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THYLAKOID-BOUND CHLOROPLAST DNA FROM SPINACH IS ENRICHED FOR REPLICATION FORKS

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Chloroplast DNA is bound to the thylakoids of spinach chloroplasts. To examine a possible role for thylakoid-bound DNA in chloroplast DNA replication, vesicles formed by treating chloroplasts in 3.5 mM $MgCl_2$ were used. Chloroplast DNA fragments are bound to the surface of these vesicles. Chloroplast DNA isolated from vesicles that had been first treated with Eco R1 contained 10% of branched fragments whereas chloroplast DNA isolated from intact chloroplasts and treated with Eco R1 contained 2% of branched fragments. This result is consistent with the growing replication fork of chloroplast DNA being associated with the chloroplast internal membrane system. Branched fragments from the chloroplast DNA digested with Eco R1 prior to the isolation from the vesicle contained fragments of unequal length. Membrane binding in chloroplasts may have a similar role in DNA replication as it does in bacteria.

When spinach chloroplast nucleoids are visualized with the fluorochrome DAPI the nucleoids appear scattered throughout the chloroplast (1). This is the scattered nucleoid distribution as defined by Kuroiwa *et al.* (2) and such an arrangement is most likely due to the binding of cpDNA to the thylakoid system (3,4). The binding of cpDNA to the thylakoid system is implicated in packaging, locating of transcripts, partitioning and replication of cpDNA (5,6). This study addresses the role of the association of cpDNA with spinach thylakoids in cpDNA replication. Morphological studies of isolated cpDNA have shown what appear to be replicative forms attached to membranes (7). More recent investigations have obtained evidence for the involvement of

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Abbreviations: cpDNA, chloroplast DNA; DAPI, 4'6-diamidino-2-phenylindole.

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thylakoid-bound proteins in supporting in vitro cpDNA synthesis (8). It has been observed that the membrane-bound fraction of DNA from E. coli (9) and B. subtilis (10) and DNA from a nuclear matrix-DNA complex from HeLa nucleoids (11) are enriched for replication forks. A similar result would be expected from the membrane-bound cpDNA fraction from chloroplasts if the chloroplast membranes had a role in cpDNA replication.

MATERIALS AND METHODS

Plant growth. The growth of spinach plants has been previously described (12). Leaves up to 5 cm in length were used.

Isolation of chloroplast and chloroplast vesicle DNA. Chloroplasts and chloroplast vesicles were isolated as previously described (4). The vesicles consist only of internal thylakoid membranes (4). DNA isolation from the chloroplasts and the vesicles has also been described (13).

Restriction endonuclease treatments. Isolated cpDNA and vesicle DNA were digested with Eco R1 as described previously (13). In one treatment the vesicles were resuspended in 10 ml of restriction buffer (14) and incubated overnight at 37°C with 16 units/ml of Eco R1 before extracting the DNA.

Spreading of DNA on a protein monolayer. The DNA was spread on a protein monolayer using a modification of the method of Kleinschmidt as described by Davis *et al.* (15). The spreading solution contained 0.5 M ammonium acetate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.4), 50% (v/v) formamide, 0.1 mg/ml cytochrome C and 0.5 ug/ml DNA. This solution was spread on a hypophase containing 0.25 M ammonium acetate and 10% (v/v) formamide (pH 8.4) in a plastic dish (113 mm x 22 mm). An acid-cleaned glass slide (stored in 0.25 M ammonium acetate at pH 8.4) was placed at an angle of 45° in the dish and allowed to drain dry. Spreading solution (50 µl) is run down the slide to form a protein film on the surface of the hypophase. A few grains of talc were used to visualise and compress the film.

