JOURNAL OF VIROLOGY, July 1997, p. 5336–5344 Vol. 71, No. 7
0022-538X/97/$04.00+0

cis-Acting Sequences in Addition to Donor and Acceptor Sites Are Required for Template Switching during Synthesis of Plus-Strand DNA for Duck Hepatitis B Virus

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Received 8 January 1997/Accepted 20 March 1997

A characteristic of all hepadnaviruses is the relaxed-circular conformation of the DNA genome within an infectious virion. Synthesis of the relaxed-circular genome by reverse transcription requires three template switches. These template switches, as for the template switches or strand transfers of other reverse-transcribing genetic elements, require repeated sequences (the donor and acceptor sites) between which a complementary strand of nucleic acid is transferred. The mechanism for each of the template switches in hepadnaviruses is poorly understood. To determine whether sequences other than the donor and acceptor sites are involved in the template switches of duck hepatitis B virus (DHBV), a series of molecular clones which express viral genomes bearing deletion mutations were analyzed. We found that three regions of the DHBV genome, which are distinct from the donor and acceptor sites, are required for the synthesis of relaxed-circular DNA. One region, located near the 3′ end of the minus-strand template, is required for the template switch that circularizes the genome. The other two regions, located in the middle of the genome and near DR2, appear to be required for plus-strand primer translocation. We speculate that these *cis*-acting sequences may play a role in the organization of the minus-strand DNA template within the capsid particle so that it supports efficient template switching during plus-strand DNA synthesis.

Many reverse-transcribing genetic elements (retroelements) replicate by copying a single-stranded RNA molecule into a double-stranded DNA molecule (for a monograph, see reference 23). For these retroelements, a process described as template switching is required for the successful synthesis of a double-stranded DNA product.

Three template switches are required for the synthesis of the relaxed-circular DNA genome in hepadnaviruses. The first template switch occurs shortly after the initiation of minus-strand DNA synthesis. Minus-strand DNA is initiated near the 5′ end of its template, the pregenomic RNA (Fig. 1A) (27, 29). The viral P protein is both the primer and polymerase for minus-strand DNA synthesis (28). Only 4 nucleotides (nt) of minus-strand DNA are synthesized before the nascent DNA strand switches templates to a complementary acceptor site (UUAC), near the 3′ end of the pregenomic RNA (Fig. 1B) (27, 29). Minus-strand DNA synthesis resumes at this position, resulting in a genome-length, minus-strand DNA (Fig. 1C and D). Accompanying the synthesis of minus-strand DNA is the degradation of the pregenomic RNA (Fig. 1C) (26). The P protein, which contains a sequence of amino acids similar to RNase H, is thought to degrade the pregenomic RNA (2, 4, 21). Upon completion of the synthesis of minus-strand DNA, the final RNase H cleavage generates the primer for the initiation of plus-strand DNA synthesis (Fig. 1D) (16). Before this 18-nt RNA segment serves as a primer, the second template switch occurs (14). The primer, which contains the 12-nt redundancy (DR1) at its 3′ terminus, is transferred to the complementary 12 nt of DR2, near the 5′ end of the minus strand (Fig. 1F). Upon basepairing with DR2, the primer is used for the initiation of synthesis of plus-strand DNA (Fig. 1G). This template switch, called primer translocation, occurs on approximately 90 to 95% of the templates (25). From 5 to 10% of plus-strand primers remain at DR1 and are used to initiate the synthesis of plus-strand DNA from DR1 (Fig. 1E). This type of plus-strand DNA synthesis, called in situ priming, results in a duplex-linear DNA genome (25). For the plus-strand DNA initiated at DR2, a third template switch occurs when the growing point of DNA synthesis reaches the 5′ end of the minus-strand DNA template (Fig. 1H). The minus-strand DNA template contains a 7- to 9-nt terminal redundancy (r). This redundancy is required for the template switch in which the 3′ end of the nascent plus strand is moved to the 3′ end of the minus strand (Fig. 1I) (15). This template switch, termed circularization, results in the relaxed-circular conformation of the genome (Fig. 1J).

Analysis of DNA isolated from cytoplasmic duck hepatitis B virus (DHBV) capsids by Southern blotting reveals three major replicative intermediates that are found in characteristic proportions (25). Synthesis of two of these forms of DNA, the relaxed-circular and duplex-linear DNAs, has been described above (Fig. 1). The third form of viral DNA detected by Southern blotting is a genome-length, single-stranded, minus-strand DNA that appears to have little, if any, plus-strand DNA associated with it. This single-stranded form may be any of the predominantly minus-strand intermediates involved in the synthesis of duplex viral DNA (Fig. 1D through I). Since the various single-stranded DNAs shown in Fig. 1 differ by 50 nt or less of plus-strand DNA, these species of viral DNA are not likely to be resolved in a Southern blot analysis.

It has been demonstrated, for DHBV, that sequences within and immediately outside of the donor site (DR1) are involved in the primer translocation template switch (25). This observation, and the notion that a specific template structure might be required for template switching, led us to question whether

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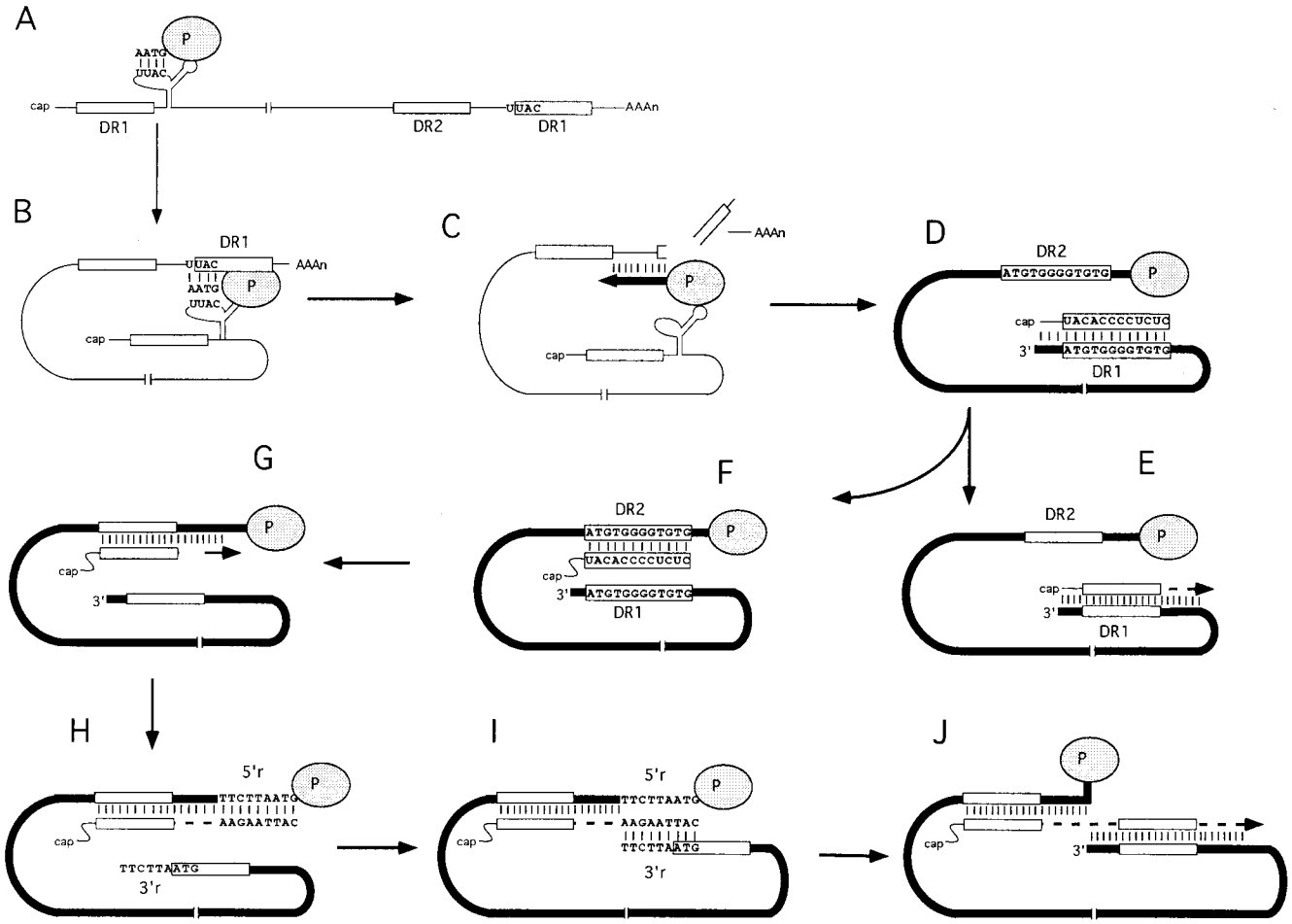

