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 ε 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.

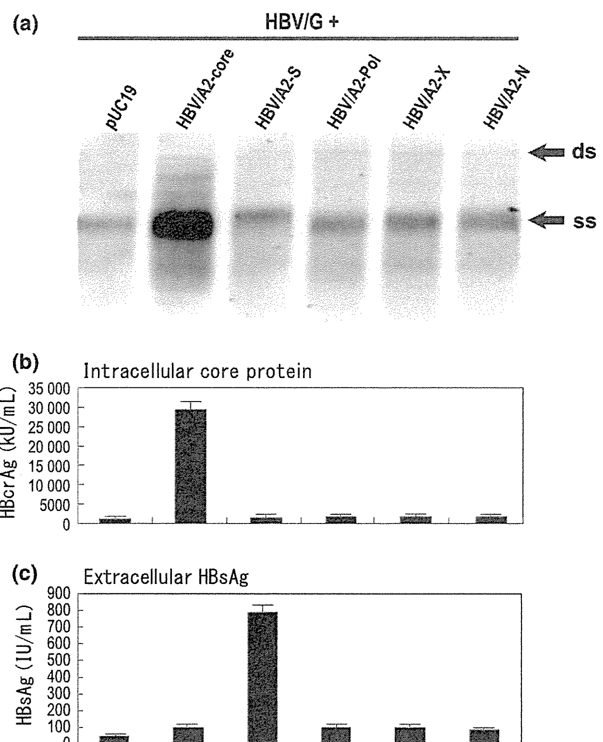

Fig. 3 (a) Southern blot analysis for replication competence of HBV/G clones co-transfected with each of the four HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X selectively expressing only one of the four viral proteins (large surface, precore/core, polymerase or X protein, 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 ε 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.

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 ε 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 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

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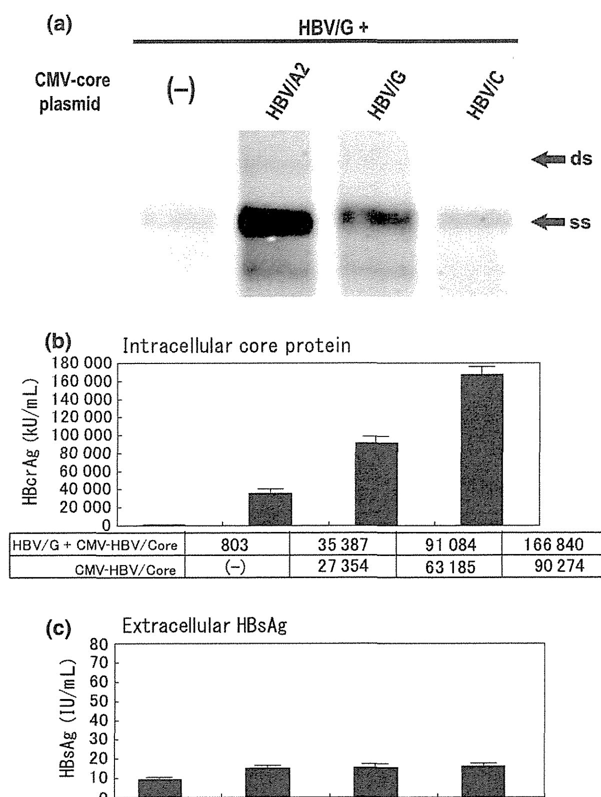

Fig. 4 (a) Southern blot analysis for the expression of intracellular HBV/G DNA during co-transfection with the three core protein expression constructs for each genotype (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding ε signal. (b) Intracellular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

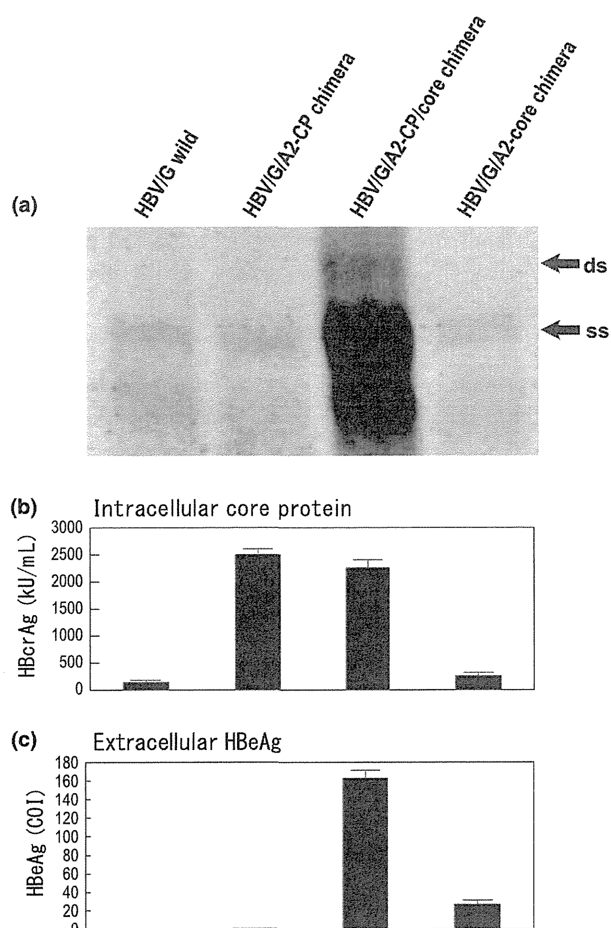

Fig. 5 (a) Southern blot analysis for HBV replication among HBV/G and three chimeric replicating constructs created by recombination of different genomic sections of HBV/G and HBV/A2 (see Materials and methods). The 'HBV/G/A2-CP' clone was a HBV/G-based construct in which the fragment containing the 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

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(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 pregenomic 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

