JOURNAL OF VIROLOGY, July 2003, p. 7673–7676 Vol. 77, No. 13
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.13.7673–7676.2003

Coexistence of Two Distinct Secretion Mutations (P5T and I97L) in Hepatitis B Virus Core Produces a Wild-Type Pattern of Secretion

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Received 23 January 2003/Accepted 2 April 2003

Unlike a Tokyo isolate of hepatitis B virus variants, we found a Shanghai isolate that secretes few virions with an immature genome despite its core I97L mutation. Core mutations P5T and I97L were found to be mutually compensatory in offsetting their respective distinct effects on virion secretion.

Hepatitis B virus (HBV) replicates by using reverse transcriptase (11, 24). Due to the low fidelity of the polymerase, error-prone replication and natural selection in vivo lead to the accumulation of multiple mutations in HBV variants predominant in chronic carriers (20, 22). The most frequent natural mutation in the HBV core protein occurs at amino acid 97 (5, 6, 9, 10, 13). It remains a challenge to elucidate the functional significance of these prevalent and predominant mutations, since there is no a priori knowledge about what kind of assays should be used. Recently, a so-called "immature secretion" phenotype was demonstrated to be a global phenotype in tissue culture by introducing a 97L mutation into the core gene of a wild-type HBV genetic background of subtype *adr* and *ayw* origins (31, 33). This phenotype of HBV variant 97L is interesting, because it represents an exception to the dogma of preferential export of virions containing mature genomes (higher-molecular-weight viral DNA in relaxed circle form) in wild-type hepadnaviruses (24). Unlike the wild-type HBV, the 97L mutant secretes similar amounts of mature and immature genomes (lower-molecular-weight viral DNA in single-strand form). This phenotype is not caused by any deficiency in reverse transcription (33) or any instability of core proteins and particles (31, 33; M. Newman, F. M. Suk, and C. Shih, unpublished results).

Although an immature secretion-like phenotype has also been found to occur in vivo in woodchuck and snow goose hepadnaviruses (4, 26), it is puzzling that it has not been found so far in natural infection in humans (F. M. Suk, M. H. Lin, and C. Shih, unpublished results). Furthermore, it remains unclear whether an immature secretion phenotype can still be observed in the genetic context of naturally occurring variants, which often contain multiple mutations throughout the genome. To address these issues, we took a reciprocal approach by reverting the natural mutation at amino acid 97 in a naturally occurring HBV variant from leucine back to isoleucine (L97I) and asked whether the predicted immature secretion phenotype can be abolished by eliminating the leucine residue at position 97 of the HBV core antigen (HBcAg).

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This parental HBV variant clone of *adr* subtype origin was isolated from a hepatocellular carcinoma patient from Shanghai, China (clone 14 in references 18 and 19). In addition to the hot spot mutation I97L, it contains multiple frequent mutations, including core mutations P5T and S87G, enhancer II mutations at nucleotides 1762 and 1764, and mutations truncating the X protein and abrogating the production of the pre-S2-containing M envelope protein (18). The 3.2-kb monomeric HBV genome in clone 14 was released from the pUC18 vector by *Sap*I digestion, religated, and linearized by *Bam*HI before being cloned into plasmid pBluescript. This HBV variant plasmid in pBluescript underwent tandem dimerization and is referred to as "Shanghai *adr*," while a wild-type *adr* HBV plasmid, obtained from K. Koike (30), was recloned and dimerized in tandem in pBluescript and is referred to here as "Tokyo *adr*."

HepG2 and Huh7 cells were transfected with 10 μg of plasmid DNA by the calcium phosphate method as previously described (31). Transfected cells were harvested on day 7 posttransfection and analyzed for HBV replication by Southern blotting. Medium was collected on days 5 and 7 posttransfection, and virus particles were purified through a 20% sucrose cushion followed by CsCl gradient ultracentrifugation. Fractions containing virion particles were collected, and HBV DNA was extracted and subjected to Southern blot analysis.

