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The sequence of the RNA primer and the DNA template influence the initiation of plus-strand DNA synthesis in hepatitis B virus

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Summary

For hepadnaviruses the RNA primer for plus-strand DNA synthesis is generated by the final RNase H cleavage of the pgRNA at DR1 during the synthesis of minus-strand DNA. This RNA primer initiates synthesis at one of two distinct sites on the minus-strand DNA template resulting in two different end products; duplex linear (DL) DNA or relaxed-circular (RC) DNA. DL DNA is made when initiation of synthesis occurs at DR1. RC DNA, the major product, is made when the RNA primer translocates to DR2 prior to initiation of DNA synthesis. We studied the mechanism that determines the site of the final RNase H cleavage in HBV. We showed that the sites of the final RNase H cleavage are always a fixed number of nucleotides from the 5' end of the pgRNA. This finding is similar to what was found previously for DHBV and suggests that all hepadnaviruses use a similar mechanism. Also, we studied the role of complementarity between the RNA primer and the acceptor site at DR2 in HBV. By increasing the complementarity we were able to increase the level of priming at DR2 over that seen in wild-type virus. This finding suggests that the level of initiation of plus-strand DNA synthesis at DR2 is sub-maximal for wild-type HBV. Finally, we studied the role of the sequence at the 5' end of the RNA primer that is outside of the DR sequence. We found that substitutions or insertions in this region affected the level of priming at DR1 and DR2.

Keywords

Hepatitis B virus; RNA primer; template switch; primer translocation; in situ priming

Introduction

Hepatitis B virus (HBV), a member of the hepadnavirus family, causes both acute and chronic infections of the liver. Approximately 2 billion people worldwide have been exposed to HBV with approximately 360 million being chronically infected (World Health Organization (2004) Hepatitis B vaccines. *Weekly epidemiological record* 79, 255–264). Individuals that are chronically infected have an increased risk of developing liver diseases such as hepatocellular carcinoma (HCC) and cirrhosis. There is a vaccine that effectively prevents HBV infection. However, the vaccine is not a treatment for those already infected. Current treatments for chronic HBV infection, such as nucleoside analogs and interferon-alpha, are not completely satisfactory. Plagued by drug resistance and undesirable side effects, these drugs are rarely effective at curing the infection¹ (World Health Organization (2004) Hepatitis B vaccines. *Weekly epidemiological record* 79, 255–264). Hence, there is a need for better treatments.

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To identify new targets for treatment, we need to better understand the mechanisms by which HBV replicates its DNA genome. All hepadnaviruses replicate their genomes by reverse transcription of an RNA precursor, the pregenomic RNA (pgRNA). Reverse transcription takes place within viral nucleocapsids in the cytoplasm of infected cells. Three template switches occur during synthesis of the major form of the genome, relaxed circular (RC) DNA. The first template switch occurs early during the synthesis of minus-strand DNA. After this template switch, the RNase H activity of the viral polymerase (P protein) degrades the pgRNA template during minus-strand DNA synthesis^{2; 3; 4} (Fig. 1a). Upon completion of minus-strand DNA synthesis, the 5' end of the pgRNA remains uncleaved and serves as primer for plus-strand DNA synthesis^{5; 6; 7; 8} (Fig. 1b). The 3' end of this RNA primer contains an 11 nucleotide (nt) sequence called DR1. In the major pathway, plus-strand DNA synthesis initiates after the RNA primer has translocated to a complementary sequence on the minus-strand template. This second template switch is called primer translocation. The complementary sequence, called DR2, is 11 nucleotides (nt) and is near the 5' end of the minus-strand DNA template⁸ (Fig. 1c). Once plus-strand DNA has been elongated to the 5' end of the template, a third template switch, called circularization, occurs. During circularization the 3' end of the nascent plus-strand DNA anneals to a short, complementary sequence at the 3' end of the minus-strand DNA^{8; 9; 10} (Fig. 1d). Plus-strand DNA synthesis resumes to ultimately make RC DNA (Fig. 1e). In the minor pathway for the synthesis of plus-strand DNA, the RNA primer is used at the site where it was generated, the 3' end of the minus-strand template. Elongation from this position on the template is called in situ priming and ultimately yields duplex linear (DL) DNA¹¹ (Fig. 1f). These two pathways of synthesis of plus-strand DNA are not equivalent for propagation of an infection. Virions containing RC DNA genomes are efficient at propagating an infection, while virions containing DL DNA genomes are not¹².

