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Chloroplast DNA replication *in vitro*: site-specific initiation from preferred templates

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ABSTRACT

An enzyme system prepared from maize chloroplasts catalyzes the synthesis of DNA from maize chloroplast DNA sequences cloned in bacterial plasmids. Cloned maize chloroplast DNA fragments Bam HI 17' (2470 bp) and Eco RI x (1368 bp) have been shown to be preferred templates for in vitro DNA synthesis catalyzed by pea chloroplast DNA polymerase preparations [Gold et al. (1987) Proc. Natl. Acad. Sci. USA 84, 194-198]. Analysis of replicative intermediates indicates that although the template activity of the recombinant plasmid pZmcBam 17' is substantially greater than that of the pZmcEco x, replication in both cases originates from within a 455 bp region which overlaps the two plasmids. The remaining approximately 1500 basepair portion of maize chloroplast BamHI fragment 17' is not more active because it contains additional origins for replication. The overlapping region shows sequence homology with a portion of the Chlamydomonas reinhardtii chloroplast chromosome that contains a replication origin. Replication is shown to proceed bidirectionally within the 455 bp origin region. Recombinant plasmid pZmc 427, which is also active in the in vitro DNA synthesis assay, promoted localized replication initiation within a 1 kbp BglII-Eco RI fragment of the chloroplast DNA insert, a region that includes the 3' terminal part of the psbA gene.

INTRODUCTION

Replicative intermediates of cpDNA have been studied by electron microscopy. Replication of higher plant cpDNA initiates by the formation of two displacement-loops (D-loops) located approximately 7 kbp apart on opposite strands of the chromosome (1-3). The D-loops elongate toward each other and fuse to form a Cairns replicative intermediate. Synthesis continues bidirectionally until the round of replication is completed. Two D-loops (6 kbp apart) have also been mapped on the Chlamydomonas reinhardtii chloroplast genome and the DNA of one of them was cloned and sequenced (4-6). On the other hand, a single Cairns intermediate has been identified in Euglena gracilis (7-10). Kolodner and Tewari (1) also observed replicative intermediates of the rolling circle type in higher plants. The origin of replication for the rolling circle replication round was found to be at or near the termination site of the Cairns round.

Analyses of albino deletion mutants of wheat and barley have also shed some light on the locations of possible cpDNA origins of replication. Up to 80% of the chloroplast genome can apparently be deleted without impairing the replication of the mutant chromosome (11,12). All of the molecules that replicate in vivo in spite of deletions contain a single common region of the genome which presumably carries at least one functional origin of replication. This region is located in the large single copy portion of the circular chromosome and contains the psbA, C and D genes in wheat.

Finally, DNA synthesis has been studied in vitro using cloned cpDNA templates and crude extracts of maize (13) and liverwort chloroplasts (14). No specific origins of replication were found in the first study (13) while in the latter several specific fragments were shown to be preferred templates (14). However, the use of crude extracts in those studies made it difficult to ascertain whether the DNA synthesis observed represented true replication or repair activity. A better defined in vitro system was established after the first chloroplast DNA polymerase was purified by McKown and Tewari (15). Recently, we have described the analysis of cloned fragments representing 94% of the maize chloroplast chromosome for their DNA synthesis template activity using partially purified pea chloroplast DNA polymerase (16). We identified two regions of the chromosome (about 180° apart) that were especially active as templates. One of these regions, cloned as the BamHI fragment 17' was very active. A subfragment, EcoRI fragment x, that includes about 450 bp of DNA also found in BamHI fragment 17', was about half to one-third as active. The latter was sequenced and found to share extensive homology with a portion of the C. reinhardtii chloroplast chromosome that contains a D-loop sequence (5,6). The homologous regions in both species code for the chloroplast ribosomal protein L16 (16). From the previous work with the cloned maize cpDNA templates BamHI fragment 17' and EcoRI fragment x, we did not know whether the former was a better template because it contained more replication initiation sites than did the subfragment x or for some other reason.

In the present paper we describe a homologous in vitro system that employs maize chloroplast DNA polymerase to catalyze replication of maize cpDNA templates. We have characterized the system with respect to its template and enzymatic requirements and we have shown that replication is initiated within the same 455 bp maize cpDNA sequence regardless of whether EcoRI fragment x or the large BamHI fragment 17' is used as a template. In

vitro DNA synthesis proceeds bidirectionally within the 455 bp fragment. We have also shown that replication from the 2 kbp BamHI-EcoRI fragment of maize cpDNA initiates from within a 1 kbp portion of it.

MATERIALS AND METHODS

1. Preparation of DNA

Recombinant plasmids pZmcEco x, pZmcBam 17' and pZmc 427 have been described previously (16). pZmc 427 consists of a 2 kbp fragment generated by EcoRI and BamHI digestion of maize cpDNA cloned in pBR322; the cloned cpDNA sequence contains the overlapping sequences between EcoRI fragment o' and BamHI fragment 8 (at about the 10 o'clock position on the map given in Ref. 17). Plasmid DNA was isolated as described (18). More than 80% of the purified plasmid was negatively supercoiled, as judged by agarose gel electrophoresis, the rest being in the open circular form. There was no detectable linear DNA. Topoisomers of pZmcBam 17' and pZmcEco x were generated according to Stirdivant et al. (19). The M13 clones employed consisted of fragments Eco x and Bam 17' cloned into the corresponding restriction sites of M13mp18. Single stranded phage DNA was isolated as described by Schreier and Cortese (20) and the orientation of the inserts determined by the "complementarity" test (21).

