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Sequence Identity of the Direct Repeats, DR1 and DR2, Contributes to the Discrimination between Primer Translocation and *in Situ* Priming During Replication of the Duck Hepatitis B Virus

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There are two mutually exclusive pathways for plus-strand DNA synthesis in hepadnavirus reverse transcription. The predominant pathway gives rise to relaxed circular DNA, while the other pathway yields duplex linear DNA. At the completion of minus-strand DNA synthesis, the final RNase H cleavage generates the plus-strand primer at direct repeat 1 (DR1). A small fraction of viruses make duplex linear DNA after initiating plus-strand DNA synthesis from this site, a process called *in situ* priming. To make relaxed circular DNA, a template switch is necessary for the RNA primer generated at DR1 to initiate plus-strand DNA synthesis from the direct repeat 2 (DR2) located near the opposite end of the minus-strand DNA, a process called primer translocation. We are interested in understanding the mechanism that discriminates between these two processes. Previously, we showed that a small DNA hairpin forms at DR1 in the avihepadnaviruses and acts as an inhibitor of *in situ* priming. Here, using genetic approaches, we show that sequence identity between DR1 and DR2 is necessary, but not sufficient for primer translocation in the duck hepatitis B virus. The discrimination between *in situ* priming and primer translocation depends upon suppression of *in situ* priming, a process that is dependent upon both sequence identity between DR1 and DR2, and the presence of the hairpin at DR1. Finally, our analysis indicates the entire RNA primer can contribute to primer translocation and is translocated to DR2 before initiation of plus-strand DNA synthesis from that site.

Keywords: hepadnavirus; template switch; primer translocation; *in situ* priming; reverse transcription

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Introduction

Hepadnaviruses and retroviruses replicate their respective genomes by reverse transcription of an RNA intermediate. The RNA templates are first converted into single-stranded DNA species (minus-strand DNA), which are subsequently used as templates for plus-strand DNA synthesis. Both classes of viruses use oligoribonucleotides as primers for plus-strand DNA synthesis, which initiate predominantly at internal locations on the single-stranded DNA. The RNA primers for retroviruses are generated as a result of short polypurine tracts being relatively resistant to degradation by RNase H,[1] whereas the primer for hepadnaviruses is generated *via* an RNase H cleavage that is a sequence-independent measurement from the 5′ end of the RNA template.[2] This 18 nt RNA primer is annealed to the 3′ end of the minus-strand DNA with the 3′ end of the primer located within the 12 nt direct repeat, DR1 (Figure 1(a)). Retroviruses initiate plus-strand DNA synthesis using the RNA primers at the sites where they were generated. In contrast, only a small fraction of the RNA primers (~1–5%) are used at the site where they are

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Abbreviations used: DL, duplex linear; RC, relaxed circular; DHBV, duck hepatitis B virus.

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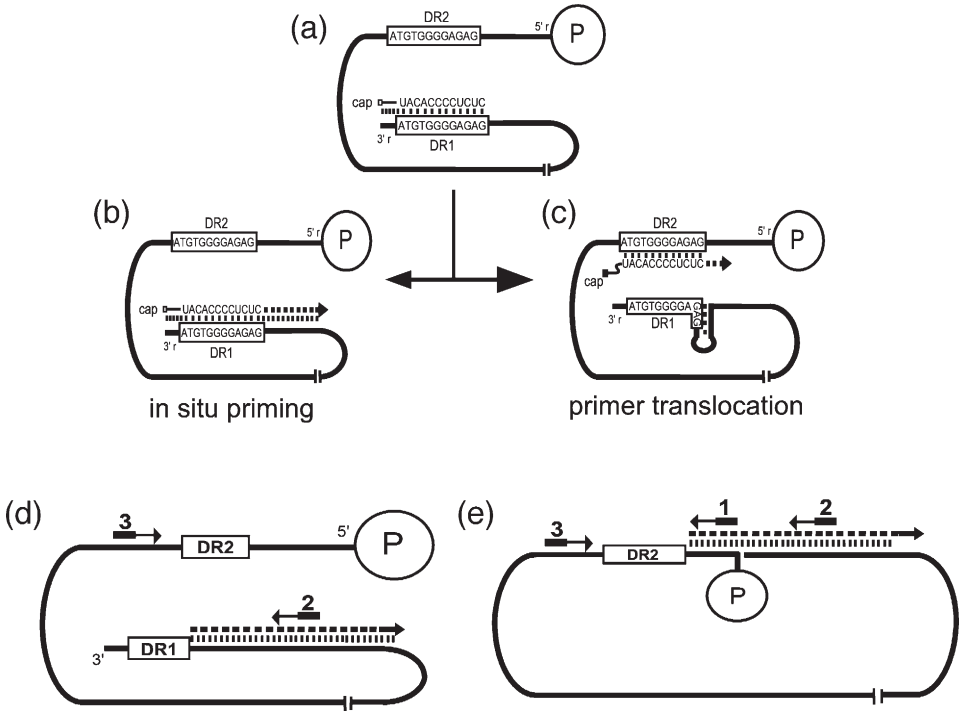

Figure 1. Two mutually exclusive pathways for initiation of plus-strand DNA synthesis. Minus-strand DNA (thick black line) is generated by reverse transcription of an RNA intermediate (pregenomic RNA) by the covalently attached P protein (circle). (a) At the completion of minus-strand DNA synthesis, the final RNase H cleavage generates an 18 nt oligoribonucleotide that serves as the primer for plus-strand DNA synthesis from one of the two sites. The 3′ end of the RNA primer is coincident with the 5′ end of DR1 in the minus-strand DNA. (b) A small fraction of viruses produce a duplex linear form of the genome by extending the primer from the site it was generated, a process called *in situ* priming. (c) The predominant pathway requires a template switch, called primer translocation, as some portion of the RNA primer is used to initiate plus-strand DNA synthesis from DR2 located near the opposite end of the minus-strand DNA. Plus-strand synthesis from DR2 results in a relaxed circular form of the genome upon completion of a second plus-strand template switch, termed circularization, which is facilitated, in part, by the small terminal redundancy in the minus-strand DNA, indicated by 5′r and 3′r. (d) and (e) The placement of the three oligonucleotides used in the primer extension analyses. (d) An *in situ-primed* molecule; (e) a molecule primed from DR2 and circularized. See Materials and Methods for description of the use of the three primers.

generated by hepadnaviruses, a process called *in situ* priming (Figure 1(b)).[3,4] Instead, the majority of plus-strand DNA synthesis initiates from the 12 nt direct repeat, DR2, located near the other end of the minus-strand DNA as a result of a process called primer translocation (Figure 1(c)).[5] The site of plus-strand priming has different consequences for hepadnaviruses. *In situ* priming results in a duplex linear (DL) DNA genome, whereas priming from DR2 can lead to the synthesis of a relaxed circular (RC) DNA genome following completion of a second template switch termed circularization. It is not clear why hepadnaviruses have this added complexity for priming plus-strand DNA synthesis, but the mechanism of primer translocation is a potential therapeutic target. As viral replication is necessary for maintenance of the hepadnavirus (including the human pathogen, hepatitis B virus) chronic carrier state, understanding replication and uncovering therapeutic targets is critical for limiting disease in carriers.

