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Evidence for Activation of the Hepatitis B Virus Polymerase by Binding of Its RNA Template

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The hepatitis B viruses replicate by reverse transcription of an RNA pregenome by using a virally encoded polymerase. A key early step in replication is binding of the polymerase to an RNA stem-loop (ε) of the pregenome; ε is both the RNA encapsidation signal and the origin of reverse transcription. Here we provide evidence that this interaction is also key to the development of enzymatic activity during biosynthesis of the polymerase. Duck hepatitis B virus polymerase expressed in *Saccharomyces cerevisiae* can synthesize DNA from ε-containing RNAs and can also end label other small RNAs. Expression of functional polymerase in *S. cerevisiae* requires interaction between the polymerase and ε during or shortly after translation for it to develop any enzymatic activity; if ε is absent during expression, the polymerase is inactive on RNAs both with and without ε. Functional duck polymerase can also be produced by in vitro translation, and synthesis of the polymerase in the presence of ε induces resistance in the polymerase to proteolysis by papain, trypsin, and bromelain. Induction of the resistance is specific for ε sequences that can support RNA encapsidation and initiation of DNA synthesis. Induction of the resistance precedes initiation of DNA synthesis and is reversible by degradation of ε. These two sets of data (i) support a model in which binding of ε to the polymerase induces a structural alteration of the polymerase prior to the development of enzymatic activity and (ii) suggest that this alteration may be required for the polymerase to mature to an active form.

Hepatitis B virus (HBV) replicates noncytolytically in the cytoplasm of hepatocytes and continuously exports newly synthesized virions from infected cells via the constitutive secretory pathway (9, 15, 19). Hepatocytes are long-lived cells that can continue to produce virus indefinitely, and therefore it is advantageous for HBV to limit its impact on the host cell in order to maximize production of progeny virions. However, because HBV replicates its genome by reverse transcription of a pregenomic RNA (pgRNA) template (14, 20), the cell is presented with a potentially hazardous situation through the introduction of a reverse transcriptase into the cytoplasm. If the HBV reverse transcriptase (P) were able to indiscriminately copy cellular RNAs into cDNAs, highly recombinogenic single-stranded DNAs would be produced, and integration of such DNAs could alter the host genome.

Many retroviruses avoid this problem by synthesizing reverse transcriptase as a precursor fused to the viral Gag protein. The retroviral polymerase usually does not become active until after it is packaged into the nascent virion and is cleaved from the Gag-Pol precursor polyprotein. Once the enzyme is safely within the viral core, it is secluded from the cellular mRNA pool and presents no direct danger to the host cell's genome. However, this mechanism cannot function for HBVs (hepadnaviruses) because these viruses synthesize their reverse transcriptase independently from the core protein (the homolog of Gag), and hence P is potentially active prior to encapsidation.

P recognizes its appropriate RNA template within the large pool of cellular mRNAs through specific binding between P and a characteristic stem-loop (ε) found on the pgRNA. However, it is unknown how P avoids synthesis of DNA from the myriad of other RNAs available to it in the cytoplasm. The lack of activity by P on cellular RNAs is only partially explained by the absence of ε, because although ε is both the hepadnaviral RNA encapsidation signal and the origin of reverse transcription (4, 23, 25), any RNA template with a primer annealed to it (for example, by hairpin formation) would be a suitable substrate for reverse transcription.

Another unexplained characteristic of the hepadnaviral P is its "template commitment"; that is, P is normally unable to release the pgRNA and then synthesize DNA from another primer-template combination (17). Other reverse transcriptases, such as the retroviral enzymes, can synthesize DNA from heterologous primer-templates, but the hepadnaviral enzyme cannot. Some of this restriction is likely due to the covalent linkage of the viral DNA to P itself that results from the protein-priming mechanism employed by P (24), but even when the viral DNA is removed by enzymatic digestion, P is still unable to synthesize DNA from exogenous DNA primer-templates (20a). The template commitment of P and the exclusion of its reverse transcriptase activity on cellular RNAs are related phenomena in that they both limit the function of P to the pgRNA, but it is unknown if the mechanisms responsible for them are related.

We have been studying the early events in hepadnaviral reverse transcription by employing functional duck hepatitis B virus (DHBV) P expressed in *Saccharomyces cerevisiae* (21). In this system, the yeast retrotransposon TY1 is used as a vector and P is expressed as a fusion protein with the TY1 capsid-like protein (TYA). The TYA-DHBV P fusion protein (TYDP) is packaged into virus-like particles (VLPs) along with an ε-containing template RNA. Within these particles, TYDP initiates reverse transcription authentically from ε and extends the resulting DNA up to 2.5 kb; TYDP retains reverse transcriptase activity within the VLPs after purification and can extend the

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preinitiated DHBV DNAs in vitro. We also employed functional DHBV P translated in vitro in rabbit reticulocyte lysates. P expressed in this manner initiates reverse transcription authentically and synthesizes short DNAs from ε-containing RNA templates (24). DHBV P synthesized in vitro can also be used for specific binding assays with RNAs containing ε (16, 26).

Here we report that interaction between the DHBV P and a functional ε RNA induces increased resistance to proteolysis in P, and we present data implying that the P-ε interaction is essential for P to develop enzymatic activity. On the basis of these data, we propose a model for the maturation pathway of the hepadnaviral polymerase.

MATERIALS AND METHODS

DNA constructs and in vitro transcription. pTYBDP-DR1/SL and its derivatives have been described previously (22, 23) and contain the DHBV type 3 P open reading frame and 3′ noncoding sequences within the yeast retrotransposon TY1-H3; TYBDP-dl1 contains a deletion of DHBV nucleotides (nt) 2531 to 2606 and deletes ε (23). Expression of these sequences is under control of the inducible *GAL1* promoter. The mutations introduced into the DR1 and ε coding sequences and are designated DRM*x*/SLM*x* (direct repeat mutant no./stem-loop mutant no., e.g., DRM3/SLM3). All mutations are downstream of the P termination codon. Plasmid pdε and its derivatives (see Table 1) contain the DHBV ε coding sequences within the transcription vector pBluescript (Promega) and have already been described (16). RNAs were transcribed with T3 RNA polymerase from the pdε plasmids linearized with *Eco*RV to produce a 172-nt transcript. Plasmid pSL-BS contains DHBV nt 2554 to 3021 within pBluescript; T3 transcription from *Sal*I-linearized DNA produces an 85-nt transcript. For the mutant derivatives of pSL-BS used in this study, see Table 1. pT7DPol contains DHBV type 3 nt 170 to 3021 within pBluescript; the allele employed contains a 33-nt insertion at nt 901 encoding the influenza virus hemagglutinin epitope (HA Tag; 13). mRNAs for DHBV P lacking ε were transcribed with T7 RNA polymerase from *Afl*II-linearized pT7DPol. pDXBdlNS contains DHBV nt 2351 to 2662 in pBluescript such that T3 transcription produces antisense RNA. Substrates for the *trans* assay were produced from plasmid pDXBdlNS in either positive or negative polarity or from pDRF (DHBV nt 2401 to 2605 pBluescript) in positive polarity (DRF positive-polarity RNA). Only the DXBdlNS positive-polarity RNA contains the complete ε. All RNAs were transcribed from linearized plasmids with the appropriate Megascript Kit (Ambion) in accordance with the manufacturer's instructions.

