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Specific Hepatitis B Virus Minus-Strand DNA Synthesis Requires Only the 5′ Encapsidation Signal and the 3′-Proximal Direct Repeat DR1*

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Human hepatitis B virus (HBV) is a small DNA virus that replicates inside the viral nucleocapsid by reverse transcription of an RNA intermediate, the pregenome. The sequences encompassing the encapsidation signal ε and the direct repeat DR1 are present in two copies on this terminally redundant transcript. We have recently shown that HBV minus-strand DNA synthesis involves transfer of a short DNA primer copied from 5′-ε to 3′-DR1 (DR1*). Using transfection of HBV genomes with lesions in 3′-ε, and 5′-DR1 and its preceding sequence, we tested whether these additional elements contribute to the specificity of the transfer reaction. However, while some mutations affected proper plus-strand DNA formation, 5′-ε and DR1* were completely sufficient for correct minus-strand DNA production.

Hepatitis B virus (HBV) is the type member of the *Hepadnaviridae*, a family of small enveloped hepatotropic DNA viruses that replicate through reverse transcription of an RNA intermediate (reference 23; for reviews, see references 12 and 14). Related viruses have been found in other mammalian (e.g., woodchuck hepatitis B virus [WHV]) and avian (e.g., duck hepatitis B virus [DHBV]) hosts. The partially double-stranded circular HBV DNA genome in extracellular virions is only about 3.2 kb in length. Internal promoter and enhancer elements, all overlapping with coding information, drive transcription of several sets of subgenomic and genomic RNAs with differing 5′ ends but common 3′ termini. The genomic transcripts are terminally redundant (Fig. 1A); hence, several essential regulatory elements are present in more than one copy. One of the genomic RNAs, the pregenome, is used to translate both core protein, forming the capsid shell, and P protein, the viral replication enzyme. Interactions of P protein with the 5′-proximal structured encapsidation signal ε (7, 16, 18a) on this RNA mediate specific packaging (6) and nucleocapsid assembly (2).

Reverse transcription, a process which appears to work properly only within the capsid (11, 28), was thought to initiate inside the direct repeat DR1* close to the 3′ end of the pregenome. Meanwhile, we have shown that, similarly as in DHBV (24, 26), HBV ε is also required for minus-strand DNA production: a bulged region inside the bipartite stem-loop structure serves as template for a short primer which, covalently bound to P protein, is transferred to DR1* at the other end of the pregenome (13). Only two nucleotides (nt) of complementary sequence at the 3′ end of the primer were sufficient for transfer to DR1*; hence, additional specificity determinants must exist.

Although mutations in 5′-ε and DR1* were dominant over the wild-type (wt) copies of 3′-ε and 5′-DR1 present on the mutant pregenomes, the latter elements, being in close proximity to the active signals, could contribute to specific transfer (Fig. 1B). 3′-ε might act as an intermediate acceptor that targets the P-primer complex formed at 5′-ε to the nearby DR1*. Alternatively, though less likely in view of previous data from the WHV (21, 22) and DHBV (4) systems, the complex could first jump to the nearby 5′-DR1, be elongated to the 5′ end of the pregenome, and then use the additional sequence complementarity for specific transfer to the identical sequence at, and preceding, DR1*. Such a jump between adjacent elements occurs if DHBV P protein is translated from mRNAs that lack the 5′ copies of DR1 and the ε-homologous region (region 1) of the bipartite DHBV encapsidation signal (3); there, a primer synthesized at 3′ region 1 is translocated to the nearby DR1* (17, 26); in this incomplete system, transfer specificity is apparently relaxed.

To explicitly examine a potential involvement of 5′-DR1 and/or 3′-ε in HBV replication, we constructed mutant HBV genomes (Fig. 1C) that either lack 5′-DR1 (construct 3122) or parts of 3′-ε (constructs Δε and Δεb) or contain a marker nucleotide in the region preceding 5′-DR1 (construct 190) that, if its complement were part of an extended primer, could be detected by a method we have developed to sequence the 5′-terminal nucleotides of minus-strand DNA (13). After transfection into Huh7 or HepG2 cells, we tested for minus-strand DNA formation by primer extension, for the accuracy of primer transfer by determining the 5′-terminal sequence of minus-strand DNA, and for plus-strand DNA formation by the endogenous polymerase reaction (see, e.g., references 11 and 18). Upon provision of deoxynucleoside triphosphates, one of which is radioactively labeled, P protein present in core particles produces a mixture of linear (L) and relaxed circular (RC) DNA (Fig. 1A) from the packaged RNA pregenome which can be sensitively detected by agarose gel electrophoresis. As shown below, none of these lesions had any dramatic effect on minus-strand DNA formation. However, removal of, and point mutations in, the 5′ region of the pregenome led to a loss of RC-DNA formation.

