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Recombination within the inverted repeat sequences of the *Chlamydomonas reinhardii* chloroplast genome produces two orientation isomers

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Summary. Two orientations of the *Chlamydomonas reinhardii* chloroplast (ct) genome are shown to be produced by recombination within the inverted repeat (IR) sequences that separate the two single copy (SC) regions. SC region 1 is bounded on its two ends by *EcoR*I restriction endonuclease fragments of 3.2 and 4.7 kilobase pairs (kb) (Rochaix 1978). The 3.2 kb *EcoR*I fragment overlaps a 51.3 kb *Bgl*II fragment spanning one of the 19.7 kb IR sequences, and the 4.7 kb *Eco*RI fragment overlaps a 42.1 kb *Bgl*II fragment spanning the other 19.7 kb IR sequence. We have shown by hybridization analysis that the 3.2 kb fragment also overlaps a *Bgl*II fragment with a predicted size of 52.3 kb, and that the 4.7 kb fragment also overlaps a *Bgl*II fragment of a predicted size of 41.1 kb. The second set of *Bgl*II fragments are isomers produced by recombination localized to the IR region. The two isomers are present in approximately equimolar ratio. Knowledge of the isomeric composition of the *C. reinhardii* ctDNA is essential for establishing a correlation between genetic and physical maps of the ct genome.

Key words: *C. reinhardii* chloroplast DNA – Orientation isomers – Inverted repeat – Recombination

Introduction

The chloroplast genome of most plants and algae so far examined is a circular DNA molecule organized into two single copy regions separated by a large repeat (reviewed in Whitfeld and Bottomley 1983). The two copies of the repeat are inverted relative to one another. In establishing the restriction endonuclease map of these molecules, it is important to determine the orientation of the two unique regions relative to one another. There are two possible orientations which can be distinguished only by analyzing DNA fragments large enough to contain an entire copy of the repeat along with identifiable segments of the unique regions which flank it. Such fragments can be obtained by using restriction endonucleases which do not have recognition sites within the repeat or by cloning an entire copy with flanking material.

Several circular DNA molecules containing inverted repeats (IR) have been analyzed in the above manner. Included are the chloroplast genomes of common bean (Palmer 1983), soybean (Palmer et al., in press 1984a), the fern *Osmunda* (unpublished results of Palmer, cited in Palmer 1983), the cyanelle DNA of *Cyanophora paradoxa* (Bohnert and Löffelhardt 1982), the mitochondrial genome of the water mold, *Achlya* (Hudspeth et al. 1983), and the 2 μm circular DNA of *Saccharomyces cerevisiae* (Guerineau et al. 1976). In each case, it has been found that the population of DNA molecules contains approximately equimolar ratios of the two orientation isomers. Similarly, the linear DNA of several herpesvirus genomes contain inverted arrangements of repeats, and all possible orientation isomers are present in equimolar quantities (Roizman 1979). Since homologous recombination within the repeat would interconvert the orientation isomers, an equimolar mixture would be expected if such recombination were active. In the case of the yeast 2 μm circular DNA, a site-specific recombination activity designated "FLP" has been demonstrated (Broach and Hicks 1980), which promotes recombination at a specific site within the repeat and maintains the equimolar ratio of the two isomers. A low level of generalized recombination occurring randomly throughout the repeat has been detected as well in this DNA (Bell and Byers 1979).

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The interconversion of DNA molecules to form orientation isomers has profound consequences which must be considered in efforts to construct genetic maps (Mets and Geist 1983). *Chlamydomonas reinhardii* is the only organism in which extensive studies of chloroplast gene recombination have been undertaken. In efforts to correlate the genetic and physical maps in this organism, it is important to know the isomeric composition of the chloroplast DNA (ctDNA). The restriction mapping done by Rochaix 1978, demonstrated that the restriction endonuclease, *Bgl* II, did not cleave within the IR, but did not sufficiently resolve the large repeat-containing fragments to establish the relative orientation of the SC regions. In this paper we show by Southern hybridization analysis of these IR containing *Bgl* II fragments that both orientation isomers exist in approximately equimolar quantities, and that the recombination responsible for the interconversion of the isomers is within or very near the IR region.

