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Fine mapping of replication origins (*ori*A and *ori*B) in *Nicotiana tabacum* chloroplast DNA

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ABSTRACT

Using a partially purified replication complex from tobacco chloroplasts, replication origins have been localized to minimal sequences of 82 (pKN8, positions 137 683–137 764) and 243 bp (pKN3, positions 130 513–130 755) for *ori*A and *ori*B respectively. Analysis of *in vitro* replication products by two-dimensional agarose gel electrophoresis showed simple Y patterns for single *ori* sequence-containing clones, indicative of rolling circle replication. Double Y patterns were observed when a chloroplast DNA template containing both *ori*s (pKN9) was tested. *Dpn*I analysis and control assays with *Escherichia coli* DNA polymerase provide a clear method to distinguish between true replication and DNA repair synthesis. These controls also support the reliability of this *in vitro* chloroplast DNA replication system. EM analysis of *in vitro* replicated products showed rolling circle replication intermediates for single *ori* clones (*ori*A or *ori*B), whereas D loops were observed for a clone (pKN9) containing both *ori*s. The minimal *ori* regions contain sequences which are capable of forming stem–loop structures with relatively high free energy and other sequences which interact with specific protein(s) from the chloroplast replication fraction. Apparently the minimal *ori* sequences reported here contain all the necessary elements for support of chloroplast DNA replication *in vitro*.

INTRODUCTION

Photosynthesis and the biosynthesis of many important metabolites occur in chloroplasts. Some of the genes for these functions are encoded in chloroplast DNA (ctDNA). Although many aspects of chloroplast gene sequence, structure and gene expression are very similar to bacterial genes, ctDNA replication is unique as compared with replication in other systems. Relatively little is known about the molecular mechanism of ctDNA replication, although a general mechanism for ctDNA replication is known (1). According to the existing model ctDNA replication begins by the introduction of two displacement loops (D loops) 6–7 kbp apart in the ctDNA (1,2). Each D loop is generated by unidirectional replication from an origin, resulting in displacement of a single-stranded region. The D loops expand toward each other and fuse to form a Cairns structure, with replication proceeding bidirectionally around the ctDNA. After the daughter molecules are synthesized replication may continue by a rolling circle mechanism. D loops of 800–900 bp were found in 15–30% of the total number of intact supercoiled ctDNA molecules examined by electron microscopy (for reviews on ctDNA replication see 2–4). It has been reported that the number and location of ctDNA replication origins may vary in different species (2). Differences in location of ctDNA replication origins may be expected as the overall size and organization of the genome varies from lower organisms to higher plants. Most plant species show low sequence conservation in some intergenic regions and most reports on ctDNA replication origins place them in intergenic regions.

Two-dimensional (2D) agarose gel electrophoresis has been successfully employed to examine *in vivo* and *in vitro* replication intermediates in several systems (5–8). Using this approach partially purified chloroplast extracts from several plant sources have been shown to prefer specific cloned regions of ctDNA as *in vitro* DNA synthesis templates, yet to date it has been difficult to distinguish between true DNA replication and random repair synthesis (2,9). Using 2D gel electrophoresis (5) and other techniques we have recently reported the identification and localization of ctDNA replication origins (*ori*A and *ori*B) in each inverted repeat (IR) of tobacco (10,11). Four *ori*s have been reported for *Oenothera* due to their location as identical pairs in each IR (12). The location of *ori*A may be conserved, as the sequence of this region shows a high degree of homology among species (2,13), whereas *ori*B shows homology only between tobacco and petunia among the sequenced genomes. The identification of minimal *ori* sequences and controlling elements may provide the basis for producing a replicating vector for chloroplast genetic engineering. Although such vectors may not be stable after introduction into chloroplasts, their copy number may increase by replication to facilitate homologous recombination into the chloroplast genome. Hence, an understanding of the molecular biology of ctDNA replication may help to address problems related to stable transformation of the chloroplast organelle. In order to achieve this it is essential to determine the minimal sequence elements required for replication. In addition, the development of a reliable *in vitro* ctDNA replication system will allow study of the role of individual proteins in replication. In this study we report identification of the minimal sequences of *ori*A (82 bp) and *ori*B (243 bp) required for *in vitro* replication of tobacco ctDNA by 2D gel electrophoresis using a partially purified chloroplast replication fraction. We demonstrate herein the

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specificity of the replication fraction by *Dpn*I and other control assays. Electron microscopy has been used to examine the mode of replication *in vitro*.