HBV pregenomes carrying lesions in 5′-DR1 and 3′-ε. All constructs used in this study are based on the previously described HBV expression plasmid pCHT-9/3091sCX/E (13). The parental transcription unit consists of the cytomegalovirus immediate-early promoter and an overlength HBV genome (subtype ayw) from nucleotide position 3091 (numbering system of Pasek et al. [15]) to position 88, i.e., after the authentic

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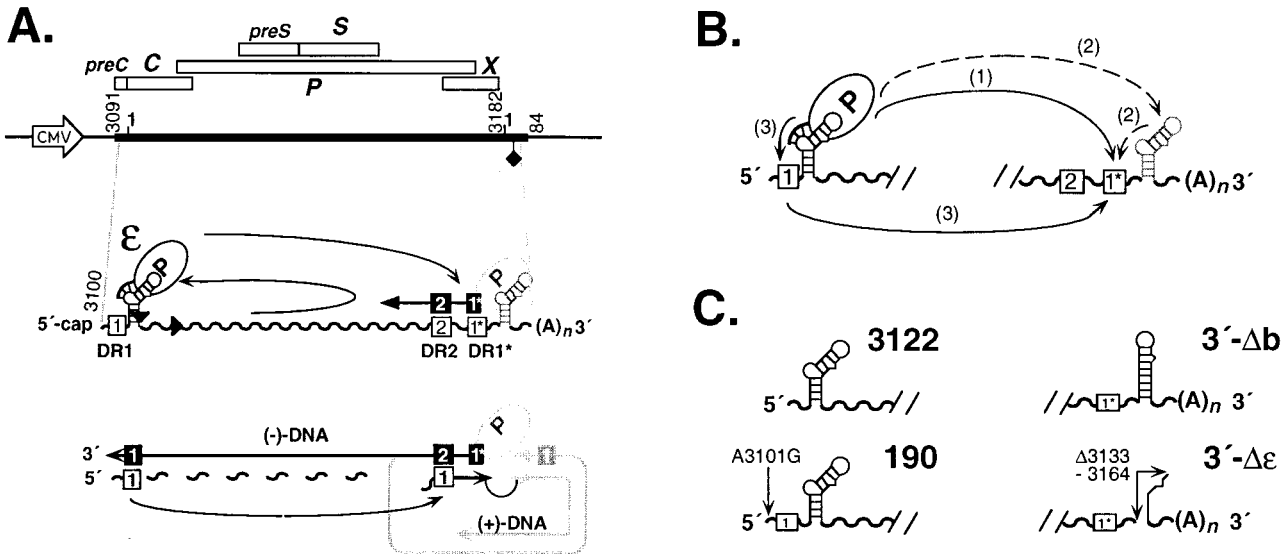

FIG. 1. Reverse transcription of the HBV pregenome. (A) Interactions of P protein with *cis* elements on the pregenome. The HBV transcription unit used in this study consists of an overlength genome (solid black line) under control of the cytomegalovirus immediate-early promoter; the diamond represents the polyadenylation signal. The bars on the top show the four major open reading frames. The pregenome (wavy line) serves as mRNA for core and P protein (start codons symbolized by triangles). P binds to 5′-ε (stem-loop) and copies part of the bulge region, and the covalent complex is transferred to DR1*. During minus-strand DNA formation, the RNA is degraded, except for a 5′-terminal oligonucleotide containing 5′-DR1. Plus-strand DNA initiated after its transfer to DR2 is extended to the 3′ end of minus-strand DNA, and a template switch to the 5′ end yields RC-DNA (shaded); in situ extension of the riboprimer gives a linear DNA. (B) Options for the involvement of 5′-DR1 and/or 3′-ε in primer transfer. (1), direct transfer; (2), 3′-ε as targeting device for the P-primer complex to DR1*; (3), initial jump to 5′-DR1 and extension to the 5′ end, giving a primer that perfectly matches over some 12 nt to the sequence preceding DR1*. (C) Mutations in the 5′- and 3′-terminal region of the pregenome used to test the transfer options. Variant 3122 lacks the 22 5′-terminal residues including 5′-DR1; variant 190 contains, inter alia, an A-to-G substitution at position 3101; in variant Δεb, the bulge of 3′-ε and, in Δε, the entire left half of the stem-loop are deleted.