2. Preparation of Maize Chloroplast DNA Polymerase Extracts

Maize chloroplast extracts containing DNA polymerase activity were prepared essentially as described by Gold et al. (16) from pea except that the phosphocellulose column fractionation was omitted. Instead the polymerase-containing fractions eluted from the heparin-Sepharose column were pooled, dialyzed and finally concentrated by either DNA agarose chromatography or Centricon 10 (Amicon, Danvers, MA) centrifugation. The polypeptide compositions of the fractions were analyzed by polyacrylamide gel electrophoresis. Slab gels were prepared according to Laemmli (22). Separating gels used for activity staining (23) also contained 150 ug/ml of activated DNA. Prior to electrophoresis, the protein fractions (100 ul) were dialyzed for 2 h at 4° C against 1 l of 10 mM Tris-HCl (pH 8), 25% (v/v) glycerol, 0.1 mM EDTA, 10 mM 2-mercaptoethanol and 2 mM phenylmethylsulfonylfluoride, in order to remove the ammonium sulfate of the storage buffer. Samples were prepared by mixing the dialyzed protein solution (20 ul) with 5 ul of 5% sodium dodecyl sulfate (SDS) containing the dyes and, in the case of the activity gels, also with 5 ul of heat-inactivated fetal calf serum (23). Each mixture was then incubated at 37°C

for 3 min, brought to room temperature and loaded. The electrophoresis buffer contained 25 mM Tris-glycine (pH 8.3), 0.5 mM EDTA and 0.1% SDS. Electrophoresis was performed at 4°C and 80 V for 16 h. Silver and activity staining of the gels followed the methods of Oakley et al. (24) and Karawya et al. (23), respectively.

3. Assay for DNA Replication

Template DNA (1-2 ug) was incubated for 20-30 min at 30°C in 50 ul of 50 mM Tris-HCl (pH 7.0); 12 mM $MgCl_2$; 120 mM KCl; 0.1 mg/ml bovine serum albumin (BSA); 1 mM 2-mercaptoethanol; 10 mM ammonium sulfate; 0.1 mM EDTA; 5% (v/v) glycerol; 20 uM of each dGTP, dCTP and dTTP; 5-10 uCi [^{32}P]dATP (5-10 Ci/mmol; 1 Ci = 37 GBq) and 1-2 x 10^{-2} units of DNA polymerase (unit as in Ref. 15). When present, rNTPs and ATP were 0.1 mM and 2 mM, respectively. The acid insoluble radioactivity was assayed as described (15).

Restriction analysis of the replication products was performed as follows: After the reaction was completed, 5 ul aliquots were withdrawn to measure acid precipitable radioactivity and the remaining reaction mixtures were diluted 1:4 with distilled water and incubated for 2 h at 50°C with 0.5 mg/ml proteinase K, followed by phenol extraction and ethanol precipitation. The samples were redissolved in water, digested with appropriate restriction endonucleases, treated again with proteinase K and phenol, and finally analyzed by polyacrylamide gel electrophoresis and autoradiography.

4. DNA Blot Hybridization

DNA blot analysis and hybridization with [^{32}P] labeled DNA probes were performed as described previously (15). Radioactive DNA newly synthesized from pZmcEco x as template was prepared by a scale-up (10x) of the replication assay described above. After the reaction was completed the samples were digested with proteinase K and extracted with phenol. Nucleic acids were precipitated with ethanol. The radioactive DNA samples were redissolved in a minimum amount of water and boiled for 3 min prior to hybridization. DNA synthesized under this protocol showed acid insoluble radioactivity corresponding to 5-8 x 10^5 cpm.

5. Materials

Activated calf thymus DNA was prepared according to Tewari (25). Centricon 10 microconcentrators were purchased from Amicon (7). Heparin-Sepharose CL6B and DNA agarose were from Pharmacia (Uppsala, Sweden). DEAE cellulose (DE 52) was from Whatman (Maidstone Kent, UK) and alpha-[^{32}P]dATP

from New England Nuclear (Boston, MA). All other reagents were of analytical grade.

RESULTS

1. Characterization of the *in vitro* DNA replication reaction.

Purified chloroplast DNA polymerase is incapable of catalyzing the replication of native supercoiled plasmid DNA (16). However, a semipurified pea extract (containing other activities presumably required for the initiation of DNA synthesis) was successfully employed to catalyze replication of recombinant plasmids containing maize cpDNA, with some inserts acting as preferred templates (16). We employed a similar purification strategy to obtain a maize chloroplast extract enriched in DNA polymerase activity in order to establish an homologous system for replication *in vitro*. The enzyme was prepared by disrupting isolated chloroplasts by Triton X-100, chromatographing in DEAE cellulose and heparin-Sepharose, and concentrating the polymerase-enriched samples by DNA agarose chromatography or Centricon centrifugation. The polypeptide composition, as revealed by SDS gel electrophoresis, after every purification step is shown in Fig. 1A. More than a dozen silver stained bands were found in the pooled DNA polymerase fractions after heparin-Sepharose purification and concentration (lane 3, left). Activity staining of the polyacrylamide gels (23) helped to establish that the relative molecular mass of the DNA polymerase that can use activated calf thymus DNA as a template is 90 ($\pm$5) x 10^3 (Fig. 1B), in fairly good agreement with the results reported by McKown and Tewari (15) for the pea enzyme. Furthermore, only one polypeptide with DNA polymerase activity could be resolved throughout the whole purification procedure (right-hand portion of lanes 1-3 in Fig. 1A). Under the same conditions, yields of the maize enzyme were usually 10-fold less than those obtained from pea.