FIG. 1. Model for DHBV reverse transcription. (A) Encapsidation of pregenomic RNA and initiation of minus-strand DNA synthesis. The thin line represents the pregenomic RNA. The direct repeats, DR1 and DR2, are represented as boxes labeled DR1 or DR2; the UUAC sequences involved in the minus-strand template switch are indicated as such. The shaded circle represents the viral P protein, which binds a stem-loop structure on the pregenomic RNA during encapsidation. The first 4 nt of minus-strand DNA is templated by the UUAC sequence within this stem-loop. Basepairing is shown as hatch marks. The P protein is the polymerase and the primer for the initiation of minus-strand DNA synthesis. (B) Minus-strand template switch. The tetranucleotide covalently linked to the P protein is moved from the stem-loop to the acceptor site during the minus-strand template switch. The sequence of the acceptor site, UUAC, is complementary to the tetranucleotide. (C) Elongation of minus-strand DNA. After the minus-strand template switch, synthesis of minus-strand DNA (bold line) resumes near the 3′ end of the pregenomic RNA. The pregenomic RNA template is degraded by the RNase H activity of the P protein. (D) Completion of minus-strand DNA synthesis and generation of the plus-strand primer. After the 5′ end of the pregenomic RNA has been copied into DNA, the synthesis of minus-strand DNA is complete. The primer for the plus strand of DNA is generated from the 5′-terminal 18 nt of the pregenomic RNA. This RNA primer contains 12 nt complementary to DR2. This complementarity is required for primer translocation. (E) Generation of a duplex-linear genome. Some of the plus-strand primers are used to initiate plus-strand DNA (dashed line) from DR1. The synthesis of a genome-length plus-strand DNA initiated at DR1 yields a duplex-linear genome. (F) Plus-strand primer translocation. Most of the plus-strand primers are not used at DR1 but, instead, are moved from DR1 to DR2. During this template switch, the plus-strand primer basepairs with the complementary 12 nt of DR2. (G) Initiation of plus-strand DNA synthesis at DR2. After translocation of the plus-strand primer from DR1 to DR2, plus-strand DNA synthesis (dashed line) is initiated at DR2. (H) Copying 5′r. Plus-strand DNA synthesis initiated at DR2 continues to the 5′ terminus of the minus-strand DNA. The sequence of the terminal redundancy of minus-strand DNA (known as 5′r and 3′r) and the complementary sequence on plus-strand DNA are shown. (I) Circularization template switch. The complementary sequence of the nascent plus-strand DNA is moved from the 5′r sequence to the complementary 3′r sequence. (J) Generation of a relaxed-circular DNA genome. After the circularization, plus-strand DNA synthesis resumes at the 3′ end of the minus-strand DNA. A relaxed-circular DNA genome is generated upon completion of plus-strand DNA synthesis.

other *cis*-acting sequences apart from the donor and acceptor sites might be involved in template switches during plus-strand DNA synthesis. We report the identification of three such regions of the DHBV genome that when deleted, almost completely inhibit the synthesis of relaxed-circular DNA. These mutants have a phenotype consistent with a defect in one of the two template switches involved in plus-strand DNA synthesis. These data are consistent with the interpretation that these three regions are involved in juxtapositioning the donor and acceptor sites during the template switches within the capsid particle.

MATERIALS AND METHODS

Plasmid constructions. Deletion mutants were constructed from the DHBV3 strain of DHBV (24), except where noted. Names of the mutant viruses refer to the nucleotide coordinates between which the sequence has been deleted (Fig. 2). All deletion junctions were verified by DNA sequencing. To distinguish a *cis*-acting phenotype from a *trans*-acting phenotype, deletions which lie within the P gene were constructed within a P^- background. The P^- plasmid, p503, contains a 1-nt deletion in the P gene at nt 424. This mutation results in a nonfunctional P protein and creates an unique *Pst*I site at nt 424 (8). A virus bearing this mutation serves as the standard, which we will call wild type, in these experiments.

(i) Deletions 2652–2912 and 2848–3018. The 2652–2912 and the 2845–3018 mutant viruses (the names reflect the DHBV sequences deleted) were created in

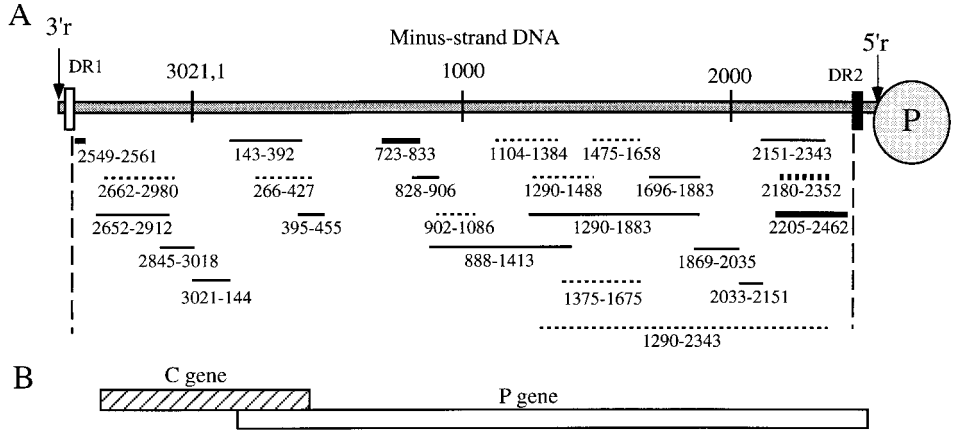

FIG. 2. (A) Map of deletion mutations. The shaded bar represents the minus-strand DNA, and the P protein (shaded oval) is covalently linked to the 5′ end of this DNA molecule. Solid lines represent deletion mutants. This set of deletions spans the genome from DR1 to DR2 (dashed vertical lines represent the inner borders of DR1 and DR2). Excluded from this set are deletions that overlap with *cis*-acting elements required for RNA stability (11) and encapsidation of the pregenome (1). The dotted horizontal lines represent deletion mutants analyzed but not presented in this report. Viruses bearing these deletions had a phenotype similar to that of viruses that contained overlapping deletions (solid lines). Heavy lines represent deletion mutations in either region 3E, M, or 5E. Deletion mutants are numbered by the convention of Mandart et al. (19) and Sprengel et al. (24). The numbers indicate the first and last nucleotides of DHBV contained at the deletion junction. (B) The C and P protein genes. The C and P genes, shown as boxes, provide the only viral proteins required for reverse transcription. Most of the deletion mutations are located within either the C or P gene. To analyze the replication of the mutant viruses, the C and P proteins were provided by a expression plasmid during transfection.

a stepwise fashion by first generating the indicated deletion in plasmid pD0.5G, which contains the DHBV sequence from nt 1658 to 3021. 2652–2912 contains a deletion between the *Eco*RV sites at nt 2562 and 2912. 2845–3018 was constructed by deleting the *Nsi*I-*Sma*I fragment in pD0.5G/SmaI. pD0.5G/SmaI is a derivative of pD0.5G that contains a *Sma*I linker (NEB 1054) inserted into the filled-in *Eco*RI site (nt 3021). Next, these plasmids containing deletions were converted into a greater-than-genome-length construct (1.5-mer) by inserting a genome-length *Eco*RI fragment into the *Eco*RI site. 1.5-mer plasmids are capable of supporting viral replication upon transfection into LMH cells (7, 17).