MATERIALS AND METHODS

Purification of the replication fraction

Two to three week old tobacco leaves were used to isolate chloroplasts. A Triton lysate mixture was prepared and the replication fraction was purified as described (14–16), except for addition of a phosphocellulose column after the DEAE–cellulose column and bound proteins were step eluted for all the columns used. Briefly, the Triton lysate was loaded onto a 60 ml pre-equilibrated DEAE–cellulose (DE-52; Whatman) column. Equlibration was with buffer A (50 mM Tris–HCl, pH 8.0, 25% glycerol, 10 mM B-mercaptoethanol and protease inhibitors: 10 mM benzamide, 1 mM PMSF, 10 mM sodium metabisulfite) containing 50 mM KCl. After loading the column was washed extensively with ~10 column vol. buffer A + 50 mM KCl. The bound proteins were eluted with buffer A containing 600 mM KCl. Ten milliliter fractions were collected and tested for ctDNA polymerase activity (11). Active fractions were pooled and dialyzed against buffer B (20 mM potassium phosphate buffer, pH 8.0, 25% glycerol, 10 mM B-mercaptoethanol and protease inhibitors: 10 mM benzamide, 1 mM PMSF, 10 mM sodium metabisulfite), with several changes of buffer. The dialyzed fraction was loaded onto a 20 ml activated phosphocellulose (Whatman) column equilibrated with buffer B. The column was washed extensively with buffer B and the bound proteins were collected as 8 ml fractions with a step elution of 400 mM phosphate buffer B. Fractions containing DNA polymerase activity were dialyzed against buffer C (50 mM Tris–HCl, pH 8.0, 25% glycerol, 1 mM $MgCl_2$, 1 mM EDTA, pH 8.0, 1 mM B-mercaptoethanol and protease inhibitors: 10 mM benzamide, 1 mM PMSF, 10 mM sodium metabisulfite) containing 100 mM KCl. The dialyzed fraction was loaded onto a 10 ml column of heparin–Sepharose (Pharmacia) equilibrated with the same buffer. After thorough washing with buffer B containing 100 mM KCl the bound proteins were collected as 5 ml fractions by step elution with buffer B containing 600 mM KCl. DNA polymerase activity was assayed for each fraction. Active fractions were tested further for replication activity with specific ctDNA clones as described (10). Fractions which showed high DNA polymerase activity and low nuclease activity were used for further studies.

In vitro DNA replication assay

In vitro DNA replication reactions were carried out as described (13) with various tobacco ctDNA clones (Fig. 2). Smaller clones were made by PCR using specific primers, with cloning into the *Sma*I site of pUC19. These clones were confirmed by standard sequence analysis to ensure the correct sequence. After initial comparison by [^{3}H]TTP incorporation *in vitro* replication products labeled with [^{32}P]dCTP were prepared and analyzed by 2D gel electrophoresis as described (11).

*Dpn*I assay

Radiolabeled *in vitro* replication products of different clones were restricted with *Dpn*I, which requires methylation of the A nucleotide in the recognition sequence for restriction. Reaction products were separated in a 1% agarose gel. After electrophoresis the gel was soaked in 5% TCA for 30 min, dried and exposed to X-ray film.

Electron microscopy

DNA spreads were done according to the Davis and Davidson method for electron microscopy (17). One hundred nanograms of purified *in vitro* replication product were mixed in a solution containing 0.2 M ammonium acetate, pH 5.5, 0.001 M EDTA, pH 7.5, and 0.0003% (w/v) cytochrome c (type VI; Sigma). A droplet of 250 µl was formed and spread DNA was picked up on a Parlodion support film on 400 mesh copper grids. DNA was stained with uranyl acetate (0.1 mM uranyl acetate in 90% ethanol) by adding 5 µl to the grid for 30 s. Grids were then dipped in 95% ethanol for 5 s, air dried and rotary shadowed at an angle of 5° with gold/palladium (60%/40%). The grids were examined with a Zeiss transmission electron microscope. Photographs were taken at various magnifications and lengths were measured using an Optimas image analyzer system.

RESULTS

Purification of the chloroplast replication complex

The progress of purification of the replication complex as measured by DNA polymerase activity through various chromatographic steps is shown in Table 1. Since the activity of the protein was underestimated in the Triton lysate due to the presence of high amounts of nucleases, the specific activity has been calculated starting from the DEAE–cellulose (DE-52) column. Though the DE-52 fraction showed DNA polymerase activity, the fraction showed some nuclease activity when incubated with DNA (data not shown). Most of the nucleases were apparently removed in later column steps. When the heparin–Sepharose column was eluted with a linear gradient of 100 mM–1 M KCl in buffer C, DNA polymerase activity was found in the latter fractions, whereas DNA binding proteins eluted in earlier fractions (data not shown). Hence, in these experiments we used a step elution for each column in order to retain necessary replication activities in the same fraction.

