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Biochemistry

Expression of functional hepatitis B virus polymerase in yeast reveals it to be the sole viral protein required for correct initiation of reverse transcription

(viral replication/retrotransposon Ty)

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ABSTRACT **Replication of hepatitis B viruses proceeds by reverse transcription of an RNA intermediate, a reaction catalyzed by the virus-encoded polymerase (P protein). The reaction product is a partially duplex DNA whose (−)-strand is covalently linked to the P protein. Efforts to understand the mechanism of the reaction have been severely retarded by an inability to express functional polymerase outside of viral particles. Here we report the successful expression of enzymatically active polymerase in yeast cells, by fusing the P gene to coding sequences of the retrotransposon Ty*1*. The enzyme initiates correctly on viral RNA in yeast cells *in vivo*, producing nascent DNA chains covalently linked to protein, exactly as found in virus-infected cells. Replication complexes isolated from these yeast are enzymatically active *in vitro*, synthesizing DNA in a reaction that is actinomycin D-resistant but sensitive to RNase pretreatment. These results indicate that P protein is the sole viral protein required for the correct priming of reverse transcription and establish a tractable system for the biochemical dissection of the reaction.**

Reverse transcription has been observed in a wide variety of genetic elements in animals, plants, and bacteria (1). Several families of viruses, many transposons, and the telomerase activity that maintains the termini of eukaryotic chromosomes employ this process (1). While the retroviral reverse transcription reaction is understood in great detail, much less is known about reverse transcription in most other elements. However, the wide variety of DNA structures found in these retroid elements suggests that an instructive diversity will be found in their replicative mechanisms.

One of the most distinctive reverse transcription reactions is that which replicates the genome of hepatitis B viruses (hepadnaviruses), small DNA viruses that cause acute and chronic hepatitis and are strongly associated with the development of hepatic cancer (2). The reaction converts an RNA template into circular, partially duplex DNA whose (−)-strand bears the viral polymerase (P) protein covalently attached to its 5′ end; this is thought to result from protein priming of DNA synthesis (3–5). Attempts to understand this reaction (and to develop inhibitors of it) have been frustrated by the inability to produce functional polymerase outside of virus particles. During viral infection, the polymerase and its RNA template are encapsidated within a nucleocapsid (or core); reverse transcription occurs principally or exclusively within these structures. Cores are impermeant to exogenous primer/templates, and even when cores are treated to make them permeable to such templates the polymerase will not engage them (6). Several explanations for this unusual behavior can be proposed. For example, the functional enzyme might be a multicomponent complex involving associations of P protein with other components of the viral core. The inability to solubilize active enzyme from these cores and the failure of all previous attempts to produce functional polymerase in recombinant expression systems (e.g., bacteria, yeast, baculovirus, or vaccinia virus) have lent support to this view. Alternatively, these properties might result from an exceptionally tight association of the enzyme with its endogenous RNA template.

Here we report that functional hepadnaviral polymerase can be produced in yeast cells by fusing the P gene to the yeast retrotransposon Ty*1*. The recombinant protein is enzymatically active and can carry out correct *de novo* initiation of reverse transcription on viral RNA, generating authentic protein-linked DNA. Our results indicate that P protein is the sole viral protein required for the initiation of reverse transcription and make possible new approaches to the study of hepadnaviral replication and its inhibition.

MATERIALS AND METHODS

Plasmids and Yeast Strains. *Saccharomyces cerevisiae* strain YH51 (*MAT***a** *GAL*$^+$ *ura3-52 spt3-202 his4-539 lys2-801*) was the gift of J. Boeke (Johns Hopkins University), as was plasmid pJEF724, which encodes a galactose-inducible Ty retrotransposon and has been described (7). pJEF724 bears a yeast replication origin and a *URA3* gene as a selectable marker for transformation into strain YH51. pTY-FS is a derivative of pJEF724; it contains a frameshift mutation at the *Sal* I site in the *TYB* gene. Plasmid pTYBDP, encoding the duck hepatitis B virus (DHBV) P protein, was produced by replacing the Ty*1* *Sal* I–*Hin*dIII region of pJEF724 with nt 167–3021 of DHBV-3. This DHBV sequence contains a 33-nt insertion at nt 901 encoding the influenza hemagglutinin epitope (HA tag) (8). Plasmid pTYBDP-FS bears a frameshift lesion at the *Kpn* I site in the DHBV P gene. pTYBDP-MS was constructed by replacing the *Hin*d-III–*Nco* I fragment of pTYBDP with the corresponding fragment of pD1.5G-YMHA (9).

