Small DNA Hairpin Negatively Regulates In Situ Priming during Duck Hepatitis B Virus Reverse Transcription

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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. Both pathways use the same RNA primer, which is capped and 18 or 19 nucleotides in length. At the completion of minus-strand DNA synthesis, the final RNase H cleavage generates the plus-strand primer. To make relaxed circular DNA, primer translocation must occur, resulting in the transfer of the primer generated at DRI to the acceptor site (DR2) near the opposite end of the minus-strand DNA. A small fraction of viruses instead make duplex linear DNA after initiating plus-strand DNA synthesis from DRI, a process called in situ priming. We are interested in understanding the mechanism of discrimination between these two pathways. Some variants of duck hepatitis B virus exhibit high levels of in situ priming due to cis-acting mutations. The mechanism by which these mutations act has been obscure. Sequence inspection predicted formation of a small DNA hairpin in the region overlapping these mutations. We have shown that substitutions disrupting base pairing potential in this hairpin led to increased levels of in situ priming. The introduction of compensatory changes to restore base pairing potential led to reduced levels of in situ priming. Thus, formation of the small DNA hairpin overlapping the 5′ end of DRI in the minus strand contributes to the regulation of primer translocation, at least, through inhibition of in situ priming by making the 3′ end of the minus-strand DNA a poor template for initiation.

Hepadnaviruses are a family of small, enveloped DNA viruses that display a narrow host range and a tropism for the liver and are capable of both acute and chronic infections in their hosts. The prototype member of the family, human hepatitis B virus (human HBV), is a major worldwide health problem, exposing ca. 350 million chronic carriers to an increased risk of developing hepatocellular carcinoma (10). As viral replication is necessary for maintenance of the chronic carrier state, elucidation of the underlying molecular mechanisms of replication may uncover therapeutic targets. The hepadnavirus family consists of a number of mammalian and avian viruses, all sharing similar genetic organization and a general replication strategy. The avian viruses duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHBV) are useful models for studying many aspects of hepadnavirus biology, including replication (reviewed in reference 4).

Hepadnaviruses are related to RNA-containing retroviruses and retroelements as they use reverse transcription to replicate their genomes. Reverse transcription commonly involves a series of template switches, the process whereby the nascent strand of DNA switches from one template to another template. This can occur either intramolecularly or intermolecularly. During hepadnavirus replication, template switches are thought to occur intramolecularly as there is only believed to be one copy of the pregenomic RNA (pgRNA) template within a core particle. The dependence upon template switching among these pathogenic viruses highlights the importance of studying their mechanistic underpinnings.

Hepadnavirus replication occurs within a newly formed core particle in the cytoplasm of the infected cell. The virally encoded P protein has numerous functions, including protein priming of DNA synthesis using its reverse transcriptase activity (26). Using a bulge in a secondary structure near the 5′ end of the pgRNA as a template, P synthesizes the first 4 nucleotides (nt) of minus-strand DNA (Fig. 1A) (18, 22, 25). The first template switch results in repositioning of the P protein and the nascent minus-strand DNA to a complementary acceptor site overlapping a 12-nt direct repeat (DR1) near the 5′ end of the pgRNA template (Fig. 1B) (22, 25). Minus-strand DNA synthesis resumes and continues to the 5′ end of the pgRNA. Concurrently, the RNase H activity of the P protein degrades the pgRNA following its use as a template, freeing the minus-strand DNA to subsequently serve as a template for plus-strand DNA synthesis (Fig. 1C) (21). Importantly, the final RNase H cleavage results in a capped oligoribonucleotide 18 or 19 bases in length that serves as the primer for the initiation of plus-strand DNA synthesis (Fig. 1D) (13, 15, 20).

