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Genetics

Nonreciprocal recombination between alleles of the chloroplast 23S rRNA gene in interspecific *Chlamydomonas* crosses

(chloroplast DNA recombination/DNA polymorphism)

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ABSTRACT **The inheritance of six polymorphic loci mapping in the rRNA-encoding (rDNA) region of the inverted repeat sequence of chloroplast DNA (cpDNA) was scored in hybrid subclones derived from reciprocal interspecific crosses between the green algae *Chlamydomonas eugametos* and *Chlamydomonas moewusii*. In order to enhance the detection of cells that had undergone recombination between parental cpDNAs, hybrids were selected that inherited a chloroplast antibiotic-resistance marker contributed by the mating-type-minus (mt^-) parent, the parent that normally contributes fewer cpDNA molecules. The major findings of this study can be summarized as follows. (*i*) The majority of the hybrids (14/17) were recombinant for cpDNA markers in the 10-kilobase-pair rDNA region under study. (*ii*) Only one allele of each polymorphic cpDNA locus was ever detected in the hybrids, thus suggesting that newly recombined rDNA sequences in one copy of the inverted repeat are rapidly spread to the other by a copy-correction mechanism. (*iii*) Chloroplast streptomycin-resistance (*sr-2*) and erythromycin-resistance (*er-nM1*) loci, although showing little or no genetic linkage, were mapped to the 16S and 23S rRNA gene regions of the cpDNA, respectively, by virtue of their perfect coinheritance with polymorphic markers within these genes. (*iv*) cpDNA markers associated with a putative intron of the *C. eugametos* 23S rRNA gene were inherited by all 17 hybrids. Such a result is similar to that observed for certain alleles of the large rRNA gene of yeast mitochondria in crosses between ω^+ and ω^- strains.**

Experimental evidence for recombination between parental chloroplast genomes during sexual reproduction is limited to the algal genus *Chlamydomonas*, for which this process has been characterized both genetically (1–4) and physically (5). Attempts to detect such recombination during sexual crosses with flowering plants have been unsuccessful, even in genera such as *Oenothera*, which transmit chloroplasts at a high frequency from both parents (6). Recently, however, recombination between parental chloroplast genomes has been demonstrated physically in an interspecific hybrid of *Nicotiana* generated by protoplast fusion (7). This particular hybrid line was rare in its being recombinant for parental non-Mendelian genetic markers. The common occurrence of recombination between parental chloroplast genomes in *Chlamydomonas* crosses is certainly related to the fact that there is fusion of the two parental chloroplasts in zygotes (8).

Differences in the restriction fragmentation patterns of chloroplast DNA (cpDNA) from the interfertile *Chlamydomonas eugametos* and *Chlamydomonas moewusii* have provided physical markers to score the inheritance of this DNA in interspecific hybrids of these algae (9, 10). *C. eugametos* and *C. moewusii* mate reciprocally and efficiently to form diploid zygospores, which undergo meiosis and germinate into four haploid products. However, survival of these meiotic products is low, with fewer than 10% giving viable progeny (11, 12). Virtually all hybrid lines recovered after meiotic germination and the completion of sorting out (about 20 mitotic generations) have proven to be homoplasmic for recombinant cpDNA digestion patterns; i.e., all of the multiple cpDNA copies in each cell appear identical (13). Most fragment markers in these recombinant patterns were derived from the mating-type-plus (mt^+) parent, a result consistent with the observation that there is preferential destruction of cpDNA derived from the mating-type-minus (mt^-) parent during the mating process (14, 15).

