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Purification and Characterization of a Gamma-Like DNA-Polymerase From *Chenopodium album* L.

K. Meißner
Humboldt-Universtät Berlin

Sabine Heinhorst
University of Southern Mississippi, sabine.heinhorst@usm.edu

Gordon C. Cannon
University of Southern Mississippi, Gordon.Cannon@usm.edu

T. Börner
Humboldt-Universtät Berlin

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Purification and characterization of a γ-like DNA polymerase from *Chenopodium album* L.

K.Meißner, S.Heinhorst[1], G.C.Cannon[1] and T.Börner*
FB Biologie, Humboldt-Universität Berlin, Invalidenstr. 43, D-10115 Berlin, Germany and [1]Department of Chemistry and Biochemistry, University of Southern Mississippi, Southern Station Box 5043, Hattiesburg, MS 39406, USA

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ABSTRACT

A DNA polymerase activity from mitochondria of the dicotyledonous angiosperm *Chenopodium album* L. was purified almost 9000 fold by successive column chromatography steps on DEAE cellulose, heparin agarose and ssDNA cellulose. The enzyme was characterized as a γ-class polymerase, based on its resistance to inhibitors of the nuclear DNA polymerase α and its preference for poly(rA)·$(dT)_{12-18}$ over activated DNA *in vitro*. The molecular weight was estimated to be 80,000 – 90,000. A 3′ to 5′ exonuclease activity was found to be tightly associated with the DNA polymerase activity through all purification steps. This is the first report of an association between a DNA polymerase and an exonuclease activity in plant mitochondria.

INTRODUCTION

Plant mitochondria contain a genome of substantially larger size (210–2400 kb) and complexity than that found in human, animal and fungal mitochondria [1, 2, 3]. According to a widely accepted model that is based on renaturation kinetics and restriction maps, plant mitochondrial DNA exists in a dynamic equilibrium between a circular chromosome representing the entire mitochondrial genome and subgenomic molecules originating from this 'master chromosome' by inter- and intramolecular recombination processes [4, 5, 6]. Despite the acceptance of the model, analysis of mitochondrial DNA by electron microscopy revealed circular molecules of the size predicted for a 'master chromosome' only in the lower plant *Marchantia polymorpha* L. [7]. On the other hand, in mitochondrial DNA preparations from several higher plants, most DNA is linear and its size is often inconsistent with the 'master circle' theory [8, 9, 10].

Although the structure of the plant mitochondrial genome apparently evolves rapidly, its sequence changes slowly compared to that of animal mitochondria, suggesting a possible difference in the mechanism of DNA synthesis in the plant organelle. One can envision that the sequence stability of the plant mitochondrial genome is based on high fidelity of DNA replication and/or efficient post-replicative repair mechanisms [11, 12]. Enzymes of DNA replication and repair and the mode of their concerted action in plant mitochondria are not known because of a lack of a simple model system. Mutants deficient in enzymes involved in DNA synthesis are not available for plant mitochondria and transformation of the organelle is at present practically impossible. In contrast to chloroplast *in vitro* systems [13, 14, 15], no specific initiation of DNA synthesis or preferential labeling of cloned mtDNA fragments could be observed in mitochondrial extracts [16, 17, 18, our own observations]. Origins of replication are largely unknown in plant mitochondrial DNA, although in some cases, DNA synthesizing activity and sequence analysis of mitochondrial DNA fragments indicate structural homology to H- and L-strand origins in mammalian mitochondria and motifs in yeast mitochondrial replication origins [19].

We are investigating DNA synthesis in mitochondria of higher plants using suspension cultures of *Chenopodium album* L. as a model system. A DNA polymerase activity in lysates of purified mitochondria was characterized previously (unpublished results), and here we report biochemical properties of the highly purified enzyme, which can be classified as a γ-polymerase [20]. In mitochondria from animal cells, DNA polymerase γ is believed to be the replicative enzyme and the only DNA polymerase in mitochondria [21, 22]. The yeast enzyme is, so far, the only organellar enzyme for which the nuclear gene has been cloned and analyzed [23].

