Nucleolar Localization of Human Hepatitis B Virus Capsid Protein†

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Wild-type human hepatitis B virus (HBV) exhibits selective export of virions containing mature genomes. In contrast, changing an isoleucine to a leucine at amino acid 97 (I97L) of the HBV core antigen (HBcAg) causes it to release immature genomes. To elucidate the structure-function relationship of HBcAg at amino acid 97, we systematically replaced the isoleucine residue at this position with 18 other amino acids via mutagenesis. Twelve of the 18 mutants exhibited no significant phenotype, while five new mutants displayed strong phenotypes. The I97D mutant had a near lethal phenotype, the I97P mutant exhibited a significantly reduced level of virion secretion, and the I97G mutant lacked the full-length relaxed circular form of viral DNA. The tip of the spike of the capsid particle is known to contain a predominant B-cell epitope. However, the recognition of this exposed epitope by an anti-HBc antibody appeared to be affected by the I97E mutation or by histidine tagging at the C terminus of mutant HBcAg, which is presumably in the capsid interior. Surprisingly, the nuclear HBcAg of mutants I97E and I97W, produced from either a replicon or an expression vector, was found to be colocalized with nucleolin and B23 at a frequency of nearly 100% by confocal immunofluorescence microscopy. In contrast, this colocalization occurred with wild-type HBcAg only to a limited extent. We also noted that nucleolin-colocalizing cells were often binucleated or apoptotic, suggesting that the presence of HBcAg in the nucleolus may perturb cytokinesis. The mechanism of this phenomenon and its potential involvement in liver pathogenesis are discussed. To our knowledge, this is the first report of nucleolar HBcAg in culture.

Hepatitis B virus (HBV) is a major human infectious pathogen that was first discovered in leukemia patients and Australian aboriginals in 1965 (3). More than one-third of the world’s population has been infected with HBV (77). Chronic active hepatitis associated with HBV infection often leads to the development of cirrhosis, liver failure, and highly malignant liver cancer (1, 2, 6, 23). In part because of the emergence of drug-resistant HBV variants, current treatments for hepatitis B have a disappointingly low efficacy compared to those for hepatitis C (9, 33). A new target, other than HBV polymerase, needs to be identified for therapeutic treatment. A better basic understanding of the life cycle of HBV, including the functional significance of HBV variants and the mechanism of virion secretion, might lead to new clinical interventions for chronic infections with HBV.

The release of HBV virions from hepatocytes is a tightly regulated event. The current dogma indicates that the mature HBV genome is preferentially exported from the intracellular compartment (24, 48, 60, 65). Recently, members of our laboratory identified an immature secretion phenotype of a highly frequent naturally occurring HBV variant containing a leucine residue at amino acid 97 of the core protein. Unlike wild-type HBV, this 97L variant secretes almost equal amounts of mature and immature genomes (73, 74). This unexpected phenomenon is not caused by instability of the core particles or by any deficiency in viral reverse transcription (35, 44, 73, 74). In addition to the I97L immature secretion variant, other naturally occurring and artificially created capsid mutants exhibit a so-called low secretion phenotype with significantly reduced virion secretion while maintaining normal intracellular viral DNA replication (12, 30, 34, 49). The mechanisms for both immature secretion and low secretion phenomena remain to be elucidated.

The expression of the HBV core antigen (HBcAg) has been studied with mammalian cell systems (42, 47, 53). HBcAg was found to be localized to the cytoplasm (47) in COS cells and predominantly to the nucleus in mouse 3T3 fibroblasts (42). HBcAg was found to be distributed in both the nucleus and the cytoplasm in HBV-producing hepatocytes and transgenic mice (11, 25, 61, 62). It has also been documented that in HBcAg-positive patients, a nucleus-dominant distribution of intracellular HBcAg is associated with minor hepatitis activity while a cytoplasmic distribution of HBcAg is associated with chronic active liver disease (12). The molecular basis for the shift from nuclear distribution and minor disease activity to cytoplasmic distribution and disease exacerbation has been unclear. Frequent mutations have been found to accumulate in HBcAg during natural infections with HBV (13, 21, 26, 28, 57). It is tempting to hypothesize that naturally occurring HBcAg variants contribute to the change in the subcellular localization of HBcAg.

