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Recombination of *Chlamydomonas* chloroplast DNA occurs more frequently in the large inverted repeat sequence than in the single-copy regions

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Summary. It is well documented that chloroplast DNA (cpDNA) recombination occurs at a relatively high frequency during sexual reproduction of unicellular green algae from the *Chlamydomonas* genus. Like the cpDNAs of most land plants, those of *Chlamydomonas* species are divided into two single-copy regions by a large inverted repeat sequence, part of which encodes the chloroplast rRNA genes. In the present study, we scored the inheritance of polymorphic loci spanning the entire chloroplast genome in hybrids recovered from reciprocal interspecific and F_1 crosses between *Chlamydomonas eugametos* and *C. moewusii*, and from these data, estimated the density of recombination junctions within each region of recombinant cpDNAs. Our results indicate that recombination junctions occur at highly variable frequencies across the three main domains of the chloroplast genome. The large inverted repeat sequence was found to exhibit at least a five-fold higher density of recombination junctions compared to one of the single-copy regions, whereas junctions in the latter region were five-fold more abundant relative to those in the other single-copy region. This marked difference in the densities of recombination junctions implies that the extent of genetic linkage between two given chloroplast loci will depend not only on their physical distance, but also on their locations within the genome.

Key words: Chloroplast DNA regions – Restriction fragment length polymorphisms – Recombination frequency – Non-Mendelian inheritance – Antibiotic resistance markers

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Introduction

Although recombination of chloroplast genetic markers has been widely documented in green algae belonging to the *Chlamydomonas* genus (Gillham 1978; Harris 1989), little is known about the nature and extent of chloroplast DNA (cpDNA) recombination itself. Efforts to correlate genetic and physical maps along the entire *Chlamydomonas* cpDNA molecule have failed so far, because most of the markers used in the construction of the genetic maps are clustered in a small portion of the chloroplast genome (Harris et al. 1987, 1989; Gauthier et al. 1988). In an attempt to resolve this problem, we have recently been tracing the inheritance of physically mapped cpDNA restriction fragment length polymorphisms in interspecific hybrids of *Chlamydomonas eugametos* and *C. moewusii* (Lemieux and Lee 1987; Lemieux et al. 1988).

Fusion of the two parental chloroplasts takes place in *Chlamydomonas* zygotes (Cavalier-Smith 1970), thus allowing recombination between opposite parental chloroplast genomes. Despite this fusion, however, most zygotes transmit exclusively or predominantly the chloroplast genetic markers derived from the mating-type plus (mt^+) parent. This predominant inheritance of mt^+ alleles is consistent with studies indicating that the multiple cpDNA copies of the mt^- parent are preferentially destroyed during the mating process (Kuroiwa et al. 1982; Coleman and Maguire 1983). Chloroplast gene recombination occurs among the progeny of zygotes transmitting the chloroplast alleles from both the mt^+ and mt^- parents. In *C. reinhardtii* (Gillham 1978) and *C. eugametos* (Lee and Lemieux 1986) intraspecific crosses as well as in *C. eugametos*/*C. moewusii* interspecific crosses (Lemieux and Lee 1987), less than 20% of the zygotes transmit chloroplast alleles from both par-

ents with a strong bias in favour of the mt^+ alleles, whereas in *C. moewusii* intraspecific crosses (Lee and Lemieux 1986) this class of zygotes, designated biparental, accounts for over 90% of the population. Segregation of cpDNA takes place rapidly among the mitotic progeny of biparental zygotes, and only 20 postmeiotic mitotic divisions are required to produce cells homoplasmic for parental or recombinant cpDNA molecules.

The chloroplast DNAs of *Chlamydomonas,* like those of most land plants, are circular and are divided into two single-copy regions by a large inverted repeat sequence (20–42 kbp) encoding the chloroplast ribosomal RNAs (Lemieux and Lemieux 1985). At 292 kbp, the *C. moewusii* cpDNA (Turmel et al. 1987) is 97 kbp and 49 kbp larger than its homologues in *C. reinhardtii* (Rochaix 1978) and *C. eugametos* (Lemieux et al. 1985 b), respectively. The overall gene organization of the *C. eugametos* and *C. moewusii* cpDNAs is essentially the same (Turmel et al. 1987, 1988), but differs tremendously from that of the *C. reinhardtii* cpDNA (Lemieux et al. 1985 a) and the consensus gene order found in most land plant cpDNAs (Turmel et al. 1988). The 49-kbp size difference between the *C. eugametos* and *C. moewusii* cpDNAs is almost totally accounted for by the presence of two large extra sequences in *C. moewusii*: a 21-kbp sequence in the inverted repeat (locus *G*; Lemieux et al. 1985 c) and a 5.8-kbp sequence in the single-copy region bordering the 16S rRNA genes (locus *R*; Turmel et al. 1987). Aside from these two major addition/deletion differences, restriction site and fragment length polymorphisms were mapped at 43 locations throughout the two algal genomes (Turmel et al. 1987).

The large inverted repeat sequence of the chloroplast genome participates in both reciprocal and non-reciprocal intramolecular recombination events. Reciprocal intramolecular recombination between the two copies of the inverted repeat sequence occurs at such a high frequency in the cpDNAs of land plants (Palmer et al. 1985) and *C. reinhardtii* (Aldrich et al. 1985) that it leads to the formation of an equal proportion of single-copy region orientation isomers. This type of intramolecular recombination, also designated flip-flop recombination, has been observed in *C. reinhardtii* cpDNA mutants carrying various deletions of the inverted repeat sequence, thus indicating that it is not restricted to a specific region of this sequence (Palmer et al. 1985). On the other hand, intramolecular gene conversion events are thought to lead to copy-correction of the inverted repeat sequence in heteroplasmic cpDNAs, i.e. cpDNAs whose copies of this sequence differ at several loci (Lemieux and Lee 1987) or cpDNAs in which point mutations (Erickson et al. 1986) or large deletions (Myers et al. 1982) are present in one of the two copies. These gene conversion events also probably account for the symmetrical restoration of inverted repeat sequences during *C. reinhardtii* transformation experiments of inverted repeat deletion mutants, with cloned cpDNA sequences encompassing the junction between the inverted repeat and one of the single copy region (Boynton et al. 1988; Blowers et al. 1989). It should be emphasized that copy-correction of inverted repeat sequences provides the only direct evidence for gene conversion events in *Chlamydomonas* chloroplasts. Although non-reciprocal recovery of chloroplast genetic and physical markers has been reported in intraspecific (VanWinkle-Swift and Birky 1978) and interspecific (Lemieux and Lee 1987; Lemieux et al. 1988) crosses of *Chlamydomonas,* this does not necessarily imply that the recombination mechanism is non-reciprocal, i.e., that it leads to the loss of alleles because of gene conversion events. Even if the mechanism of cpDNA recombination were solely reciprocal, a biased output of certain alleles could be caused by interspecific incompatibilities (Lemieux and Lee 1987) or the random drift of cpDNA sequences (Birky et al. 1981).