MATERIALS AND METHODS

Chemicals

[^{3}H]TTP (1.5–2.5 TBq/mmol) was purchased from ICN; nonradioactive deoxynucleotides (dNTPs), M13mp18 (+ strand), DNase-free bovine serum albumin (BSA) for *in vitro* assays, $(dT)_{12-18}$, poly(rA) and poly(dA) were from Pharmacia. [α^{32}P]dNTPs (110 TBq/mmol), [γ^{32}P]ATP (185 TBq/mmol) and Klenow Fragment of DNA polymerase I from *E.coli* (4 U/μl) were purchased from Amersham International. T4 polynucleotide kinase (10 U/μl) was purchased from Stratagene. The protease

* To whom correspondence should be addressed

inhibitors phenylmethylsulfonyl fluoride (PMSF) and *p*-toluene sulfonylfluoride (PTSF), dithiothreitol (DTT), single stranded (ss) DNA cellulose and activated calf thymus DNA were from Sigma Chemical Corporation. Diethyl amino ethyl (DEAE) cellulose was purchased from Whatman (DE52). Heparin agarose was purchased as a 5 ml cartridge from BioRad.

Plant material

Chenopodium album L. suspension culture C.9.1. was grown as previously described [24]. Cells were harvested 7–8 days post transfer, in a phase of active growth and division.

Preparation of mitochondrial extracts

All preparation steps were carried out at 4°C. Approximately 400 g (wet weight) of cells were harvested and homogenized three times with mortar and pestle in grinding buffer (400 mM sucrose, 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 3.5 mM 2-mercaptoethanol, 0.1% BSA) with the addition of glass beads. The homogenate was filtered through cheesecloth and miracloth. Cell debris was recovered as a pellet by two centrifugation steps at 1,900×g. The supernatant was centrifuged 15 min at 20,000×g. The mitochondrial pellet was resuspended in a minimal amount of grinding buffer.

During the development phase of the final purification scheme, the mitochondria were subsequently purified in a sucrose step gradient, which was later omitted. The mitochondrial suspension was loaded on a three-step sucrose gradient (30%, 52%, 60% sucrose in 350 mM sorbitol, 50 mM Tris–HCl, 5 mM EDTA, pH 8.0) and after 1 h centrifugation at 100,000×g, the mitochondria were collected from the 30%/52% interphase and washed once in 30 mM mannitol, 50 mM Tris–HCl, pH 8.0, 10 mM NaCl, 2 mM 2-mercaptoethanol, then resuspended in a minimal amount of washing buffer, frozen in liquid nitrogen and stored at −80°C.

Lysis of mitochondria from several pooled preparations was achieved after addition of one volume of DEAE buffer containing 300 mM NaCl and 0.1% Triton X-100 on ice for 30 min on a shaker. After centrifugation for 20 min at 20,000×g, the supernatant was dialyzed five times against 500 ml DEAE buffer for a total of 2 h and loaded onto a DEAE cellulose column.

DEAE cellulose chromatography

A BioRad Econo System was used for all column chromatography steps. The cleared mitochondrial lysate was loaded onto a DEAE cellulose column (4–10 mg protein/ml bed volume) which had previously been equilibrated with DEAE buffer (30 mM Tris–HCl, pH 8.0, 20 mM NaCl, 10% [v/v] glycerol, 0.3 mM PMSF, 0.3 mM PTSF, 1 mM DDT). The column was washed with two column volumes of DEAE buffer and a linear gradient to 500 mM NaCl in DEAE buffer was applied.

Heparin agarose chromatography

Fractions eluted from the DEAE cellulose column that were highly active in DNA polymerization were pooled, dialyzed against HEPA buffer (50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 100 mM $(NH_4)_2SO_4$, 10% [v/v] glycerol, 50 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.1 mM PTSF) and loaded onto a 5 ml heparin agarose column (BioRad) equilibrated with HEPA buffer. After washing the column with two volumes of HEPA buffer, the DNA polymerase activity was eluted with a linear gradient to 500 mM $(NH_4)_2SO_4$ in HEPA buffer.

ssDNA cellulose chromatography

The peak fractions of DNA polymerase activity obtained from the heparin agarose column step were pooled and dialyzed against SSDNA buffer (10 mM potassium phosphate, pH 8.0, 20 mM KCl, 10% [v/v] glycerol, 0.5 mM dithiothreitol (DTT), 0.1 mM PMSF, 0.1 mM PTSF). This fraction was loaded onto a ssDNA cellulose column (0.5 mg protein/ml column volume) equilibrated with SSDNA buffer. The column was washed with two volumes of SSDNA buffer containing 100 mM KCl and the DNA polymerase was eluted with a linear gradient to 700 mM KCl in SSDNA buffer.

