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Mutations within DR2 Independently Reduce the Amount of both Minus- and Plus-Strand DNA Synthesized during Duck Hepatitis B Virus Replication

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The initial aim of this study was to examine the role of complementarity between the plus-strand primer and the minus-strand DNA template for translocation of the plus-strand primer in hepadnaviral replication. We show that when a 5-nucleotide substitution was placed in either DR1 or DR2, translocation of the primer at a detectable level did not occur. Placing the mutation in both DR1 and DR2 did not restore primer translocation, which indicates that complementarity is not the sole determinant for primer translocation. These mutants, in which primer translocation has been inhibited, have been additionally informative. The mutation in DR1 led to efficient synthesis of plus-strand DNA, albeit primed in situ. In contrast, the mutation in DR2 resulted in a reduction in the amount of plus-strand DNA synthesized per unit of minus-strand DNA. These findings were interpreted as indicating that a mutation at DR2, the primer acceptor site, can inhibit both primer translocation and in situ priming. Lastly, we show that mutations within DR2 can result in a reduction in the synthesis of minus-strand DNA and that this reduction is occurring at an early phase of the process. We speculate that this reduction in the amount of minus-strand DNA synthesized could be due to an inhibition of the template switch during minus-strand DNA synthesis.

Hepatitis B virus (HBV) is a human pathogen whose primary site of infection is the liver. HBV infection can cause a necroinflammatory disease of variable severity and duration, which can ultimately result in hepatocellular carcinoma (for a review, see reference 5). HBV belongs to the family *Hepadnaviridae*; this family of viruses, which display a narrow host range, includes several additional members which infect either woodchucks (27), ground squirrels (17), ducks (18), or herons (23). Hepadnaviruses contain a 3.0-kbp circular DNA genome (20), which is replicated via a reverse transcription pathway (26).

All reverse transcription reactions, including hepadnaviral reverse transcription, must solve a common set of problems. These problems include the initiation of DNA synthesis for both minus-strand and plus-strand DNA. Solutions to these problems involve either the recruitment or the generation of specific primers. Typically, the primer for plus-strand DNA synthesis is derived from the original RNA template and is generated as a consequence of an RNase H cleavage during minus-strand DNA synthesis. Hepadnaviruses use such a strategy to generate their plus-strand primer (15). However, before a hepadnavirus initiates plus-strand DNA synthesis, an additional step occurs. The plus-strand primer translocates to a new position on the minus-strand template before the initiation of plus-strand DNA synthesis (13). The primer translocation process is unique to hepadnaviral reverse transcription.

Covalently closed circular DNA, the transcriptional template for the synthesis of pregenomic RNA (29), contains two copies of a 12-nucleotide direct repeat, named DR1 and DR2. The pregenomic RNA, as a consequence of being terminally redundant, contains two copies of DR1 and one copy of DR2 (Fig. 1, step 1). Two early events during hepadnaviral DNA synthesis are the coencapsidation of the pregenomic RNA and viral P protein (1, 2, 10, 21) and initiation and synthesis of the first four nucleotides of minus-strand DNA (19, 28, 31, 32). The viral P protein is both a primer and a reverse transcriptase for the initiation of synthesis of minus-strand DNA with an RNA stem-loop (epsilon) serving as the template for the synthesis of the first four nucleotides of minus-strand DNA (Fig. 1, step 1) (30). Next, the four nucleotides of minus-strand DNA, linked to the P protein, switch template to base pair with a UUAC sequence overlapping the copy of DR1 near the 3′ end of the pregenomic RNA (Fig. 1, step 2) (28, 31). Elongation of minus-strand DNA continues to the 5′ terminus of the pregenomic RNA (14), with the viral RNase H degrading the copied RNA template (Fig. 1, steps 3 to 5) (26). The last RNase H cleavage is thought to generate the RNA primer for plus-strand DNA synthesis (Fig. 1, step 5) (15). This primer is approximately 18 nucleotides in length and contains the 12-nucleotide DR1 sequence at its 3′ terminus (13). Then the primer is translocated to the DR2 position, which is near the 5′ terminus of the minus-strand DNA template (Fig. 1, step 6) (13). This translocation is the subject of this study. Because of the sequence identity between DR1 and DR2, the primer can anneal to the complementary DR2 site, thus permitting the initiation of plus-strand DNA synthesis (Fig. 1, step 7). After an intrastrand template switch (Fig. 1, step 8), plus-strand DNA synthesis proceeds to copy the minus-strand template, which ultimately results in a relaxed circular molecule (Fig. 1, step 9). Previous studies had indicated that base substitutions at the primer donor site could inhibit primer translocation (25). In these studies, inhibition of primer translocation was accompanied by an increase in a phenomenon called in situ priming (Fig. 1, step 10). In situ priming results when the plus-strand primer initiates plus-strand synthesis at the primer donor site (DR1). In situ priming results in a duplex linear genome (Fig. 1, step 11) rather than a relaxed circular genome.

