Hepadnavirus Reverse Transcription Initiates within the Stem-Loop of the RNA Packaging Signal and Employs a Novel Strand Transfer

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Replication of the hepadnavirus genome occurs by reverse transcription of an RNA pregenome and is mediated by the viral polymerase; the polymerase is also required for packaging of the pregenome through interaction with the RNA packaging signal, ϵ . Previous work suggested that reverse transcription of minus-strand DNA initiates within the sequence element DR1 (direct repeat 1) and that disruption of DR1 activates a cryptic initiation site in a downstream copy of ϵ . However, using active duck hepatitis B virus polymerase expressed in a yeast Ty vector system, we demonstrate that synthesis of minus-strand DNAs with 5' ends at DR1 requires the stem-loop of ϵ , whereas the production of DNAs mapping to ϵ does not require DR1. Mutations at ϵ that remove homology between ϵ and DR1 eliminate reverse transcripts with 5' ends in DR1, and restoring homology at DR1 to a mutant ϵ partially restores DNAs mapping to DR1. Insertions of one nucleotide into the bulge region of the ϵ stem-loop increase the length of minus-strand DNA whose 5' ends map to DR1 by one nucleotide. Thus, very short minus-strand primers are initiated within ϵ , rather than in DR1 as previously supposed; they are then transferred to a four-nucleotide homology in DR1. Transfer was also observed *in vivo* during replication of duck hepatitis B virus in avian cells; in this case, transfer is from the 5' copy of ϵ to the 3' copy of DR1. This minus-strand transfer reaction is likely to be a general feature of all hepadnaviruses.

The hepadnaviruses are unique among the animal DNA viruses in that they replicate through reverse transcription of an RNA intermediate (19). The reverse transcription mechanism of these viruses is highly distinctive and differs in many important respects from the well-established paradigm of RNA-directed DNA synthesis found in retroviruses and many retrotransposons. For example, hepadnaviruses employ a protein rather than a tRNA primer for the first (minus)-strand DNA synthesis; second (plus)-strand priming involves the cleavage and translocation of a capped RNA primer, and the resulting DNA product is circular rather than linear (reviewed in references 8 and 13). Despite limited sequence homologies to its retroviral counterparts, the hepadnavirus polymerase (P) is also very distinctive. P itself is the primer for minus-strand synthesis (3, 5, 21) and, unlike other reverse transcriptases, is required for packaging of the RNA pregenome into the viral cores (2, 9). Thus, a fuller understanding of hepadnavirus replication should contribute importantly to our understanding of the diversity of RNA-directed DNA synthesis.

In this report, we examine the nature of minus-strand initiation in the duck hepatitis B virus (DHBV). The current view of this process is outlined in schematic form in Fig. 1. The RNA template for the reaction is a terminally redundant RNA of 3.3 kb that is generated by read-through transcription from the circular viral DNA. Included in the terminal redundancy are two important *cis*-acting motifs: (i) ϵ , an RNA stem-loop structure required for RNA packaging (10, 11, 15); and (ii) direct repeat 1 (DR1), a 12-nucleotide (nt) element thought to be important in minus-strand initiation. As noted above, pregenomic RNA encapsidation requires both P and the ϵ

RNA target site. Importantly, although ϵ is present twice in genomic RNA, only the 5' copy is functional in encapsidation (10, 11): deletion of the 5' ϵ ablates RNA packaging and DNA production, while deletion of the 3' ϵ allows both reactions to proceed (17). Following encapsidation, minus-strand DNA is synthesized. The 5' end of minus-strand DNA maps within DR1 (nt 2537) (7, 12, 14), and deletions of DR1 greatly reduce viral DNA synthesis (7, 17); these and other data have suggested that DR1 is the principal initiation site for minus-strand synthesis. There are two copies of DR1 on the RNA template; examination of genetically marked hepadnaviruses has suggested that only the 3' copy is functional in reverse transcription (17). On the basis of these results, the reverse transcription reaction is classically depicted as initiating within the 3' copy of DR1 (Fig. 1, step 2).

