Roles of the Three Major Phosphorylation Sites of Hepatitis B Virus Core Protein in Viral Replication

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Hepatitis B virus (HBV) core protein is a phosphoprotein. Its three major phosphorylation sites have been identified at the serine residues located at amino acids 157, 164, and 172. In order to investigate the role of core protein phosphorylation in HBV replication, these three serine residues were mutated to alanine to mimic nonphosphorylated serine or to glutamic acid to mimic phosphoserine. The nonphosphorylated core protein analog did not package the HBV pregenomic RNA, and the phosphorylated analog packaged the pregenomic RNA but failed to support viral DNA replication. These results indicate that the core protein phosphorylation may be important for pregenomic RNA packaging and that its dephosphorylation may be important for viral DNA replication. The individual roles of these three major phosphorylation sites in HBV replication were further investigated by being mutated to alanine in different combinations. The results showed that the serine residue at amino acid 157 was not essential for pregenomic RNA packaging, whereas the serine residues at amino acids 164 and 172 were more important. Furthermore, the serine residue at amino acid 157 was not essential for viral DNA replication or viral maturation.

INTRODUCTION

Hepatitis B virus (HBV) is a small DNA virus with a genome size of only 3.2 kb. Its genome encodes four different genes named C, S, P, and X genes. The C gene encodes two related proteins named precore protein and core protein. The precore protein is the precursor of the circulating e antigen found in the sera of HBV patients, and the core protein is the viral capsid protein. The S gene encodes the three different viral envelope proteins named surface antigens. The P gene encodes the viral DNA polymerase, which is also a reverse transcriptase, and the X gene encodes a transcriptional activator (for reviews, see Ganem, 1995; Yen, 1993).

The C gene directs the transcription of two mRNAs named precore RNA and core RNA, which encode precore protein and core protein, respectively (for a review, see Ou, 1997). After its synthesis, the core protein packages its own mRNA, which is also known as the pregenomic RNA, to form the core particle. The viral DNA polymerase that is also packaged in the core particle then converts the pregenomic RNA to the viral genomic DNA. The replication of viral DNA is essential for the core particle to be enveloped to form the mature virion, which is secreted from cells (Wei et al., 1996).

The core protein is a phosphoprotein (Machida et al., 1991; Yeh and Ou, 1991). Studies conducted in vitro indicate that the core protein can be phosphorylated by protein kinase C (Kann et al., 1997; also unpublished observation), the cell cycle-regulated kinase cdc2 (Yeh et al., 1993), the kinase activity of glyceraldehyde-3-phosphate dehydrogenase (Duclos-Vallee et al., 1998), and a 46-kDa serum kinase (Kau and Ting, 1998). However, these are indeed the kinases responsible for the phosphorylation of the core protein during natural infection remains to be determined. The three major phosphorylation sites in the HBV core protein sequence have been mapped to the serine residues located at amino acids 157, 164, and 172 near the C-terminus (Liao and Ou, 1995). These three serine residues reside in the three adjacent SPRRR repeats and overlap the nuclear localization signal (Yeh et al., 1990; Eckhardt et al., 1991).

The role of the core protein phosphorylation in the life cycle of HBV remains unclear. Studies conducted with the distantly related duck hepatitis B virus (DHBV) indicated that the core protein present in the DHBV virion in the serum was hypophosphorylated and that that present in the intracellular core particle was hyperphosphorylated (Pugh et al., 1989). This observation suggests that phosphorylation or dephosphorylation of the core protein may play an important role in viral replication. In this report, we have investigated the possible roles of the three major phosphorylation sites of the HBV core protein in viral replication. Our results indicate that the conversion of these phosphorylation sites to alanine to mimic dephosphorylation would abolish the packaging of the pregenomic RNA. In contrast, the conversion of
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FIG. 1. Immunoprecipitation analysis of core and precore proteins expressed by the HBV DNA constructs. Huh7 cells grown in a 60-mm dish were transfected with 10 μg of the following plasmids: pUC-HBV2 (lane 1), pUC19 (lane 2), or pHBV-Cmt (lane 3). Cells were labeled with 200 μCi [35S]methionine, lysed 48 h after transfection, and immunoprecipitated with rabbit anti-core antibody using our previous procedures (Yeh and Ou, 1991). The protein samples were then analyzed by 12.5% SDS–PAGE. Lane M is the core protein marker, which was synthesized using rabbit reticulocyte lysates and labeled with [35S]methionine. The arrowhead marks the location of the core protein band, and dots mark the locations of the precore protein derivatives (Ou, 1997).