Much of our current knowledge of primer translocation has been obtained using an avian member of the hepadnavirus family, duck hepatitis B virus (DHBV), which has proven to be a useful model for understanding hepadnavirus biology, in particular replication.[4,6,7] Although a number of *cis*-acting sequences have been shown to contribute to primer translocation during DHBV replication, the mechanism is not understood incompletely. A secondary structure has been proposed that may facilitate juxtaposition of the donor and acceptor sites for the plus-strand template switches, at least to the same general vicinity.[6,8] These base-pairing interactions in the minus-strand DNA were shown to be critical for efficient primer translocation. In addition, a small DNA hairpin was shown to form near the 3′ end of the minus-strand DNA overlapping the 5′ end of DR1 (Figure 1(c)).[4] Substitutions that disrupt base-pairing within the hairpin result in priming from DR1 at the expense of priming from DR2 (Figure 1(b)), suggesting the hairpin contributes to primer translocation by inhibiting the process of *in situ* priming. It is not known whether the hairpin is sufficient to inhibit *in situ* priming, nor is it known what fraction of each RNA primer translocates to DR2 before plus-strand priming. Our aim is to understand the process discriminating between these two types of

priming events (Figure 1) and the mechanism directing priming at each site.

An investigation into the role complementarity between the RNA primer and DR2 plays in primer translocation was previously undertaken using DHBV.[9] In that study, when five of the 12 nt in either DR1 or DR2 (5/12 DR1, 5/12 DR2, respectively; see viruses 66 and 60)[9] were altered to reduce complementarity between the primer and DR2, no detectable primer translocation occurred. When the two mutations were combined to restore complementarity (5/12 DR1/2; virus 67),[9] albeit to a mutant sequence, a concomitant restoration of primer translocation was not observed. Thus, it was not clear to what extent, if any, complementarity between the primer and DR2 contributed to this process. The recent identification of the hairpin that inhibits *in situ* priming indicated a contribution of the DR1 sequence to the discrimination between *in situ* priming and primer translocation.[4] The overlap between the mutation in the 5/12 DR1 variants and the hairpin indicated a need to re-examine the extent to which complementarity between the RNA primer and DR2 contributes to primer translocation.

We present evidence that sequence identity between the DRs (DR1 and DR2) is important to the process of primer translocation. A reduction in sequence identity of the DRs leads to a reduction in primer translocation, and results in increased levels of *in situ* priming. We show that both the hairpin at DR1 and the sequence identity between the DRs play important roles in discriminating between *in situ* priming and primer translocation. We also present evidence that sequence identity between the DRs and the presence of the hairpin at DR1 are not the only determinants in the DR sequence to facilitate the primer translocation process.

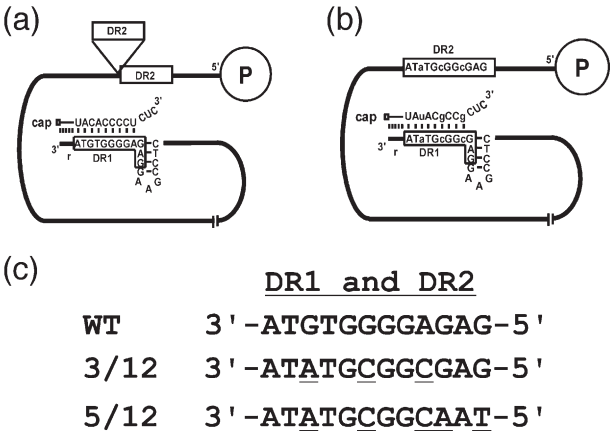

Figure 2. Representation of variants used to examine the role of complementarity between the RNA primer and DR2 without altering the hairpin at DR1. (a) A DHBV variant was created that contained a second copy of the 12 nt direct repeat inserted adjacent to the normal copy of DR2 as shown. The remaining minus-strand DNA (thick black line) was wild-type in sequence, including the 3′ end. The small DNA hairpin overlapping the 5′ end of DR1 in the minus-strand is indicated. (b) The capped RNA primer is shown annealed to the 3′ end of the minus-strand DNA. Depicted is a DHBV variant (3/12 DR1/2) that contains a 3 nt substitution in both DR1 and DR2. Mutations that affect the 3′ end of the minus-strand also affect the RNA primer as indicated by lower-case letters. The 3/12 DR1 and 3/12 DR2 variants contain the 3 nt substitution at one site (DR1 or DR2, respectively) and the wild-type sequence at the other. (c) The sequence of the wild-type direct repeats is shown (minus-sense polarity). The 3 nt substitution (3/12) used in this work is depicted with the altered nucleotides identified by underscores (minus-sense polarity). The 5/12 mutation described in the text and elsewhere is shown for comparison.[9]

Results

Rationale and experimental design

The 5/12 DR1 and 5/12 DR2 variants described earlier (Figure 2(c)) had significantly lower levels of primer translocation compared with a wild-type reference, but did not have identical phenotypes.[9] Of the plus-strand DNA synthesized, the 5/12 DR1 variant synthesized a much higher fraction of DL DNA than the 5/12 DR2 variant, and the DR2 variant synthesized lower levels of total minus-strand DNA. The double variant (5/12 DR1/2) had both a low level of total minus-strand DNA and a high relative fraction of DL DNA. Thus, it appeared that the mutations introduced into DR2 interfered with the ability to make both minus-strand and plus-strand DNA, whereas the mutations in DR1 affected both the level and initiation site of plus-strand DNA synthesis. More recent work showed that a small hairpin forms near the 3′ end of the minus-strand DNA overlapping DR1 and contributes to the discrimination between primer translocation and *in situ* priming by inhibiting priming from DR1 (Figure 1).[4] In that study, it was shown that variants retaining sequence identity between DR1 and DR2, while abrogating the hairpin, predominantly primed plus-strand DNA synthesis from DR1. Those findings suggested that DR1 was the preferred site for initiation of plus-strand DNA synthesis in the absence of an inhibitor of *in situ* priming. We rationalized that high levels of *in situ* priming occurred in the 5/12 DR1 and 5/12 DR1/2 variants because two of the nucleotides altered in DR1 interfered with base-pairing in the hairpin (Figure 2). Thus, the role of complementarity between the RNA primer and DR2 in primer translocation could not be evaluated because the hairpin was disrupted. Therefore, we chose to examine the contribution of complementarity between the RNA primer and DR2 in primer translocation without disrupting the hairpin overlapping DR1.

In one approach, two potential acceptor sites were provided for primer translocation in the context of a normal 3′ end of the minus-strand DNA. This was done by generating a variant of DHBV containing a second copy of the 12 nt DR2 sequence inserted

immediately adjacent to the normal copy (Figure 2(a)). After establishing that both copies of DR2 were readily used as acceptor sites for primer translocation, mutagenesis of each copy independently allowed us to assess the effect of complementarity on site selection (see below). A second approach evaluated primer translocation in the context of a 3 nt substitution (3/12) in either DR1, DR2 or both (Figure 2(b) shows combined mutation). The three nucleotides chosen for mutagenesis were derived from the 5/12 variants, where the two nucleotides that altered the base-pairing potential in the hairpin at DR1 were wild-type in sequence (Figure 2(c)). This same 3 nt mutation was used whenever the DR sequences are referred to here as 3/12.

