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Sequence Identity of the Terminal Redundancies on the Minus-Strand DNA Template Is Necessary but Not Sufficient for the Template Switch during Hepadnavirus Plus-Strand DNA Synthesis

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The template for hepadnavirus plus-strand DNA synthesis is a terminally redundant minus-strand DNA. An intramolecular template switch during plus-strand DNA synthesis, which permits plus-strand DNA elongation, has been proposed to be facilitated by this terminal redundancy, which is 7 to 9 nucleotides long. The aim of this study was to determine whether the presence of identical copies of the redundancy on the minus-strand DNA template was necessary and/or sufficient for the template switch and at what position(s) within the redundancy the switch occurs for duck hepatitis B virus. When dinucleotide insertions were placed within the copy of the redundancy at the 3′ end of the minus-strand DNA template, novel sequences were copied into plus-strand DNA. The generation of these novel sequences could be explained by complete copying of the redundancy at the 5′ end of the minus-strand DNA template followed by a template switch and then extension from a mismatched 3′ terminus. In a second set of experiments, it was found that when one copy of the redundancy had either three or five nucleotides replaced the template switch was inhibited. When the identical, albeit mutant, sequences were restored in both copies of the redundancy, template switching was not necessarily restored. Our results indicate that the terminal redundancy on the minus-strand DNA template is necessary but not sufficient for template switching.

Reverse transcription, a phenomenon found throughout nature, is the process in which RNA is used as a template to synthesize DNA (for a monograph, see reference 18). The reverse transcription schemes of retroviruses (for a review, see reference 22), hepadnaviruses (for a review, see reference 10), and caulimoviruses (for a review, see reference 1) are among the best understood. For these divergent families of viruses, the major end product of reverse transcription is a linear (retroviruses) or a circular (hepadna- and caulimoviruses) double-stranded DNA molecule. Although the specific mechanisms of reverse transcription of these three virus families differ, they share common features. One such commonality is a process known as a template switch or strand transfer. Because initiation of both minus- and plus-strand DNA synthesis usually occurs at an internal position on the template (near the 5′ terminus of the template strand), a template switch is necessary for elongation of the DNA strand being synthesized. A template switch occurs when the growing point of DNA synthesis reaches a position at, or near, the 5′ end of the template strand and switches to a position at, or near, the 3′ end of the template strand, thus allowing DNA synthesis to resume. In general, the donor and acceptor templates contain a repeated sequence, usually at the termini, that is thought to be necessary for the template switching. The length of the repeated sequence varies with the virus and the template being switched. Typically, a reverse-transcribing virus has an obligate template switch during both minus- and plus-strand DNA synthesis. Examples of intramolecular and intermolecular template switching have been found (6, 15).

Retroviruses and caulimoviruses have similar strategies for the priming of minus- and plus-strand DNA synthesis, and they also have similar strategies for template switching. For example, for the template switch during minus-strand DNA synthesis, RNase H activity is thought to degrade the 5′ end of the RNA template, thus facilitating annealing of the nascent minus-strand DNA to the 3′ end of the RNA template (3). Similarly, during plus-strand DNA synthesis, the tRNA primer attached to the 5′ end of the minus-strand template is thought to be cleaved to allow annealing of the nascent plus strand to the 3′ end of the minus-strand template (14).

The template-switching strategies of hepadnaviruses are different from those of the retro- and caulimoviruses. For example, the template switch during hepadnavirus minus-strand DNA synthesis occurs in the absence of an RNase H cleavage of the RNA template, and the donor and acceptor sites are internal on the RNA template (13, 21, 24). Also, the template switch during plus-strand DNA synthesis probably uses a mechanism different from those of the retro- and caulimoviruses, because hepadnaviruses lack an RNA moiety at the 5′ terminus of the minus-strand DNA template (4, 5, 12).

Hepadnaviruses initiate plus-strand DNA synthesis at a position (DR2) near the 5′ end of the minus-strand DNA template, as a consequence of primer translocation (8, 17) (Fig. 1, part 6). Synthesis of plus-strand DNA proceeds a short distance before reaching the 5′ terminus of the template (Fig. 1, part 7). For continuation of plus-strand DNA synthesis, an intramolecular template switch must occur. It has been proposed that the 7- to 9-nucleotide terminal redundancy (called r) facilitates the template switch during synthesis of plus-strand DNA (9, 17, 25) (Fig. 1, part 7). After switching of the templates, synthesis of plus-strand DNA resumes, resulting in a relaxed circular DNA genome (Fig. 1, parts 8 and 9).

We have examined the role of the terminal redundancy in the template switch during synthesis of plus-strand DNA for duck hepatitis B virus (DHBV). We show that the redundancy

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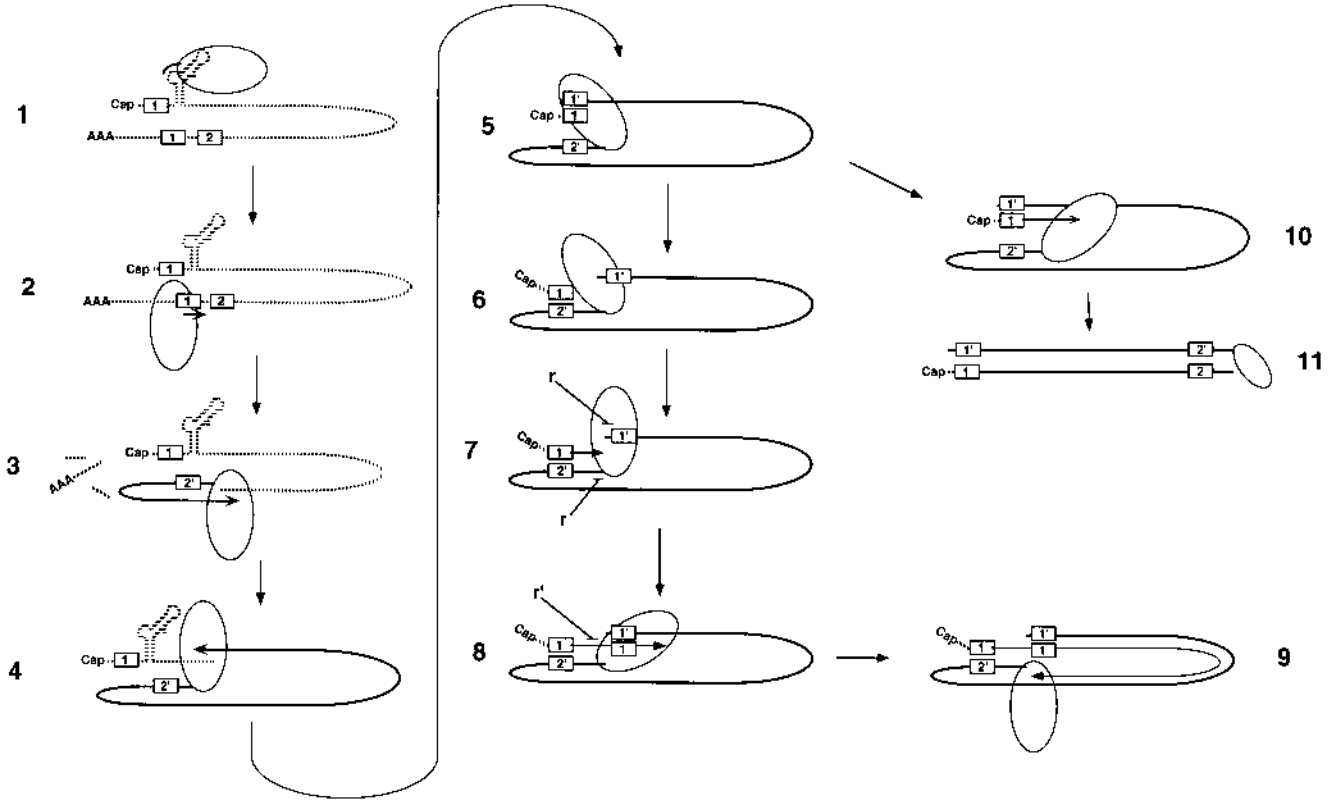

