CHLOROPLAST GENETICS OF CHLAMYDOMONAS. I. ALLELIC SEGREGATION RATIOS

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Manuscript received October 20, 1975
Revised copy received January 27, 1976

ABSTRACT

This paper presents allelic segregation data from a series of 16 crosses segregated for nuclear and chloroplast genes. By means of pedigree analysis, segregants of chloroplast markers occurring in the zygote have been distinguished from those occurring in zoospore clones. The genes *ac1*, *ac2*, and *tm1* showed little if any deviation from 1:1 either in zygotic segregation or in zoospore clones. The genes *sm2*, *ery*, and *spc* showed a significant excess of the allele from the *mt*+ parent in zygotes. However, in zoospores, *mt*+ excess was seen only when that allele was the mutant (resistant) form but not when it was wild type (sensitive).

These results show that the extent of preferential segregation differs in zygotes and in zoospores, and that preferential segregation is influenced by map location and by allele specificity. A comparison of progeny from zygotes mated after 0, 15″, 30″, and 50″ UV irradiation of the *mt*+ gametes demonstrated the lack of an effect of UV upon allelic segregation ratios. In total, these results exclude the multi-copy model of chloroplast genome segregation suggested by GILLHAM, BOYNTON and LEE (1974) and support the diploid model we have previously proposed (SAGER and RAMANIS 1968, 1970; SAGER 1972).

THE analysis of chloroplast genetics in Chlamydomonas began with the discovery of the first gene pair showing non-Mendelian segregation in this organism: a mutant gene conferring resistance to streptomycin, and its wild-type allele, conferring sensitivity (SAGER 1954). Subsequently, many other genes were found showing the same pattern of transmission in crosses (summarized in SAGER 1972); and the first linkage map including seven of these gene pairs was reported (SAGER and RAMANIS 1970). On the basis of extensive genetic studies, (SAGER 1960, 1962; SAGER and RAMANIS 1963, 1965, 1967, 1968, 1970; and SAGER and RAMANIS, unpublished) a genetically circular and diploid model of the chloroplast genome was proposed (SAGER 1972) in which segregation and recombination resulted from exchanges at a four-strand stage similar to that seen in nuclear mitotic crossing over (PONTECORVO 1958).

This paper is the first in a series that presents the detailed genetic evidence upon which the circular diploid model of the Chlamydomonas chloroplast genome

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is based. As in Mendelian genetics, the ratios of parental alleles recovered in the progeny of crosses provide fundamental information necessary for the interpretation of segregational and recombinational data. Consequently, this paper will be concerned with the allelic segregation ratios observed in a series of crosses. The same crosses will be further analyzed in subsequent papers in which a number of special methods that we have developed will be utilized to establish gene order and intergenic distances, and to support the circular diploid model.

Genes located on chloroplast DNA have been distinguished from nuclear genes by their non-Mendelian pattern of inheritance in crosses (SAGER 1954, 1972; GILLHAM 1969, 1974). This pattern is predominantly maternal, in that the alleles coming from the *mt*+ parent, designated female, are transmitted to all progeny in most zygotes, whereas the corresponding alleles from the *mt*⁻ (male) parent are lost in the zygote and never reappear. Exceptions to the usual maternal inheritance are of two sorts: biparental zygotes in which the chloroplast genomes of both parents are transmitted, and paternal zygotes, in which the maternal complement is lost. Thus, the pattern of transmission is zygote-specific; and in a single cross, all three types of transmission may occur.

The relative frequencies of the three types can be greatly influenced by UV irradiation of the *mt*+ parent before mating (SAGER and RAMANIS 1967), by pre-treatments of *mt*+ and *mt*⁻ gametes with various growth inhibitors, (SAGER and RAMANIS 1973), by the nuclear genes *mat-1* and *mat-2* linked to the mating-type locus (SAGER and RAMANIS 1974), and by genetic factors that influence the spontaneous frequency of exceptional zygotes (SAGER and RAMANIS 1976).

