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Transfer of the Minus Strand of DNA during Hepadnavirus Replication Is Not Invariable but Prefers a Specific Location

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The current model for replication of duck hepatitis B virus has reverse transcription initiating and copying a UUAC motif within the encapsidation signal, epsilon, near the 5′ end of the RNA template. This results in synthesis of four nucleotides of DNA. This short minus-strand DNA product is then transferred to a complementary position, at DR1, near the 3′ end of the RNA template. Elongation of minus-strand DNA then ensues. We have examined the transfer of minus-strand DNA during replication of duck hepatitis B virus in cell culture. The initial aim of this work was to examine the effect of mutations at DR1 on the transfer process. We found that when mutations were introduced into the UUAC motif overlapping DR1, the 5′ end of minus-DNA no longer mapped to position 2537 but was shifted two or four nucleotides. Mismatches were predicted to exist at the new sites of elongation. Elongation from nucleotide 2537 could be restored in these mutants by making compensatory changes in the UUAC motif within epsilon. This finding led us to examine limitations in the shifting of the site of transfer. When the UUAC motif in epsilon was changed to six different tetranucleotide motifs surrounding position 2537, transfer of minus-strand DNA shifted predictably, albeit inefficiently. Also, when multiple UUAC motifs were introduced near DR1, the UUAC motif at nucleotide 2537 was used preferentially. Overall, our findings confirm the current minus-strand DNA transfer model and demonstrate a marked preference for the site of the transfer.

Hepatitis B viruses are a family of viruses, also known as hepadnaviruses, that can cause diseases of the liver, including cancer (for a review, see reference 8). These enveloped viruses contain a 3.0-kbp circular DNA genome which is replicated via reverse transcription of an RNA intermediate, the pregenomic RNA (for a review, see reference 14). DNA synthesis for this virus occurs within a nucleocapsid (core particle) in the cytoplasm before secretion of the mature virion (21). Prior to DNA synthesis, the pregenomic RNA serves as mRNA for translation of the viral C (core) and P (polymerase) proteins (4, 17). The C protein assembles to form a capsid particle. An early step in DNA replication is coencapsidation of the P protein and pregenomic RNA (1, 2, 9). A stem-loop structure, near the 5′ end of the pregenomic RNA which is conserved in all hepadnaviruses, has been shown to be either sufficient (human hepatitis B virus; 11, 15) or necessary (duck hepatitis B virus [DHBV]; 3, 10, 16) for encapsidation of the pregenomic RNA (Fig. 1A and B). This stem-loop structure is referred to as epsilon. Recent findings have led to a model which mechanistically couples encapsidation of the pregenomic RNA and initiation and synthesis of the first four nucleotides of minus-strand DNA (22–24). In this model, the P protein uses a six-nucleotide bulge within epsilon as a template to synthesize the first four nucleotides of minus-strand DNA (Fig. 1C). The P protein acts as both primer and reverse transcriptase for this reaction, resulting in covalent linkage of the first four nucleotides of minus-strand DNA to the P protein (23). This protein-linked tetramer is then transferred to base pair with a UUAC motif overlapping the DR1 element near the 3′ end of the pregenomic RNA (Fig. 1D). Minus-strand DNA synthesis then reinitiates and proceeds to copy completely the RNA template, resulting in a full-length minus-strand DNA molecule. After a series of equally complex but distinctly different steps, plus-strand DNA synthesis is achieved, resulting in a relaxed circular DNA genome (for a review, see reference 14).

The mechanism for transfer of the first four nucleotides of minus-strand DNA is poorly understood. Clearly, complementarity between the minus-strand DNA and the UUAC motif overlapping DR1 on the RNA plays a role in the process, as demonstrated by mutagenesis studies (7, 18, 22, 24). But complementarity is not sufficient to explain why minus-strand DNA transfers to the UUAC motif overlapping DR1 and not to the other 13 UUAC motifs on the pregenome. We propose that additional forces or interactions are part of the mechanism and, therefore, specificity of transfer of minus-strand DNA. Tertiary structure of the RNA template and P-protein interactions with the RNA template could be involved in the mechanism of minus-strand DNA transfer. In this report, we show results which are consistent with these two notions. We show that complementarity although preferred, is not an absolute requirement for successful transfer. We also show that the site of transfer can be shifted by changing the sequence of minus-strand DNA but that transfer to the wild-type site is preferred, even over transfer to another site only three nucleotides away.

