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Wheat DNA Primase[1]

RNA Primer Synthesis in Vitro, Structural Studies by Photochemical Cross-Linking, and Modulation of Primase Activity by DNA Polymerases

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DNA primase synthesizes short RNA primers used by DNA polymerases to initiate DNA synthesis. Two proteins of approximately 60 and 50 kD were recognized by specific antibodies raised against yeast primase subunits, suggesting a high degree of analogy between wheat and yeast primase subunits. Gel-filtration chromatography of wheat primase showed two active forms of 60 and 110 to 120 kD. Ultraviolet-induced cross-linking with radioactive oligothymidilate revealed a highly labeled protein of 60 kD. After limited trypsin digestion of wheat (*Triticum aestivum* L.) primase, a major band of 48 kD and two minor bands of 38 and 17 kD were observed. In the absence of DNA polymerases, the purified primase synthesizes long RNA products. The size of the RNA product synthesized by wheat primase is considerably reduced by the presence of DNA polymerases, suggesting a modulatory effect of the association between these two enzymes. Lowering the primase concentration in the assay also favored short RNA primer synthesis. Several properties of the wheat DNA primase using oligoadenylate [oligo(rA)]-primed or unprimed polythymidilate templates were studied. The ability of wheat primase, without DNA polymerases, to elongate an oligo(rA) primer to long RNA products depends on the primer size, temperature, and the divalent cation concentration. Thus, Mn^{2+} ions led to long RNA products in a very wide range of concentrations, whereas with Mg^{2+} long products were observed around 15 mM. We studied the ability of purified wheat DNA polymerases to initiate DNA synthesis from an RNA primer: wheat DNA polymerase A showed the highest activity, followed by DNA polymerases B and CII, whereas DNA polymerase CI was unable to initiate DNA synthesis from an RNA primer. Results are discussed in terms of understanding the role of these polymerases in DNA replication in plants.

The requirement for a separate activity to initiate nascent DNA derives from the inability of DNA polymerases to initiate DNA chains de novo. Initiation of DNA synthesis by cellular DNA polymerases is accomplished only after DNA primase has synthesized a short RNA primer. The latter RNA is further elongated by a replicative DNA polymerase. Primer RNA has a relatively specific size, consisting of approximately 10 nucleotides, but its sequence is highly variable (for a review, see Kornberg and Baker, 1992).

Prokaryotic and eukaryotic cells are characterized by the presence of multiple DNA polymerases. Five DNA polymerases (α, β, γ, δ, and ϵ) have been studied in animal cells (Table I) (for a recent review, see Wang, 1991). DNA polymerases α, δ, and ϵ seem to be involved in DNA replication (Budd and Campbell, 1993), whereas DNA polymerase β and probably DNA polymerase ϵ are involved in DNA repair (Popanda and Thielmann, 1992; Wang et al., 1993). DNA polymerase γ replicates the mitochondrial DNA.

In animal cells DNA primase is tightly associated with DNA polymerase α. Both enzymes co-purify during several steps including immunoaffinity chromatography (Chang et al., 1984; Wang et al., 1984). In the case of yeast and plant cells, DNA primase can be found, at least partially, to be free of DNA polymerase activity. Thus, in the yeast *Saccharomyces cerevisiae*, a fraction of DNA primase is associated with DNA polymerase α (Singh and Dumas, 1984). These activities have been separated by conventional and immunoaffinity purification procedures (Plevani et al., 1984). In virtually all eukaryotic organisms analyzed so far, DNA primase activity is associated with a heterodimer of 48- and 58-kD subunits encoded by two different genes (for reviews, see Wang, 1991; Kornberg and Baker, 1992).

Our laboratory has characterized four extramitochondrial DNA polymerases (A, B, CI, and CII) and a DNA primase from wheat (*Triticum aestivum* L.) germ. Some of their biochemical characteristics are summarized in Table I. We found that DNA polymerase CII has some properties of DNA polymerase α, whereas DNA polymerase B is very similar to DNA polymerase δ, since it is highly resistant to butyl-phenyl dGTP and possesses a 3′-5′ exonuclease activity (Richard et al., 1991). DNA polymerase CI is similar to animal DNA polymerase β: it is the only low mol wt DNA polymerase in wheat and it is resistant to aphidicolin and highly sensitive to ddTTP (Castroviejo et al., 1991). DNA polymerase A is an difficult enzyme to classify and shows some properties of DNA polymerase γ (Tarrago-Litvak et al., 1975). Although it recognizes poly(rA)-oligo(dT) very efficiently, enzyme A is not found in the mitochondrial compartment (Christophe et al., 1981; Ricard et al., 1983) or in wheat chloroplasts (P.

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Abbreviations: ddTTP, dideoxy TTP; FPLC, fast protein liquid chromatography; PCNA, proliferating cell nuclear antigen; rA, riboadenylate.

Table I. *Comparison of some biochemical characteristics of wheat and mammalian DNA polymerases*

Mit, Mitochondrial. nd, Not determined. inh, Inhibited. not, Not inhibited. med, Medium. NEM, *N*-ethylmaleimide. BuPhedGTP, Butylphenyl-dGTP.

	Wheat Polymerases					Mammalian Polymerase				
	CII	CI	A	B	Mit	α	β	γ	δ	ε
Corresponding yeast polymerases						I		Mit	III	II
kD	150	60	150	120	180	165	40	140	125	255
Associated Activity										
Exonuclease	−	−	−	+	nd	−	−	+	+	+
Primase	−	−	+	−	nd	+	−	−	−	−
Processivity										
Inherent	low	low	high	low	nd	med	low	high	low	high
+ PCNA	high	low	high	high	nd	med	low	high	high	high
Inhibitor										
Aphidicoline	inh	not	not	inh	not	inh	not	not	inh	inh
NEM	inh	inh	inh	inh	inh	inh	not	inh	inh	nd
BuPhedGTP	inh	inh	inh	not	nd	inh	not	not	not	not
ddTTP	not	not	inh	not	inh	not	inh	med	not	not

Laquel, unpublished results). In addition, unlike polymerase γ, DNA polymerase A is able to reverse transcribe natural and synthetic RNA templates (Laquel et al., 1990b). Moreover, a fraction of wheat germ DNA primase activity is found associated with DNA polymerase A. Both enzymes co-elute during the first steps of the purification procedure and co-migrate after centrifugation in a glycerol gradient (Graveline et al., 1984), but they are separated on phosphocellulose and DNA cellulose columns (Laquel et al., 1990a).

We have previously analyzed the effect of the PCNA on the processivity of wheat DNA polymerases (Laquel et al., 1993). The effect of PCNA suggests that DNA polymerase A is similar to animal DNA polymerase α, since it is highly processive and not stimulated by PCNA; DNA polymerases B and CII are similar to DNA polymerases δ and ε, since their processivity is increased in the presence of PCNA. DNA polymerase CI resembles animal DNA polymerase β, since it is fully distributive under all conditions tested.