(ii) Deletion 2206–2462. Deletion mutant 2206–2462 was constructed by introducing an *Nco*I site at nt 2461 of pD0.5G, via an oligonucleotide-directed mutagenesis procedure (13), and then removing the *Sgr*AI (nt 2206)-*Nco*I (nt 2462) fragment. The 2206–2462 deletion mutant was then converted into a 1.5-mer by inserting a genome-length *Pml*I fragment into the *Pml*I site (nt 2151).

(iii) Deletion 395–456. The 395–456 deletion mutant was created by removing the *Bgl*II-*Bsg*I fragment of a wild-type 1.5-mer plasmid (nt 391 to 456).

(iv) Deletion 723–833. The 723–833 deletion mutant was constructed by inserting the *Pst*I-*Eco*RV fragment (nt 424 to 723) of p503 into the same sites of pBluescript IISK plasmid (Stratagene) and then inserting the *Aat*II–blunted-*Kpn*I fragment (nt 833 to 1290) into *Cla*I fill-in and *Kpn*I sites. The 723–833 deletion mutant was converted into a 1.5-mer by replacing the *Pst*I-*Kpn*I fragment (nt 424 to 1290) of p503 with the *Pst*I-*Kpn*I fragment of the phagemid.

(v) Deletions 1290–1883 and 1290–2343. The 1290–1883 and 1290–2343 deletion mutants were converted in a 1.5-mer by the method of Hoheisel and Pohl (10). Briefly, a 1.5-mer was digested with *Kpn*I and *Bam*HI and then treated with exonuclease III and S1 nuclease. The termini were blunted and ligated in the presence of a *Sma*I linker.

(vi) Deletion 1696–1883. The 1696–1883 deletion mutant was constructed by replacing the *Bgl*II-*Sma*I fragment of 1290–1883 with a *Bgl*II-*Msl*I fragment (nt 391 to 1696).

(vii) Deletion 1869–2035. The 1869–2035 deletion mutant was constructed in a 1.5-mer that had an A residue inserted at nt 2035, which creates an unique *Nhe*I site. The *Bgl*II-*Nhe*I fragment (nt 391 to 2033) was replaced with the *Bgl*II-*Bsp*HI blunted fragment (nt 391 to 2035).

(viii) Deletion 2033–2151. The 2033–2151 deletion mutant was also constructed in the 1.5-mer containing the *Nhe*I site. The *Nhe*I-*Nde*I fragment (nt 2033 to 3698) was replaced with a *Pml*I-*Nde*I fragment (nt 2151 to 3698).

(ix) Deletion 2151–2344. The 2151–2344 deletion mutant was constructed by replacing the *Bgl*II-*Sma*I fragment of 1290–2343 with a *Bgl*II-*Pml*I fragment (nt 391 to 2151).

(x) Deletion 2549–2561. The 2549–2561 deletion mutant was made as described by Hirsch et al. (9) and was referred to as Δ2 in their report.

(xi) Deletions 3021–144, 143–392, 828–906, and 88–1413. The 3021–144, 143–392, 828–906, and 88–1413 deletion mutants were made in DHBV16 and were provided by Calvert and Summers (1).

(xii) P and C protein expression plasmid. A 1.5-mer plasmid which expressed an encapsidation-deficient pregenome via the endogenous viral promoter was used as a donor to provide P and C proteins in *trans*. The pregenomic RNA was encapsidation negative as a result of a deletion in the encapsidation signal, from nt 2549 to 2580. This mutant was made as described by Hirsch et al. (9) and was referred to as Δ5 in their report. In addition, the pregenomic RNA expressed from the donor plasmid was designed to contained another deletion which would inhibit replication of the encapsidation deficient RNA. This deletion, which was created by means of an oligonucleotide-directed mutagenesis procedure (13), removed the 12 nt of the 3′ copy of DR1 (16).

Cell culture and transfections. The chicken hepatoma cell line LMH was used to replicate DHBV transiently (6, 12). LMH cells were cultured in vitro (17). Transfections were performed by calcium phosphate precipitation (3). The precipitate, which contained 3 μg of the plasmid containing the wild-type or mutant viral genome and 1.5 μg of the C and P donor plasmid, was added to the culture medium of a 60-mm-diameter dish containing LMH cells at about 50% confluency.

Isolation and analysis of viral DNA. Viral DNA from cytoplasmic capsids was isolated from the LMH cells 3 days after the transfection as described by Calvert and Summers (1).

Southern blot analysis. Southern blot analysis was performed on a fraction (1/10 to 1/2) of the total viral DNA isolated from a transfected plate of LMH cells. The viral DNA was electrophoresed through a 1.25% agarose gel at 40 V for 16 h in a Tris-borate-EDTA buffer (22). Next, viral DNA in the agarose gel was transferred by capillary action to a membrane (Hybond-N^+; Amersham) in a solution of 1.5 M NaCl and 0.5 M NaOH. The membrane was dried and exposed to 120 mJ of 254-nm-wavelength UV light per cm^2. The membrane was prehybridized and hybridized as described by Church and Gilbert (5), and a genome-length, minus-strand-specific, RNA probe (nt 2526 to 2526) was used to detect viral DNA. Phosphorimages of the different forms of viral DNA were quantified with a Molecular Dynamics PhosphorImager (model 445 SI). The ratio of relaxed-circular DNA to duplex-linear DNA was determined from at least three independent experiments. To detect the 3′ terminus of the mature minus-strand DNA, membranes were prehybridized in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's–0.5% sodium dodecyl sulfate (SDS)–50 mg of sheared salmon sperm DNA per ml at 65°C for 1 h (22). Next, 5 pmol of a ^{32}P-end-labeled oligonucleotide derived from nt 2530 to 2549 of the DHBV genome was added to the prehybridization solution. The hybridization temperature was raised to 70°C for 15 min and then lowered to 48°C for 3 h. The membrane was washed with 6× SSC twice at room temperature for 15 min each and twice at 50°C for 15 min each. Positive and negative DNA controls were included to demonstrate the specificity of hybridization.

Primer extension analysis. Primer extension analysis was performed on approximately 1 ng of viral DNA isolated from the cytoplasmic capsids of transfected LMH cells. The amount of viral DNA detected by primer extension was measured relative to a DNA standard that was added to the viral DNA samples at the start of the analysis. This internal standard was a DHBV plasmid doubly digested with *Ava*I and *Eco*RV. The mixture of viral and internal standard DNAs was treated with NaOH (25). An equal fraction of this sample was used in three different primer extension reactions. The end-labeled oligonucleotides used in these reactions were named 2622 (complementary to DHBV nt 2599 to 2622), 2537 (complementary to DHBV nt 2520 to 2537), and 2489 (derived from DHBV nt 2489 to 2507). Primer extension reactions were performed with oligonucleotide 2489 to quantify the amount of 5′ termini of minus-strand DNA located at nt 2537. In addition, oligonucleotide 2537 was used to quantify the amount of 5′