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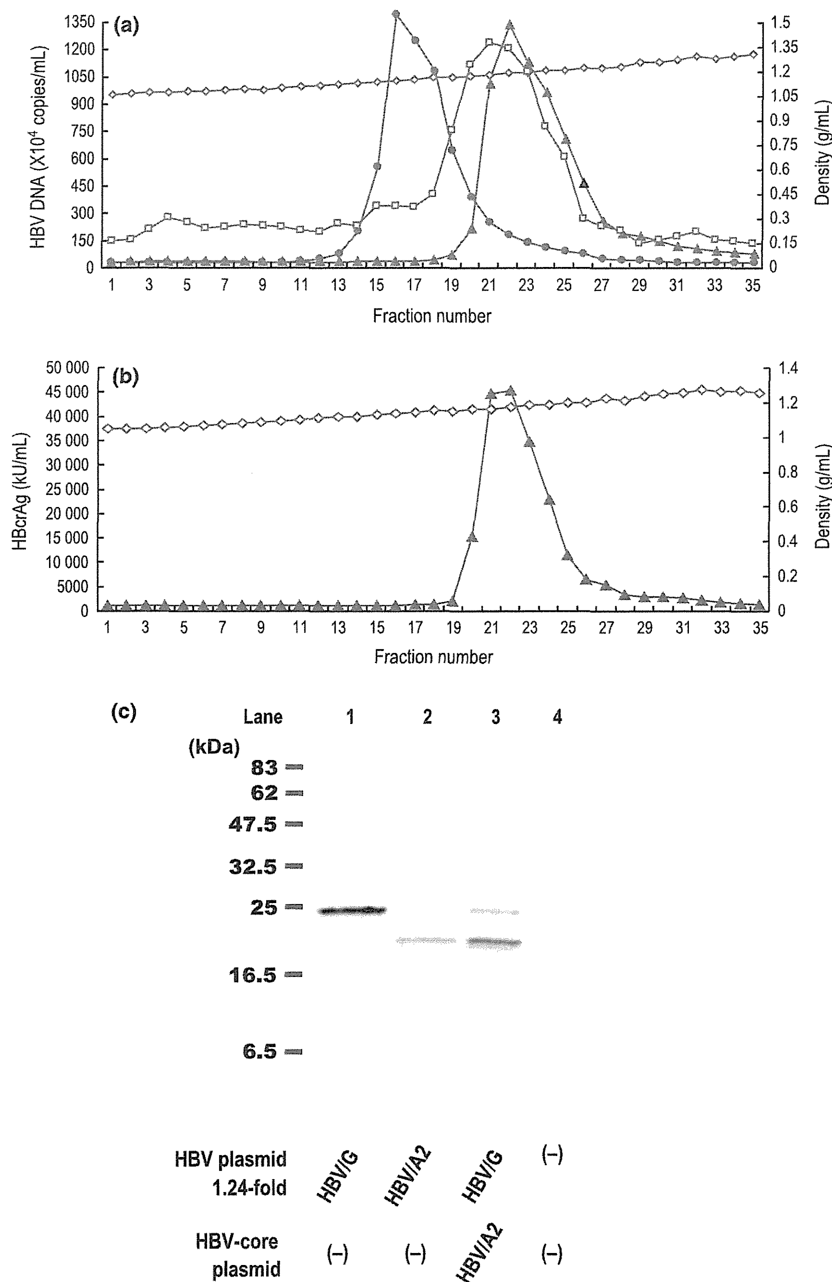

Fig. 6 (a) Sucrose gradient analysis of the culture media of Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid. It was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient. Density of each fraction is shown as a line with diamond 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

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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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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Plasmid construct of HBV/G.

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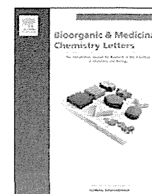

2′-Fluoro-6′-methylene-carbocyclic adenosine phosphoramidate (FMCAP) prodrug: In vitro anti-HBV activity against the lamivudine–entecavir resistant triple mutant and its mechanism of action

Ravindra K. Rawal [a], Uma S. Singh [a], Satish N Chavre [a], Jianing Wang [a], Masaya Sugiyama [b], Wai Hung [a], Rajgopal Govindarajan [a], Brent Korba [c], Yasuhito Tanaka [b], Chung K. Chu [a,*]

[a] The University of Georgia, College of Pharmacy, Athens, GA 30602, USA
[b] Nagoya City University Graduate School of Medical Sciences, Nagoya 467 8601, Japan
[c] Georgetown University Medical Center, WA 20057, USA

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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).[1] 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.[2]

Currently, there are several nucleos(t)ide analogues available to treat chronic hepatitis B virus infection.[3–6] 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 (LVDr) 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.[7] 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.[8] 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,[8,9] which

* Corresponding author. Address: Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602, USA. Tel.: +1 706 542 5379; fax: +1 706 542 5381.
E-mail address: DCHU@rx.uga.edu (C.K. Chu).

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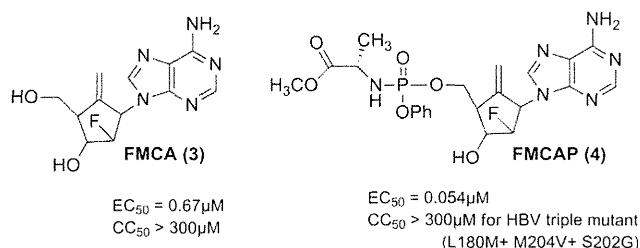


Figure 1. Structures of 2′-fluoro-6′-methylene-carbocyclic adenosine (FMCA; **3**) and its prodrug (FMCAP; **4**).

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.[10,11] 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.[12] 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)[13] was synthesized using a known method in the literature,[14,15] 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.

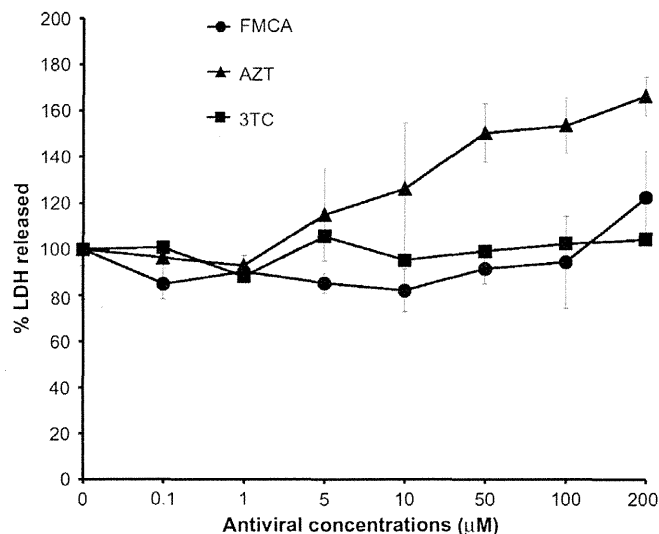
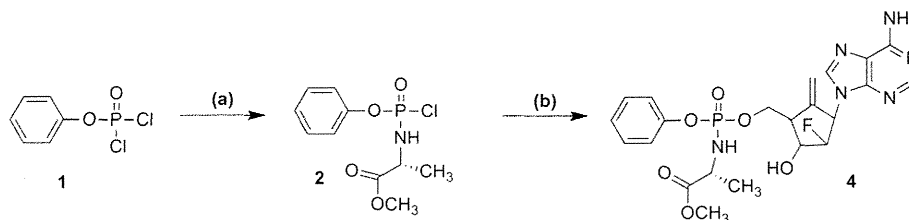


Figure 2. Mitochondrial toxicity of FMCA **3**, AZT and 3TC through lactate dehydrogenase release (LDH) assay.

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



Scheme 1. Reagent and conditions: (a) L-alanine methyl ester hydrochloride, Et_3N, CH_2Cl_2; (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			
	Wild-type			L180M + M204V + S202G
	EC_{50} (µM)	EC_{90} (µM)	CC_{50} (µM)	EC_{50} (µM)
FMCA **3**	0.548 ± 0.056	6.0 ± 0.400	>300	0.67
FMCAP **4**	0.062 ± 0.011	0.46 ± 0.060	>300	0.054
Lamivudine	0.056 ± 0.003	0.142 ± 0.008	>300	>500[17]
Entecavir	0.008	0.033	28	1.20[16]