Further biochemical characterization of wheat DNA primase will help us understand the crucial step of the initiation of DNA synthesis in plant cells. In previous reports we observed that wheat primase devoid of DNA polymerase activity could synthesize long RNA molecules (Graveline et al., 1984; Laquel et al., 1990a). The biochemical characterization of primase from animal and yeast cells that are able to produce short RNA primers has been performed with a DNA polymerase α-primase complex. This work has been devoted to the study of new structural and biological properties of wheat DNA primase and its interactions with DNA polymerases. Thus, the subunit structure of the enzyme has been studied by immunological and photochemical approaches. Furthermore, we have gained further insight into the ability of wheat DNA polymerases to modulate DNA primase activity and to initiate DNA synthesis from an RNA primer. We also studied the modulatory effect of primase concentration, temperature, divalent cation concentration, and the influence of oligonucleotide primer length on the RNA primer synthesis catalyzed by wheat primase.

MATERIALS AND METHODS

Materials

Wheat (*Triticum aestivum* L.) embryos were prepared from the variety Marius (Brosse Monceaux Agronomical Center, Monceaux, France). Freshly prepared commercial wheat germ was a kind gift from Les Grands Moulins de Paris (Bordeaux, France). Unlabeled nucleotides were obtained from Sigma or P.L. Pharmacia. Labeled precursors were from Amersham. The synthetic polynucleotides poly(dT), poly(dA), and oligo(dT)$_{5,10}$ were from Sigma. Oligo(rA)$_n$, prepared by hydrolysis of poly(rA), was a generous gift of Dr. G.A. Nevinsky (Institut of Bioorganic Chemistry, Russian Academy of Sciences, Novosibirsk, Russia). Oligonucleotide sizing markers (8–32 bases in length) were purchased from Pharmacia. *Escherichia coli* DNA polymerase I was from Boehringer Mannheim. Alkaline phosphatase and T4 polynucleotide kinase were from Pharmacia. Yeast primase-specific antibodies against the subunits p58 and p48 were a kind gift of Dr. P. Plevani (University of Milan, Milan, Italy). Sephadex G-25, Mono S (HR 5/5), gel filtration Superose 12, and Fast Desalting columns were from Pharmacia. ^{125}I-labeled protein A was from New England Nuclear.

Purification of Wheat DNA Primase and Wheat DNA Polymerases

The detailed purification procedures of DNA primase (Laquel et al., 1990a) and DNA polymerases A, B, CI, and CII have been published previously (Castroviejo et al., 1979, 1991; Laquel et al., 1990b; Richard et al., 1991).

FPLC Superose 12 Filtration of Wheat Primase

The pool of enzyme activity from Heparin-Sepharose was filtered on a 20-mL FPLC Superose 12 column equilibrated with 150 mM KCl in buffer A (50 mM Tris-HCl, pH 7.9, 1 mM 2-β-mercaptoethanol, 0.1 mM EDTA, 20% glycerol, and 0.1

mM PMSF). Wheat primase activity was detected by using the direct assay described below.

DNA Primase Assay

The incubation mixture for the direct assay of primase activity contained, in a final volume of 50 μL, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM DTT, 0.48 A_{260}/mL poly(dT) as template, and 10 μM [^{3}H]ATP (500–1500 cpm/pmol) as substrate. Incubations were for 30 min at 20°C in the presence of primase (5 μg of protein). The reaction volume was directly precipitated with 1 mL of 10% (w/v) cold TCA plus 0.1 M PPi. The precipitate was filtered onto nitrocellulose filters and dried and the radioactivity was counted in a 2,5-diphenyloxazole:1,4-bis(2-(5-phenyloxazolyl)benzene): toluene (4 g:0.1 g:1 L) scintillation mixture.

Immunoblot Method

Transfer of proteins to nitrocellulose sheets was done as described (Towbin et al., 1979). The blots were saturated using 1% (w/v) gelatin in TBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 h at 37°C, then incubated with antiserum (1:1000 dilution) for 2 h at 37°C and subsequently washed with TBS (five washes of 200 mL for 10 min each). Detection of antigen-antibody complexes was done with ^{125}I-labeled protein A in 1% gelatin in TBS (1:1000 dilution) for 1 h at 37°C, followed by washing and autoradiography.

UV Cross-Linking of Primase with ^{32}P-Labeled Oligo(dT)$_{10}$

Oligo(dT)$_{10}$ Labeling

One A_{260} of oligo(dT)$_{10}$ was previously dephosphorylated in the presence of bacterial alkaline phosphatase and 5′-end labeled in the presence of the T4 polynucleotide kinase and 20 μCi of [γ-^{32}P]ATP (30 Ci/mmol) as described (Maniatis et al., 1983). The labeled oligo(dT)$_{10}$ was separated from the unincorporated nucleotides by filtration on a 1-mL Sephadex G-25 column. Thirty microliters of the labeled primer (30×10^6 cpm) used in each labeling assay correspond to 0.06 A_{260} of oligo(dT)$_{10}$.

UV Cross-Linking of Primase

Oligo(dT)$_{10}$ cross-linking labeling of wheat primase was performed by incubating 10 μg of the enzyme in a 100-μL reaction volume at 20°C in 50 mM Tris-HCl (pH 7.9), 10% glycerol, 6 mM $MgCl_2$, 2 mM DTT, and 0.06 A_{260} of 5′-end-labeled oligo(dT)$_{10}$. After preincubation of primase with the labeled primer for 30 min, the incubation mixture was exposed for 15 s at 100 J at 254 nm in the UV-Stratalinker 1800 (Stratagene) (3000 μW/cm^2). Proteins were precipitated in the presence of 10% cold TCA, resuspended in 20 μL of the loading buffer, and electrophoresed on a 9 to 15% polyacrylamide gel as described (Laemmli, 1970). After electrophoresis, proteins were stained with Coomassie blue and the gel was dried and autoradiographed at −80°C.

