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A Bulged Region of the Hepatitis B Virus RNA Encapsidation Signal Contains the Replication Origin for Discontinuous First-Strand DNA Synthesis

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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. Encapsidation of this RNA pregenome is mediated by the interaction of the viral replication enzyme P with the structured 5′-proximal RNA element ε; replication was thought to start in the 3′-proximal direct repeat DR1*. However, recent data obtained with the duck hepatitis B virus indicated a novel, discontinuous mechanism of negative-strand DNA synthesis. Here we demonstrate, using DNA transfection of complete HBV genomes, that the 3′-half of a 6-nucleotide bulge in HBV ε whose primary sequence is not important for encapsidation serves as template for a short DNA primer that is subsequently transferred to DR1*. Apparently, P protein copies any template sequence that does not interfere with ε structure; however, altered primary sequences can induce polymerase stuttering, resulting in extended primers containing more than one equivalent of the template sequence. The importance of the bulged structure is emphasized by the dependence of primer length on bulge size. Transfer specificity is in part controlled by sequence complementarity. The strategy of using the 5′ encapsidation signal as the origin of replication for discontinuous negative-strand DNA synthesis, common to mammalian and avian hepadnaviruses, suggests the evolutionary origin of hepatitis B viruses to lie between that of modern retroviruses and primitive retroelements like the Mauriceville retroplasmid.

Hepatitis B virus (HBV), the causative agent of B-type hepatitis in humans, is the type member of the *Hepadnaviridae*. These small enveloped hepatotropic DNA viruses replicate through reverse transcription of an RNA molecule, the pregenome (42). Despite this fundamental relationship to retroviruses, they exhibit distinct characteristics (for a review, see reference 30) and hence are classified as pararetroviruses (for a review, see reference 39). Their tiny 3-kb genome is compactly organized, with many coding and regulatory functions overlappingly arranged on the same nucleotide sequence (Fig. 1A). Two of the few primary translation products are directly involved in assembly of replication-competent nucleocapsids: core protein from the pre-C/C open reading frame (ORF) forms the protein shell of the icosahedrally symmetrical core particle (10, 16); P protein, specified by the P ORF, is the viral replication enzyme. Both proteins are separately translated from the same terminally redundant genomic RNA (Fig. 1A). Of all viral transcripts, exclusively this RNA serves also as pregenome; i.e., it is packaged into nucleocapsids and reverse transcribed into the circular, partially double-stranded DNA genome found in extracellular virions.

Copackaging of pregenome and replication enzyme is mediated by interaction between P protein (1, 14) and, for HBV, a short 5′-proximal sequence on the RNA, called ε (15), that acts as an encapsidation signal. On a subset of slightly longer genomic transcripts that contain the complete pre-C/C ORF, 5′ ε is apparently inactivated by translating ribosomes (29). For unknown reasons, the 3′ copy of ε present on all viral transcripts is inactive (15). Hence, all available evidence points to an initial binding of P to 5′ ε (Fig. 1A) as the triggering event for pregenome encapsidation (3).

Reverse transcription, however, involving covalent attachment of P protein to the 5′-terminal nucleotide of negative-strand DNA (2), requires targeting of P to the 3′-proximal RNA region (Fig. 1B). It was thought that one of three copies of a direct repeat element, DR1*, close to the 3′ end (nucleotides [nt] 2872 to 2882), represents this origin of replication (40, 54). Accordingly, hepadnaviruses would produce their first DNA strand in a continuous fashion, in contrast to almost all other known retroelements. However, recent data obtained for duck hepatitis B virus (DHBV) indicated that negative-strand DNA synthesis initiates with the production of a short DNA primer within the RNA region corresponding to HBV ε and its subsequent transfer to DR1* (34, 44, 46, 49, 50). Unfortunately, only predictions are available for the structure of the ε-homologous region in DHBV; the structure most closely resembling that of HBV ε (15) differs substantially from that predicted to be the most stable one. Also, in DHBV, this RNA element is not by itself sufficient for encapsidation but depends on a second region several hundred nucleotides apart (6).

By contrast, the secondary structure of HBV ε is well defined: it adopts a bipartite stem-loop structure with a 6-nt bulge, a 6-nt loop, and a single unpaired U residue (Fig. 2). Deletion studies had indicated that these subelements are critical for the encapsidation function (12, 18, 33). By selection for encapsidation-competent individuals from pools of partially randomized ε sequences, we recently obtained evidence that, surprisingly, the importance of primary sequence in the ε bulge was distinctly different in its 5′ and 3′ parts: the wild-type (wt) nucleotides C and U at positions 1 and 2 of the bulge were strongly selected for, while almost all nucleotides were tolerated at the following positions. From these data, we proposed a tentative structural model for the P protein-ε interaction, with the salient feature that only the 5′ part of the bulge is in

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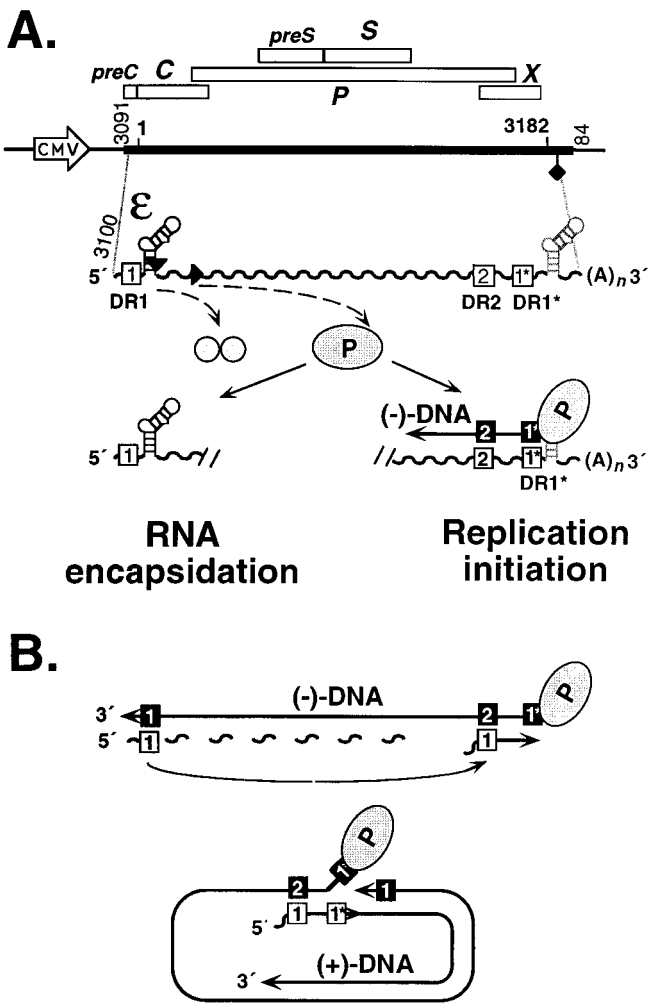


