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Localization of Replication Origins in Pea Chloroplast DNA

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The locations of the two replication origins in pea chloroplast DNA (ctDNA) have been mapped by electron microscopic analysis of restriction digests of supercoiled ctDNA cross-linked with trioxalen. Both origins of replication, identified as displacement loops (D-loops), were present in the 44-kilobase-pair (kbp) *Sal*I A fragment. The first D-loop was located at 9.0 kbp from the closest *Sal*I restriction site. The average size of this D-loop was about 0.7 kbp. The second D-loop started 14.2 kbp in from the same restriction site and ended at about 15.5 kbp, giving it a size of about 1.3 kbp. The orientation of these two D-loops on the restriction map of pea ctDNA was determined by analyzing *Sma*I, *Pst*I, and *Sal*I-*Sma*I restriction digests of pea ctDNA. One D-loop has been mapped in the spacer region between the 16S and 23S rRNA genes. The second D-loop was located downstream of the 23S rRNA gene. Denaturation mapping of recombinants pCP 12-7 and pCB 1-12, which contain both D-loops, confirmed the location of the D-loops in the restriction map of pea ctDNA. Denaturation-mapping studies also showed that the two D-loops had different base compositions; the one closest to a *Sal*I restriction site denatured readily compared with the other D-loop. The recombinants pCP 12-7 and pCB 1-12 were found to be highly active in DNA synthesis when used as templates in a partially purified replication system from pea chloroplasts. Analysis of in vitro-synthesized DNA with either of these recombinants showed that full-length template DNA was synthesized. Recombinants from other regions of the pea chloroplast genome showed no significant DNA synthesis activity in vitro.

Chloroplast DNA (ctDNA) in higher plants exists as multimers of homogeneous sequence of about 120 to 160 kilobase pairs (kbp) (8, 13). The molecular events in the replication, recombination, and segregation of organelle DNA are poorly understood. Replicative intermediates of ctDNA have been well defined by Kolodner and Tewari (7, 9), using electron microscopy (EM). Replication of pea and maize ctDNAs has been shown to proceed by the introduction of displacement loops (D-loops) in the supercoiled ctDNA. Detailed analysis of pea ctDNA replicative intermediates has shown that two D-loops expand toward each other and initiate formation of Cairns replicative forked structures. The small Cairns forked structures expand bidirectionally until termination takes place at a site that is 180° around the circular molecule from the initiation site. Electron microscopic studies have further shown that ctDNA molecules also replicate by rolling-circle-type structures. Denaturation mapping studies (10) on the tails of rolling-circle molecules showed that the initiation of the rolling-circle intermediates lies at or near the termination site of the Cairns round of replication.

The mapping of the initiation sites of Cairns replicative intermediates in *Euglena gracilis* has recently been reported by Koller and Delius (6) and Ravel-Chapuis et al. (15). They have identified an apparent single origin of replication by electron microscopic analysis of replication forks about 5 to 6 kbp upstream of the 5′ end of the extra or supplementary 16S rRNA gene and in the close vicinity of a previously described polymorphic region known to contain a variable number of short repeats (5). Waddell et al. (19), using another unicellular alga, have mapped the replication origins in *Chlamydomonas* ctDNA by using EM. One replication origin was mapped at about 10 kbp upstream of the 5′ end of a 16S rRNA gene. The second origin was spaced 6.5 kbp apart from the first origin and was about 16.5 kbp upstream of the same 16S rRNA gene. In a recent publication, Wu et al. (21) have sequenced the insert of pSC3-1, which includes the 0.42-kbp D-loop region, as well as 0.2 kbp to the 5′ end and 0.43 kbp to the 3′ end of the D-loop region. The sequence was found to be A+T rich and contained four large stem-loop structures. Wu et al. (21) have also used clones SC3-1 and R-13 as templates in a crude in vitro replication system from *Chlamydomonas reinhardtii*. Both recombinants were found to act as templates. The authors did not report on the template activity of other recombinants containing ctDNA. A similar approach, to identify initiation sites in the ctDNA of a higher plant, has been taken by Gold et al. (4). Maize ctDNA sequences representing 94% of the chloroplast genome have been surveyed for their activity as autonomously replicating sequences in yeasts and as templates for DNA synthesis in vitro by a partially purified chloroplast DNA polymerase. The smallest of the active subfragments was the 1,368-base-pair (bp) *Eco*RI X fragment, and it has been sequenced.

In this paper, we report on the physical mapping of the replication origins (D-loops) in the ctDNA of pea, a higher plant, by electron microscopy. The identified replication origins have been confirmed by in vitro replication studies by using D-loop-containing ctDNA recombinants as templates in a DNA polymerase system that contains DNA polymerase, DNA-binding proteins, and other yet unidentified proteins.

MATERIALS AND METHODS

Isolation and restriction of DNA. Pea ctDNA used in these experiments was isolated as covalently closed, supercoiled DNA by three successive CsCl-ethidium bromide gradients by the procedure of Kolodner and Tewari (7, 10). Purified DNA preparations were stored as individual, small samples (1 to 2 μg each) that were kept frozen at −20°C until immediately before use. Supercoiled ctDNA isolates were used in EM experiments within 2 weeks. DNA concentrations were estimated by direct comparison of restriction

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band intensities in ethidium bromide-agarose gels against known amounts of restricted marker DNA. Where appropriate, the DNA concentration was calculated from the measured A_{260}.