RNA Primer Synthesis with Primed or Unprimed Poly(dT) Template Catalyzed by Wheat Primase

The assay mixture (50 μL) contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 1 A_{260}/mL poly(dT), and 3 nM [α-^{32}P]ATP as the labeled substrate. When poly(dT) is present in the assay, 0.05 A_{260}/mL oligo(rA)$_n$ (n = 3–16; ratio primer:template = 1:20) was added as primer. Reactions were initiated with 5 μg of primase (Heparin-Sepharose fraction), incubated at 30°C for 60 min, and stopped by the addition of EDTA to a final concentration of 20 mM. Proteins were extracted twice with phenol, and nucleic acids were precipitated with ethanol, using calf thymus DNA as carrier. Centrifuged pellets were dried and redissolved in formamide containing 0.5% xylene cyanol, 0.5% bromphenol blue, and 40% Suc. After denaturation at 85°C for 1 min, RNA primer size was determined by electrophoresis on 14% denaturing polyacrylamide gels containing 100 mM Tris-borate (pH 8.3), 7 M urea, and 2 mM EDTA (Sanger et al., 1977). Electrophoresis was carried out at 1200 V until the bromphenol blue tracking dye had migrated to about three-fourths of the gel length. After electrophoresis, gels were exposed at −80°C using Kodak XAR films and an intensifying screen.

Replication of RNA-Primed DNA Templates by Wheat DNA Polymerases

The assay mixture contained, in 50 μL, 50 mM Tris-HCl (pH 8.0) or 50 mM Hepes (pH 6.5), 10 mM $MgCl_2$, 16 mM DTT, 1 A_{260}/mL poly(dT) or poly(dA), 0.05 A_{260}/mL oligo(rA)$_9$ or oligo(rU)$_8$, and 3 nM [α-^{32}P]dATP or [α-^{32}P]dTTP as labeled precursor. Reactions were started by the addition of equivalent DNA polymerases units [10 pmol incorporated dTMP (30 min)$^{-1}$ (30°C)$^{-1}$ when tested in the presence of poly(dA)-oligo(dT)$_{10}$] corresponding to 1 to 5 μg of protein. Incubation was performed at 25°C with poly(dT)-oligo(rA)$_9$ and at 30°C with poly(dA)-oligo(rU)$_8$ for different times. DNA size was analyzed by electrophoresis on sequencing gels as described above. Protein concentration was determined by the method of Bradford (1976) using BSA as standard.

RESULTS

Characterization of Wheat Primase Subunits

Immunodetection of Wheat Primase

We analyzed the cross-reactivity of wheat primase with specific antibodies raised against the yeast primase subunit p58. Autoradiography of the immunoblot is shown in Figure 1. Two major wheat bands of 60 and 50 kD, very similar in molecular mass to *S. cerevisiae* primase subunits, were recognized by yeast antibodies. Two minor bands of 110 and 82 kD were also detected. The same results were obtained with the specific antibodies against the p48 subunit of yeast primase (not shown), whereas no proteins were revealed by the nonimmune serum.

UV Cross-Linking Labeling of Wheat Primase

Labeling of wheat DNA primase by UV cross-linking was used to determine the subunit(s) carrying the active site(s) of

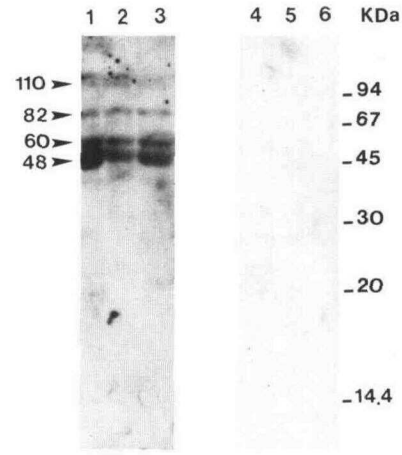

Figure 1. Immunoblotting of wheat primase with yeast-specific antibodies. Proteins were blotted for 3 h at 300 mA on Immobilon P sheets (Millipore) (Towbins et al., 1979). Incubation was performed overnight at 37°C with antibodies against yeast primase as described in "Materials and Methods." Lanes 1 and 4, Heparin-Sepharose enzyme fraction; lanes 2 and 5, Mono S fraction; lanes 3 and 6, ATP-Sepharose peak of activity. Lanes 1 to 3 were incubated in the presence of immune serum and lanes 4 to 6 were incubated in the presence of nonimmune serum. Electrophoretic mobility of the molecular mass markers is indicated on the left of the figure.

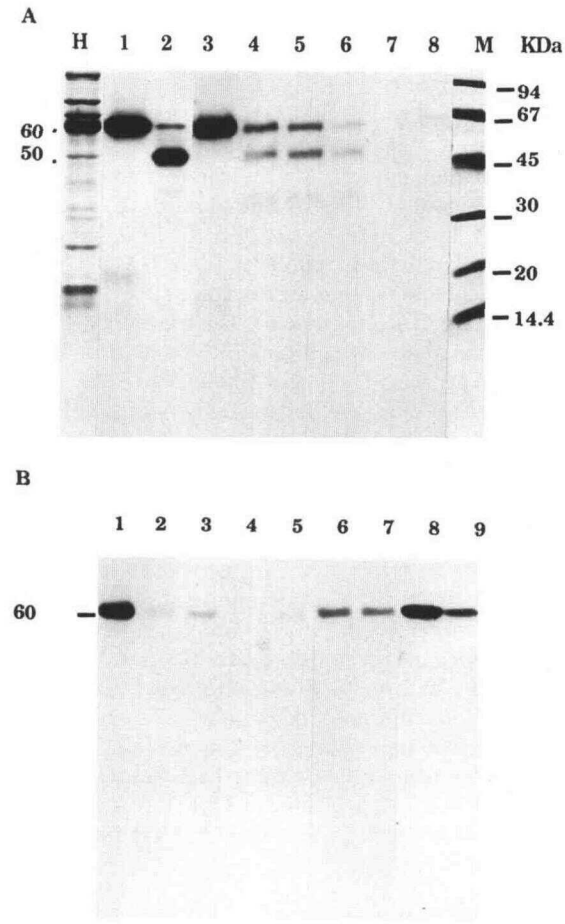

Figure 2. UV cross-linking labeling of wheat DNA primase. The detailed procedure is described in "Materials and Methods." Similar amounts of protein were detected in all fractions tested after TCA precipitation. Lane H, Total proteins of the Heparin-Sepharose fraction; lane M, molecular mass marker proteins stained with Coomassie blue. The numbered lanes correspond to the UV cross-linking of primase to the labeled oligo(dT)$_{10}$ and gel autoradiography. A, Lanes 1 and 3 show the labeling of primase immediately after purification (control), and lane 2 shows labeling of an old primase preparation. Lanes 4 to 7, Digestion of primase by 20 μg of trypsin for 1, 5, 10, or 15 min at 37°C. Lane 8, Autoradiography of the cross-linking assay with the low molecular mass standard proteins. B, Lanes 1 and 8, Control reaction with the native enzyme. Lanes 2 and 4, Preincubation of primase with 3% SDS (lane 2) or 10% TCA (lane 4) before cross-linking. Lane 3, Heat denaturation of primase for 10 min at 80°C before incubation and cross-linking with oligo(dT)$_{10}$. An excess ratio of unlabeled poly(dT) (1500:1) (lane 6) or unlabeled oligo(dT)$_{10}$ (500:1) (lane 7) to the labeled oligo(dT)$_{10}$ was used compared with the control assay (lane 8). Lane 9, UV exposure at 254 nm for 10 s compared with the control cross-linking performed for 20 s (lane 8). Lane 5, UV cross-linking of wheat primase with oligo(dT)$_5$ for 20 s.