these sites to glutamic acid to mimic phosphorylation had no significant effect on the packaging of the pregenomic RNA. The phosphorylated analog, however, was not able to support viral DNA replication. Further analysis indicated that the serine residue at amino acid 157 by itself was not essential for pregenomic RNA packaging, viral DNA replication, and viral maturation.

RESULTS

Packaging of HBV pregenomic RNA by the phosphorylated but not the nonphosphorylated analog of the core protein

To investigate the possible effects of core protein phosphorylation on the packaging of the pregenomic RNA, an HBV genomic dimer, which contained a missense mutation in the initiation codon of the core protein gene, was constructed. As shown in Fig. 1, this mutant, pHBV2-Cmt, could express the precore protein derivatives properly but could not express the core protein. This genomic mutant was then cotransfected with the core protein-expressing plasmid pCMV-CM1 or pCMV-CM2 into Huh7 hepatoma cells for transcomplementation studies. pCMV-CM1 and pCMV-CM2 expressed the core protein mutants CM1 and CM2, respectively. CM1 contained the serine-to-alanine mutation at the three major phosphorylation sites and mimicked the nonphosphorylated core protein, and CM2 contained the serine-to-glutamic acid mutation at the same sites and hence mimicked the phosphorylated core protein (Table 1). The control pRc/CMV vector and pCMV-C, a plasmid that expressed the wild-type core protein, were also used in the transcomplementation study to serve as controls. The total cellular RNA and the RNA extracted from the core particles were analyzed by primer extension. As shown in Fig. 2A, the pregenomic RNA, the precore RNA, and the core RNA expressed from the cytomegalovirus (CMV)-derived vector could all be detected when the total cellular RNA was analyzed. However, as shown in Fig. 2B, the pregenomic RNA was the predominant RNA species detected in the core particle fraction. This result, which showed that the pregenomic RNA was selectively packaged, is in agreement with the previous reports (Enders et al., 1987; Nassal et al., 1990). Interestingly, while the transcomplementation with the CM1 mutant reduced the amount of pregenomic RNA to an undetectable level (Fig. 2B, lane 4), the transcomplementation with the CM2 core protein reduced only slightly the amount of pregenomic RNA packaged (Fig. 2B, lanes 3 and 5). This result was not due to different levels of pregenomic RNA expressed, as similar levels of pregenomic RNA were expressed by pHBV2-Cmt whether it was transcomplemented with the wild-type core protein, CM1, or CM2 (Fig. 2A). Nor was this result due to the differential stability of the wild-type core protein, CM1, and CM2 in cells, because similar amounts of these proteins were detected in Western-blot analysis (data not shown). Thus, the results shown in Fig. 2 indicated that the conversion of the three major phosphorylation sites, Ser-157, Ser-164, and Ser-172, of the core protein to alanine to mimic dephosphorylation had a significant, negative effect on pregenomic RNA packaging, while the conversion of these major phosphorylation sites to glutamic acid to mimic phosphorylation had only a marginal effect.