Restriction of the ctDNA for EM experiments was carried out in the buffers recommended for each restriction enzyme. Bovine serum albumin and reducing agent were omitted from the reaction mixture to simplify the spreading procedure. The restriction digestions of trioxalen-cross-linked DNAs (Tx-DNA) were performed at 37°C for 4 to 16 h, using a 20-fold unit-time excess of enzymatic activity. For restrictions of uncross-linked ctDNA, the reactions were carried out with similar enzymic excess but for a period of 1 to 2 h at 23°C. The specificities of restriction endonuclease site recognition were checked by comparison of agarose gel bands produced by reactions done at 23 and 37°C, using limit digest conditions for the reaction at 37°C. Unless otherwise noted in the figure legends, the reactions were performed in a manner which produced at least 80% complete digestion as determined by EM.

Cross-linking of DNA. Tx cross-linking of ctDNA was performed on supercoiled DNA samples by using the conditions given by Cech and Pardue (1), except that the UV irradiation exposure was performed for 6 instead of 30 min. This gave an estimated extent of cross-linking of about 1 cross-link per 0.5 to 1 kbp. After cross-linking and dialysis of the DNA samples, the restricted Tx-ctDNA showed unaltered agarose gel electrophoresis bands. For the EM experiments, the Tx-DNAs were restricted, digested with 50 μg of proteinase K per ml (self-digested) in 0.1% sodium dodecyl sulfate for 2 to 4 h at 37°C and then dialyzed overnight against 1× TE (10 mM Tris hydroxide, 1 mM disodium EDTA [pH 7.5]), using Spectrapor 6 (Spectrum Medical Industries, Inc.) dialysis tubing with a 50-kilodalton cutoff value.

Electron microscopy. Electron microscopy spreadings were carried out essentially as done by Kolodner and Tewari (10). For the experiments involving uncross-linked ctDNA, the freshly restricted preparations were immediately made up into the spreading buffer: 10× TE, marker DNA (0.02 μg/ml), ctDNA (0.05 to 0.08 μg/ml), cytochrome *c* (30 μg/ml), and 50% formamide. In the denaturation-mapping studies, the formamide concentrations were adjusted as indicated in the figure legends, starting from the basic conditions used by Kolodner and Tewari (10). Details are given in the figure legends. The EM grid replicas were examined in a Zeiss EM 10 (CR) electron microscope using 80-kV accelerating voltage. The electron micrographs were projected onto a translucent glass digitizing tablet (20 by 20 cm; Scriptel SPD 202OT) with a Leitz/Wetzlar Makro Promar projector. The digitizing tablet was connected to a Zenith Z-241 PC computer in conjunction with Sigma-Scan software from Jandel Scientific Corp. for interpretation and analysis of the digital output of the tablet.

Subcloning of ctDNA fragments. The 44-kbp *Sal*I A fragment was subcloned, and two plasmids were used for further analysis. These plasmids were the clone containing the *Pst*I 12.5-kbp ctDNA fragment, termed pCP 12-7, and the clone termed pCB 1-12, containing the 10-kbp *Bam*HI ctDNA fragment. Denaturation loop mapping of supercoiled plasmid DNAs was done by using the conditions of Cech and Pardue (1) and Wu et al. (21). Extensive cross-linking was done at 4°C by using various NaCl concentrations from 0.15 M down to 0.05 M. "Hot" glyoxal denaturation treatment was then carried out after the supercoiled plasmid DNAs were Tx cross-linked and had been dialyzed overnight into 10 mM sodium phosphate buffer (pH 8.2). The Tx-DNAs were adjusted to 5% in fresh glyoxal (40% stock solution) and incubated for 30 min at 37°C and then dialyzed against 1× TE at 4°C overnight. Control tests (20) of the trioxalen-NaCl conditions were done by examining the results obtained with the supercoiled vector pBR322 and pCP 11.7, a clone of a pea ctDNA *Pst*I fragment about 29 kbp removed from the D-loop region of *Sal*I-A. The cross-linking conditions which yielded only one or two denaturation looped regions in the supercoiled ctDNA plasmid clones, and essentially none in either the pBR322 or pCP 11.7 DNAs, were used to produce Tx-DNA for restriction, proteinase K digestion, dialysis, and spreading as described above. Size markers for restricted plasmids were either simply the excised pBR322 vector DNA fragments which were recognizably smaller than the ctDNA fragments or linearized marker DNA as indicated.

In vitro replication. A crude replication complex was prepared as follows. Triton X-100-disrupted chloroplasts were obtained as described before (18), and the supernatent material was loaded on a 50-ml DEAE-cellulose column equilibrated with buffer A (25% glycerol, 50 mM Tris hydrochloride [pH 8.0], 10 mM 2-hydroxyethylmercaptan, 0.2 mM phenylmethylsulfonyl fluoride) plus 25 mM $(NH_4)_2SO_4$. After extensive washings with the same buffer, the bound proteins were eluted with buffer A plus 0.5 M $(NH_4)_2SO_4$. The fractions were assayed for topoisomerase (B. Nielsen and K. Tewari, personal communication), DNA polymerase (12), and RNA polymerase (17) activities. All of the fractions containing the three enzymes were pooled together and loaded on a 10-ml heparin-Sepharose column equilibrated with buffer B (buffer A, 0.1% Triton X-100, 0.1 mM EDTA) plus 0.1 M $(NH_4)_2SO_4$. The column was washed with buffer B plus 0.3 M $(NH_4)_2SO_4$, and the bound proteins were eluted from the column with 0.5 M $(NH_4)_2SO_4$ in buffer B. All three enzyme activities were found to be present in the same fractions. The peak enzyme fractions were pooled, dialyzed against buffer A plus 0.1 M $(NH_4)_2SO_4$, and loaded on a phosphocellulose column equilibrated with the same buffer. The enzyme activities were eluted from the column with 0.4 M $(NH_4)_2SO_4$ in buffer A. The phosphocellulose fraction containing all of the three activities was used in the replication system. Replication assays were carried out in a 100-μl reaction mixture containing 50 mM Tris hydrochloride (pH 7.0); 12 mM $MgCl_2$; 100 mM KCl; 100 μM each of dATP, dCTP, and dGTP; 1 μCi of [^{3}H]TTP; and 10 μM each of ATP, GTP, UTP, and CTP along with 1 μg of supercoiled DNA templates.

