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Purification and properties of a pea chloroplast DNA polymerase

(circular DNA/DNA replication)

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ABSTRACT **A DNA polymerase has been purified >3,000-fold from the chloroplasts of pea plants by chromatography on DEAE-cellulose, phosphocellulose, single-stranded DNA-agarose, and sedimentation in a glycerol gradient. Electrophoretic analysis on polyacrylamide gels in the presence of sodium dodecyl sulfate indicates that the final fraction contained a single discernible protein band of 90,000 daltons. Gel filtration on Sephacryl S-200 and glycerol gradient sedimentation under nondenaturing conditions demonstrate that the chloroplast DNA polymerase has a native molecular mass of approximately 87,000 daltons. The purified polymerase lacks any associated nuclease activity. The enzyme activity is inhibited by *N*-ethylmaleimide (74% at 1.0 mM) and ethidium bromide (90% at 0.23 mM) and is resistant to aphidicolin. The purified enzyme is totally dependent on the presence of added DNA, has an absolute requirement for Mg^{2+} (12 mM optimal), is stimulated by K^+ (120 mM optimal), and requires all four deoxynucleoside triphosphates for maximum activity. Native DNA which has been degraded to a limited extent with DNase I is the most efficient template.**

The chloroplast genome in higher plants exists as circular DNA molecules of 130–150 kilobase pairs (kbp). The chloroplast DNAs (ctDNAs) from higher plants have been found to be largely homogeneous in sequence organization as shown by renaturation kinetics, electron microscopy, denaturation mapping, and restriction endonuclease digestions (1–3). The replication of ctDNA has been studied by analyzing the replicating ctDNA molecules with electron microscopy (4, 5). These studies have shown that the replication of ctDNA initiates with the formation of two displacement loops. The two displacing strands are hydrogen bonded to the opposite parental DNA strands. They expand towards each other, forming a structure that looks like a Cairns type of replicative intermediate when the two displacing strands elongate past each other. Electron microscopic studies have further shown that ctDNA molecules also replicate by a rolling-circle type of structure. The initiation site for the rolling-circle round of replication was found to lie at or near the termination site of the Cairns round of replication, suggesting that the rolling-circle intermediates are initiated by the Cairns round of replication.

The enzymatic mechanisms involved in the replication of ctDNA have not yet been investigated, even though the presence of a DNA polymerase in isolated chloroplasts has been demonstrated (6). Recently Sala *et al.* (7) have partially purified a γ-like DNA polymerase from spinach chloroplasts. In this paper we report a purification procedure that results in an essentially homogeneous preparation of a DNA polymerase from pea chloroplasts. The DNA polymerase from pea chloroplasts consists of a single polypeptide of 90,000 daltons.

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MATERIALS AND METHODS

Isolation of Chloroplasts. One kilogram of 7- to 10-day-old pea leaves were razor chopped with 2 liters of STM buffer containing 0.5 M sucrose, 0.05 M Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride. After filtration with four layers of cheesecloth and four layers of Mira-cloth, the filtrate was centrifuged at 1000 × *g* for 10 min. The pellet was suspended in 250 ml of STM buffer and centrifuged again at 1000 × g for 10 min. This procedure was repeated once more. The final pellet was suspended in a volume of 200 ml with STM buffer, and Triton X-100 was added to a final concentration of 2.5%. The mixture was allowed to stand for 30 min at 4°C. The lysed chloroplasts were centrifuged at 5000 × *g* for 30 min to remove nuclear contamination. The Triton X-100-disrupted chloroplasts were found to be essentially free of nuclear contamination. This procedure results in the isolation of pure undegraded ctDNA by substituting 5 mM EDTA for 5 mM $MgCl_2$ in the STM buffer.