Since the sequence of the RNA primer cannot be altered without changing the sequence of the 3′ end of minus-strand DNA, which includes DR1, it is difficult to distinguish between contributions made as a result of complementarity between the RNA primer and DR2 *versus* contributions due to the need for sequence identity between the DRs. For the sake of clarity, we describe our results in terms of complementarity, but the implications of this limitation in the experimental design will be considered in Discussion.

Complementarity between the RNA primer and DR2 influences which acceptor site is used for primer translocation

The DHBV variant tDR2 containing a second copy of the DR2 sequence inserted adjacent to the native copy of DR2 is depicted in Figure 3(a). The two copies of DR2 are referred to as A and B. The plasmids expressing this variant, and all subsequent derivatives, were transfected into LMH cells and, three days later, viral DNA replication intermediates were isolated for analysis by Southern blot and primer extension. Southern blotting indicated the normal discrete bands indicative of RC DNA, DL DNA and single-stranded (SS) DNA with similar proportions to the wild-type reference (Figure 3(b)).

Primer extension was performed on the viral DNA sample to determine the relative utilization of the acceptor sites. Prior to primer extension, alkaline treatment was used to remove the RNA primers from the plus-strand DNA termini. Thus, primer 1 (see Materials and Methods) detected the 5′ termini of all plus-strand DNA that initiated from either copy of DR2 and elongated at least to the 5′ end of the minus-strand DNA. Two distinct sets of bands were observed that represent the priming events from copy A and copy B, which were separated by

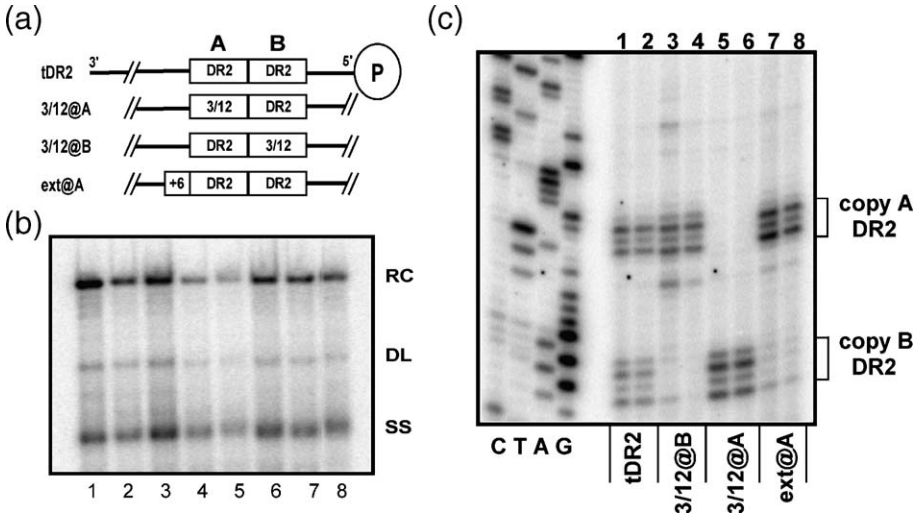

Figure 3. Acceptor site selection for primer translocation is influenced by the extent of complementarity between the RNA primer and the acceptor site. (a) The tDR2 variant contains two identical copies of DR2 inserted in a head-to-tail orientation as shown in the minus-strand DNA (thick black line) with the P protein (circle) attached to the 5′ end. The two copies are labeled A and B. The 3/12@A and 3/12@B variants contain the 3/12 mutation (Figure 2(c)) in either copy A or copy B, respectively. The sequence adjacent to copy A is substituted by 6 nt that increase the complementarity between copy A and the RNA primer from 12 to 18 nt in the ext@A variant, where copy B retains the normal 12 nt of complementarity. (b) Southern blotting of viral DNA from LMH cells isolated three days post-transfection. The positions of the prominent DNA replication intermediates (RC, DL, and SS) are indicated. The blot was hybridized with a genomic-length, minus-strand+specific RNA probe. Lane 1, wild-type reference; lanes 2 and 3, tDR2; lanes 4 and 5, 3/12@B; lanes 6 and 7, 3/12@A; lane 8, ext@A). (c) Primer extension with primer 1 can distinguish between the 5′ ends of plus-strand DNA initiating from either copy of DR2. The 5′ ends are detected primarily at acceptor sites wild-type in sequence or containing extended complementarity with the RNA primer. Ends mapping to copy A or copy B are denoted next to the gel image. Using this assay, it is normal to detect bands at four consecutive positions, where the fastest migrating species corresponds to the position just outside of DR2. The two fastest migrating bands represent authentic 5′ ends of viral DNA.[6] The third and fourth band are thought to be derived from the two fastest migrating bands as a consequence of non-templated, 3′-end additions during the primer extension reaction. A sequencing ladder was generated using primer 1 and a wild-type DHBV template.

the size (12 nt) of the inserted DR sequence (Figure 3(c)). Both copies of DR2 were used for initiation and elongation of plus-strand DNA in the tDR2 variant, although there appeared to be a slight preference for copy A (Figure 3(c), lanes 1 and 2).

Next, the 3/12 mutation (Figure 2(c)) was introduced into either copy A or copy B in the context of the tDR2 variant (Figure 3(a); 3/12@A and 3/12@B, respectively). The same primer extension strategy was used to determine the effect of a 3 nt mismatch in either DR2 sequence upon acceptor site selection and subsequent DNA synthesis. The input viral DNA for each primer extension reaction was normalized to the amount of full-length, minus-strand DNA detected by Southern blot for each sample. Few, if any, plus-strands were detected that originated from copy A of the DR2 sequence when that acceptor site contained the 3 nt mismatch with the RNA primer (3/12@A); whereas, copy B had the wild-type DR sequence and was used in the same variant (Figure 3(c), lanes 5 and 6). The reciprocal effect was observed when the 3/12 mutation was introduced into copy B (3/12@B). In this case, relatively few priming events were detected from copy B, whereas copy A was still used (Figure 3(c), lanes 3 and 4). Therefore, introduction of a 3 nt mismatch within the interior of either copy of the DR2 sequence reduced plus-strand initiation from that location.

To determine whether the observed phenotypes were a result of an overall loss of priming because of the mutated copy of DR2 and/or a preferential usage of the wild-type copy of DR2, we used a quantitative primer extension reaction to measure the total RNA priming events that occurred from both copies of DR2 relative to the total minus-strand DNA in the reaction. Primer extension was performed using primer 1 and primer 3 (see Materials and Methods) on the same preparation of viral DNA sharing a common internal standard (data not shown). There was no difference in the total number of priming events (A + B) that occurred from DR2 in the 3/12@A variant relative to the tDR2 variant (100% of tDR2), indicating the loss of priming at copy A was accompanied by increased priming from copy B. A reduction in the total amount of priming was measured in the 3/12@B variant (70% of the tDR2 reference); however, an increase in the fraction of priming events from the wild type copy of DR2 (copy A) in the 3/12@B variant still occurred (1.2-fold higher than copy A in tDR2). The reduction in priming from DR2 was not associated with a concomitant increase in priming from DR1 (data not shown). Thus, the fate of the undetected RNA primers is not known. Therefore, plus-strand priming occurred preferentially from acceptor sequences containing the wild-type DR2 sequence and/or those that retained the normal 12 nt of complementarity between the RNA primer and the acceptor site.

