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# Hepatitis B Virus DNA Replication Is Coordinated by Core Protein Serine Phosphorylation and HBx Expression

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**The hepatitis B virus (HBV) core protein forms the capsid of viral particles and is essential for viral genome DNA replication and maturation. The C terminus of core protein contains three serines at positions 155, 162, and 170, phosphorylation of which is important for viral DNA replication. We demonstrate that the phosphorylation of these serines is stimulated by the viral HBx protein, a regulatory protein that activates signal transduction pathways and viral replication. HBx is therefore shown to stimulate HBV replication by increasing core serine phosphorylation. Mutational, biochemical, and mixing studies of C-terminal core serine mutants demonstrate that multiple serine phosphorylations occur on the same core protein. Mutation of individual core protein serines is shown to inhibit HBV replication at distinct stages corresponding to encapsidation of viral pregenomic RNA, reverse transcription, and restriction to synthesis of specific DNA replicative intermediates. We therefore demonstrate that a primary target of HBV replication that is regulated by HBx protein corresponds to increased phosphorylation of the viral core protein. We also demonstrate that core phosphorylation mediated by HBx promotes sequential progression of viral replication through the assembly of capsids primed for different stages of DNA synthesis.**

Hepatitis B virus (HBV) is a pathogen that chronically infects 350 million people worldwide, a large percentage of whom will develop virus-related liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. While many of the steps of HBV replication are now known, there is little detailed knowledge of the regulation of viral replication. HBV particles contain a 3.2-kb partially double-stranded circular DNA molecule bound to the virally encoded polymerase protein, which is within a protein capsid. The capsid is composed entirely of the viral core protein surrounded by an envelope made of lipids and the three viral envelope proteins. Once HBV infects a liver cell, the viral DNA enters the nucleus where the DNA plus strand is synthesized to completion, the polymerase is detached from the DNA minus strand, and the gaps are repaired to generate a covalently closed circular DNA (cccDNA) (reviewed in reference 13). cccDNA functions as a nonreplicative, nonintegrating plasmid-like template for viral transcription by cellular RNA polymerase II. Among the viral transcripts synthesized from cccDNA, the 3.5-kb pregenomic RNA (pgRNA) encodes the core and polymerase proteins and carries, at its 5′ end, a stem-and-loop structure (ε) that directs its encapsidation into the viral replication complex. The polymerase protein recognizes the RNA ε structure in the context of a ribonucleoprotein complex that also includes cellular heat shock proteins (15) and then reverse transcribes the pgRNA into the first nucleotides of the DNA minus strand (41). This short DNA sequence is then translocated onto direct repeat 1 (DR1) at the 3′ end of the pgRNA, from which DNA minus-strand synthesis proceeds (40).

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HBV DNA replication takes place in the cytoplasm inside viral capsids, icosahedral structures formed from 120 dimers of the core protein (42). The human HBV core protein is a 183-amino-acid polypeptide composed of two moieties (29): a 140-amino-acid N-terminal domain that is responsible for core protein dimer formation and assembly into capsid structures and a C-terminal region, rich in arginine residues, which has nucleic acid binding activity. The HBV core protein contains three serine-proline (Ser-Pro) residues embedded in the C-terminal basic domain, each of which is phosphorylated (26). The identity of the kinase(s) that phosphorylates these residues has not been firmly established, and there may be several involved. Studies conducted in vitro have proposed both SRPK members (12) and protein kinase C as candidate kinases (18, 19). It is postulated that as core protein dimers assemble into capsids they recognize the viral pgRNA/polymerase complex (16). Importantly, it has not been determined whether core protein phosphorylation partially regulates the sequential steps of HBV DNA replication or how it might do so. Certainly core protein phosphorylation is critical for generating viral particles fully capable of viral replication (25).

HBV in vivo replication requires the expression of its regulatory protein, known as HBx (7, 13). Indeed, viral infection cannot be established in woodchucks injected with HBx-deficient woodchuck hepatitis B virus DNA (11, 44). In the HepG2 human hepatoma cell line, an HBx-deficient virus replicates at much lower levels than does $HBx^+$ virus (8, 28), again supportive of an important role for HBx in stimulating HBV replication. HBx has been shown to possess at least several activities including moderate transcriptional activation, stimulation of cytoplasmic signal transduction pathways, and other activities that could impact viral replication (7).

In this study, we show that phosphorylation occurs on each C-terminal core protein serine, that each serine specifically contributes to and coordinates the stepwise completion of ma-

ture HBV DNA genomes, and that all three serines are necessary to generate capsids harboring mature viral DNA. Furthermore, HBx protein was found to stimulate core serine phosphorylation and increase HBV DNA synthesis at multiple steps, a finding which for the first time connects HBx activity to specific functions in HBV replication. We also show that capsids containing HBV DNA replicative intermediates are selectively found in the cytoplasm, whereas capsid mutants which fail to synthesize DNA are localized equally in the nucleus and in the cytoplasm. This suggests that encapsidation of HBV DNA, whose synthesis is stimulated by C-terminal core protein serines, provides a cytoplasmic retention signal and initiates a "maturation signal" that allows nucleocapsids to acquire an envelope and subsequently to be secreted from the cell (32, 38). Finally, we show that coassembly of hybrid wild-type (WT) and serine mutant core proteins into capsids interferes at multiple levels with successful HBV DNA replication. This result suggests that phosphorylation of all three serine residues occurs on the same core molecule and that all three serines are essential for progression of HBV DNA replication. Hence, HBV core phosphorylation promoted by HBx constitutes a key event in HBV replication and may represent a target for the development of novel antiviral agents.

## MATERIALS AND METHODS

**Vectors.** Mutations described in the text were introduced by site-directed mutagenesis using the pAlter system (Promega), and the mutagenized DNA fragment was subsequently exchanged into the vector payw1.2, in either $HBx^+$ or $HBx^-$ genetic backgrounds (37). Cytomegalovirus immediate-early promoter expression vectors were constructed that direct the synthesis of the following: an $HBx^-$ pgRNA containing wild-type core protein or mutated core; a pgRNA in which the core initiation codon was mutated from AUG to ACG, thereby abolishing core protein synthesis; the HBx open reading frame (ORF); and wild-type or mutant core ORFs.

**Cell culture.** The HepG2 cell line (American Type Culture Collection) was grown in minimal essential medium with Earle's salt and L-glutamine (Mediatech; Cellgro) containing sodium pyruvate, nonessential amino acids, penicillin-streptomycin, and 10% fetal calf serum. Cells were grown at 37°C in a humidified atmosphere of 5% $CO_2$ on dishes coated with bovine dermal collagen (Vitrogen 100; Cohesion). DNA was prepared using the QIAGEN Maxiprep kit (QIAGEN). Cells were grown to 95% confluency and transfected with plasmid DNA using FuGENE6 lipid mix, according to the manufacturer's instructions (Roche), and harvested 2 days later.