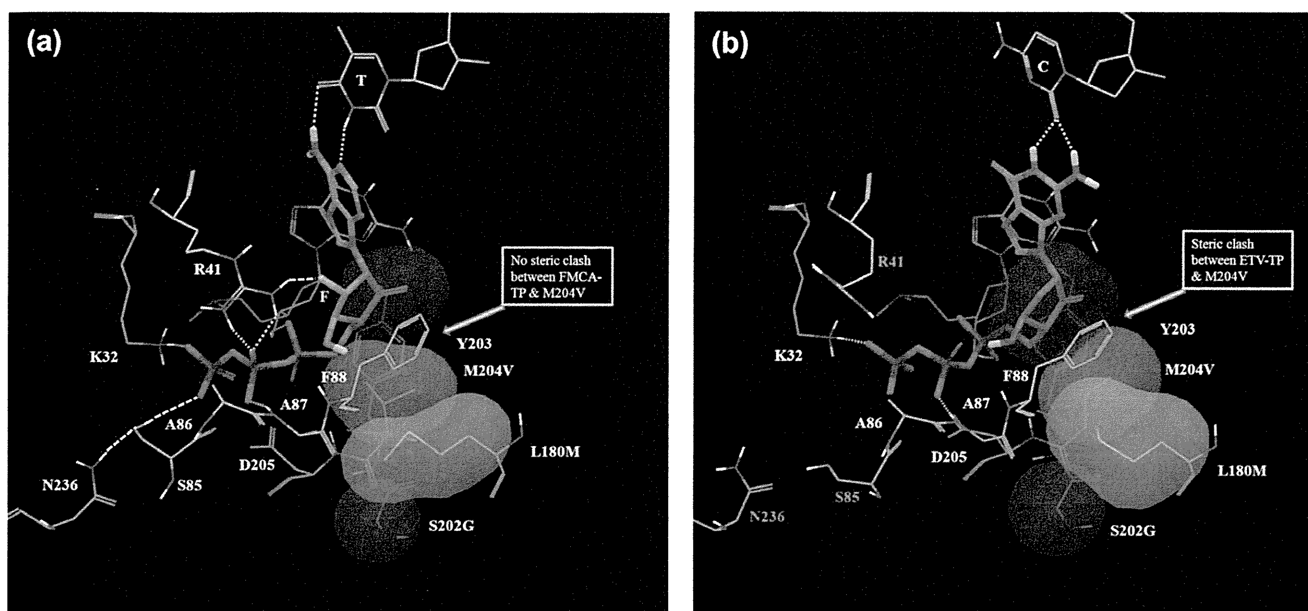


Figure 3. (a) FMCA-TP binding mode in ETVr (L180M + M204V + S202G); and (b) ETV-TP binding mode in ETVr (L180M + M204V + S202G) and there is a steric hindrance. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å). The Van der Waals surface of L180M is colored yellow. The Van der Waals surface 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[21] and energy minimization[22]

Strains	Compounds	Energy difference results (ΔE, kcal/mol)		
		Total energy	VdW[a]	Electrostatic
Wild-type	FMCA-TP	−588.05	375.78	−6341.08
	ETV-TP	−597.25	350.35	−6009.65
ETVr	FMCA-TP	−591.54	359.91	−6245.68
(L180M + M204V + S202G)	ETV-TP	−320.28	248.82	−4831.12

[a] Van der Waals interaction.

was reduced by 150-fold (EC_{50} 1.2 μM) in comparison to wild type.[16]

In the preliminary mitochondrial toxicity studies in HepG2 cells by measuring the lactic dehydrogenase release,[18] FMCA **3** did not exhibit any significant toxicity up to 100 μM 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.[12] 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.[19] A previously described homology model was used to further explore the impact of the ETVr to the HBV-RT.[12] The homology model of HBV-RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD).[20]

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)[22] 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[21] 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,[12] whereas ETV-TP lose the hydrogen bonding with R41 & S85. The γ-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, γ-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.

Acknowledgment

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13. *Compound* **4**: ^{1}H NMR (500 Mz, $CDCl_3$) δ 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}F NMR (500 MHz, $CDCl_3$) δ −192.86 (m, 1F); ^{13}C NMR (125 MHz, $CDCl_3$) δ 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}P NMR (202 MHz, $CDCl_3$): δ 2.67, 2.99. Anal. Calcd For $C_{22}H_{26}FN_6O_6P.0.5H_2O$: C, 49.91; H, 5.14; N, 15.87; Found C, 49.84; H, 5.06; N, 15.22.
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Journal of Medical Virology 84:587–595 (2012)

Multiple Intra-Familial Transmission Patterns of Hepatitis B Virus Genotype D in North-Eastern Egypt

Mostafa Ragheb,[1] Abeer Elkady,[2] Yasuhito Tanaka,[2]* Shuko Murakami,[2] Fadia M. Attia,[3] Adel A. Hassan,[1] Mohamed F. Hassan,[1] Mahmoud M. Shedid,[1] Hassan B. Abdel Reheem,[1] Anis Khan,[2] and Masashi Mizokami[4]

[1]*Department of Endemic and Infectious Disease, Suez Canal University, Ismailia, Egypt*
[2]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan*
[3]*Department of Clinical Pathology Faculty of Medicine, Suez Canal University, Ismailia, Egypt*
[4]*Research Centre for Hepatitis and Immunology, International Medical Centre of Japan Konodai Hospital, Tokyo, Japan*

The transmission rate of intra-familial hepatitis B virus (HBV) and mode of transmission were investigated in north eastern Egypt. HBV infection was investigated serologically and confirmed by molecular evolutionary analysis in family members (N = 230) of 55 chronic hepatitis B carriers (index cases). Hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) prevalence was 12.2% and 23% among family members, respectively. HBsAg carriers were prevalent in the age groups; <10 (16.2%) and 21–30 years (23.3%). The prevalence of HBsAg was significantly higher in the family members of females (19.2%) than males (8.6%) index cases ($P = 0.031$). HBsAg and anti-HBc seropositive rates were higher significantly in the offspring of females (23%, 29.8%) than those of the males index cases (4.3%, 9.8%) ($P = 0.001$, 0.003), as well as higher in the offspring of an infected mother (26.5, 31.8%) than those of an infected father (4.7%, 10.5%) ($P = 0.0006$, 0.009). No significant difference was found in HBsAg seropositive rates between vaccinated (10.6%) and unvaccinated family members (14.8%). Phylogenetic analysis of the preS2 and S regions of HBV genome showed that the HBV isolates were of subgenotype D1 in nine index cases and 14 family members. HBV familial transmission was confirmed in five of six families with three transmission patterns; maternal, paternal, and sexual. It is concluded that multiple intra-familial transmission routes of HBV genotype D were determined; including maternal, paternal and horizontal. Universal HBV vaccination should be modified by including the first dose at birth with (HBIG) administration to the newborn of mothers infected with HBV. ***J. Med. Virol. 84:587–595, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBV genotype D; intra-familial transmission; vaccine

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major health problem worldwide and is affecting approximately 350 million individuals [Lee, 1997]. Infection with HBV may lead to chronic state of hepatitis in 5–10% of patients who acquired the infection in the adult life and in 80–90% of patients who acquired the infection in the infancy [Chen, 1993]. Infection with HBV can lead to a progressive liver disease including liver cirrhosis and hepatocellular carcinoma (HCC) with approximately 1 million HBV-associated deaths from HCC every year [Seeger and Mason, 2000; Kao and Chen, 2002].

Based on the proportion of the population who are seropositive for hepatitis B surface antigen (HBsAg),

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Mostafa Ragheb and Abeer Elkady contributed equally to this study.

*Correspondence to: Yasuhito Tanaka, MD, PhD, Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho, Nagoya 467-8601, Japan. E-mail: ytanaka@med.nagoya-cu.ac.jp

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the world is divided conceptually into zones of high, intermediate, and low HBV endemic areas [Lavanchy, 2004]. In countries where the HBV infection is endemic, most infections result from the vertical transmission from the mother to the child in the peripartum period or from the infection in the early childhood. In the low HBV endemic regions, the neonatal or the childhood HBV infection is rare or even sporadic and the transmission of HBV occurs primarily among unvaccinated adults through the sexual transmission and injecting drug use [Custer et al., 2004].