Analysis of recombinant cpDNA digestion patterns from *C. eugametos* × *C. moewusii* hybrids gives no information on the number and position of recombinational events without knowledge of the restriction maps of the parental cpDNAs as well as the alignment of these maps. Such maps and their alignment, now available (16–18), reveal fragment length and restriction site polymorphisms that are both numerous and well-dispersed throughout the chloroplast genome. In the present study, we have focused on the inheritance of six polymorphic loci in the rRNA gene (rDNA) region of the inverted repeat sequences of the *C. eugametos* and *C. moewusii* cpDNAs. Reciprocal crosses were performed between *C. eugametos* with a non-Mendelian mutation for resistance to streptomycin (*sr-2r*) and *C. moewusii* with a non-Mendelian mutation for resistance to erythromycin (*er-nM1r*). The *sr-2* locus has already been shown to map to the chloroplast 16S rRNA gene (13), whereas the *er-nM1* locus is shown in the present study to map to the chloroplast 23S rRNA gene. In order to enhance the detection of hybrids recombinant for the physical markers examined, cells were selected that contained the antibiotic-resistance allele derived from the mt^- parent, the parent that normally contributes fewer cpDNA molecules. With this approach, proposed by Mets and Geist (3), we have measured the extent of linkage retention between the selected genetic marker and the unselected genetic and physical cpDNA markers. The results show that almost half of the hybrids were recombinant for the unselected genetic marker and most were recombinant for one or more of the physical ones. In addition, a polymorphic cpDNA region mapping in the chloroplast 23S rRNA gene was inherited, without exception, from the *C. eugametos* parent. Such a nonreciprocal recovery of recombinants is similar to that observed for certain alleles of the large rRNA gene of yeast mitochondria in crosses between ω^+ and ω^- strains (19). A portion of this work has been published in preliminary form (20).

MATERIALS AND METHODS

Strains, Crosses, and Genetic Analysis. The origin of the mt^+ and mt^- strains of *C. eugametos* carrying the streptomycin-resistance marker (*sr-2r*) and of the mt^+ and mt^- strains of *C. moewusii* carrying the erythromycin-resistance marker (*er-nM1r*) has been described, as have the procedures

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Abbreviations: cpDNA, chloroplast DNA; rDNA, DNA that encodes rRNA; *mt*, mating type.

used for crosses and genetic analysis (4). Recombination frequencies between the *sr-2* and *er-nM1* loci were measured according to the paternal (mt^-) marker selection method of Mets and Geist (3).

DNA Isolation. Whole-cell DNA was isolated from *C. eugametos*, *C. moewusii*, and interspecific hybrid strains as described previously (10), except that after the extraction with phenol, nucleic acids were precipitated twice with ethanol in the presence of 2.5 M ammonium acetate and redissolved in 10 mM Tris·HCl, pH 8.0/1 mM EDTA.

Southern Blot Hybridization. DNA restriction fragments separated by electrophoresis in 0.75–1.5% agarose gels were transferred to nitrocellulose filters and hybridized with nick-translated ^{32}P-labeled probes as described by Rochaix and van Dillewijn (21).

RESULTS

The genetic history of the 17 hybrid subclones scored for the inheritance of chloroplast rDNA polymorphisms is shown in Table 1. These subclones were derived from reciprocal interspecific crosses between *C. eugametos sr-2^r er-nM1^s* and *C. moewusii sr-2^s er-nM1^r*. In each cross, most zygospore colonies expressed only the resistance marker of the mt^+ parent (UP+ zygospore clones); many expressed the resistance markers of both parents (BP zygospore clones); and rare ones expressed only the resistance marker of the mt^- parent (UP− zygospore clones). There was a higher fraction of BP and UP− zygospore clones in cross I, where *C. eugametos* was the mt^+ parent, than in the reciprocal cross (cross II). The usual two-sample test for equality of binomial probabilities shows that each of these differences between the two crosses is unlikely to have occurred by chance ($P < 10^{-4}$). Fifty-seven subclones with the mt^--derived resistance marker were each recovered from independent BP and UP− zygospore clones without regard to the antibiotic-resistance marker contributed by the mt^+ parent. About 40% of these subclones later proved recombinant for the mt^+-derived resistance marker. For DNA analysis, 8 subclones from cross I and 9 from cross II were chosen randomly from the 57 subclones. With further testing, all of these proved stable and homoplasmic for their indicated non-Mendelian phenotype;

Table 1. Genetic history of hybrid subclones

	Cross I: Ce mt^+ (*sr-2^r er-nM1^s*) × Cm mt^- (*sr-2^s er-nM1^r*)	Cross II: Cm mt^+ (*sr-2^s er-nM1^r*) × Ce mt^- (*sr-2^r er-nM1^s*)
	*Frequency of zygospore colonies**	
UP+	0.905 (987)	0.707 (176)
BP	0.089 (97)	0.253 (63)
UP−	0.006 (7)	0.040 (10)
	No. of independent subclones with selected marker†	
	35 (*er-nM1^r*)	22 (*sr-nM1^r*)
	Fraction of subclones recombinant for unselected marker	
	46%	36%
	Hybrid subclones employed‡	
	S^{s}E^r: 23, 30, 43, 46, 48	S^{r}E^s: 2, 6, 7, 15, 18
	S^{r}E^r: 27, 50, 54	S^{r}E^r: 1, 3, 4, 5

Ce, *C. eugametos*; Cm, *C. moewusii*.