To determine if extending the potential for base-pairing between the RNA primer and the acceptor site influenced the primer translocation process, a variant was made where the 6 nt located 3′ of copy A in the minus-strand DNA were changed to be complementary to the 5′ end of the RNA primer. Thus, copy A had 18 nt of complementarity with the RNA primer, while copy B retained the original 12 nt of complementary (Figure 3(a); ext@A). Both copies of DR2 in this variant were wild-type in sequence, as was the RNA primer. In the presence of extended complementarity between the RNA primer and copy A, very few plus-strand initiation events were detected from copy B (Figure 3(c), lanes 7 and 8). The total amount of priming that occurred from DR2 was the same as in the tDR2 reference (100% of tDR2), indicating the extended complementarity resulted in a shift in acceptor site usage, from copy B to copy A. Thus, complementarity between the RNA primer and the acceptor site, and not just the presence of a wild-type DR2 sequence, appeared to influence the primer translocation process.

The RNA primer is generated at DR1 and thus contains complete complementarity with the 3′ end of the minus-strand DNA, except for the ultimate nucleotide of the RNA primer, which is not thought to template DNA synthesis.[10] To determine if the level of *in situ* priming decreased as a result of competition between the 3′ end of the minus-strand DNA and the extended DR2 (copy A), Southern blotting was used to calculate *in situ* priming (Figure 3(b)). Only a marginal decrease in the level of *in situ* priming was measured compared with the tDR2 reference (~10% lower than tDR2 reference; $P < 0.08$).

Complementarity between the RNA primer and DR2 is important, but not sufficient, for primer translocation

As mentioned earlier, substantial reductions in primer translocation were observed for the 5/12 DR1, 5/12 DR2 and 5/12 DR1/2 variants with much higher levels of *in situ* priming in the variants containing mutations in DR1 and the hairpin at that site.[9] To determine if complementarity between the RNA primer and DR2 was sufficient for primer translocation when the hairpin at DR1 was left unaltered, three variants were made containing the 3/12 mutation (Figure 2(c)) in either DR1, DR2 or in both sequences (3/12 DR1, 3/12 DR2, and 3/12 DR1/2, respectively; Figure 4(a)). These plasmids were expressed in LMH cells and the viral DNA was isolated three days post-transfection for analysis. The proportions of the major DNA replication intermediates were analyzed by Southern blotting (Figure 4(b)). The phenotypes of the 3/12 DR1 and 3/12 DR2 variants appeared similar, except that the 3/12 DR2 variant synthesized much lower levels of total minus-strand DNA (Figure 4(b), lanes 2 and 3, respectively). Most notably, neither variant synthesized a significant amount of RC DNA (~2% of the wild-type reference; Figure 4(d), gray bars). The 3/12 DR1/2 restoration variant was able to synthesize significantly higher levels of RC DNA (~20-fold;

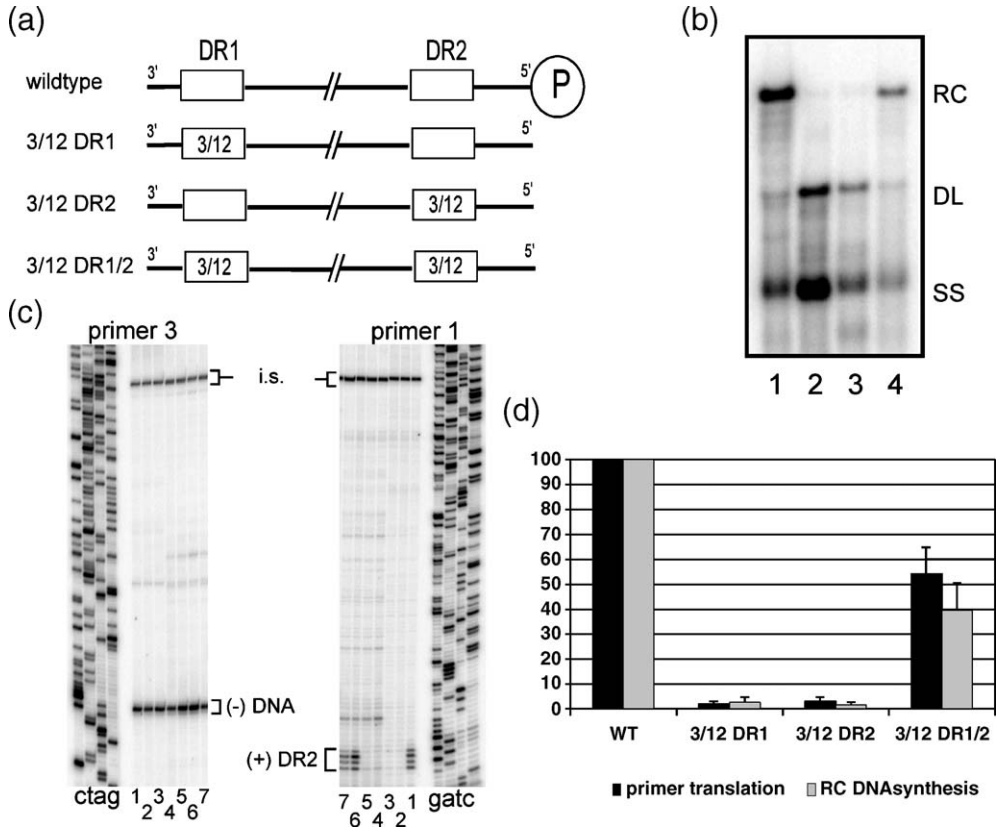

Figure 4. Complementarity between the RNA primer and DR2 is necessary, but not sufficient for primer translocation. (a) Description of the 3/12 variants with the location of the 3/12 mutation (Figure 2(c)) indicated in each case. DR1 and DR2 are indicated as boxes on the schematic of minus-strand DNA (thick black line) with P protein (circle) attached. (b) Southern blot of viral DNA isolated from LMH cells three days post-transfection. Lane 1, wild-type reference; lane 2, 3/12 DR1; lane 3, 3/12 DR2; lane 4, 3/12 DR1/2). The positions of the prominent DNA replication intermediates (RC, DL, and SS) are indicated to the left. The blot was hybridized with a genomic-length, minus strand-specific, RNA probe. (c) Primer extension with primer 3 measured the 5′ termini of minus-strand DNA {(–) DNA} and the internal standard DNA (i.s.). Primer extension with primer 1 measured the amount of plus-strand DNA initiated from DR2 and elongated at least to the 5′ end of minus-strand DNA {(+) DR2} and the internal standard DNA (i.s.). Lane 1, wild-type reference; lanes 2 and 3, 3/12 DR1; lanes 4 and 5, 3/12 DR2; lanes 6 and 7, 3/12 DR1/2). Sequencing ladders were generated using their respective primers and a wild-type DHBV template. (d) The percentage of RC DNA synthesis (Southern blot) and primer translocation (primer extension) were calculated as described in Materials and Methods. Measurements were normalized to a wild-type reference, which was set to 100. For each virus, the mean value is presented with error bars indicating one standard deviation. Each virus was analyzed multiple times from independent transfections.

