Effect of mutating the two cysteines required for HBe antigenicity on hepatitis B virus DNA replication and virion secretion

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Received 17 September 2004; returned to author for revision 17 October 2004; accepted 22 November 2004
Available online 24 December 2004

Abstract

Hepatitis B virus (HBV) variants with impaired expression of e antigen (HBeAg) frequently arise at the chronic stage of infection, as exemplified by precore and core promoter mutants. Since an intramolecular disulfide bond maintains the secondary structure of HBeAg, we explored effect of missense mutations of either cysteine codon. Consistent with earlier reports, substitution of each cysteine rendered HBeAg nearly undetectable. With underlying nucleotide changes at the loop of pregenome encapsidation signal, the C-7 mutants were severely impaired in pregenomic RNA packaging and hence DNA replication. Although none of the missense mutations at C61 reduced DNA replication, replacement with arginine, but not alanine, aspartic acid, phenylalanine, or serine, blocked virion secretion. Consistent with the detection of C61R genome from a patient serum, secretion block of the C61R mutant could be overcome by co-expression of wild-type core protein. In conclusion, point mutations of the C61 codon may generate viable HBeAg-negative variants.

Keywords: Disulfide bond; HBV variant; HBeAg; Pregenome encapsidation signal; DNA replication; Virion secretion

Introduction

Hepatitis B e antigen (HBeAg) is an auxiliary protein of the hepatitis B virus (HBV) that is not essential for viral replication in vitro yet crucial for establishing persistent infection in vivo, as experimentally demonstrated for the related woodchuck hepatitis virus (Chen et al., 1992; Tong et al., 1991). It has been proposed that HBeAg expression during neonatal transmission promote immune tolerance (Milich et al., 1990). Furthermore, it may buffer immune response against the related core protein (Milich and Liang, 2003). Expression of HBeAg requires the coding capacity of both the precore region and the core gene. Of the 29 residues specified by the precore region, the first 19 residues serve as a signal peptide to target the protein to the endoplasmic reticulum, where the peptide is cleaved (Bruss and Gerlich, 1988; Ou et al., 1986). Subsequently, the arginine-rich carboxyl terminus of the protein is clipped away by a furin-like endopeptidase in the secretory pathway before the mature protein of 17 kDa is released into the blood stream (Messageot et al., 2003). Therefore, HBeAg contains 10 extra amino acids at its N-terminus than the core protein but lacks about 30 residues at the C-terminus. Consistent with the nonessential nature of HBeAg for HBV life cycle, the development of anti-HBe antibodies in the late stage of HBV infection often correlates with emergence of viral variants with impaired expression of HBeAg. The replacement of the wild-type virus by the HBeAg variants is possibly associated with the ability of the variants to avoid clearance mediated by anti-HBe immunity (Zhang and Summers, 1999). Among the known HBeAg variants, the core promoter mutants...
express less HBeAg owing to transcriptional down
regulation of its mRNA (Buckwold et al., 1996; Li et
al., 1999; Okamoto et al., 1994; Parekh et al., 2003;
Scaglioni et al., 1997; Tang et al., 2001; Yu and Mertz,
2003; Zheng et al., 2004), whereas precore mutants fail to
express HBeAg because of a nonsense or frameshift
mutation that terminates translation prematurely (Carman
et al., 1989; Li et al., 1990; Tong et al., 1990). In addition,
HBeAg translation can be prevented by a point mutation of
the precore ATG codon, or mitigated by mutations of its
Kozak sequence (Ahn et al., 2003; Raimondo et al., 1990).
Considering the many steps involved in the biosynthetic
pathway for HBeAg, it is worthwhile to explore whether
there are additional avenues whereby the virus can evade
the anti-HBe immunity of its host (Fig. 1). This is
especially relevant for genotype A and certain strains of
genotypes C and F, which rarely develop the HBeAg-
minus G1896A mutation due to base-pairing requirement
between nucleotides 1896 and 1858 in the pregenome
encapsidation signal (e signal) (Alestig et al., 2001; Arauz-
Ruiz et al., 1997; Li et al., 1993; Lok et al., 1994;
Rodriguez-Frias et al., 1995).

