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A Domain of the Hepadnavirus Capsid Protein Is Specifically Required for DNA Maturation and Virus Assembly

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Mutations introduced into the capsid gene of duck hepatitis B virus (DHBV) were tested for their effects on viral DNA synthesis and assembly of enveloped viruses. Four classes of mutant phenotypes were observed among a series of deletions of covering the 3′ end of the capsid open reading frame. Class I mutant capsids were able to support normal single-stranded and relaxed circular viral DNA synthesis; class II mutant capsids supported normal single-stranded DNA synthesis but not relaxed circular DNA synthesis; class III mutant capsids resembled class II capsids, but viral DNA synthesis was inhibited 5- to 10-fold; and class IV capsids were severely restricted in their ability to support viral DNA synthesis. Class I capsids were assembled into enveloped virions, but class II, III, and IV capsids were not. Viral DNA synthesized inside class II capsids was normal with respect to minus-strand DNA initiation, plus-strand DNA initiation, and circularization of the DNA, but plus strands failed to be elongated to mature 3-kb DNA. The results suggest that a function of the capsid protein specifically required for viral DNA maturation is also required for assembly of nucleocapsids into envelopes. Thus, class II mutants appear to be defective in the appearance of the "packaging signal" for virus assembly (J. Summers and W. Mason, Cell 29:403–415, 1982).

Hepadnaviruses are small DNA-containing enveloped viruses that cause persistent infections in hepatocytes. Hepadnaviruses with a narrow host range specificity for humans (7), woodchucks (27), ground squirrels (14), ducks (16), and European herons (24) have been identified and extensively studied with regard to mechanisms of replication. Hepadnavirus DNA, unlike that of other DNA viruses, is replicated conservatively, by transcription of an RNA genome, followed by reverse transcription to produce new DNA (28).

Hepadnavirus genes are found in persistently infected cells as covalently closed circular DNA molecules in the nucleus (15, 16, 22, 29). These molecules serve as transcriptional templates for production of the RNA genome, or pregenome. The pregenome is packaged in the cytoplasm into an initially immature viral capsid consisting of 180 molecules of a single virus-encoded protein and displaying icosahedral symmetry (3). In addition to the pregenome, the immature capsid contains products of the viral P gene (1, 8), which carry out priming of viral DNA synthesis (2, 4, 18), reverse transcription of the pregenome to generate the minus-strand DNA, degradation of the pregenome, and synthesis of the viral plus-strand DNA (5, 21). Only after plus-strand elongation has occurred are mature viral capsids assembled into viral envelopes and secreted from the cell. A small fraction of viral nucleocapsids are also utilized to augment the levels of viral covalently closed circular DNA (cccDNA) in the nucleus (29). This "amplification" of cccDNA is necessary for high levels of virus production (29), but is under strict control of the large viral envelope protein (P36 in the duck hepatitis B virus [DHBV]), which inhibits further cccDNA synthesis in normal hepatocytes after a copy number of 10 to 50 per cell is attained (25, 26).

Current knowledge of the replication cycle of hepadnaviruses suggests that the viral capsid protein may have functions other than simply to provide a protein shell in which the viral nucleic acids are sequestered. In addition to assembling into a capsid, the capsid protein probably has a role in mediating recognition of the P protein gene product(s) for coassembly with the pregenome, in recognizing the envelope proteins for assembly into virions, in participating in nuclear transport of viral DNA for cccDNA formation and amplification, and in controlling cccDNA amplification through interaction with the pre-S envelope protein. The function of the capsid protein that is required for viral DNA synthesis is less clear. Although mutational analysis of hepadnavirus genomes have established that capsid protein is required for viral DNA synthesis (9), this requirement could be indirect, for example, for RNA or P protein packaging.

Viral nucleocapsids in the cytoplasm of infected cells do not appear to be equivalent substrates for assembly into enveloped virions. While many capsids contain nascent minus-strand DNA, only those capsids containing relaxed circular double-stranded DNA are actually secreted from cells as enveloped virus. This observation has been used to postulate the existence of a "packaging signal," which appears on the surface of the viral capsid at some point in the maturation of its DNA (28). The appearance of this signal, which is necessary for assembly into envelopes, would in some way be correlated with maturation of the DNA within the capsid. Therefore, an additional function of the capsid protein might be to transduce a signal from the DNA to the surface of the nucleocapsid.