For HBV, the mechanisms that control the site of the final RNase H cleavage as well as the processes of in situ priming (to generate DL DNA) and primer translocation (to generate RC DNA) have not been studied directly. These mechanisms have been characterized more extensively in duck hepatitis B virus (DHBV). In DHBV the final RNase H cleavage to generate the RNA primer for plus-strand DNA synthesis occurs a fixed number of nucleotides (15–18 nt) from the 5' end of pgRNA⁶. DHBV has a mechanism to inhibit this RNA primer from initiating plus-strand DNA synthesis at DR1. A small DNA hairpin forms in the minus-strand DNA at the site of initiation of in situ priming, displacing the 3' end of the RNA primer from the template^{13; 14}. An equivalent, small hairpin does not occur in HBV. However, based on the disparity between the level of plus-strand DNA synthesis initiating at DR1 versus DR2, it stands to reason that all hepadnaviruses, including HBV, have mechanisms to inhibit in situ priming. The mechanism has not been identified for mammalian hepadnaviruses. In DHBV, sequence identity between the donor and acceptor sites for primer translocation is necessary for efficient priming at DR2¹⁵. Additionally, the nucleotide composition of the 5' end of the pgRNA influences priming at DR1 and DR2 in DHBV¹⁶. Analogous studies of the effect of the sequence at the primer translocation donor (DR1) and acceptor (DR2) site on in situ priming and primer translocation have not been reported for HBV.

We studied the mechanism that determines the site of the final RNase H cleavage for HBV. Also, we characterized the roles of the nucleotide sequence of DR1 and DR2 in the priming of plus-strand DNA synthesis at both sites. We present evidence that, like DHBV, the sites of the final RNase H cleavage are determined by the number of nucleotides from the 5' end of the pgRNA⁶. Also, we put forth evidence that the level of priming from DR2 is sub-maximal for the wild-type virus. Lastly, we show that the sequence adjacent to DR1 influences the level of priming at DR1 and DR2.

Results

Characterization of the RNA primers of WT HBV

To study the mechanism that determines the site of the final RNase H cleavage in HBV, we needed to establish the positions of the final cleavages for the wild-type virus. Huh7 cells were transfected with plasmids that expressed our wild-type HBV reference. We analyzed replicative intermediates from cytoplasmic nucleocapsids by primer extension with and without the RNA primer still attached to the 5' end of plus-strand DNA. Leaving the RNA primer intact allowed us to determine the 5' end of the RNA primer. Removing the RNA primer allowed us to identify the positions of the RNA:DNA junctions, corresponding to the sites of the final RNase H cleavage. By comparing the locations of all the 5' and 3' ends of the RNA primers within a sample, we could deduce the range of sizes (see Fig. 2a).

Characteristic of all hepadnaviruses studied to date, we found 5' ends of plus-strand DNA at multiple positions at both DR1 and DR2, indicating that the final RNase H cleavage occurs over a small range of sites (Fig. 3a and b)⁶. For DL DNA, the 5' ends of the plus-strand DNA corresponded to cleavages that occurred within the DR1 sequence (Fig. 3b). The RNA primers ranged in length from 11–15 nt with the longest RNA primer containing 10 out of the 11 nt of DR1. In contrast to DR1, one 5' end of plus-strand DNA at DR2 was consistently more prominent than the other ends (see Fig. 3b). This 5' end corresponded to an RNA primer containing all 11 nucleotides of DR1. Overall, RNA primers initiating plus-strand DNA synthesis at DR2 ranged in length from 12–16 nt. Most of the RNA primers initiated plus-strand DNA synthesis at both DR1 and DR2. However, the longest RNA primer, which contained all 11 nt of DR1, was observed only at DR2. This finding suggests that this RNA primer does not initiate plus-strand DNA synthesis at DR1, or does so at a greatly reduced level. This observation was not seen in DHBV⁶.

