LINKAGE OF A KNOWN CHLOROPLAST GENE MUTATION TO THE UNIPARENTAL GENOME OF *CHLAMYDOMONAS REINHARDII*

LAURENS J. METS* AND LOIS J. GEIST†

**Department of Biology, University of Chicago, Chicago, Illinois 60637; and †School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106*

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ABSTRACT

Data are presented that associate three new markers with the uniparental linkage group in *Chlamydomonas reinhardii*. One of these, mutant 10-6C, is a genetic marker for the structural gene of the large subunit of ribulose bisphosphate carboxylase. These results provide the first direct link between the uniparental gene map and the physical map of chloroplast DNA. The other two markers, Dr2 (DCMU resistant) and 8-36C (deficient in photosystem II activity), map to a single locus. The data suggest that mixing in zygotic chloroplasts may not be complete so that input genomes do not have equal opportunities to recombine. The data are not compatible with simple linear or circular maps but can be explained on the basis of the known physical structure of chloroplast DNA.

CHLAMYDOMONAS reinhardii has become a valuable organism for studies of chloroplast gene function because it is the only organism in which putative chloroplast genes have been associated to recombine (GILLHAM 1978; KIRK and TILNEY-BASSETT 1978). Several different types of analysis of the observed recombination have been used to construct genetic maps (for reviews, see SAGER 1977; GILLHAM 1978). If these maps can be correlated with the physical map of chloroplast DNA, then it will be possible to use genetic mapping to locate interesting mutants on the DNA and to use DNA sequencing to characterize the primary lesion in such mutants.

The work described in this report is part of a general effort to correlate the physical and genetic maps. It had three specific objectives: (1) to test for linkage between a known chloroplast gene marker and the other markers in the genetic map; (2) to extend the present genetic map to include more markers with diverse, well-characterized phenotypes; and (3) to develop a genetic mapping method that could be used for mapping biochemical markers that are relatively laborious to score. Our motivations for adopting these objectives are as follows.

There are numerous genetic markers in *C. reinhardii* that show a non-Mendelian, predominantly uniparental pattern of inheritance (SAGER 1977; GILLHAM 1978). Because of this genetic property, they are known as uniparental markers. The phenotypes of those that have been well studied involve alterations in chloroplast components. It has long been presumed that they represent

mutations in chloroplast genes, and, for convenience, they are frequently called chloroplast markers. A consolidating force behind this presumption is the observation that all of the markers that have been studied by recombination analysis seem to be part of a single linkage group (SAGER 1977; GILLHAM 1978). Chloroplast DNA is known to have the same general pattern of inheritance (GRANT, GILLHAM and BOYNTON 1980; LEMIEUX, TURMEL and LEE 1980; METS 1980) and it could, therefore, carry this linkage group. However, the data do not exclude other uniparentally inherited DNA species as possible carriers (METS 1980). Our strategy has been to isolate a genetic marker for a known chloroplast gene. A demonstration of genetic linkage to this standard marker would then provide positive evidence that other uniparental genetic markers are also carried on chloroplast DNA.

The chloroplast gene we chose for use as a standard marker was that encoding the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase/oxygenase. We obtained a mutant (10-6C) that produces an inactive enzyme with a more acidic isoelectric point of the LS (SPREITZER and METS 1980). It requires acetate and darkness for growth. DNA sequencing shows a transition mutation leading to a gly → asp substitution at residue 171 of the LS gene (DRON *et al.* 1983). This is the first chloroplast gene mutant for which the DNA sequence is known. In this paper, we show that mutant 10-6C maps as part of the uniparental linkage group.

Most of the uniparental gene mapping effort in *C. reinhardii* has focused on markers that confer resistance to inhibitors of chloroplast protein synthesis (*e.g.*, erythromycin, streptomycin and spectinomycin) (CONDE *et al.* 1975; HARRIS *et al.* 1977; SAGER 1977; GILLHAM 1978; BARTLETT *et al.* 1979). This poses two separate classes of problems for efforts to correlate the genetic and physical maps. First, we cannot guess, at the outset, the quantitative relationship between physical distances along the chloroplast DNA molecules and apparent recombination frequencies. It is conceivable that the antibiotic resistance markers are clustered in a small portion of the chloroplast genome. To be certain that we have markers covering the entire chloroplast genome, we must study markers in other phenotypic classes. Second, interactions among altered ribosome components in the various antibiotic-resistant mutants could affect ribosome function and change the viability of specific marker combinations. In particular, ribosomes bearing two different resistance alterations in the same subunit can show a defect in function or assembly that was not evident with either resistance by itself. Low viability has been reported for strains bearing two uniparentally determined resistances in the small subunit of the chloroplast ribosome (VANWINKLE-SWIFT 1976; VANWINKLE-SWIFT and BIRKY 1978) as well as for strains bearing two nuclear mutations affecting resistance of the LS (METS and BOGORAD 1971). If we want to obtain apparent recombination frequencies that depend upon physical distances between markers on the chloroplast DNA rather than upon phenotypic effects, it is necessary to use markers that are expressed without epistatic interactions affecting cell viability.

The three new markers we analyze in this paper will help deal with both of these problems. The first is mutant 10-6C discussed earlier. We do not expect

it to have allelic interactions with the antibiotic resistance markers. The second, mutant Dr2, is resistant to the photosynthetic electron transport-inhibiting herbicide diuron (3,4-dichlorophenyl-1,1-dimethyl urea, or DCMU). The phenotype of Dr2 involves a change in the thylakoid membranes that reduces their affinity for DCMU (GALLOWAY and METS 1982). This phenotype is expressed independently and can be scored in any combination with the antibiotic resistance markers and the 10-6C mutation. Mutant 8-36C, the third new marker we have analyzed, is nonphotosynthetic and requires acetate for growth. It has a specific lesion in photosystem II activity (SPREITZER and METS 1981) and is missing a set of thylakoid membrane polypeptides (L. J. METS, unpublished data) which is also missing in several Mendelian photosystem II mutants studied by CHUA and BENNOUN (1975) and MAROC and GARNIER (1981). These same polypeptides are present in photosystem II-enriched thylakoid membrane subfractions (DINER and WOLLMAN 1980). Mutant 8-36C is, therefore, defective in the assembly of a photosystem II membrane complex. It also can be scored in any combination with the antibiotic resistance and 10-6C markers.