Isolation of Ty-Related Particles. Ty virus-like particles (VLPs) were isolated by a modification of the method of Gabriel and Boeke (7). Yeast strain YH51 containing pJEF724 or pTYBDP or their derivatives was grown in 200 ml of synthetic complete medium lacking uracil (SC−ura) with 1% glucose to an OD_{590} of 2. Cells were collected and diluted 2.5-fold into SC−ura with 1% raffinose and grown at 30°C for 3 hr. Cultures were induced by addition of galactose to 2% and grown at 23–25°C for 18–20 hr. All further steps were at 4°C or on ice. Cells were collected and suspended in 6 ml of B/EDTA (15 mM KCl/10 mM Hepes pH 7.8/5 mM EDTA) with 3 mM dithiothreitol, 2 mM phenylmethanesulfonyl fluoride, and 0.1 trypsin-inhibiting unit of aprotinin per ml. Cells were lysed by vortex mixing with glass beads (12 g) for 3 min; the lysate was clarified by centrifugation at 11,000 × *g* for 12

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Abbreviations: DHBV, duck hepatitis B virus; VLP, virus-like particle.

min. The supernatant was loaded onto a step gradient of 20%/30%/70% sucrose in B/EDTA (18 ml/6 ml/5 ml) and centrifuged for 180 min at 100,000 × *g*. The gradient was fractionated and fractions from the 30%/70% interface were concentrated by dilution into B/EDTA and centrifugation for 60 min at 200,000 × *g*. The pellet was resuspended in B/EDTA with 5% sucrose and stored at −70°C. For Western blot analysis the proteins were resolved by SDS/7% PAGE and transferred to nitrocellulose; DHBV P products were detected with monoclonal antibody 12CA5 and anti-mouse IgG conjugated to alkaline phosphatase, followed by nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Reverse Transcriptase Assay *in Vitro*. Yeast spheroplasts were prepared (10) and VLPs were partially purified as above. VLP extracts (7.5 μg of protein) were incubated for 75 min at 24°C in 30 μl containing α-^{32}P-labeled dCTP, dGTP, and dTTP [1 μCi (37 kBq) each], 62 mM Tris (pH 8.0), 12.5 mM $MgCl_2$, 6.2 μM dATP, and 2.5% (vol/vol) 2-mercaptoethanol. Reactions were terminated by adding 8 μl of 5× Laemmli sample buffer (11) and heating to 95°C for 3 min, the reaction products were resolved in 7% polyacrylamide gels, and the gels were fixed, dried, and autoradiographed. Where noted, actinomycin D (0.2 μg/μl; Boehringer Mannheim) was added to the reaction mixture. Where indicated, VLP extracts were treated with RNase A (0.13 μg/μl; Sigma) at 24°C for 20 min or with 20 units of micrococcal nuclease (Boehringer Mannheim) and 7 mM $CaCl_2$ at 24°C for 30 min prior to the labeling reaction. Micrococcal nuclease activity was terminated by adding EGTA (15 mM) prior to the labeling reaction. Where indicated, the labeled products of the polymerase reaction were treated with DNase I (1 mg/ml) for 90 min at 37°C after completion of labeling.

RESULTS

Expression Strategy. To express functional P protein of DHBV, we employed a yeast retrotransposon Ty*1* vector (12). This element carries two overlapping genes, *TYA* and *TYB* (Fig. 1*A*), expressed from a single genomic mRNA. *TYA* encodes a structural protein that directs formation of a VLP (13); *TYB* encodes a multifunctional product with protease, reverse transcriptase, and integrase activities. *TYB* is initially expressed as a TYA–TYB polyprotein by ribosomal frameshifting (14, 15). This polyprotein, together with Ty genomic RNA, is then incorporated into the VLP and is processed by the protease activity encoded at the N terminus of TYB to liberate TYB protein (16). Earlier work has shown that TYB determinants can be replaced by coding regions for a variety of heterologous proteins, which can then be expressed as fusion proteins with TYA and readily purified as VLPs (7, 17, 18). We chose Ty as the expression system for hepadnaviral polymerase because we suspected that earlier failures to express functional P protein might have been due to toxicity of the enzyme and that such toxicity might be limited by sequestration within a VLP. Additionally, VLP-based expression would provide a simple means for purification of the enzyme and should ensure local concentration of the enzyme and its RNA template.