**Nucleic acid purification and analysis.** To prepare cell lysates, cells were harvested by scraping from plates into ice-cold phosphate-buffered saline, recovered by centrifugation for 5 min at 2,500 rpm, and resuspended in 1 ml per $10^7$ cells of lysis buffer (100 mM NaCl, 10 mM magnesium acetate, 50 mM Tris-HCl, pH 8.0, 0.5% NP-40). Cells were kept on ice for 5 min with intermittent mixing, and then debris was removed by centrifugation at 14,000 rpm at 4°C in a microcentrifuge. Cytoplasmic HBV DNA was then purified (39), by incubation with 200 μg/ml DNase I and 100 μg/ml RNase A at 37°C to degrade plasmid DNA and cellular RNA. To purify replicative DNA, capsids and other proteins were then degraded by adjusting lysates to 1.0 mM EDTA, 0.5% (wt/vol) sodium lauryl sulfate, 5 mM $CaCl_2$, and 50 μg/ml proteinase K. Lysates were incubated for 1 h at 52°C and then subjected to phenol-chloroform extraction until clean, and DNA was precipitated with 2 volumes of ethanol and resuspended in different buffers. For electrophoretic analysis, DNA was resolved on a 1.2% agarose gel in Tris-buffered EDTA buffer. DNA was transferred to a positively charged nylon membrane (GeneScreen Plus; Perkin-Elmer Life Sciences) in 10× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.7]) and hybridized to probes prepared from the 3.2-kb HBV DNA with $^{32}$P-labeled deoxynucleoside triphosphates and the Rediprime II kit (Amersham Biosciences). For isolation of total RNA, cells were lysed and RNA was extracted using Trizol (QIAGEN Corp.) according to the manufacturer's instructions. Northern blot RNA analysis was performed by formaldehyde-1% agarose gel electrophoresis, transfer of RNA to nylon membrane, and hybridization with $^{32}$P-labeled probes prepared from the entire HBV genome. For RNA protection analysis (RPA) assay, core proteins/capsids in cell lysates prepared as described above were immunoprecipitated using polyclonal anti-HBc antibodies (Dako) coupled to protein A-Sepharose beads for 2 h at 4°C. Beads were gently pelleted, washed extensively in lysis buffer, and then incubated with 5 μg DNase I (Sigma) at 37°C for 1 h to degrade plasmid DNA. The encapsidated pgRNA was extracted with Trizol (Invitrogen) followed by an additional digestion with 25-U/ml RQ1 RNase-free DNase I (Promega) at 37°C for 1 h. DNA was then extracted by phenol-chloroform and recovered by ethanol precipitation. A riboprobe was generated using $^{32}$P-labeled deoxynucleoside triphosphates and SP6 RNA polymerase, transcribing the antisense orientation of an HBV BspEI-XbaI fragment within the core ORF (nucleotides 2327 to 2139, using the EcoRI site GAATTC, with nucleotide position one corresponding to C). RPA was performed according to the RPAIII kit instructions (Ambion). Protected RNA was then resolved by electrophoresis on a denaturing 8 M urea-5% polyacrylamide gel. For the endogenous polymerase analysis, capsids were concentrated by centrifugation of lysates through a 20% (wt/vol) sucrose cushion using a TLS55 rotor and an Optima ultracentrifuge (Beckman Coulter) for 1 h at 50,000 rpm. The pellet was resuspended in 80 μl of reaction mix (50 mM Tris-HCl, pH 8.0, 40 mM $MgCl_2$, 50 mM $NH_4Cl$, 0.3% 2-mercaptoethanol, 12.5 μM dTTP-dATP-dGTP, 0.2 μl [$^{32}$P]dCTP/sample), at 37°C for 2 h, followed by chase with unlabeled 12.5 μM dCTP for 1 h (29). Capsid DNA was purified by sodium dodecyl sulfate (SDS)-proteinase K digestion and phenol-chloroform extraction as described above, concentrated by ethanol precipitation, and resolved on a 1% agarose–Tris-acetate-EDTA–1% SDS gel in Tris-acetate-EDTA–1% SDS (29). The gel was dried and exposed for autoradiography. For analysis of the priming reaction, total lysates of transfected cells were incubated as for the endogenous polymerase assay in the presence of [$^{32}$P]dCTP or [$^{32}$P]dGTP as described above, followed by chase with the respective cold deoxynucleotide. Particles containing labeled DNA were directly visualized following electrophoresis on native agarose gels. Alternatively, labeled core-associated viral DNA was extracted and separated on a 1.2% agarose gel.

**Capsid analysis by native agarose gels.** Cell lysates were prepared by lysis of cells and preparation of DNase- and RNase-treated capsids as described for nucleic acid purification above. Fifty micrograms of protein was resolved on 1.2% agarose gels in Tris-buffered EDTA buffer at 50 V for 2 h. The gel was transferred to GeneScreen Plus in TNE buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) and probed by immunoblotting with polyclonal anti-HBc serum (1:500; Dako) followed by detection of secondary antibody by chemiluminescence. Following protein detection, the same membrane was denatured in 0.2 M NaOH, 150 mM NaCl for 5 min and neutralized by being soaked in 0.2 M Tris-HCl, pH 7.5, 1.5 M NaCl for 5 min, and then nucleic acids were cross-linked on the membrane by UV irradiation (35, 43). Hybridization was performed, as described above, for capsid-associated DNA.

**Cell labeling.** Transfected HepG2 cells were labeled with 200 μCi [$^{32}$P]orthophosphate (New England Nuclear) per $10^7$ cells for 2 h, rinsed in ice-cold phosphate-buffered saline, and lysed in 500 μl lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 0.5% NP-40, 50 mM NaF, 25 mM β-glycerophosphate, 2 mM sodium vanadate, and protease inhibitors). Lysates were precleared for 1 h at 4C° with 4 μg rabbit anti-mouse immune globulins and protein A-Sepharose and then immunoprecipitated with 1 μl rabbit anti-HBc antiserum for 2 h at 4°C and 40 μl protein A-Sepharose. Beads were sedimented and washed four times in lysis buffer. Beads were resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer and resolved by 15% SDS-PAGE, transferred to a polyvinylidene difluoride Immobilon-P membrane (Millipore), exposed for autoradiography, and probed with an anti-HBc antiserum as described above.

## RESULTS

**Serine 162 in the HBV core protein is necessary and sufficient for encapsidating HBV pgRNA.** HepG2 cells were transfected with the replication-competent (replicon) vector payw1.2 (37) that expresses WT or mutated core proteins containing only one of three serines at position 155 (SAP mutant), 162 (ASP mutant), or 170 (AAS mutant) (Fig. 1A). Other core mutants contain two out of three serines at positions 155 and 170 (SAS mutant) or 162 and 170 (ASS mutant). The substitution of alanine (Ala) for serine prevents phosphorylation and produces a minimal change in protein structure, whereas substitution of a proline at the serine 170 site was chosen out of