To further investigate the mechanism of HBV virion secretion and HBcAg subcellular localization in HBcAg variants, we systematically introduced different amino acids at position 97 of HBcAg and analyzed their phenotypic consequences via several different functional assays. In addition to using virus replication and virion secretion assays, we examined the potential relationship between nuclear targeting and virion secre-
tion by immunofluorescence microscopy. Although we detected no significant phenotypic changes for 12 of 18 mutants, we observed strong phenotypes for 6 mutants. Most intriguingly, the I97E mutant appeared to have an increased accumulation of nuclear HBcAg. Furthermore, the nuclear HBcAg of mutants I97E and I97W usually colocalized with nucleolin. In summary, like the delta antigen of hepatitis D virus (5, 31, 40, 63), the HBcAgs of both wild-type and mutant HBV can localize to the nucleolus, albeit at a lower frequency and to a limited extent for wild-type HBV. The mechanism of this unexpected nucleolar localization of HBcAg and its potential biological significance in non-immunity-mediated liver injury during natural infection are discussed (10, 18, 43).

MATERIALS AND METHODS

Plasmid constructs. (i) HBV core protein expression vectors. Plasmids pSVC-I97H (carries wild-type adr), pSVC-I97A, pSVC-I97C, pSVC-I97D, pSVC-I97E, pSVC-I97F, pSVC-I97G, pSVC-I97K, pSVC-I97M, pSVC-I97N, pSVC-I97P, pSVC-I97Q, pSVC-I97R, pSVC-I97S, pSVC-I97T, and pSVC-I97W are wild-type and mutant core antigen expression vectors with a simian virus 40 early enhancer and promoter. These vectors were constructed via site-directed mutagenesis by PCR amplification of the core gene from wild-type pSVC-197H (68) (Table 1). All mutants were confirmed by DNA sequencing. Plasmids pSVC-I97L and pSVC-I97F were described previously (74).

(ii) Histidine tagging of HBcAg. For the construction of pSVC His-tag derivatives, two primers were designed to amplify core gene fragments containing six consecutive histidine residues at the C terminus of HBcAg. The upstream primer (5'-AGA GGC CTA AAA ATC AGA CAA CTA TGG TGG) and the downstream primer (5'-AGA GAG CTC CTA TGG AGA CAA CCA CTG TGG TGG) contained HBV plus-strand DNA sequences from nucleotides nt 1877 to 1897 with a HindIII cleavage site (underlined). The amplified wild-type or mutant core expression vector DNA was transfected into human Huh7 hepatoma cells. The cell lysates were harvested at 3 days posttransfection, and core protein expression was detected by Western blot analysis using a diluted (1:500) rabbit anti-HBcAg antibody (Dako Cytomation Co., Carpinteria, Calif.). The detection of His-tag fusion proteins was performed by use of a His-tag stop codon downstream of the HBcAg initiation codon, we used a 3.2-kb HBV monomer (adr) in a pBluescript plasmid as a template and PCR amplified a monomer fragment by using a pair of complementary primers (sense primer, 5'- TTACA CAGCATTGTTACACTT CTC TCT 3'). The amplified mutant monomer genome was cut with BamHI and dimerized in tandem in pBlue-script. Wt EKO and mutant I97W EKO of HBV adr origin were confirmed by DNA sequencing. They were replication competent and secreted little HBcAg in the medium, as measured by an enzyme-linked immunosorbent assay (low signals were detected due to released naked core particles [data not shown]).

TAA mutant 1903. Mutant 1903 bears an ablated AUG initiation codon of HBcAg in both copies of the env HBV tandem dimer. This mutant is thus replication defective due to the absence of core protein production (73). Mutant 1903 can be rescued to replicate if the core protein is provided by trans-complementation. Immuno blot analysis of core and His-tag fusion proteins. Ten micrograms of wild-type or mutant core expression vector DNA was transfected into human Huh7 hepatoma cells. The cell lysates were harvested at 3 days posttransfection, and core protein expression was detected by Western blot analysis using a diluted (1:500) rabbit anti-HBcAg antibody (Dako Cytomation Co., Carpinteria, Calif.). The detection of His-tag fusion proteins was performed by use of a His-tag.

FIG. 1. Intracellular viral DNA replication of HBV mutants containing different amino acids at position 97 of HBcAg. The core-deficient replicon plasmid 1903 was cotransfected into Huh7 cells with a second plasmid, 197*, which expressed the various mutant HBcAgs (*, amino acids other than isoleucine). HBV DNAs were harvested at 5 days posttransfection and examined by Southern blot analysis. Full-length RC DNA at the 4.6-kb position and single-stranded (SS) DNA at the 1.5-kb position are indicated by arrows. Wt, wild type. The I97G mutant lacked the full-length RC form and exhibited an overall reduction in replication. The I97D mutant was almost lethal, with barely detectable replication. The I97F mutant exhibited a normal pattern of DNA replication and was not included in this figure (74). (a) First set of nine mutants. (b) Second set of nine mutants.