MATERIALS AND METHODS

Construction of mutant DHBV genomes. The European strain DHBV3 of DHBV was used in all experiments (19). Mutations were introduced into a plasmid clone containing the 1,364-nucleotide *Bam*HI-*Eco*RI fragment (nucleotides 1658 to 3021) by using the oligonucleotide-directed mutagenesis procedure of Kunkel et al. (13). Mutations were identified by DNA sequencing. The absence of fortuitous mutations was ensured by sequencing the appropriate region of each mutant plasmid. These mutant plasmids, which contain the 3′ half

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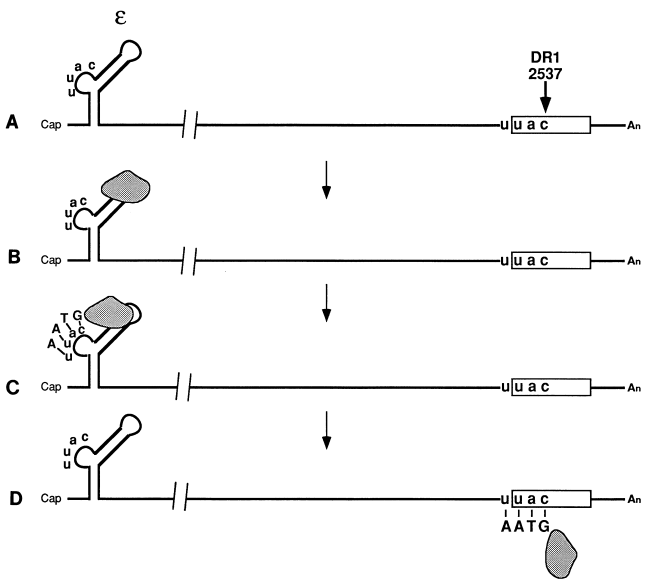

FIG. 1. Model for the synthesis and transfer of the first four nucleotides of DHBV minus-strand DNA. (A) The solid line represents pregenomic RNA which is capped and polyadenylated. The stem-loop structure (designated ε) near the 5′ terminus is part of the encapsidation signal. The bulge within the stem contains the sequence uuac. Near the 3′ terminus of the pregenomic RNA is DR1, which is represented by the rectangle. Overlapping DR1 is the tetranucleotide sequence uuac. The cytosine residue of the uuac motif is at nucleotide coordinate 2537. The pregenomic RNA is approximately 3,300 nucleotides long. The copy of epsilon found near the 3′ end of the pregenomic RNA is not shown because removal of it does not affect viral DNA synthesis (data not shown). (B) The P protein (shown as a shaded amoeboid object) binds to the stem-loop structure as a prerequisite to encapsidation and initiation of synthesis of minus-strand DNA. (C) The P protein, acting as both the primer and reverse transcriptase, synthesizes the first four nucleotides of minus-strand DNA by using the uuac motif within the bulge of the stem-loop as the template. (D) Transfer of the P protein covalently linked to the first four nucleotides of minus-strand DNA to the uuac motif overlapping DR1.

of the genome (0.5-mer), were converted to overlength forms (1.5-mer), which are competent to support viral replication upon transfection into LMH cells. This was done by cloning a monomer of the genome into the 0.5-mer. For mutations within the encapsidation signal, a 3,021-nucleotide *Nsi*I-*Nsi*I fragment was cloned into the *Nsi*I site (nucleotide 2845) of the 0.5-mer. This results in the presence of the mutation only within the 5′ redundancy of the pregenomic RNA. For mutations at and surrounding the 3′ copy of DR1, a 3,021-nucleotide *Nco*I-*Nco*I fragment was cloned into the *Nco*I site (nucleotide 2351) of the 0.5-mer. This results in the presence of the mutation only within the 3′ redundancy of the pregenomic RNA. For the ε1, ε2, ε3, ε5, and ε6 mutants (see Fig. 4), which have mutations within the 5′ stem-loop of the pregenomic RNA, a partial deletion of the 3′ stem-loop was introduced. This step was done to prevent a plasmid containing a wild-type 3′ stem-loop from recombining a plasmid containing a mutant 5′ stem to give rise to a DNA molecule with a wild-type 5′ stem-loop. This partial deletion of the 3′ stem-loop by itself does not interfere with viral DNA replication (data not shown).

Cell culture and DNA transfections. The chicken hepatoma cell line LMH (6, 12) was used in all cell culture experiments and grown in a 1:1 mixture of Dulbecco's modified Eagle medium and Nutrient F-12 (GibcoBRL catalog no. 12500-070) supplemented with 5% fetal bovine serum. Transfections were performed by the calcium phosphate protocol of Chen and Okayama (5). Typically, 7.5 to 10 μg of plasmid DNA was transfected into LMH cells which were at approximately 50% confluency on a 100-mm-diameter plate.

Nucleic acid manipulations. Isolation of viral DNA from cytoplasmic core particles was performed 3 days posttransfection as described by Staprans et al. (20). Primer extension analysis of minus-strand DNA was performed on 1/30 to 1/5 of the viral DNA isolated from a single transfection. An end-labeled oligonucleotide derived from nucleotides 2425 to 2447 of DHBV was used. The primer extension reaction mixture contained 20 mM Tris-HCl (pH 8.8); 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 5 mM $MgSO_4$; 200 μM each dATP, dCTP, dGTP, and TTP; 0.1% Triton X-100; 1 U of Vent exo$^-$ DNA polymerase; and 0.6 pmol of the end-labeled oligonucleotide in a final volume of 10 μl. The reaction mixture was placed in a thermal cycler and incubated at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 20 cycles and then incubated at 72°C for 5 min. Samples were electrophoresed on a 6% polyacrylamide sequencing gel, dried, and autoradiographed (20). Analysis of the autoradiographic image was performed on a Molecular Dynamics Phosphorimager 445 SI.