A detailed understanding of the biochemistry of the hepadnavirus reverse transcription mechanism has been greatly hampered by the fact that active P cannot be solubilized from viral cores (16) and that P has been difficult to express in an enzymatically active form in heterologous systems (1). We have recently succeeded in expressing active DHBV P in yeast cells, using the retrotransposon Ty as a vector (20). In this system, P is expressed as a fusion protein with the TyA structural protein (DP, analogous to a retroviral *gag-pol* fusion) and is packaged along with its chimeric Ty-DHBV mRNA into a virus-like particle (VLP). The encapsidated RNA contains both DR1 and ϵ at its 3' end. Expression of this clone in yeast cells results in the synthesis of protein-linked minus-strand DHBV DNA templated by this transcript. Primer extension analysis of the product DNA indicates that the 5' ends map to two start sites: the correct position (nt 2537) within DR1 and a cryptic site (nt 2576) within a 6-nt bulge in the ϵ stem-loop (20). Viral DNAs originating at this cryptic site

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FIG. 1. Classical hepadnavirus reverse transcription mechanism. Pregenomic RNA (dashed line; step 1) is capped and polyadenylated and has a large terminal redundancy (R). The locations of DR1 and DR2 are indicated with numbered boxes, and the ϵ stem-loops are diagrammed. Pregenomic RNA is packaged into cores along with polymerase (P; shaded oval) through interaction with ϵ . P initiates reverse transcription at the 3' DR1 (step 2), and the DNA is extended. The pregenomic RNA is degraded by the RNase H activity of P (step 3) until P reaches the 5' end of the template, where it leaves an RNA oligomer annealed the 5' copy of a short terminal duplication in minus-strand DNA (r; step 4). The RNA oligomer is transferred to DR2, where it primes plus-strand DNA synthesis (lower solid line; step 5). During elongation, a second template transfer circularizes the genome and the plus-strand is extended for a variable length (step 6) to yield mature progeny viral DNA.

had been previously observed in DHBV-infected cells, but only when DR1 was deleted and then only at low levels (7). The significance of these species mapping within ϵ was unclear, since the 3' copy of ϵ was known to be dispensable for reverse transcription *in vivo*. However, all *in vivo* analyses of this matter are complicated by the fact that a second copy of the ϵ sequences must be preserved at the 5' end of the genome to allow genomic RNA packaging.

Our Ty-polymerase fusion system is not dependent on ϵ for packaging either the polymerase or its RNA template. We therefore used this system to analyze the *cis* requirements for initiation of minus-strand DNA in the absence of a wild-type copy of these signals. Here we show that the origin of reverse transcription is within the stem-loop of ϵ and not within DR1 as was previously believed. Nascent chains initiated within ϵ are transferred to DR1, where they are extended to their mature length. Although the ϵ sequences used in the Ty system are supplied from the 3' position, in authentic DHBV replication it is the 5' copy of these sequences that is the functional origin. These data, and similar recent results independently obtained by Wang and Seeger (22), radically alter our view of the early events in hepadnavirus DNA synthesis.

MATERIALS AND METHODS

Plasmid construction. pTYBDP is a yeast shuttle expression plasmid containing the DHBV3 P gene within a galactose-inducible Ty1 element (20). pTYBDP-dl1 was created by deletion loop mutagenesis of pTYBDP to remove DHBV nt 2531 to 2606. All other mutant derivatives of pTYBDP were constructed by PCR; the mutant fragments were sequenced and inserted into pTYBDP-dl1 by standard techniques. Plasmids constructed to analyze the initiation sites contain the mutation DRM1 (nt 2533 to 2537, ATTAC to CACGT) or DRM2 (nt 2533 to 2535, ATT to TCC), dlBulge (nt 2572 to 2576 deleted), SLM2 (nt 2572 to 2576, TTTAC to CACGT, creating a *Pml*I site), SLM5 (A2575G), SLM6 (T2574C), SLM8 (insert C after nt 2575), or SLM9 (insert A after nt 2575). These mutations were introduced singly or in combination, as indicated in the construct name. Alterations at DR1 and the stem-loop of ϵ were combined by use of a *Hind*III site created between these elements by mutating nt 2555 to 2556 from CT to AA; creation of this site had no effect on the minus-strand replication activity (data not shown). D1.5G is an overlength DHBV3 expression construct cloned in pBS(-)