HBV polyadenylation signal (Fig. 1A). Transcription starts at position 3100 and yields a terminally redundant genomic RNA essentially identical to the authentic pregenome. A *Cla*I (position 7)-to-*Xba*I (position 241) restriction fragment of the core open reading frame is derived from a synthetic core gene; the nucleotide exchanges do not measurably influence replication (10). A single point mutation (A-3123→G) creates a unique *Esp*3I restriction site between DR1* and 3′-ε. The relevant mutations in this study are schematically shown in Fig. 1C: in variant 3122, genomic RNA is expected to start at position 3122 because of deletion of the preceding nucleotides; hence, the entire 5′-DR1 region is absent. In variant Δεb, the bulge region of 3′-ε is removed; this lesion, if present in 5′-ε, abolishes encapsidation competence (7, 16). A more drastic deletion was produced by removing the *Esp*3I-to-*Sty*I restriction fragment carrying the left half of the 3′-ε sequence. This mutation should not at all allow for a similar secondary structure as present in wt-ε. Construct 190 bears several point mutations in the 5′-proximal region of the pregenome (see Fig. 3A). The first pair of substitutions creates a *Hin*dIII site (C-3099→A; A-3101→G). A genomic RNA starting at the authentic position 3100 should carry the signature G residue at position 3101. A second pair of mutations (T-3126→G; C-3127→A) generates a *Cla*I site (position 3123) immediately preceding the lower stem of ε; these mutations are not expected to be relevant for replication.

Normal minus-strand DNA formation in the absence of 5′-DR1 and 3′-ε. A possible influence of the above-described deletions on minus-strand DNA synthesis was monitored by conventional primer extension analysis (Fig. 2). Three days posttransfection with the corresponding constructs, core particles were harvested by anticore immunoprecipitation, and nucleic acid was extracted by proteinase K digestion and phenol extraction as previously described (11); after RNA hydrolysis by alkali treatment, primer extension was performed with a 5′-^{32}P-labeled plus-sense oligonucleotide corresponding to the HBV sequence from positions 2978 to 2995 (Fig. 3B, primer P2). For calibration, a sequencing ladder was generated by using the same primer oligonucleotide on cloned HBV DNA as template. Products were separated on a denaturing gel containing 6% polyacrylamide.

In accord with previous analyses, all constructs gave essentially the same band pattern as the wt, i.e., a major band migrating at position 3108 and a weaker product longer by 1 nt. The signal from construct 3122 was consistently lower than that from wt. This decrease, however, coincided with a two- to threefold lower core protein production, as measured by en-

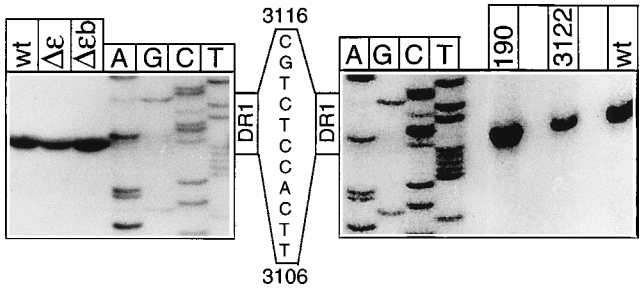

FIG. 2. Influence of 5′- and 3′-proximal pregenome lesions on minus-strand DNA synthesis. The 5′ ends of minus-strand DNA from constructs Δε, Δεb, 3122, and 190 were mapped by primer extension with 5′-^{32}P-labeled primer P2 (cf. Fig. 3B); wt refers to the products from the previously described construct pCHT-9/3091sCX/E (13). Extension products were separated on a denaturing gel containing 6% polyacrylamide. Lanes A, G, C, and T show a sequencing ladder obtained on cloned HBV DNA with the same primer. The primer extension products were also used for determination of the 5′-proximal minus-strand DNA sequences (cf. Fig. 3B).

