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ISOLATION AND CHARACTERIZATION OF AN IN VITRO DNA REPLICATION SYSTEM FROM MAIZE MITOCHONDRIA

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An in vitro DNA replication system from maize mitochondria has been isolated and characterized. Maize mtDNA polymerase activity was purified about 1100-fold through DEAE cellulose and Heparin-Sepharose columns. In addition to the DNA polymerase activity, this in vitro replication system also contained topoisomerase I, DNA primase and RNA polymerase activities. Optimal conditions for enzyme activity, preferred templates and inhibitors were determined in order to further characterize this in vitro replication system; this system was devoid of any detectable extramitochondrial activity as determined by: a) the mt origin of the DNA polymerase activity as evidenced by studies using different templates and inhibitors, b) absence of chloroplast or nuclear DNA, glucose -6-P-dehydrogenase (known to be present only in the cytosol and chloroplasts) and photosynthetic pigments in the mitochondrial fraction and c) the ability of maize mt topoisomerase I to relax positively supercoiled DNA.

Mitochondrial DNA polymerase has been partially purified and characterized from a number of plants. Plant mtDNA polymerases [1-6] are resistant to aphidicolin and are inhibited by N-ethylmaleimide, ethidium bromide and high ratios of ddNTPs to dNTPs; they are generally most active on poly(dA)-oligo(dT_{12-18}) and on activated DNA templates and exhibit only a low activity on poly(rA)-oligo(dT_{12-18}) even in the presence of Mn^{2+} ions [2]. The turnip [3], petunia [4] and maize [this study] mtDNA polymerases show similar characteristics.

It is important to know the number and the location of replication origin(s) in the plant mitochondrial genome in order to gain a basic understanding of replication events in this compartment of the plant cell. Precise determination of DNA sequences that are required for a replication event would involve the use of in vivo and in vitro DNA replication systems; lack of such systems have greatly hampered progress in this field. More recently, Daniell et al. [7]

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Abbreviations: mt, mitochondria; ct, chloroplast.

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have reported a new approach to study DNA replication in vivo; in this approach foreign plasmids containing oris may be introduced into cultured cells (using the Gene Gun) and transformed cells may be permeabilized using the detergent L-lysophosphatidyl choline to specifically label introduced foreign DNA. Some of the enzymes that participate in DNA replication have been isolated and partially characterized in the past (crude lysates have been occasionally used); this study reports the first isolation and partial characterization of an in vitro DNA replication system from maize mitochondria that has been successfully used to isolate a putative replication origin (Zheng and Daniell, in review).

MATERIALS AND METHODS

a) Isolation of the enzyme system: Mitochondria were prepared by homogenization of etiolated maize seedlings (genotype B-37, obtained from Pioneer Hibred Inc.) in STM buffer (0.5M sucrose, 50mM Tris-HCl, pH 8.0, 5mM $MgCl_2$, 5mM 2-mercaptoethanol, 0.2 mM PMSF) with three bursts of 10 sec each in a blender and filtration of the homogenate through four layers of cheesecloth and three layers of miracloth (Calbiochem). The mitochondria-enriched fraction was obtained by differential centrifugation at 15,000xg, after discarding the nuclei (at 40xg) and chloroplasts (at 1500xg). Isolated mitochondrial pellets were frozen and stored at -80°C. Thawed mitochondrial pellets were resuspended in a suitable volume of STM buffer containing 2.5% of Triton X-100. After lysis and solubilization of mitochondria at 4°C for 30 min, membrane debris was removed by a 5 min centrifugation at 3000xg. The resulting supernatant was loaded onto a DEAE column as described in the following section.

