

Characterisation and mode of *in vitro* replication of pea chloroplast *OriA* sequences

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A partially purified replicative system of pea chloroplast that replicates recombinant DNAs containing pea chloroplast origin sequences has been characterised. Polymerisation by this system is very fast and insensitive to chain terminators like dideoxynucleotides, arabinosylcytosine 5'-triphosphate, etc. Both strands of template DNA are synthesized and single-stranded DNA templates undergo more than one round of replication.

When sequences of either of the two chloroplast origins of replication (*OriA* or *OriB*) are used as templates, the replicative intermediates are found to have sigma structures. Electron microscopic analysis of the sigma structures restricted with various enzymes reveals that the initiation site of *in vitro* replication maps near the displacement-loop regions where replication initiates also *in vivo*. Although the observed replication initiation in the *OriA* recombinant template is chloroplast-DNA-specific, the mode of replication is different from that observed *in vivo* with intact ctDNA. However, when the template DNA contains both the *OriA* and *OriB* sequences, the *in vitro* replication proceeds in the theta mode, the mode of replication usually observed *in vivo*.

The complex process of DNA replication has been defined for only a few biological systems such as plasmids, bacteriophages, bacteria and viruses [1–6]. So far, little is known about the DNA replication processes in plant systems except for few reports in the field of plant organelles [7–11]. Chloroplast DNA (ctDNA) of higher plants seems to be an attractive system for investigation of the molecular details of replication. The chloroplast genomic size of about 150 kb is large but not too large for recombinant manipulations and the genome exists in multiple copies. Understanding the molecular biology of ctDNA replication would not only reveal the detailed characteristics of this system [12–14] but also help solve problems related to stable transformation of the chloroplast organelle. Thus, development of an *in vitro* replication system derived from higher plant chloroplasts is important both in helping to define DNA metabolism in general as well as advancing the technology of transgenic plant production [15–18].

Replicative intermediates of ctDNA from pea leaves have been well characterised using electron microscopic techniques [19]. In pea and maize ctDNA, it was shown that replication begins by forming two displacement loops (D-loops) which expand towards each other to form a theta structure. This small Cairns' structure then proceeds bi-directionally until termination takes place. Meeker et al. [20] have mapped the two replication origins or D-loops by electron

microscopic analyses of restriction digests of supercoiled DNA from pea chloroplasts. *OriA* (or sequences where the left D-loop is formed during replication *in vivo*) is mapped in the spacer region between 16S and 23S rRNA genes while *OriB* (or right D-loop) is located at the 3' end of 23S rRNA gene. Daniell et al. [21] have constructed chloroplast expression vectors containing *OriA* and delivered them biolistically into cultured *Nicotiana Tabacum* cells. These clones express the reporter gene product efficiently only in the chloroplast of bombarded cells and the vectors containing *OriA* sequences are more effective in expression than the vectors without *OriA* regions. They have hypothesised that *OriA*-containing clones probably have transiently replicated in the tobacco chloroplasts. So it needs to be tested directly whether vectors containing only *OriA* sequences are capable of replicating by themselves.

We have now characterised the *in vitro* replication system of pea ctDNA. This pea chloroplast replication system is quite capable of replicating constructs carrying the *OriA* sequence. The rate of replication is very fast and apparently not very sensitive to chain terminators like dideoxynucleotides (ddNTPs) and arabinosylcytosine 5'-triphosphate (aCTP). Many topoisomers are generated as end products of the reaction if ATP is added. During replication, both strands of template DNA are copied and the replication process continues beyond at least one round, if a single-stranded DNA (ssDNA) is used as the source of template. Although we do not observe formation of any D-loop during the initiation of *in vitro* replication of *OriA*-containing clones, the *in vitro* replication initiation sequences match reasonably well with those seen *in vivo*, as judged by electron microscopic analyses of the restricted replicative intermediates. The *in vivo* mode, i.e.

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Abbreviations. aCTP, arabinosylcytosine 5'-triphosphate; ctDNA, chloroplast DNA; ddNTP, dideoxynucleoside triphosphate; D-loop, displacement loop; *Ori*, origin; ssDNA, single-stranded DNA.

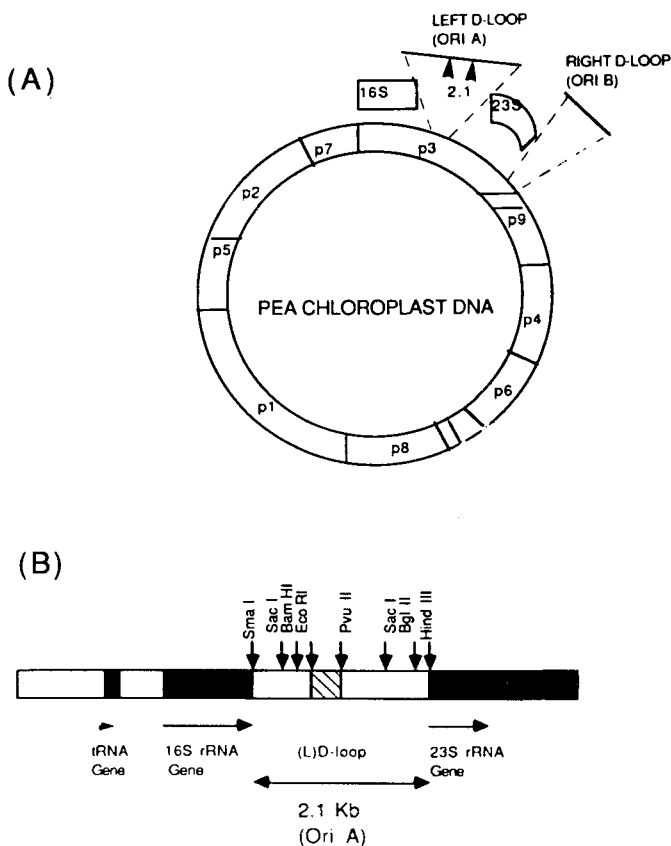


Fig. 1. Replication origin of pea chloroplast DNA. (A) Relative locations of *OriA* and *OriB* in the circular map of pea chloroplast DNA. *PstI* fragments are shown as *pn*. The fragment p3 is 12.5 kb long and contains both *Ori* regions. *OriA* is spaced between two rRNA genes 16S and 23S. (B) The restriction map of *OriA* region. The initiation of replication in the *OriA* region begins by forming a D-loop in the area marked as a hatched box. The figures are not drawn to scale. Several restriction sites have been marked by arrows.