Standard DNA polymerase assay

DNA polymerase activity was determined in a 50 µl standard assay containing 50 mM Tris–HCl, pH 8.0, 125 mM KCl, 0.5 mM $MnCl_2$, 0.1 mM DTT, 140 µg/ml BSA, 64 µg/ml primed homopolymer poly(rA)·$(dT)_{12-18}$ (molar ratio of template to

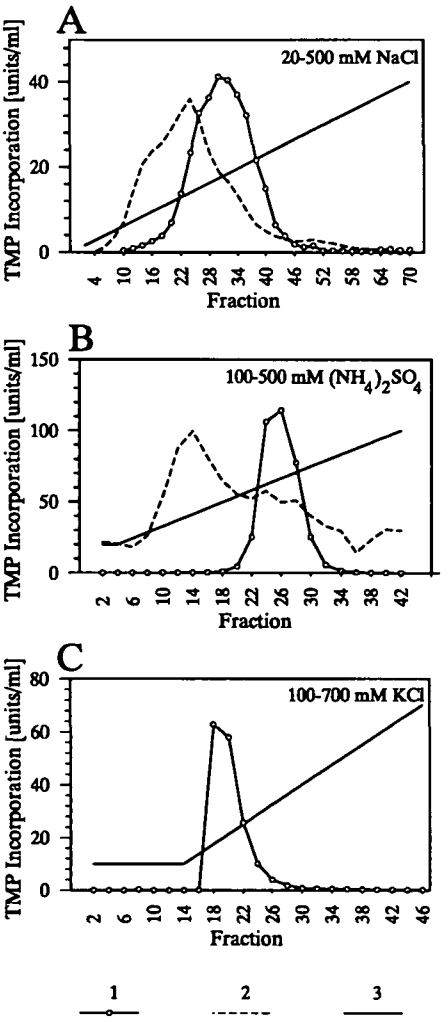

Figure 1. Elution profile of the mitochondrial DNA polymerase from **A:** DEAE cellulose, **B:** heparin agarose and **C:** single stranded DNA cellulose. The enzyme activity (1), calculated as units/mg of protein, was measured using the standard assay conditions described in Materials and Methods. The protein profile (2) was measured by the method of Bradford. The columns were eluted with linear salt gradients (3).

primer was 4:1) and 33 μM dTTP/[^{3}H]dTTP at a specific radioactivity between 300 and 1000 dpm/pmol. The reaction was started with the addition of a 5 μl enzyme aliquot and carried out at 37°C for 30 minutes. Aliquots of 40 μl were spotted onto DE81 2×2 cm filter squares. The filters were washed four times for 5 minutes each at room temperature in 0.5 M Na-phosphate buffer, pH 7.0, once in 80% ethanol and dried under a heat lamp. Radioactivity bound to each filter was measured in an LS 6000 SC (Beckman) liquid scintillation counter in 4 ml Ready Safe scintillation fluid (Beckman).

3′-5′ exonuclease assay

The gel purified [25] synthetic oligodeoxynucleotide (18mer: 5′ GAC TCT AGA GGA TCC CCG 3′) was 5′-end labeled with T4 polynucleotide kinase. The 20 μl reaction contained 10 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 μCi [γ-^{32}P]ATP, 100 pmoles oligodeoxynucleotide and 5 units of T4 polynucleotide kinase. After 30 min incubation at 37°C the reaction was terminated by boiling the sample for 10 min.

The 3′-5′ exonuclease assay (20 μl) contained 50 mM Tris−HCl, pH 8.0, 7.5 mM $MgCl_2$, 1 mM DTT, 0.7 pmol 5′-labeled oligodeoxynucleotide and a 2 μl enzyme aliquot per assay. After incubation for 30 min or 1 h at 37°C, 10 μl of formamide containing 0.1% bromophenol blue and 0.1% xylene cyanol were added and the mixture was heated for 10 min at 65°C. Aliquots were separated on a 0.4 mm denaturing polyacrylamide sequencing gel (20% polyacrylamide, 7 M urea in TBE buffer [25]). The gel was dried on a gel dryer (BioRad) and autoradiographed.