Materials and methods

Growth Conditions. C. reinhardii strains 2137 mt^+, 1374 mt^-, or CC 125 mt^- were grown mixotrophically in 8 l of tris-acetate-phosphate medium (Surzycki 1971) in a 13 l Bellco flask. Cultures were bubbled with sterile air and were maintained on a rotary platform shaker at ambient room temperature (23 °C) under continuous room fluorescent illumination.

Isolation of Chloroplast DNA. Cells grown to a density of $1-5 \times 10^6$/ml were collected by centrifugation and processed 2 l at a time. Pellets were resuspended, using a soft bristle brush, in 10 mM Tris-acetate (pH 6.5) and 1 mg/ml bovine serum albumin (lysis buffer) (Tamaki et al. 1981). Resuspended cells were pooled and adjusted to a final volume of 100 ml with lysis buffer, and 10 ml of autolysin-containing supernatant from mating gametes (collected previously and maintained at −70 °C) was added to remove the cell walls (Tamaki et al. 1981). Following incubation at room temperature for 30 min, the cells were collected by centrifugation and resuspended in 20 ml of 20 mM Tris/100 mM NaCl/100 mM EDTA, pH 8.0. After resuspension the nuclease inhibitors aurintricarboxylic acid (Hallick et al. 1977) and ethidium bromide (EBr) (Kislev and Rubenstein 1980) were added to 2 mM and 400 μg/ml, respectively. Sodium dodecylsulfate (SDS) was added to a final conc. of 1%, and the cell lysate was incubated with gentle shaking for 30 min. Solid CsCl was gradually added to obtain a refractive index of 1.3990. The lysates were centrifuged in a Sorvall TV 865B vertical rotor for 12–14 h at 58,000 rpm, 22 °C. The single DNA band that formed was collected, recentrifuged using the same conditions and was destained by extracting twice with CsCl saturated 1-butanol. The chloroplast DNA (ctDNA) was separated from other cellular DNAs by adding Hoechst dye 33258 (Calbiochem Behring Corp) from a stock solution of 10 mg/ml in water to a final conc. of 200 μg/ml (Aldrich and Cattolico 1981) and the samples were centrifuged in the Sorvall TV 865 rotor at 58,000 rpm, 22 °C 12–14 h. The top band (ctDNA) was collected and recentrifuged using the same conditions. Dye was removed from the collected band by extracting with CsCl-saturated 1-butanol. Dialysis against 1 mM Tris/0.1 mM EDTA, pH 8.0 with 3 changes over 24 h followed. Aliquots of the DNA were stored at −70 °C.

Some ctDNA isolations were also prepared according to the method of Grant et al. 1980a.

Restriction Endonuclease Digestion and Gel Electrophoresis of ctDNA. Digestion of ctDNA with restriction endonucleases was according to the manufacturer's directions (Bethesda Research Labs), except that all reactions were carried out at 37 °C for 3 h using 1–2 μg ctDNA with 10–20 units of endonuclease. Gel electrophoresis in 1% or 0.2% agarose gels proceeded as previously described (Aldrich et al. 1982). Transfer of DNA from 1% agarose to nitrocellulose was as described by Southern (1975), and transfer of DNA from 0.2% agarose gels differed only in the mechanics of transfer. A Bethesda Research Labs slab gel electrophoresis unit designed for low percentage gels was used. This unit permitted construction of a shallow "agarose gel box" made of 2% agarose. The agarose box was filled with 0.2% agarose and the DNA samples were separated by electrophoresis in this 0.2% agarose. The buffer used for electrophoresis was Tris acetate pH 8.1 (Maniatis et al. 1982). Following electrophoresis of the DNA in the 0.2% agarose, the entire gel was placed upon 5 sheets of wicking paper (Bethesda Research Labs) wetted with 20X SSC (SSC: 0.15 M NaCl, 0.15 M sodium citrate). Then water-soaked nitrocellulose paper, three sheets of wicking paper wetted with 2X SSC and 3" dry wicking paper were placed over the depression containing the 0.2% agarose. After an hour a flat weight was placed on top of the wicking paper and the transfer continued for another 3 h.

Hybridization of DNAs to Filter-bound DNA. Fragments of ctDNA for nick translation were isolated from agarose gels after electrophoresis of DNA fragments generated from restriction endonuclease digestion of the ctDNA. Plasmids containing ctDNA fragments were also used (kindly provided by J. Palmer, J. Boynton and N. Gillham, Duke Univ.) The DNAs were nick-translated with ^{32}P dCTP and hybridized using stringent conditions to ctDNA restriction fragments bound to nitrocellulose paper (Maniatis et al. 1982).