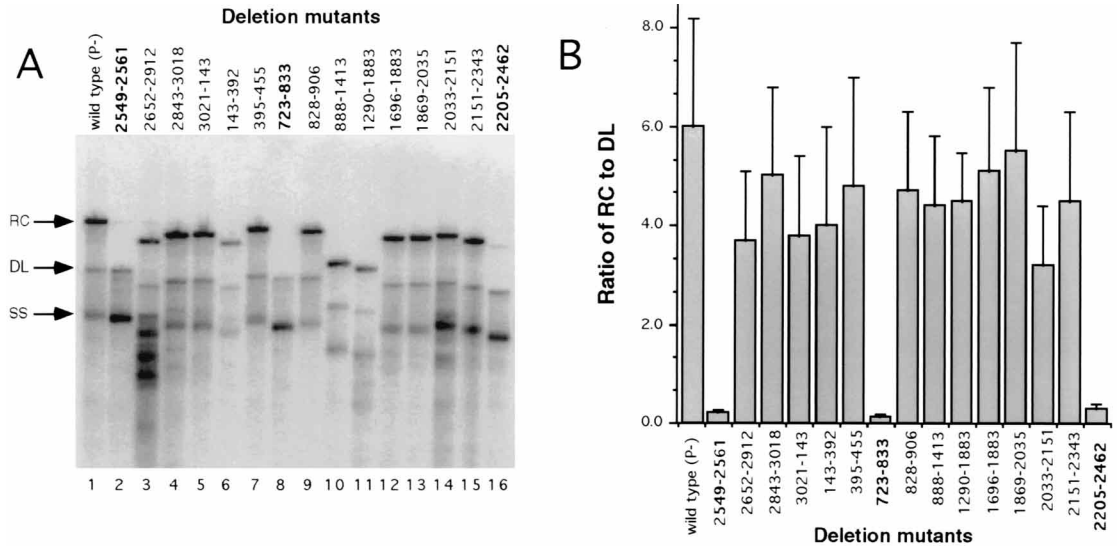

FIG. 3. Three deletion mutants inhibit relaxed-circular DNA synthesis. (A) Cytoplasmic viral DNA isolated from transfected cells was analyzed by Southern blotting. A genome-length, minus-strand-specific probe was used to detect viral DNA. The positions of relaxed-circular (RC), duplex-linear (DL), and single-stranded (SS) DNA forms are indicated for the wild-type virus. (B) Ratio of relaxed-circular DNA to duplex-linear DNA. The amount of relaxed-circular and duplex-linear DNA for each of the mutants was quantified with a PhosphorImager in at least three experiments. The mean ratio for each virus is plotted on the histogram, and the error bars indicate 1 standard deviation.

termini of plus-strand DNA located at DR2 before the circularization step, and oligonucleotide 2622 was used to determine the amount of 5′ termini of plus-strand DNA located at DR1 and DR2. The plus-strand DNA which was detected at DR2 with oligonucleotide 2622 has circularized and synthesized at least 135 nt of plus-strand DNA. The primer extension reaction mixtures contained 1× Vent DNA polymerase buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.5), 2 mM $MgSO_4$, 0.1% Triton X-100], 0.2 mM (each) deoxynucleoside triphosphate, 1 U of Vent exo$^-$ DNA polymerase (New England BioLabs), 0.6 pmol of 5′-end-labeled oligonucleotide, and approximately 330 pg of viral DNA. To resolve a sequence compression which was near the 3′ end of DR1, 7-deaza-dGTP was included in the 2622 primer extension reaction mixture instead of dGTP. The reaction mixtures were incubated in a thermocycler (Perkin-Elmer GeneAmp PCR system 9600). The thermocycling parameters were 15 cycles of 95°C for 30 s, 46°C for 30 s, and 72°C for 30 s for oligonucleotide 2622. The annealing temperatures for oligonucleotides 2537 and 2489 were 40 and 30°C, respectively. To demonstrate that the signal quantified by the PhosphorImager analysis was directly proportional to the amount of DNA added to the primer extension reaction mixture, serial dilutions of the viral DNA samples were analyzed (data not shown). DNA sequencing ladders were generated by using the same thermocycling parameters with a molecular clone of DHBV. Primer extension products were electrophoresed on a 40-cm, 6% denaturing-polyacrylamide gel, and then dried. Phosphorimages of the primer extension products were visualized and quantified with a Molecular Dynamics PhosphorImager. The amount of plus-strand DNA relative to the amount of minus-strand DNA was calculated for each mutant by the following equation; (+/is)/(−/is), where (+) represents the amount of 5′ termini of plus-strand DNA detected from a primer extension reaction mixture containing either oligonucleotide 2537 or 2622, (−) is the amount of 5′ termini of minus-strand DNA detected from a primer extension reaction mixture containing oligonucleotide 2489, and (is) represents the amount of primer extension product detected from the internal standard DNA. Viral DNA from three independent transfections was analyzed for each mutant.

Viral DNA analyzed by primer extension with both oligonucleotides 2537 and 2546 simultaneously was treated as described above, except that the annealing temperature during thermocycling was 28°C and the internal standard contained a 12-nt deletion of DR2. The plasmid used as the internal standard in these primer extension reactions was also used to generate the sequencing ladder.

RESULTS

Three *cis*-acting regions of the genome are involved in the synthesis of relaxed-circular DNA. To determine whether *cis*-acting sequences apart from the donor and acceptor sites were involved in the synthesis of relaxed-circular DNA, we analyzed the viral DNA synthesized from a set of molecular clones of the DHBV genome, which we refer to as viruses, that contain a series of deletion mutations. Collectively, the set of deletion mutations spanned most of the genome (Fig. 2). To determine whether a virus bearing a deletion mutation could synthesize relaxed-circular DNA, LMH cells were transfected with both the mutant virus (a molecular clone of the viral genome containing a deletion mutation) and a plasmid capable of providing the P and C proteins in *trans*. The viral DNA produced after transfection was isolated from cytoplasmic capsids and examined by Southern blot analysis (Fig. 3A shows the results obtained from the analysis of a representative set of mutant viruses). To measure the effect of the deletion mutations on the synthesis of relaxed-circular DNA, the ratio of relaxed-circular DNA to duplex-linear DNA was calculated for each of the mutant viruses presented in Fig. 3A. For most of the mutant viruses, the mean ratio of relaxed-circular DNA to duplex-linear DNA, which was calculated from at least three experiments, was within 1 standard deviation of the mean ratio for the wild-type virus (Fig. 3B). This result was interpreted to indicate that plus-strand DNA synthesis was not significantly perturbed by the absence of sequences deleted in these viruses. For three of the mutant viruses (2549–2561, 723–833, and 2205–2462) there was a reduction in the amount of the relaxed-circular DNA form and an increase in the amount of the single-strand DNA form (Fig. 3A, lanes 2, 8, and 16).

The locations of the deleted sequences in mutants 2549–2561, 723–833, and 2205–2462 that disrupted relaxed-circular DNA formation (Fig. 2), are distinct from the DR1/DR2 and the r sequences, which have been shown previously to be involved in the synthesis of relaxed-circular DNA (7, 15, 18, 25).

Two of these replication-impaired viruses (723–833 and 2205–2462) could potentially generate a mutant form of the P protein (Fig. 2B). Since these deletions were constructed into a P-null background, it appears that the unusual pattern of replicative intermediates produced by these molecular clones results from the deletion of *cis*-acting sequences rather than the generation of a dominant-acting mutant of the P protein.

The inability of these three mutant viruses to synthesize relaxed-circular DNA efficiently may result from the creation of novel sequences at the deletion junction that inhibit the replicative process. To address this possibility, two additional

TABLE 1. Mean distribution of viral DNA detected by Southern blot from three transfections

Virus	Percentage (mean ± SD) of:		
	Relaxed-circular DNA	Duplex-linear DNA	Single-stranded DNA
Wild type	68 ± 3	14 ± 4	18 ± 5
2459–2561	3 ± 1	20 ± 3	77 ± 3
723–833	1 ± 1	16 ± 5	83 ± 6
2205–2462	3 ± 1	21 ± 4	76 ± 5

mutations were studied. In one mutant, nt 722 to 833 was replaced with the 125-nt *Hin*dIII fragment from bacteriophage lambda, and in the second mutant, nt 2180 to 2352 was removed (Fig. 2). These mutant viruses had a phenotype that was similar to the phenotype of viruses containing the original mutations (data not shown). Therefore, the deleted sequences appear to contain necessary *cis*-acting sequences that act positively to support viral plus-strand DNA synthesis. We refer to the regions containing these sequences as region 3E (located near DR1, close to the 3′ end of minus-strand DNA), region M (located in the middle of the genome), and region 5E (located near DR2, close to the 5′ end of minus-strand DNA).