Protein determination

Protein was determined by a modification of the dye binding method [26] with BSA as standard according to the manufacturer's (BioRad) instructions.

Denaturing polyacrylamide gel electrophoresis

Polypeptides were resolved by denaturing polyacrylamide gel electrophoresis (7.5% T, 2.67% C) in the presence of sodium dodecylsulfate (SDS) [27]. The proteins in the gel were usually stained with silver according to the manufacturer's (BioRad) instructions.

Glycerol gradient sedimentation

After each step of purification, 150 μl of protein fraction active in DNA polymerization mixed with 30 μl of internal protein standards (10 mg/ml in 50 mM Tris−HCl) or 30 μl of 50 mM Tris−HCl, pH 8.0, were layered onto a 15−35% linear glycerol gradient in 50 mM Tris−HCl, pH 8.0, 250 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 0.1 mM PTSF. Prior to centrifugation, Ultraclear tubes (Beckman) were coated with a total protein extract (2 mg/ml) of *Chenopodium album* that had been denatured by incubation for 60 h at 65°C. Centrifugation was carried out at 4°C for 65 h at 30,000 rpm in a Beckman SW-40 rotor. Gradients were fractionated from the bottom of the tube into 220 μl fractions and assayed for DNA polymerase activity. The marker protein composition was assessed by SDS-PAGE. Molecular weight standards were BSA (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000) either run in parallel gradients or as internal markers in the same gradient as the enzyme fraction.

Table 1. Purification of the mitochondrial DNA polymerase from *Chenopodium album* L. suspension culture cells

Purification Step	Protein [mg]	Activity [units]	Spec. Act. [units/mg]	Purification [x-fold]
Crude	442	157	0.36	1
DEAE Cellulose	12	585	49	137
Heparin Agarose	0.085	118	1393	3924
ssDNA Cellulose	0.020	63	3139	8843

A unit of activity is defined as 1 nmole of [^{3}H]TTP incorporated into poly(rA)·dT_{12-18} in 60 min at 37°C with Mn^{2+} at 0.5 mM as cofactor.

RESULTS

Mitochondrial lysates

The procedure for preparation of mitochondria described in Materials and Methods was optimized for maximum DNA synthesis activity with respect to cell source (culture vs. leaves) and time of cell harvest (growth phase vs. stationary phase) [24]. Isolated mitochondria can be stored at −80°C for several months with only little loss of DNA polymerase activity. The capacity for DNA synthesis of crude mitochondrial lysates was tested under various conditions *in vitro*. Aphidicolin, a strong inhibitor of nuclear DNA polymerase α in eukaryotes, had no effect on the enzyme activity in lysates. N-ethylmaleimide, a sulfhydryl blocking reagent, as well as the chain terminator ddTTP reduced DNA synthesis, which agrees with the characteristics of DNA polymerases found in animal mitochondria. The crude mitochondrial lysate was used as a source for subsequent purification of the DNA polymerase.

Table 2. Biochemical properties of the *Chenopodium* mitochondrial DNA polymerase

Test Parameter	
Mn^{2+} optimum	0.5 mM
Mg^{2+} optimum	10 mM
KCl optimum	125 mM
Aphidicolin (20 μg/ml)	Resistent
araCTP (0.1 mM)	Resistent
ddTTP/TTP (10:1)	60−70% Inhibited
NEM (1 mM)	Strongly Inhibited
KPO_4	Strongly Inhibited

Table 3. Template preferences of the *Chenopodium* mitochondrial polymerase

Template	% Activity [0.5 mM Mn^{2+}]	% Activity [10 mM Mg^{2+}]
Poly(rA)·dT_{12-18}	100	0.5
Poly(dA)·dT_{12-18}	1	40
Poly(dA)	0	0
Activated DNA	8	60
pBR322 Circular	0	0
M13 (+) Strand with Single Primer	0	5
mtRNA·$(dNMP)_5$	0	0

An aliquot of the purified DNA polymerase was incubated in a 50 μl assay for 30 min at 37°C with different nucleic acids as templates in the presence of Mn^{2+} or Mg^{2+}. The activity with the preferred template, poly(rA)·dT_{12-18}, was set as 100%.