DNA Polymerase Assay. The enzymatic activity was assayed in a 0.2-ml mixture containing 50 mM Tris·HCl (pH 7.0); 12 mM $MgCl_2$; 120 mM KCl; 0.001 mM each of dATP, dCTP, dGTP, and TTP; and 1 μCi (1 Ci = 37 GBq) of [^{3}H]TTP (75 Ci/mmol) in the presence of 5 μg of calf thymus DNA activated by the method of Kornberg (8). Incubation was carried out for 30 min at 37°C. The precipitated radioactivity was determined as described (6). One unit of DNA polymerase activity is defined as 1 nmol of total deoxyribonucleotide incorporated into acid-insoluble product in 30 min.

Column Chromatography. DEAE-cellulose (DE-52) was obtained from Whatman and used without further treatment. Cellulose phosphate P11 was purchased from Whatman and pretreated by first washing with 0.5 M HCl in 50% ethanol, followed by repeated washings with water and finally with 0.5 M NaOH. After the alkali treatment, the phosphocellulose was washed with H_2O until neutral. Single-stranded DNA-agarose was from Bethesda Research Laboratories and used without any further treatment.

Nucleic Acids. Isolation of ctDNA, restriction digests of ctDNA, and DNA·DNA hybridizations were carried out as described (3).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Polypeptides were analyzed by polyacrylamide gel electrophoresis; 0.5 × 10 cm cylindrical gels were prepared with 10% separating and 4% stacking concentrations of acrylamide. The electrophoresis buffer contained 25 mM Tris·glycine (pH 8.3) and 1% NaDodSO$_4$. Electrophoresis was performed at 2.5 mA per gel (constant current) until the tracking dye was 1.0 cm from the bottom.

Exonuclease Assay. Exonuclease activity was assayed essentially as described by Richardson (9). One microgram of

Abbreviations: ctDNA, chloroplast DNA; kbp, kilobase pair(s); Da, dalton(s).

single-stranded ^{3}H-labeled pea DNA (1×10^5 cpm/μg of DNA) was mixed with 0.01 ml of sample in 0.2 ml of standard buffer containing 0.05 M Tris·HCl (pH 8.0), 10 mM 2-mercaptoethanol, and 10 mM $MgCl_2$. After incubation for 1 hr at 37°C, 50 μg of calf thymus DNA was added as carrier, and 0.3 ml of ice-cold 10% trichloroacetic acid was mixed with the reaction sample. The mixture was allowed to precipitate on ice for 5 min and then centrifuged for 5 min at $15,000 \times g$; 0.4 ml of the supernatant was removed and added to 5 ml of liquid scintillation fluor (PS II from Amersham). Radioactivity was measured in a Beckman model LS-230 scintillation counter. When enzyme was omitted from the reaction, <1% of the labeled DNA was acid soluble.

RESULTS

DNA Polymerase Activity of Chloroplasts. Triton-disrupted chloroplasts were found to contain an active DNA polymerase activity that was essentially dependent upon endogenous DNA. Addition of exogenous DNA increased the DNA polymerase activity only by about 20%. If the Triton-disrupted chloroplasts were incubated at 4°C, the endogenous DNA was progressively degraded, and the DNA polymerase activity was correspondingly dependent upon the exogenous DNA. In order to find out whether Triton-disrupted chloroplasts were using ctDNA as a template in the DNA polymerase reaction, the *in vitro* synthesized DNA was isolated without adding exogenous DNA as a template. The Triton-disrupted chloroplasts were able to polymerize deoxynucleotides almost linearly for >1 hr. A large-scale DNA polymerase reaction was carried out with [^{32}P]TTP, and the enzymatic reaction was stopped after 30 min by adding 2% $NaDodSO_4$ (final concentration). The lysed chloroplast preparations were treated with protease K (50 mg/ml) that had been extracted with phenol twice, and the DNA was precipitated by adding 2 volumes of ethanol and 50 mg of purified calf thymus DNA per ml as a carrier. The precipitated DNA was dissolved in 15 mM NaCl (pH 7.5) and dialyzed against the same buffer for 48 hr. Isolated DNA contained >50% of the acid-insoluble radioactivity present in the incubation mixture. The *in vitro* synthesized radioactive DNA was hybridized to pea ctDNA digested with the endonucleases *Sal* I and *Xba* I. The autoradiographs of the Southern blots are shown in Fig. 1. All of the ctDNA fragments produced by these endonucleases hybridized to the *in vitro* synthesized DNA. Thus, the DNA polymerase activity of the chloroplasts uses endogenous ctDNA as a template. These studies again confirm the presence of a DNA polymerase in chloroplasts and demonstrate that the Triton X-100-disrupted chloroplasts contain a polymerase that actively synthesizes ctDNA *in vitro*.