FIG. 1. Basic aspects of HBV replication. (A) Genome organization and interactions of P protein with the pregenome. The solid black line represents a linear version of the HBV DNA genome that, in the virus, is a partially double-stranded circular molecule of 3,182 nt (subtype ayw). Transcription of an authentic RNA pregenome can also be driven by foreign promoters (arrowhead), e.g., the cytomegalovirus (CMV) immediate-early promoter used in this study, from linear overlength DNA constructs. The diamond symbolizes the 3′-proximal polyadenylation signal. The open bars on the top show the four ORFs of HBV. The RNA pregenome (wavy line) starts at nt 3100 (numbering system according to reference 32) and, after one unit length, ends with a terminal redundancy of about 130 nt; hence, DR1 and ε (stem-loop structure) are present at both ends. The pregenome serves as mRNA for core (small spheres) and P protein whose binding to 5′ ε mediates RNA packaging; replication was thought to initiate de novo inside DR1*. (B) Classical model of HBV reverse transcription. Concomitantly with continuous negative-strand DNA [(−)-DNA] synthesis, the RNA template is degraded except for the 5′-terminal oligonucleotide including 5′ DR1; this positive-strand DNA [(+)-DNA] primer is transferred to DR2 and extended to the 5′ end of negative-strand DNA. A short terminal redundancy allows for a template switch to the 3′ end of negative-strand DNA and formation of the circular DNA genome.

close contact with P while the following nucleotides are much more flexibly arranged (37). In HBV isolates from patients, by contrast, the corresponding nucleotides are highly conserved (20, 22), indicative of an important function other than encapsidation, i.e., possibly as template for a short P-protein-linked DNA primer as in DHBV.

In the absence of an appropriate in vitro system for HBV, we relied on analytical improvements in the established methodology of transfecting cloned HBV DNA into suitable human liver cell lines to test the central prediction of the revised replication model, namely, that the 5′-proximal nucleotides of negative-strand DNA are derived from ε rather than DR1*. By sequencing primer extension products obtained on negative-strand DNA from mutant HBV genomes, we directly demonstrate complementarity of the negative-strand DNA 5′ end to the mutated ε-bulge nucleotides. Hence, HBV, under in vivo-like conditions, i.e., on an authentic RNA pregenome and in the presence of core protein, uses 5′ ε as replication origin for discontinuous first-strand DNA synthesis. The bulged structure is essential for primer formation, and the fact that only its 3′ part serves as a template is in accord with our structural model. Unexpectedly, not only bulge size but also primary sequence is a determinant for primer length, suggesting that the wt sequence is optimized for efficient primer synthesis and transfer.

MATERIALS AND METHODS

Plasmid constructs. The parental construct used to produce complete HBV pregenomes carrying mutations in their ε and DR1* sequences was plasmid pCHT-3091sCX (18); in brief, the plasmid contains a slightly overlength HBV genome under control of the cytomegalovirus immediate-early promoter. Replacement of A-3123 in the 3′ redundancy of the cloned HBV genome by G created a unique *Esp*3I restriction site (CgTCTC; mutated position in lowercase) between DR1* and 3′ ε. Mutated 5′-ε sequences were introduced into the resulting construct pCHT-3091sCX/E as *Sal*I-*Cla*I restriction fragments after PCR amplification of the corresponding regions from the pCHG-3122 plasmids; the 5′-terminal *Sal*I site and the HBV sequence from positions 3091 to 3121 were provided by the upstream PCR primer. Mutations in DR1* were introduced by PCR amplification using suitable primers and cloned via the *Esp*3I site plus an appropriate upstream restriction site. All mutations were confirmed by sequencing the relevant region on the plasmids by using Sequenase (USB/Amersham, Braunschweig, Germany).

Cells, transfections, and isolation of core particles. HepG2 cells were transfected with the appropriate plasmid constructs (15 μg of CsCl gradient-purified DNA per 10-cm-diameter dish) by the calcium phosphate coprecipitation technique and lysed by detergent 3 days posttransfection. Nuclei were removed by low-speed centrifugation, and core particles were immunoprecipitated from the supernatant by using a polyclonal rabbit antiserum, bound to protein A-Sepharose, elicited against recombinant HBV core protein. Details of these procedures have been previously described (27).

Primer extension analysis. Nucleic acid prepared from immunoprecipitated core particles (29) was used as the template for avian myeloblastosis virus (AMV) reverse transcriptase (RT) (Stratagene, Heidelberg, Germany)-catalyzed

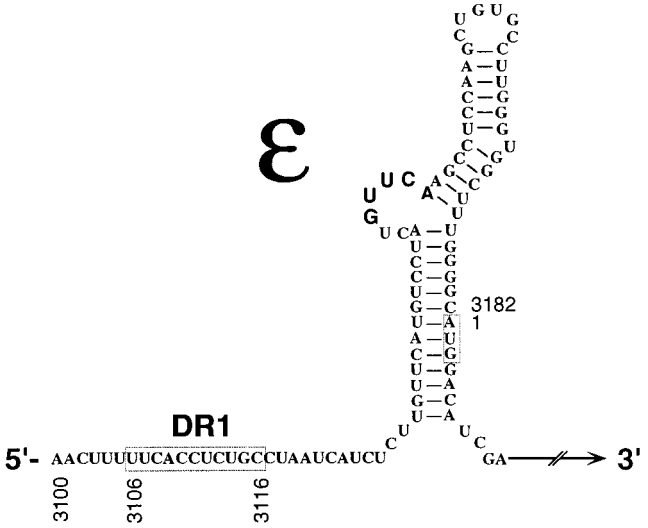


FIG. 2. Nucleotide sequence and secondary structure of HBV ε. The 5′-terminal sequence of the authentic RNA pregenome is shown. Position 1 marks the translational start for the core protein. The primary sequence in the 3′ part of the bulge that is not important for encapsidation but conserved in most natural HBV isolates is shown in boldface.

primer extension with a 5′-^{32}P-phosphorylated oligonucleotide corresponding to HBV positions 2978 to 2995 as previously described (27). For calibration, sequencing ladders were obtained by using the same 5′-labeled oligonucleotide on cloned HBV DNA as the template. Analytical gels were exposed on X-ray film or a PhosphorImager (Molecular Dynamics, Krefeld, Germany).

PCR-assisted sequencing of primer extension products. Primer extensions were performed as described above, usually with the core nucleic acid equivalent to one 10-cm-diameter dish; for the weak products obtained from most mutants with nonmatching 5′ ε and DR1*, up to four dishes were used. The products were separated on denaturing 6% polyacrylamide gels, cut out after exposure of the wet gels on a PhosphorImager, and eluted with a buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.3 M sodium acetate, 0.1% sodium dodecyl sulfate, 1 mM EDTA (pH 5.2). DNA was precipitated with glycogen and 3′ tailed with either dC or dG, using the corresponding nucleoside triphosphate and terminal transferase (Boehringer, Mannheim, Germany) as recommended by the manufacturer. After phenol extraction and ethanol precipitation, one-fifth of the DNA was used as the PCR template with a primer matching HBV positions 2996 to 3017 and a primer complementary to the corresponding homo-oligomer tail that also provided a 5′-proximal *Eco*RI site, e.g., CGGCGAATTCATG$_{10}$. Sometimes, no visible products were generated in a single 35-cycle PCR run with *Pfu* polymerase (Stratagene); however, consistent results were obtained by reamplification of a 40% aliquot of the first reaction with *Taq* polymerase and the same primers. Temperatures for denaturation, annealing, and extension were 95, 37, and 72°C. The products were blunt ended by T4 DNA polymerase and then digested with *Eco*RI. After purification on low-melting-point agarose gels, the blunt-end–*Eco*RI fragments were cloned into pBSIISK(−) cut with *Sma*I and *Eco*RI. Sequence analysis was performed with the HBV primer used for the PCR.