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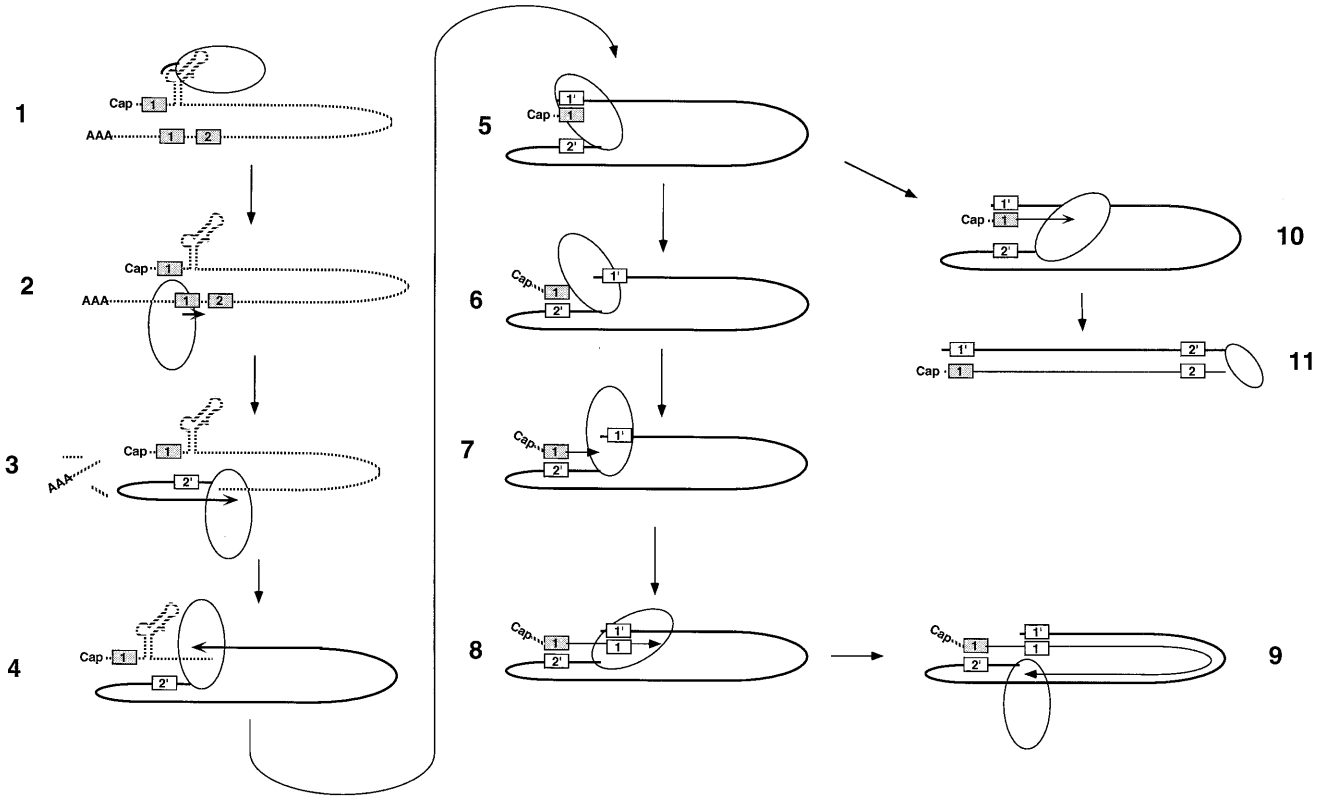


FIG. 1. Model for hepadnavirus DNA synthesis. (1) Encapsidation and initiation of minus-strand DNA synthesis. Pregenomic RNA which is capped and polyadenylated (dashed line) and DR1 and DR2 (boxes 1 and 2, respectively) are indicated. DR1 is present twice on pregenomic RNA. The P protein (oval) binds to the stem-loop structure near the 5′ end of the RNA to facilitate encapsidation and initiation of minus-strand DNA synthesis. Four nucleotides of minus-strand DNA (thick black line) are copied from a bulge within the RNA stem-loop. (2) Minus-strand template switch. A tetranucleotide linked to the P protein switches template so that the first four nucleotides of minus-strand DNA are base paired with sequence overlapping the 3′ copy of DR1. Synthesis of minus-strand DNA recommences. (3 and 4) Synthesis of minus-strand DNA. As minus-strand DNA (thick black line) is polymerized, P-gene-encoded RNase H activity degrades RNA pregenome. Box 2′, DR2 on minus-strand DNA. (5) Completion of minus-strand DNA synthesis and generation of RNA plus-strand primer. Minus-strand DNA synthesis proceeds to the 5′ end of the RNA template. Box 1′, DR1 on minus-strand DNA. The final RNase H cleavage generates the plus-strand primer, which is derived from the first 18 nucleotides of pregenome. The primer starts at the cap structure and proceeds to the 3′ end of DR1. Upon completion of minus-strand DNA synthesis, the primer donor (DR1) and primer acceptor (DR2) sites are juxtaposed. (6) Translocation of the plus-strand primer from donor to acceptor site. Primer, which was annealed for 18 nucleotides at DR1, is now base paired for 12 nucleotides at DR2. (7) Initiation and elongation of plus-strand DNA synthesis to the 5′ end of the template. Approximately 50 nucleotides of plus-strand DNA are synthesized. (8) Intrastrand template switch and elongation of plus-strand DNA synthesis. The growing point of synthesis of plus-strand DNA switches templates from the 5′ end to the 3′ end of minus-strand DNA. An 8-nucleotide terminal redundancy on the minus-strand is important for the switch. Plus-strand DNA is then elongated. The minus strand is now in a circular conformation. (9) Elongation and completion of plus-strand DNA synthesis results in a relaxed circular DNA. (10) In situ priming of plus-strand DNA synthesis. A fraction (ca. 5%) of plus strands is not initiated at DR2 but instead at DR1. The plus strands initiated at DR1 arise when the plus-strand primer is not translocated but instead used at the primer donor site. (11) Elongation of an in situ-primed plus strand results in a duplex linear DNA.