In this article we report that mutations in the C terminus of the DHBV capsid protein can affect the ability of the nascent viral DNA within capsids to mature to the point at which the nucleocapsid would normally be assembled into envelopes and secreted as infectious virus. The inhibition of DNA maturation was due to a defect in elongation of the viral plus strand. The same mutations resulted in the inhibition of assembly of capsids into envelopes. We discuss the possible relationship between the inhibition of DNA maturation and the inhibition of capsid assembly into envelopes.

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MATERIALS AND METHODS

Plasmids and construction of mutants. The expression cassette of plasmid pCMV/core consisted of the immediate-early cytomegalovirus (CMV) promoter (*Stu*I-*Hin*dIII fragment), including the cap site for the promoter, isolated from pBC12/CMV/IL2 (6) and blunt-end ligated to a 3.4-kbp fragment of DHBV DNA, beginning with the start codon of the capsid open reading frame (nucleotide 2647, according to the numbering scheme of Mandart et al. [13]) and ending at the *Eco*RI site (nucleotide 3021). This cassette was cloned between the *Hin*cII and *Eco*RI sites of pUC119 so as to produce plus-strand DNA after infection with bacteriophage M13 K07 (30). The plasmid pSPDHBV.RV2650, consisting of pSP65 containing an *Eco*RI dimer of a DHBV genome defective in capsid protein production, has been described before (9).

C-terminal deletion mutants were generated from pCMV/core by cleavage at the unique *Bgl*II site (nucleotide 391), limited Bal 31 digestion, and blunt-end formation with DNA polymerase I large fragment (Klenow), followed by ligation to a 12-bp linker containing termination codons in all reading frames (CTAGCTAGCTAG). Plasmids were screened for the linker by *Nhe*I digestion, and the extent of the deletion was determined by sequencing. The internal deletion mutant C7B was constructed by fusion of the N-terminal sequence from mutant 246 with the C-terminal sequence of the capsid gene from the *Bgl*II site after cleavage and blunt-end formation with DNA polymerase. This fusion resulted in the substitution of glutamic acid for amino acids 247 through 254. The substitution mutant C4S was constructed by oligonucleotide-directed mutagenesis by the method described by Kunkel et al. (11). A synthetic oligonucleotide was used to substitute the sequence CCTCTCCCACGTGCAGCTGCAG CTCACCATAGATCT for the wild-type sequence between nucleotides 361 and 396.

Cells and transfections. DNA was transfected into the chicken hepatoma cell line LMH (10) by the calcium phosphate coprecipitation procedure described previously (25). Transfections were carried out with 10 μg of DNA per 60-mm dish. Cotransfection of pSPDHBV.RV2650 and pCMV/core was performed with 5 μg of each DNA.

Assay of transfected cells for viral replicative intermediates. Extraction of replicative intermediates, purification of enveloped virus from culture supernatants, and assay of viral DNA by agarose gel electrophoresis and blot hybridization have been described previously (25). When viral DNA was assayed in the nucleus, the nuclear fraction of transfected cells lysed with 1% Nonidet P-40 was washed once with TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.4]), and total DNA was isolated by protease digestion, phenol extraction, and ethanol precipitation.

Mapping of 5′ ends of plus- and minus-strand DNAs. The ends of plus- and minus-strand DNAs were mapped by measurement of the sizes of the single-stranded fragments produced from each strand by restriction enzyme digestion and denaturation. Sizes were determined by electrophoresis through a 1.5% agarose gel after glyoxylation of the DNA and blot hybridization with riboprobes specific for the plus and minus strands, as previously described for the assay of viral RNA (26).

Primer extensions. 5′ ends were further mapped by primer extension. DNA (estimated at 1 ng) was denatured by heating at 96°C, and primers were annealed by slow cooling between 65 and 35°C during 30 min in the presence of 6 mM $MgCl_2$. Primers were extended with T7 DNA polymerase (Sequenase 2.0; USB), and the extension products were analyzed on an 8% polyacrylamide sequencing gel and compared with a sequence ladder generated on cloned DHBV DNA with the same primer.