FIG. 2. Minus-strand DNAs mapping to DR1 are dependent on the stem-loop. (A) The RNA structures produced from the mutants are diagrammed; indicated are the stop codon for the P open reading frame (UAA), DR1, and the stem-loop. Leftward-pointing arrows represent the 5' ends of the minus-strand DNAs. Gaps and dashed lines represent deletions and substitutions of heterologous sequences, respectively. The columns at right indicate the presence (+) or absence (-) of minus-strand DNAs with 5' ends at DR1 or the stem-loop (SL). (B) Primer extension analysis of the minus-strand DNAs produced from the constructs diagrammed in panel A. The mutants examined in each lane are indicated above the corresponding lanes. The positions of 5' ends mapping to DR1 and the bulge of the stem-loop are marked at the right, and a sequencing ladder of pTYBDP primed with D2453+ is to the left. DP-MS is a derivative of pTYBDP containing missense mutations in the reverse transcriptase active site (YMDD to YMHA [6]).

(Stratagene Cloning Systems) and contains a 5' duplication of nt 1658 to 3021. Plasmids D1.5Gc2574 and D1.5Gc2575 are derivatives of D1.5G and contain mutations in the 5' copy of the ϵ sequences (T2574C and A2575G, respectively).

TyDP VLP isolation. TyDP fusion VLPs were isolated from the *spt3 Saccharomyces cerevisiae* strain YH51, as the endogenous Ty elements are not expressed in this background (4). Following induction of VLP expression with galactose, spheroplasts were prepared and lysed by agitation with glass beads, and VLPs were partially purified by sucrose gradient centrifugation as described previously (20).

VLP DNA purification and primer extension. DNA was isolated from the VLP preparations by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation (20). The 5' ends of the DHBV minus-strand DNAs were determined by reiterative primer extension, using a thermal cycler. 32 P-end-labeled oligonucleotide D2453+ (1.5 ng, 5'-CCGCCTGATTGGACGGCTTTTC) was mixed with DNA isolated from 1 μ g of VLP protein extract and subjected to 35 to 55 cycles of extension (94°C for 1 min, 51°C for 1.5 min, 73°C for 2.5 min) at 1.5 mM MgCl₂ with 1.0 U of *Taq* DNA polymerase in a 50- μ l reaction volume. The product DNAs were extracted with 1:1 phenol-chloroform, ethanol precipitated, resolved on a 6% acrylamide-7 M urea sequencing gel, and detected by autoradiography.

Cell culture, DHBV core DNA isolation, and Southern blotting. LMH cells (a chicken hepatoma cell line) were grown in 1:1 Dulbecco minimal essential medium-Ham nutrient mixture F12 supplemented with 10% fetal bovine serum (Gibco Laboratories). LMH cells (3×10^6) were transfected by calcium phosphate coprecipitation as described previously (18). DHBV DNA was extracted 4 days posttransfection from

cytoplasmic cores as described previously (18), with the following modification. Transfected plasmid DNA in the lysates was digested by adding CaCl₂ to 5 mM and micrococcal nuclease (200 U; Boehringer Mannheim Biochemicals) and incubating the mixture at 37°C for 1 h; digestion was terminated by adding EGTA to 7.5 mM. DHBV DNAs were resolved on 1% agarose-Tris-borate-EDTA gels without ethidium bromide, transferred to Hybond N (Amersham), and probed with DHBV sequences as described earlier (18).