b) DEAE cellulose column chromatography: The Triton X-100 supernatant was loaded onto a 30 ml DEAE cellulose column (Whatman DE52) at 30 ml/hr, previously equilibrated with buffer A (50mM Tris-HCl, pH 8.0, 50mM 2-mercaptoethanol, 0.2mM PMSF, 25% glycerol and 50mM NaCl). The column was washed with 10 volumes of buffer A and eluted with buffer A containing 0.6M NaCl. The fractions were assayed for DNA polymerase activity using activated calf-thymus DNA as the template in 100μl reactions containing: 50mM Tris, pH 7.0, 150mM KCl, 7.5mM $MgCl_2$, 100μM dCTP, dGTP, dATP, 1μCi [H^3]-TTP (84 Ci/mmol), 5μg of activated calf thymus DNA and 10μl of the enzyme fraction. Reactions were incubated for 30 min at 37°C and the products were precipitated in 5% TCA. Acid-insoluble products were collected on GF/A filters, and the filters were washed with 5% TCA and 70% ethanol, dried, and the incorporated radioactivity was determined in a toluene-based scintillation fluid. Active fractions were dialyzed against buffer B (buffer A plus 0.1mM EDTA and 0.1% Triton X-100) containing 0.1M NaCl.

c) Heparin-Sepharose column chromatography: Active, dialyzed fractions were loaded onto a 6-7 ml Heparin-Sepharose (Pharmacia) column (at about 10 ml/hr) which had been equilibrated with buffer B containing 0.1M NaCl. After washing the column with 10 volumes of buffer B containing 0.1M NaCl and 0.3M NaCl successively, the DNA polymerase activity was eluted with buffer B containing 0.6M NaCl. The fractions were assayed for DNA polymerase activity using activated calf-thymus DNA as the template. Active fractions were dialyzed against buffer B and loaded again onto a 6-7 ml Heparin-Sepharose column which had been equilibrated with Buffer B containing 0.1M NaCl. After washing the column with 10 volumes of Buffer B containing 0.1M and 0.3M NaCl successively, the DNA polymerase activity was finally eluted with a linear (0.3-0.6M NaCl) gradient in buffer B. Active fractions dialyzed against Buffer A were used for in vitro replication studies. Fractions were stored at -80°C.

d) Assay for DNA primase activity: DNA primase activity was determined as the ability to prime in vitro DNA replication using a single stranded DNA template. Reactions were carried

out in a 100 μl volume containing 50mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 1mM dithiothreitol, 100 μg/ml BSA, 2mM ATP, 0.1mM each of dCTP, dGTP, dATP, 0.2 mM each of CTP, UTP, GTP, 1 μCi of [^{3}H]-TTP (84 Ci/mmol), 1 μg of single stranded M13 mp19 and 10 μl of the Heparin-Sepharose peak enzyme fraction. Incubations were carried out for 30 min at 37°C and the incorporated radioactivity was determined as described above.

e) Assay for RNA polymerase activity: The enzymatic activity was assayed in a 100 μl reaction mixture containing 50 μM of ATP, GTP, CTP, 1 μCi of [H^3]-UTP (37.2 Ci/mmol), 10mM $MgCl_2$, 10mM Tris-HCl pH 8.5, 10 μl of the Heparin-Sepharose peak enzyme fraction and 1 μg of the desired template. The incubation was carried out at 37°C for 30 min and the incorporated radioactivity was determined as described above.

f) Assay for topoisomerase I activity: Topoisomerase I activity was assayed using 0.5 μg of supercoiled pUC19 DNA in a 20 μl volume containing 50mM Tris-HCl pH 8.0 and 10 μl of the Heparin-Sepharose peak enzyme fraction, in the presence or absence of $MgCl_2$ (10 mM) and/or KCl (150 mM). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 4 μl of 20% ficoll containing 0.5% SDS and 0.5% bromophenol blue. The samples were heated for 2 min at 65°C and separated in a 0.8% agarose gel using 1X TBE buffer. After electrophoresis the gel was stained with ethidium bromide and photographed on a UV transilluminator.