In order to make relaxed circular (RC) DNA, the form of the genome found in most virions, two template switches are necessary during plus-strand DNA synthesis. In contrast to retroviruses, the plus-strand RNA primer is used at a site other than where it was generated, a process termed primer translocation. This requirement poses an interesting mechanistic challenge to the virus. Primer translocation results in, at least, some portion of the RNA primer being transferred from DR1, the donor site, to a partially complementary acceptor site, direct repeat 2 (DR2), near the 5′ end of the minus strand (Fig. 1E) (13). Following initiation of DNA synthesis from DR2, the
nascent plus-strand DNA is extended to the 5' end of the minus-strand DNA template (Fig. 1F). At this point the third template switch, termed circularization, facilitated in part by a small terminal redundancy (r) in the minus-strand DNA, allows annealing of the 3' end of the nascent plus strand to the 3' end of the minus strand, where its extension leads to the synthesis of RC DNA (Fig. 1G) (14). Although plus-strand DNA synthesis initiates from DR2 during production of the RC DNA species, a low level of initiation occurs from DR1, a process termed in situ priming, which results in a duplex linear (DL) form of the genome (Fig. 1H) (20). Although infection with this form can lead to covalently closed circular DNA formation through a process of nonhomologous recombination, it is rapidly outcompeted by virus able to efficiently synthesize the RC DNA species. This highlights the importance of efficient primer translocation during reverse transcription (27, 28, 30). As plus-strand DNA priming can occur from DR1, it is likely that determinants exist that promote priming from DR2 at the expense of priming from DR1 (20). We are interested in understanding the mechanism underlying this discrimination.

Previous studies found the overall level of in situ priming increased following mutation of the sequence within or adjacent to DR1 (20). These observations indicate that in situ priming is regulated and the nucleotides within and adjacent to DR1 participate in that regulation. It has been shown that complementarity between the primer and DR2, although likely important, is not sufficient to promote preferential priming from DR2 (17). The RNase H cleavage generating the RNA primer is independent of sequence as mutations within and adjacent to DR1 did not affect the position of the cleavage sites. Rather, cleavage occurs at one of two sites, 18 or 19 nt from the 5' end of the pgRNA (15). Thus, increased in situ priming is not attributed to alterations in the primer length due to variations in the RNase H cleavage sites. Until now, the mechanism responsible for increased in situ priming has remained enigmatic. In this study, we present evidence for the
formation of a small stem-loop structure in the minus-strand DNA and its role as a cis-acting negative regulator of in situ priming during DHBV replication.

MATERIALS AND METHODS

Nomenclature. Extending the nomenclature originated by Staprans and colleagues (20), we refer to the base pairing partners in the hairpin according to their position with respect to DR1. As outlined in Fig. 2B, the mutations at the base of the stem are referred to as the DR10 series as they include mutations in the 10th nt of DR1. The other series are similarly named in an incremental fashion, progressing towards the top of the stem (DR11, DR12, and DR13). The DR13 series does not alter the sequence of either DR1 or DR2, as its mutations lie outside of DR1. Sequences depicted in this manuscript are of minus-sense polarity, except where noted. Variants containing mutations in the 3' region of the stem, and DR2 where necessary, are referred to as Watson (W) variants, whereas those in the 5' half are referred to as Crick (C) variants. Restoration (R) variants combine the mutations from the W and C variants, thus restoring the base pairing potential. An additional series of variants, designated the S4 series, substitutes each side of the stem-loop independently to their exact complements as indicated by dashed lines. (B) Design of the genetic analysis to test the role of the putative hairpin in regulating in situ priming. Single nucleotide substitutions were independently introduced as diagrammed at each position of the putative stem (W and C variants [see Materials and Methods for nomenclature]). These mutations disrupt 1 bp in the putative hairpin. Variants were constructed to restore base pairing potential (R variants; not shown) by combining the mutations from the W and C variants. The thin line indicates the capped oligoribonucleotide, which serves as the primer for plus-strand DNA synthesis. The thick line indicates the mature minus-strand DNA template for plus-strand synthesis. The 3' and 5' ends of the minus-strand DNA are indicated with the P protein (circle) covalently attached to the 5' end of the minus-strand DNA.