Cairn's mode of replication, is restored by having both the *OriA* and *OriB* sequences in *cis* configuration in the template.

MATERIALS AND METHODS

Templates

Most of the replication reactions in this study were carried out using a template containing the 2.1-kb *OriA* sequence cloned into *SmaI* and *HindIII* sites of pUC19 vector (2.1/pUC19). The location of the 2.1-kb fragment in the restriction map of pea ctDNA with respect to the two *Ori* regions is shown in Fig. 1. A 12.5-kb *PstI* fragment of pea ctDNA containing both *OriA* and *OriB* regions was cloned in either pACYC177 or pUC19. The 2.1-kb ctDNA fragment was also cloned into M13 DNA as described in the text and legends to figures.

Replicative system

The soluble replicative system from pea chloroplasts was prepared by the protocol of Meeker et al. [20] with minor modifications. Intact pea chloroplasts were isolated and disrupted by Triton X-100 [17]. In brief, endogenous DNA was removed either by DEAE-cellulose chromatography or by

precipitation with poly(ethyleneimine). A 30–70% $(\text{NH}_4)_2\text{SO}_4$ fraction of the material devoid of DNA was made. Subsequently, DNA-binding proteins were enriched by heparin–Sepharose and/or phosphocellulose chromatography. The phosphocellulose or heparin–Sepharose fractions were further subjected to velocity sedimentation at 4°C and 195000 g in a 20–50% glycerol gradient in the presence of 100 mM NaCl for 40 h. The 0.5-ml fractions rich in DNA polymerase activity were pooled and could be used for replication reactions. Unless mentioned otherwise, replication reactions were carried out with the glycerol-gradient-purified enzymes.

Replication *in vitro*

Reactions were carried out essentially as described by Meeker et al. [20]. Replicative products were analysed using denaturing or non-denaturing agarose gel electrophoresis. When topoisomers were looked for, non-denaturing gels were used and run in the absence of ethidium bromide.

Slot-blot hybridisation

DNA samples were blotted onto a Gene-Screen Plus membrane in a PR 600 slot-blot apparatus manufactured by Hoefer Scientific Instruments. Hybridisation and washes were carried out following Du-Pont Company protocols.

Enzymic assays

Published protocols were used to assay the presence of the individual activities of the enzyme fraction, i.e. DNA polymerase [22, 23], polymerase-primase complex [24] and topoisomerase I [25]. The presence of helicase was detected by a standard strand-displacement assay [26].

Electron microscopy

Replicated products were purified by treatment with protein denaturants and subsequent gel-filtration chromatography [27]. DNA molecules were prepared for microscopy by following essentially the method of Davis et al. and others [28, 29]. DNA samples (concentrations $\approx 10 \mu\text{g/ml}$ in Tris/EDTA buffer) were mixed with cytochrome *c* (0.01%) and 50% formamide and then spread on water. This monolayer of DNA molecules containing cytochrome *c* was gently picked up onto carbon-coated grids (400 mesh), stained with uranyl acetate (stock: 5 mM in 50 mM HCl, freshly diluted 100-fold with 95% ethanol) and washed in 95% ethanol. The grids were rotary shadowed with Pt/Pd (80:20) and examined under a Philips EM 410 transmission electron microscope. Photographs were taken at various magnifications. Length was measured either by using a map measurer or by a marked tape after magnification by projection.

RESULTS

Replicative system consists of various activities

The glycerol-gradient fractions were analysed for competency of replication of supercoiled plasmid DNA using mainly 2.1/pUC19 DNA as a source of template. Replicated nascent strands of the size of template (4.8 kb) were generated only with fractions 16 and 17 (data not shown). Optimal replication activity was observed at 75 mM NaCl and 10 mM

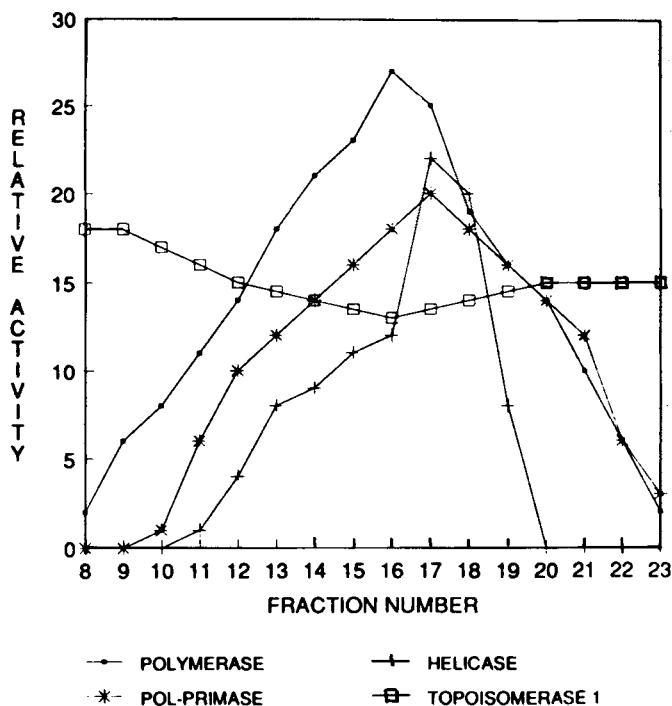


Fig. 2. Activity profile of the various enzymes. All fractions of the glycerol gradient were dialysed against buffer B (50 mM Tris, pH 7.5, 50 mM NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA) and tested for individual enzyme activities. The highest activity of each enzyme was assigned an arbitrary value.