Despite their extensive overlap of the primary sequenc-
es, HBeAg and core protein adopt very different higher
structures. For example, HBeAg does not oligomerize to
form particles. The unique secondary structure of secreted
HBeAg has been attributed to its N-terminal 10 residues,
which are derived from the precore region and hence
absent in core protein. Indeed, C23 of the precore
peptide (4th residue of mature HBeAg, -7 position
relative to residue 1 specified by the core gene) forms
an intramolecular disulfide bond with C61 specified by
the core gene (Nassal and Rieger, 1993; Wasenauer et
al., 1993). Because of this intra-molecular S–S bond
between C-7 and C61, molecular dimerization via C61–
C61 disulfide bridge, as occurs in the core protein, is
prevented. Mutation of either cysteine residue is known
to greatly reduce HBe antigenicity (Nassal and Rieger,
1993; Wasenauer et al., 1993). In addition, mutating C61
impairs secretion of the variant HBeAg molecule (Nassal
and Rieger, 1993). Mutants of C-7 have been found
during chronic HBV infection (Erhardt et al., 2000).
However, a comparative study of HBV DNA between
the tumorous vs. nontumor parts of the liver revealed
exclusive distribution of the missense mutations of the C-7
codon in the tumor tissue, where viral replication has
ceased (Manzin et al., 1992). To determine whether the C-7
and C61 mutants are viable, we generated site-directed
mutants and tested their biological properties by trans-
fection experiments in the Huh7 human hepatoma cells.

Results and discussion

Missense mutations of the C-7 codon markedly impair
genome replication

Based on the previous reports of naturally occurring
mutations at the C-7 codon (Erhardt et al., 2000; Manzin et
al., 1992), four site-directed mutants were generated: C-7F,
C-7G, C-7S, and C-7R (Table 1). The parental construct for
this study, N4, is characterized by high replication capacity
as well as efficient expression of both hepatitis B surface
antigen (HBsAg) and HBeAg (Khan et al., 2004). Tandem
dimers of the constructs were generated for transfection into
Huh7 hepatoma cells using the lipofectamine method,
together with a plasmid encoding secreted alkaline
phosphatase (SEAP). Cells and culture supernatants were
harvested at day 5 post-transfection. The transfection
efficiencies were comparable among various constructs, as
indicated by similar levels of SEAP and HBsAg in culture
supernatant (Fig. 2A). According to assays performed with a
commercial radioimmunoassay, all the four mutants pro-
duced negligible HBeAg in culture supernatant (Fig. 2A).
The lack of detectable HBeAg is most likely caused by
conformational change of the protein, rather than due to a

Fig. 1. Schematic view of the HBeAg biosynthesis pathway and its
modulation by naturally occurring mutations. Although a G1862T mutation
was suspected to reduce HBeAg expression at the step of signal peptide
removal, our recent work failed to support this hypothesis (Guarnieri et al.,
in preparation). On the other hand, naturally occurring mutations in the
RRDRGR motif, the furin cleavage site, have been found to reduce HBeAg
secretion (Kim et al., in preparation).

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide</th>
<th>Amino acid</th>
<th>DNA replication</th>
<th>Virion secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>N4</td>
<td>WT</td>
<td>WT</td>
<td>++++</td>
<td>OK</td>
</tr>
<tr>
<td>G5</td>
<td>G1881T</td>
<td>C-7F</td>
<td>+</td>
<td>OK</td>
</tr>
<tr>
<td>G33</td>
<td>T1880G</td>
<td>C-7G</td>
<td>+</td>
<td>OK</td>
</tr>
<tr>
<td>G34</td>
<td>T1880A</td>
<td>C-7S</td>
<td>+</td>
<td>OK</td>
</tr>
<tr>
<td>G35</td>
<td>T1880C</td>
<td>C-7R</td>
<td>++</td>
<td>OK</td>
</tr>
<tr>
<td>G6</td>
<td>T2081C</td>
<td>C61R</td>
<td>++++</td>
<td>defective</td>
</tr>
<tr>
<td>G7</td>
<td>G2082C</td>
<td>C61S</td>
<td>++++</td>
<td>OK</td>
</tr>
<tr>
<td>G17</td>
<td>T2081G/G2082C</td>
<td>C61A</td>
<td>++++</td>
<td>OK</td>
</tr>
<tr>
<td>G18</td>
<td>T2081G/G2082A</td>
<td>C61D</td>
<td>++++</td>
<td>OK</td>
</tr>
<tr>
<td>G19</td>
<td>G2082T</td>
<td>C61F</td>
<td>++++</td>
<td>OK</td>
</tr>
</tbody>
</table>

NA: not applicable.
true loss of protein expression (Nassal and Rieger, 1993; Wasenauer et al., 1993).