FIG. 2. Design of experiment based on previously published variants. (A) Previously published variants containing mutations within or adjacent to DR1 that were shown to have increased in situ priming (18). Sequence of the 3' end of the minus strand is shown with DR1 indicated by a box. The altered nucleotides for each variant are indicated by dashed lines. (B) Design of the genetic analysis to test the role of the putative hairpin in regulating in situ priming. Single nucleotide substitutions were independently introduced as diagrammed at each position of the putative stem (W and C variants [see Materials and Methods for nomenclature]). These mutations disrupt 1 bp in the putative hairpin. Variants were constructed to restore base pairing potential (R variants; not shown) by combining the mutations from the W and C variants. The thin line indicates the capped oligoribonucleotide, which serves as the primer for plus-strand DNA synthesis. The thick line indicates the mature minus-strand DNA template for plus-strand synthesis. The 3' and 5' ends of the minus-strand DNA are indicated with the P protein (circle) covalently attached to the 5' end of the minus-strand DNA.
DNA that initiated from DR1 and had extended approximately 80 nt had sequence complementary to nucleotide coordinates 2629 to 2598 (primer 2629; annealing temperature, 58°C). The end-labeled primer used to measure the level of plus-strand DNA that initiated from DR2 and elongated at least to the 5′ end of minus-strand DNA had complementarity to nucleotide coordinates 2537 to 2520 (primer 2537; annealing temperature, 37°C). See Fig. 4 for a schematic of the primer extension strategy. The primer extension reaction mixtures contained 1× ThermoPol Buffer [10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO4, 0.1% Triton X-100 (New England BioLabs), 2 U of Vent DNA polymerase (New England BioLabs), 0.2 mM each deoxynucleoside triphosphate, ~0.7 pmol of end-labeled primer, internal standard plasmid DNA, and viral DNA. Ten cycles were performed for each reaction, and the products were electrophoresed using 6% polyacrylamide, 7.6 M urea gels. The gels were dried, exposed onto Molecular Dynamics phosphorimaging cassettes, and scanned using the Molecular Dynamics STORM PhosphorImager for visualization and quantitation using ImageQuant version 5.1.

RESULTS

Rationale for model and experimental design. An explanation for how mutations adjacent to DR1 can affect the site of DNA initiation without altering the final RNase H cleavage site had been elusive. Inspection of the primary sequence in this region uncovered the potential for a small DNA hairpin to form in the minus strand overlapping the 5′ end of DR1. We hypothesized that formation of the hairpin could act as a negative regulator of in situ priming by making DR1 an inefficient site for initiation of plus-strand DNA synthesis. Previously described DHBV variants, containing mutations within the putative hairpin sequence (Fig. 2A), were shown to have increased in situ priming (20). The phenotypes of these variants were consistent with the hypothesis, as they would be expected to destabilize the hairpin. However, those variants did not directly test the model.

To determine if this putative DNA hairpin contributed to the regulation of in situ priming, we used a genetic approach to test for base pairing between nucleotides within the stem sequence. The model predicted that disruption of base pairing should destabilize the hairpin, leading to increased levels of minus-strand DNA, whereas restoration of base pairing to a mutant sequence should reduce the level of in situ priming by restabilization of the hairpin. Three of the four previously published variants shown in Fig. 2A contain a single point mutation within the putative stem sequence and had measurable increases in the relative level of minus-strand DNA. The data set by individually mutating each of the 8 nt within the predicted stem sequence (Fig. 2B) (see Materials and Methods for nomenclature). The substitutions were designed such that upon combination of the mutations from the W and C variants a third variant (R) was generated, which restored the potential for 4 bp to form in the stem. Mutations introduced into DR1 altered the sequence of the RNA primer and reduced complementarity between the primer and DR2. As the contribution and necessity of complementarity to the process of primer translocation has not been clearly established, mutations were concomitantly made in DR1 and DR2 to circumvent any uncertainties (includes W and R variants, Fig. 2B). By introducing single nucleotide mutations, we generated four independent sets of mutants to rigorously test the hypothesis. We were also able to determine the necessity for base pairing at each position within the stem.