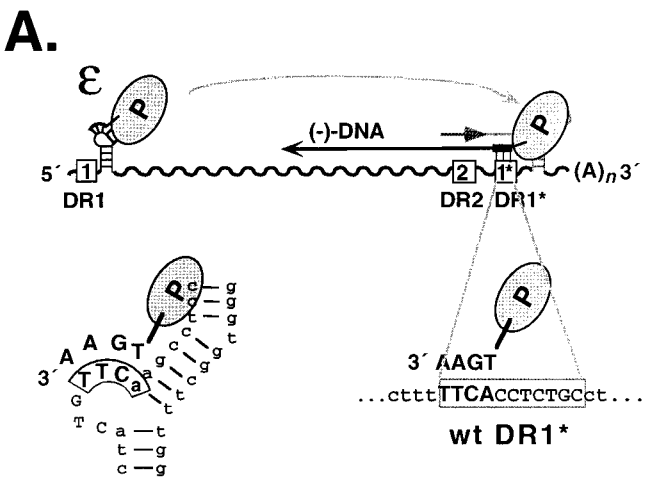
RESULTS

Mutational analysis of the role of HBV ε in negative-strand DNA synthesis. Three direct sequence repetitions are essential *cis* elements for hepadnaviral reverse transcription: 5′ DR1, immediately preceding 5′ ε; 3′ DR1 (DR1*), in the terminal redundancy of the pregenome; and DR2, located at the end of the P ORF (Fig. 1A). Previous primer extension analyses (27, 40, 54) had suggested that the negative-strand DNA initiation site is at nt 3108 and/or 3109 (UUCA; the indicated positions are underlined) within DR1*. The 3′ half of the ε bulge plus the first nucleotide of the upper stem also comprise the motif UUCA. Hence, copying of a DNA primer complementary to this motif followed by transfer to DR1* would yield the same 5′ end of negative-strand DNA (Fig. 3A). According to this model, mutations in the ε bulge should profoundly influence primer synthesis and/or translocation, whereas de novo initiation of negative-strand DNA synthesis in DR1* should not be affected.

Genetic evidence for the involvement of the HBV encapsidation signal in negative-strand DNA initiation. To analyze a potential role of ε in replication in the context of the complete HBV genome, we replaced the 5′-ε sequence in the expression plasmid pCHT-9/3091E with cassettes from several bulge variants (listed in Fig. 2B) which had been selected for encapsidation competence in ε-*lacZ* fusions (37). Hence, potentially negative results would arise not from a packaging but rather from a replication defect. The plasmid contains a 1.1-unit-length HBV genome under control of the cytomegalovirus immediate-early promoter (Fig. 1A). Upon transfection, it produces a genomic RNA essentially identical to the authentic HBV pregenome.

Negative-strand DNA production was analyzed by primer extension on nucleic acid isolated from core particles produced in transfected HepG2 cells, using a positive-sense oligonucleotide primer corresponding to nt 2978 to 2995 (Fig. 3A). We note that only negative-strand DNAs starting downstream of nt 2995, e.g., at the authentic position, but not grossly aberrant initiation events are detected by this primer. As previously observed, the wt HBV construct gave a major extension product mapping to position 3108 and a weaker band longer by 1 nt (Fig. 4, lanes wt); however, none of the mutants except mutant 1 showed a signal corresponding in intensity and position to that of the wt (Fig. 4A). Longer exposures revealed weak but specific bands for most of the other variants, at positions a few to many nucleotides away from the wt product (e.g., bands marked by arrows in Fig. 4). Hence, mutations inside the bulge region of 5′ ε influence efficiency and accuracy of negative-strand DNA synthesis from DR1*. These data support the proposed template function of the ε bulge; they also demonstrate that the wt copy of ε at the 3′ end, present in all constructs, does not rescue proper replication.

To differentiate between defects in primer synthesis and primer transfer, we constructed ε-DR1* double mutants (marked



Construct	5′-ε bulge sequence	3′-DR1* sequence
wt	taCTGTTCaa	ctttTTCACCTCTGCct
#1 #1dr	taC**G**GTT**G**aa dto.	wt ctttTT**G**ACCTCTGCct
#2 #2dr	taC**CAAC**Caa dto.	wt ctttA**A**CACCTCTGCct
#13 #13dr	taCTG**GGA**aa dto.	wt ctttG**GA**ACCTCTGCct
#17 #17dr	ta**T**TG**CGG**aa dto.	wt ctttC**GG**ACCTCTGCct
#20 #20dr	taC**AGATT**aa dto.	wt ctttA**TT**ACCTCTGCct
#bs2 #bs2dr	taC**CACTT**aa dto.	wt ctttC**TT**ACCTCTGCct
#bl2 #bl2dr	taCCACTTAATTaa dto.	wt ctttC**TT**ACCTCTGCct

FIG. 3. Genetic analysis of ε function in HBV negative-strand DNA synthesis. (A) Discontinuous model for first-strand DNA synthesis in HBV. P protein, bound to 5′ ε, uses the 3′ part of the bulge as the template for a short DNA oligonucleotide that is transferred to the DR1* and extended from there. The blowup view below shows the primary sequences (as DNA) of the ε bulge and DR1* (in capitals; flanking sequences in lowercase) in wt HBV. The motif TTCa is present in ε (boxed) and in the 5′ part of DR1*. The rightward-pointing arrow on negative-strand DNA [(−)-DNA] represents the HBV-specific sense oligonucleotide used for the primer extension analyses shown in Fig. 4. (B) HBV mutants used to analyze negative-strand DNA initiation. The first column contains the construct designations; primary sequences in and around the 5′ ε bulge and DR1* are given in the following columns. Bulge and DR1* nucleotides are shown in capitals; sequence alterations are highlighted in boldface. Variants without the suffix "dr" contain a wt DR1*, in the others, the first 3 nt of DR1* are adjusted to the 3′ part of the ε bulge.

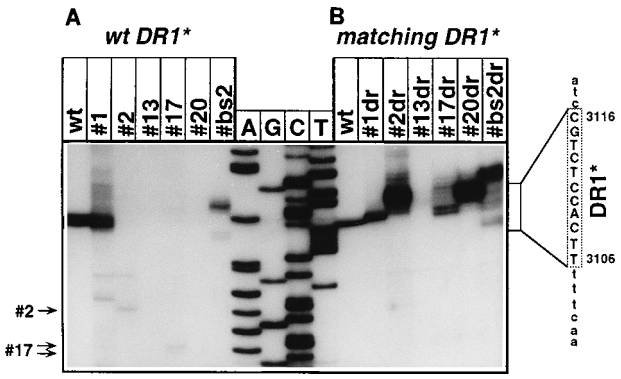


FIG. 4. Influence of sequence alterations in 5′ ε and DR1* on negative-strand DNA synthesis. Production of negative-strand DNAs was monitored by primer extension analysis. For calibration, the same oligonucleotide was used to generate a sequence ladder on cloned HBV DNA (lanes marked A, G, C, and T). Except for variant 1, most mutants with wt DR1* gave only very weak signals, while DNA production was restored in most double mutants; however, product mobilities were frequently distinct from those in wt HBV. Weak products specific for variants 2 and 17 are marked by arrows.

by the suffix "dr"; Fig. 3B) in which the 5′ nucleotides of DR1* are identical to those in the mutated bulge. ε-derived primers should now be able to base pair to the mutant DR1*. As shown in Fig. 4B, most of the double mutants gave easily visible signals close to the position observed for the wt.