Figure 4(d)) than either the 3/12 DR1 or 3/12 DR2 variant. However, the restoration of RC DNA synthesis was only partial (~40%, Figure 4(d); $P < 0.005$) compared with the wild-type reference.

In addition to primer translocation, synthesis of RC DNA requires successful circularization of its genome, the final template switch during reverse transcription. To determine whether the 3/12 DR1/2 variant was defective for one or both of these processes, we used the primer extension assay described previously. Primer 1 measured the relative amount of plus-strand DNA that had initiated from DR2, whereas primer 3 measured the relative amount of minus-strand DNA in the reaction (Figure 4(c)). As expected, primer translocation occurred more readily in the 3/12 DR1/2 variant than either single mutant (~25-fold higher, Figure 4(d) black bars). However, primer translocation did not occur to the level of the wild-type reference (~55%, Figure 4(d); $P < 0.03$). A third primer, primer 2, was used to measure the fraction of plus-strand DNA that had initiated from DR2 and had also circularized their genomes (gel image not shown). The 3/12 DR1/2 variant was also defective for circularization (~60% of the wild-type reference; $P < 0.03$) consistent with the difference between RC DNA synthesis and primer translocation (Figure 4(d)). Thus, both primer translocation and RC DNA synthesis occurred at a higher level when the RNA primer and DR2 were complementary throughout the DR sequence.

The observation that the overall level of minus-strand DNA synthesis appeared lower in the 3/12 DR2 and 3/12 DR1/2 variants (Figure 4(b), lanes 3 and 4) compared with the wild-type reference and the 3/12 DR1 variant (Figure 4(b), lanes 1 and 2) was consistent with a previous report showing that mutations in DR2 can lead to a reduction in minus-strand DNA synthesis by an unknown mechanism.[9]

Reduced primer translocation is associated with increased *in situ* priming in variants containing reduced complementarity between the RNA primer and DR2

The 3/12 DR1 and 3/12 DR2 variants indicated that primer translocation did not occur to a significant degree when a 3 nt mismatch existed between the RNA primer and DR2 (Figure 4(d)). To determine if the lack of priming at DR2 led to an increase in priming from DR1, we measured the level of *in situ* priming for each variant. *In situ* priming was calculated using both Southern blotting and primer extension. The increase in the level of *in situ* priming was similar for the two variants (Figure 5(a); threefold to fourfold for 3/12 DR1; twofold to fourfold for 3/12 DR2). The hairpin at DR1 did not appear to be compromised by the 3/12 DR1 mutation, as the 3/12 DR1/2 restoration variant had wild-type levels of *in situ* priming (Figure 5(a)). Thus, the phenotypes of the 3/12 DR1 and 3/12 DR2 variants revealed that reduced complementarity between the RNA primer and DR2 could also increase the level of *in situ* priming.

To determine if increased *in situ* priming was seen routinely when complementarity between the RNA primer and DR2 was reduced, we analyzed four more variants with changes in DR2. Variants were chosen that would result in mismatches between the 3′ end of the RNA primer and DR2 as the 5′ portion (ext@A, Figure 3) and the middle region of the RNA primer (3/12 DR analysis, Figure 4) had already been evaluated. The variants analyzed contained either 1 nt or 3 nt substitutions within DR2 (Figure 5(b)). The 3′ end of the minus-strand DNA, including DR1, was wild-type in sequence in each of the variants. Primer extension was used to measure primer translocation and elongation for each of the variants. Single-nucleotide mutations were sufficient to lower the frequency with which primer translocation and elongation occurred, albeit to different degrees (Figure 5(c)). Primer extension with primer 1 revealed the characteristic pattern of four bands at DR2 (data not shown). The DR2-12 variant initiated DNA synthesis from DR2 at very high levels (~90% of the wild-type reference). However, the single-nucleotide mutations in the DR2-10 and DR2-11 variants had greater impacts upon primer translocation and elongation (45% and 65% of the wild-type reference, respectively). Little, if any, primer translocation occurred in the DR2-s3 variant containing a 3 nt substitution at the 5′ end of DR2 in the minus strand (Figure 5(b)), similar to the other variants containing 3 nt mismatches (3/12 DR1 and 3/12 DR2, Figure 4(d)). The reduction in primer translocation and elongation was correlated with increased levels of *in situ* priming (Figure 5(c) and (d); $P < 0.05$, two-sided).

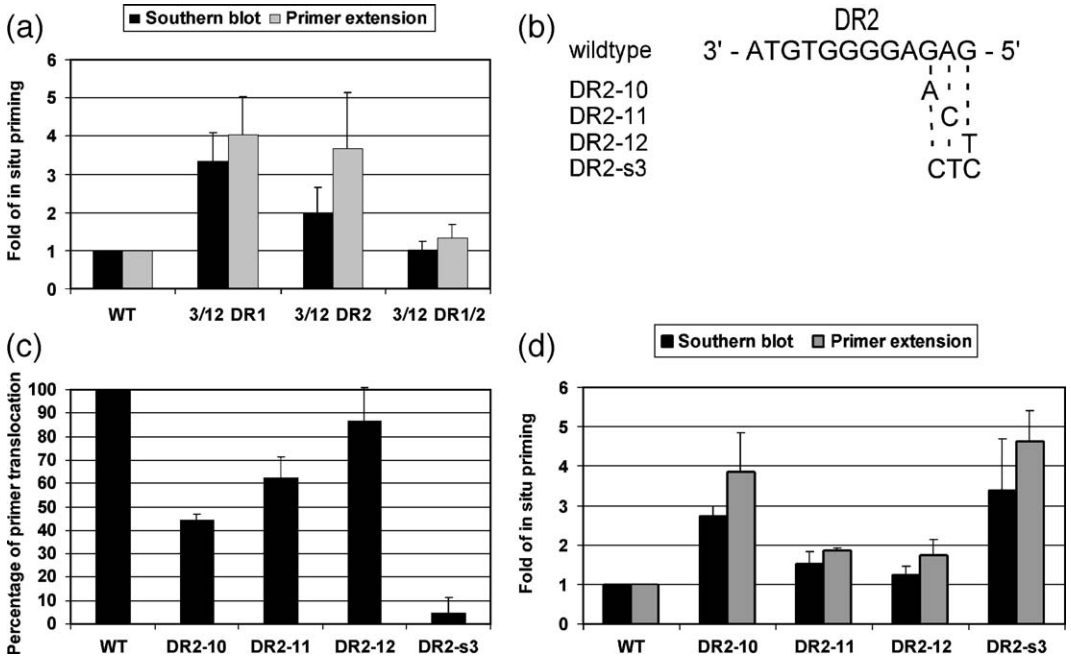

Figure 5. Reduced complementarity between the RNA primer and DR2 affect both primer translocation and *in situ* priming levels. (a) *In situ* priming of the 3/12 DR set of variants was measured using both Southern blot (black bars) and primer extension (gray bars) and calculated as described in Materials and Methods. (b) Description of variants containing 1 nt or 3 nt mutations in the DR2 sequence. The wild-type sequence (minus-sense polarity) is provided as a reference. (c) Primer translocation was calculated by primer extension using primers 1 and 3 as described for Figure 4(c) and (d) (gels not shown). (d) *In situ* priming was calculated using both Southern blot (black bars) and primer extension (gray bars) as described in Materials and Methods. Samples in (a), (c) and (d) were normalized to a wild-type reference (primer translocation = 100, *in situ* priming = 1) and presented as the mean with error bars to indicate the standard deviation. Each virus was analyzed multiple times from independent transfections.