MgCl₂. Addition of bacterial polymerase I to other fractions, for example 11, did not restore the replication competence. The replication-proficient fractions (i.e. 17) also contained the maximal activities of DNA polymerase, DNA primase, DNA helicase but not topoisomerases I as seen in Fig. 2. Topoisomerase I [30] of this system could be inhibited by 60 µg/ml camptothecin or berenil and the inhibited enzyme did not support replication. DNA polymerase responded very poorly to the chain terminators like ddNTPs and aCTP. Polymerisation activity was reduced only 50% when the ddNTP/dNTP ratio was maintained at the level of 100:1. The replication-competent fractions had at least 24 polypeptides, most of which were functionally unidentified.

Kinetics of DNA replication

The 2.1-kb fragment containing template DNA of various forms was incubated with the replication-competent extract as mentioned above. At various time intervals, aliquots of replicative products were withdrawn and analysed in alkaline agarose gels. Fig. 3 shows the autoradiograph of the synthesised DNA using various templates. Even at 15 s the full-length template-sized product, 9.1 kb long, was made, when the ssDNA (2.1/M13mp19) was used as the template as shown in Fig. 3 (i). Hence the polymerisation was very fast and must have occurred at a rate of at least 650 nucleotides/s. The recovered 9.1-kb product was found to hybridise with the ssDNA template. Below we describe biochemical and physical evidence showing that the ssDNA templates were converted to duplex DNA during the replication reaction. Thus the observed 9.1-kb product must have formed as a result of replication of ssDNA template and not due to some kind of artefactual end labelling of the starting ssDNA template.

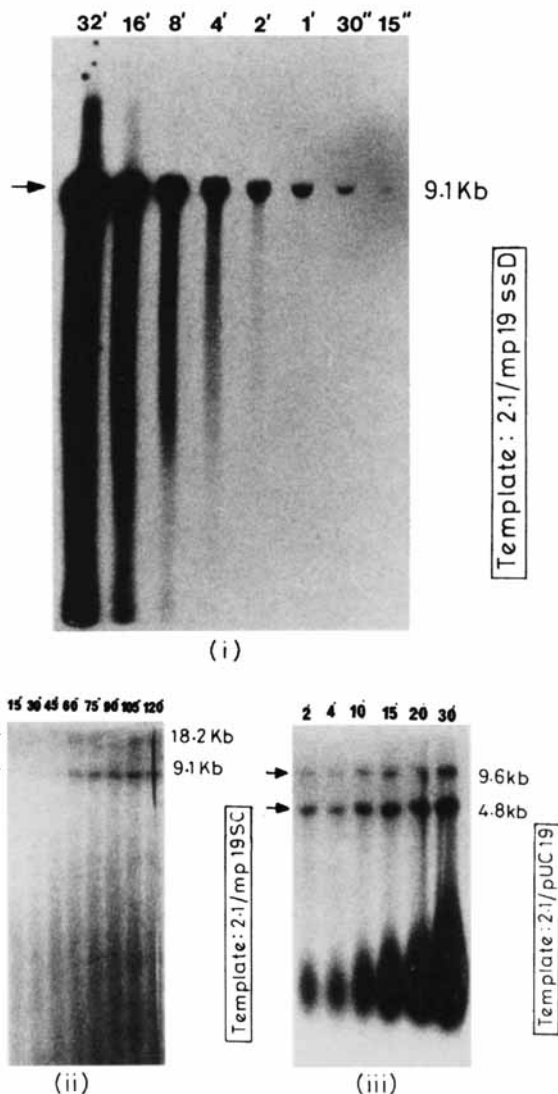


Fig. 3. Rate of replication. Denatured replicated products of various time periods were analysed in an alkaline-agarose gel and subsequently autoradiographed. 2.1/M13mp19 ssDNA, 2.1/M13mp19 supercoiled (SC) form I DNA, 2.1/pUC19 supercoiled DNA were used as template DNA as shown in (i), (ii) and (iii), respectively. The template-sized nascent DNA has been shown by arrows and the appropriate sizes are shown in each set. On the top of each lane, the time of each replication reaction is given (32 min to 15 s). The reversed order of time course in (i) has no significance. The particular batch of DNA preparation used for (iii) contained about 25% dimeric species.

However, when 2.1/M13mp19 supercoiled DNA was used as the template, it took about 30 s to make the 9.1-kb polymeric chain and 60 s for the 18.2-kb one (Fig. 3, ii). Obviously, the fork movement was a little slower when both the strands of the duplex DNA were present as templates.

Besides the formation of full-length products, a uniform streaking in the gel, indicating incomplete synthesis, was also observed when ssDNA was used as a template (Fig. 3, i). In addition to uniform streaking, heavy synthesis of chains ranging from 150 to 1250 nucleotides in length was also seen when supercoiled DNA was used as template (Fig. 3, ii and iii).