TABLE 1. Oligonucleotides for construction of HBcAg amino acid mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Oligonucleotide sequence (5' to 3')</th>
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<tbody>
<tr>
<td>I97I (Wt)</td>
<td>ATG GGC CTA AAA ATC AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97G</td>
<td>ATG GGC CTA AAA GGA AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97A</td>
<td>ATG GGC CTA AAA TGC AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97V</td>
<td>ATG GGC CTA AAA CGT AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97M</td>
<td>ATG GGC CTA AAA ATG AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97C</td>
<td>ATG GGC CTA AAA TGC AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97Q</td>
<td>ATG GGC CTA AAA CAG AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97N</td>
<td>ATG GGC CTA AAA AAC AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97P</td>
<td>ATG GGC CTA AAA CCA AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97W</td>
<td>ATG GGC CTA AAA TGG AGA CAA CTA TGG TGG</td>
</tr>
<tr>
<td>I97C</td>
<td>ATG GGC CTA AAA TGC AGA CAA CTA TGG TGG</td>
</tr>
</tbody>
</table>

a Mutated nucleotides are underlined.
b Wt, wild-type HBV of the adr subtype.

c I97I (Wt) derived plasmid 1903 was cotransfected into Huh7 cells with a second plasmid, 197*, which expressed the various mutant HBcAgs (*) amino acids other than isoleucine. HBV DNAs were harvested at 5 days posttransfection and examined by Southern blot analysis. Full-length RC DNA at the 4.6-kb position and single-stranded (SS) DNA at the 1.5-kb position are indicated by arrows. Wt, wild type. The I97G mutant lacked the full-length RC form and exhibited an overall reduction in replication. The I97D mutant was almost lethal, with barely detectable replication. The I97F mutant exhibited a normal pattern of DNA replication and was not included in this figure (74). (a) First set of nine mutants. (b) Second set of nine mutants.
were drained and then fixed in ice-cold acetone-methanol for 5 min. Alternatively, cells were fixed with freshly prepared 4% paraformaldehyde in PBS for 10 min, washed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature, and finally washed again four times with PBS. HBcAg was stained with either a diluted (1:500) rabbit anti-HBcAg antibody (Dako Co.) or a mouse monoclonal antibody (Hyb-3120; Institute of Immunology, Tokyo, Japan). The Hyb-3120 mouse monoclonal antibody recognizes a capsid conformation-specific epitope (15). Goat anti-rabbit–fluorescein isothiocyanate (FITC) and goat anti-mouse immunoglobulin G (IgG)–tetramethylrhodamine isocyanate (TRITC) or rabbit anti-goat–TRITC and goat anti-mouse IgG–FITC were used as secondary antibodies for the experiments shown in Fig. 5 to 8. For the experiment shown in Fig. 9b, goat anti-rabbit IgG–TRITC was used. Nucleolin was stained with a diluted (1:8) supernantant of a hybridoma culture containing the mouse monoclonal antibody CC98 (7). B23 was stained with a goat anti-B23 polyclonal antibody (sc-6013; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). The nuclei of the cells were counterstained with 10 μg of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, Mo.). The results were visualized under a Zeiss LSM 510 confocal laser scanning microscope. Serial sections of 12 to 15 slices per sample were usually examined, with a 0.50- to 0.73-μm thickness per section.

Transfection, viral replication, and virion secretion assays. For DNA transfection, 10 μg of psCV and 10 μg of mutant 1903 plasmid DNAs were adjusted to a final total of 35 μg with carrier DNA and then transfected into the Huh7 human hepatoma cell line by the calcium phosphate coprecipitation method (56). The preparation of intracellular core particles and viral DNA and gradient centrifugation analyses of secreted viral particles were performed as detailed elsewhere (73).

RESULTS

A total of 18 different HBcAg mutants containing different substitutions at amino acid 97 were analyzed for their capabilities in DNA replication, virion secretion, and HBcAg nuclear accumulation. Briefly, Huh7 cells were cotransfected with mutant 1903 (core-deficient) and different HBcAg-expressing plasmids containing various mutations at amino acid 97. Assays for DNA replication and virion secretion were performed at 5 days posttransfection, while the nuclear accumulation of
HBcAg was visualized at approximately 3 to 5 days posttransfection.