At present, the methods for analyzing uniparental gene recombination require scoring the phenotypes of thousands of progeny in order to obtain stable maps (HARRIS *et al.* 1977; SAGER 1977; GILLHAM 1978). This precludes the mapping of markers such as DNA restriction site differences or deletions (MYERS *et al.* 1982) and protein electrophoretic polymorphisms (CHUA 1976), which would require a major effort to score. We are attempting to develop mapping methods that would yield statistically reliable data from the analysis of many fewer progeny. If the number could be brought down near 100, then it would be feasible to map molecular markers. This paper described experience with one such method: paternal marker selection. It is a modification of the zygote clone-mapping method (see CONDE *et al.* 1975) and will be described in detail.

PATERNAL MARKER SELECTION MAPPING

To reduce the number of cells that must be analyzed to obtain reliable mapping data, we have used two modifications of zygote clone analysis. First, we analyze only one cell from each biparental zygote clone. This ensures that each cell scored has a genotype that is determined independently from every other. Standard zygote clone analysis involves scoring 64 or more cells randomly selected from each zygote clone (CONDE *et al.* 1975). It has been observed, however, that the genotypes within a clone are highly correlated (GILLHAM, BOYNTON and LEE 1974; BIRKY *et al.* 1981). This correlation reduces the contribution of each scored cell to improving the precision of recombination estimates. In practice, it has been necessary to analyze thousands of cells (from 100 or so zygote clones) in order to obtain reproducible maps (HARRIS *et al.* 1977). One advantage of deriving recombinant frequencies from the analysis of cells that are known to be of independent origin is that standard statistical methods can be employed for establishing confidence limits on measured frequencies. Such confidence limits cannot be determined from data de-

rived from standard zygote clone analysis until the degree of correlation within a clone is quantified. Until that is done, we will not know the exact relative numbers of cells that must be scored by the two methods to achieve similar precision in recombination estimates, but it is certain that many fewer cells are required when they are all from independent zygotes. The second modification is based upon the observation that biparental zygote clones carry biased genotypic composition favoring alleles contributed by the maternal parent (GILLHAM, BOYNTON and LEE 1974; HARRIS *et al.* 1977; FORSTER *et al.* 1980; BIRKY *et al.* 1981). Usually, more than half of the total cells analyzed carry the maternal parental (nonrecombinant) genotype. The work involved in scoring these cells contributes no information about relative recombination frequencies. Therefore, we eliminate these cells from consideration by selecting cells that carry at least one marker from the paternal parent. In doing so, we determine the extent of linkage retention among markers contributed to the zygote by the paternal parent in the cross.

Paternal marker selection mapping is similar to zygote clone analysis in that it determines the frequency of recombinant cells in a zygote clone rather than the recombination frequency in the usual sense. It is, therefore, sensitive to factors other than genetic linkage relationships. For instance, if cells of different genotypes have different growth rates, then their relative frequencies among cells in zygote clones will be biased away from that determined by recombination. For this reason, we have arranged our analysis so that the various genotypes to be scored have approximately equal growth rates. The recombinant frequencies we determine following paternal marker selection are based upon only a subset of the progeny analyzed by zygote clone analysis. Because of the bias favoring transmission of maternal markers, we do not expect recombinant frequencies determined by the two methods to be equal. However, the two methods should have equal validity for detecting linkage effects in the observed recombinant frequencies.

MATERIALS AND METHODS

Strains and culture conditions

Strain 10-6C, mt^+ carries the 10-6C mutation at the uniparental locus *rcl-u-1* (SPREITZER and METS 1980). The strain used in these experiments was isolated from a maternal tetrad in a cross of the original mutant with wild-type strain 137C, mt^- (from R. P. LEVINE). Strain 8-36C, mt^+ was also obtained from a maternal tetrad in a cross of the original photosystem II-deficient mutant (SPREITZER and METS 1981) with 137C, mt^-. Both of these strains were grown on 10 mM sodium acetate-supplemented minimal 1.5% agar medium (SURZYCKI 1971) in the dark.

Strain Ar2-3D, mt^- was used as the mating type minus parent in crosses with the other two strains. It carries three uniparental markers: *sr-u-sm2*, *er-u-11* and Dr2 (to be designated *hrb-u-1-Dr2* on the basis of results reported in this paper) and two nuclear markers: *arg-2* and *msr-1*. The *sr-u-sm2* marker confers resistance to streptomycin and was originally isolated by SAGER (1954). The small subunit of the chloroplast ribosome shows *in vitro* resistance (SCHLANGER and SAGER 1974) and contains an altered protein detectable by carboxymethyl-cellulose chromatography (OHTA, SAGER and INOUYE 1975). The *er-u-11* marker confers resistance to erythromycin. It is identical with *ery1* (SAGER 1977; HARRIS *et al.* 1977) and tightly linked or allelic with *er-u-1a* (HARRIS *et al.* 1977). The latter mutant has altered *in vitro* binding of erythromycin to the large subunit of the chloroplast ribosome (METS and BOGORAD 1971) and an alteration in a large subunit

protein detectable by two-dimensional electrophoresis (METS and BOGORAD 1972). The properties of the Dr2 marker are discussed in the text. Both the *sr-u-sm2* and *er-u-11* markers have been used extensively in mapping experiments (SAGER 1977; GILLHAM 1978) and were chosen as standards for these experiments. The nuclear markers in this strain (*arg-2*, arginine requiring, and *msr-1*, resistance to methionine sulfoximine) were used in the crossing procedure to provide positive identification of colonies derived from germinated zygotes (see following data). This multiply marked strain was constructed in the following way. Strain GB221 [*sr-u-sm2*, *er-u-11*, mt^- (HARRIS *et al.* 1977)] was crossed with the mutant Dr2, and a mt^+ cell resistant to erythromycin, streptomycin and DCMU was selected from among the progeny of a biparental zygote (GALLOWAY 1982). This strain was then crossed with strains carrying the nuclear markers, and an *arg-2*, *msr-1*, mt^- product was chosen from a dissected tetrad showing maternal inheritance of the three uniparental markers. This strain was maintained on media containing 50 mg/liter of arginine. A similar strain (LG418B6D, mt^-) carrying the same uniparental genome and nuclear marker *nic-13* (nicotinamide requiring) instead of *arg-2* was also constructed and used in collecting some data from crosses with strain 10-6C, mt^+.

Crossing procedure

Cells were handled for gametogenesis, mating and replica plating as described by LEVINE and EBERSOLD (1958). Just before mating, mt^+ gametes were irradiated with UV light from a F15T8 Westinghouse sterilamp at 10 J m^{-2} sec^{-1} for 0, 15 or 30 sec. Gametes were immediately mixed and allowed to mate in the dark for 1 hr. The young zygotes were then plated on minimal 4% agar plates, incubated in the light (4000 lux cool-white fluorescent) for 1 day and in the dark for 6 days. Mature zygotes were then replated onto fresh arginine + acetate medium. The plates were exposed to chloroform vapors for 30 sec to kill any unmated cells and then plated in the light (4000 lux) overnight to induce germination. They were then incubated in the dark until colonies large enough to replica plate were formed (about 2 wk).