The sites of the final RNase H cleavage are a fixed number of nucleotides from the 5' end of pgRNA

For DHBV, the final RNase H cleavage occurs a fixed number of nucleotides from the 5' end of pgRNA and is not dependent on a specific sequence at the site of cleavage⁶. If HBV uses a similar mechanism, we predicted that changing the number of nucleotides between the 5' end of pgRNA and DR1 should shift the sites of the final RNase H cleavage relative to DR1. To test this prediction, we created variants InsA, InsB, InsC, and $\Delta 3$ (Fig. 4b). This hypothesis also predicts that substitutions at the normal site of cleavage should not alter the cleavage. To this end, we created variant SubD which has 6 nts at the wild-type cleavage sites altered (Fig. 4b).

The positions of the 5' ends of plus-strand DNA at DR1 and DR2 were determined for all five variants by primer extension (Fig. 2b, oligos 1 and 2; Fig. 4). When nucleotides were deleted or inserted between the 5' end of pgRNA and DR1, there was a corresponding shift in the position of the 5' end of plus-strand DNA relative to DR1 (Fig. 4). Changing the sequence at the wild-type cleavage sites, on the other hand, did not affect the position of the 5' end of plus-strand DNA (SubD, Fig. 4). Additionally, we determined that the 5' end of the RNA primer was the same as the wild-type reference for all variants (data not shown). Knowing the 5' and 3' ends of the RNA primers allowed us to conclude that the RNA primers for each variant were 11–16 nt in length, as was observed for the wild-type reference. For variants InsA and InsB longer RNA primers were used more frequently at DR2 while shorter RNA primers were used more frequently at DR1. A similar correlation was not seen with the other variants. Additionally, for InsA, InsB, and InsC, unlike the wild-type reference virus, the longest RNA primer was used at DR1 and DR2. For variants SubD and $\Delta 3$, the longest RNA primer was used only at DR1. This was likely due to the loss of complementarity at the 3' end of the longest

RNA primer with the DR2 site. Taken together, this data indicates that in HBV the final RNase H cleavage occurs a fixed number of nucleotides from the 5' end of pgRNA and is independent of the sequence at the site of the cleavage. Hence, HBV and DHBV appear to use a similar mechanism to define the 3' end of the RNA primer for plus-strand DNA synthesis⁶.

The level of priming from DR2 is proportional to the number of base pairs between the RNA primer and the minus-strand template

Although the longest RNA primer is 16 nt, it is annealed to the DR2 site through only 11 base pairs. Its 5'-terminal nucleotides are not annealed. We wanted to know whether altering the sequence adjacent to DR2 to increase the number of base pairs between the RNA primer and DR2 would increase priming from this site over the wild-type level. Related to this idea, we wanted to understand the impact of reducing complementarity between the RNA primer and DR2. To address these questions, we created a series of variants in which the amount of complementarity between the RNA primer and DR2 was increased or decreased (Fig. 5a). These variants were analyzed by Southern blotting and primer extension. Southern blotting was used to measure the level of RC DNA that each variant made. The level of RC DNA can be an informative, though indirect, measure of the level of priming from DR2. Primer extension analysis was used to directly measure the level of priming at DR2 (Fig. 2b, oligos 3 and 4).

Variants DR2-14 and DR2-16 were designed to increase the number of base pairs between the RNA primer and the DR2 site by 3 nt and 5 nt, respectively. Both variants had increased levels of priming from DR2 (160% and 177% the levels of the wild-type reference, respectively) (Fig. 5). Variants DR2-10, DR2-9, DR2-8, and DR2-7, which were designed to have progressively fewer base pairs between the RNA primer and the acceptor site, had decreasing levels of priming from DR2 (88%, 74%, 42%, and 25% the levels of the wild-type reference, respectively). Clearly, more base pairs between the RNA primer and DR2 increased priming while fewer base pairs reduced priming (Fig. 5b). These results indicate that complementarity is important for efficient priming at DR2. More interestingly, increasing complementarity beyond the wild-type level of 11 nt increased priming from DR2. This result suggests that the level of initiation of plus-strand DNA synthesis at DR2 is sub-maximal in the wild-type virus.

