Chimeric constructs between two hepatitis B virus genomes confirm transcripational impact of core promoter mutations and reveal multiple effects of core gene mutations

Adrienne Tsai1,2, Shigenobu Kawai1,3, Karen Kwei4, Dina Gewaily, Alexander Hutter, David R. Tong, Jisu Li, Jack R. Wands, Shuping Tong*

Liver Research Center, Rhode Island Hospital, The Warren Alpert School of Medicine, Brown University, Providence, RI 02906, USA

ARTICLE INFO

Article history:
Received 27 November 2008
Returned to author for revision 17 December 2008
Accepted 3 March 2009
Available online 26 March 2009

Keywords:
Core gene
Core promoter
Core protein
DNA replication
Hepatitis B virus
Imature secretion
Naturally occurring mutations
Pre genomic RNA
Trans-complementation

ABSTRACT

Hepatitis B virus (HBV) clone 4B replicated much more efficiently than clone 2A of the same genotype. Introduction of its T1753C, A1762T, G1764A, and C1766T core promoter mutations into the 2A genome greatly enhanced genome replication and suppressed HBeAg expression. Here we show that these effects are mediated by transcriptional up regulation of pregenomic RNA and suppression of precore RNA. Analysis of chimeric constructs suggested that the 5′ end of the 2A core gene conferred higher level of pregenomic RNA, but less core protein and genome replication relative to the 4B sequence. Genome maturity of secreted virions was reduced by mutations present in the core protein of the 2A genome but enhanced by mutations found in the 4B core protein. The 4B core protein migrated faster than that of clone 2A. The possible links among the various phenotypes and the responsible mutations remain to be established.

© 2009 Elsevier Inc. All rights reserved.

Introduction

The hepatitis B virus (HBV) has a small DNA genome of 3.2 kb, which contains four open reading frames termed X, P, core, and envelope (Seeger et al., 2007). The P gene overlaps partially with the X and core genes and completely with the envelope gene. It specifies DNA polymerase (P protein), the enzyme implicated in viral genome replication. The core gene encodes core protein, which forms capsids shielding viral genomic RNA or DNA. In addition, it forms part of the coding sequence for hepatitis B e antigen (HBeAg), a secreted protein involved in modulation of host immune response. From the envelope gene three co-terminal envelope proteins: large (L), middle (M), and small (S) are generated through alternative translation initiation sites. The P gene translation initiation site is located about 500 nucleotides downstream of the 5′ end of the pg RNA. It sits downstream of the core gene initiation codon but about 150 nucleotides upstream of the core gene termination codon. Therefore, P protein translation is achieved by ribosomal leaky scanning of the core gene initiator and several internal AUGs (Fouillot et al., 1993; Hwang and Su, 1998). This low rate of P protein expression is compatible with the need for just one molecule of P protein per capsid, which is assembled from 180 or 240 copies of core protein. As expected, experiments in the related duck
hepatitis B virus (DHBV) revealed that increasing the efficiency of core protein translation reduced P protein expression, whereas ablating core protein expression increased P protein translation (Sen et al., 2004).

HBV genome replication is driven solely by the pg RNA, which serves not only as the messenger for both core and P proteins, but also as the genome precursor. The 5′ end of this 3.5-kb mRNA, which is missing in the shorter transcripts, forms a stem–loop structure that functions as the encapsidation signal. It is believed that one molecule of pg RNA and P protein is packaged into the capsid, where the P protein synthesizes the minus strand DNA from the pg RNA template by reverse transcription, using a domain of the P protein as the primer. The P protein subsequently degrades pg RNA via its RNase H domain, and synthesizes plus stranded DNA through the template of minus strand DNA. The capsid is subsequently released from the cell by budding and in doing so acquires its envelope and the three envelope proteins. Virion-associated HBV genome contains full-length minus strand DNA, and a plus strand DNA of variable lengths (from 50 to 100%). In contrast, both double stranded DNA and DNA: RNA hybrid (single stranded DNA) can be detected inside intracellular capsids. This difference in genome maturity suggests the presence of a “maturation signal” for selective or preferential envelopment of capsids containing double stranded DNA genome. In this regard, virion-associated DHBV core protein is hypophosphorylated relative to the intracellular core particles (Pugh et al., 1989). However, mutating the three phosphorylation sites of HBV core protein affected the efficiencies of pg RNA encapsidation and genome replication (Gazina et al., 2000; Lan et al., 1999; Melegari et al., 2005), not necessarily the stringency of virion secretion. A more recent study in the DHBV model suggests that alanine substitutions (to mimic unphosphorylated form) blocked reverse transcription, while aspartate substitutions (to mimic phosphorylated form) blocked plus strand DNA synthesis (Basagoudanavar et al., 2007). On the other hand, a naturally occurring I97L mutation in the core protein caused an “immature secretion phenotype” of predominantly single stranded DNA (Yuan et al., 1999). We found that the extra 12-amino acid residues unique to the core protein of genotype G reduced virion secretion efficiency but increased genome maturity of secreted virions (Li et al., 2007).