Phosphorylation of oligonucleotide primers. Primers were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Approximately 50 pmol of [γ-^{32}P]ATP (3,000 to 6,000 Ci/mmol) and 50 pmol of oligonucleotide were incubated in a 20-μl reaction mix containing 50 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 0.02 mM spermidine, 1 mM dithiothreitol, 0.01% Triton X-100, and 20 U of polynucleotide kinase (New England BioLabs). After 30 min at 37°C, the reaction was stopped by the addition of 2 μl of 0.5 M EDTA and heating at 65°C for 5 min. Labeled primers were quantitatively recovered by precipitation with cetyltrimethyl ammonium bromide (19). The reaction was diluted to 0.4 ml with TE containing 0.125 M NaCl and 50 μg of carrier tRNA, and 50 μl of 1% cetyltrimethyl ammonium bromide was added. After 5 min at room temperature, the precipitate was collected by microfuge centrifugation and washed three times with a mixture of 70% ethanol–30% 0.1 M magnesium acetate and one time with absolute ethanol. The pellet was dried in a vacuum and dissolved in TE with heating at 65°C for 10 min.

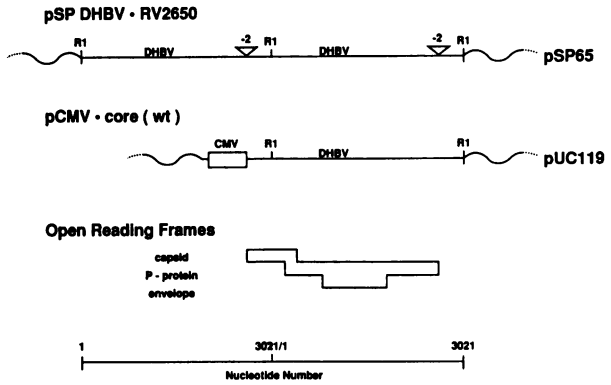
Sequencing. Sequence reactions were performed by the dideoxy chain termination method on denatured double-stranded plasmid DNA with a commercial kit (Sequenase 2.0) according to the supplier's directions.

RESULTS

The C terminus of the capsid open reading frame overlaps the N terminus of the P open reading frame by 245 nucleotides. Therefore, mutations introduced into this region can cause mutation of the P protein and possible virus inactivation. In order to test the effects of mutations in the capsid protein without complications arising from interfering with P protein expression, we relied on a cotransfection system in which replication of a viral genome was dependent on *trans* complementation by the capsid protein. Two plasmids were used in this analysis (Fig. 1a). pSPDHBV. RV2650, an *Eco*RI dimer of DHBV DNA, was used to produce, after transfection, an intact pregenome from the DHBV promoter as well as wild-type envelope and P proteins. This plasmid, however, is unable to encode a capsid protein due to a 2-bp deletion following the first codon of the capsid open reading frame and therefore is totally dependent on capsid protein supplied in *trans* for DNA synthesis. A second plasmid, pCMV/core, used to supply capsid protein, consisted of the immediate-early CMV promoter cloned into pUC119 and driving transcription of DHBV DNA. A terminally redundant 3.4-kbp piece of DHBV DNA was inserted so that the capsid protein AUG was the first initiation codon in the CMV-driven mRNA and the normal DHBV polyadenylation cleavage signal would be used to generate a 3.2-kb mRNA. This transcript expressed capsid protein but could not itself be rescued as DNA by complementation (data not shown).

C terminus of DHBV capsid protein is required for synthesis of mature double-stranded DNA but not single-stranded DNA. pCMV/core was used to generate a series of deletion mutations in the C terminus of the capsid protein (Fig. 1b). Since the C terminus of the human hepatitis B virus (HHBV) capsid protein is not required for capsid assembly (3), we wanted to know whether other functions could be ascribed to this domain. Progressive C-terminal deletions were generated by Bal 31 digestion and ligation of a linker containing

a. Plasmids



b. Mutants

mutant	C-terminal amino acid sequence
	230 240 250 260
	221-tvvygrrrsksrerraptpqragsplprsssshhrspsprk-261
wt	.....
256	.....
252	.....
246	.....
245	.....
243	.....
239	.....las
237	.....
236	.....s
231	.....s
225	.....ts
C4S	.....aaaa.....
C7B	.....e .....