Hirao and colleagues (7, 8, 29) identified structural features of similar small DNA hairpins with unusually high thermostability in which both the stem and loop sequences contributed to the hairpin’s thermostability. In particular, trinucleotide loops containing the sequence 5′-GNA-3′ were shown to be particularly stable, with sheared base pairs forming between the G and A in the loop. The putative hairpin in DHBV contained a 5′-GAA-3′ loop, lending credibility that such a small DNA hairpin could form with sufficient thermostability. Hirao and colleagues have also shown a correlation between the mobility in gel electrophoresis and the thermodynamic Tm of the DNA molecules. They found that single-stranded DNA with abnormally fast mobility on denaturing polyacrylamide gels, containing 7 M urea, corresponded to DNA containing a secondary structure with a Tm greater than 60°C (8). We performed a similar biochemical analysis (band compression on DNA sequencing gels; data not shown) and determined that the hairpin sequence could form a stable hairpin. Furthermore, using this assay, we were able to confirm that, at least under these conditions, single nucleotide mutations (W or C) were sufficient to decrease the hairpin stability, whereas hairpin stability increased in the restoration (R) variants relative to the W and C variants. With these results in hand, we decided to analyze the influence of these mutations (Fig. 2B) on DHBV DNA replication in cell culture.

Analysis of DNA replication in cell culture indicates that the hairpin negatively regulates in situ priming. Molecular clones encoding each of the viral variants depicted in Fig. 2B were expressed in the LMH cell line. The intracellular DNA replicative intermediates were isolated 3 days posttransfection for analysis using two independent methods, Southern blotting and primer extension. Southern blotting (Fig. 3A) allows discrimination of the major forms of DNA replicative intermediates (RC, Fig. 1G; DL, Fig. 1H; SS, Fig. 1D, 1E, and 1F). DL DNA, which is synthesized as a result of plus-strand DNA initiation from DR1, was used as an indicator of the level of in situ priming. The level of in situ priming for each virus was calculated as the percent of DL DNA relative to mature minus-strand DNA, as indicated in Fig. 3A. In this calculation, all mature minus-strand DNA was used in the denominator as it is the template for plus-strand DNA synthesis. Restricting analysis to the mature minus-strand DNA ensures deficiencies at earlier steps in replication would not influence the analysis. As shown in Fig. 3A and expressed quantitatively in Fig. 3B, in each of the four independent series (DR10, DR11, DR12, and DR13), the individual W and C variants had increased relative levels of DL DNA compared to the wild-type virus. Analyses of the DR10 series found similar increases in DL DNA accumulation regardless of which side of the stem contained the mutation (compare DR10W and DR10C). The restoration variant (DR10R) combining the mutations from the W and C variants had lower relative levels of DL DNA production than either of the individually mutated variants, showing a partial restoration. The observation that the DR10R variant had lower relative levels of in situ priming than both the DR10C and DR10W variants is evidence that these nucleotides contribute to function, at least in part, by base pairing. The DR11 series showed a similar trend; however, there was a pronounced difference between the relative levels of DL DNA in the DR11W and DR11C variants. The reason for this difference is not known, but it is consistent with the observation that the substitution in the DR11C variant had a greater affect.
on hairpin stability using the band compression assay mentioned previously (data not shown). In the restoration variant, DR11R, the relative level of DL DNA was again reduced compared to either of the individually mutated variants. The single mutations introduced to generate the DR12 and DR13 series led to similar increases in relative DL DNA synthesis. Compared to either of the individually mutated variants. The single mutations introduced to generate the DR12 and DR13 series led to similar increases in relative DL DNA synthesis. The results of the Southern blot analyses. Primer extension allowed quantitation of all molecules priming from DR1 that have synthesized at least 80 nt of plus-strand DNA. This assay does not require complete synthesis of the DL DNA species for detection. One preparation of viral DNA was used for two separate primer extension reactions, one measured the amount of minus-strand DNA, and the other measured the amount of plus-strand DNA that had initiated from DR1, while the middle set of bands indicated the plus-strand DNA termini (Fig. 4 shows the primer extension strategy). Primer extension with an oligonucleotide, 2425+, was used to quantitate minus-strand DNA. A single band corresponding to the 5’ terminus of the minus-strand DNA is indicated in Fig. 5A. As shown in Fig. 5B, primer extension with an oligonucleotide, 2629−, which hybridized to plus-strand viral DNA, resulted in two sets of bands. The lower set of bands indicates the plus-strand DNA that initiated from DR1, while the middle set of bands indicates plus-strand DNA initiating from DR2 that has successfully circularized and extended. The relative level of in situ DNA priming was defined as the amount of priming that occurs from DR2. Coordinate of minus-strand DNA 5’ termini, 2537; plus-strand DNA initiating from DR2, 2489; plus-strand DNA initiating from DR1, 2547. (A) A single-stranded (SS) intermediate, in which plus-strand DNA synthesis has not occurred, is detected by primer 2425+, but not 2537− or 2629−. (B) DL DNA is detected by primers 2425+ (minus-strand DNA) and 2629− (plus-strand DNA initiating from DR1). Primer 2537− will not produce a signal as it hybridizes at the 3’ end of plus-strand DNA. (C) RC DNA is detected by all three primers.
DNA hairpin contributes to regulation of in situ priming. The analysis, up to this point, was performed by examining single base pairing positions within the hairpin. Although four independent sets of analyses provided compelling evidence that the hairpin negatively regulated in situ priming, the magnitude of the increase for each substitution variant was modest. To determine whether larger increases in the level of in situ priming could be elicited, we analyzed a series of progressively larger substitutions starting from the base of the stem. The substitutions were placed in the 5’ half of the stem, leaving DR1 and DR2 unaltered (S1C, S2C, S3C, S4C; Fig. 6A). Southern blotting indicated that as the number of base pairing partners within the stem was reduced from four to zero, the level of in situ priming increased progressively (Fig. 6C). Clearly, very high levels of in situ priming could be elicited by changing the sequence within the hairpin shifting the primary site of plus-strand DNA priming to DR1 (primer extension; data not shown).