PCR amplification of viral DNA. Cytoplasmic DHBV core DNA was digested with *DpnI* to reduce the level of contaminants of the input plasmids, and the digestion mixture was amplified by PCR (25 cycles; 94°C for 1 min, 50°C for 2 min, 72°C for 1 min); primers were 5'-GTAGAAGCTTCTGCAGCG AAAGCAATATATATTCCACATAGG and 5'-AGAAGGTA CCGAATTCTAAAGCATCTTTTGCATCTCTAAC. The PCR products were digested with *PstI* and *EcoRI* and cloned into pBS(-), and the sequences of DR1 and ϵ were determined in multiple individual clones.

RESULTS

Dependence of start sites at DR1 on the stem-loop of ϵ . To determine if the dominant start site within DR1 and the cryptic site within the stem-loop were used independently of each other, we deleted the elements individually or in combination (Fig. 2A) and determined the 5' ends of the resulting minus-strand DNAs by primer extension. DNAs from the wild-type construct pTYBDP gave the expected products mapping to DR1 and the bulge of the stem-loop; these products were not made when DP carried missense mutations in the reverse transcriptase active site (Fig. 2B, lanes 1 and 2). Minus-strand

FIG. 3. Minus-strand DNA at DR1 requires homology to a site in the stem-loop. (A) Sequences flanking the 5' ends of minus-strand DNAs are shown for wild-type and mutant constructs (nt 2531 to 2548 for DR1 and nt 2569 to 2584 for the bulge of the stem-loop [SL]). DR1 and bulge sequences are boxed, mutant nucleotides are underlined, and observed DNA 5' ends produced by each construct are indicated with leftward arrows. Nucleotide numbers for DNA 5' ends observed with wild-type constructs are indicated above the sequence. (B) Primer extension analysis of the minus-strand DNAs produced from the constructs in panel A. The positions of 5' ends mapping to DR1 and the bulge of the stem-loop are marked at the right, and a sequencing ladder of pTYBDP primed with D2453+ is to the left. Lanes 8 and 10 show primer extension products from 2 ng of pTYBDP-SLM2 cut with *Pml*I. Lanes 9 and 10 are a darker exposure from a different experiment to better show the signal at DR1 for DP-DRM2/SLM2.

DNAs were not detected when both DR1 and the stem-loop were deleted (DP-dl1; lane 3). When DR1 was replaced with heterologous sequences, no DNAs were found mapping to the DR1 position, but there was no change in products mapping to the bulge (DP-SL; lane 4). As expected, 5' ends were not detected at the bulge when the stem-loop was deleted. Unexpectedly, however, none were detected at DR1 either (DP-DR1; lane 5). To confirm this observation with a less radical mutation, 5 nt were deleted from the bulge; again, no minus-strand DNAs were detected at DR1 (DP-dlBulge; lane 6). (Very weak signals whose 5' ends flank the bulge deletion [nt 2571 and 2579] upon prolonged exposure of these autoradiograms could be detected; no 5' ends were observed at DR1 even at this more sensitive level [data not shown].) These results indicate that minus-strand DNAs with 5' ends mapping

FIG. 4. Minus-strand DNA primers are transferred from the stem-loop to DR1. (A) Sequences flanking the 5' ends of minus-strand DNAs are shown for wild-type and mutant constructs as described in the legend for Fig. 3A. The downstream start sites for SLM5 and SLM6 have not been determined precisely; the potential locations of the 5' ends are indicated by AC above the arrow. (B) Primer extension analysis of the minus-strand DNAs produced from the constructs in panel A. The positions of 5' ends mapping to DR1 and the bulge of the stem-loop are marked at the right, and a sequencing ladder of pTYBDP primed with D2453+ is to the left. Lane 6 shows primer extension products from 2 ng of pTYBDP-SLM2 cut with *Pml*I.

to DR1 are dependent on the stem-loop, whereas those DNAs mapping to the stem-loop are independent of DR1.