RESULTS

Mapping of D-loops in pea ctDNA. When ctDNA was digested to completion with the restriction enzyme *Sal*I, there were 10 identifiable ctDNA fragments generated, as determined by gel electrophoresis (14). These fragments were as follows: (i) the 44-kbp *Sal*I-A; (ii) the 22.8-kbp *Sal*I-B; (iii) the 16-kbp *Sal*I-C; (iv) the 12.4-kbp *Sal*I-D; (v) the 10.3-kbp *Sal*I-E; (vi) the 10-kbp *Sal*I-F; (vii) the 2.3-kbp *Sal*I-G; and (viii) three fragments of approximately 1 kbp in size, all of which were clustered between the *Sal*I C and *Sal*I A fragments (Fig. 1A, top portion). All of these DNA fragments could be identified by electron microscopic measurements of *Sal*I-digested ctDNA. To locate the D-loops in pea ctDNA, supercoiled ctDNA molecules were digested with *Sal*I and examined for D-loop-containing fragments. In Fig. 2A and 2B, we present some representative micro-

graphs of typical D-loop-containing *Sal*I-digested DNA fragments. As can be seen (Fig. 2A), there were two D-loops present in one *Sal*I fragment. Most of the *Sal*I ctDNA fragments, however, had only one D-loop. An analysis of a series of these *Sal*I fragments is presented in Fig. 3B. The bottom group of 13 individual fragments in Fig. 3B can be seen to have a D-loop structure which has a predominant start site at approximately 10 kbp in from the shortest end of the *Sal*I ctDNA fragment. The middle group of 15 measured fragments in Fig. 3B appears to have single D-loop structures beginning at about 15 kbp in from the near end of the fragment. The positioning of both replicative structures at the same end of the *Sal*I fragment in these initial D-loop mapping experiments was used to be consistent with the data of Kolodner and Tewari (7), whose results showed that these two structures are always found within about 7 kbp of each other in the circular pea ctDNA. Further evidence to support this positioning is seen in the group of four *Sal*I fragments shown at the top of Fig. 3B. These fragments can clearly be seen to contain two D-loop structures (an example is seen in Fig. 2A). The sites of these structures within these double D-loop *Sal*I fragments appear to show very good agreement with the positions found for the single D-loop fragments, which were oriented independently by the procedure above.

The cumulative histogram of D-loop locations is presented in Fig. 3A. This histogram has an overall bimodal character. The statistical analysis of the size of *Sal*I ctDNA fragments containing D-loops is presented in Table 1. The data clearly identify the fragment containing the D-loops to be a pea ctDNA *Sal*I fragment of 45 kbp, i.e., *Sal*I-A. No other clearly defined smaller-size *Sal*I fragments were found to contain a D-loop structure.

The data presented above have identified the single *Sal*I fragment of pea ctDNA which contains the D-loops. To

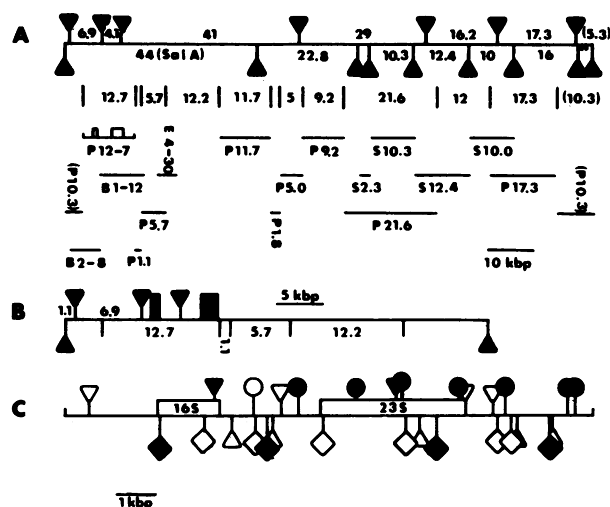

FIG. 1. Simplified restriction map of pea ctDNA and map of cloned recombinants used in the in vitro replication assays. (A) Map of three restriction endonuclease enzyme recognition sites in the intact chloroplast genome, oriented with the largest *Sal*I fragment (*Sal*I-A, shown as Sal A) on the left. Beneath the restriction map are shown the positions of the various ctDNA inserts of the recombinants used in Table 3. Abbreviations: P 12-7, pCP 12-7; S 10.3, pCS 10.3; E 4-30, pCE 4-30. □, Left to right, the positions of the 16S and 23S rRNA genes on P 12-7. (B) Enlargement of the *Sal*I A fragment including the *Sma*I and *Pst*I restriction sites. (C) Detailed restriction site map of the 12.5-kbp *Pst*I ctDNA insert fragment in pCP 12-7. Symbols: ▲, *Sal*I; ▼, *Sma*I; ⊤, *Pst*I; ♀, *Bam*HI; ▽, *Bgl*II; ◇, *Eco*RI; ●, *Hin*dIII; ◆, *Pvu*II; △, *Sac*I; ■, D-loops.

