JOURNAL OF VIROLOGY, Dec. 1994, p. 8437–8442
0022-538X/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 68, No. 12

Role of RNA in Enzymatic Activity of the Reverse Transcriptase of Hepatitis B Viruses

GUANG-HUA WANG, FABIEN ZOULIM,† ERNEST H. LEBER, JEREMY KITSON,‡ AND CHRISTOPH SEEGER*

Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Received 16 June 1994/Accepted 15 August 1994

The hepadnavirus reverse transcriptase is a multifunction enzyme. In addition to its role in DNA synthesis, the polymerase is required for RNA packaging and also functions as the primer for minus-strand DNA synthesis. Previously, we demonstrated that the protein-priming activity of the polymerase requires a viral RNA segment, termed epsilon, which serves as a template for the synthesis of a short DNA oligomer that is covalently attached to the reverse transcriptase (G.-H. Wang and C. Seeger, J. Virol. 67:6507–6512, 1993). We now report that epsilon is sufficient for activation of the reverse transcriptase to prime DNA synthesis through the formation of a stable RNA-protein (RNP) complex. We also demonstrate that the binding reaction depends on sequence-specific determinants on epsilon. Moreover, our results indicate that two genetically separated domains of the reverse transcriptase are required for formation of the RNP complex. Finally, we show that the polymerase has a DNA polymerase activity in the absence of epsilon which does not depend on the protein-priming mechanism.

The reverse transcriptases of hepatitis B viruses (hepadnaviruses) polymerize DNA from viral RNA or DNA templates in subviral core particles (21). However, in contrast to their retroviral counterparts, encapsidated polymerases of hepadnaviruses cannot be solubilized and therefore do not accept exogenous RNA or DNA templates for DNA synthesis. Recently we showed that the reverse transcriptase of duck hepatitis B virus (DHBV) can prime minus-strand DNA synthesis with the hydroxyl group of a tyrosine residue which is located near the N terminus of the polymerase gene product (27). As a consequence of this reaction, the 5′ end of minus-strand DNA becomes covalently linked to the reverse transcriptase (3, 7, 26, 27). The protein-priming reaction requires a viral RNA segment, termed epsilon, as a template for the synthesis of the first four nucleotides of minus-strand DNA (22, 24). Although two copies of epsilon are present on pregenomic RNA, only the 5′ copy functions as a template for the priming reaction (Fig. 1) (24). Following initiation of minus-strand DNA synthesis, the DNA oligomer is transferred by an unknown mechanism to the 3′ end of pregenomic RNA, where it can anneal with complementary sequences at a sequence motif called DR1 (19). Reverse transcription then continues toward the 5′ end of the RNA template.

The 5′ copy of epsilon also acts as a packaging signal for the incorporation of pregenomic RNA into subviral core particles (11). RNA packaging depends on the presence of the polymerase (2, 9). It is not yet known whether the polymerase binds directly to epsilon or whether other host proteins are required for this interaction, i.e., as described for the interaction of the Tat protein with the TAR signal on the human immunodeficiency virus genome (14, 15). However, biochemical and genetic analysis of the epsilon signal established that this RNA segment has the ability to fold into a stem-loop structure with a bulge, in good agreement with the predicted secondary structure of this sequence motif (11, 12, 17). It is possible that the priming reaction and the packaging reaction in binding to epsilon are part of a single process. However, in DHBV, assembly of viral particles depends in addition to epsilon on a second signal on pregenomic RNA, suggesting that the two reactions may be distinct (4, 8). To obtain a better understanding of the interaction between the polymerase and epsilon for the protein-priming reaction, we investigated how viral RNA affects the enzymatic activity of the hepadnavirus reverse transcriptase. In addition, we sought to identify the domains on the polymerase polypeptide that are important for interactions with epsilon.

Epsilon is necessary and sufficient for the DNA-priming activity of hepadnavirus reverse transcriptase. Previous experiments showed that the enzymatic activity of the in vitro-translated reverse transcriptase required a viral RNA segment that was located downstream of the polymerase gene between positions 2527 and 3021 (Fig. 1B) (25). This fragment contained sequences corresponding to the 5′ end of minus-strand DNA (position 2537) and epsilon (Fig. 1B). Since the UUAC motif at position 2576 in epsilon is the template for the synthesis of the first four nucleotides of minus-strand DNA, we surmised that the RNA sequences corresponding to epsilon would be sufficient for the protein-priming reaction. As predicted, the activity of the polymerase to incorporate deoxynucleoside triphosphates (dNTPs) required the presence of the epsilon RNA segment, which was provided in *trans* to the in vitro translation reaction (Fig. 2A). The activity of the reverse transcriptase was dependent on the concentration of epsilon and was maximal at an RNA concentration of 1 μM. On the basis of the efficiency of the in vitro translation system, we estimated that, at this concentration, the RNA was present in a 100-fold molar excess over the polymerase polypeptide (lane 3). The activity of the polymerase was up to 10-fold higher under conditions in which epsilon was present during the in vitro translation reaction compared with conditions in which it was added after expression of the polypeptide (Fig. 2A, lanes 2 and 6). This difference in enzymatic activity

* Corresponding author. Phone: (215) 728-4312. Fax: (215) 728-3616. Electronic mail address: seeger@cs.fccc.edu.
† Present address: INSERM U 271, Lyon 69003, France.
‡ Present address: Virology Department, Glaxo Group Research Limited, Middlesex, UB6 0HE, United Kingdom.