Some of the mutant viruses (2652–2912, 2843–3018, 1290–1883, and 2033–2151) appeared to accumulate viral DNA that migrates faster than the single-stranded DNA form (Fig. 3A, lanes 3, 4, 11, and 14). Further characterization of these forms of viral DNA will not be presented in this report. Since these mutant viruses did not have an altered ratio of relaxed-circular DNA to duplex-linear DNA, the process of plus-strand DNA synthesis appears to be unperturbed in these viruses.

A reduction in the level of relaxed-circular DNA correlates with an increase in the level of single-stranded DNA. For viruses with mutations in region 3E, M, or 5E, a reduction in the level of relaxed-circular DNA could result from the disruption of the process of primer translocation (Fig. 1F). Staprans et al. (25) described DHBV mutants that were defective for primer translocation. Such mutants synthesized higher than wild-type levels of in situ-primed plus-strand DNA molecules (Fig. 1E). Visual inspection of the Southern blot indicated that the viruses with mutations in region 3E, M, or 5E were not simply accumulating duplex-linear DNA at the expense of relaxed-circular DNA (Fig. 3, lanes 2, 8, and 16). To determine whether deletion of sequence within region 3E, M, or 5E caused an increase in the amount of plus-strand DNA primed in situ, we performed two different analyses. First, we measured the proportions of the three major replicative intermediates which were detected by Southern blot analysis (Table 1). An increase in the amount of in situ priming would result in the accumulation of duplex-linear DNA at the expense of relaxed-circular DNA. The viruses with mutations in region 3E, M, or 5E had only a marginal increase in the amount of duplex-linear DNA when compared to the wild-type virus (Table 1). For the wild-type virus, 14% of the three major replicative intermediates was duplex-linear DNA, and for the mutant viruses, this value ranged from 16% (for the region M mutant), to 21% (for the region 5E mutant). This result indicated that the deletion mutations caused only a slight increase in the proportion of duplex-linear DNA.

In the second analysis, primer extension was used to determine whether the mutations within region 3E, M, or 5E resulted in an increase in the amount of plus-strand DNA primed in situ. The annealing sites for the oligonucleotides used in this analysis (primers 2622 and 2489) are shown in Fig. 4A. The number of 5′ termini of plus-strand DNA primed at DR1 (Fig. 4B) was determined relative to the amount of minus-strand DNA (Fig. 4C). As seen in Fig. 4C (lanes 5 to 8), the three mutant viruses have 5′ termini of minus-strand DNA at the wild-type position (nt 2537) and at a level comparable to that of the wild-type virus. The same amount of viral DNA that was used in the minus-strand primer extension analysis was then used in the plus-strand primer extension analysis (Fig. 4B). As can be seen in Fig. 4B (lanes 1 through 4), the mutant and wild-type viruses each had detectable levels of 5′ termini at DR1. Next, the amount of plus-strand DNA initiated at DR1 was plotted relative to the amount of minus-strand DNA (Fig. 4D). For the wild-type virus, 2.5% of the cytoplasmic capsids that elongated minus-strand DNA from nt 2537 also synthesized in situ-primed plus-strand DNA. Compared to the wild type, there was no significant difference in the amount of 5′ termini at DR1 for the region 3E and M mutants, and there was an approximately threefold increase in the amount of 5′ termini at DR1 for the region 5E mutant (Fig. 4D). Therefore, the reduction in the relative number of relaxed-circular DNA molecules for the viruses with mutations in region 3E, M, or 5E observed by Southern blot analysis (Table 1) was not accompanied by a proportional increase in the number of in situ-primed DNA molecules (Fig. 4D).

Table 1 shows that the decrease in the relative level of relaxed-circular DNA for the three mutant viruses accompanies a proportional increase in the amount of the single-stranded form of viral DNA. The experiments described below were designed to determine why these mutant viruses accumulated a single-stranded DNA at the expense of relaxed-circular DNA.

The single-stranded DNA intermediate in the mutant viruses is a full-length minus-strand DNA. In our analysis of the replicative intermediates, we noted that the viruses with mutations in region 3E, M, or 5E accumulated single-stranded forms of DNA (Table 1). One reason why these viruses could accumulate single-stranded DNA at the expense of relaxed-circular DNA may be that minus-strand DNA synthesis was incomplete and completion of the synthesis of minus-strand DNA may be required for the initiation of the synthesis of plus-strand DNA. Termination of minus-strand DNA synthesis close to the 5′ end of the RNA template would result in the accumulation of single-stranded DNA. Since Southern blot analysis (Fig. 3A) may not resolve nearly full-length and full-length single-stranded DNAs, we asked if a probe complementary to the last 20 nt of the 3′ terminus of minus-strand DNA could hybridize to the single-stranded DNA of the mutants (Fig. 5A). This probe detected the single-stranded DNA for the mutant and wild-type viruses (Fig. 5A). Furthermore, we observed no significant difference in the relative proportions of the different forms of viral DNA that were detected with the 3′-terminal probe when compared to a genome-length probe (Fig. 5B). Therefore, incomplete synthesis of minus-strand DNA cannot account for the block in the synthesis of plus-strand DNA.

Reduced detection of plus-strand DNA initiated from DR2 for the viruses mutant in region M and in region 5E. The accumulation in the amount of full-length minus-strand DNA at the expense of relaxed-circular DNA could occur if the plus-strand primers that normally translocated to DR2 were instead lost or not elongated following translocation. The mutant viruses would then synthesize very little plus-strand DNA with 5′ termini located at DR2. To measure the amount of 5′ termini at DR2 for the mutant viruses, a primer extension analysis was performed (Fig. 6). To distinguish between the absence of DNA plus strands and the presence of very short