RESULTS

Experimental design. In this work, we studied the transfer of the first four nucleotides of minus-strand DNA, which is linked to the P protein, from the 5′ end to the 3′ end of the pregenomic RNA during hepadnaviral reverse transcription. We studied this reaction during replication of DHBV. This was done by transfecting chicken hepatoma cell line LMH with cloned plasmid DNA of DHBV which was competent to support pregenomic RNA transcription. After transfection, pregenomic RNA was transcribed from the plasmid DNA and the reverse transcription pathway commenced. In this study, primer extension analysis was performed on minus-strand viral DNA replicated in cell culture. From the position of the 5′ end of these minus-strand molecules, we deduced the position of the minus-strand transfer. We refer here to the position of transfer as an acceptor site. For the wild-type virus, the nascent minus strand appears to base pair with a UUAC motif at positions 2534 through 2537 (these coordinates are from the published DHBV sequence [19]) after the transfer. Throughout this report, the phrase "transfer to site 2537" refers to the first residue of minus-strand DNA that is opposite the C residue at position 2537 on the RNA template after the transfer process.

Mutations within the UUAC motif at DR1 change the site of transfer. Complementarity between the first four nucleotides of minus-strand DNA and the acceptor site on the pregenomic RNA exists. We reasoned that if base pairing is important for efficient transfer, we should disrupt the transfer by introducing mutations within the UUAC motif overlapping DR1. To this end, we examined the minus-strand transfer reactions for four different mutants (Fig. 2A). In three of the mutants (Fig. 2A, clones M1, M5, and M7), there were nucleotide insertions within the UUAC motif, while in the fourth (Fig. 2A, clone M3), five nucleotides were substituted, one of which changes the UUAC motif to AUAC. Replication of these mutants was assayed by transfecting LMH cells with DNA clones of each mutant and then isolating viral DNA from cytoplasmic subviral core particles 3 days posttransfection. Southern blot analysis of each of the four mutants revealed the presence of viral DNA, at a level not less than 0.5 times that of the wild type (data not shown). This measurement meant that these mutations had not dramatically reduced the process of minus-strand transfer. To determine the site of the minus-strand transfer, a primer extension analysis of these mutant viral DNAs was performed. The results of the primer extension analysis (Fig. 2B) indicated that each of the mutants transferred its minus-strand DNA to a new site, within two to four nucleotides of the original site 2537. The position of the 5′ end of minus-strand DNA of the M1 mutant (Fig. 2B, lane 12) indicates that the transferring minus-strand DNA moiety (5′-dGTAA-3′) paired with the 5′-rUUCC-3′ sequence on the RNA template. This assignment of the transfer site argues that a dT · rC mispairing (corresponding to the second position of DNA) was tolerated. Analysis of the other three mutants (M3, M5, and M7) argues for additional unusual pairings between the transferring minus DNA moiety and the RNA template (Fig. 2A shows the assignments of the transfer positions). An additional example is clone M7 (Fig. 2B, lane 6). The new acceptor site is 5′-rUUGG-3′, which argues for a dGT · rGG pairing (corresponding to the first two positions of DNA). Lastly, each mutant contained secondary sites of transfer, 3′ of DR1. The most prominent of these was the UUAC motif within the bulge of the stem-loop structure 3′