These maize chloroplast DNA polymerase preparations were used to catalyze the replication of the recombinant plasmids pZmcEco x, pZmcBam 17' and pZmc 427 *in vitro* (illustrated in Fig. 2). The requirements for this reaction are shown in Table 1. A standard replication assay is described under Materials and Methods. BSA was found to stimulate the reaction by preventing thermal denaturation of the DNA polymerase which results in loss of linearity due to irreversible inactivation of more than 50% of the enzyme activity during the 30 min assay (Table 1). Accordingly, the polymerase was slightly more active at 30°C than at 37°C. It was rapidly

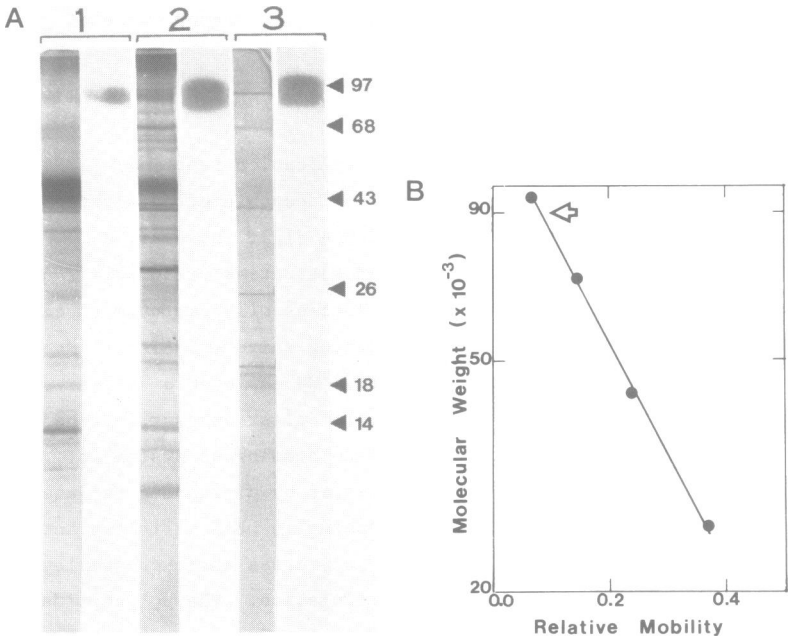

Figure 1. Polypeptide composition of chloroplast DNA polymerase fractions at different stages of purification as analyzed by SDS polyacrylamide gel electrophoresis. Experimental conditions are described in Materials and Methods. For details of the purification procedure see Gold et al. (16). (A) Lane 1, Triton X-100 extract; Lane 2, DEAE cellulose eluate; Lane 3, heparin-Sepharose eluate after concentration on DNA agarose. In each lane, the left-hand member of each pair shows the polypeptide pattern in the gel as visualized by silver staining. The right-hand member of the pair is an autoradiogram showing the location of DNA synthetic activity of the same fractions. (B) Determination of the approximate molecular weight of maize chloroplast DNA polymerase in the activity gels. The electrophororetic mobility of the polymerase was taken at the middle of the activity band. Molecular weight standards (BRL, Maryland) were: phosphorylase b, 97,400; bovine serum albumin, 68,000; ovalbumin, 43,000; alpha-chymotrypsinogen, 25,700; beta-lactoglobulin, 18,400; lysozyme, 14,300.

and irreversibly inactivated at 45°C even in the presence of BSA (data not shown). Other requirements are similar to those described for the pea enzyme (15).

The specificity of the reaction was drastically reduced when a mixed population of linear and relaxed circular, some probably nicked, plasmid DNA (obtained as the uppermost band after agarose gel electrophoresis and electro-elution) was used as template (data not shown), suggesting that the nicks might act as sites of unspecific starts for repair activity or that the normal site(s) for replication initiation became unrecognizable by the

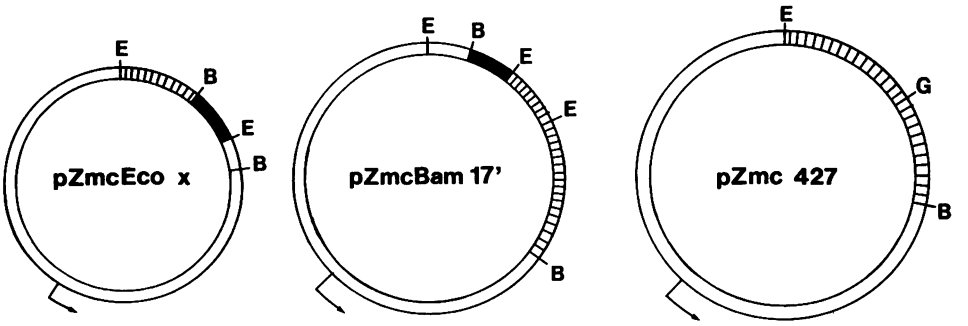

Figure 2. Plasmid templates used for in vitro replication by maize chloroplast DNA polymerase. Open and hatched bars indicate pBR322 and chloroplast sequences, respectively. The solid bars of pZmcEco x and pZmcBam 17' represent the 450 bp BamHI-EcoRI fragment that is shared by both plasmids. The arrows in each plasmid indicate the origin and direction of replication of pBR322. Relevant restriction sites are indicated: B, BamHI; E, EcoRI; G, BglII.

enzyme. On the other hand, relaxation of the plasmids with topoisomerase I, as described by Stirdivant et al. (19), had no effect on either the extent or the specificity of the DNA replication (Table 1).