Transfer of minus-strand DNA from the stem-loop to DR1. Inspection of the sequences at the stem-loop and around DR1 revealed that there was an identity of 4 nt between these sites (TTAC; Fig. 3A). We tested whether this identity might serve to direct a homology-mediated strand transfer reaction of nascent minus-strand DNA from the stem-loop to DR1. In such a reaction, sequences reverse transcribed from the stem-loop would be the donor and those in DR1 would be the acceptor. This notion was made attractive by the key observation of Wang and Seeger (21) that the principal product of in vitro reverse transcription by DHBV polymerase is a P-linked

FIG. 5. Mutations in the 5' stem-loop of ϵ are transferred to DR1 during viral replication in vivo. (A) Southern blot of cytoplasmic core DNA 4 days posttransfection showing the relaxed circular, double-stranded (DS) linear, and single-stranded (SS) linear viral DNA forms. DNAs used were wild-type D1.5G (WT), 5' ϵ mutants T2574C and A2575G, and the reverse transcriptase active site mutant (MS). (B) Pregenomic RNA contains the mutations (X) in the bulge of the 5' ϵ stem-loop (SL). After initiation at the 5' stem-loop (curved arrow), the nascent minus-strand DNAs are transferred to the 3' copy of DR1 (straight arrow) and extended. Completion of reverse transcription yields circular viral DNA containing a single copy of each DR1 and the ϵ coding sequences in the orientation shown. The three possible configurations for the DNA are shown, with the positions of the mutations indicated with X's. The number of clones in each configuration obtained for the mutants following PCR amplification of ϵ is shown at the right.

tetranucleotide corresponding to the minus-strand complement of this sequence.

The mutations constructed to test this hypothesis are shown in Fig. 3A, and primer extension analysis of these mutants is shown in Fig. 3B. Primer extension on DNA from a construct carrying a 5-nt substitution in the bulge of the stem-loop (DP-SLM2; Fig. 3A) reveals two dominant ends mapping within the stem-loop, nt 2574 and 2582 (Fig. 3B, lane 2). Start sites at nt 2582 are also seen with the wild-type stem-loop, although less efficiently, and nt 2574 is the nearest C in the mutant bulge to the normal start site at nt 2576. When this mutation was paired with DR1 (DP-DR1/SLM2; Fig. 3B, lane 3), a moderate signal was observed at DR1; however, inspection of the sequence at the downstream stem-loop start site at nt 2582 revealed a 3-nt homology (TAC) with the proposed DR1 acceptor site (cf. Fig. 3A). We therefore altered DR1 to remove homology to both upstream sites (DRM1; Fig. 3A); no minus-strand DNAs at DR1 were detected from this construct when it was expressed alone (DP-DRM1; Fig. 3B, lane 4), paired with SLM2 (DP-DRM1/SLM2; lane 5), or paired with the wild-type stem-loop (data not shown). We then restored homology at DR1 to the novel initiation site at nt 2574 in SLM2 (mutant DRM2; Fig. 3A). No 5' ends were observed at DRM2 when it was expressed alone (Fig. 3B, lane 6); however, a weak signal was detected when it was paired with SLM2 (Fig. 3B, lane 7, and a darker exposure from another experiment in lane 9).

The recovery of 5' ends at DR1 when homology was restored to a site in the stem-loop is consistent with a strand transfer from the stem-loop to DR1, but the poor efficiency of the transfer with the mutant donor to the homologous mutant

acceptor led us to examine the reaction in another manner. We inserted either a C (DP-DR1/SLM8) or an A (DP-DR1/SLM9) into the bulge between nt 2575 and 2576 to increase the size of the minus-strand DNA primers (Fig. 4A). Primer extension of DNA from these constructs showed doublets at the start sites in the stem-loop for both mutants (Fig. 4B, lanes 4 and 5), with the lower band corresponding to initiation at the inserted nucleotide (the normal position relative to the 5' side of the bulge) and the upper band (carrying the inserted nucleotide) due to initiation at the normal position, nt 2576. Importantly, both mutants had a doublet of 5' ends at DR1; one end was at the wild-type position (nt 2537), and the second was 1 nt larger at nt 2538 (Fig. 4B, lanes 4 and 5). The larger of these DNAs can be explained only by transfer of the lengthened primer from the stem-loop to DR1. The smaller product is likely due to transfer from the lower band in the stem-loop.