Together, these genetic data strongly support the primer transfer model for HBV. Accordingly, in the wt, the 3′-proximal sequence UUC in the ε bulge, and possibly the following A residue, constitutes the template for the production of a complementary DNA primer (3′-AAG-5′ or 3′-AAGT-5′) that is transferred to the matching sequence present at the beginning of DR1*. However, the often altered mobilities and heterogeneity of the primer extension products from most double mutants were surprising. To resolve this problem and to directly prove the template function of the ε bulge, we used a procedure allowing to obtain sequence information from the 5′-proximal nucleotides of negative-strand DNA.

Determination of the 5′-proximal sequence of HBV negative-strand DNA. The yields of replication-competent HBV cores obtained by transfection are prohibitively low for a direct sequence determination of the negative-strand DNA. Furthermore, its 5′ end is blocked by the covalently linked P protein, probably via a Tyr residue as in DHBV (52, 55). For a direct PCR approach (46, 49), only the positive strand bridging the discontinuity in relaxed circular DNA is a suitable template (Fig. 1B), the formation of which depends on sequence identity between 5′ and 3′ ends of negative-strand DNA.

We therefore established a method that is independent of replication steps occurring after negative-strand DNA formation. Since the 5′-terminal sequence of the negative-strand DNA is stored as its complement in the 3′-proximal sequence of the primer extension products described above, we adopted an anchored PCR protocol to clone and sequence the primer extension products (Fig. 5A). The regions containing the product band(s) were cut out from polyacrylamide gels, and the extracted cDNAs were tailed with a homo-oligomer and amplified by PCR using the complementary homo-oligomer as one primer and an HBV-specific oligonucleotide as the second primer. The sequence immediately preceding the homo-oligomer tail reveals the sequence of the 5′-terminal nucleotides in negative-strand DNA. Of course, resolution cannot be higher than that of primer extension itself. The linkage to P protein, even after proteinase K digestion, could affect the ability of AMV RT to proceed to the very 5′-terminal nucleotides of the template, and AMV RT can add nontemplated nucleotides to a blunt-end DNA substrate (9). Another possible limitation is that individual sequences obtained by PCR may reflect rare initiation events; therefore, they were correlated with the intensity of the original primer extension products. Together, the data presented below provide clear-cut evidence for the proposed primer transfer mechanism.

In an initial control experiment (Fig. 5B), the primer extension products obtained from the wt HBV construct were tailed with oligo(dG) and oligo(dC). The majority of sequences ended with TTTTTC in front of the homo-oligomer, in accord with the position of the major primer extension product at C-3108; a substantial fraction contained an additional A residue. As both the nucleotide following the ε bulge and nt 3109 in DR1* are A residues, they could originate from templated synthesis, but nontemplated addition by AMV RT cannot be excluded. In view of the transfer model, this finding indicates that a primer complementary to TTC, or TTCA, is copied from the 3′-terminal bulge nucleotide (C-3147) and possibly the first or sometimes second A residue in the upper stem; it must end opposite the U residue at position 4 or 5 of the bulge, as the preceding G residue does not match the sequence in DR1*.

Mutations in the 5′ ε bulge are transferred to the 5′ end of negative-strand DNA. Of the bulge variants with a wt DR1*, only mutant 1 (taCGGTTGaa; bulge region in capitals and flanking sequences in lowercase) had given easily visible primer extension products with wt-like mobility, mapping to position 3107 (1 nt shorter than the wt) and 3108 (Fig. 5C). The sequences of most products (6 of 10) ended with cttTT G(c) (from here on, the nucleotides most likely derived from ε are shown in capitals; the lowercase letter in parentheses represents the first nucleotide identical to the homo-oligomer), corresponding to the extension product 1 nt shorter than the wt. Additional sequences were ctttTTG(c) (2 of 10) cttttTT G(c) (1 of 10), and cttTT(c) (1 of 10). None of these products conforms to de novo initiation, but all conform to the synthesis of a DNA primer from the mutant bulge which is then transferred to DR1*, either to the authentic TTC triplet or to the preceding TTT. In the double mutant 1dr (DR1*, aactttTTG ACC..; DR sequence in capitals, flanking sequence in lower case, and mutation underlined) with matching DR1*, one major primer extension product was observed (Fig. 5C). Most sequences ended with ctttTTG(c) (6 of 10), suggesting that the adjusted triplet TTG in DR1* served as an efficient acceptor site. Additional minor sequences contained an extra A (as seen for the wt) and AAG, respectively, which corresponds to the sequence immediately following the ε bulge; this finding suggests an aberrant primer initiation event in the upper stem of ε. Two sequences with no G at or close to the 3′ end were probably descendants of incompletely elongated primer extension products. Together, these data provide convincing evidence for the primer transfer model. They also indicate that a primer with only two of three bases matching the acceptor site can still be transferred to DR1* (mutant 1), but transfer specificity is increased if three matches are possible (mutant 1dr). However, sequence complementarity alone cannot determine transfer site selection. The perfect target sequence TTG occurs six times in the readable range of the primer extension gel, but at none of these sites were extension products seen.

Mutations in the bulge of 5′ ε influence accuracy and efficiency of negative-strand DNA primer synthesis and transfer to DR1*. As in the wt, the critical sequences of the ε-DR1* double mutants are identical and should not allow for a distinction between primer transfer and de novo initiation. However, only variant 17dr gave rise to primer extension products