FIG. 1. Plasmids and mutants used in the analysis of capsid protein function. (a) Plasmids. The replication-defective dimer-containing plasmid pSPDHBV.RV2650 was described previously (9). The position of the 2-bp deletion is indicated. pCMV/core (wild type [wt]) is shown relative to open reading frames for the capsid, P protein, and envelope genes. (b) Mutants. The amino acid sequence of the C terminus of the wild-type capsid protein is displayed. Amino acids are numbered from the AUG of the capsid protein at nucleotide 2647 according to Mandart et al. (13). For each of the mutant capsid proteins depicted below, identity of amino acids to the wild-type sequence is marked by a solid circle. In some mutants amino acids encoded by the linker are present, as indicated. C-terminal deletion mutants are designated by the number of the farthest C-terminal wild-type amino acid.

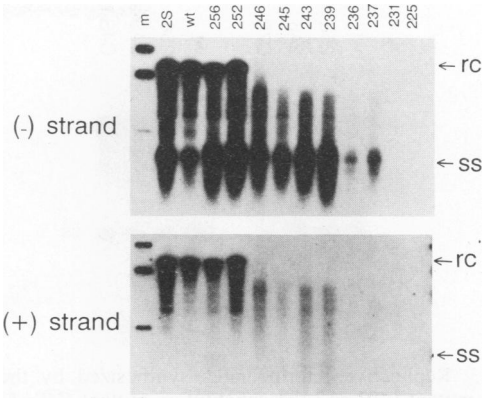


FIG. 2. Replicative DNA intermediates synthesized within wild-type and mutant capsids. Plates (60 mm) of LMH cells were cotransfected with pSPDHBV.RV2650 and the indicated mutant. One plate (2S) was cotransfected with a wild-type (wt) DHBV dimer plasmid, pSPDHBV5.1(2X) (9), and pCMV/core. DNA replicative intermediates were extracted and subjected to 1% agarose gel electrophoresis, followed by transfer to a nylon membrane (Hybond-N). The membrane was hybridized first with a ^{32}P-riboprobe specific for detection of the minus strand (top panel). After exposure of the autoradiogram, the probe was stripped from the filter by incubation in 0.2 N NaOH at 65°C for 30 min. The filter was washed and hybridized with a riboprobe specific for detection of plus strands (lower panel). Hybridization markers (lane m) consisting of 1 ng of pSPDHBV5.1(2X) digested with *Bam*HI (fragment sizes, 4.6, 3.0, and 1.4 kbp) was included in the gel. The migration positions of relaxed circular (rc) and single-stranded (ss) viral DNA are indicated.

termination codons in all three reading frames. Mutant capsid proteins were tested for their ability to substitute for wild-type capsid protein in complementing the pSPDHBV.R2650 mutant for DNA synthesis.

Replicative viral DNA intermediates were detected in cotransfections with mutants lacking up to 25 amino acids from their C termini (Fig. 2). However, deletions of more than nine amino acids resulted in abnormal patterns of replicative intermediates or in reduced amounts of viral DNA. Four distinct phenotypes for DNA synthesis could be distinguished in this series of mutants, and these phenotypes were associated with the removal of successively greater portions of the capsid C terminus. Class I mutants consisted of those mutant capsids in which the formation of single-stranded and relaxed circular viral DNA was apparently normal. Two mutants in which five and nine amino acids were deleted had this phenotype. Class II mutant capsids accumulated normal amounts of minus-strand DNA, but accumulation of double-stranded intermediates was reduced and the formation of mature relaxed circular DNA was not detected. Deletion of between 15 and 22 amino acids from the capsid protein C terminus resulted in this phenotype. Class III mutants resembled class II mutants, but total levels of viral DNA synthesized were reduced by 5- to 10-fold. Class III mutants resulted from the deletion of 24 to 25 amino acids from the C terminus. Class IV mutants were severely defective in synthesis of viral DNA. This phenotype was observed when 30 or more amino acids were deleted.