We have recently traced the inheritance and recombination of 23 polymorphic cpDNA loci among randomly selected homoplasmic hybrids issued from reciprocal *C. eugametos*/*C. moewusii* interspecific crosses (Lemieux et al. 1988). Analysis of the inheritance patterns revealed that most loci display the allele of the mt^+ parent, a result which is consistent with the predominantly uniparental inheritance of individual chloroplast genetic markers in such crosses. Whatever the mating type of the parental strains, however, three loci (*C, G* and *R*) featured only the long alleles derived from *C. moewusii* or *C. eugametos*; i.e., the 5.8- and 21-kbp extra sequences of *C. moewusii* and an optional group I intron in the large subunit rRNA gene of *C. eugametos*. These unidirectional patterns of inheritance are likely to be the results of gene conversion events. Most recombination junctions identified in F_1 hybrid cpDNAs were mapped in the vicinity of the unidirectionally inherited loci, suggesting that co-conversion of alleles at adjacent loci occurs over a relatively short distance. A limited number of recombination junctions were identified within cpDNA regions that are distant from the unidirectionally inherited loci *C, G* and *R*. As these junctions cannot be explained by co-conversion of alleles, they most probably result from the generalized cpDNA recombination system. To facilitate studies of this recombination system, one could attempt to maximize the chances of detecting cells that are recombinant for cpDNA loci by selecting progeny with a chloroplast antibiotic resistance marker derived from the mt^- parent, i.e., the parent that normally contributes fewer cpDNA molecules. Using this approach, Lemieux and Lee (1987) easily recovered, from reciprocal interspecific crosses, hybrids that were recombinant for several of the six polymorphic loci they analyzed within the rDNA operon.

In the present study, we asked if generalized cpDNA recombination occurs at the same frequency throughout the *Chlamydomonas* chloroplast genome. To answer this question, we scored the inheritance of polymorphic cpDNA loci in the *C. eugametos*/*C. moewusii* hybrids that Lemieux and Lee (1987) selected for the presence of the chloroplast antibiotic resistance marker contributed by the mt^- parent, as well as in F_2 hybrids that we selected in a similar fashion from a cross in which both F_1 parents shared the three unidirectionally inherited cpDNA sequences, but differed for the remaining polymorphi loci. Our results indicate that the single-copy regions are far less recombinant than the inverted repeats, an observation that complicates the task of aligning the genetic and physical maps of the *Chlamydomonas* chloroplast genome.

Materials and methods

Strains, crosses and genetic analysis

The genetic history of the 17 F_1 hybrids has been described by Lemieux and Lee (1987). These hybrids were derived from reciprocal crosses between a *C. eugametos* strain carrying the chloroplast streptomycin resistance mutation *sr-2* and a *C. moewusii* strain carrying the chloroplast erythromycin resistance mutation *er-nM1*. After about 20 post-meiotic mitotic divisions on non-selective medium, individual zygote colonies expressing the chloroplast antibiotic resistance marker contributed by the mt^- parent were resuspended in minimal medium, and an aliquot from each colony was plated on non-selective agar medium. For each zygote, one subclone carrying the mt^- resistance marker was randomly picked and tested for the inheritance of chloroplast polymorphic markers and the chloroplast genetic marker contributed by the mt^+ parent.

The F_2 hybrids were obtained by crossing two of the aforementioned F_1 hybrids: the mt^+ F_1 hybrid 18 carrying the *sr-2* mutation and the mt^- F_1 hybrid 46 carrying the *er-nM1* mutation. This cross as well as the genetic analysis of the F_2 meiotic progeny were performed as described by Lee and Lemieux (1986). Recombination frequency between the *sr-2* and *er-nM1* loci was determined in the meiotic progeny by the paternal (mt^-) selection method of Mets and Geist (1983). For cpDNA analysis, isolated zygotes were allowed to germinate and divide mitotically on minimal agar plates until they formed a colony. A sample of each zygote colony was transferred to minimal agar medium containing 100 µg/ml erythromycin for the detection of resistant cells. Liquid suspensions of eight resistant zygote colonies were plated on non-selective medium and a single resistant subclone from each zygote colony was randomly chosen for DNA isolation.

Inheritance of cpDNA polymorphic loci

To determine the inheritance of polymorphic cpDNA loci, Southern blots of total cellular DNA digests from the hybrids and the *C. eugametos* and *C. moewusii* parents were hybridized with cloned cpDNA fragments containing the loci of interest. The parental origin of alleles at a given polymorphic region was determined by comparing the electrophoretic mobilities of hybridizing fragments from hybrids relative to those of the parental strains. Table 1 presents the overall hybridization strategy employed as well as the position and nature of the polymorphic loci examined.