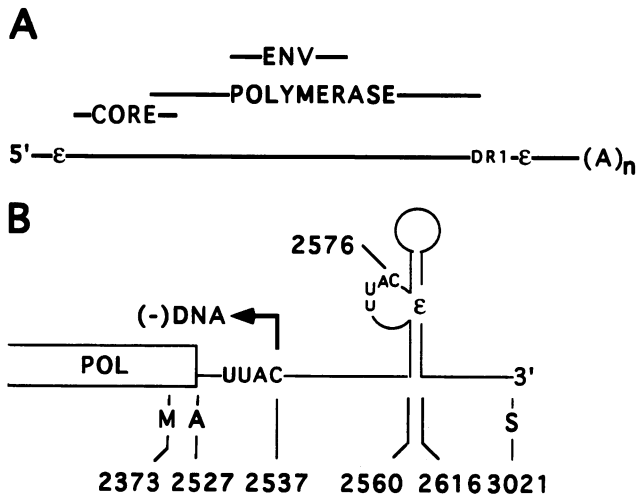

FIG. 1. Physical map and structure of DHBV pregenomic RNA. (A) Pregenomic RNA with the two copies of epsilon (ε) near the 5′ and 3′ ends and DR1. The positions of the core, polymerase, and envelope (ENV) genes are indicated. (B) Detailed map of the 3′ end of the polymerase (POL) gene and the positions of sequences critical for the initiation of reverse transcription, as described in the text. M, *Msc*I; A, *Afl*II; S, *Sal*I.

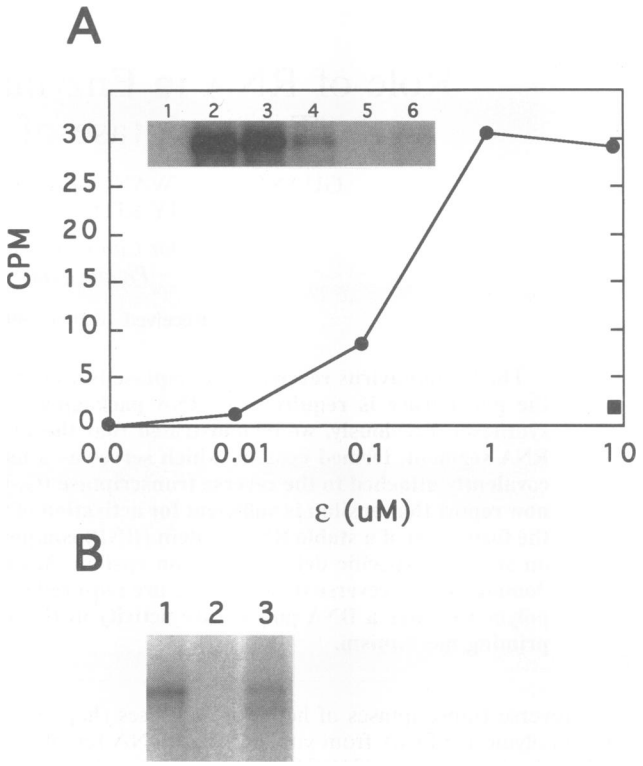

FIG. 2. Epsilon is required for the protein-priming reaction. (A) The polymerase was translated from an RNA template transcribed from pHTP (27), with 3′ ends corresponding to the *Afl*II site at position 2527 on the DHBV genome (Fig. 1B) (25). The translation reaction was supplemented with RNA corresponding to the epsilon signal (positions 2560 to 2616). Epsilon RNA was transcribed from an 82-nucleotide-long DNA oligomer spanning positions 2560 to 2616 on the DHBV genome. In addition the DNA oligomer contained 25 nucleotides corresponding to the SP6 promoter at the 3′ end. To create a functional SP6 promoter, the oligomer was annealed to a 25-nucleotide-long DNA oligomer (5′-GGATTTAGGTGACACTA TAGAATAC-3′). After the translation reaction, the polymerase was incubated with [^{32}P]dATP and dNTPs. The concentration of epsilon in the translation reaction was 10 μM (lane 2), 1 μM (lane 3), 0.1 μM (lane 4), and 0.01 μM (lane 5). The polymerase was expressed without epsilon (lanes 1 and 6), and epsilon was added after translation at a final concentration of 10 μM (lane 6). The amount of radioactivity incorporated into the polymerase was quantitated with an Ambis 4000 Imager (Ambis Inc.) and plotted as a function of the concentration of epsilon (ε, μM). The solid square represents the quantitation of the signal shown in lane 6 of the inset. (B) The polymerase was expressed from an RNA template transcribed from pHP (27), with 3′ ends corresponding to the *Sal*I site at position 3021 (Fig. 1B). The polymerase polypeptide was assayed for its ability to incorporate [^{32}P] dGTP without incubation with RNase A (lane 1). The RNase A (10 ng/μl)-treated polymerase polypeptide was assayed for enzymatic activity after the addition of RNasin (2 U/μl) (lane 2) or RNasin (2 U/μl) and epsilon (10 μM) (lane 3) to the reaction mixture.

appeared to vary with the concentration of magnesium ions in the DNA-priming reaction mixture and was maximal at high (10 mM) concentrations of magnesium (Fig. 2A, lane 6 and results not shown).