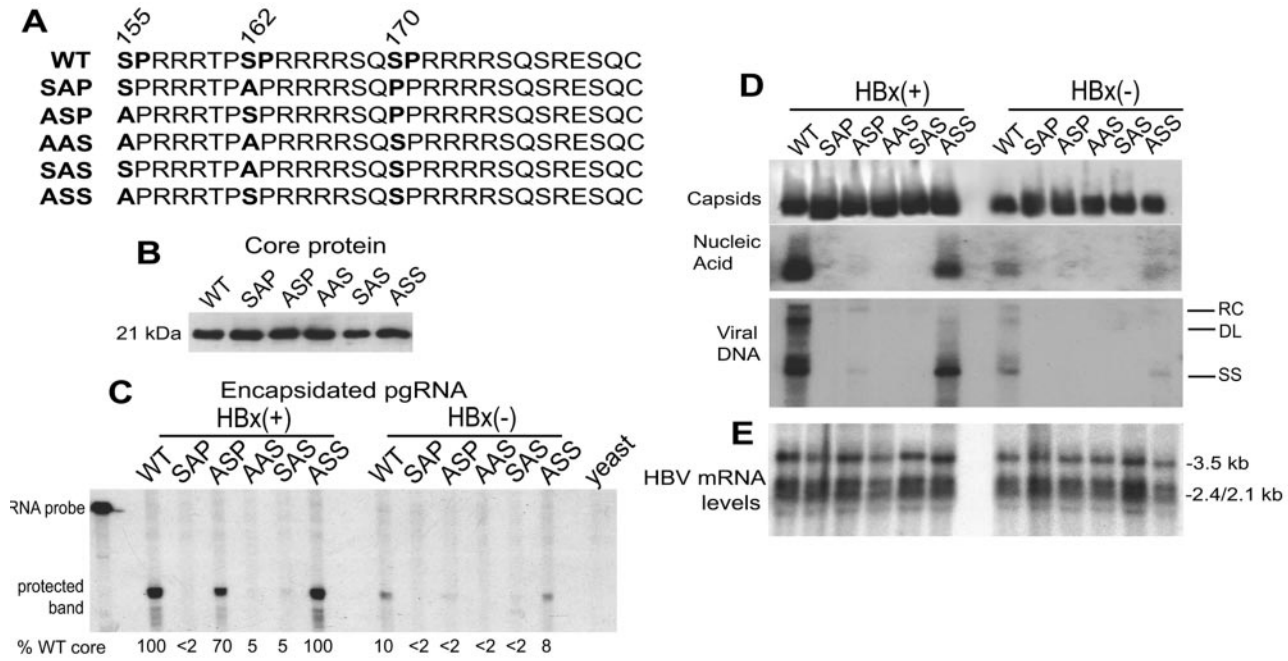


FIG. 1. Role of individual serines in HBV pgRNA encapsidation and DNA replication. HepG2 cells were transfected with 1.2-mer HBV replicon constructs expressing WT or core mutants, in an $HBx^+$ or $HBx^-$ genetic background. (A) Schematic representation of the amino acid sequence of the C-terminal region of WT and serine substitution mutants of core protein. Mutant nomenclature corresponds to the three phosphoserines at positions 155, 162, and 170. (B) WT and mutant capsids were recovered from equal amounts of HepG2 cell lysates by immunoprecipitation 2 days posttransfection, resolved by denaturing SDS-15% PAGE, and detected by immunoblotting. (C) RPA was performed on extracted pgRNA from capsids purified as described above. The 225-nucleotide antisense riboprobe encompasses the HBV DNA sequence of nucleotide positions 2139 to 2327, present exclusively in the pgRNA. The protected 188-nucleotide fragments were resolved on denaturing 8 M urea-5% polyacrylamide gels, visualized by autoradiography, and quantified by densitometry. Data were normalized to the value of the $HBx^+$ WT core samples, which was set at 100%. (D) (Top panel) Equal amounts of cytoplasmic lysates were subjected to native agarose gel electrophoresis and immunoblotting using anticore antibodies. (Middle panel) Nucleic acids within capsids were detected by hybridization after alkali denaturation. (Bottom panel) Southern blot DNA hybridization analysis of core-associated HBV genomic DNA. DNA was extracted from cytoplasmic capsids and analyzed by Southern blot DNA hybridization. (E) Cytoplasmic RNAs were extracted from cells transfected as described above, resolved by formaldehyde agarose gel electrophoresis, and detected by Northern blot hybridization for HBV mRNAs. For all panels, representative results are shown from at least three independent studies. Results were quantified by densitometry of autoradiograms.

necessity, to leave the overlapping polymerase ORF unchanged. A triple mutant was not engineered because it had been previously shown that mutating all three serines (Ser 155, 162, and 170) to alanine almost fully abolishes HBV core protein phosphorylation (26), leaving only a residual weak phosphorylation signal at alternate sites (12). Moreover, several studies have now shown that the triple mutation abolishes encapsidation of pgRNA and viral DNA replication (24, 25). The defect of the triple mutant was also recently linked to the enhanced encapsidation of a defective short spliced RNA derived from pgRNA (24). We therefore did not include this aberrantly defective mutant in these studies. HBV replicons were generated in the background of $HBx^+$ or $HBx^-$ genomes (28).

HepG2 cells transfected with HBV replicon constructs exhibited similar expression levels of WT and mutant core proteins, indicating that the mutations did not destabilize or alter core protein abundance (Fig. 1B). It should be noted that, even on an SDS-15% polyacrylamide gel, WT and mutant human HBV core proteins migrate as a single band of identical size, regardless of core protein phosphorylation status. Therefore, conventional SDS-PAGE does not allow direct assessment of HBV core phosphorylation. Capsids comprised of the mutant core proteins also sediment identically to wild-type capsids in 20 to 50% (wt/vol) sucrose gradients (data not shown). There is also no reason to suspect significant alterations in the stabilities of capsids containing mutant core proteins, as similar levels (within twofold) were generally observed in the studies presented, and a previous report did not show significant changes in stability of similar mutants (14).

To determine whether capsids comprised of mutant core proteins were capable of encapsidating pgRNA, HepG2 cells were transfected with the different HBV replicons, capsids were purified by immunoprecipitation using a polyclonal antiserum to the core protein (anti-HBc), and the encapsidated RNA was extracted. The level of core particle encapsidated RNA was examined by RPA using an RNA probe complementary to the core region in pgRNA. The protected labeled fragments were subsequently resolved by denaturing 8% urea-5% PAGE and visualized by autoradiography (Fig. 1C). In the HBx-expressing cells, encapsidated pgRNA of the predicted size was present at similar levels in the WT and ASS core capsids and at slightly lower levels for the ASP mutant (70% of WT), whereas it was almost undetectable in capsids derived from the SAP, AAS, and SAS core mutants (between 2 and 5% of WT). It is unlikely that the proline at position 170 in the

SAP mutant affects the ability of the polymerase/pgRNA complex to assemble within capsids, since this same amino acid is present in the ASP mutant, which was proficient in pgRNA encapsidation. In addition, the AAS and SAS core protein constructs that specifically lacked serine 162 were defective in pgRNA encapsidation, yet they contain serine 170. Furthermore, the ASS mutant that contains serines 162 and 170 did encapsidate pgRNA. These data therefore indicate that serine 162 is necessary for encapsidation of the viral pgRNA.

The replicons deficient in HBx expression demonstrated that only the ASS mutant and WT core protein particles encapsidated nearly similar levels of pgRNA, which were reduced ~10-fold compared to replicons expressing HBx. However, the ASP core particle mutant in the $HBx^-$ samples contained very low levels of pgRNA relative to ASP core in the $HBx^+$ samples. As addressed below in this study, the serine 170 site is more significantly influenced by HBx-mediated phosphorylation than are the other sites in this system. The lower levels of encapsidated pgRNA in the absence of HBx expression are due in part to the decreased synthesis of pgRNA and core protein, as discussed below.

**Both serine 162 and serine 170 are necessary to produce HBV DNA replicative intermediates in an HBx-dependent manner.** To dissect the function of the core protein C-terminal serines, HepG2 cells were transfected with WT or core mutant HBV replicons (+/− HBx expression) (Fig. 1D). Cytoplasmic extracts were prepared and analyzed for capsid levels (top panel), total encapsidated viral nucleic acids (pgRNA and DNA; middle panel), and core-associated viral DNA replication intermediates (bottom panel). Similar levels (within twofold) of intact capsids were found for WT and mutant core proteins, as determined by native agarose gel electrophoresis and immunoblot identification with an anti-HBc antibody (Fig. 1D, top panel). In the absence of HBx, less core protein was synthesized (data not shown) and approximately two- to threefold-lower levels of core particles were formed compared to wild-type replicons. Immunoblots were denatured and hybridized to a $^{32}$P-labeled HBV probe to detect encapsidated nucleic acids. A significant signal was found only in capsids derived from WT or ASS mutant core proteins in HBx-expressing replicons, whereas a very weak signal was detected in the ASP core mutant, and much fainter signals overall were found for the $HBx^-$ constructs expressing WT or ASS core proteins (Fig. 1D, middle panel).