Isolation of VLPs. VLPs were isolated as described earlier (21). Briefly, yeast cultures containing TYDP expression plasmids were induced by addition of galactose, and 20 to 22 h later, cells were collected and lysed. The clarified lysate was layered onto a three-layer sucrose step gradient (60, 30, and 20%) and centrifuged at 100,000 × *g* for 3 h. Particulate matter at the interface between the 60 and 30% sucrose steps was collected, concentrated by centrifugation, and suspended at a concentration of 1 μg/μl; such extracts are highly enriched for VLPs.

VLP reverse transcriptase assays. Reverse transcriptase activity for which the template is RNA within the VLP (the *cis* assay) was measured by incubating 5 μg of protein from the VLP extracts with 50 mM Tris (pH 8.0)–100 mM NaCl–0.1% Nonidet P-40–10 mM $MgCl_2$–2.5% 2-mercaptoethanol–4 U of RNasein (Promega)–40 μM each dATP, dCTP, and dTTP–2 μCi of [α-^{32}P]dGTP (3,000 Ci/mmol) in a total volume of 20 μl at 37°C for 1 h. The reactions were terminated by addition of Laemmli loading buffer, the samples were boiled for 4 min, and the radioactive products were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 7% polyacrylamide gels. The gels were dried, and the radioactive products were detected by autoradiography.

DNA polymerase activity employing exogenous RNA substrates (the *trans* assay) was detected by first digesting 5 μg of protein from the VLP extracts with 30 U of micrococcal nuclease (Boehringer Mannheim) in the presence of 5 mM $CaCl_2$ at 30°C for 15 min to destroy the endogenous RNA. The micrococcal nuclease was then inactivated by addition of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to 7.5 mM, the reaction conditions were adjusted to those employed for the *cis* assay (25-μl total volume), and 0.5 μg of substrate RNA was added. The reaction mixture was incubated at 37°C for 90 min, and DNA synthesis was terminated by addition of Laemmli buffer and boiling. The products were resolved and detected as for the *cis* assay. SDS-PAGE gels were used in the analysis because the large quantity of protein in the *trans* reaction interfered with electrophoresis on standard acrylamide-Tris-borate-EDTA gels. Control experiments employing purified DRF positive-polarity RNA on polyacrylamide gels indicated that the *trans* product comigrated with the substrate RNA under these more traditional electrophoretic conditions.

The substrate RNA was dephosphorylated (see Fig. 1B) by incubating 5 μg of DRF positive-polarity RNA with 10 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) in 100 μl of the manufacturer-supplied buffer with 80 U of RNasein (Promega) at 37°C for 70 min. The RNA was then extracted with phenol and chloroform and precipitated with ethanol. The phosphatase was omitted from the mixture for the Mock CIP control. Dephosphorylation was confirmed prior to use in the *trans* assay by incubating a sample of the RNA with [γ-^{32}P]ATP and polynucleotide kinase and observing the labeling of the RNA.

The 3′ end of the RNA was modified with ddGTP and ddCTP by incubating 4 μg of DRF positive-polarity RNA with 20 U of terminal deoxynucleotidyl transferase (Promega) in 40 μl of the manufacturer-supplied buffer containing 125 μM either ddGTP or ddCTP at 37°C for 70 min. The $CoCl_2$ within the reaction buffer allows tailing of the 3′ hydroxyl of any polynucleotide. The RNA was then extracted with phenol and chloroform and precipitated with isopropanol. The transferase was omitted from a mixture containing ddGTP for the Mock TdT control.

In vitro translation. ^{35}S-labeled DHBV P was translated in vitro by employing the micrococcal nuclease-treated Rabbit Reticulocyte Lysate System from Promega in a 20-μl total volume employing [^{35}S]methionine (>1,000 Ci/mmol; Amersham) in accordance with the manufacturer's instructions. Translation was terminated by addition of cycloheximide to 40 μM. The mRNA employed did not contain ε; however, ε RNAs (125 ng) were added during translation as indicated in Results.

Partial proteolysis of P-ε complexes. Translation mixtures (3.5 μl) were placed on ice, and 6.5 μl of an ice-cold protease solution (freshly diluted in diethylpyrocarbonate-treated water containing 0.15 μg of papain [0.0038 U; Sigma], 0.05 μg of trypsin [0.64 U; Sigma], or 0.5 μg of bromelain [0.00065 U; Sigma]) was added. The mixtures were incubated at 14°C for 10 min, and proteolysis was terminated by placing the mixtures on ice and adding 20 μl of 1.5× Laemmli buffer. The samples were run on SDS–12% PAGE gels, the gels were dried, and the partial digestion products were detected by autoradiography. When indicated in Results, RNA in the translation reaction mixtures was destroyed (subsequent to addition of cycloheximide but prior to proteolysis) by adding of 10 μg of RNase A or 190 U of RNase T_1 and incubating the mixtures at 30°C for 10 min. For DNA priming conditions (see Fig. 8), translation was terminated, $MgCl_2$ was added to 4 mM, deoxynucleoside triphosphates (dNTPs) were added to 20 μM, and the samples were incubated at 30°C for 15 min prior to proteolysis. Vigorous DNA priming occurs under these conditions, which are based on those of Wang and Seeger (24).

DNA priming. DNA priming was detected by translating P in the presence of ε and terminating translation by addition of cycloheximide. $MgCl_2$ was added to 4 mM, and 10 μCi of [α-^{32}P]dGTP was added. Samples were incubated at 37°C for 30 min, and the reactions were terminated by addition of Laemmli loading buffer prior to resolution by SDS-PAGE.

ε binding. Binding assays were performed as described by Pollack and Ganem (16).

Northern (RNA) blots. RNAs were isolated from the translation mixtures by phenol and chloroform extraction followed by ethanol precipitation. RNAs were resolved on 1.2% agarose–TBE gels and transferred to Hybond N (Amersham). The probe was prepared by random-primed labeling of the DHBV sequences within pSL-BS. The hybridization and wash conditions used were previously described (2).

RESULTS

Enzymatic activity of TYDP on exogenous RNAs. TYDP can synthesize DNA in vitro from an ε-containing RNA that is copackaged with the polymerase into the VLPs. This *cis* activity is authentic reverse transcription and extends DHBV DNAs that were previously initiated within the yeast cells prior to VLP isolation (21). In the course of unsuccessful attempts to detect in vitro initiation by TYDP, it was noted that the enzyme can also synthesize DNA from exogenously added RNAs in *trans* (Fig. 1). This *trans* activity is weak, and to detect it the RNA within the VLPs must be removed prior to addition of the exogenous RNA substrate to eliminate the signal from the more vigorous *cis* activity. Micrococcal nuclease is employed to destroy the endogenous RNA because this enzyme requires Ca^{2+}, and hence it can be specifically inactivated by addition of the calcium chelator EGTA.