Table 1. Hybridization strategy used to determine the inheritance of the polymorphic cpDNA loci in hybrids

Fragment probe[a]	Total DNA digest	Polymorphic loci		
		Name	Position[b]	Nature[c]
E21m	EcoRI	A	2	+400 bp Cm
E14e	EcoRI	C	8	1 EcoRI site Ce
E14e	AvaI	B, D	6, 8	+500 bp Ce, +330 bp Ce
E18e	EcoRI	C, D	8	1 EcoRI site, +330 bp Ce
E29′m	AvaI	E[d], F	10, 12	+150 bp Cm, +100 bp Cm
E29′m	EcoRI	F	12	+100 bp Cm
S7m	EcoRI	G	15	4 EcoRI fragments Cm (11.5 kbp)
R15,4r	BstEII	H, I	19, 21	2 BstEII sites Cm
E3m	BstEII	J, K	26, 30	2 BstEII sites Cm
E23′m	EcoRI	L	38	+100 bp Cm
E6′m	AvaI	M	46	1 AvaI site Ce
E8e	AvaI	N	50	+400 bp Cm
E21e	EcoRI	O	72	+100 bp Cm
E11m	AvaI	P	135	+200 bp Ce
E22m	EcoRI	Q	152	+300 bp Cm
E26m	EcoRI	R	164	1 EcoRI fragment (1.8 kbp)
E19m	BstEII	S	172	1 BstEII site Cm
E5m	AvaI	T	190	1 AvaI site Ce
E16m	EcoRI	U	208	1 EcoRI site Cm
R3r	BstEII	V, W	212, 216	2 BstEII sites Ce

[a] Each fragment probe is designated by a capital letter (E, S or R) followed by a number and a lower case letter (e, m or r). E and S refer to the EcoRI and SalI restriction fragments of *C. eugametos* and *C. moewusii* cpDNAs, while R refers to the *C. reinhardtii* cpDNA fragments EcoRI 3 (Rochaix 1978) and HindIII 15.4 (Dron et al. 1982). The *C. eugametos* and *C. moewusii* fragments are numbered according to the nomenclature used by Lemieux et al. (1985b, c) and Turmel et al. (1987). The lower case letter indicates the origin of the probe, e, m, and r referring to *C. eugametos*, *C. moewusii* and *C. reinhardtii*, respectively. The papers of Lemieux et al. (1985b) and Turmel et al. (1987) should be consulted for further details on the comparison of the *C. eugametos* and *C. moewusii* cpDNA restriction maps

[b] These positions were determined relative to the EcoRI site of fragment 21 at the junction of the inverted repeat and the single-copy region SCR2 (Fig. 1). Note that polymorphic loci *A* through *I* are within the inverted repeat; the second set of these loci is located between positions 108 and 130

[c] The + followed by a number indicates the size in bp of the additional sequences present in *C. eugametos* (Ce) or *C. moewusii* (Cm) cpDNAs. Additional restriction sites or fragments of *C. eugametos* or *C. moewusii* origin are also indicated. The additional *C. moewusii* fragments in the polymorphic loci *G* and *R* reside within the extra 21-kbp and 5.8-kbp sequences, respectively

[d] The inheritance of polymorphic locus *E* cannot be determined unambiguously by hybridization of the E29′m probe to AvaI DNA digests. Hybridization of this probe to EcoRI digests is also required to score the inheritance of this locus

For preparation of total cellular DNA, liquid cultures (30 ml) of *C. eugametos*, *C. moewusii*, and interspecific hybrids were grown at 20 °C in the minimal medium of Gowans (1960) supplemented with 3% CO_2 in air under alternating 12-h light (30,000 lx)/12-h dark periods. When they reached a density of

2.5×10^6 to 1×10^7 cells/ml at the onset of the light period (L-0), from 5×10^7 to 2.5×10^8 L-0 cells were cooled on ice, pelleted at $6{,}000 \times g$ for 10 min at 4 °C, resuspended in 1 ml of buffer A (10 m*M* TRIS-HCl, pH 8.0, 10 m*M* EDTA, 10 m*M* NaCl), repelleted in a microcentrifuge at $13{,}000 \times g$ for 30 s at 4 °C, and finally resuspended in 450 µl of buffer A. After addition of 25 µl of proteinase K (1 mg/ml) and 25 µl of 10% SDS, each of the cell suspensions was incubated at 50 °C for 1 h and extracted successively with 500 µl of phenol, 500 µl of phenol/chloroform (1 : 1), and 500 µl of chloroform, according to Maniatis et al. (1982). The aqueous phases were made 200 m*M* NaCl and the nucleic acids were ethanol precipitated. Each of the pellets was dissolved in 200 µl of TE (10 m*M* TRIS-HCl, pH 8.0, 1 m*M* EDTA), and 2 µl of 2.5 mg/ml DNAse-free RNAse A were added. After incubation at 37 °C for 15 min, the resulting mixtures were then made 2.5 *M* NH_4OAc, centrifuged at $13{,}000 \times g$ for 30 s at room temperature to remove the flocculent precipitates, and the DNA from the supernatants was ethanol precipitated at −70 °C. This DNA precipitation step in the presence of NH_4OAc was repeated twice, and the final pellets were washed with 70% ethanol, 33 m*M* NaCl. DNA samples were dried and redissolved in 40 µl of TE. About 20 µg of total cellular DNA was usually obtained from 10^8 cells.

Aliquots containing about 1 µg of total cellular DNA were restricted with either EcoRI, AvaI or BstEII. Restriction fragments were separated on 0.8% or 1.5% agarose gels and transferred to Hybond-N (Amersham) nylon membranes according to the method of Southern (1975). The filters were hybridized with nick-translated (Maniatis et al. 1982) clones of cpDNA fragments (Table 1), as described previously (Lemieux et al. 1988).

Results

Chloroplast DNA recombination in F_1 hybrids

We determined the inheritance of 17 polymorphic cpDNA loci mapping outside the rDNA region in each of the 17 F_1 hybrid subclones isolated by Lemieux and Lee (1987). These subclones were derived from distinct zygotes; those recovered from the *C. eugametos, mt*$^+$, *sr-2* × *C. moewusii, mt*$^-$, *er-nM1* cross were selected for the *er-nM1* erythromycin resistance marker, while those from the *C. moewusii, mt*$^+$, *er-nM1* × *C. eugametos, mt*$^-$, *sr-2* cross were selected for the *sr-2* streptomycin resistance marker. These two antibiotic resistance loci are less than 10 kbp apart and map within the rDNA region. The *sr-2* locus is located within the small subunit rRNA gene only 9 bp upstream of polymorphic locus *A*, and the *er-nM1* locus within the large subunit rRNA gene only 70 bp upstream of polymorphic locus *E* (Gauthier et al. 1988). Consistent with these molecular data, Lemieux and Lee (1987) noted a strict correlation between the inheritance of polymorphic loci *A* and *E* and that of the genetic loci *sr-2* and *er-nM1*, respectively, in the 17 interspecific hybrids.