RESULTS AND DISCUSSION

Mitochondrial DNA polymerase was partially purified from etiolated maize seedlings as described in Materials and Methods. DNA polymerase activity eluted as a single peak from the DEAE cellulose column when assayed using activated calf-thymus DNA as the template. DNA polymerase activity more than doubled when the Triton supernatant was passed through the DEAE-cellulose column and resulted in a 17-fold purification (21.9 u/mg specific activity). Peak fractions obtained from the DEAE-cellulose column were passed through Heparin-Sepharose columns twice. The first elution was done with 0.6M NaCl in buffer B; this step elution resulted in 53% yield of DNA polymerase activity (140 u/mg) and 109-fold purification of the enzyme. The second elution was done with a linear (0.3-0.6M) NaCl gradient in buffer B. Peak DNA polymerase activity eluted at 0.55 M NaCl concentration; this gradient purification step did not decrease the enzyme yield but resulted in about 1110-fold purification (1432 u/mg). Peak fractions had no detectable DNA polymerase activity in the absence of exogenously added template (Table I). No DNase activity was detected when templates were incubated with the peak Heparin-Sepharose enzyme fraction in the presence of $MgCl_2$ at 37°C (Fig. 1). RNase activity was also not observed (data not shown).

DNA polymerase: The maize mtDNA in vitro replication system was optimized using appropriate templates for maximal incorporation of [H^3]-TTP into DNase sensitive, RNase insensitive TCA-insoluble products. The incorporation of [H^3]-TTP was linear up to 60 min at 37°C and pH 7.0. DNA polymerase required an optimal concentration of 140mM KCl. Magnesium was preferred over manganese by all the templates tested (Table I).

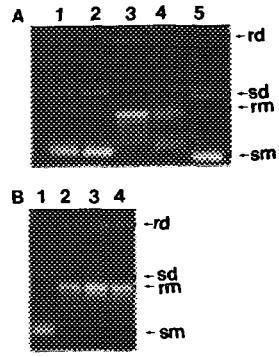

Figure 1. Maize mitochondrial topoisomerase I activity. Reactions were carried out as described in Materials and Methods. A: Salt requirement. Lane 1: +KCl, +$MgCl_2$; Lane 2: -$MgCl_2$, +KCl; Lane 3: +$MgCl_2$, -KCl; Lane 4:-KCl, -$MgCl_2$; Lane 5: control pUC19; final concentration: 150 mM KCl; 10 mM $MgCl_2$. B: Relaxation of positively supercoiled DNA. Lane 1: control pUC19; Lane 2: minus ethidium bromide; Lane 3: 0.5 μg/ml ethidium bromide; Lane 4: 1μg/ml ethidium bromide. sm: supercoiled monomer; sd: supercoiled dimer; rm: relaxed monomer; rd: relaxed dimer.

Table I shows efficiency of the maize mtDNA polymerase on various templates in the presence of Mg^{2+} or Mn^{2+}. Maize mtDNA polymerase preferred poly (dA)-oligo (dT_{12-18}) over all other synthetic and natural templates. This observation is in agreement with other reports on plant organellar DNA polymerases but is different from the results obtained by Cannon's group [5,6] who reported that poly (rA)-oligo (dT_{12-18}) was a preferred template of mtDNA polymerase

Table I. Characterization of the maize mtDNA polymerase. Reactions were carried out in a 100-μl volume containing 50 mM Tris-HCl,pH 8.0, 140 mM KCl, 100μm dNTPs (except dTTP), 7.5 mM $MgCl_2$, 1 μCi ^{3}H-TTP, 1 μg of the indicated template and 10 μl of the peak Heparin-Sepharose fraction, and the reactions were incubated for 30 min at 37°C. 100% activity was 277,106 cpm with poly(dA)-oligo (dT_{12-18}) and 197,368 cpm with pHD32 as template, respectively. The clone pHD32 is pUC19 containing a putative maize mtDNA ori.

Assay conditions	% Optimal activity
Poly(dA)-oligo(dT_{12-18}) + $MgCl_2$	100
Poly(dA)-oligo(dT_{12-18}) + $MnCl_2$	38
Activated calf thymus DNA + $MgCl_2$	43
Activated calf thymus DNA + $MnCl_2$	17
Poly(rA)-oligo(dT_{12-18}) + $MgCl_2$	0.3
Poly(rA)-oligo(dT_{12-18}) + $MnCl_2$	0.2
Minus template + $MgCl_2$	0.03
Minus template + $MnCl_2$	0.01
Poly(dA)-oligo(dT_{12-18}) + 15mM N-ethylmaleimide	9
pHD32 + 100μg/ml aphidicolin in DMSO	89
pHD32 + DMSO only	87
pHD32 + ara CTP:dCTP(40:1)	95
pHD32 + ddNTP:dNTP(20:1)	22

from soybean and Chenopodium; this may be due to differences in the template/primer preparations.