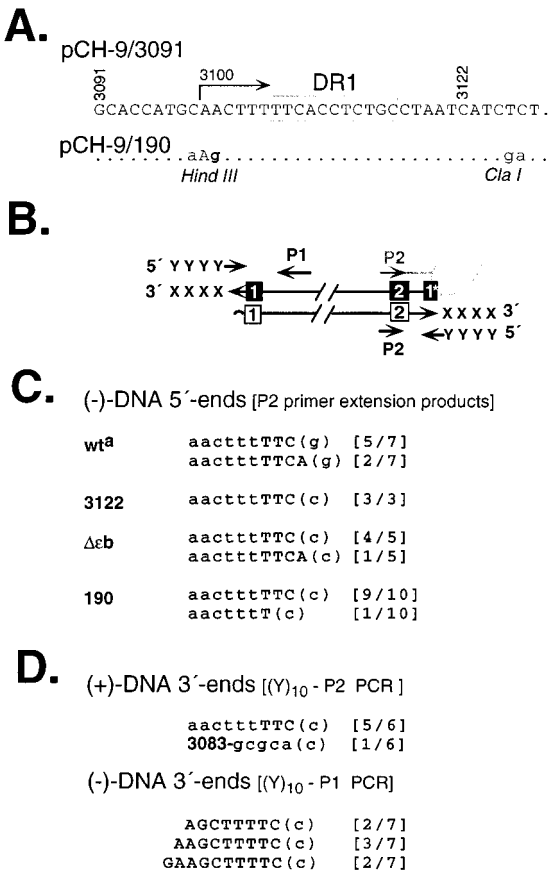

FIG. 3. Influence of 5′- and 3′-proximal mutations on transfer specificity. (A) 5′-terminal HBV sequences in constructs pCHT-9/3091sCX/E and pCH-9/190. Cloned HBV DNA starts at position 3091; cytomegalovirus promoter-driven transcription starts at nt 3100; nt 3122 is the expected start site in construct pCH-9/3122. In pCH-9/190, nucleotide exchanges C-3099→A and A-3101→G generate a *Hin*dIII site and T-3026→G and C-3027→A generate a *Cla*I site. (B) PCR-assisted sequencing of terminal regions in minus- and plus-strand DNA. cDNA obtained by primer extension with primer P2, after homo-oligomer tailing and amplification, gives the sequence at the 5′ end of minus-strand DNA (13). Similarly, L-DNA from an endogenous polymerase reaction can be tailed with a homo-oligomer (XX) at both 3′ ends. Amplification with a complementary homo-oligomer (YY) and primer P1 followed by cloning reveals the 3′-terminal sequence of minus-strand DNA, and amplification with P2 reveals that of plus-strand DNA. (C) Complement to 5′-terminal minus-strand DNA sequences derived by primer extension sequencing from constructs 3122, Δεb, and 190. Uppercase letters show ε-bulge-derived sequence; the nucleotide in parentheses indicates the first residue identical to the homo-oligomer tail. Numbers in brackets show the frequency of a specific sequence in the total number of isolates analyzed. Sequences obtained with the wt construct are shown for comparison (13). (D) Complement to 3′-terminal sequences in L-DNA from construct 190. The 3′ ends of minus-strand DNA contain the expected nucleotide exchanges; they are centered around the expected pregenome start site at nt 3100. The majority of plus-strand DNA 3′ ends correspond to the 5′ end of minus-strand DNA; one sequence, ending after nt 3087, was derived from an incompletely extended plus-strand DNA.

zyme-linked immunosorbent assay; also, the construct lacks the 5′-terminal sequence of the authentic pregenome that can stimulate encapsidation (16). Hence, the mutations had no detectable influence on the accuracy of primer transfer and only marginally, if at all, affected its efficiency.

To corroborate this result, we used a technique that we have recently established to determine the sequence of the 5′-terminal nucleotides in minus-strand DNA: by tailing with a homo-oligomer, the gel-purified primer extension products can be cloned and sequenced (13); the sequence preceding the homo-oligomer tail corresponds to the 3′ end of the primer extension product and hence to the 5′-end of minus-strand DNA. The results were again identical to those obtained with the wt construct, i.e., the majority of the products ended with TTC, in agreement with their position on the primer extension gels (Fig. 3C).