Results

Isolation of ctDNA was facilitated (Aldrich and Cattolico 1981) by using Hoechst dye 33258 – CsCl equilibrium density gradient centrifugation (Fig. 1). The top band was shown to be ctDNA by restriction endonuclease DNA fragment pattern analyses (Fig. 4) identical to those obtained by Rochaix 1978. The ctDNA isolated in this manner was linear and in the range of 100–200 kb as measured in 0.2% agarose gels (data not shown). Yields of ctDNA obtained by the method of Grant et al. 1980a, were greater (15–20 μg ctDNA) than those obtained by the method detailed here (5–10 μ ctDNA). The latter method, however, requires less preparation time before CsCl-Hoechst dye centrifugation than the former.

Digestion of the ctDNA with *Bgl* II yielded two DNA fragments of 42.1 and 51.3 kb that span the two IR-containing regions (Fig. 2) (Rochaix 1978). Cleavage of ctDNA isolated by either of the above two methods with *Bgl* II results in a DNA fragment profile following electrophoresis in 0.2% agarose gels that clearly shows these

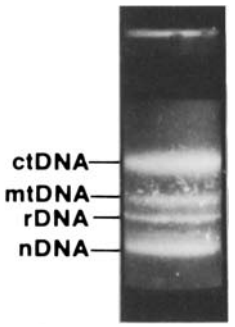

Fig. 1. CsCl-Hoechst 33258 dye gradient of cellular DNA of *C. reinhardii*. Total cell DNA isolated by the method detailed in materials and methods or by the method of Grant et al. 1980a, is resolved into the four bands characteristic of *C. reinhardii* (Behn and Herrmann 1977) on CsCl – Hoechst 33258 dye gradients: ctDNA (chloroplast DNA), mtDNA (mitochondrial DNA) rDNA (ribosomal DNA) and nDNA (nuclear DNA). The ctDNA and mtDNA fractions were further identified by their characteristic restriction endonuclease fragment patterns (Rochaix 1978; Grant and Chiang 1980b)

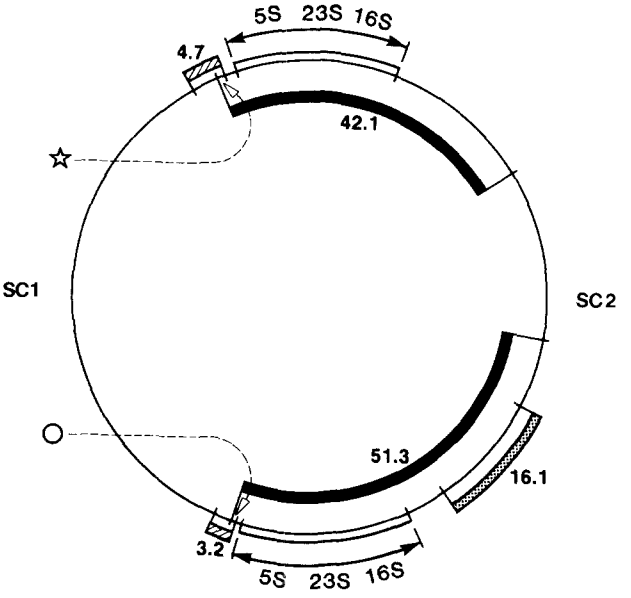

Fig. 2. Schematic diagram of the *C. reinhardii* chloroplast genome. Two large *Bgl*II fragments (solid bars) span the IR sequences (open bars) of the *C. reinhardii* chloroplast genome: the larger fragment is 51.3 kb and the smaller is 42.1 kb (adapted from Rochaix 1978). Single copy (SC) regions 1 and 2 bordering the IR sequences are defined. The positions of the *EcoR*I-*Bgl*II (0.5 kb, ○, and 1.3 kb, *) fragments derived from the 3.2 kb and 4.7 kb *EcoR*I fragments, respectively (*cross-hatched*), and the 16.1 kb *BamH*I fragment (*dotted*) used in hybridization analyses are shown

two fragments (Lane 2, Fig. 4). However, recombination within the IR of these two large fragments would generate recombinant fragments (Form II, Fig. 3) that differ in size by only 1 kb from the original fragments (Form I, Fig. 3), a size difference that cannot be resolved even on low percentage agarose gels. The resulting recombinant