Figure 1 summarizes the inheritance patterns of the 17 polymorphic loci analyzed here as well as those of the 6 polymorphic rDNA loci examined by Lemieux and Lee (1987). In both reciprocal crosses, a substantial proportion of the loci revealed alleles derived from the *mt*$^-$ parent. This proportion was significantly higher in the *C. eugametos, mt*$^+$, *sr-2* × *C. moewusii, mt*$^-$, *er-nM1* cross a situation that could result from inherent differences in the transmission of cpDNA sequences in reciprocal crosses or more simply from selection of hybrid progeny with a higher input of chloroplast genomes from the *mt*$^-$ parent. In any case, the degree of linkage retention between the selected antibiotic resistance marker in the rDNA region and the remaining cpDNA polymorphic loci must be strongly influenced by the strict unidirectional inheritance of alleles from opposite parents at two loci (*C* and *G*) in the immediate vicinity of these resistance markers. As previously observed in randomly selected F_1 hybrids (Lemieux et al. 1988), the long allele of locus *C* originating from *C. eugametos* and the long alleles of loci *G* and *R* originating from *C. moewusii* were unidirectionally inherited in the 17 hybrids, whereas the remaining polymorphic loci were inherited from one or the other species. During their study of the 17 F_1 hybrids, Lemieux and Lee (1987) observed that each of the rDNA polymorphic loci carries identical alleles in the two copies of the inverted repeat sequence, indicating that this sequence is rapidly homogenized through copy-correction. Here, we extend this observation to include three additional polymorphic loci (*G*, *H*, and *I*) within the inverted repeat.

In all hybrids, multiple recombination junctions were identified between the polymorphic loci, particularly within the inverted repeat and the sequences in the vicinity of the boundaries between the inverted repeat and the single-copy regions. Table 2 shows the proportion of potential recombination sites that were actually observed in the two sets of hybrids and compares the average densities of recombination junctions within various cpDNA regions. The inverted repeat sequence of hybrids recovered from both reciprocal crosses reveals a 5- to 30-fold higher density of recombination junctions as compared to the single-copy regions. Few recombination junctions were noted within the single-copy regions, with the fewest in the region containing loci *J*, *K*, *L*, *M*, *N*, and *O* (SCR1). Junctions may have been missed and consequently underestimated in SCR1 because of the rarity of polymorphic loci in a substantial portion of single-copy region. However, we consider this possibility unlikely because in all hybrids, with the exception of hybrid 27 from the *C. eugametos* × *C. moewusii* cross, all six loci scored in SCR1 exhibit the same parental allele.

The recombination junctions in the vicinity of the four boundaries between the inverted repeat and the single-copy regions (regions *I*–*J*, *I*–*O*, *A*–*P*, and *A*–*W*) seem to represent the highest density class of recombination junctions (Fig. 1). For instance, in hybrids from the *C. moewusii, mt*$^+$ × *C. eugametos, mt*$^-$ cross, the density of junctions within the 7-kbp DNA segment delimited by