The *trans* activity requires an exogenously added RNA (Fig. 1A, lanes 1 and 2) but has no discernible sequence specificity for the substrate RNA (all of the small RNAs tested to date support the activity to various levels; 20a). The product of the *trans* assay cannot be removed from the aqueous phase by phenol extraction (Fig. 1A, lane 5), indicating that it is not covalently attached to P and, hence, that it does not result from protein-primed initiation. The *trans* product (which is labeled with radioactive dNTPs) can be partially degraded by RNase

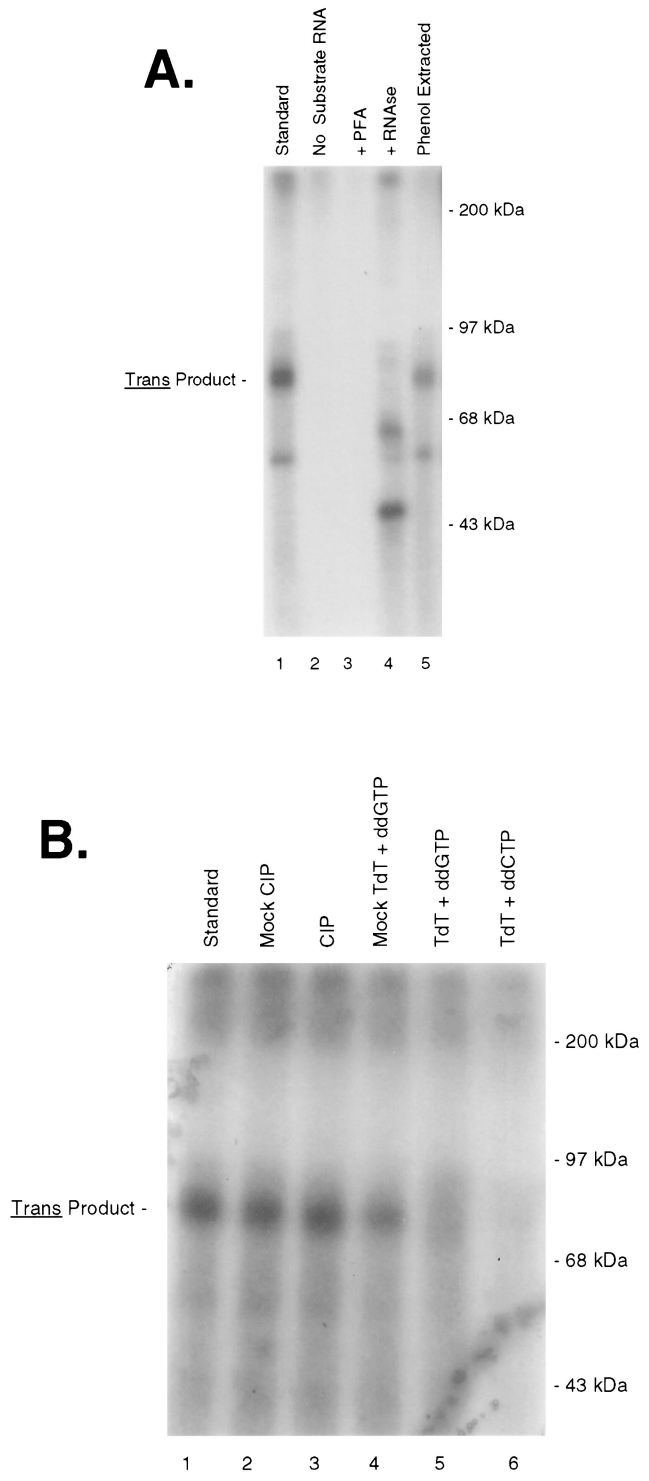

FIG. 1. Characterization of the *trans* product. (A) Characterization of the *trans* product. TYDP VLPs were treated with micrococcal nuclease and Ca^{2+} to remove the endogenous RNA, and then the nuclease was inactivated by addition of EGTA. Enzymatic activity was detected by addition of a substrate RNA (positive-polarity DRF) and radioactive dNTPs. Additional experimental conditions are listed above the lanes. The standard conditions are shown in lane 1, and the exogenous RNA substrate was omitted in lane 2. Inhibition of the *trans* activity by 2 mM phosphonoformic acid is shown in lane 3. Lane 4 contains the RNase T_1 digestion products of a standard *trans* assay, and the sample in lane 5 was extracted with phenol and chloroform after the *trans* assay. The products were resolved by SDS-PAGE and detected by autoradiography. The mobility of protein molecular mass markers and the location of the *trans* product are shown. (B) Labeling of the *trans* product at the 3′ end. The *trans* reaction was performed as described for panel A with DRF positive-polarity substrate RNA. Lanes: 1, standard reaction; 2, *trans* reaction with RNA that had been mock dephosphorylated; 3, *trans* reaction with RNA that had been dephosphorylated with calf alkaline phosphatase prior to the assay; 4, *trans* reaction with RNA that had been incubated prior to the *trans* reaction in terminal transferase buffer with ddGTP but without the transferase; 5, *trans* reaction with RNA incubated with terminal transferase and ddGTP; 6, *trans* reaction with RNA incubated with terminal transferase and ddCTP. The figure was produced electronically and printed by dye sublimation. The original autoradiographs were digitized with a MicroTek ScanMaker 600Z scanner, the size and resolution of the image were adjusted with Microsoft Imager, and the figure was labeled with Microsoft Powerpoint.

(Fig. 1A, lane 4), indicating that it is an RNA-DNA chimera. The RNase digestion was incomplete because the substrate RNA (DRF positive-polarity RNA) contains extensive secondary structure and RNase T_1 attacks G residues in single-stranded regions. Terminal digestion of the RNA and resolution of the residual DNA on high-percentage sequencing gels indicate that the DNA portion of the molecule is heterogeneous in length and contains less than 10 nt (20a). Finally, the DNA appears to be added to the 3′ end of the RNA molecule because dephosphorylation of the substrate RNA has no effect on the *trans* reaction (Fig. 1B, lanes 1 to 3), whereas adding ddGTP or ddCTP to the 3′ end of the substrate RNA with terminal deoxynucleotidyl transferase inhibits the *trans* reaction (Fig. 1B, lanes 4 to 6). However, it is unknown if the 3′ or 2′ hydroxyl of the RNA is involved in priming of the *trans* reaction (bacterial reverse transcriptases employ an internal 2′ hydroxyl as a primer for DNA synthesis; 10).

This chimeric RNA-DNA molecule could be produced by TYDP through either template-free addition of nucleotides to the RNA in a terminal transferase-like reaction (limited template-free polymerization activity is common with polymerases lacking a 3′ to 5′ proofreading exonuclease activity; 3) or by hairpin formation by the substrate RNA to create a suitable primer-template. In either case, the *trans* product itself is unlikely to be physiologically relevant, but there are two observations that indicate that the *trans* reaction accurately reflects the catalytic activity of the polymerase. First, the *trans* reaction is inhibited by phosphonoformic acid, an inhibitor of the hepadnaviral reverse transcriptase (Fig. 1A, lane 3), and second, mutation of TYDP to inactivate the polymerase active site eliminates the *trans* activity (Fig. 2A, lane 8). These two observations indicate that the *trans* activity employs the same catalytic site as does the physiologically relevant *cis* activity and, consequently, that the *trans* activity is relevant to the enzymology of the hepadnaviral polymerase extension reaction.

