In vivo selection of a hepatitis B virus mutant with abnormal viral protein expression

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We have investigated the molecular basis for the in vivo selective advantage of a hepatitis B virus (HBV) mutant. We have determined the complete nucleotide sequences of the major HBV forms identified at the beginning (B1-83) and end (B1-89) of a 6 year follow-up of a chronically infected patient. The B1-89 sequence showed marked nucleotide rearrangements (a nucleotide divergence of 11.3% compared with the adw2 subtype), but sequence comparison showed that both viral molecules were of common origin (62/138 mutations were found on both molecules, compared to adw2). In vitro transfection of Huh7 cells showed important modifications in B1-89 viral protein expression. We observed a decrease in B1-89 envelope protein expression associated with a modification of the migration pattern of the large envelope protein. For the B1-89 capsid protein, an insertion of 36 nucleotides at the 5' end of the C gene resulted in increased expression of a core-specific protein of abnormal size (24 kDa versus 22 kDa). Finally, our data also suggest an increase in the trans-complementation efficiency of the mutated B1-89 polymerase protein. Thus, we were able to demonstrate distinct intrinsic properties of HBV DNA molecules isolated from a chronic carrier with virus multiplication at different times during infection. Modifications of viral protein expression in the mutated form illustrate strategies used by the virus to prevent clearance and to contribute to viral persistence.

Introduction

Hepatitis B virus (HBV) is associated with a number of different clinical states, ranging from an apparently healthy carrier to acute self-limited or fulminant hepatitis and chronic liver disease. The events leading to clearance or persistence of HBV in an infected individual are still poorly understood. As with other viral infections, the outcome of HBV infection reflects a balance between the host's immune response and the ability of the virus to escape it. Indeed, a number of nucleocapsid and envelope epitopes for cytotoxic T cell responses have been described which might be implicated in the immune response to the virus (Ferrari et al., 1994; Guilhaud et al., 1992). HBV has a small (3-2 kb) circular, partially double-stranded DNA genome with four open reading frames (ORFs) coding for the capsid, envelope, polymerase and X proteins (Gerlich & Heerman, 1991). HBV replication requires reverse transcription of a pregenomic RNA (Ganem & Varmus, 1987). This reverse transcription step probably accounts for the greater genetic variability of the HBV genome compared with other DNA viruses. Indeed, on the basis of comparative sequence analysis of HBV DNA isolated at different times from a single chronic HBV carrier, it has been estimated that the rate of substitution in the HBV core gene is about 2.2 × 10−5 per nucleotide per year, a figure about 100-fold higher than for herpesviruses but 10-fold lower than for retroviruses (Okamoto et al., 1987). HBV variants probably arise during active viral replication. This is illustrated by the frequent occurrence of viral genomes containing major deletions in patients chronically infected with HBV. Thus, mutations that occur during the replication cycle permit the emergence of new viruses into the initial pool for natural selection.

The in vivo occurrence of genetic HBV variants has been extensively studied and the potential implications of mutant forms of HBV DNA are now under investigation (Carman et al., 1994; Kremsdorf & Brechot, 1994). Various naturally occurring mutations in the preS/S gene have been described, including deletions and point mutations, leading to subtypic changes (Fernholz et al., 1993; Gerken et al., 1991; Norder et al., 1992; Okamoto et al., 1989; Santantonio et al., 1992; Tran et al., 1991). One of the best characterized naturally occurring mutations detected so far affects the group-specific
determinant ‘a’ (Carman et al., 1990; Harrison et al., 1991; Yamamoto et al., 1994). Indeed, it has been demonstrated that amino acid substitutions may change the conformation of the ‘a’ determinant and lead to HBV infection despite the presence of anti-HBs (referred to as HBV ‘escape’ mutants). In the preC region the mutations most frequently identified introduce a stop codon, resulting in the inability to produce HBeAg (Carman et al., 1989). These mutations have been frequently observed in HBV carriers with persistent HBV multiplication (Brunetto et al., 1990; Carman et al., 1989; Santantonio et al., 1991).

A causal relationship between a given mutation and a biological effect is difficult to establish since most viral genomes carry more than one mutation and most individuals are infected by more than one variant. To overcome these difficulties, in vitro expression of genetically defined mutants has been investigated (Hasegawa et al., 1994; Melegari et al., 1994). However, no data are currently available on in vitro expression of HBV mutants clearly selected in vivo during the course of chronic infection.