Table 1. Purification steps for the replication complex from tobacco chloroplasts

Source	Protein (mg)	Volume (ml)	Specific activity[a]
Triton lysate	402	150	Not determined
DEAE–cellulose	26.8	70	60.38
Phosphocellulose	10.5	32	805
Heparin–Sepharose	0.73	15	7647.8

[a]One unit of DNA polymerase activity is defined as the amount of enzyme that will catalyze the polymerization of 1 µmol [^{3}H]TTP using activated calf thymus DNA as template at 37°C in 30 min. Specific activity is expressed as units DNA polymerase/mg protein.

2D gel analysis of replication intermediates

Neutral/neutral 2D agarose gel electrophoresis (5,11,18) was used to detect and analyze replication intermediates from *in vitro* reactions. A schematic diagram for established patterns generated by this technique is shown in Figure 1. A theoretical discussion of the type of patterns which may be observed by 2D gel

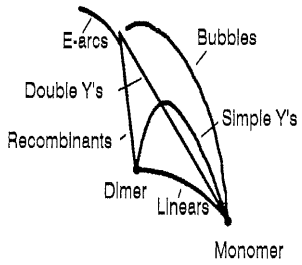

Figure 1. Schematic representation of replication intermediate patterns observed by 2D gel electrophoresis (based on refs 5,8). Linear monomer and dimer spots, the connecting diagonal of broken linear molecules and replication intermediate patterns are indicated. The E arc represents atypical patterns expected for D loops due to the inability of restriction enzymes to digest single-stranded regions.

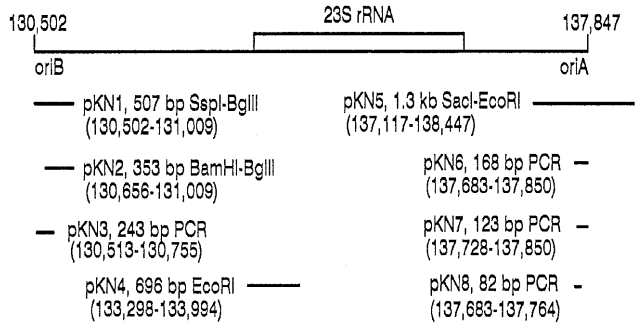

Figure 2. Map of the 23S rRNA gene region including flanking regions and clones used in this study. Coordinates indicate tobacco ctDNA sequences from IR_A (28); these sequences are duplicated in IR_B.

electrophoresis of D loop-containing molecules has recently been reported (8) and includes a proposed extended arc (E arc) pattern (Fig. 1). E arcs show atypical patterns as a result of the inability of restriction enzymes to cleave single strands and contain long single-stranded regions joined to double-stranded DNA at different locations relative to the ends of the linearized fragments, making it difficult to predict the pattern which would be generated (8). Specific tobacco subclones (Fig. 2) were tested as templates for *in vitro* DNA replication using the partially purified tobacco chloroplast fraction and the results are shown in Figures 3–5. Only simple Y replication patterns along with X-shaped recombination patterns are expected for small clones containing a single D loop region (see Discussion), as seen in Figures 3 and 4. For most templates a continuous diagonal signal representing broken linear molecules was also observed. The 507 bp template pKN1 from *ori*B exhibited a high level of *in vitro* DNA replication activity as detected by the generation of a strong simple Y pattern from the linearized product (Fig. 3A). However, the 353 bp pKN2 clone, which lacks 154 bp containing upstream sequences and most of the stem–loop structure at one end of pKN1, was found to support little or no replication activity (Fig. 3B), whereas the 243 bp PCR clone pKN3, which contains the complete stem–loop structure, showed a strong simple Y pattern (Fig. 3C). Similarly, the pKN5 template from *ori*A, which contains a 1.3 kb insert, showed high replication activity and showed the expected simple Y pattern in the 2D gel (Fig. 4A). However, for smaller subclones from pKN5, such as pKN6, pKN7 and pKN8 (Fig. 4B, C and D respectively), there is some variation in the intensity of the signal though the input amount of template DNA in each reaction was identical. The exposure time for pKN7 was 4 days longer than for pKN6 and pKN8. The 82 bp pKN8 clone, which spans only the stem–loop structure and minimal flanking sequences, showed very high activity as compared with the other templates. No replication intermediates were detected for a clone (pKN4) from a region between the two *ori*s (Fig. 3D) or for pKN1 incubated with either Klenow fragment (Fig. 3E) or with DNA polymerase I (Fig. 3F) from *Escherichia coli* instead of the chloroplast replication fraction complex. These results suggest that the stem–loop sequences in *ori*A and *ori*B (10,11) play a necessary role for replication *in vitro* and that the patterns observed are not due to repair synthesis.