The most interesting feature of the *trans* reaction is not the activity itself but rather which TYDP constructs can perform it. Figure 2A indicates that only those TYDP molecules that are synthesized in the presence of a functional ε (and that can therefore initiate authentic reverse transcription [the *cis* reaction]) are active on exogenously supplied RNAs (the *trans* reaction). TYDP must interact with ε within the yeast cells prior to isolation to be active in the *trans* assay, because TYDP synthesized in the absence of a functional ε RNA is inactive even when the substrate RNA for the *trans* assay contains a functional ε (Fig. 2B). A perfect quantitative correlation has been observed between the activity of TYDP in the *cis* and *trans* assays with 15 ε sequences. The dependence of the *cis* assay on ε is not surprising, because ε is the origin of reverse transcription. However, the dependence of the *trans* assay on ε is unexpected for two reasons: (i) the ε-containing RNAs are digested with micrococcal nuclease prior to addition of the

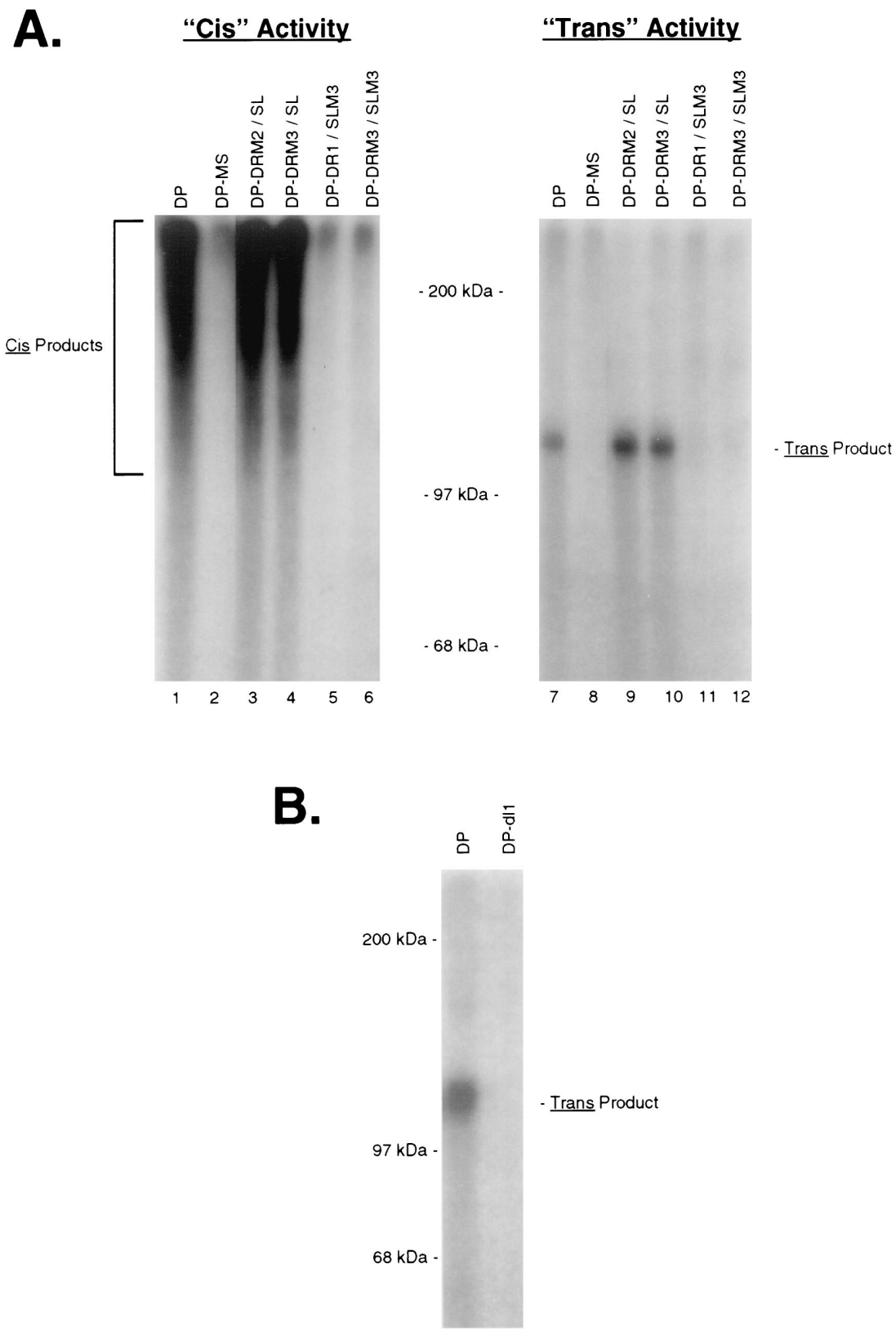

FIG. 2. Correlation between *cis* and *trans* activities and requirement for ε during synthesis of P. (A) Correlation between *cis* and *trans* activities. The left panel ("Cis" Activity) shows the reverse transcriptase activity on the endogenous RNA within the VLPs, and the right panel ("Trans" Activity) shows the corresponding activity for the same TYDP constructs on an exogenously added RNA substrate (DXBdlNS negative-polarity RNA). The TYDP constructs employed are indicated above the gels: DP represents wild-type TYDP, DP-MS contains mutations in the reverse transcriptase active site and lacks all polymerization activity, DRM2 and DRM3 contain mutations within the DR1 sequence, and SLM3 contains mutations within ε. The products were resolved on SDS-PAGE gels and detected by autoradiography. The mobility of protein molecular mass markers and the locations of the *cis* and *trans* products are shown. (B) TYDP must be synthesized in the presence of ε to be active in the *trans* assay. TYDP was synthesized in the presence (lane DP) or the absence (lane DP-dl1) of ε, and these polymerases were tested for *trans* activity on an exogenously added RNA containing a functional ε (DXBdlNS positive-polarity RNA). The products were resolved on SDS-PAGE gels and detected by autoradiography. The mobility of protein molecular mass markers and the location of the *trans* product are shown. The figure was produced electronically as described in the legend to Fig. 1.