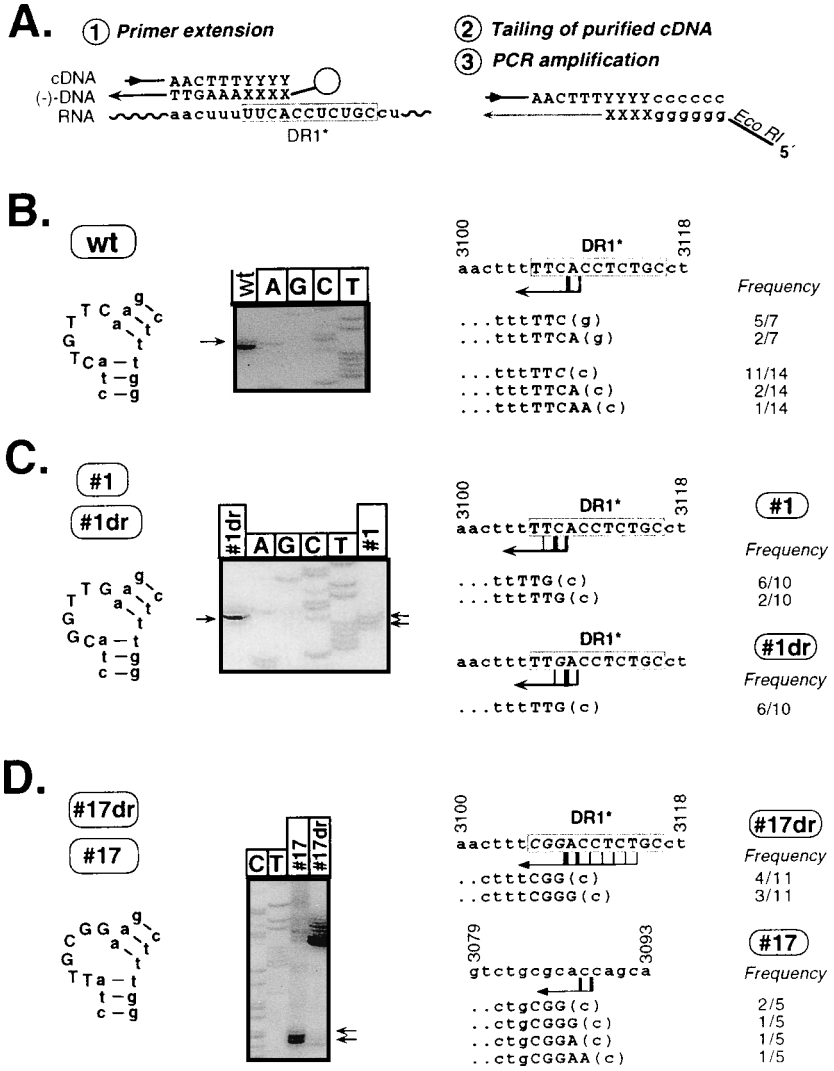


FIG. 5. Sequence evidence for primer transfer from 5′ ε to DR1*. (A) Principle of the primer extension product sequencing protocol. To reveal the unknown sequence at the 5′-end of negative-strand DNA [(−)-DNA] (XX), primer extension (step 1) was used to produce a cDNA that should end with the complementary sequence (YY). Products isolated from preparative sequencing gels were 3′ tailed (step 2) by using terminal transferase [here oligo(dC)] and then amplified by PCR (step 3) using an HBV-specific and an oligo(dG)-containing primer. PCR products were blunted on one end, cut with *Eco*RI at the other end, and then cloned. The following panels show, from left to right, the sequence of the relevant part of 5′ ε, the primer extension product(s) next to an appropriate sequencing ladder, and the sequence of DR1*; the leftward-pointing arrows below DR1* indicate the positions and approximate intensities of the apparent negative-strand DNA start sites. The sequences below show the 3′-terminal nucleotides in the primer extension products and, in parentheses, the first nucleotide identical to the homo-oligomer tail; nucleotides probably derived from 5′ ε are shown in capitals. The last column gives the fraction of specific sequences found in the total number of sequenced clones; infrequently found sequences are mentioned in the text. (B) wt HBV. The most frequent sequence corresponds to a primer complementary to TTC; a minor, longer product (arrow) corresponds to TTCA. (C) Variants 1 and 1dr. (D) Variants 17 and 17dr. Note the mobility shift between single and double mutants.

with a mobility similar to that of the wt; the other mutants produced mostly bands of lower mobility, sometimes with one or two prominent products (e.g., mutants 2dr and 20dr) and sometimes with distinct groups of bands (e.g., mutant bs2dr). Obviously, these products originated from negative-strand DNAs with extended 5′ ends, either from elongated primers targeted to the authentic site, from wt-analogous short primers transferred to a slightly different acceptor site in DR1*, or from a combination of both.

The strongest primer extension product from mutant 17dr (bulge, aTTGCGGaag; DR1*, aactttCGGACC..) mapped to the same position as the wt product, with a major additional band 1 nt longer (Fig. 5D). In accord with this finding, the most frequently found sequences ended with aactttCGG(c) (four of 11) and aactttCGGG(c) (3 of 11); minor sequences were aactt tCGGA(c) and products longer by 2, 3, or 4 nt, all of which contained sequence repetitions of the motif CGG. Thus, the major product is compatible with production of a wt-analogous, bulge-derived primer complementary to CGG, sometimes with an extra G or A residue; the longer products were suggestive of the production of extended primers (see below). In construct 17 with its wt DR1*, similar primers were made but apparently transferred to a CGC target at positions 3084 to 3086. As this is the only occurrence of a CG motif in the relevant region of the pregenome, target site selection is apparently governed by sequence complementarity at the first two positions.

Sequence alterations in the 5′ ε bulge can induce predominant formation of extended primers. Variant 2dr (bulge, aCC AACCaa; DR1*, tttACCACCTC..) produced a strong band