We inserted the coding region for DHBV P protein (and 3′ noncoding sequences) into a plasmid-borne copy of Ty*1*, just downstream of the *TYB* protease domain and in frame with *TYB* (Fig. 1*B*). This plasmid also bears a yeast replication origin to allow its stable episomal maintenance in yeast. Expression of this cassette should generate a TY–DHBV P fusion protein (DP) via ribosomal frameshifting in the TYA–TYB overlap. The P gene used here is a functional mutant allele that incorporates an 11-amino acid epitope from influenza hemagglutinin into the hypervariable "spacer" domain of the polymerase; when recombined into the DHBV genome this allele is fully replication-competent (D. Loeb and D.G., unpublished data). The recombinant expression cassette is driven by the galactose-inducible *GAL1* promoter (Fig. 1 *A* and *B*). Two mutant derivatives of this vector were also

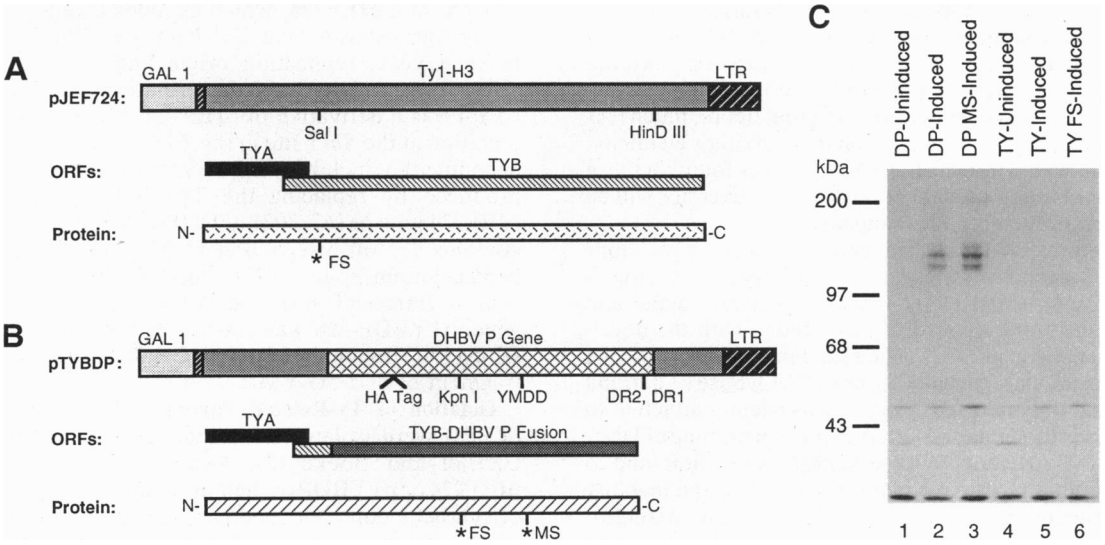

FIG. 1. Organization and expression of Ty-DHBV recombinant plasmids. (*A*) Plasmid pJEF724 (top line) contains a Ty*1* element cloned downstream of the galactose-inducible promoter of the *GAL1* gene; at the 3′ end of the Ty coding region is the long terminal repeat (LTR) of Ty*1*. Below this are depicted the *TYA* and *TYB* open reading frames (ORFs) and a schematic representation of the TYA–TYB fusion protein resulting from ribosomal frameshifting. Asterisk indicates the position of a frameshift mutation (FS) in a separate plasmid derivative. (*B*) Plasmid pTYBDP encodes an epitope-tagged DHBV P protein. Shown are the DHBV sequence (cross-hatched box), location of the inserted hemagglutinin epitope (HA tag), position of the conserved YMDD amino acid motif at which a missense lesion (YMDD → YMHA) has been created (mutant DP-MS), and a *Kpn* I site at which a frameshift lesion was introduced (mutant DP-FS) in separate plasmid derivatives. DR2, DR1 denotes position of direct repeats of 12 nt of DHBV DNA that play important roles in initiation of DHBV DNA replication (2, 4). At the bottom is a depiction of the predicted primary translational frameshift product; asterisks indicate positions of MS and FS lesions in mutant derivatives. (*C*) Expression of Ty-DHBV recombinant plasmids. VLPs generated from the indicated constructs were analyzed by SDS/PAGE and immunoblotting with monoclonal antibody 12CA5, which is specific for the hemagglutinin epitope. DP and DP MS, products of pTYBDP and pTYBDP-MS, respectively; TY and TY FS, products of pJEF724 and pJEF724-FS, respectively; uninduced, cells grown without galactose; induced, cells grown with galactose.