In a previous study, we demonstrated the emergence of, and gradual takeover by mutated HBV DNA forms in an HBV chronic carrier (Tran et al., 1991). This indicated that in vivo selection of the mutant forms occurred. In the present work, we took advantage of this observation to investigate the molecular basis for this in vivo selection of mutant forms. Thus, we analysed the complete nucleotide sequence of the HBV forms characterized during the follow-up of the patient in question. In addition, in an attempt to evaluate their relative potential in the course of HBV infection, in vitro studies on replication competence and expression of viral proteins were performed. We were able to demonstrate that the HBV DNA molecules selected during chronic infection show marked modifications in expression of viral proteins.

**Methods**

**Patient.** Serial serum samples were obtained during a 6 year follow-up, from an HbsAg+ HBeAg- and HBV-DNA-positive chronic carrier with moderately active chronic hepatitis, previously referred to as patient B1 (Tran et al., 1991). Clinical status and interferon or adenine arabinoside treatments were described previously (Tran et al., 1991). Based on preS/S and preC/C nucleotide sequence analysis, different HBV DNA molecules were detected in this patient. At the beginning of the follow-up we identified, as the major form, an HBV genome (B1-83) with minor modifications compared with adw2, whereas, 6 years later, the major HBV form (B1-89) showed marked rearrangements (Tran et al., 1991).

**Plasmids.** From a 10 ml pool of serum samples obtained in a 1 year period from 1988–1989, viral particles were pelleted by ultracentrifugation (20 h at 200000 g at 4 °C) through 1 ml of 20 % sucrose. HBV DNA was extracted using protease K (1 mg/ml) in a buffer consisting of 200 mM-NaCl, 40 mM-EDTA (pH 8) and 1.5 % SDS. B1-83 and B1-89 molecules were cloned into the Bluescript vector (Stratagene). Plasmid B1-83 contains a head-to-tail dimer of 3.2 kb unit length HBV DNA cloned via the unique EcoRI site into Bluescript. Plasmid B1-89 contains a head-to-tail HBV DNA dimer cloned via the SpeI (nt 2116) site into Bluescript. The construction containing the HBV polymerase gene under the control of the metallothionein promoter was a gift from H. Schaller. The B1-89 polymerase plasmid was obtained by replacing the HBV FspI–BspEI fragment (nt 519–3211) of the HBV polymerase construct with the B1-89 FspI–BspEI fragment. The construction of head-to-tail defective HBV, corresponding to the cDNA of the singly spliced 2.2 kb RNA, has been described elsewhere (Rosmorduc et al., 1995).

**Nucleotide sequence analysis.** The complete nucleotide sequence of B1-83 and B1-89 molecules was determined by the dideoxy chain termination method (Sequenase, USB). For specific regions sequence data were confirmed by using the Applied Biosystems sequencing method (ESGS).

**Cells and transfection.** The human hepatocarcinoma HuH7 cell line was used for transfection experiments (Nakabayashi et al., 1982). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal calf serum, glutamine and antibiotics (penicillin and streptomycin) at 37 °C with 5 % CO2. DNA transfection was performed by the calcium phosphate precipitation method as previously described, at a dose of 10–15 μg of HBV plasmid DNA per 2.5 × 106 cells per Petri dish (Chang et al., 1987). In some experiments, a β-galactosidase vector (3 μg) was used to measure the efficiency of transfection (Herbomel et al., 1984). The HepG2 cell line stably transfected with HBV was used as a control (Sells et al., 1987).

**Analysis of cytoplasmic viral nucleic acids.** For Northern blot analysis of viral RNA, total RNA was extracted by the guanidium thiocyanate method (RNAzol) 2 days after transfection. Ten μg of total RNAs were separated on a denaturing 1.5 % agarose gel and transferred to a nitrocellulose membrane (Hybond-C extra). The membranes were hybridized with different 32P-labelled HBV specific probes: an S PCR fragment (nt 134–437), a B1-83 specific oligonucleotide (5′ GTGTGTGATTTTTGTACAATATGATCC, nt 235 (1231) and a B1-89 specific oligonucleotide (5′ TTGCTTAATTTCTGTGGTGT GTGTTC, nt 237–2349). Standardization of RNA was achieved using a β2-microglobulin probe. To reprobe, the membranes were first stripped by boiling in a solution containing 0.1 % SDS for 5 min.