To our surprise, as shown in Fig. 1, the parental Shanghai *adr* isolate, abbreviated "P5T/I97L" here, does not exhibit any immature virion secretion, despite the presence of a leucine residue at HBcAg position 97 (31, 33). One interpretation of this intriguing result is that a leucine residue at amino acid 97 is not always dominant for the immature secretion phenotype. Indeed, we observed a suppression effect on immature secretion by a core natural mutation, P130T (32), or a pre-S1 artificial mutation, A119F (16). However, when the published DNA sequences of this Shanghai *adr* variant were examined, there were no P130T or A119F mutations (18) (GenBank accession no. AF411408). It is therefore tempting to speculate that there is another unknown compensatory mutation present somewhere in this Shanghai *adr* variant.

As mentioned earlier, this Shanghai *adr* variant contains a number of mutations, including core mutations P5T, S87G, and I97L. This P5T mutation, changing a highly conserved pro-

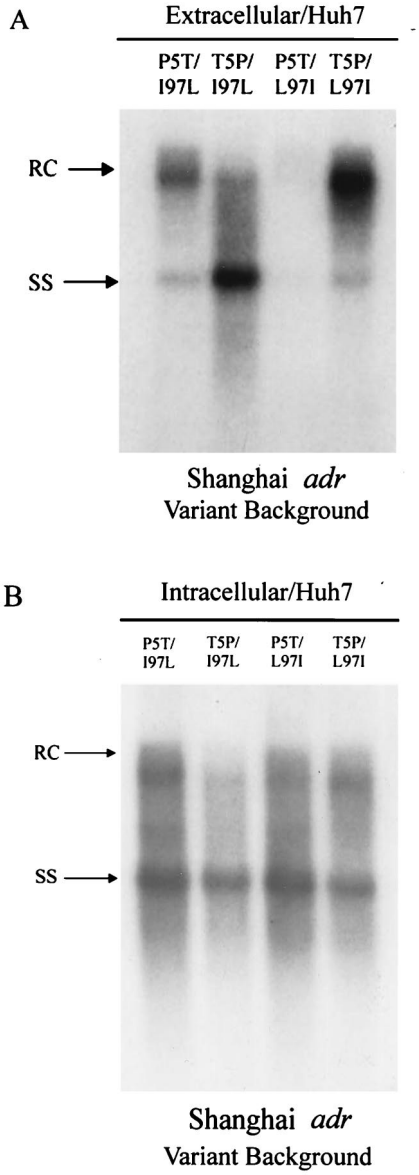

FIG. 1. A frequent HBV core mutation, P5T, is responsible for the absence of the immature virion secretion phenotype of a naturally occurring variant containing another frequent core mutation, I97L, in the variant genetic background of a Shanghai *adr* strain. Culture media from Huh7-transfected cells were analyzed for secreted virion particles (A), while HBV core-associated DNAs from transfected cell lysate were subjected to Southern blot analysis (B). RC, relaxed circular; SS, single-stranded HBV DNA replicative intermediates.

line to threonine at codon 5, was initially discovered during our sequencing studies of the e antigen mutation (13). In the genetic context of wild-type HBV, the naturally occurring core mutation P5T resulted in a low level of virion secretion (17). A similar phenotype of reduced level of virion secretion was observed when certain artificial mutations were introduced into HBcAg (e.g., P79A) (14, 21).

To test if the core mutation P5T could be compensatory for 97L-induced immature secretion, we introduced mutations into the Shanghai *adr* variant at core amino acids 5 and 97 by using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, Calif.). The oligonucleotides used for mutagenesis are shown in Table 1. The HBV monomers were then dimerized in tandem to mimic the circular configuration of an HBV genome as previously described (31). All mutations were confirmed by sequencing.

Interestingly, when the mutation T5P was introduced into the Shanghai *adr* gene, immature secretion was revealed in mutant T5P/I97L-transfected Huh7 cells (Fig. 1). In contrast, mutant P5T/L97I exhibited a "low-secretion" phenotype with almost no detectable virion secretion, a result consistent with our previous report of mutant P5T in the wild-type Tokyo *adr* background (17). Finally, when both positions 5 and 97 of HBcAg in Shanghai *adr* were replaced by wild-type amino acids, mutant T5P/L97I exhibited normal mature secretion at increased intensity (Fig. 1A). It should be noted here that there is no apparent correlation between the diverse virion secretion profiles of various genotypes in Fig. 1A and their respective intracellular activities of HBV replication in Fig. 1B. Such a lack of correlation strongly suggests that these extracellular phenotypes of virion secretion are not the result of any minor difference, if any, in their intracellular viral replication or capsid stability. Taken together, while the mutation P5T can rescue the immature secretion induced by mutation I97L, the low-secretion phenotype induced by the P5T mutation can be rescued by the I97L mutation. In other words, the P5T and I97L mutations appear to be mutually compensatory in the genetic background of the Shanghai *adr* variant.