Solubilization of DNA Polymerase. The RNA polymerase activity of chloroplasts is known to be tightly bound to ctDNA (10). Recently it has been possible to remove the RNA polymerase activity by self-digesting the endogenous ctDNA (11). The DNA polymerase activity of chloroplasts, on the other hand, has not been found to be tightly bound to ctDNA. It has been possible to remove ctDNA from Triton X-100-disrupted chloroplasts by passing the solution through DEAE-cellulose. The DNA polymerase activity from the DEAE-cellulose column has been found to be totally dependent upon exogenous DNA.

Purification of the DNA Polymerase. The Triton X-100-disrupted chloroplasts were either fractionated by ammonium sulfate or directly fractionated on a DEAE-cellulose column. For ammonium sulfate fractionation, disrupted chloroplasts were treated with ammonium sulfate up to 30% saturation. At this ammonium sulfate concentration, all of the chloroplast membranes were found to precipitate. The supernatant from this fraction contained most of the DNA polymerase activity. Ammonium sulfate was added to this fraction to 70% saturation, and the pellet was collected and dialyzed against a 0.01 M potassium phosphate buffer (pH 8.0) containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 25% (vol/vol) glycerol. The precipitated fraction contained about 70% of the DNA polymerase activity of the Triton X-100-disrupted chloroplasts.

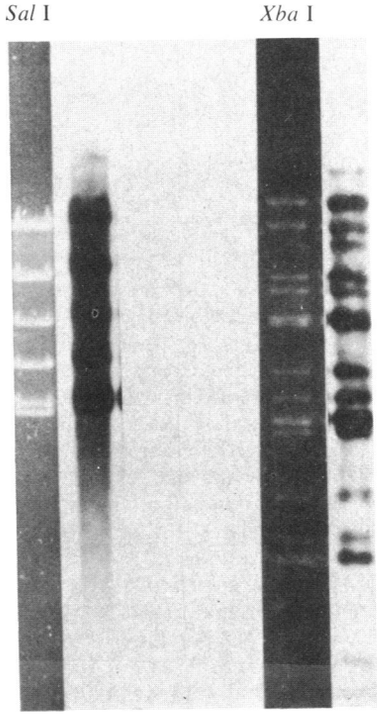

FIG. 1. Hybridization of *in vitro* synthesized DNA with restriction digests of pea ctDNA. Lysed chloroplasts were incubated by using [α-^{32}P]-labeled TPP as described without adding exogenous DNA. After a 30-min incubation, *in vitro* synthesized DNA was extracted and hybridized to Southern blot transfers of *Sal* I and *Xba* I restriction digests of pea ctDNA.

The Triton X-100-disrupted chloroplasts or the 70% ammonium sulfate fraction obtained from 1 kg of leaves was applied to a 2.5×20 cm DEAE-cellulose column equilibriated with 0.01 M phosphate buffer (pH 8.0). Seventy to 80% of the DNA polymerase activity was bound to the column. The column was washed extensively with 0.05 M phosphate buffer and then was eluted in one step with 0.35 M phosphate buffer. It was possible to recover about 50% of the original enzyme activity of the Triton X-100-disrupted chloroplasts from the DEAE-cellulose column. It was not possible to purify the enzyme on a DEAE-cellulose column by gradient elution. Therefore, a one-step elution with 0.35 M phosphate was used to further purify the enzyme. The 0.35 M phosphate fraction from DEAE-cellulose was pooled and extensively dialyzed against 0.01 M phosphate buffer (fraction II).