To identify encapsidated HBV DNA, cytoplasmic fractions of 3 day transfected cells were prepared as described previously (Melegari et al., 1994). To hydrolyse any residual transfecting DNA, DNase I (100 μg/ml) and magnesium acetate (10 mm) were added. After incubation for 30 min at 37 °C, viral capsids were immunoprecipitated using polyclonal anti-HBc (gift from H. Schaller) or anti-HBe/c. Protease K (1 mg/ml) was added and incubation was continued for 4 h at 55 °C. Viral DNA was obtained from the lysate by phenol-chloroform extraction. In order to normalize the results, the efficiency of each transfection was measured using a β-galactosidase reporter vector. A colorimetric assay was used to measure the β-galactosidase activity in cytoplasmic extracts from transfected cells (Herbomel et al., 1984). Replicative forms were separated on 0.8 % agarose gels and transferred to nylon membranes (Hybond-N+). Membranes were hybridized with a 32P-labelled HBV-specific probe.

**Viral protein analysis.** Secretion of envelope and capsid proteins into the culture medium was measured by radioimmunoassay (RIA). For envelope protein detection, rabbit polyclonal anti-HBs IgGs were used on the solid phase and monoclonal antibodies directed against HBs (F39.20), preS2 (F124) or preS1 (F35.25) in the detector phase, as described in detail elsewhere (Petit et al., 1990). HBeAg was determined using a commercial RIA from Abbott Laboratories. For analysis of
released viral particles, the culture medium was centrifuged at 200000 g for 2 h and the pellet analysed by SDS-PAGE using the monoclonal preS1 (F35.25) antibody. Binding was detected by incubation with the 125I-labelled F(ab')2 fragment of anti-rabbit IgGs (Amersham) (Gerken et al., 1991; Tran et al., 1991).

For cellular capsid protein analysis, cells were harvested 3 days after transfection and the supernatants immunoprecipitated with human anti-HBe/c IgGs. The immune complexes were analysed by Western blot. After transfer, the filters were incubated with rabbit polyclonal antibodies against HBe/c and binding was detected by incubation with the 125I-labelled F(ab')2 fragment of anti-rabbit IgGs. For immunohistochemical analysis, transfected cells were collected 3 days after transfection and spotted onto slides with a Shandon Cytospin-2. An immunoperoxidase based assay was then performed using monoclonal antibodies against preS1 (F35.25), preS2 (F124), HBs (F39.20) and HBe (F8), as previously described (Gerken et al., 1991; Tran et al., 1991).

Results

Sequence analysis

In a previous study of serial serum samples from an HBsAg- and HBeAg-positive HBV chronic carrier we demonstrated, using PCR, the emergence of and takeover by HBV DNA molecules with nucleotide rearrangements in the preS/S and preC/C domains (Tran et al., 1991). Serum samples were obtained during a 6 year follow-up (1983, 1985, 1988 and 1989). The availability of serial serum samples from the same chronically infected individual allowed us to observe the kinetics of appearance of rearranged HBV DNA molecules. During the first 2 years of the follow-up only molecules with minor rearrangements were identified, whereas analysis of serum samples obtained 2, 5 and 6 years later showed the gradual emergence of and takeover by mutated HBV molecules (Tran et al., 1991).

In order to determine the complete nucleotide sequence and to investigate expression in vitro, the major molecular forms observed at the beginning (B1-83) and end (B1-89) of the follow-up were cloned as indicated in Methods. The complete nucleotide sequences of B1-83 and B1-89 are shown in Fig. 1. Compared with the adw2 subtype a total nucleotide divergence of 4.3% (138/3221) and 11.3% (363/3221) was observed for B1-83 and B1-89, respectively (Ono et al., 1983). Sequence comparison of B1-83 and B1-89 showed 81 point mutations located at the same sites, of which 62 were identical. Thus, 44.9% of the point mutations detected in isolate B1-83 were also found in isolate B1-89, indicating that the B1-89 isolate had emerged during replication of the B1-83 isolate. Furthermore the B1-89 isolate contained, in addition to point mutations, an insertion of 36 bp (nt 92–128), deletions of 6 bp (after nt 578) and 3 bp (after nt 1102), and three instances of a nucleotide deletion associated with the insertion of one nucleotide (deletions after nt 841, 1226 and 3075; insertions at nt 838, 1230 and 3082) (Fig. 1). When these sequences were compared with sequences that we have previously published for the B1-83 and B1-89 preS/S and preC/C regions, few nucleotide sequence differences were observed (data not shown) (Tran et al., 1991). The major modification was an insertion of one nucleotide (nt 1230) after a 1 bp deletion in B1-89 (Fig. 1). This new insertion restores the preS1 and polymerase encoding capacity. This modification was not detected in the partial sequence previously published. This discrepancy, which resulted from technical problems, was solved by the use of a different sequencing technique. Thus, with the exception of HBeAg, none of the rearrangements led to the expression of interrupted viral proteins.

