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DNA Replication in Chloroplasts

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

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

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COMMENTARY

DNA replication in chloroplasts

Sabine Heinhorst and Gordon C. Cannon

Department of Chemistry and Biochemistry, University of Southern Mississippi, Southern Station Box 5043, Hattiesburg, MS 39406-5043, USA

SUMMARY

Chloroplasts contain multiple copies of a DNA molecule (the plastome) that encodes many of the gene products required to perform photosynthesis. The plastome is replicated by nuclear-encoded proteins and its copy number seems to be highly regulated by the cell in a tissue-specific and developmental manner. Our understanding of the biochemical mechanism by which the plastome is replicated and the molecular basis for its regulation is limited. In this commentary we review our present understanding of chloroplast DNA replication and examine current efforts to elucidate its mechanism at a molecular level.

Key words: chloroplasts, DNA replication, plastome

INTRODUCTION

Of the three compartments in the higher plant cell that contain their own DNA, the chloroplast harbors the least complex genetic material. The chloroplast genome (plastome) exists as a covalently closed, double stranded circle ranging in size from 120 kilobase pairs (kbp) in some species to over 200 kbp in others (reviewed by Palmer, 1985). With few exceptions, higher plant chloroplast (ct) DNA contains two inverted, exact repeats of 20 to 30 kbp, which are separated by a small and a large single copy region (Fig. 1). Among other genes, one copy of the ribosomal RNA genes is located on each repeat. The complete nucleotide sequences of the ctDNA from tobacco (Shinozaki et al., 1986), the liverwort *Marchantia polymorpha* (Ohyama et al., 1986) and rice (Hiratsuka et al., 1989) have been determined, and work on the maize chloroplast genome is near completion (H. Kössel, personal communication). The estimated coding capacity of the plastome, including open reading frames and known genes, is approximately 140 genes, most of which are components of the organellar transcriptional and translational apparatus, or are involved in photosynthesis. A recurring theme among organellar multisubunit proteins is the distribution of their genes between nuclear and organellar genomes, as exemplified by the CO_2-fixing enzyme ribulose-1,5-bisphosphate carboxylase. While the gene for its large subunit is located on the plastome, its nuclear-encoded small subunit is synthesized in the cytosol and post-translationally imported into the chloroplast as a larger precursor. Evidence obtained from plants lacking functional chloroplast ribosomes (Walbot and Coe, 1979; Herrmann and Feierabend, 1980; Scott et al., 1982), as well as studies with inhibitors of chloroplast RNA and protein synthesis (Leonard and Rose, 1979; Heinhorst et al., 1985a; Rapp and Mullet, 1991) indicate that most, and probably all, genes for proteins involved in ctDNA replication are located in the nucleus. Considering this evidence together with the limited coding capacity of the plastome, the nuclear-cytosolic compartment is the most likely entity controlling chloroplast DNA replication. Environmental factors such as light (Tymms et al., 1982; Cannon et al., 1985), as well as tissue type and developmental stage of the plant (Lawrence and Possingham, 1986; Lamppa and Bendich, 1979; Boffey and Leech, 1982; Tymms et al., 1983), also play a role in this regulation, although its molecular mechanism is not known. It is clear, however, that syntheses of nuclear and chloroplast DNA are not directly coupled. While nuclear DNA is replicated only once during the cell cycle, plastome replication does not seem to be limited to the S-phase (Rose et al., 1975; Lawrence and Possingham 1986; Heinhorst et al., 1985a,b; Sala et al., 1981).

Progress towards unraveling the cellular “players” and molecular mechanisms that govern ctDNA replication has been difficult and would be facilitated by elucidation of plastome replication mechanisms.

PLASTOME COPY NUMBERS DURING LEAF DEVELOPMENT

The number of plastome copies per organelle and cell varies in a developmental and tissue-specific manner (Lamppa and Bendich, 1979; Boffey and Leech, 1982; Tymms et al., 1983; Scott and Possingham, 1983; Lawrence and Possingham, 1986). By a combination of techniques that include