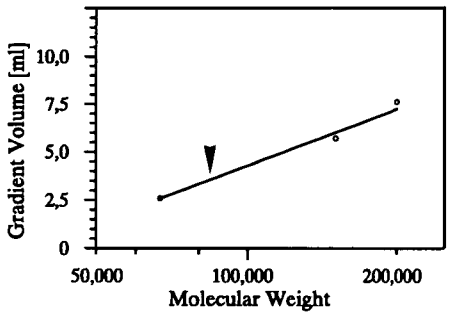

Figure 2. Glycerol gradient velocity sedimentation of *Chenopodium* mitochondrial DNA polymerase. Protein fractions were separated in a 15–35% glycerol gradient. Marker proteins run in the same gradient were: bovine serum albumin (67,000), alcohol dehydrogenase (150,000) and β-amylase (200,000). The estimated molecular weight of the DNA polymerase is 80,000–90,000 (indicated by an arrow).

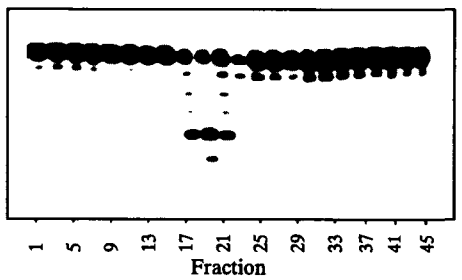

Figure 3. 3′-5′exonuclease activity profile eluted from ssDNA cellulose. Every other column fraction (see panel C in Fig. 1) was screened for 3′to 5′exonuclease activity using the gel assay described in Materials and Methods. Incubation time was for 60 min at 37°C.

Purification of the mitochondrial DNA polymerase

The DNA polymerase from isolated mitochondria of *Chenopodium album* was highly purified by sequential column chromatography on DEAE cellulose, heparin agarose and ssDNA cellulose. Polymerase activity eluting from these columns was routinely detected with the standard assay which contains poly(rA)·dT_{12-18} as a template and 0.5 mM Mn^{2+}. The DEAE cellulose column fractions were additionally screened with poly(dA)·dT_{12-18} and activated DNA in the presence of Mg^{2+} in order to rule out contaminating nuclear DNA polymerase activity and to possibly detect further mitochondrial enzymes that might have been present in the organellar lysate. No additional DNA polymerase activities were detected with either of these assay regimes. The DNA polymerase activity was eluted from DEAE cellulose at 250 mM NaCl as a single, broad peak after the main protein peak. On heparin agarose the enzyme was separated from the bulk protein and eluted at approximately 350 mM $(NH_4)_2SO_4$. The polymerase activity was recovered from ssDNA cellulose at 300 mM KCl as a sharp peak (Fig. 1). Table 1 summarizes a representative preparation of the mitochondrial DNA polymerase, carried through the three chromatographic purification steps. The enzyme activity was purified more than 8800 fold, yielding 20 μg of DNA binding proteins from 440 mg of solubilized mitochondrial protein (Table 1). The polypeptides separate into seven prominent bands in the 40,000 to 68,000 molecular weight range and a faint band of 98,000 Dalton on silver stained SDS-polyacrylamide gels (not shown). In the early stages of this study, a phosphocellulose chromatography step was included in the purification procedure, but since the activity of the mitochondrial DNA polymerase was lost during this step, probably because of the extreme sensitivity of the enzyme to phosphate in the elution buffer, this step was later omitted.

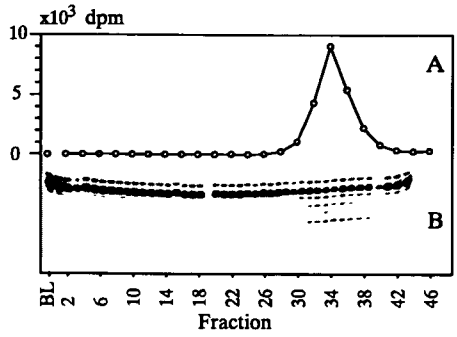

Figure 4. Activity profile of the γ-DNA polymerase peak fraction recovered from ssDNA cellulose, after sedimentation through a 15–35% glycerol gradient. **A:** Scan for DNA polymerase activity using the standard DNA polymerase assay. **B:** 3′-5′exonuclease activity profile.