orient the D-loops within this restriction fragment of pea ctDNA (Fig. 1A), single and double digestions of pea ctDNA with the enzymes *Sma*I and *Sal*I were examined. When the large ctDNA restriction fragments generated by complete *Sma*I digestion were analyzed (Fig. 2C; Fig. 4), only one D-loop was found in the ctDNA fragment of 41 kbp. This observation appeared to be consistent with the D-loops being positioned at the end of the *Sal*I A fragment, which has a cluster of three *Sma*I sites (i.e., the 1.1-kbp *Sma*I-*Sal*I fragment and the two *Sma*I fragments of 6.9 and 4.1 kbp). The other D-loop was located in the 4.1-kbp *Sma*I fragment found at the left end of *Sal*I-A (Fig. 2E; Table 1). The D-loop structure in the 4.1-kbp fragment could only be visualized by using Tx-ctDNA. Thus, from our results and from the published data (14) on restriction site location, it becomes apparent that the D-loops must be at the left end of the 44-kbp *Sal*I fragment of pea ctDNA. From the above conclusion, it would be expected that the 12.5-kbp *Pst*I fragment of pea ctDNA would be an excellent candidate to contain at least one, if not both, of the D-loop structures. A micrograph of a ctDNA fragment, containing a D-loop, derived from *Pst*I-restricted ctDNA is shown in Fig. 2F. Figure 4A shows the cumulative histogram for the *Pst*I-generated DNA fragments containing D-loops. From Fig. 4A and Table 1, a clear bimodal distribution for the position of the D-loops can be observed in the 12.5-kbp *Pst*I fragment. The orientation of D-loops presented in Fig. 1B for the *Sal* DNA fragment A is the only one which fits the observed data (Table 2). These results then place the first D-loop between the *Bam*HI and the *Bgl*II sites located between the 16S and 23S rRNA genes, and the second D-loop is placed near the *Sac*I site at the far right of the ctDNA *Pst*I 12.5-kbp fragment (Fig. 1C).

With the above information, it became apparent not only that the recombinant pCP 12-7 was essentially certain to contain the two D-loop regions, but also that pCB 1-12 was an excellent nominee to contain the DNA sequences that could code for one or both of the D-loop structures (Fig. 1A). Wang et al. (20) have shown that the D-loop region in ctDNA has a higher A+T content than the sequences surrounding it. To test whether the pea ctDNA D-loop sequences were A+T rich, it was decided to examine supercoiled plasmids that had been extensively trioxalen cross-linked at various salt concentrations. At 0.10 M NaCl, both plasmids showed a single denaturation loop (Fig. 2G and 2H) when spread in the presence of formamide after hot glyoxal denaturation. Using these conditions, we did not observe denaturation loops to occur in either pBR322 or the ctDNA clone pCP 11.7. To find the physical location of these A+T-rich regions in the plasmids, we decided to use these experimentally determined conditions to prepare the supercoiled pCP 12-7 and pCB 1-12 DNAs for restriction digestion. Cumulative histograms from denaturation loop mapping of plasmids pCB 1-12 and pCP 12-7 are shown in Fig. 5A. From this figure, it can be seen that the plasmid DNAs tend to show a single denaturation region that maps almost precisely within the region of the leftward D-loop (Fig. 5A, line C) and that the rightward D-loop, under these conditions, shows essentially no tendency to denature. These histograms then support the distribution of denaturation looping in the plasmids as being unimodal in character with an A+T-rich region centered about the leftward D-loop position.

A further base composition comparison was performed by using denaturation mapping (10) of the ctDNA *Sal*I A fragment (Fig. 5B). The 11 molecules shown schematically in Fig. 5B, line e, were generated by using the same formamide-denaturing conditions described above. At this relatively