The colonies growing on these plates could be derived from one of several origins. First, they could have grown from unmated gametes that survived chloroform treatment. Such colonies occur only rarely but must be identified so that their cells are not mistakenly included in the analysis. Colonies of the mt^+ parental cells in both crosses are unable to grow on media lacking acetate, whereas colonies from the mt^- parent are unable to grow on media lacking arginine (or nicotinamide in the case of strain LG418B6D). The mt^+ parents are also sensitive to methionine sulfoximine. Second, they could have been derived from vegetative diploids. Such cells arise when a zygote formed in the mating reaction does not enter meiosis but continues to divide mitotically as a diploid. This happens to a few percent of the zygotes formed (EBERSOLD 1967). Progeny from vegetative diploids can include cells with recombinant uniparental gene combinations, and their analysis can be used for uniparental gene mapping (VANWINKLE-SWIFT 1976; VANWINKLE-SWIFT and BIRKY 1978), but the frequencies of various recombinant cell types are not the same as those obtained from germinated zygotes. For valid statistical analysis, it is important that the cells studied be derived from a single population, either from vegetative diploids or from meiotic zygotes. Therefore, we have chosen to eliminate vegetative diploids from consideration. Diploids from these crosses are heterozygous for the *msr-1* marker, which is recessive and, therefore, cannot grow on media containing methionine sulfoximine. Third, a colony could be derived from a zygote that germinates following meiosis. In this case, the colony contains clonal descendants of all of the haploid products derived from a single meiosis. Some of these cells can grow in the absence of arginine, and some can grow in the presence of methionine sulfoximine, and so every zygote colony will show growth under both conditions. Consideration of the uniparental markers in the cross indicates that most colonies are derived from maternal zygotes that transmit only markers from the mt^+ parent. These colonies do not contain cells that can grow in the absence of acetate. Only colonies from exceptional zygotes, which transmit at least some markers from the mt^- parent, contain cells that can grow on minimal medium (A^+ phenotype). Among these exceptional zygotes, some transmit only markers from the mt^- parent. Such paternal zygotes contain no acetate-requiring cells (A^-). Biparental zygotes, on the other hand, transmit markers from both parents, and their colonies contain both A^+ and A^- cells. Only in biparental zygotes do uniparental genetic

markers have an opportunity to recombine, and our analysis is based upon scoring one cell from among the segregated progeny of each biparental zygote.

We used the following procedure to select the cells for study. The original colonies were replica plated onto plates containing arginine + acetate, arginine alone, acetate alone, and arginine + acetate + 300 mg/liter of L-methionine-L-sulfoximine (Sigma Chemical Company). The replica on arginine alone was incubated at 4000 lux, and the other replicas were returned to the dark until differential growth was evident (about 1 wk). Colonies that show growth on the acetate alone, as well as on the methionine sulfoximine replicas, are known to be derived from germinated zygotes (as opposed to unmated gametes or vegetative diploids). Colonies also appearing on the arginine-alone replica are derived from exceptional zygotes (biparental or paternal). These colonies were picked from the arginine acetate replica and replated on the same medium to obtain single cells. Colonies grown in the dark (about 2 wk) were replica plated to arginine alone. Zygote colonies giving mixtures of cells, some of which could grow in the absence of acetate (A^+) and some of which could not (A^-), were known to be derived from biparental zygotes. One A^+ cell from each biparental zygote was selected at random and streaked to medium containing arginine plus 100 mg/liter of streptomycin, 250 mg/liter of erythromycin or 3×10^{-6} M DCMU for scoring its resistance phenotypes.

Each of the crosses was repeated on several different occasions, and the data were pooled for analysis. No significant differences among the repeats were detected.

Data analysis

The phenotype of each cell in our sample is determined by independent random processes. Therefore, the binomial distribution is an exact model for the phenotypic distribution in the sampled population. Confidence limits (quoted at the 95% level) on estimates of recombinant frequencies were calculated using the normal approximation of the binomial distribution and a correction for continuity.

RESULTS

UV effect

Two crosses were performed, each using the same mt^- strain, which has full photosynthetic competence (A^+) and carries uniparentally inherited resistances to erythromycin (Er), streptomycin (Sr) and DCMU (Dr). In cross I, the mt^+ parent was the carboxylase mutant 10-6C that requires acetate for growth (A^-) and is sensitive to each of the inhibitors (Es,Ss,Ds). In cross II, the mt^+ parent was the photosystem II mutant 8-36C (A^-,Es,Ss,Ds). In each cross, aliquots of mt^+ gametes were treated with UV light for 15 or 30 sec just prior to mating, which increases the frequency of biparental zygotes (SAGER and RAMANIS 1967). The spontaneous biparental zygote frequency was great enough (about 5%) to allow data to be collected from zygotes obtained without UV pretreatment as well. From each biparental zygote clone, a single cell carrying the A^+ marker from the mt^- parent was selected and then scored for the three resistance markers. The results for cross I are presented in Table 1 and for cross II in Table 2.

As was noted by SAGER and RAMANIS (1967, 1976), the UV treatment does not have a major effect on the frequencies of cells observed in each of the phenotypic classes. If we hypothesize that the UV treatment has no effect, the frequency of the A^+ErSrDr nonrecombinant class following 30 sec of UV treatment in cross II (Table 2) appears somewhat depressed relative to expectation (17 *vs.* 25.1). By pooling the 0- and 15-sec UV data in this cross, we

TABLE 1

Cross I: 10-6C, mt$^+$ *($A^-EsSsDs$) × Ar2-3D,* mt$^-$ *($A^+ErSrDr$)*

Progeny phenotypes	UV irradiation dose (sec)			Combined
	0	15	30	
$A^+ErSrDr$	29	31	42	102
$A^+ErSrDs$	0	1	3	4
$A^+ErSsDr$	3	4	5	12
$A^+ErSsDs$	1	3	1	5
$A^+EsSrDr$	1	1	2	4
$A^+EsSrDs$	1	0	1	2
$A^+EsSsDr$	0	2	3	5
$A^+EsSsDs$	2	5	11	18
Total	37	47	68	152

Each cell scored was obtained from a different biparental zygote and was selected for A^+ phenotype as described in MATERIALS AND METHODS.