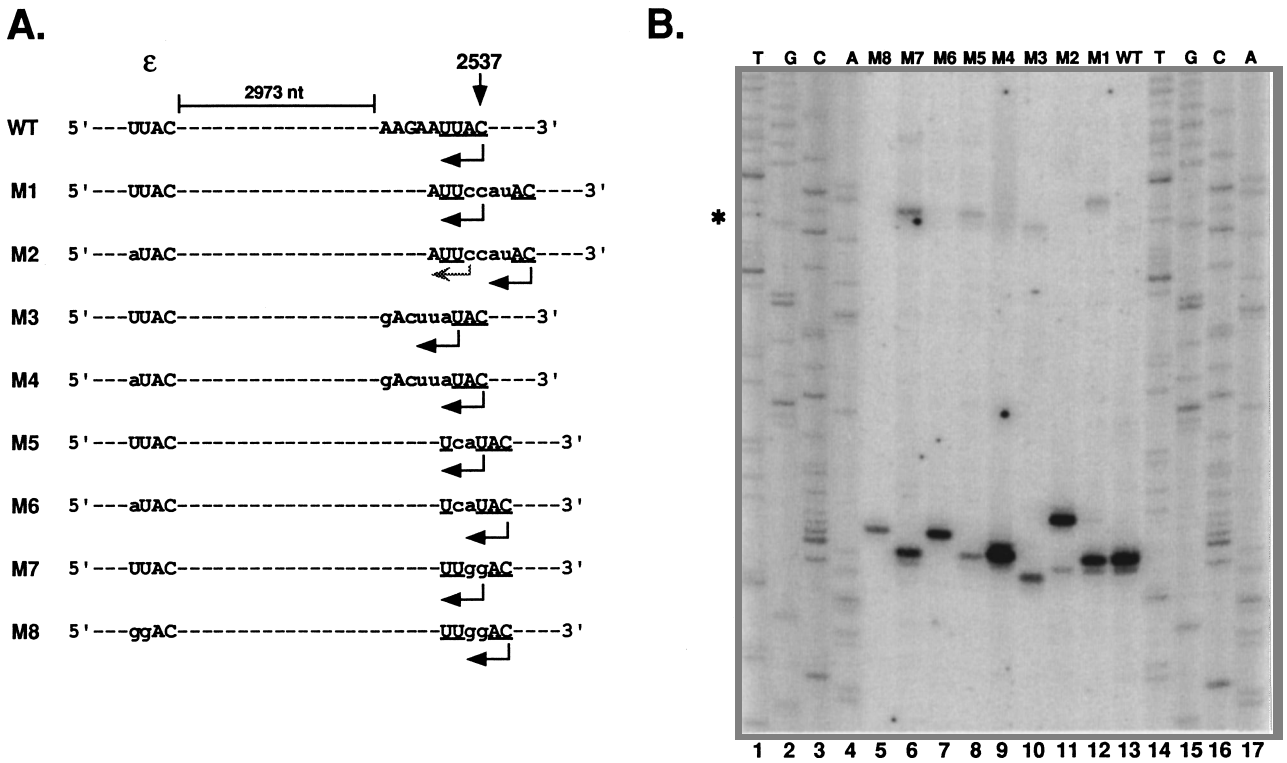

FIG. 2. Mutations within the acceptor site shift the site of transfer. Subsequent mutations in epsilon shift the transfer back to the original location. (A) Representation of pregenomic RNA sequences of the wild type (WT) and mutants M1 through M8. The tetranucleotide near the 5′ end of the RNA represents the bulge sequence within epsilon that serves as the template for the four-nucleotide minus-strand primer. The wild-type sequence, UUAC, is in uppercase letters. Mutant nucleotides are lowercase letters. The sequence near the 3′ end of the pregenomic RNA represents the nucleotides surrounding the acceptor site, and UUAC is underlined. Mutations within the acceptor site are in lowercase letters. Insertions within the UUAC motif are not underlined. Positions of 5′ ends of minus-strand DNA, as determined by primer extension, are indicated by black arrows. The M2 mutant has a second, less abundant 5′ end that is indicated by a grey arrow. (B) Primer extension analysis of minus-strand DNAs in panel A. Lanes: 1 to 4 and 14 to 17, sequencing ladder of wild-type DHBV DNA; 5, M8; 6, M7; 7, M6; 8, M5; 9, M4; 10, M3; 11, M2; 12, M1; 13, wild type. The asterisk indicates the position of a secondary site of transfer.

of DR1 (Fig. 2B, asterisk). This acceptor site, which is not used by the wild-type virus, was previously noted by Condreay et al. (7) when DR1, and therefore the UAC of the UUAC motif, was deleted.

Compensatory changes in the encapsidation element shift the site of transfer back to the wild-type location. In each of the four mutants, a new tetranucleotide motif existed at what was the original site 2537. We reasoned that if the UUAC motif in the encapsidation signal were changed to match the new tetranucleotide motif at site 2537 in each of the mutants, then transfer should shift to this new site. In the mutant with the four-nucleotide insertion (M2), the new transfer was predicted to shift four nucleotides in the 3′ direction. In the other three mutants (M4, M6, and M8), the transfer was predicted to shift two nucleotides in the 3′ direction. The UUAC motif within the encapsidation signal was changed to either AUAC or GGAC and then recombined with the appropriate mutations within the UUAC motif at the 3′ copy of DR1 (Fig. 2A). Viral DNA was isolated from cytoplasmic core particles from transfected cells, and then a primer extension analysis of minus-strand DNA was done. In each of the double mutants, the length of minus-strand DNA was longer as predicted (Fig. 2B, compare lanes 5 and 6, 7 and 8, 9 and 10, and 11 and 12). Also, the use of the secondary or cryptic sites of transfer, noted in the single mutants, was either reduced or eliminated (Fig. 2B, asterisk).