In order to determine what type of 2D gel pattern would be generated for a clone containing both *ori*s together, template pKN9 was constructed. This was done by inserting the 4.5 kb

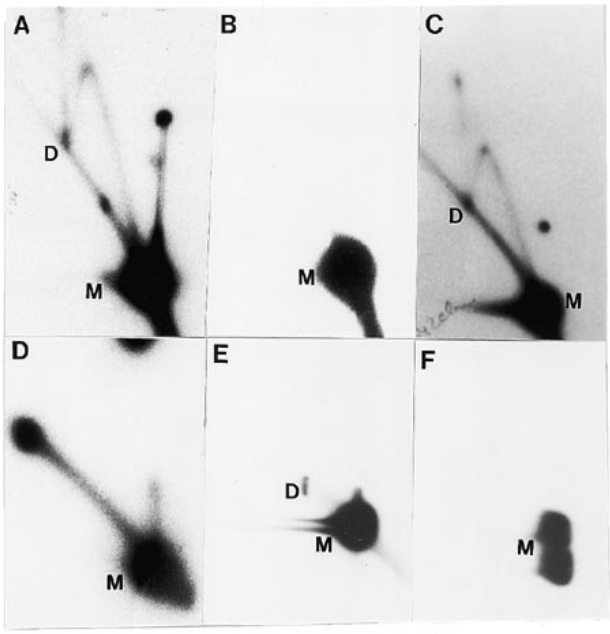

Figure 3. *In vitro* replication products were analyzed by 2D gel electrophoresis as described in Materials and Methods. For each template products were linearized with *Pst*I. (**A**) pKN1, (**B**) pKN2, (**C**) pKN3 and (**D**) pKN4. As controls *in vitro* reactions were performed with pKN1 using *E.coli* Klenow fragment (**E**) or DNA polymerase I (**F**), instead of the chloroplast replication fraction. Electrophoresis in the first dimension is from left to right and the second dimension is from top to bottom. Each gel was exposed to X-ray film for 3 days.

*Eco*RI fragment from *ori*A into the *Eco*RI site of pKN1. The resultant pKN9 template was restricted with *Sac*I after the *in vitro* reaction for 2D gel analysis. This clone showed a high level of *in vitro* replication activity (Fig. 5). A double Y pattern will be generated if two replication forks are converging from both ends of the fragment, such as may occur after the two expanding D loops have fused to form a Cairns intermediate (18). *Sac*I restriction of pKN9 gives two bands (3.1 kb containing *ori*B and 4.5 kb containing *ori*A) and two steep straight diagonal lines similar to double Y patterns are observed by 2D gel analysis (small arrowhead in Fig. 5). In addition to the double Y, a pattern somewhat similar to replication bubbles but extending past dimer size was observed (large arrowhead in Fig. 5). This atypical

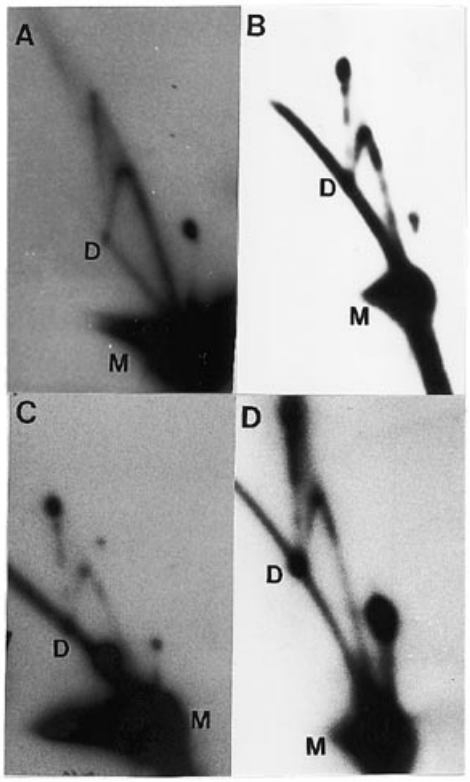

Figure 4. 2D gel analysis of *in vitro* replication products for *ori*A templates. (**A**) pKN5, (**B**) pKN6, (**C**) pKN7 and (**D**) pKN8. Conditions were the same as in Figure 3, except that the gel in (C) was exposed to X-ray film for 7 days.

pattern may include an E arc, as suggested by Han and Stachow (8), representing a large bubble structure not restricted by the enzyme, which may occur prior to fusion of the D loops.