Our initial aim in the studies presented in this paper was to examine whether complementarity between the primer and the DR2 was sufficient for successful primer translocation. In the course of answering this question, we discovered that mutations in DR2 decrease the amount of minus-strand DNA synthesized. We also learned that a mutation in DR2 can inhibit both primer translocation and in situ priming.

MATERIALS AND METHODS

Construction of mutant DHBV genomes. The DHBV3 strain (24) of duck HBV (DHBV) and derivatives thereof were used in all experiments. The substitution of 5 of 12 nucleotides (TA**T**AC**G**CC**GTTA** for TACACCCCTCTC) (5/12 mutation) in DR1 and/or DR2 and the precise deletion of DR2 mutations were introduced into a M13 clone containing the 1,364-nucleotide *Bam*HI-*Eco*RI fragment (nucleotides 1658 to 3021) by the oligonucleotide-directed mutagenesis procedure of Kunkel et al. (12). Mutations were identified by DNA sequencing, and a restriction fragment containing the mutation (either a *Bal*I-*Nsi*I fragment [nucleotides 2370 to 2845] or a *Ava*I-*Acc*I fragment [nucleotides 2410 to 2577]) was transferred from the mutant M13-DHBV clone into a plasmid containing the 1,364-nucleotide *Bam*HI-*Eco*RI fragment (nucleotides 1658 to 3021). The absence of fortuitous mutations was ensured by sequencing the transferred restriction fragment. These plasmids (0.5-mer), which contain approximately the 3′ half of the genome, were converted into overlength plasmids (1.5-mer) which were competent to support transcription of pregenomic RNA in LMH cells. For the plasmid expressing the 60 virus (5/12 substitution in DR2), a 3,021-nucleotide *Nco*I-*Nco*I fragment of DHBV was inserted into the *Nco*I site (nucleotide 2351) of the DR2 mutant 0.5-mer plasmid. For the plasmid expressing the 66 virus (5/12 substitution in DR1), a 3,021-nucleotide *Eco*RI-*Eco*RI fragment of DHBV was inserted into the *Eco*RI site (nucleotide 3021) of the DR1 mutant 0.5-mer plasmid. The 66 virus was previously described (15). For the plasmid expressing the 67 virus, a 3,021-nucleotide *Eco*RI-*Eco*RI fragment containing the 5/12 mutation in DR2 was inserted into the *Eco*RI site of the 0.5-mer containing the 5/12 mutation in DR1. For the plasmid expressing the 270 virus (precise deletion of DR2), a 3,021-nucleotide *Nco*I-*Nco*I fragment of DHBV was inserted into the *Nco*I site of the DR2 mutant 0.5-mer plasmid. In addition, the plasmid expressing the 270 virus contained a frameshift mutation (deletion of nucleotide 424 [10]) within the 5′ portion of the P gene so that translation of the P gene terminated prematurely, thus eliminating any potential influence of the deletion of DR2 on the function of the P protein. The two additional P^- viruses (WT/P^- [e.g., see Fig. 4B, lane 4] and 60/P^- [e.g., see Fig. 4B, lane 3) used in these studies contained the P-gene frameshift mutation described above. For the plasmid expressing the +4 virus, two mutations were introduced. First, the *Afl*II site (nucleotide 2526) near the 3′ end of the genome was cleaved, filled in with Klenow fragment of *Escherichia coli* DNA polymerase I, ligated, and then cloned, thus creating a 4-nucleotide insertion. Second, the C gene of the +4 virus

contained a mutation so that functional capsid protein was not expressed. This was accomplished by creating a 4-nucleotide deletion at the *Nsi*I site (nucleotides 2846 to 2849) within the C gene. The expression plasmid used to donate P protein (in the experiments whose results are shown in Fig. 2B, lanes 5 and 6) contained a deletion within epsilon and a precise deletion of the 3′ copy of DR1, thus preventing replication of the expressed RNA.

Cell culture and DNA transfections. The chicken hepatoma line LMH (7, 11), used in all experiments, was cultured as previously described (16). DNA transfections were performed by the calcium phosphate protocol of Chen and Okayama (4). Typically, LMH cells which were at approximately 50 to 70% confluency on a 60-mm-diameter plate were transfected with 3 to 4 μg of plasmid DNA.

Southern blot analysis of viral DNA. Viral DNA was isolated from cytoplasmic core particles 3 days posttransfection as previously described (25). Electrophoresis of viral DNA for Southern blot analysis was performed with 1.25% agarose using 1× Tris-borate-EDTA as a buffer. A radiolabeled, minus-strand-specific, genome-length RNA was used as a nucleic acid hybridization probe. Hybridization of Southern blot membranes was carried out by the method of Church and Gilbert (6). Quantification of autoradiographic images was performed with a Molecular Dynamic Phosphorimager 445 SI.

Determining the ratio of plus-strand DNA to minus-strand DNA (+/− ratio). Viral DNAs were denatured with 0.2 N NaOH and then neutralized with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Viral DNA samples were then split into two equal portions and applied to a nylon membrane via vacuum filtration through a slot blot manifold so that duplicate membranes were made. Serial dilutions of cloned DHBV DNA were also applied, in duplicate, to the membrane to serve as mass standards. The viral DNA was then cross-linked to the nylon membrane with UV light. The two membranes were hybridized to either a genome-length, minus-strand-specific or plus-strand-specific radiolabeled RNA probe. Quantification of autoradiographic images was performed with a Molecular Dynamic Phosphorimager 445 SI.