constructed: one (DP-FS) bears a frameshift mutation at a *Kpn* I site within the P gene, while the other (DP-MS) contains two missense changes in a highly conserved sequence (Tyr-Met-Asp-Asp, YMDD) believed to be essential for reverse transcriptase activity (19). Both mutations abolish polymerase activity *in vivo* (9). The 3′ noncoding sequences in all constructs contain the normal initiation site for DHBV reverse transcription; the RNA bearing these sequences will be packaged into the VLP by *TYA* gene products (12).

These constructs were transfected into *S. cerevisiae* cells bearing a mutation in the chromosomal *SPT3* locus (to diminish transcription of endogenous Ty*1* elements, which are not under galactose control; ref. 20), and stable transformants were selected. After induction with galactose, cells were harvested and lysates were fractionated by discontinuous sucrose gradient sedimentation (7). VLP-containing fractions were collected, concentrated, and examined by SDS/PAGE. The presence of TYA protein in these fractions was readily detectable by Coomassie staining (data not shown). The separated proteins were then transferred to nitrocellulose filters and probed with a monoclonal antibody (12CA5) to the hemagglutinin epitope. Two bands of 138 and 131 kDa were seen in preparations from cells bearing the wild-type P gene; these proteins were seen only after galactose induction and were absent from wild-type Ty particles (Fig. 1*C*). The missense mutant P protein is expressed as efficiently as the wild-type protein, indicating that the mutations did not affect its stability. These species are larger than the native DHBV P protein, presumably due to the presence of Ty determinants at their N termini. The doublet is most likely due to proteolysis, since neither band is the size expected for the primary translation product (≈155 kDa); the extent of Ty sequences in these proteins is not known.

Viral DNA Synthesis *in Vivo*. To look for evidence of polymerase activity *in vivo*, we examined the nucleic acids in the VLP preparations. VLP-containing fractions were purified from cells bearing Ty-DHBV plasmids and were extracted with phenol/chloroform, either with or without prior treatment with proteinase K. The resulting nucleic acids were examined by agarose gel electrophoresis and Southern blotting with ^{32}P-labeled DHBV DNA probes. All such preparations contained contaminating plasmid DNA (Fig. 2). However, samples from constructs bearing the wild-type P gene also contained an array of DHBV DNA molecules from 300 to 2500 nt long (Fig. 2, lane 3), and these species were absent from comparable preparations made from cells harboring the frameshift (lanes 5 and 6) or missense (data not shown) mutant P genes. Thus the production of these heterogeneous DNAs required the presence of a functional P gene. Probing similar blots with strand-specific DHBV probes (data not shown) indicated that this material was of (−)-strand polarity. No annealing was observed to Ty probes, indicating that initiation was not occurring randomly on the template, but toward the 3′ end of the DHBV insert. Importantly, these species were removed from the aqueous phase by phenol extraction (lane 4) unless the preparations were first digested with proteinase K (lane 3). This highly unusual behavior is characteristic of the covalent linkage of DNA to the viral terminal protein, which causes its attached (−)-strand DNA to partition into the phenol (22); such behavior is the distinctive signature of correctly initiated products of hepadnaviral reverse transcription *in vivo* (3, 22).