Since RNA used for translation of the polymerase gene was still present in the in vitro polymerase reaction, we could not yet conclude that epsilon by itself could activate the polymerase. To directly examine whether epsilon RNA alone was sufficient for the DNA-priming activity, the polymerase gene was expressed from an RNA template with 3′ ends at position 3021 that contained epsilon. This template directed the expression of reverse transcriptase that displayed enzymatic activity (Fig. 2B, lane 1). Incubation of the translated polymerase polypeptide with RNase A prior to the addition of dNTPs to the polymerase reaction abolished the ability of the enzyme to initiate minus-strand DNA synthesis (lane 2), indicating that the RNA in the in vitro translation reaction was digested by the RNase. However, when the reaction mixture was supplemented with epsilon RNA after RNase A treatment, the polymerase gained enzymatic activity (lane 3). The presence of a second, radioactively labeled band most likely represents a truncated polymerase product, which may be the result of proteolytic degradation that occurred during RNase treatment of the reaction mixture. These results confirmed previous observations that indicated a requirement of viral RNA for the activity of the hepadnavirus reverse transcriptase to prime DNA synthesis and identified epsilon as the RNA segment necessary for this reaction.

The reverse transcriptase forms a stable ribonucleoprotein (RNP) complex with epsilon. Our results suggested that the polymerase binds to epsilon to prime reverse transcription of minus-strand DNA. To examine the specificity of this interaction, we tested the enzymatic activity of the polymerase in the presence of epsilon sequences derived from human hepatitis B virus (HBV) and heron hepatitis B virus (HHBV) and also with a recombinant epsilon signal, termed REC, in which the lower stem was derived from HBV and the bulge, loop, and upper stem were derived from DHBV (Fig. 3A). On the basis of their predicted structures, the DHBV and HBV epsilon sequences are very similar despite substantial differences in their primary nucleotide sequences. In contrast, the epsilon sequences of the avian hepadnaviruses are almost identical, with the exception of the region encompassing the upper portion of the upper stem. As a consequence of these differences, epsilon of HHBV may have a larger loop region compared with epsilon of DHBV or HBV (Fig. 3A). Unlike

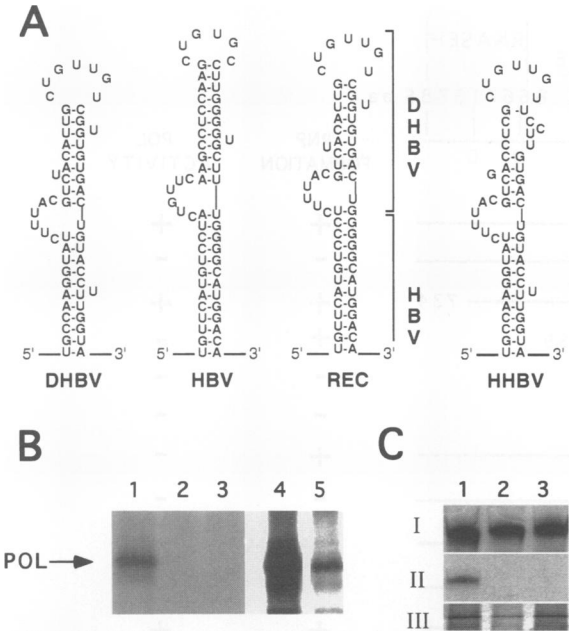

FIG. 3. Polymerase and epsilon form a stable complex. (A) Nucleotide sequence and predicted secondary structure of epsilon (11) derived from DHBV, HBV (23), HHBV (20), and REC. REC is a DHBV epsilon sequence in which the lower stem was replaced with the corresponding sequences from HBV. (B) The polymerase was expressed from RNA transcribed from pHTP, with 3′ ends corresponding to the *Afl*II site at position 2527 (Fig. 1B) in the presence of epsilon from DHBV (lanes 1 and 4), HBV, REC, and HHBV (lanes 2, 3, and 5). The autoradiograph shows the results from the DNA-priming reaction performed in the presence of [^{33}P]dATP and unlabeled dNTPs as described previously (25). (C) The polymerase was translated with [^{35}S]methionine in the presence of the [^{32}P]UTP-labeled epsilon of DHBV, HBV, and REC. The polymerase polypeptides were immunoprecipitated from the lysate with the help of a monoclonal antibody (12CA5-I, BAbco) directed against an influenza virus hemagglutinin epitope present in the spacer region of the polymerase (Fig. 4A), and the precipitated RNA and protein were analyzed on a 7.6 M urea–8% acrylamide gel and on a 0.1% sodium dodecyl sulfate–10% polyacrylamide gel, respectively. Immunoprecipitation of the polymerase polypeptide was performed essentially as described by Persing et al. (16). I, ^{33}P-labeled RNAs of epsilon from DHBV, HBV, and REC (lanes 1 to 3) that were included during the in vitro translation reactions; II and III, immunoprecipitated ^{33}P-labeled DHBV, HBV, and REC epsilon RNAs (lanes 1 to 3) and [^{35}S]methionine-labeled DHBV polymerase polypeptides.