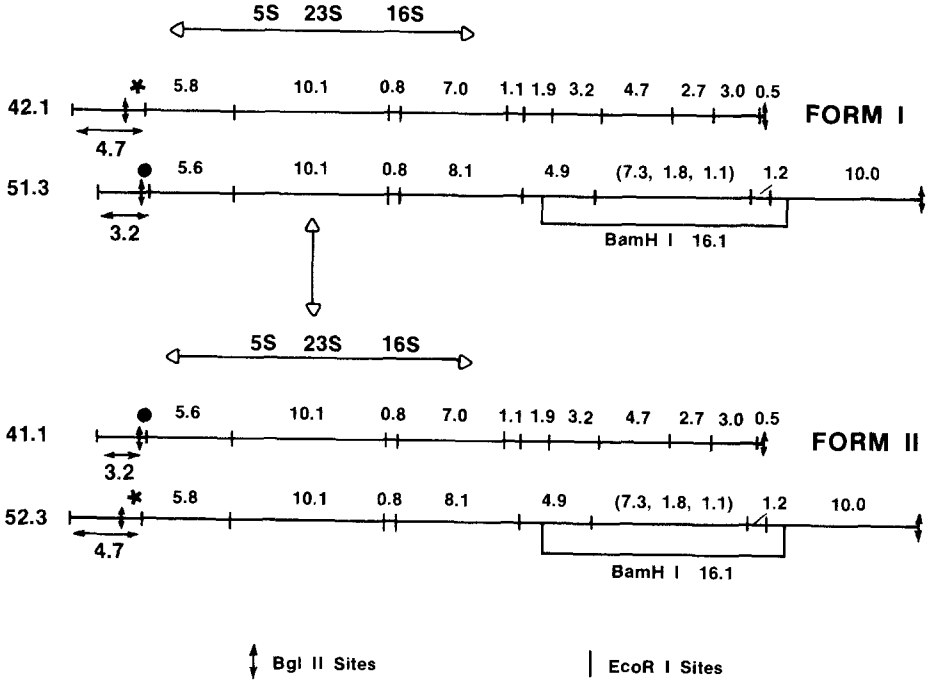

Fig. 3. Alignment of the two *Bgl*II DNA fragments and the predicted consequence of recombination between them. Form I fragment sizes are 42.1 and 51.3 kb (adapted from Rochaix 1978). Form II *Bgl*II fragments, generated if recombination occurs between the *Bgl*II fragments of Form I have sizes of 41.1 and 52.3 kb. If recombination occurs, the 1.3 kb *Bgl*II *EcoR*I fragment (*) will be located within the 42.1 kb fragment of Form I configuration as well as within the 52.3 kb fragment of Form II. Likewise, the 0.5 kb *Bgl*II-*EcoR*I fragment (•) will be located within the 51.3 kb fragment of Form I as well as within the 41.1 kb fragment of Form II. However, the 16.1 kb *BamH*I fragment will be found only within the 51.3 and 52.3 kb fragments if recombination is within the IR. The order of the *EcoR*I fragments internal to the 16.1 kb *BamH*I fragment is from Vallet et al. (1984)