Significant replication defect of mutants I97G and I97D. As shown in Fig. 1, most of the substitutions at position 97 had no apparent effect on HBV DNA replication, as detected by Southern blot analysis. However, when isoleucine-97 was changed to an acidic residue, aspartic acid (D), the replication activity was completely abolished in at least two independently derived bacterial clones (data not shown). The low level of expression of the I97D mutant was not due to a poor transfection efficiency since secreted HBsAg and HBcAg, both of which were produced from the cotransfecting plasmid 1903, were present at normal levels (data not shown). When isoleucine-97 was changed to the smallest amino acid, glycine (G), the replication activity was significantly reduced. An even more prominent phenotype of the I97G mutant was the lack of the full-length relaxed circle (RC) DNA form at the 4.0-kb position (Fig. 1). The results shown in Fig. 1 are representative of at least five independent transfection experiments.

Virion release deficiency of I97 mutants. In addition to the replication assay, we performed assays of virion release into the medium. As shown in Fig. 2, we found no deficiency in virion release for the I97 mutants, except for the I97P mutant. Although the level of intracellular viral DNA replication of the I97P mutant was slightly lower than that of the wild type (Fig. 1), this difference was too small to account for the large deficiency in virion secretion (Fig. 2). Consistent with their intracellular phenotypes of reduced viral DNA replication (Fig. 1), the I97G mutant exhibited significantly lower levels of virion-associated HBV DNA, with none of the full-length RC form, and the I97D mutant had a barely detectable level of HBV DNA in the medium. As reported previously (12, 34, 35, 59, 73, 74, 75), the I97L mutant exhibited an immature secretion phenotype by releasing more virions containing single-stranded DNA into the medium (Fig. 2). The results shown in Fig. 2 are representative of at least five independent transfection experiments.

Were the mutant phenotypes caused by an alteration of epitope recognition or by protein instability? To determine if any of the aforementioned phenotypes were caused by alterations in mutant protein stability, we performed an immunoblot analysis to determine the steady-state level of HBcAg. As shown in Fig. 3a, the majority of mutants exhibited similar levels of HBcAg. In contrast, the I97P, I97G, I97D, and I97E mutants exhibited reduced steady-state levels of HBcAg (Fig. 3b). The reduced signal from mutant HBcAg may have been caused either by altered recognition of a dominant conformational epitope by the anti-core antibody (54) or by reduced protein stability. To address this issue, we tagged the HBcAg of the wild type and those of the mutants at the C terminus with six histidine (His) residues. As shown in Fig. 4, the I97D mutant was intrinsically unstable and little signal could be detected when it was either probed with an anti-core antibody (Fig. 4a) or reprobed with an anti-His antibody (Fig. 4b). Although the I97E mutant core protein exhibited a significantly lower signal than the wild type (panel a), the difference in signal intensity between the Wt-His and I97E-His proteins was minimal when the same filter was reprobed with an anti-His antibody (panel b). These results suggest that the I97E mutation probably does not change the protein’s stability. Instead, the I-to-E mutation may affect the recognition of a distal dominant conformational epitope around amino acids 78 to 82 at the tip of the spike (4,
FIG. 5—Continued.
FIG. 6. Colocalization of nucleolin and HBcAg was also observed when HBcAg was produced from an HBeAg-negative replicon (EKO). The assay procedures were the same as those described in the legend to Fig. 5. The granular staining pattern observed with the I97W SVC expression vector (Fig. 5) was also present in the I97W EKO replicon. The cytoplasmically dominant staining pattern observed with the Wt SVC expression vector was also present in the Wt EKO replicon. The explanations for the colors in the subpanels (blue, green, red, and yellow) are the same as those for Fig. 5.
14, 16, 54, 66). The effect of the I97G and I97P mutations on epitope recognition by the anti-core antibody was not as strong as that of the I97E mutant (Fig. 4). Finally, it was most intriguing that histidine tagging at the C terminus of mutant HBcAg almost completely abolished the recognition of the distal dominant B-cell epitope by the anti-core antibody (Fig. 4a).