the DHBV epsilon sequence, the HBV signal did not activate the polymerase to initiate minus-strand DNA synthesis at detectable levels (Fig. 3B, lanes 1 and 2). Also, the hybrid structure REC failed to function as a template for the DNA-priming reaction, suggesting that the lower stem bears sequence specific determinants required for polymerase activity (Fig. 3B, lane 3). In contrast, epsilon of HHBV could substitute for the DHBV signal albeit with an approximately fivefold reduced efficiency (Fig. 3B, lanes 4 and 5).

Thus, our results suggested that the activity of the polymerase to prime DNA synthesis required the specific interaction of epsilon sequences with the reverse transcriptase. We next assessed whether the polymerase and epsilon would form a stable RNP complex. For this purpose, we developed an assay for the binding of epsilon to the polymerase, which is based on the immunoprecipitation of ^{33}P-labeled epsilon RNA with a monoclonal antibody directed against a synthetic influenza virus hemagglutinin epitope in the spacer region of the polymerase polypeptide (Fig. 4A) When the polymerase was translated in the reticulocyte lysate in the presence of the radioactive epsilon RNA from DHBV, RNA could be coimmunoprecipitated with the polymerase (Fig. 3C, lane 1). Quantitation of the immunoprecipitated ^{33}P-labeled RNA revealed that roughly equal molar amounts of epsilon RNA and polymerase were recovered in the pelleted material (results not shown). However, under the same conditions, the HBV and REC epsilon sequences could not be detected after immunoprecipitation from the lysate (Fig. 3A, lanes 2 and 3). We estimated that the RNA binding activity of the HBV and REC epsilon sequences for the DHBV polymerase was at least 13 times lower than that of the DHBV epsilon sequence. Hence, these results indicated that the polymerase forms a stable RNP complex with its cognate epsilon sequence and that this interaction is required for the activity of the reverse transcriptase to initiate DNA synthesis.

Binding of the reverse transcriptase to epsilon is controlled by two genetically separated domains on the polymerase polypeptide. To better understand the nature of the interaction between epsilon and the reverse transcriptase, we sought to identify the domains on the polymerase polypeptide that were required for this interaction. The model for the priming of reverse transcription predicts that a tyrosine residue at position 96 of the polymerase is the substrate for the incorporation of the first nucleotide, dGMP, and that the template for this reaction is located in epsilon (22, 24, 26, 27). For this reason, it is likely that the binding of epsilon with the polymerase polypeptide occurs through domains near the catalytic site of the enzyme as well as in the amino-terminal domain close to tyrosine 96 (Fig. 4A).

To identify the domains on the reverse transcriptase that were required for the binding of epsilon we assayed polymerase polypeptides with truncations at their N and C termini for the capacity to form a complex with ^{33}P-labeled epsilon RNA (Fig. 4A). Deletion analyses at the N-terminal region revealed that the first 74 amino acids were dispensable for RNA binding activity. However, a polymerase variant with a deletion extending beyond tyrosine residue 96 to amino acid 126 failed to form a complex with epsilon (Fig. 4A, rows TP1 and TP2, and 4B, lanes 4 to 6). Deletion analyses at the C-terminal region showed that the last 226 amino acids of the polymerase polypeptide are not required for the RNA binding activity (Fig. 4A, rows MM and MB, and 4B, lanes 1 and 2). In contrast, polymerase mutants with deletions spanning beyond amino acid 559 lost the ability to bind epsilon RNA (Fig. 4A, rows MA and MX, and 4B, lanes 3 and 7).