A phosphocellulose column of 1.5×5 cm was prepared and equilibriated with 0.01 M phosphate buffer. The dialyzed fraction II was applied to the column at a rate of 10 ml/hr. Most of the protein of fraction II did not bind to phosphocellulose, but 70–80% of the DNA polymerase activity was retained by the column (Table 1). The phosphocellulose column was developed with a 250-ml linear gradient of 0.1–0.35 M phosphate buffer. About 90% of the bound polymerase activity was eluted as a single peak at 0.25 M phosphate and was used in further purification (Fig. 2). However, there was a small but reproducible amount of DNA polymerase activity that was eluted at about 0.2 M phosphate and appeared as either a shoulder or a small distinct peak of activity. At this

Table 1. Purification of chloroplast DNA polymerase

Fraction	Step	Protein, mg	Activity, units	Specific activity, units/mg	Yield, %	Purification
I	Lysed chloroplasts	973	375	0.39	100	1
II	DEAE-cellulose	107	175	1.64	47	4
III	Phosphocellulose	0.84	48	57.1	13	146
IV	Single-stranded DNA-Agarose	0.053	21	396	6	1015
V	Glycerol gradient	0.012	15	1250	4	3205

time, it is difficult to conclude whether there is another DNA polymerase enzyme in chloroplasts or whether the small amount of activity eluting as a shoulder to the main peak is because of some nuclear contaminants in the preparation. The DNA polymerase activity was found to undergo about 30- to 40-fold purification at the phosphocellulose step (Table 1). The major peak containing the DNA polymerase activity was pooled, concentrated by ultrafiltration with an Amicon S-12 ultrafiltration cell having a YM5 Diaflo membrane and dialyzed against 0.01 M phosphate buffer (fraction III).

Fraction III was applied to a 5-ml single-stranded DNA-agarose column equilibriated with 0.01 M phosphate buffer. About 75–85% of the DNA polymerase activity was found to be retained by the column. After a 0.1 M phosphate buffer wash, the absorbed activity was eluted from the column by using a 30-ml linear gradient of 0.1–0.5 M phosphate buffer. The DNA polymerase was eluted as a single peak at 0.35 M phosphate. The peak fractions of activity were collected, concentrated by ultrafiltration (fraction IV), and centrifuged in a linear 30–50% (vol/vol) glycerol gradient containing 50 mM Tris·HCl (pH 8.0), 1 M NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride. The glycerol gradient was centrifuged for 16 hr at 50,000 rpm in an SW56 rotor at 4°C. The DNA polymerase activity sedimented as a single peak. The fractions containing the DNA polymerase activity were pooled and concentrated as described before (fraction V).

Molecular Size of the Native DNA Polymerase. The native molecular size of the DNA polymerase was determined by gel filtration on a Sephacryl S-200 column and sedimentation on a glycerol gradient under nondenaturing conditions. The chloroplast DNA polymerase was eluted from a Sephacryl column between bovine serum albumin [66,200 daltons (Da); stokes radius, 35 Å] and alkaline phosphatase (100,000 Da; stokes radius, 42 Å). From the elution profiles of these proteins, the stokes radius of the chloroplast DNA polymerase was calculated to be 38 Å. The velocity sedimentation gradient showed that the chloroplast DNA polymerase sedimented between bovine serum albumin ($s^0_{20,w}$, 4.3) and alkaline phosphatase ($s^0_{20,w}$, 6.2). From these data, the chloroplast DNA polymerase was found to have an $s^0_{20,w}$ of 5.2. From the stokes radius and sedimentation coefficient, the approximate native molecular weight of chloroplast DNA polymerase has been calculated to be 87,000 Da by the method of Siegel and Monty (12).