HBcAg of wild-type HBV and those of mutants I97E and I97W are targeted for the nucleus at different frequencies. In our studies of naturally occurring secretion variants, we speculated that a low secretion behavior might be caused by an increased efficiency of nuclear targeting and, conversely, that an immature secretion variant might cause a decreased efficiency of nuclear targeting. In other words, we wished to test whether nuclear targeting and endoplasmic reticulum budding are two competing pathways for the same pool of capsid particles. Furthermore, we wanted to ask whether HBcAg variants are responsible for the shift from a cytoplasm-dominant to a nucleus-dominant distribution of HBcAg. To these ends, we performed immunofluorescence microscopy of Huh7 cells that were transiently transfected with wild-type or mutant plasmids in the context of either an expression vector or a replicon.

The immunostaining patterns of HBcAgs from most mutants (Table 1; data not shown) and from the wild type (Fig. 5) were cytoplasmically dominant, which is consistent with results in the existing literature (62, 70, 71). Surprisingly, we noted that the HBcAgs of mutants I97E and I97W were clearly dominant in the nucleus, particularly when fixed with acetone-methanol. Even more surprisingly, an almost perfect colocalization of HBcAg and nucleolin was frequently observed for the I97E and I97W mutants. In contrast, for wild-type SVC (Fig. 5), only two of six nucleoli (highlighted by white arrows) exhibited visible colocalization with HBcAg, even after extensive searching of serial sections by confocal microscopy. Unlike the case for the I97E and I97W mutants, only a portion of these two colocalizing nucleoli was occupied by HBcAg when they were examined in serial sections by confocal microscopy (see Fig. S2 in the supplemental material). Another example of serial confocal sections of wild-type HBcAg, which again exhibited partial colocalization with nucleolin, is shown in Fig. S1 in the supplemental material. Overall, relative to the very extensive and near perfect colocalization of the mutant I97W and I97E HBcAgs with nucleolin, the colocalization of wild-type HBcAg was more limited. The nucleolar localization of HBcAg was also confirmed by use of a goat antibody that was specific for another independent nucleolar marker, B23 (Fig. 5).

This nucleolar localization phenomenon was observed even at a lower dose of input DNA for transfection (0.25 μg of DNA per 35-mm-diameter dish) (data not shown). This was observed with either a rabbit anti-core polyclonal antibody or a capsid conformation-specific mouse monoclonal antibody (Hyb-3120) (data not shown). Finally, this phenomenon was also observed in the context of either an expression vector (pSVC) or a replicon (EKO), indicating that no other viral components are required for HBcAg to colocalize with nucleolin (Fig. 6). As a side note, the HBV tandem dimer replicon can produce both the core protein and HBeAg. HBeAg is known to be nonessential for HBV replication (23). Since HBeAg and the core protein are immunologically cross-reactive, the EKO (HBeAg knock out) mutant was used for the study of HBeAg, which avoids this complication.

It is also worth mentioning that in most cases, we used the acetone-methanol method for fixation (see Materials and Methods) (Fig. 5 and 6) because this method seemed to give a lower signal and background in both the cytoplasm and the nucleus. As such, the colocalization between HBcAg and nucleolin was more easily visualized. In contrast, when the paraformaldehyde method was used for fixation (Fig. 7), it was more difficult to visualize colocalization due to the higher overall background signal. On the other hand, the nucleolar necklace, or doughnut-shaped structure, seemed to be better preserved by the paraformaldehyde method (Fig. 7, I97E SVC).

The nucleolin-colocalizing phenotype of HBcAg was not necessarily associated with the phenotype of nucleus-dominant staining. We also observed very strong nucleolin colocalization in some cells with a cytoplasm-dominant staining pattern (data not shown).

HBcAg of the I97E mutant does not colocalize with splicing factor SC35. Previously, it was reported that the duck hepatitis B virus (DHBV) core protein can be localized to the periphery of a spliceosome compartment (39). As shown in Fig. 8, we found no perfect colocalization between the splice factor SC35 and the HBcAg of the I97E mutant. Although some of the HBcAg (green dots) appeared to be near the periphery of the SC35 signals (red dots), given the fact that the SC35-specific red dots were so abundant in the nuclei of human Huh7 cells, we are not certain about the spatial relationship between HBcAg and SC35. These results demonstrate that the HBcAg in HBV and the core protein of DHBV have different subnuclear locations.