When the supercoiled template molecules with non-*OriA/OriB* sequences or without *OriA* sequences were incubated

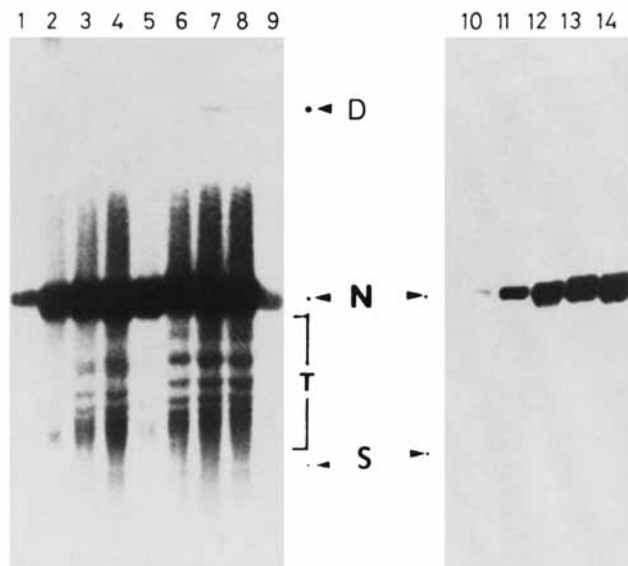


Fig. 4. Effect of ATP and stabilising proteins in the enhancement of replication synthesis. Replicative products at the end of a 20-min reaction were deproteinised with proteinase K followed by phenol purification and run in 1% agarose gel without ethidium bromide and subsequently autoradiographed. Individual and combined influences of ATP and ligase have been shown in lanes 1–9. Control synthesis (lanes 1 and 9) gave rise to nicked species only. In lanes 2–4 only ATP was added at 0.25 mM, 1 mM and 4 mM, respectively, in normal replication reactions. In lanes 5–8, ligases (1 unit) and varying amounts of ATP (0, 0.25 mM, 1 mM, 4 mM, respectively) were added. Lanes 10–14 represent a separate set of replication reactions. Lane 10 shows the control synthesis. Bovine serum albumin was added in lanes 11 and 12 at 500 µg/ml and 5 mg/ml, respectively. Gelatin was added similarly in lanes 13 and 14 at 5 and 10 mg/ml, respectively. N, T, S and D represent monomeric nicked, topoisomers, supercoiled and the nicked dimeric species, respectively.

with the replicative system, observed DNA synthesis as measured by the tritium incorporation assay [20] was 5–6-fold less compared to template molecules bearing the *Ori* sequences. Analysis of the denatured nascent products revealed that the template-sized strands were few in number while the majority of the products was of small molecular size. Such observations were in agreement with the findings reported by Nielson et al. [31].

Replicative products analysed in non-denaturing conditions

The majority of the products of replication with *OriA*-containing supercoiled DNA template were of nicked-circle structures at early stages of reaction. Similar products were observed irrespective of the size of starting template DNA. It appears that the initiation proceeds with a nick in the supercoiled DNA and this is supported by subsequent electron microscopic observations of replicative products. With increasing time of incubation, molecules of indistinct masses heavier than the monomeric structures of template DNA, as well as a very few monomeric topoisomers, were visible as end products.

Addition of ATP or stabilising proteins had a dramatic positive effect on the rate of synthesis. Lanes 1 and 9 of Fig. 4 show control synthesis without ATP where mostly nicked structures were seen. With increasing concentrations

of ATP from 0.1 mM to 4 mM, topoisomers along with nicked structures were generated (lanes 2–4). At 4 mM ATP, the total amount of topoisomers and nicked circles formed were in a ratio of 3:7. In lane 5, ligase alone was used without any ATP. Enhanced synthesis of nicked circle structures occurred. Addition of ligase along with increasing amounts of ATP resulted in the formation of higher amounts of topoisomers (lanes 6–8). The ratio of the total amounts of topoisomers and nicked circles was 6:4 at an ATP concentration of 4 mM (lane 8). If one assumes that covalently closed circular DNA should be the end product of *in vitro* replication reactions (DNA isolated from pea chloroplasts exists mostly in the supercoiled form), it is likely that ATP-dependent ligase-like or type II topoisomerase activities were present in limiting amounts in the replicative system under consideration.

In Fig. 4, lanes 10–14, synthesis is shown using a separate set of replication reactions. In lane 10, control synthesis without any exogenous reagent is displayed; 500 µg/ml and 5 mg/ml bovine serum albumin and 5 mg/ml and 10 mg/ml gelatin were additionally used in lanes 11–14, respectively. Addition of stabilising proteins significantly enhanced replication synthesis but did not generate any topoisomers.

Both strands of duplex DNA are synthesised

The slower polymerization rate on duplex DNA template as seen in Fig. 3 (ii) could result from coordination control between the synthesis of both strands. As a result, we needed to verify directly whether both the strands were made simultaneously during replication of a double-stranded template DNA. M13 ssDNA containing either strand of the 2.1-kb ctDNA sequence along with ssDNA of vector M13 DNA were blotted onto nitrocellulose membranes; 1–4 µg 2.1/M13mp19 ssDNA, 1–4 mg 2.1/M13mp18 ssDNA and 3–12 µg control M13 mp19 ssDNA were loaded in the lanes 1, 2 and 3, respectively, of Fig. 5. These DNAs were hybridised with radioactively labelled (specific activity 3×10^7 cpm/µg) linear *OriA* fragment derived from *in-vitro*-replicated plasmid DNA containing *OriA*. Both the strands containing sequences of the 2.1-kb ctDNA piece hybridized with the probe so that the replicated 2.1-kb fragment must have incorporated radioactive label in both strands. The non-specific binding of the control DNA largely disappeared (Fig. 5B) with the stringent wash conditions. Since similar results were obtained with probes from replication reactions carried out either for short or long periods of time, both the strands were probably synthesized simultaneously.