Given that restoration variants did not completely restore the level of in situ priming to that of the wild-type virus (Fig. 3B and 5B), it was apparent that the wild-type nucleotide sequence made a contribution. This point was emphasized when we analyzed the S4C, S4W, and S4R variants. The S4C and S4W variants contained mutations that completely abrogated base pairing within the stem by changing all 4 nt to their complement (S4C, S4W, and S4R; Fig. 6A). As measured by Southern blotting (Fig. 6B), both the S4W and S4C variants had increased proportions of DL DNA compared to the S4C variant, it remained slightly higher when we analyzed the S4C, S4W, and S4R variants. The S4C and S4W variants contained mutations that completely abrogated base pairing within the stem by changing all 4 nt to their complements (Fig. 6A). Note that DR2 was altered where necessary to retain DR1 and DR2 sequence identity. As measured by Southern blotting (Fig. 6B), both the S4W and S4C variants had increased proportions of DL DNA with higher levels in the S4C variant (Fig. 6C). Although the restoration variant, S4R, had a dramatically reduced proportion of DL DNA compared to the S4C variant, it remained slightly higher than that of the S4W variant. These results were corroborated using the primer extension method of analysis described earlier (data not shown). Combined, these results indicate that regulation of in situ priming is extremely sensitive to mutations

FIG. 5. Primer extension analyses also indicate that the DNA hairpin contributes to regulation of in situ priming. Increased in situ priming was detected in each virus compared to the wild type. The relative level of in situ priming was lower in the restoration variants (R) than in either of the W or C variants. A mixture of viral DNA and internal standard DNA was split into two primer extension reactions (see Materials and Methods). (A) Primer extension with primer 2425 measured the 5’ termini of minus-strand DNA (labeled minus). The amount of viral DNA was normalized to the internal standard (i.s.). The DNA sequencing ladder was generated using primer 2425+. (B) Primer extension with primer 2629− measured the amount of plus-strand DNA initiated from DR1 (labeled DR1) and the level of circularized plus-strand DNA initiated from DR2 (not used in this analysis). Each viral sample was normalized to the internal sample (i.s.). The DNA sequencing ladder was generated using primer 2629+. (C) The amount of plus-strand DNA initiated from DR1 relative to the amount of 5’ termini of minus-strand DNA located at position 2537 was calculated as described in Materials and Methods. For each virus, the mean value is presented, with error bars indicating 1 standard deviation. Each virus was analyzed multiple times from independent transfections (wild type [WT], n = 20; DR13W, DR13C, and DR13R, n = 5; DR12W, DR12C, and DR12R, n = 4; DR11W, DR11C, and DR11R, n = 3; DR10W, DR10C, and DR10R, n = 2).
within the hairpin sequence and that simply having the base pairing potential to form a 4-nt stem is necessary but not sufficient to regulate the site of plus-strand DNA initiation to wild-type levels.