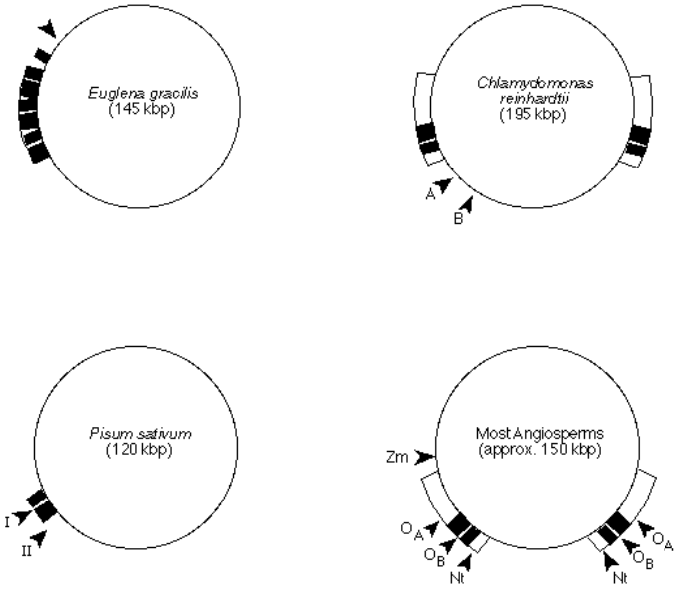

Fig. 1. Chloroplast DNA structures. The filled large and small bars depict the locations on the plastome of the 23 S and 16 S rRNA genes, respectively. Inverted repeat regions are indicated by wide, open bars and arrows mark origins of replication. A,B, *ori*A and *ori*B, respectively (*Chlamydomonas reinhardtii*). I,II, D-loop initiation sites (*Pisum sativum*). O_A,O_B, *ori*A and *ori*B, respectively (*Oenothera*). Nt, *ori* (*Nicotiana tabacum*). Zm, *ori* (*Zea mays*).

quantitative molecular hybridization and microfluorometric DNA estimation in situ, it was determined that plastome copies are greatly amplified in mesophyll cells of the developing leaf and can reach values of more than 20,000 per cell (Lawrence and Possingham, 1986). In those spinach leaf regions, where the mesophyll cells are merely increasing in size but do not divide, the amount of ctDNA per cell stays constant, while the mature organelles themselves are actively dividing. Here, the rate of chloroplast division surpasses that of plastome replication, resulting in a reduction of chloroplast ploidy (Lawrence and Possingham, 1986). In older leaves of barley, this decrease of ctDNA copy number per organelle might also involve DNA degradation in addition to re-distribution of existing plastomes among the chloroplast progeny (Baumgartner et al., 1989). In epidermal cells of the spinach leaf, on the other hand, the number of plastomes per organelle stays constant and does not seem to be developmentally modulated (Lawrence and Possingham, 1986). Bendich (1987) suggested that the elevated ctDNA copy numbers in the developing mesophyll chloroplasts are needed to increase the dosage of plastome-encoded genes. This, in turn, would augment the organelle's translational capacity and allow it to keep in pace with the increased demand for photosynthesis in the developing leaf. While there is no direct proof for this postulate, it remains an attractive working hypothesis and is consistent with most of the existing data on chloroplast development.

MODELS OF ctDNA REPLICATION

The currently prevailing model of DNA replication in chloroplasts is based on electron microscopic examination of replication intermediates in isolated pea and maize ctDNA, and was put forth by Kolodner and Tewari over a decade ago (Kolodner and Tewari 1975a,b,c). Replication begins at two displacement (D-) loop initiation sites located on opposite strands approximately 7 kbp apart (Fig. 2). The D-loops expand unidirectionally toward each other until the advancing forks pass the D-loop initiation site on the opposite strand, at which point discontinuous replication begins, resulting in two Cairns-type, bidirectional forks moving away from each other. Presumably the forks meet at some point approximately 180 degrees from the starting point and give rise to two daughter molecules. Occurrence of sigma-type structures in significant frequencies has led to the extension of the model to include a rolling circle mode of replication initiated at the termination of the Cairns cycle (Kolodner and Tewari, 1975c). The exact mechanism for conversion of one replication mode to another is still not understood, and direct evidence that the sigma structures are products of a rolling circle mode initiated by a round of Cairns-type replication has not been found.

Electron microscopic examination of ctDNA from other plant species has supported this model to various degrees. In *Chlamydomonas* and *Oenothera*, two D-loops 7 and 4 kbp apart, respectively, have been mapped on the plastome (Waddell et al., 1984; Chiu and Sears, 1992). However, conversion to discontinuous synthesis apparently occurs soon after D-loop initiation, since mainly forks with exclusively double-stranded arms have been observed in preparations of replication intermediates. It is unknown whether each D-loop represents an origin for one strand, and if both origins are used simultaneously in a single round of replication.