Patients with chronic hepatitis B are considered to be the major reservoirs for the transmission of HBV. High incidence of infection with HBV is observed within the household contacts of chronic HBV carriers and it is not rare to have several members of the same household who have evidence of infection with HBV [Milas et al., 2000; Thakur et al., 2002]. However, the precise mechanisms of intra-familial spread have not been established clearly.

Different prophylactic strategies for controlling the HBV infection have been used by different countries depending on the prevalence of the HBV infection in each country [Poland and Jacobson, 2004]. The widespread immunization program against hepatitis B, which was implemented in more than 100 countries, was capable of dramatic reduction in the occurrence of chronic HBV infection and HCC [Zuckerman, 1997]. In Egypt, the HBV vaccine was included in 1992 in the Expanded Program of Immunization with injection at 2, 4, and 6 months of age [El Sherbini et al., 2006]. This program resulted in a significant reduction in the rate of acute symptomatic hepatitis B among the children in the age group eligible to receive the vaccine [Zakaria et al., 2007].

At least eight HBV genotypes have been identified based on the divergence of 8% or more of the entire nucleotide sequence and most of the HBV genotypes have a distinct geographical distribution [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000]. Accumulated evidences indicated the difference in the virological characteristics among different HBV genotypes, which is reflected by the difference in the clinical outcome of infection with hepatitis B according to the infecting genotype [Miyakawa and Mizokami, 2003; Schaefer, 2005; Ozasa et al., 2006; Sugiyama et al., 2006]. However, data regarding the specificity of the transmission routes of each genotype is still scarce globally and need to be clarified.

The prevalence of HBV ranges between 2% and 6% in Egypt with the predominance of infection with HBV genotype D [Zekri et al., 2007]. It is widely known that Egypt is one of the countries with highest prevalence rate of infection with HCV in the world [el-Zayadi et al., 1992; Arthur et al., 1993; el Gohary et al., 1995]. However, the burden of HBV related progressive liver disease including liver cirrhosis and HCC in Egypt is observable either single or in a dual infection with HCV [Abdel-Wahab et al., 2000; el-Zayadi et al., 2005].

This study aimed to evaluate the prevalence of infection with HBV within the families of chronic HBV carriers in north Eastern Egypt. In addition, the intra-familial mode of transmission of HBV genotype D was also examined in the current cohort by the molecular evolutionary analyses. The impact of the HBV immunization programme in protecting this high-risk group was also investigated.

PATIENTS AND METHODS

Patients

The present study was conducted between January 2008 and June 2008 at the Communicable Disease Research and Training Centre, in Suez city. The study protocol was approved by the ethics committees of the participating institution and an informed consent was obtained from the included subjects.

Chronic HBV carriers were defined as individuals whose serum samples tested positive for HBsAg for at least 6-months period. Patients who fulfilled the criteria of chronic HBV carriers and were first detected within their families, were defined as the index cases (n = 55). The index cases included 40 (72.7%) men and 15 (27.3%) women. Their mean age (± SD) was 41 ± 10.7 years and all the index cases were negative for HBeAg.

A total of 230 household contacts of the index cases were included in the study and defined as family members group. Data regarding their family relationship to the index cases, age, and the HBV vaccination history have been obtained.

According to the kinship of the family members to the index case group, the family members included 139 offspring, 4 parents, 46 spouses, 15 siblings, and 26 defined as other relatives who are living in the same house with the index cases.

Serological Methods

Serum samples were collected from the index cases and family members groups.

The Serum samples were examined for HBsAg, anti-HBc, anti-HBs, and HBeAg by the chemiluminescence enzyme immunoassay with the commercial assay kits (Fujirebio, Inc., Tokyo, Japan). The examination of the serum samples for anti-HCV and HIV was conducted using commercial kits (Abbott Laboratories, Abbott Park, IL).

Molecular Evolutionary Analysis

The HBV/DNA was extracted from 200 μl of serum samples positive for HBsAg using the QIAamp DNA MiniKit (QIGEN, Inc., Hilden, Germany), and re-suspended in 100 μl of a storage buffer (provided by the kit manufacturer).

The entire preS2 and S regions of the HBV genome (799 nucleotides; nucleotide positions 34–833) were amplified using the primers set and the conditions described previously [Sugauchi et al., 2001].

The amplified products were sequenced using Prism Big Dye (Pekrin–Elmer Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer according to the manufacturer's protocol. The sequences were aligned together with the CLUSAL X software programme [Thompson et al., 1994].

The phylogenetic tree was constructed using the neighbor joining method with Tamura-Nei's distance correction model using the Online Hepatitis Virus database (http://s2as02.genes.nig.ac.jp/) [Shin et al., 2008]. The Bootstrap values were determined on 1000 database resampling tests. The sequences of other HBV isolates used for the construction of the phylogenetic tree were retrieved from the DDBJ/EMBL/GenBank sequence database and were indicated in their accession numbers. The new nucleotide sequences data that were reported in this manuscript will appear in the DDBJ/EMBL/GenBank sequence database with accession numbers AB561825-AB561856.

Statistical Analysis

Statistical analysis was performed with the Fisher's exact probability test and the independent t-test for the continuous variables using the SPSS software package (SPSS, Chicago, IL). P-values (two-tailed) <0.05 were considered to be significant statistically.

RESULTS

The family member included 96 (41.7%) males and 134 females (58.3%). Their mean age ($\pm$SD) was 20.6 ± 14.6. The rate of seropositivity for HBsAg and anti-HBc was 12.2% (28/230) and 23% (53/230) of the family members group with no statistical significant difference between the males and females members.

Age Group Distribution of HBV Infection Within the Family Members Group

Figure 1 illustrates the HBsAg and anti-HBc prevalences among different age groups of the family members. The highest prevalence of HBsAg seropositive cases was observed in the age group, 21–30 years old; (10/43; 23.3%) followed by the age group, 0–10 years old; (11/68; 16.2%). No statistical significant difference was found in the HBsAg seropositive rates between these two age groups. The prevalence of HBsAg was 7.7% (5/65), 3.4% (1/29), and 4% (1/25) in the age groups; 11–20, 31–40, and $\geq$41 years old, respectively. The prevalence of anti-HBc seropositive cases was significantly increasing with the age and the highest rate was observed in the age group $\geq$41 years old. The prevalence of anti-HBc was 8.8% (6/68), 20% (13/65), 25.6% (11/43), 37.9% (11/29), and 48% (12/25) in the age groups; 0–10, 11–20, 21–30, 31–40, and $\geq$41 years old, respectively.

The HBsAg and anti-HBc seropositive rates were analyzed in the family members with respect to their relationship to the index cases (Fig. 2A). As overall, the HBsAg was positive in 6.5% (3/46) spouse of index cases, 10.8% (15/139) of the offspring, 25% (1/4) of the parents, and 40% (6/15) of the siblings (Fig. 2A).