Characterization of the mitochondrial DNA polymerase

To characterize the mitochondrial enzyme from *Chenopodium*, only the peak fraction of DNA polymerase activity eluted from ssDNA cellulose was used. A divalent cation was required for enzyme activity, and depending on the template, Mg^{2+} or Mn^{2+} fulfilled this requirement best (Tables 2 and 3). The enzyme was stimulated by KCl to an optimum concentration of 125 mM, and was inhibited at higher salt concentration. Aphidicolin and araCTP, inhibitors of nuclear DNA polymerase α [28, 29, 30], did not have an effect on the enzyme from *Chenopodium* mitochondria, but the DNA polymerase γ inhibitor, dideoxythymidine triphosphate (ddTTP) [31, 32] reduced the polymerase activity to 30–40% at a ddTTP:TTP ratio of 10:1 (Table 2). The enzyme was further inhibited by the sulfhydryl reagent N-ethyl maleimide (NEM) and was extremely sensitive to phosphate at concentrations of less than 5 mM (Table 2).

The purified enzyme preferred poly(rA)·$(dT)_{12-18}$ with Mn^{2+} as cofactor over activated DNA and poly(dA)·$(dT)_{12-18}$ in the presence of Mg^{2+}, a feature of γ-class DNA polymerases [21, 22]. The mitochondrial DNA polymerase was not able to incorporate deoxynucleotides into supercoiled double stranded or unprimed single stranded DNA. Single-primed M13 ssDNA was not utilized very efficiently by the plant mitochondrial DNA polymerase. *Drosophila* mitochondrial DNA polymerase, on the other hand, is much more active on this template [47]. No reverse transcriptase activity could be observed with $(dT)_{12-18}$-primed poly(A^+)RNA or with randomly primed mitochondrial RNA (Table 3).

DNA polymerase fractions from various stages of purification were sedimented in 15–35% glycerol gradients and the molecular weight of the enzyme was estimated from the sedimentation data of marker molecules, assuming a globular protein conformation. In all instances, the mitochondrial DNA polymerase was resolved as a single peak corresponding to a molecular weight of

80,000–90,000 (Fig. 2). Detection of DNA polymerase activity in SDS-polyacrylamide gels after renaturation was attempted under various experimental conditions which employed Mn^{2+} and the preferred template poly(rA)·$(dT)_{12-18}$, Mg^{2+} and poly(dA)·$(dT)_{12-18}$ or activated DNA, as well as several different batches of SDS [33, 34]. Except for the control enzyme *E.coli* DNA polymerase I and its large fragment, no polymerizing activity could be observed in any of the mitochondrial DNA polymerase fractions. Modified protocols of the photolabelling procedures developed by Schulte and Lambowitz [35] and Insdorf and Bogenhagen [36], likewise, were unsuccessful in detecting polypeptides with DNA polymerase activity in gels.

Co-purification of a 3′-5′ exonuclease activity

Fractions eluted from heparin agarose and ssDNA cellulose that contain DNA polymerase activity were screened for 3′-5′ exonuclease activity by a gel assay described in Materials and Methods. Two 3′-5′ exonuclease activities were separated on heparin agarose, one of which copurifies with the mitochondrial DNA polymerase while the second one elutes with the main protein peak (not shown). From ssDNA cellulose, the 3′-5′ exonuclease activity eluted as a single peak at 300 mM KCl, coinciding with the DNA polymerase activity (Fig. 1 and 3). No 5′-3′ exonuclease activity was observed in any of the fractions eluting from this column under the same assay conditions. The tight association between the exonuclease and the polymerase activity was also maintained on glycerol gradients, where DNA polymerase that had been purified through the ssDNA cellulose step and the 3′-5′ exonuclease co-migrated as a single peak in the molecular weight range of 80,000–90,000 (Fig. 4).

DISCUSSION

We previously demonstrated that lysates of purified mitochondria of *Chenopodium album* L. contain activities of enzymes involved in DNA replication, like DNA polymerase, DNA primase, 5′-3′ exonuclease, 3′-5′ exonucleases (unpublished results) and DNA topoisomerase I [37] and are active in DNA synthesis *in vitro*. In this paper we describe the purification of the DNA polymerase from plant mitochondria and extensively characterize the purified enzyme, since to date only a few reports exist that are concerned with the biochemical properties of DNA polymerases from plant organelles [38, 39, 40, 41], and of these only two describe plant mitochondrial enzymes [40, 41]. In all cases, the enzyme was either not highly purified or was not characterized extensively.