After 1 min the film on the surface of the hypophase was picked up on a 200 mesh copper grid coated with 3.5% parlodion (Ladd Research Industries, Burlington, VT., USA). The grid was then dipped in 90% (v/v) ethanol, stained with uranyl acetate for 2 min and rinsed in 90% (v/v) ethanol. The grids were rotary shadowed with 80% Pt/20% Pd wire (Agar Aids, Sydney, Aust.) using a casting angle of 4°, a casting distance of 10 cm and 60 rpm (Denton Vacuum DV-502 vacuum coater). The grids were viewed in a Jeol JEM 100 CX electron microscope. Following observations on fragment types Eco R1 fragments were scored for the proportion of branched fragments (100 fragments from three grids for each treatment).

RESULTS

Preliminary investigations were carried out on DNA isolated from whole chloroplasts and restricted with Eco R1, to determine if

branched fragments characteristic of Cairns-type replicative intermediates could be visualised. Such fragments were observed and are shown in Figs. 1 and 2. The fragments contain two branches of equal length. A single stranded connection (arrow) on one of the branches occurs at the branch junction. This single-stranded connection is characteristic of the lagging

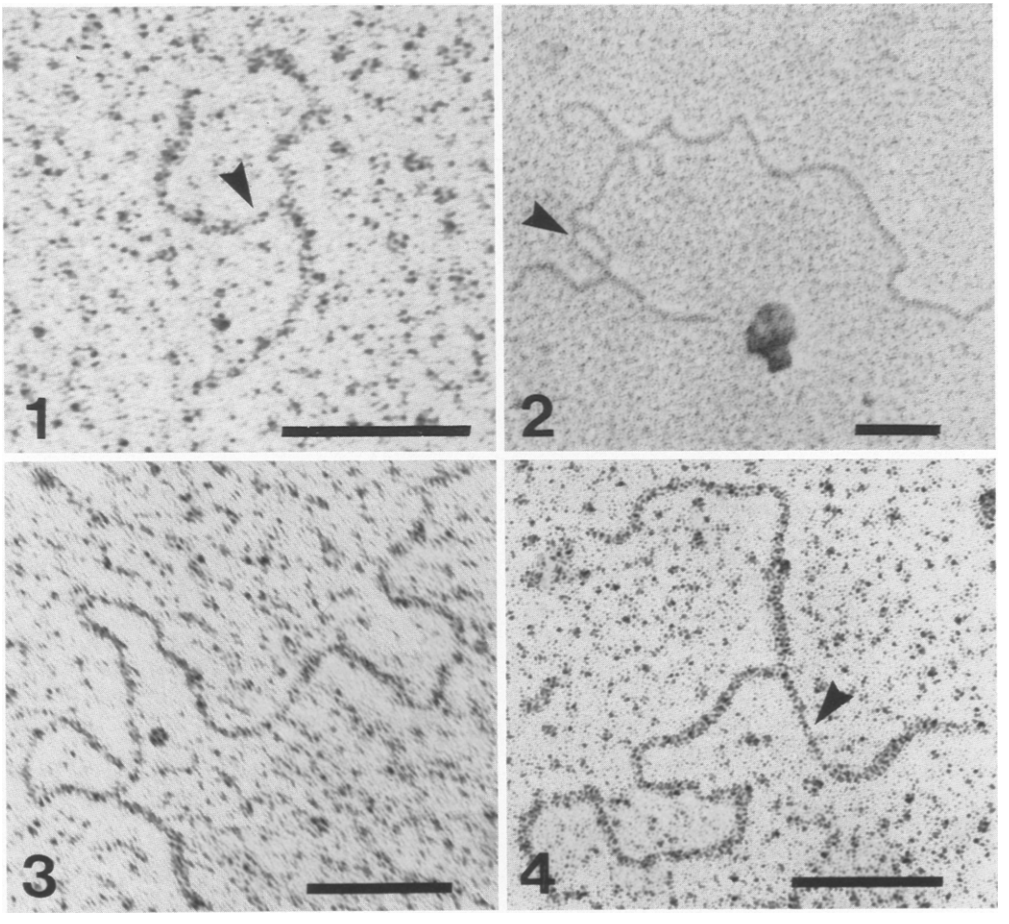

Fig.1. Branched cpDNA fragment from Eco R1 digestion of cpDNA isolated from intact spinach chloroplasts. Arrowhead indicates single stranded connection. Branches are of equal length. Scale bar = 100 nm.

