Transformation of Chloroplast Ribosomal RNA Genes in Chlamydomonas: Molecular and Genetic Characterization of Integration Events

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ABSTRACT

Transformation of chloroplast ribosomal RNA (rRNA) genes in Chlamydomonas has been achieved by the biolistic process using cloned chloroplast DNA fragments carrying mutations that confer antibiotic resistance. The sites of exchange employed during the integration of the donor DNA into the recipient genome have been localized using a combination of antibiotic resistance mutations in the 16S and 23S rRNA genes and restriction fragment length polymorphisms that flank these genes. Complete or nearly complete replacement of a region of the chloroplast genome in the recipient cell by the corresponding sequence from the donor plasmid was the most common integration event. Exchange events between the homologous donor and recipient sequences occurred preferentially near the vector:insert junctions. Insertion of the donor rRNA genes and flanking sequences into one inverted repeat of the recipient genome was followed by intramolecular copy correction so that both copies of the inverted repeat acquired identical sequences. Increased frequencies of rRNA gene transformants were achieved by reducing the copy number of the chloroplast genome in the recipient cells and by decreasing the heterology between donor and recipient DNA sequences flanking the selectable markers. In addition to producing *bona fide* chloroplast rRNA transformants, the biolistic process induced mutants resistant to low levels of streptomycin, typical of nuclear mutations in Chlamydomonas.

RECOMBINATION occurs between introduced donor fragments of chloroplast DNA and the recipient chloroplast genome during biolistic transformation of the Chlamydomonas chloroplast (BLOWERS *et al.* 1989; BOYNTON *et al.* 1988, 1990a). To study the mechanism and site specificity of intermolecular chloroplast gene recombination in Chlamydomonas, we have developed methods for transformation of the 16S and 23S ribosomal RNA (rRNA) genes located in the inverted repeat region of the chloroplast genome (BOYNTON *et al.* 1990a). Mutant alleles exist at five different sites within the 16S rRNA gene that confer resistance to streptomycin, spectinomycin, and neamine/kanamycin and at two loci in the 23S gene that confer resistance to erythromycin, and the base pair changes that cause each mutation have been determined (HARRIS *et al.* 1989). The entire chloroplast rRNA gene operon has been sequenced, including the intergenic spacer and flanking regions, and the operon has been shown to be transcribed in the 16S–23S gene order (DRON, RAHIRE and ROCHAIX 1982; LEMIEUX *et al.* 1989; ROCHAIX and DARLIX 1982; ROCHAIX and MALNOË 1978; SCHNEIDER *et al.* 1985; SCHNEIDER and ROCHAIX 1986). In progeny of meiotic zygotes exhibiting biparental inheritance of chloroplast antibiotic resistance mutations, recombination within and between 16S and 23S chloroplast genes from opposite parents occurs following organelle fusion (LEMIEUX and LEE 1987; S. M. NEWMAN, E. H. HARRIS, N. W. GILLHAM and J. E. BOYNTON, in preparation). These events have been particularly well characterized genetically in *Chlamydomonas reinhardtii* (HARRIS *et al.* 1977) where a physical map of *ca.* 7 kb of the chloroplast genome can be correlated with the detailed genetic map obtained by recombination of antibiotic resistance mutations (HARRIS *et al.* 1989).

Localization of individual exchange events during recombination to specific sites on the chloroplast genome is made possible through the use of restriction fragment length polymorphisms (RFLPs) that distinguish the chloroplast genomes of *C. reinhardtii* and the interfertile strain *Chlamydomonas smithii* (HARRIS, BOYNTON and GILLHAM 1987; PALMER *et al.* 1985). RFLPs surrounding the rRNA genes have been characterized by fine structure mapping and in some cases by sequence analysis. Differences in the number and spacing of small (0.1–0.5 kb) dispersed repeat (SDR) sequences located in the intergenic regions (BOYNTON *et al.* 1990b; SCHNEIDER *et al.* 1985; SCHNEIDER and ROCHAIX 1986) and marked by *Kpn*I and *Aat*II restriction sites generate most of the RFLPs that distinguish the chloroplast genomes of these two strains (PALMER *et al.* 1985).

In this paper we more fully document our prelimi-

nary report of chloroplast rRNA gene transformation (BOYNTON *et al.* 1990a) and demonstrate the utility of this system for studying chloroplast gene recombination. Transformation frequencies were maximal when the recipient cells were grown in 5-fluorodeoxyuridine (FdUrd) before bombardment to reduce the chloroplast genome copy number (WURTZ, BOYNTON and GILLHAM 1977) and when sequence heterology between the donor and recipient DNAs was minimized. The most common integration event resulted in complete or nearly complete replacement of the resident chloroplast DNA sequence by the corresponding sequence in the donor plasmid without integration of vector sequences. Positions of exchange events between the homologous recipient and donor sequences were biased and tended to occur near the insert:vector junctions. Furthermore the integrated donor sequences were present in both inverted repeat elements of all copies of the chloroplast genome in the transformed recipient cells, consistent with the presence of an active copy correction mechanism for maintaining the homogeneity of the inverted repeat (MYERS *et al.* 1982; PALMER *et al.* 1985). We also found that the process of bombarding wild-type Chlamydomonas cells can induce mutations, especially those conferring resistance to low levels of streptomycin, which are typical of nuclear *sr-1* mutants (HARRIS 1989).

MATERIALS AND METHODS

Strains: All Chlamydomonas strains were obtained from the Chlamydomonas Genetics Center, Department of Botany, Duke University. CC-124 and CC-125 are wild-type mt^- and mt^+ strains, respectively (HARRIS 1989). CC-1852 is a mt^- wild-type F_1 hybrid between *C. reinhardtii* and *C. smithii* that contains the organelle genomes of *C. smithii* (GILLHAM, BOYNTON and HARRIS 1987). CC-227 is a *C. reinhardtii sr-u-2-60 spr-u-1-6-2 er-u-37* mt^+ strain that carries streptomycin (*sr*) and spectinomycin (*spr*) resistance mutations in the 16S rRNA gene (nucleotide numbers 474 and 1123, respectively; HARRIS *et al.* 1989) and a mutation that confers resistance to erythromycin (*er*) located in the 23S rRNA gene at a position equivalent to nucleotide number 2057 of *Escherichia coli*, now shown to be position 2067 in the *C. reinhardtii* gene (LEMIEUX *et al.* 1989). CC-130 is a *C. reinhardtii sr-u-2-60 nr-u-2-1* mt^+ strain that possesses a kanamycin/neamine resistance (*kr*) mutation at nucleotide 1341 in the 16S rRNA gene (HARRIS *et al.* 1989), as well as the *sr-u-2-60* mutation, while CC-110 is a *C. reinhardtii* strain carrying the spectinomycin resistance mutation *spr-u-1-6-2*. CC-2477 is a spectinomycin resistant isolate from CC-1852, containing a mutation in the *C. smithii* chloroplast genome that alters the same *Aat*II restriction site in the 16S rRNA gene as do the other spectinomycin resistant mutants that have been sequenced in *C. reinhardtii* (HARRIS *et al.* 1989).

Culture conditions: For DNA isolation cells were grown phototrophically as described by HARRIS *et al.* (1989) in shaking cultures of 300 ml HS medium (SUEOKA 1960) bubbled with 5% CO_2 under continuous cool white fluorescent light (200 $\mu E/m^2$ per second) at 25°. For transformation, cells were grown mixotrophically to mid log phase (*ca.* 3–5 × 10^6 cells/ml) at 200 $\mu E/m^2$ per second and 25° in HSHA medium (HS medium supplemented with 29.4 mM sodium acetate; HARRIS 1989) and bubbled with air. A filter-sterilized solution of FdUrd was added to some cultures (final concentration 0.5 mM) at a cell density of 5 × 10^4/ml and the cultures grown for *ca.* 6 cell generations (*cf.* HOSLER *et al.* 1989).

Cloning of donor DNA fragments: Chloroplast DNA was isolated by sodium iodide gradient centrifugation as previously described (GRANT, GILLHAM and BOYNTON 1980). Restriction fragments containing 16S and 23S rRNA genes from *C. reinhardtii* (CC-227, CC-110) or *C. smithii* (CC-2477) were cloned into pUC vectors (see Figure 1) using standard methods (MANIATIS, FRITSCH and SAMBROOK 1982) and designated P-183, P-228 and P-229, respectively. Plasmid DNA used in the transformation experiments was isolated as the covalently closed circular band from cesium chloride gradients (MANIATIS, FRITSCH and SAMBROOK 1982).