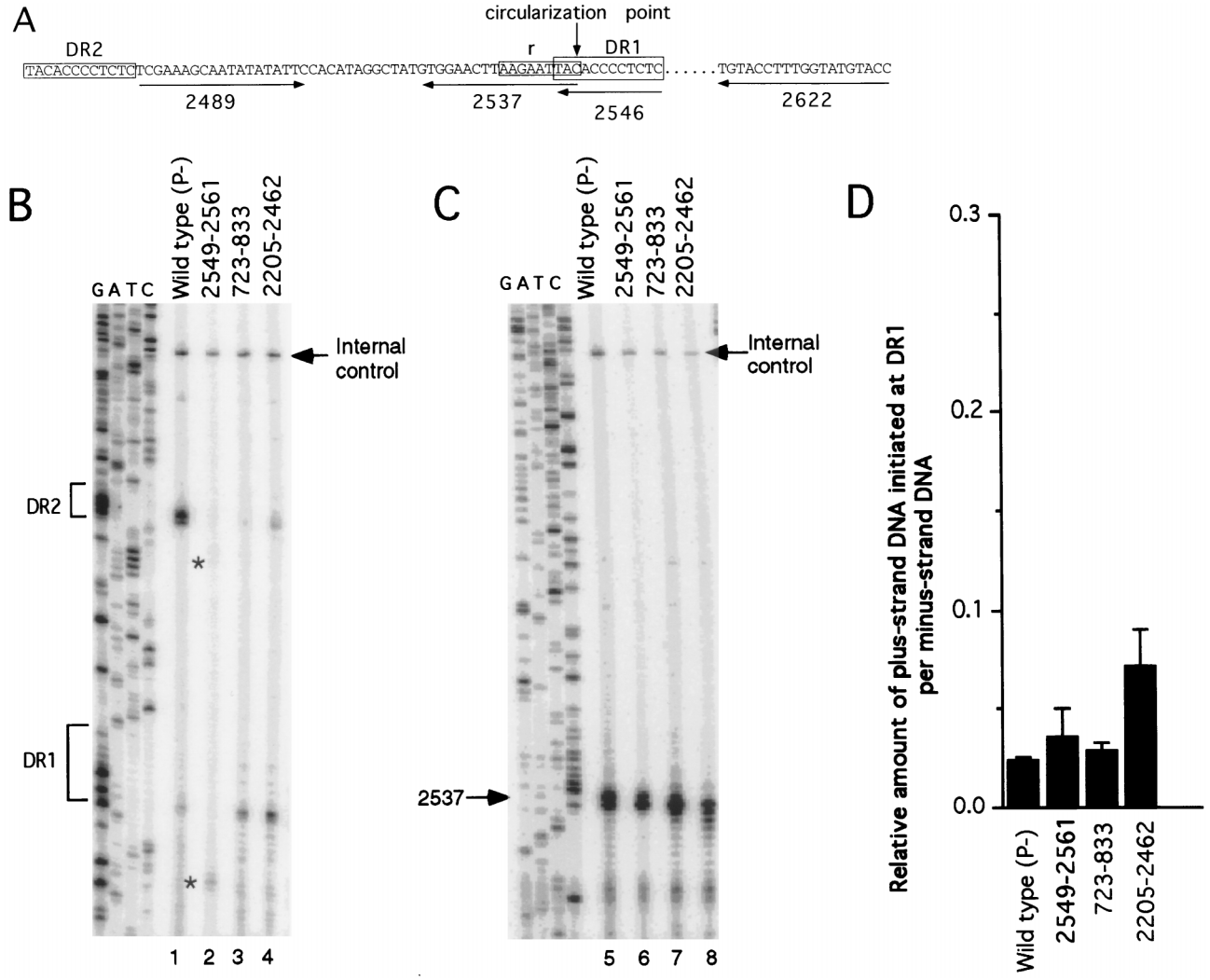

FIG. 4. (A) Position of annealing sites of oligonucleotides used in primer extension reactions. The plus-strand sequence of viral DNA (from nt 2477 to 2622) is shown; the dotted line indicates omitted sequence (from nt 2547 to 2603). DR1, DR2, and r are boxed and labeled. The annealing sites of the oligonucleotides used in the primer extension analysis are shown as arrows; arrowheads indicate the 3′ end of the oligonucleotides, and numbers indicate the position of the 5′ end of the oligonucleotides. The circularization point, which is also the position of the 5′ end of the minus-strand DNA, is marked. (B) Detection of the 5′ termini of plus-strand DNA. Oligonucleotide 2622 was used to determine the amount of plus-strand DNA initiated at DR1 and DR2. The primer extension products detected in lane 6 (marked with asterisks) were 11 nt smaller due to the 11-nt deletion in the virus bearing the 2459–2561 mutation. A sequencing ladder generated with primer 2622 flanks the primer extension reactions. (C) Detection of 5′ termini of minus-strand DNA. Oligonucleotide 2489 was used to quantify the amount of 5′ termini of minus-strand DNA located at position 2537 for the mutant and wild-type viruses. A sequencing ladder generated with primer 2489 flanks the primer extension reactions. (D) Amount of plus-strand DNA initiated at DR1 per minus-strand DNA in mutant and wild-type viruses. The amount of 5′ termini of plus-strand DNA located at DR1 relative to the amount of 5′ termini of minus-strand DNA located at position 2537 was calculated as described in Materials and Methods. For each virus, the mean value of the ratio of plus-strand DNA to minus-strand DNA from three different transfections was plotted on the histogram, and the error bars represent 1 standard deviation.

DNA plus strands initiated at DR2, we used an oligonucleotide that could anneal to plus-strand DNA at a site that is within 50 nt of DR2 and before the circularization point (Fig. 4A, oligonucleotide 2537). Because we were measuring the relative levels of the 5′ termini of plus-strand DNA at DR2, it was necessary to normalize these measurements to the level of minus-strand DNA in each of the samples. The plus-strand primer extension analysis (Fig. 6A) was performed on the same viral DNA samples as were used in the minus-strand primer extension analysis (Fig. 4C). For the plus-strand primer extension analysis, a large amount of 5′ termini located at DR2 in the wild type and the virus mutated in region 3E was detected (Fig. 6A, lanes 1 and 2). In contrast, far fewer 5′ termini located at DR2 were detected for the viruses mutated in region M or 5E (lanes 3 and 4). The amount of 5′ termini of plus-strand DNA detected at DR2 relative to the amount of 5′ termini of minus-strand DNA detected at nt 2537 was calculated for the wild-type virus and each of the mutant viruses (Fig. 6B). For the virus mutated in region 3E, the amount of plus-strand DNA made before circularization was about the same as that for wild-type virus (Fig. 6B). In contrast, the viruses mutated in region M or region 5E synthesized less plus-strand DNA initiated from DR2 before circularization, about 10% of the wild-type value. Clearly, a defect in primer translocation could not solely account for the inability of the virus mutated in region 3E to synthesize relaxed-circular DNA. These data are consistent with the interpretation that the mutation in region 3E does not significantly affect primer translocation. In addition, these data are consistent with the interpretation that the viruses mutated in region M or 5E do not use

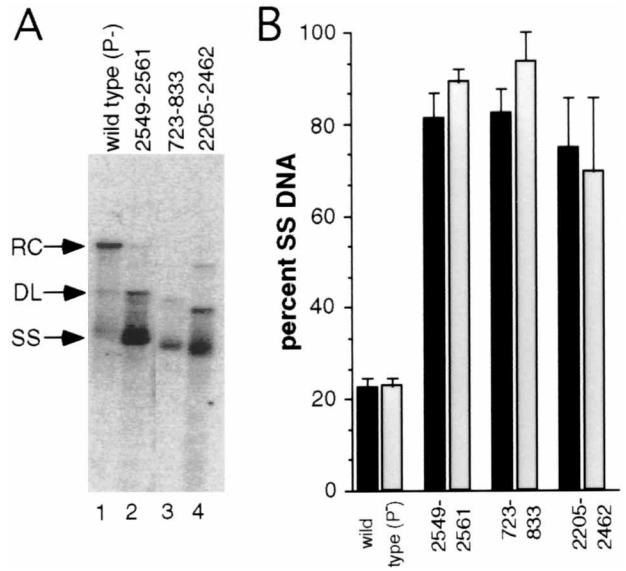

FIG. 5. Single-stranded DNA species contains full-length minus-strand DNA. (A) Southern blot analysis with a 3′ terminal probe. A membrane containing viral DNA isolated from transfected LMH cells was probed with a 3′-terminal probe. This probe, complementary to the last 20 nt of minus-strand DNA, was used to detect full-length minus-strand DNA. RC, relaxed circular; DL, duplex-linear; SS, single stranded. (B) Proportion of single-stranded DNA detected with two different probes. The amount of viral DNA migrating at the position of the single-stranded DNA relative to the three forms of viral DNA detected by Southern blotting was determined with a 3′-terminal probe (shaded bars) and a genome-length probe (solid bars). The mean proportion of single-stranded DNA was calculated from two different transfections with both the 3′-terminal probe and the genome-length probe. These values were plotted on the histogram, and error bars indicate 1 standard deviation.

the primers which are normally translocated to DR2 to initiate plus-strand DNA at DR2.