this enzyme. The enzyme was cross-linked to 5′-end-labeled oligo(dT)$_{10}$ by exposure to a 254-nm wavelength source. Labeled polypeptides were identified upon electrophoresis on a polyacrylamide gel. A single polypeptide of the wheat primase (Heparin-Sepharose fraction) with a molecular mass of 60 kD was labeled (Fig. 2). In old primase preparations (kept for 2 years at −20°C in 50% glycerol), the 60-kD band was still labeled, but a strong band of 48 kD, which is very faint in fresh preparations, was detected. To determine if the 48-kD protein was derived from the proteolytic cleavage of the 60-kD protein, we tested the effect of trypsin after UV cross-linking labeling. After proteolytic digestion of primase for different lengths of time at 37°C, labeling of the 60-kD band decreased, whereas 48-, 38-, and faint 17-kD protein bands, which may correspond to degradation products of the 60-kD protein, were detected. The native enzyme was required for the specific labeling, since no cross-linking was observed when samples were preincubated with 3% SDS, 10% TCA or heat denatured for 10 min at 80°C.

The specificity of the labeling was followed with a chase experiment by incubating the enzyme in the presence of an excess of unlabeled poly(dT) (ratio 1500:1). The intensity of the 60-kD band was reduced by 50%. The same result was obtained with unlabeled oligo(dT)$_{10}$ but with lower amounts of unlabeled competitor (ratio 500:1), indicating that the enzyme may have a stronger affinity for oligo(dT)$_{10}$ than for poly(dT). We also used 5′-end-labeled oligo(dT)$_5$ template for UV cross-linking and observed that oligo(dT)$_5$ was less efficient than oligo(dT)$_{10}$ for labeling the primase. Similar labeling of the primase subunit was obtained with oligo(rA) used as a primer. Oligo(rA)$_3$ and oligo(rA)$_5$ were more effi-

cient than oligo(rA)$_{10}$ and oligo(rA)$_{15}$ for labeling the 60-kD protein (data not shown). The absence of labeling of wheat primase observed with oligo(dG)$_{10}$ may be correlated to the lack of recognition of poly(dG) as template by wheat primase. Lowering the UV exposure by half led to a concomitant decrease in primase labeling, demonstrating that cross-linking of labeled oligo(dT)$_{10}$ to wheat primase depends on the UV dose used.

FPLC Superose Gel Filtration

A detailed wheat primase purification procedure has been previously published (Laquel et al., 1990a). By FPLC chromatography on a MonoS column, we observed significant enrichment of two bands of 60 and 50 kD. They coincided with the peak of activity and are similar in mol wt to yeast primase subunits, suggesting that they correspond to the wheat primase subunits. Very similar results were obtained by ATP-Sepharose affinity chromatography (results not shown). However, because we observed a significant loss of activity after these chromatographic purification steps, we have used the Heparin-Sepharose fraction of the wheat primase for all the studies described in this work.

Proteins from the Heparin-Sepharose fraction were separated by FPLC gel filtration on Superose 12. As shown in Figure 3, primase activity coincided with the position of a 60-kD monomer protein (arrow B), as well as with the 110- to 120-kD dimer (arrow A).

Biochemical Properties of Wheat Primase

Effect of Protein Concentration on Poly(dT) Initiation

Previous studies of RNA primer synthesis catalyzed by the isolated wheat DNA primase, free of DNA polymerases, indicated the ability of the enzyme to synthesize long RNA products (Laquel et al., 1990a). Nevertheless, this result could not be accurately compared with those obtained with primase from either yeast or animal cells, since in the latter cases the enzyme is tightly associated with DNA polymerase α and synthesizes short RNA primers (10–15 nucleotides).

It seemed interesting to study whether primase concentration had any influence on the size of the RNA primer products. By using poly(dT) as template and labeled ATP as substrate, we analyzed the size of RNA products by varying the wheat primase concentration in the assay. As shown in Figure 4, at relatively high protein concentrations wheat primase synthesized rather long RNA products. Decreasing the amount of protein in the in vitro assay to approach as much as possible the concentrations found under physiological conditions led to the synthesis of shorter RNA molecules.

Primase Activity in the Presence of Wheat DNA Polymerases

The close association of DNA primase and DNA polymerase α in the replication fork suggests a coordinated action of these two enzymes in the synthesis of the Okazaki fragments of the lagging strand. We studied the influence of the presence of wheat DNA polymerases on the ability of the plant primase to synthesize long RNA products during initiation of DNA replication using unprimed poly(dT) as template. As shown in Figure 5, synthesis of long RNA molecules was abolished in the presence of wheat DNA polymerases A, B, CI, and CII and animal DNA polymerase α. In the presence of each of these DNA polymerases, wheat DNA primase synthesized short RNA primers, ranging in size from 5 to 15 nucleotides.

Use of Primed and Unprimed Templates

The activity of wheat primase is usually assayed with unprimed poly(dT) as template. The enzyme initiates poly(dT) replication by synthesizing an oligo(rA)$_n$ primer. However, the enzyme can also elongate an oligo(rA)$_n$ annealed to the poly(dT) template. Thus, the influence of the oligo(rA)$_n$ primer length on the ability of wheat primase (Heparin-Sepharose step) to initiate the replication of a duplex poly(dT)-oligo(rA)$_n$ was studied. As shown in Figure 6,