TABLE 2

Cross II: 8-36C, mt$^+$ *($A^-EsSsDs$) × Ar2-3D,* mt$^-$ *($A^+ErSrDr$)*

Progeny phenotypes	UV irradiation dose (sec)			Combined
	0	15	30	
$A^+ErSrDr$	32	58	17	107
$A^+ErSsDr$	32	42	29	103
$A^+EsSrDr$	2	8	3	13
$A^+EsSsDr$	63	93	52	208
Total	129	201	101	431

Each cell scored was obtained from a different biparental zygote and was selected for A^+ phenotype as described in MATERIALS AND METHODS. No Ds progeny were observed.

can test to determine whether the overall frequency of recombinant *vs.* nonrecombinant cells is contingent upon the additional UV treatment in the 30-sec group. The difference is just significant at the 95% confidence level (χ^2 = 4.5; 1 d.f.). However, the elevated frequency of parental types in the pooled data is entirely within the 15-sec treatment group, and so there is no directional trend caused by UV treatment. There is no other significant deviation from the hypothesis that UV treatment has no effect. In cross I, the frequency of the nonrecombinant class appears slightly elevated in the unirrdiated sample (29 observed *vs.* 24.8 expected). In this case, a test to determine whether the frequency of recombinant *vs.* nonrecombinant cells is contingent upon UV treatment (untreated *vs.* pooled 15- and 30-sec data) is not significant at the 95% level (χ^2 = 2.8; 1 d.f.). Thus, there is a slight suggestion that UV treatment may reduce the frequency of parental cell types, but the pattern of the trend is not consistent between the two crosses. GILLHAM, BOYNTON and LEE (1974) noted that UV treatment affects the ratio of reciprocal recombinant classes observed in zygote clone analysis. In the absence of UV, they observed

a bias favoring classes carrying a majority of maternal alleles over their reciprocals carrying a majority of paternal alleles. UV treatment effectively abolished this bias. We would not have detected this effect in our experiments, since we did not score reciprocal classes. Overall, the analysis suggests that the data from the three UV treatment classes can be combined without serious error, and the analyses that follow are based upon the combined data.

Cross I

Inspection of the data from cross I (Table 1) reveals that the parental phenotypic class ($A^+ErSrDr$) is the most common among the progeny scored. This result is qualitatively similar to results that have been obtained by conventional zygote clone analysis (HARRIS *et al.* 1977). All four markers have linked inheritance, even in biparental zygotes. The data add two new markers (10-6C and Dr2) to the uniparental genetic linkage group. Mutant 10-6C is the first marker in the group for which the physical location on chloroplast DNA is known, and so these data provide the first direct link between the physical and genetic maps of the chloroplast genome.

The observed frequencies of cells recombinant for each marker pair are listed in Table 3 along with the calculated 95% confidence intervals. These have been used to construct the fragmentary maps depicted in Figure 1. Considering the three loci *A*, *E*, and *S*, a consistent linear map is obtained with E in the middle. The observed least frequent phenotypic class corresponds to the expected double recombinant class predicted by this map. Coincidence is slightly positive (six observed doubles *vs.* 4.4 expected) but not statistically significant. A similar picture is obtained from the analysis of the three markers A, D and S, with D as the middle marker and the same values for coincidence. Unfortunately, the data cannot be resolved into a simple linear map containing all four markers. The two consistent linear maps would place D and E very close to one another, yet they show a highly significant recombinant frequency. Three-point analyses containing both D and E do not have a significantly least frequent class that might correspond to a double recombinant class in a linear map, and so the relative orders of the markers cannot be decided. If we force such a three-point map to the data (giving D and E either possible orientation relative to A or S), calculated values for coincidence are much higher (2.6), the hypothetical double events being as frequent as single ones. The overall data do not contain a class that is significantly less frequent than any other. However, if we use the numerically least frequent class ($A^+EsSrDs$) as a possible indicator of the presumptive triple recombinant class, neither of the possible four-point orders is consistent with both of the three-point maps. (S would have to be between D and E.) A simple circular map is also not consistent with the data.

In a pool of multiple DNA molecules undergoing recombination, the maximum recombination frequency between any pair of markers can be less than 50% if the number of rounds of pairing for recombination is limited or if mixing of the parental molecules is incomplete. A recombination limit of this sort has been well documented for mitochondrial markers in *Saccharomyces*

TABLE 3

Recombinant frequencies from cross I

Marker pair	Frequency of recombinants[a]
A(10-6C)-E	0.191 ± 0.062
A(10-6C)-S	0.263 ± 0.070
A(10-6C)-D	0.191 ± 0.062
E-S	0.151 ± 0.057
E-D	0.118 ± 0.051
S-D	0.151 ± 0.057

[a] Listed range is 95% confidence limit calculated from the normal approximation of the binomial distribution and corrected for continuity.

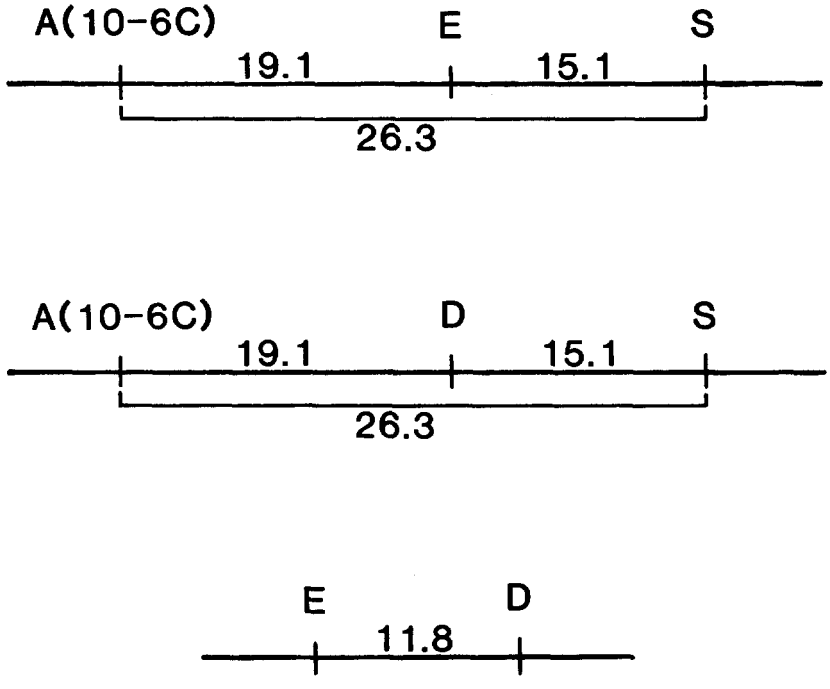

FIGURE 1.—Map fragments from cross I. Distances are given as recombinant frequency (×100).