FIG. 1. Model for hepadnavirus DNA synthesis. (1) Encapsidation and initiation of minus-strand DNA synthesis. The dashed line represents pregenomic RNA which is capped and polyadenylated. The shaded rectangular boxes labeled 1 and 2 represent DR1 and DR2, respectively. DR1 is present twice on pregenomic RNA. The P protein (transparent 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) is synthesized by copying the bulge within the stem-loop. (2) Minus-strand template switch. The tetranucleotide linked to the P protein switches templates such 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-encoded RNase H activity degrades the RNA pregenome. The white rectangular box labeled 2′ represents 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. The white rectangular box labeled 1′ represents DR1 on minus-strand DNA. The final RNase H cleavage generates the plus-strand primer which is derived from the first 18 nucleotides of the 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. The 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 template. Approximately 50 nucleotides of plus-strand DNA is synthesized. The terminal redundancy on minus-strand DNA, of 8 or 9 nucleotides, is labeled r. (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. The terminal redundancy on the minus strand is important for the switch. Plus-strand DNA is then elongated. Present in plus-strand DNA is a single complementary copy of the redundancy, r′. 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 are not initiated at DR2 but instead are initiated at DR1. The plus strands initiated at DR1 arise when the plus-strand primer is not translocated but instead is used at the primer donor site. (11) Elongation of an in situ-primed plus strand results in a duplex linear DNA.

at the 5′ terminus of minus-strand DNA can be completely copied into plus-strand DNA. In addition, upon transfer of the plus strand of DNA to the 3′ redundancy of minus-strand DNA, resumption of DNA synthesis from a mispaired 3′ terminus occurs readily. Also, we show that the template switch can be inhibited when the terminally redundant sequences on the minus-strand template are not identical and that the presence of identical mutant copies of the redundancy is not necessarily sufficient for restoration of template switching.

MATERIALS AND METHODS

Construction of mutant DHBV genomes. The DHBV3 strain (19) and derivatives thereof were used in all experiments. Mutations were created by using a site-specific, oligonucleotide-directed mutagenesis procedure (7). Mutations were identified by DNA sequencing. The absence of unwanted mutations was confirmed by DNA sequencing. Plasmids competent to express virus contained 1.5 copies of the DHBV genome (11). These expression plasmids were constructed such that only one copy of the redundancy, where appropriate, contained the mutation. In addition to the mutation(s) within the redundant region, the T101 and T559 viruses had the template for the synthesis of the first four nucleotides of minus-strand DNA (the sequence UUAC in the bulge within the encapsidation signal [21, 24]) changed to AUAC so that the sequence of the 5′ end of mature minus-strand DNA would be 5′-GTATAAGTC-3′.

Cell culture, viral DNA isolation, and Southern blot analysis. Transfection of LMH cells was carried out as described previously (11). Isolation of viral DNA from cytoplasmic capsids was carried out as described by Staprans et al. (20) or Calvert and Summers (2). Southern blot analysis was performed as described previously (20).

DNA cloning and sequencing of plus-strand DNA. (i) Virus 160. A modified 5′ RACE (rapid amplification of cDNA ends) protocol was employed (16). Primer extension was performed on viral DNA (approximately 1 ng of relaxed circular DNA) with 2 pmol of an oligonucleotide complementary to nucleotides 2828 to 2845. The primer extension reaction mixture contained 20 mM Tris-HCl (pH 8.8); 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 200 μM (each) dATP, dCTP, dGTP, and TTP; 0.1% Triton X-100, and 1 U of Vent exo$^-$ DNA polymerase, in a final volume of 20 μl. The reaction mixture was placed in a thermocycler and incubated at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 20 cycles followed by 72°C for 5 min. The primer extension product was purified from unincorporated primer, deoxynucleoside triphosphates, and proteins by a protocol utilizing a GlassMAX spin cartridge (Gibco BRL). A homopolymeric deoxycytidine 3′ tail was added to the primer extension product in a reaction

mixture which contained 10 mM Tris-HCl (pH 8.5), 25 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM dCTP, and 10 U of terminal transferase, which was incubated at 37°C for 5 min followed by 65°C for 10 min to inactivate the terminal transferase. This tailed primer extension product was then PCR amplified with 4 pmol of a dC homopolymeric-specific oligonucleotide (CUACUACUACUAGAATTCTA GAGCTCGAGGGGIIGGGIIGGGIIG [a kind gift from Walter Hubert and Paul Lambert]) and 8 pmol of a DHBV-specific oligonucleotide complementary to nucleotides 2678 to 2700. The PCR mixture contained 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, and 1 U of *Taq* DNA polymerase. The reaction mixture was placed in a thermocycler and incubated at 95°C for 60 s, 57°C for 60 s, and 72°C for 60 s for 35 cycles, followed by 72°C for 5 min. The PCR products were extracted with phenol-chloroform, and DNA in the aqueous phase was precipitated with ethanol. To render them suitable for DNA cloning, the PCR products were digested with *Eco*RI (cleaves at homopolymeric termini) and *Eco*RV (cleaves at DHBV nucleotide 2650). The restriction digest was electrophoresed through an agarose gel, and DNA migrating around the position of 200 bp was removed and extracted from the gel. This material was cloned into a plasmid vector, and positive clones were identified by hybridization with an oligonucleotide complementary to DHBV nucleotides 2513 to 2532. DNA sequence analysis of the positive clones was performed with a an oligonucleotide complementary to DHBV nucleotides 2599 to 2622 as a primer. Only clones with 5′ termini of DHBV within DR2 were included in the analysis. Wild-type DHBV DNA was analyzed in a similar fashion to demonstrate the ability of the 5′ RACE procedure to accurately yield the sequence of plus-strand DNA. All eight wild-type clones analyzed contained the wild-type sequence.