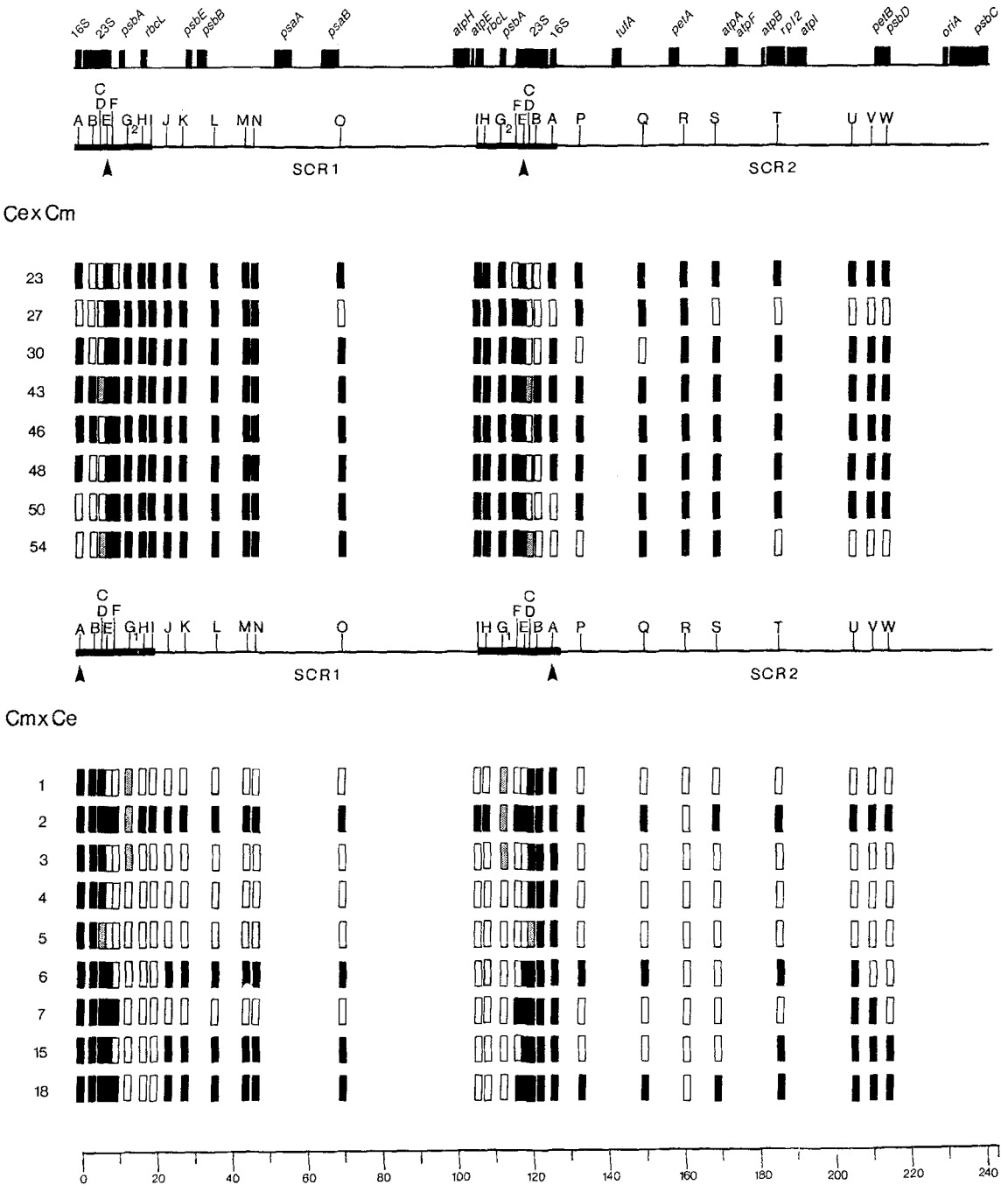

Fig. 1. Inheritance of 23 polymorphic cpDNA loci (*A*–*W*) in 17 F_1 hybrids selected for the presence of a chloroplast antibiotic resistance marker contributed by the *mt*$^-$ parent. The hybrids are identified by numbers and are divided into two groups, depending on the cross from which they were derived. Ce × Cm represents the cross *C. eugametos, mt*$^+$, *sr-2* × *C. moewusii, mt*$^-$, *er-nM1*, and Cm × Ce the cross *C. moewusii, mt*$^+$, *er-nM1* × *C. eugametos, mt*$^-$, *sr-2*. *Open* and *filled boxes* below the diagrams showing the positions of polymorphic and gene loci indicate the parental origin of the alleles inherited by hybrids: an *open box* denotes the allele of the *mt*$^+$ parent and a *filled box* that of the *mt*$^-$ parent. *Shaded boxes* in the inverted repeat region (*thicker line*) indicate nonparental alleles, i.e., the deleted version of the *C. moewusii* extra 21-kbp sequence at locus G_1 in hybrids from the Cm × Ce cross (Fig. 2) and the nonparental allele of locus *D* (note that locus *C* revealed only the *C. eugametos* allele; Lemieux and Lee 1987). The parental alleles at locus G_1 feature a 21-kbp difference, while those at locus G_2 feature a 17-kbp difference (Fig. 2). Hybrids from the Ce × Cm cross were selected for the presence of the *er-nM1* resistance marker which is tightly linked to locus *E* (*arrowhead*), while hybrids from the Cm × Ce cross were selected for the presence of the *sr-2* resistance marker which lies very close to locus *A* (*arrowhead*). This figure shows only one of the two orientation isomers of the single-copy regions (SCR1 and SCR2) (Aldrich et al. 1985; Palmer et al. 1985). Note that the inheritance of polymorphic loci *A* through *F* has been previously reported by Lemieux and Lee (1987). The *sr-2* and *er-nM1* mutations were mapped by Gauthier et al. (1988) and positions of the chloroplast gene loci were taken from Turmel et al. (1988)

Table 2. Number and average density of recombination junctions in various regions of hybrid cpDNAs

Cross[a]	No. of hybrids	No. of junctions[b]				Density of junctions ($\times 10^{-3}$)[c]		
$mt^+ \times mt^-$		IR	SCR1	SCR2	IR/SCR	IR	SCR1	SCR2
Selected F_1 hybrids								
Ce × Cm	8	17 (5/8)	1 (1/5)	4 (4/7)	5 (3/4)	106	3	6
Cm × Ce	9	12 (6/8)	0 (0/5)	10 (6/7)	18 (4/4)	67	0	13
Random F_1 hybrids								
Ce × Cm	8	10 (3/8)	1 (1/5)	16 (4/7)	5 (2/4)	60	3	25
Cm × Ce	8	18 (5/8)	2 (1/5)	2 (2/7)	4 (2/4)	110	5	3
Selected F_2 hybrids								
F_1 (18) × F_1 (46)	8	5 (3/4)	0 (0/5)	3 (3/6)	6 (3/4)	31	0	5
Total	41	62 (8/8)	4 (2/5)	35 (7/7)	38 (4/4)			

[a] Data for the random F_1 hybrids were derived from the cpDNA inheritance patterns reported by Lemieux et al. (1988). It should be noted that the selected and random F_1 hybrids were recovered from independent crosses. Ce, *C. eugametos, sr-2*; Cm, *C. moewusii, er-nM1*; F_1 (18), F_1 hybrid 18, *sr-2* originating from the Cm × Ce cross; and F_1 (46), F_1 hybrid 46, *er-nM1* originating from the Ce × Cm cross

[b] For each region the number of recombination sites identified relative to the total number of potential recombination sites is indicated in parenthesis. IR, inverted repeat; SCR1, single-copy region bordering the 23S rRNA genes; SCR2, single-copy region bordering the 16S rRNA genes; IR/SCR, cpDNA sequences in the vicinity of the boundaries between the inverted repeat and single-copy regions

[c] This value was obtained by dividing the total number of junctions detected in a given set of hybrids by the number of hybrids analyzed and by the length of cpDNA sequences (in kbp) spanning the polymorphic markers in the region of interest (IR = 20 kbp, SCR1 = 48 kbp, and SCR2 = 81 kbp)

loci *A* and *P* is 50% higher than that observed within the adjacent inverted repeat sequence. Since such junctions lie in cpDNA sequences delimited by outermost polymorphic loci in the inverted repeat (*I* or *A*) and outermost ones in single-copy regions (*J, O, P,* or *W*), they cannot be unambiguously assigned to one or the other region of the chloroplast genome and, therefore, were disregarded for our estimation of density of recombination junctions (Table 2). However, as most of the unassigned recombination sites occur in symmetric pairs at the same end of the inverted repeat within a given recombinant DNA molecule, it is likely that they lie in the inverted repeat sequence and originate from gene conversion events involving the edges of this region.