Complementarity is necessary, but not necessarily sufficient for efficient primer translocation

Next, we wanted to know whether complementarity between the RNA primer and DR2 was sufficient for priming of plus-strand DNA synthesis at DR2. To this end, we analyzed seven sets of variants in which the same substitution was placed into DR1, DR2, or both sites (Table 1). For each of the single mutations at DR1 or DR2, we expected to see reduced levels of priming at DR2 due to the reduced complementarity between the RNA primer and template. More importantly, if complementarity was sufficient for efficient priming at DR2, we expected to see the level of priming at DR2 restored to the level of our wild-type reference in the double substitutions.

As predicted, for all seven sets of variants, substitutions at DR1 or DR2 led to reductions in priming from DR2 (Table 1). More importantly, we found that restoration of complementarity between the RNA primer and minus-strand template completely restored priming at DR2 for some but not all variants (see Table 1). For variant sets A, B, and C when the mutations were present at DR1 and DR2 the level of priming at DR2 was similar to the wild-type reference (Table 1), indicating that complementarity between the RNA primer and DR2 was sufficient for restoration of primer translocation to wild-type levels. However, the other four sets of variants had only partial restoration in the level of priming from DR2 (ranging from 52% to 79% of the level of the wild-type reference; sets D, E, F, and G, Table 1). The lack of complete

restoration with the double mutants of these sets indicates the sequence of the DR makes a contribution to either the process of primer translocation or the inhibition of in situ priming.

The sequence between the 5' end of pgRNA and DR1 influences plus-strand priming

During the course of our analysis of the final RNase H cleavage, we noticed that variants InsA and InsB had 3-fold higher levels of in situ priming than the wild-type reference (Fig. 6b). This result suggested the insertions in these variants disrupted a *cis*-acting element that normally inhibits priming at DR1. However, InsC, which also contained an insertion at the same location, did not have an increased level of priming at DR1. This led us to question why variants InsA and InsB had increased priming at DR1 while variant InsC did not. Variants InsA and InsB inserted GCG and CGC, respectively, while variant InsC inserted UUU. Therefore, it was possible that the difference in the level of priming at DR1 between the variants was due to altering the number of G and C nucleotides in the sequence adjacent to DR1.

To test this idea we created variants SubA, SubB, and SubC (Fig. 6). These variants contained substitutions at the same location as the insertions in variants InsA, InsB, and InsC. The substitutions in SubA, SubB, and SubC mimicked the insertions in InsA, InsB, and InsC, respectively, except that variant SubB substituted GGG instead of CGC like InsB (see Fig. 6a). This alteration was done to avoid creating additional complementarity between the RNA primer and DR2, which could increase the level of priming at DR2 (refer to Fig. 5d). If the increase in priming from DR1 for InsA and InsB was due to increasing the number of G and C nucleotides in the sequence adjacent to DR1, then we would predict SubA and SubB to have increased priming at DR1 and SubC to have normal levels of priming at DR1.

Variants SubA, SubB, and SubC were analyzed by primer extension (see Fig. 2b). Unlike variants InsA and InsB, variants SubA, SubB, and SubC did not have increased priming at DR1 (see Fig. 6b). In fact, variant SubB had reduced priming from DR1 (39% that of the wild-type reference) (Fig. 6b). Therefore, the increased priming from DR1 for variants InsA and InsB was not caused by increasing the number of G and C nucleotides in the sequence adjacent to DR1. Nor was it caused by inserting three nucleotides at this location, as InsC did not have increased priming from DR1.

Additionally, all six of these variants were analyzed for their level of priming at DR2 (see Fig. 2b; Fig. 6c). For the insertion variants, the final RNase H cleavage site was shifted by 3 nt. Shifting the site of the final cleavage resulted in reduced complementarity between the RNA primer and DR2 for InsA and InsC. Therefore, it was predicted that these variants would have reduced priming at DR2. Indeed, InsA and InsC primed from DR2 at 28% and 77% the level of the wild-type reference, respectively (Fig. 6c). InsB, which did not have a reduction in complementarity between the RNA primer and DR2, had wild-type levels of priming at DR2 (Fig. 6c). The substitutions in variants SubA, SubB, and SubC also did not affect the complementarity between the RNA primer and DR2 and thus were predicted to have wild-type levels of priming at DR2. However, SubA, SubB, and SubC had reduced levels of priming at DR2 (28%, 26%, and 76% the level of the wild-type reference, respectively) (Fig. 6c).