These results strongly suggested that the recombinant P protein could mediate the correct initiation of reverse transcription in the absence of any other viral protein. To further validate this notion we mapped the 5′ ends of the DHBV (−)-strands generated in this reaction to see whether they corresponded to the sites of initiation used *in vivo*. In authentic viral replication, (−)-strand DNA synthesis begins principally at nt 2537 within the DR1 sequence, a direct

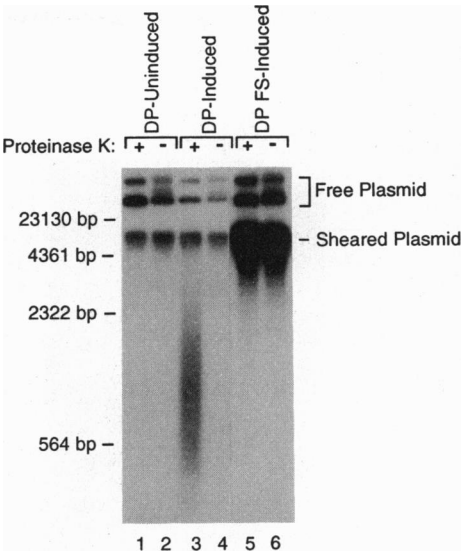

FIG. 2. DHBV DNA is synthesized by P protein in yeast cells *in vivo*. Cells bearing pTYBDP (DP) or its P-gene frameshift mutant derivative (DP-FS) were grown in the absence (uninduced) or presence (induced) of galactose, and VLPs were prepared. VLPs were resuspended in 10 mM Tris/1 mM EDTA, pH 7.5, with (+) or without (−) proteinase K (500 μg/ml). After 3 hr at 37°C, samples were extracted twice with phenol and once with chloroform, ethanol-precipitated, and examined by electrophoresis through 1% agarose gels. Following transfer to nylon filters, samples were hybridized to ^{32}P-labeled DHBV DNA prepared by random-primer extension on cloned DHBV DNA, and filters were washed and autoradiographed as described (21).

repeat of 12 nt found twice in viral genomic RNA (23). In addition, a secondary initiation site at nt 2576 can be utilized at lower efficiency when the primary site is deleted (24). (This region is present at the 3′ end of the DHBV insert in plasmid pTYBDP; Fig. 1*B*.) To map the ends generated by the Ty-DHBV P enzyme, DNAs prepared as in Fig. 2 were examined by primer extension, using a 5′ end-labeled primer homologous to nt 2477–2497, some 40 nt downstream of the correct initiation site in DR1. Primers annealed to this DNA were extended with avian myeloblastosis virus polymerase and the products were sized by electrophoresis alongside a sequencing ladder generated from the same primer. With DNA made by wild-type P protein, two sets of ends were observed (Fig. 3); these map precisely to the primary and secondary initiation sites previously described (24). Again, these species were seen only after galactose induction (Fig. 3, lanes 1 and 3) and were removed from the preparation when DNA was phenol-extracted without prior proteolysis (lane 4). No such products were detectable in DNA from constructs bearing the frameshift (lanes 5 and 6) or missense (data not shown) mutant P genes.

Polymerase Activity *in Vitro*. The further utility of this system for studying reverse transcription depends upon its retention of enzymatic activity *in vitro*. Accordingly, VLP-containing fractions were examined for endogenous polymerase activity by incubation with [α-^{32}P]dNTPs in the absence of exogenously supplied primer/templates. Initial efforts to detect incorporated cpm by trichloroacetic acid precipitation were inconsistent, presumably due to a high background adhering nonspecifically to macromolecules in these relatively impure fractions. This problem was bypassed by boiling the products in Laemmli sample buffer (11) and analyzing them by SDS/PAGE and autoradiography (Fig. 4 *Left*). Strong incorporation was directed by particles harboring wild-type P protein (lane 3); this incorporation was virtually abolished by the missense mutation in P (lane 4) but