In an earlier study (Lemieux et al. 1988), we scored the inheritance of 23 polymorphic cpDNA loci in 16 F_1 hybrid subclones that were randomly recovered from reciprocal crosses between the parental strains used here. Comparison of the inheritance patterns of the random and selected F_1 hybrids (Table 2) reveals that selection of progeny for the antibiotic resistance marker contributed by the mt^- parent effectively increases the proportion of polymorphic cpDNA loci inherited from the mt^- parent, as well as the proportion of recombination junctions that cannot be explained by co-conversion of alleles in the vicinity of the unidirectionally inherited loci *C, G,* and *R*. This selection procedure, however, does not significantly increase the total number of recombination junctions. With the exception of SCR2, all cpDNA regions display similar densities of recombination junctions in random and selected F_1 hybrids. The abundant recombination junctions in the SCR2 region of random F_1 hybrids from the *C. eugametos*, $mt^+ \times$ *C. moewusii*, mt^- cross map in the immediate vicinity of locus *R* and may be simply attributed to unidirectional gene conversion of the long allele of this locus.

Comparison of the inheritance patterns of the polymorphic cpDNA loci followed in this study with the map positions of known genes on the *C. eugametos* and *C. moewusii* chloroplast genomes strongly suggests that subunits from three of the four macromolecular complexes within thylakoid membranes can be inherited from opposite parents in individual hybrids (Fig. 1). For instance, cpDNAs of hybrids 27 and 54 from the *C. eugametos*, $mt^+ \times$ *C. moewusii*, mt^- cross are clearly recombinant with respect to genes encoding subunits of

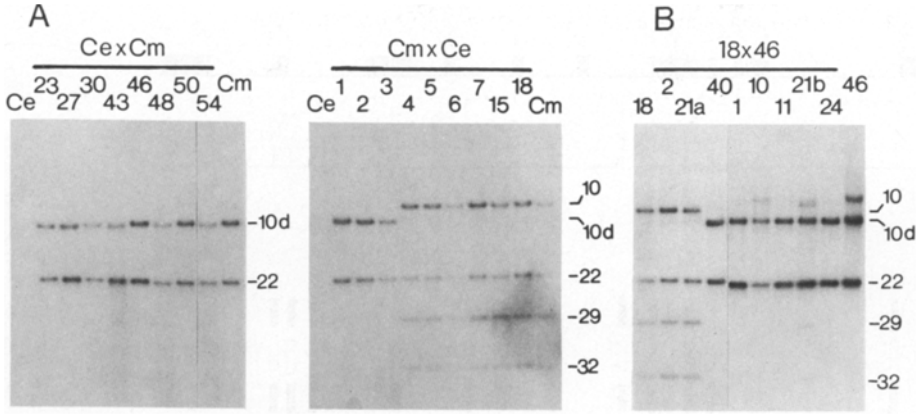

Fig. 2 A and B. Hybridizations showing the inheritance of polymorphic loci G_1, G_2, and G_3 in the 17 F_1 hybrids (**A**) and 8 F_2 hybrids (**B**) analyzed in this study. Ce × Cm represents the cross *C. eugametos, mt*$^+$, *sr-2* × *C. moewusii, mt*$^-$, *er-nM1*, and Cm × Ce the cross *C. moewusii, mt*$^+$, *er-nM1* × *C. eugametos, mt*$^-$, *sr-2*; 18 × 46 denotes the cross between F_1 hybrid 18, *mt*$^+$, *sr-2*, and F_1 hybrid 46, *mt*$^-$, *er-nM1*. Southern blots of EcoRI-digested total cellular DNA from the hybrids and the parents were hybridized with the *C. moewusii* SalI fragment 7 (designated S7m in Table 1), a probe that recognizes specifically the 21-kbp insertion sequence identified in the wild-type strains of *C. moewusii*. Two hybridization patterns were observed among the hybrid progeny: the one displaying the EcoRI fragments 10, 22, 29, and 32 is characteristic of the 21-kbp sequence, whereas the one displaying fragment 22 and the partially deleted fragment 10 (designated 10d) reveals a 17-kbp version of this sequence. Hybridizing fragments are numbered according to the nomenclature of Lemieux et al. (1985c). Note that the fragment larger than fragment 10, which was detected in F_1 hybrid 46 and two F_2 hybrids (**B**), results from partial DNA digestion

the photosystem II (*psb*), the cytochrome b_6/f (*pet*), and the ATP synthase (*atp*) complexes. Hybrid 27, in particular, most likely inherited *psbC, psbD, petB, atpA, atpB, atpF,* and *atpI* from *C. eugametos* and *psbA, psbB, psbE, petA, atpE,* and *atpH* from *C. moewusii*. Recombinant patterns for genes encoding subunits of the photosystem I complex (*psa*) could not be demonstrated, as no polymorphic locus is available between the only two *psa* genes (*psaA* and *psaB*) that have been mapped so far. Overall, our results indicate that certain combinations of subunits from *C. eugametos* and *C. moewusii* can form functional complexes in interspecific hybrids. The mapping of additional chloroplast genes on the two algal chloroplast genomes as well as the analysis of a large number of recombinant chloroplast genomes will be required to determine if all combinations of subunits are possible.

A 4-kbp cpDNA region encompassing EcoRI fragments 29, 32, and part of fragment 10 within the *C. moewusii* extra 21-kbp sequence (locus *G*) appears to be prone to deletion during sexual crosses. Deletion of this 4-kbp region was detected in three hybrids from the *C. moewusii, mt*$^+$, *er-nM1* × *C. eugametos, mt*$^-$, *sr-2* cross as well as in one of the *C. moewusii* parents, the *mt*$^-$, *er-nM1* strain (Fig. 2). The *C. moewusii, mt*$^+$, *er-nM1* stain revealed the full-length version of the 21-kbp sequence. As the *mt*$^-$, *er-nM1* strain was obtained by crossing the original *mt*$^+$, *er-nM1* mutant to the *mt*$^-$, wild-type strain (Lee and Lemieux 1986), both of which had a full-length 21-kbp sequence, the mutational event that led to the deleted form of the 21-kbp sequence most probably occurred during this cross. The frequent occurrence of a 4-kbp deletion within locus *G* is likely to involve tandemly repeated sequences at the borders of the deleted sequence. Deletion may simply occur through recombination between the repeats.