Mutations that alter HBV replication capacity may have a fundamental impact on its biology and pathogenesis. In this regard, both the pg RNA, the only transcript required for genome replication, and the pc RNA (the HBeAg messenger) are under the control of the core promoter. They are products of imprecise transcription initiation, although distinct sequence elements within the core promoter can differentially regulate the transcription of pc RNA vs. pg RNA (Yu and Mertz, 1996). The core promoter can be further divided into the core upstream regulatory sequence and basal core promoter (Honigwachs et al., 1989; Yuh et al., 1992), where mutations frequently arise during the later stage of chronic HBV infection (Kidd-Ljunggren et al., 1997; McMillan et al., 1996; Okamoto et al., 1994). The hot-spot double mutation in the core promoter, A1762T/G1764A, was found by site-directed mutagenesis to moderately increase genome replication and decrease HBeAg expression in transiently transfected human hepatoma cell lines (Buckwold et al., 1996; Li et al., 1999; Scaglioni et al., 1997). In a previous study, we compared in vitro replication capacity in transiently transfected human hepatoma cell lines (Buckwold et al., 1996; Li et al., 1999; Scaglioni et al., 1997). The impact of the 1814–2330 fragment can also be observed among constructs lacking core promoter mutations, as evidenced by higher pc RNA and pg RNA levels produced by chi9 than chi9 and chi10 among 4B-based constructs (Fig. 1C, left panel), as well as lower RNA levels produced by chi12 and chi13 relative to 2A itself (right panel).

Sequence variations in the core gene in addition to the core promoter mutations affect the steady state levels of pc RNA and pg RNA.

Fig. 1C shows the result of a representative primer extension assay of total RNA extracted from Huh7 cells at day 4 posttransfection. Clone 2A displayed a pc RNA/pg RNA ratio of about 1:1.5, while clone 4B produced 2–4 times higher levels of the pg RNA but a negligible amount of pc RNA. The transcriptional difference between the two genomes could be mapped to the 1-kb RsrII–Apal restriction fragment, which covers the core promoter region, the entire core gene, and the 5′ end of the P gene (Fig. 1B). The determinants for these phenotypes were further narrowed down using 9 additional constructs for which the basal core promoter region (positions 1570–1813), the 5′ 2/3rd of the core gene (1814–2330), or the 3′ end of core gene/5′ end of P gene (2331–2599) was selectively exchanged by overlap extension PCR (Fig. 1A, chi8–chi16).

Functional analysis revealed many unexpected features. Surprisingly, three chimeric constructs with core promoter mutations produced more pg RNA than clone 4B (Fig. 1C, chi8, chi11, chi15), and they share a 2A-derived 5′ core gene (1814–2330 fragment) (Fig. 1A). The impact of the 1814–2330 fragment can also be observed among constructs lacking core promoter mutations, as evidenced by higher pc RNA and pg RNA levels produced by chi9 than chi9 and chi10 among 4B-based constructs (Fig. 1C, left panel), as well as lower RNA levels produced by chi12 and chi13 relative to 2A itself (right panel).

Both core promoter and core gene mutations regulate core protein expression.