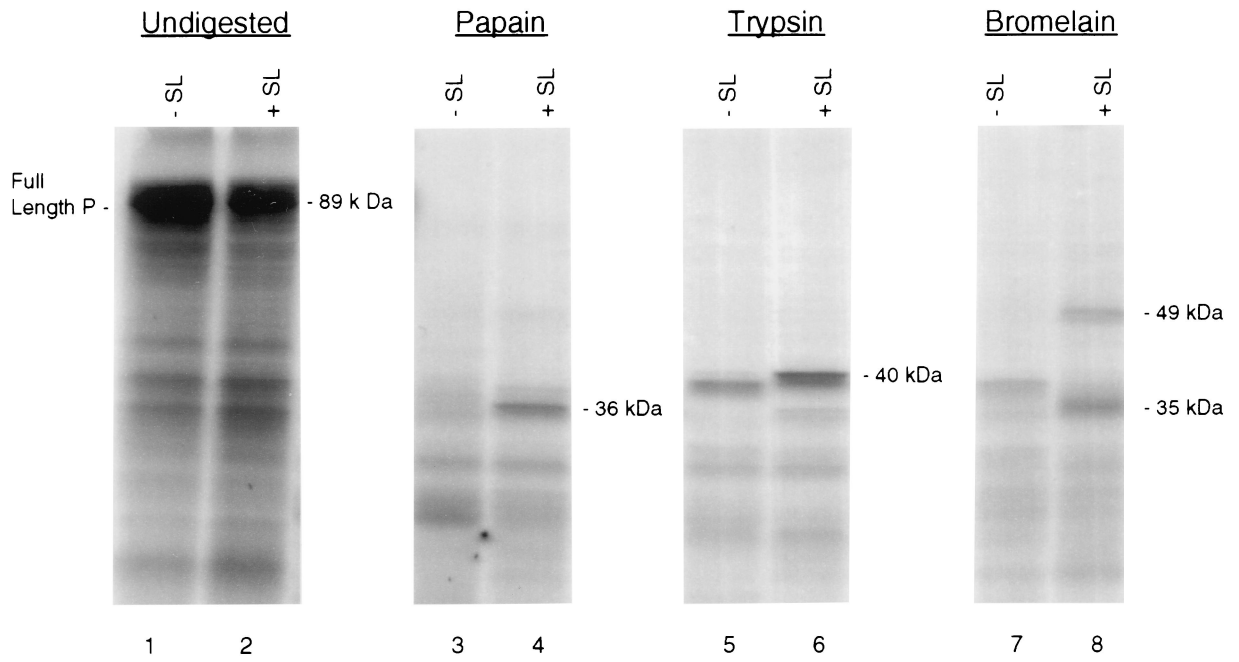

FIG. 3. Translation of DHBV P in the presence of ε (SL) increases resistance to proteolysis. DHBV P was translated in rabbit reticulocyte lysates in the presence (even-numbered lanes) or absence (odd-numbered lanes) of ε prior to partial digestion with papain (lanes 3 and 4), trypsin (lanes 5 and 6), or bromelain (lanes 7 and 8). The sizes of the undigested full-length P and the primary protease resistance fragments are indicated. The figure was produced electronically as described in the legend to Fig. 1.

dNTPs and the substrate RNA, and (ii) the ε sequences are located 3′ to the P open reading frame in the TYDP constructs. Consequently, the primary sequences of the polymerases are identical in these constructs and the RNA that contains the differences between them is destroyed prior to the start of the *trans* assays. Therefore, the dramatic difference in the abilities of these enzymes to support the *trans* activity suggests that the ε sequences are required during the biosynthesis of the polymerase to produce catalytically active protein.

Protease digestion profiles of in vitro synthesized DHBV P. The preceding data suggest that an interaction between TYDP and ε during or shortly after translation enables the enzyme to perform the *trans* assay. An attractive model to account for this observation is that ε induces a posttranslational alteration of the polymerase. There are many candidates for this putative alteration, such as complex formation with cellular proteins, chemical modification, or conformational isomerization. As ε-dependent isomerization of P is plausible and readily testable, we probed the conformation of P-ε complexes through partial proteolysis. In these experiments, we employed in vitro-synthesized P because it is enzymatically active, binds specifically to ε, and can be easily detected through incorporation of [^{35}S]methionine during translation. DHBV P was synthesized in rabbit reticulocyte lysates with or without ε, and translation was terminated by addition of cycloheximide. Aliquots of the translation mixtures were then subjected to limited digestion with papain, trypsin, or bromelain, and the digestion products were resolved on high-percentage SDS-PAGE gels prior to detection by autoradiography. Figure 3 shows that fragments of P with significantly increased resistance to proteolysis are detected when P is synthesized in the presence of ε and that these fragments are absent when ε is omitted from the translation mixtures. Although the resistant fragments in lanes 4, 6, and 8 appear much fainter than does the primary translation product in lane 2, they represent a very large proportion of the P molecules that are capable of binding to ε because only about 10% of the molecules synthesized in the reticulocyte lysates can bind ε (16, 20a), and because the fragments are much smaller than is the full-length P and hence contain fewer labeled methionine residues. All of the subsequent experiments were performed with all three proteases and yielded identical results, and therefore only the data obtained by papain digestion are presented in the remaining figures.

Production of protease-resistant fragments requires functional ε. Induction of protease resistance in P is specific for ε because irrelevant RNAs (a transcript from the plasmid pBluescript) or a DHBV sequence lacking ε (either positive or negative polarity) failed to induce protease resistance (Fig. 4). We next determined the ability of mutant ε RNAs to induce the protease resistance (Fig. 5A). dε-dlBulge, dε-Loop3,4, and dε-Loop5,6 failed to induce protease resistance in P (lanes 5, 6, and 7); dε-dlU1 and dε-LowerL/R induced low levels of resistance (lanes 8 and 9); and SLM2 induced wild-type protease resistance (lane 10). The induction of protease resistance by the mutant ε RNAs correlates very well with the ability of the ε RNAs to support pgRNA encapsidation and priming of DNA synthesis (Table 1; binding, packaging, and priming data for the dε series are from reference 16). However, simple binding of P to ε was insufficient to induce protease resistance in P because both dε-Loop3,4 and dε-Loop5,6 bound to P yet failed to induce resistance (Fig. 5A, lanes 5 and 6, and Table 1). This key point is emphasized in Fig. 5B, where the bromelain digestion patterns for dε and dε-Loop5,6 sequences are shown. ε (lane 1) efficiently induced bromelain resistance, whereas dε-Loop5,6 (lane 2) did not. Also shown in Table 1 are binding, priming, and proteolysis data for eight additional mutant ε RNAs that expand the correlation of ε function with the induction of protease resistance.

Production of the protease-resistant fragments requires the continual presence of ε RNA. The alteration to P that results

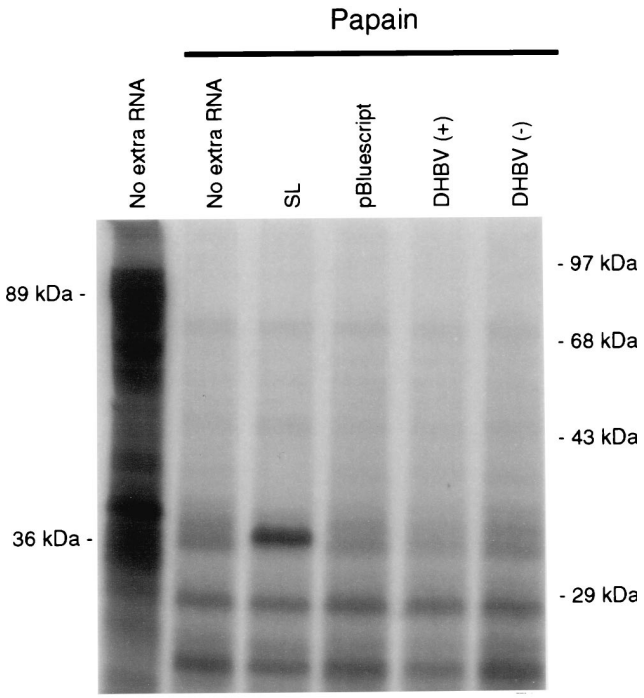

FIG. 4. Induction of protease resistance is specific for DHBV ε (SL). DHBV P was translated in vitro in the presence of no extra RNA (lanes 1 and 2), ε (lane 3), nt 924 to 737 encoded by plasmid pBluescript (lane 4), DHBV positive-polarity RNA lacking the entire ε (nt 2401 to 2662; lane 5), or the same DHBV sequences of negative polarity (lane 6). Samples 2 to 6 were partially digested with papain. The mobilities of the full-length P and the primary protease-resistant fragments are indicated at the left, and the mobility of molecular mass markers is indicated at the right. The figure was produced electronically as described in the legend to Fig. 1.