Capsid gel analysis of intact capsids. Capsid gel analysis was performed as described by Calvert and Summers (3), with several minor modifications. Transfected LMH cells were lysed 3 days posttransfection with 0.5 ml of lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Nonidet P-40 [pH 8.0]). Nuclei were removed by microcentrifugation, magnesium acetate and DNase I were added to 6 mM and 100 μg/ml, respectively, and the mixture was incubated at 37°C for 30 min to degrade transfected plasmid DNA. For each transfection, 12 and 6 μl of lysate were loaded onto a 0.8% agarose gel prepared in 1× Tris-acetate-EDTA buffer and electrophoresed at 35 V for 4 h. At the completion of electrophoresis, the gel was blotted onto either nitrocellulose (for immunodetection) or nylon (for plus-strand nucleic acid detection) by capillary action in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–150 mM NaCl. The nitrocellulose membrane was immunostained for capsids with a rabbit anti-core antibody (a kind gift of Jesse Summers) followed by a donkey anti-rabbit immunoglobulin G antibody conjugated with ^{35}S (Amersham SJ.434). Encapsidated plus-strand nucleic acids were detected on the nylon membrane by using a strand-specific RNA probe that would detect plus-strand sequences from nucleotides 2845-(3021/1)-902.

Primer extension analysis of minus-strand DNA. Minus-strand primer analysis was performed as previously described (16).

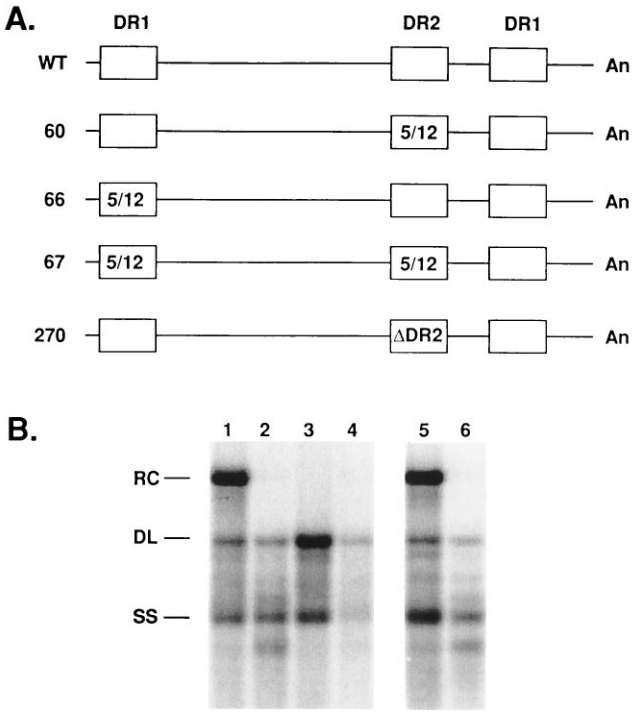


FIG. 2. Southern blot analysis of DR1 and DR2 mutants. (A) Structure of mutant viruses. Wild-type DHBV pregenomic RNA is represented at the top (line), with DR1 and DR2 indicated (boxes). Mutants 60, 66, 67, and 270 are each depicted below the wild type with the mutated DR sequences indicated. ΔDR2 indicates a precise deletion of DR2. (B) Southern blot of viral DNA from cytoplasmic capsids of wild-type and mutant viruses. On the left, the positions of relaxed circular (RC), duplex linear (DL), and single-strand minus (SS) DNAs are indicated. Lane 1, wild-type DHBV; lane 2, 60 virus; lane 3, 66 virus; lane 4, 67 virus; lane 5, A P-protein null version of wild-type virus complemented with P protein; lane 6, 270 virus (which is P-protein null) complemented with P protein.

RESULTS

The initial aim of this study was to evaluate the role of complementarity between the plus-strand primer and the acceptor site, DR2, on the minus strand of DNA in primer translocation. To study translocation of the plus-strand primer, we examined the DNA genomes of mutant DHBVs replicated in cell culture. This was done by transfecting LMH cells with plasmids competent to support transcription of viral pregenomic RNA, which subsequently led to viral reverse transcription. Three days after transfection of the plasmid DNAs, cytoplasmic capsid particles were harvested, and viral nucleic acid was purified from them and analyzed.

Complementarity between the plus-strand RNA primer and the DR2 site on the minus-strand template is not sufficient for translocation of the primer. After translocation, the plus-strand RNA primer is base paired with minus-strand DNA at DR2. Because the 3′ terminus of the plus-strand primer is derived from DR1, an RNA-DNA duplex of 12 nucleotides is predicted to exist at the acceptor site. It can be reasoned that complementarity between the primer and the template is necessary for translocation of the primer. We questioned whether complementarity between the primer and the template was sufficient for primer translocation. To this end, we attempted to disrupt primer translocation by 5/12 mutation of either DR1 (66 virus) or DR2 (60 virus). If primer translocation were disrupted, we would then test whether primer translocation could be restored when both DR1 and DR2 contained the 5/12 mutation (67 virus). Towards this end, we placed the 5/12 mutation in either DR1 or DR2 and then within both DR1 and DR2 and examined the ability of these mutant viruses to translocate the plus-strand primer (Fig. 2A). Plasmid DNAs encoding these mutant viruses were transfected into LMH cells, and 3 days later, viral DNA from cytoplasmic capsid particles was isolated. To examine translocation of the plus-strand primer, a Southern blot analysis was carried out (Fig. 2B). This type of analysis was informative because translocation of the plus-strand primer is required for the synthesis of a relaxed circular DNA genome. Previous studies (25) have established that mutations at the primer donor site (DR1) can inhibit primer translocation with a concomitant increase in the number of plus strands of DNA initiated from DR1. This type of plus-strand priming leads to the synthesis of a duplex linear DNA genome and has been termed in situ priming (25). Relaxed circular and duplex linear DNA genomes were then distinguished by a Southern blot analysis. When the 5/12 mutation was present either in DR1 or in DR2, relaxed circular DNA could not be detected, indicating that primer translocation failed (Fig. 2B, lanes 2 and 3). When the 5/12 mutation was present in both DR1 and DR2, relaxed circular DNA also could not be detected, indicating that translocation had not been restored (Fig. 2B, lane 4). Two additional observations