The DNA polymerase from maize mitochondria was completely resistant to aphidicolin, an inhibitor of nuclear DNA polymerases [α,δ;8]; aphidicolin was not inhibitory even at 100 μg/ml (Table I) whereas nuclear DNA polymerase α is inhibited at 10 μg/ml. Similarly, ara CTP was not inhibitory even at a ratio of 40:1 (ara CTP:dCTP, Table I). Though no significant inhibition was observed at an equimolar ratio of ddNTP:dNTP, at higher ratios of ddCTP:dCTP (5:1 to 20:1) detectable inhibition of maize mtDNA polymerase activity was observed (Table I). N-ethylmaleimide (NEM) inhibited mtDNA polymerase activity indicating its need for reduced sulfhydryl groups for activity (Table I). However, the inhibition observed with NEM did not follow a linear decrease probably because of some residual 2-mercaptoethanol from the enzyme fraction in the reaction mixture resulting in the lack of inhibition at lower concentrations of NEM. Thus, maize mtDNA polymerase appears to be a γ-like enzyme but is distinct from animal mtDNA polymerase in that it does not prefer poly (rA)-oligo (dT_{12-18}) as a template.

Topoisomerase I: Typical assays of topoisomerase I activity are shown in Fig 1. The maize mitochondrial topoisomerase I activity was sensitive to high salt concentrations but required $MgCl_2$. Maximal topoisomerase I activity was observed in the absence of KCl and in the presence of 10 mM $MgCl_2$ (Fig 1A, lane 3). Absence of $MgCl_2$ significantly reduced topoisomerase I activity (Fig 1, lanes 2,4). KCl (150 mM) significantly inhibited topoisomerase I activity in the presence (Fig 1A, lane 1) or absence (Fig 1A, lane 2) of $MgCl_2$. It is known that the interaction between topoisomerase I and DNA template is essentially electrostatic and therefore the salt concentration is a major determining factor of topoisomerase I activity [9].

A characteristic feature that distinguishes eukaryotic nuclear and mt topoisomerase I from the prokaryotic type I is the ability of eukaryotic topoisomerase I to remove positive superhelical turns. Relaxation of positively supercoiled DNA by maize mt topoisomerase I present in the in vitro DNA replication system is shown in Fig. 1B; since ethidium bromide was not extracted relaxed templates still appear as slower moving species after gel electrophoresis. All of these features are similar to the mt topoisomerase I isolated from wheat embryos [10].

DNA primase: In general, most DNA polymerases are incapable of initiating DNA replication in the absence of an oligonucleotide primer which provides a free 3'-OH group that serves as a starting point for DNA replication (except in cases where termini binding proteins may provide the 3'-OH group). DNA primase and RNA polymerase are both capable of synthesizing primers for DNA replication. Maize mtDNA primase activity was assayed in the Heparin-Sepharose enzyme fraction using single-stranded M13mp19 DNA as the template. About 50% of optimal DNA primase activity was observed in the absence of exogenously added rNTPs (Table II); this may be due to the presence of DNA primers or rNTPS in the enzyme preparation. The

TABLE II. Maize mitochondrial RNA polymerase and DNA primase. Reactions were carried out as described in Materials and Methods. In RNA polymerase assays the following final concentrations were used: KCl, 150 mM; ethidium bromide, 50 μg/ml. DNA primase activity is described as pmoles of [H^3]-TTP incorporated per hour at 37°C using M13mp19 as the template. In all assays 1 μg of the indicated template was used.