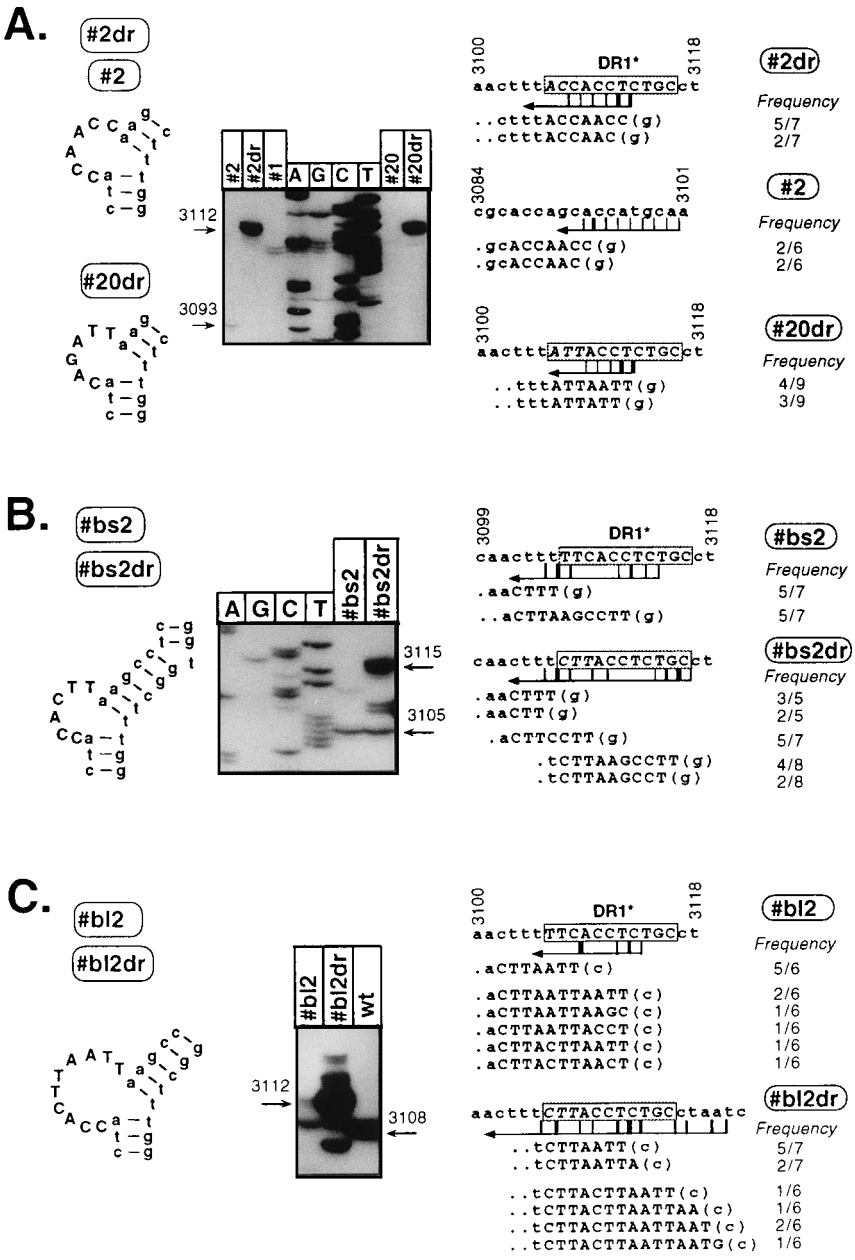


FIG. 6. Sequence evidence for extended primers from variants with altered 5′-ε bulge. (A) Variants 2, 2dr, 20, and 20dr. Products from variants 2dr and 20dr are several nucleotides longer than those from the wt. The autoradiogram is overexposed to show the weak signal at position 3093 from variant 2 which on even longer exposures appears as a series of bands. The doublet of bands from variant 1 (see Fig. 6) may be used as a marker. (B) Variants bs2 and bs2dr. Both mutants produce a distinct pattern of bands. (C) Variants b12 and b12dr. The autoradiogram is overexposed to show the minor, more slowly migrating products from mutant b12 around position 3112. Of the various products from variant b12dr, only those migrating more slowly than the wt product (position 3108) were analyzed.

mapping to position 3112 (Fig. 6A). After tailing with dG, most sequences (five of seven) ended with tttACCAACC(g), corresponding to position 3112; a minority (two of seven) lacked the terminal C residue. After tailing with dC, ttACCAA(c) (four of eight), tttACCA(c) (two of eight), and tttACCAAA(c) (two of eight) were found; probably, in most of them one or two of the terminal C residues were already present in the primer extension product. Thus, the major negative-strand DNA species generated has a 5′-terminal sequence complementary to tttA CCAACC. This sequence is incompatible with a bulge-derived primer corresponding to only the triplet ACC but is plausibly explained if the primer is longer than in the wt and comprises the complement of the entire bulge (CCAACC).

Analysis of products from the parental construct 2 supported this notion (Fig. 6A). The major species ended with cgcACC AACC(g) (two of six) and cgcACCAAC(g) (two of six), while the target sequence is CGCACCAGCA (positions 3084 to 3093). Selection of this site by an extended primer containing the complement to ACC at its 3′ terminus is much more likely than transfer of a short ACC primer to the nonmatching GCA triplet.

Sequence evidence for slippage and reinitiation during primer synthesis. Mutant 20dr (bulge region, taCAGATTaa; DR1* region, tttATTACCTC..) produced two major products mapping to positions 3111 and 3112 (Fig. 6A). Two predominant sequences were found: tttATTAATT(g) (four of nine) and ttt

ATTATT(g) (three of nine). Minor species were tttAATA(g) (one of nine) and tttATTA(g) (one of nine). A 3-nt primer complementary to only ATT matches neither the sequence at positions 3109 to 3111 (ACC) nor that at positions 3110 to 3112 (CCT), which would be required to explain the lengths and sequences of the primer extension products; however, a longer primer such as the complement to ATTAATT or ATT ATT could base pair to the ATT at the 5′ end of the mutated DR1*. The involvement of these 5′-terminal DR1* residues in accepting the primer is also obvious from the negative result with mutant 20 with its wt DR1*. In contrast to variant 2, the extended primers from variant 20dr can arise not from copying the entire bulge but rather by repeated use of the same template region as in the wt, i.e., by slippage and reinitiation. A slight inaccuracy in repositioning the initial 3-nt primer can plausibly explain the finding that about half of the sequences contain two neighboring internal A residues.

Distinct pathways for primer synthesis can coexist. Variants bs2 (bulge region, taCCACTTaa) and bs2dr (DR1* region, ttt CTTACCTCTGCca) gave rise to complex primer extension patterns (Fig. 6B), suggesting the concurrent production of different classes of primers. The most prominent product from variant bs2 mapped to position 3105, with companion bands differing by 1 nt; products with identical mobility were also present in variant bs2dr. The major sequences were aaCTT T(g) (five of seven) for variant bs2 and aaCTTT(g) (three of five) and aaCTT(g) (two of five) for variant bs2dr. This result is compatible with a bulge-derived, wt-analogous primer complementary to CTT, but with frequent addition of an extra T; transfer had apparently occurred to the triplet CTT preceding DR1* (positions 3102 to 3104). A second group of bands, unique to variant bs2, mapped to position 3111; surprisingly, the most frequently found sequence was aaCTTAAGCCTT(g) (five of seven), which, except for the terminal T residue, is exactly the sequence following the ε bulge. Similarly, the products from variant bs2dr mapping to position 3115 ended with tttCTTAAGCCTT(g) (four of nine) and tttCTTAAGCCT(g) (two of nine). The only likely explanations are an aberrant initiation in the upper ε stem or slippage of an initial CTT primer to this region and reinitiation. The resulting primers are transferred to the CTT triplet preceding DR1* in variant bs2 and to the CTT triplet present in the mutated DR1* in variant bs2dr. Almost all of the minor sequences, including those derived from the doublet at positions 3108 and 3109 unique to variant bs2dr, were similar to these elongated primers but lacked 1 to 3 nt of the internal AAG sequence.

Together, these analyses revealed an unexpected complexity in the details of HBV negative-strand DNA primer generation whenever the wt bulge sequence of ε was altered. However, several general conclusions can be drawn: first, all data are compatible with primer synthesis from the bulge region of 5′ ε but not with de novo initiation; second, P protein apparently copies any sequence from the bulged RNA template; third, this sequence can profoundly affect the final primer length and composition. However, the predominant initiation and stop sites on the template were the same in most mutants as in the wt, i.e., the last and the fourth nucleotides of the ε bulge. This finding suggested that primer synthesis is arrested by some kind of mechanism. To address this question, we analyzed a mutant with a bulge of increased size.

The size of the 5′ ε bulge is a determinant for negative-strand DNA primer length. Two possible outcomes for primer synthesis from a larger bulge might be expected: either the primers contain only the complement to the three most 3′-proximal bulge nucleotides or synthesis proceeds to the fourth 5′-proximal nucleotide of the bulge. A fortuitous unique *Afl*II restriction site in the bulge region of variant bs2 (taCCACTT aag; *Afl*II site underlined) was used to generate a 4-nt insertion. According to computer prediction, this will lead to a larger bulge (uaCCACTTAATTaag) in an otherwise unaltered secondary structure. Primers should then be complementary to ATT or to CTTAATT. This mutant was analyzed in the context of a wt DR1* (variant bl2) or a mutated DR1* (variant bl2dr; DR1* region; tttCTTACCTCTGCct), providing a CTT target site at its 5′ end. Both variants gave clearly detectable though heterogeneous primer extension products (Fig. 6C), indicating their encapsidation competence. The most prominent products from variant bl2 mapped to positions 3108 and 3112; the predominant band from variant bl2dr mapped to position 3112. The majority of products at position 3108 had the terminal sequence aaCTTAATT(c) (five of six). Only a primer including the complement to CTT matches this site at the CTT triplet preceding DR1* (nt 3102 to 3104). Similarly, the major product from mutant bl2dr ended with tttCTTAA TT(c) (five of seven) and hence results from an identical primer targeted to the CTT triplet in the mutated DR1*. The sequences from the minor primer extensions products were heterogeneous, but all contained partial repetitions of the bulge sequence; possibly they originated from similar slippage-reinitiation events as described above. These data show that bulge size is critical for primer length. Apparently, the start and stop sites are defined by the most 3′-proximal nucleotides and the fourth nucleotide from the 5′ end of the bulge, in accord with the relatively flexible arrangement of these nucleotides in our structural model.