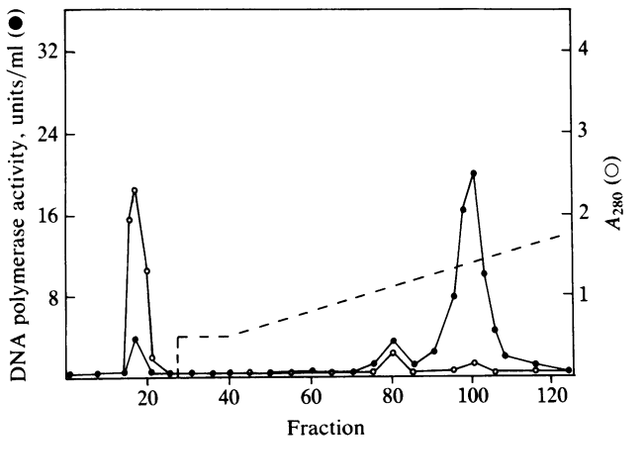

FIG. 2. Phosphocellulose chromatography of DNA polymerase. Fraction II (20 ml) containing 125 units was applied to the phosphocellulose column and washed with 0.1 M potassium phosphate. The column was developed with a 250-ml linear gradient from 0.1 to 0.35 M potassium phosphate (dashed line). The activity was pooled, concentrated, and dialyzed against 0.01 M phosphate buffer (fraction III).

Polypeptide Composition of the DNA polymerase. At every step of the purification, polypeptide composition of the fractions was analyzed by $NaDodSO_4$ gel electrophoresis. Fraction III (Table 1) contained a large number of polypeptides along with a polypeptide of 90,000 Da (Fig. 3, lane 1). Fraction IV (DNA-agarose-purified fraction) contained essentially a 90,000-Da polypeptide along with some faint polypeptides of <50,000 Da. DNA polymerase from the glycerol gradient contained a single polypeptide of about 90,000 Da (Fig. 3, lane 2). The molecular size of the native DNA polymerase along with the molecular size of the denatured protein is consistent with the idea that the chloroplast DNA polymerase is made up of a single polypeptide.

Properties of the Chloroplast DNA Polymerase.

Nuclease Activity. The nuclease activity of the various fractions was assayed by using radioactivity-labeled DNA. The lysed chloroplasts were found to degrade labeled DNA (Table 2), and fraction II (DEAE-cellulose) also contained

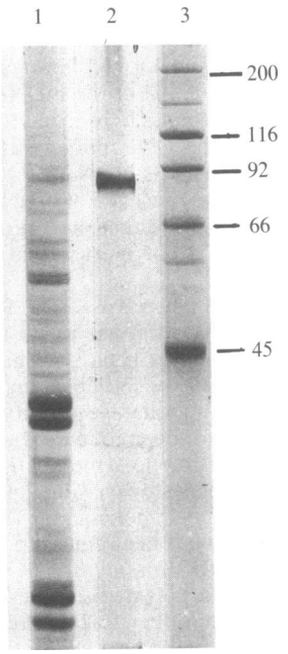

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis. Lanes: 1, 0.1 ml of fraction III enzyme stained with Coomassie brilliant blue (13); 2, 0.1 ml of fraction V enzyme visualized with silver stain (14); 3, 2.5 μg of each molecular weight standard stained with Coomassie blue; sizes are shown in kDa.