Since RNA priming is a general feature of initiation of DNA synthesis in both eucaryotes and procaryotes, it was surprising to observe that the addition of the four ribonucleoside triphosphates had only a minor stimulatory effect on the replication reaction (Table 1). However, this lack of a strict requirement for rNTPs has been observed recurrently with other in vitro cpDNA synthesis systems derived from maize (13), liverwort (14) and C. reinhardtii (6).

2. Analysis of replication products

As reported earlier (16), DNA synthesized from templates pZmcEco x, pZmcBam 17' and pZmc427 appeared to be the size of the entire plasmid when analyzed by alkaline agarose gel electrophoresis (data not shown). We tried to establish whether replication of each of these plasmids in vitro starts at a single or at numerous sites and to further map the origin(s) by determining the pattern of labeling of parts of template sequence (i.e. various restriction fragments of the template) during in vitro synthesis. Specifically, we (16) had found pZmcBam 17' to be a much more active template than pZmcEco x; the maize cpDNA sequences cloned in these two plasmids have 455 bp in common. We were especially interested to determine whether the fragment BamHI 17' contains more sites for the initiation of

DNA replication than does EcoRI fragment x as a first step in understanding the difference in their behavior as templates. For these experiments, it was necessary to enrich the reaction mixtures for partially replicated intermediates. This was accomplished by impairing elongation of the newly synthesized DNA chains by the addition to the reaction mixtures of various amounts of the chain terminators 2', 3'-dideoxycytidine 5'-triphosphate (ddCTP) and cytosine-beta-D-arabinofuranoside-5'-triphosphate (ara-CTP) so that elongation following each replication initiation was limited to different average extents.

Products of in vitro DNA synthesis were digested with the appropriate restriction enzymes and subjected to electrophoresis on polyacrylamide gels. Staining the gels with ethidium bromide confirmed the presence of equimolar amounts of restriction fragments in each band, regardless of the length of the replication product (data not shown). However, autoradiographs indicated that the newly synthesized DNA did not appear in equimolar amounts in each restriction fragment (Figs. 3,4). Moreover, as the average length of the replication product was decreased by including

Table 1. Requirements for in vitro DNA synthesis using supercoiled plasmids as templates.

Components	DNA synthesis activity (%)
Complete, pZmcEco x	100
- DNA	>5
- dNTPs	>5
- rNTPs	81
- ATP	92
- bovine serum albumin	44
Complete, relaxed pZmcEco x	116
Complete, pBR322	12
Complete, pUC19	7
Complete, pZmcBam 17'	244
Complete, relaxed pZmcBam 17'	226
Complete, pZmc 427	31

DNA replication was measured as described in Materials and Methods. The concentration of each component is also given there. Plasmid DNA concentration was adjusted in all cases to 20 ug/ml. The structures of the recombinant plasmids are shown in Fig. 2. The 100 % value equals 12.2 pmol of dNMP incorporated.

higher concentrations of the chain terminators in the reaction mixture, labeling was progressively confined to unique, specific bands.

Figure 3A shows the pattern of products when pZmcEco x served as the template. Even in the absence of ddCTP (first lane of Fig. 3A), the most heavily labeled band was the 455 bp fragment generated by EcoRI and Bam HI