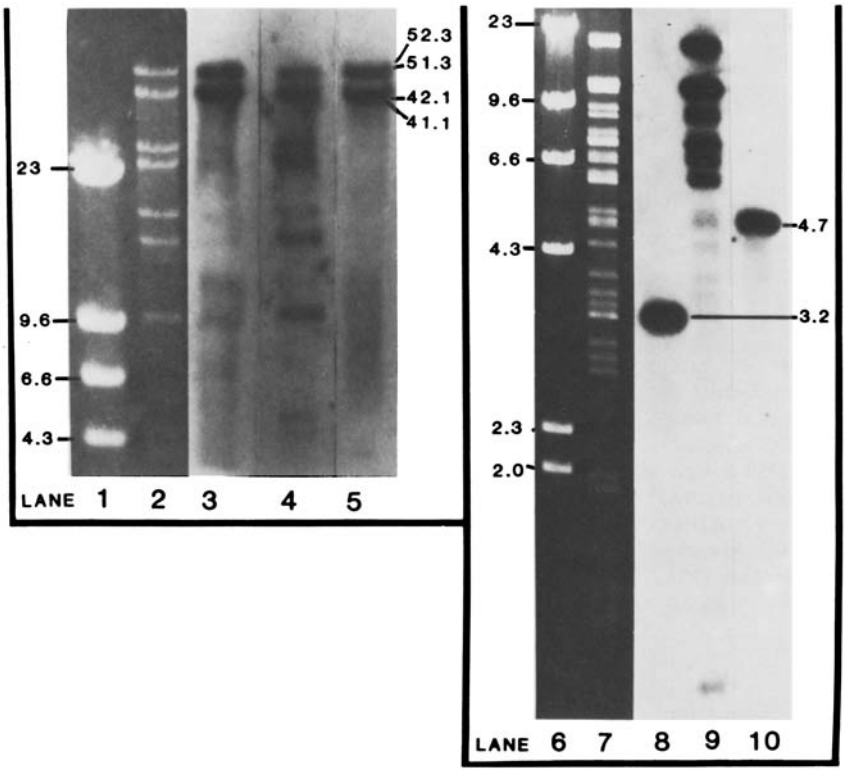

Fig. 4. Hybridization of the 0.5 and 1.3 kb *EcoR*I-*Bgl*II DNA fragments to ctDNA digested with *Bgl*II or *EcoR*I. Filter-bound *Bgl*II cleaved ctDNA transferred from 0.2% agarose gels in lanes 3–5 was hybridized to ^{32}P-labelled 0.5 kb *EcoR*I-*Bgl*II fragment (lane 3), to ^{32}P-labelled 1.3 kb *EcoR*I-*Bgl*II fragment (lane 5), and to ^{32}P-labelled total ctDNA (lane 4). The same DNA fragments were hybridized to *EcoR*I cleaved ctDNA transferred from 1% gels: 0.5 kb (lane 8) and 1.3 kb (lane 10). Filter-bound *EcoR*I cleaved ctDNA hybridized to total ctDNA is shown in lane 9. Ethidium bromide stained fragments of λ-*Hind*III (lanes 1 and 6), ctDNA cleaved with *Bgl*II (lane 2) or *EcoR*I (lane 7) are shown for comparison

fragments would therefore comigrate with the original fragments such that each of the two largest *Bgl*II bands would contain two fragments that differ by only 1 kb. Detection of two comigrating fragments and, hence, recombination, within each band is possible if DNA fragments that overlap the ends of the *Bgl*II fragments and SC1 are used in hybridization analyses of the two *Bgl*II bands. Two *EcoR*I fragments of 3.2 and 4.7 kb overlap the two *Bgl*II fragments and extend into SC1 (Figs. 2 and 3), yet both are outside the IR regions (Rochaix 1978). Thus, both *EcoR*I-*Bgl*II fragments of 0.5 and 1.3 kb generated from these two *EcoR*I fragments should hybridize to both *Bgl*II bands if recombination occurs (Fig. 3). The results presented in Fig. 4 clearly show that the two *EcoR*I-*Bgl*II fragments of 0.5 and 1.3 kb hybridize equally to both *Bgl*II bands (lanes 3 and 5, Fig. 4) and have sequences in common only with the *EcoR*I fragments from which they were derived (lanes 8–10). However, hybridization of a 16.1 kb *BamH*I fragment (Fig. 2) from SC2 is only to the larger of the two *Bgl*II bands (Fig. 5, lane 3), showing that recombination is localized to the IR region. Some cross hybridization to smaller *Bgl*II fragments (Fig. 5, lane 1) is also evident as expected from the results of Rochaix 1978 and 1981.

Discussion

The only conclusion consistent with these hybridization results and with the map of Rochaix 1978, is that both *Bgl*II bands are mixtures of the two comigrating DNA fragments which would result from a recombination event to the left of the *BamH*I fragment and to the right of the *EcoR*I-*Bgl*II fragments. Recombination is thus within or very near the IR region. The resulting isomerization must be a frequent event judging from the near equal stoichiometry of the two *Bgl*II fragments following hybridization to the two *EcoR*I-*Bgl*II fragments (Fig. 4). The two orientations of the chloroplast genome of *C. reinhardii* are shown in Fig. 6. A consequence of inversion isomerization on the physical linkage map is shown by comparing the positons of rbcl and D2, genes for the large subunit of ribulose bisphosphate carboxylase oxygenase and a thylakoid membrane protein, respectively (Malnoe et al. 1979; Rochaix 1981). The two gene loci are differently positioned in the two isomers. Recently, Palmer et al. (in press, 1984b) have obtained new restriction endonuclease mapping data that also shows the existence of both isomeric forms of the ctDNA of *C. reinhardii.*