Table 2. Nuclease activity

Enzyme	Acid-soluble radioactivity, cpm
None	562
Chloroplast DNA polymerase	
Lysed chloroplasts (fraction I)	30,885
DEAE-cellulose (fraction II)	5,864
Phosphocellulose (fraction III)	991
Single-stranded DNA-agarose (fraction IV)	483
Escherichia coli DNA polymerase I	17,416
Exonuclease III	27,596

significant nuclease activity. However, fraction III from phosphocellulose contained only a negligible amount of the nuclease activity, and fraction IV from DNA-agarose did not contain any nuclease activity. The nature of the nuclease activity in chloroplasts was determined by using supercoiled dimer pBR322 DNA (Fig. 4, lane 1). The incubation of supercoiled pBR322 with lysed chloroplasts relaxed all the supercoiled DNA (Fig. 4, lane 2). Similarly, incubation of plasmid DNA with the concentrated ammonium sulfate fraction not only relaxed supercoiled DNA but degraded the plasmid DNA into small fragments (Fig. 4, lane 3). The degradation of plasmid DNA was not seen by the Triton X-100 supernatant because a very small amount of the enzyme was used. Similarly, the DEAE-cellulose fraction was found to relax and degrade the plasmid DNA (Fig. 4, lane 4) However, the enzyme from the phosphocellulose column (fraction III) did not have any observable effect on the plasmid DNA (Fig. 4, lane 5). Exonuclease III, as expected, degraded the relaxed plasmid DNA but did not relax the supercoiled form (Fig. 4, lane 6). Thus, the purified chloroplast DNA polymerase contains neither an endonuclease nor an exonuclease activity.

General properties. The purified chloroplast DNA polymerase showed complete dependence on Mg^{2+}. The optimum concentration of Mg^{2+} was found to be 12 mM. Mn^{2+} could replace Mg^{2+}; but, at the optimum Mn^{2+} concentration of 0.5 mM, the activity was about 20% of that found with Mg^{2+}. The purified DNA polymerase was stimulated by K^+ or Na^+ (optimum concentration, 120 mM). In the absence of KCl or NaCl, DNA polymerase showed only 32% of the optimum activity. The maximum activity of the enzyme was found to require the presence of all four deoxynucleoside triphosphates (Table 3). The enzyme showed a broad pH optimum and was found to be highly sensitive to *N*-ethylmaleimide; at 1.0 mM its activity was inhibited by 75% of the control. Ethanol at the concentration of 10% inhibited the polymerase activity by about 90%. Similar inhibition was seen with ethidium bromide. Aphidicolin, on the other hand, had no effect on the chloroplast DNA polymerase (Table 3).

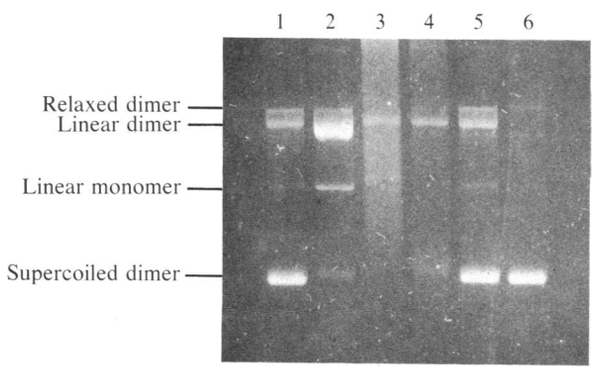

FIG. 4. Nuclease activity. Supercoiled dimer pBR322 DNA (0.5 μg) was mixed with 0.01 ml of sample in 0.05 ml of standard buffer containing 0.05 M Tris·HCl (pH 8.0), 10 mM 2-mercaptoethanol, and 10 mM $MgCl_2$. After incubation for 30 min at 37°C, the reactions were terminated with 2% $NaDodSO_4$ and 5 mM EDTA. The reaction mixtures were loaded on a 0.8% agarose gel, and electrophoresis was conducted at 50 mA for 16 hr. The gel was stained with ethidium bromide. Lanes: 1, no enzyme addition; 2, 48 μg of fraction I enzyme; 3, 250 μg of ammonium sulfate fraction; 4, 127 μg of fraction II enzyme; 5, 1.7 μg of fraction III enzyme; 6, exonuclease III.