We used two antibodies to detect core protein expression. A polyclonal rabbit antibody (Dako) efficiently detects the wild-type core protein, but it is less reactive with the core protein of clone 4B due to an E77Q mutation (Kim K et al., unpublished). Binding of this antibody to core protein of 4B origin is subject to the washing
conditions (probably the duration of washing and/or speed of shaking), which explains the higher intensity of the Q77 variant in the left panel of Fig. 1E over the right panel or Fig. 2C. This antibody is useful in comparing core protein expression levels among constructs with the same sequence at residue 77. Comparison of the expression levels between the E77 and Q77 variants requires a monoclonal

A

B

nucleotide

BCP

pol

core

4B-based

2A-based

C

D

E

F

G

Abcam

Dako

3.2kb

1.7kb

1.5kb

RC

DL

SS

3.2kb

1.7kb

1.5kb

3.2kb

1.7kb

1.5kb

pe RNA

pg RNA

294nt

200nt

pe RNA

pg RNA

pe RNA

pg RNA

pe RNA

pg RNA
Fig. 2. Comparison of pg RNA, pc RNA transcription and core protein expression among core promoter mutants. (A) Sequence at positions 1753, 1762, 1764, and 1766 of the core promoter, with the mutations shown in boldface. Chi11 was called Ex2 in the previous study (Parekh et al., 2003). (B) Primer extension assay of pg RNA and pc RNA. (C) Western blot analysis of core protein expression. Probing the same blot with GAPDH antibody revealed similar amounts of protein loading (data not shown).

Both core promoter and core gene mutations alter genome replication capacity

The 1-kb RsrII–ApaI fragment was implicated as the major determinant for differential replication capacities: inserting the 2A sequence into the 4B background nearly abolished genome replication (Fig. 1F, left panel, compare 4B with chi3), whereas the reverse exchange greatly enhanced replication (right panel, 2A vs. chi7). Further mapping experiments revealed that the core promoter mutations alone were sufficient to maintain the high replication capacity of clone 4B (left panel, chi8 vs. 4B) and to confer enhanced replication to clone 2A (right panel, chi11 vs. 2A). However, even with wild-type core promoter sequence chi9 and chi10 with 4B background continued to display much higher replication capacity than clone 2A (left panel). Similarly, chi12 and chi13 showed higher replication capacity than clone 2A, although to a much smaller extent (right panel).

Thus, either the 1814–2330 fragment of clone 4B enhances genome replication, or the cognate fragment of clone 2A impairs genome replication. These mutations most likely work in cis, because the 2A and 4B core proteins supported genome replication to similar extents when co-transfected with the 4B core–mutant (Figs. 3A and D).

The 1814–2599 fragment controls the coupling between genome maturation and virion secretion

Considering that Huh7 cells secrete both virions and naked core particles into culture supernatant, we used a horse polyclonal anti-HB antibody to selectively immunoprecipitate virions. This antibody failed to precipitate any HBV DNA from culture supernatant of cells transfected with an HBV mutant defective in envelope protein expression (data not shown). Virion secretion can be monitored easily for constructs with high replication capacities. Presence of 4B core gene sequence (1814–2599 fragment) correlated with much higher proportion of double stranded DNA inside virions (Fig. 1G, chi1, chi2, chi7, chi12) than the core gene of 2A origin (chi8; chi11). Simultaneous detection of virions and naked core particles following their separation through CsCl gradient revealed that genome maturity associated with clone 4B and chi7 was restricted to virions: their naked core particles contained predominantly single stranded DNA similar to naked core particles released by chi8 and chi11 (Fig. 4). For chi8 and chi11, single stranded DNA was found inside both virions and naked core particles, suggesting a loss of quality control during virion formation. Treatment of DNA samples with heat prior to electrophoresis, which denatures the double stranded DNA into single stranded form but has no affect on the single stranded DNA, confirmed immature secretion phenotype of chi8 and chi11 (Fig. 5). Constructs with a mosaic core gene secreted virus particles with an intermediate degree of genome maturity (Fig. 1G, right...
panel, chi14 and chi15), suggesting that both the 5′ and 3′ ends of the core gene affect the maturity of virion-associated DNA.