Transformation procedures: Cells grown to mid log phase were concentrated by centrifugation at 20° to a density of 1.14 × 10^8 per ml in HSHA medium, diluted 1:1 with 0.2% Difco agar in HS medium at 42° and 0.7-ml aliquots containing 4 × 10^7 cells were rapidly dispersed onto the surfaces of 10 cm petri dishes containing 1.5% agar in HSHA medium. Cells will not withstand 42° for more than a few minutes. Sixty milligrams of tungsten M10 microprojectiles (Du Pont) were resuspended in 1 ml of 95% EtOH, vortexed for 2 min to deglommerate, and spun 2 min in the microfuge the morning of each experiment. Particles were washed two times by carefully removing the supernatant (except for residual 80–100 μl), and resuspending them in 1 ml of sterile distilled H_2O by vigorous vortexing. Twenty-five microliters of resuspended tungsten particles were added to a microfuge tube, immediately followed by 2.5 μl of the donor plasmid DNA at 1 μg/ml, 25 μl of 2.5 M $CaCl_2$ and 10 μl of 0.1 M spermidine (free base). Tubes were finger flicked 8–10 times to mix, allowed to sit at room temperature 8–12 min, spun 30 sec in the microfuge and 50 μl of supernatant discarded, leaving enough particle suspension for three bombardments. The DNA-coated particles were resuspended by vortexing vigorously and 2 μl immediately loaded per macroprojectile. Blank charges (0.22 caliber gray, power level #1) were used as accelerators for bombardment in a Biolistic PDS-1000 Particle Delivery System (Du Pont).

Bombarded plates were respread within 2 hr or incubated in darkness overnight and respread the next morning onto selective HSHA media containing 100 μg/ml streptomycin, 100 μg/ml spectinomycin or 250 μg/ml erythromycin singly or in various combinations. To reduce the possibility of bacterial contamination, all media contained 50 μg/ml ampicillin which has no effect on the growth of Chlamydomonas (HARRIS 1989). Colonies, usually visible after 1 to 2 weeks, were retested for growth on selective media. Putative transformants were scored for their ability to grow on the nonselected antibiotics to which the donor DNA conferred resistance or sensitivity. In addition, certain transformants were tested for growth on 500 μg/ml streptomycin or 100 μg/ml kanamycin. The frequency of transformants was calculated by dividing the number of colonies appearing on selective plates by the number of cells in the spray pattern of the gun (14% of the 4 × 10^7 cells on a 10-cm shot plate) and by the fraction of cells (25%) recovered after biolistic bombardment and dilution replating on nonselective medium. Both values were determined empirically for conditions used in our laboratory and represent constants applied to all experiments.

TABLE 1

Recovery of antibiotic-resistant isolates after biolistic bombardment

Selection	Frequency of resistant colonies ($\times 10^{-6}$)[a]				
	Unbombarded control	Bombarded, no DNA	Bombarded, P-17 (*atpB*)	Bombarded, P-183 (*sr spr er*)	Phenotype
Streptomycin (100 μg/ml)	0.013	19.8	17.0	42.7	19.5–sr[b] 19.6–sr spr 3.6–sr spr er

[a] In each treatment, 4.2×10^7 cells were bombarded and 3.9×10^8 cells were plated on 100 μg/ml streptomycin for unbombarded controls.

[b] Ninety percent were resistant only to 100 μg/ml streptomycin, suggestive of nuclear mutations rather than chloroplast transformants, which are resistant to 500 μg/ml.

DNA isolation and characterization: A rapid procedure for isolation of total cell DNA from Chlamydomonas colonies growing on solid media was developed by modifying the methods of GRANT, GILLHAM and BOYNTON (1980) and GILLHAM, BOYNTON and HARRIS (1987). Cells were spread by toothpick into 1–2-cm^2 patches on HS or HSHA-YA agar plates and grown 3–7 days in the light (80 $\mu E/m^2$ per second). Each patch was scraped up with a sterile toothpick and resuspended in a 1.5 ml microfuge tube containing 1.0 ml cold TEN buffer (150 mM NaCl, 10 mM Na_2EDTA, 10 mM Tris-HCl, pH 8.0), pelleted at room temperature and resuspended in 0.4 ml TEN, to which 40 μl 20% SDS, 40 μl 20% Sarkosyl and 35 μl of 50 mg/ml heat-treated Pronase (Boehringer Mannheim) were added. Samples were gently vortexed, and rotated for 5 min at 4° and then extracted with 0.65 ml TEN-saturated phenol:chloroform:isoamyl alcohol 25:24:1 (v:v) for 10 min at room temperature with gentle intermittent vortexing. The top aqueous phase (<0.5 ml) was recovered after a 10 min centrifugation at room temperature and the nucleic acids precipitated with 1.0 ml ice-cold 95% ethanol at −20° for 30 min. The precipitate was collected by centrifugation for 10 min at room temperature, washed with cold 70% ethanol, dried, resuspended in 0.05 ml TE (10 mM Tris, pH 8.0, 1 mM EDTA) and 5–10-μl aliquots digested. All single and double restriction digests were carried out according to the manufacturer's specifications. Fragments were separated by electrophoresis in 0.8, 1.0, or 1.2% agarose gels and blotted to nitrocellulose, hybridized with ^{32}P nick-translated probes and washed under stringent conditions as described by GILLHAM, BOYNTON and HARRIS (1987).

RESULTS

Induction of mutations by microprojectile bombardment: Experiments indicate that the biolistic transformation process using tungsten particles without DNA is mutagenic to Chlamydomonas. When wild-type *C. reinhardtii* cells were bombarded with either uncoated tungsten microprojectiles or microprojectiles coated with P-17 DNA encoding the chloroplast *atp*B gene (BOYNTON *et al.* 1988), the frequency of colonies capable of growth on 100 μg/ml streptomycin increased over 1000-fold compared to the unbombarded control (Table 1). When the P-183 plasmid containing the *er spr sr* markers was used as the donor DNA, the frequency of streptomycin resistant colonies increased an additional twofold. About half of the latter streptomycin resistant isolates were resistant to one or both of the unselected antibiotic resistant markers (*spr* or *spr er*) carried by the donor plasmid. Furthermore, all of the *sr spr* and *sr spr er* isolates were resistant to 500 μg/ml streptomycin, characteristic of the *sr* resistance allele in the donor plasmid, whereas only 10% of the isolates resistant to streptomycin alone grew on the higher concentration of this antibiotic. Our data suggest that the vast majority of the isolates resistant to only 100 μg/ml streptomycin were in fact new mutations equivalent to the *sr* isolates obtained following bombardment with tungsten particles carrying no DNA or *atp*B DNA. These low level streptomycin resistant isolates are very likely new mutations in the single nuclear *sr-1* gene (HARRIS 1989), whereas the *sr spr* and *sr spr er* isolates resistant to 500 μg/ml streptomycin from the P-183 bombardment are almost certainly *bona fide* transformants. Genetic analyses of representative low level streptomycin resistant isolates from a second experiment involving P-183 bombardment of wild type showed that the vast majority exhibited biparental transmission of that phenotype typical of nuclear mutations (data not shown).

Transformation strategies: Our experimental designs utilized donor fragments and recipient chloroplast genomes that differed by both genetic and physical markers. This allowed us to rule out the possibility that antibiotic resistant colonies appearing on the bombarded plates arose from antibiotic resistance mutations induced by the transformation process itself (Table 1) or by growth of the recipient cells in FdUrd (Table 2; WURTZ *et al.* 1979).