These results indicated that both the N-terminal and reverse transcriptase domains of the polymerase polypeptide are required for the RNA binding reaction and that the RNase H domain was dispensable for the RNA binding activity. These results were confirmed with the analysis of polymerase polypeptides with single amino acid changes or small deletions and insertions in these two domains. For example, when two charged amino acids at positions 183 and 186 in the terminal protein domain were substituted with alanine, the RNA binding activity of the polymerase was lost (Fig. 4A, row M6, and 4B, lane 10). Similarly, mutations in the reverse transcriptase domain at amino acid positions 378 to 385 and 456 also blocked the formation of an RNP complex (Fig. 4A, rows M28 and M68, and 4B, lanes 12 and 14). In contrast, substitution of tyrosine residue 96 with phenylalanine, which abrogates the protein-priming reaction, did not interfere with the RNA binding reaction (27) (Fig. 4A, row Y96F, and 4B, lane 9). As

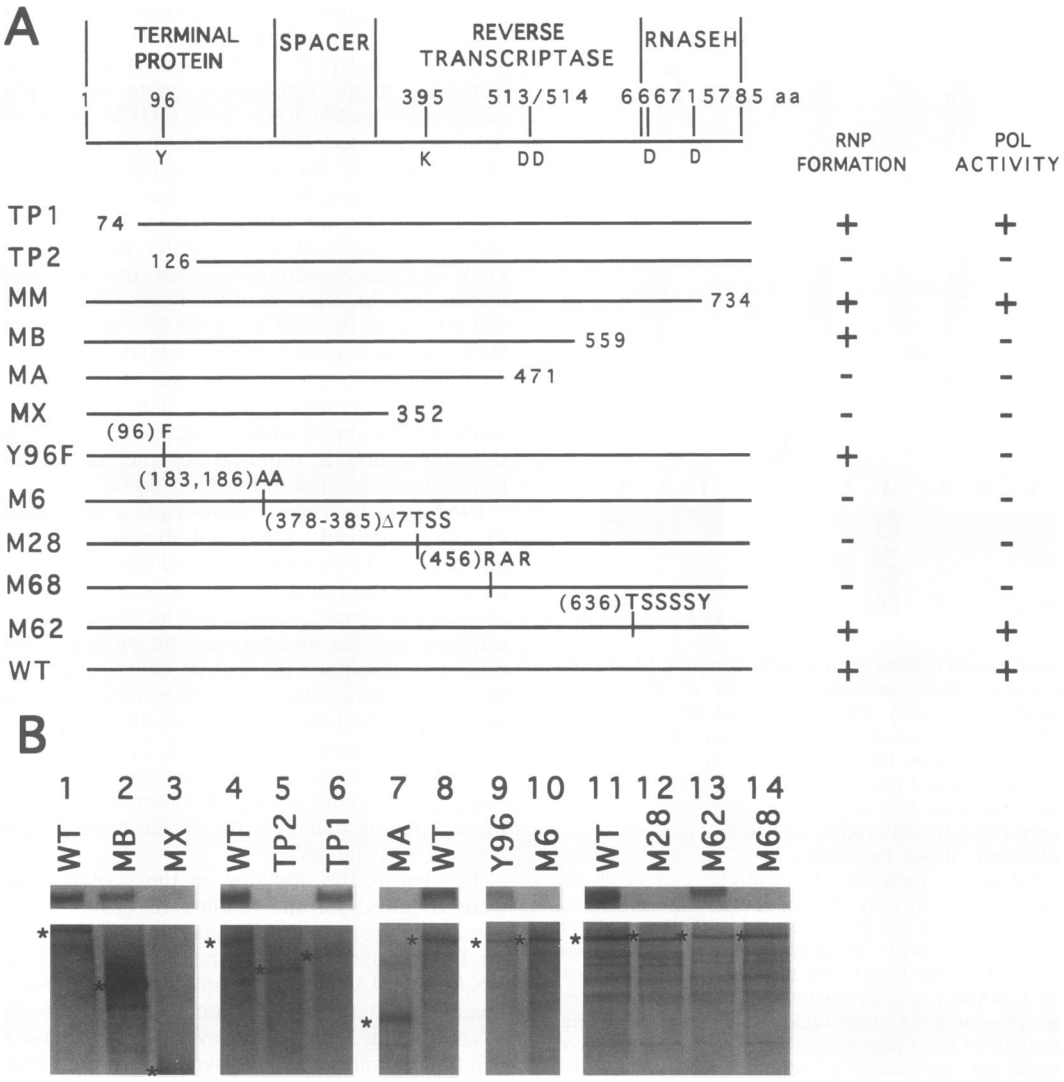

FIG. 4. Identification of the determinants on polymerase polypeptide that are required for epsilon binding. (A) Physical map of the DHBV polymerase gene. Amino acid residues that are conserved in the reverse transcriptase of hepadnaviruses and other retroviruses are indicated (1, 10). Tyrosine (Y) residue 96 is required for formation of the covalent bond between the reverse transcriptase and minus-strand DNA (26, 27). The structures of the polymerase genes with truncations at the 5′ and 3′ termini (TP1, TP2, MM, MB, MA, and MX) and with amino acid substitutions and insertions (Y96F, M6, M28, M68, and M62) are shown below. Variant M28 has a deletion of 7 amino acids (Δ7). The column labeled "RNP formation" summarizes the results shown in panel B. The column labeled "Pol activity" indicates the competence of the different polymerase mutants to initate protein-primed DNA synthesis (results not shown). (B) The polymerase mutants were examined for the ability to bind to ^{33}P-labeled epsilon RNA from DHBV as described in the legend to Fig. 3C. Immunoprecipitated ^{33}P-labeled RNAs and [^{35}S]methionine-labeled polymerase polypeptides (asterisks) are shown in the upper and lower gels, respectively.

expected from previous results, a polymerase variant with a mutation in the RNase H domain remained competent for RNP formation (Fig. 4A, row M62, and 4B, lane 13).