(ii) Viruses G245 and G251. A PCR protocol was employed to amplify plus-strand DNA sequence, followed by DNA cloning and sequencing. In the PCR procedure, one primer was derived from DHBV nucleotides 2507 to 2526, while the second primer was complementary to DHBV sequences from nucleotide 2678 to 2700. The plus strand of DNA is continuous between this pair of primers, while the minus strand of DNA is not. First, viral DNA was digested with *Dpn*I (site at position 2686) to cleave any residual bacterial DNA in the viral DNA preparation. Next, 15 pmol of the 5′-phosphorylated form of each primer was used to amplify the *Dpn*I-digested viral DNA. The PCR mixture contained 20 mM Tris-HCl (pH 8.8); 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 200 μM (each) dATP, dCTP, dGTP, and TTP; 0.1% Triton X-100, and 2 U of Vent exo$^-$ DNA polymerase, in a final volume of 100 μl. The reaction mixture was placed in a thermocycler and incubated at 94°C for 60 s, 48°C for 60 s, and 72°C for 60 s for 20 cycles followed by 72°C for 5 min. To make the termini of the amplified DNA fragment blunt, 3 U of T4 DNA polymerase was added and incubated at 16°C for 15 min. The PCR DNA was then extracted with phenol-chloroform and precipitated with ethanol. A 193-nucleotide fragment was then cloned into a plasmid vector and sequenced with the same primer as used for sequencing the virus 160 RACE clones. To convince ourselves that the PCR procedure was amplifying relaxed circular plus-strand DNA, we performed the procedure on a mutant virus which makes no detectable relaxed circular DNA but instead makes duplex linear DNA and found little to no PCR amplification.

Primer extension analysis of plus-strand DNA. Approximately 500 pg of viral DNA was mixed with 400 pg of the plasmid D1.5G (the wild-type DHBV expression plasmid) cleaved with *Ava*I (which cleaves at nucleotide 2411) in 10 mM EDTA. NaOH was added to 0.2 N and incubated at 95°C for 5 min to hydrolyze the plus-strand RNA primer. The solution was neutralized by the addition of Tris hydrochloride to a final concentration of 0.33 M. DNA was precipitated with ethanol in the presence of 20 μg of carrier glycogen. The DNA was resuspended in 12 μl of Tris-EDTA. For primer extension, usually 4 μl of the NaOH-treated viral DNA was used in a reaction mixture containing 20 mM Tris-HCl (pH 8.8); 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 200 μM (each) dATP, dCTP, dGTP, and TTP; 0.1% Triton X-100; 1 U of Vent exo$^-$ DNA polymerase; and 0.65 pmol of the end-labeled oligonucleotide in a final volume of 10 μl. The oligonucleotide that anneals to plus-strand DNA before the template switch (oligonucleotide B) is complementary to nucleotides 2511 to 2528 (annealing temperature of 37°C), while the oligonucleotide that anneals to plus-strand DNA after the template switch (oligonucleotide A) is complementary to positions 2547 to 2567 (annealing temperature of 60°C). Experiment 3 in Table 1 used an oligonucleotide complementary to positions 2599 to 2622 (annealing temperature of 60°C) to detect plus-strand DNA after the template switch. Primer extension reaction mixtures were placed in a thermocycler and incubated at 95°C for 30 s, at an annealing temperature specific for each oligonucleotide for 30 s, and then at 72°C for 30 s, for 10 cycles. Samples were electrophoresed as previously described (11). Quantification of autoradiographic images was performed with a Molecular Dynamics 445 SI PhosphorImager. Under these primer extension conditions, the PhosphorImager values obtained were directly proportional to the amounts of viral DNA added to the reaction, indicating that the assay was quantitative.

Calculation of the relative efficiency of template switching. To normalize for variations in the primer extension procedure, cloned plasmid DNA cleaved at the *Ava*I site was added to each viral DNA prior to the NaOH treatment. For each viral DNA analyzed by primer extension, a value representing the amount of viral 5′ termini at DR2 divided by the amount of *Ava*I-cleaved (IC) plasmid termini was calculated; for example, $DR2_A/IC_A = A_{wt}$ (wild-type virus analyzed with oligonucleotide A). This kind of value was calculated for wild-type and each mutant virus upon analysis with oligonucleotides B and A, and then this value calculated for oligonucleotide A was divided by this value calculated for oligonucleotide B; for example, A_{wt}/B_{wt} (wild-type virus). This last value represents the fraction of plus strands that carried out the template switch for wild-type DHBV. The value determined for the wild-type virus was normalized to 100%, and the *A*/*B* values for the mutant viruses were expressed as percentages of that for the wild type.

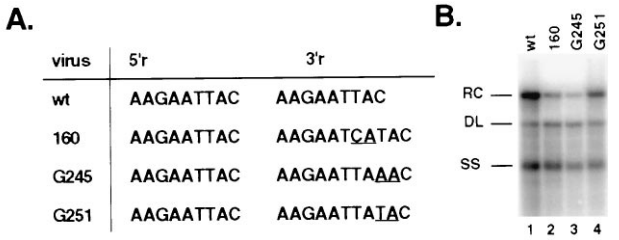

FIG. 2. (A) The 5′r and 3′r regions within minus-strand DNA of wild-type (wt) and mutant viruses. Sequences are represented as plus-strand polarity. Viruses 160, G245, and G251 contain dinucleotide insertions with the 3′r region. Inserted sequences are underlined. (B) Southern blot analysis of viral DNA from cytoplasmic capsids. RC, DL, and SS represent the mobilities of the relaxed circular, duplex linear, and minus-strand single-stranded molecules, respectively. The hybridization probe was genomic length and minus-strand specific.

RESULTS

The aim of this study was to examine the role of the terminal redundancy on the minus-strand DNA template for the intramolecular template switch during synthesis of DHBV plus-strand DNA. Specifically, we wanted to determine whether sequence identity of both copies of the redundancy was necessary and/or sufficient for the template switch and at what position(s) within the terminal redundancy the switch occurred. To study this intramolecular template switch, we introduced mutations into plasmids competent to express viruses with changes in either the 5′ copy, the 3′ copy, or both copies of the redundancy on the minus-strand DNA template. We then transfected the mutant plasmids into cells competent to support DHBV DNA synthesis. Three days after the transfection, cytoplasmic capsids that contained the newly replicated viral DNA were harvested and viral DNA was purified. Analysis of plus-strand DNA was then performed.