Specificity of the *in vitro* reaction

To ensure that the purified replication complex is specific to the presence of *ori* sequences *Dpn*I assays were carried out after the *in vitro* reactions (Fig. 6). The restriction endonuclease *Dpn*I recognizes the sequence GATC and restricts between the A and T nucleotide positions only if the A is methylated in both DNA strands. During *in vitro* replication the newly synthesized strand is not methylated. Hence, *Dpn*I cannot restrict the newly synthesized strand. From Figure 6 it is clear that *in vitro* replication products from the pKN1, pKN6, pKN7 and pKN8 (lanes 1, 4, 7 and 9 of Fig. 6A and B) templates showed resistance to *Dpn*I, whereas pUC19 and pKN1 incubated with Klenow fragment (data not shown) and the non-*ori* clones (Fig. 6, lane 5) were completely restricted. For *ori*B pKN1 showed complete resistance to *Dpn*I, while pKN2 showed complete digestion by *Dpn*I, reflecting the same specificity as observed by 2D gel analysis. For *ori*A clones some DNA is completely restricted, suggesting that only a portion of the template molecules are replicated *in vitro*. Some radioactive incorporation is observed with all clones, suggesting a certain level of random incorporation. This assay, coupled with assays using Klenow fragment or DNA polymerase I as discussed in the previous section, is a powerful tool to detect specific replication activity.

Figure 5. *In vitro* replication products of clone pKN9 containing both *ori*s were analyzed by 2D gel electrophoresis after *Sac*I restriction. The small arrowhead points to the double Y pattern, whereas the large arrowhead points to the bubble-like pattern. Conditions are as in Figure 3.

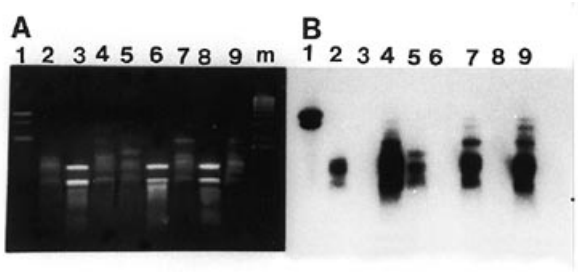

Figure 6. *Dpn*I assays of *in vitro* replication products to distinguish replication from repair activity. (**A**) Ethidium bromide stained agarose gel of *Dpn*I-restricted reaction products. (**B**) Autoradiograph of the same gel. Lane 1, pKN1; lane 2, pKN2; lane 3, cold pKN1; lane 4, pKN6; lane 5, pKN4; lane 6, cold pKN6; lane 7, pKN7; lane 8, cold pKN8; lane 9, pKN8. Clones in lanes 3, 6 and 8 were not subjected to the *in vitro* reactions, as controls, and show complete digestion. Lane m, 1 kb ladder as a molecular weight marker.

Electron microscopic analysis

In vitro replication products of different clones with unlabeled dNTPS were processed for electron microscopic observation. Clones containing a single *ori* (either *ori*A or *ori*B) formed σ-like structures (Fig. 7A and B), indicative of rolling circle replication. The length of the tail from the σ structure reflects the extent of replication, with some longer than unit length. Reaction products with templates carrying non-*ori* sequences (Fig. 7C) or clones having an *ori* sequence but treated with no protein (data not shown) showed only circular molecules. Clones containing both *ori*s (pKN9) showed the presence of D loop structures (Fig. 7D). Observation of a D loop structure suggests that the mode of replication of pKN9 is different from other clones containing only one *ori* and having tails, as suggested by 2D gel analysis.

DISCUSSION

Two-dimensional agarose gel electrophoresis is a powerful technique to identify replication intermediates and has been utilized in various systems (see Introduction). We report here the usefulness of this technique to identify minimal sequences required for replication *in vitro* of *ori*A and *ori*B from tobacco