Chloroplast DNA recombination in F_2 hybrids

The increased density of recombination junctions in the inverted repeat of F_1 hybrid cpDNAs could be a consequence of the unidirectional inheritance of polymorphic loci *C* and *G*. As the long alleles of these loci are unidirectionally inherited from opposite parents, most probably as the result of unidirectional gene conversion events, a recombination junction is necessarily generated within the inverted repeat during interspecific crosses. To test the influence of these putative conversion events on the generalized recombination system, we determined the inheritance of polymorphic loci in an F_1 cross. If the conversions at loci *C* and *G* are solely responsible for the increased density of recombination junctions in the inverted repeat region of F_1 hybrid cpDNAs, then a uniform density of junctions would be predicted throughout the chloroplast genomes of F_2 hybrids. Conversions at loci *C* and *G* cannot occur in a typical F_1 cross simply because the cpDNAs of both F_1 parents contain the long alleles of these loci.

Among the F_1 hybrids examined in the previous section, only hybrids 18 and 46 could be used as parents of a cross in which it is possible to measure the density of recombination junctions in progeny selected for a chloroplast resistance marker contributed by the *mt*$^-$ parent.

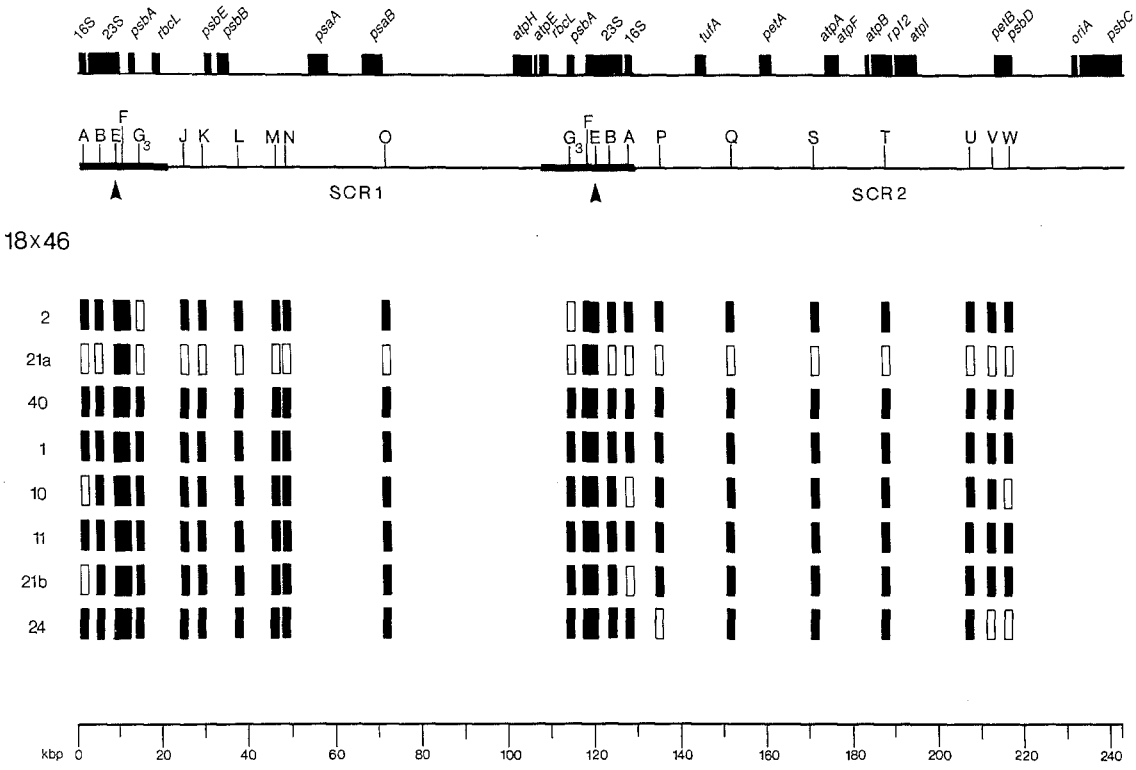

Fig. 3. Inheritance of 18 polymorphic cpDNA loci in eight F_2 hybrids selected for the presence of a chloroplast erythromycin resistance marker (*er-nM1*) contributed by the mt^- parent. These hybrids originate from a cross in which the parental strains were F_1 hybrid 18, mt^+, *sr-2*, and F_1 hybrid 46, mt^-, *er-nM1* (Fig. 1 for their cpDNA inheritance patterns). *Open* and *filled boxes* below the diagram showing the positions of polymorphic and gene loci indicate the parental origin of the alleles inherited by hybrids: an *open box* denotes the allele of the mt^+ parent and a *filled box* that of the mt^- parent. The parental alleles at locus G_3 display a 4-kbp difference (Fig. 2). The *arrowhead* points to the polymorphic locus (*E*) that is tightly linked to the selected *er-nM1* mutation (Gauthier et al. 1988). Only one of the two orientation isomers of the single-copy regions (SCR1 and SCR2) is represented in this figure (Aldrich et al. 1985; Palmer et al. 1985)

These two hybrids display opposite mating types and contain the three unidirectionally inherited sequences, while they show different alleles for most of the remaining polymorphic loci, including locus G_3 as well as the *er-nM1* and *sr-2* loci. The parental alleles at locus G_3 feature a 4-kbp difference in the extra 21-kbp sequence characteristic of *C. moewusii* (Fig. 2). Only loci *C, D, H, I,* and *R* cannot be scored for their inheritance in the F_2 progeny.

The cross F_1 hybrid 18, mt^+, *sr-2* × F_1 hybrid 46, mt^-, *er-nM1* produced zygotes that germinated with high frequency but, as in reciprocal interspecific crosses (Cain 1979; Lemieux et al. 1981; Lemieux et al. 1984; Lemieux and Lee 1987), high lethality was observed among the meiotic progeny (Table 3). Approximately one-third of the zygotes (21/57) yielded a single surviving product per tetrad, while most of the remaining ones produced no viable products. The *er-nM1* resistance marker contributed by the mt^- parent was inherited much more frequently in the F_2 meiotic progeny than in the F_1 progeny from reciprocal interspecific crosses (Table 3). We observed almost equal frequencies for the three classes of meiotic products identified, i.e., those uniparental for the *sr-2* marker of the mt^+ parent (UP^+), biparental for the resistance markers (BP), and uniparental for the *er-nM1* marker of the mt^- parent (UP^-). Recombination between the *sr-2* and *er-nM1* loci was scored by the mt^- marker selection procedure of Mets and Geist (1983). Of the 19 F_2 hybrid subclones that were randomly selected for the *er-nM1* marker among individual BP- or UP-meiotic products, 9 were found to be recombinant for the unselected *sr-2* marker, thus revealing a recombination frequency of 0.47. A similar value, indicating at best weak linkage, was noted between these markers in reciprocal interspecific crosses (Lemieux and Lee 1987).