cerevisiae (DUJON, SLONIMSKI and WEILL 1974). Under these circumstances, three markers could all show the maximum recombination frequency relative to one another and, therefore, their relative order would be ambiguous. As outlined in DISCUSSION, mixing may be incomplete in biparental zygotes from cross I, and so we expect that the maximum possible recombination frequency could be less than 50%. We must consider whether the difficulty in ordering markers derives from an approach to a recombination limit. Analyzing three markers at a time, the relative orders for the triples A-E-D and E-S-D are statistically ambiguous from our data, in spite of pairwise recombination frequencies well below 50%. However, the pairwise E-S, E-D, and S-D recombi-

nant frequencies given in Table 3 are also all significantly less than the measured A-S frequency. This is judged by using χ^2 tests to determine whether the observed number of recombinants for the A-S pair (40) is significantly different from the number observed for the E-S, E-D and S-D pairs (23, 18 and 23, respectively). The differences are all significant with greater than 95% confidence. Thus, the ambiguous orderings only arise in cases that include pairwise recombinant frequencies that are less than the maximum frequency observed in the cross. Therefore, these ambiguities in establishing relative marker order do not arise from an approach to this type of recombination limit. There must be some other mechanism causing the ambiguity in marker order. Because we do not have a set of three markers that fits the expectations of models for recombination limits in recombining DNA pools, we cannot tell from our data whether the maximum recombinant frequency we have observed (0.26) is close to a limit or even whether a limit below 50% will be observed for chloroplast genes in Chlamydomonas.

In *S. cerevisiae*, markers that give ambiguous three-point maps because of approach to the recombination limit always yield a significantly least frequent recombinant class in four-point analyses (SLONIMSKI and TZAGOLOFF 1976). This class corresponds to the class that arises from the greatest number of physical exchanges. As mentioned before, cross I did not yield a least frequent class, but many more recombinant progeny would have to be analyzed to prove whether or not such a class exists. Further analysis of this mapping problem is given in DISCUSSION.

Cross II

Cross II yielded only four of the possible eight phenotypic classes (Table 2). There were no Ds progeny at all, indicating no recombination between Dr2 and the photosystem II mutant 8-36C. This result assigns mutant 8-36C to the uniparental linkage group, since cross I shows linkage between mutant Dr2 and the standard Er and Sr markers. Since the Dr2 mutation affects the binding of DCMU to the photosystem II complex and mutant 8-36C is defective in photosystem II activity, it is reasonable to assume that they could be mutations in the same gene. The strong linkage between the two mutants makes this possibility very likely. We propose the name *hrb-u-1* for the locus of these mutations.

The results of cross II are qualitatively quite different from those of cross I because the parental phenotype is not the most frequenct among the progeny. Thus, it is not possible to use standard recombinant frequency analysis to construct maps. The data can be analyzed like a transduction experiment, however, if we regard the mt^+ parent as the recipient and the mt^- parent as the donor. Upon selection of the A^+ allele of the 8-36C locus, the probability of cotransmission of Dr = 1.0; Er = 0.487 (±0.048); Sr = 0.278 (±0.043). These data give the qualitative map depicted in Figure 2. If Er and Sr were transmitted independently of one another (not linked), the expected proportion of the ErSr phenotype among the progeny would be (0.487) × (0.278) = 0.135. The observed frequency is 0.248 (±0.041). In particular, Er has a high prob-

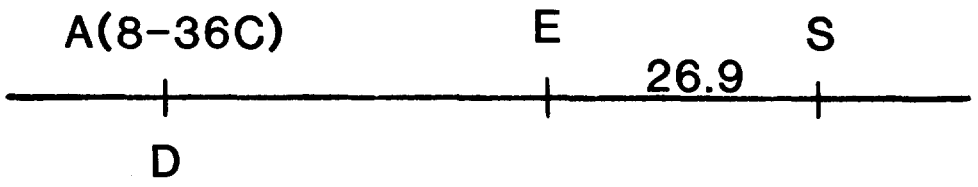

FIGURE 2.—Qualitative map from cross II. Map constructed from cotransmission frequencies, as described in RESULTS. The E-S map distance is given as the recombinant frequency (×100).

ability of being included when both A^+ and Sr are retained. This result is also consistent with E being a middle marker between A and S. For the E-S marker pair, a recombinant frequency of 0.269 (±0.043) can be calculated to yield the map distance listed on Figure 2.

DISCUSSION

Evaluation of mapping method

The paternal marker selection method we have tested achieves the objective of producing reliable maps from the scoring of relatively few progeny cells. This is emphasized by comparing results of cross II at each of the three UV doses (Table 2). The maps from each dosage subset of the data are qualitatively identical and quantitatively quite similar. Allelic fractions occur well within the random fluctuation expected for independent samples from a binomial distribution. Thus, in cross II, scoring of 100 progeny is sufficient to provide an unambiguous map. In cross I, the relative confidence limits on the observed recombinant frequencies are wider. This is because the total frequency of recombinant individuals is lower. Also, the total observations in cross I are distributed among all eight possible phenotypic classes, rather than just four. Thus, it would take more total recombinants scored to achieve the same precision in estimating frequencies of individual classes. Nevertheless, with 150 progeny scored, the fragmentary maps presented in Figure 1 have high statistical validity, and it can be judged with confidence that the data are incompatible with simple linear or circular maps.

One of the disadvantages of the paternal marker selection method is that only half of the expected phenotypic classes issuing from a cross can be observed. This is because all classes showing the maternal allele of the selected marker are eliminated from consideration. Although this practice reduces the number of cells that must be analyzed to obtain maps, it prevents a direct comparison of the relative frequencies of reciprocal recombinant classes. Such comparisons can be useful indicators of factors that must be considered in deriving recombination frequencies from the observed frequencies of recombinant phenotypes. For instance, in zygote clone analysis (which does allow detection of all phenotypic classes), the frequencies of reciprocal classes are often quite unequal (GILLHAM, BOYNTON and LEE 1974; HARRIS *et al.* 1977; BARTLETT *et al.* 1979), particularly when they involve two markers that affect the antibiotic resistance properties of the same ribosomal subunit. This partic-

ularly inequality can be explained on the basis of allelic interactions that reduce the viability of cells carrying two modifications in the same subunit (VANWINKLE-SWIFT 1976; VANWINKLE-SWIFT and BIRKY 1978). We have been careful to try to eliminate this problem by using markers for which we do not expect allelic interactions. The Er-Sr marker pair does, in fact, yield nearly equal frequencies of reciprocal recombinant classes in zygote clone analysis (HARRIS *et al.* 1977). The colony growth rates of cells with each of the marker combinations we have scored in these crosses are all approximately equivalent (GALLOWAY 1982). However, since reciprocal phenotypic classes are not scored, comparisons of their relative frequencies cannot be used as direct internal checks for the effects of allelic interactions, and we cannot exclude the possibility that such effects influence our mapping data.