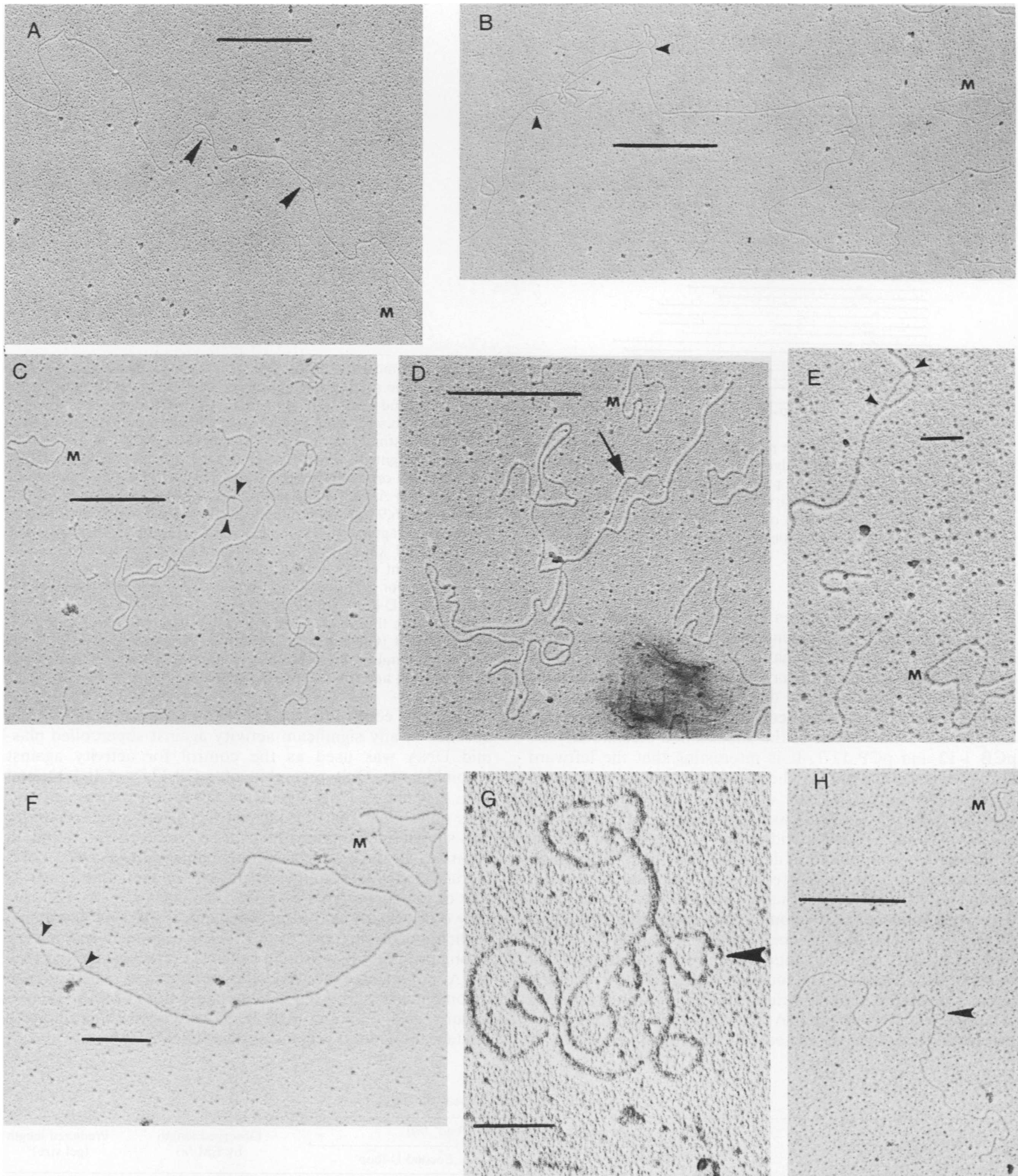

FIG. 2. Electron micrographs of pea chloroplast replicative DNA regions, using formamide/cytochrome *c* monolayer spreading. (A) A *Sal*I-generated ctDNA fragment containing two D-loop structures. Bar, 5 kbp. (B) An expanding D-loop structure in a *Sal*I ctDNA fragment. Bar, 5 kbp. (C) The large ctDNA fragment containing a single D-loop generated by *Sma*I digestion. Bar, 5 kbp. (D) A double-digest fragment produced by *Sma*I-*Sal*I. Bar, 5 kbp. (E) The small *Sma*I D-loop fragment. Bar, 1 kbp. (F) *Pst*I single D-loop-containing fragment. Bar, 2 kbp. (G) Recombinant pCP 12-7 DNA heavily trioxalen cross-linked at 0.10 M NaCl showing a single, small denaturation loop (arrow). Bar, 1 kbp. (H) *Pst*I-digested, Tx-hot glyoxal-denatured fragment of pCP 12-7. Bar, 5 kbp. The arrow indicates the denaturation loop region. The letter 'M' in panels A to D, F, and H indicates cospread marker molecules. The single large arrow in panel D points to the single-stranded DNA side of the D-loop. The small arrowheads in panels B, C, E, and F point out the forks of the D-loop structures.

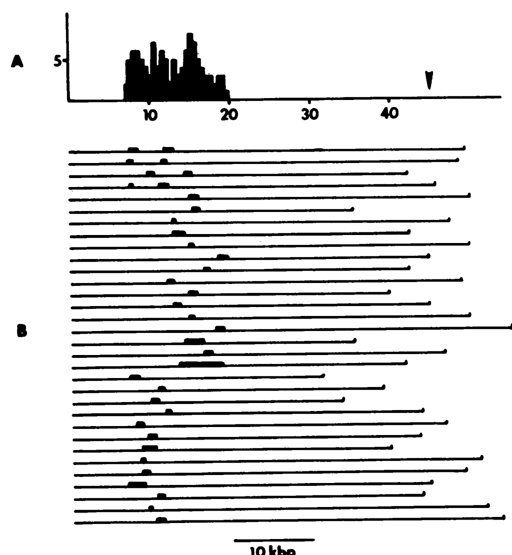

FIG. 3. (A) Histogram of the position distribution of the D-loop structures found in the *Sal*I digestion fragments of pea ctDNA. Position numbers are in kilobase pairs. (B) Linear schematics of *Sal*I A fragments containing D-loop structure(s) generated from complete *Sal*I digests. All fragments are oriented in relation to the D-loop structure(s) found in the molecule, with the nearest *Sal*I site put on the left. Symbols: ■, D-loop(s); ▼, *Sal*I site.