Deletion of serine-rich sequence causes defect in DNA maturation; phosphorylation of serines in this region is not required for DNA maturation. Since the boundary between the generation of class II and class I phenotypes was located between amino acids 246 and 252, we asked whether deletion or mutation of a small number of amino acids within this region would generate the class II phenotype. Two mutant capsid genes were produced (Fig. 1b). In mutant C7B, an in-frame deletion of eight amino acids was constructed, and this mutant retained the C-terminal seven amino acids of the wild-type protein. A second mutation changed the four serines within this region to alanines (mutant C4S). The phenotype of this mutation was of interest since it is known that the C-terminal domain of the DHBV capsid protein is phosphorylated (20, 23), and we wanted to know whether phosphorylation at one or more of these serines was required for DNA maturation.

As shown in Fig. 3, deletion of an eight-amino-acid domain resulted in the class II phenotype. However, muta-

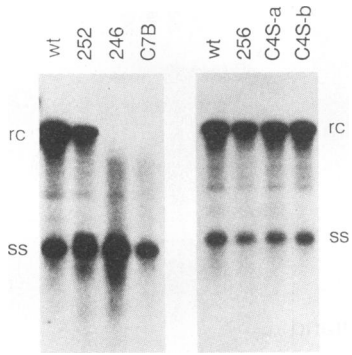


FIG. 3. Replicative intermediates synthesized by the internal deletion mutant C7B and the substitution mutant C4S. LMH cells were transfected and replicative intermediates were assayed as described in the legend to Fig. 1. The blot was hybridized with a riboprobe specific for detection of the minus strand. Wild-type (wt) capsid protein was compared with the class I mutants 252 and 256, the class II mutant 246, C7B, and two independent clones of mutant C4S, a and b. rc, Relaxed circular DNA; ss, single-stranded DNA.

tion of the four serines to alanines at amino acid positions 250 to 253 had no effect on normal DNA maturation.

Mutants defective in DNA maturation are also defective in production of extracellular enveloped virus. We tested whether the phenotype of class II mutants was due to depletion of mature DNA forms from the cell by abnormally rapid assembly into enveloped particles and secretion. A series of class I, II, and III mutants were tested for the release of enveloped DNA-containing particles into the culture medium of cotransfected cells. Enveloped particles were assayed by buoyant density centrifugation in cesium chloride gradients, extraction of DNA from each fraction, and assay of viral DNA by agarose gel electrophoresis and blot hybridization. Enveloped particles banded in the gradient, while free nucleocapsids were pelleted.

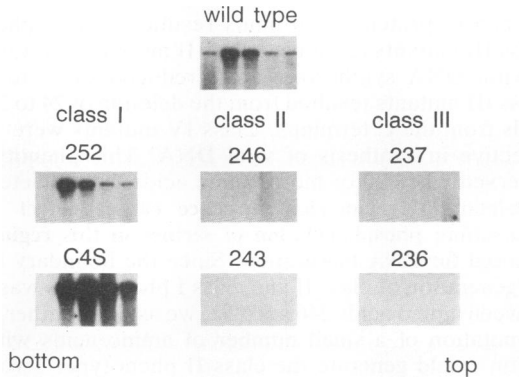


FIG. 4. Cesium chloride gradient centrifugation of extracellular enveloped virus from wild-type- and mutant-transfected cells. Culture fluids from LMH cells cotransfected with the wild type and class I, class II, and class III mutants were harvested at 4 and 5 days posttransfection (total volume, 8 ml), concentrated by precipitation with polyethylene glycol, and centrifuged to equilibrium in CsCl in a Beckman VTi80 rotor, as described previously (26). Fractions were collected from the bottom of the tubes and analyzed for viral DNA by extraction, agarose gel electrophoresis, and blot hybridization with a riboprobe specific for detection of the minus strand. Enveloped virus particles banded in the gradient, while naked cores pelleted on the outside of the tube and were usually seen as contaminants of the final fraction (top).