First, wild-type cells carrying the chloroplast genomes of *C. reinhardtii* (CC-124) and *C. smithii* (CC-1852), respectively, were grown with or without FdUrd for 5–7 cell generations and bombarded with the plasmid P-183 carrying the *er spr* and *sr* mutations (Figure 1). Putative transformants selected on medium containing both spectinomycin and streptomycin were subsequently scored for resistance to erythromycin. Double *sr spr* resistant CC-124 and CC-1852

TABLE 2

Transformation frequency for Chlamydomonas chloroplast rRNA genes

Experiment No.	Donor plasmid	Recipient strain	Selection	Pre-growth in FdUrd	Total cells ($\times 10^7$)	Transformants		
						No.	Frequency ($\times 10^{-6}$)	Percent with unselected donor marker
1	P-183	CC-124	*sr spr*	No	6.8	7	0.41	29
1	P-183	CC-124	*sr spr*	Yes	6.8	94	5.5	27
1	P-183	CC-1852	*sr spr*	No	6.8	0	<0.12	NA
1	P-183	CC-1852	*sr spr*	Yes	6.8	34	2.0	21
2	P-183	CC-125	*er*	Yes	5.1	52	4.1	21
3	P-228	CC-130	*spr*	No	3.4	310	36.3	89
4	P-228	CC-130	*spr*	No	3.4	503	58.8	ND
3	P-229	CC-130	*spr*	No	3.4	20	2.3	85
4	P-229	CC-130	*spr*	No	5.1	4	0.31	75

No spontaneous CC-124 or CC-1852 mutants doubly resistant to streptomycin (*sr*) and spectinomycin (*spr*) were observed (frequency $<0.25 \times 10^{-8}$). The frequencies of spontaneous erythromycin (*er*) resistant CC-125 and spectinomycin-resistant CC-130 mutants were calculated to be 8.7×10^{-8} and 1.4×10^{-9} respectively. NA, not applicable, ND, not determined.

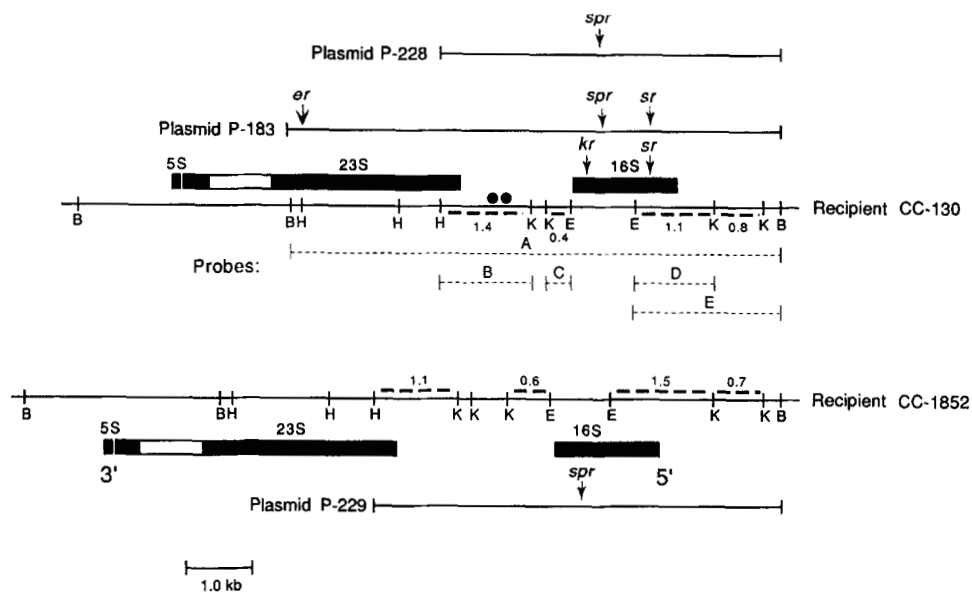


FIGURE 1.—Physical and genetic maps of the rRNA region of the inverted repeat of the chloroplast genomes of *C. reinhardtii* strain CC-130 and hybrid strain CC-1852 containing the chloroplast genome of *C. smithii* that were used as transformation recipients. Chloroplast DNA fragments were cloned into pUC vectors for use as donor plasmids as indicated by the solid bars above or below the restriction maps of strains CC-130 and CC-1852. Positions of the antibiotic resistance alleles carried by the chloroplast inserts in P-183 (*er*, *spr*, *sr*) and P-228 and P-229 (*spr*) plasmids and the *kr* and *sr* alleles of the CC-130 recipient strain are shown. Locations of the tRNA genes for isoleucine (*trn*I) and alanine (*trn*A) on the chloroplast genome of *C. reinhardtii* are indicated by ●. The position and size of the RFLPs scored in this study are marked by dashed lines on the restriction map. The unique 0.5-kb *Kpn*I fragment present between the 16S and 23S rRNA genes in *C. smithii* but absent in *C. reinhardtii* was not scored. For several of the RFLPs shown, the length changes responsible have been further localized (see Figure 6). Transcription of the rRNA gene operon is initiated 72 nucleotides upstream of the 16S rRNA and continues through the 23S gene (SCHNEIDER *et al.* 1985). The origins of the *C. reinhardtii* probes A–E used to detect the RFLP variation in the transformants are shown by dotted lines. B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; E, *Eco*RI.

transformants occurred at frequencies of 2 and 5×10^{-6}, respectively, following bombardment, whereas no spontaneous isolates resistant to both antibiotics were detected in 4×10^8 unbombarded cells of either CC-124 or CC-1852 plated directly on selective medium (Table 2).

Second, the plasmids P-228 and P-229, carrying *spr* markers from *C. reinhardtii* and *C. smithii*, respectively (Figure 1), were used to transform *C. reinhardtii* strain CC-130. This strain is sensitive to spectinomycin but carries *kr* and *sr* mutations flanking the *spr* mutation in the donor fragment. Colonies resistant to spectinomycin were selected, and tested for growth on kanamycin and streptomycin. Spectinomycin resistant colonies appeared at frequencies of 36 to 59 and 0.3 to 2.3×10^{-6} when CC-130 cells were bombarded with P-228 and P-229 respectively (Table 2). Virtually all these *spr* isolates were *bona fide* transformants since they carried both the donor kr^+ and sr^+ or the kr^+ alleles. In two experiments, only one spectinomycin resistant colony arose spontaneously on unbombarded control plates containing a total of 1.4×10^9 CC-130

cells (Table 2). This is not surprising since the frequency of nuclear mutations resistant to spectinomycin is much lower than the frequency of nuclear mutations resistant to streptomycin (HARRIS 1989).

Effects of chloroplast DNA sequence heterology on the transformation frequency of chloroplast rRNA genes: The extent of homology between the donor plasmid and recipient chloroplast genomes appears to affect the frequency of rRNA gene transformation (Table 2). When the donor plasmid contained an insert homologous to the recipient, except for three single base pair changes that confer the antibiotic resistant phenotypes, the frequency of transformation was several to many fold higher compared to a donor plasmid containing a chloroplast DNA insert which had some heterologous intergenic regions with respect to the recipient genome (Table 2). For example, cotransformation frequencies for *sr* and *spr* were two- to threefold higher when P-183 (chloroplast insert from *C. reinhardtii*) was used with CC-124 (*C. reinhardtii* chloroplast genome) than with CC-1852 (*C. smithii* chloroplast genome). An even larger increase was observed when CC-130 (*C. reinhardtii* chloroplast genome) was transformed to spectinomycin resistance with P-228 (chloroplast insert from *C. reinhardtii*) rather than P-229 (chloroplast insert from *C. smithii*). Transformation frequencies using a specific donor plasmid and recipient cell combination were reasonably consistent between experiments (*e.g.*, P-228/CC-130, Table 2). However, transformation frequencies for different plasmids varied considerably. For example, CC-130 cells bombarded with P-228 and selected for spectinomycin resistance yielded a 5–10-fold higher transformation frequency than when CC-124 cells were bombarded with P-183 and selected for streptomycin and spectinomycin resistance, even though the recipient CC-130 strain was not grown in FdUrd. This is probably not due to preferential selection for a single marker (*spr*) rather than two closely linked markers (*spr sr*) since bombardment of FdUrd-grown CC-125 with P-183 resulted in similar transformation frequencies whether erythromycin or streptomycin + spectinomycin selection was imposed (Table 2). Although we cannot exclude differences between recipient strains or in the quality of the plasmid DNA preparations used, the largest differences in transformation frequency were observed between physically different donor sequences containing the same selectable markers.