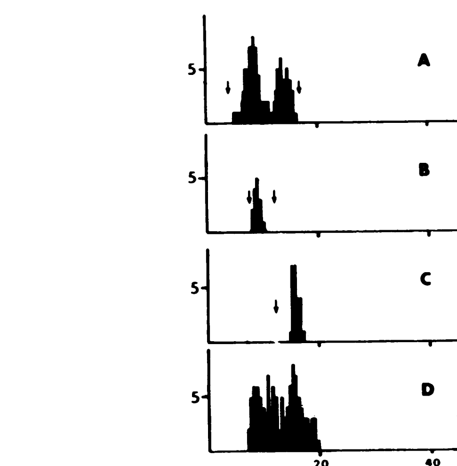

FIG 4. Cumulative histograms of restriction digest data for *Pst*I (A), *Sma*I (the small fragment only) (B), *Sal*I-*Sma*I (large fragment only) (C), and *Sal*I (D). Positions of histograms are adjusted according to restriction fragment sizes and order in the map; i.e., for *Sal*I no adjustment is made of the left restriction site of the *Sal*I 44-kbp A fragment, which is set at zero; for the *Sal*I-*Sma*I large fragment, the original histogram data had 12.1 kbp added to it (i.e., from the *Sal*I-A left end at zero, add 1.1 kbp to the first *Sma*I site and then the 6.9-kbp *Sma*I and the 4.1-kbp *Sma*I fragments); for the *Sma*I small fragment (the complete *Sma*I digest of the first two *Sma*I sites) add 1.1 kbp + 6.9 kbp, and the D-loop position within this small fragment works best by putting it to the left of the fragment; and, finally, for *Pst*I add 4 kbp. Arrows indicate restriction sites for the various D-loop-containing fragments i.e., the *Pst*I 12.5-kbp fragment (A); the *Sma*I 4.1-kbp fragment (B); the *Sal*I-*Sma*I 31.9-kbp fragment (C); and the *Sal*I 44-kbp fragment (D). The *y* axis shows the number of molecules containing a D-loop at that particular position, and the *x* axis is in kilobase pairs.

low denaturation level, it can be seen quite clearly that the region of the *Sal*I A fragment which includes the leftward D-loop (Fig. 5B, line f) denatures consistently and, therefore, must be assumed to have a high A+T content. This particular orientation of the denaturation-looped fragments is assumed from data obtained through Tx cross-linking and hot glyoxal denaturation of the supercoiled plasmid DNAs, pCB 1-12 and pCP 12-7. It is interesting that the leftward D-loop lies within one of the six major denaturation loop regions that were observed at 2.5% average denaturation (10) (Fig. 5B, line g). Also, as seen in Figure 5B, line e, the region of *Sal*I-A corresponding to the rightward D-loop position was rarely found to denature with the 37% formamide conditions and therefore can be assumed to have a significantly higher G+C content than the leftward D-loop.

In vitro replication of recombinants containing D-loop regions. The DNA polymerase activity obtained by using various ctDNA recombinants and the replication complex are shown in Table 3. The location of each of the recombinants is indicated in the restriction endonuclease map of pea ctDNA presented in Fig. 1A. The nonspecific DNA polymerase activity against nicked calf thymus DNA served as the positive control for the ctDNA polymerase activity. The absence of any significant activity against supercoiled plasmid DNA was used as the control for activity against recombinants. Only recombinants pCP 12-7, pCB 1-12, and pCE 4-30 showed any significant DNA polymerase activity. It is quite clear that the highest DNA polymerase activity was shown by recombinants pCP 12-7 and pCB 1-12, and, in contrast, all the other 14 recombinants showed DNA polymerase template activities that were in the same range as that of the supercoiled pBR322. The plasmid pCE 4-30, the only other clearly active recombinant, is known to contain a replication origin type of A+T-rich sequence and a very strong promoter of a protein gene (11). The specificity in the DNA polymerase activity was totally lost when nicked recombinants were used as templates. Both of these recombinants, pCP 12-7 and pCB 1-12, have been shown here to contain the D-loop regions of pea ctDNA.

TABLE 1. D-loop localization data[a]

Restriction enzyme(s)	Position of D-loop [start[b]/end[c] (*n*)]		Observed length by EM (*n*)	Predicted length (gel size)[d]
	First D-loop	Second D-loop		
*Sal*I	9.0 ± 1.3/9.7 ± 1.4 (18)	14.2 ± 2.1/15.5 ± 2.3 (20)	45 ± 5.7 (33)	44
*Sal*I-*Sma*I (large fragment)		2.3 ± 1.3/3.5 ± 1.4 (12)	33.2 ± 3.0 (12)	31.9
*Sma*I, *Sma*I-*Sal*I (small fragment)	0.8 ± 0.5/1.6 ± 0.7 (7)		3.7 ± 1.3 (7)	4.1
*Pst*I	3.1 ± 1.1/5.3 ± 1.6 (10)	9.4 ± 0.8/10.6 ± 0.7 (12)	12.6 ± 0.9 (19)	12.5

[a] All values are in kilobase pairs.
[b] Start of D-loop structure which was measured as closest to the short end of the fragment.
[c] Distal end of the D-loop structure.
[d] As determined by Palmer and Thompson (14).

TABLE 2. Restriction map orientation of D-loops

Restriction enzyme(s)	Position of left D-loop[a] (mean)		Position of right D-loop (mean)	
	Start	End	Start	End
*Sal*I	9	9.7	14.2	15.5
*Sma*I-*Sal*I (large fragment)			14.4	15.6
*Sma*I, *Sma*I-*Sal*I (small fragment)	8.8	9.6		
*Pst*I	8.1	9.3	13.4	14.6

[a] Distance(s) (in kilobase pairs) derived as explained in text and figure legends.