Nucleotide and amino acid divergence, in the different HBV genes, of the B1-83 and B1-89 forms relative to the adw2 subtype is summarized in Table 1. In the preS/S region, sequence divergence of B1-83 and B1-89 from adw2 was 3.3% and 10.3% for nucleotide sequence, and 4.3% and 13.3% for amino acid sequence. The highest divergence was in the preS1 and preS2 domains (Table 1). In the preC/C region, nucleotide sequence divergence from the adw2 nucleotide sequence was 3.9% (preC, 2.3; C, 1.1) for B1-83 and 10.3% (preC, 4.6; C, 11.2) for B1-89 (Table 1). For the X and polymerase genes, the genetic variability was located in the central part of the X protein, and in the C-terminal part of the terminal protein and the N-terminal part of the spacer domain of the polymerase (Fig. 2).

Analysis of viral transcript synthesis in B1-83 and B1-89 transfected Huh7 cells

To compare viral transcript synthesis from B1-83 and B1-89 HBV molecules, head-to-tail dimers of both forms were constructed and transfected into Huh7 cells. B1-83 and B1-89 viral transcripts were detected by Northern blot. Using an S HBV DNA probe which revealed both B1-83 and B1-89 transcripts, all the expected viral transcripts (3.5, 2.6 and 2.1 kb) were detected (Fig. 3a). After semi-quantification of the total RNA with a β-globin probe it appeared that the level of B1-83 viral transcripts was higher than that of B1-89 viral transcripts (Fig. 3a). To confirm the specificity of the viral transcripts the membrane was rehybridized with two different oligonucleotide probes specific for B1-83 and B1-89 sequences (see Methods). As expected, specific hybridization with no cross-hybridization was observed (Fig. 3b, c). Additional bands observed in all the transfection experiments correspond, as show by hybridization with a plasmidic probe, to readthrough events (data not shown). These results demonstrated the capacity of both B1-83 and B1-
Fig. 1. For legend see opposite.
Table 1. Nucleotide and amino acid sequence divergence between isolates HBV B1-83 or B1-89 and the adw2 subtype

<table>
<thead>
<tr>
<th></th>
<th>PreS1</th>
<th>PreS2</th>
<th>S</th>
<th>PreC</th>
<th>C</th>
<th>X</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1-83/HBV adw2</td>
<td>4.5 (7-6)</td>
<td>7.3 (10.9)</td>
<td>1.6 (6-9)</td>
<td>2.3 (3-4)</td>
<td>4.1 (1-1)</td>
<td>28 (3-9)</td>
<td>4.4 (5-6)</td>
</tr>
<tr>
<td>B1-89/HBV adw2</td>
<td>20.4 (24-4)</td>
<td>13.9 (21.8)</td>
<td>3.8 (5-3)</td>
<td>4.6 (4-)</td>
<td>11.2 (5-4)</td>
<td>15.2 (18-2)</td>
<td>10.4 (12-6)</td>
</tr>
</tbody>
</table>

* The PreC amino acid divergence was not determined because of the presence of a stop codon in the preC region.

89 to synthesize 3-5, 2-6 and 2-1 kb viral transcripts. Owing to their location in the genome, the probes used here were unable to detect the X transcript.

**HBV replicative capacity in B1-83 and B1-89 transfected Huh7 cells**

In order to determine the replicative capacity of the B1-83 and B1-89 forms the amounts of encapsidated viral DNAs were measured. HBV particle associated DNA was isolated from the cytoplasm of transfected cells and subjected to Southern blot analysis, as described in Methods. After hybridization with an HBV probe, signals for encapsidated viral DNA replicative intermediates were observed for both B1-83 and B1-89 forms (Fig. 4a). We repeatedly observed a slightly higher level of B1-89 DNA replicative molecules.