The r sequence at the 5′ end of minus-strand DNA can be completely copied into plus-strand DNA. By virtue of being terminally redundant, minus-strand DNA has the same sequence at its 5′ end (5′r) and 3′ end (3′r). Plus-strand DNA has a single, complementary copy of the redundancy (r′), which is located approximately 50 nucleotides from its 5′ terminus. In this report, we have defined the redundancy as the 9-nucleotide sequence 5′-AAGAATTAC-3′ (plus-strand sense). To determine which nucleotides in the 5′r and 3′r were used as a template for the synthesis of r′, which would indicate the site of the template switch, we introduced mutations into the 3′r on the minus-strand DNA template and asked whether the mutation was copied into plus-strand DNA. If the switch to the 3′r template occurs before the position of the mutation, the mutation will be incorporated into plus-strand DNA. If the switch to the 3′r template occurs after the position of the mutation, the mutation will not be incorporated into plus-strand DNA. In the first experiment, two nucleotides were inserted into the 3′r template to give the sequence AAGAATCATAC (Fig. 2A, virus 160). Throughout this report, the plus-strand sequence polarity has been used to represent the redundancy. Southern blot analysis of viral DNA from virus 160 detected relaxed circular DNA (Fig. 2B, lane 2), indicating that the mutation in the 3′r had not completely inhibited the template switch. Be-

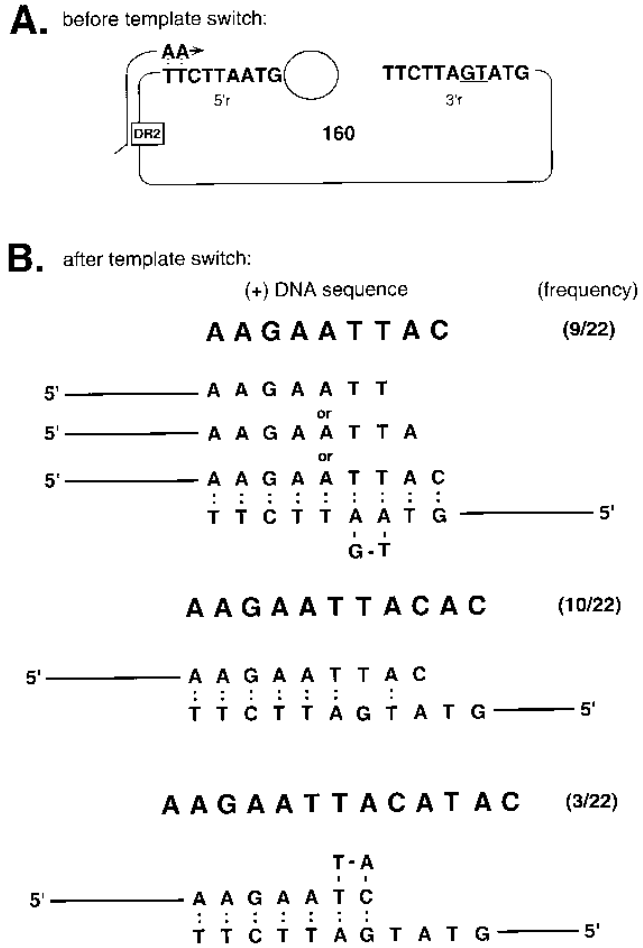

FIG. 3. Sequence of the r′ region in plus-strand DNA for virus 160. (A) Representation of the replicative intermediate before the template switch. The insertion mutation within the 3′r on the minus strand is underlined. (B) Three different sequences of the r′ region in plus-strand DNA were found. The sequence of the r′ region in the plus-strand DNA is indicated. The frequency of each sequence is on the right. Hypothetical intermediates after the template switch but before the resumption of plus-strand DNA synthesis are below each determined sequence. Plus-strand DNA is on the top, while minus-strand DNA is on the bottom. Base pairs are indicated by colons. Bulges in plus and minus strands are indicated above or below the respective strands.

cause the template switch was occurring readily, we wanted to know the sequence of the plus-strand r′. To determine this sequence, individual plus strands of DNA were amplified by a PCR procedure followed by molecular cloning and DNA sequencing of 22 individual clones. For virus 160, three different r′ sequences were recovered (Fig. 3B). Nine examples of the wild-type sequence (AAGAATTAC) were found. Recovery of the wild-type sequence was interpreted to indicate that either AAGAATT, AAGAATTA, or AAGAATTAC was copied from the 5′r template and that after the template switch, the 3′r template contained a 2-nucleotide bulge and the terminus of the plus-strand DNA was base paired for either 1, 2, or 3 nucleotides (Fig. 3B). Ten examples of the second sequence, AAGAATTACAC, and three examples of the third sequence, AAGAATTACATAC, were recovered. Both of these sequences are novel because they do not exist in the expression plasmid DNA. A plausible scenario (Fig. 3B) to generate the sequence AAGAATTACAC is that the 5′r template was copied completely, and upon template switching, a 3′-terminal mispairing existed between the plus-strand DNA and the 3′r, followed by resumption of DNA synthesis from the mispaired terminus. Generation of the third sequence, AAGAATTACATAC, could also involve complete copying of the 5′r template and then a switch to a different position within the 3′r template such that the two nucleotides adjacent to the 3′ terminus of the plus-strand DNA are not base paired with the template but instead bulge.

Extension from a mismatched terminus can occur after a template switch. It was unexpected to find that extension from a terminal mismatch had occurred. Also, the results described above indicated that the template switch occurred after the 5′r was completely copied. To corroborate and extend these interpretations, we tested two additional mutants that harbored mutations within the 3′r template. The mutant G251, with the sequence AAGAATTATAC, had a TA dinucleotide insertion (Fig. 2A). The mutant G245, with the sequence AAGAATTA AAC, had an AA dinucleotide insertion (Fig. 2A). Southern blot analysis of viral DNA from viruses G251 and G245 showed the presence of relaxed circular DNA, indicating that the two mutants supported the template switching (Fig. 2B, lanes 3 and 4). Cloning and sequencing of eight individual plus-strand DNAs of virus G251 (AAGAATTATAC) indicated that two different sequences were recovered, six examples of AAGAA TTACAC and two examples of AAGAATTATAC (Fig. 4). The six examples of AAGAATTACAC, which is a sequence that does not exist in the expression plasmid, could have arisen if the 5′r was completely copied followed by the template switch and extension from a terminal mismatch. The two examples of AAGAATTATAC sequence for virus G251 could