To examine whether the ability of the polymerase to form an RNP complex correlated with the protein-priming activity, we assayed the polymerase variants for the potential to prime minus-strand DNA synthesis. With the exception of mutants MB and Y96F (described in reference 27), all variants that displayed RNA binding activity were also positive in the protein-priming assay (Fig. 4A).

The reverse transcriptase displays a polymerase activity that does not require epsilon. Since plus-strand DNA synthesis is primed by the 3′ hydroxyl group of an RNA oligomer that is base paired with minus-strand DNA (13, 19), we asked whether the in vitro-synthesized reverse transcriptase could prime DNA synthesis in the absence of epsilon. Evidence for DNA synthesis that could occur independently of the protein-priming reaction was obtained with polymerase reactions carried out with RNA A in the absence of epsilon. RNA A spans positions 2373 to 2567 on the DHBV genome and thus lacks epsilon but contains the UUAC motif at position 2537 that corresponds to the natural 5′ end of minus-strand DNA (Fig. 1B). Under these conditions, DNA strands with scattered 5′ ends were synthesized as determined by primer extension analysis performed on DNA synthesized in the in vitro reaction (Fig. 5A, lane 3). Although a DNA band representing minus strands with 5′ ends at position 2537 could be observed, it did not represent the major product from the DNA extension

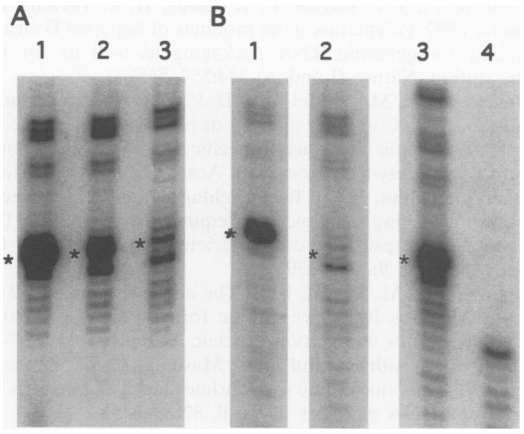

FIG. 5. Reverse transcriptase activity that does not depend on epsilon. (A) DNA products obtained from primer extension reactions that were carried out on DNA synthesized in the in vitro DNA polymerase assay. The extension reactions were primed with a DNA oligomer corresponding to positions 2472 to 2448 on the DHBV genome and carried out as described previously (25). The polymerase was translated from an RNA template transcribed from pHP (27), with 3′ ends corresponding to the *Afl*II site at position 2527. Epsilon (10 μM, final concentration) was present during the translation reaction (lane 1) or was added after the translation of the polymerase to the DNA synthesis reaction (lane 2). RNA A, spanning positions 2373 to 2567 (Fig. 1B), was added after the translation reaction (lanes 1 to 3). (B) DNA products from primer extension reactions obtained with wild-type polymerase (lanes 1 and 3) or the polymerase mutants Y96F and DHA (lanes 2 and 4) (25, 27). Epsilon was present during in vitro translation of the polymerase polypeptides. After translation, RNA A was added to the polymerase reaction mixture. Asterisks depict the DNA products that correspond to minus strands with 5′ ends at position 2537.

reaction. However, when epsilon and RNA A were added together to the polymerase reaction, minus-strand DNA species with 5′ ends mapping to position 2537 were the most prominent reaction products (lane 2). The synthesis of minus-strand DNA with 5′ ends at position 2537 was optimal under conditions in which epsilon was present during the translation reaction, as was the case for the protein-priming reaction (Fig. 5A, lane 1, and Fig. 2A, inset, lanes 2 and 6). The intensity of the labeled band corresponding to minus strands with 5′ ends at position 2537 was approximately 10-fold higher in the presence of epsilon than the signal obtained without epsilon (Fig. 5A, lanes 1 and 3).

Direct evidence for the potential of the reverse transcriptase to initiate DNA synthesis without the protein-priming mechanism was obtained with the help of mutant Y96F, which as described above, lost the capacity to form a covalent bond with DNA and hence to act as a protein primer for minus-strand DNA synthesis (27). When the Y96F polymerase variant was assayed under the same conditions as the wild-type polymerase in the presence of epsilon and RNA A, the reaction yielded DNA strands with apparently random 5′ ends, similar to the pattern observed when the wild-type polymerase was assayed in the absence of epsilon (Fig. 5A, lane 3, and 5B, lanes 1 and 2). It is conceivable that RNA oligomers present in the reticulocyte lysate served as primers for these reactions. As expected, the synthesis of these DNA products was dependent on the expression of enzymatically active reverse transcriptase in the reticulocyte lysate. Mutant DHA, which carries a mutation in a conserved sequence motif (YXDD) among reverse transcriptases (25), was deficient in the synthesis of DNA products observed with the Y96F mutant or with wild-type polymerase (Fig. 5B, compare lanes 1 to 3 with lane 4).