Analysis of the sequence inserted or substituted for each of these six variants does not reveal a simple pattern or explanation for the effect on priming at DR1 and DR2. However, one common feature for all six variants is the location of the changes in the sequence. Therefore, this region between the 5' end of pgRNA and DR1 appears to make a *cis*-acting contribution that both inhibits priming at DR1 and promotes priming at DR2.

Discussion

We have shown that the final RNase H cleavage that generates the RNA primer for plus-strand DNA synthesis occurs at multiple adjacent sites and that these sites are a fixed number of nucleotides from the 5' end of pgRNA. This result also was seen for DHBV⁶, and suggests that most, if not all, hepadnaviruses use a similar mechanism. Unlike in DHBV, we found that the longest RNA primer in HBV initiates plus-strand DNA synthesis only at DR2 (see Fig. 3). We can offer two explanations for this observation in HBV. First, the longest RNA primer could be translocated very efficiently to DR2 such that no priming from DR1 takes place. Second, the longest RNA primer may be completely inhibited from priming at DR1.

We have shown that the extent of complementarity between the RNA primer and DR2 affects the level of priming at DR2. Progressively reducing the complementarity reduced the level of priming at DR2 (Fig. 5d). More interestingly, increasing complementarity resulted in levels of priming from DR2 greater than the wild-type reference (Fig. 5d). The ability to increase the level of priming at DR2 suggests that the process of primer translocation and/or initiation of plus-strand DNA synthesis at DR2 in the wild-type virus is sub-maximal. This begs the question as to why the wild-type virus does not have higher levels of priming from DR2. One explanation is that the nucleotide sequence immediately upstream of DR2 encodes an important but different function for the virus. For example, this sequence lies within the P and X genes. It is possible that there are selective pressures to maintain the amino acid sequence of one or both of these proteins. Another possible reason the virus does not initiate more DNA synthesis at DR2 may be that higher than normal levels of RC DNA are incompatible with sustaining an HBV infection in a host.

Although complementarity between the RNA primer and DR2 is important for efficient primer translocation, it is not necessarily sufficient, as was shown with variant sets D, E, F, and G (Table 1). In these sets, the variants that restored complementarity between the RNA primer and DR2, albeit with different nucleotides, did not have wild-type levels of priming at DR2. The inability of these variants to initiate plus-strand DNA synthesis at DR2 as efficiently as the wild-type reference, despite 11 nts of predicted complementarity, suggests that the sequence of DR1, DR2, or both makes *cis*-acting contributions in addition to complementarity.

The process of initiation of plus-strand DNA synthesis in HBV involves multiple steps including generation of the RNA primer, re-orientation of the polymerase on the template DNA, and priming. In addition, the RNA primer must translocate to DR2 prior to initiation of synthesis in order to form RC DNA. Each of these processes likely requires specific interactions between the polymerase and the RNA primer:DNA template duplex. We speculate that these interactions require that the RNA primer:DNA template maintain a specific conformation. There is precedence for this idea in HIV-1. Crystal structure, CD spectra, and chemical staining studies in HIV-1 have shown that the RNA primer:DNA template duplex has an unusual conformation that does not fit the conventional A or B forms and contains an unzipped region^{17; 18; 19}. This conformation was found to be dependent upon the sequence¹⁸ and multiple interactions between the polymerase and the RNA primer:DNA template duplex were detected¹⁷. Similar studies have not been performed on hepadnaviruses. Therefore, it is difficult to predict the conformation of the HBV RNA primer:DNA template duplex or specific nucleotide interactions with the polymerase. However, there are similarities between HBV's and retroviruses' RNA primer:DNA template duplex. All mammalian hepadnaviruses have a 5 nt poly(U) tract between the 5' end of the pgRNA and DR1 which includes the first 2 nt of DR1²⁰. The presence of a U-rich sequence immediately 5' to the plus-strand primer is also a common feature among retroviruses including human immunodeficiency virus type 1 (HIV-1), Rous sarcoma virus, and Moloney murine leukemia virus (Mo-MLV)^{21; 22; 23}. In Mo-MLV, substitutions and deletions in this region affect plus-strand DNA priming^{21; 24}. In HBV, we

found that substitutions and insertions within the poly(U) tract can influence the level of priming at DR1 and/or DR2 (Fig. 6). It is possible that this region in HBV has a similar function in initiation of plus-strand DNA synthesis. Establishing the conformation of the HBV polymerase-RNA primer:DNA template complex would aid in understanding the impact of our mutations on initiation of plus-strand DNA synthesis at DR1 and DR2.