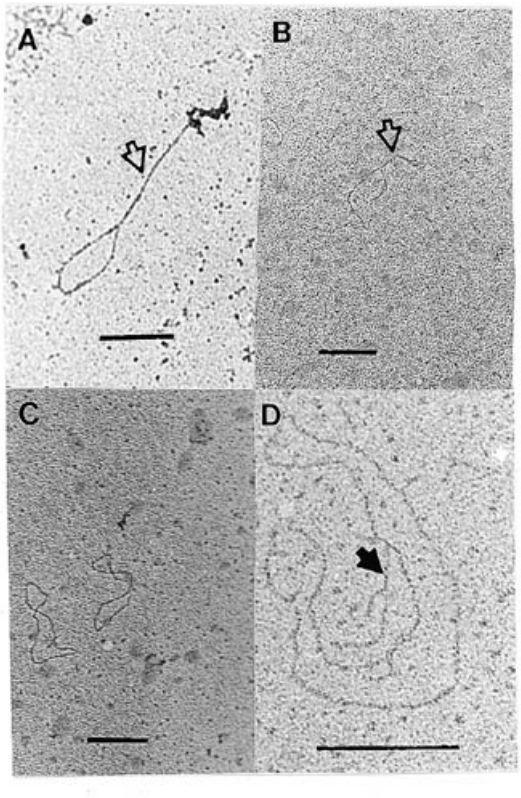

Figure 7. Electron microscopy analysis of *in vitro* ctDNA replication products. (**A**) Clone pKN1 (*ori*B) shows a rolling circle replication intermediate. (**B**) Clone pKN8 (*ori*A) shows a rolling circle replication intermediate. (**C**) A control template (pKN2) shows only circular DNA. (**D**) Clone pKN9 containing both *ori*s shows a D loop intermediate. Open arrows indicate tails whereas the filled arrow shows the D loop. Bar 1 kb.

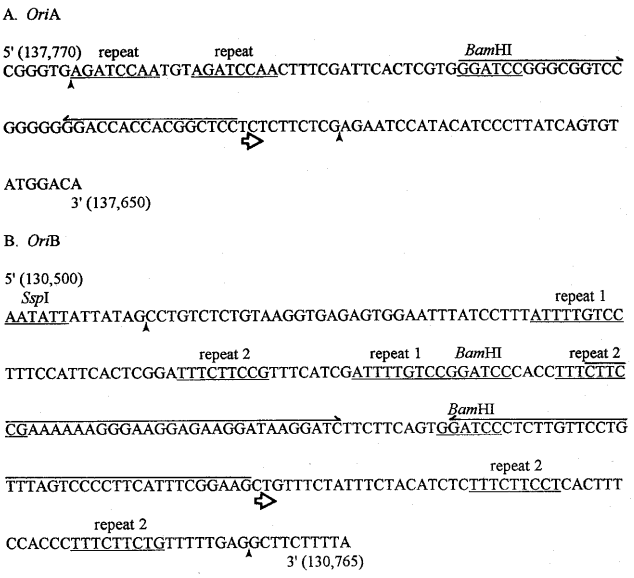

Figure 8. Stem–loop structure-forming regions of *ori*A (**A**) and *ori*B (**B**) from tobacco chloroplast DNA. The long horizontal arrows on top of the sequence indicate the stem–loop-forming structures. The large open arrows indicate the direction of replication from *ori*A and *ori*B. Pertinent restriction sites are shown and direct repeat elements are underlined. The ends of the minimal *ori* sequences from *in vitro* replication experiments are indicated by small vertical arrowheads (82 bp for *ori*A and 243 bp for *ori*B). Sequence coordinates for IR_A are shown at each end of the *ori* sequences from the published tobacco ctDNA sequence (28; EMBL accession no. Z00044). These sequences are also present in IR_B.

ctDNA. Although there are a few previous reports on *in vitro* replication analysis using partially purified chloroplast protein fractions (2,9,13), it is not always clear whether the activity observed reflected a true replicative type of DNA synthesis or repair synthesis. In addition, a reliable method for the purification of a faithful replication system is necessary to carry out biochemical studies. We report here for the first time a reliable and reproducible method to isolate a chloroplast replication complex and show the capability of this complex to distinguish between true replication and non-specific or repair DNA synthesis *in vitro*. The availability of a faithful *in vitro* ctDNA replication system will facilitate further study of the role of individual proteins in replication.

We have earlier reported the identification and localization of *ori* regions in the IR of tobacco ctDNA (10,11). In this study we describe detailed *in vitro* 2D gel analysis of *ori* regions of tobacco ctDNA using a partially purified tobacco chloroplast DNA replication complex. This fraction contains DNA polymerase, topoisomerases, DNA primase, single-stranded DNA endonuclease and helicase, which have been identified and purified to homogeneity from pea chloroplasts (16,19–23). Recently a 43 kDa DNA binding protein from pea chloroplasts has been purified which exhibits non-specific DNA binding and stimulates ctDNA polymerase activity (24). Analysis by 2D gel electrophoresis of *in vitro* replication products from *ori*A or *ori*B clones (pKN1, pKN2, pKN3, pKN5, pKN6, pKN7 and pKN8) showed simple Y patterns (Fig. 4). However, clones containing non-ori sequences (pKN4) did not produce any simple Y pattern (Fig. 3D). Only simple Y patterns are expected for clones containing a single ctDNA replication origin. As shown earlier (10,11), such clones replicate by a rolling circle mechanism (25).