Apoptosis. We noted that a substantial fraction of the nucleolus- and HBcAg-colocalizing cells seemed to be apoptotic, adjacent to an apoptotic-like nucleus, or binucleated (Fig. 9a). The nuclei of these apoptotic cells often exhibited abnormal morphologies and were often stained poorly by DAPI. To investigate the potential cause-effect relationship between apoptosis and the colocalization phenomenon, we used annexin V and TUNEL assays to confirm apoptosis. As shown in Fig. 9b, panel I97W SVC, although two of the HBcAg-positive cells (red) were indeed apoptotic, with very weak DAPI staining and strong TUNEL positivity (green), there were also other HBcAg-positive, nonapoptotic cells (no yellow areas in the merged picture). Neither annexin V nor TUNEL assays revealed any
FIG. 8. HBcAg of the I97E mutant did not seem to colocalize with splicing factor SC35, as examined by confocal immunofluorescence microscopy. The experimental procedures used were the same as those described in the legend to Fig. 6. The majority of the nuclear HBcAgs of the wild type and the I97E mutant was not colocalized with the splicing factor SC35 in the nuclei of Huh7 cells. (A) DAPI staining for nuclei (blue). (B) Rabbit anti-HBcAg (Dako; 1:500) staining (green). (C) Mouse anti-SC35 (Sigma; 1:4,000) (red). (D) Merged pictures, with inserts enlarged in the corners.
significant difference in the rate of apoptosis between the wild type and the I97E and I97W mutants (data not shown).

**DISCUSSION**

Interestingly, as summarized in Fig. 10, among all of the possible amino acids that were substituted at position 97 of HBcAg (Table 2), only leucine (L) led to an immature secretion phenotype (Fig. 2) (73, 74), only aspartic acid (D) resulted in a lethal phenotype (Fig. 1), only glycine (G) gave rise to a replication profile devoid of the 4.0-kb full-length RC form of DNA (Fig. 1), only proline (P) displayed a low secretion phenotype (Fig. 2) (34, 49), and only tryptophan (W) and glutamic acid (E) exhibited granular nuclear staining with HBcAg and nucleolin colocalization (Fig. 5, 6, and 7). Other amino acid substitutions did not produce a strong phenotypic effect in the assays described in this paper (Table 2).

**Hydrophobic neighborhood around amino acid 97.** According to the known structure of the T=4 capsid of HBcAg 1-149, amino acid 97 of the HBV core protein is located on the α4b helix (4, 14, 66) and is near the kink at amino acids 90 to 92 (Fig. 11). A computer-aided structural analysis of the HBV capsid revealed a hydrophobic pocket within the 5-Å neighborhood of amino acid 97 which includes amino acids Val-27, Leu-31, Ala-58, Ile-59, Cys-61, Trp-62, Met-93, Lys-96, Leu-100, and Leu-101 (Fig. 11). While Cys-61 is a polar amino acid, Lys-96 is the only charged amino acid in this neighborhood. We hypothesize that adequate interactions between amino acid 97 and these neighboring hydrophobic amino acids play an important role in maintaining the stability of the α-helical hairpin and the normal behavior of HBV capsids. For example, the tryptophan of the I97W mutant is probably too bulky and the glycine of the I97G mutant is probably too small for normal functioning. In the case of mutants I97P and I97G, both proline and glycine may serve as helix breakers for the α4b helix of HBcAg (4, 14, 66). In the case of mutants I97D and I97E, since both aspartic acid (D) and glutamic acid (E) are acidic, it is puzzling that the I97D mutant has a near lethal phenotype while the I97E mutant is viable, with a nucleolin-colocalizing phenotype. A related question is why mutants I97K and I97R, which contain a positive charge at position 97, do not have the same phenotype as I97D and I97E, which contain a negative charge at position 97. Further structural studies may help to address this issue.

**Alteration of epitope recognition and its implication in genome maturation signal hypothesis.** A dominant B-cell epitope of HBcAg has been mapped to the tip of the spine of the capsid particle (54, 66). Since the tip of the spine is spatially distal to both the C terminus and position 97 of HBcAg, we did not anticipate that the recognition of this epitope in the I97E mutant and of His-tagged HBcAg by an anti-core polyclonal antibody would be dramatically reduced or totally lost, as shown by immunoblot analysis (Fig. 4). The DHBV core protein contains serine residues which are hypophosphorylated in virions (50). In addition, the phosphorylation status of HBcAg is important for nuclear import (29, 51, 71). It is tempting to propose that the C terminus of HBcAg can sense the growing genome maturity via phosphorylation or dephosphorylation. The genome maturation status may be transmitted via a signal to the surfaces of capsids en route through amino acid 97. When amino acid 97 is a wild-type isoleucine, the signal transduction pathway is normal, and the epitope at the tip of the spike can be recognized by the anti-core polyclonal antibody (Fig. 4). However, when amino acid 97 is mutated from an I into a D or E, the signal transduction pathway from the C terminus to the tip of the spike and, presumably, to other parts of the capsid particles may be interrupted. As such, aberrant behaviors were observed for mutants I97D and I97E.