Implications for the protein-priming reaction. We demonstrated that the activity of the hepadnavirus reverse transcriptase to initiate DNA synthesis depends on a specific interaction with epsilon, the packaging signal on pregenomic RNA. The reverse transcriptases of hepadnaviruses are expressed by de novo initiation of translation from pregenomic RNA, in contrast to their retroviral counterparts, which are expressed as inactive Gag-Pol fusion proteins (5, 18). Thus, the interaction between epsilon and the polymerase has two separate functions: the first is to activate the polymerase and the second is to create a packaging signal for the incorporation of the RNA-polymerase complex into viral capsids. However, differences in requirements for primary sequence and structure of epsilon for RNA packaging and protein priming may exist. For example, in the DHBV system RNA packaging depends not only on the presence of epsilon but also on an additional RNA segment located approximately 1 kb downstream of the 5′ end of pregenomic RNA (4). In contrast, the protein-priming activity appears to depend only on epsilon (Fig. 2B). Genetic analyses of the HBV epsilon sequence suggested that in the lower stem, primary sequence may not be required for RNA packaging (17). Our results indicated that, at least in DHBV, the structure of the lower stem by itself is not sufficient for the formation of a stable complex with the polymerase and for the DNA-priming activity (Fig. 3). Furthermore, it is notable that the predicted structure of the epsilon sequence of HHBV forms a very short upper stem with a large loop (Fig. 3A). Yet, despite the differences in primary sequence and predicted structure between the epsilon sequences of HHBV and DHBV, epsilon of HHBV could activate the DHBV polymerase polypeptide (Fig. 3B). Further investigations are necessary to understand the significance of the predicted stem-loop structure of epsilon for the protein-priming reaction and for RNA packaging.

Comparisons of the predicted amino acid sequences of the viral polymerase polypeptides with the sequences of proteins known to form RNP complexes has, so far, not revealed significant similarities that could be used to assign this polypeptide to a particular family of RNA binding proteins. So far, our results suggested that two separate domains of the reverse transcriptase are required for binding to epsilon (Fig. 4). Since epsilon functions as the template for the initiation of DNA synthesis and since this reaction depends on a tyrosine residue near the N terminus and an internal domain of the polymerase polypeptide, the RNA hairpin may have to bind simultaneously to both domains. However, it would be premature to conclude that amino acids in one or the other polymerase domain actually provided contact points for the interaction with epsilon. It is certainly plausible that host factors are required for this interaction as was shown previously for the interaction of the Tat protein with the TAR element on human immunodeficiency virus RNA (14, 15). RNA packaging, however, may require additional domains on the reverse transcriptase that are not required for its ability to bind with epsilon and to prime DNA synthesis. For example, a mutation in the RNase H domain (amino acid 711) of the DHBV polymerase apparently prevents RNA packaging but, according to our results (Fig. 4), would still remain competent to bind epsilon and prime DNA synthesis (6).

A critical step subsequent to the initiation of minus-strand DNA synthesis is the translocation of the short DNA primer to the 3′ end of pregenomic RNA where minus-strand DNA synthesis continues. This step would require an exchange of RNA templates in the active site of the polymerase. Epsilon

must be released so that the 3′ end of pregenomic RNA with the UUAC motif at position 2537 (Fig. 1B) can gain access to the catalytic site of the polymerase. We demonstrated that this reaction can occur in vitro under our selected assay conditions and that, as expected, elongation of minus-strand DNA with natural 5′ ends (position 2537) depends on the presence of epsilon (Fig. 5). The genetic criteria for this transfer reaction are still elusive. We tested a model which predicts that a second binding site for the polymerase is present between positions 2373 and 2563 on RNA A and thus facilitates the switch of RNA templates required to continue DNA synthesis (Fig. 1B). However, we found that elongation could occur equally well from heterologous RNA fragments provided that they contained a UUAC sequence motif for the annealing of the four-nucleotide-long GTAA sequence motif that is covalently linked to the polymerase (reference 24 and results not shown). Although we cannot exclude that such a signal exists further upstream on pregenomic RNA, it is quite possible that the precise arrangement of pregenomic RNA and polymerase in viral capsids accounts for the specificity of this transfer reaction.

We are grateful to William S. Mason and Richard Katz for critical reviews of the manuscript. We acknowledge the help of Sanam Roder during a part of this study and thank Jesse Summers for providing a DHBV genomic library for the isolation of polymerase mutants. We thank Dan Loeb and Haiyan Jiang for the communication of unpublished results, which were critical for the design of the experiments shown in Fig. 5.

This work was supported by Public Health Service grants from the NIH and by an appropriation from the Commonwealth of Pennsylvania.