Cytoplasmic viral DNA replicative intermediates were analyzed by Southern blot hybridization of DNA purified from isolated cytoplasmic capsids, and the major DNA intermediate forms were identified (Fig. 1D, bottom panel). Analysis of viral DNA showed that, when core protein contains only serine 162 (ASP), very little HBV DNA was synthesized. However, this mutant was capable of encapsidating pgRNA at ~70% the level of WT core protein, as shown earlier (Fig. 1C). Thus, serines 155 and 170 are important for viral DNA replication. Interestingly, the ASS core mutant was capable of generating replicative single-stranded DNA intermediates, but not the most mature genome forms, known as relaxed circular (RC) and double-stranded linear (DL) DNAs. Only $HBx^-$ replicons encoding WT core protein, and to a lesser extent the ASS core mutant, produced detectable HBV DNA replicative intermediates at a level ~10% of that of $HBx^+$ samples. Thus, these data indicate the importance of serine 162 for pgRNA encapsidation and serine 170 for formation of HBV DNA, in conjunction with HBx to stimulate viral DNA replication. The much lower levels of encapsidation by the mutant core replicons were not due primarily to the decreased availability of HBV RNA for encapsidation, since viral mRNA levels were generally reduced only two- to threefold from $HBx^-$ replicons, including pgRNA (Fig. 1E). Therefore, at most, approximately half the decreased encapsidation of pgRNA could result from decreased expression of pgRNA. These data therefore suggest that HBx expression promotes encapsidation of pgRNA and/or viral DNA replication.

**HBx promotes phosphorylation of core serine residues.** HBx stimulates cytoplasmic signal transduction pathways and HBV replication (7). Since the studies presented above showed the importance of C-terminal core serines for stimulation of viral DNA replication, we investigated whether HBx activity promotes phosphorylation of these residues. HepG2 cells were transfected with WT or mutant core $HBx^-$ replicons, with and without *trans*-complementation by cotransfected HBx, and then cells were labeled with $^{32}PO_4$. Capsids were purified by immunoprecipitation, and core protein was resolved by SDS-15% PAGE and transferred to membranes. Core protein phosphorylation was detected by autoradiography, followed by immunoblot analysis of the same membranes to determine total core protein levels (Fig. 2A). Autoradiograms were quantified by densitometry, and the ratio of $^{32}$P-labeled core protein to steady-state core protein levels for each mutant was calculated and normalized relative to the WT core protein, $HBx^-$ sample (Fig. 2B). HBx expression increased the overall phosphorylation of WT core protein by twofold compared to $HBx^-$ replicon counterparts. However, HBx strongly increased serine phosphorylation at specific sites in the C terminus of core protein. Stimulation of phosphorylation by HBx was strongest for serines 162 and 170 combined (mutant ASS, 18-fold increase), followed by single serine position 170 (mutant AAS, approximately sixfold increase) and single serine position 162 (mutant ASP, approximately fourfold increase), whereas serine 155 demonstrated the weakest stimulation (mutant SAP, approximately threefold increase). These data indicate that each serine in the C terminus of core is a substrate for HBx-induced phosphorylation to different extents, and all are phosphorylated in the absence of HBx, but also to different extents.

**HBV core serine 155 is required for formation of relaxed circular DNA intermediates.** Studies were next performed to determine whether phosphorylation of specific core protein serines influences the functional state of the polymerase within capsids. Similar levels of native capsids were produced for all core protein mutants from the HBV ($HBx^+$) replicons and at approximately half that level for $HBx^-$replicons, as determined by agarose gel electrophoresis and immunoblot staining of total capsids (Fig. 3A). An endogenous polymerase assay was then performed using core particles from $HBx^+$ and $HBx^-$ replicons expressing WT or mutant core proteins. This assay detects the activity of the viral polymerase within capsids by providing a tracer-labeled [$^{32}$P]dCTP and unlabeled deoxynucleotides, thereby allowing completion of the initiated DNA strand. Analysis of replicated viral DNA within capsids showed that the wild-type virus yielded the expected two major

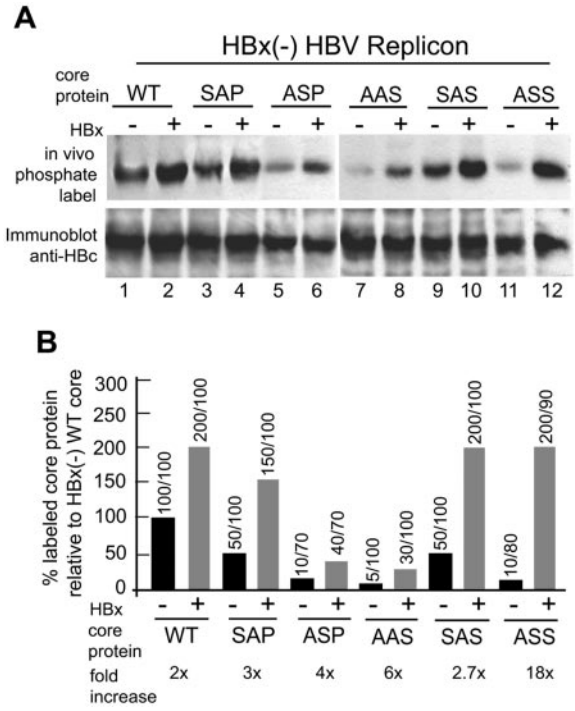


FIG. 2. HBx expression induces core protein phosphorylation. (A) HepG2 cells were transfected with WT or core mutant $HBx^-$ constructs, *trans*-complemented with or without an HBx expression vector, and then labeled at 2 days with $^{32}PO_4$. To facilitate comparisons of $^{32}PO_4$ incorporation, capsids and core protein were immunoprecipitated with anti-HBc antibody using levels of cell lysates that roughly normalized core protein levels between $HBx^+$ and $HBx^-$ samples. Precipitates were denatured; core protein was resolved by SDS-15% PAGE, transferred to membrane, and exposed for autoradiography; and then immunoblot analysis was performed using an anti-HBc antibody and representative results are shown. (B) Autoradiograms were quantified by densitometry. The ratios of $^{32}PO_4$ label incorporated relative to the $HBx^-$ WT sample (numerator) were normalized to total core protein steady-state levels relative to $HBx^-$ WT sample (denominator in parentheses) to derive the percent labeled core protein values shown along the *y* axis. Fold values listed under the *x* axis represent the fold increase in core protein phosphorylation with each core mutant set (+/− HBx expression).

DNA forms (Fig. 3B), corresponding to an RC and DL genomic DNA (shown in higher resolution in the Southern blot DNA analysis in Fig. 3C). Earlier results demonstrated that the SAP and AAS core mutants did not encapsidate pgRNA (Fig. 1C), and accordingly, they generated only very low levels of viral DNA by endogenous polymerase assay. The ASP core mutant, which is proficient in encapsidation of the pgRNA but deficient in HBV DNA synthesis (Fig. 1C and D), also did not generate significant levels of the replicative DNA forms under the extended synthesis conditions provided by the endogenous polymerase reaction. Similarly, *trans*-complementation of a replicon that cannot produce core protein, with a construct that synthesizes an ASP mutant core protein, did not restore polymerase activity (data not shown). Thus, the defect is specific to the core mutant protein and not a secondary result of mutation of the HBV genome. Since ASP core capsids package pgRNA (Fig. 1C), these data indicate that the HBV polymerase packaged within capsids is in an early defective, nonprocessive state. It is likely that the polymerase is incapable of using the ε RNA signal in the pgRNA to prime the reverse transcription of minus-strand DNA (see below), or it cannot transfer the short DNA replication primer onto DR1, thereby inhibiting DNA minus-strand synthesis.