**Colocalization of nucleolin and HBcAg of mutants I97E and I97W.** Cytoplasmic nucleocapsids of hepadnaviruses can deliver the viral genome to the nucleus and thus amplify the pool of intranuclear covalently closed circular DNA (64). The DHBV core protein has been found to be distributed in the nucleus as distinct nuclear bodies (38, 39). Using indirect immunofluorescence, Mabit et al. (39) demonstrated that the DHBV core protein is highly concentrated in the so-called nuclear core bodies, which are always localized at the periphery of the spliceosome compartment of infected duck hepatocytes. The functional significance of this phenomenon remains unclear. In our study, we also found brightly staining nuclear core body-like spots in the nuclei of HuH7 cells that were transfected with either the I97E or I97W mutant, and on less frequent occasions, with wild-type HBV. However, there was no convincing evidence to exclude the occasional colocalization of SC35 and some of the HBcAg of a wild-type or mutant HBV (Fig. 8). This was in part because the SC35 signals were so abundant that it was sometimes difficult to distinguish between a bona fide colocalization and a coincidence. Instead of SC35, to our surprise, we found near perfect colocalization of HBcAg and nucleolin (Fig. 5 to 7). It will be interesting to ask in the future whether mutant HBcAg can physically bind to B23, nucleolin, or any other nucleolar proteins.

The capsid proteins of the I97E and I97W mutants that accumulated in the nucleolus were not monomers or dimers of HBcAg since they could be immunostained with a monoclonal antibody (Hyb-3120) specific for the HBV capsid conformation (data not shown) (15). At present, it remains unclear whether these nucleolus-associated capsids are imported through the nuclear pore complex from the cytoplasm without prior disassembly or are reasssembled from imported capsid protein monomers and dimers. On some rare occasions, we found some small nuclear foci of HBcAg that did not appear to colocalize with nucleolin. However, examinations of further sections by confocal microscopy often revealed that these foci also colocalized with nucleolin (data not shown). We speculate here that these small HBcAg foci probably occurred when the newly capsids just passed through the nuclear pore or when all of the existing nucleoli had already been saturated with HBcAg.

Several viral proteins have been localized to the nucleolus, including the coronavirus nucleoprotein (8), the UL3 protein of herpes simplex virus type 2 (69), the E7 protein of human papillomavirus type 16 (76), the delta antigen of hepatitis D virus (31, 63), and the Rev and Tat proteins of human immunodeficiency virus (HIV) (19, 36). The Rev protein is well known for its role in nuclear RNA export (17, 22, 27). Since we did not detect colocalization of HBcAg and nucleolin in every HBcAg-positive cell that was transfected with the wild-type replicon EKO, a more sensitive method for detecting colocalization will be needed to address this issue.
Mechanism of nucleolin and HBeAg colocalization. The highly frequent colocalization of mutant HBeAg with nucleolin may be caused by several possibilities, including (i) an increased import of mutant capsids from the cytoplasm to the nucleus, (ii) increased trafficking or binding of mutant HBeAg from the nucleoplasm to the nucleolus, (iii) an increased tendency of forming mutant protein aggregates in the nucleolus, (iv) an increased induction of apoptosis, which in turn some-
how contributes to colocalization, and (v) combinations of the above.

The fourth possibility can be excluded because we found no difference in the rates of apoptosis, as measured by annexin V and TUNEL assays, between wild-type and mutant HBcAg-transfected cultures (data not shown). We noted that a significant fraction of colocalizing cells were binucleated or apoptotic. It is quite possible that when wild-type or mutant HBcAg is expressed at a high level, it is cytotoxic to host hepatocytes (45, 52, 72; P. Chua and C. Shih, unpublished results). This may be another mechanism for non-immunity-mediated liver injury during natural infection (18).