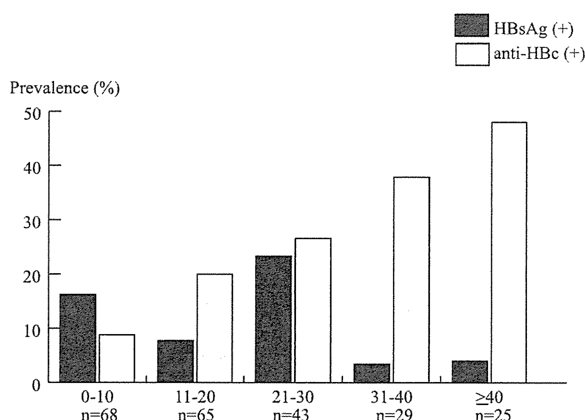

Fig. 1. Age distribution and HBV serological status among family members.

The prevalence of anti-HBc was 34.8% (16/46) in the spouse of index cases, 17.3% (24/139) in the offspring, 50% (2/4) in the parents, and 46.7% (7/15) in the siblings of the index cases (Fig. 2A).

Interestingly, the prevalence of HBsAg and anti-HBc was significantly higher in the family members of the females (19.2%, 15/78) than that of the males index cases (8.6%, 13/152; $P = 0.034$) and a trend of higher incidence of anti-HBc in the family members of the females than the males index cases (Fig. 2B). Among the offspring group, HBsAg and anti-HBc seropositive rates were significantly higher in the offspring of the females index cases (HBsAg; 23%, 11/47, anti-HBc; 29.8%, 14/47) cases than in the offspring of the males index cases (HBsAg; 4.3%, 4/92, antiHBc; 9.8%, 9/92), ($P = 0.001$, 0.003 respectively; Fig. 2C).

Further analysis was performed regarding the HBsAg seropositive rate in the offspring according to HBV infection of both one and two parents and the parent gender who is infected with HBV. Significantly higher rate of HBsAg positive (26.5%, 13/49) and anti-HBc positive (31.8%, 14/49) off spring were found in families where the mother was positive for HBsAg compared with families where the father was HBsAg positive (HBsAg; 4.7%, anti-HBc; 10.5%), ($P = 0.0006$, 0.009 respectively) (data not shown).

The seropositive rate of HBsAg was higher in the non-sexual contacts (13.6%, 25/184) of the index cases (parents, offspring, siblings, and cousins) than the sexual contacts (spouses; 6.5%, 3/46) with no statistical significant difference. Anti-HBc seropositive cases were observed more frequently in the sexual contacts (spouses) than in the non-sexual contacts (parents, offspring, siblings cousins) of the index cases. (Sexual vs. non-sexual contacts, 34.8% vs. 20.1%, $P = 0.049$) (data not shown).

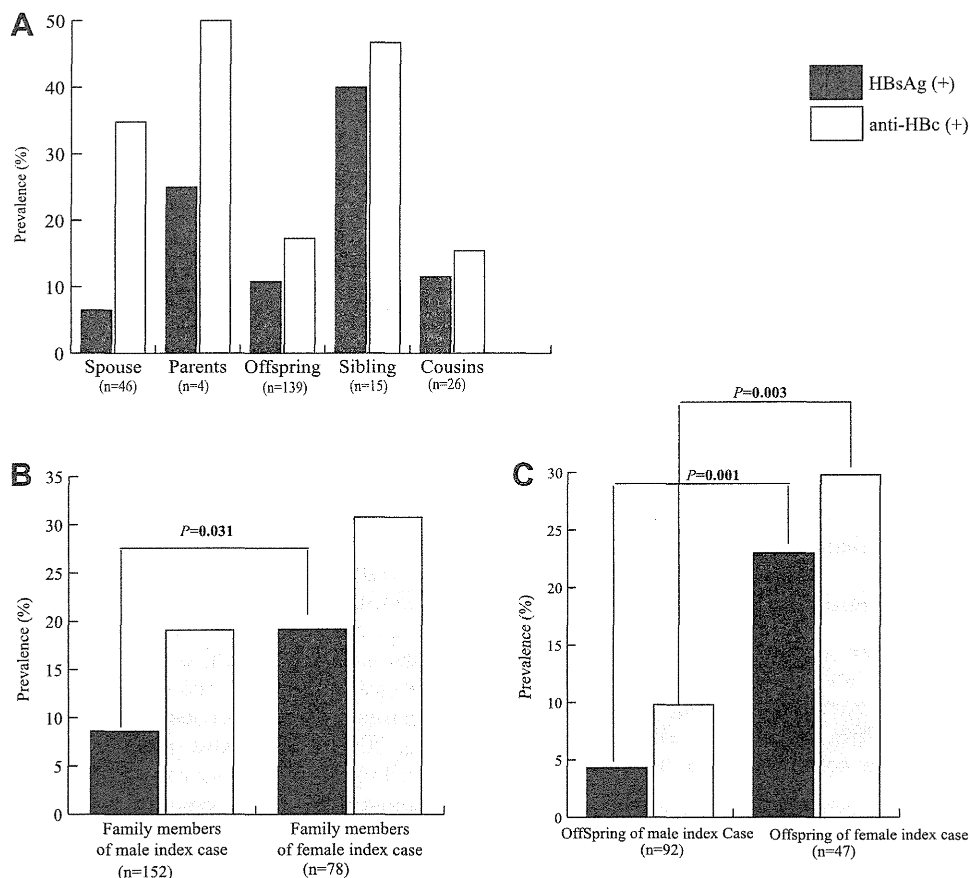

Fig. 2. Prevalence of HBsAg and anti-HBc within family members stratified by relationship to the index cases (**A**). HBV serological status of family members according to gender of the index case (**B**), and HBV serological status of the offspring according to HBV infected parent (**C**).

Molecular Evolutionary Analysis and Transmission Pattern of Hepatitis B in the Family Members Group

Eighteen index cases out of 55 (32.7%) were found to have at least one family member positive for HBsAg. The age range of these index cases was 26–56 years and 50% (9/18) of them were male (Table I). Twenty-eight family members were found to be positive for HBsAg. The data regarding the degree of relativity of each family member infected with HBV to the index case, the age of the infected family member, and the vaccination status were indicated in Table I. The mean age (±SD) of the family members with active HBV infection was 17.8 ± 13.0 years old (Table I).

The HBV genomic region of 799-nt length and spanning PreS2 and S region was amplified in 44% (8/18) of the index cases and in 50% (14/28) of the family members infected with HBV. However, the target genomic region could be amplified and sequenced simultaneously in the index cases and their related family members in six subjects. These six subjects are defined in the present report, Table I and Figure 3 as F 3, F4, F19, F35, F37, and F 43 (Table I, Fig. 3).

To confirm the family clustering, a phylogentic tree was constructed by (1) the previous mentioned sequences (2) sequences isolated from the index cases whose family members were negative for HBsAg (3) HBV nucleotide sequences isolated from HBV chronic carriers residing in different districts in Egypt (North and South) either retrieved from the data base band or further included in the present study.