On an ssDNA template replication synthesis continues beyond one round

ssDNA containing the *OriA* sequence (2.1/M13mp19) was used as the template for a 30-min or 60-min *in-vitro*-replication reaction. Replicated products were digested with *SmaI* and *HindIII* restriction enzymes to recover the radiolabelled ctDNA fragment as before and used as the probe for hybridisation with various kinds of unlabelled ssDNA spotted onto nylon-based nitrocellulose membranes (Gene Screen). Of the three different kinds of ssDNA spotted, the ssDNA having either strand of *OriA* sequences hybridised with radiolabelled probe (data not shown). The probe did not hybridise under stringent conditions with the parent vector

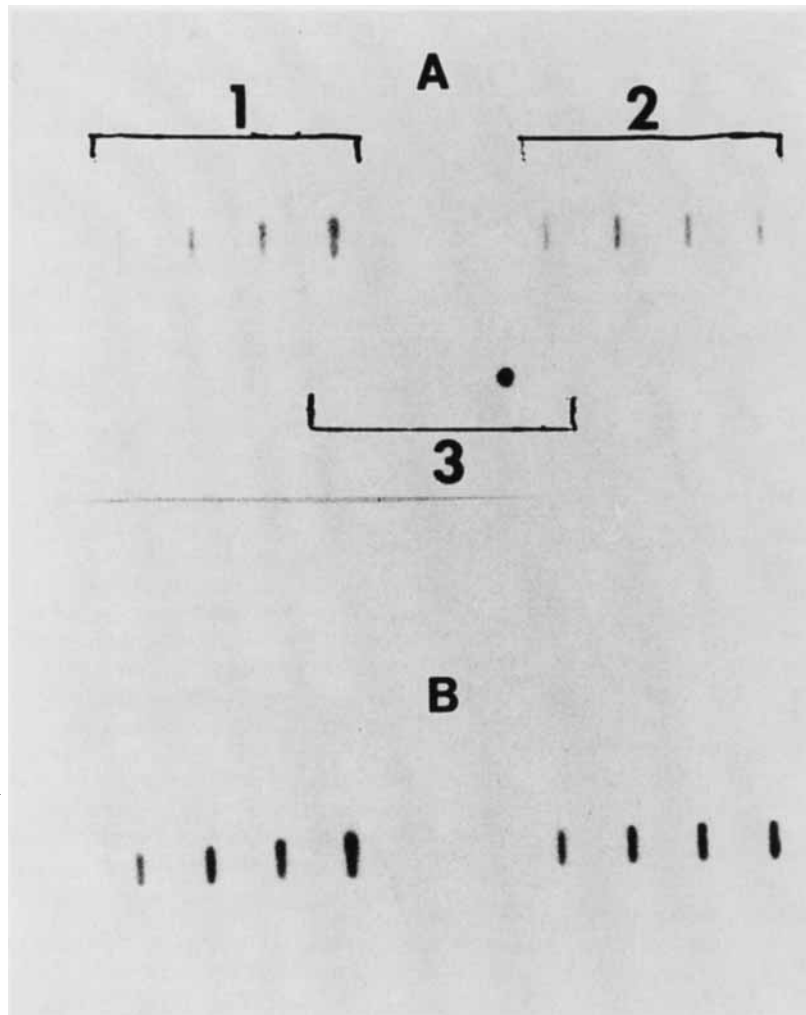


Fig. 5. Hybridisation of replicated 2.1 ctDNA to various templates. 2.1/pUC19 supercoiled DNA was allowed to replicate for 30 min with [32 P]dCTP substrate and others. Replicative products were digested with *Sma*I and *Hind*III restriction enzymes and the recovered 2.1-kb ctDNA was used as the probe for hybridisation. In panels (1–3), ssDNA of 2.1/M13mp19, 2.1/M13mp18 and M13mp19 template, respectively, was used. Each panel consisted of four spots of graded amounts of DNA as mentioned in the text. Hybridization was carried out at 45°C in 0.9 M NaCl, 0.09 M sodium citrate, pH 7; corresponding autoradiogram is shown in A. Results of a stringent wash at 50°C with 15 mM NaCl, 1.5 mM sodium citrate, pH 7, are displayed in B.

M13 DNA. The nature and intensity of the hybridisation were almost identical for either time of replication reaction (30 or 60 min). These data show that both the strands of ctDNA were radiolabelled during the replication reaction. Since the spots of 2.1/M13mp19 unlabelled ssDNA were brighter than the corresponding spots of 2.1/M13mp18 ssDNA, the amount of synthesised DNA was certainly higher (more than double) in the strand complementary to the one used as the template for replication reaction. These observations rule out the possibility of several rounds of replication during DNA synthesis (in which case ssDNAs of either strand should have had an almost equal intensity). The data also clearly indicate that at least a substantial portion of the double-stranded forms of DNA, generated during synthesis on ssDNA template, must have undergone one more additional round of replication. Electron microscopic visualisation of the replicated products (Fig. 7, below) also clearly points out that the *in vitro* replication synthesis must have cycled beyond just a single round on the starting ssDNA template.

Electron microscopy of replicative intermediates of duplex DNA templates

2.1/pUC19 supercoiled DNA molecules were allowed to replicate and the products were processed for microscopic observations. In Fig. 6, molecules have been displayed according to the gradient of replication. All the replicative molecules assumed sigma-like structures. The length of the tail of sigma structures reflects the extent of replication. The replicative molecules shown in Fig. 6A and B show 5% and 65% replication of the genome, respectively. Some very rarely occurring replicative molecules (1/300) were also seen where tails are definitely much bigger than the template genomic size. In Fig. 6C, the tail is four times the genomic size. The existence of tails larger than the genomic size possibly rules out the nucleolytic conversion of theta structures to sigma structures. It is worth mentioning that the clone containing *OriB* sequences (3.75/pUC19) also replicated in a similar sigma mode (data not shown).