DISCUSSION

All retroelements are related by the fundamental process of reverse transcription, but the ways in which they express and propagate their genetic information are exceedingly diverse. Hepadnaviruses, like retroviruses, form extracellular infectious particles, but their replication characteristics are distinct in many aspects (for reviews, see references 28 and 30). For instance, virions contain DNA rather than RNA, and the protein components of the nucleocapsid are separately expressed and apparently not processed. As a consequence, HBV uses the interaction of P protein with the 5′-proximal ε signal to ensure coencapsidation of replicase and RNA template, while in retroviruses, Gag binds genomic RNA and Gag-Pol is incorporated via its Gag domain. Initiation of reverse transcription in hepadnaviruses seemed to represent another conspicuous exception (23): while most retroelements require a tRNA primer to initiate discontinuous first-strand DNA synthesis, hepadnaviruses appeared to produce, by protein priming (2, 48), negative-strand DNA in a continuous fashion, a view recently challenged by data obtained in studies of the avian hepadnavirus DHBV (46, 49).

The improved analytical techniques used in this study allowed a detailed analysis of the ε-P-protein mediated negative-strand DNA initiation mechanism in the type member of the hepadnaviruses, HBV, and thus complement and extend, under in vivo-like conditions, the DHBV data. In particular, they allow for a correlation of the specific replication function of HBV ε with its known secondary structure. In accord with our structural model, the more flexibly arranged 3′ half of the ε bulge serves as template for a DNA primer, while its 5′-proximal nucleotides, apparently in closer contact with P protein (37), are likely to have a role in arresting primer elongation (Fig. 7A). The short primer, covalently fixed to P protein, is then specifically transferred to the 3′-proximal DR1* (Fig. 7B). Hence, the same structured RNA element functions as an

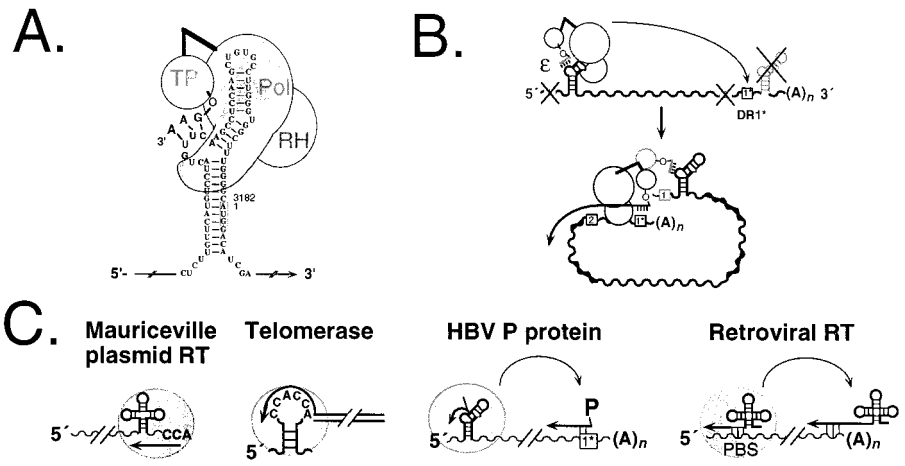


FIG. 7. Probable mechanism of negative-strand DNA synthesis in HBV and its evolutionary implications. (A) Higher-resolution model of P protein-ε interaction. This model correlates the functional importance of ε for primer generation with the three-domain structure of P protein (35). Covalent attachment of the primer occurs to a Tyr residue in the terminal protein (TP) domain of the P protein of DHBV and probably also HBV. As the ε bulge is used as a template, the active site of the polymerase domain (Pol) must be in close proximity; no evidence exists for a direct involvement of the RNase H domain (RH). (B) P protein-primer transfer on an authentic HBV pregenome. Primer synthesis occurs only at 5′ ε, and transfer is specific for DR1* (38), suggesting that 5′ and 3′ ends of the RNA pregenome are in close proximity. (C) Initiation characteristics of RNA-dependent DNA polymerases. HBV reverse transcription is intermediate in complexity between that of the Mauriceville retroplasmid, which uses a 3′-proximal *cis*-acting RNA element as a positioning device for continuous daughter strand synthesis, and the retroviruses employing a *trans*-acting tRNA primer for discontinuous negative-strand DNA production; it also bears features found in telomerase. See text for details.

encapsidation signal and defines the origin of HBV replication. Hepadnaviral negative-strand DNA synthesis is therefore discontinuous and in this respect more closely related to synthesis in other retroelements than previously thought. However, using a 5′-proximal *cis*-acting RNA element as a positioning device and part of its as template is a unique replication strategy that combines features found in RNA viruses and primitive retroelements with those seen in modern retroviruses.

HBV negative-strand DNA synthesis initiation: copying from a bulge. Most 5′-ε-bulge mutations combined with an authentic DR1* compromised negative-strand DNA production. By contrast, adjusting the 5′-terminal nucleotides of DR1* to those in the mutated bulge, in general, restored efficient negative-strand DNA synthesis, suggesting a decisive role for ε in HBV replication. Direct evidence for the synthesis of a short DNA primer from the 5′ ε bulge and its subsequent transfer to the 3′-proximal DR1* was obtained by our primer extension product sequencing protocol that also enabled us to uncover some of the mechanistic aspects underlying the perplexing length variation observed for negative-strand DNAs from many bulge mutants.

Our assay reflects the influence of ε mutations on both primer synthesis and transfer; however, a clear distinction between whether ε or the sequence preceding DR1* served as the template for a given nucleotide in negative-strand DNA is possible whenever they are different; also, a minimal sequence match between primer and target site appears to be required for transfer (see below). Plausibly then, the ε-derived part of negative-strand DNA corresponds to the unique sequence plus at least two matching nucleotides. All data are in accord with the primer transfer model; i.e., HBV negative-strand DNA synthesis in vivo is absolutely ε dependent. For the wt, the major primer is apparently a trinucleotide complementary to the 3′ half of the ε bulge; in 10 to 20% of the sequences, we found an extra A residue. Preliminary experiments with ε mutants containing a nucleotide different from A at the corresponding position suggest that also this nucleotide arises from templated synthesis. Currently, it cannot be excluded that this longer product corresponds to the actual major primer.

A surprising finding was the profound influence of the primary bulge sequence on primer length. Only in mutants 1/1dr and 17/17dr were the sequences compatible with a wt-analogous primer of 3 or 4 nt, while the other mutants predominantly produced extended primers, possibly by copying the entire bulge sequence (variants 2 and 2dr), aberrant initiation in the upper stem of ε (part of the products from variants bs2 and bs2dr) as was recently suggested to also occur in DHBV (45), or repeated use of the ε-bulge template (e.g., variant 20dr). A plausible explanation for the latter products is the initial synthesis of a short primer, as in the wt, which prior to transfer to DR1* slips back on the template and reinitiates synthesis inside the ε bulge. We favor this as the dominant mechanism for generating extended primers, as it can explain all phenotypes observed (see below). Apparently, in some mutants these pathways can coexist and generate distinct populations of primers.