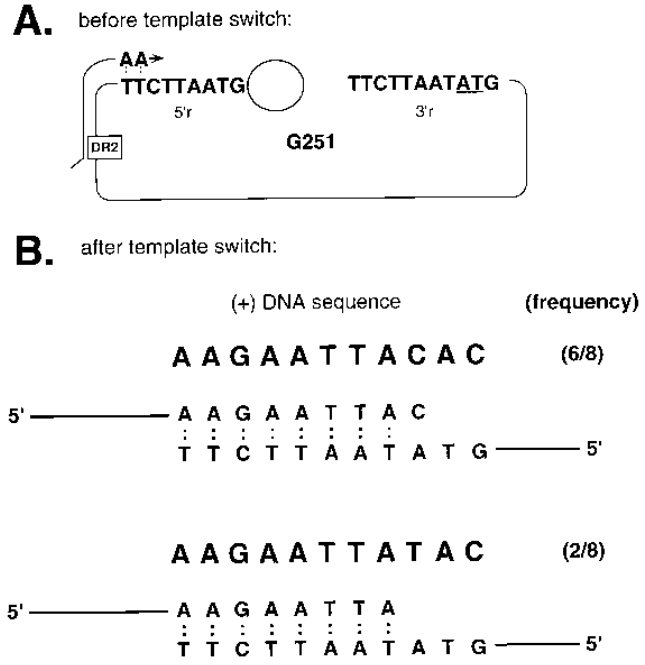

FIG. 4. Sequence of the r′ region in plus-strand DNA for virus G251. (A) Representation of the replicative intermediate before the template switch. The insertion mutation within the 3′r on the minus strand is underlined. (B) Three different sequences of the r′ region in plus-strand DNA were found. The sequence of the r′ region in the plus-strand DNA is indicated. The frequency of each sequence is on the right. Hypothetical intermediates after the template switch but before the resumption of plus-strand DNA synthesis are below each determined sequence. Plus-strand DNA is on the top, while minus-strand DNA is on the bottom. Base pairs are indicated by colons. Bulges in plus and minus strands are indicated above or below the respective strands.

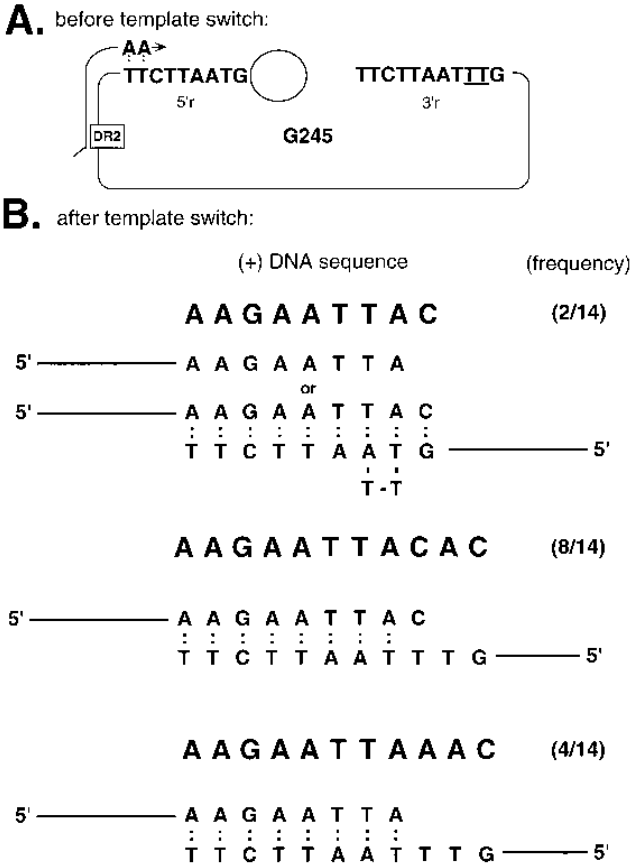

FIG. 5. Sequence of the r′ region in plus-strand DNA for virus G245. (A) Representation of the replicative intermediate before the template switch. The insertion mutation within the 3′r on the minus strand is underlined. (B) Two different sequences of the r region in plus-strand DNA were found. The sequence of the r region in the plus-strand DNA is indicated. The frequency of each sequence is on right. Hypothetical intermediates after the template switch but before the resumption of plus-strand DNA synthesis are below each determined sequence. Plus-strand DNA is on the top, while minus-strand DNA is on the bottom. Base pairs are indicated by colons. Bulges in plus and minus strands are indicated above or below the respective strands.

have arisen during a template switch which occurred before the copying of the 5′-terminal nucleotide of the minus-strand DNA template. These interpretations of virus G251 suggest that although complete copying of the 5′r sequence before the template switch is preferred, switching before copying to the 5′ end of minus-strand DNA can occur.

Cloning and sequencing of 14 plus-strand DNAs from virus G245 (AAGAATTAAAC) yielded three different sequences (Fig. 5). The most prevalent sequence (eight examples) was AAGAATTACAC. As found earlier, this sequence was not present in the expression plasmid. Its synthesis was interpreted to occur when the 5′r template was completely copied followed by switching to the 3′r template and extension from a terminal mismatch. Four examples of the sequence AAGAATTAAAC were found and were interpreted to have arisen as a consequence of a template switch before the copying of the 5′ nucleotide of the minus-strand DNA template. Two examples of the sequence AAGAATTAC were found and were interpreted to indicate complete copying of the 5′r template followed by transfer of the plus-strand DNA such that the terminal two nucleotides (5′-AC-3′) were base paired with the 3′-TG-5′ at the 5′ boundary of the 3′r template, yielding a 2-nucleotide bulge on the template (Fig. 5).

The interpretation of all the above-described results is that (i) the template switch could occur when the 5′r and 3′r were not identical; (ii) the template switch could occur after the r sequence at the 5′ end of minus-strand DNA was completely copied; and (iii) following the template switch, DNA synthesis could extend from a terminal mismatch. Next, we investigated to what extent disruption of complementarity between the DNA of the nascent plus strand and the 3′r inhibits the template switch. If the template switch was inhibited, we would then determine whether template switching was restored when both the 5′r and 3′r contained the same mutation.