*UP+, uniparental inheritance of resistance marker from the mt^+ parent; BP, biparental inheritance of resistance markers; UP−, uniparental inheritance of resistance marker from the mt^- parent. The number of zygote colonies in each category is indicated in parentheses.

†Selected marker is indicated in parentheses.

‡Hybrid subclones are designated by numbers and grouped into three phenotypic classes (S^{s}E^r, *sr-2^s er-nM1^r*; S^{r}E^s, *sr-2^r er-nM1^s*; S^{r}E^r, *sr-2^r er-nM1^r*).

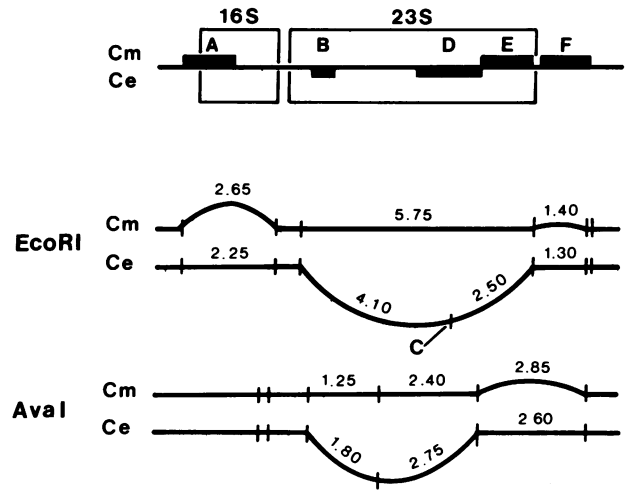

FIG. 1. Positions of six polymorphic loci (A–F) within the rDNA region of the *C. eugametos* (Ce) and *C. moewusii* (Cm) chloroplast genomes. The *C. eugametos* and *C. moewusii* *Eco*RI and *Ava* I restriction maps of this rDNA region are shown together with the sizes (in kbp) of homologous fragments detected in hybridization experiments (17). Polymorphisms of A, B, D, E, and F result from fragment length differences of 0.40, 0.55, 0.35, 0.15, and 0.10 kbp, respectively. The extra sequences causing these length differences are located in *C. eugametos* or *C. moewusii* somewhere within the cpDNA segment delimited by the thick lines. Polymorphisms of C differ for the presence or absence of an *Eco*RI restriction site. The boxes indicate the positions of the 16S and 23S rRNA genes.

i.e., additional subcloning revealed no segregation of alleles for resistance and sensitivity.

Fig. 1 reviews the aligned restriction maps of the rDNA regions of the *C. eugametos* and *C. moewusii* cpDNAs as well as the positions of the six polymorphic loci followed here. Five of these loci consist of restriction-fragment length differences ranging from 0.12 to 0.55 kilobase pair (kbp) (A, B, D, E, and F) and the other (C) is an *Eco*RI restriction-site difference. The probing strategy employed to score the inheritance of these polymorphisms is summarized in Table 2. Cloned *Eco*RI cpDNA fragments spanning the polymorphic regions were hybridized to Southern blots of *Ava* I and *Eco*RI digests of whole-cell DNA from the 17 hybrids and from *C. eugametos* and *C. moewusii* (Fig. 2). By comparing sizes of the hybrid and parental cpDNA fragments that were detected in the hybridizations (Table 3), it was possible to unambiguously score the parental origin of the regions A, B,

Table 2. Hybridization strategies employed to score the inheritance of chloroplast rDNA polymorphisms in hybrid subclones

Hybridization strategy	*Eco*RI fragment probes*	Filter-bound whole-cell DNA fragments	Polymorphic region(s) scored†
1	2.65 m	*Eco*RI	A
2	4.10 e	*Ava* I	B, D
3	4.10 e	*Eco*RI	C, (B), (D)
4	2.50 e	*Eco*RI	C, (D), (E)
5	1.40 m	*Eco*RI	F
6	1.40 m	*Ava* I	(E), (F)

*The probes are pBR322 clones of *C. moewusii* (m) or *C. eugametos* (e) *Eco*RI cpDNA fragments. These probes are designated by the size (kbp) of their cpDNA insert.