TABLE 1. +/− ratios for the 5/12 DR mutants relative to wild-type virus as determined from Southern blot analysis

Virus	+/− ratio[a]			
	Expt 1	Expt 2	Expt 3	Expt 4
Wild type	100	100	100	100
66 (DR1)	96	90	88	93
60 (DR2)	31	24	15	56
67 (DR1/DR2)	75	60	62	87

[a] Values for the mutant viruses are relative to the wild type, which has been normalized to 100.

were made. First, when the 5/12 mutation was present in DR2 (60 and 67 viruses; Fig. 2B, lanes 2 and 4), the amount of viral DNA detected was less than when DR2 was wild type in sequence (wild type and 66 viruses; Fig. 2B, lanes 1 and 3). This diminution did not appear to be a result of less-efficient transfection of these plasmids (see below). The DR2 mutants appeared to make less minus-strand DNA, indicating a defect at or prior to the synthesis of minus-strand DNA. Second, visual inspection of the Southern blot raised the question whether the 60 virus (DR2 mutation) contains less plus-strand DNA per unit of minus-strand DNA than either the wild type or the 66 virus (DR1 mutation). We have answered this question below (next section).

To corroborate the findings from the Southern blot analysis, a primer extension analysis (15, 25) was performed with the three mutant viruses to determine the position of the 5′ end of plus-strand DNA (data not shown), using an oligonucleotide that would simultaneously detect 5′ termini of plus-strand DNA at both DR2 and DR1. For the three mutants (60, 66, and 67), most, if not all, of the 5′ ends mapped to DR1, indicating in situ priming. The nucleotide positions of the 5′ ends of the plus strands for the three mutants at DR1 were the same as in the wild type, indicating that primer cleavage was normal. To eliminate the possibility that these mutants translocated their plus-strand primer but failed to carry out the subsequent intramolecular template switch (Fig. 1, steps 7 and 8), we carried out a primer extension analysis with an oligonucleotide that annealed to plus-strand DNA between DR2 and DR1 (data not shown; see reference 15 for the strategy). The 60 and 66 viruses had no detectable 5′ termini at DR2, while the 67 virus had a small number of 5′ termini that were detectable. The number of 5′ ends at DR2 relative to DR1 for the 67 virus was much lower (estimated to be <5%; data not shown), indicating that primer translocation had not been efficiently restored for the double mutant. Overall, the primer extension analysis corroborated the Southern blot analysis presented in Fig. 2.

The mutation in DR2 leads to a reduction in the amount of plus-strand DNA synthesized per unit of minus-strand DNA synthesized. During hepadnaviral DNA synthesis, minus-strand DNA synthesis is completed before the initiation of synthesis of plus-strand DNA, and the template for the synthesis of plus-strand DNA is the minus-strand DNA. If a mutant virus is defective for plus-strand DNA synthesis, it will contain less plus-strand DNA per unit of minus-strand DNA than the wild-type virus. We examined whether the 60 virus (DR2 mutation) made less plus-strand DNA per unit of minus-strand DNA than the wild type and the 66 virus (DR1 mutation). To do this, we quantified the amounts of plus-strand DNA and minus-strand DNA for wild-type virus and the 60, 66, and 67 viruses and then calculated the +/− ratio. We used two methods to calculate this ratio.

In the first method, we quantified the +/− ratio from Southern blot analyses, such as the data presented in Fig. 2B. This can be done because each molecule in the relaxed circular and duplex linear bands contains one minus strand and one plus strand and each molecule in the single-strand minus band contains one minus strand. To measure this ratio, the relative amounts of DNA in the relaxed circular, duplex linear, and single-strand minus bands were determined, and then the +/− ratio was calculated for each of the viruses. The ratio for wild-type virus was normalized to a value of 100, and the ratios relative to the wild type for the three mutants were calculated from four independent transfections (Table 1).

In the second method, viral DNA from the wild type and each of the three mutants was bound to duplicate membranes, which were then subjected to plus- and minus-strand-specific probing. From this, a +/− ratio was calculated, and the values were normalized, with the wild type set at 100. Six independent transfections were analyzed by the second method (Table 2).