Effects of nonphosphorylated and phosphorylated core protein analogs on HBV DNA replication

The possible effects of nonphosphorylated and phosphorylated core protein analogs on the replication of HBV DNA were also analyzed. HBV core particles were isolated from Huh7 cells cotransfected with pHBV2-Cmt and one of the following core protein-expressing plasmids: pCMV-C, pCMV-CM1, or pCMV-CM2. The wild-type

<table>
<thead>
<tr>
<th>Core protein construct</th>
<th>Ser-157*</th>
<th>Ser-164*</th>
<th>Ser-172*</th>
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<tbody>
<tr>
<td>C (wild type)</td>
<td>Serine</td>
<td>Serine</td>
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<tr>
<td>CM1</td>
<td>Alanine</td>
<td>Alanine</td>
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<tr>
<td>CM2</td>
<td>Glutamate</td>
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<tr>
<td>CM3</td>
<td>Serine</td>
<td>Alanine</td>
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<td>CM4</td>
<td>Alanine</td>
<td>Serine</td>
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<td>CM5</td>
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<td>CM6</td>
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<td>CM7</td>
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<tr>
<td>CM8</td>
<td>Alanine</td>
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* Ser-157, Ser-164, and Ser-172 indicate the serine residues located at amino acids 157, 164, and 172, respectively. They were mutated to either alanine or glutamic acid, depending on the core protein constructs.
HBV genomic dimer pWTD was also used in the transfection studies to serve as a positive control. The HBV DNA extracted from these core particles was then subjected to Southern-blot analysis. As shown in Fig. 3, the 3.2-kb linear genomic DNA could be detected in cells transfected with the wild-type HBV genome pWTD; lanes 2–5, cells cotransfected with pHBV2-Cmt and one of the following plasmids: the control vector pRc/CMV (lanes 2), pCMV-C that expressed the wild-type core protein (lanes 3), pCMV-CM1 (lanes 4), or pCMV-CM2 (lanes 5). In (A), the cDNA bands of core (pregenomic) RNA, precore RNA, and the core RNA derived from the CMV vector are marked by core, precore, and core*, respectively. In (B), the arrowhead denotes the pregenomic RNA band, and the asterisk marks the location of the core RNA band derived from the CMV vector. Since the transcomplementation experiment reduced significantly the viral replication rate, the primer-extension sample loaded in lane 1 in (B) was one-tenth of the amount of the samples loaded in the other lanes. M, molecular weight markers.

FIG. 2. Primer-extension analysis of the pregenomic RNA expressed by nonphosphorylated and phosphorylated core protein analogs. (A) Analysis of the total Huh7 cell RNA. (B) Analysis of the RNA extracted from the core particles isolated from Huh7 cells. Lanes 1, cells transfected with the wild-type genome pWTD; lanes 2–5, cells cotransfected with pHBV2-Cmt and one of the following plasmids: the control vector pRc/CMV (lanes 2), pCMV-C that expressed the wild-type core protein (lanes 3), pCMV-CM1 (lanes 4), or pCMV-CM2 (lanes 5). In (A), the cDNA bands of core (pregenomic) RNA, precore RNA, and the core RNA derived from the CMV vector are marked by core, precore, and core*, respectively. In (B), the arrowhead denotes the pregenomic RNA band, and the asterisk marks the location of the core RNA band derived from the CMV vector. Since the transcomplementation experiment reduced significantly the viral replication rate, the primer-extension sample loaded in lane 1 in (B) was one-tenth of the amount of the samples loaded in the other lanes. M, molecular weight markers.

Individual roles of Ser-157, Ser-164, and Ser-172 of the core protein in pregenomic RNA packaging

To investigate the individual roles of Ser-157, Ser-164, and Ser-172 of the core protein in the packaging of the pregenomic RNA, another set of transcomplementation experiments was conducted. In this set of studies, Ser-157, Ser-164, and Ser-172 of the core protein were converted to alanine in different combinations. The plasmids that expressed these core protein mutants were individually cotransfected with pHBV2-Cmt into Huh7 cells. Similar to the experiments shown in Fig. 2, both the cellular RNA and the RNA associated with core particles were analyzed. As shown in Fig. 4A, similar levels of the pregenomic RNA were expressed in cells by pHBV2-Cmt in different transcomplementation experiments. However, when the pregenomic RNA packaged in core particles was analyzed, different amounts of the pregenomic RNA were detected (Fig. 4B). The difference in packaging efficiency was not due to the differential stability of the core protein mutants, as these proteins had similar stability in cells (Liao and Ou, 1995). The core protein mutant CM4, which contained Ala-157, Ser-164, and Ser-172 (Table 1), packaged the pregenomic RNA with efficiency similar to that of the wild-type core protein. This result indicates that Ser-157 of the core protein is not essential shown in Fig. 3 suggest a role for core protein dephosphorylation in HBV DNA replication.