The mitochondrial DNA polymerase from *Chenopodium album* was purified several thousand fold through three chromatography steps that are based on the principles of ion exchange and affinity, and a final sedimentation velocity gradient. As also seen with other plant organellar DNA polymerases, a significant increase in activity was observed after chromatography on DEAE cellulose which probably reflects the removal of competing, non-productive nucleic acid templates and factors that inhibit the enzyme activity in the crude lysate [40]. In view of the increase in enzyme activity that apparent after the first chromatography step, the extent of purification reported in Table 1 is probably an overestimate. As shown in Table 2, KPO_4 is a potent inhibitor of the DNA polymerase, and we therefore avoided use of phosphate buffers during the purification. This phenomenon undoubtedly was the cause for over 90% loss of activity on phosphocellulose so that this column material, although probably an efficient purification step [42, 43], had to be excluded from the purification scheme. All chromatography steps that were ultimately adopted resulted in separation of the DNA polymerase from the bulk protein and brought about a significant increase in specific enzyme activity (Fig. 1, Table 1) to a final value of over 3,000 units/mg of protein. Despite the greater than 8,000 fold purification of the enzyme, a value that is in the high range for organellar DNA polymerases [40], a homogeneous preparation was not achieved. As judged by SDS-polyacrylamide gel electrophoresis, even the most highly purified fraction still contained several polypeptides (not shown).

A divalent cation, which can be either Mg^{2+} or Mn^{2+} depending on the *in vitro* template, was essential for activity of the mitochondrial DNA polymerase (Tables 2 and 3). Although the enzyme was unable to use poly(rA)·dT_{12-18} in the presence of Mg^{2+}, this primed homopolymer was the preferred template when Mn^{2+} was the cation in the assay (Table 3). Activated DNA, the best *in vitro* template for nuclear DNA polymerases of the α type [20], could only be used by the mitochondrial enzyme when Mg^{2+} was the cofactor. The DNA polymerase from chloroplasts and mitochondria of a soybean suspension cell line [40] and the chloroplast enzyme from spinach [38] show similar template preferences as the mitochondrial DNA polymerase described here. The ability to use oligo(dT)-primed poly(rA) as a template is a *per definitionem* feature of DNA polymerases belonging to the γ-class [20] and supports classification of these organellar enzymes as γ-type enzymes. By contrast, the mitochondrial enzyme from wheat embryos [41] utilizes the primed ribohomopolymer poorly with Mn^{2+} or Mg^{2+} and prefers poly(dA)·dT_{12} instead, a reason for the authors to propose placing this mitochondrial DNA polymerase in a class other than γ. Likewise, a DNA polymerase isolated from pea chloroplasts [39] was reportedly unable to utilize poly(rA)·dT_{12-18}, but in this case the assay was performed in the presence of Mg^{2+}. While these discrepancies may reflect fundamentally different enzymes that are present in the organelles of the plant species in question, variations in assay conditions, particularly divalent cation species and salt concentration, might explain the observed differences. The low activity of the plant mitochondrial DNA polymerase on single-primed M13 ssDNA, which is in contrast to the efficient use of this template by the analogous enzyme from *Drosophila* [48] is noteworthy and needs to be further examined by an analysis of the product of this reaction.

Resistance of the DNA polymerase from *Chenopodium* mitochondria to inhibitors of DNA polymerase α (aphidicolin and araCTP), its inhibition by NEM, stimulation by KCl and the preference for primed polyadenylate (Tables 2 and 3) clearly are properties of γ-class DNA polymerases [20, 44] and agree with the characteristics ascribed to most DNA polymerases from animal and plant cell organelles [38, 40, 45]. The inhibition of mitochondrial DNA polymerases from plants by ddTTP, however, is much less pronounced than that seen with the enzyme from animal sources. While the plant enzymes retain between 30 and 40% of their activity at a ddTTP to TTP ratio of 10:1 (Table 2) [40], DNA polymerases from animal mitochondria are practically inactive under these conditions [46, 47, 48]. Likewise, the sensitivity of the *Chenopodium* enzyme to phosphate (Table 2) seems to be a distinguishing characteristic of organellar DNA polymerases from plants [40], since DNA polymerases γ from animal sources are much less affected by this anion [42].

