not enhance replication, even though its core protein

production was significantly increased (Figs 5a,b). Although it was previously reported that the 36-nt insertion of the HBV/G core gene was required for both efficient core protein expression and HBV/G replication [13], in this study, the discrepancy between viral replication and core production of the HBV/G/A2-CP clone might indicate insufficient virion assembly due to the larger core protein of HBV/G (12-aa unique insertion). *Trans*-complementation experiments carried out by Gutelius *et al.* [14] demonstrated an association between enhanced core protein level and reduced replication capacity only when the core and polymerase proteins are expressed from the same RNA template. Thus, it was indicated that HBV/G itself could not replicate sufficiently due to a defect in its core protein and/or the core promoter of HBV/G.

Finally, we investigated whether HBV/G utilises the core protein of HBV/A2 for virion packaging. Dane particles obtained from the culture supernatants of cells that had been co-transfected with HBV/G and CMV-HBV/A2/core were assessed by Western blotting, and it was found that the Dane particles of HBV/G contained HBV/A core proteins. Thus, it was implied that HBV/G replication is enhanced by the core protein of HBV/A because it is more suitable for virion packaging than its own core protein, suggesting that the core protein of HBV/A is a key element enhancing the replication of HBV/G during co-infection. Interestingly, our experiments demonstrated that there were large differences in core protein expression among the CMV-core constructs of different genotypes, despite the fact that all of the CMV-core constructs had the same CMV promoter (Fig. 4b). In a previous report, it was speculated that the core protein binds to its own mRNA to influence

protein translation [13]. For example, dihydrofolate reductase protein has been found to downregulate its own translation by binding to cognate mRNA [27,28]. Therefore, we predict that the core protein of HBV/A2 enhances HBV/G replication by affecting viral promoters or transcription in addition to its role in virion packaging.

In conclusion, enhanced replication of HBV/G requires the HBV/A2 core protein during co-infection with HBV/A2. Our findings provide a possible explanation that the core protein of HBV/A2 is more suitable for virion packaging rather than that of HBV/G, and the replication of HBV/G occurs at a very low level, which may be due to defects in its core protein functions and/or core promoter activity. Further experiments are warranted to clarify the detailed roles of the enhanced HBV/G replication by co-infection with the other genotype and the clinical manifestation of HBV/G infection.

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#### CONFLICT OF INTEREST STATEMENT

None declared.

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