Only in *Euglena* has a single origin of ctDNA replication been observed (Ravel-Chapuis et al., 1982; Koller and Delius, 1982). Electron microscopic analysis of replication intermediates suggested that bidirectional replication proceeds from the origin via Cairns forks. Although a specific model has not been put forth, the mechanism of *Euglena*

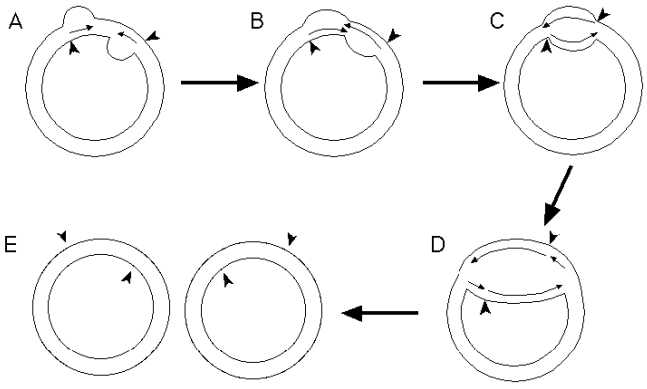

Fig. 2. The dual D-loop model for the initiation of chloroplast DNA replication. (A) Unidirectional elongation of nascent strand initiated from both origins. (B) Unidirectional fork movement toward each other. (C) Fusion of D-loops to Cairns-type intermediates. (D) Bidirectional, semi-discontinuous replication. E, Resulting daughter molecules. The arrows denote the two D-loop initiation sites (origins).

ctDNA replication seems to be different from those proposed for the other plastomes studied so far.

Results obtained in vitro with maize and pea chloroplast extracts (see below) indicated that a region of the maize plastome homologous to *ori*A from the *Chlamydomonas* plastome could act as an origin of replication, but unlike earlier results obtained by electron microscopic observation of maize ctDNA replication intermediates (Kolodner and Tewari, 1975a), replication in vitro seemed to proceed bidirectionally after initiation (Gold et al., 1987; Carrillo and Bogorad, 1988).

Data other than electron microscopic observations that would permit the formulation of a more general model, or to determine if, in fact, the plastomes of the plant species studied so far are all replicated by different mechanisms, are scarce. The advent of pulsed-field gel electrophoresis has allowed the separation of large DNA molecules, and when such an analysis was performed on ctDNA a distribution of molecular sizes was found that represented oligomeric forms (Bendich and Smith, 1990; Deng et al., 1989). In a wide range of species, the multiple forms migrated as a series from monomer to trimer or tetramer with an abundance inversely proportional to size. In spinach the multimers were shown to be true oligomers and not multimeric catenanes. Two possible mechanisms can explain the existence of these oligomeric forms. The first is the production of multimeric forms by the rolling circle mode of replication, if the nascent tail portion of the replicative structure is not tailored to monomeric units immediately after formation. This possibility was argued against by Gruissem and coworkers (Deng et al., 1989) because the relative abundance of each form remained unchanged throughout leaf development. Since mature leaves replicate little new ctDNA in comparison to young ones, if rolling circle replication was the source of the oligomers then older leaves should contain a higher percentage of monomeric units than younger leaves. An alternative possible mechanism is intramolecular recombination taking place dynamically among the multiple copies of the plastome within each chloroplast and resulting in an equilibrium of oligomeric forms. If recombination is continuing at a constant rate regardless of age and developmental status of the leaf cell, a constant ratio of the oligomeric plastomes would be expected as was, indeed, observed. Recent reports of a RecA-like activity in pea and *Arabidopsis* chloroplasts (Cerutti et al., (1992); Pang et al. (1992)) in addition to the well documented intramolecular recombination between inverted repeats (Palmer 1983), support this possibility, but do not yet provide a satisfactory overall model relating the observed replication intermediates, the two-phase replication model and the existence of multimeric plastomes.

Perhaps the replication of the 2 μm plasmid in yeast can be regarded as an analogous model (Kornberg and Baker, 1992). The plasmid can replicate once during the cell cycle by initiation from a single origin, followed by bidirectional, discontinuous elongation via the Cairns mode. Amplification of the plasmid takes place when site-specific recombination occurs between the two inverted repeats after one fork has passed through one, but not yet the other, repeat. This phenomenon gives rise to a "double rolling circle" with multimeric intermediates that are subsequently converted into monomers. This mechanism allows the plasmid to be amplified without the necessity for multiple initiation events during the yeast cell cycle.