Here we have shown that, while there are some similarities between DHBV and HBV regarding the initial steps of plus-strand DNA synthesis, there are also several differences. Both DHBV and HBV appear to use a similar mechanism to establish the site of the final RNase H cleavage to generate the RNA primer for plus-strand DNA synthesis, and both require complementarity between the RNA primer and DR2 for efficient initiation of synthesis at DR2^{6; 15}. However, the mechanisms by which HBV and DHBV inhibit *in situ* priming and promote primer translocation appear to be different. In avian hepadnaviruses, such as DHBV, a hairpin forms in the minus-strand DNA at the 3' end of the RNA primer¹³. This hairpin inhibits *in situ* priming. A similar secondary structure has not been identified in HBV. Additionally, previous studies in DHBV showed a direct relationship between the number of G and C nucleotides adjacent to DR1 and the level of priming at DR1 and DR2¹⁶. The greater the number of G and C nucleotides, the higher the level of priming at DR1 and the lower the level of priming at DR2. Our HBV analyses with variants SubA, SubB, and SubC do not show a relationship between the number of G and C nucleotides and the level of priming at DR1 and/or DR2 (see Fig. 6). The apparent difference in the role of the sequence surrounding DR1 in avian versus mammalian hepadnaviruses suggests that the two groups developed different mechanisms for discriminating between priming at DR1 and DR2.

Materials and Methods

Molecular Clones

All HBV molecular clones were derived from HBV subtype *ayw* (GenBank accession number V01460). Nucleotide position 1 is the C of the EcoRI site (GAATTC). The wild-type (WT) reference for $\Delta 3$, InsA, InsB, InsC, and SubD was LJ144. LJ144 is derived from pHc-9/3091 (kindly provided by Michael Nassal). LJ144 expresses HBV pgRNA under the control of the cytomegalovirus (CMV) immediate early promoter and does not express the envelope proteins²⁵. The WT reference for DR2-16, DR2-14, DR2-10, DR2-9, DR2-8, DR2-7, variant sets A through G, SubA, SubB, and SubC was NL84, which expresses HBV pgRNA under the control of the CMV immediate early promoter and does not express P, C, X, or envelope proteins²⁶. For both LJ144 and NL84 the transcription start sites of pgRNA are identical to those found with the endogenous HBV promoter²⁵. These sites correspond to nt 1817 and nt 1818. The expression plasmid for the HBV replication proteins, LJ96, has been described²⁵.

Mutations at DR2 were made using the linker-tailing method with two complementary oligonucleotides containing the desired mutation and spanning from the RsrII to BmgBI sites²⁷. Mutations at DR1 were created by oligonucleotide-directed mutagenesis. The upstream primer incorporated the desired mutation and the BsiWI restriction site, which is located at the junction of the CMV promoter and HBV sequence in our plasmids. The mutagenic primer was used with a primer downstream of BspEI to generate a DNA fragment via PCR that then was digested with BsiWI and BspEI and substituted into NL84 or LJ144. All variants analyzed in the LJ144 background were also analyzed in the NL84 background. The results were similar in both backgrounds.

Cell culture and isolation of viral replicative intermediates

The human hepatoma cell line, Huh7, was used in all analyses. These cell cultures were grown in DMEM-F12 medium supplemented with 5% fetal bovine serum at 37°C. Transfection of

the cell cultures was performed on 100mm or 60mm dishes that were approximately 90% confluent using a calcium phosphate precipitation method²⁸. When NL84 or one of its derivatives was transfected, LJ96 was co-transfected to express the replication proteins. The medium on each cell culture was changed daily until the cultures were harvested at day 5.

Viral replicative intermediates were isolated from cytoplasmic capsids as described²⁶ with the following modifications. After removal of the nuclei, the cell lysate was adjusted to 2mM CaCl₂, then incubated with 44U of micrococcal nuclease, and 200 ng of RNase at 37°C for 2 hours. After pronase digestion viral replicative intermediates were extracted with a mixture of phenol-chloroform, followed by chloroform and either stored in ethanol or precipitated with ethanol and resuspended in 10mM Tris, 0.1mM EDTA.