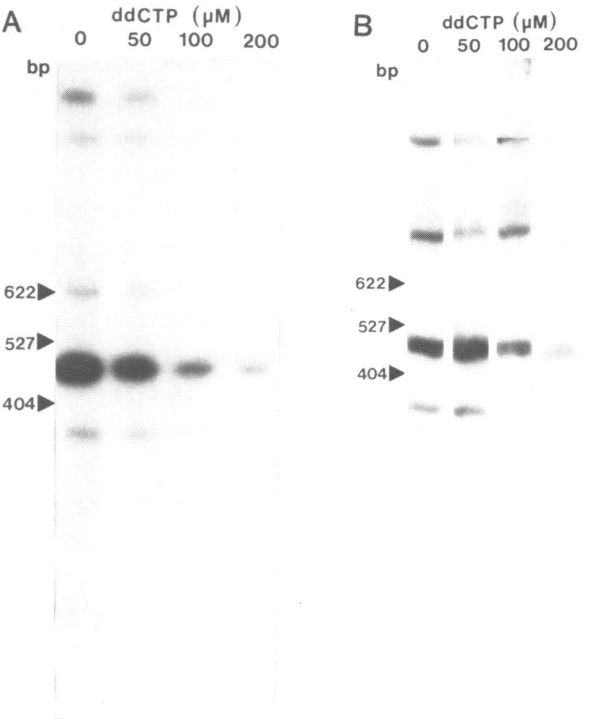

Figure 3. DNA synthesis by DNA polymerase extracts was carried out in the presence of various amounts of ddCTP and 20 uM dCTP. Acid insoluble radioactivity was determined in 5 ul aliquots. Autoradiograms show the distribution of incorporated radioactivity among various restriction fragments after in vitro DNA synthesis. After the reaction was completed the samples were treated as described in Materials and Methods, digested with BamHI and EcoRI, and finally subjected to electrophoresis on a 5% polyacrylamide gel (acrylamide to bisacrylamide ratio 30:1) in 90 mM Tris-borate (pH 8.4), 1 mM EDTA at 25 C and 40 mA for 2-3 h. Gels were dried and autoradiographed. Numbers over each lane indicate the concentration of ddCTP added to the reaction mixture. The molecular size standard used was pBR322 digested with HpaII. In (A) the DNA replication activities were 9.8 (100%); 8.2 (84%); 6.6 (67%) and 4.5 (46%) pmol of dNMP incorporated in the presence of 0, 50, 100 and 200 M ddCTP, respectively. In (B) the activities were 27.4 (100%); 20.3 (74%); 15.9 (58%) and 11.8 (43%) pmol of dNMP incorporated with 0, 50, 100 and 200 M of ddCTP, respectively.

digestion (see restriction map in Fig. 2). At 48% of control replication this fragment is the only one which shows significant labeling (fourth lane in Fig. 3A).

The effect is even more obvious when pZmcBam 17' is used as a template instead of pZmcEco x. pZmcBam 17' contains the 455 bp BamHI-EcoRI portion of Eco x plus the adjacent 1.5 kbp sequence that includes the 5' end of the gene for the maize chloroplast ribosomal protein L16 (Fig. 2 and Ref. 16) and it is 2-3 times more active as a template than pZmcEco x (Table 1). Fig. 3A shows that in spite of the additional DNA sequence present in pZmcBam 17' vs pZmcEco x, the 455 bp BamHI-EcoRI fragment is still preferentially labeled when compared to the other restriction fragments, suggesting that the increase in activity is not due to the use of additional replication origins in the cpDNA insert of pZmcBam 17'.

To study replication of pZmc 427, we used ara-CTP instead of ddCTP as chain terminator, in order to obtain a higher degree of inactivation, since ddCTP inhibition levels off at 60-70% even at ddCTP/dCTP ratios of 100 (not shown). The amount of labeling in the absence of ara-CTP increased with the size of the restriction fragment (Fig. 4, lane 2), as should be expected if the whole plasmid were uniformly labeled. However, addition of the chain terminator to the reaction mixture drastically increased the relative specific activity of the 1 kbp BglII-EcoRI fragment (Fig. 4). This portion of the insert includes the 3' end of the psbA gene (I. Larrinua, personal communication).

Most of the chloroplast genome was inactive in the in vitro assay (16). When, as a control, one of these inactive fragments, subclone 1a from BamHI fragment 6 (17), was provided as a template (20 ug DNA instead of 1-2 ug in the reaction mixture) in an ara-CTP inhibited reaction and the products were subjected to restriction analysis, the restriction pattern did not show any preferentially labeled fragment and radioactivity from both plasmid and cpDNA derived restriction fragments declined at about the same rate with increasing concentrations of ara-CTP (data not shown). This result suggests that replication of this plasmid did not start at specific sites.

It is also noteworthy that the fragment containing the pBR322 origin of replication (uppermost band in the gels of Figs. 3,4, see also map in Fig. 2) was not highly labeled (except in the case of pZmc 427, Fig. 4) and that labeling of this fragment declined faster than the preferentially labeled fragments as the concentration of chain terminators was increased

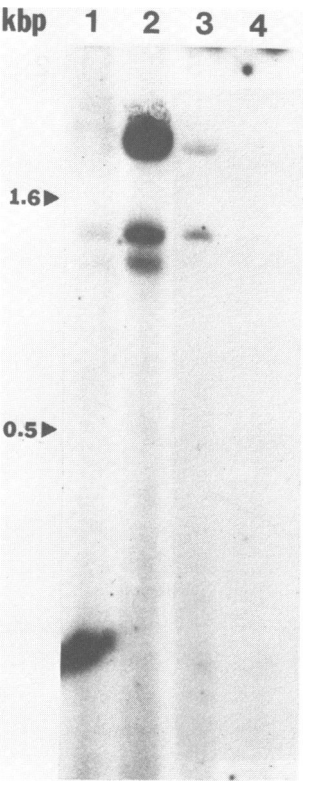

Figure 4. Inhibition of <u>in vitro</u> replication of pZmc 427 by ara-CTP. Conditions for DNA synthesis, assay of the activity and processing of the samples were as in Fig. 3. The products of <u>in vitro</u> replication were digested with BamHI, EcoRI and BglII and analyzed by polyacrylamide gel electrophoresis as described before. Lane 1, the reaction was carried out for 3 min in the absence of ara-CTP; lane 2, 20 min of reaction, no ara-CTP; lane 3, 20 min reaction, 4 mM ara-CTP; lane 4, 20 min reaction, 10 mM ara-CTP. Acid insoluble radioactivity was determined in 5 ul aliquots: 0 ara-CTP, 4.1 pmol dNMP incorporated = 100%; 4 mM, 46% inhibition; 10 mM, 89% inhibition. Molecular size standard set was pBR322 digested with HinfI.