Phenotypic analysis of rRNA gene transformants: The presence of unselected donor alleles allowed us to estimate how much of the 23S and 16S rRNA genes were transferred from the donor to the recipient chloroplast genome. The *er* mutation in P-183 (Figure 1) was transferred to 21%–29% of the CC-124 or CC-1852 transformants selected for spectinomycin and streptomycin resistance (Table 2). This suggests that integration at this end of the donor DNA resulted from an exchange event in the 200 bp interval (<3% of the 7-kb insert sequence) between the *er* mutation and the *Bam*HI site in the 23S gene that marks the end of the donor sequence. The majority of the P-183 transformants had an er^+ *spr sr* phenotype, which is consistent with an exchange event occurring in the 3.8-kb interval between the *er* and *spr* mutations. Transformants with the donor *er spr sr* phenotype following P-183 bombardment of *C. reinhardtii* strains CC-124 and CC-125 were remarkably constant in frequency regardless of whether *spr-sr* or *er* were used as the selectable markers (Table 2).

Two phenotypic classes of spectinomycin resistant CC-130 transformants were obtained following bombardment with P-229 which carries the 16S rRNA gene with the *spr* marker and flanking RFLPs from *C. smithii*. Seventeen of the 20 *spr* isolates had a kr^+ $spr\ sr^+$ phenotype indicating that they had received most or all of the 16S gene from the donor. Two transformants had a kr^+ *spr sr* phenotype suggesting the occurrence of an exchange event within the 16S gene between the *spr* and *sr* mutations, which are 649 bp apart. One isolate was resistant to all three antibiotics, as was the spontaneous spectinomycin resistant mutant of CC-130 that grew on non-bombarded spectinomycin control plates. The kr^+ $spr\ sr^+$ isolates were presumed to be true transformants since the probability of three independent mutations occurring in the 16S rRNA gene of a CC-130 *kr* spr^+ *sr* recipient cell is exceedingly low. Analysis of the RFLP markers flanking the rRNA genes confirmed that they were *bona fide* transformants (see below). Several hundred spectinomycin resistant CC-130 isolates, obtained following transformation with P-228 which carries the 16S rRNA gene from *C. reinhardtii* with the *spr* marker but no RFLP differences, were analyzed for the phenotypes of the flanking *kr* and *sr* loci. Over 89% of the *spr* transformants had a kr^+ $spr\ sr^+$ phenotype, which would be expected if the resident 16S rRNA gene were replaced by the homologous sequence on P-228. Therefore, the vast majority of CC-130 transformants (75–89%) possess the unselected markers (kr^+ and sr^+) from the donor that flank the selectable *spr* allele in the 16S rRNA gene.

Molecular analysis of rRNA gene transformants: Analysis of the P-183/CC-1852 and P-229/CC-130 rDNA transformants indicated that the major phenotypic class in each case reflected replacement of much or all of the resident rRNA genes by sequences introduced on the donor plasmid. This was confirmed by molecular analysis of RFLP markers (Figure 1) flanking the 16S and 23S rRNA genes from representative transformants. SOUTHERN (1975) blot hybridizations of *Bam*HI digests of total cellular DNA

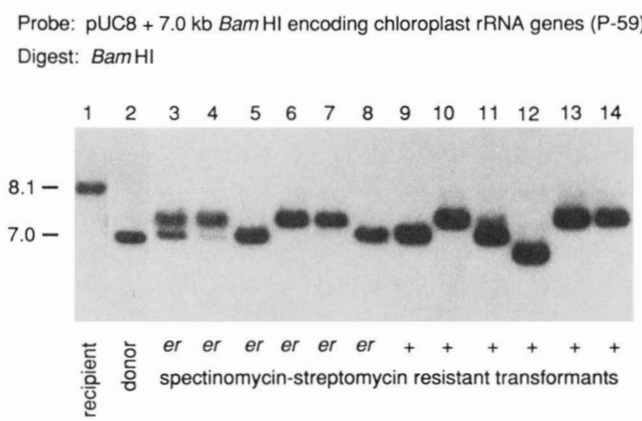


FIGURE 2.—RFLP analysis of P-183/CC-1852 transformants. Total cell DNA was prepared from the recipient strain CC-1852 (lane 1), CC-227 from which the P-183 donor plasmid was derived (lane 2), six *er spr sr* (lanes 3–8) and six er^+ *spr sr* (lanes 9–14) transformants. DNA samples were digested with *Bam*HI, separated on 0.8% agarose gels and blotted to nitrocellulose before hybridization with a cloned Bam 11/12 fragment from wild type *C. reinhardtii* (probe A, Figure 1).

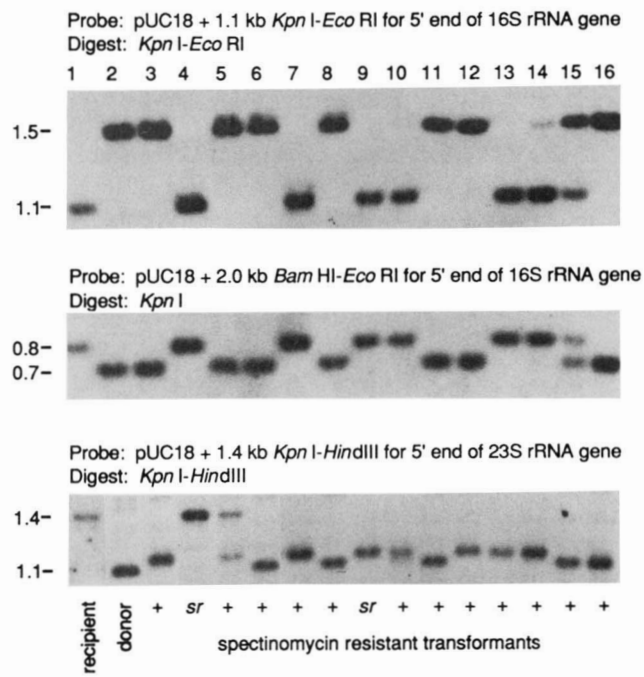
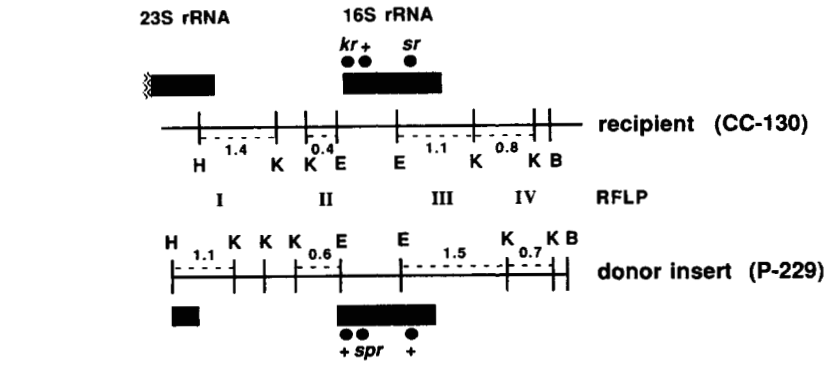


FIGURE 3.—RFLP analysis of P-229/CC-130 transformants. Total cell DNA from the recipient CC-130 (lane 1), CC-2477 from which the P-229 donor plasmid was derived (lane 2), 12 kr^+ *spr* sr^+ (lanes 3, 5–8, 10–16) and two kr^+ *spr sr* (lanes 4, 9) transformants was isolated, digested with *Kpn*I-*Eco*RI (top panel), *Kpn*I (middle panel), or *Kpn*I-*Hin*dIII (bottom panel) and fractionated on 1.0% agarose gels, blotted to nitrocellulose. Probes B and C were used for the 1.4/1.1-kb *Kpn*I-*Hin*dIII and 0.4/0.6-kb *Kpn*I-*Eco*RI RFLPs in the 16S–23S spacer whereas probes D and E were used for the 1.1/1.5-kb *Eco*RI-*Kpn*I and 0.8/0.7-kb *Kpn*I RFLPs upstream of the 16S gene as shown in Figure 1.

from 20 *sr spr* er^+ or *sr spr er* P-183/CC-1852 transformants revealed that none contained the 8.1-kb Bam 11/12 fragment typical of the *C. smithii* CC-1852 recipient strain (Figure 2). In seven transformants (*e.g.*, lanes 5, 8, 9) the Bam 11/12 fragment was indistinguishable from the 7.0-kb Bam 11/12 fragment of the donor. Nine transformants (*e.g.*, lanes 6, 7, 10, 13, 14) had a Bam 11/12 fragment that was intermediate in length between the 8.1-kb recipient and the 7.0-kb donor fragments. We determined that the Bam 11/12 fragments of intermediate length are actually chimeras, containing the RFLPs just beyond the 5′ end of the 16S gene of the CC-1852 recipient and the RFLPs from the 16S–23S spacer region of the P-183 donor plasmid (see below). Three transformants (lanes 3, 4, 11) were mixed at the time of analysis, having both the donor Bam 11/12 fragment and a second fragment of intermediate length in variable proportions. Analysis of a set of 12 single cell clones from each of these three transformants demonstrated that all three transformants were mixtures of cells pure for one or the other RFLP type. All subclones were considered to be *bona fide* transformants because they contained the appropriate phenotypic markers and the donor form of at least one RFLP that was scored. One transformant (Figure 2, lane 12) had a Bam 11/12 fragment smaller than the 7.0-kb donor fragment.