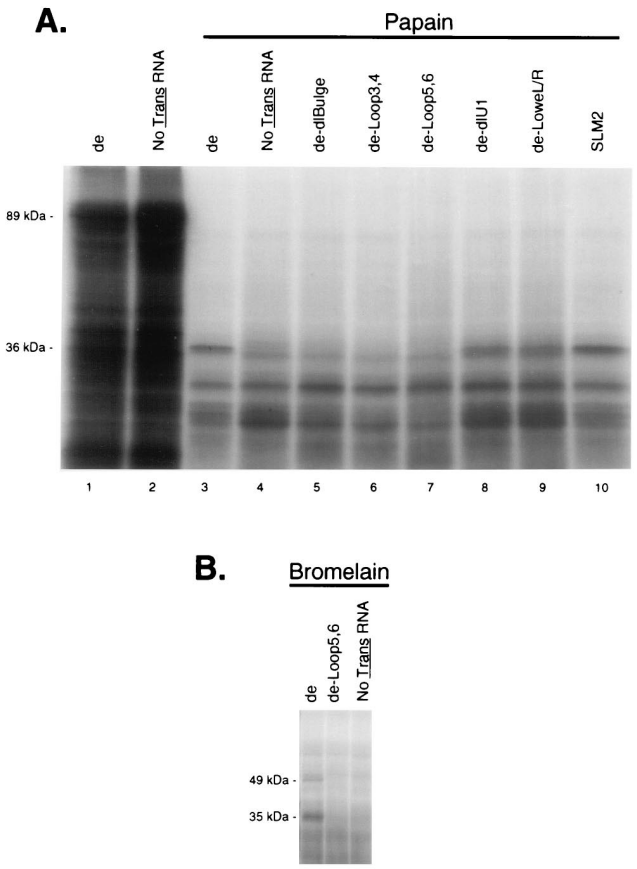

FIG. 5. Only functional ε sequences can induce protease resistance. (A) Papain digestion with mutant ε sequences. DHBV P was translated in vitro in the presence of the wild-type ε (lanes 1 and 3), no ε RNA (lanes 2 and 4), and mutant ε sequences (lanes 5 to 10) prior to partial digestion with papain (lanes 3 to 10). The identities of the ε sequences are indicated above the gel (de represents ε), and the mobilities of the full-length P and the primary protease-resistant fragment are indicated to the left. (B) Bromelain digestion with ε and ε-Loop5,6 sequences. DHBV P was translated as for panel A with ε (lane 1) or ε-Loop5,6 (lane 2) or without ε (lane 3) and was partially digested with bromelain prior to electrophoresis. The figure was produced electronically as described in the legend to Fig. 1.

in protease resistance could be dependent on the continual presence of ε, or it could be irreversible. To distinguish between these possibilities, DHBV P was synthesized in vitro with or without ε and translation was terminated by addition of cycloheximide. Aliquots of the translation mixture were removed and treated with water (Fig. 6, lanes 3 and 4), RNase A (lanes 5 and 6), or RNase T_1 (lanes 7 and 8) prior to proteolysis. The ε-dependent protease resistance was efficiently generated (and was unaffected by the mock RNase treatment), but the resistance was abolished by both RNases, implying that P must remain bound to ε for the protease resistance to be produced.

The protease-resistant state of P does not reform following removal of ε. Attempts to reform the protease-resistant state in P by supplying additional ε RNA following digestion of the ε RNA were unsuccessful (Fig. 7). DHBV P was synthesized in the presence of ε, and aliquots were removed and placed on ice for the mock digestion and standard papain digestion samples (Fig. 7, lanes 1 and 2). The RNAs in the remaining translation mixture were then digested with RNase A, and an aliquot was removed (lane 3). RNasein (Promega) was then added to inhibit the RNase A, and additional ε RNA was added. This sample was incubated at 30°C for 15 min to allow the P-ε complex to reform, and an aliquot was removed (lane 4). The RNA in the mixture was then digested with RNase T_1 (which is not inhibited by RNasein), and the final aliquot was removed (lane 5). Half of each aliquot was then used for the protease digestion experiment shown in the top portion of Fig. 7, and RNA was isolated from the remaining half of each sample. The DHBV ε RNA was detected by Northern blot analysis by using random-primed ε sequences from plasmid pSL-BS (Fig. 7, bottom). Identical results were obtained when these experiments were repeated with micrococcal nuclease instead of RNase A (data not shown). The protease-resistant state of P was efficiently generated in the translation reaction, but it could not be reformed following removal of ε. This observation implies that the protease-resistant state may be able to form only once for each polymerase molecule.

The protease digestion pattern is not further altered by DNA synthesis. Hepadnaviral reverse transcription is primed by a tyrosine residue in the N-terminal domain of P (Y-96 in DHBV; 27, 28), and hence the negative-polarity DNA is covalently linked to P itself. This posttranslational addition of DNA to P might also affect the conformation or structure of the protein. To explore this possibility, the proteolytic profiles of P before and after priming of DNA synthesis were compared by synthesizing P with or without ε and terminating translation with cycloheximide; $MgCl_2$ was then added to 4 mM to enable DNA synthesis, and aliquots of the translation mixture were removed. dNTPs (20 μM each) were added to

TABLE 1. Correlation of binding, packaging, and priming activities with the induction of protease resistance for mutant ε sequences[a]

ε	Nucleotide change(s)	Location of mutation	P binding	RNA packaging	DNA priming	Protease resistance
SL	None	Not applicable	++	++	++	++
dε-dlBulge	Δ2570–2576	Bulge	−	−	−	−
dε-Loop3,4[b]	2589–2590 AC	Loop	+	−	±	−
dε-Loop5,6[b]	2591–2592 CA	Loop	++	−	±	−
dε-dlU1[b]	Δ2580	Upper stem	+	±	+	+
dε-LowerL/R[b]	2567–2569 CAU 2606–2608 AUG	Lower stem	+	++	+	+
SLM2	2572–2576 CACGU	Bulge	++	++[c]	++	++
SLM4	2576 U	Bulge	++	ND[d]	+[e]	++
SLM7	2573 C	Bulge	++	ND	++	++
SLM8	Inserted C after 2575	Bulge	++	ND	++	++
SLM11	SLM2 plus 2582 U, 2599 A	Bulge, upper stem	++	ND	++	++
SLM13	2590 C	Loop	−	ND	−	−
SLM15	2587–2588 UC	Loop	++	ND	++	++
SLM16	2593 C	Loop	++	ND	+	+
SLM17	Δ2590	Loop	++	ND	+	+

[a] Binding, packaging, priming, and induction of protease resistance activities are indicated as follows: ++, 70 to 100% of wild-type levels; +, 10 to 70% of wild-type levels; ±, 1 to 10% of wild-type levels; −, no detectable activity.
[b] Priming and packaging data are from reference 16.
[c] Packaging was not directly tested, but wild-type activity was inferred from wild-type levels of negative-polarity DNA synthesis when a DHBV expression vector carrying these mutations was transfected into LMH cells (20a).
[d] ND, not determined.
[e] Priming detected with labeled dATP because this mutation alters the template for the first nucleotide of negative-polarity DNA from C to U. No priming was detected with labeled dGTP.

half of the samples, and the mixtures were incubated at 30°C for 15 min prior to proteolysis. Vigorous DNA priming occurs under these conditions (the experiment was also done with [^{35}S]methionine–[α-^{32}P]dGTP dual labeling to confirm priming activity; data not shown). Comparison of lanes 5 and 7 of Fig. 8 reveals no difference between the proteolytic profiles of P before and after DNA priming. This result and the observation that the protease resistance is efficiently generated in the absence of dNTPs (Fig. 3 to 6) indicate that the induction of protease resistance in P occurs prior to priming of reverse transcription.