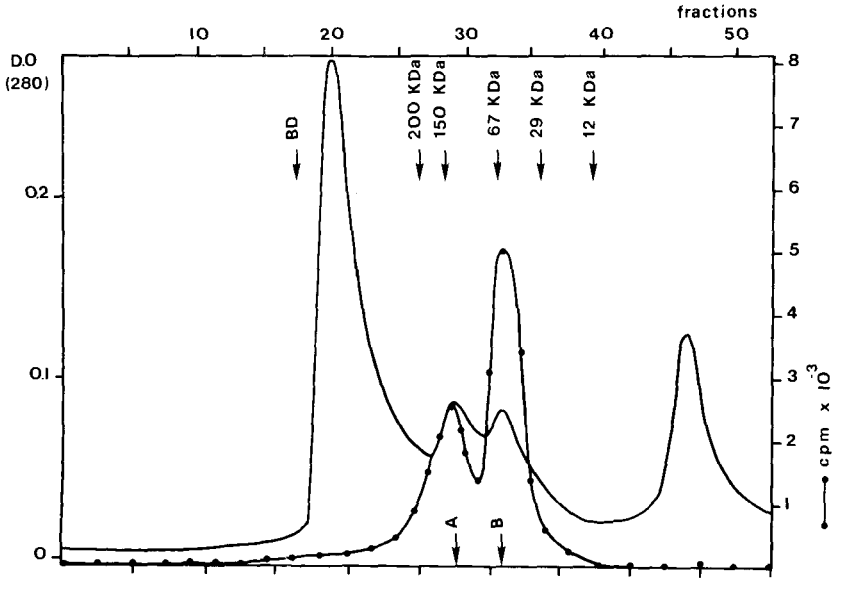

Figure 3. FPLC Superose 12 gel filtration of wheat primase. An aliquot (200 μL) of the primase pool from the Heparin-Sepharose step was loaded onto an FPLC Superose 12 column in the presence of 150 mM KCl, eluted and assayed for primase activity (●) as described in "Materials and Methods." Primase size was estimated from elution of markers, which were used for the calibration of the same column (dextran blue, β-amylase [200 kD], alcohol dehydrogenase [150 kD], BSA [67 kD], carbonic anhydrase [29 kD], and Cyt c [12 kD]). Arrows A and B indicate the elution of the two peaks of primase activity: A, 110 to 120 kD; B, 60 kD.

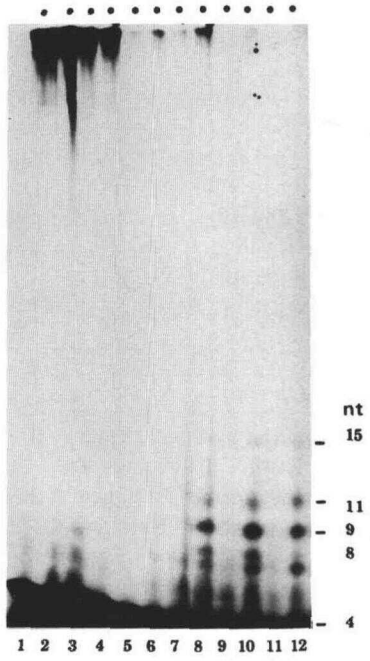

Figure 4. Effect of protein concentration on RNA primer synthesis with poly(dT) template. Different concentrations of wheat primase (Heparin-Sepharose fraction) were tested in a 50-μL reaction volume as described in "Materials and Methods." Enzyme was incubated for 10 min (lanes 1, 3, 5, 7, 9, 11) or 30 min (lanes 2, 4, 6, 8, 10, 12) at 30°C in the presence of poly(dT) template and [α-^{32}P]-ATP as the labeled substrate. The RNA product size was analyzed by electrophoresis on a 16% polyacrylamide sequencing gel. Primase concentrations were 100 μg/mL (lanes 1 and 2), 50 μg/mL (lanes 3 and 4), 20 μg/mL (lanes 5 and 6), 10 μg/mL (lanes 7 and 8), 2 μg/mL (lanes 9 and 10), and 0.2 μg/mL (lanes 11 and 12).

the size of RNA products synthesized by wheat primase changed with the primer length, with the temperature, and with the divalent cation concentration.

Effect of Temperature on Poly(dT)-Oligo(rA)$_n$ Replication

The optimal temperature and divalent cation concentrations for primer synthesis were determined. The optimal temperature was 20°C in the presence of either Mg or Mn. The effect of temperature on the ability of wheat primase to synthesize short or long RNA molecules was analyzed. Temperature varied from 10 to 42°C and the radiolabeled elongation products were analyzed on a sequencing gel. The poly(dT) template was sparsely primed with oligo(rA) (ratio 20:1). As can be seen in Figure 6A, the effect of temperature varied with the primer size. When small RNA products were obtained, the optimum was 30°C with oligo(rA)$_3$ and increased with longer primers [i.e. 30–37°C with oligo(rA)$_7$ and 37–42°C with oligo(rA)$_{16}$]. For the synthesis of long RNA products the temperature optimum was around 20 to 25°C for all primers tested, but long RNA synthesis was observed essentially at 20°C with short primers, e.g. oligo(rA)$_3$. A broad range of temperature [between 20 and 30°C with oligo(rA)$_7$ and 10 and 42°C with oligo(rA)$_{16}$] was obtained with longer primers. In addition, we observed that the length of the RNA product increased with the primer size. Thus, with oligo(rA)$_3$ heterogeneous products ranging in size from about 30 to about 500 nucleotides were obtained, whereas with oligo(rA)$_{16}$ RNA products ranged essentially from about 150 to 500 nucleotides. Similar results were obtained with unprimed poly(dT) as template (data not shown).

Effect of Divalent Cation Concentration on Poly(dT)-Oligo(rA)$_n$ Initiation

We examined the influence of $MgCl_2$ or $MnCl_2$ concentrations on the reaction catalyzed by DNA primase. No incorporation was observed in the absence of divalent cation. Optima were determined in the presence of oligo(rA)$_5$-primed or unprimed poly(dT) templates. The optimal concentrations were broader with primed poly(dT) template than with unprimed poly(dT) template.

Wheat primase was very active in the presence of $MnCl_2$ compared with $MgCl_2$. We monitored the size of the primase reaction products on a sequencing gel (Fig. 6B). Primase catalyzed the synthesis of long RNA in a broad range of

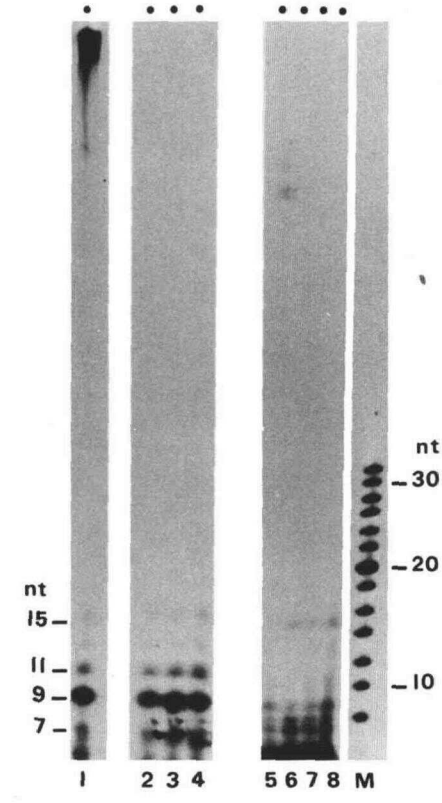

Figure 5. Influence of wheat DNA polymerases on wheat primase activity tested with unprimed poly(dT). Wheat primase (100 μg/mL) was incubated in the presence of poly(dT) and labeled ATP as substrate for 45 min at 30°C in the absence (lane 1) or presence of wheat DNA polymerase A, B, CI, or CII (lanes 5–8) or with the animal DNA polymerase α (lanes 2–4) for 5 (lane 2), 15 (lane 3), or 45 min (lanes 4–8) at 30°C. RNA primer size was determined as described above.