Similar results were obtained when 20 representative P-229/CC-130 *spr* transformants were analyzed in the same way, with the exception that one *spr* transformant possessed a *Bam*HI-*Hin*dIII fragment identical in size to the recipient (data not shown). Therefore, in every homoplasmic transformant but one, both resident copies of the rRNA genes were replaced by all or part of the homologous sequences from the donor plasmid. Sequences homologous to the pUC vectors were not detected in total cell DNA preparations from the 40 rRNA gene transformants analyzed. These results indicate that the vector is not stably integrated into the recipient chloroplast (or nuclear) genome nor is the plasmid replicating autonomously in the transformants. The transformation process is apparently limited to sequences homologous between the donor and recipient, since alterations are seen only in Bam 11/12, while the adjacent *Bam*HI fragments (Bam 14/15 and Bam 10) in the P-183/CC-1852 transformants are identical in size to those of the CC-1852 recipient strain (data not shown).

Detailed mapping of the exchange events in rRNA gene transformants: The length differences between the insert sequence in the transforming plasmids (P-183 and P-229) and the homologous sequences in the recipient chloroplast genomes (CC-1852 and CC-130) represent the sum of five easily scorable RFLPs (Figure 1). Three or four RFLPs were analyzed in the transformants (Figures 3–5) to determine where specific exchange events occurred during the process of integrating the donor fragment into the recipient genome. Twenty P-229 transformants of strain CC-130 were analyzed for the 1.4/1.1-kb



Region of Exchange	Resistance genotype	RFLP I	II	III	IV	Number	Class
	+ spr sr	R	R	R	R	1	A
	+ spr +	D	D	D	D	8	B
	+ spr sr	X	D	R	R	1	C
	+ spr +	X	D	D	D	4	D
	+ spr +	X	D	R	R	4	E
	+ spr +	D	D	R	R	2	F

FIGURE 4.—Diagrammatic interpretation of postulated exchange events necessary to generate the different classes of transformants in the P-229/CC-130 experiment. The donor plasmid insert and recipient chloroplast genome are labeled at the top of the figure. Diagrams are presented for the origin of each class of transformants, with the antibiotic resistance genotype, pattern of RFLPs and numbers of each class indicated to the right. Abbreviations for restriction enzyme sites are identified in the legend to Figure 1. The size (kb) and location of the RFLPs that were scored are indicated and the positions of the antibiotic resistance markers are designated by ●. Recombinant restriction fragments that are intermediate in length between either RFLP type (classes C, D, E) are designated by X. One member of class B and one member of class D were initially mixed for RFLPs 5′ to the 16S gene. Single cell cloning resulted in isolates pure for the two RFLP types.

*Kpn*I-*Hin*dIII RFLP spanning the 5′ end of the 23S rRNA gene, and for the adjacent 1.1/1.5-kb *Eco*RI-*Kpn*I and 0.8/0.7-kb *Kpn*I RFLPs at the 5′ end of the 16S gene (Figures 3, 4).

Twelve transformants inherited both RFLPs in the 5′ region of the 16S gene from the donor, indicating that an exchange event occurred between the polymorphism in the *Kpn*I 0.7/0.8-kb fragment and the *Bam*HI site *ca.* 500 bp away that marks the end of the insert sequence (*e.g.*, Figure 3, top and middle panels, lanes 3 and 5; Figure 4, classes B and D). The 0.8-kb recipient form of this RFLP and the adjacent 1.1-kb *Kpn*I-*Eco*RI RFLP were observed in eight transformants (*e.g.*, Figure 3, lanes 4 and 7; Figure 4, classes A, C, E, F). In these transformants an exchange event occurred between the *spr* locus in the 16S gene and the polymorphism immediately 5′ to the gene in the *Kpn*I-*Eco*RI fragment. Two transformants were mixed for both the 1.1/1.5- and 0.8/0.7-kb RFLPs, containing donor and recipient forms of each restriction fragment (Figure 3, top and middle panels, lanes 14 and 15); subcloning of the initial colonies resulted

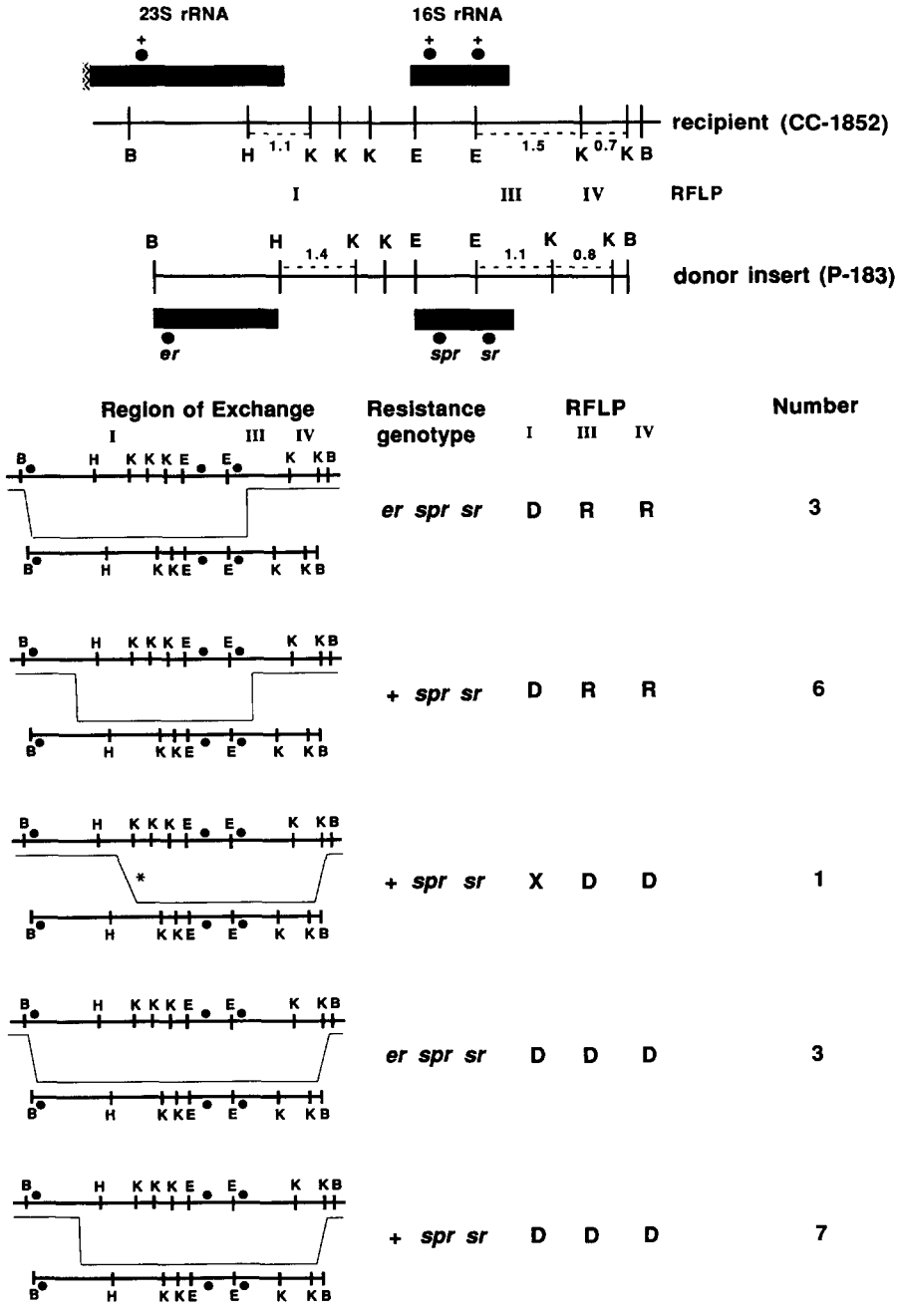


FIGURE 5.—Diagrammatic interpretation of postulated exchange events necessary to generate the different classes of transformants in the P-183/CC-1852 experiment. Symbols are explained in the legend to Figure 4. (A) Recombinant restriction fragment longer than the donor RFLP (class C) is indicated by X. The location of the putative illegitimate exchange event between multiple SDR elements that generates this non-parental form of the *Kpn*I-*Hin*dIII RFLP is designated by *. Two members of class D and one member of class E were initially mixed for RFLPs 5′ to the 16S gene. Single cell cloning resulted in isolates pure for the two RFLP types.