**Hairpin sequence can inhibit DNA initiation in cis at an alternative site.** To assess the ability of the hairpin to negatively influence priming in cis, we asked whether it could reduce levels of initiation from an alternate site. We introduced the hairpin sequence in the analogous position at DR2 and measured the effect on priming from that site. Introduction of the hairpin sequence, fortuitously, resulted in base pairing potential for a 5-nt stem rather than the 4-nt stem found at DR1 (Fig. 7A). However, when sequence capable of forming a 5-nt stem at DR1 was analyzed, the level of in situ priming in that virus, as measured by Southern blotting, was not significantly different (6%, data not shown) from that in the wild-type virus.

Primer extension was used to measure the amount of plus-strand DNA priming occurring at each of the sites, DR1 and DR2 (Fig. 4). In situ priming was measured as described earlier using oligonucleotide 2629/H11002, while initiation of plus-strand DNA synthesis from DR2 was measured using oligonucleotide 2537/H11002, which hybridized to viral plus-strand DNA just prior to the point of circularization. Minus-strand DNA was measured as described earlier. Akin to the DR1 measurements, plus-strand priming from DR2 was measured as the amount of priming detected at DR2 divided by the amount of minus-strand DNA 5’ termini, after adjusting for the internal standard (gels not shown). Introduction of the hairpin sequence at DR2 modestly increased the amount of plus-strand priming from DR1 compared to the wild type (Fig. 7B). However, priming from DR2 was dramatically reduced in the presence of the hairpin sequence, decreasing from 89 to 9%. In fact, plus-strand DNA priming, in general, was severely defective in this variant, suggesting a general inhibition of priming results when both sites are occupied with the hairpin sequence. An independent variant containing a 13-nt substitution just downstream of the 3’ boundary of DR2 did not alter the proportions of DNA replicative intermediates (data not shown), indicating that we were not disrupting sequence necessary for efficient primer translocation. This analysis is consistent with the hairpin acting as a negative regulator of DNA synthesis in cis but does not exclude additional roles for the hairpin or its primary sequence.

**Mechanism conserved in a related avian hepadnavirus.** Next, we asked whether other avian hepadnaviruses use this hairpin to regulate in situ priming. Phylogenetic analysis indicated that HHBV4 is the most distantly related avian hepadnavirus to the Western country DHBV isolates, which include DHBV3 (24). HHBV4 shares ca. 77% nucleotide identity with DHBV3 and is unable to infect ducks (9). As depicted in Fig. 8A, the HHBV4 sequence has the potential to form a 4-nt

FIG. 6. Sequence of the hairpin plays a prominent role in regulating in situ priming. (A) Diagrams indicate the sequence of each hairpin variant. Substituted nucleotides are enclosed in circles. Variants S1C to S4C progressively decrease base pairing potential. S4W is the complementary mutation to S4C. The S4R variant combines the mutations from the S4W and S4C variants. (B) Southern blotting of viral DNA isolated from LMH cells 3 days posttransfection. The blot was hybridized with a genomic-length, minus-strand-specific RNA probe. Positions of the prominent forms of DNA replicative intermediates (RC, DL, and SS) are indicated. (C) Proportions of DL DNA were calculated as described in Fig. 4. As shown by the first five bars (wild type [WT], S1C, S2C, S3C, and S4C), the level of in situ priming increased as the number of substitutions increased (base pairing partners decreased). The S4R variant reduced the level of in situ priming compared to S4C, but its level of priming was slightly higher than that of S4W. Note the S1C variant is the same variant previously referred to as DR10C (Fig. 2B).
stem, but instead of the trinucleotide (5'-GAA-3') loop found in DHBV3, the heron virus contains a tetranucleotide loop (5'-GAAT-3'). We asked if the hairpin contributed to the regulation of in situ priming in HHBV4 by analyzing mutations that were analogous to the DR13 series of DHBV (Fig. 2B). As shown in Fig. 8A, the predicted Watson loop-closing nucleotide was changed to its complement (G to C) to generate the HDR13W, while the converse (C to G) was done to generate the HDR13C variant. The restoration variant, HDR13R, combined the mutations from the two variants to restore base pairing potential. The molecular clones were expressed in LMH cells, and replicative intermediates were analyzed by Southern blotting. A summary of the Southern blot analyses is presented (Fig. 8B). The HDR13W and HDR13C variants had increased DL DNA proportions compared to the wild-type virus. Consistent with the hairpin contributing to the regulation of in situ priming, the HDR13R restoration variant had reduced relative levels of DL DNA compared with either of the single mutation variants. These data suggest the mean of two independent analyses with the standard deviation indicated.