The template switch can be inhibited, and an identical redundancy does not necessarily restore the template switch. Next, we attempted to inhibit the template switch by introducing multiple base substitutions in one copy of r while leaving the other copy unchanged. To this end, three nucleotides were changed, resulting in the mutant r sequence AGGCCTTAC. Two plasmids, competent to express virus, in which either the 5′r (virus 238) or the 3′r (virus 250) contained this mutation were made and transfected into LMH cells (Fig. 6A). Three

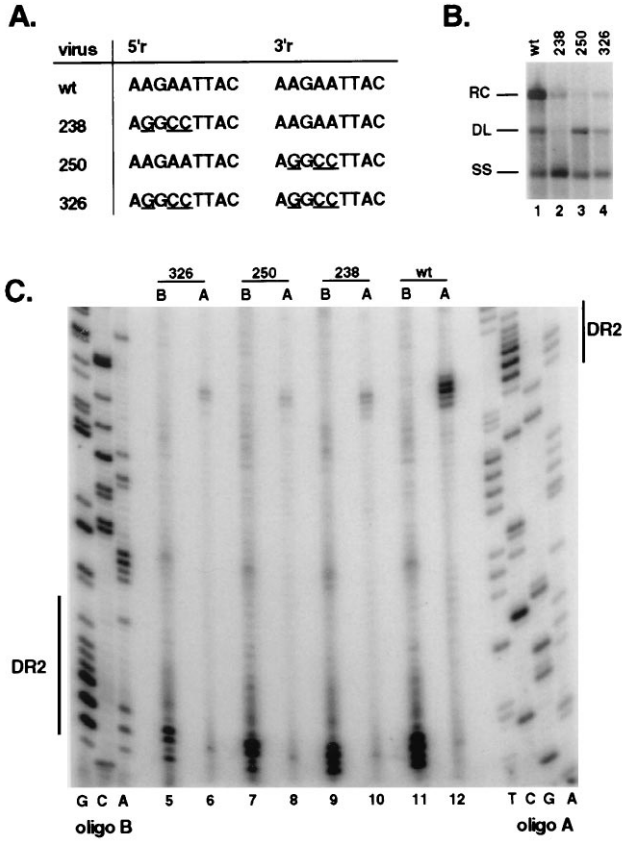

FIG. 6. Analysis of template switching for viruses with an AGGCCTTAC r region. (A) The 5′r and 3′r regions of the wild-type (wt) and mutant viruses. Sequences are represented as plus-strand polarity with base substitutions underlined. (B) Southern blot analysis of viral DNA from cytoplasmic capsids. RC, DL, and SS represent the mobilities of the relaxed circular, duplex linear, and minus-strand single-stranded molecules, respectively. The hybridization probe was genomic length and minus-strand specific. (C) Primer extension analysis to measure the extent of template switching. A and B, oligonucleotides A and B, respectively. Sequencing ladders generated with oligonucleotides A and B are on the right and left, respectively. The positions of DR2 on the sequencing ladders are indicated. The 5′ termini of plus-strand DNA are found at four consecutive positions at the 3′ end of DR2.

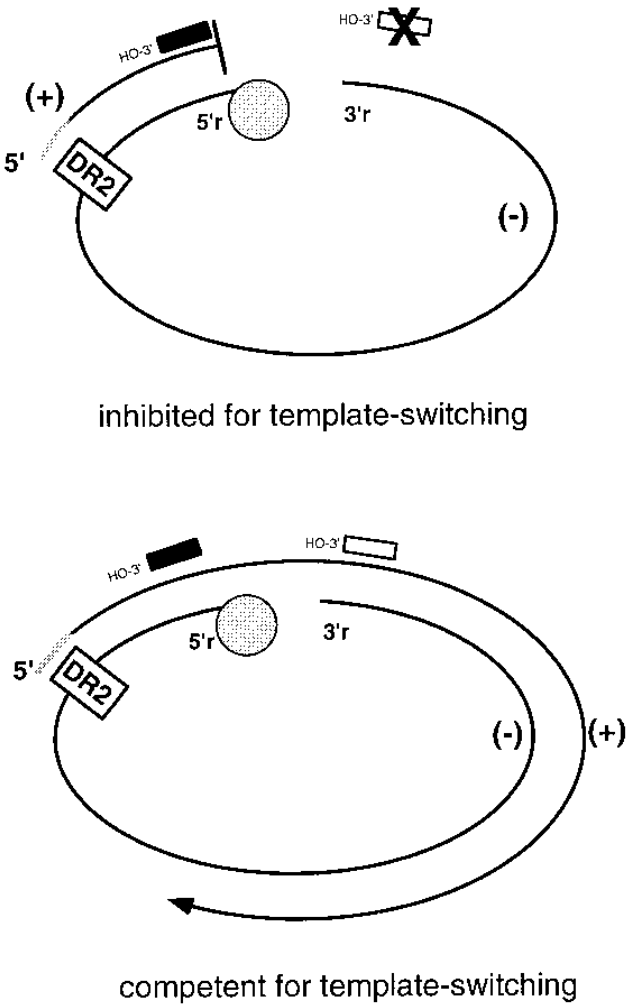

FIG. 7. Strategy for measuring the template switch. Two different replicative intermediates are represented. The replicative intermediate on the bottom was competent for the template switch, while the one on the top was inhibited for the template switch. Both replicative intermediates have a full-length minus-strand DNA (inner, black ovoid), with the 5′r and 3′r, P protein (shaded circle), and DR2 (rectangle) indicated. Plus-strand DNA is initiated from an RNA primer (grey line) annealed to DR2. The oligonucleotides used in the primer extension analyses are indicated by the black rectangle (oligonucleotide B, which detects plus strands before the template switch) and the white rectangle (oligonucleotide A, which detects plus strands after the template switch). HO-3′ indicates the 3′ termini of the oligonucleotides. Oligonucleotide A cannot anneal to the replicative intermediate that is inhibited for the template switch.

days after transfection, viral DNA from cytoplasmic capsids was harvested and analyzed by Southern blotting (Fig. 6B). If the template switch was inhibited, we would expect a reduction in the proportion of relaxed circular DNA in the sample and an accumulation of a DNA species that would be similar in structure to the immediate precursor prior to the template switch (Fig. 1, part 7). Such a DNA species would likely migrate at a position similar to that of single-stranded minus-strand DNA (SS position) in the Southern blot analysis. When virus 238 (5′r mutant) was analyzed by Southern blotting, it indeed revealed a decrease in the relative amount of relaxed circular DNA (in comparison to that for wild-type DHBV) with a proportional increase in the level of DNA migrating at the SS position (Fig. 6B, lane 2 compared to lane 1). Southern blot analysis of virus 250 (3′r mutant) also revealed a decrease in the relative amount of relaxed circular DNA with increases in both duplex linear DNA and the DNA species migrating at the SS position (Fig. 6B, lane 3). The mutation in virus 250 is also present at the 5′ end of the RNA pregenome and therefore in the plus-strand primer. We think that this mutation inhibited primer translocation (20), resulting in an increase in in situ priming from DR1 (primer extension data not shown), thus accounting for the relative increase in duplex linear DNA found for virus 250. Nonetheless, consistent with an inhibition of template switching, virus 250 had a higher proportion of DNA species migrating at the SS position than did the wild type. Analysis of the Southern blot indicated that the sequence AGGCCTTAC inhibited template switching when present within either the 5′ or 3′ copy of r. To corroborate and extend these interpretations, we developed an assay that quantifies the extent of inhibition of template switching of a mutant relative to that of the wild type. This strategy uses primer extension and is depicted in Fig. 7. Two different oligonucleotides are used in the primer extension analysis to quantify the number of 5′ termini of plus-strand DNA at DR2. The first oligonucleotide (oligonucleotide B) anneals to a sequence on plus-strand DNA at a position before the template switch, and the second oligonucleotide (oligonucleotide A) anneals to a sequence on plus-strand DNA at a position after the template switch. This comparison is informative because a virus that is deficient for the template switch will yield a smaller amount of 5′ termini at DR2 as detected by oligonucleotide A. Therefore, by comparing the relative amount of 5′ ends at DR2 detected with oligonucleotide A to the amount of 5′ ends detected with oligonucleotide B, the extent of template-switching can be determined. The extent of template switching by mutant viruses can be compared to that by wild-type DHBV. This type of analysis of viruses 238 and 250 indicated that the template switch was inhibited. Template switching for the 3′r mutant (virus 238) occurred at 17 to 29% of wild-type levels, while template switching for the 5′r mutant (virus 250) was reduced to 5 to 8% of wild-type levels (Fig. 6C and Table 1).