in only pure colonies of the two types. One of these transformants contained a donor and one an intermediate length form of the *Kpn*I-*Hin*dIII RFLP from the 16S–23S spacer (Figure 3, bottom panel, lanes 14 and 15). No evidence was observed for physical exchange of donor and recipient chloroplast sequences between the adjacent 1.1/1.5-kb *Eco*RI-*Kpn*I and 0.8/0.7 *Kpn*I RFLPs immediately upstream of the 16S rRNA gene (Figures 3, 4).

When the RFLPs in the 16S–23S rRNA spacer region of the P-229/CC-130 transformants were analyzed, a strong bias was observed for integration of the donor form of the *Kpn*I-*Hin*dIII fragment. Ten kr^+ *spr* sr^+ transformants of CC-130 (Figure 4, classes B, F) contained the donor form of both the 1.4/1.1-kb *Kpn*I-*Hin*dIII (*e.g.*, Figure 3, bottom panel, lanes 6 and 8) and 0.4/0.6-kb *Kpn*I-*Eco*RI intergenic RFLPs (data not shown). This is indicative of an exchange event between the polymorphism in the 1.4/1.1-kb *Kpn*I-*Hin*dIII fragment and the *Hin*dIII site marking the end of the insert. Only one transformant (kr^+ *spr sr*) had the recipient forms (Figure 3, bottom panel, lane 4) of the intergenic RFLPs (Figure 4, class A). This genotype and RFLP pattern is consistent with an exchange event having occurred between the *kr* locus and the polymorphism in the small *Kpn*I-*Eco*RI fragment near the 3′ end of the 16S rRNA gene. The putative *kr spr sr* transformant with the same phenotype as the spontaneous spectinomycin resistant mutant isolated from the CC-130 control plates, also

possessed the recipient forms of all four RFLPs. This isolate, which was not included in the tabulation presented in Figure 4, very likely resulted from a spontaneous spectinomycin resistance mutation on one of the bombarded plates rather than from a double exchange event with ends in the *kr-spr* and *spr-sr* intervals in the 16S gene.

Nine transformants (Figure 4, classes C, D, E), which possessed donor forms of the intergenic *Kpn*I-*Eco*RI RFLP (data not shown), had *Kpn*I-*Hin*dIII fragments that were intermediate in length (*ca.* 1.15 kb) compared to the donor and recipient RFLPs (*e.g.*, Figure 3, bottom panel, lanes 3 and 7) and were likely of recombinant origin. One transformant (kr^+ *spr* sr^+) was mixed for both the *Kpn*I-*Hin*dIII RFLP (Figure 3, bottom panel, lane 5) and for the *Kpn*I-*Eco*RI RFLP (data not shown), containing recipient and intermediate types of the former RFLP and donor and recipient forms of the latter. Although this isolate contained donor forms of the RFLPs (*Kpn*I-*Eco*RI 1.1/1.5 and *Kpn*I 0.8/0.7) upstream of the 16S rRNA gene (and therefore is a *bona fide* transformant), it may represent the only example of a transformant in which an exchange event occurred between the *Kpn*I-*Hin*dIII and *Kpn*I-*Eco*RI RFLPs in the 16S–23S spacer region. An additional exchange event between the *Kpn*I-*Eco*RI RFLP and the *kr* locus needs to be postulated to account for its kr^+ *spr* sr^+ phenotype. Single cell cloning of this isolate was not performed and therefore it is not included in Figure 4.

Comparable results were obtained when these RFLPs were analyzed in 14 er^+ *spr sr* and 6 *er spr sr* transformants of strain CC-1852 bombarded with P-183 (Figure 5). However one transformant with an er^+ *spr sr* phenotype (Figure 5, class C) contained a non-parental 1.7-kb form of this RFLP that was larger than either the donor or recipient restriction fragment (see BOYNTON *et al.* 1990a, Figure 5). Since the overall length of the Bam 11/12 fragment in this transformant is smaller than the recipient form by *ca.* 300 bp (Figure 2, lane 12), and the RFLPs at the 5′ end of the 16S gene are identical to the recipient, one or more illegitimate exchange events most likely occurred in the cluster of SDR elements found in the 16S–23S spacer.

DISCUSSION

Microprojectile bombardment can be mutagenic: Bombardment of wild-type *C. reinhardtii* cells with tungsten microprojectiles results in over a 1000-fold increase in the frequency of mutants resistant to low levels of streptomycin, which is characteristic of nuclear *sr-1* mutations (HARRIS 1989). In a second transformation experiment involving P-183/CC-124, 48 isolates resistant only to low levels of streptomycin were tested genetically. All exhibited a biparental pattern of inheritance typical of nuclear mutations, indicating that the biolistic process is indeed mutagenic. The frequency of uniparentally inherited mutants resistant to high levels of streptomycin was also increased somewhat above the control levels in this experiment, suggesting that the biolistic process also induces chloroplast mutations.

Strategies for biolistic transformation of chloroplast rRNA genes: Our experiments have taken advantage of the existence of multiple antibiotic resistance markers in the 16S and 23S rRNA genes and easily detectable RFLPs that distinguish the chloroplast genomes of the interfertile *C. reinhardtii* and *C. smithii* strains. Both the P-183/CC-1852 and P-229/CC-130 donor/recipient combinations, which differ by diagnostic chloroplast RFLPs flanking the selectable markers, yielded transformants at frequencies of $\sim 2 \times 10^{-6}$ (1–2 transformants per plate of bombarded cells). In contrast, the recipient CC-130 strain yielded only one *spr* isolate (frequency 1.4×10^{-9}) when unbombarded cells were plated on spectinomycin and unbombarded cells of CC-1852 yielded no colonies resistant to spectinomycin plus streptomycin. Donor plasmids containing two or more selectable markers obviate the problems of antibiotic resistance mutations that arise either spontaneously or as a consequence of particle gun bombardment. Analogous methods have recently been used to transform the chloroplasts of tobacco cells (SVAB, HAJDUKIEWICZ and MALIGA 1990). Transformants, selected for a spectinomycin resistance marker in the 16S rRNA gene, were scored for a flanking streptomycin resistance marker and a novel restriction site constructed in the donor DNA. As in our experiments with Chlamydomonas, integration of donor sequences occurred by homologous replacement, followed by copy correction, and segregation to yield isolates homoplasmic for transformed chloroplast genomes.

Effect on transformation frequencies of sequence heterology in intergenic regions between donor and recipient: When the plasmid DNA insert was completely homologous to the corresponding region of the recipient genome (with the exception of the single base pair changes which confer the antibiotic resistance phenotypes, for example P-183/CC-124 and P-228/CC-130), higher frequencies of transformation were observed than when the donor DNA possessed several sequence heterologies dispersed throughout intergenic regions of the plasmid insert, for example, P-183/CC-1852 and P-229/CC-130 (Table 2). These heterologies are primarily insertions and deletions of 50–400-bp SDR elements (PALMER *et al.* 1985). Between the regions of heterology, both the coding and non-coding sequences are quite homologous, exhibiting only *ca.* 2–4% mismatch over stretches of several hundred base pairs (S. M. NEWMAN, unpublished

data). Sequence heterology between the donor DNA and the recipient chloroplast genome may reduce the efficiency of pairing, and the probability of exchange events leading to integration, thus decreasing the frequency of chloroplast rRNA gene transformation.