The striking structural similarities between the chloroplast DNA molecule and the 2 μm plasmid, as well as the circumstantial evidence described above, make it tempting to speculate that ctDNA replication involves a similar mixture of replication modes and recombination. However, direct evidence or even an obvious experimental design to test this model as a hypothesis is so far not forthcoming.

IN VITRO ctDNA REPLICATION SYSTEMS

As in other DNA replication studies, an important goal of several laboratories has been to assemble a defined in vitro ctDNA replication system consisting of a suitable template and a set of replication enzyme activities that is capable of specific initiation and elongation of ctDNA replication.

The earliest attempts towards this goal employed isolated organelles (Bohnert et al., 1974; Zimmermann and Weissbach, 1982; Mills and Baumgartner, 1983; Overbeeke et al., 1984; Tanaka et al., 1984). Photosynthetically active, intact chloroplasts from pea and spinach were shown to incorporate radiolabeled thymidine into acid-insoluble material in a light-dependent fashion, while chloroplasts from maize, *Petunia*, the liverwort *Marchantia polymorpha*, as well as osmotically shocked plastids from spinach, were able to take up deoxynucleoside triphosphates (dNTPs) but did not demonstrate light-dependence of DNA synthesis. With the exception of ATP-mediated stimulation of reaction rates in maize and *Petunia* chloroplasts, ribonucleotides were not required in any of these systems. Crude soluble chloroplast extracts from maize (Zimmermann and Weissbach, 1982), *Petunia hybrida* (de Haas et al., 1987) and *Marchantia polymorpha* (Tanaka et al., 1984) were capable of in vitro DNA synthesis with exogenous templates. Although there is some evidence for site specific initiation, the limited product analysis, when performed, did not allow differentiation between true replication and repair-type synthesis, emphasizing the limitations of in organello and crude lysate systems for investigations of chloroplast DNA replication.

Recently, research efforts toward the goal of developing a site-specific initiation system have focused on more refined, soluble chloroplast extracts (Wu et al., 1986; Gold et al., 1987; Carrillo and Bogorad, 1988; Meeker et al., 1988). In general, these are salt or detergent extracts that have been purified through at least a diethyl-aminoethyl (DEAE)-cellulose chromatography step in order to remove endogenous ctDNA. In most cases, additional purification steps were performed in order to reduce the activity of nucleases, which are often prevalent in chloroplast protein preparations. Activity of ctDNA polymerase was usually used to define fractions active in DNA synthesis. The main criterion for specificity in these lysates is template preference for selected ctDNA clones from a complete library, and increased incorporation rates are taken to mean that these clones are likely to contain a ctDNA origin of replication. Like the *ori*C-dependent in vitro DNA replication system from *E. coli* (Kornberg and Baker, 1992), the

chloroplast extracts seem to depend on supercoiled templates and lose specificity with relaxed plasmid templates. It is interesting to note that dNTP incorporation does not require exogenous ribonucleotides, suggesting that residual ribonucleotides remained in these extracts or that the priming activity involved can also utilize dNTPs.

Wu et al., (1986) prepared a combined cellular and thylakoid protein extract from *Chlamydomonas* which demonstrated some discrimination between supercoiled vector DNA and plasmids containing cloned ctDNA fragments that had previously been shown to contain D-loops associated with one of the replication origins (Waddell et al. 1984). Initiation in vitro from a site close to *ori*A was verified by limiting chain elongation to a region close to the synthesis start site with dideoxynucleotides. The chain terminator approach is, however, not easily applicable to higher plant chloroplast DNA replication systems, since, in contrast to the ctDNA polymerase from the alga *Chlamydomonas* (Wang et al., 1991), the enzyme from higher plants is largely insensitive to this inhibitor (Heinhorst et al., 1990a). A cleared, partially purified detergent extract from pea chloroplasts displayed five- to ten-fold template discrimination between individual cloned maize chloroplast DNA fragments (Gold et al., 1987). Of the four ctDNA regions preferred as templates in vitro, one area extending over 9 kbp was particularly active. Analysis of smaller subfragments from within this region revealed considerable homology between a 1.33 kbp *Eco*RI fragment and the *Chlamydomonas* ctDNA D-loop site at *ori*A (Wang et al., 1984). In order to verify the preferential replication of these maize ctDNA fragments in a homologous system, Carrillo and Bogorad (1988) subsequently developed a similar chloroplast extract from maize and confirmed the high template activity of the 1.33 kbp *Eco*RI fragment. The presumed origin region was fine-mapped to a 455 bp region by chain termination with ddCTP and araCTP at very high concentrations. Radiolabeled, newly synthesized DNA hybridized to both strands of the cloned 1.33 kbp *Eco*RI fragment, suggesting that DNA synthesis in vitro from this initiation site was bidirectional. The pea chloroplast extract was also used by Meeker et al. (1988) to assess template activity in vitro of cloned pea ctDNA fragments. Regions of the pea ctDNA that had been mapped as D-loop sites were preferred four- to ten-fold over other cloned DNA fragments or the vector alone, and when analyzed on alkaline agarose gels, the products of the in vitro reaction were full length.