We therefore determined the importance of mutations in the polymerase gene on replication efficiency in trans-complementation experiments. Cotransfection experiments were performed to bypass a possible difference in the efficiency of core particle immunoprecipitation, as a result of major variations in B1-89 core amino acid sequences. The cotransfected constructions were the wild-type or B1-89 polymerase genes under the control of the metallothionein promoter and a head-to-tail dimer of a defective HBV DNA (dHBV). This defective HBV DNA, deficient for replication and encapsidation, corresponds to the cDNA of the singly spliced 2-2 kb RNA which showed a deletion from the last codon of the core gene to the middle of the S gene (Rosmorduc et al., 1995). As we have previously demonstrated, owing to the lack of complete ORFs encoding the polymerase and envelope proteins, this defective HBV DNA requires helper HBV for its replication (Rosmorduc et al., 1995). In order to make the assays semi-quantitative, the results were normalized by taking into account the level of β-galactosidase expression. The amount of replicative form was significantly and reproducibly higher (about 2-fold) in the cotransfection that included the B1-89 polymerase gene, as compared to the cotransfection that included the wild-type polymerase gene (Fig. 4b, lanes 2 and 4). In addition, after RNase H treatment, no modification of the replicative intermediate forms was observed (Fig. 4c). This indicated that in both cotransfections reverse transcription had occurred and given rise to single- and double-stranded DNA. These experiments also suggested an increased trans-complementation efficiency of the B1-89 mutated polymerase.

**Detection of viral proteins in B1-83 and B1-89 transfected Huh7 cells**

HBV antigens were assayed in the culture medium of transfected cells by RIA. Envelope proteins were detected using specific monoclonal antibodies against preS1 (F35.25), preS2 (F124) and S (F39.20). HBe antigen was detected using a commercial RIA (Abbott). The level of secreted preS1, preS2 and HBeAg, but not of HBsAg, was strongly reduced (about 80%) in cells transfected with the HBV mutated form, B1-89, compared with B1-83.
corresponding to the forms of the large envelope protein 

Western blot analysis of capsid proteins in transfected cells showed expression of the usual 22 kDa major core protein for B1-83 (Fig. 5c, lane 2) and a predominant abnormal 24 kDa protein for B1-89 (Fig. 5c, lane 3). We observed that the B1-89 24 kDa protein was very strongly expressed, and that minor bands at 27 and 30 kDa were also detected (Fig. 5c, lane 3).

Finally, immunohistochemical analysis of viral antigens expressing core (F8), preS1 (F35.25), and preS2 (F124)
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3.5
2.6
2.1

(a) (b) (c)

Fig. 3. Northern blot of B1-83 and B1-89 viral transcripts. Total RNA was extracted from Huh7 cells transfected with B1-83 (lanes 1) or B1-89 (lanes 2) DNAs. The membrane was sequentially hybridized with an S probe (a) or B1-83 or B1-89 specific oligonucleotides (b and c, respectively). Semi-quantification of RNA was performed with a β2-microglobulin probe (β2m).

and S (F39.20) epitopes in B1-83 and B1-89 transfected cells showed no clear differences in viral protein localization (Fig. 5d).

Discussion

In this report we have described the molecular basis for the in vivo selective advantage of an HBV mutant during the course of chronic infection. Indeed, we were able to demonstrate that certain viral protein modifications might account for this advantage.

Analysis of the complete sequence data showed that most of the mutations were point mutations. The different deletions observed do not modify the ORFs. Indeed, either the deletions had conserved the ORF or, if not, were associated with an insertion which restored the initial ORF. Sequence comparison showed that 62 of the HBV B1-83 and B1-89 point mutations were identical when compared to the adw2 subtype. These conserved point mutations occurred throughout the genomes. This confirmed that B1-89 was derived from B1-83. However, a striking observation was the high sequence divergence (10.9%) between the two viral DNAs. Taking into account the available data on the rate of HBV genetic variability (about 1.45 × 10⁻⁵ non-synonymous substitutions per site per year) (Orito et al., 1989), it seems unlikely that the sequence evolution of B1-83 to B1-89 had occurred in patient B1; this patient was probably infected 5 years before initiation of the follow-up. Thus, the only explanation could be that the patient was infected by a mixed viral population and that the B1-89 form underwent a selective expansion with time. In this regard, it is also quite plausible that antiviral treatment and/or specific host immune system effects favoured the selection and emergence of the mutated form in this patient. This hypothesis is reinforced by our previous finding, using PCR with sets of primers specific for B1-83 or B1-89 sequences, of the B1-89 form in the patient’s serum before antiviral treatment (Tran et al., 1991).