For reasons unclear at present, the immature secretion phenotype was much less pronounced in HepG2 cells (Fig. 2). The diminished phenotype of immature secretion of mutant T5P/I97L in the Shanghai *adr* background in HepG2 cells could be related to both host factors (HepG2) and viral factors (e.g., X and M protein deficiency) (18). Previously, we noted that both the extracellular immature secretion phenotype and the intracellular replication advantage phenotype tend to be more pronounced in Huh7 cells than in HepG2 cells (23, 33). However, host factors alone cannot entirely explain this phenomenon, since mutant I97L in the Tokyo *adr* genetic background can still exhibit immature virion secretion in HepG2 cells (Fig. 3A). On the other hand, viral factors alone cannot entirely explain this phenomenon either, since mutant T5P/I97L in the Shanghai *adr* genetic context can exhibit immature virion secretion in Huh7 cells (Fig. 1A).

The compensatory effect of mutation P5T on immature virion secretion was confirmed in the genetic background of the wild-type Tokyo *adr* HBV in HepG2 (Fig. 3) and Huh7 cells (data not shown). Instead of using the genetic context of

TABLE 1. Oligonucleotides used for mutagenesis

Oligonucleotide[a]	Sequence[b]
T5P (S)	5′-ATGGACATTGAC**CCC**TATAAAGAATTTGGA-3′
T5P (AS)	5′-TCCAAATTCTTTATA**GGG**GTCAATGTCCAT-3′
L97I (S)	5′-ATGGGCCTAAAA**ATC**AGACAACTACTGTGG-3′
L97I (AS)	5′-CCACAGTAGTTGTCT**GAT**TTTTAGGCCCAT-3′
P5T (S)	5′-ATGGACATTGAC**ACG**TATAAAGAATTTGGA-3′
P5T (AS)	5′-TCCAAATTCTTTATA**CGT**GTCAATGTCCAT-3′

[a] S, sense polarity; AS, antisense polarity.
[b] Mutated codons are underlined, mutation sites are in boldface.

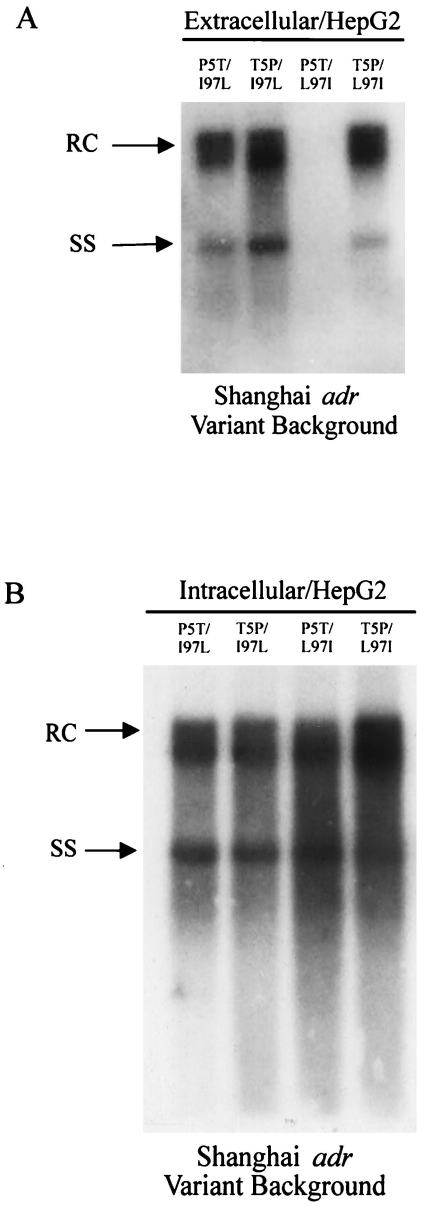

FIG. 2. The immature virion secretion phenotype of core mutation I97L in the genetic background of a Shanghai *adr* strain is not pronounced in HepG2 cells. Culture media from HepG2-transfected cells were analyzed for secreted virion particles (A), while HBV core-associated DNAs from transfected cell lysate were subjected to Southern blot analysis (B). RC, relaxed circular; SS, single-stranded HBV DNA replicative intermediates.