These findings indicate that mismatches between the RNA primer and DR2 lead to reduced primer translocation along with increased *in situ* priming. Furthermore, the position and size of the mismatch both appear to influence the severity of the defect.

Suppression of *in situ* priming requires complementarity between the RNA primer and DR2 as well as the hairpin at DR1

To determine whether the hairpin and complementarity between the RNA primer and DR2 made distinct, but additive, contributions to the suppression of *in situ* priming, variants were made which mutated the hairpin but maintained complementarity between the RNA primer and DR2 (DR1/2 variants; Figure 6(a)), created a mismatch between the RNA primer and DR2 but maintained the wild-type hairpin (DR2 variants; Figure 5(b)), or had both a mutation in the hairpin and a DR2 mismatch (DR1 variants; Figure 6(a)). A full description of each set of variants is provided in Figures 5(b) and 6(a).

Southern blotting was used to measure the levels of *in situ* priming for the four sets of variants (gel images not shown). In each analysis, the level of *in situ* priming for the wild-type reference was set to 1 and the fold increase was reported for each variant (Figure 6(b)). In all but one set of variants (DR12), the level of *in situ* priming was significantly higher in the variants altering both the hairpin and introducing a mismatch than in the variants containing either a hairpin or a mismatch mutation. The level of *in situ* priming was not considered statistically different ($P = 0.5$) for the DR1/2-12 and DR1-12 variants, consistent with the nominal increase in the level of *in situ* priming measured for the DR2-12

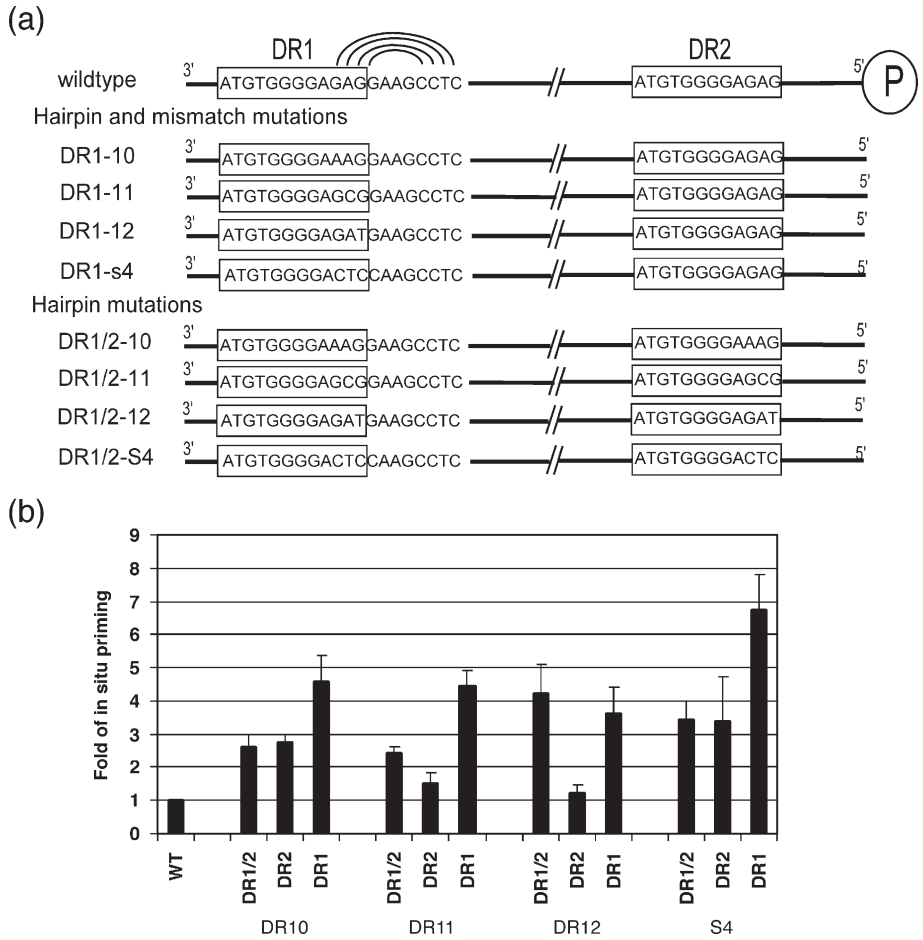

Figure 6. Complete suppression of *in situ* priming requires the hairpin at DR1 and complementarity between the RNA primer and DR2. (a) A schematic of the wild-type minus-strand DNA (thick black line) indicates the sequence of the direct repeats, DR1 and DR2, and the hairpin that resides in overlapping DR1. The four arcs indicate the base-pairing partners in the stem of the predicted hairpin. The four variants containing only DR1 (DR1-*x*, where $x = 10, 11, 12, s4$) mutations reduce base-pairing in the hairpin and introduce mismatches between the RNA primer and DR2. The four variants containing mutations in both DR1 and DR2 (DR1/2-*x*, where $x = 10, 11, 12, s4$) reduce base-pairing in the hairpin, but retain complementarity between the RNA primer and DR2. The mutations that leave the hairpin intact, but disrupt complementarity between the RNA primer and DR2 are shown in Figure 5(b) (DR2-, where $x = 10, 11, 12, s3$). (b) *In situ* priming was calculated from Southern blots as described in Materials and Methods. Samples were normalized to a wild-type reference (set to 1) and presented as the mean with error bars to indicate the standard deviation. Each virus was analyzed multiple times from independent transfections. Values for DR2-*x* variants (Figure 5(d)) are reproduced here for clarity.

variant (1.2-fold increase over wild-type reference). Thus, both the hairpin at DR1 and sequence identity between the DRs was necessary for complete suppression of *in situ* priming.

Discussion

It has been shown that a sequence within DR1 is involved in forming a small DNA hairpin that acts to inhibit the process of *in situ* priming, thereby promoting primer translocation.[4] Here, we have shown that a sequence within the direct repeats, in addition to the nucleotides participating in the hairpin, contributes to the discrimination between *in situ* priming and primer translocation. In particular, there appears to be a requirement for sequence identity between the DRs and/or complementarity between the 12 nt at the 3′ end of the RNA primer and DR2. As mentioned previously, a limitation of our experimental design is that it does not allow us to discriminate between these two possibilities, as the sequence and production of the RNA primer is dependent upon DR1. Thus, complementarity between the RNA primer and DR2 is dependent upon sequence identity of the DRs.

In our analyses, measurements of the primer translocation frequency are based on the levels of plus-strand DNA synthesis that occurs following the translocation and has reached at least the 5′ end of the minus-strand DNA template. It is conceivable that some primers that are translocated to DR2 but fail to prime plus-strand DNA synthesis. Such translocation events would not be scored by our approach. Indeed, these events would be nearly impossible to measure.