While DNA synthesis in these partially purified chloroplast extracts is, indeed, suggestive of site-specific initiation, a more thorough product analysis is needed in order to obtain convincing evidence that these highly active templates correspond to origins used in vivo. Time-dependent conversion of short, recently initiated replication intermediates to longer products, or changes in labeling pattern with time of restriction fragments surrounding the presumed initiation site, have yet to be demonstrated in most of the higher plant in vitro DNA synthesis systems. Furthermore, the bidirectional replication mode observed in vitro by Carrillo and Bogorad (1988) does not agree with the original observations of maize ctDNA by Kolodner and Tewari (1975a) and may reflect missing components in the in vitro system.

REPLICATION ENZYMES

Based on other pro- and eukaryotic, as well as viral DNA replication systems (Kornberg and Baker, 1992), one can assume that a basic set of enzymes is necessary to replicate the plastome. These include DNA polymerase(s), DNA unwinding activity (helicase), primase and/or RNA polymerase, single stranded DNA binding protein, topoisomerase I and II, origin recognition protein(s). Of those isolated from chloroplasts, by far the best characterized ones are the DNA polymerases. First described by Spencer and Whitfield (1969), ctDNA polymerases have been characterized in crude extracts from a number of plant sources. The DNA polymerases from pea, spinach, soybean and *Chlamydomonas* chloroplasts have been purified extensively (McKown and Tewari, 1984; Sala et al., 1980; Heinhorst et al., 1990b; Wang et al., 1991). While some discrepancy exists in the literature concerning the enzymes' preferred in vitro templates and their ability to utilize primed synthetic homopolymers, all ctDNA polymerases have an estimated molecular mass of between 90,000 and 120,000 and are resistant to the DNA polymerase α inhibitor, aphidicolin. In contrast to the mitochondrial DNA polymerases from animal sources, the chloroplast enzymes, which also belong to the γ-class of eukaryotic DNA polymerases, are quite resistant to the chain-terminating dideoxynucleotides. An exception seems to be the ctDNA polymerase from *Chlamydomonas*, which is much more susceptible to dideoxynucleotides than its higher plant counterparts. Keim and Mosbaugh (1991) demonstrated that the ctDNA polymerase from spinach contains a 3′- to 5′-exonuclease activity which resides on a polypeptide of molecular mass approximately 20,000 that is tightly associated with the one harboring the polymerase activity. The nuclease was able to remove matched and mismatched 3′ primer termini, with some preference for the latter. To date, there is no unequivocal proof for the involvement of these chloroplast DNA polymerases in ctDNA replication, and the possibility exists that they are repair enzymes whereas the true replicative enzymes have yet to be described. Evidence favoring a role of these DNA polymerases in DNA replication includes the fact that they are highly active (and the only ones detected so far) in the in vitro replication systems described above. Furthermore, electron microscopic localization of radiolabeled thymidine incorporation to the chloroplasts of cultured rice cells in the presence of aphidicolin implicates the γ-like DNA polymerase in ctDNA replication. Complicating the current picture are the multiple forms of ctDNA polymerases eluting at different salt concentrations from DEAE-cellulose in some higher plant species (Heinhorst et al., 1990b; Tewari et al., 1976). Closer analysis of the biochemical properties of the two enzyme forms from soybean chloroplasts revealed no differences with respect to optimal assay requirements, template preferences, effects of various inhibitors and estimated molecular mass, suggesting that the two forms might be related to one another by covalent modifications or minor partial proteolytic degradation. Further work is needed to distinguish more clearly the DNA polymerase forms and to establish their roles in ctDNA replication and/or repair.