(Figs. 3,4), indicating that chloroplast DNA polymerase, although probably replicating the entire plasmid template (full length products), was not able to use the plasmid origin of replication to initiate DNA synthesis <u>in vitro</u> efficiently, if at all. This result is in good agreement with the lack of template activity of pBR322 and pUC19 in the <u>in vitro</u> assay (Table 1).

The preceding results strongly suggest that <u>in vitro</u> DNA synthesis

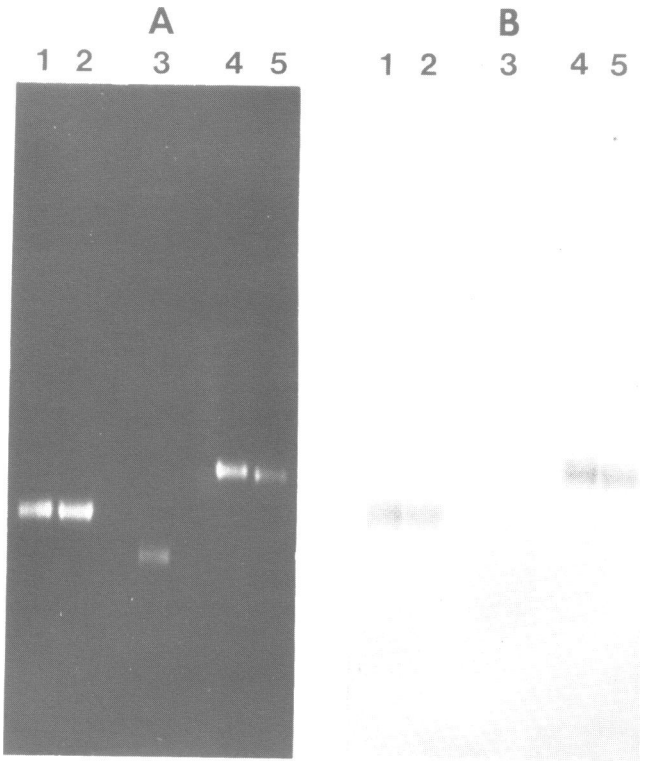

Figure 5. Hybrydization of in vitro synthesized DNA with single stranded cpDNA fragments of Eco x and Bam 17' cloned in M13mp18. Recombinant M13 clones were obtained and purified as described in the text. The single stranded samples were subjected to electrophoresis on a 1.2% agarose gel in 40 mM Tris-acetate (pH 8.3), 1 mM EDTA, at 40 V and 25 C for 14 h. DNA was visualized by UV illumination of the ethidium bromide-stained gel (A). The gel was blotted onto nitrocellulose filters (16), hybridized to newly synthesized DNA (see Materials and Methods for experimental details) and the filter was autoradiographed with intensifying screen (B). Lanes 1 and 2, M13mp18Eco x in the (+) and (-) orientations, respectively; lane 3, M13mp18 (no insert); lanes 4 and 5, M13mp18Bam 17' in the (+) and (-) orientations, respectively. The (+) and (-) orientations are arbitrary, but any given orientation represents the same strand in both the Eco x and Bam 17' fragments, determined by the complementarity test (21).

using these preferred templates starts at a unique site within the cpDNA inserts although the possible existence of several replication origins within the 455 bp BamHI-EcoRI fragment can not be ruled out by these experiments.

3. Direction of replication of pZmcEco x

Analysis of the labeling patterns of Figs. 3,4 shows that the specific

radioactivity associated with the restriction fragments decreases with higher concentrations of chain terminator at approximately the same rate at both sides of the preferentially labeled region, suggesting that DNA synthesis proceeds sequentially and bidirectionally from the replication origin.

In order to confirm this suggestion, we studied the direction of *in vitro* DNA replication using pZmcEco x as template. For this purpose, we cloned the Eco x and Bam 17' fragments of maize cpDNA in M13mp18 and selected clones in the two possible orientations by using the "complementarity" test (21). Samples of M13mp18 carrying opposite strands of Eco x and Bam 17' were subjected to agarose gel electrophoresis (Fig. 5A) and the gel was blotted onto nitrocellulose paper. DNA replicated *in vitro* from pZmcEco x was used as a hybridization probe for the blotted single stranded fragments.

Fig. 5B shows that the newly synthesized DNA hybridized to both strands of Eco x and Bam 17' cloned into M13 (lanes 1, 2, 4 and 5), indicating that in this case replication was bidirectional. Signals were very weak and the filters had to be exposed for long periods, presumably due to the presence in the hybridization mixture of unlabeled template DNA. The probe did not hybridize with phage DNA that carries no insert (Fig. 5B, lane 3), indicating that hybridization was specific for chloroplast-derived sequences.

4. Bending analysis of Eco x

Bent DNA has been found at viral and plasmid DNA replication origins (26-29) and in a yeast autonomous replicating sequence (30). Analysis of EcoRI fragment x by polyacrylamide gel electrophoresis of permuted restriction fragments of Eco x (34) showed the existence of a bend site at or beyond the 3' end of the L16 gene coding region (Fig. 6). These results are consistent with the conclusion that the 455 bp BamHI-EcoRI fragment may contain a functional origin of replication, probably located at the 3' end of the L16 gene or in the intergenic region.