The ASS core protein mutant was found to encapsidate pgRNA to the same extent as WT core protein capsids (Fig. 1C) but was replication impaired both in the endogenous polymerase assay (Fig. 3B) and by Southern blot DNA analysis (Fig. 1D). As shown by higher-resolution Southern blot DNA analysis (Fig. 3C), the $HBx^+$ ASS core mutant replicon generated much lower levels of HBV DNA intermediates and only the single-stranded to double-stranded linear forms. The mature relaxed circular DNA form was not evident. Indeed, the polymerase assay for this mutant showed that the ASS mutant could produce only the DL HBV DNA form at lower levels than WT, with no evidence for the RC DNA form (Fig. 3B). These data suggest that core serine 155, which is mutated to alanine in this construct, is required to promote the transfer of the 5′-terminal RNA oligonucleotide containing the DR1 repeat onto the DR2 repeat in the newly synthesized minus-strand HBV DNA. If the oligonucleotide RNA is not transferred, in situ priming generates only DL DNA (36). As expected, the SAP and AAS core mutants, which are deficient in pgRNA encapsidation (Fig. 1C), were also deficient in endogenous polymerase activity, whether in $HBx^+$ or $HBx^-$ genomic backgrounds (Fig. 3B). Both the RC and DL RNA forms were generated by the $HBx^-$ replicon construct expressing WT core protein, but at overall lower levels, as shown by both endogenous polymerase assay and Southern blot DNA analysis (Fig. 3B and C). However, the $HBx^-$ ASS core mutant was able to produce a very low level of double-stranded DNA molecules detectable by endogenous polymerase assay that were not detectable by Southern blot analysis. These data again indicate that in the absence of HBx expression there is a quantitative defect in the DNA synthesis reaction. It should be noted that the significant inhibition of HBV pgRNA encapsidation and DNA replication in the absence of HBx expression results from the combined reductions in viral mRNA and pgRNA levels as well as reduced core protein phosphorylation.

The data presented suggest that the viral polymerase packaged into mutant ASP core protein capsids is not proficient for DNA elongation. We therefore determined whether the polymerase in ASP core capsids is capable of performing the priming reaction on the encapsidated pgRNA. In the priming reaction, the first nucleotides reverse transcribed by the viral polymerase are 5′-GAA. This primer then becomes covalently linked to the polymerase on its tyrosine 63 residue. The primer-polymerase complex is then translocated to the complementary sequence on DR1 at the 3′ end of the pgRNA, where elongation of the DNA minus strand proceeds (27). If the polymerase is functional for priming but not for elongation, then labeled dGTP is incorporated into the primer and the primer-elongation product can be detected by electrophoresis of capsids on native agarose gels and autoradiography (20). The dCTP control measures elongation of the precommitted polymerase. Cells were transfected with constructs expressing HBV pgRNA containing WT or mutant core proteins, and capsids were isolated (Fig. 3D, top panel), and then lysates were incubated with [$^{32}$P]dCTP or [$^{32}$P]dGTP to measure en-

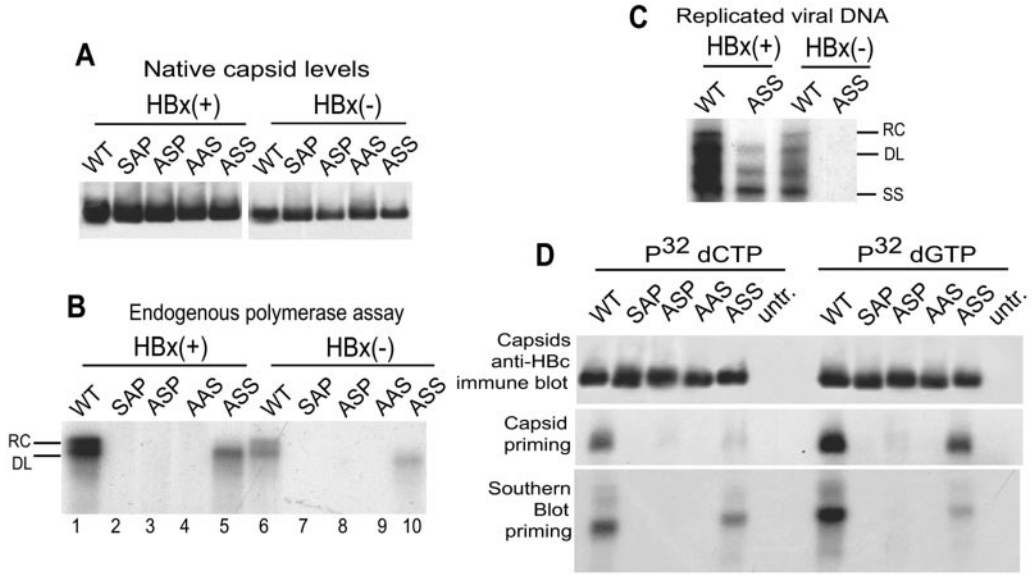


FIG. 3. Core protein serine 155 is required for formation of the mature RC HBV replicative DNA form. Representative results are shown for HepG2 cells transfected with plasmids expressing $HBx^+$ or $HBx^-$ replicons and WT or mutant core proteins. Two days later lysates were analyzed for (A) capsids by native agarose gel electrophoresis and immunoblot analysis; (B) the ability of encapsidated polymerase to replicate HBV DNA, as determined by the endogenous polymerase assay for polymerase function; and (C) capsid-associated HBV DNA, determined by Southern blot DNA hybridization of DNA extracted from core particles. (D) Autoradiogram of endogenous polymerase reaction carried out with [$^{32}$P]dGTP compared to incorporation of [$^{32}$P]dCTP. (Top panel) Immunoblot of capsids obtained from equal amounts of lysates and resolved by native agarose gel electrophoresis. (Middle panel) Endogenous capsid priming assay and autoradiography (see Materials and Methods for experimental details). (Lower panel) Southern blot DNA hybridization analysis of capsid-associated HBV DNA.

dogenous polymerase activity (Fig. 3D, middle panel). Under these conditions there was a very faint DNA replication signal in the ASP capsids after dGTP incubation, whereas no signal was detected in the dCTP reaction, even after prolonged overexposure of films. Labeled DNA was extracted from the endogenous polymerase reaction mixture and resolved by electrophoresis (Fig. 3D, bottom panel). The only signal detectable in the native agarose gel was for WT and ASS core protein mutants, not the ASP mutant. These data therefore indicate that the polymerase in capsids composed of the ASP core protein mutant is highly impaired in the initial priming reaction. The short primer that is likely synthesized in the ASP mutant would be too small to be retained in capsids during electrophoresis and is therefore not observed. From these data we can conclude that the C terminus of core protein coordinates multiple steps in HBV DNA replication through the action of the phosphoserine residues. Phosphorylation of core protein at the C-terminal serines might functionally interact with components of the HBV ribonucleoprotein replication complex, thereby activating the viral polymerase. HBx expression was shown to promote core phosphorylation, which in turn facilitates reverse transcription and genome maturation.