It is known that the nucleolus can sequester cell cycle regulatory molecules, including p53 (46). Furthermore, telome-
HBcAg contributes to its nucleolar targeting in addition to its protein was identified (58). This sequence motif is very rich in nucleolar or nucleolar trafficking and increased binding of mutant HBcAg to the nucleolus. Previously, a nucleolar localization sequence of the type I human T-cell leukemia virus pX protein was identified (58). This sequence motif is very rich in arginine and proline and contains serine and/or threonine (Fig. 12a). At the C terminus of HBcAg, an arginine- and proline-rich domain also contains several serine and/or threonine residues (Fig. 12a). The striking similarity between the reported nucleolar localization sequence and the unusually long stretch of the arginine- and proline-rich motifs at the C terminus of HBcAg strongly suggests that the C-terminal domain of HBcAg contributes to its nucleolar targeting in addition to its

**FIG. 11.** Topological relationships of the side chain of residue Ile-97 with side chains of neighboring residues within a 6- to 7-Å distance. The image is a schematic representation of the fold of the HBV capsid protein monomer derived from the published crystal structure (accession number 1QGT; http://www.rcsb.org/pdb) by use of the Swiss-PdbViewer, version 3.7b2, program. The dimer interface is nearest the viewer. Red, residue Ile-97; blue, residues Val-27 and Leu-31 in α2a helix; yellow, residues Ala-58, Ile-59, Cys-61, and Trp-62 in α3 helix; green, residues Met-93, Lys-96, Leu-100, and Leu-101 in α4b helix. Other residues that are not shown here include residue Glu-64 from the counterpart monomer of the same dimer. N, N terminus; Thr-142 is seven amino acids away from the C terminus.

**TABLE 2.** Characterizations of HBcAg codon 97 mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Replication</th>
<th>Virion secretion</th>
<th>Core Ag Western blot result</th>
<th>Core Ag IFA result</th>
<th>Phenotype</th>
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<tr>
<td>I97P</td>
<td>++</td>
<td>+</td>
<td></td>
<td>C ≥ N</td>
<td>N</td>
</tr>
<tr>
<td>I97G</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>C ≥ N</td>
<td>No full-length RC form</td>
</tr>
<tr>
<td>I97A</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>C ≥ N</td>
<td>Immature secretion</td>
</tr>
<tr>
<td>I97V</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97L</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97F</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97M</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97C</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97W</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>N &gt; C</td>
<td>Nucleolar colocalization</td>
</tr>
<tr>
<td>I97P</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>C ≥ N</td>
<td>Low secretion</td>
</tr>
<tr>
<td>I97N</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97Q</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97S</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97T</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>C ≥ N</td>
<td></td>
</tr>
<tr>
<td>I97Y</td>
<td>++</td>
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<td>+</td>
<td>C ≥ N</td>
<td></td>
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<tr>
<td>I97H</td>
<td>++</td>
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<td>+</td>
<td>C ≥ N</td>
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</tr>
<tr>
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<td>+</td>
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<tr>
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<td>C ≥ N</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>N &gt; C</td>
<td>Nucleolar colocalization</td>
</tr>
<tr>
<td>I97D</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>C ≥ N</td>
<td>Near lethal</td>
</tr>
</tbody>
</table>

*a* I97T represents the wild-type adr subtype.

*b* C, cytoplasm dominant; N, nucleus dominant. The relative distribution of HBcAg between the nucleus and the cytoplasm also depended on the timing of immunostaining after transfection. The cells were fixed by the acetone-methanol fixation method.
known role in nuclear targeting (20, 29, 70, 71). It is common that nuclear and nucleolar localization sequences overlap, and the latter has been reported to be longer and more stringent than the former (37).

However, the different extents of nucleolar colocalization between wild-type HBCAg and those of the I97E and I97W mutants still cannot be explained by their having the same sequences in the vicinity of amino acid 97 of HBCAg. Bold letters represent conserved positions.

FIG. 12. (a) Sequence comparison between a known nucleolar localization signal of the human T-cell leukemia virus type 1 (HTLV-1) pX protein (58) and the arginine-rich domain of HBCAg at the C terminus. Sequence similarities are highlighted by underlining. (b) Sequence comparisons between the vicinity of a known nucleolin binding site of hepatitis delta antigen (32), nuclear localization sequences of adenovirus protein V (41) and SV40 Tag (67), and the vicinity of amino acid 97 of HBCAg. Bold letters represent conserved positions. The negatively charged amino acid E is italicized.

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REFERENCES


ERRATUM

Nucleolar Localization of Human Hepatitis B Virus Capsid Protein

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Volume 78, no. 24, p. 13653–13668, 2004. Page 13659: The lower panel of Fig. 6 should appear as shown below.