Our results are similar to those from transformation and recombination experiments in *Saccharomyces cerevisiae* (SMOLIK-UTLAUT and PETES 1983), *Escherichia coli* (SHEN and HUANG 1986) and mammalian cells (WALDMAN and LISKAY 1987) where recombination in specific intervals is decreased by increasing the extent of sequence heterology. Although the effect of donor/recipient heterology on chloroplast transformation frequencies is striking, we have reason to believe that heterologies do not always alter the pattern of exchange events between the donor and recipient sequences (see below). Furthermore, the RFLPs generated by the sequence heterologies provide physical markers critical for documenting unequivocally chloroplast rRNA gene transformation.

Temporary heteroplasmic nature of transformants: A minority of the transformants were initially mixed for both forms of some RFLPs at the time the DNA of the clones was first isolated and analyzed (*e.g.*, Figure 3, top and middle panels, lanes 14 and 15). In all cases subclones proved to be homoplasmic for one of the two types of RFLPs. The failure to detect persistent heteroplasmons is consistent with the existence of an efficient copy correction mechanism for ensuring homoplasmicity of the inverted repeat in chloroplast DNA (MYERS *et al.* 1982; PALMER *et al.* 1985) and the rapid segregation of chloroplast genomes (BOYNTON *et al.* 1990b). Integration of donor DNA into one copy of the inverted repeat on the recipient chloroplast genome by homologous replacement of recipient sequences, which results in the formation of transient heteroplasmic genomes, is followed by intramolecular copy correction that yields homoplasmic molecules with either the donor or recipient form of a given RFLP in both inverted repeat elements. The transient heteroplasmic genome might also undergo intermolecular copy correction, thus spreading the introduced sequences to additional molecules that could then undergo intramolecular copy correction. Novel polymorphisms arising following recombination between donor and recipient RFLPs in the inverted repeat are also efficiently copy corrected to the other repeat (Figure 3, bottom panel). Less frequently, two integration events or two copy correction events with different endpoints may occur in the same recipient cell and result in a colony that is initially mixed for the diagnostic RFLPs. A precedent exists for multiple transformation events occurring within the nucleus of the same yeast cell bombarded with a mixture of two different plasmids, although most recipient cells are penetrated by only a single particle (ARMALEO *et al.* 1990). Therefore the chloroplast rDNA transformants in this study presumably represent the product of two or three distinct kinds of recombination events followed by segregation of the transformed genomes to yield homoplasmic single cell isolates.

Preferential localization of exchange events during integration of donor DNA: Exchange events leading to rRNA gene transformation are not randomly distributed along the length of the donor and recipient sequences. This conclusion stems from the following observations. (I) Recipient forms of all the RFLPs flanking the rDNA region were observed in only one (Figure 4, class A) out of 40 transformants examined from P-183/CC-1852 and P-229/CC-130. Exchange events that generate restriction fragments of intermediate length between the donor and recipient appeared to occur within ~500 bp of the 5′ end of the 23S rRNA gene in the 1.4/1.1-kb *Kpn*I-*Hin*dIII RFLP (Figures 4, 5) or ~500 bp from the 5′ end of the 16S gene (see below). (II) Eighteen out of 20 CC-130/P-229 transformants were phenotypically kr^+ *spr* sr^+, and, as a minimum, must represent replacement of virtually all of the resident 16S gene by the corresponding sequence in the P-229 plasmid (Figure 4). (III) The frequency of the *spr sr* transformants that carried the unselected *er* marker (7/34) from CC-1852/P-183 (Table 2) was high considering that the *er* locus is only *ca.* 200 bp from the end of the 7.0-kb insert in P-183. Although this distance represents only 5% of the length from the *Bam*HI site at the end of the plasmid to the selectable *spr* marker, over 20% of the exchange events are observed in this interval. Collectively these data suggest that the exchange events resulting in rRNA gene transformation do not occur randomly over the regions of homology between the donor insert and the recipient chromosome.

Since the positions of the length differences responsible for each RFLP are reasonably well known, the observed recombination frequency between RFLPs can be determined for the transformants and compared to the expected frequency of recombination (Figure 6). For each interval between scorable physical or genetic markers, a predicted recombination frequency has been calculated based on the assumption that one exchange event occurs (100% probability) between the selectable marker and each end of the donor insert and that the recombination frequency per unit length is constant. Substantial bias is observed for the exchange events to occur between the restriction sites at the plasmid:insert junction and the first available marker which is either the unselected *er* mutation in the P-183/CC-1852 transformation or the polymorphism in the *Kpn*I-*Hin*dIII fragment spanning the 5′ end of the 23S rRNA gene for the P-229/CC-130 experiments. A similar bias is also ob-

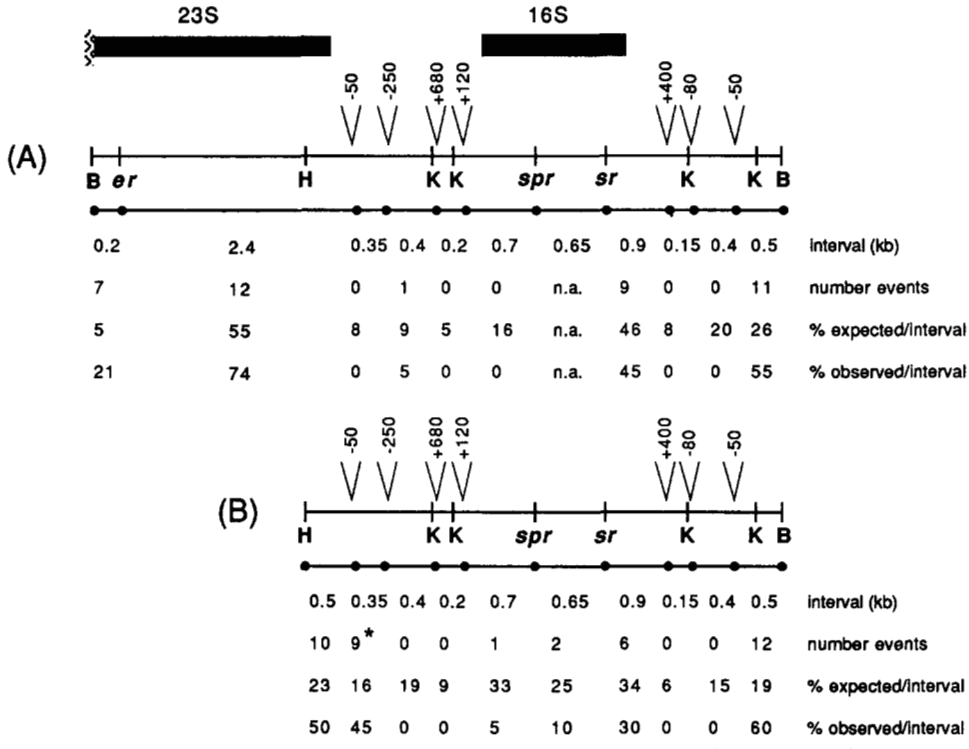


FIGURE 6.—Comparison of the expected and observed frequencies of exchange events for (A) P-183/CC-1852 and (B) P-229/CC-130 transformants. Relative positions of restriction sites, antibiotic resistance loci and size of heterologies responsible for the RFLPs are based on fine structure mapping and sequence analysis. The unique 0.5-kb *Kpn*I fragment of *C. smithii* is included as part of the 680-bp polymorphism in the 16S–23S rRNA gene interval. Abbreviations are as in Figure 1 legend. The expected percent recombination per interval is calculated separately for the two halves of the map, from the *Bam*HI sites to *sr* or *spr* for (A) and from *spr* to the *Bam*HI or *Hin*dIII sites for (B), as being directly proportional to the physical distance between pairs of genetic and physical markers. Events marked by the * occur within the polymorphism, generating a RFLP of intermediate (*ca.* 1.15 kb) length (*e.g.*, Figure 3, bottom panel). Number and percent of events occurring between the *Bam*HI site and *er* are derived from phenotypic analysis of 34 *spr sr* P-183/CC-1852 transformants, although the RFLP patterns of only 20 isolates (6 *er spr sr* and 14 er^+ *spr sr*) were determined. Since *sr* and *spr* alleles were used as the co-selectable markers in P-183/CC-1852 transformation experiments, recombination frequencies in that interval were not applicable (n.a.).

served for integration events near the 5′ end of the 16S rRNA gene. In both cases this is seen when the percent of exchange events expected in a given interval is compared to the percent of all exchange events that actually occur in that interval (Figure 6, A and B). Based on an equal probability of exchange per unit length, a strong preference for exchange at both ends of the inserts from P-183 and P-229 is observed compared to internal intervals nearest the selectable markers. A small increase in the expected *vs.* observed frequency of exchange is also detected in the 2.4-kb interval from the *er* locus to the polymorphism immediately 5′ to the 23S rRNA gene (Figure 6A). The increases in the observed *vs.* expected values for exchange events at each end of the donor fragments range from 2.1- to 4.2-fold.