To determine if a template switch could be restored by making both copies of the r region identical but mutant, the plasmid 326 was constructed to express a virus with the sequence AGGCCTTAC present at both the 5′r and 3′r (Fig. 6A). Southern blot analysis of virus 326 indicated aberrant proportions of the three replicative intermediates, suggesting that template switching had not been completely restored (Fig. 6B, lane 4). Primer extension analysis indicated that virus 326 was still inhibited for its template switch (Fig. 6C, lanes 5 and 6; Table 1). In two experiments the level of template switching for virus 326 (5′r and 3′r mutations) was less than that found for virus 238 (5′r mutation), while in a third experiment virus 326 switched templates slightly better than virus 238 (Table 1).

In summary, when three of the nine nucleotides of r were replaced, the assayed template switch was inhibited. The de-

TABLE 1. Extent of template switch for mutant viruses relative to wild-type virus

Expt no.	wt[a]	Mutant virus					
		238	250	326	T101	252	T559
1	100	17	5	29			
2	100	29	8	26			
3	100	25	5	23			
4	100				27	24	99
5	100				45	16	111
6	100				37	13	137

[a] Value for wild-type (wt) virus has been normalized to 100.

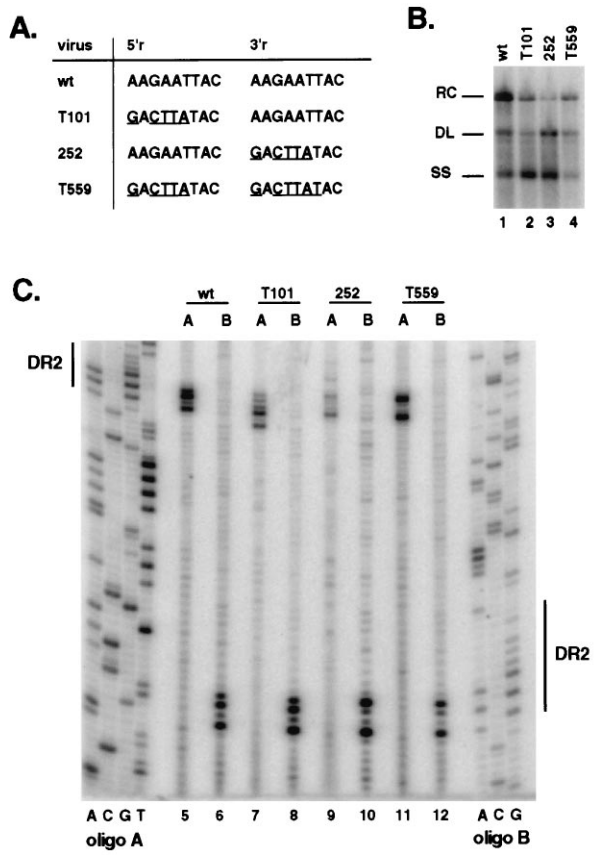

FIG. 8. Analysis of template switching for viruses with a GACTTATAC r region. (A) The 5′r and 3′r regions of the wild-type (wt) and mutant viruses. Sequences are represented as plus-strand polarity with base substitutions underlined. (B) Southern blot analysis of viral DNA from cytoplasmic capsids. RC, DL, and SS represent the mobilities of the relaxed circular, duplex linear, and minus-strand single-stranded molecules, respectively. The hybridization probe was genomic length and minus-strand specific. (C) Primer extension analysis to measure the extent of template switching. A and B, oligonucleotides A and B, respectively. Sequencing ladders generated with oligonucleotides A and B are on the left and right, respectively. The positions of DR2 on the sequencing ladders are indicated. The 5′ termini of plus-strand DNA are found at four consecutive positions at the 3′ end of DR2.

gree of inhibition was greater when the mutation was present at the 3′ end than when it was present at the 5′ end of the minus-strand DNA template. Making both copies of the template identical albeit mutant led to only a modest or no restoration of the template switch, indicating that the presence of a terminal redundancy on the minus-strand DNA template is not the sole requirement for template switching.

To corroborate the finding that the presence of identical mutant copies of r was not sufficient for normal levels of template switching and to corroborate the finding that mutants with mutations in the 3′r were inhibited for template switching to a greater degree than those with mutations in the 5′r, we analyzed a second set of mutant viruses that now had five of the nine nucleotides of r replaced (GACTTATAC). Plasmids expressing the three mutants viruses (5′r, T101; 3′r, 252; 5′r and 3′r, T559) were made (Fig. 8A). Southern blot analysis of the three mutant viruses showed aberrant proportions of the three major replicative intermediates, suggesting inhibition of template switching (Fig. 8B). Primer extension analysis indicated that template switching was inhibited (Fig. 8C and Table 1). Template switching for the 5′r mutant virus (T101) had occurred at 27 to 45% of wild-type levels (Table 1). The 3′r mutant virus (252) was inhibited to a greater extent, switching templates at 13 to 24% of wild-type levels (Table 1). This rank order of the 5′r and 3′r mutants was also found in the previous set of mutants (see above and Fig. 6). When both copies of r were mutant (T559), the level of template switching was similar to that of wild-type virus (Table 1). This restoration in template switching for virus T559 was in contrast to the inability of virus 326 to restore template switching.