Can the minus strand transfer to sites distal to nucleotide 2537? The preceding experiments indicated that transfer of the minus strand could be shifted, either two or four nucleotides, as a function of the sequence of the minus strand of DNA. This flexibility in the site of transfer is a reflection of the mechanism of transfer. Determining limitations to this flexibility in the site of transfer will lead to a better understanding of the acceptor site, which should ultimately help us to understand the mechanism of transfer. We used the following two strategies to determine how far from site 2537 efficient transfer can occur: (i) change the tetranucleotide motif in epsilon, and therefore the first four nucleotides of minus-strand DNA, to different tetranucleotides derived from the sequence surrounding nucleotide 2537 at DR1 and determine whether transfer to this new site can occur and (ii) introduce other and/or additional UUAC motifs at new sites surrounding the UUAC motif at nucleotide 2537 and determine whether the minus strand of DNA can transfer to these new UUAC motifs.

Changing the sequence of the first four nucleotides of minus-strand DNA. DR2 is a 12-nucleotide sequence which is identical to DR1 and located 58 nucleotides 5′ of DR1 on the pregenomic RNA (Fig. 3A). We investigated whether minus-strand DNA can transfer to DR2. Normally, this transfer does not occur (Fig. 3B, lane 2), presumably because the potential acceptor site at DR2 has the sequence AUAC instead of UUAC. To determine whether, with complete complementarity, a minus strand could transfer to DR2, we changed the UUAC motif in the encapsidation signal to AUAC and looked for transfer of the minus strand of DNA to DR2. Examination of viral DNA from the AUAC mutant by Southern blot anal-

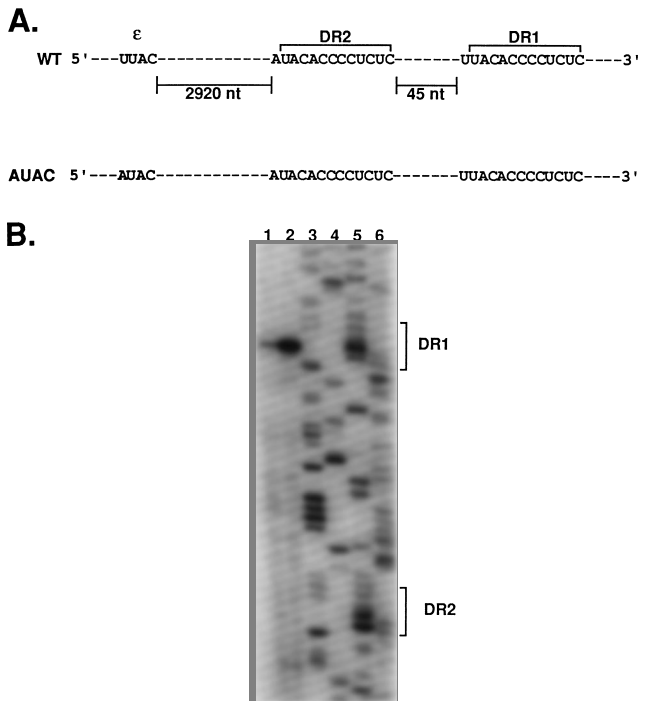

FIG. 3. DR2 does not serve as an acceptor site. (A) Schematic representation of wild-type (WT) and AUAC mutant pregenomic RNAs. ε indicates the UUAC motif within epsilon. DR2 is located 2,922 nucleotides 3′ of ε. DR1 is located 48 nucleotides 3′ of DR2. Overlapping DR2 is an AUAC motif in the position analogous to that of the UUAC motif overlapping DR1. AUAC indicates the mutant which has an AUAC motif in ε, thus changing the sequence of the fourth nucleotide of minus-strand DNA. (B) Primer extension analysis of minus-strand DNA from the wild type and the AUAC mutant. Lanes: 1, AUAC; 2, wild type; 3 to 6, T, G, C, and A of a DNA sequence ladder of a cloned wild-type DHBV plasmid. Sequences of DR2 and DR1 are indicated by brackets on the right.

ysis indicated a pattern of replicative intermediates qualitatively similar to those of wild-type DNA but reduced in abundance (data not shown). Primer extension analysis of minus-strand DNA indicated that (i) transfer to DR1 predominated (Fig. 3, lane 1) and (ii) transfer to DR2 did not occur appreciably (Fig. 3, lane 1). From this finding, we conclude that DR2 cannot efficiently serve as an acceptor site for the minus-strand transfer reaction. Interestingly, transfer and elongation of minus-strand DNA from the UUAC motif at position 2537 was detected despite a predicted mispairing at the 3′ terminus of the minus-strand DNA primer.