Region 3E is required for circularization. For the virus with mutations in region 3E, the amount of plus-strand DNA initiated from DR2 relative to the amount of minus-strand DNA was within twofold of that in the wild-type virus (Fig. 6B), indicating that plus-strand primers were translocated to DR2 and used to initiate plus-strand DNA synthesis in this virus. However, in a primer extension analysis with an oligonucleotide that annealed to plus-strand DNA 135 nt downstream of DR2, fewer 5′ termini of plus-strand DNA were detected at DR2 (Fig. 4B, lane 2). These results indicated that the virus mutated in region 3E was deficient in the elongation of plus-strand DNA initiated from DR2. To determine whether this mutant was specifically defective in the circularization process, we performed a primer extension analysis with a set of oligonucleotides (Fig. 4A, oligonucleotides 2546 and 2537) that anneal to plus-strand DNA immediately before and after the circularization point (Fig. 7). This primer extension reaction was performed with both oligonucleotides simultaneously. For the wild-type virus, 5′ termini at DR2 were detected with both primers, as expected for a virus competent to carry out the template switch mediating circularization (Fig. 7, lanes 3 and 4). For the virus mutated in region 3E, 5′ termini were undetectable at DR2 after circularization despite the detection of 5′ termini at DR2 before circularization (lanes 1 and 2). Therefore, region 3E appears to be required for the circularization process during plus-strand DNA synthesis.

DISCUSSION

We have identified in DHBV three *cis*-acting sequences that are required for the synthesis of relaxed-circular DNA within three different regions of the genome. These three regions are distinct from the direct repeats, DR1 and DR2, and the terminal redundancy, r, on the minus-strand template, which are involved in the primer translocation and circularization steps, respectively. Region 3E, located near the 3′ end of the minus-strand DNA template, is required for the template switch that circularizes the genome (Fig. 6). Our primer extension analysis indicates that the virus mutated in region 3E accumulates a plus-strand DNA intermediate. This finding is consistent with a defect in the template switch needed to circularize the genome. The other two regions are located near the middle of the genome (region M) and near DR2 (region 5E). Viruses containing deletions within region M or region 5E have a similar phenotype, which is distinct from the phenotype of the virus mutated in region 3E. In our primer extension analysis, viruses mutated in region M or region 5E contain very few 5′ termini of plus-strand DNA located at DR2 (Fig. 6). In addition, the decrease in the amount of 5′ termini detected at DR2 for the region M and region 5E mutants was not accompanied by a proportional increase in the amount of 5′ termini detected at DR1. These results are consistent with the interpretation that deletion of either region M or region 5E results in the plus-strand primers normally translocated to DR2 being incapable of priming plus-strand DNA synthesis at either DR2 or DR1.

The location of region 3E, which is near the 3′ terminus of the minus-strand DNA template, is intriguing. It seems rea-

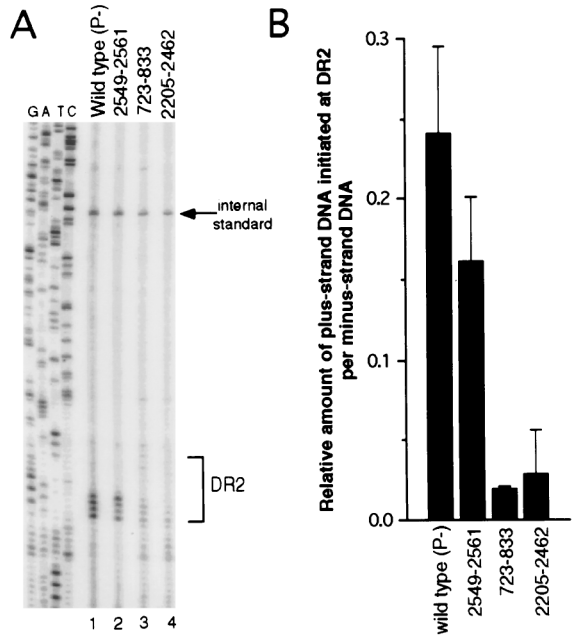

FIG. 6. (A) Detection of plus-strand DNA before circularization. The amount of 5′ termini of plus-strand DNA at DR2 was determined by primer extension analysis with oligonucleotide 2537. This oligonucleotide anneals to plus-strand DNA before the circularization point (Fig. 4A). (B) Amount of plus-strand DNA initiated at DR2 before circularization relative to the amount of minus-strand DNA. The amount of 5′ termini of plus-strand DNA located at DR2 before circularization relative to the amount of 5′ termini of minus-strand DNA located at position 2537 (Fig. 4B) was calculated as described in Materials and Methods. The mean ratio of plus-strand DNA to minus-strand DNA from three different transfections was plotted on the histogram, and error bars represent the standard deviation.

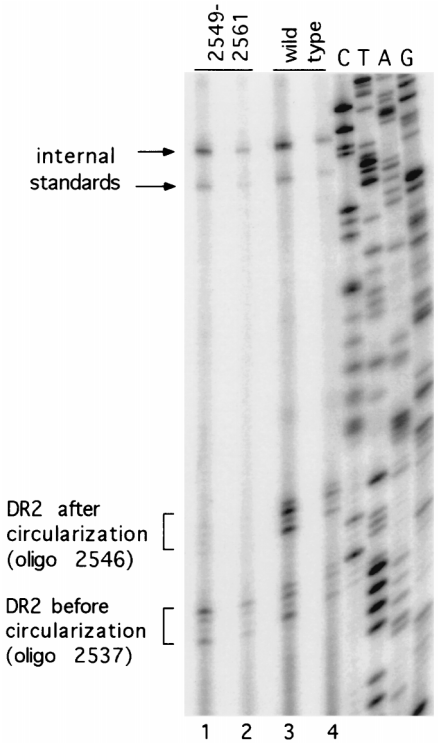

FIG. 7. The virus mutated in region 3E is defective for circularization. Primer extension was performed on viral DNA from the wild type and the region 3E mutant viruses. Oligonucleotides 2537 and 2546 (Fig. 4A) were included simultaneously to detect plus-strand DNA immediately before and immediately after circularization. Two dilutions of each viral DNA are shown. Cloned DHBV DNA added to the viral DNA samples before the primer extension reaction served as an internal standard.

sonable that during the circularization process, the 5′ and 3′ copies of r are juxtaposed within the capsid. This positioning may be required for the efficient switching of the nascent plus-strand DNA to the acceptor site (3′r). Region 3E may play a role in this positioning if, for example, the sequence of region 3E is the binding site for a protein, viral or cellular, that is involved in bringing together the two copies of r. One speculation is that the P protein moiety covalently linked to the 5′ end of minus-strand DNA binds to region 3E and thus brings together or juxtaposes the ends of the minus-strand DNA. Alternatively, the DNA sequence of region 3E may directly interact with a DNA sequence near the 5′ terminus of minus-strand DNA to juxtapose the 5′ and 3′ copies of r. A genetic analysis, however, has not revealed a *cis*-acting sequence required for circularization near the 5′ end of the minus-strand DNA template (nt 2464 to 2476 and 2490 to 2528 were altered in this analysis [data not shown]).

Unlike the case for the virus with a deletion in region 3E, few 5′ termini of plus-strand DNA were detected at DR2 for the viruses mutated in region M or region 5E. The reduction in the amount of 5′ termini of plus-strand DNA detected at DR2 may be due to the inability of the mutant virus to complete one of three steps during replication: (i) translocation of the plus-strand primer, (ii) initiation of plus-strand DNA synthesis, or (iii) elongation of plus-strand DNA. The observation that the viruses containing deletions in region M or region 5E are capable of normal initiation and elongation of plus-strand DNA synthesis from DR1 (Fig. 4C) supports the notion that the mutant viruses are defective for primer translocation and are not defective for the initiation or elongation of plus-strand DNA.

The region M sequence is located near the middle of the minus-strand DNA template. Although it is surprising to find a *cis*-acting sequence at this location, which is involved in an early step of plus-strand DNA synthesis at DR2, this finding is not without precedent. A molecular clone of heron hepatitis B virus which contains a mutation in this region has been demonstrated to be defective for the synthesis of relaxed-circular DNA in cell culture (20). It is not clear how the region M sequence, which is located approximately 1,200 nt from the donor site (DR1) and 1,650 nt from the acceptor site (DR2) on the linear template, is involved in the synthesis of plus-strand DNA. Our analysis is consistent with the idea that this element is involved in translocation of the plus-strand primer to DR2. The similar phenotypes of viruses with deletions in region M or region 5E indicate that the sequences within region M and region 5E play a role at the same step during the synthesis of plus-strand DNA. The sequences within region M and region 5E could interact, directly or indirectly, with each other to mediate primer translocation. We have observed a small region of complementarity between region M and 5E, whose functional significance is currently being investigated. Alternatively, the sequences within region M and region 5E might independently interact with proteins during primer translocation.