Southern blot analysis of intracellular core particles revealed greatly reduced viral DNA replication of the C-7 mutants, although there is no difference in the pattern of HBV DNA bands (Fig. 2B). Reflecting the sequential steps involved in HBV replication, the major bands in the Southern blot correspond to single-stranded (SS), partially double stranded (PDS), and the mature relaxed circular (RC) DNA forms. Based on phosphoimage analysis, replication capacity of the mutants ranged from 20% of the wild-type level (C-7R) to a complete loss of replication (C-7G) (Fig. 2A). The C-7F and C-7S constructs displayed intermediate replication capacity, at about 10% of the wild-type level. The mutations did not affect virion secretion, as evidenced by similar degree of reduction in DNA signals.
in culture supernatant as inside core particles (Fig. 2B, compare “Medium” with “Cell”). The transfection experiments were repeated at least two more times and very similar results were obtained (data not shown). The reduced viral replication may well be caused by the underlying nucleotide changes, rather than by amino acid substitutions in HBeAg protein, which is dispensable for viral replication in vitro (Tong et al., 1991). Although the precore region specifies the signal peptide of HBeAg protein when present at the 5' end of HBeAg mRNA, it has no coding function for pregenomic RNA due to the lack of precore AUG codon. Instead, it forms a stem-loop structure that directs the packaging of pregenomic RNA into core protein particles, a step preceding viral DNA replication (Fig. 3, left panel) (Junker-Niepmann et al., 1990). As shown in Table 1, the C-7G, C-7S, C-7R, and C-7F substitutions are achieved by single point mutations at nucleotide 1880 or 1881 of the loop of the \( \varepsilon \) signal, which is crucial for RNA packaging and extremely sensitive to perturbation. Single nucleotide substitutions in the loop have been found to abolish packaging of pregenomic RNA and hence viral replication (Knaus and Nassal, 1993; Pollack and Ganem, 1993; Tong et al., 1992).

The replication defect of the C-7 mutants lies at the step of pregenome packaging

Consistent with the cis effect of the C-7 mutations, nucleotide transition at position 1880 (T1880C/C-7R) impaired viral genome replication less dramatically than nucleotide transversions (T1880G/C-7G and T1880A/C-7S) (Fig. 2A and Table 1). Primer extension assay of total cellular RNA revealed similar levels of precore and pregenomic RNAs produced from cells transfected with the wild-type genome, T1880C, T1880A, and T1880G mutants (Fig. 3, right panel). However, pregenomic RNA associated with core particles was reduced in the three mutants progressively, with virtually no such RNA in cells transfected with the T1880G and T1880A mutants. Thus, nucleotide changes at 1880 indeed impair viral replication at the step of pregenomic RNA encapsidation.

Mutations of the C61 codon do not compromise viral replication

We have recently characterized dozens of naturally occurring genotype A isolates of HBV (Parekh et al., 2003). While analyzing full-length clones derived from

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![Diagram](https://via.placeholder.com/150)

Fig. 3. The replication defect of the C-7 mutants lies at the step of pregenome encapsidation. (Left panel) The secondary structure of pregenome encapsidation signal. Mutations of the C-7 studied here cover nucleotides 1880 and 1881, which are located in the loop of the pregenome encapsidation signal. Of the three point mutations involving nucleotide 1880, the nucleotide transition (T1880C) incurred least reduction in viral replication. (Right panel) Transcription and encapsidation of pregenomic RNA from the C-7 mutants. Total RNA was isolated from transfected cells by Trizol reagent. To extract encapsidated pregenomic RNA, intracellular core particles were concentrated from cell lysate by ultracentrifugation through sucrose cushion. The precore and pregenomic RNA species were detected by primer extension analysis. Positions of the two RNA species are indicated. End-labeled HaeIII fragments of \( \phi x174 \) DNA served as size markers.
these samples, we came across a clone (13.1) with no HBeAg expression despite the lack of core promoter or precore mutation. Sequence analysis revealed a single T2081C nucleotide change that converted codon 61 of the core gene from cysteine (TGC) into arginine (CGC). This C61R mutation and an artificial C61S mutation were separately introduced into clone N4 (Table 1). Transfection experiments revealed greatly reduced HBeAg levels of both mutants consistent with the loss of HBe antigenicity (Fig. 2A). In contrast to the C-7 mutants, neither mutant of C61 was compromised in DNA replication (Fig. 2B). In this regard, Nassal (1992) observed sustained viral replication when C61 and three additional cysteines (C48, C107, C183) were mutated to serines. Thus, although C61 is thought to mediate core protein dimerization, its substitution by serine does not affect core protein functions such as particle formation, pregenome packaging, and DNA replication. However, results presented below revealed impairment of virion secretion by the C61R mutation.