the wild-type HBV, to our knowledge, this was the first study of the functional significance of HBV core mutations using the genetic context of a naturally occurring variant. Our study demonstrates that not only viral replication, but also the immature secretion phenotype, can occur in Huh7 cells independent from the expression of an M envelope and full-length wild-type X proteins (18).

In the literature, out of 65 sequences containing 97L mutations, 10 (15.4%) have concurrent P5T mutations (1, 2, 5, 9, 12, 13, 15, 25, 27, 28). Previously, we reported a compensatory core mutation, P130T, which also can rescue the immature secretion phenotype induced by the I97L mutation (32). In the sequences mentioned above, a total of 43 out of 65 (66.2%) sequences were found to contain both 97L and P130T. Of these, only 3 out of 65 (4.6%) sequences contain all three mutations 97L, P5T, and P130T. Taken together, approximately 76% of HBV 97L variants isolated from chronic carriers are found to contain compensatory mutations at codon 5 or

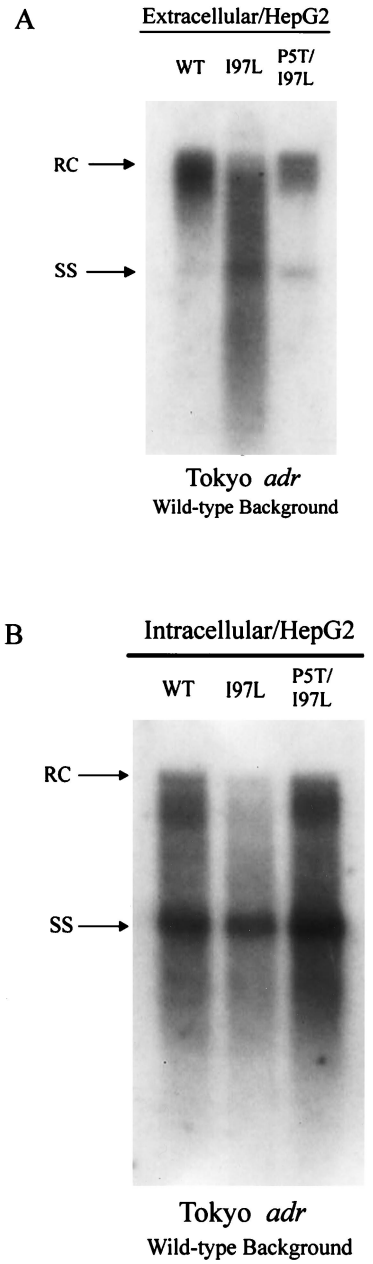

FIG. 3. A frequent core mutation, P5T, is compensatory for the immature virion secretion caused by another frequent core mutation, I97L, in the wild-type (WT) HBV genetic background of a Tokyo *adr* strain. Southern blot analysis for secreted virion particles from culture media of transfected HepG2 cells (A) and assay of intracellular viral replication of transfected human HepG2 cells (B) were performed as described in the text. RC, relaxed circular; SS, single-stranded HBV DNA replicative intermediates.

130. It is theoretically possible that some of the naturally compensatory mutations for 97L immature secretion may eventually be found in the envelope genes (16).

At present, the mechanisms of the compensatory effect on immature secretion by either core mutation P5T or P130T remain unclear. Based on the known three-dimensional structure of HBV capsid particles (3, 7, 8, 29), both amino acids 5 and 130 appear to be distant from amino acid 97 in the monomeric structure. Further examination of the core-envelope interaction (16), as well as the spatial and dynamic relationships between core amino acids 5, 97, and 130 in the structural context of dimer, 180-mer, 240-mer, or replicating icosahedral particles, is warranted.

This work was funded by NIH grants R01 CA 70336 and CA 84217 to C.S.

We thank colleagues in C. Shih's laboratory for careful reading of the manuscript.

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