REFERENCES

1. **Argos, P.** 1988. A sequence motif in many polymerases. Nucleic Acids Res. **16:**9909–9916.
2. **Bartenschlager, R., M. Junker-Niepmann, and H. Schaller.** 1990. The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. J. Virol. **64:**5324–5332.
3. **Bartenschlager, R., and H. Schaller.** 1988. The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. EMBO J. **7:**4185–4192.
4. **Calvert, J., and J. Summers.** 1994. Two regions of an avian hepadnavirus RNA pregenome are required in *cis* for encapsidation. J. Virol. **68:**2084–2090.
5. **Chang, L.-J., P. Pryciak, D. Ganem, and H. E. Varmus.** 1989. Biosynthesis of the reverse transcriptase of hepatitis B viruses involves de novo translation initiation not ribosomal frameshifting. Nature (London) **337:**364–368.
6. **Chen, Y., W. S. Robinson, and P. L. Marion.** 1992. Naturally occurring point mutation in the C terminus of the polymerase gene prevents duck hepatitis B virus RNA packaging. J. Virol. **66:**1282–1287.
7. **Gerlich, W. H., and W. S. Robinson.** 1980. Hepatitis B virus contains protein attached to the 5′ terminus of its complete DNA strand. Cell **21:**801–809.
8. **Hirsch, R., D. D. Loeb, J. Pollack, and D. Ganem.** 1991. *cis*-acting sequences required for encapsidation of duck hepatitis B virus pregenomic RNA. J. Virol. **65:**3309–3316.
9. **Hirsch, R. C., J. E. Lavine, L. J. Chang, H. E. Varmus, and D. Ganem.** 1990. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. Nature (London) **344:**552–555.
10. **Johnson, M. S., M. A. McClure, D.-F. Feng, J. Gray, and R. F. Doolittle.** 1986. Computer analysis of retroviral pol genes: assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. Proc. Natl. Acad. Sci. USA **83:**7648–7652.
11. **Junker-Niepmann, M., R. Bartenschlager, and H. Schaller.** 1990. A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J. **9:**3389–3396.
12. **Knaus, T., and M. Nassal.** 1993. The encapsidation signal on the hepatitis B virus RNA pregenome forms a stem-loop structure that is critical for its function. Nucleic Acids Res. **21:**3967–3975.
13. **Lien, J., C. E. Aldrich, and W. S. Mason.** 1986. Evidence that a capped oligoribonucleotide is the primer for duck hepatitis B virus plus strand DNA synthesis. J. Virol. **57:**229–236.
14. **Madore, S. J., and B. R. Cullen.** 1993. Genetic analysis of the cofactor requirement for human immunodeficiency virus type 1 Tat function. J. Virol. **67:**3703–3711.
15. **Marciniak, R. A., B. J. Calnan, A. D. Frankel, and P. A. Sharp.** 1990. HIV-1 tat protein trans-activates transcription in vitro. Cell **63:**791–802.
16. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1986. Antibodies to pre-S and X determinants arise during natural infection with ground squirrel hepatitis virus. J. Virol. **60:**177–184.
17. **Pollak, J. R., and D. Ganem.** 1993. An RNA stem-loop structure directs hepatitis B virus genomic RNA encapsidation. J. Virol. **67:**3254–3263.
18. **Schlicht, H. J., G. Radziwill, and H. Schaller.** 1989. Synthesis and encapsidation of duck hepatitis B virus reverse transcriptase do not require formation of core-polymerase fusion proteins. Cell **56:**85–92.
19. **Seeger, C., D. Ganem, and H. E. Varmus.** 1986. Biochemical and genetic evidence for the hepatitis B virus replication strategy. Science **232:**477–484.
20. **Sprengel, R., E. F. Kaleta, and H. Will.** 1988. Isolation and characterization of a hepatitis B virus endemic in herons. J. Virol. **62:**3832–3839.
21. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29:**403–415.
22. **Tavis, J. E., S. Perri, and D. Ganem.** 1994. Hepadnavirus reverse transcription initiates within the stem-loop of the RNA packaging signal and employs a novel strand transfer. J. Virol. **68:**3536–3543.
23. **Valenzuela, P., M. Quiroga, J. Zaldivar, P. Gray, and W. J. Rutter.** 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes, p. 57–70. *In* B. Fields, R. Jaenisch, and C. F. Fox (ed.), Animal virus genetics. Academic Press, Inc., New York.
24. **Wang, G.-H., and C. Seeger.** 1993. Novel mechanism for reverse transcription in hepatitis B viruses. J. Virol. **67:**6507–6512.
25. **Wang, G. H., and C. Seeger.** 1992. The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. Cell **71:**633–670.
26. **Weber, M., V. Bronsema, H. Bartos, A. Bosserhoff, R. Bartenschlager, and H. Schaller.** 1994. Hepadnavirus P protein utilizes a tyrosine residue in the TP domain to prime reverse transcription. J. Virol. **68:**2994–2999.
27. **Zoulim, F., and C. Seeger.** 1994. Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase. J. Virol. **68:**6–13.