The phylogenetic analysis of the preS2 and S regions of the HBV genome revealed that the HBV isolates were of subgenotype D1 (Fig. 3). Using the phylogenetic analysis, in family 4 (F4), a high homology was detected between the HBV strains isolated from the grandmother together with her daughters and her grandchildren (Fig. 3). In the Family 35 and Family 43 (F35, and F43), the father and the child harbored very closely related HBV isolates and the phylogenetic analysis suggesting that the father may have been the source of infection for his child in Family 35 (F35) and Family 43 (F43). Similarly, very closely related HBV isolates were also detected in the

TABLE I. Descriptive Analysis of the Family Members Positive for the HbsAg

Subject	Relation (gender)	Age	HBV-vaccine[a]	PreS2 + S
F3	Index (F)	42		(+)
F3-1[b]	Daughter	13	Yes	(+)
F10	Index (F)	30		(−)
F10-1	Daughter	3	Yes	(+)
F11	Index (F)	33		(+)
F11-1	Daughter	8	Yes	(−)
F11-2	Cousin	10	Yes	(−)
F30	Index (F)	42		(−)
F30-1	Son	8	Yes	(−)
F34	Index (F)	30		(−)
F34-1	Son	7	Yes	(+)
F34-2	Son	9	Yes	(+)
F48	Index (F)	30		(−)
F48-1	Son	5	Yes	(−)
F35	Index (M)	29		(+)
F35-1[b]	Daughter	5	Yes	(+)
F39	Index (M)	33		(−)
F39-1	Daughter	5	Yes	(−)
F43	Index (M)	47		(+)
F43-1[b]	Daughter	12	Yes	(+)
F55	Index (M)	56		(+)
F55-1	Daughter	12	Yes	(−)
F37	Index (M)	45		(+)
F37-1[b]	Wife	26	Yes	(+)
F36	Index (M)	31		(−)
F36-1	Brother	26	No	(−)
F36-2	Brother	28	No	(−)
F36-3	Brother	22	No	(+)
F36-4	Mother	63	No	(+)
F4	Index (F)	54		(+)
F4-1	Daughter	35	No	(+)
F4-2	Daughter	20	No	(+)
F4-3	Grandchild	6	Yes	(+)
F4-4[b]	Grandchild	4	Yes	(+)
F19	Index (M)	29		(+)
F19-1[b]	Wife	27	No	(+)
F40	Index (M)	26		(−)
F40-1	Relative	24	No	(−)
F40-2	Relative	29	No	(−)
F41	Index (F)	53		(−)
F41-1	Daughter	23	No	(−)
F41-2	Daughter	17	No	(−)
F45	Index (M)	33		(+)
F45-1	Wife	27	No	(−)
F50	Index(F)	27		(−)
F50-1	Sister	25	No	(−)

[a]HBV vaccination history is provided for the family member.
[b]Index and family members who are positive simultaneously for the PreS2and S region.

man and his wife in Families 19 and 37 (F19 and F37) (Fig. 3). The molecular evolutionary analysis of the sequences isolated from the mother and her daughter in Family 3 (F3), yielded two separate but distinct groupings of the HBV isolates, suggesting that the presence of two different HBV viral isolates infecting the mother and her daughter (Fig. 3).

Serological Markers of HBV Infection in the Vaccinated and Unvaccinated Family Members

The family members group was subdivided into two subgroups according to the history of full regimen schedule of HBV vaccination as shown in Table II; (1) A group of vaccinated family members which includes a total of 142 subjects, who received the complete HBV vaccine regimen. (2) A group of unvaccinated family members, which included 88 subjects with no previous history or incomplete regimen of HBV vaccination.

The family members in the unvaccinated group were significantly older (mean ± SD; 32.5 ± 12.5 years old) than in the vaccinated group (mean ± SD; 13.3 ± 10.4, $P = 0.012$). No statistical significant difference was found in the male gender distribution between the two groups. The anti-HBs seropositive rate was significantly higher in the vaccinated group than the unvaccinated group [69.8% (99/142) vs. 33% (29/88), respectively, $P < 0.0001$] (Table II). The mean anti-HBs titre was significantly higher in the vaccinated than unvaccinated family members (70.1 ± 129.7 vs. 21.6 ± 51.7 mIU/ml, respectively $P < 0.0001$).

The prevalence of anti-HBc was significantly higher in the unvaccinated family members compared to vaccinated groups (37.5% vs. 14.1% respectively, $P < 0.0001$). Interestingly, no statistical significant difference was detected between the vaccinated and the unvaccinated groups regarding the prevalence of HBsAg [vaccinated vs. unvaccinated; 10.6% (15/142) vs. 14.8% (13/88), $P = 0.4$] (Table II). The HBV DNA was detected in 50% of family members positive for HBsAg with no statistical significant difference between the vaccinated (53%, 8/142) and unvaccinated groups (46.2%, 6/88) (Table II).

Mutations in the "a" determinant region. The available nucleotide sequences spanning the S gene of HBV isolated from the nine vaccinated and five unvaccinated members were translated into amino acid and aligned in correspondence to the reference sequences. The amino acid substitutions in the "a" determinant region that was reported to be associated with vaccine escape mutation were not detected. However, an amino acid substitution at the second loop of "a" determinant region (T143L) was clustered in the family subject F37 (F37 and F37-1) and found in one unvaccinated family member (F4-1). Another substitution was detected in the second loop of "a" determinant region (T140I) in an unvaccinated member (F36-1). P127A substitution in first loop of the "a" determinant region was clustered in the family 43(F43 and F43-1; Fig. 4).

DISCUSSION

The investigation of the intra-familial transmission in a particular region usually reveals valuable information about the routes of HBV spread in general and may help in exploring the HBV spread problem and local peculiarities. This study is the first one in Egypt done to explore the intra-familial spread of HBV infection and inclusively HBV genotype D transmission routes in Egypt. An evaluation of the impact of the universal HBV vaccination on the intra-familial transmission of HBV was also done.