We next investigated whether other tetranucleotide sequences, closer to nucleotide 2537, could serve as acceptor sites. The sequence of the UUAC motif in the encapsidation signal was changed to six different tetranucleotides derived from the sequence surrounding nucleotide 2537 (Fig. 4). Southern blot analysis of viral DNA indicated levels of replicative intermediates lower than the wild-type level (data not shown). This decrease was not due to a defect in encapsidation, since all of these mutants encapsidated their pregenomic RNAs to extents similar to that of the wild-type virus (data not shown). Primer extension analysis of the minus-strand DNAs of these six mutants was then performed. In general, primer extension analysis of minus-strand DNA of each mutant indicated that minus-strand DNA had transferred to the new tetranucleotide in a predictable fashion, but several additional observations are noteworthy. The ε1 mutant (GAAC) did not transfer to its corresponding tetranucleotide, 11 nucleotides 5′ of position 2537, but instead transferred to a GAAU motif 3 nucleotides 5′ of position 2537 (Fig. 4C, lane 11). Secondly, in clones ε4, ε5, and ε6 (Fig. 4C, lanes 4 to 6), not only were 5′ ends of minus-strand DNA mapped to the predicted positions but additional 5′ ends were mapped to nearby positions. These additional 5′ ends could be understood by invoking a single mismatch between the DNA and RNA after transfer to the nearby site. For both mutants ε2 and ε3, not only was a 5′ end at the predicted position but 5′ ends were also observed at several consecutive positions. These additional 5′ ends cannot be understood by invoking one or two mismatches between the DNA and RNA after the transfer. We are at a loss to explain their origin. In summary, changing the sequence of the first four nucleotides of minus-strand DNA resulted in transfer of this tetranucleotide to a new site in a predictable manner.

Transfer to the UUAC motif at position 2537 is preferred over transfer to other, adjacent UUAC motifs. An alternative approach to delimiting the sequences on the pregenomic RNA which can serve as acceptor sites is to introduce multiple UUAC motifs surrounding position 2537. This approach has

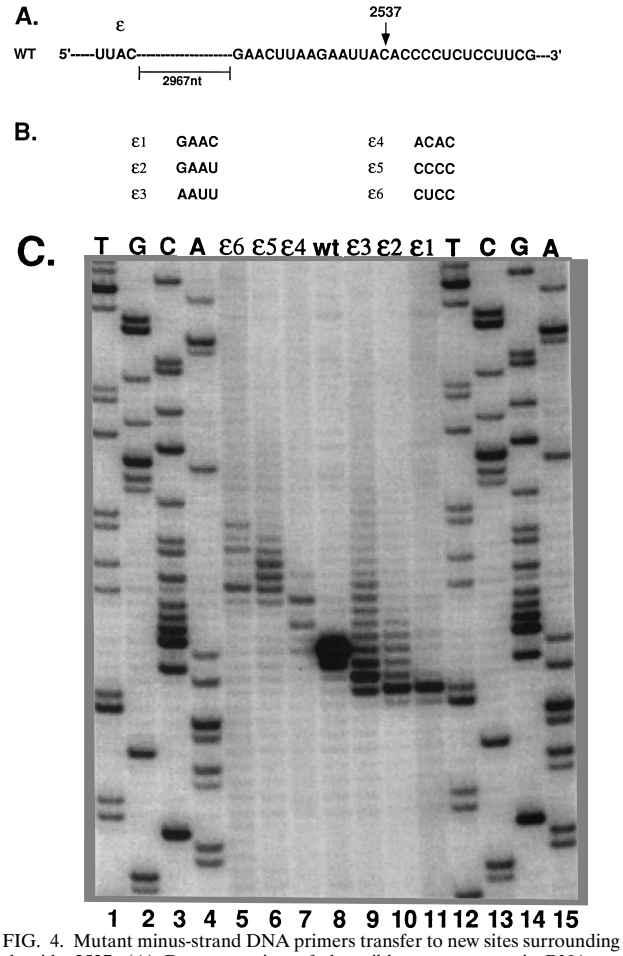

FIG. 4. Mutant minus-strand DNA primers transfer to new sites surrounding nucleotide 2537. (A) Representation of the wild-type pregenomic RNA sequence. UUAC near the 5′ end of the RNA represents the bulge sequence within epsilon that serves as a template for the four-nucleotide minus-strand primer. The sequence near the 3′ end of the pregenomic RNA represents the 29 nucleotides surrounding the acceptor site. A sequence of 2,967 nucleotides separates the 5′ and 3′ sequences. The vertical arrow indicates the C residue at position 2537 within the UUAC acceptor site which overlaps DR1. (B) Sequences of six different mutations ε1 through ε6) within epsilon. (C) Primer extension analysis of minus-strand DNAs of the six epsilon mutants. Lanes: 1 to 4 and 12 to 15, sequencing ladders of wild-type DNA; 5, ε6; 6, ε5; 7, ε4; 8, wild type; 9, ε3; 10, ε2; 11, ε1.