DNA topoisomerase I and II activities have been

observed in higher plant chloroplasts (Siedlecki et al., 1983; Lam and Chua, 1987; Nielsen and Tewari 1988; Fukata et al., 1991). The type II enzyme from pea chloroplasts is sensitive to low levels of novobiocin, an inhibitor of *E. coli* DNA gyrase. The first DNA topoisomerase I activity from higher plant chloroplasts was reported by Siedlecki et al. (1983). The partially purified enzyme was classified as a prokaryotic type, based on its inability to relax positive supercoils and its dependence on a divalent cation for activity. A similar, prokaryotic type ctDNA topoisomerase I from pea, when purified to apparent homogeneity, displayed a single polypeptide band of 112 kDa. A recent report has challenged this classification, since topoisomerase I activities from spinach and cauliflower chloroplasts (Fukata et al., 1991), upon re-examination under a different assay regime, appeared to be stimulated, but not absolutely dependent on Mg^{2+}. Furthermore, the DNA topoisomerases type I from cauliflower and spinach chloroplasts were found to relax positive as well as negative supercoils, using a different assay scheme. In order to gain insight into the possible role of ctDNA topoisomerase I in replication of the plastome, an in vitro ctDNA replication system largely devoid of topoisomerase I activity was prepared and the effect of added enzyme on replication rates was determined (Nielsen and Tewari, 1988). Using recombinant plasmid DNA containing the pea ctDNA D-loop regions as templates, replication was stimulated between 1.8- and 6-fold.

Nielsen and colleagues (1991) described a priming activity that was partially purified from pea chloroplasts. With an estimated size of 115-120 kDa, the DNA primase is considerably smaller than the multisubunit, high-molecular-mass chloroplast RNA polymerase, and could be distinguished from the latter by its insensitivity to the inhibitor tagetitoxin and the lack of cross-reactivity to antibodies raised against purified chloroplast RNA polymerase. In vitro, the enzyme can prime a single-stranded DNA template, and product analysis from a coupled priming-replication assay in vitro suggests that the enzyme preferentially synthesizes primers at the origin-containing, cloned pea ctDNA D-loop sites. An in vitro system devoid of DNA primase, but containing ample RNA polymerase activity, did not support significant DNA synthesis on these single-stranded DNA templates.

Upon determining classical replication activities in an initiation-competent chloroplast extract from soybean tissue culture cells, we have detected all of the above activities, an RNA polymerase, as well as several DNA-binding activities, one of which showed specificity for single-stranded templates (unpublished observations). Briat and colleagues (1984) have found that a DNA-binding protein partially purified from chloroplast nucleoids cross-reacted with polyclonal antiserum raised against the histone-like protein HU from *E. coli* and from a cyanobacterium. The protein appeared to be composed of two subunits whose molecular mass was estimated to be 17,000.

We have partially purified and characterized a DNA-dependent ATPase (helicase) from soybean chloroplasts (Cannon and Heinhorst, 1990). The enzyme required a divalent metal cofactor, preferably Mg^{2+}, and a nucleoside triphosphate. While strand displacement activity was highest in the presence of ATP, many other ribo- and deoxyribonucleotides were able to support helicase activity. The chloroplast DNA helicase was active on templates with a single stranded 3′ or 5′ tail and could also displace fully matched primers from a single stranded circular template. Its role in DNA replication and the possible presence of other helicases in chloroplasts have not yet been closely examined.

Apart from biochemical characterization and indirect indications of their possible function in plastome replication, no further insight has yet been gained into the roles of these and other potential DNA replication proteins. Central to understanding both mechanism and regulation of replication initiation would be the characterization of factors that recognize and bind the ctDNA origin, analogous to the DnaA protein in *E. coli* and the SV40 T antigen (Kornberg and Baker, 1992). In *Chlamydomonas*, three proteins have been detected in fragment retention assays that specifically bound cloned fragments containing *ori*A (Nie et al., 1987). Subsequent work identified one of these proteins, a chloroplast gene product, as an iron sulfur protein related to NADH dehydrogenase (Wu et al., 1989). The authors offered some suggestions for a possible function of this association, but direct evidence linking the protein to initiation of ctDNA replication is lacking.

ORIGINS OF REPLICATION

As described above, the chloroplast genome exhibits a great deal of conservation between plant species in both gene order and sequence homology. It is reasonable, then, to expect that some similarities would exist also between plastome replication origins from various species. However, experimental evidence so far has provided little in the way of a consensus sequence for the origin and does not point to a map location that is preferred as an initiation point.