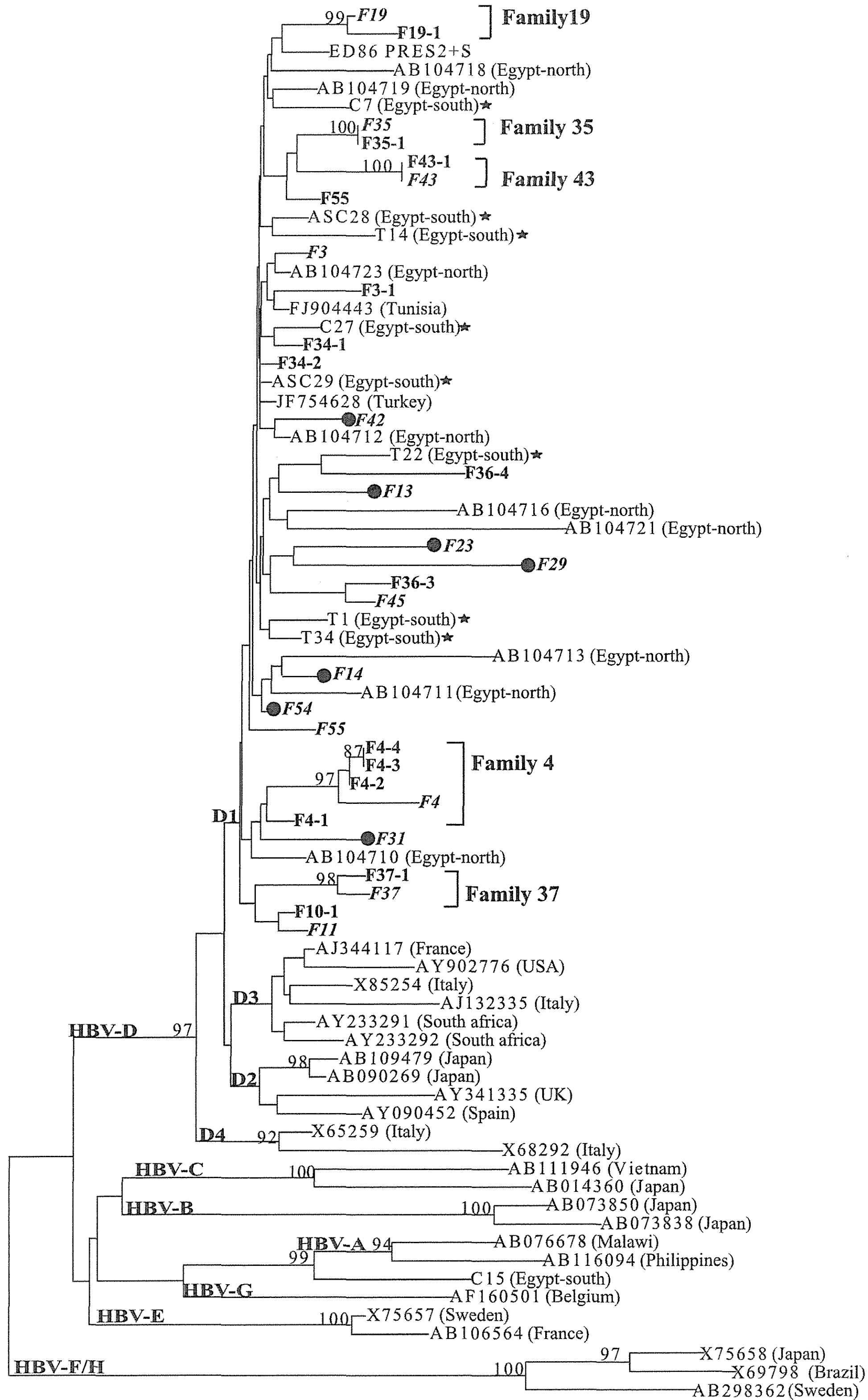

Fig. 3. Phylogenetic tree constructed by the nucleotide sequences of the partial PreS2 and S HBV genomic region. The phylogenetic tree is constructed by the neighbor joining method and significant bootstrap values (>75%) are indicated in the tree roots. HBV sequences isolated from index cases and family members are indicated in italic bold and bold fonts respectively. Reference sequences retrieved from the GenBank/EMBL/DDBJ are indicted in their accession numbers. Solid black rounds indicate sequences from index cases with family members negative for HBsAg. (★) Strains isolated from chronic hepatitis B carriers residing in Egypt south. The country origin of the reference sequences are indicated in brackets. HBV genotypes A–H are indicated in the cluster roots.

TABLE II. Comparison of Hepatitis B Serological Markers in Vaccinated Versus Unvaccinated Family Members Group

	Total (N = 230)	Vaccinated group (N = 142)	Unvaccinated group (N = 88)	*P*-value
Age[a]	20.6 ± 14.6	13.3 ± 10.4	32.5 ± 51.7	<0.0001
Gender (Male)[b]	96(41.7)	64 (45.1)	32 (36.4)	NS
Anti-HBc (+)[b]	53 (23)	20 (14.1)	33 (37.5)	<0.0001
HBsAg (+)[b]	28 (12.2)	15 (10.6)	13 (14.8)	NS
Anti-HBs (+)[b]	128 (55.7)	99 (69.8)	29(33)	<0.0001
HBV-DNA (+)[b]	14 (50)	8 (53.3)	6 (46.2)	NS

[a]Mean ± SD.
[b]N (%).

In the present study, 12.1% of the family members were infected with HBV. This incidence was much higher than that detected among the blood donors (1.4%) resident in the same area in Egypt (data not shown). Clustering of the HBV infection within the families has been described in nearby countries located within the same zone of the HBV endemicity but with different incidences; 30% in Turkey, 15.8% in Greece, and 11.9% in Iran [Alizadeh et al., 2005; Zervou et al., 2005; Ucmak et al., 2007]. An important risk factor was found to be implicated in acquiring the infection among the family was the presence of female infected with HBV. Furthermore, the higher incidence of HBsAg positive rate among the offspring of the females' index cases than that of males index cases illustrates clearly the role of the mother in the transmission of HBV. Similarly, Salkic et al. [2007] reported the same observation in his study from Bosnia [Salkic et al., 2007]. However, in Taiwan no significant difference was found in the HBsAg positivity among the offspring of the two groups, suggesting the importance of the paternal as well as the maternal transmission for the HBV intra-familial spread in Taiwan [Lin et al., 2005].

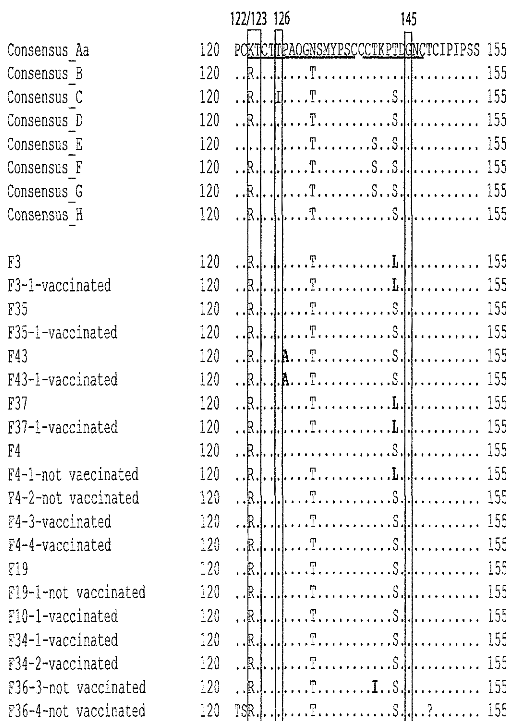

Fig. 4. The alignment of amino acid sequences of the HBV partial surface gene encompassing the "a" determinant region in the HBsAg positive family members. The upper eight sequences are consensus of the corresponding HBV genotypes Aa/A1, B, C, D, E, F, G, and H reference strain retrieved from DDBJ/GenBank database. Dots in alignment indicate identity of amino acids to the consensus sequence of genotype Aa/A1. First and second loop positions are underlined in the consensus sequence of the genotype Aa/A1 and positions of previously reported vaccine escape mutants are indicated in numbers and included in boxes.

Despite being a tedious and labor-intensive method, sequencing of the viral genomes isolated from different individuals, with the subsequent homology comparison and the phylogenetic analysis remains the golden approach for demonstrating the HBV transmission in a given population [Dumpis et al., 2001; Zampino et al., 2002; Tajiri et al., 2007].

The full length HBV sequence analysis is the gold standard for this purpose but remains a cost approach [Datta et al., 2007]. Highly variable HBV genomic region is recommended by some investigators to study the transmission event. Variability of the genomic region is affected by several factors one of which is the clinical characteristics of the studied cohort [Wu et al., 2005]. PreC/C region exhibit high variability in the cases of acute or fulminant hepatitis and thus analysis of this region is preferable for investigating the chain of recent/nosocomial fulminant cases [Bracho et al., 2006; Ozasa et al., 2006]. However, a high S gene variability is documented among the chronic hepatitis B carriers and their families, thus investigating the genotype, subgenotype, subtypes, and mutations by the sequence analysis of the S gene with further analysis by testing the constructed phylogenetic tree with the bootstrap resampling maximum-likelihood test, may provide enough confidence to prove the transmission event in the case of chronic HBV carriers [Thakur et al., 2003]. Hence, in the present study, the phylogenetic analysis of the HBV nucleotide sequences spanning the entire preS2 and S HBV genomic regions and isolated from chronic hepatitis B carriers which include index cases and their family members revealed the infection with HBV genotype D which coincides with the previous

data regarding the predominance of infection with HBV genotype D in Egypt [Saudy et al., 2003]. In addition, the phylogenetic analysis documented the presence of three different patterns of HBV genotype D transmission within the families in Egypt; maternal transmission (from mother to child as in the family 4), paternal transmission (from father to child as in family 35 and family 43) and spousal transmission (between spouses as in family 19 and family 37). This was different from the transmission pattern characteristics of genotype D in Uzbekistan where the horizontal transmission was the predominant route of infection with HBV genotype D within a family [Avazova et al., 2008].