DISCUSSION

In most of the systems described so far to study chloroplast DNA replication *in vitro* (13,14), it has been unclear whether the reactions observed reflected a replicative type of DNA synthesis or a repair synthesis in which large stretches of DNA are replaced. Our system does, however, satisfy several criteria for *in vivo* DNA replication, namely:

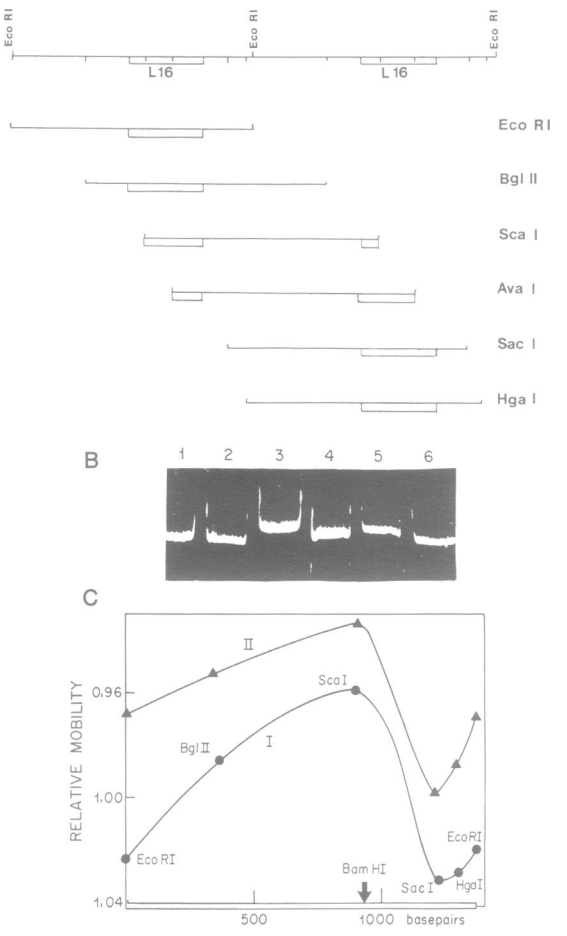

Figure 6. (A) DNA fragment analyzed for bending. A tandem duplication of EcoRI fragment x was constructed by partial digestion of pZmcEco x with EcoRI and insertion of an additional Eco x fragment. Transformants were isolated and analyzed for the proper orientation by restriction mapping. The DNA was then digested with restriction enzymes that cut only once within Eco x to generate a series of fragments, each 1368 bp in length. The samples were used for migrational analysis either directly or after purification from polyacrylamide gels. The upper line shows the duplicated DNA; the blocks indicate DNA sequences coding for ribosomal protein L16 (16). The fragments generated are shown below the restriction map and are designated by the enzyme used to produce them. (B) Analysis of permuted fragments by polyacrylamide gel electrophoresis.The fragments, generated as described above, were separated electrophoretically essentially as described by Koepsel and Kahn (27) except that gels were run for 10 rather than for 4 hours. Lanes 1-3, 5 and 6 contain fragments generated by HgaI, SacI, ScaI, BglII and EcoRI, respectively. Lane 4 contains a 1371 bp marker fragment generated by digestion of lambda DNA with BstEII. (C) Relative

migration of the permuted fragments. The electrophoretic mobilities of the fragments analyzed above are compared with the migration of the 1371 bp marker fragment that was used as a standard. The results are plotted against the position of the restriction enzyme cleavage sites (as measured from the right-hand end of the fragment as shown in A above). Curve I, DNA without DNA polymerase preparation. Curve II, DNA was incubated for 5 min. at 30°C with DNA polymerase preparation (5-10 X 10^{-2} units of polymerase activity) in the same medium as used for the activity assay except that dNTPs were omitted. In both cases the lowest point of the plot identifies the bend site (29).

requirements for dNTPs and for cpDNA inserts in the template plasmids (Table 1) together with the preferential incorporation of radioactivity into a single restriction fragment upon increasing the concentrations of chain terminators (Figs. 3,4). The latter result suggests that site-specific initiation of DNA synthesis is occurring.

RNA priming of DNA synthesis in eucaryotes and procaryotes is well documented (31). Addition of ribonucleoside triphosphates to our system only slightly enhanced DNA replication activity (Table 1), a result already observed with other systems from algal and plant chloroplasts (6, 13, 14). The lack of absolute dependence on added ribonucleotides for duplex templates does not rule out an essential role for priming in the initiation of DNA synthesis. Results in other *in vitro* replication systems show that lack of strict dependence ribonucleotides can reflect either high endogenous ribonucleotide concentrations or the fact that primases can use deoxyribonucleoside triphosphates under some conditions. It is noteworthy in this context that highly purified chloroplast DNA polymerase is unable to catalyze DNA replication of supercoiled plasmid templates (16), suggesting that site-specific initiation of DNA synthesis as observed here depends upon other proteins in the extract besides DNA polymerase. Proof of this suggestion requires further study.