The sequence preceding 5′-DR1 is not involved in minus-strand DNA initiation. The 5′ truncation in variant 3122 is a rather severe mutation; we therefore analyzed whether the more subtle change in primary sequence of variant 190 (Fig. 3A) would give an indication for a two-step primer transfer mechanism (Fig. 1B, option 3). A prerequisite for possibly finding the indicator nucleotide, the complement to G-3101, in the 5′-proximal region of minus-strand DNA is its presence on the genomic transcript from construct 190; the two nucleotide exchanges on the DNA in immediate vicinity to the expected transcription start might influence the actual start site. Therefore, we determined the sequence of the 3′ end of the minus-strand DNA produced from construct 190 by a modification of the PCR-assisted sequencing protocol (Fig. 3B): full-length DNA obtained by the endogenous polymerase reaction (see below) was isolated from the agarose gel and tailed with a homo-oligomer; this provides a PCR anchor sequence at the 3′ ends of both minus- and plus-strand DNA. By using an HBV oligonucleotide of appropriate polarity as second primer (Fig. 3B, primers P1 and P2), the terminal sequences of both DNA strands can be selectively amplified, cloned, and sequenced. As shown in Fig. 3D, we found a slight length variation at the 3′ end of minus-strand DNA, but all sequences analyzed contained the expected indicator nucleotide. Conversely, the clones derived from the 3′ end of plus-strand DNA, which is copied from the 5′ end of minus-strand DNA, had the wt sequence. In agreement with this result, none of the sequences derived from the primer extension product sequencing procedure contained the G residue in question (Fig. 3C); hence, primer transfer from 5′-ε to DR1* is direct.

Lesions in the 5′-terminal pregenome region but not in 3′-ε influence RC-DNA formation. Formation of the RC form of the HBV DNA genome (Fig. 1A) requires transfer of an oligoribonucleotide derived from the 5′ end of the pregenome comprising 5′-DR1 to DR2 on the newly generated minus-strand DNA, extension of plus-strand DNA toward the 5′ end

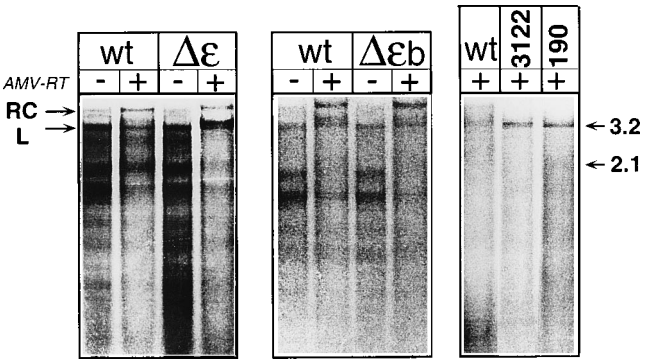

FIG. 4. Influence of 5′- and 3′-proximal lesions in the pregenome on HBV DNA formation. Core particles obtained by transfection of the indicated constructs were subjected to the endogenous polymerase reaction. DNA products were analyzed by agarose gel electrophoresis, either without (−) or with plus an additional in vitro fill-in reaction with avian myeloblastosis virus reverse transcriptase (11, 18). The position of L and RC wt genomes, and of labeled DNA size markers of 3.2 and 2.1 kb in length, is indicated. To account for some variability in the amounts of RC-DNA, the products obtained from parallel transfections with the wt construct pCHT-9/3091sCX/E are shown for all experiments.

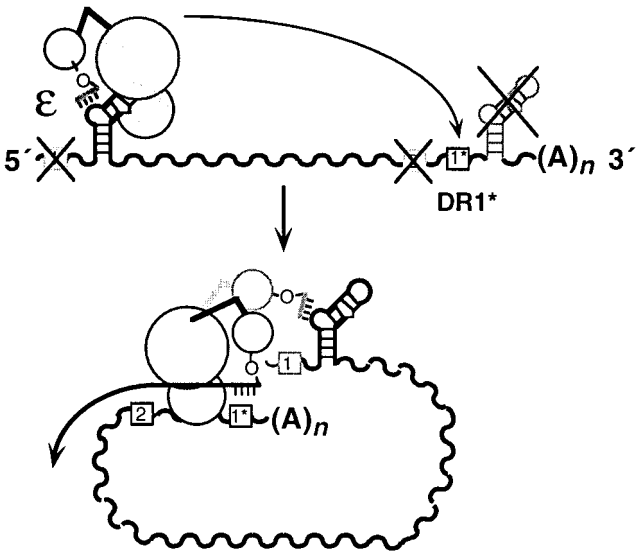

FIG. 5. Model for P protein-primer transfer from 5′-ε to DR1*. All available data are consistent with a direct transfer mechanism. Circularization of the pregenome would plausibly explain how transfer from 5′-ε at one end to DR1* at the opposite end of the pregenome occurs. The ends may be brought together by direct RNA-RNA interactions but, more likely, by the RNA interacting with viral and/or cellular proteins.