The C61R mutant is defective in virion secretion

In HuH-7 cells transfected with HBV DNA, both virions and naked core particles can be detected in culture supernatant. However, secreted virions contain the more mature forms of viral genome (RC and PDS), while viral DNA inside naked core particles remain in the immature single-stranded state (Parekh et al., 2003). Pattern of HBV DNA present in the culture supernatant of the C61S mutant is similar to the N4 parental clone (Fig. 2B, lower panel). This finding is in agreement with the previous report that the C48S/C61S/C107S/C183S quadruple mutant is secretion competent (Nassal, 1992). In contrast, the extracellular particles of the C61R mutant were devoid of the PDS and RC DNA forms. Further separation of extracellular particles by ultracentrifugation in cesium chloride gradient confirmed such single-stranded genome as part of naked core particles, which have higher densities than virions (Fig. 4). Particles with density of virions and PDS/RC DNA forms were observed for the C61S but not C61R variant.

The ability of a single C61R missense mutation in the core gene to abolish virion secretion is striking. Nevertheless, the neighboring L60V mutation has been found to reduce virion secretion while the L60A mutation totally blocked virion secretion (Le Pogam et al., 2000; Ponsel and Bruss, 2003). To determine the specificity of the arginine mutation on virion secretion, we generated three additional mutants of this residue: C61A, C61D, and C61F. Transfection experiments revealed normal DNA replication and virion secretion capacities of all the three new constructs (Fig. 2), suggesting that a positive (but not negative) charge at residue 61 is incompatible with virion secretion. In this regard, a short linear sequence in the preS1/Pre-S2 region of the L protein is essential for virion formation and possibly involved in physical contact with core particles (Bruss, 1997). This sequence contains several conserved arginine residues. We speculate that C61 of core protein is positioned close to an arginine residue of the L protein, and the C61R mutation repels core particle contact with L protein, thus impeding virion formation. If this hypothesis is correct, then a compensatory mutation in the pre-S domain of L protein may restore virion formation in the C61R mutant.

Secretion of viral particles containing the C61R mutant genome through co-expression of wild-type core protein

From the patient serum where the C61R mutant was derived, other clones contained wild-type sequence at the C61 codon (data not shown). To determine how the C61R genome could be released into patient serum, we performed co-transfection experiment of the C61R mutant with two expression constructs of wild-type core protein (in terms of residue 61). Artificial constructs 2A p<sup>−ɛ−</sup> and 4B p<sup>−ɛ−</sup> are incapable of replication due to a null polymerase gene and nonfunctional ɛ signal, although they continue to express “wild-type” core protein. Such replication deficient constructs could each rescue the virion secretion defect of the C61R mutant, as suggested by the appearance of PDS and RC DNA forms in association with particles concentrated from culture supernatant (Fig. 5A). Furthermore, CsCl<sub>2</sub> gradient centrifugation of extracellular particles derived from C61R/4Bp<sup>−ɛ−</sup> co-transfected cells followed by Southern blot analysis of gradient fractions confirmed virion secretion (Fig. 5B). The more potent effect of the 4B p<sup>−ɛ−</sup> construct in rescuing virion secretion may be related to its higher level of core protein. Clone 4B is a core
promoter mutant (Parekh et al., 2003) and expresses high levels of core protein (unpublished observation). Our findings indicate that the mutant core protein, albeit unable to support virion secretion, does not block virion formation mediated by the wild-type core protein. Certainly, we do not know whether mosaic core particles with both wild-type core protein and C61R mutant were formed and enveloped.

In conclusion, single missense mutations of the C61 codon may effectively destroy HBe antigenicity without compromising viral genome replication or virion secretion, as exemplified by the C61S (G2082C) and C61F (G2082T) mutants. Single point mutations of the C-7 codon will greatly impair packaging of pregenomic RNA, an essential step in viral replication cycle. Such a defect may explain why C-7 mutations were found in liver cancer tissues lacking active viral replication but not in adjacent non-tumorous tissues, where viral replication persisted (Manzin et al., 1992). Since genotype A of HBV is compromised in conversion into HBeAg-minus mutants through the G1896A nonsense mutation in the precore region (Li et al., 1993), missense mutations of C61 may represent an alternative avenue of escaping the anti-HBe immunity. Further studies are needed to evaluate the prevalence of the C61 mutation in genotype A strains at the anti-HBe stage of infection.