FIG. 3. Effects of nonphosphorylated and phosphorylated core protein analogs of the HBV core protein on viral DNA replication. Core particles were isolated from Huh7 cells 48 h after transfection (see Materials and Methods for experimental details). The genomic DNA was then extracted from core particles and analyzed by Southern blot. Lane 1, cells transfected with pWTD; lanes 2–5, cells transfected with pHBV2-Cmt and one of the following core protein-expressing plasmids: pRc/CMV vector (lane 2), the pCMV-C that expressed the wild-type core protein (lane 3), pCMV-CM1 (lane 4), or pCMV-CM2 (lane 5). The location of the 3.2-kb linear HBV genome is indicated (also see Fig. 5). Note that the relaxed circular (RC) form of the HBV DNA in this particular assay was not detected. The reason for this is unclear. It may be because the RC DNA packaged in the core particles was sensitive to nuclease used for the purification of the viral DNA (see Materials and Methods for details). Similar results were observed in previous reports (Bchini et al., 1990; Lamberts et al., 1993).
for packaging the pregenomic RNA. For the rest of the core protein mutants, there was a positive correlation between the number of phosphorylation sites retained and the efficiency of pregenomic RNA packaging. CM3, CM5, and CM8, which contained two serine-to-alanine mutations and hence retained only one phosphorylation site (Table 1), packaged little pregenomic RNA. CM6 and CM7, which both contained a single serine-to-alanine mutation and hence retained two phosphorylation sites, packaged a small but yet detectable amount of the pregenomic RNA.

Individual roles of Ser-157, Ser-164, and Ser-172 of the HBV core protein in viral DNA replication

In order to analyze the individual roles of Ser-157, Ser-164, and Ser-172 of the core protein in HBV replication, Huh7 cells were again cotransfected with pHBV2-Cmt and the plasmid that expressed one of the following core proteins: the wild-type core protein (lanes 1), CM3 (lanes 2), CM4 (lanes 3), CM5 (lanes 4), CM6 (lanes 5), CM7 (lanes 6), and CM8 (lanes 7). In (A), the cDNA bands derived from the core (pregenomic) RNA, the precore RNA, and the core RNA expressed by the CMV vector are marked with core, precore, and core*, respectively. In (B), the arrowhead denotes the pregenomic RNA band, and the asterisk marks the location of the core RNA derived from the CMV vector. M, molecular weight markers.

![Image](https://example.com/image1)

**FIG. 4.** Effects of individual phosphorylation sites in the HBV core protein on pregenomic RNA packaging. (A) Primer extension of the total cellular RNA. (B) Primer extension of the pregenomic RNA extracted from core particles. All of the cells were cotransfected with pHBV2-Cmt and the plasmid that expressed one of the following core proteins: the wild-type core protein (lanes 1), CM3 (lanes 2), CM4 (lanes 3), CM5 (lanes 4), CM6 (lanes 5), CM7 (lanes 6), and CM8 (lanes 7). In (A), the cDNA bands derived from the core (pregenomic) RNA, the precore RNA, and the core RNA expressed by the CMV vector are marked with core, precore, and core*, respectively. In (B), the arrowhead denotes the pregenomic RNA band, and the asterisk marks the location of the core RNA derived from the CMV vector. M, molecular weight markers.