†The inheritance of markers in parentheses cannot be determined unambiguously by hybridization strategies 3, 4, or 6. The results of hybridization strategies 5 and 6 are necessary to follow the inheritance of polymorphic region E.

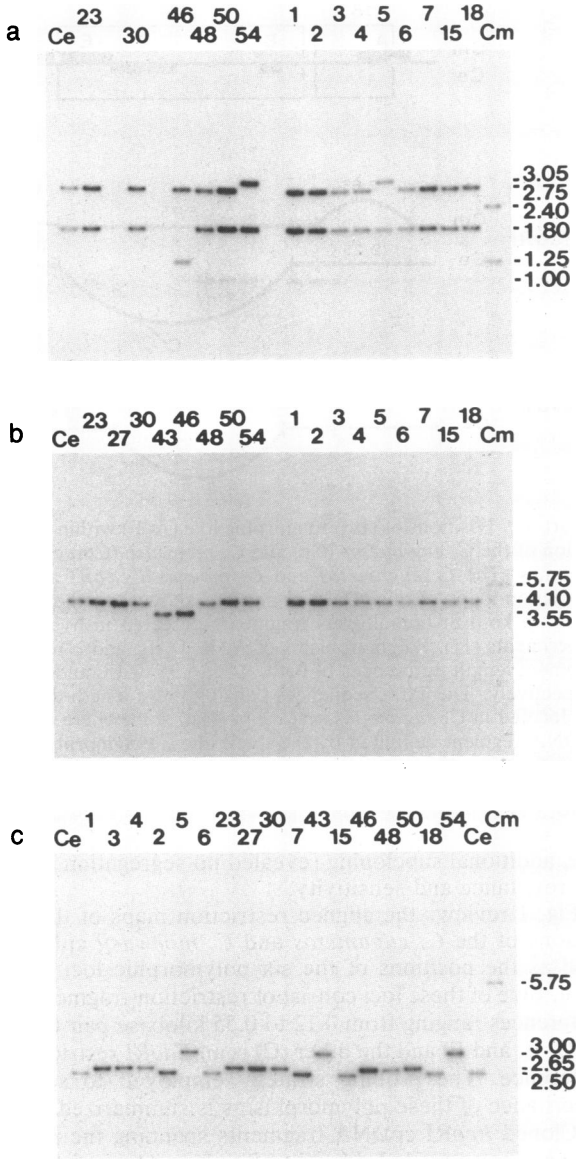

FIG. 2. Hybridization of cloned *Eco*RI cpDNA fragments to restriction digests of whole-cell DNA from *C. eugametos* (Ce), *C. moewusii* (Cm), and 17 hybrid subclones. (*a*, *b*, and *c*) Results of hybridization strategies 2, 3, and 4, respectively (see Table 2). Sizes of hybridizing fragments are given in kbp on the right of the autoradiograms.

C, E, and F in all 17 hybrids. The parental origin of region D, however, could not always be determined because hybrids 5, 43, and 54 inherited a nonparental form of this region. This is most clearly illustrated by probing strategy 2, where the *C. moewusii* and *C. eugametos* alternatives are easily detected as 2.40- and 2.75-kbp hybridizing *Ava* I fragments, respectively, while the nonparental allele is detected as a 3.05-kbp *Ava* I fragment. Probing strategies 3 and 4 indicate that the extra DNA associated with the nonparental allele is positioned between the *Eco*RI site polymorphism (site C) and the downstream *Ava* I site. The remaining 14 hybrids all inherited the *C. eugametos* allele of region D.

Fig. 3 summarizes the phenotypes of the 17 hybrids with respect to the streptomycin- and erythromycin-resistance markers and the five cpDNA polymorphisms whose parental origin could be scored in all hybrids. The results indicate that only one allele of each polymorphic DNA region was ever detected in each of the 17 hybrids. This supports genetic data that sorting out in these hybrids was complete; i.e., that the cells did not contain a mixture of parental and recombinant chloroplast genomes. It also indicates that the two copies of the rDNA region from each hybrid are identical with respect to the markers followed. The symmetrical occurrence of mutational events in the large inverted repeat sequence of chloroplast genomes is well-documented in the recent literature (22, 23). This phenomenon is generally believed to result from a copy-correction mechanism that maintains sequence homogeneity in the two repeats. Because the rDNA region studied here is part of this repeated sequence and because only one allele of each polymorphic region was ever detected in particular hybrids, newly recombined rDNA sequences in one copy of the repeat are likely to have spread rapidly to the other through such a copy-correction mechanism.