The synthesised products with the templates carrying non-*Ori* or no *Ori* sequences did not exhibit characters of

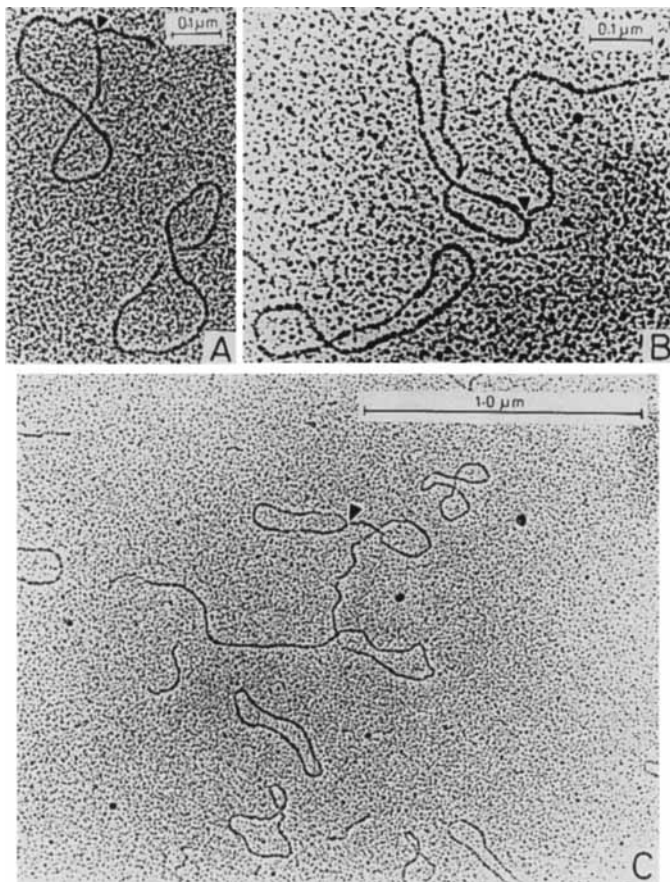


Fig. 6. Structure of the replicated molecules. Tails of various sizes of the lariats are shown with the forkpoints marked by single arrows. Corresponding unreplicated molecules are also shown for dimensional comparisons. Scales are at the top of each figure.

true replicative intermediates. Some of them had small bubbles and others had protruding single-stranded small tails ranging from one to five/circular molecule (data not shown). In other words, these products did not have the structures specific for replicative intermediates.

The number of molecules replicated is limited to 5% only

Applying dimer statistics to the molecules in the electron micrographs, the number distribution of early replicating molecules has been found to be 5% of the starting template molecules. In a particular batch of unreplicated DNA sample, supercoiled dimers occurred at a frequency of 8% of total molecules. In the replicated sample, we counted the number of molecules whose sizes varied between monomer and dimer only. The number of molecules of this chosen size was about 13% of the total molecules as seen in Table 1. The increase of 5% must, therefore, result from replication of the DNA template.

Replicative intermediates of ssDNA templates also have sigma structures

Replicative intermediates of 2.1/M13mp19 ssDNA templates, prepared as described before, were examined. In Fig. 7, (i) and (ii) represent two independent single-stranded unreplicated parental molecules. Fig. 7 (iii) shows the presence of a replicative intermediate which is of duplex form (as judged from thickness) and sigma-like in appearance. The ssDNA template had probably been converted to a duplex form as a result of a single round of replication. The resulting duplex DNA might have assumed the sigma-shape at a subsequent step of ongoing replication. Thus the presence of sigma molecules as the intermediate products of the replication reaction clearly supports the view point that the *in vitro* replication synthesis must have proceeded beyond just a single round on the starting DNA templates.

Replication originates around the *OriA* area and moves unidirectionally

The free end of the tail of a lariat should contain the sequences of the replication origin. The free end was mapped by restriction of the very early replicative intermediates and subsequent measurement and analysis of the arms of the resultant branched structures. In Figs 6 and 7, each replicating molecule had a single fork, indicating unidirectionality of replication. When intermediates are cut with the restriction enzyme *SspI*, most molecules were scored with two (out of

Table 1. Number and size of replicating template molecules. 2.1/pUC19 supercoiled DNA were allowed to replicate in four independent sets of reactions for the indicated periods of time in the presence or absence of ddNTP. Replicated DNA (including fraction 17) or unreplicated DNA (without fraction 17) was visualised in the electron microscope. Unreplicated DNA samples consisted of monomeric and dimeric species only whereas, in the replicated sample, the whole spectrum of species between monomer and dimer was present. In both kinds of sample the distribution of species of dimensions higher than the monomers was looked for.

Pre- paration	ddNTP	Unreplicated DNA		Replicated DNA		Replication	
		no. molecules	no. (%) dimer	no. molecules	no. (%) dimer	time	efficiency
						min	%
1	+	400	32(8)	298	45(15)	15	7.0
	-	516	37(7)	432	70(15)	15	8.0
2	+	312	25(8)	335	47(14)	30	6.0
	-	427	32(7.5)	402	60(15)	30	7.5
3	+	277	22(8)	300	38(13)	45	5.0
	-	329	27(8)	427	60(14)	45	6.0
4	+	409	33(8)	425	64(15)	60	7.0
	-	403	34(8)	457	69(15)	60	7.0