During the course of this study, we have examined ctDNA isolated from cultures of three different strains (one mt^+ and two mt^-) of *C. reinhardii.* In each case, the large scale cultures were begun from single cloned vegetative cells, and each strain showed the presence of inversion isomers. Genetic studies of cells containing mixtures of differentially marked ct genomes have shown that chloroplast markers segregate during vegetative cell division, eventually yielding progeny cells which are

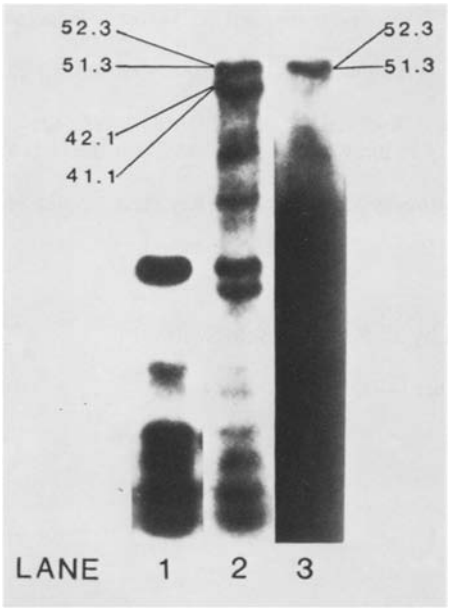

Fig. 5. Hybridization of *BamHI* 16.1 kb fragment to ctDNA digested with *BglII*. *BglII* cleaved ctDNA fragments separated by electrophoresis in 0.2% gels were transferred to nitrocellulose paper. Hybridization with ^{32}P-labelled *BamHI* 16.1 kb fragment (lanes 1 and 3) compared with hybridization to ^{32}P-labelled ctDNA (lane 2). Lane 3 represents a longer exposure of the autoradiogram than lane 1

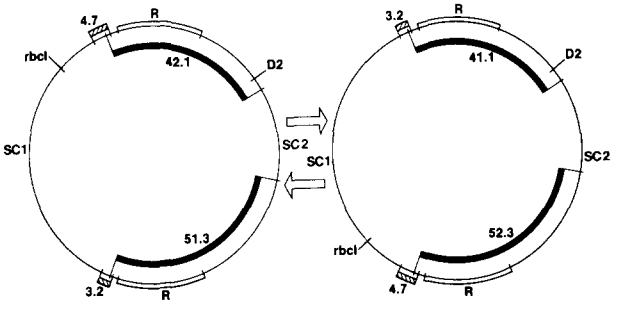

Fig. 6. Molecular isomers of the *C. reinhardii* chloroplast genome. Inversion of SC1 relative to SC2 is shown. The locations of the genes for the large subunit of ribulose bisphosphate carboxylase-oxygenase (rbcl) (Malnoe et al. 1979) and a thylakoid membrane protein (D2) (Rochaix 1981) are shown for comparison

homoplasmic: containing chloroplast genomes which are genetically identical (see reviews by Sager 1977 and Gillham 1978). If a cell containing a mixture of ctDNA isomers were to replicate and segregate ctDNA in the absence of recombination, it would produce progeny cells which contain purely one ctDNA isomer or the other. The occurrence of both isomers in the DNA samples implies that the single cells from which the cultures were started either contained both isomers initially or carried the capacity to generate both isomers. In either case, recombination must be occurring in vegetative cells. The presence of equilibrium equimolar mixtures of isomers shows that the recombination rate at least keeps pace with segregation.

We do not know whether the recombination events that produce the isomerization are general or site-specific, or whether intra and/or intermolecular recombination events are involved. Genetic studies in *C. reinhardii* demonstrate that homologous recombination is active in post-meiotic vegetative cells (Sager 1977; Gillham 1978), diploid vegetative zygotes (VanWinkle-Swift and Birky 1978), and somatic fusion products formed from vegetative cells (Matagne and Hermesse 1980; Matsuda et al. 1983; Galloway and Holden 1984). In each case the recombination machinery seems to be quite general, since it causes the reassortment of numerous genetic markers. It is reasonable to assume that similar recombination enzymology is active and sufficient to promote ctDNA isomerization within the *C. reinhardii* vegetative cells that we have studied.

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