Linkage of physical and genetic maps

The results of cross I provide the first direct evidence that the uniparental linkage group in Chlamydomonas is actually associated with chloroplast DNA, as has long been presumed. Mutant 10-6C is the first marker at a known site on chloroplast DNA that has been mapped genetically. The evidence that associates it with the uniparental linkage group is qualitatively the same as that which established the identity of the group initially. That is, even in biparental zygotes that yield recombinant progeny, the parental combination of markers tends to be retained. Physical location on the same DNA molecule is one mechanism that could produce linked inheritance of the markers, although the results of cross II suggest another, which must be given careful consideration (see following data).

SAGER (1977) has proposed that the uniparental linkage group might be genetically diploid. The known cpDNA of Chlamydomonas is present in 50–200 copies per cell (BEHN and HERRMANN 1977; ROCHAIX 1978). This raises the question of whether there exists a physically diploid component of cpDNA that could carry a diploid linkage group but has otherwise escaped attention (WELLS and SAGER 1971). Our data show that a genetic marker (mutant 10-6C), known to be located on the multicopy cpDNA, maps as part of the linkage group. Given this positive example, all of the linkage group markers could be physically located in the multicopy cpDNA, without the need to postulate the existence of a separate diploid segment of cpDNA. Whether or not this multicopy cpDNA has special transmission mechanisms to produce effectively diploid genetic behavior is a separate question, which is not addressed in these experiments. However, many of the results from zygote clone analysis cannot be explained on the basis of an effectively diploid behavior of the linkage group (see GILLHAM 1978), and multicopy models can provide adequate explanation of SAGER'S pedigree analysis data (FORSTER *et al.* 1980; METS 1973; VANWINKLE-SWIFT 1980).

Assuming that all of the markers in cross I are syntenic (all located on the same DNA molecule—the 190-kb circular cpDNA where mutant 10-6C is located), how are they arranged on the molecule? One intriguing possibility is presented in Figure 3. Chloroplast DNA in Chlamydomonas (ROCHAIX 1978)

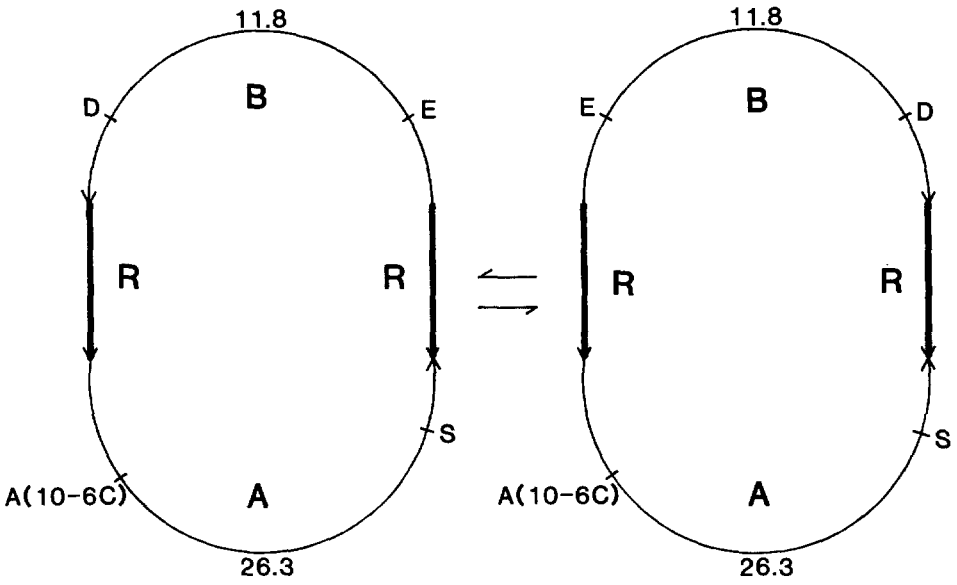

FIGURE 3.—Possible correspondence between the physical map of cpDNA and the genetic results of cross I. The 20-kb inverted repeat regions (R) are depicted by thick arrows drawn in the direction of transcription of the rRNA genes they contain (ROCHAIX 1978). The arrowhead indicating the orientation of the 78-kb unique region "A" is drawn in the direction of transcription of the carboxylase LS gene (DRON, RAHIRE and ROCHAIX 1983). The arrowhead indicating the orientation of the 72-kb unique region "B" is placed arbitrarily. Marker A(10-6C) is located in the LS gene in region A (DRON *et al.* 1983) about 15 kb from the boundary with the repeat region (MALNOE, ROCHAIX and CHUA 1979). The two molecular isomers generated by intramolecular recombination within the repeat would also be isomers of the genetic map as shown. The map distances given are from Figure 1. Other map distances would be averages of the two isomers.

and most higher plants (KOLODNER and TEWARI 1979; PALMER and THOMPSON 1982) contains a large inverted repeat that separates two unique regions in the circular map. Presumably, any mechanism that catalyzes homologous intermolecular recombination [as must be occurring in heteroplasmic Chlamydomonas cells (LEMIEUX, TURMEL and LEE 1981)] would also catalyze intramolecular recombination between the two copies of the repeat (in region R, Figure 3). This would generate two isomers of the molecule that differ only in the relative orientation of the two unique regions. In the one plant that has been critically tested for the presence of these isomers, *Phaseolus vulgaris*, both were detected in approximately equal amounts (PALMER 1983). Such isomerization would generate a genetic map in which recombination frequencies do not correspond linearly to physical distances spanning the repeat. Specifically, it is possible to arrange two markers in one unique region that are equal recombination distances from markers in the other and yet have substantial distance between them. This is because the genetic distance would be determined by the average of the physical distances in the two isomers. Thus, markers E and D could be located in one unique region, about equal physical distances from the repeat boundary, and markers A(10-6C) and S could be in

the other, at somewhat unequal distances from the boundary. The arrangement of markers proposed in Figure 3 is based on the known location of mutant 10-6C in the carboxylase LS gene (DRON *et al.* 1983), with the other markers placed on the basis of our genetic data in accord with the aforementioned rationale. The repeat region is expected to be a localized site of high coincidence. Although this model for the correspondence between the physical and genetic maps is sufficient to explain the results of cross I, we are not certain that it is unique. However, it does serve to underscore the factors that must be considered in efforts to correlate the two maps.