Investigations of the effect of topoisomerases on chloroplast DNA synthesis have yielded conflicting data. Inhibitors of topoisomerase II impaired DNA synthesis by isolated chloroplasts from *E. gracilis* (32) and *C. reinhardtii* (33) but not from maize (13). In addition, Zimmermann and Weissbach (13) reported that DNA synthesis on plasmid templates catalyzed by a crude extract of maize chloroplasts resulted in the replication of only the open circular or linear forms of the plasmid DNA and concluded that they were studying a repair enzyme. We have also observed that this type of synthesis is of the repair type and does not represent a true replicase activity (16). Site-specific initiation of DNA synthesis occurs

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CCCCGAATAT TGGGTAGCCG TTGTTAAACC AGGTCGAATA CTTTATGAAA TGAGCGGAGT ATCCGAAACT  70

GTAGCTAGAG CAGCTATCTC TATAGCTGCC AGTAAAATGC CCATACGAAG TCAATTTCTT CGATTAGAGA 140
    3' rpl 16
TATAGAACCC CAAAAAGGAA TATTGAAGAT AAAAAACCCC TAGTTTTTCT TTCTGGAAAG ACAATATTTC 210
                                                5' rpl 14
TTTCATCCTT TTGCATTTGA AATAACAAAT GGAAACCAAA TAATATGATT CAACCTCAGA CCCTTTTAAA 280

TGTAGCAGAT AACAGTGGAG CTCGAAAATT GATGTGTATT CGAGTCATAG GAGCCGCTGG TAATCAGCGA 350

TATGCTCGTA TTGGTGATGT TATTATTGCT GTAATCAAAG ACGCAGTGCC CCAAATGCCT CTAGAAAGAT 420

CCGAAGTAAT TCGAGCTGTA ATTGTACGTA CACG 455
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Figure 7. The nucleotide sequence of the 455 bp BamHI-EcoRI fragment of maize chloroplast DNMA (16) within which replication has been shown to initiate in these experiments. The terminal of rpl 16 is indicated by (3'). The initial nucleotide of the coding sequence for rpl 14 is marked (5') as well. The underlined region is a sequence that can form a stable stem-loop structure (16).

only on covalently closed templates. In our hands, relaxation of the plasmids with topoisomerase I affected neither the activity nor the specificity of the replication process (Table 1).

Incorporation of radioactive dATP into DNA in the presence of chain terminators allowed us to narrow down the putative origin of replication. When the highly active templates pZmcEco x and pZmcBam 17' were used, labeling of the 455 bp BamHI-EcoRI fragment persisted up to the highest concentration of ddCTP used (Fig. 3), indicating that it is within this sequence that replication occurs first in both plasmids. This fragment is the only portion of cpDNA shared by the two templates (Fig. 2) and appears to carry putative origins of replication of the chloroplast genome. Initiation of DNA synthesis proceeds bidirectionally within this 455 bp sequence (Fig. 5). The fragment contains DNA coding for the C-terminal portion (48 triplets plus one nucleotide of another triplet) of the L16 ribosomal protein gene, a 109 bp intergenic region and 67 codons for the N-terminal part of the L14 ribosomal protein gene (Ref. 16, see also Fig. 7). The gene for L14 is absent from this region of C. reinhardtii chloroplast DNA but the 145 bp stretch belonging to the L16 gene is homologous to a part of the D-loop containing region of C. reinhardtii (6, 16) and the DNA sequence from the 3' end of rpl 16 to the start of the coding sequence of rpl 14 in maize is about 33% homologous to the corresponding region in C. reinhardtii. A bend site was localized beyond the 3' end of L16 coding region (Fig. 6). This is in accord with the probability that a true origin

of DNA synthesis is somewhere in this neighborhood, in view of the proposed role played by DNA bending in replication (26-29). In summary and taking into account the various considerations discussed here, this origin is likely to be upstream of rpl 14 and either in the intergenic region between rpl 16 and 14 or within the 3' region of rpl 16; from the BamHI site of the 455 bp fragment to the nucleotide pair before the start of the first codon of rpl 14 is 245 bp. Our data do not exclude the possibility that there are multiple initiation sites within the 455 bp fragment or that a site is within the rpl 14 sequence.

pZmcBam 17' was 2-3 times more active than pZmcEco x in the in vitro assay. However, Fig. 3 shows that both plasmids initiate replication in the same region of the cpDNA insert, namely the 455 bp BamHI-EcoRI fragment. Other sequence elements in pZmcBam17' apparently account, in an unknown manner, for the enhancement of the template activity of pZmcBam17' with respect to pZmcEco x, without altering the initiation site.

Replication of pZmc 427 initiated in the 1 kbp fragment generated by BglII and EcoRI digestion (Figs. 2, 4) which encodes the C-terminal and the 3' flanking region of the psbA gene.

The available data do not allow us to match cpDNA sequences that are active in the in vitro assay with any of the D-loops mapped in the maize chloroplast chromosome (1-3). We think that a more thorough understanding of the DNA synthesis process in vivo will come from the combination of different approaches, namely, the identification and mapping of D-loop regions in the chloroplast genome, and the use of in vitro systems of defined composition (with respect to both enzyme and template requirements) that faithfully mimic the in vivo replication process. The system described here satisfies several criteria of in vivo DNA replication and may help to define the minimal sequences required to initiate the de novo synthesis of the chloroplast chromosome.

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