The core gene rather than the P gene determines the secretion phenotype

The 3′ end of the core gene overlaps with the 5′ end of the P gene, which is mutated in clone 4B (D16V, T18A) but not 2A (Fig. 1B). To verify whether mutations in the core or P protein determine the stringency of virion secretion, we generated core− and ϵ−/p− mutants of both 2A and 4B genomes. The core− mutants will express P protein and their pg RNA is capable of encapsidation and conversion to DNA. In contrast, the ϵ−/p− mutants serve as the source of core

Fig. 3. Reconstitution of HBV genome replication and virion secretion between 4B core− dimer and a CMV promoter-driven core protein construct of 2A, 4B, or 6.2 origin. Huh7 cells were co-transfected with 1 μg of 4B core− dimer and 0.2–1 μg of CMV-core construct (panels A–C), or 0.5 μg of 4B core− dimer and 0.3–1.5 μg of CMV-core construct (panels D and E). As a positive control, cells were transfected with 1 μg or 0.5 μg of 4B dimer. (A, D) Southern blot analysis of genome replication at day 5 posttransfection. DL: double stranded linear DNA; SS: single stranded DNA. (B, E) Southern blot analysis of virion secretion at day 5 posttransfection. (C) IP-Western blot analysis of core protein expression. Core protein was immunoprecipitated from cell lysate using the Dako antibody, and detected in Western blot sequentially with the Dako and Abcam antibodies. Positions of antibodies used in IP (lg) and core protein are indicated. (F) Comparison of mobility of core protein expressed from EcoRI dimers of 2A, 4B, and 6.2 genomes.

Fig. 4. Comparison of genome maturity of virions and naked core particles among constructs with 2A- and 4B-derived core gene. Virions and naked core particles were separated by ultracentrifugation through CsCl gradient, and about 11 fractions were taken from the top. Fractions 3–10 were analyzed by Southern blot. RC: relaxed circular; DL: double stranded linear; SS: single stranded. The density of each fraction is provided at the bottom.

Fig. 5. Verification of virion-associated double and single stranded DNA via heat treatment. Virions were immunoprecipitated from culture supernatant by an anti-HBs antibody, and DNA extracted was divided into two equal parts. One part was heated at 95 °C for 5 min and cooled down on ice. DNA was separated in 1.5% agarose gel followed by Southern blot analysis. DS: double stranded; SS: single stranded; RC: relaxed circular; DL: double stranded linear.
Neither the core− nor e−/p− mutants were replication competent when transfected alone (data not shown). Co-transfection of 1.5 μg of 4B e−/p− mutant with 0.5 μg of its core− mutant reconstituted much higher genome replication than co-transfection at a 0.5 μg/1.5 μg ratio (Fig. 6A, lane 12 vs. lane 4). In contrast, co-transfection of 4B e−/p− mutant with 2A core− mutant at 0.5 μg/1.5 μg ratio reconstituted slightly more genome replication than co-transfection at a 1.5 μg/0.5 μg ratio (lane 6 vs. lane 14). Co-transfection of 2A e−/p− mutant with 4B core− mutant did not lead to efficient genome replication at any ratio. Importantly, use of 2A e−/p− mutant as the source of core protein invariably led to the secretion of virions containing single stranded DNA, whether in conjunction with 2A or 4B core− mutant (Fig. 6B, odd numbered lanes). In contrast, the 4B e−/p− mutant always conferred secretion of predominantly double stranded DNA, with a very small proportion of single stranded DNA (even numbered lanes).

The 2A- and 4B-derived core proteins respectively reduce and enhance maturity of virion-associated genomes

A variable in the trans-complementation experiments between the 2A and 4B genomes is the efficiency of genome replication, as most odd numbered lanes in Fig. 6 had lower replication capacity than even numbered lanes. To further validate the role of the core protein in the virion secretion phenotype, we inserted the 0.6-kb core gene of clones 2A, 4B, and 6.2 (wild-type) into an expression vector driven by the CMV promoter, and performed co-transfection experiments with the 4B core− mutant. Efficient reconstitution of genome replication required a high dose of the core protein construct (compare Fig. 3A with Fig. 3D), but there was little difference between the three core protein constructs. At all the doses tested, the 4B core protein was invariably associated with a higher degree of genome maturity of secreted virions than the wild-type core protein of 6.2, whereas the 2A core protein was associated with a lower degree of genome maturity than 6.2 (Figs. 3B and E). This finding, together with the results of the mapping experiments, establishes that amino acid substitutions at the N-terminus of the 2A core protein (A367T or G63V) reduce the stringency of virion secretion, whereas amino acid changes at the C-terminus of the 4B core protein (T147A, R151C, D153G, or Q179K) enhance genome maturity of secreted virions.