MYERS *et al.* (1982) have recently described a series of cpDNA deletion mutants that are linked to genetic locus *ac-u-c*. These are physically located in the opposite unique region from mutant 10-6C near the boundary with the repeat. Genetic studies have not yet linked this locus to other markers in the uniparental linkage group, and it will be interesting to see whether such a linkage can be established. It will also be interesting to determine how these deletions map genetically relative to the D, E and S markers. Such an analysis could be performed with the paternal marker selection method, scoring not only the acetate-requiring phenotype of the *ac-u-c* marker, but also the cpDNA deletion phenotype.

Mechanism of recombination in cross II

In the transduction-like results of cross II, markers from the mt^- parent are dispersed among cells otherwise retaining intact genomes from the mt^+ parent. In a genetic sense, it appears that the genome from the mt^- parent becomes fragmented, either before or during the recombination process. These two distinct mechanisms for fragmentation are diagrammed in Figure 4. According to SAGER'S model for the mechanism of uniparental inheritance, cpDNA contributed to the zygote by the mt^- parent is enzymatically degraded (SAGER and RAMANIS 1973). The biparental zygotes that form the basis of our genetic analysis may arise from incomplete degradation which leaves DNA fragments large enough to recombine with the intact molecules from the mt^+ parent (Figure 4A). The resulting marker rescue could yield the data obtained in cross II. On the other hand, fragmentation of the genome from the mt^- parent could occur *during* the recombination process (Figure 4B). If there were an excess of genomes from the mt^+ parent, and if individual molecules had several opportunities to recombine before segregating from the recombining pool of molecules, markers from the mt^- parent would become distributed among multiple molecules that are predominantly derived from the mt^+. In zygote clone analysis, the output of markers is generally biased in favor of markers from the mt^+ parent (GILLHAM, BOYNTON and LEE 1974; HARRIS *et al.* 1977; BIRKY *et al.* 1981). This output bias has been interpreted to indicate that the input of molecules into the zygote is also unequal (GILLHAM, BOYNTON and LEE 1974; WURTZ, BOYNTON and GILLHAM 1977; ADAMS 1978), as would be required in this second model for the transduction-like process. Our data do not discriminate between these two possible mechanisms.

An adventitious feature of both of these models is that they would explain

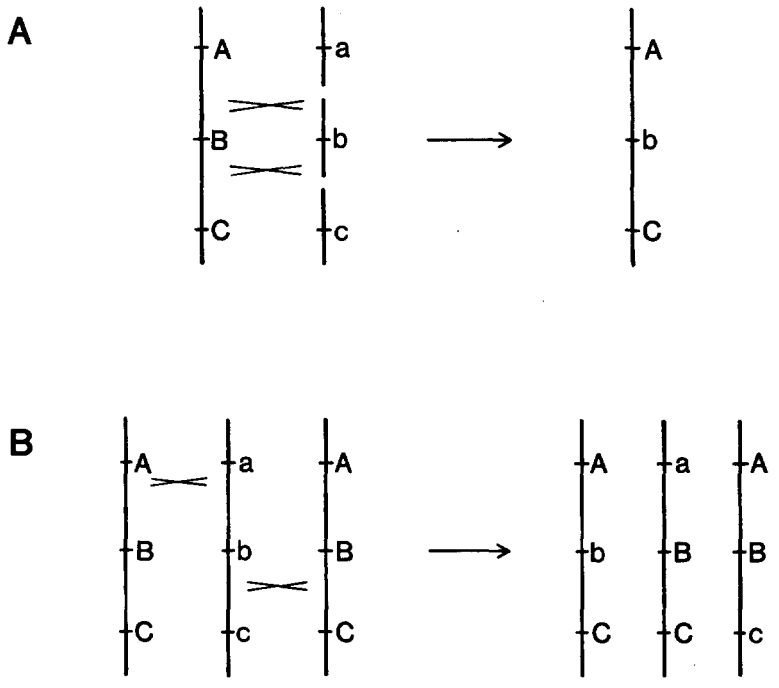

FIGURE 4.—Possible molecular mechanisms for genetic fragmentation of markers from the mt^- parent in cross II. Upper and lower case symbols refer to alleles from the mt^+ and mt^- parents, respectively. In A, the mt^- genome becomes physically fragmented before recombination, whereas that from the mt^+ parent does not. Recombination then yields molecules that predominantly carry markers from the mt^+. In B, fragmentation occurs as a *consequence* of recombination of a small number of mt^- genomes with a larger number of mt^+ genomes.

the observed nonreciprocality of recombinant cell production in zygote clones (GILLHAM 1965; SAGER and RAMANIS 1976; VANWINKLE-SWIFT and BIRKY 1978). Individual zygote clones that yield a high frequency of a particular recombinant cell type are no more likely to yield cells of the reciprocal class than are other zygote clones (VANWINKLE-SWIFT and BIRKY 1978). In the first model discussed, recombination results in the replacement of segments of DNA and does not produce genetically reciprocal molecules. In the second, reciprocals from one recombination event would be disguised by additional events involving different molecules. In either case, individual zygote clones would not be expected to produce reciprocal classes in a correlated manner. For this explanation of nonreciprocality to have general validity, it must be true that the recombination processes evident in the rather unusual results of cross II are also operating in more usual crosses that do not require a transduction-like model for analysis. Whether or not this can be the case will be discussed in more detail.

It is important to recognize that UV treatments used to increase the relative frequency of biparental zygotes do not substantially affect the results of cross II (or its qualitative differences from cross I). UV treatment of mt^+ gametes is

usually regarded as causing some form of defect in the normal uniparental mechanism, either by preventing degradation of molecules from the mt^- (SAGER and RAMANIS 1973), or by changing the relative heritability (GILLHAM, BOYNTON and LEE 1974) or input (WURTZ, BOYNTON and GILLHAM 1977; ADAMS 1978) of genomes contributed by the two parents. None of these models for the effect of UV treatment is strictly compatible with the models we have considered for the transduction-like recombination process in cross II. If recombination involves fragments of DNA from the mt^- parent, then treatments that increase fragment size should increase cotransmission frequencies. Likewise, treatments that decrease the relative input of molecules from the mt^+ parent should decrease recombinational dispersion of markers from the mt^-. By either of these models, UV treatment should *increase* the apparent linkage among markers from the mt^- parent, but our results detect no such progressive change. Unfortunately, our understanding of the molecular effects of UV treatment and of the mechanisms of chloroplast genome recombination are still insufficient to resolve these discrepancies.