DISCUSSION

We have found that for DHBV, (i) disruption of the redundant nature of the minus-strand DNA can partially inhibit template switching during plus-strand DNA synthesis; (ii) the 5′r can be copied completely before the template is switched; (iii) after switching of the templates, extension from a mispaired 3′ terminus can occur readily; and (iv) sequence identity of the 5′r and 3′r is not sufficient for the template switch. The first and second findings verify that the 5′r serves as a template for the synthesis of the r′ region in plus-strand DNA to create a situation in which the nascent plus-strand DNA can base pair with the 3′r (acceptor template) to facilitate the template switch. In addition, our other findings indicate that more than conventional Watson-Crick base pairing dictates the process of template switching.

We interpret the results of our first set of experiments (Fig. 2 to 5) to argue that before the template switch, the 5′r is frequently completely copied into plus-strand DNA. We interpret these same results to also argue that upon transfer, the nascent plus-strand DNA can be mispaired with its template, the 3′r, without abrogating the resumption of DNA synthesis.

In the second set of experiments in this study (Fig. 6 to 8), we found that disruption of the identity between r sequences inhibited the template switch. These findings verify the earlier predictions (9, 17, 25) that the terminal redundancy in minus-strand DNA plays a role in the template switch.

Also, in the second set of experiments (Fig. 6 and 8; Table 1), we found that sequence identity of the r region is necessary but not sufficient for the process of template switching. We offer two explanations for the lack of restoration of template switching for virus 326. In the first explanation, the G+C content of the r region of virus 326 (AGGCCTTAC) is greater than those of virus T559 (GACTTATAC) and wild-type virus (AAGAATTAC). Upon polymerization of the 5′ copy of r into plus-strand DNA, the newly synthesized duplex of virus 326 would be more thermostable than the wild-type duplex. If the amount of energy normally dedicated to the process of template switching cannot disrupt this duplex of virus 326, then template switching would be inhibited. However, because the G+C content of the r sequence for virus T559 is similar to that for the wild type, the degree of template switching for virus T559 would be expected to be closer to that for the wild-type virus, which was experimentally observed (Table 1). A second explanation for the difference in template switching for viruses 326 and T559 would be that the mechanism of template switching has specific sequence requirements within r. These sequence requirements are not met by virus 326 but are met by virus T559.

Also, in the second set of experiments, we found that the degree of inhibition of the template switch was greater when a virus contained only a mutant 3′r than when a virus contained only a mutant 5′r. This trend was observed with the two sets of mutants examined (Fig. 6 and 8; Table 1) and suggests that mutations within the 3′r are more deleterious for template switching than those within the 5′r, although a larger sample

size would be necessary to positively indicate this trend. Nonetheless, we can offer two explanations for this putative trend. In the first explanation, juxtaposition of the donor template (5′r) and the acceptor template (3′r) is necessary for template switching. A protein complex, which includes the P protein linked to the 5′ end of the minus strand and therefore the 5′r, is involved in the mechanism of juxtapositioning. The 5′r is positioned via its covalent linkage to P protein, while the 3′r is positioned via a specific protein-DNA interaction. The viruses with mutations only in the 3′r (viruses 250 and 252) would be deficient for this specific protein-DNA interaction, which would contribute to the observed inhibition of template switching. This explanation would also predict that viruses with identical albeit mutant copies of r would be deficient for template switching. However, this model would not predict the apparently near-normal level of template switching seen for the T559 double mutant. A second explanation for the observed trend would be that it is a coincidence. It would be a coincidence because the sequences of the heteroduplexes after the template switch would be different for a 5′r mutant and a 3′r mutant. If the instability of the heteroduplex contributes to the observed inhibition of template switching, then the degree of inhibition of template switching could be different for a 5′r mutant and a 3′r mutant.

Mechanism of the template switch during plus-strand DNA synthesis. We think it is reasonable to postulate that the 5′ and 3′ termini of the minus strand are specifically juxtaposed as part of the mechanism of template switching. If so, then we expect the existence of *cis*-acting sequences, other than r, to be involved in this juxtapositioning of the minus-strand termini. We also think that a protein(s), either viral or cellular, could be involved in the colocalization of the minus-strand termini.

Our results indicate that the ultimate 5′ nucleotide of minus-strand DNA can serve as a template for plus-strand DNA synthesis, meaning that the P-protein linkage does not sterically occlude the use of the 5′-terminal nucleotide of the minus strand as a template. This observation is more remarkable if the P protein responsible for polymerizing this nucleotide of plus-strand DNA is the same P protein which is linked to the 5′ terminus of the minus-strand DNA template.

The sequences of plus-strand DNA for several mutants indicated that after the template switch, extension from a 3′-terminal mismatch occurred. This capacity for the plus-strand DNA to be extended from a 3′ terminus which is mismatched was found in all three mutants examined in this study, albeit to variable degrees. This ability of viruses 160, G245, and G251 to extend from a terminal mismatch suggests the involvement of forces other than conventional Watson-Crick base pairing participating in the mechanism of the template switch and subsequent elongation. This ability to extend from a terminus which is not base paired with its template has been observed previously during two other steps during DHBV DNA replication: the minus-strand template switch (11) and the translocation of the plus-strand primer (20). That the three template switches and strand transfers during hepadnavirus DNA replication share this feature suggests a shared mechanism.

We suggest that the mechanism of the plus-strand template switch for hepadnaviruses differs from those of retro- and caulimoviruses as a consequence of the different mechanisms of their priming of minus-strand DNA synthesis. Consider the nature of the 5′ termini of the respective minus-strand templates. The 5′ redundancy on the retro- and caulimovirus minus-strand template is contributed by the portion of the tRNA primer that is complementary to the primer binding site. After copying of this portion of the tRNA into plus-strand DNA, an RNase H activity is thought to cleave the tRNA primer from the 5′ terminus of minus-strand DNA (14). According to this model, the cleaved tRNA primer then dissociates from the plus-strand DNA. The single-stranded plus-strand DNA then anneals to the complement of the primer binding site at the 3′ terminus of the minus-strand DNA, resulting in the plus-strand template switch. Key to this mechanism is the 5′ minus-strand terminus being RNA. Because hepadnaviruses employ a protein priming mechanism to initiate minus-strand DNA synthesis (23), no RNA moiety is found at the 5′ terminus of the minus strand. After initiation of plus-strand DNA synthesis at DR2, the minus-strand template, according to our findings, is likely to be copied to the 5′ terminus. This copying would result in a DNA-DNA duplex the length of r. Clearly, a mechanism different than the one employed by retro- and caulimoviruses would be needed to allow the 3′ terminus of the hepadnavirus minus-strand template to base pair with the nascent plus strand to permit resumption of DNA synthesis. Possibilities would include a strand invasion mechanism, a helicase activity to melt the original duplex, or a triple-stranded intermediate. In vitro recapitulation of this template switch should allow us to distinguish these possibilities.

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