The results from the two methods were similar. The 66 virus has a +/− ratio similar to that of the wild-type virus. The +/− values for both the 60 and the 67 viruses were less than those for both the wild-type and the 66 viruses, with the value for the 67 virus consistently being higher than that for the 60 virus. The rank orders of the values for the three viruses were the same in all experiments. Because of six determinations in Table 2, we determined that the rank order observed is statistically significant ($P > 0.016$; Wilcoxon signed rank test). In summary, the 5/12 mutation in DR2 (60 virus) resulted in the virus synthesizing less plus-strand DNA per mass of minus-strand DNA. The defect was less severe when the 5/12 mutation was present at both the 5′ copy of DR1 and the copy of DR2. The 5/12 mutation in DR1 (66 virus) resulted in levels of plus-strand DNA similar to that of the wild-type virus.

Mutations in DR2 cause a defect during the initial phase of minus-strand DNA synthesis, thus reducing the amount of minus-strand DNA synthesized. Inspection of the Southern blot in Fig. 2 indicated that the 60 and 67 viruses synthesized less viral DNA than the wild-type or 66 virus. In addition to a reduction in plus-strand synthesis for 60 and 67 viruses (as discussed above), there appears to be a reduction in the amount of minus-strand DNA synthesized. Consistent with this observation, when performing the analysis presented in Table 2, we measured less minus-strand DNA isolated from transfected cells for the 60 and the 67 viruses than for the wild-type virus (data not shown). To determine if the defect leading to this reduction in the amount of minus-strand DNA synthesized for the DR2 mutants occurred at or prior to packaging of the pregenomic RNA, encapsidation assays were performed on the wild-type and 66, 60, and 67 viruses. The results of an example of an encapsidation assay (3) are presented in Fig. 3. Cytoplasmic capsid particles were electrophoresed through a nondenaturing agarose gel and then transferred to a nitrocellulose

TABLE 2. +/− ratios for the 5/12 DR mutants relative to the wild type as determined by slot blot analysis

Virus	+/− ratio[a]					
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6
Wild type	100	100	100	100	100	100
66 (DR1)	107	87	99	92	83	106
60 (DR2)	40	29	45	42	40	57
67 (DR1/DR2)	58	54	76	74	52	71

[a] Values for the mutant viruses are relative to the wild type, which has been normalized to 100.

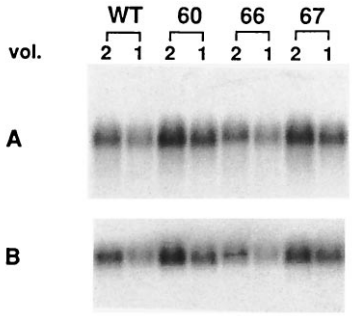
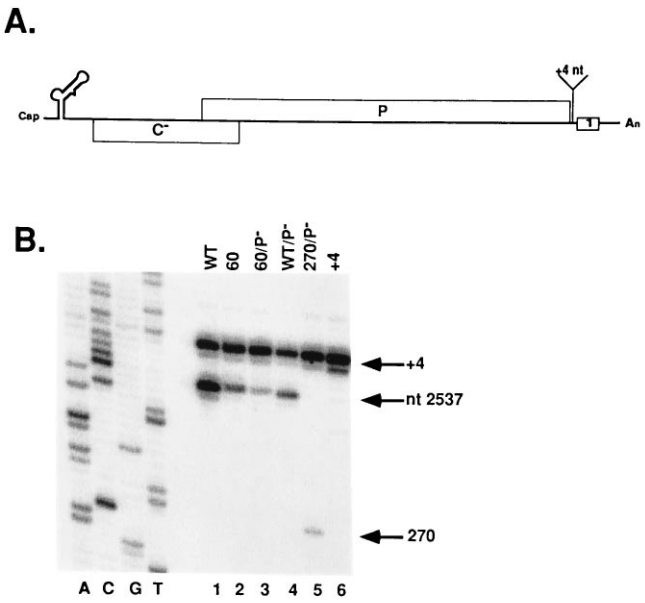


FIG. 3. Encapsidation analysis of RNA for wild-type (WT), 60, 66, and 67 viruses. LMH cells were transfected with plasmids expressing the indicated viruses. Cytoplasmic capsids were analyzed by electrophoresis through nondenaturing agarose gels and transfer to a membrane. (A) Immunostaining of capsids. (B) Nucleic acid hybridization detection of encapsidated plus-strand nucleic acids within capsids. For each virus, two volumes, 12 and 6 μl (indicated as 2 and 1, respectively) were analyzed.

membrane and a nylon membrane. Capsid particles are quantified on the nitrocellulose membrane by detection with an antibody reactive against DHBV capsid protein, which was subsequently detected with a ^{35}S-labeled antibody reactive towards the anti-DHBV capsid antibody (Fig. 3A). We then measured the plus-strand-specific nucleic acid contents of the capsids on the nylon membrane by denaturation of the capsids followed by hybridization with a probe specific for the detection of viral plus strands (Fig. 3B). For 60 and 67 viruses, the ratio of plus-strand nucleic acid to capsid particles was similar to that for the wild type and the 66 virus, indicating that steps up to and including encapsidation are not affected by the 5/12 mutation in DR2. This result, in conjunction with the result in Fig. 2B which showed a reduction in the amount of minus-strand DNA, suggests that a mutation in DR2 can lead to a defect in minus-strand DNA synthesis.