The earliest attempts to define a ctDNA replication origin were made in *Euglena gracilis*. The plastome of this unicellular alga lacks the inverted repeat morphology, but does contain tandem copies of the rRNA genes in addition to a third 16 S rRNA gene that is located upstream from the other two copies (Fig. 1). By electron microscopic mapping of replication intermediates, the origin was localized independently by two groups to a region approximately 6 kbp upstream from the supplementary 16 S rRNA gene (Ravel-Chapuis et al., 1982; Koller and Delius, 1982).

The replication origin of *Chlamydomonas reinhardtii* ctDNA, which does contain inverted repeats and uses the D-loop initiation mode, was mapped by Wu and colleagues (Waddell et al., 1984) to the small, unique region close to one of the inverted repeats (A,B in Fig. 1). One of the D-loop initiation sites, *ori*A, is located 10 kbp and the second, *ori*B, 16.5 kbp upstream from the 16 S rRNA genes. *ori*A has been more precisely mapped by heteroduplex analysis and in vitro initiation of replication to a 420 bp fragment which also contains the coding sequence for *rpl*16, the gene for a protein from the large ribosomal subunit (Lou et al., 1987). Sequence analysis demonstrated that *ori*A contains, like all putative ctDNA origins so far reported, numerous regions with potential for stem-loop structures (Wu et al., 1986).

When the previously observed D-loop-containing fragments from pea chloroplasts were mapped by electron microscopy, they were found to be located in proximity to the rRNA genes (Meeker et al., 1988). One D-loop initiation site is located within the spacer region, and the other approximately 7 kbp away, downstream of the 23 S rRNA gene (I,II in Fig. 1).

Chiu and Sears (1992) recently mapped two D-loop regions to the inverted repeats of the *Oenothera* plastome by electron microscopic analysis of replicative intermediates. *ori*A was located in the spacer region between the rRNA genes, close to the 16 S rRNA gene and *ori*B was found 4 kbp away, upstream of the 16 S rRNA gene (O_A,O_B in Fig. 1). This arrangement is remarkable in that it would result in four D-loop origins for each plastome molecule. Whether both pairs of origins can initiate simultaneously is unknown. While *Oenothera* is the only higher plant reported to begin replication via a possible dual D-loop mechanism within the inverted repeat region, proplastids from a tobacco suspension culture were shown by electron microscopic mapping and preferential in vivo labeling to have one D-loop initiation site within the inverted repeat (Nt in Fig. 1), resulting in a net two halves of two D-loop initiating pairs according to the two-phase replication model (Takeda et al., 1992). These are the first experiments in a higher plant that provide evidence for a ctDNA replication origin in vivo.

Another approach to the identification of origins has been to determine the autonomous replication ability of cloned ctDNA fragments in yeast (ARS sequences). Several such sequences were found for the plastomes from *Chlamydomonas* (Rochaix et al., 1984; Vallet and Rochaix, 1985), tobacco (Ohtani et al., 1984), maize (Gold et al., 1987) and *Petunia* (Overbeeke et al., 1984). In *Chlamydomonas*, most of the DNA fragments displaying ARS activity did not coincide with the mapped D-loop origins, and in the case of maize were not efficient in vitro templates. One *Petunia* chloroplast ARS which contains regions that are highly homologous to the *ori* region on the *Euglena* plastome was reported to direct site-specific nucleotide incorporation in *Petunia* chloroplast extracts (de Haas et al., 1986, 1987). The *Petunia* ARS is located in the small unique region of the plastome in close proximity to one of the inverted repeats.

To date, fine mapping of origin regions on chloroplast DNA has not progressed sufficiently to allow implication of a distinct sequence that is used as an actual origin. Homology exists between the 420 bp fragment containing *ori*A of the *Chlamydomonas* plastome and a 410 bp region of the 1.3 kbp maize ctDNA fragment that supports in vitro dNTP incorporation (Gold et al., 1987; Carrillo and Bogorad, 1988). However, this region contains the highly conserved *rpl*16 gene and may be only circumstantially involved with any origin function. Another homology exists between a 100 bp region of a cloned 5.9 kbp DNA fragment from *Petunia* chloroplasts that contains the ARS element described above, and the putative origin of plastome replication from *Euglena* (de Haas et al., 1986). Also in this case, finer mapping is required to determine if the homology is fortuitous or represents actual sequence conservation between true origin elements.