**DISCUSSION**

The observation that the sequences near DR1 contribute to the regulation of in situ priming was first made by Staprans and colleagues (20). Our results have shown that at least one contribution of that sequence is to provide base pairing partners necessary to form a secondary structure, which acts as a negative regulator of in situ priming. The inability of any of the restoration variants to lead to complete restoration of wild-type levels of in situ priming emphasizes the requirement for more than one hairpin with four base pairing partners. The sequence constraints may be due to structural contributions. Consistent with previous studies suggesting the sequence of the stem in small DNA hairpins influences its structural stability (6–8), there may be inherent properties of the wild-type not clear. To ascertain whether this could be explained by the HHBV loop providing additional structural stability, we substituted the DHBV trinucleotide loop with the HHBV 4-nt loop. Introduction of the HHBV loop into DHBV did not reduce the proportion of in situ priming (data not shown). We interpret this result to indicate that other virus-specific contributions are responsible for the different relative levels of DL DNA synthesized by the two viruses.
DHBV sequence making it more or less stable than the restoration hairpins. Although measuring the thermostability of the wild-type hairpin and various derivatives can be done in vitro, these analyses may be misleading as they will not accurately simulate the environment within the capsid. Other factors, such as surrounding sequence and the presence of the RNA primer as well as other trans-acting factors, may influence hairpin formation and stability. Alternative structural contributions are also possible. For example, the hairpin may be a binding site for a trans-acting factor involved in either facilitating primer translocation or the inhibition of in situ priming. These ideas are not mutually exclusive. Currently we are unable to distinguish between these possibilities.

We have shown that introduction of the hairpin sequence at DR2 in the minus-strand DNA template resulted in a reduction of plus-strand priming from that site (Fig. 7). This variant, containing sequence capable of forming a hairpin at both DR1 and DR2, had a defect in primer utilization from both sites. This result shows the hairpin sequence acts as a cis-acting negative regulator of priming. Furthermore, the phenotypes of the variants shown in Fig. 6 are consistent with the notion that in the absence of a negative regulator of in situ priming, DR1 is the predominant site for initiation of plus-strand DNA synthesis.

A comprehensive understanding of the mechanism of primer translocation is not yet at hand. We have provided evidence for one contribution to the mechanism, in the formation of the hairpin. Our evidence suggests formation of the hairpin is a cis-acting negative regulator of in situ priming. As it has not been established whether all or just the 3′ end of the RNA primer is translocated to DR2 prior to plus-strand DNA synthesis, it is possible the role of the hairpin is to displace just the 3′ end for hybridization and extension from DR2. Clearly, this model relies on other mechanisms that would precisely juxtapose the donor and acceptor sites in order to facilitate this reaction (e.g., triple-helix formation). We would classify this type of process as a primer displacement mechanism (Fig. 9B). Primer displacement may, alternatively, be the precursor to a mechanism involving facilitated transport of the primer. For instance a protein, such as the P protein, may actively participate in moving the primer from DR1 to DR2. In the absence of the hairpin, the primer may be used, instead, from DR1. An alternative model includes a role for the DNA hairpin in facilitating complete displacement of the primer from DR1, termed primer ejection (Fig. 9C). This model, which is an extreme form of the primer displacement model, is consistent with the ideas proposed by Condrea and colleagues (3), who suggested that the primer is in a state of dynamic equilibrium between DR1 and DR2. In a state of dynamic equilibrium, the hairpin at DR1 may decrease the rate at which the primer returns, thus driving the reaction towards priming from DR2. Further studies are necessary to distinguish between these and other models. In particular, investigation of the variant containing a copy of the hairpin sequence at both locations (Fig. 7) may provide insight into which of these models, if any, are operative. Due to the defect in primer utilization in this variant, the location of the unused primers may be informative.