Discussion

In the previous study we observed marked differences in replication capacity among naturally occurring HBV isolates belonging to genotype A, and found a correlation between the high replication capacity and presence of core promoter mutations (Parekh et al., 2003). The most common core promoter mutations, A1762T/G1764A, had been found by other investigators to moderately increase genome replication (Buckwold et al., 1996; Moriyama et al., 1996; Scaglioni et al., 1997). However, we observed much greater variations in replication capacity among the natural HBV isolates, with the highest replicating clones harboring additional point mutations in the core promoter. Thus, clone 4B contains a combination of T1753C, A1762T, G1764A, and C1767T mutations in the core promoter and replicated about 10–20 times more efficiently than clone 2A with a wild-type core promoter sequence. Simply introducing the quadruplue mutation or just the 1762/1764/1766 triple mutation into clone 2A conferred about a 10-fold increase in genome replication, while the 1753/1762/1764 triple mutation and the 1762/1764 double mutation increased genome replication by about 4-fold and 2-fold, respectively. In addition, the 1762/1764, 1753/1762, 1764/1766, and 1753/1762/1764/1766 mutations reduced HBeAg expression by 20–80% (Parekh et al., 2003). In the present study, we found that the quadruple and 1762/1764/1766 triple mutation caused the greatest increase in pg RNA transcription and inhibition of pc RNA transcription, whereas the 1762/1764 double mutation was least effective (Fig. 2B). The expression of core protein followed a similar trend as the pg RNA (Fig. 2C). Therefore, the core promoter mutations studied here increase genome replication by up regulation of pg RNA transcription; they suppress HBeAg expression through reduced pg RNA transcription.

A core promoter mutant implicated in an outbreak of fulminant hepatitis also exhibited a much higher replication capacity in cell culture than a wild-type clone of the same genotype (Hasegawa et al., 1994; Liang et al., 1991). The high replication capacity was mapped to nucleotides 1574–1986, where 7 core promoter mutations are present: G1751T, T1753A, G1757A, A1762T, G1764A, C1767T, and T1768A. Remarkably, introduction of the C1766T/T1768A double mutation alone into a wild-type background reproduced a 15-fold increase in replication capacity (Baumert et al., 1996). Elegant studies were followed to elucidate the mechanism involved. The double mutation incurred merely a 2-fold increase in total pg RNA level and P protein expression (based on a reporter assay), but increased core protein expression by 15 fold and greatly increased pg RNA encapsidation (Baumert et al., 1998). High replication capacity could be reconstructed by co-transfecting the e−/p− mutant of the core promoter mutant with the core−/p− mutant of the wild-type virus, or its e−/p− mutant with the core− mutant of the wild-type virus. In contrast, co-transfection of its core−/p− mutant with the e− mutant of the wild-type virus, or its e−/core− mutant with the p− mutant of the wild-type virus, did not yield high replication capacity. Our trans-complementation experiments between the 2A and 4B genomes gave rise to very similar results when the e−/p− mutant was co-transfected with the core− mutant at a ratio of 1:1 (Fig. 6, lanes 9 and 10). However, trans-complementation between a core promoter mutant and a wild-type virus at a 1:1 ratio would inevitably lead to the assignment of the high replication phenotype to the core gene, even though expression of the
P protein, as well as the pg RNA available for encapsidation, are also increased. We found that the optimal ratio between the $e^{-}/p^{-}$ mutant and core $^{-}$ mutant of the same genome for DNA replication was 3:1, not 1:1 or 1:3, as exemplified by clone 4B (Fig. 6A, lanes 12, 8, 4). The requirement for less core $^{-}$ mutant for genome replication could be explained by the fact that only about 1/10th of the pg RNA is encapsidated, and that P protein expression is enhanced by 2–3 fold in the absence of core gene translation, at least in the DHBV model (Sen et al., 2004). Therefore, elucidating the mechanism of the high replication capacity of a core promoter mutant by trans-complementation assay would require experiments under a range of ratios between the complementing constructs such that each component is tested as a limiting factor for replication. The fact that co-transfecting 4B $e^{-}/p^{-}$ mutant with 2A core $^{-}$ mutant at 1:3 ratio was as efficient in reconstituting genome replication as co-transfection at 3:1 ratio (Fig. 6, lane 6 vs. lane 14) is consistent with clone 4B’s higher pg RNA transcription rate.