Effect of heterology on the location of exchange events: Heterology can have several distinct effects on the location of exchange events. Such events probably cannot occur beyond the end of the donor insert because of the lack of homology between vector and recipient DNA sequences (LIEB, TSAI and DEONIER 1984). Heteroduplexes would not extend into these dissimilar DNA sequences and therefore branch migration would not proceed into the vector. Presumably resolution of any heteroduplex between donor and recipient sequences would occur in a region of homology near the insert:vector junction. This hypothesis would explain why a *Kpn*I-*Hin*dIII RFLP of intermediate size is observed in rDNA transformants of CC-130 generated with P-229 (Figure 4), but not with P-183/CC-1852 (Figure 5). In the case of the P-183/CC-1852 transformants (Figure 6A), no recombination between the −50 and −250 RFLPs in the 1.4-kb *Kpn*I-*Hin*dIII fragment is seen compared to the 8% expected, whereas in the P-229/CC-130 transformants (Figure 6B) recombination in this same interval is now more than two-fold higher than expected. The −50 and −250 RFLPs in the *Kpn*I-*Hin*dIII fragment (Figures 1, 6) may be close enough to the *Hin*dIII end of the P-229 insert (but too far from the *Bam*HI end in the 23S rRNA gene of the P-183 insert) to be involved in the resolution step of the exchange event leading to integration. This difference in the frequency of exchange events for the same 0.35-kb region in the *Kpn*I-*Hin*dIII fragment clearly

shows that position rather than sequence is responsible for the variation. Other polymorphisms (0.1–0.5-kb additions and deletions of SDR elements) distributed throughout the rDNA region of the chloroplast genome do not appear to stimulate the active exchange process that is observed near the insert:vector junctions (Figure 6).

The tendency for exchange events to occur repeatedly within a specific region may also be related to the distribution of heterologous and homologous sequences. In those transformants with an intermediate size *Kpn*I-*Hin*dIII RFLP, this fragment is always *ca.* 1.15 kb. If the polymorphism that generates the 300-bp length variation in the 1.4/1.1-kb *Kpn*I-*Hin*dIII fragment is the sum of separate 50-bp and 250-bp polymorphisms, then an exchange event in the homologous sequence between those two polymorphisms could generate a single intermediate size class of 1.15-kb *Kpn*I-*Hin*dIII fragments containing the 50-bp polymorphism (Figure 6).

Tracts of homology less than some minimum length may not promote efficient heteroduplex formation needed for recombination. This would be manifested as apparent linkage between adjacent RFLPs, when, in fact, heteroduplex formation necessary for exchange events could not be initiated. The minimum length of homologous DNA required for efficient recombination varies from 23 to 90 bp in prokaryotes (SHEN and HUANG 1986) to between 200 and 400 bp in eukaryotic nuclear DNA (BOLLAG, WALDMAN and LISKAY 1989), although recombination between homologous sequences as short as 20 bp has been observed in several systems (WATT *et al.* 1985). In addition, single base pair changes which disrupt stretches of perfect homology can affect initiation of recombination (WALDMAN and LISKAY 1988) although propagation of heteroduplexes through sequences of greater heterogeneity readily occurs (BOLLAG, WALDMAN and LISKAY 1989). Therefore in localized regions flanking the chloroplast rRNA genes of Chlamydomonas, homologous sequences may not be long enough nor exhibit sufficient homology to permit the formation of heteroduplexes. At present we are unsure of the extent and distribution of sequence homology required for heteroduplex formation initiating chloroplast DNA exchange events in Chlamydomonas. Clearly heteroduplex formation occurs frequently in the 200-bp interval between the *er* marker and the *Bam*HI site marking the vector:insert junction of P-183 to generate *er spr sr* transformants with CC-1852. However this short interval is adjacent to a long region of homologous 23S rRNA gene coding sequence which may enhance pairing.

Exchange near the vector:insert junctions may also be enhanced by recombinational suppression immediately adjacent to the *Kpn*I sites upstream and downstream of the 16S rRNA gene. No physical evidence for exchange was observed between the adjacent RFLPs upstream of the 16S rRNA gene in either the P-183/CC-1852 or P-229/CC-130 transformants or, with one exception, between the RFLPs separated by clusters of *Kpn*I sites in the 16S–23S intergenic spacer region of these transformants. These RFLPs cosegregate (*e.g.*, Figure 3, top and middle panels) and therefore appear to be tightly linked genetically. While the tight linkage of these RFLPs may be the effect of heterology (see above) and not the cause of preferential recombination at vector:insert junctions, there may also be a strong selection for a mechanism in *C. reinhardtii* that reduces exchange events between SDR elements in the chloroplast genome. This would minimize the number of deletion and inversion mutations arising from illegitimate pairing and crossing over between these regions of homology present in most intergenic regions (*cf.* BOYNTON *et al.* 1990b, PALMER *et al.* 1985). Such a mechanism would serve to increase the relative recombination frequencies in those regions of the chloroplast genome lacking SDR elements in sexual crosses and during integration of donor fragments that generate chloroplast transformants.

Integration events involving the 0.9-kb region at the 5′ end of the 16S rRNA gene located *ca.* 1.4 kb from the vector:insert junction occur at the expected frequency compared to the strong suppression of exchange events observed for all other internal regions in common between the donor fragment and the recipient genome (Figure 6). This is likely not the result of recombination suppression between tightly linked RFLPs promoting recombination in an adjacent region of homology, since the comparably sized 0.7-kb interval at the 3′ end of the 16S gene, which is also flanked by tightly linked RFLPs, shows strong suppression of exchange events. The normal level of exchange events occurring in the 0.9-kb interval upstream of the 16S gene in a region of recombination suppression could result from unique properties of the 400-bp region of heterology or could be related to transcription of the rRNA operon, which is initiated 72 bp 5′ to the 16S gene (SCHNEIDER *et al.* 1985). Transcriptionally induced exchange events are observed in the promoter region of the *S. cerevisiae* nuclear rDNA repeat unit (VOELKEL-MEIMAN, KEIL and ROEDER 1987). Since transformants containing the rDNA promoter region from the recipient strain do not appear to be favored over those having the donor promoter (Figures 4, 5), recombinants are not being selected to maintain a particular promoter-rRNA gene combination. Preferential localization of exchange events upstream of the 16S rRNA gene and in a particular region of the 16S–23S rRNA spacer is also seen in 60 recombinant meiotic progeny from crosses of strains carrying the *C. reinhardtii* and *C.*

smithii chloroplast genomes (S. M. NEWMAN, E. H. HARRIS, N. W. GILLHAM and J. E. BOYNTON, in preparation). In contrast, crosses involving *C. reinhardtii* strains carrying different chloroplast antibiotic resistance markers in the 16S and 23S rRNA genes, show remarkably uniform recombination over a 7-kb region containing the rRNA genes (HARRIS *et al.* 1989), and there seems to be no recombinational suppression between markers in the 16S and 23S rRNA genes.

In conclusion, recombination events leading to homologous replacement of recipient sequences by cloned donor fragments during chloroplast transformation opens the possibility of manipulating specific chloroplast sequences as a means of beginning to understand the mechanisms of chloroplast DNA recombination. RFLP differences between donor and recipient have been shown to be useful for defining the sites of exchange surrounding chloroplast genes introduced by transformation. The extent and distribution of heterologous and homologous regions between the recipient chloroplast genome and donor insert clearly influence the frequency, and probably the distribution, of exchange events between chloroplast genomes.

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