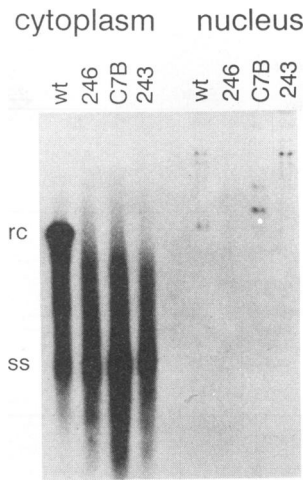


FIG. 5. Assay for viral DNA in the cytoplasm and nucleus of wild-type- and mutant-transfected cells. LMH cells were cotransfected with pSPDHBV.RV2650 and either wild-type (wt) or mutant pCMV/core plasmids. After 5 days, cells were lysed in buffer containing 1% Nonidet P-40, and replicative intermediates were isolated from the cytoplasmic fraction as described in the text. The nuclear pellet was washed one time in 10 mM Tris-HCl, 1 mM EDTA (pH 7.4) and dissolved in a solution containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM NaCl, 0.5% sodium dodecyl sulfate and 0.5 mg of pronase per ml. After 1 h at 37°C, nucleic acids were isolated by phenol extraction and ethanol precipitation. The cytoplasmic and nuclear DNA fractions were analyzed by 1% agarose gel electrophoresis and blot hybridization. rc, Relaxed circular DNA; ss, single-stranded DNA.

All class I mutant capsids (including the C4S mutant) were able to be assembled into enveloped particles and secreted into the culture medium (Fig. 4). These enveloped particles, like wild-type virus, contained nucleocapsids with only mature viral DNA. Class II and class III mutant capsids, however, were defective in the assembly and release of enveloped virus, indicating that mature forms of viral DNA were not depleted from the cell by assembly and secretion. Moreover, immature forms of viral DNA were also absent from enveloped particles in the culture medium, demonstrating a correlative defect in maturation of nucleocapsids, assembly, and secretion.

Mature viral DNA absent from cytoplasm and nucleus of class II mutant cotransfected cells. Our assay for replicative intermediates involved the selective extraction of encapsidated viral DNA from the cytoplasm of transfected cells. In order to determine whether mature viral DNA had been depleted from the cytoplasm by transport to the nucleus, we fractionated cells into cytoplasmic and nuclear fractions and assayed both fractions for total virus-specific DNA. The cytoplasmic fraction, being heavily contaminated with plasmid DNA from the transfection, was treated with DNase I before protease digestion and purification of DNA. The nuclear fraction was not heavily contaminated and therefore was protease treated and extracted directly.

In cells expressing the wild-type capsid protein, the majority of viral DNA was found in the cytoplasmic fraction, with the nuclear fraction containing a small amount of relaxed circular DNA, not visible in the exposure (Fig. 5). Cells expressing class II mutant capsid proteins likewise

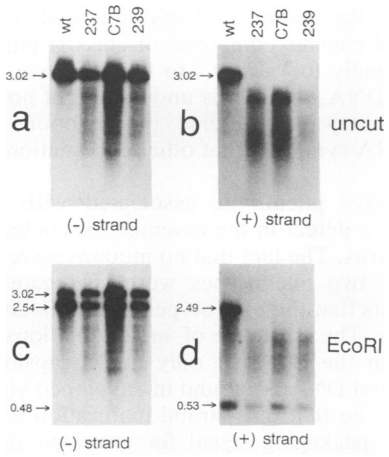


FIG. 6. Agarose gel analysis of denatured plus- and minus-strand DNAs synthesized in wild-type and class II mutant capsids. DNA purified from the capsids used for cotransfection of LMH cells with wild-type (wt) and class II mutants was separated into two portions. One portion was digested with *Eco*RI, and then the untreated portion (a and b) and the digested portion (c and d) were denatured, glyoxylated, and analyzed in duplicate by electrophoresis through 1.5% agarose gels. The gels were transferred to nylon membranes and hybridized with riboprobes specific for the detection of either minus strands (a and c) or plus strands (b and d). The expected *Eco*RI-generated fragments from the minus strand (5′ end, 2.54 kb; 3′ end, 0.48 kb) and from the plus strand (5′ end, 0.53 kb; 3′ end, 2.49 kb) are indicated by arrows.

contained large amounts of cytoplasmic single-stranded viral DNA, but no relaxed circular DNA was present, nor was relaxed circular DNA seen in the nuclear fraction. Therefore, in class II mutants, mature forms were not depleted from the cytoplasm by transport to the nucleus. These combined data strongly suggest that mature forms of viral DNA failed to be synthesized in class II capsid mutants.

Class II mutants initiate viral minus- and plus-strand DNA at the correct sites but fail to elongate plus strands to maturity. The nature of the defect in the production of mature viral DNA was investigated by examining the extent to which viral minus strands and plus strands were initiated at the correct sites. Failure of minus- or plus-strand priming to be limited to the correct sites could result in the inability of much of the viral DNA to circularize during plus-strand synthesis and consequent failure of the plus strands to be fully elongated.