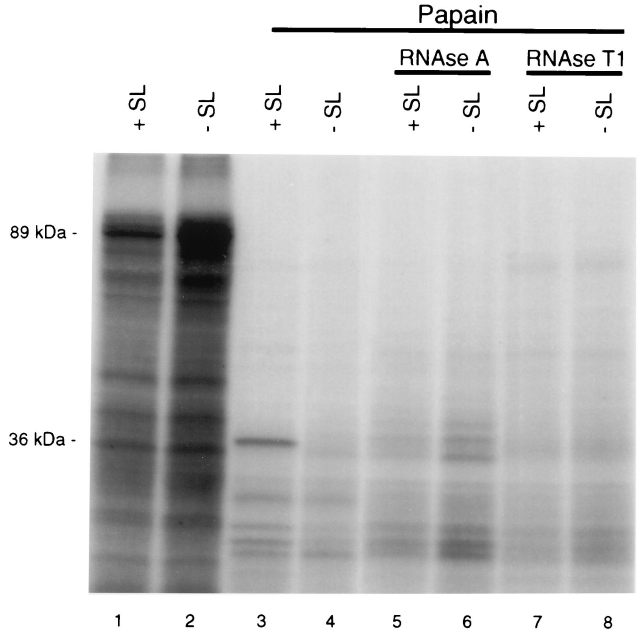

FIG. 6. Protease resistance of P can be reversed by digestion of ε (SL) with RNase. DHBV P was translated in vitro in the presence or absence of ε. Following termination of translation, aliquots were removed and treated with water (lanes 1 to 4), RNase A (lanes 5 and 6), or RNase T_1 (lanes 7 and 8) prior to partial digestion with papain (lanes 3 to 8). The presence or absence of ε RNA is indicated above the lanes, and the mobilities of the full-length P and the primary protease-resistant fragment are indicated. The figure was produced electronically as described in the legend to Fig. 1.

DISCUSSION

We employed functional DHBV P, both expressed in *S. cerevisiae* and translated in vitro, to analyze the earliest stages of the reverse transcription pathway, the interaction of P with the pgRNA, and the priming of DNA synthesis. We detected a polymerization activity on exogenous RNAs that is dependent on the reverse transcriptase active site through the analysis of TYDP expressed in *S. cerevisiae*. Unexpectedly, this *trans* activity is also dependent on the interaction between TYDP and a functional ε RNA during or shortly after translation of TYDP, although the ε RNA associated with TYDP is not the substrate in the *trans* assay. We also detected an increased resistance of the DHBV P to proteolysis when it is translated in vitro in the presence of a functional ε RNA and determined that binding between P and ε is necessary but not sufficient to induce the protease resistance.

Model for the maturation pathway of the hepadnaviral polymerase. These data can be incorporated into a model for the maturation pathway of the hepadnaviral polymerase (Fig. 9). In this model, P is synthesized from the pgRNA (its mRNA) and then binds to ε on the pgRNA. If ε is a functional element, an alteration is induced in P, and following the alteration, P becomes enzymatically active. The alteration in this model is detected experimentally as an increased resistance to proteolysis.

The partial proteolysis data demonstrate that the alteration occurs prior to the development of DNA synthesis activity and that it is dependent on a functional ε RNA, but these data do not address the possibility that the alteration is integrally involved in the maturation of P. However, the data obtained with

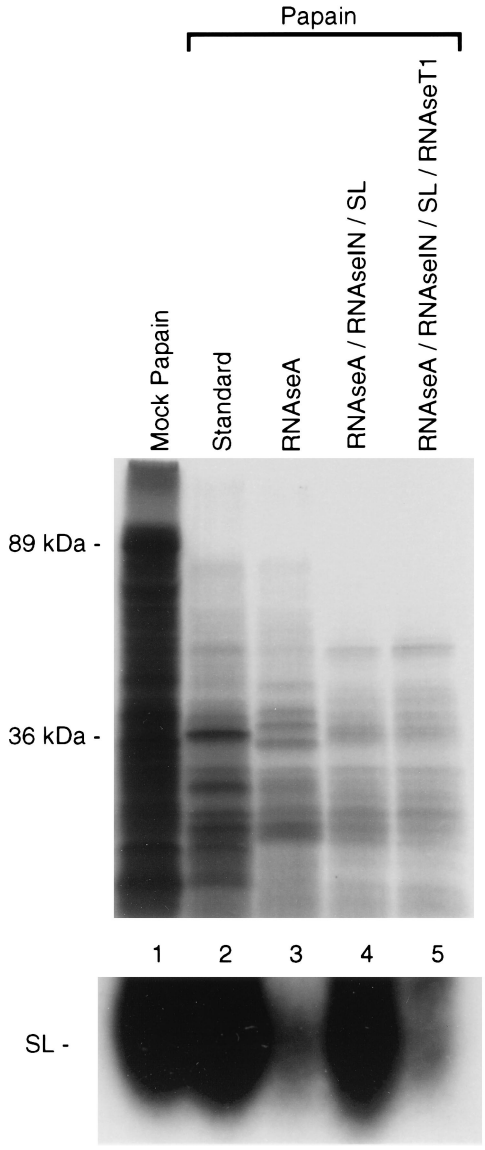

FIG. 7. The protease-resistant state of P does not reform following digestion of ε (SL) with RNase. DHBV P was translated in vitro in the presence of ε, and aliquots were removed for lanes 1 and 2. The remaining mixture was treated with RNase A, and an aliquot was removed (lane 3). RNasein and additional ε RNA was added, and the P-ε complex was given time to reform before the aliquot for lane 4 was removed. The RNA was then digested with RNase T_1, and the final aliquot was removed (lane 5). Half of each aliquot was subjected to mock papain digestion (lane 1) or papain digestion (lanes 2 to 5) prior to SDS-PAGE. RNA was isolated from the remaining half of each aliquot, and ε was detected by Northern analysis (bottom). The figure was produced electronically as described in the legend to Fig. 1.