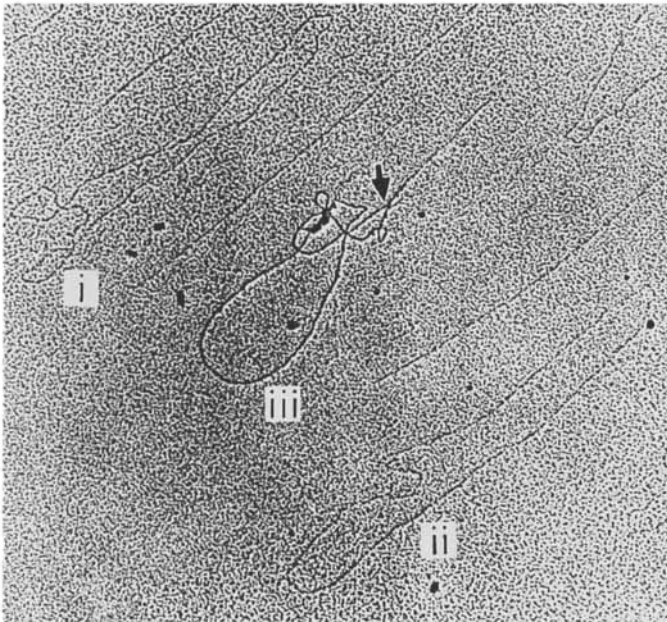


Fig. 7. Replicated DNA of ssDNA template as visualised in the electron microscope. Molecules shown directly above the areas marked (i) and (ii) are unreplicated ssDNA templates which assumed the typical kinky appearance. In (iii), a sigma-like molecule is shown which was the product of replication. The single arrow represents the fork point.

three) identical arms but few molecules had all the three arms of unequal lengths. If a replicative intermediate was such that its forkpoint had travelled beyond the *SspI* site, cutting that molecule with *SspI* would result in a branched structure having two (of the three) arms of exactly equal length. When these early replicative molecules were cut with *BglII* enzyme, 80% of the digested molecules had three unequal arms and two of the arms differed by a constant amount in all the cases.

Fig. 8 shows a circular map of the template molecule along with a summary of restriction analysis. Schematic diagrams of the cut early intermediates are seen in the middle of Fig. 8. The junction of the three arms represents the fork-point. Since branched molecules having two arms of equal dimension were obtained more with *SspI* restriction than with *BglII* digestion, the replication fork must have traversed the *SspI* site prior to the *BglII* site. So the fork possibly moves in a counter-clockwise direction initiating anywhere within the *BglII*–*SspI* segment carrying the D-loop sequences. C represents the tail or initiation site of replication. The length (B+C) from the *SspI* site in a clockwise sense, and similarly the length (B–C) from the *BglII* site in the counter-clockwise sense, should map to the free end of the tail or initiation site of replication. The result of the above-mentioned analysis has been shown pictorially at the bottom of Fig. 8. With the *SspI*-cut molecules, the initiation point mapped to the left of the D-loop area (about 100–250 nucleotides away from the left

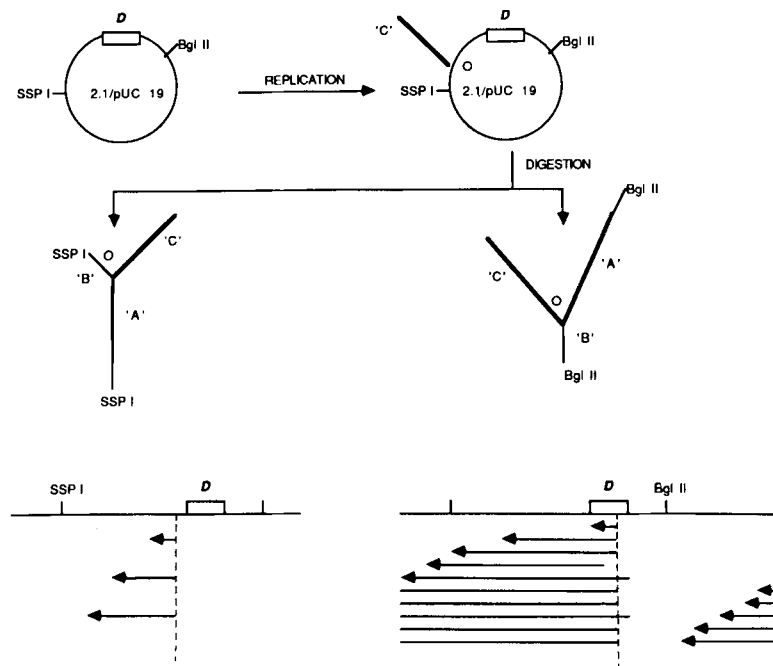


Fig. 8. Localisation of origin and directionality of replication. The top part of the diagram shows how the template 2.1/pUC19 supercoiled molecule assumes a sigma-like structure during early *in vitro* replication. D represents location of the D-loop and C indicates the tail of the lariat. The middle part shows the structure of linearised replicative intermediate. An O represents the fork point and the restriction ends are marked by the names of the corresponding enzymes. A, B and C represent the dimensions of the branches of linear replicative intermediates. The dotted lines in the bottom part indicate the initiating regions of the *in vitro* replication. The extent of replication of the analysed, digested intermediates are represented by single horizontal lines with the arrowheads indicating both the direction and forkpoint of replication.