Similar to the fulminant hepatitis strain (Baumert et al., 1998; Baumert et al., 1996), we found a small (2–4 fold) difference in the levels of total pg RNA between clones 2A and 4B despite a much greater difference in their replication capacities (Figs. 1C and E). Simply introducing the core promoter mutations into clone 2A led to higher pg RNA level than achievable by clone 4B (chi11, Fig. 1C). Analysis of all the chimeric constructs suggests that the 5$'$ end of the 4B core gene (nucleotides 1814–2330), which contains 4 nucleotide changes (Fig. 1B), suppresses pg RNA production or its accumulation. Alternatively, the 5$'$ end of the 2A core gene, which contains 2 nucleotide changes, increases pg RNA production or prolongs its half life. Interestingly, the 2A sequence at the 5$'$ end of the core gene was associated with reduced core protein level, as well as reduced genome replication, relative to 4B sequence. Thus, discordance between the pg RNA and replicating DNA levels were sometimes observed: achi12 and ch13 had higher replication capacity than clone 2A despite lower levels of pg RNA and pg RNA. Similarly, ch17 and ch18 had similar level of pg RNA as 2A, but much higher replication capacity (Figs. 1C and F). We hypothesize that the rate of core particle assembly is greatly enhanced with increasing core protein concentration. The core promoter mutations increase core protein (as well as P protein) expression leading to more efficient pg RNA encapsidation and conversion to DNA, thus reducing the pool of free pg RNA. Such an encapsidation-mediated reduction in free pg RNA is disrupted by mutations in the 2A genome, which reduce core protein expression to a concentration insufficient for core particle assembly and pg RNA conversion. Our hypothesis can be tested by comparing transcript levels between clone 4B (or 2A) and its $e^{-}/p^{-}$ mutant or core $^{-}$ mutant. An alternative hypothesis is suggest by a recent elegant study in the DHBV model, where the P protein was found to inhibit reporter protein expression at the transcriptional level (Cao and Tavis, 2006). Further analysis demonstrated ability of the P protein to feed back and reduce its own transcript, the pg RNA as well (Cao and Tavis, 2006). This hypothesis can explain why the level of pc RNA, which is not encapsidated, is affected by mutations in the 5$'$ end of core gene as well.

The virion secretion phenotype of clone 2A has not been well characterized due to its low replication capacity. Simply augmenting the replication of clone 2A by core promoter mutations revealed envelopment and secretion of core particles harboring primarily single stranded DNA (Fig. 1G, chi11), in sharp contrast to the double stranded DNA secreted by clone 4B. Constructs with hybrid core gene showed intermediate phenotypes (chi14 and chi15, Fig. 1G), suggesting a contribution of both the 5$'$ and 3$'$ ends of the core gene. The two types of trans-complementation assays shown in Figs. 3 and 6 clearly indicate that amino acid changes in the core protein are responsible for altered genome maturity. Moreover, together with the results of chimeric constructs they suggest that mutations present at the N-terminus of 2A core protein (A367 or G63V) reduce the stringency of virion secretion, whereas mutations present at the C-terminus of the 4B core protein (T147A, R151C, D153G, or Q179K) enhance genome maturity of secreted virions. In this regard, other naturally occurring sequence variations in the core gene have been found to modulate virion secretion. An F97L mutation causes the immature secretion phenotype, which could be overcome by a P130T mutation (Yuan et al., 1999; Yuan and Shih, 2000). In contrast, a P75 mutation impairs virion secretion when present alone and restores wild-type secretion phenotype when combined with the I97L mutation (Chua et al., 2003). The 12-aa insertion unique to the core protein of genotype G reduces the efficiency of virion secretion but enhances genome maturity, as does the myristoylation signal in the L envelope protein (Gripion et al., 1995; Li et al., 2007).