The region 5E sequence is near DR2, yet its contribution to DNA synthesis is also obscure. Since viruses containing mutations in DR2, region M, or region 5E all appear to be inhibited for primer translocation in a similar manner (18; see above), we speculate that these sequences may be working together to position the plus-strand primer at DR2. Region 5E and DR2 appear to be distinct *cis*-acting elements, since alteration of the 20 nt between region 5E and DR2 does not affect the synthesis of relaxed-circular DNA (data not shown). Whether region 5E needs to be a certain number of nucleotides from DR2 to function normally remains to be determined. Whether region 5E and region M are binding sites for proteins involved in primer translocation also remains to be determined.

In conclusion, we have identified *cis*-acting sequences outside of the donor and acceptor sites which are required for plus-strand primer translocation and circularization. The mechanisms of these two template switches are largely unknown. It seems likely that the donor and acceptor templates are juxtaposed during these processes. It follows that the template, outside of the donor and acceptor sites, could have a specific conformation or structure, through either nucleic acid-nucleic acid or nucleic acid-protein interactions, which is required for the juxtaposition of the donor and acceptor sites. This reasoning leads us to suggest that regions 3E, M, and 5E are involved in establishing a conformation of the template that is integral to the mechanisms of the respective template switches.

Template switching also occurs during retrovirus and caulimovirus reverse transcription. A reasonable extension of our findings is that retroviruses and caulimoviruses also use *cis*-acting sequences distinct from their donor and acceptor sites to facilitate template switching.

ACKNOWLEDGMENTS

We thank Ru Tian and Karolyn Gulya for assistance in constructing some of the deletion mutants. Jackie Calvert and Jesse Summers generously gave us the deletion mutants mentioned and shared with us their observation that a 719–833 deletion mutant does not make relaxed-circular DNA. We thank Haiyan Jiang, Jill Slansky, Lou Mansky, Karlyn Mueller-Hill, Mike Schwartz, Ashok Aiyar, Paul Ahlquist, Nito

Panganiban, Norman Drinkwater, Bill Sugden, and Paul Lambert for helpful discussions and criticisms of the manuscript.

This work was supported by NIH grants GM50263, CA09135, and CA07175. M.B.H. is a Cremer Scholar. D.D.L. is the recipient of an American Cancer Society Junior Faculty Research Award (JFRA-651).

REFERENCES

1. **Calvert, J., and J. Summers.** 1994. Two regions of an avian hepadnavirus RNA pregenome are required in *cis* for encapsidation. J. Virol. **68:**2084–2090.
2. **Chang, L.-J., R. C. Hirsch, D. Ganem, and H. E. Varmus.** 1990. Effects of insertional and point mutations on the functions of the duck hepatitis B virus polymerase. J. Virol. **64:**5553–5558.
3. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
4. **Chen, Y., W. S. Robison, and P. L. Marion.** 1994. Selected mutations of the duck hepatitis B virus P gene RNase H domain affect both RNA packaging and priming of minus-strand DNA synthesis. J. Virol. **68:**5232–5238.
5. **Church, G. M., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
6. **Condreay, L. D., C. E. Aldrich, L. Coates, W. S. Mason, and T.-T. Wu.** 1990. Efficient duck hepatitis B virus production from an avian liver tumor cell line. J. Virol. **64:**3249–3258.
7. **Condreay, L. D., T. T. Wu, C. E. Aldrich, M. A. Delaney, J. Summers, C. Seeger, and W. S. Mason.** 1992. Replication of DHBV genomes with mutations at the sites of initiation of minus- and plus-strand DNA synthesis. Virology **188:**208–216.
8. **Hirsch, R. C., J. E. Lavine, L. J. Chang, H. E. Varmus, and D. Ganem.** 1990. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. Nature **344:**552–555.
9. **Hirsch, R. C., D. D. Loeb, J. R. Pollack, and D. Ganem.** 1991. *cis*-acting sequences required for encapsidation of duck hepatitis B virus pregenomic RNA. J. Virol. **65:**3309–3316.
10. **Hoheisel, J., and F. M. Pohl.** 1986. Simplified preparation of unidirectional deletion clones. Nucleic Acids Res. **14:**3605.
11. **Huang, M., and J. Summers.** 1994. *pet*, a small sequence distal to the pregenome cap site, is required for expression of the duck hepatitis B virus pregenome. J. Virol. **68:**1564–1572.
12. **Kawaguchi, T. K., K. Nomura, Y. Hirayama, and T. Kitagawa.** 1987. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. Cancer Res. **47:**4460–4464.
13. **Kunkel, T. A., J. D. Roberts, and R. A. Zabour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
14. **Lien, J. M., C. E. Aldrich, and W. S. Mason.** 1986. Evidence that a capped oligoribonucleotide is the primer for duck hepatitis B virus plus-strand DNA synthesis. J. Virol. **57:**229–236.
15. **Loeb, D. D., K. Gulya, and R. Tian.** 1997. Sequence identity of the terminal redundancies on the minus-strand DNA template is necessary but not sufficient for the template switch during hepadnaviral plus-strand DNA synthesis. J. Virol. **71:**152–160.
16. **Loeb, D. D., R. C. Hirsch, and D. Ganem.** 1991. Sequence-independent RNA cleavages generate the primers for plus strand DNA synthesis in hepatitis B viruses: implications for other reverse transcribing elements. EMBO J. **10:**3533–3540.
17. **Loeb, D. D., and R. Tian.** 1995. Transfer of the minus strand of DNA during hepadnavirus replication is not invariable but prefers a specific location. J. Virol. **69:**6886–6891.
18. **Loeb, D. D., R. Tian, and K. Gulya.** 1996. Mutations within DR2 independently reduce the amount of both minus- and plus-strand DNA synthesized during hepatitis B virus replication. J. Virol. **70:**8684–8690.
19. **Mandart, E., A. Kay, and F. Galibert.** 1984. Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. J. Virol. **49:**782–792.
20. **Mueller-Hill, K., and D. D. Loeb.** 1996. Previously unsuspected *cis*-acting sequences for DNA replication revealed by characterization of a chimeric heron/duck hepatitis B virus. J. Virol. **70:**8310–8317.
21. **Radziwill, G., W. Tucker, and H. Schaller.** 1990. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. J. Virol. **64:**613–620.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Skalka, A. M., and S. P. Goff (ed.).** 1993. Reverse transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Sprengel, R., C. Kuhn, H. Will, and H. Schaller.** 1985. Comparative sequence analysis of duck and human hepatitis B virus genomes. J. Med. Virol. **15:**323–333.
25. **Staprans, S., D. D. Loeb, and D. Ganem.** 1991. Mutations affecting hepadnavirus plus-strand DNA synthesis dissociate primer cleavage from translocation and reveal the origin of linear viral DNA. J. Virol. **65:**1255–1262.
26. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of and RNA intermediate. Cell **29:**403–415.
27. **Tavis, J. E., S. Perri, and D. Ganem.** 1994. Hepadnavirus reverse transcription initiates within the stem-loop of the RNA packaging signal and employs a novel strand transfer. J. Virol. **68:**3536–3543.
28. **Wang, G. H., and C. Seeger.** 1992. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. Cell **71:**663–670.
29. **Wang, G. H., and C. Seeger.** 1993. Novel mechanism for reverse transcription in hepatitis B viruses. J. Virol. **67:**6507–6512.