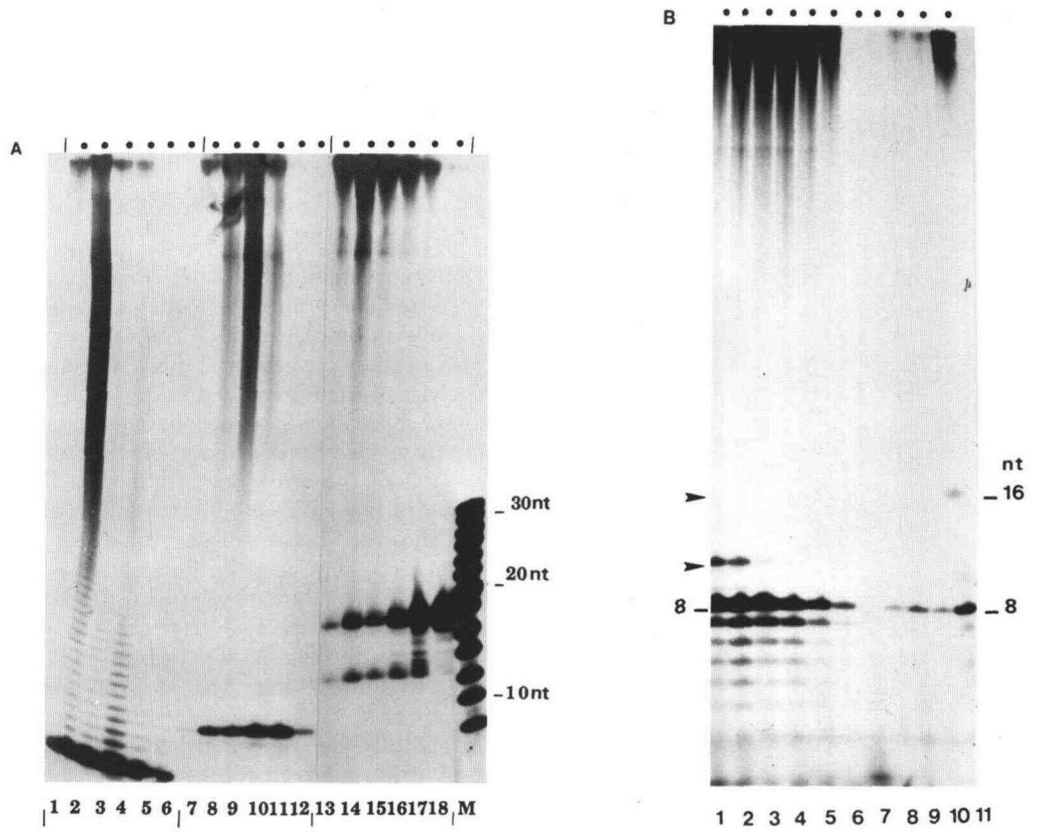

Figure 6. Effect of temperature and salt concentration on primase activity. A, Influence of temperature on wheat DNA primase processivity with poly(dT)-oligo(rA)$_n$. Five micrograms of primase (Heparin-Sepharose fraction) were tested in the presence of sparsely primed poly(dT)-oligo(rA)$_n$ (ratio 20:1) and 3 nM [α-^{32}P]ATP as the labeled substrate, for 60 min in a 100-μL reaction volume as described in "Materials and Methods" at 10°C (lanes 1, 7, 13), 20°C (lanes 2, 8, 14), 25°C (lanes 3, 9, 15), 30°C (lanes 4, 10, 16), 37°C (lanes 5, 11, 17), or 42°C (lanes 6, 12, 18). The oligo(rA)$_n$s used were oligo(rA)$_3$ (lanes 1–6), oligo(rA)$_7$ (lanes 7–12), and oligo(rA)$_{16}$ (lanes 13–18). Product length was determined by electrophoresis on a 14% denaturing polyacrylamide gel. Lane M, 8- to 32-oligonucleotide size markers 5′ end labeled with [γ-^{32}P]ATP as described (Maniatis et al., 1983). B, Influence of divalent cation concentration on wheat DNA primase processivity. Wheat primase was incubated for 60 min at 30°C in the presence of poly(dT)-oligo(rA)$_n$ (20:1) and 3 nM [α-^{32}P]ATP in the presence of various concentrations of $MgCl_2$ and $MnCl_2$. Product lengths were determined by electrophoresis on a 14% polyacrylamide gel. Lane 1, 0.05 mM Mn^{2+}; lane 2, 0.1 mM Mn^{2+}; lane 3, 0.25 mM Mn^{2+}; lane 4, 0.5 mM Mn^{2+}; lane 5, 1 mM Mn^{2+}; lane 6, 2 mM Mn^{2+}; lane 7, 0 mM Mg^{2+}; lane 8, 0.5 mM Mg^{2+}; lane 9, 2.5 mM Mg^{2+}; lane 10, 5 mM Mg^{2+}; lane 11, 15 mM Mg^{2+}.

Mn^{2+} concentrations, and the optimum was 250 to 500 μM. The optimum for small RNA synthesis was observed at lower concentrations of $MnCl_2$ (50–100 μM). We observed a discrete but significant band of 11 nucleotides when primase was incubated in the presence of oligo(rA)$_7$ with low concentrations of Mn^{2+}, probably corresponding to a pause in the incorporation of the labeled substrate (see lower arrowhead in Fig. 6B).

The effect of $MgCl_2$ was markedly different. The enzyme incorporated only a few residues at a wide concentration range of this cation. Under these conditions the bulk of the enzyme probably dissociates from the template-primer. Nevertheless, a low but significant amount of product of about 200 to 250 nucleotides was observed, essentially at 15 mM $MgCl_2$. A discrete band of 16 nucleotides was detected in this case, also indicated by the upper arrowhead in Figure 6B. This pause was more pronounced when a longer primer [i.e. oligo(rA)$_{10}$] was used (data not shown). Similar results were observed with unprimed poly(dT) template.