We can envision two mechanisms to alter the genome maturity of secreted virions. First, a mutation directly facilitates or impedes plus strand DNA synthesis. Such a mutation can occur in the core gene or the P gene and is expected to alter the genome maturity of both replicative DNA inside cells and virion-associated DNA. Mutations in the phosphorylation sites of the core protein may have such an effect (Basagoudanavar et al., 2007). Second, a mutation in the core gene or envelope gene reset the time point of capsid envelopment, thus altering the genome maturity of virions but not necessarily intracellular capsids. The myristoylation signal in the L protein and the 12-aa insertion in the core protein probably ensure delayed envelopment of core particles. Since the mature genome represents a small fraction of intracellular capsids, possibly due to reduced capsid stability during genome maturation (Kock et al., 2003), a delay in the envelopment often results in reduced efficiency of virion formation. In the present study, the defect in minus strand DNA synthesis was seen mostly with secreted virus particles rather than with intracellular naked core particles (chi 7 vs. chi11, compare Fig. 1F with Fig. 1G), but at low concentration the 4B core protein was associated with higher genome maturity even for intracellular core particles (Figs. 3A and D, 0.2 pg and 0.3 pg of CMV-core constructs, respectively). Also, the virion secretion efficiency was not reduced by the 4B core gene or increased by the 2A sequence. These features are more consistent with the first mechanism.

Most studies aimed at characterizing the functional consequences of naturally occurring mutations in the HBV genome employ the approach of site-directed mutagenesis of a wild–type clone. While this approach is straightforward and the results obtained are easily interpretable, they fail to identify complex interactions among coevolving mutations. An alternative approach is to generate and characterize chimeric constructs between two HBV isolates, which is necessary when an HBV isolate possesses an unusual phenotype (such as high replication or failure of virion secretion) but the underlying mutation cannot be deduced from the sequence information. This approach is extremely time consuming and data interpretation can be complicated if both genomes contain mutations. Nevertheless, we have successfully used this approach to identify an G199E mutation in the envelope gene as responsible for impaired virion secretion of an HBV isolate, and also revealed ability of an M133T mutation in the envelope gene of clone 4B to overcome an I110M mutation, which would otherwise block virion secretion (Khan et al., 2004). In the present study, characterization of 16 chimeric constructs between the 2A and 4B genomes revealed pleiotropic effects of sequence variations in the core gene on RNA transcription, core protein expression, genome replication, and virion secretion. Further investigation will attempt to connect these phenotypic changes and identify novel regulatory mechanisms in the HBV life cycle.

**Methods**

**Chimeric constructs, site-directed mutants, and expression constructs**

Chimeric constructs between 2A and 4B genomes were generated by restriction fragment exchange, using the unique AvrII, EcoRV, Rsrl, and Apal sites present on the HBV genome (Fig. 1A). Overlap extension PCR was used to replace a fraction of the sequence within the Rsrll–
Apal fragment. Since restriction fragment exchange converts a dimer into a monomer, the chimeric HBV genome was released from the pUC18 vector by EcoRI digestion, and ligated with EcoRI-cutt, dephosphorylated pUC18 DNA at high insert:vector ratio. Clones harboring tandem dimers were identified by colony hybridization with an oligonucleotide probe and confirmed by restriction enzyme digestion as described previously (Parekh et al., 2003). Site-directed mutants were created by overlap extension PCR followed by restriction fragment exchange. The core−/− mutants contain a C2044A change that converts codon 48 from TGC to TGA. The ε−/− mutants harbor G1879T/T1880A double mutation that abolishes pg RNA encapsidation (Bang et al., 2005) as well as a C2589T nonsense mutation of the 59th codon of P gene. The CMV promoter-driven core protein constructs were generated by inserting a 0.6-kb sequence encompassing the core gene into the Xhol–EcoRI sites of the pcDNA3.1 zeo(−) vector (Li et al., 2007).

**Transient transfection and analysis of HBV genome replication and virion secretion**

Detailed experimental procedures have been described elsewhere (Bang et al., 2005; Guarneri et al., 2006; Khan et al., 2004; Li et al., 2007; Parekh et al., 2003) except for some minor modifications. Briefly, HuH7 cells seeded in six-well plates at 50–80% confluency were transfected with 2 μg of HBV dimer DNA together with 5 μg of CDNA encoding secreted alkaline phosphatase (SEAP) using the LT1 reagent (Mirus). Cells and culture supernatant were harvested 5 days posttransfection. The SEAP activity was measured from an aliquot of

This was performed essentially as described before (Bang et al., 2005) using a commercial kit (Promega). HuH7 cells were lysed at day 4 posttransfection using Trizol (Gibco/BRL), and total cellular RNA was extracted with isopropanol precipitation in the presence of glycogen, HBV DNA was quantified by running an aliquot in 5% polyacrylamide gel until the 21-kb core protein was about 1 cm to the bottom of the gel as judged from the molecular size markers. The gel was dried and radioactive