In initial experiments we mapped the 5′ ends of the minus and plus strands of class II mutants compared with the wild type by restriction enzyme digestion and size determination of the separated single strands after glyoxylation. In Fig. 6, the 5′-terminal fragments derived from the minus strand or from the plus strand of wild-type DNA are compared with equivalent fragments from DNA synthesized by three class II mutants. In Fig. 6a and b, it can be seen that the DNA synthesized in wild-type capsids contained full-length minus and plus strands, while that synthesized in class II mutant capsids contained full-length minus strands but only immature plus strands. Examination of the 5′ fragments of the two strands generated by *Eco*RI digestion (Fig. 6c and d) revealed the presence of fragments of the expected sizes for both wild-type and class II mutants (2.54 kb for the minus strand and 0.53 kb for the plus strand), indicating that initiation of both strands occurred at the proper sites (12). Approximately two-thirds of the minus strands from the wild-type and one-half of the minus strands from class II mutant capsids were cut by *Eco*RI, corresponding to the fraction of minus strands that were duplexed with plus strands at the *Eco*RI site. The expected fragment derived from the 3′ end of the plus strands (2.49 kb) was readily observed in DNA from wild-type but not from class II mutant capsids. These results suggest that class II mutants were able to carry out normal initiation and elongation of minus strands and normal plus-strand initiation, but failed to elongate plus strands to completion.

In order to confirm that minus- and plus-strand initiation was indeed occurring at DR1 and DR2, respectively, we carried out primer extension reactions with synthetic oligonucleotides complementary to sequences downstream from DR2 on the plus strand and DR1 on the minus strand. In the case of the plus-strand-specific primer, only those strands that were initiated at DR2 and elongated past the 5′ end of the minus strand, resulting in circularization of the genome, would have been detected.

We included two additional primer extension reactions that would measure the relative number of molecules in which plus strands were duplexed at other sites in the genome downstream of DR2. These sites were detected by cleavage of the double-stranded DNA with either *Hin*dIII, at position 1093, or *Bam*HI, at position 1658, followed by denaturation and primer extension with an oligonucleotide that hybridized to the plus strand at positions downstream of each cleavage site (Fig. 7). This assay measured the number of molecules in which plus strands were duplexed between the cleavage sites and the primer-binding sites relative to the number in which plus-strand DNA was initiated at DR2.

DNA from cells expressing the wild-type capsid and DNA from those expressing class II mutant capsids contained equivalent amounts of minus strands initiated at DR1 (Fig. 7, primer 4) as well as equivalent amounts of plus strands initiated at DR2 (primer 1). With DNA from wild-type capsids as the template, the signal due to primer extension products at the *Hin*dIII and at the *Bam*HI sites was roughly equivalent to that due to extension products representing the 5′ end of the plus stands at DR2. This result is consistent with the interpretation that most plus strands had been initiated at DR2 and elongated through position 1825 (primer 3 binding site). In contrast, plus strands in DNA synthesized in class II mutant capsids were initiated at DR2 and elongated through position 1178 (primer 2 binding site) at wild-type efficiency, but no plus strands elongated as far as position 1825 (ca. 2,160 nucleotides from DR2) were detected.

DISCUSSION

The results reported here demonstrate that the C-terminal domain of the DHBV capsid protein is required for the production of a mature viral plus-strand DNA. The defect in plus-strand synthesis was not due to a defect in plus-strand priming at DR2 or circularization, in synthesis of the correct DNA minus strand, or in earlier steps required for DNA synthesis. The same mutations that produced this defect also produced a defect in the assembly of nucleocapsids into enveloped virus.