**HBx-dependent HBV replication does not require increased core protein expression.** As shown earlier, HBx expression moderately stimulates viral transcription in the HBV replicon system. For $HBx^-$ replicons, the 3.5-kb precore and pgRNAs were expressed at approximately 50% of the level of wild-type replicons (Fig. 1E). To determine whether the lower levels of encoded core protein are limiting for assembly of nucleocapsids and therefore replication, HepG2 cells were transfected with $HBx^+$ or $HBx^-$ replicons, with or without a plasmid expressing WT core protein in *trans*. Core protein overexpression led to a marked increase in capsid abundance and core protein levels (Fig. 4). However, the level of replicated viral DNA was not increased despite the enhanced levels of capsids. The slight decrease in viral DNA replication observed with overexpression of core protein is not a reproducible finding (e.g., Fig. 5, lane 1). Moreover, it was irrelevant whether the pgRNA was derived from $HBx^+$ or $HBx^-$ genomes, since for either virus there was no change in encapsidated DNA with overexpression of core protein. These data indicate that the

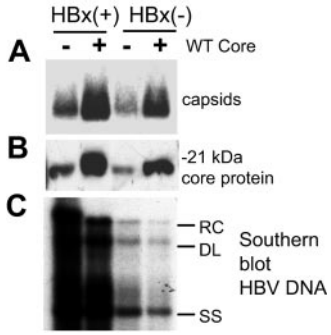


FIG. 4. Core protein level is not a limiting factor in assembly of replicative active nucleocapsids. $HBx^+$ and $HBx^-$ replicons were cotransfected into HepG2 cells with a construct expressing WT core protein in *trans*. Lysates were analyzed for (A) capsids by native agarose gel electrophoresis and immunoblotting, (B) core protein levels by immunoblotting of equal amounts of lysates, and (C) Southern blot DNA hybridization of core-associated HBV DNA. Typical results of at least three independent studies are shown.

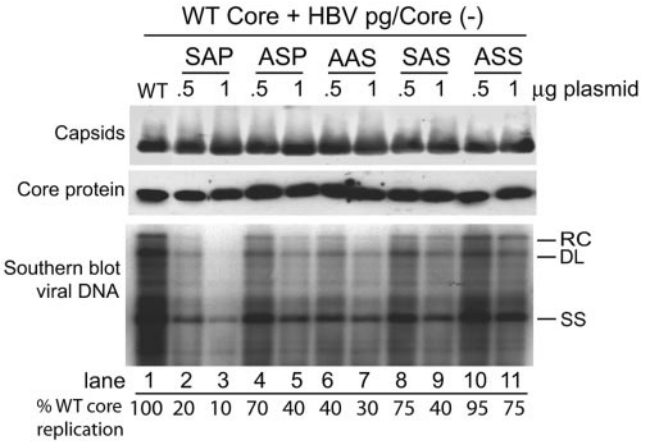


FIG. 5. Mutant core proteins defective in serine phosphorylation exert a dominant-negative effect on WT HBV replication. HepG2 cells were transfected with a $pgRNA^{+}/core^{-}$ HBV replicon (lanes 1 to 11) and plasmids expressing WT (lanes 2 to 11) or serine phosphorylation mutant core proteins (lanes 3 to 11) at different concentrations (0.5 or 1 μg plasmid DNA). Capsids were analyzed by native agarose gel electrophoresis of equal amounts of cell lysate and detected by immunoblot analysis. Total core protein levels were examined by SDS-PAGE and immunoblot analysis. Viral DNA levels in cytoplasmic capsids were analyzed by Southern blot DNA hybridization. Similar levels of core proteins and capsids were produced. Autoradiograms were quantified by densitometry, and results of DNA replication are displayed below lane numbers relative to the DNA replication level of the sample containing only WT core protein (lane 1).

level of core protein is not limiting for HBV DNA replication in this system.

At the concentration of replicon plasmid used in this study, HBx expression modestly increased 3.5-kb pgRNA and capsid levels (Fig. 1). Given the more profound requirement for HBx in viral DNA replication than for viral transcription in this system (reference 8 and shown here), it is likely that HBx expression might increase the level of phosphorylated nucleocapsids that are more efficient in replicating HBV DNA, possibly by promoting interaction between the polymerase and the ε RNA element with the core protein. However, it is also possible that HBx expression might activate the polymerase protein through phosphorylation or aid in spatial orientation of the replicative complexes within the phosphorylated capsids. The high levels of capsids devoid of pgRNA/polymerase (Fig. 4) exclude the possibility that those capsids might be unstable.

**Serine core mutants have a dominant-negative effect on HBV replication.** We have shown that capsids composed of core proteins containing a single C-terminal serine are not capable of supporting HBV replication. Moreover, only a limited number of serines appear to be phosphorylated per capsid (14). We therefore asked whether WT core proteins could rescue HBV DNA replication in capsids that also contain serine mutant core proteins or whether core protein with serine mutations acts in a dominant-negative manner on viral DNA replication when partially incorporated into viral capsids. HepG2 cells were cotransfected with a core-deficient replicon donor and WT core (Fig. 5, lanes 1 to 11) or mutant core protein expression constructs, at either 0.5-to-1 or 1-to-1 ratios of mutant to WT core plasmid DNAs (Fig. 5, lanes 2 to 11). Similar levels of capsids and core proteins were produced in all samples (Fig. 5, top and middle panels). Levels of viral DNA replicative intermediates in capsids were determined by Southern blot DNA analysis of viral DNA extracted from purified cytoplasmic core particles and quantified by densitometry. Viral DNA levels were found to be reduced by coexpression of any of the single serine core proteins with WT core protein. In particular, DNA replication was most impaired by the SAP core mutant, averaging only 10 to 20% of that of WT core protein capsids (Fig. 5, bottom panel, lanes 2 and 3). Incorporation of the ASS core protein into capsids had the least effect (reduction of 5 to 25% of that of WT core protein). In general, the incorporation of serine mutant core proteins into capsids impaired HBV DNA replication to an extent representative of their individual effects. It is not known how many mutant core proteins must be incorporated into capsids to inhibit viral DNA replication. However, since mutant core proteins have a dominant-negative effect on replication, and it is known that hybrid capsids resulting from two core donor constructs can occur (10), we can conclude that WT and mutant core protein mixing take place at a significant enough level to block DNA replication.

**Phenotypic mixing of single serine core mutants does not reconstitute HBV replication.** Our results demonstrate that each single serine residue within the C terminus of core protein has a distinct function in HBV DNA replication. We therefore asked whether wild-type HBV DNA replication could be recovered by coexpressing the three single serine-containing core mutants or the two serine-containing core mutants in the absence of WT core protein. Cells were transfected with a core-deficient HBV replicon and plasmids encoding the three different mutant core proteins capable of phosphorylation of just one serine (serine 155, SAP; serine 162, ASP; serine 170, AAS) or two serines (serine 155/170, SAS; serines 162 and 170, ASS). Similar levels of capsids were detected in all samples expressing core proteins (Fig. 6, top panel), and similar transfection efficiencies were ensured by inclusion of a green fluorescent protein expression plasmid (data not shown). When WT core protein was provided in *trans*, all DNA replicative intermediates (RC, DL, and single stranded) were readily detected (Fig. 6, lane 2). However, the simultaneous cotransfection of SAP, ASP, and AAS core mutants did not restore HBV DNA synthesis (Fig. 6, lane 9), providing only ~5% of the DNA replication level of WT core protein. In addition, the DNA replicative intermediates generated by cotransfection with the ASS construct primarily generated the DL form of viral DNA, and at a level similar to that of WT core protein, indicating that the defect in DNA synthesis is in fact due to the *trans*-complemented mutant core protein (Fig. 6, lane 10). Simultaneous expression of single serine mutants with the ASS construct further reduced the level of DNA replicative intermediates to 10 to 20% of that of WT core protein (Fig. 6, lanes 11 to 13). The direct interpretation of these results suggests that core protein mixing occurred, which generated a dominant-negative effect on HBV replication. These data also suggest that the three serine residues must be located on the same core protein to coordinate encapsidation and reverse transcription of viral pgRNA and that core protein serine phosphorylation and dephosphorylation take place in a coordinated and controlled fashion.