Materials and methods

Site-directed mutants

Clone N4 is based on the naturally occurring core promoter mutant called 3.4 (Parekh et al., 2003), with the AvrII–EcoRV fragment replaced by sequence from clone 4B (Khan et al., 2004). This replacement restored efficient HBsAg detection. The site-directed mutants generated for this study are listed in Table 1. Each mutation was introduced by overlap extension polymerase chain reaction (PCR), using High Fidelity PCR system (Roche). First, two separate PCR reactions of 50 \(\mu\)l were performed: the 1501 sense primer (5'-GTCTGCCGTTCCAGCCA-3') together with the mutagenic antisense primer, and in another tube, mutagenic sense primer in conjunction with 2664 antisense primer (5'-GGATAGAATCTAGGCA-3'). Each PCR reaction employed 100 \(\mu\)M of dNTP, 1 \(\mu\)M of primers, 100 ng of template DNA, and 1 u of polymerase. Samples were denatured at 95 °C for 5 min, followed by 23 cycles of amplification (95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2.5 min). The two separate PCR reactions of 50 \(\mu\)l were performed: the 1501 sense primer (5'-GTCTGCCGTTCCAGCCA-3') together with the mutagenic antisense primer, and in another tube, mutagenic sense primer in conjunction with 2664 antisense primer (5'-GGATAGAATCTAGGCA-3'). Each PCR reaction employed 100 \(\mu\)M of dNTP, 1 \(\mu\)M of primers, 100 ng of template DNA, and 1 u of polymerase. Samples were denatured at 95 °C for 5 min, followed by 23 cycles of amplification (95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2.5 min). The two PCR products were gel purified, and a 1/25th were combined as templates for the second round of PCR (25 cycles), which employed 1501 and 2664 primers. The PCR products were double digested with either RsrII–BspE1 or RsrII–ApaI, and cloned into the same sites of clone N4. The PCR-derived region of each mutant was verified by customer sequence analysis at the Keck Laboratory of Yale University. Each mutant construct was converted into tandem dimer version in the EcoRI site of pUC18 vector (Parekh et al., 2003). The plasmid DNA was purified by a commercial kit (Marligen), further extracted with phenol and chlorofom/isooamyl alcohol, and dissolved in TE buffer.

Two replication-deficient dimers capable of core protein expression were used for the rescue of virion secretion defect.
of the C61R mutant. Constructs 2A p^-/e^- and 4B p^-/e^- were derived from 2A and 4B genomes, respectively (Parekh et al., 2003), by introduction of G1879T/T1880A double mutation into the loop of the ε signal, as well as a C2589T nonsense mutation into the polymerase gene. The mutants were converted into tandem dimer in the EcoRI site of pUC18 vector. Due to the defective ε signal, these two genomes cannot be packaged into core particles.

Transfection

The HBV dimers were transfected into Huh7 hepatoma cells via TransIT Transfection system (Mirus), together with SEAP cDNA to control for variation in transfection efficiency. Serum-free MEM medium (150 μl) was mixed with 4 μl of trans-LT1 reagent by vortex, and incubated at room temperature for 15 min with additional vortex. Following addition of 1.5 μg of HBV dimer DNA and 0.1 μg of pSEAP2 DNA (Clontech), samples were further incubated for 15 min, and added dropwise to Huh7 cells grown in 6-well plates (9.4 cm²/well). Cells had been seeded the day before transfection at around 4 × 10⁵/well using complete MEM medium (containing 10% fetal bovine serum), and were 50–80% confluent at the time of transfection. Following overnight incubation, cells were washed once, and cultured in complete MEM medium at 37 °C for 4 additional days before harvesting. No medium change was performed during this period. As a negative control for replication, HBV clone 5.4, which is defective in genome replication and HBeAg expression due to a single nucleotide deletion in the core gene (Parekh et al., 2003), was transfected in parallel. For the trans-complementation assays shown in Fig. 5, a total of 2 μg of DNA (for example, 1 μg of C61R DNA plus 1 μg of 2A p^-/ε^- DNA) was transfected to each well of the 6-well plates.