Table 3. Properties of the purified chloroplast DNA polymerase

Reaction conditions	Nucleotides incorporated, units/ml	Activity, %
Complete	5.4	100
$-Mg^{2+}$	0.2	4
$-K^+$	1.7	32
−DNA	0.1	2
−dATP	2.4	45
−dATP, dCTP	1.9	34
−dATP, dCTP, dGTP	1.1	21
Inhibitory effects		
Complete	7.2	100
N-Ethylmaleimide (1.0 mM)	1.9	26
Ethanol (10%)	0.9	13
Aphidicolin (0.1 mM)	7.1	99
Ethidium bromide (0.025 mM)	0.7	10
Template–primer		
Complete	6.7	100
Denatured calf thymus DNA	1.7	25
Native calf thymus DNA	1.4	21
Supercoiled pBR322 DNA	0.1	1
Oligo(dT)·poly(rA)	0.1	1
Oligo(dT)·poly(dA)	0.8	12
Poly(dA·dT)	1.5	23

The enzyme required an activated template for its optimum activity. The purified enzyme did not show any preference for ctDNA in place of calf thymus DNA. Native and denatured DNA were poor templates showing only 25% of the optimal activity. Supercoiled DNA did not act as a template with the purified chloroplast DNA polymerase. The chloroplast enzyme also did not show any enzymatic activity when oligo(dT)·poly(rA) was used as a template.

DISCUSSION

DNA polymerase activity of freshly isolated pea chloroplasts utilizes endogenous ctDNA as a template. This also was found to be the case in isolated chloroplasts from maize (15). The hybridization of *in vitro* synthesized DNA with restriction digests of pea ctDNA showed that practically all of the ctDNA was involved as a template in the DNA polymerase reaction (Fig. 1). The DNA polymerase activity was found to dissociate easily from the endogenous ctDNA either by DEAE-cellulose chromatography or by self-digestion of ctDNA with endogenous nucleases. We have been able to purify the enzyme to a single homogeneous protein. The enzyme was found to be pure at about 3,000-fold purification. The most effective steps in the purification were chromatography on phosphocellulose and single-stranded DNA-agarose. The purified enzyme was found to have a specific activity of 1250 units/mg, which compares reasonably well with those reported for eukaryotic DNA polymerases (16–19). The chloroplast DNA polymerase has a marked preference for Mg^{2+} and is strongly inhibited by *N*-ethylmaleimide. The pea chloroplast DNA polymerase was found to be unable to copy oligo(dT)·poly(rA) when assayed under a variety of experimental conditions. However, pea chloroplast DNA polymerase can utilize oligo(dT)·poly(dA) as a primer template and even more efficiently the double-stranded polymer poly(dA·dT). Sala *et al.* (7) have carried out a 33-fold purification of spinach chloroplast DNA polymerase, which shows preference for the synthetic primer template oligo(dT)·poly(rA).

The enzyme is also more active in the presence of Mn^{2+} than Mg^{2+}.

Misumi and Weissbach (20) have purified an α-class eukaryotic DNA polymerase from spinach. The enzyme has a molecular weight of 160,000 ± 10,000 Da and is markedly inhibited by aphidocolin and *N*-ethylmaleimide. The pea chloroplast DNA polymerase differs strikingly from the α class of polymerases. It has a native molecular weight of about 90,000 Da, and its enzymatic activity is not inhibited by aphidicolin. In contrast to the α class of DNA polymerases, pea chloroplast DNA polymerase requires 120 mM KCl for optimum activity. The pea chloroplast DNA polymerase does share some features that are common to all eukaryotic DNA polymerases. For example, the purified pea chloroplast enzyme has no associated nuclease activity, aggregates at low salt concentrations, and fills gaps in double-stranded DNA. At this point, it cannot be categorically stated that this DNA polymerase is the only enzyme involved in chloroplast DNA replication.

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