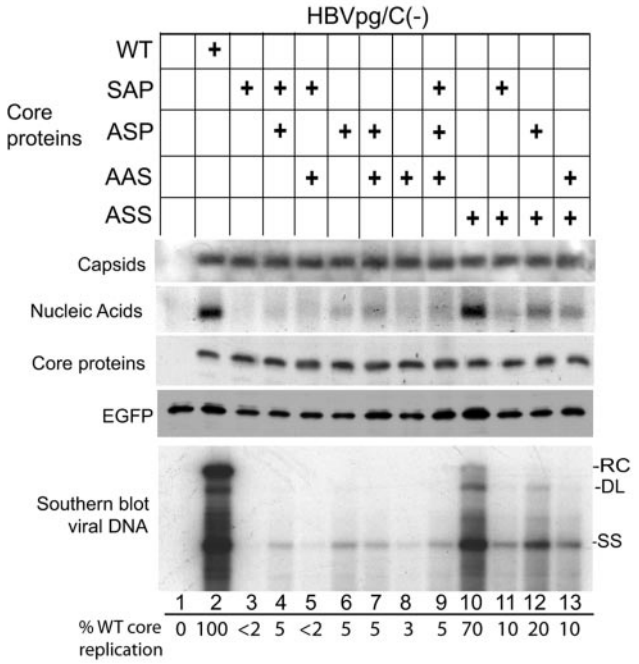


FIG. 6. Mixing of individual serine core protein mutants cannot reconstitute the wild-type HBV DNA replication phenotype. HepG2 cells were transfected with plasmids expressing a pgRNA$^+$/core$^-$ pgRNA (lanes 1 to 13) and WT (lane 2) or mutant core proteins, individually or in different combinations (lanes 3 to 13). Two days later lysates were analyzed for native capsid levels, total nucleic acids within capsids, total core protein levels, reporter enhanced green fluorescent protein (EGFP) levels (by immunoblot analysis), and capsid-associated HBV DNA by Southern blot DNA hybridization analysis. Similar levels of core proteins and capsids were produced. Autoradiograms were quantified by densitometry, and results of DNA replication are displayed below lane numbers relative to the DNA replication level of the sample containing only WT core protein (lane 2).

**HBV DNA synthesis signals the retention of replicon capsids in the cytoplasm.** The data presented above show that the simultaneous presence of serines 162 and 170 allows HBV DNA synthesis to proceed but without generating significant amounts of the RC form, which is the mature viral DNA form. Other mutant capsids either encapsidated almost undetectable levels of pgRNA or failed to reverse transcribe it. We therefore asked whether one of the serine phosphorylation-dependent steps defines a cue for "particle maturation" whereby nucleocapsids that contain DNA are provided with an envelope (17, 19, 23, 32, 34, 38).

HepG2 cells were transfected with WT or core mutant replicon constructs, and then cytoplasmic and nuclear extracts were prepared by two different extraction procedures (Fig. 7, top panels). Both approaches yielded similar fractionation results. Immunoblot analysis of equal amounts of lysates with antitubulin antibody confirmed that there was only a minimal contamination of cytoplasmic proteins in the nuclear extracts (Fig. 7, lower panel). All constructs produced similar levels of cytoplasmic capsids, as demonstrated by native agarose gel electrophoresis and immunoblot analysis. However, capsids were preferentially found in the nuclear extracts of cells expressing the SAP, ASP, or AAS core mutants which failed to synthesize DNA.

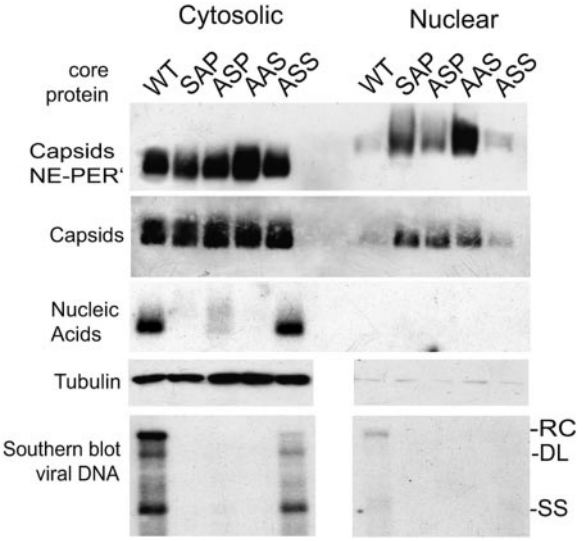


FIG. 7. Capsids that do not support HBV DNA synthesis localize to a nuclear compartment. HepG2 cells were transfected with WT or core protein mutant HBV replicons. Two days later cytoplasmic and nuclear extracts were produced using two different methods (see Materials and Methods for experimental details), equal amounts of proteins were resolved by native agarose gel electrophoresis, and capsids were detected by immunoblot analysis for core protein. Total nucleic acids and capsid-associated HBV DNA were analyzed as described in Materials and Methods. An immunoblot assay was performed using an antitubulin antibody to quantify cytoplasmic contamination of nuclear extracts.

To ensure that the core protein mutants which failed to replicate HBV DNA were not simply redirecting capsids containing nucleic acid to the nucleus, the nucleic acid content and DNA forms in capsids were probed using equal amounts of cytoplasmic and nuclear protein extracts. Very low levels of HBV DNA were found only in WT and ASS core protein capsids obtained from nuclear lysates, which was most likely due to a minor contamination of the nuclear extracts with cytoplasmic capsids (Fig. 7, bottom "Nuclear" panel). Nuclear capsids from the other core mutants did not have detectable levels of DNA, as found in immature capsids (34). The absence of HBV DNA could therefore provide or unmask a nuclear localization signal linked to specific serine residues and their phosphorylation, while capsids that support DNA synthesis are cytoplasmically retained for maturation.

## DISCUSSION

Core protein serves several essential roles in formation of HBV replication complexes. Core is the only protein that forms the capsid, and it has a nucleic acid binding activity in the C terminus that is responsible for encapsidating the viral pgRNA (2, 29). In this paper we demonstrate that each serine phosphorylation site in core protein promotes a distinct function in HBV replication and that all phosphorylation events are inducible to different extents by the viral regulatory protein HBx. Collectively, all three serines coordinate and facilitate the proper progression of HBV replication, possibly by acting in part on the viral polymerase. pgRNA encapsidation specifically requires serine 162, and phosphorylation of this serine is

in part dependent on HBx expression. Furthermore, both serines 162 and 170 must be present for polymerase priming and elongation. However, it is only in the presence of all three serines that the viral RC DNA (which is the mature form) is generated within virions. Mixing of the single serine mutant core proteins failed to recapitulate wild-type HBV DNA replication, providing none of the immature DNA forms at significant levels. These results suggest that phosphorylation of the three serine residues must occur on the same core protein to promote HBV DNA replication. Thus, these results define unique and independent functions for the core protein C-terminal serines in HBV replication, RNA encapsidation, reverse transcription, and DNA synthesis, and they link pgRNA encapsidation and DNA replication to HBx function and its ability to stimulate core protein phosphorylation.