The results of Fig. 3 further indicate that most of the 17 hybrid subclones had undergone recombination between cpDNA markers in the region under study. Moreover, a recombination was detected between all cpDNA markers in this region; hence, our results agree with the results of Palmer *et al.* (24), which indicate that recombinational events between inverted repeats of *Chlamydomonas reinhardtii* cpDNA are not limited to one or a few sites. Only hybrid subclones 2, 7, and 18 from cross II showed no recombination in the rDNA region; however, these hybrids recently proved recombinant for cpDNA polymorphisms outside this region (B. Lemieux, M. Turmel and C.L., unpublished observations). The data also show perfect linkage between the *er-nM1* locus and polymorphic region E at the 3′ end of the 23S rRNA gene, regardless of whether or not hybrids were selected to contain *sr-2*^{r} from *C. eugametos* (cross II) or *er-nM1*^{r} from *C. moewusii* (cross I). In a similar fashion, the inheritance of the *sr-2* locus was strictly correlated with that of polymorphic region A near the 5′ half of the 16S rRNA gene.

Finally, the results in Fig. 3 show some striking differences in the gradient of linkage between the selected marker of each cross, indicated by the vertical boxes, and the unselected ones. In cross II, no recombination was detected between the selected *sr-2*^{r} marker (tightly linked with region A) and

Table 3. Sizes of cpDNA fragments from *C. eugametos* (Ce), *C. moewusii* (Cm), and the 17 hybrid subclones

Probe	Digest	Fragment size, kbp																		
		Ce	Cm	23	30	43	46	48	27	50	54	1	3	4	5	2	6	7	15	18
2.65 m	*Eco*RI	2.25	2.65	2.65	2.65	2.65	2.65	2.65	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25
4.10 e	*Ava* I	2.75	2.40	2.75	2.75	3.05	2.75	2.75	2.75	2.75	3.05	2.75	2.75	2.75	3.05	2.75	2.75	2.75	2.75	2.75
		1.80	1.25	1.80	1.80	1.25	1.25	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
4.10 e	*Eco*RI	4.10	5.75	4.10	4.10	3.55	3.55	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10	4.10
2.50 e	*Eco*RI	2.50	5.75	2.65	2.65	3.00	2.65	2.65	2.65	2.65	3.00	2.65	2.65	2.65	3.00	2.50	2.50	2.50	2.50	2.50
1.40 m	*Eco*RI	1.30	1.40	1.30	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.30	1.40	1.30	1.40	1.30
1.40 m	*Ava* I	2.60	2.85	2.75	2.85	2.85	2.85	2.85	2.85	2.85	2.85	2.85	2.85	2.85	2.85	2.60	2.70	2.60	2.70	2.60

Fragments were detected by use of the hybridization strategies described in Table 2.