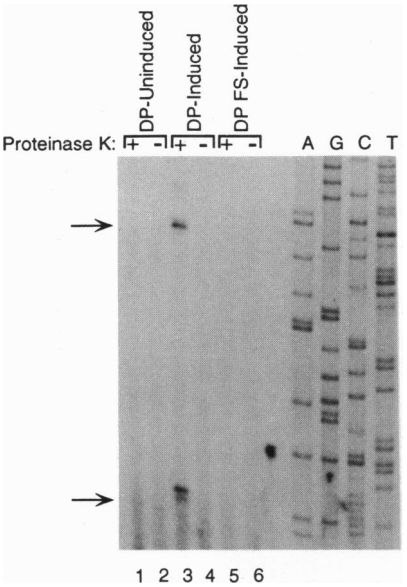

FIG. 3. Localization of 5′ ends of (−)-strand DHBV DNA synthesized by P protein in yeast. The indicated VLP DNAs prepared as in Fig. 2 were heat-denatured and annealed to a 5′-end-labeled oligonucleotide spanning nt 2477–2497 and primer extension was carried out as described (21). Products were sized in a 6% acrylamide/8 M urea gel alongside a sequencing ladder (lanes A, G, C, and T) generated from cloned DHBV DNA by using the same primer. Two sets of doublet bands are seen in DNA synthesized by wild-type P protein (lane 3). Similar doublets have been reported in virus-infected hepatoma cells (24); the lower bands of the doublets correspond to authentic viral initiation sites at nt 2537 and nt 2576 (used by wild-type and DR1-mutant viruses, respectively). The upper band results from the nontemplated addition of a nucleotide; this also occurs when cloned restriction fragments are used as templates (24).

was resistant to actinomycin D (lane 5) under conditions in which incorporation by the Klenow fragment of DNA polymerase I was inhibited by 90% (data not shown). This strongly suggested that incorporation was templated by the endogenous Ty-DHBV RNA [and implies that little (+)-strand synthesis occurred]. Consistent with this, incorporation was greatly impaired when the VLPs were treated with RNase (compare lanes 7 and 11) or micrococcal nuclease (lanes 7 and 13) prior to labeling. Incorporation was sensitive to EDTA (lane 9), implying a requirement for divalent cation, and the product was sensitive to digestion with DNase I (lane 15). Incorporation was also sensitive to phosphonoformate (data not shown), an inhibitor of the viral polymerase (25).

To determine whether the labeled P product was linked to protein, the labeled product was extracted with phenol/chloroform either with or without pretreatment with proteinase K and then examined by conventional PAGE in Tris borate buffer. (This allows more accurate sizing of the DNA species than the Laemmli gel system in Fig. 4 *Left* and *Center*). The DNA was efficiently removed by phenol extraction in the absence of protease pretreatment (Fig. 4, lanes 16 and 6). When pretreated with proteinase K and then phenol-extracted, the DNA product remained in the aqueous phase, running as a heterogeneous array of fragments ranging from ≈50 nt to >1000 nt (lane 18). These species migrate more rapidly and heterogeneously than the unproteolyzed product (lane 3) because the linked protein significantly retards their mobility. Thus, chains labeled *in vitro* also bear linked terminal protein and presumably have initiated at the correct viral replication origin. Consistent with this interpretation, deletion of DR1 and surrounding sequences (including the secondary initiation site) resulted in a major reduction in incorporation of label *in vitro* (J.E.T., unpublished results). In our *in vitro* polymerase assays the labeled chains could have resulted from *de novo* initiation *in vitro* or from elongation of chains that had been initiated *in vivo* (or both).

DISCUSSION

These data show that DHBV P protein can correctly initiate reverse transcription in the absence of all other viral proteins. Our findings accord well with the recent work of Wang and Seeger (5), who have independently shown that hepadnaviral