Initial knowledge about the mechanism of ctDNA replication was based on electron microscopic examination of replication intermediates and the occurrence of D loops. We were interested to know the mechanism of *in vitro* replication of the clones containing one or both *ori*s by electron microscopic studies. Electron microscopic analysis of *in vitro* replication products showed circular molecules with tails for single *ori* sequence-containing clones, whereas only circular molecules were seen for non-*ori* sequence clones (Fig. 7A–C). The observation of D loops in pKN9 (Fig. 7D) and rolling circle intermediates with clones containing either *ori* (*ori*A or *ori*B) alone suggest that the mechanism of ctDNA replication *in vitro* and *in vivo* are the same. However, it is not known what causes these changes in the mode of replication. Apparently the distance between the two *ori*s is important to determine the mode of replication used. Further experiments are in progress to examine this.

In order to demonstrate the specificity of the replication fraction we carried out *Dpn*I resistance assays. A *Dpn*I resistance assay has recently been used to characterize autonomous replication of plasmids in chicken cells (26). *Dpn*I restricts pUC19, which is our basic vector for cloning different ctDNA inserts, into 16 fragments, of which the largest is 955 bp. In addition, the insert from pKN1 (*ori*B) contains six recognition sites, whereas the insert in pKN8 (*ori*A) contains three sites for *Dpn*I. If the newly

synthesized DNA is not methylated during the *in vitro* replication reaction, the template will not be completely restricted by *Dpn*I. Specific *ori* sequence clones did not show complete restriction (Fig. 6, lanes 1, 4, 7 and 9). However, the non-*ori* clone pKN4, which contains two *Dpn*I sites in the insert in addition to the sites in the vector, showed complete restriction (Fig. 6, lane 5), although some radioactive label was incorporated. As a control experiment we used template DNA not subjected to the *in vitro* reaction, which showed complete restriction (Fig. 6A, lanes 3, 6 and 8). Thus it is clear that *in vitro* DNA synthesis by the chloroplast fraction represents true replication rather than DNA repair synthesis. Although we cannot rule out the possibility that the fraction contains some DNA repair synthesis activity (i.e. the monomer spot in Fig. 3D and incorporation of radioactivity in the non-*ori* sequence template in Fig. 6B, lane 5), it is clear that the replication fraction contains specific replication activity for the *ori* sequences, for the following reasons. (i) *In vitro* replication reactions using either *E.coli* Klenow fragment or DNA polymerase I in place of the chloroplast replication fraction did not show any simple Y structure by 2D gel analysis (Fig. 3E and F) though the template pKN1 contains *ori*B sequences. Similarly, the *ori*A clone pKN8 also did not show any simple Y pattern with either of the *E.coli* enzymes (data not shown). (ii) *In vitro* replication reactions using the chloroplast replication fraction with non-*ori* sequence clones showed only a monomer spot by 2D gel analysis (Fig. 3D) and these products were restricted completely by *Dpn*I (Fig. 6, lane 5), reflecting random repair incorporation.

The smallest *ori*A clone which showed high *in vitro* replication activity, pKN8, contains 82 bp (137 683–137 764) and is ~1.2 kb from the reported autonomously replicating NICE1 sequence (27). Interestingly, the 82 bp sequence reported here contains the entire stem–loop structure and the two 8 bp direct repeats identified earlier (Fig. 8A; see 10). It has been observed that some protein(s) from the chloroplast fraction binds specifically to this region (Kunnimalaiyaan *et al*., unpublished observation). Analysis of *in vitro* DNA replication products from a set of tobacco ctDNA subclones (Fig. 3A–C) by 2D gel electrophoresis indicated that the 154 bp region from the *Ssp*I site at the end of the IR to the second *Bam*HI site (positions 130 502–130 656) is essential for replication activity. For *ori*B minimal required sequences were localized to 243 bp (Fig. 8B). Though the *ori*B clones pKN1 and pKN3 showed high levels of *in vitro* replication activity, the level of activity was less than that obtained with a clone from the *ori*A region (pKN7, compare Fig. 3A and C with 4D). This may suggest that *ori*A is more active than *ori*B for initiation of replication. Further studies are needed to determine what activates replication from each of these two *ori*s. Multiple binding sites for sequence-specific ctDNA binding proteins in pKN1 and pKN3 have been reported (11). In earlier studies we found that this region has a relatively strong stem–loop structure, four nearly identical 9 bp AT-rich direct repeats and two exact copies of a different 9 bp sequence (Fig. 8B; see 11). These sequences are not found elsewhere in the IR. Our next step in this area of research is to identify and purify the *ori*-specific binding protein(s) and preliminary experiments are in progress in our laboratory. In addition, the minimal *ori* sequences are potential candidates for construction of vectors for chloroplast transformation.