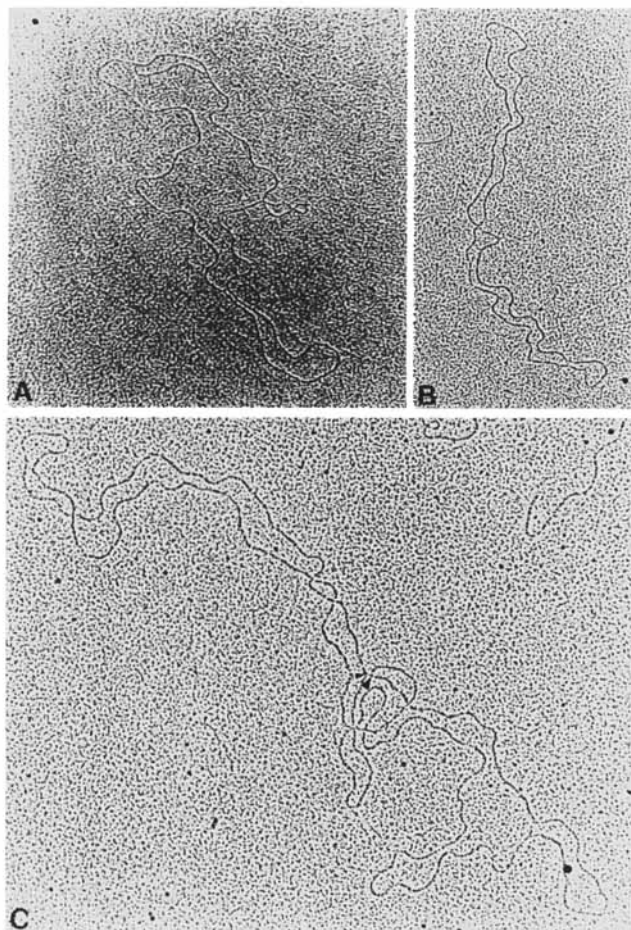


Fig. 9. Electron micrographs of replicative intermediates of 12.5/pACYC177 templates. Molecules enclosing the smallest and largest replication bubbles are shown in A and C. B shows a molecule of monomeric dimension. Two arrows in C represent the terminal forkpoints. Note the presence of single-stranded regions near the forkpoints of the replicating molecules as shown in both A and C.

border of the D-loop). But for the *Bgl*III-cut molecules, the initiation site mapped in the middle of the D-loop area, the region from which the pea ctDNA replication was shown to begin *in vivo* [25]. The fork point of a replicative intermediate is shown by an arrowhead.

Clones containing both *OriA* and *OriB* sequences of pea chloroplast DNA replicate in theta mode

Since *OriA* (or *OriB*) clones replicated in a nonphysiological sigma mode, it could be argued that the replicative enzyme system lacked protein(s) responsible for the *in vivo* replication mode. To test the possibility, *in vitro* replication was carried out with a template which contains both *OriA*, *OriB* and the spacer-sequences. Replicated molecules were visualised and these are displayed in Fig. 9. In Fig. 9A, a molecule is shown whose bubble size is 5% of the genome. A small ssDNA stretch was visible at one end of the bubble. Two arrows in Fig. 9C delimit the little unreplicated zone of the parental molecule on one side. The other extended side points out the presence of the large bubble. The molecule shown in B represents the monomeric size of the template and the extent of replication in molecule C is about 100%. In other words, the *in vitro* replication proceeded in the theta

mode and could generate products of dimeric size. When the replicated products were analysed in nondenaturing agarose gels in the absence of ethidium bromide we have also observed that supercoiled monomers were present as 60% of the finished products in contrast to the abundance of the nicked species with the replicated *OriA* (or *OriB*)-containing templates (data not shown).

Performing the dimer-statistical analysis, we found that the number of replicating intermediates was only 0.5%. Although the frequency of replicating molecules was extremely small, 95% of the intermediates were of theta and the rest of sigma structures. Since the mode of *in vitro* replication of constructs containing both the *Ori* sequences mimicked the physiological *in vivo* mode, it could be inferred that the glycerol-gradient fraction 17 contained the essential enzyme activities that were necessary and sufficient for specific replication of ctDNA. This *in vitro* replication system might still miss some other factors which are necessary to restore the *in vivo* rates of DNA synthesis, copy number of replicated molecules, etc.

DISCUSSION

The replicative system from pea chloroplast recognises *OriA* and *OriB* in recombinant plasmids *in vitro*. Using *OriA*, the data presented in this paper show that the templates replicate *in vitro* and the observed results are not due to any repair synthesis. This contention is supported by the fact that this replicative system can utilise ssDNA template to synthesize fully double-stranded DNA molecules. In addition, the replication on ssDNA template goes beyond one round of synthesis; when ATP is included in the substrates, topoisomers of various superhelicities are generated as end products. These data could not be interpreted in terms of a simple repair reaction. Furthermore, the replication of the template DNA is confirmed by observing replicative intermediates in the electron microscope.

It was reported that the recombinants containing *OriA* sequences only could be used for organellar transformation and the recombinants replicated transiently in the tobacco chloroplast following transformation [21]. This observation is surprising in the light of the fact that the *in vivo* mechanism [20] of ctDNA replication requires two origins to produce replicating molecules with theta structures. This discrepancy may be explained by an anomalous behaviour of the pea *OriA* sequence in the heterologous tobacco system.

The observation of theta mode of replication in the replicative intermediates of the templates containing both *Ori* sequences strongly suggests that the *in vitro* replication system of pea chloroplasts is capable of mimicking the *in vivo* mode of replication. It would be extremely interesting to identify what causes the switch in the mode of replication with *OriA* (or *OriB*) sequence-containing templates. It would also be worth finding out the role of spacer sequences between *OriA* and *OriB* in restoring the physiological mode of replication.

The *in vitro* system reported here has its own novel and complex set of characteristics which deserve further attention. The molecular nature of intense smeary bands of small mass in Fig. 4 (iii), poor activity of the chain terminators, site-specificity of the nuclease, if any, acting as an initiator of sigma mode of replication, etc. should be investigated and probed further in order to reveal the mechanism of initiation and propagation of DNA replication within and along the pea chloroplast *OriA* sequences. However, we have provided

straightforward evidence that a soluble pea chloroplast system could be developed which recognises chloroplast *Ori* sequences in recombinant templates and initiates replication from the sequences which are also used for *in organello* DNA replication. This *in vitro* system should facilitate further identification and purification of all the necessary and accessory factors involved, thereby permitting the eventual reconstitution of pea chloroplast DNA replication in a defined manner.

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