The need for sequence identity was clearly established by the variants containing the 3/12 mutation in either DR1 or DR2. Both variants were shown to be severely defective for primer translocation. When the 3/12 mutations were combined to restore sequence identity, albeit to a mutant sequence, a 25-fold increase in primer translocation was observed (Figure 4(d)). Sequence identity cannot be the only determinant as primer translocation was only ~55% of the wild-type reference in the 3/12 DR1/2 restoration variant (Figure 4(d)). The level of *in situ* priming for the 3/12 DR1/2 variant was similar to that of the wild-type, indicating that the incomplete restoration of primer translocation was not due to increased *in situ* priming (Figure 5(a)). Thus, in addition to the need for sequence identity of the DRs, the primary sequence of one or both DRs appears to be important for primer translocation.

When mutations were introduced into DR2 alone, reduced primer translocation was associated with increased *in situ* priming. This finding suggests that either RNA primers alone or in complex with the 3′ end of the minus-strand DNA have had an opportunity to interact with or sample DR2 to determine it was an unsuitable acceptor site before initiating plus-strand synthesis from DR1. We will consider our results in terms of two general models for primer translocation as depicted in Figure 7.

We have presented evidence that the 5′ portion of the primer can influence acceptor site selection in the ext@A variant (Figure 3(b)). We have shown that the 3/12 mutation in the central region of the RNA primer influences both site selection (Figure 3(b)) and the ability of primer translocation to occur (Figure 4(d)). Finally, mismatches between the 3′ end of the RNA primer and DR2 lead to reductions in primer translocation (Figure 5(c)). Thus, it appears that a sequence encompassing the entire length of the RNA primer can influence primer translocation, and that the entire RNA primer is translocated to DR2 before plus-strand initiation. Taken together, these results are consistent with a dynamic equilibrium model for primer translocation where the RNA primer has an intermediate state free from the minus-strand DNA (Figure 7(b), primer ejection). In this model, the role of the hairpin at DR1 may be to contribute to the ejection of the RNA primer and/or limit the frequency with which RNA primers return to DR1, a process that may be overcome when mismatches prevent the RNA primers from annealing to DR2. It has been shown that a positive correlation exists between the number of G and C residues within the non-DR1 portion of the RNA primer (5′ end) and the level of *in situ* priming.[11] This correlation is consistent with an increase in stability between the RNA primer and the 3′ end of the minus-strand DNA, which may either decrease the fraction of RNA primers that are ejected or increase the ability of the 3′ end to compete for the free RNA primers.

There remain a number of observations that cannot be readily explained by the primer ejection model. For instance, it is unclear why primer translocation occurred only at a level ~55% of the wild-type reference in the 3/12 DR1/2 variant. It is possible the reduction in primer translocation is due to decreased stability between the RNA primer and DR2 or an increased stability between the RNA

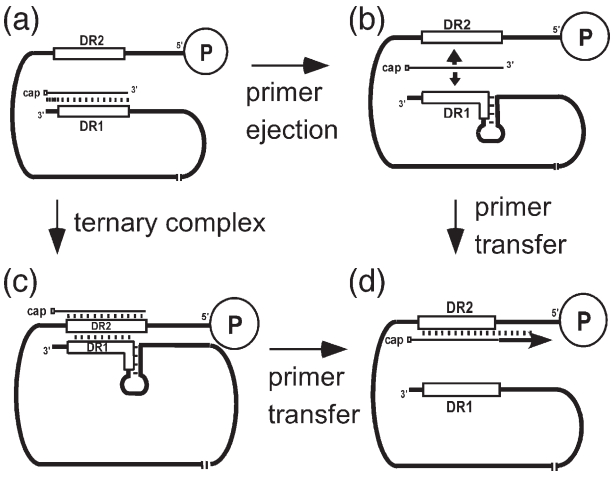

Figure 7. Two general models for primer translocation. See Discussion for thorough description of these models.

primer and the 3′ end of the minus-strand DNA as a result of changing 3 nt in the 3/12 mutation, even though the potential for base-pairing was unchanged. However, the overall G+C content of the DR sequence is conserved by the 3/12 mutation (Figure 2(c)) and an algorithm incorporating nearest-neighbor effects (IDT Oligoanalyzer 3.0) predicts a slightly higher T_m for the 3/12 sequence than that for the wild-type sequence (40.5 °C *versus* 37.6 °C, respectively; DNA/DNA hybrid). Additionally, the level of *in situ* priming did not increase, as would be expected if primer translocation was reduced as a result of increased stability between the RNA primer and the 3′ end of the minus-strand DNA (Figure 5(a)). It was surprising that the defect in primer translocation would be so great in the presence of a single-nucleotide mismatch, and that it would increase as the mismatch was moved further toward the interior on the RNA primer rather than near the 3′ end where plus-strand initiation occurs (Figure 5(c)). A prediction of the primer ejection model is that increasing the number of copies of DR that can act as acceptor sites should lead to increased competition for the free RNA primers. However, introducing an additional copy of wild-type DR2 sequence or a copy with extended base-pairing potential with the RNA primer did not result in a significant reduction in the fraction of primers that initiated from DR1 (tDR2 and ext@A, respectively; Figure 3(a) and (b)). This finding may indicate that a small subset of the RNA primers are never ejected and, thus, not susceptible to competition by acceptor sites. Alternatively, this finding may indicate the competition for an RNA primer is not between a free RNA primer and the acceptor sites, rather between the complex of DR1 and the RNA primer with one copy of DR2.

Although many of the results presented here are consistent with complementarity between the RNA primer and DR2 being the operative mechanism, we cannot exclude an independent and/or additional role for sequence identity between DR1 and DR2 in the minus-strand DNA. For example, it remains tenable that some sort of ternary intermediate is involved in transferring the primer from DR1 to DR2 (Figure 7(c)). The schematic depicted in Figure 7(c) is designed to illustrate an example of a ternary complex, not to infer a specific interaction, as none can be predicted from our results. In such a model, the primary sequence and/or identity of the DRs may be important for facilitating this interaction, which may then take advantage of complementarity between the RNA primer and DR2 to actually transfer the RNA primer. Such an intermediate may serve a number of purposes. It might result in a precise juxtaposition of the donor and acceptor sites, which could increase the efficiency of the primer translocation process. If the sequence involved in, or necessary for, this interaction was located in the middle of the DRs it might explain why we observed only a partial restoration of primer translocation with the 3/12 DR1/2 variant (Figure 4(d)). Similarly, it might explain why the phenotype was more severe as the site of the mismatch was moved closer to the middle of DR2 (Figure 5(c)). In this type of model, mismatches between DR2 and either DR1 or the RNA primer could inhibit the complex from forming, resulting in primers having increased residency time at DR1, which could allow them to eventually overcome the block to *in situ* priming. Alternatively, the hairpin could facilitate transfer of the RNA primer from within the ternary complex by displacing the 3′ end. It is not clear why extending sequence identity between the DRs (ext@A; Figure 3(c)) would have resulted in such a dramatic shift in acceptor site usage using this model, unless DR2 is sampled multiple times before the actual transfer of the RNA primer. Thus, the extended base-pairing potential between the RNA primer and DR2 may decrease the number of times DR2 is sampled before transfer of the RNA primer. Although both models are intriguing, a number of questions remain, which indicates that primer translocation is not fully understood and may involve a more complicated mechanism than that proposed here. Further investigation is necessary to distinguish between these and other models.