Fig.2. Another branched fragment as for Fig.1. Scale bar = 100 nm.

Fig.3. Branched cpDNA fragment from Eco R1 digestion of membrane bound cpDNA. Branches are of unequal length. No clear single stranded connection at the branch point. Scale bar = 100 nm.

Fig.4. Branched cpDNA fragment from Eco R1 digestion of membrane bound cpDNA. Branches are of unequal length. Arrowhead indicates single stranded connection. Scale bar = 100 nm.

strand of the growing replicative fork (16). Eco R1 restriction produces the branches of equal length as it would cut at the same site in the daughter molecules.

Some branched fragments were observed (Fig.3) that did not contain a noticeable single stranded region. In these fragments it is possible that the single stranded connection has been replicated or almost replicated and is not long enough to be easily visualized. Such a situation would still be consistent with discontinuous replication of the lagging strand via Okazaki fragments (17).

Following on from our preliminary morphological studies branched fragments were utilised as an indicator of active DNA replication in different cpDNA fractions. Three different DNA fractions are compared in Table 1. Chloroplast DNA was isolated from whole chloroplasts then cleaved with Eco R1, cpDNA was isolated from chloroplast vesicles then cleaved with Eco R1 and cpDNA was isolated from chloroplast vesicles that had been first treated with Eco R1. An enrichment of branched fragments was observed in the latter treatment where chloroplast vesicles were first incubated with Eco R1 prior to cpDNA isolation and examination by electron microscopy.
The different treatments shown in Table 1 also showed qualitative differences in the replication forks observed. Chloroplast DNA from intact chloroplasts contained branched fragments with two segments of equal length. Chloroplast DNA which was digested with Eco R1 before isolation from chloroplast vesicles contained predominantly branched fragments with segments of unequal length (Figs.3 and 4). Chloroplast DNA digested with Eco R1 after isolation from chloroplast vesicles contained both types of fragments.

While some nuclear contamination may occur in chloroplasts isolated by sucrose gradients, there is no evidence that this is responsible for the results obtained. Gel electrophoresis of cpDNA from intact chloroplasts and chloroplast vesicles indicates there is less nuclear contamination in the vesicle preparations than in the chloroplast preparations (envelopes with any adhering DNA from broken nuclei are all lost) and most branched fragments occur in DNA from Eco R1 treated vesicles. There is an enrichment in this latter fraction irrespective of whether the

Table 1

Percentage of branched fragments in Eco R1 digests of cpDNA isolated from intact spinach chloroplasts and spinach chloroplast vesicles

Treatment	Branched fragments (%)
1. cpDNA isolated from intact spinach chloroplasts and digested with Eco R1	2
2. cpDNA isolated from spinach chloroplast vesicles and digested with Eco R1	4
3. cpDNA digested with Eco R1 before isolation from spinach chloroplast vesicles	10

comparisons are against vesicle or chloroplast cpDNA preparations.

DISCUSSION

Chloroplast DNA in higher plants appears to replicate by the formation of two displacement loops on opposite strands which elongate towards each other to form a Cairns replicative intermediate (18). The rolling circle type of intermediate has also been observed (18). The branched structures we have observed would result from the cleavage of the Cairns-type replicative intermediate with Eco R1 (Fig. 1). The growing points of the Cairns intermediates often exhibit single-stranded connections which are almost always associated with daughter strands (16,17).