![Image](https://example.com/image2)

**FIG. 5.** Effects of individual phosphorylation sites in the HBV core protein on viral DNA replication. As described in the legend to Fig. 3, the replicated HBV DNA was extracted from core particles and analyzed by Southern blot. All of the cells were transfected with pHBV2-Cmt and the plasmid that expressed one of the following core proteins: wild-type core protein (lane 1), CM1 (lane 2), CM3 (lane 3), CM4 (lane 4), CM5 (lane 5), CM6 (lane 6), CM7 (lane 7), and CM8 (lane 8). A small amount of a linearized 3.2-kb HBV DNA was included in lane M to serve as a marker.
The identity of the PCR product was verified by Southern-blot analysis. The arrowhead marks the location of the 607-bp PCR product.

We have also analyzed the individual roles of Ser-157, Ser-164, and Ser-172 of the core protein in HBV DNA replication. CM4, which contained only the serine-to-alanine mutation at Ser-157, could support viral DNA replication (Fig. 5). In contrast, CM6 and CM7, which contained the serine-to-alanine mutation at Ser-172 and Ser-164, respectively, could package some viral pregenomic RNA but could not produce a detectable level of viral DNA (Fig. 5). These results indicate that Ser-157 is not essential for HBV DNA replication while both Ser-164 and Ser-172 are critical. This finding is interesting, as these three serine residues served as the phosphorylation acceptor sites with the same efficiency in Huh7 cells (Liao and Ou, 1995).

As mentioned above, CM2, the phosphorylated analog of the core protein, could not support viral DNA replication. Therefore, dephosphorylation of the core protein is likely required for viral DNA replication. This may be due to the need of the hydroxyl group of the serine residues, because CM6 and CM7, which contained one serine-to-alanine mutation at either Ser-164 or Ser-172, could not support HBV DNA replication despite their ability to package the pregenomic RNA. When the serine residue is not phosphorylated, its free hydroxyl group in the SPXX sequence can form a $\beta$-turn structure for DNA binding (Suzuki, 1989). Since both Ser-164 and Ser-172 are in the SPPRR repeat, the $\beta$-turn structures formed by them may be important for the interaction with the viral DNA to facilitate DNA replication.

The mutation of Ser-157 to alanine (i.e., the CM4 mutant) not only did not prevent pregenomic RNA packaging and viral DNA replication, it also did not prevent the release of the virion from cells (Fig. 6). Yu and Summers (1994) demonstrated that one of the four major phosphorylation sites in the DHBV core protein was important for the initiation of infection and two others were needed for the synthesis of the covalently closed circular DNA for viral mRNA transcription. It is conceivable that Ser-157, which is highly conserved among mammalian hepadna-
viruses (Yeh et al., 1990), plays an important role in the early stage of HBV infection.

In conclusion, in this report we demonstrated the possible importance of core protein phosphorylation in pregenomic RNA packaging and dephosphorylation in viral DNA replication. We also found that Ser-157 of the core protein was not essential for pregenomic RNA packaging unless either Ser-164 or Ser-172 was also mutated to alanine. While Ser-164 and Ser-172 are both critical for viral DNA synthesis, Ser-157 is not essential for viral DNA replication and virion release from cells.

MATERIALS AND METHODS

Cell lines and DNA plasmids

Huh7 cells, a well-differentiated human hepatoma cell line, were used for transfection studies. This cell line was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The construction of pWTD, a plasmid that contains the head-to-tail dimer of the HBV DNA genome of the adw2 subtype, has been previously described (Buckwold et al., 1996). pHBV2-Cmt was identical to pWTD, except that the initiation codon of the core protein coding sequence had been mutated from ATG to GCC. Thus, pHBV2-Cmt was an HBV genomic mutant that could not produce the core protein. The construction of pCMV-C, pCMV-CM1, pCMV-CM3, pCMV-CM4, pCMV-CM5, pCMV-CM6, pCMV-CM7, and pCMV-CM8 has been previously described (Liao and Ou, 1995). pCMV-C contained the wild-type core protein sequence, and the rest of the DNA plasmids contained the core protein sequence with different combinations of the serine-to-alanine mutation at amino acids 157, 164, and 172 (see Table 1). The expression of the core protein sequences in these DNA plasmids was under the control of the immediate early promoter of the CMV. The parental vector used for constructing the core protein-expressing plasmids was pRC/CMV (Invitrogen). In the DNA plasmid pCMV-CM2, amino acids 157, 164, and 172 were all mutated from serine to glutamic acid to mimic phosphoserine. This plasmid was constructed by the same procedures used to construct the other core protein plasmids, except that in the CM2 case, the oligonucleotide which contained the sequence 5′-CCGAGGAGATCT-TCTGCGACGCGGCTCTTGAGACCTGCGTCTGCGAG-3′ was used as the downstream primer for the polymerase chain reaction (PCR). The mutations created were confirmed by nucleotide sequencing.