To determine if the defect seen for the 60 and 67 viruses was manifested early during minus-strand DNA synthesis, we employed a primer extension assay. Primer extension analysis was informative because only minus-strand DNA molecules longer than 112 nucleotides would contain the annealing site of the oligonucleotide used in the primer extension analysis and therefore would be detected in the primer extension analysis. For example, if the reduction in minus-strand DNA synthesis for the 60 virus occurred prior to the synthesis of the annealing site for the oligonucleotide used in the primer extension analysis, then a decreased amount of 5′ termini of minus-strand DNA would be detected for the 60 virus in comparison with that for the wild-type virus. In this analysis, we also included an internal standard to eliminate variations in measurements of minus-strand DNA caused by variations in transfection efficiency, in preparation of viral DNA, and in efficiency of primer extension. The internal standard consisted of a virus with a 4-nucleotide insertion (+4 virus; Fig. 4A) near the 5′ end of minus-strand DNA so that its 5′ terminus could be clearly distinguished from the 5′ terminus of a virus which did not contain the 4-nucleotide insertion (Fig. 4B, lane 1, for example). In this strategy, a plasmid expressing the +4 virus was cotransfected with a plasmid expressing wild-type virus (or a P$^-$ version of the wild-type virus). Minus-strand primer extension analysis was then performed on the subsequently reverse-transcribed viral DNAs, and the ratio of the 5′ ends of the wild-type virus to those of the +4 virus was determined (Fig. 4B, lane 1). This value, determined in each experiment, was normalized to 100 (Fig. 4C, virus WT). During the same experiment, in a second cotransfection, plasmids expressing the 60 virus and the +4 virus were introduced into LMH cells. Reverse-transcribed viral DNA was isolated and primer extension analysis of minus-strand DNA was performed (Fig. 4B, lane 2), and a ratio of 60 virus to +4 virus was determined (Fig. 4C). The ratio derived for the 60 virus was then expressed as a percentage of the wild type. The value for the 60 virus was approximately 20% of the wild type, indicating that the minus-strand defect occurred prior to the synthesis of the 112th nucleotide of minus-strand DNA. This fivefold reduction for the 60 virus observed in the primer extension assay was of the magnitude of the reduction observed for the Southern blot assay (Fig. 2). This defect in minus-strand DNA synthesis could not be attributed to a mutant form of the P protein, because the nucleotide substitutions in DR2 for the 60 virus



C.

virus	ratio $\frac{\text{virus}}{+4}$	% WT
WT	1.30	
60	0.27	21%
WT/P$^-$	0.395	
60/P$^-$	0.076	19%
270/P$^-$	0.028	7%

FIG. 4. Minus-strand primer extension analysis of DR2 mutants. (A) Structure of the +4 virus, which serves as an internal control during the analysis. Pregenomic RNA with the stem-loop indicating epsilon (thick line), DR1 (rectangle 1), and the C and P genes (rectangles C$^-$ and P) are indicated. The C gene contains a mutation so that functional capsid protein is not expressed. The position of the 4-nucleotide insertion 5′ of DR1 (+4 nt) is indicated. (B) Minus-strand primer extension analysis. All viral DNAs were generated by cotransfection of the indicated viruses with the +4 virus. Lanes A, C, G, and T, sequencing ladder generated with the end-labeled oligonucleotide used in the primer extension reactions; lane 1, wild-type DHBV; lane 2, 60 virus; lane 3, P-protein null derivative of 60 virus; lane 4, P-protein null derivative of wild-type virus; lane 5, P-protein null derivative of 270 virus; lane 6, +4 virus only. The position of the 5′ end of minus-strand DNA for the 270 virus is 12 nucleotides shorter as a consequence of the deletion of DR2. (C) Determination of ratio of 5′ ends and percentage of the wild type. The virus/+4 ratio is the ratio of 5′ ends at position 2537 to 5′ ends for the +4 virus.

did not lead to amino acid changes in the P protein. To rule out completely any unforeseen contribution to the mutant phenotype from the P protein expressed from the 60 virus, we complemented a derivative of the 60 virus deficient for P-protein expression (60/P$^-$) with a wild-type P protein. Analysis of this complementation (Fig. 4B, lanes 3 and 4) again indicated that the mutation in DR2 reduced synthesis of minus-strand DNA to 22% of the wild-type level (Fig. 4C). If DR2 is a *cis*-acting element for minus-strand DNA synthesis, we reasoned that a more severe mutation in DR2 could result in a more drastic reduction in minus-strand synthesis than the 5/12 mutation. To this end, we made a virus with a precise deletion of DR2 (270 virus; Fig. 2) and determined the ability of this virus to synthesize minus-strand DNA (Fig. 4B, lane 5). Complementation with the +4 virus as an internal standard followed by primer extension analysis of minus-strand DNA was performed. The deletion of DR2 resulted in a 15-fold reduction in minus-strand DNA synthesis. The results with the 60 and 270 viruses indicated that mutations within DR2 resulted in *cis*-acting defects active after encapsidation and before the synthesis of the 112th nucleotide of minus-strand DNA.

DISCUSSION

In this report, we show that mutation of DR2 can independently reduce the amounts of both plus- and minus-strand DNA synthesized. We show that mutation of DR2 results in decreased synthesis of plus-strand DNA per unit of minus-strand DNA. Also, DR2 mutations lead to less synthesis of minus-strand DNA, implying a previously unsuspected role for DR2 during minus-strand DNA synthesis. This work also shows that complementarity between the plus-strand primer and the DR2 sequence on the minus-strand DNA template is not sufficient for translocation, a result observed previously (25).