Marker transfer from DHBV 5' ϵ to DR1 in vivo. We next considered whether this primer transfer process might also operate during authentic DHBV replication in vivo. Paradoxically, earlier work had shown that the 3' copy of ϵ was dispensable for minus-strand DNA synthesis (17). However, as mentioned earlier, in these studies a wild-type copy of ϵ was retained at the 5' end of the RNA (to ensure proper RNA encapsidation). We therefore examined whether this 5' copy of ϵ might in fact serve as the template for the true primer in vivo. To do this, two mutations (T2574C and A2575G) were inserted individually into the 5' copy of ϵ in the overlength DHBV expression construct D1.5G. If minus-strand DNA initiates in the 5' stem-loop and extends for at least 2 (A2575G) or 3 (T2574C) nt prior to transfer, the mutation in the stem-loop will be transferred to DR1 in the resulting viral DNA.

FIG. 6. Proposed hepadnavirus reverse transcription mechanism. The symbols are the same as those used for Fig. 1. Minus-strand initiation (Fig. 1, step 2) now consists of two steps. P initiates reverse transcription at the 5' stem-loop and extends minus-strand DNA (solid line) for 3 to 4 nt (step 2a). Then P and the covalently attached nascent DNA are transferred to the 3' DR1 (step 2b), and the DNA is extended. The subsequent steps of the mechanism are as described in Fig. 1.

These mutant plasmids were transfected into LMH cells, and 4 days later cytoplasmic core DNA was examined by Southern blotting (Fig. 5A). Both mutants were replication competent, although they replicated less efficiently than did wild-type DHBV (32 and 23% for T2574C and A257G, respectively). As expected from earlier work (7, 12, 14), primer extension of these core DNAs detected 5' ends at DR1 only; no extension products mapped to the stem-loop (data not shown). (In the yeast Ty system, constructs carrying the identical mutations did produce DNAs with 5' ends at both the stem-loop and at DR1 [DP-DR1/SLM5 and DP-DR1/SLM6; Fig. 4A and B, lanes 2 and 3]; see Discussion.) The DR1 ϵ region (nt 2490 to 2750) from the cytoplasmic DNAs was then amplified by PCR and cloned, and the sequences at DR1 and the stem-loop were determined for multiple clones from each mutant (Fig. 5B). The results for T2574C were definitive: 21 of the 22 PCR clones sequenced had the T-to-C mutation at both the original position in the stem-loop and

also at DR1 (T2535C). The remaining clone carried the mutation at neither location and was a PCR product of either (i) the 3' end of the input plasmid DNA or (ii) a DNA primed by transfer to DR1 of a primer initiated at the 3' stem-loop. The A2574G mutation was transferred to DR1 (A2536G) in 14 of the 22 clones sequenced; 6 of the remaining clones were PCR contaminants from the 3' end of the input plasmids (or were transfer products from the 3' stem-loop). Two clones carried the mutation at the stem-loop only. These clones represent either initiation at DR1 without transfer (i.e., the classical model of initiation, as in Fig. 1) or are false negatives resulting from PCR amplification of the 5' end of the plasmid or from circularization of the template during plus-strand synthesis prior to copying the transferred lesion in DR1 (our PCR strategy relies on amplifying the viral plus-strand DNA because the primers flank the gap in the minus-strand DNA).

Given these possibilities for the origin of clones with wild-

type DR1 sequences, the observed transfer frequencies represent minimal measurements of the relative frequency of initiation at the 5' stem-loop versus DR1. Since all 21 unambiguous reverse transcription products for T2574C initiated at the marked stem-loop and transferred the mutation to DR1, binomial probability analysis indicates that at 95% confidence, no more than 13% of the initiation events could have occurred at DR1 in the course of normal viral replication.