Comparison of results from cross I and cross II

Qualitatively, the results of crosses I and II are so different that they require different formal models for analysis. Recombination is so frequent in cross II that the parental genotype is in the minority, whereas it is so infrequent in cross I that the parental class accounts for ⅔ of the progeny. The frequency of recombination of the E and S markers in cross II is twice that found in cross I (Figures 1 and 2). Since the same mt^- strain was used in both crosses, the difference in results must be attributable to some difference between the two mt^+ strains. We will consider three general models that could be the mechanistic basis for this difference.

First, it is possible that there is a difference in the input of cpDNA from the 10-6C and 8-36C strains. If strain 8-36C contributed an abnormally large amount of cpDNA to the zygote, then, in a panmictic recombining pool, the markers from the mt^- strain would become relatively dispersed, as we have seen in cross II. The ratio of chloroplast to nuclear DNA in vegetative cells of these two strains is not noticeably different (data not shown). Also, as mentioned, UV treatments that are effective in changing the relative genetic input (ADAMS 1978) do not affect the qualitative difference between crosses I and II. Thus, it seems unlikely that a difference in cpDNA input is the major factor causing the difference between the two cross results.

A second possibility is that biparental zygotes derived from the two strains differ in the extent of mixing of the contents of the zygotic chloroplast. If mixing were incomplete (in zygotes derived from mutant 10-6C—cross I), then some parental cpDNA molecules would never have an opportunity to recombine and would be transmitted intact. If this were the case, then recombination among molecules in mixed regions of the stroma could be as intense as in cross II and could involve the same molecular mechanisms. In this model, the frequent recombination observed in cross II is a consequence of more thorough mixing. Incomplete mixing should generate positive coincidence, since

all of the recombination events are confined to a subset of the heritable genomes. Such positive coincidence has been observed in standard zygote clone analysis (HARRIS *et al.* 1977) and is not excluded by the data of cross I. A much larger sample of progeny from cross I must be examined to obtain a strong statistical test for coincidence in this type of analysis. VANWINKLE-SWIFT (1980) has noted that the observed organization of cpDNA into nucleoids could lead to incomplete genetic mixing. She has developed this model to explain many of the known features of chloroplast genome recombination. The model is also consistent with our data.

The ultrastructure of mutant 8-36C suggests a plausible mechanism for a difference in stroma mixing in these two crosses. As is true of other photosystem II-deficient mutants (GOODENOUGH and LEVINE 1969), vegetative cells of mutant 8-36C lack typical stacking between thylakoid membranes (Dennis KEEFE, unpublished observations). It is easy to imagine that the normal stacking found in wild-type and mutant 10-6C cells would effectively compartmentalize cpDNA nucleoids into separate regions and present a barrier to random mixing. To test this model, it will be important to perform crosses involving other photosystem II-deficient mutants in our collection (SPREITZER and METS 1981) as well as other mutants with different alterations in plastid ultrastructure.

If cytoplasmic mixing is truly incomplete, as suggested in the model, then the mechanistic basis for linkage among the uniparental markers must be carefully reevaluated. Markers located in the same sector of unmixed cytoplasm could show linked inheritance whether or not they are carried on the same DNA molecule. In principle, chloroplast and mitochondrial markers could show linked inheritance if mixing of the total zygotic cytoplasm were restricted. However, the plausible mechanism for the difference between apparent mixing in crosses I and II would only explain differences in mixing of chloroplast contents. If this proposed mechanism is correct, then the affected markers would most likely be localized in the chloroplast. If cross II represents the fully mixed state, the Er and Sr markers still show an apparent effect of linkage (see RESULTS), and the Dr and A(8-36C) markers appear absolutely linked. Thus, it still seems that physical location on the same DNA molecule is the most likely cause of linked inheritance of these markers. A critical test for molecular linkage would involve the selective analysis of progeny cells that are recombinant for at least one pair of markers. The genomes of such cells must have been derived from a region of cytoplasm that was mixed. A study of this type is in progress.

A third possibility is that the difference between the parental strains, which is responsible for the difference in overall recombination frequencies, is unassociated with the known phenotypes of the mutants. For example, certain types of DNA sequence differences, such as *omega* on the *S. cerevisiae* mitochondrial DNA (DUJON 1980) and *chi* on bacteriophage lambda DNA (STAHL 1979), can have dramatic effects on recombination frequencies without other phenotypic effects. In both of these cases, differences in the presence of these sequences have been found between strains with different natural origins. Mutants 8-36C and 10-6C were derived by mutagenesis of the same wild-type strain

(SPREITZER and METS 1981), and so they could not carry natural differences of this sort. However, they may carry induced deletions that could conceivably produce effects analogous to those of *omega*. Disturbed recombination frequencies are noticed only in heteropolar (*omega*$^+$ × *omega*$^-$) crosses, where the two parents differ in the presence or absence of the *omega* insertion in the large ribosomal RNA gene (DUJON 1980). In Chlamydomonas, the use of fluorodeoxyuridine during mutagenesis has been recognized to produce strains carrying cpDNA deletions (MYERS *et al.* 1982). These deletions may or may not be linked to a known phenotype (GRANT, GILLHAM and BOYNTON 1980; MYERS *et al.* 1982). Since fluorodeoxyuridine was used in the experiments that yielded mutants 8-36C and 10-6C (SPREITZER and METS 1981), it is conceivable that either or both carry cpDNA deletions. Analysis of cpDNA restriction patterns has not detected a difference in fragment sizes among the strains, however (data not shown), and would limit the size of any deletion to less than 500 base pairs. At present, this class of explanation cannot be excluded, but it is subject to experimental test. A site responsible for enhancing recombination frequencies may be genetically separable from the site of the 8-36C mutation. Also, it may not be present in other induced photosystem II-deficient mutants.

In spite of the major differences in overall recombinant frequencies between crosses I and II, the three markers common to the two crosses show the same map order. That is, the Er marker appears more tightly linked to both the Sr and Dr markers than these two markers are to each other. Although this agreement is encouraging, three-point maps might agree by chance at a fairly high frequency. What is urgently needed is an increased collection of markers with diverse and independently expressed phenotypes. Especially if more markers can be located on the physical map of cpDNA, genetic analyses of larger numbers of markers are likely to resolve many of the ambiguities that remain in the chloroplast genetics of Chlamydomonas.

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Corresponding editor: J. E. BOYNTON