The Data regarding the difference of transmission routes of HBV infection between different genotypes are controversial and scarce. Based on the findings that the patients infected with HBV genotype C may exhibit delayed HBeAg seroconversion decades later than the patients infected with other genotypes, Livingston et al. [2007] speculated that genotype C is the most responsible for the perinatal transmission and that the other genotypes (A, B, D, and F) are mainly transmitted horizontally [Livingston et al., 2007]. A recent study has shown a different data through exploring that both genotypes B and C can be transmitted by maternal and horizontal routes [Wen et al., 2011]. Whether different HBV genotypes have different transmission routes remains a question, which needs further global studies to clarify this interesting and important issue.

In an attempt to evaluate the influence of the universal vaccination on the intra-familial HBV infection, it was surprising to find a high prevalence rate of HBsAg among the vaccinated members with no significant difference when compared to the unvaccinated group. In an agreement with the present data, El Sherbini et al. [2006] reported the unchangeable prevalence of HBsAg among the vaccinated school children across a decade despite the significant decrease of the anti-HBc rate [El Sherbini et al., 2006]. The possible explanation for this vaccine failure is the acquiring of the HBV infection in the lag period between the birth and the time of receiving the first HBV vaccine dose at the age of 2 months. Supporting our explanation is the recent data coming from Taiwan where a different HBV infection prophylactic strategy is applied by administrating the first dose of the HBV vaccine at birth with the administration of the hepatitis B immunoglobulin to the infants born to the HBeAg positive mother within 24 hr after birth. The recent study has clearly demonstrated that the current HBV prophylactic strategy in Taiwan was capable of reducing the intra-familial HBV transmission and reducing the overall HBsAg positive rate among the infants [Mu et al., 2011]. In Japan, the extension of the active and passive immunization to the babies born to HBeAg negative mother had greatly reduced the HBsAg prevalence to 0.2% of blood donors younger than 19 years old [Noto et al., 2003; Matsuura et al., 2009]. The present study recommends the changing of the current HBV prophylactic policy in Egypt. It would be needed to provide the first dose of the HBV vaccine at birth together with screening for HBV infection markers prenatally and administration of the HBIG to the infants born from HBeAg-positive mothers. The documented role of the HBV spousal transmission in the present study by the phylogenetic analysis (Family 19 and Family 37), coincides with the recent data conducted in Egypt that the first sexual contact with an infected spouse was a significant risk factor for infection with HBV among females and may further emphasize the importance of the premarital screening for HBV in Egypt [Paez Jimenez et al., 2009]. Investigating the " a" determinant region of viral isolates retrieved from the vaccinated members infected with HBV provides no evidence of breakthrough infection by previously reported vaccine escape mutant virus [Carman et al., 1990].

In conclusion, the present study has clearly explored the role of the HBV intra-familial transmission and spread in north Eastern Egypt. Three patterns of HBV transmission were determined in the current cohort infected with HBV genotype D; maternal, paternal, and spousal. The present study recommends the change of the current prophylactic policy against the HBV infection in Egypt by including the first dose of HBV vaccine at birth, screening of pregnant women for HBsAg and the administration of HBIG to the infants born from HBeAg positive mothers within 24 hr after birth. Further studies are needed globally to determine the transmission patterns of different HBV genotypes and locally in different districts in Egypt to explore the impact of familial transmission in HBV infection in Egypt.

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Structure-guided mutagenesis for the improvement of substrate specificity of *Bacillus megaterium* glucose 1-dehydrogenase IV

Taiki Nishioka[1], Yoshiaki Yasutake[2], Yoshiaki Nishiya[3,*] and Tomohiro Tamura[1,2]

1 Graduate School of Agriculture, Hokkaido University, Sapporo, Japan
2 Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo, Japan
3 Tsuruga Institute of Biotechnology, Toyobo Co. Ltd, Japan

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Bacillus megaterium; crystal structure; glucose 1-dehydrogenase; short-chain dehydrogenase/reductase; site-directed mutagenesis; substrate specificity

Correspondence
T. Tamura, Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1, Tsukisamu-higashi, Toyohira, Sapporo 062-8517, Japan
Fax: +81 11 857 8980
Tel: +81 11 857 8938
E-mail: t-tamura@aist.go.jp

***Present address**
Department of Life Science, Setsunan University, 17-8, Ikeda-Nakamachi, Neyagawa, Osaka 572-8508, Japan

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Bacillus megaterium IAM 1030 (*Bacillus* sp. JCM 20016) possesses four D-glucose 1-dehydrogenase isozymes (BmGlcDH-I, -II, -III and -IV) that belong to the short-chain dehydrogenase/reductase superfamily. The BmGlcDHs are currently used for a clinical assay to examine blood glucose levels. Of these four isozymes, BmGlcDH-IV has relatively high thermostability and catalytic activity, but the disadvantage of its broad substrate specificity remains to be overcome. Here, we describe the crystal structures of BmGlcDH-IV in ligand-free, NADH-bound and β-D-glucose-bound forms to a resolution of 2.0 Å. No major conformational differences were found among these structures. The structure of BmGlcDH-IV in complex with β-D-glucose revealed that the carboxyl group at the C-terminus, derived from a neighboring subunit, is inserted into the active-site pocket and directly interacts with β-D-glucose. A site-directed mutagenic study showed that destabilization of the BmGlcDH-IV C-terminal region by substitution with more bulky and hydrophobic amino acid residues greatly affects the activity of the enzyme, as well as its thermostability and substrate specificity. Of the six mutants created, the G259A variant exhibited the narrowest substrate specificity, whilst retaining comparable catalytic activity and thermostability to the wild-type enzyme.

Database
The atomic coordinates and structure factor amplitudes for BmGlcDH-IV in ligand-free form, in complex with NADH, in complex with D-glucose, G259A mutant in ligand-free form, and A258F mutant in complex with D-glucose and NADH were deposited in the RCSB Protein Data Bank (http://www.rcsb.org) under the accession codes 3AUS, 3AUT, 3AUU, 3AY6 and 3AY7, respectively

Structured digital abstract
- BmGlcDH-IV and BmGlcDH-IV bind by x-ray crystallography (View Interaction: 1, 2)

Introduction

NAD(P)$^+$-dependent glucose 1-dehydrogenase (GlcDH) isozymes from *Bacillus megaterium* (EC 1.1.1.47; BmGlcDHs) catalyze the oxidation of β-D-glucose to D-glucono-1,5-lactone, using NAD(P)$^+$ as a cofactor.

Abbreviations
BmGlcDH, *Bacillus megaterium* glucose 1-dehydrogenase; GlcDH, glucose 1-dehydrogenase; SDR, short-chain dehydrogenase/reductase.