Replication of RNA-Primed DNA Templates by Wheat DNA Polymerases

During the replication of the lagging strand of DNA, short RNA primers synthesized by DNA primase are elongated by one or more replicative DNA polymerases. In addition to providing the biochemical identification of the enzyme(s) associated with DNA primase, we corroborated the replicative function of DNA polymerases by showing the ability of these DNA polymerases to replicate an RNA-primed DNA template. Figure 7 illustrates our analysis of the replication of poly(dT)-oligo(rA)$_9$ and poly(dA)-oligo(rU)$_8$ by wheat DNA polymerases. Template was sparsely primed (ratio of template:primer = 20:1). Both temperature and pH of the

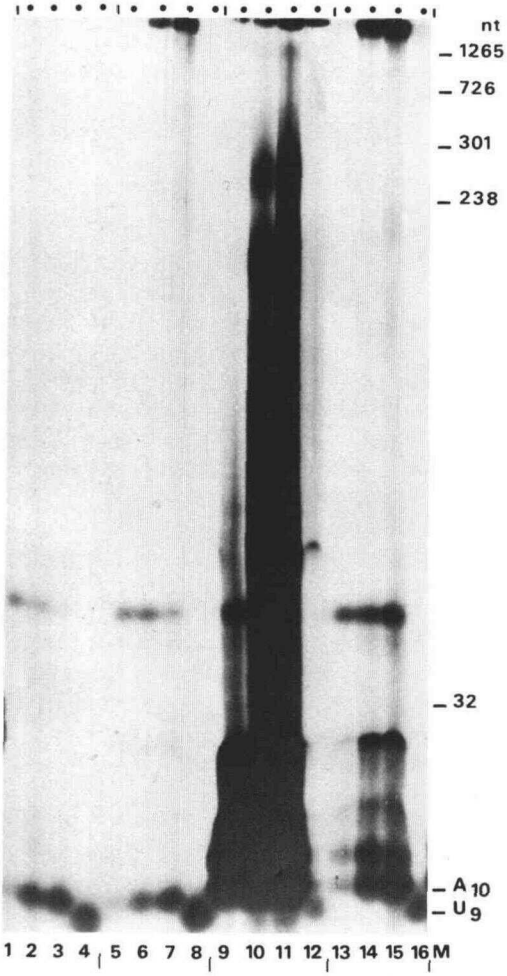

Figure 7. Replication of RNA-primed DNA templates by wheat DNA polymerases. Purified wheat DNA polymerases were assayed as described in "Materials and Methods" with poly(dT)-oligo(rA)$_9$ (ratio 20:1). DNA polymerases CI (lanes 1–3), CII (lanes 5–7), A (lanes 9–11), and B (lanes 13–15) were incubated for 5 min (lanes 1, 5, 9, 13), 20 min (lanes 2, 6, 10, 14), or 60 min (lanes 3, 7, 11, 15). DNA polymerases CI (lane 4), CII (lane 8), A (lane 12), and B (lane 16) were incubated for 60 min in the presence of poly(dA)-oligo(rU)$_8$ (ratio 20:1) at pH 6.3 and at 30°C with 3 nM [α-^{32}P]dTTP as the labeled substrate. Electrophoretic migration of oligonucleotide markers is indicated on the right of the figure (lane M).

reaction mixture were optimized for replication of these template-primer complexes. For all tested enzymes, the best conditions were 25°C and pH 8.0 with poly(dT)-oligo(rA)$_9$ and 30°C and pH 6.5 with poly(dA)-oligo(rU)$_8$. As can be seen in Figure 7, DNA polymerase CI was not able to replicate these templates, but DNA polymerases A, B, and CII were able to replicate poly(dT)-oligo(rA)$_9$. The most active enzyme was DNA polymerase A, which was also able to recognize poly(dA)-oligo(rU)$_8$ as illustrated by the high mol wt material not entering the gel.

Because of the possible effect of PCNA on wheat polymerase activities and/or processivity (Laquel et al., 1993), we analyzed the effect of PCNA in these experiments. No effect of PCNA was observed on the activity of any of these DNA polymerases with these template-primer complexes (results not shown). Similar-sized products were synthesized by wheat polymerases and animal DNA polymerase α (data not shown).

DISCUSSION

DNA primase is a key enzyme involved in the initiation of DNA replication. Several important questions remain to be answered concerning the mode of action of this enzyme in the overall process of DNA replication in plant cells. In the present study we have performed a structural and functional analysis of the enzyme purified from wheat embryos. After FPLC chromatography of wheat primase on a Mono S column, a single peak of activity was detected. Gel densitometric analysis of the active fractions revealed the significant increase of two bands of approximately 60 and 50 kD (results not shown). The strong coincidence between the peak of primase activity and the enrichment of these two bands suggested that they may be related to wheat primase subunits. Gel filtration of wheat primase in the presence of high ionic strength revealed two peaks of wheat primase activity (60 and 110–120 kD). This result indicates that the 60-kD monomer seems sufficient for RNA primer synthesis. It remains to be determined whether the high molecular mass peak corresponds to the association of the 60- and 50-kD proteins, as in the case of yeast.

Several eukaryotic organisms are characterized by the presence of dimeric primases (Wang, 1991; Kornberg and Baker, 1992). DNA primase from yeast cells is composed of two subunits of 58 and 48 kD, the latter carrying the catalytic site (Plevani et al., 1984, 1985; Badaracco et al., 1985). The yeast 48-kD subunit (p48), free of the 58-kD subunit (p58), has been purified and is sufficient for RNA primer synthesis (Santocanale et al., 1993). Interestingly, we found a significant cross-reactivity of wheat DNA primase with yeast primase antibodies raised against the p58 and p48 proteins. Two major bands of 60 and 50 kD of the wheat primase preparation were revealed by these antibodies, suggesting a significant analogy between yeast and wheat primase subunits. This analogy opens the way for the search for the plant primase gene using the available heterologous yeast probe. An interesting difference is observed between the yeast and wheat primases. The two subunits that form the yeast primase enzyme (p58 and p48) are encoded by two different genes (Lucchini et al., 1987; Foiani et al., 1989a; Francesconi et al., 1991) and do not show an immunological cross-reaction. However, in the case of the wheat primase the two putative plant subunits (p60 and p50) both react with both yeast antibodies. Further experiments focused on the search for the wheat DNA primase genes will be necessary to clarify whether our immunological results may be related to a different evolution of a common gene ancestor of the dimeric primase.

To search for the active polypeptide site of the wheat DNA primase, UV cross-linking experiments were performed. UV-induced photochemical cross-linking has been demonstrated for a number of nucleic acid binding proteins including adenovirus DNA binding protein (Cleghon and Klessig,

1992), DNA polymerase III (Biswas and Kornberg, 1984), DNA polymerase γ from *Xenopus laevis* (Insdorf and Bogenhagen, 1989), *E. coli* RNA polymerase (Bartholomew et al., 1990), δ-helicase from calf thymus (Li et al., 1992; Siegal et al., 1992), and yeast DNA primase (Foiani et al., 1989b).