Analysis of HBV DNA replication and virion secretion

The details of these assays have been described elsewhere (Parekh et al., 2003). Briefly, HBV genome replication was measured by Southern blot analysis of DNA associated with intracellular core particles. Extracellular viral particles (both naked core particles and enveloped Dane particles) were concentrated by ultracentrifugation through 10–20% sucrose cushion, and DNA was extracted for Southern blot analysis. In experiments where further separation of Dane particles from core particles was necessary, concentrated particles were resuspended in 4.5 ml of TEN buffer, CsCl (1.5 g) was added and dissolved, and samples were spun in a Sorvall SW65 rotor at 46,000 rpm, 12 °C for 48 h. Aliquots of 400 μl were taken from the top, weighed, and dialyzed against TEN buffer to remove CsCl. CaCl₂ and MgCl₂ were added to final concentrations of 8 and 6 mM, respectively, and samples were treated with 1 U of DNaseI and 1.5 U of mung bean nuclease at 37 °C for 15 min. Next, samples were treated with 0.5 μg/ml of proteinase K in the presence of 25 mM Tris (pH 7.5), 10 mM EDTA, and 0.5% SDS. DNA was extracted by phenol, precipitated with ethanol using glycogen as a carrier. HBV DNA was detected by Southern blot analysis. Quantification of viral DNA in the Southern blots was achieved by phosphoimage analysis, using model STORM820 (Molecular Dynamics) and ImageQuant software.

Primer extension analysis of total and encapsidated pregenomic RNA

Huh7 cells from one well of 6-well plates were transfected with 2 μg of HBV dimer DNA. RNA was harvested at day 3 post-transfection with 1 ml of Trizol (Invitrogen), extracted with chloroform, and precipitated with isopropanol. The RNA pellet was dissolved in nuclease-free water, and 10 μg was incubated at 58 °C for 30 min with the antisense primer 5′-GACTCTAAGGGTCTCTGATACAGAG-3′ in the AMV reverse transcriptase buffer (the AMV reverse transcription kit from Promega). The oligonucleotide had been labeled with γ-32P ATP using polynucleotide kinase. Following cooling down for 10 min at room temperature, the annealed oligonucleotide was extended by AMV reverse transcriptase at 42 °C for 30 min. The product was heated at 90 °C for 10 min before separation in a 4% acrylamide gel containing 7M urea. As a molecular size marker, HaelIII-digested φX-174 DNA was end labeled with γ-32P ATP and run in parallel. The gel was dried and DNA signals were revealed by autoradiography.

For analysis of encapsidated pregenomic RNA, cells derived from two wells of 6-well plates of Huh7 cells were harvested at day 3 post-transfection, and lysed with 400 μl of a buffer containing 10 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 1% NP40. The lysate was layered on top of 20% sucrose solution prepared in TEN buffer, and centrifuged overnight at 46,000 rpm in the Sorvall SW65 rotor to pellet core particles. The solution was removed except for the bottom 100 μl, and the pellet was resuspended by vortex. The volume was brought up to 150 μl with TEN buffer, and non-encapsidated RNA was degraded by Staphylococcus aureus nuclease (2 μg/ml) at 37 °C for 40 min, in the presence of 2.5 mM CaCl₂. Next, 240 μl of proteinase K buffer (25 mM Tris, pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) was added, and core particles were digested at 37 °C for 1 h with proteinase K (50 μg/ml). Nucleic acids were extracted with phenol, and precipitated with isopropanol in the presence of glycogen. Following resuspension in water, primer extension assay was performed in the same manner as described for total RNA.

Measurement of HBsAg, HBeAg, and SEAP

HBeAg present in culture supernatant was detected by the EBK 125I Radioimmunoassay Kit (DiaSorin) using 5 μl of samples. The values shown in Fig. 2A are enzymatic activities ×10-7. The HBsAg was measured from 5 μl of
culture supernatant by the Auszyme Monoclonal HBsAg Kit (Abbot Laboratories). Secreted alkaline phosphatase was measured from 12 μl of culture supernatant, using the Great EscAPE SEAP reporter system (BD Biosciences). The values shown in Fig. 2A are enzymatic activities \(\times 10^{-7}\).

Acknowledgments

We thank Michael Nassal, University of Freiburg, Germany for stimulating discussions. This work was supported by grants AI54535, CA35711, DK62857, and p20RR15578 from the National Institutes of Health, an Undergraduate Teaching and Research Assistantship from Brown University (to G.B.), and by the Tan Yan Kee Foundation, Incorporated.

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