Identification and characterization of a number of *cis*-acting sequences that contribute to primer translocation have been shown to contribute to circularization.[4,6,11–13] These findings are consistent with the primer translocation and circularization processes sharing mechanisms. The 3/12 DR1/2 variant described here also had defects in both of these processes, ca 55% and 60% of the wild-type reference for primer translocation and circularization, respectively. Although this finding is intriguing, it is not clear whether this indicates another example supporting a mechanistic linkage or whether the defect is solely a result of the 1 nt difference between the 5′r and 3′r sequences in these variants as a result of the 3/12 DR1 mutation.

It is interesting to consider whether the production of the DL DNA species is important to the hepadnavirus life-cycle or is simply a byproduct of an inefficient primer translocation process. Perhaps the fact that hepadnaviruses use primer translocation to move their RNA primers before plus-strand priming rather than generating their RNA primers using a polypurine tract at DR2, akin to retroviruses, indicates a need for this additional complexity and the DL DNA species itself.

Materials and Methods

Molecular clones

All molecular clones were derived from DHBV, type 3.[14] Two molecular clones were used to express the wild-type reference virus. D1.5G contains 1.5 copies of the genome, sufficient for generation of the terminally redundant pgRNA. 503-3 is a P-null version of D1.5G containing a frameshift mutation in the P protein open reading frame. Mutations that would alter the amino acid sequence of the P protein were constructed in the 503-3 background. All

other variants were constructed in the D1.5G background. All plasmids in the P-null background were co-transfected with a P protein donor plasmid (G308-2) whose RNA was not competent for encapsidation due to a deletion within the packaging signal, epsilon, and a deletion of the 3′ copy of DR1. Here, we report the results of the molecular clones, D1.5G and/or 503-3, as wild-type in all cell culture analyses. All of the clones described herein produce wild-type core protein.

Oligonucleotide directed mutagenesis was used to generate both 0.5-mer and 1.5-mer plasmids containing mutations within the 5′ copy of DR1 (3/12 DR1, DR1-10, DR1-11, DR1-12, DR1-S4) as described.[4] A megaprimer protocol[15] was used to generate 1.5-mer plasmids containing mutations in the 3′ copy of DR2 (3/12 DR2, DR2-10, DR2-11, DR2-S3) as described.[4] Variants containing both DR1 and DR2 mutations were generated by inserting an EcoRI monomer from the 1.5-mer plasmids containing DR2 mutations into the 0.5-mer plasmids containing the appropriate mutations in DR1. The resulting 1.5-mer plasmids contained mutations in the 5′ copy of DR1 and the 3′ copy of DR2 (3/12 DR1/2, DR1/2-10, DR1/2-11, DR1/2-12, DR1/2-S4). The DR2-12 molecular clone is a 2.0-mer plasmid containing two identical monomers in a tandem arrangement as described.[3]

A megaprimer protocol was used to generate a molecular clone (J211-1; 1.5-mer) with a second copy of DR2 inserted adjacent (5′) to the original copy. This plasmid also contained a SfoI restriction enzyme site at position 2467 just upstream of the introduced copy of DR2, in a P-null background. The tandem DR2 variants described in Figure 3(a) were made by oligonucleotide directed mutagenesis using a downstream primer that annealed to a sequence within the vector sequence. The mutagenic primers were phosphorylated before PCR to produce a 5′ end competent for blunt-end ligation. The PCR product was digested with the restriction enzyme XhoI to produce a blunt-sticky product that was swapped with the SfoI to XhoI sequence of J211-1. The mutagenic primer had wild-type DHBV sequence at the 5′ end, which restored the SfoI restriction site to the wild-type sequence. All molecular clones were verified by DNA sequencing.

Cell culture and isolation of viral DNA

The chicken hepatoma cell line LMH was used to replicate DHBV.[16,17] Cells were cultured as described.[18] Calcium phosphate transfections were performed. Viral DNA was isolated from cytoplasmic capsids three days post transfection as described.[4]

Primer extension analyses

Primer extension was performed as described.[4,8,11,13] Typically, 1–4 ng of viral DNA was processed for use in up to three separate primer extension reactions. Each viral DNA sample was mixed with approximately 1 ng of a DHBV-containing plasmid (pD0.5G) digested with NcoI (cleavage at nucleotide 2351) and EcoRV (cleavage at nucleotide 2650) to serve as an internal standard (i.s.) and allow for normalization between reactions. The end-labeled oligonucleotide (primer 1) used to measure the level of plus-strand DNA that initiated from DR2 and had extended at least to the 5′ end of the minus-strand (total priming from DR2) was derived from nucleotides 2537–2520 (minus-sense polarity; annealing temperature, 37 °C). Primer 1 was also used to distinguish between priming events in tDR2 and its derivatives (see Figure 3). The end-labeled oligonucleotide (primer 2) used to measure the level of plus-strand DNA that initiated from DR2, successfully circularized, and elongated by at least 90 nt (post-circularization) was derived from nucleotides 2629–2598 (minus-sense polarity; annealing temperature, 58 °C). Primer 2 was also used to measure the level of plus-strand DNA that initiated from DR1 and elongated at least 80 nt. The end-labeled oligonucleotide (primer 3) was used to measure the amount of 5′ termini of minus-strand DNA that had extended at least 112 nucleotides and was derived from nucleotides 2425 to 2447 (plus-sense polarity; annealing temperature, 58 °C).

Southern blotting analyses

Southern blotting was performed as described[19] for DHBV replication intermediates using 1.25% (w/v) agarose gels containing TBE. Blots were probed with genomic-length, plus-strand RNA probes specific for DHBV. Membranes were exposed and scanned using Molecular Dynamics phosphorimaging cassettes and the Molecular Dynamics STORM. Visualization and quantification were performed using Molecular Dynamics ImageQuant Software.

Calculations

Southern blotting was used to measure DL DNA and RC DNA synthesis relative to the full-length minus-strand DNA, including those that had served as a template for plus-strand DNA synthesis.

- RC DNA synthesis = 100 × RC DNA/total full-length minus-strand DNA
- DL DNA synthesis = 100 × DL DNA/total full-length minus-strand DNA

The quantitative primer extension assay described previously was used to measure the relative efficiencies of the template switches for each variant.[4,8,11,13]

In situ priming = 100 × (priming from DR1/i.s.)/(minus-strand DNA 5′ termini/i.s.)

Primer translocation = 100 × (total priming from DR2/i.s.)/(minus-strand DNA 5′ termini/i.s.)

Circularization efficiency = 100 × (post-circularization/i.s.)/(total priming from DR2/i.s.)

All measurements were normalized to a wild-type reference sample (wild-type = 1 for *in situ* priming; wild-type = 100 for all other measurements).

Statistical tests

The Wilcoxon rank sum test was used to test variants for difference in location compared with the reference virus. Kendall's rank correlation test was used to test for a correlation between primer translocation and *in situ* priming.

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