RNA polymerase assay conditions	[H^3]-UTP CPM	% ACTIVITY
-DNA	629	3%
Poly [(dAdT).(dAdT)]	22,272	100%
Poly [(dAdT).(dAdT)] + KCl	7,960	36%
Poly [(dAdT).(dAdT)] + Ethidium Bromide	1,310	6%
pUC19	2,085	
pHD32	6,284	

DNA primase assay conditions	DNA synthesis (p moles)	% ACTIVITY
-DNA	0.08	0.4%
+ M13mp19	11.72	51%
+M13mp19 and 100μM rNTPs	17.85	77%
+M13mp19 and 200μM rNTPs	23.08	100%
+M13mp19 and 500μM rNTPs	16.64	72%

concentration of exogenously added rNTPs was found to be crucial in stimulating or inhibiting DNA primase activity. Maximal DNA primase activity was observed at 200 μM rNTP; at 500 μM rNTP only 70% of the maximal activity was observed. In the absence of exogenously added template, less than 1% of the maximal DNA primase activity was observed. These observations suggest the presence of DNA primase activity in the maize mtDNA in vitro replication system.

RNA polymerase: The Heparin-Sepharose peak fraction was tested for RNA polymerase activity, using poly [(dAdT)).(dAdT)] as template (Table II). In the presence of 150 mM KCl, RNA polymerase activity was inhibited by about 60%, while ethidium bromide (at 50 μg/ml) almost completely abolished RNA polymerase activity. It should be pointed out here that in contrast to DNA polymerase where activity is enhanced by KCl, RNA polymerase activity is sensitive to higher salt concentrations. Less than 3% of the maximal activity was observed in the absence of any exogenously added template. The RNA polymerase was found to be about three-fold more active with a clone containing a putative maize mtDNA ori compared to pUC19 (Table II), suggesting that the presence of the maize mtDNA insert (containing several potential promoter sequences, data not shown) may be responsible for the 3-fold enhanced activity of the enzyme. DNA primase and RNA polymerase may both be involved in replication of maize

mtDNA, with DNA primase being responsible for priming the lagging strand which becomes transiently single stranded as the replication fork opens up.

Mitochondrial origin of the DNA replication system: In order to verify the mitochondrial origin of the enzymes present in the in vitro DNA replication system, the following investigations were made. Inhibitor studies have already shown that maize mt DNA polymerase activity was insensitive to aphidicolin (Table I) whereas most plant nuclear DNA polymerases are inhibited by aphidicolin [8]. In addition, glucose-6-phosphate dehydrogenase activity was not detected in the mitochondrial fraction. Glucose 6-P dehydrogenase is known to be present in both the chloroplast and the cytosol, but not in mitochondria [11]. The extent of possible plastid contamination was further checked by analysis of pigment absorption spectra in different fractions. No detectable carotenoid absorption (A_{450}) was observed in the mitochondrial fraction indicating the absence of any significant plastid contamination. Chloroplast topoisomerase I does not relax positively supercoiled DNA [12] whereas maize mt topoisomerase I is capable of relaxing positively supercoiled DNA (Fig. 1) confirming that the enzyme is not of chloroplast origin. Finally, ct or nuclear DNA was not present in the mt fraction as evidenced by Southern hybridization studies; when probed with rbcL restriction profiles were very different in ct and mt DNA fractions (Fig. 2); when probed with rbcS, no restriction fragment was detected (data not shown). Based on all these observations we conclude that the enzyme activities observed were devoid of any detectable extramitochondrial activity.

In conclusion, this study reports the presence of DNA polymerase, RNA polymerase, DNA primase and topoisomerase I activities in the in vitro maize mtDNA replication system. This in vitro replication system is as efficient as the in vivo replication system and has been used

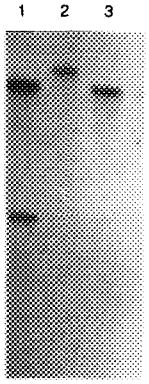

Figure 2. Southern blot of maize ct and mt DNA probed with rbcL. Isolation of ct and mt DNA was done according to Daniell et al. (ref 7). Probe was labeled with [^{32}P]dCTP by using random primer labeling procedure. Lane 1: ctDNA digested with BamHI; Lane 2: ctDNA digested with XhoI and Lane 3: mtDNA digested with BamHI.

to isolate a putative replication origin from the maize cmsT mt genome (Zheng and Daniell, 1995, in review). Results obtained from these studies should serve as a basis for further characterization of DNA replication in the maize mitochondrial genome.

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