We performed the labeling of wheat primase with different oligonucleotides, since this enzyme has been shown to initiate DNA synthesis with multiple synthetic and natural nucleic acids (Graveline et al., 1984; Laquel et al., 1990a). A major band of 60 kD of the purified wheat primase was very efficiently labeled after UV exposure with [^{32}P]oligo(dT)$_{10}$. Oligo(rA) used as primer was significantly less efficient than oligo(dT) in labeling the 60-kD protein, and no cross-linking of wheat primase was obtained with oligo(dG)$_{10}$. Thus, the extent of labeling is highly influenced by the nature of the oligonucleotide used. The oligonucleotide optimal size for primase labeling was 10 residues with oligo(dT) and 5 residues with oligo(rA). The efficiency of labeling with various sized molecules raises the question of the topology of the primase active site. When primase is incubated with a significant excess of unlabeled oligo(dT)$_{10}$, the labeling of the 60-kD protein with [^{32}P]oligo(dT)$_{10}$ was reduced by only 50%, suggesting a great efficiency of the UV-induced cross-linking of primase with oligo(dT). This is probably due to the strong chemical reactivity, after UV exposure, of thymidines with the amino acid residues of the template-primer domain of the enzyme (Smith, 1977). Native enzyme is required for labeling the 60-kD protein, since incubation with SDS or TCA or digestion with trypsin abolished the cross-linking. After trypsin digestion, or in old wheat primase preparations, a second band of 48 kD and two minor bands of 38 and 17 kD were revealed that may be degradation products of the 60-kD protein. Gel-filtration results demonstrate that the 60-kD subunit seems to be sufficient for RNA synthesis (see above).

The UV cross-linking results may be related to the ability of primase to synthesize short RNA primers. Thus, wheat DNA primase behaves differently depending on the size of the RNA primer available in the assay and on the temperature or divalent cation concentration. The enzyme was essentially distributive, incorporating efficiently one or two nucleotides. Nevertheless, a significant fraction of the small RNA primer could be elongated into long RNase-sensitive products (Laquel et al., 1990a). Little is known about the topography of the interaction between the primer-template duplex and the polymerase-primase complex. It may be speculated that in the presence of primase, the primer site of the DNA polymerase catalytic center can hold a rather short primer oligonucleotide (about 5–10 nucleotides), as also suggested by the greater efficiency of small oligo(rA) for primase labeling; shorter primers may lead to an unstable template-primer duplex, whereas the binding of longer primers may be impossible by steric hindrance, which would then interfere with the process of elongation by DNA polymerase.

During DNA replication, primase must act in close coordination with the replicative DNA polymerase(s) and other proteins involved in the progression of the replication fork. RNA primers synthesized by primase from animal cells or yeast, which are closely associated with the replicative DNA polymerase α, are of relatively short size, consisting of 9 to 15 nucleotides. However, depending on the presence or absence of a wheat DNA polymerase and on incubation length times, the wheat enzyme is able to synthesize longer RNA primers with either poly(dT) or M13 DNA as templates. Several observations described previously (Graveline et al., 1984; Laquel et al., 1990a) argue against the possibility that the wheat primase activity described here corresponds to one of the orthodox nuclear DNA-dependent RNA polymerases.

An important question concerns the factor(s) involved in the regulation of the size of the RNA primer synthesized by a primase. Thus, DNA primase activity may be modulated by the presence of an associated replicative DNA polymerase to produce an RNA primer of a specific size. The ability of wheat DNA primase to synthesize long RNA molecules when tested alone may reflect the absence of such regulation. We have analyzed the ability of wheat DNA polymerases to modulate DNA primase activity. In the absence of DNA polymerases, long RNA product synthesis was predominant, whereas in the presence of wheat DNA polymerases A, B, CI, and CII, or animal DNA polymerase α, we observed that wheat primase synthesized short RNA primers. The sizes of these primers correspond to those observed in vivo during DNA replication. These results strongly suggest that the interaction of wheat primase with DNA polymerase may play a role in the regulation of short primer RNA synthesis.

Enzyme concentration may be another way of regulating primase activity. In the presence of low amounts of primase, short RNA synthesis is favored, whereas in the presence of increasing amounts of primase, long RNA accumulates. Higher concentrations of primase may stabilize its activity or facilitate primase rebinding to a primer-template complex, giving rise to longer RNA products. This observation may be correlated to the influence of primer size and the effect of temperature and divalent cation concentration on the ability of wheat primase to synthesize long RNA products, as shown in Figure 6. Thus, the stability of the duplex poly(dT)-oligo(rA) can be influenced by the parameters mentioned above (temperature, divalent cation, primer size), which must be optimal to avoid the dissociation of wheat primase from the template-primer duplex after each incorporation event.

Having shown that DNA polymerases are able to modulate the size of the primase RNA product, it seemed interesting to identify the wheat DNA polymerase(s) able to initiate DNA replication from RNA-primed templates. As described in previous articles (Graveline et al., 1984; Laquel et al., 1990a), wheat DNA polymerase A was extremely efficient in elongating an RNA primer, whereas the responses with the other three DNA polymerases were not significant. However, under the incubation conditions described in this work, DNA polymerases B and CII were also able to use an RNA primer, whereas DNA polymerase CI proved reluctant to initiate from an RNA primer. It is puzzling that DNA polymerase CI, although unable to initiate from RNA primers, can reduce the size of the RNA products synthesized by wheat DNA primase (Fig. 5). It is possible that this polymerase interacts with primase and/or the template in such a way that elongation is hampered.

The ability of wheat polymerases B and CII to initiate from RNA primers, although to a different extent (Fig. 7), is an important breakthrough in our work. We have recently

shown that wheat DNA polymerase B is a δ-polymerase (Richard et al., 1991). In the presence of PCNA this enzyme displays a high degree of processivity in DNA synthesis (Laquel et al., 1993). The high processivity of DNA polymerase B in the presence of PCNA, associated with its 3′-5′ exonuclease activity, seems suitable for the continuous synthesis of the leading strand, whereas DNA polymerase A, with its associated primase activity and the ability to initiate DNA synthesis from RNA primers, may fulfill the function needed for the elongation of RNA primers during discontinuous synthesis of the lagging strand of the DNA replication fork. In that sense wheat DNA polymerase A may play a role similar to DNA polymerase α of other eukaryotes. In addition, the weak efficiency of DNA polymerase CII in replicating RNA-primed DNA templates suggests that this enzyme and DNA polymerase B play different roles. DNA polymerase CII may be more related to animal DNA polymerase ε, which has been proposed to be involved in DNA elongation of the lagging strand together with the α-like DNA polymerase (Budd and Campbell, 1993). The results presented in this work are an important step toward understanding the roles of plant DNA polymerases and DNA primase at the level of the replication fork. Further experiments are in progress in our laboratory with the goal of understanding the function of the enzymes involved in the plant DNA synthetic machinery. For that purpose the development of an in vitro DNA synthesis system constitutes our current goal.

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