While it is true that no particular position on the plastome presently seems to be favored as an origin location, it is remarkable that most of the putative origins reported so far are in close proximity to rRNA genes on the ctDNA (Fig. 1). It is interesting to note that in nuclear DNA of sea urchin, *Tetrahymena*, yeast and *Physarum*, initiation sites have been observed in the spacer regions between rRNA genes (Kornberg and Baker, 1992).

CONCLUSIONS

As evident from the above discussion, the current state of DNA replication studies in chloroplasts is rather a confusing one. Lack of a genetics system in plastids has prevented mutant analysis that has been instrumental in identifying DNA replication enzymes in bacteria and yeast. Likewise, difficulty in synchronizing ctDNA replication and the impermeability of the organelle to immediate DNA replication precursors has hindered studies that would unambiguously define regions of the plastome acting as origins in vivo. This leaves electron microscopic observation of ctDNA replication intermediates and in vitro DNA synthesis with rather crude chloroplast components as the bulk of the evidence that has accumulated so far. Results obtained with diverse plant types and with plastomes of different morphologies do not yet allow us to present a generalized picture of ctDNA replication. What can be stated with confidence is that in higher plants the ctDNA replication machinery seems to be encoded in the nucleus, which would place regulation of plastome synthesis under cellular control. Furthermore, all higher plant chloroplasts so far studied initiate replication of their genome at a minimum of two sites, but the exact mode of replication following initiation is as yet unclear. Probably all of the enzymatic activities one would expect to be necessary for DNA replication are present in chloroplasts, although detailed biochemical studies of any but DNA polymerase have not been conducted for most plant species. While partially purified chloroplast extracts from several plant sources have been shown to prefer particular cloned regions of ctDNA as in vitro DNA synthesis templates, to date no evidence of true in vitro DNA replication, when compared to evidence presented in better defined replication systems, has been presented.

It seems then that much additional work will be needed to elucidate completely the mechanism and regulation of ctDNA replication. Several new techniques offer potential as tools in this pursuit. Pulsed-field gel electrophoresis has already been shown to be capable of resolving plastomes and plastome multimers (Bendich and Smith, 1990; Deng et al., 1989), and perhaps further refinement of its application to ctDNA replication will allow direct study of largely intact replication intermediates that would be invaluable in determining the mode of replication used by chloroplasts.

Two-dimensional agarose gel electrophoresis has been successfully employed to determine in vivo replication origins in several animal systems and in yeast (Umek et al., 1989; Vaughn et al., 1990; Fangman and Brewer, 1991). The method, pioneered by Huberman and colleagues (Huberman et al., 1987) and by Brewer and Fangman

(1987), allows separation and identification of replication intermediate types based on their shape, which is then used to pinpoint origins by homologous hybridization with probes specific to various regions of the DNA in question. Preliminary evidence obtained with ctDNA from soybean suspension cells indicated that a region near the *rpl*16 gene may be involved in replication initiation, since intermediates have been detected by two-dimensional electrophoretic analysis that are not consistent with those expected if this region were the site of a simple replication fork passing through (Cannon et al., 1992).

Biolistic transformation of higher plant chloroplasts may provide a direct method for the identification of sequence elements that are able to act as replication origins in vivo. The technique involves penetration of cell and chloroplast with tungsten beads coated with the DNA of choice and has already been successfully employed to transform tobacco and *Chlamydomonas* chloroplasts (Boynton et al., 1988; Blowers et al., 1989; Daniell et al. 1990; Svab et al., 1990). Examination of the state of the DNA that was introduced into the tobacco organelles in this manner revealed that, rather than existing as an independently replicating entity, the foreign DNA was integrated into the plastome. A systematic study of potential origin-containing DNA fragments, and subsequent mutational analysis of those regions that are able to support plastome-independent replication in vivo, may help to identify sequence elements that are necessary for origin function in the chloroplast.

Finally, use of cultured plant cells provides a consistent, high volume source of actively replicating chloroplasts that can be used to purify replication components and intermediates. Cultures offer the additional potential for manipulation, such as synchronization of plastome replication, as recently demonstrated by Takeda and colleagues (1992) for tobacco.

Use of these emerging techniques, together with continued application of already existing methods that have been successful in other systems, will undoubtedly allow the formulation of a model that incorporates all existing and, at present often confusing, data.

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