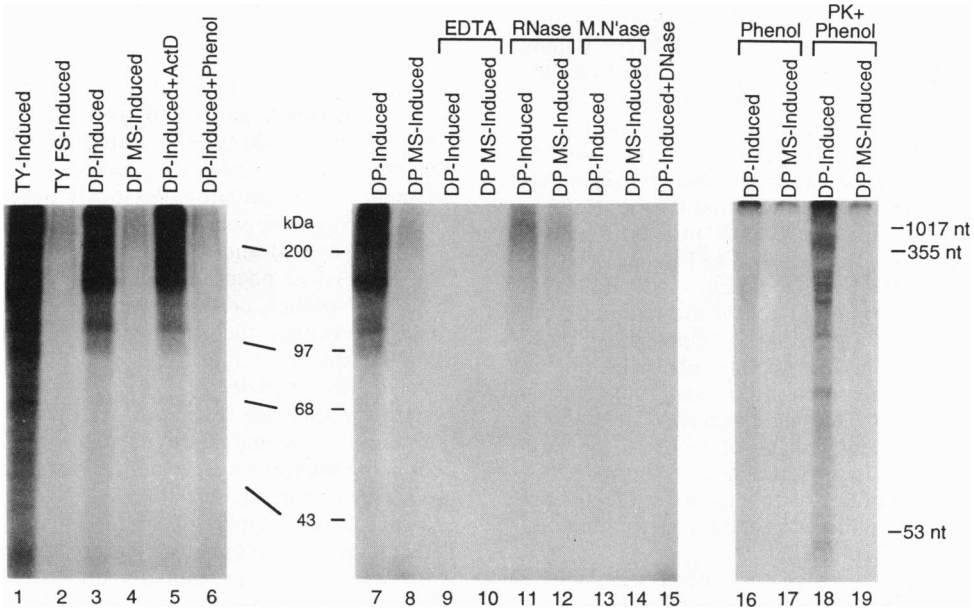

FIG. 4. TY-DHBV P protein is enzymatically active *in vitro*. (*Left* and *Center*) VLP extracts were incubated with ^{32}P-labeled dNTPs, mixed with Laemmli sample buffer (11), and denatured at 95°C; the labeled products were resolved by SDS/7% PAGE and detected by autoradiography. The VLP polymerase is indicated above each lane; abbreviations are as in Fig. 1. Where indicated, VLPs were pretreated with RNase A or micrococcal nuclease (M.N'ase); for lane 15, product DNA was treated with DNase I prior to electrophoresis. ActD, actinomycin D. (*Right*) The indicated labeled DNA samples were phenol-extracted with (lanes 18 and 19) or without (lanes 16 and 17) predigestion with proteinase K (PK, 500 μg/ml); products were ethanol-precipitated and examined in 10% polyacrylamide gels in Tris borate buffer.

P protein produced by translation *in vitro* can correctly initiate viral DNA synthesis in reticulocyte lysates. Taken together, these studies exclude models that require participation of C protein or other viral factors in the initiation reaction. It also appears unlikely that host proteins will be required for this step, as such factors would have to be functionally conserved in hosts as divergent from vertebrates (the normal viral hosts) as yeast. The simplest model consistent with these findings is that P protein determinants alone recognize the initiation site and serve as primers. In accord with this, Wang and Seeger (5) observed the addition of dNTPs to P protein chains *in vitro*.

While clearly not required for (−)-strand initiation, other host or viral proteins could influence or regulate this step. For example, the efficient use of the secondary initiation site by the recombinant P protein suggests that in the viral core particle, use of this site is suppressed; this could result from RNA–core (C) protein or C–P protein interactions.

Our results do not exclude roles for additional viral or host proteins at other P protein-dependent steps in genomic replication [e.g., genomic RNA packaging (26, 27), (−)-strand elongation or (+)-strand synthesis (21, 28)]. Packaging of the RNA template into the VLP is mediated by Ty functions in our system; this allows analysis of polymerase functions without requiring P protein participation in RNA packaging. DNA chains made *in vivo* can be up to 2–2.5 kb long (Fig. 2), substantially longer than those described by Wang and Seeger (5) *in vitro* but still shorter than full length. The recombinant P protein may be less processive than its counterpart in authentic cores, perhaps due to its added N-terminal sequences, to interference by Ty capsid proteins, or to the absence of C protein or other factors.

The absence of (+)-strand synthesis in our preparations is somewhat surprising. While our constructs do not contain all the cis-acting signals required for correct initiation of (+)-strand synthesis, deletion of these signals in the viral genome does not abrogate (+)-strand DNA synthesis but leads to the use of aberrant primers (21, 28). The failure to observe (+)-strands in our system could be due to (*i*) an inability to generate such primers, (*ii*) an inability to elongate these primers, or (*iii*) a lack of processivity of the recombinant enzyme [since such aberrant primers are generated only upon completion of (−)-strand synthesis].

A detailed understanding of hepadnaviral genomic replication requires *in vitro* systems that faithfully reproduce the entire reaction. The expression system described here represents an important initial step in this direction and should facilitate rapid progress toward this goal. In addition, the availability of enzymatically active reverse transcriptase provides a system for screening for inhibitors of this reaction; such inhibitors could provide novel approaches to antiviral therapy.

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