TYDP indicate that the interaction between P and a functional ε RNA is necessary for DNA synthesis on RNAs containing ε (the *cis* assay) and also on RNAs that do not contain ε (the *trans* assay). This implies that the alteration may not simply precede the development of enzymatic activity but may be required for its development. These two sets of data were obtained with different systems, and consequently the requirement for ε in the *trans* assay is not proven to result from the induction of the alteration to TYDP. However, because both sets of data reveal a fundamental dependence of the hepadnaviral polymerase on interaction with ε, they may result from

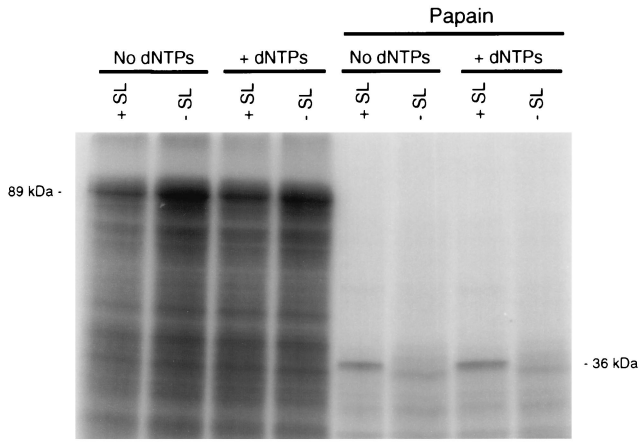

FIG. 8. DNA synthesis does not alter the partial proteolysis pattern. DHBV P was translated in vitro in the presence or absence of ε (SL). Following termination of translation, Mg^{2+} was added to 4 mM and aliquots were removed. All four dNTPs were added to half of the samples (lanes 3, 4, 7, and 8), and DNA synthesis was allowed to occur prior to partial proteolysis with papain (lanes 5 to 8). The presence or absence of ε RNA is indicated above the lanes, and the mobilities of the full-length P and the primary protease-resistant fragment are indicated. The figure was produced electronically as described in the legend to Fig. 1.

a common mechanism. For this reason, we propose that the alteration may be causally involved in the development of the enzymatic activity of P.

Seeger and colleagues have observed that posttranslational addition of ε to the in vitro translation mixtures reduces priming activity up to 10-fold relative to parallel reactions in which ε was added cotranslationally (26). This observation has been interpreted to imply an activation function of ε for the polymerase similar to that proposed here. However, this large difference in priming induced by co- or posttranslational addi-

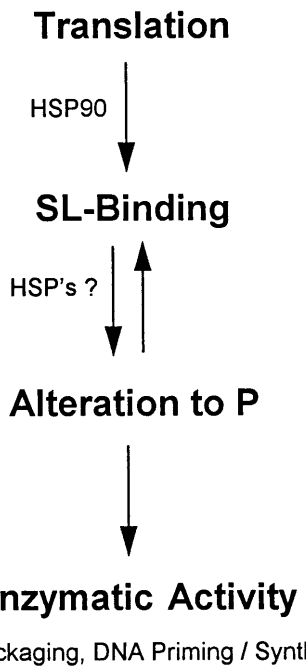

FIG. 9. Model for the maturation pathway of the hepadnavirus polymerase. HSP, heat shock protein; SL, stem-loop.

tion of ε was not observed in several other laboratories, and Seeger's group has recently published an experiment showing no great difference in the priming activity when ε was present either co- or posttranslationally (8). Therefore, the significance of this observation is unclear.

Occasionally, weak priming activity can be detected in reactions containing ε-Loop5,6 and ε-Loop3,4 (Table 1 and reference 20a), although no protease-resistant fragments are detected when P interacts with these RNAs. Also, Seeger and coworkers have shown that DHBV P translated in vitro can synthesize limited amounts of DNA in the absence of ε (26). These observations may indicate that P possesses a low-level basal activity prior to the proposed alteration or that the proposed inactive (protease-sensitive) and active (protease-resistant) forms of the polymerase exist in equilibrium and that binding to ε shifts the balance to the resistant (active) state. Since the enzymatic assays are more sensitive than are the proteolysis experiments, they could more easily detect a small fraction of functional P chains. DNA polymerization activity has also been detected with the human HBV polymerase in the absence of the HBV ε when P is expressed in *Xenopus* oocytes (18), although this activity is not bona fide HBV-specific reverse transcription. This activity could also result from a basal ε-independent activity or from an equilibrium between active and inactive states. Recently, functional HBV polymerase capable of bona fide HBV DNA priming was expressed in baculovirus. Interestingly, a functional enzyme could not be produced unless the ε sequences were present in the expression vector; this strongly underscores the importance of the P-ε interaction for production of enzymatically active hepadnaviral polymerase.

The reversibility of the protease resistance observed in Fig. 6 implies either that ε remains bound to P throughout the replication cycle or that an analogous alteration to the native P within the viral particles is not as readily reversible. No data are available for the persistence of the requirement of P upon the ε for activity within native virions; however, the *trans* activity of TYDP is detected following treatment with micrococcal nuclease. A small fraction of ε remains detectable in Northern blots of the micrococcal-nuclease-treated VLP extracts (data not shown). This residual RNA may be involved in the *trans* activity, but this has not been demonstrated.

What is the alteration? There are many changes to P that may cause the alteration that we detected as increased resistance to proteolysis, including covalent modification, complex formation with cellular proteins, or isomerization of P itself. The trivial explanation that RNA binding occludes protease sites is excluded by the fact that some ε mutants (e.g., ε-Loop5,6) can bind P normally but do not confer protease resistance on P. We feel that covalent modification is unlikely because the protease resistance can be rapidly reversed by addition of RNase. Formation of a complex with cellular proteins is a more attractive alternative; however, two observations argue against the notion that the protease resistance results from simple steric occlusion of the protease-sensitive sites in P. (i) The resistant fragments of P are most prominent very late in the digestion cascade, and an occlusion mechanism would require that binding between P and its putative cellular partner persist through extensive digestion of each protein. (ii) The protease resistance is observed with three proteases with different recognition sequences, and so if the resistance resulted from occlusion, three sets of sites in P would have to be protected. Therefore, we feel that the most likely cause of the alteration is isomerization of P following binding to ε; this isomerization would rearrange a domain(s) of the enzyme and would alter the accessibility of the protease recognition sites. Such allosteric conformational changes of proteins are well documented following ligand binding (1, 7, 12).

Complex formation with cellular proteins and allosteric isomerization of P are by no means mutually exclusive possibilities, especially as Hu and Seeger have recently shown that HSP90 binds to P (8). Heat shock proteins can act as molecular chaperones to mediate correct folding of proteins and to hold newly synthesized proteins in an inactive state until they interact with a suitable ligand (5, 6, 11, 12). Heat shock proteins are also attractive candidates for cellular partners with P because they are highly conserved through evolution (e.g., the human and yeast HSP90 proteins can complement each other; 12) and, hence, may provide functions similar to the maturation of P in yeast, duck, and rabbit (the source of the reticulocyte lysates) cells. It is therefore plausible that HSP90 and, possibly, other cellular chaperones bind to P and actively mediate its isomerization following ε binding. However, a putative involvement of a molecular chaperone in the proposed isomerization of P would be distinct from the activity of HSP90 detected by Hu and Seeger that is involved in P-ε binding, because mutant ε sequences (such as ε-Loop5,6) can bind to P without inducing the protease resistance that is a marker for the alteration of P.

In the model proposed here, P is inactive until bound to ε, whereupon it is encapsidated safely away from the cellular mRNA pool. If P were to then dissociate from ε-containing RNA, the isomerization would reverse itself and inactivate P. Because the polymerase appears to be unable to reform the protease-resistant state, the situation would appear experimentally as template commitment. Consequently, this model provides a common mechanism for both the restriction of the hepadnaviral reverse transcriptase activity to the appropriate pgRNA and for the template commitment observed with the mature enzyme.

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