Southern Blot analysis

Southern blotting was performed as described²⁹. Membranes were hybridized with a genome-length riboprobe that detected minus-strand DNA. Autoradiography and quantitation were performed using a Molecular Dynamics Storm PhosphorImager and ImageQuant5.2 software.

Primer extension analysis

All oligonucleotides were end labeled with [γ -³²P]ATP. To determine the position of the RNA:DNA junctions at the 5' end of plus-strand DNA, viral replicative intermediates were treated with NaOH to hydrolyze the RNA primer, prior to primer extension analysis. To determine the positions of the 5' end of the RNA primer, replicative intermediates were analyzed with the RNA primer intact. To determine the positions of the 5' ends of plus-strand DNA at DR1, oligo 1, which is complementary to nt 1948-1926, was used. To determine the positions of the 5' ends of plus-strand DNA at DR2, oligo 2, which is complementary to nt 1661-1638, was used.

To determine the level of priming at DR1 and DR2 primer extension with two oligos in each reaction was performed. In every reaction oligo 3, which anneals to minus-strand DNA at coordinates 1772 to 1788, was used to measure the level of minus-strand DNA in the sample. To determine the level of priming at DR1 oligo 3 was paired with oligo 1. To determine the level of priming at DR2 oligo 3 was paired with oligo 4, which is complementary to nt 1680-1661 on the plus-strand DNA. By measuring the level of both minus-strand DNA and plus-strand DNA in the same reaction we could directly compare the two values without concern for variations in the manipulation of the sample or during the loading of the gel. To account for variation in end labeling and primer extension efficiency of the two oligonucleotides we had a separate reaction with linearized plasmid DNA. Products of the primer extension reactions were electrophoresed on a 6% denaturing polyacrylamide gel. Autoradiography was done with a Storm PhosphorImager. Quantification was performed using ImageQuant5.2 software.

Calculations

The percent of RC DNA relative to total full length minus-strand DNA was calculated using Southern blotting as follows: %RC = [RC/(total full length (-) DNA)]. The value RC was the measurement of the DNA from labeled RC. The value "total full length (-) DNA" was the measurement of all DNA forms between and including RC and SS DNA. Primer extension was used to calculate the level of plus-strand priming at DR1 and DR2 as follows: priming efficiency at DR1 = [(priming at DR1/oligo 1 control)/(minus-strand DNA 5' termini/oligo 3 control)] $\times$ 100; priming efficiency at DR2 = [(priming at DR2/oligo 4 control)/(minus-strand DNA 5' termini/oligo 3 control)] $\times$ 100. Each of these values was then normalized to a wild-type reference (wild type = 100).

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Figure 1.

The reverse transcription pathway of HBV. (a) The pgRNA (thin line) is the template for the synthesis of minus-strand DNA. As the minus-strand DNA (thick grey line) is synthesized, the RNase H activity of the P protein degrades the pgRNA. (b) The final RNase H cleavage leaves the 5' end of pgRNA, which will then serve as primer for plus-strand DNA synthesis. (c) Primer translocation. The RNA primer translocates to a complementary sequence at DR2, which is near the 5' end of the minus-strand DNA template. (d) Plus-strand DNA synthesis (thick black line) begins and proceeds to the end of the template. (e) Circularization. The 3' end of the nascent plus-strand DNA anneals to a complementary sequence, 3' r, on the minus-strand

template and plus- strand DNA synthesis resumes. RC DNA subsequently is made. (f) In situ priming. Less frequently, plus-strand DNA synthesis initiates at DR1. DL DNA is synthesized.

Figure 2.

Schematic representations of primer extension assays. (a) Primer extension analysis to determine 5' and 3' end of RNA primers to deduce their lengths; minus strand DNA indicated by thick grey line, plus-strand DNA indicated by thick black line, RNA primer indicated by thin black line; arrow represents oligo with dotted line indicating product generated by primer extension; to determine the 5' end of the RNA primer, primer extension analysis was performed with the RNA primer still present (product A); to determine the 3' end of the RNA primer samples were treated with NaOH prior to primer extension analysis to remove the RNA (product B); RNA primer lengths (C) were determined by subtracting the length of each A product by the length of each B product. (b) Representation of all primer extension oligos used

and their templates; oligos 1 and 2 were used to determine the 5' and 3' ends of the RNA primer at DR1 (oligo 1) and DR2 (oligo 2); oligos 1, 3, and 4 were used to determine priming efficiency at DR1 (oligos 1 and 3 in same reaction) or DR2 (oligos 3 and 4 in same reaction).