Our in vitro studies underlined important modifications in B1-89 viral protein expression and viral replication. We observed a significant decrease of viral B1-89 envelope protein secretion, probably explained by a decrease in the 2.1 and 2.6 kb HBV transcripts. This down-regulation of the envelope transcripts might be due to mutations in transcriptional control elements. Indeed, we found several nucleotide modifications in a region (nt 1200–1650) which was recently reported to be essential for high-level expression of the major envelope protein (Huang & Liang, 1993).

For the large envelope protein, a modified migration pattern was observed (two bands at 43 and 45 kDa). The
same modification pattern was also observed, in vivo, in comparison with the circulating envelope protein in the serum of patient B1 obtained in 1989 (Tran et al., 1991). The large protein exists in two unglycosylated (p39) and monoglycosylated (gp42) forms (Ganem & Varmus, 1987; Gerlich & Heerman, 1991). Glycosylation takes place on Asn-146 of the major protein (Heermann et al., 1984). The pattern of the B1-89 large envelope protein was compatible with the existence of mono- and diglycosylated forms. Indeed, it might be hypothesized that preS1 amino acid changes would have induced modification in the protein topology and that hence Asn-4 of the preS2 protein became accessible for glycosylation. This amino acid is usually glycosylated in the middle envelope protein and not in the large envelope protein (Heermann et al., 1984). One might speculate that such modification of the conformation of the preS1 envelope protein could modify the host immune response to the virus and/or the biological cycle of the virus.

For the B1-89 capsid protein, the insertion of 36 nucleotides at the 5′ end of the C gene resulted in the expression of a core-specific protein of abnormal size (24 kDa versus 22 kDa). This insertion might induce modifications of both the encapsidation signal sequence and the core protein structure. In addition, strong expression of this modified capsid protein in B1-89 transfected cells was observed when compared to the wild-type capsid protein. Accumulation of this 24 kDa protein also occurred in cotransfection experiments (B1-83/B1-89, data not shown). At present, we do not know whether the intracellular accumulation of this viral protein is related to impaired morphogenesis of complete virions, increased synthesis of the protein, modifications of its degradation or decreased secretion. Interestingly,
Hasegawa et al. (1994) have demonstrated that an HBV mutated genome isolated from a patient with fulminant hepatitis induced, in transient expression experiments, a twofold increase in the half-life of the capsid protein. It should be pointed out, however, that in our case the mutated genome was isolated from a chronic patient without severe liver disease. This therefore indicates that capsid protein accumulation is not sufficient by itself to account for the severity of acute or chronic hepatitis.

A paradoxical observation was the detection of
HBeAg despite the presence of a stop codon in the preC region of the B1-89 strain. This might be explained by conformational antigenic modifications induced by the genetic rearrangements in the C coding region, and/or by weak cross-reactivity of the anti-HBe antibody with the capsid protein.

It is now well established that core and polymerase proteins are required for pregenomic RNA encapsidation (Bartenschlager & Schaller, 1992; Hirsch et al., 1990). The rearrangement that we detected in the mutated polymerase protein did not affect the replication competence of B1-89. In fact, most of the amino acid changes were located in the spacer domain. This confirmed that this region was not essential for viral polymerase activity. Interestingly, in cotransfection experiments we observed. When compared to wild-type polymerase protein, that the mutated B1-89 polymerase leads to higher levels of pregenomic RNA encapsidation was previously reported in an HBV strain associated with fulminant hepatitis, and the authors suggested that HBV mutants with enhanced viral replication may be important in the pathogenesis of fulminant hepatic failure (Hasegawa et al., 1994). However, as shown in Fig. 4(a) (lanes 1 and 2), transfections with full-length B1-83 and B1-89 HBV DNA only led to a minor, yet reproducible, increase in B1-89 replicative intermediates. It should be noted that this was observed despite a low level of B1-89 transcription; this favours the hypothesis that the enhancement of viral replication might result from a more efficient encapsidation step. It might illustrate modulation of phenotypic effects of some mutations in the context of a whole genome.

In conclusion, we were able to demonstrate distinct intrinsic properties of HBV DNA molecules isolated from a replicative chronic carrier during the course of infection. It is plausible that antiviral treatments might have played a role in the selection of pre-existing mutated viral DNA capable of escaping the immune host response. Modifications of viral protein expression of the mutated B1-89 form illustrate strategies used by the virus to prevent clearance and to contribute to viral persistence. Altogether, this combination of in vivo and in vitro approaches should provide a better understanding of the effect of HBV genetic variability on viral persistence.

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