Translocation of the plus-strand primer and in situ priming can be inhibited by a mutation at the primer acceptor site. The 66 virus, which has the 5/12 mutation in the copy of DR1 at the 5′ end of the pregenome and therefore at the plus-strand primer generation site, does not synthesize detectable amounts of relaxed circular DNA. We interpret this as indicating that primer translocation is inhibited. To the extent that translocation is inhibited, the 66 virus seems to have a proportional increase in the number of primers used in situ, leading to the accumulation of duplex linear DNA. We surmise that the phenotype of the 66 virus indicates that primer translocation is inhibited and that the primers which normally would be translocated are instead used in situ. This interpretation of this phenotype has previously been described (25) and leads to the following general hypothesis: mutations within the primer generation or donor site (DR1) result in an inhibition of primer translocation with a corresponding increase in in situ priming. It is as if the primers never leave or leave and then return to DR1 to allow an increase in in situ priming.

The 60 virus, which has the 5/12 mutation in DR2 and therefore has a mutation at the primer acceptor site on the minus-strand DNA template, also fails to synthesize detectable levels of relaxed circular DNA. Not surprisingly, a mutation within the primer acceptor also inhibits primer translocation, a result observed previously (8). However, the amount of in situ priming for the 60 virus is not increased to an extent similar to that for the 66 virus. For the 60 virus, it seems that some fraction of the plus-strand primers is not used either at DR2 or at DR1. We interpret this result as indicating that a mutation in the primer acceptor site can inhibit both primer translocation and in situ priming.

The 67 virus has the 5/12 mutation at the donor (DR1) and acceptor (DR2) sites. The +/− ratios for the 67 virus are intermediate to the +/− ratios for the 66 and 60 viruses. Although primer translocation is apparently inhibited for the double mutant, the degree of inhibition of in situ priming is not as large as observed for the acceptor site mutant (60 virus). We offer two interpretations for the increase in in situ priming of the double mutant (67 virus) over the acceptor site mutant (60 virus). The compensatory change at DR1 allows some primers, which are not used for the 60 virus, to be used at DR1 for the 67 virus. This restoration in the ability to in situ prime for the double mutant (67 virus) would indicate an interaction between the primer donor and acceptor sites. The second interpretation states that although the effects of mutations at the primer donor site (66 virus) and primer acceptor site (60 virus) differ, they occur at the same time during the process of translocation, and the phenotype of the double mutant is simply a mixture of the two single-mutant phenotypes.

An alternative possibility for the reduced +/− ratio calculated for capsids isolated from the cytoplasms of cells expressing the 60 and 67 viruses has proportionally more of their duplex DNA that is ultimately made being either secreted as virions or transported to the nucleus. In theory, an increase in virion secretion or nuclear transport could proportionally deplete the cytoplasmic pool of capsids containing mature plus-strand DNA. A recognition signal(s) on the capsid is hypothesized to be required for capsids with mature plus-strand DNA to be secreted as virions or recycled to the nucleus (26, 34). According to this alternative possibility, the 5-nucleotide substitution within DR2 for viruses 60 and 67 would cause an increase in these cytoplasmic capsids being secreted as virions and/or transported to the nucleus. When viral DNAs from the media of cells expressing the wild-type, 60, 66, and 67 viruses were examined, the relative proportion of the plus-strand DNA between viruses was similar to the relative proportion seen within the cytoplasm, indicating that a proportional increase in virus release for the 60 and 67 viruses had not occurred (data not presented). Because LMH cells do not support the efficient conversion of duplex linear DNA to covalently closed circular DNA (33), experimental examination of an increase in nuclear transport for the 60 and 67 viruses is not trivial. Nonetheless, we think that this alternative possibility is unlikely.

DR2 is a *cis*-acting element for one of the early steps of minus-strand DNA synthesis. The observation that mutations in DR2 reduce the amount of minus-strand DNA synthesized is unexpected. Current models for the synthesis of minus-strand DNA do not indicate a role for DR2 in this process. We think that the likeliest roles for DR2 during minus-strand DNA synthesis would be during the minus-strand template switch or during the initial phase of minus-strand elongation after the template switch. We have shown that encapsidation appears normal for the DR2 mutants. Previous studies (32) indicate that epsilon is sufficient for the synthesis of the first four nucleotides of minus-strand DNA in vitro; therefore, it seems unlikely that the DR2 mutation is affecting synthesis of the first four nucleotides of minus-strand DNA. The template switch during minus-strand DNA synthesis is a process that is not well understood. Previous work indicates that this process is highly specific (16, 22), yet the basis of the specificity is not clear. If DR2 is a *cis*-acting element for the minus-strand DNA template switch, then mutations at DR2 would inhibit this process. In this interpretation, DR2 would contribute to the switching of the minus-strand DNA template by influencing the structure of the RNA template or because DR2 is recognized by *trans*-acting factors involved in the template switch. A

second model to account for the observed decrease in minus-strand DNA for the DR2 mutants would be a failure to elongate during the initial phase of minus-strand DNA synthesis. A putative elongation defect would need to occur before the synthesis of the 112th nucleotide of minus-strand DNA, because the defect is detected in our primer extension analysis. Although there is not a precedent for a specific *cis*-acting defect during minus-strand DNA elongation in hepadnaviral reverse transcription, we cannot now eliminate this possibility.

The discovery that DR2 contributes to minus-strand DNA synthesis coupled with our understanding that DR2 is the acceptor site for the plus-strand primer indicates that the 12 nucleotides of DR2 have two *cis*-acting roles during hepadnaviral DNA synthesis and illustrates again the utility for hepadnaviruses to have the same genetic information function in quite different processes. This efficiency is truly impressive.

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