The subunit composition of γ-class DNA polymerases from animal sources seems to be variable. While the mitochondrial

enzymes from mouse myeloma cells and from chick embryos are homotetramers consisting of 47,000 Dalton subunits [42, 43], those from *Drosophila melanogaster* embryo [48] and *Xenopus laevis* mitochondria [36] are heterodimers composed of a large catalytic subunit of molecular weight greater than 100,000 and an associated smaller subunit. In yeast, the mitochondrial enzyme consists of a single, 140,000 Dalton subunit [23]. The *Chenopodium* DNA polymerase in preprations at various degrees of purification was estimated by glycerol gradient velocity sedimentation to have a molecular weight of 80,000–90,000 (Fig. 2), which agrees with values reported for other plant organellar enzymes [38, 40]. At present, an assignment of subunit composition of the native mitochondrial enzyme from *Chenopodium* is impossible, because detection of catalytic subunits by UV crosslinking [35, 36] or enzyme activity assays in polyacrylamide gels [33, 49] were so far unsuccessful. It should be noted that the final purified enzyme fraction contains a polypeptide band of an estimated molecular weight of 98,000 (not shown) which is consistent with the molecular weight range estimated for the native enzyme by glycerol gradient sedimentation and might represent the DNA polymerase. Since we have no means of verifying that the polypeptide in question has enzymatic activity, a correlation between this band and the mitochondrial DNA polymerase, at this point, is mere conjecture.

Mitochondrial DNA polymerases from a variety of sources are associated with an 3′-5′ exonuclease activity, which seems to be a general characteristic of these enzymes [22, 50, 51, 52, 53]. Fractions eluted from each chromatography step employed in the purification of the mitochondrial DNA polymerase from *Chenopodium* were screened for a 3′-5′ exonuclease activity. Throughout all purification steps the peak of DNA polymerase activity coincided with a 3′-5′exonuclease activity, as shown for the ssDNA cellulose column eluate (Fig. 1 and 3) and for the glycerol gradient profile of the peak fraction from this eluate (Figure 4). Both peaks of activity were superimposed at an estimated molecular weight of 80,000–90,000. The fact that both activities remain tightly associated despite the high salt concentration necessary to elute the DNA polymerase and 3′-5′ exonuclease activities from ssDNA cellulose (300 mM KCl) and to prevent formation of protein aggregates during ultracentrifugation (250 mM KCl) suggests that the DNA polymerase and the 3′-5′ exonuclease activities are carried on a single polypeptide. A tight association between these enzyme activities was also reported for the mitochondrial DNA polymerase from *Drosophila* embryos [54] and yeast [53]. In contrast, Keim et al. [55] found that a small subunit of molecular weight 26,500 harboring the exonuclease activity, after repeated centrifugation and during SDS-polyacrylamide gel electrophoresis dissociated from a DNA polymerase/3′-5′ exonuclease complex that was purified from spinach chloroplasts. In this case, the molecular weight of the enzyme complex is higher (105,000) than that of the enzyme from *Chenopodium*. The relatively low molecular weight of the native mitochondrial DNA polymerase from *Chenopodium*, which remained the same throughout all purification steps, further substantiates the assumption that both enzyme activities are located on one polypeptide chain.

The biochemical characteristics of the DNA polymerase from *Chenopodium* mitochondria are similar to other enzymes of the γ type [38, 40, 41] and are distinct from plant nuclear DNA polymerases α and β [56, 57]. Since only a single peak of activity was detected during chromatographic purification of a lysate obtained from purified mitochondria, it seems reasonable to assume, analogous to DNA polymerases from animal and fungal mitochondria, that this DNA polymerase constitutes the replicative enzyme in the plant organelle as well. Studies are currently underway to determine if the 3′-5′ exonuclease activity of the DNA polymerase influences the fidelity of nucleotide incorporation during DNA synthesis, as was shown for the γ-polymerase from mammalian mitochondria, chick embryos and *Drosophila* embryos and from yeast. In these cases, the 3′to 5′ exonuclease activity functions in proofreading during DNA synthesis [53, 56, 58, 59]. Although at present it is impossible to assign a function in DNA replication and/or repair to the enzyme from *Chenopodium*, a highly accurate plant mitochondrial enzyme might help to explain the strong sequence stability of the plant mitochondrial genome [11].

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