of minus-strand DNA, and a template switch to the short terminal redundancy present at the 3′ end of minus-strand DNA. Plus-strand riboprimers failing to be transferred can initiate in situ plus-strand DNA synthesis from their original location, generating linear double-stranded molecules, both in DHBV (8) and in HBV (11). When the above-described mutants were analyzed in the endogenous polymerase assay, variants Δεb and Δε produced a mixture of L and RC-DNA, comparable to that from the wt construct (Fig. 4); in all cases, the fraction of RC-DNA relative to replicative intermediates could be increased by a subsequent fill-in reaction with avian myeloblastosis virus reverse transcriptase (11, 18). Hence, this assay confirmed that 3′-ε has no detectable function in the basic HBV replication cycle. However, both construct 3122 and construct 190 gave rise only to L-DNA (Fig. 4, right panel). For 3122, this result was expected, as the riboprimer, defined by distance from the 5′ end of the pregenome (8), lacks the DR1 sequence required for transfer to DR2; hence, only linear DNA is formed. The RC defect in mutant 190 is less clear; at least a fraction of the minus-strand DNA 3′ ends and hence pregenome 5′ ends was longer than expected (see above; Fig. 3D). Such extended riboprimers, shifted by 1 or more nt toward the 5′ end, may not comprise a sufficiently large part of DR1 for transfer to DR2. However, the nonidentity in the terminal redundancy in minus-strand DNAs from the construct may also affect the template switch, during plus-strand DNA synthesis, to the 3′ end of minus-strand DNA of riboprimers that were translocated to DR2.

Conclusions. The activity of several *cis* elements that are present more than once on the terminally redundant hepadnaviral pregenome is regulated by their respective positions. As encapsidation signal, ε is active only at a 5′-proximal position (6); probably as a consequence, the same copy is used as replication origin for minus-strand DNA initiation; productive primer transfer, however, occurs specifically to the 3′-proximal DR1*. While the data presented above confirm the roles of 5′-DR1 and its preceding sequence in plus-strand DNA and RC-DNA formation, they demonstrate that both this region and 3′-ε are not involved in proper minus-strand DNA production. Hence, the mechanism underlying the specific primer transfer from one end of the pregenome to the other remains to be established. The exclusive use of the 3′-proximal polyadenylation signal has been explained by the absence of upstream activating elements from, and promoter proximity of, its 5′ counterpart (19, 20). Similarly then, transfer-activating elements upstream of DR1* and/or inactivation of 5′-DR1 could be responsible for the exclusive primer acceptor function of DR1*. We consider it unlikely, however, that such regulatory signals are effective on a stretched-out, linear pregenome; rather, we favor the view that 5′-ε and DR1*, though far apart in primary sequence, are in fact in close spatial proximity, probably by the RNA adopting some kind of circularized shape (Fig. 5). Whether RNA-RNA and/or RNA-protein interactions would bring the ends together will require further investigations. Candidates are a second binding site for P protein in the vicinity of DR1* and cellular proteins that interact with 5′- and 3′-proximal mRNA regions (9), but also the specific environment provided by the core particle as, in vivo, encapsidation and replication of the pregenome appear to be tightly coupled. While it has been stressed that only P protein and pregenome are required for minus-strand DNA initiation (24), proper replication as a whole depends on the functional integrity of the core particle (11, 28). In addition, though the sequence of events has not been firmly established, RNA encapsidation is likely to precede reverse transcription: P protein with a defective polymerase domain still supports encapsidation (1, 5); hence, primer formation is not a prerequisite for RNA packaging. Appropriately supplementing the recently introduced in vitro translation system for active DHBV P protein (17, 25–27) with its relaxed primer transfer specificity should allow definition of the factors that determine the exquisite position dependence observed under in vivo-like conditions.

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