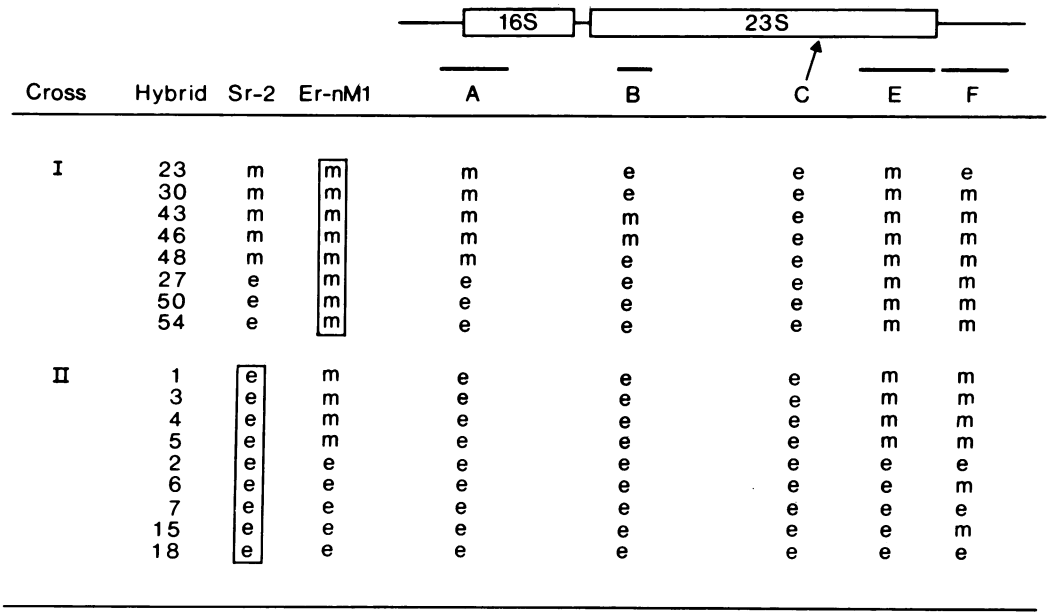

FIG. 3. Summary of the inheritance of non-Mendelian antibiotic-resistance markers and cpDNA polymorphisms in hybrid subclones. Vertical boxes surround the selected antibiotic-resistance marker of each cross. The *C. eugametos* and *C. moewusii* alleles of the genetic and physical loci are represented by e and m, respectively. Cross I, *C. eugametos sr-2^r er-nM1^s mt$^+$* × *C. moewusii sr-2^s er-nM1^r mt$^-$*; cross II, *C. moewusii sr-2^s er-nM1^r mt$^+$* × *C. eugametos sr-2^r er-nM1^s mt$^-$*.

downstream regions B and C. Regions E and F further downstream, however, appear unlinked with the selected marker. In cross I, little recombination was noted between the selected *er-nM1^r* marker (tightly linked with region E) and downstream region F, but all hybrids were recombinant for region C only several hundred base pairs upstream of the selected marker; i.e., all hybrids inherited the *C. eugametos* allele of region C. The frequency of recombination between region C and the more upstream regions B and A, in the same cross, increases with their distance from C to the point where A appears unlinked to C. We stress that only the *C. eugametos* allele of polymorphic region C was detected in the 17 hybrids.

DISCUSSION

This report describes the physical mapping of an erythromycin-resistance locus to a chloroplast genome. The locus, *er-nM1*, was mapped to the 3′ half of the chloroplast 23S rRNA genes of *C. eugametos* and *C. moewusii*, a result consistent with the position of base-pair changes associated with erythromycin resistance in *Escherichia coli* (25) and in yeast (*Saccharomyces cerevisiae*) mitochondria (26). We have also confirmed our earlier mapping (13) of the *sr-2* locus to the 16S rRNA gene of these algae. Recently, a specific site for streptomycin resistance has been identified within the chloroplast 16S rRNA gene of *Euglena* (27).

The mapping of the *sr-2* and *er-nM1* loci to genes within the chloroplast rDNA region of the inverted repeat sequence of *C. eugametos* and *C. moewusii* raises questions about the physical significance of chloroplast genetic maps from *C. reinhardtii* (1, 2). As loci for resistance to streptomycin and erythromycin are among the most distantly separated on these genetic maps, it is possible that all or many of the mapped markers are clustered within the inverted repeat region of the *C. reinhardtii* chloroplast genome. This possibility is strengthened by the fact that most of these markers are functionally related by conferring antibiotic resistance to chloroplast ribosomes.

It is of particular interest that only the *C. eugametos* allele of the *Eco*RI restriction-site polymorphism (C) was inherited by the hybrid subclones. The *C. eugametos* long allele of polymorphic region D was also inherited preferentially: 14 of the 17 hybrid subclones inherited this allele. The remaining three hybrids inherited a nonparental allele of region D that was longer than either parental allele. Recent sequence analysis of the *C. eugametos* chloroplast 23S rRNA gene indicates the presence of a putative 953-base-pair intron near the middle of this gene in polymorphic region D (20). The unidirectionally inherited *Eco*RI restriction-site marker was shown to be centrally located in this putative intron. As there is only a 0.35-kbp addition/deletion difference between the *C. eugametos* and *C. moewusii* alleles of region D, it is likely that *C. moewusii* has a smaller intron either at the same position or at another position within region D. We favor the second possibility because recombinational events combining two optional introns, one from *C. eugametos* that contains the *Eco*RI site and one from *C. moewusii* slightly downstream in region D, would explain the origin of three hybrids with an allele of region D that is larger than either parental allele. Proof or disproof of this possibility must await further sequence analysis. To our knowledge, the only other interrupted chloroplast rRNA gene known to date is that coding for the 23S rRNA of *C. reinhardtii* (28). Little homology, however, was detected between the 870-base-pair intron of this gene and the *C. eugametos* and *C. moewusii* 23S rRNA genes (C.L., unpublished observations).