To analyze whether the incorporation of [^{3}H]TTP in the three recombinants reflects true replication of full-sized DNA molecules, in vitro DNA synthesis experiments were carried out as described before except that 25 μCi of [α-^{32}P]TTP was used instead of [^{3}H]TTP, the concentration of dATP, dGTP, and dCTP was decreased to 20 μM, and 2 μM cold TTP was included. After 30 min at 37°C, the reaction was stopped by adding 10 μl of 3 M sodium acetate, 20 mM EDTA, and 5 μl of 10% sodium dodecyl sulfate. The mixture was extracted with phenol-chloroform and precipitated by ethanol, and the sample was analyzed on a 0.8% alkaline agarose denaturing gel (2). The size of single-stranded DNA synthesized in vitro is shown in Fig. 6. The radioautographs clearly show the synthesis of DNA fragments of about 17, 14.5, and 9 kilobases (kb), corresponding to full-length pCP 12-7, pCB 1-12, and pCE 4-30, respectively. No synthesis of a unit length fragment of about 9.4 kb is seen when pCP 5.0 is used as a template (Fig. 6, lane 4). Full-length DNA strands contained 25 to 40% of all incorporated radioactivity.

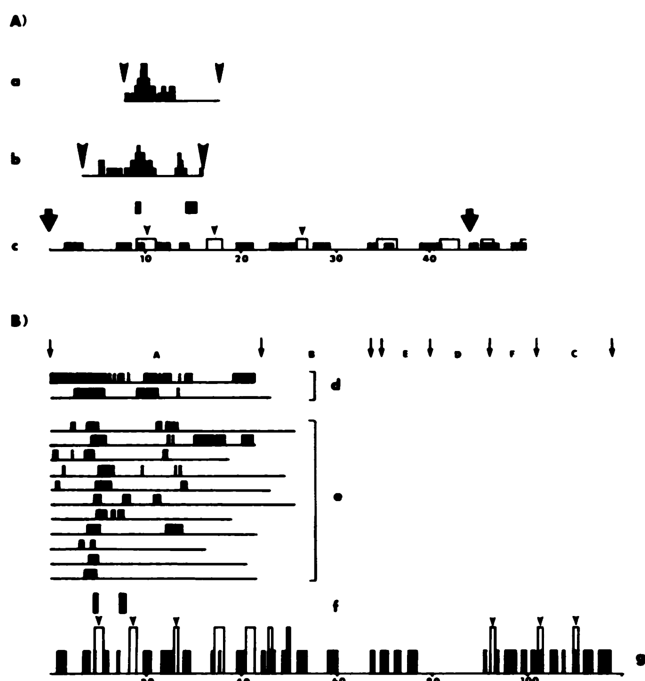

FIG. 5. (A) Cumulative histograms of the results of denaturation loop mapping (1, 21) of pCB 1-12 (line a) and pCP12-7 (line b); line c shows the Kolodner and Tewari (10) denaturation loop map of whole pea ctDNA. Restriction site symbols: line a, *Bam*HI (▼); line b, *Pst*I (▼); line c, *Sal*I (⧫). Scale is in kilobase pairs. (B) Denaturation loop mapping of fragment A of the *Sal*I digests of pea ctDNA, presented as a best-fit orientation to the denaturation loop map produced by Kolodner and Tewari (10). Line d, Map of some representative denaturation-looped *Sal*I A fragments produced with a 47% formamide hypophase and a 77% formamide spreading buffer solution. Line e, *Sal*I-A molecules denaturation looped at 37% formamide hypophase and 67% formamide spreading buffer. Line f, D-loop positions as determined from Table 1. Line g, Denaturation loop map of Kolodner and Tewari (10) oriented with the 44-kbp *Sal*I A fragment placed at the left. Symbols: ↓, *Sal*I restriction sites; ▮, D-loop structures (in lines c and f); □, denaturation loop regions seen at 2.5% denaturation (10) (in line g); ■, denaturation loop regions at 22% (10) (in line g); ▼, the six major denaturation loop regions observed at 2.5% denaturation of the intact pea ctDNA molecule (10) (in line g).

DISCUSSION

The data presented in this paper have confirmed the presence of two D-loops in pea ctDNA and mapped their locations on the restriction map of pea ctDNA. The first, or leftward, D-loop was found to begin 9.0 kbp from the closest *Sal*I site of the ctDNA fragment and to end at 9.7 kbp, thus giving an average size for this particular D-loop of about 0.7 kbp. The beginning of the second D-loop was found at 14.2 kbp in from the left *Sal*I site in ctDNA *Sal*I-A and ended at 15.5 kbp. These results are fully consistent with those of Kolodner and Tewari (7) both in the average distance between the distal ends of the two D-loops found in the *Sal*I A fragment (6.5 kbp in this study versus 7.1 kbp in reference 9) and in the average sizes of the individual D-loop structures themselves (700 bp and 1.3 kbp). The distribution of the two D-loops also indicated that there was no bias in favor of one D-loop over another (Fig. 3B). Since the observed overall ratio of fragments containing two D-loops to single-D-loop fragments was essentially the same as that found by Kolodner and Tewari (7) with intact pea ctDNA, our experimental conditions for visualizing restricted fragments did not seem to introduce any artifacts. Furthermore, we also observed some expanding D-loop molecules (Fig. 2B). Such molecules were rarer than those observed with whole pea ctDNA, but this is to be expected, since restriction will cause those molecules whose replication structures have expanded past one or more restriction sites to be seen either as large forked structures or simply as whole fragments lacking a D-loop. In fact, we saw restriction fragments

TABLE 3. Chloroplast replication system assays[a]

Template	Activity (cpm)	Template	Activity (cpm)
Control DNA	930	pCP 1.8	7,440
Activated calf thymus DNA	99,950	pCP 17.3	4,950
Supercoiled pBR322	3,100	pCP 21.6	3,100
pCB 2-8	7,600	pCP 10.3	9,700
pCB 1-12	35,960	pCP 9.2	5,500
pCP 12-7	99,400	pCE 4-30	18,520
pCP 1.1	6,260	pCS 12.4	2,750
pCP 5.7	4,000	pCS 10.3	2,680
pCP 11.7	3,170	pCS 10.0	3,300
pCP 5.0	1,700	pCS 2.3	2,050
pCP 12.0	1,830		

[a] Assays for incorporation of [^{3}H]TTP on the specified plasmid DNA templates (1 μg each), using an enzyme fraction which had been purified through DEAE-cellulose, heparin-Sepharose, and phosphocellulose columns. This enzyme fraction contained DNA polymerase, RNA polymerase, topoisomerase, and probably other enzymes required for replication.