DNA transfection and the analysis of viral RNA

For transcomplementation studies, 70–80% confluent Huh7 cells in a 10-cm petri dish were cotransfected with 10 μg pHBV2-Cmt and 10 μg core protein-expressing plasmid. Forty-eight hours after transfection, cells were lysed. The viral RNA was extracted with RNAzol (Tel-Test, Inc.) following the manufacturer’s procedures. The RNA was then used for primer extension. Details of the primer-extension procedures have been previously described (Li et al., 1999). For analysis of the pregenomic RNA packaged in core particles, cells were rinsed twice with TBS (10 mM Tris–HCl, pH 7.0, 150 mM NaCl) and lysed with 1 ml TBS containing 0.1% Nonidet-P40 (NP-40). After a brief centrifugation in a microcentrifuge to remove the nuclei, MgCl₂ and CaCl₂ were added to the cytoplasmic lysates to final concentrations of 10 and 5 mM, respectively. The cytoplasmic lysates were then treated with 10 μg DNase I (Boehringer Mannheim) and 1 unit micrococcal nuclease (Boehringer Mannheim) at 37°C for 1 h. Core particles were pelleted by ultracentrifugation in a Beckman SW55Ti rotor at 50,000 rpm for 1 h and then resuspended in 0.5 ml RNAzol for the extraction of the pregenomic RNA. The RNA thus extracted was used for primer extension.

Analysis of HBV DNA replicative intermediate (RI)

For the analysis of the HBV RI DNA, cells transfected with the DNA plasmids were lysed with TBS containing 0.1% NP-40 and treated with DNase I and micrococcal nuclease as described above. The nuclease reaction was stopped with 20 mM EDTA, 1% sodium dodecyl sulfate, and 200 μg proteinase K, and the sample was further incubated at 37°C for 2 h. The RI DNA was isolated by extraction with phenol and chloroform and analyzed by Southern blot using a purified 3.2-kb HBV genomic DNA fragment as the probe.

PCR analysis of the HBV virion DNA

The incubation media of cells were harvested 48 h after transfection. A 10-μl aliquot was mixed with 90 μl 10 mM Tris–HCl, pH 7.5, containing 10 mM MgCl₂, 10 mM CaCl₂, 5 units micrococcal nuclease, and 10 μg DNase I and incubated at 37°C for 30 min to remove any contaminating HBV DNA. The nuclease reaction was stopped by the addition of 10 μl 0.5 M EDTA, 6 μl 10% SDS, 10 μg yeast tRNA carrier, and 50 μg proteinase K. Alternatively, a 1-ml aliquot of the medium was treated with the nuclease followed by immunoprecipitation with the anti-HBsAg antibody and the treatment with the proteinase K. After an incubation at 60°C for 2 h, the virion DNA was extracted with phenol and chloroform, ethanol precipitated twice, and resuspended in 10 μl TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). The PCR was carried out using the Taq polymerase (Life Technologies) following the manufacturer’s procedures. The sense primer used was 5′-TCTGTGCCAAAGTGTTTGCTGA-3′, and the antisense primer used was 5′-CTAATACAAAGACCTTTAACCTA-3′. The PCR product, which contained most of the X protein coding sequence, was 607 bp in length.
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