The conservation of the hairpin as an inhibitor of in situ priming in the avian hepadnaviruses was shown through a genetic analysis of the distantly related avian hepadnavirus, HHBV4. Just as with DHBV, the restoration variant did not result in a complete restoration of the levels of in situ priming compared to the wild-type virus. In addition to suggesting a conserved role for the hairpin in two distantly related avian hepadnaviruses, this result further supports the contention that the native sequence is important. Using ClustalW software (23) to align all of the avian hepadnavirus complete genome sequences from the GenBank database along with DHBV3 (19), it was found that 20 of the 24 sequences precisely matched the hairpin sequence shown for DHBV3 in Fig. 8A while the other four sequences were identical to the HHBV4 sequence as shown in Fig. 8A (data not shown). This conservation of sequence and therefore predicted structure strongly suggests that these hepadnaviruses use the DNA hairpin as a means to regulate in situ priming.

Although a majority of our experiments have focused on the contributions of the stem of the hairpin, we have made several insights into the role of the loop. Based upon previous studies of other small DNA hairpins, a 5′-GNA-3′ trinucleotide loop is thought to contribute to structural stability of the hairpin through sheared base pairing between the G and A residues (29). The DHBV loop, 5′-GAA-3′, has the correct sequence to make a sheared base pair, and the mutant loop, 5′-AAA-3′, has increased in situ priming (data not shown). Although these data are consistent with a sheared base pair contributing to function, additional analyses will be needed to clarify the role of the loop and its mechanism of action. This point is emphasized upon consideration that the HHBV loop is 4 nt instead of 3 nt, while the HHBV and DHBV stems are identical in sequence (Fig. 8A). In fact, HHBV has lower levels of in situ priming than DHBV (1 versus 5%). Under the guise of a different hypothesis, we asked whether the loop sequence was responsible for the different levels of in situ priming. Introduction of the HHBV tetraloop into DHBV did not reduce the level of in situ priming (data not shown). This result suggests

![Figure 9](http://jvi.asm.org/...)

FIG. 9. Models for the possible role of the DNA hairpin. (A) Following the final RNase H cleavage, a capped oligoribonucleotide remains for priming plus-strand DNA synthesis. (B) Primer displacement occurs as a result of the hairpin forming in the region overlapping the 3′ end of the primer at DR1. The 3′ end of the primer is inefficiently extended from DR1 in this reaction. (C) Primer ejection occurs as a result of the hairpin forming in the 3′ end of the minus strand. The RNA primer is predicted to be free of a minus-strand DNA template following this reaction.
there are other virus-specific contributions to the regulation of primer translocation and/or in situ priming. The question arises whether the use of a small DNA hairpin to negatively regulate in situ priming is a universal feature of hepadnavirus DNA replication. We have provided compelling evidence that this is likely to be the case for avian hepadnaviruses. However, the minus-strand nucleotide sequence of the mammalian hepadnaviruses (e.g., HBV and WHY) does not appear to contain a similar hairpin at the 5′ end of DR1. This observation invokes two considerations. (i) Inhibition of in situ priming through a DNA secondary structure is an underlying principle of hepadnavirus DNA replication, and the mammalian family members use a different DNA structure. (ii) As the mechanism of primer translocation is likely to have positive-acting components in addition to negative regulation of in situ priming, the mammalian viruses could have more efficient positive contributions. More needs to be learned about positive-acting mechanisms in primer translocation. In addition, a rigorous comparison of the relative efficiencies of priming from DR2 versus DR1 between the mammalian and avian hepadnaviruses remains to be performed.

In conclusion, we present evidence that, at least in avian hepadnaviruses, one contribution to the preferential priming of plus-strand DNA at DR2 is provided by a cis-acting structure in the minus-strand DNA acting as an inhibitor of priming from DR1.

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