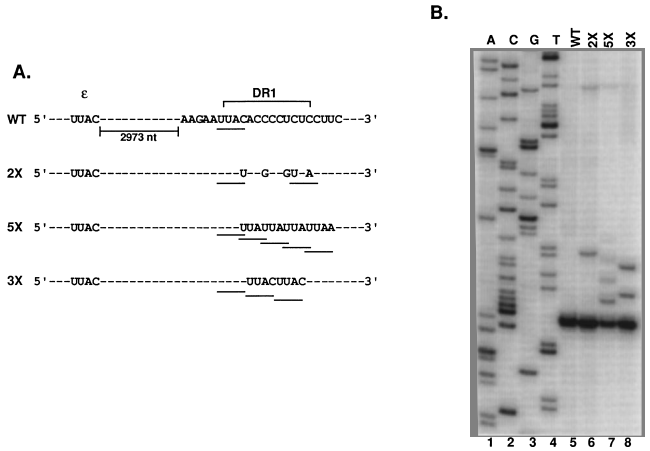

FIG. 5. Preference for the acceptor site at nucleotide 2537. (A) Schematic representation of wild-type (WT) and multiple acceptor site mutant pregenomic RNAs. The UUAC motif near the 5′ end of the wild-type pregenomic RNA represents the bulge sequence within epsilon and therefore the template for the synthesis of the first four nucleotides of minus-strand DNA. The sequence near the 3′ end of the RNA represents the wild-type acceptor site with the UUAC motif underlined. For each of the mutants (2×, 3×, and 5×), base changes were introduced within the sequence surrounding the acceptor site. Changes relative to the wild-type sequence are indicated, and identities are represented by dashes. Predicted acceptor sites in the mutants are underlined. (B) Primer extension of minus-strand DNA of the multiple acceptor site mutants. Lanes: 1 to 4, sequencing ladder of wild-type DHBV DNA with the same oligonucleotide as used for primer extension analysis; 5 to 8, wild-type, 2×, 3×, and 5× viral DNAs, respectively.

the benefit of eliminating any influence mutations within epsilon might have on the transfer process. We can then determine which, if any, of the introduced sites are used and whether there is a preference. To do this experiment, we constructed three different mutants which had, in addition to the site at nucleotide 2537, potential alternative sites of transfer nearby (Fig. 5A). The first mutant tested (Fig. 5A, 2×) had an additional UUAC introduced at position 2547. Also in this mutant, the C at nucleotide 2537 was changed to a U, creating a UUAU motif at this position. Southern blot analysis of viral DNA indicated wild-type levels of minus-strand DNA (data not shown). Primer extension analysis of minus-strand DNA mapped the 5′ end to both locations but with a greater than 10-fold preference for the mispaired site at nucleotide 2537. To verify and extend this observation, two additional mutants were studied. The wild-type sequence UUACACCCCTCTCC TT was changed to UUAUUAUUAUUAUUAA. This mutant, called 5× (Fig. 4A), contained five overlapping motifs that could serve as acceptor sites. Each motif would have one mismatch with the first nucleotide of the minus-strand DNA. Our previous analysis of the 2× mutant indicated that such a mismatch has no effect on the efficiency of the transfer. Mutant 3× (Fig. 5A) has three consecutive UUAC motifs. For both mutants 5× and 3×, the array of tetranucleotide motifs begins at the wild-type position and progresses toward the 3′ end. Southern blot analysis of viral DNAs of mutants 5× and 3× revealed wild-type levels of minus-strand DNA (data not shown). Primer extension analysis of both mutants indicated transfer to all sites but with a preference for the site at nucleotide 2537 and with decreasing use as the distance from nucleotide 2537 increased (Fig. 5B). These data indicate that the minus-strand DNA strongly prefers to transfer to the site at nucleotide 2537 over another site only three nucleotides away.

DISCUSSION

We have examined the transfer of minus-strand DNA during replication of DHBV in cell culture. Our results indicate that complementarity between the transferring minus strand of DNA and the RNA template, although important, is not an absolute requirement. The sequence of DR1 and five of the six nucleotides immediately upstream of DR1 can be changed without affecting the efficiency of transfer to nucleotide 2537. In addition, the position of transfer is not invariant but can occur over a small range of nucleotides surrounding position 2537, although transfer to position 2537 is strongly preferred.

The role of complementarity. In our first set of experiments, we showed that when the UUAC motif overlapping DR1 (the acceptor site) is disrupted by a mutation, transfer of minus-strand DNA occurs, but to a new position which is within several nucleotides of the original position. The new site of transfer is predicted to have mispairings between the transferred four nucleotides of minus-strand DNA and the RNA template. The ability to apparently tolerate mismatches is a peculiar feature of this process and one we did not expect on the basis of the thermostability of a mispaired four-nucleotide RNA-DNA duplex. Therefore, we propose that forces other than Watson-Crick base pairing are part of the mechanism of transfer and placement of the minus-strand DNA primer. The AUAC mutant from Fig. 3 illustrates this point. The ability of this mutant virus to transfer and elongate minus-strand DNA from the UUAC sequence at position 2537 suggests that even a mismatch in the 3′-terminal position of the minus-strand primer is tolerated. We interpret this to mean that conventional Watson-Crick base pairing is not the sole factor in the transfer and subsequent extension of the minus-strand DNA primer.