Schlicht et al. (23) reported on the effect of two mutations in which premature termination codons in the capsid gene of DHBV resulted in the production of capsid proteins lacking 12 or 24 amino acids from the C terminus. Removal of 12 amino acids did not destroy the synthesis of viral DNA or

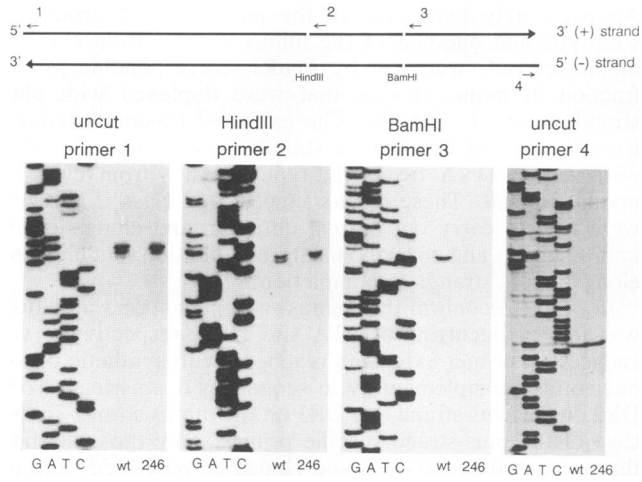


FIG. 7. Primer extension analysis of initiation and elongation of the minus and plus strands synthesized in a wild-type and a class II mutant capsid. Replicative DNA intermediates isolated from LMH cells cotransfected with wild-type (wt) or mutant 246 plasmids were isolated, and adventitious primers were removed by preparative gel electrophoresis through low-melting-point (SeaPlaque) agarose. Portions of the two DNAs were digested with *Hin*dIII or *Bam*HI and used for primer extension analysis. Extensions of primers 1, 2, and 3 were performed with ^{32}P-labeled synthetic oligonucleotides and unlabeled nucleoside triphosphates. Extension of primer 4 was performed with unlabeled primer and a reaction mix containing ^{35}S-deoxyadenosine-5′-(α-thio)triphosphate. A sequence ladder with cloned viral DNA was performed in parallel with each primer extension. Nucleotide coordinates for the 5′ to 3′ direction were as follows: primer 1, 2622 to 2606; primer 2, 1221 to 1207; primer 3, 1821 to 1807; primer 4, 2471 to 2494. (Note: Since these primer extensions were carried out with modified T7 DNA polymerase [Sequenase 2.0] lacking 3′ exonuclease activity, extension products are one nucleotide longer than the templated size. Sequenase 2.0 adds one nontemplated nucleotide to the 3′ end of a blunt-end duplex DNA molecule. This effect can be seen by comparing the extension products of primers 2 and 3 with their corresponding sequence ladders.)

the assembly of nucleocapsids into enveloped virus particles. This mutation would fall within the boundary region between our class I and class II mutants, and the phenotype they reported is consistent with our results. The removal of 24 amino acids resulted in an ablation of viral DNA synthesis but not RNA packaging. This mutant falls near the boundary between class III and class IV mutants, which synthesize little or no DNA. The existence of class II phenotypes reported here demonstrates that mutations in the capsid protein can also affect specific steps in viral DNA synthesis.

The function of the capsid protein that is required specifically for elongation and maturation of the plus-strand DNA is unclear. Mutants defective in elongation did not accumulate plus strands of discrete size, but rather appeared to generate relatively normal amounts of intermediates of different sizes, except for the more mature forms, which were virtually absent from transfected cells and supernatants. The lack of accumulation of a single immature species would indicate that the defect in plus-strand elongation was due to a progressive inability to elongate the plus strand to maturity. Such progressive inhibition might result from the imposition of steric constraints on the amount of DNA synthesis that could occur in capsids composed of mutant proteins. Such steric constraints could be generated either by the formation by the mutant proteins of a smaller capsid or by the failure of capsids composed of class II mutant proteins conformationally to "adjust" to the presence of increasing amounts of DNA. So little is understood of how replicative complexes carry out reverse transcription and double-stranded DNA synthesis that other explanations are equally possible.

A correlative phenotype associated with the class II mutants was a defect in the assembly of nucleocapsids into enveloped virus. The fact that no mutants were generated in which these two phenotypes were segregated from each other suggests that the phenotypes were functionally related in some way. The existence of such a relationship might be inferred from the fact that only nucleocapsids containing "mature" viral DNA are found in enveloped virus particles. While it may be that plus-strand maturation is required for the putative packaging signal for envelope recognition to appear on the surface of the capsid, it is equally possible that the appearance of the packaging signal is required for plus-strand maturation to take place. Class II mutants may provide an opportunity to investigate biochemically the nature of this capsid function specifically required for DNA maturation and virion assembly.

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