We scored the inheritance of 18 polymorphic cpDNA loci in eight F_2 hybrid subclones that were derived from distinct zygotes (Fig. 3). As for the F_1 subclones analyzed in this study, zygote clones and subclones were selected for the presence of the *er-nM1* resistance marker from the mt^- parent. Although a significant portion of the polymorphic loci showed the allele of the mt^- parent in the F_2 progeny, cpDNA recombination junctions were detected less frequently than in the F_1 hybrids. Three F_2

Table 3. Compared viability and inheritance of chloroplast genetic markers among meiotic products from an F_1 cross between hybrids 18 and 46 [$F_1(18) \times F_1(46)$] and an interspecific *C. eugametos* × *C. moewusii* (Ce × Cm) cross

Cross	Viable products per tetrad (no. of tetrads)					Surviving products	Inheritance pattern of resistance markers[a]		
$mt^+ \times mt^-$	4	3	2	1	0		UP^+	BP	UP^-
$F_1(18) \times F_1(46)$	0	0	5	21	36	13%	0.39 (12)	0.39 (12)	0.23 (7)
Ce × Cm[b]	0	0	3	38	81	9%	0.96 (42)	0.02 (1)	0.02 (1)

[a] The number of meiotic products in each class is given in parenthesis. UP^+ = uniparental inheritance of resistance marker from the mt^+ parent; BP = biparental inheritance of resistance markers; UP^- = uniparental inheritance of resistance markers from the mt^- parent

[b] Data taken from Lemieux et al. (1981)

hybrids revealed cpDNAs indistinguishable from that of the parental mt^- chloroplast genome, while the cpDNAs of the remaining five hybrids were recombinant for only a few of the polymorphic markers. The latter hybrids resemble the F_1 hybrid progeny in displaying a higher density (six-fold) of recombination junctions in the inverted repeat as compared to the single-copy regions. Thus, there is no correlation between the unidirectional inheritance of long alleles at loci *C* and *G* in F_1 hybrids and the increased density of junctions within the inverted repeat.

Discussion

In this study, we determined the inheritance patterns of polymorphic loci spanning the entire chloroplast genome in 25 hybrids recovered from interspecific and F_1 crosses between *C. eugametos* and *C. moewusii*. The most important finding that emerges from our data is that recombination junctions occur at highly variable frequencies across the three main domains of the chloroplast genome. The inverted repeat sequence was found to exhibit at least a five-fold higher density of recombination junctions compared to the SCR2 region, whereas junctions in SCR2 were detected at a five-fold higher frequency relative to those in SCR1. In a similar study on the inheritance of polymorphic cpDNA loci in a *Nicotiana* somatic hybrid, Medgyesy et al. (1985) identified a total of six recombination junction but, because of the lack of markers within the inverted repeat and the small single-copy regions, they were unable to detect a differential density of recombination junctions.

Several hypotheses can explain the high density of recombination junctions within the inverted repeat region of *Chlamydomonas* cpDNA. One of these argues that certain sequences within the inverted repeat promote recombination events, and that the increased density of junctions we observed is simply the result of multiple rounds of intermolecular recombination which occurred between opposite parental cpDNAs at different sites within the inverted repeat before cells became homoplasmic. These events could be initiated in zygotes at the time of chloroplast fusion or in the few generations of heteroplasmic mitotic progeny following meiosis. An alternative hypothesis, which we favor, assumes that the highly recombinant inverted repeat results mainly from successive intramolecular recombination events between the two copies of the inverted repeat sequence in cpDNAs carrying opposite parental alleles of loci within this region. Such cpDNAs, also called heterotypic cpDNAs, are expected to be the major products of intermolecular recombination events involving the inverted repeat sequence, because both copies of this sequence are unlikely to participate simultaneously in these events. The unimolecular kinetics of intramolecular recombination obviously predict that this type of recombination is more prevalent than intermolecular recombination.

The marked differences between the densities of recombination junctions within the inverted repeat and the single-copy regions imply that the extent of genetic linkage between two given chloroplast loci will depend not only on the physical distance separating them, but also on their location within the genome. Markers within the inverted repeat are expected to show a weaker linkage relative to others equally distant within the single-copy regions. Since the *sr-2* and *er-nM1* resistance loci are genetically unlinked in reciprocal interspecific crosses as well as in the F_1 cross, two markers separated by at least 9 kbp in the inverted repeat should appear unlinked in *C. eugametos*/*C. moewusii* hybrids. Recombination between the selected *er-nM1* marker (locus *E*) and the remaining four polymorphic markers within the inverted repeat occurred at a frequency of 3% – 4% per kbp in the F_1 cross. Recombinational activity of the inverted repeat was found to be significantly higher in interspecific cross-

es, probably because the putative gene conversion events at loci *C* and *G* contribute to the generation of recombination junctions.

In contrast, antibiotic resistance loci within the inverted repeat of the *C. reinhardtii* chloroplast genome show a remarkably tight linkage as defined by classical recombination criteria (Harris et al. 1977). By aligning the recombination map of these resistance loci with the physical map of the corresponding *C. reinhardtii* rDNA region, Harris et al. (1989) found that the 7-kbp DNA segment separating the small and large subunit rRNA genes represents 7 map units, i.e., only 1% recombination per kbp. This may suggest that there are fundamental differences in the rates of chloroplast gene recombination in the *C. eugametos*/*C. moewusii* and *C. reinhardtii* systems. Alternatively, the use of different mapping methods may account for the apparent differences in recombination. Additional comparisons of genetically and physically derived map distances between markers within the inverted repeat and most importantly within the single-copy regions are clearly needed in both systems to understand more fully the rules governing recombination of cpDNA in *Chlamydomonas*. In any case, it is obvious that the alignment pattern of genetic and physical maps along the entire chloroplast genome will be complex and non-linear, due to the enhanced frequency of recombination within the large inverted repeat sequence.

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