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REFERENCES

1 Kolodner,R.D. and Tewari,K.K. (1975) *Nature (Lond.)*, **256**, 708–711
2 Kunnimalaiyaan,M. and Nielsen,B.L. (1997) *J. Plant Biochem. Biotechnol*., **6**, 1–7.
3 Tewari,K.K. (1988) In Bryant,J.A and Dunham,V.L. (eds), *DNA Replication in Plants*. CRC Press, Boca Raton, FL, pp. 69–116.
4 Heinhorst,S. and Cannon,G.C. (1993) *J. Cell Sci*., **104**, 1–9.
5 Brewer,B.J., Sena,E.P. and Fangman,W.L. (1988) *Cancer Cells*, **6**, 229–234.
6 Dijkwel,P.A., Vaughn,J.P. and Hamlin,J.L. (1991) *Mol. Cell. Biol*., **11**, 3850–3859.
7 Martin-Parras,L., Hernandez,P., Martinez-Robles,M.L. and Schvartzman,J.B. (1992) *J. Biol. Chem*., **267**, 22496–22505.
8 Han,Z. and Stachow,C. (1994) *Chromosoma*, **103**, 162–170.
9 Hedrick,L.A., Heinhorst,S., White,M.A. and Cannon,G.C. (1993) *Plant Mol. Biol*., **23**, 779–792.
10 Lu,Z., Kunnimalaiyaan,M. and Nielsen,B.L. (1996) *Plant Mol. Biol*., **32**, 693–706.
11 Kunnimalaiyaan,M., Shi.,F. and Nielsen,B.L. (1997) *J. Mol. Biol*., **268**, 273–283
12 Chiu,W.-L. and Sears,B.B. (1992) *Mol. Gen. Genet*., **232**, 33–39.
13 Nielsen,B.L., Lu,Z. and Tewari,K.K. (1993) *Plasmid*, **30**, 197–211.
14 Meeker,R., Nielsen,B.L. and Tewari,K.K. (1988) *Mol. Cell. Biol*., **8**, 1216–1223.
15 Carrillo,N. and Bogorad,L. (1988) *Nucleic Acids Res*., **16**, 5603–5620.
16 Nielsen,B.L., Rajasekher,V.K. and Tewari,K.K. (1991) *Plant Mol. Biol*., **16,** 1019–1034.
17 Davis,R.W. and Davidson,N. (1968) *Proc. Natl. Acad. Sci. USA*, **60**, 243–250.
18 Brewer,B.J. and Fangman,W.L. (1988) *Cell*, **55**, 637–643.
19 McKown,R.L. and Tewari,K.K. (1984) *Proc. Natl. Acad. Sci. USA*, **81,** 2354–2358.
20 Nielsen,B.L. and Tewari,K.K. (1988) *Plant Mol. Biol*., **11**, 3–14.
21 Mukherjee,S.K., Reddy,M.K., Kumar,D. and Tewari,K.K. (1994) *J. Biol. Chem*., **269**, 3793–3801.
22 Kumar,D., Mukherjee,S.K., Reddy,M.K. and Tewari,K.K. (1995) *J. Exp. Bot*., **46**, 767–776.
23 Tuteja,N., Phan,T.N. and Tewari,K.K. (1996) *Eur. J.Biochem*., **238**, 54–63.
24 Chen,W., Gaikwad,A., Mukherjee,S.K., Choudhury,N.R., Kumar,D. and Tewari,K.K. (1996) *Nucleic Acids Res*., **24**, 3953–3961.
25 Reddy,M.K., Choudhury,N.R., Kumar,D., Mukherjee,S.K. and Tewari,K.K. (1994) *Eur. J. Biochem*., **220**, 933–941.
26 Bogdanova,A.N., Razin,S.V. and Vassetzky,Y.S. (1995) *Biochemie*, **77**, 880–887.
27 Staub,J. and Maliga,P. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 7468–7472.
28 Shinozaki,K., Ohme,M., Tanaka,M., Wakasugi,T., Hayashida,N., Matsubayashi,T., Zaita,N., Chunwongse,J., Obokata,J., Yamaguchi-Shinozaki,K., Ohto,C., Torazawa,K., Meng,B.Y., Sugita,M., Deno,H., Kamogashira,T., Yamada,K., Kusuda,J., Takaiwa,F., Kato,A., Tohdoh,N., Shimada,H. and Suguira,M. (1986) *EMBO J*., **5**, 2043–2049.