In a formal genetic sense, the nonreciprocal recovery of certain chloroplast 23S rDNA markers in the interspecific crosses described here strongly resembles the well-characterized phenomenon of polarity of recombination in yeast mitochondrial DNA. This phenomenon was first observed as a widely unequal recovery of reciprocal recombinants between certain markers in crosses between ω^+ and ω^- yeast strains (29). It is now known that the region showing polarity of recombination is in the large rRNA gene near an optional group I intron (r1) and that ω^+ and ω^- strains differ with respect to the presence or absence of this intron (19, 30). More recent evidence indicates that a translation product of

the intron encodes information at least partly responsible for the efficient conversion of ω^- to ω^+ strains (31, 32). This event coconverts markers flanking the intron insertion site at a frequency that decreases sharply with their distance from this site (33); markers only a few kilobase pairs from this region show no polarity of recombination. Other, less well-characterized examples of locus-specific, biased inheritance of mitochondrial DNA markers have been reported in yeast (34) and *Neurospora* (35, 36).

Our operating assumption is that the molecular basis for the unidirectional inheritance of cpDNA markers described here is similar to that of the r1 intron of yeast. This view is supported by the observation that the *Chlamydomonas* markers showing this unidirectional inheritance map in or around a putative intron of the chloroplast large rRNA gene and by the indication that the algae being crossed differ with respect to the size or presence of this intron. In the absence of direct molecular proof for such an intron-mediated conversion mechanism, it is difficult to eliminate explanations based on intra- or intercellular selection. We now know, however, that the low survival associated with the interspecific crosses is unrelated to a requirement for cells to inherit the putative *C. eugametos* intron. In a high-viability cross between *C. moewusii* mt^+ and a mt^-, fifth-generation backcross hybrid with the putative *C. eugametos* intron, there was quantitative recovery, in all 17 zygospore clones scored, of only the alleles associated with this putative intron (unpublished observations).

The majority of the interspecific hybrids analyzed in this study proved to be recombinant for the rDNA polymorphic markers. The different level of recombination of physical markers in the reciprocal crosses is certainly related to the fact that the hybrids from one cross were selected to contain *er-nM1*^{r} from the *C. moewusii* mt^- parent, while the *C. eugametos* mt^+ parent contributed the unidirectionally inherited putative intron sequence (cross I). In the reciprocal cross (cross II) the unidirectionally inherited sequence and the selected *sr-2*^{r} marker came from the same *C. eugametos* mt^- parent. Nevertheless, recombination between the ends of the region under study was quite common in both crosses, as revealed by the frequency of recombination between the *er-nM1* and *sr-2* loci. The frequency of 46% in cross I and 36% in cross II may approach the upper level of recombination for chloroplast markers as detected with the methods employed (3). This high frequency of recombination between chloroplast rDNA sites separated by no more than 10 kbp may not be unique to the interspecific *C. eugametos* × *C. moewusii* crosses; in intraspecific *C. moewusii* (4) and *C. reinhardtii* (2, 3) crosses, recombination between chloroplast markers for streptomycin resistance and erythromycin resistance is similarly high. Among the markers used in these latter crosses, however, only *er-nM1*^{r} of *C. moewusii* is known to map in the inverted repeat region of cpDNA.

Finally, the difference between the reciprocal interspecific crosses with respect to the inheritance of antibiotic-resistance markers in zygospore colonies might be explained by weak linkage between the unidirectionally inherited *C. eugametos* cpDNA sequence and one or both antibiotic resistance loci. Alternatively, this difference could be related to known differences between patterns of chloroplast gene transmission in *C. eugametos* and *C. moewusii* crosses (4).

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