We showed that HBx expression promotes phosphorylation of all critical serine residues in core protein. Since HBx stimulates HBV DNA replication, this effect likely occurs through core protein phosphorylation events. However, HBx has multiple functions, as it also activates signal transduction pathways that affect cell cycle progression and acts on the basal and inducible transcription machinery, and possibly on apoptosis (7, 13). In transformed cell lines, HBx modulates mitochondrial calcium release (6, 8, 9, 31), leading to activation of the Src family of tyrosine kinases (21, 22), as well as Ras and Jun N-terminal protein kinase pathways (5, 30). HBx expression would then be expected to be particularly potent in untransformed and quiescent cells, such as hepatocytes. One of the HBx-induced signal transduction pathways therefore likely includes activation of the core protein serine kinase(s).

The identity of the serine kinases that phosphorylate the C terminus of core protein is unknown or at least unconfirmed. Scanning the core protein for consensus phosphorylation motifs in the C terminus identifies sites for protein kinases C and A, cyclin-dependent kinase 2 (cdk2), and other kinases. However, the use of inhibitors of these kinases in HepG2 cells did not alter HBV replication levels or core protein phosphorylation, regardless of whether the cells were grown in high or low serum concentration (M. Melegari and R. J. Schneider, unpublished results). Moreover, in vitro studies with activated cdk2, the key $G_1$/S transition kinase, did not detect phosphorylation of recombinant core protein or core protein derived from capsids (data not shown).

Several studies have investigated the role of phosphorylation of the serines embedded within the positively charged C terminus of core protein. One report found a requirement for both serines 162 and 170 to support viral DNA replication, as did we, but the authors did not investigate the independent roles of serines 155 and 162 (25). Another study, using a catalytically inactive polymerase and *trans*-complementation assays, proposed functions consistent with those reported here for serines 162 and 162/170, but the authors were not able in this system to evaluate the steps downstream of pgRNA encapsidation (14). Interestingly, core protein mutants containing glutamic acid substituted for serine, which mimics a fixed phosphorylation conformation, generated the lowest encapsidation levels (14). Our results build on these reports to establish important distinct functions in progression and regulation of HBV replication controlled by the C-terminal serine residues and link these effects to promotion by HBx protein.

A very recent study has also examined in greater depth the function of C-terminal core protein phosphorylation (24). It is difficult to compare this study with ours as there is only one core protein mutant in common, the serine 155 mutant, ASS. For that mutant our results are identical in that serine 155 was found to be somewhat reduced in polymerase activity and viral DNA replication. However, we found that serine 155 is essential for production of the mature relaxed circular DNA form of the HBV genome (Fig. 3), suggesting that it promotes the transfer of the DR1 oligonucleotide primer to DR2 in minus-strand synthesis. Kock et al. (24) did not investigate this aspect of core mutant activity. On the other hand, Kock et al. (24) found that certain core protein C-terminal mutants displayed an altered capsid conformation, as detected by increased sensitivity of encapsidated pgRNA to nuclease and increased encapsidation of a short, spliced viral transcript. We are certain that none of the core protein mutants described in our study which fail to encapsidate pgRNA result from greater sensitivity to nucleases. We obtained identical results for pgRNA encapsidation regardless of DNase addition, and addition of exogenous RNase had no effect on the phenotype of the core protein mutants (data not shown). In principle, the lack of HBV nucleic acids (pgRNA and DNA) detected in the SAP, AAS, and SAS capsid mutants might be due to a decrease in capsid stability and degradation of encapsidated RNA and DNA, as shown for a duck HBV core mutant (23). However, we excluded this possibility for the following reasons. First, identical results were obtained when cytoplasmic extracts were examined for capsids by native agarose gel hybridization, whether or not samples were treated with RNase or DNase. Therefore, the absence of nucleic acids in the mutant capsids did not result from postlysis nucleases or from increased susceptibility of certain mutant capsids, as discussed above. Second, similar amounts of capsids were found for WT and mutant core proteins. Third, even at high salt concentrations, mutant core proteins were still assembled into capsids, indicating that they readily and efficiently self-assemble. With respect to the reported preferential encapsidation of spliced HBV RNA by capsid mutants (24), we did not detect this outcome with the different groups of mutants that we tested. However, we did not examine capsids composed of core protein containing mutations in all three serines, the form which predominantly encapsidated spliced RNA (24), since it had been shown to be defective in pgRNA encapsidation (14).

Mutagenesis of core protein and analysis of the ASP core mutant replicons identified a disparity between pgRNA encapsidation, previously shown to require the presence of polymerase (1), and polymerase activation. Analysis of capsid-endogenous polymerase activity has shown it to be inhibited if the polymerase is mutated in its catalytic site, if the pgRNA is deleted of the ε element or the DR1 and DR2 regions, or if core protein is deleted in the C-terminal 20 amino acids (29). In addition, polymerase activation in vitro assays involve conformational changes produced by several heat shock proteins (3, 4, 16), where the DNA priming and initial DNA synthesis steps are carried out independently of core protein. In the ASP core mutant, following pgRNA encapsidation, the polymerase is most likely present in a nonprocessive state that cannot efficiently initiate the primer synthesis, transpose it on DR1, and elongate the minus-strand DNA. Moreover, the ASS core

protein HBV replicon failed to generate the mature RC DNA form. This had been shown to occur in genomes containing a specific deletion within the encapsidation signal or DR1 sequences (36). Here we have shown that core protein serine phosphorylation controls the second template switch by the polymerase in *trans* and that this event is phosphorylation regulated and stimulated by HBx.

It had been proposed that, following genome maturation, the core C terminus extrudes from capsids and that the associated charge modification and/or DNA association masks a nuclear import signal (42). Accordingly, we found that single serine core protein replicon constructs were also distributed in the nucleus, whereas replicons that synthesize DNA were retained in the cytoplasm. We propose that these two serines (160 and 170) and/or their phosphorylated forms, or possibly the HBV DNA molecule itself, mask the nuclear localization signal of core protein and constitute part of the "maturation signal" of the virus particle.

Core phosphorylation has also been proposed to facilitate the import of core protein dimers and/or capsids with their viral nucleic acids into the nucleus (19, 23, 34), although it is not clear whether this modification might simply favor capsid disassembly. Indeed, in duck HBV, core protein is present in different phosphorylated states within the infected cell but only as dephosphorylated protein in the secreted virion (33). This would indicate that, during viral maturation, phosphorylation is lost or active dephosphorylation occurs. HBV core serine phosphorylation, induced by HBx expression, could be transient, and the serine residues might become dephosphorylated upon completion of HBV DNA synthesis. Conversely, phosphorylation might facilitate recycling of the capsids to the nucleus for viral cccDNA maintenance. In transfected HepG2 cells, viral nucleoparticle recycling does not occur at a significant level, since there is little or no formation of cccDNA. Therefore, our data do not necessarily oppose the view that core phosphorylation facilitates nuclear import. Rather, these results suggest that core phosphorylation is integral to particle maturation and DNA synthesis. Since specific serine mutants generate nucleocapsids that are locked at different steps in HBV replication, which requires the polymerase to perform a template switch, it is likely that individual phosphorylation events operate on those transitions. Our data are consistent with a dual role for HBx in HBV DNA replication, one being exerted at the transcriptional level and one being exerted at the posttranscriptional level, which promotes core phosphorylation that in turn coordinates and directs polymerase function. Finally, our data suggest that specific inhibition of HBV core phosphorylation, by either drugs, competing peptides, or inhibitors of HBx signal transduction activity, could be an effective strategy to block HBV replication.

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