In our second set of experiments, the DR1 mutants (Fig. 2A, M1, M3, M5, and M7) were manipulated to change their epsilon sequences, resulting in minus-strand DNA primers that are perfectly complementary to the tetranucleotide motif derived from the original site 2537. This modification resulted in transfer of the minus-strand DNA to site 2537 as predicted. This shift was either two or four nucleotides in the 3′ direction. We interpret these results to indicate that the minus strand of DNA prefers to transfer to a site of perfect complementarity even if it means moving to a new position several nucleotides away. This movement indicates that the acceptor site is not invariable but tolerates some shifting in its position, at least up to a range of four nucleotides.

Is there a *cis*-acting element at DR1 for transfer? The UUAC motif overlapping DR1 could potentially play two roles during the transfer process. In addition to providing complementarity to the transferring minus-strand molecule, the UUAC motif could be part of a *cis*-acting element that is involved in the transfer process. We think that this explanation does not apply, because the M2, M4, M6, and M8 clones have mutations within the UUAC motif and they support wild-type levels of minus-strand DNA transfer (Fig. 2B). Mutant M4 is additionally informative because four of the five nucleotides immediately 5′ of the UUAC motif are not wild type yet minus-strand DNA transfers efficiently. This finding suggests that these five nucleotides are not part of a *cis*-acting element for the transfer process. Mutants 2×, 3×, and 5× (Fig. 5) synthesize minus-strand DNA at a level similar to that of the wild type. The sequence of DR1 in these mutants has been radically changed, yet the efficiency of the minus-strand transfer process has not decreased. These data support models in which the DR1 sequence, per se, is not involved in the transfer process. We propose that *cis*-acting determinants for transfer lie outside of the region immediately surrounding the acceptor site.

Variability in the site of transfer. Our experiments demonstrate that the transfer of minus-strand DNA can be shifted as a function of the sequence of the minus strand of DNA. This flexibility in the site of transfer is not unlimited. DR2 cannot serve as an acceptor site even when the minus strand of DNA transferring is completely complementary to DR2. Our experiments indicate that there is a 5′ limit to the site of transfer between 3 and 11 nucleotides upstream of nucleotide 2537, as demonstrated by the GAAC bulge mutant (Fig. 4, mutant ε1). Efficient transfer in the 3′ direction is similarly limited, as most clearly demonstrated by mutant 2× (Fig. 5). Even though there is flexibility in the site of transfer, there is a marked preference for transfer to site 2537. Preferred transfer to the wild-type position, even over a site only three nucleotides away, as in mutant 5×, clearly demonstrates this bias. This limited flexibility in the site of transfer could be a reflection of the region of RNA that is exposed to the transferring DNA molecule.

The transferring minus strand is four nucleotides long. Wang and Seeger (23, 24), utilizing an in vitro system to study the synthesis of the first four nucleotides of minus-strand DNA, argued that the DNA molecule copied from the bulge sequence within epsilon is either three or four nucleotides long. We propose that the transferring molecule is four nucleotides long. Consider mutants M3 and M4 in Fig. 2. If a trinucleotide (5′-dGTA-3′) is competent to be efficiently transferred, then mutants M3 would have an opportunity to transfer to a perfect complement, resulting in a minus strand two nucleotides longer than that seen. In addition, mutant M4, which only changes the sequence of the template for the fourth nucleotide of minus-strand DNA, has a resultant shift in the site of transfer (relative to M3), arguing that the transferring minus strand has this fourth nucleotide.

What is the mechanism of the minus-strand DNA transfer? We cannot offer a detailed model of the mechanism of transfer but do emphasize two points and suggest two interpretations. First, complete complementarity between the transferring minus-strand DNA molecule and RNA template is not required, suggesting that forces other than Watson-Crick base pairing are involved in the transfer process. Since the P protein is part of the transferring minus-strand molecule, the P protein could be actively involved in the transfer process, thus providing a means to stabilize a mispaired DNA-RNA duplex. Second, the minus strand strongly prefers transfer to site 2537. The tertiary structure of the pregenomic RNA could be such that epsilon and site 2537 are spatially juxtaposed. This could explain the preference for transfer to site 2537. How epsilon and site 2537 could be juxtaposed is unclear.

Many of our findings are similar to findings made by Seeger and Maragos (18) when they studied the sequence requirements at DR1 for the synthesis of minus-strand DNA in woodchuck hepatitis virus. For woodchuck hepatitis virus, the trinucleotide UUC at DR1 is proposed to be the site of transfer. When Seeger and Maragos (18) introduced base changes in the UUC motif at DR1, the 5′ end of the minus-strand DNA mapped to a new location several nucleotides away, depending on the mutant. This finding is very similar to our findings obtained with mutants M1, M3, M5, and M7. Seeger and Maragos (18) also found that when an additional UUC motif was introduced five nucleotide 3′ of the wild-type UUC motif, transfer occurred at the introduced position but there was a strong preference for transfer to the wild-type site, a finding we have noted with DHBV. These similar findings on DHBV and woodchuck hepatitis virus are consistent not only with the idea that similar minus-strand transfer reactions operate in all hepadnaviruses but also with the idea that the requirements of the reactions are similar.

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