Core protein detection

Half of the cell lysate was used for Western blot analysis. Proteins were separated overnight in SDS-12% polyacrylamide gel until the 21-kd core protein was about 1 cm to the bottom of the gel as judged from the prestained molecular size markers. After transfer, the blot was incubated at room temperature for 30 min with 3% bovine serum albumin (BSA) dissolved in TBST solution (50 mM Tris, pH 7.5, 125 mM NaCl, 0.1% Tween 20) followed by incubation at 4 °C for 2–3 nights with the monoclonal antibody against core protein (14E11, Abcam) at 1:2000 dilution in 3% BSA–TBST. The blot was washed at room temperature for 40 min (10 min × 4 times) with TBST solution, and further incubated at room temperature for 1 h with a 1:40,000–60,000 dilution (in 3% BSA–TBST) of rabbit anti-mouse antibody conjugated with horseradish peroxidase (HRP). Following another wash of 40 min, signals were revealed by enhanced chemiluminescence (ECL). The antibodies were removed by shaking the blot at 37 °C for 20 min in the stripping buffer (Pierce), followed by extensive rinse with water, and wash with TBST solution for 40 min. The blot was blocked again with 3% BSA-TBST solution, and incubated at 4 °C overnight with the polyclonal rabbit antibody against core protein (Dako) at 1:2000 dilution. The remaining steps are similar to that for the monoclonal antibody, except that the HRP-conjugated goat anti-rabbit antibody (Upstate) was used at 1:100,000 dilution. The blot was stripped again, and used for the detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a mouse monoclonal antibody (Chemicon) at 1:10,000 dilution, and anti-mouse antibody at 1:40,000 dilution.

In some experiments, core protein was detected by immuno-precipitation followed by Western blot analysis. In this case, the cell lysate was diluted with PBS to a final volume of 400 μl and incubated at 4 °C overnight with a 1:1000 dilution of the Dako antibody. After addition of 10 μl of protein G-Agarose beads and a further incubation of 4 h, the immunoprecipitate was collected by low speed centrifugation, washed once with PBS, and resuspended in protein loading buffer. The subsequent Western blot analysis was the same as described above.

**Primer extension assay for pc RNA and pg RNA**

This was performed essentially as described before (Bang et al., 2005) using a commercial kit (Promega). Huh7 cells were lysed at day 4 posttransfection using Trizol (Gibco/BRL), and total cellular RNA was extracted with isopropanol. RNA concentration was determined by spectrometer and confirmed by running an aliquot in agarose gel. Ten microgram of RNA was annealed at 58 °C for 20 min with an antisense oligonucleotide 5′-GACACTTACGCTTTCGATG-3′ (positions 2033–2010) that had been end labeled with γ-32P ATP using T4 polynucleotide kinase. The annealed oligonucleotide was extended by AMV reverse transcriptase (Promega). The 5′-end labeled RNA and total cellular RNA was extracted with chloroform, and precipitated with isopropanol.

**Separation of virions from naked core particles by ultracentrifugation through CsCl gradient**

As described previously (Parekh et al., 2003), precleared culture supernatant (9 ml) was layered on top of 1 ml each of the 20% and 10% sucrose in TEN buffer, and centrifuged in a Sorvall TH-641 rotor at 39,000 rpm for 20 h. The pellet was dissolved in 4.8 ml of TEN buffer, supplemented with 1.6 g of CsCl, and centrifuged in a Sorvall AH-650 rotor at 46,000 rpm for 66 h. Fractions of 400 μl each were collected from top, weighed, and dialyzed against TEN buffer. Each sample was supplemented with 8 mM CaCl2 and 6 mM MgCl2 and digested with 4 U of DNase I and 6 U of mung bean nuclease at 37 °C for 20 min. Tris (pH7.5), EDTA (pH8.0), SDS, and proteinase K were added to final concentrations of 25 mM, 10 mM, and 0.5%, 0.5 mg/ml, respectively, followed by digestion at 37 °C for 2 h. After phenol extraction and isopropanol precipitation in the presence of glycogen, HBV DNA was analyzed by Southern blot.

**Acknowledgments**

This work was supported by the American Cancer Society grant RSG 06-059-01-MBC, and NIH grants CA35711, CA109733, CA133976, AA08169, and DK066950.