DISCUSSION

Using a yeast Ty expression system that is not dependent on ϵ for template packaging, we have demonstrated that initiation of DHBV reverse transcription occurs within the stem-loop and not at DR1 as was previously supposed. The viral DNAs whose 5' ends map to DR1 are derived not by de novo initiation there but by transfer of short nascent P-linked chains initiated within the ϵ stem-loop in a reaction mediated in part by homology between the primer and the acceptor site in DR1 (see below). While the 3' copy of ϵ serves this origin function in our Ty constructs, marker transfer experiments in the context of the viral genome indicate that it is the 5' copy of ϵ that carries out this function in vivo. Independently of this work, Wang and Seeger have obtained closely similar results, using both DHBV P made in vitro and marker transfer experiments in vivo (22). Together, these results overturn earlier models of hepadnavirus reverse transcription in which the priming site was assigned to DR1 and establish the existence of a previously unsuspected template transfer reaction in minus-strand synthesis. This new model is summarized in Fig. 6. The conservation of homology between DR1 and the bulge in all hepatitis B viruses strongly argues that this mechanism will be applicable to all members of this virus family.

The previous identification of the initiation site as nt 2537 within DR1 was based on data gathered by analysis of reverse transcription products produced in vivo. The 3' copy of DR1 was taken to be the initiation site on the basis of marker transfer studies very similar in outline to those reported here (17). Mutations just upstream of the 5' DR1 were not incorporated into the 5' end of viral DNA, while identical lesions placed near the 3' DR1 were. In retrospect, however, we now know that these mutations were outside of the sequences that are actually involved in the template transfer and hence failed to detect the transfer. In the context of our present understanding (Fig. 6), what these prior studies actually establish is (i) that the 3' copy of DR1 is the acceptor site for the transfer and (ii) that the transferred primer can be no longer than 5 nt.

The transfer of the minus-strand primer usually employs a short region of homology. However, homology alone is not sufficient to explain all features of the transfer: there appear to be sequence specificity and/or context effects that are also important. Transfer can occur when 1 of the 4 nt of homology between the sites is altered (T2574C and A2575G) or when the primer is lengthened by 1 nt (DP-DR1/SLM8 and DP-DR1/SLM9). Such changes would be expected to significantly affect hybridization of a 4-nt nucleic acid. More compellingly, the inefficient transfer of DP-DRM2/SLM2 despite 6 nt of homology between the donor and acceptor sites in this construct argues strongly that homology per se is not the sole determinant of transfer. Finally, the fidelity with which the transfer reaction selects nt 2537 as the 5' end of the acceptor (instead of other UAC or UUAC motifs near the 3' DR1) cannot be explained simply by homology considerations and suggests that other structural determinants may affect acceptor site choice.

A more detailed analysis of these features of the transfer reaction is in progress.

The results of the marker transfer experiment in avian cells (Fig. 5) establish that the vast majority of priming events occur at the 5' copy of ϵ but do not exclude the possibility of a low level of initiation within the 3' copy of DR1. However, if this occurs in vivo, it is unlikely to contribute more than a few percent of the total products of reverse transcription. These experiments also suggest that either the 3' stem-loop is rarely used for initiation in vivo or, if it is, products from this site are neither extended beyond a few nucleotides nor transferred to DR1. How this 3' ϵ copy is silenced as a productive initiation site in wild-type DHBV is not understood, but its use in DR1 deletion mutants (7) indicates that its capacity to function in vivo is not irreversibly abrogated.

DHBV P initiates reverse transcription by interacting with the same copy of ϵ that is employed for RNA packaging. The primary sequence motifs for binding and recognition by P lie outside of the bulge in the stem-loop (15a), and the bulge appears primarily to provide an appropriately structured template for initiation (which appears to strongly prefer to utilize a C). This is reminiscent of the role of the bulge of the human hepatitis B virus stem-loop in pregenomic RNA packaging (15); in fact, detailed mutational analysis of the *cis* requirements for both the packaging and priming reactions reveals them to be remarkably similar (15a). These findings suggest the satisfyingly parsimonious model that only a single RNA recognition event may be required to trigger both RNA encapsidation and reverse transcription.

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