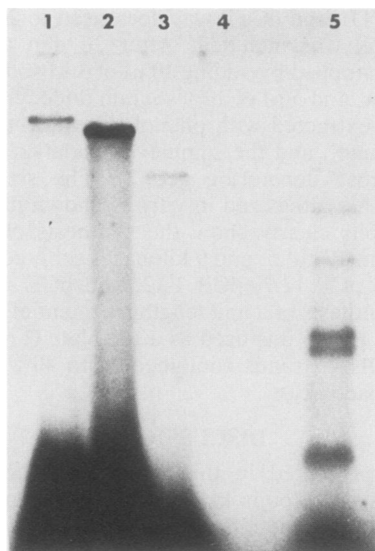

FIG. 6. Alkaline denaturing gel of in vitro-synthesized DNA. In vitro DNA synthesis was carried out as described in the text, with recombinants pCP 12-7, pCB 1-12, pCE 4-30, and pCP 5.0 as templates. Synthesized DNA was analyzed in a 0.8% alkaline agarose gel as described previously (2), with lambda DNA digested with *Hin*dIII and end labeled as size markers. Lanes: 1, pCP 12-7, with a total size of 17 kb; 2, pCB 1-12, 14.5-kb total size; 3, pCE 4-30, 9-kb total size; 4, pCP 5.0, 9.4 kb; 5, *Hin*dIII-digested lambda DNA size markers of 9.4, 6.7, 4.4, 2.3, 2.0, and 0.56 kb (from top to bottom).

containing forked structures with some frequency, but since it was not possible to determine how they arose (i.e., by an expanded D-loop mechanism or as a rolling-circle replication intermediate), these fragments were not scored. The Tx-glyoxal denaturation studies with recombinants pCP 12-7, pCB 1-12, and *Sal*I DNA fragments have shown that one D-loop (present in the 4.1 kbp *Sma*I pea ctDNA fragment) was found to denature much more readily than the other D-loop (present in the 31.9-kbp *Sal*I-*Sma*I ctDNA fragment; Fig. 2D), demonstrating a base compositional difference between the two D-loops. These findings were well supported by the direct formamide denaturation mapping of the pea ctDNA *Sal*I A fragment.

The recombinants pCP 12-7 and pCB 1-12, which have been shown to contain D-loop regions, were found to be very active in an in vitro replication system. The DNA synthesized with these recombinants was found to represent full-length sizes of the recombinants. Other recombinants that were not active in the in vitro system were found to synthesize no discernable DNA in alkaline agarose gels. The preliminary data on the in vitro replication strongly suggest that the D-loop-containing recombinants are used to produce full-length DNA copies in our replication system. Also, the specificity of replication is lost when nicked ctDNA recombinants are used at templates. Wu et al. (21) have likewise shown in vitro replication of D-loop-containing recombinants from *C. reinhardtii* and a crude chloroplast extract.

The location of the D-loops in pea ctDNA, which does not have an inverted repeat region, is clearly different than the location of the ctDNA D-loops which have been mapped in *C. reinhardtii* (19). That study (19) found one D-loop (termed *ori*A) to be 10 kbp upstream from one 16S rRNA gene, with the second D-loop (*ori*B) found at 16.5 kbp upstream from that same 16S rRNA gene. Roughly the same configuration was determined for the ctDNA D-loop region of *Euglena gracilis* (6) in which the D-loop was mapped at about 6.5 kbp upstream of the supplementary 16S rRNA gene. Our results, however, differ from these studies in that the leftward D-loop maps in the spacer region between the 16S and 23S rRNA genes (Fig. 1C) at or near the *Bam*HI restriction site. This location of the leftward D-loop is thus to be found downstream of the 16S rRNA gene. The precise location of the second or rightward D-loop is less clear, but as can be seen from Fig. 1, its location, as mapped by us, must be several kilobase pairs downstream of the 23S rRNA gene. The D-loop regions in pea ctDNA also differ from those reported in maize ctDNA. Gold et al. (4) have observed a putative *ori* region in corn ctDNA that mapped near the ribosomal protein L16. This region maps outside of the inverted repeat sequences of maize ctDNA.

In summary, the positioning of the *ori*-specific sequences in ctDNA may have a general characteristic of proximity to the rRNA genes but a flexible positioning relative to them. Also, those reports (3, 4, 16, 21) on the base composition of the chloroplast-specific *ori* regions have, in general, found them to be A+T rich, and our data from a higher plant are clearly in agreement with this property. It will be highly interesting to determine and explore the precise base sequences of the two pea ctDNA D-loop regions mapped here and then to compare the various properties of a higher plant ctDNA replicative region with the properties of its counterpart in the algal replicative region. Towards this end, the subcloning of the D-loop-specific regions of the pea ctDNA inserts of pCP 12-7 and pCB 1-12 is in progress. These subclones will be used to study in greater detail the biochemical properties of the pea ctDNA D-loop sequences with a more highly purified pea chloroplast replicative system now being developed in our laboratory.

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