Figure 3.

Characterization of sites of the final RNase H cleavage to generate the RNA primer for plus-strand DNA synthesis for wild-type HBV. (a) Primer extension analysis with oligo 1 after removal of RNA primer. Arrows indicate positions of 5' ends of plus-strand DNA initiated at DR1. (b) Primer extension analysis with oligo 2 after removal of RNA primer. Arrows indicate positions of 5' ends of plus-strand DNA initiated at DR2. (c) 5' end of pgRNA, DR1 sequence underlined; arrows represent sites of final RNase H cleavage based on RNA:DNA junction sites at DR1 or DR2. Grey arrow represents cleavage site seen at DR1 only ~50% of the time.

Figure 4.

The final RNase H cleavage occurs a set number of nucleotides from the 5' end of pgRNA. (a) Primer extension analysis with oligo 1 after removal of RNA primer showing 5' ends of plus-strand DNA that initiated synthesis at DR1. (b) 5' end of pgRNA for each variant; DR sequence is underlined; insertions and substitutions indicated in bold, deletion denoted by dotted line. Arrows indicate all RNA:DNA junction sites seen at DR1 and/or DR2; grey arrows indicate 5' ends of plus-strand DNA only seen at DR2 and believed to be caused by aberrant translocation events.

Figure 5.

Priming at DR2 for wild type HBV is sub-maximal. (a) Sequence of pgRNA at 5' end and acceptor site for each variant. DR sequence is boxed, substitutions are bolded. Variant names reflect the number of predicted base pairs between the RNA primer and DR2 (b) Southern blot probed with genome-length, minus-strand-specific riboprobe. RC, DL, and full-length minus-strand DNA (SS DNA) indicated on left. (c) Primer extension analysis with oligos 3 and 4 to determine priming efficiency at DR2. RNA primer removed from samples prior to primer extension analysis. Oligo 4 product, representing 5' end of plus-strand DNA at DR2, indicated on left by (+). Oligo 3 product, representing 5' end of minus-strand DNA, indicated on left by (-). Number of predicted base pairs indicated at bottom (d) Priming efficiency at DR2 for each variant; values normalized to WT reference; WT value set at 100.

Figure 6.

Sequence between 5' end of pgRNA and DR1 influences priming efficiency at DR1 and DR2. (a) Sequence of pgRNA for each variant. DR sequence is boxed, insertions and substitutions are bolded. (b) Level of priming at DR1 as determined by primer extension. All variants were normalized to the wild-type reference, which was set to a value of 100. (c) Level of priming at DR2 as determined by primer extension. All variants were normalized to the wild-type reference, which was set to a value of 100.

Table 1

Complementarity is not always sufficient for efficient primer translocation

Variant set	DR Sequence: UUCACCUCUGC	Location of substitutions	Priming efficiency at DR2 (% WT) ¹
A ³	UUCACCAGACG	DR1	15 ± 17
		DR2	20 ± 16
		DR1/2	96 ± 18
B ³	AAGUGGAGACG	DR1	13 ± 6
		DR2	18 ± 6
		DR1/2	112 ± 18
C, ²³	UUCAGGACUGC	DR1	11 ± 11
		DR2	19 ± 4
		DR1/2	90 ± 12
D ³	AAGUGCUCUGC	DR1	23 ± 6
		DR2	13 ± 6
		DR1/2	65 ± 12
E ⁴	CCUGUUCUCAU	DR1	8 ± 3
		DR2	14 ± 6
		DR1/2	61 ± 16
F ³	GGCACCCUGC	DR1	14 ± 8
		DR2	11 ± 3
		DR1/2	56 ± 9
G ⁵	UUAACAUUUAC	DR1	3
		DR2	4
		DR1/2	79

Substitutions indicated in bold

¹ values based on primer extension analysis² values for set C based on Southern blotting analysis³ each variant analyzed at least 7 times⁴ each variant analyzed 4 times⁵ each variant analyzed twice