The branched fragments then represent the site of the actively replicating cpDNA. These fragments are present in the largest numbers when vesicles with associated cpDNA are restricted with Eco R1 and the cpDNA then isolated (Table 1). The degree of enrichment in replication forks obtained corresponds with what would be expected if the replication forks were associated with a thylakoid-bound cpDNA fraction. Chloroplast vesicles retain about 45% of the cpDNA (4) and Eco R1 digestion reduces this to about 18%. An approximate five times increase in replication forks would therefore be expected in the vesicle fraction treated with Eco R1 compared to total cpDNA. Most of the replication forks in

this latter fraction also have uneven branches, possibly due to restriction site blockage of one of the sites because of membrane binding. Vesicle formation would however result in some indiscriminate shearing.

The simplest interpretation of our data is that the cpDNA replication complex is membrane-bound and is the site of the growing replication fork. This is consistent with the idea that morphological structures are involved in in vivo DNA replication in other systems. In the case of B. subtilis and E. coli this is the cell membrane (9,10,19); and in the mammalian nucleus it is the nuclear matrix (11,17,20). There is little detailed information on this point in chloroplasts. However there are reports of membrane-associated DNA polymerase activity (21) and thylakoid proteins are required for in vitro cpDNA replication in Chlamydomonas (8).

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REFERENCES

1. Possingham, J., Chaley, N., Robertson, M., and Cain, P. (1983) Biol. Cell. 47, 205-212.
2. Kuroiwa, T., Suzuki, T., Ogawa, K., and Kawano, S. (1981) Plant Cell Physiol. 22, 381-396.
3. Rose, R.J., and Possingham, J.V. (1976) J. Cell Sci. 20, 341-355.
4. Rose, R.J. (1979) J. Cell Sci. 36, 169-183.
5. Rose, R.J. (1988) In The Division and Segregation of Organelles (S.A.Boffey and D.Lloyd.Eds.) pp.171-195. Cambridge University Press, Cambridge U.K.
6. Kiseleva, E.V., Dudareva, N.A., Dikalova, A.E., Khristoliubova, N.B., Salganik, R.I., Laktionov, P.P., Roshke, V.V. and Zaichikov, E.F. (1989) Plant Science 62, 93-103.
7. Bisalputra, T., and Burton, H. (1970) J. Microscopie 9, 661-666.
8. Nie, Z.Q., Chang, D.Y., and Wu, M. (1987) Mol. Gen. Genet. 209, 265-269.
9. Valenzuela, M.S., and Inman, R.B. (1978) Molec. Gen. Genet. 166, 245-249.
10. Valenzuela, M.S., Aguinaga, M.D.P. and Inman, R.B. (1981) Molec. Gen. Genet. 181, 241-247.
11. Valenzuela, M.S., Mueller, G.C., and Dasgupta, S. (1983) Nucl. Acids Res. 11, 2155-2164.
12. Rose, R.J. (1980) Aust.J.Plant Physiol. 7, 713-725.
13. Lindbeck, A.G.C., and Rose, R.J. (1987) J. Plant Physiol. 129, 425-434.
14. Rose, R.J., Johnson, L.B., and Kemble, R.J. (1986) Plant Mol. Biol. 6, 331-338.

15. Davis, R.W., Simon, M., and Davidson, N. (1971) In Methods in Enzymology, Vol.21, Nucleic Acids, Part D (L.Grossman and K. Moldave. Eds) pp.413-428. Academic Press, New York USA.
16. Schnos, M. and Inman, R.B. (1970) J. Mol. Biol. 51, 61-73.
17. Younghusband, H.B., and Inman, R.B. (1974) Annu. Rev. Biochem. 43, 605-619.
18. Kolodner, R.D., and Tewari, K.K. (1975) Nature 256, 708-711.
19. Firshein, W. (1989) Annu. Rev. Microbiol. 43, 89-120.
20. Zehnbauer, B.A., and Vogelstein, B. (1985) BioEssays 2,52-54.
21. Herrmann, R.G., Kowallik, K.V., and Bohnert, H.J. (1974) Portugaliae Acta Biologica Serie A. 14, 91-110.