Another important parameter for primer length is the size of the bulge. Most negative-strand DNAs from variants bl2 and bl2dr are derived from primers containing the complement to the additional 4 nt present in their ε bulges. Similarly, the terminal residues in most other variants corresponded to the last nucleotide of the bulge and the fourth residue from the start of the bulge, as in the wt. We favor the view that primer elongation is arrested by the close interaction of the nucleotides at positions 1 and 2 of the bulge with P protein, as proposed in our model, and the base pairing in the lower stem of ε.

Hence, the bulge, embedded into the larger ε structure forming a binding site for P protein, is exquisitely suited to allow for the synthesis of a short specific DNA primer: its 3′-proximal part provides an exposed initiation site that obviates the need for melting double-stranded structures; as in DNA replication, or transcription by RNA polymerase II (for a review, see reference 7), this would require a helicase activity. Interestingly, the requirement for the helicase activity associated with basal transcription factor TFIIH is overcome in artificial DNA templates containing a mismatched, heteroduplex initiation site (43). The absence of helicase activity, at least

during the initial DNA primer production in HBV, would also explain primer elongation arrest once the polymerase domain of P encounters the structurally rigid lower part of ε.

Specificity of primer transfer. One determining factor in primer transfer is sequence complementarity between the 3′ end of the primer and the target site, as shown by the different acceptor sites used in mutants with matching and with nonmatching ε and DR1*. Complementarity of at least two 3′-terminal nucleotides of the primer appears to be required and sufficient. Mutant primers with no sequence match at or close to DR1* were translocated to better-fitting aberrant sites; the major primer from mutant 1, bearing the complement to TTG, was transferred to the authentic TTC or the overlapping TTT triplet at wt DR1*. The predominant transfer to the exactly matching TTG triplet in DR1* of mutant 1dr suggests that the third base from the 3′ end of the primer also influences targeting.

However, additional mechanisms accounting for the specificity for DR1* must exist, as even the tetranucleotide TTCA occurs 18 times on the unit-length HBV genome. This is emphasized by transfer of the primer from mutant 1 to DR1* rather than any of the six TTG triplets in the analyzed pregenome region. Candidates for such recognition elements are a second binding site for P or the P-primer complex on the pregenome or for the formation of a transfer complex in which 5′ ε and DR1* are held close together (Fig. 7B). The possible importance of overall RNA structure is also stressed by the observation that on the authentic HBV pregenomes used here, 3′ ε was completely silent whereas in the DHBV in vitro systems, the corresponding region is functional. Similarly, 5′ DR1, in close proximity to the active 5′ ε, is excluded from serving as primer acceptor (38).

Transfer requires that the P-primer complex be released from its initial binding site at 5′ ε, possibly by structural changes induced by base pairing of the primer with its template, and then engage in a new interaction at DR1* which is also influenced by base pairing. Hence, the primary sequences of template and primer together with RNA-protein interactions contribute to the overall energetics of both release and transfer; this could explain the generation of extended primers by slippage and reinitiation once the sequence in the ε bulge is altered. Probably, reinitiation is just a special case of aberrant transfer in which rebinding of the initial P-primer complex to 5′ ε is energetically favored. As in the proper translocation reaction, reinitiation site selection will be influenced by the sequence of the initial primer. Interestingly, slipping back and/or jumping back of DNA polymerases are found as integral parts of protein-primed replication initiation, e.g., in adenovirus (17) and phage ϕ29 (25) and also during primer shifting and "inchworming" of *Escherichia coli* RNA polymerase (31, 41). Of course, the bulge mutations themselves may induce subtle changes in the ε structure that influence P-protein positioning and primer synthesis. Analysis of further mutants in our system should help to disclose the mechanistic details underlying primer synthesis; the recently described baculovirus expression system for HBV P protein (19) may be suitable to complement such studies in vitro. The complex dependence of the primer on primary sequence in the bulge may also explain why in a recent report apparently no intramolecular complementation was observed in two HBV ε mutants with sequence-adjusted DR1* when DNA synthesis was monitored by the endogenous polymerase reaction (12).

The short length of the HBV primer will per se facilitate release and hence transfer; moreover, generation of a longer DNA-RNA hybrid of some 15 or more nt could, in an untimely fashion, engage the RNase H activity of P protein (21) and destroy the 5′-proximal region of the pregenome which is essential as template for a complete, circularization-competent negative-strand DNA.

Evolutionary implications of the HBV replication strategy. The evolutionary origin of viruses, and in particular retroviruses, is still much disputed (for reviews, see references 11 and 26). Retroviruses might be descendants of cellular DNA elements (47). Alternatively, viruses replicating through RNA might be molecular fossils of a previous RNA world, with those using reverse transcription marking the transition to the present-day DNA world. Many RNA viruses such as brome mosaic virus use a 3′-proximal *cis*-acting tRNA-like element to position their replicase (36); such a replication element is also present in the positive RNA strand of the double-stranded L-A virus of the yeast *Saccharomyces cerevisiae*, which, for RNA packaging, appears to cooperate with a second hairpin that is recognized by the replicase (13, 53). Progeny RNA is copied continuously, and the positioning element as a whole serves as a template. According to the genomic tag hypothesis (23), these tRNA-like structures later evolved into the *trans*-acting tRNA primers of retroviruses, now active in a 5′-proximal position.

The recently characterized replication mechanism of an unusual retroelement, the Mauriceville plasmid of *Neurospora* spp. mitochondria (51), suggested an evolutionary link between RNA viruses and contemporary retroviruses (for a review, see reference 24). Its genomic RNA carries a *cis*-acting 3′-proximal tRNA-like structure that defines the start site for continuous negative-strand DNA synthesis (Fig. 7C) and contains the 8 nt that are universally conserved in all tRNAs (GUUCGACUC; conserved residues underlined). Most unusually, the retroplasmid RT does not require a primer for initiation, a property thought to be exclusively confined to RNA polymerases.

The salient features of hepadnaviral replication are the use of a *cis*-acting RNA structure as replication origin, as in RNA viruses and the retroplasmid; the positioning element is, in part, also template. Its 5′-proximal location, however, gives rise to discontinuous daughter strand synthesis as in the vast majority of retroelements. These characteristics suggest that hepadnaviruses may evolutionarily link the Mauriceville plasmid and modern retroviruses (Fig. 7C). This view is supported by the ε sequence encompassing the initiation site which contains six of the eight invariable tRNA residues (GUUCAAGCC). That only part of ε serves as template is a feature shared by another unusual RT, telomerase (for reviews, see references 5 and 8), which uses an exposed region (4) of a tightly associated RNA as template to add, by slippage-reinitiation, the telomeric repeats to the ends of eukaryotic chromosomes (Fig. 7C). Finally, we wish to point out that the covalent linkage between hepadnaviral P proteins and negative-strand DNA does not prove a chemical requirement for an extendable 3′ end in the initiation reaction but might as well reflect the necessity to prevent the exceedingly short ε-derived negative-strand DNA primer from being lost by diffusion and to ensure its proper transfer to DR1*.

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