Genetic analysis of cytoplasmic genomes requires the prior sorting out of zygote classes, since only the biparental zygotes are useful for examining segregational and recombinational events. In our strains, the spontaneous frequency of biparental zygotes is only about 0.1%. Selection, required to recover this class of zygotes, leads to distortion of allelic ratios and difficulties in interpretation of recombination data. The UV method (SAGER and RAMANIS 1967) in which biparental zygotes are recovered without selection, carries the potential disadvantage that the UV irradiation may itself influence the outcome. In this paper, a graded series of UV doses are examined and shown to have a negligible effect upon allelic segregation ratios.

Another important feature of the genetic behavior of the chloroplast genome is the occurrence of segregational and recombinational events not only in the zygote but also, and more extensively, in the mitotic growth of zoospore clones after meiosis and germination. We found it necessary to distinguish zygotic from post-zygotic events, because we found that the patterns of segregation and recombination differ in these two stages of the life cycle. Our method of pedigree analysis (SAGER and RAMANIS 1970; SAGER 1972) was originally developed to distinguish between reciprocal and nonreciprocal exchanges, a distinction which provides powerful data for genetic analysis. In addition, pedigree analysis provides a means to distinguish events occurring in the zygote from those occurring post-zygotically.

MATERIALS AND METHODS

Our stocks are descendants of a single zygote isolated by G. M. Smith and designated C137. Two strains involved in this work come from the Gillham-Boynton (G-B) laboratory, one carrying a mutation conferring resistance to neamine (*nea*-r) and the other a mutation to spectinomycin resistance (*spc* 1-27-3, designated *spc2* in this paper). To our knowledge the G-B stocks also came from Smith's C137 mating pair but have been maintained separately from ours for at least twenty years.

Media and methods are as previously described (SAGER and RAMANIS 1965, 1970). Antibiotics were used to assay progeny by direct or replica plating on agar at the following concentrations: streptomycin; 100 μg/ml for *sm2*, *sm4*, and *sm5*, 50 μg/ml for *sm3*; erythromycin, spectinomycin, spiramycin, carbomycin, cleocin, and oleandomycin, each 50 μg/ml; neamine, 150 μg/ml. In crosses involving acetate-requiring strains, all steps of the procedure were carried out with acetate-containing media.

Heterozygotes segregating for drug-resistance and sensitivity were identified by the presence of mixtures of dead and living cells detected by microscopic examination of colonies replicated on drug-containing media. Heterozygotes segregating for temperature-resistance and sensitivity were similarly identified by mixtures of dead and living cells in replicated colonies incubated at 37°C. Heterozygotes for acetate requirement were identified by direct plating on minimal medium on which mixtures of cells carrying *ac*+ and either *ac1* or *ac2* are easily distinguished from pure clones. This procedure has been tested by restreaking and is reliable because hets segregate close to 1:1.

Crossing procedure: Parental strains, started from single colonies, are grown on a nitrogen-deficient minimal agar medium in continuous light until they approach stationary growth. Cells are washed off into a five-fold diluted nitrogen-free minimal medium (SAGER and GRANICK 1954) at pH 8 and left overnight at room temperature. When the parental strains are mixed the next day, zygote formation is fast (about one hour to completion) and the yield is usually over 90%. Zygotes are plated at dilution onto minimal medium plus 0.1% acetate-containing agar, incubated overnight in the light, and then kept in the dark 5–8 days. When mt+ cells are UV-irradiated before mating, the mating mixture is kept in the dark for two hours, then plated and incubated overnight in the light, and stored in the dark as above. Zygotes germinate synchronously following incubation for about 18 hours in continuous light. Vegetative cells contaminating zygote plates are killed by 30-second exposure to chloroform vapor, usually the day before zygote germination.

Preparation of pedigrees: Germinating zygotes are transferred individually to fresh minimal agar at the four-cell (tetrad) stage, and at the eight-cell stage, cells are spread over the petri dish. After one further doubling, each pair is locally respread, so that after growth, 16 colonies are expected from each zygote. Occasional losses are more than offset by the ease of the method, which permits routine preparation of about 40–50 zygotes per hour.

The four products of meiosis, the zoospores, are distinguished from each other by the segregation of three pairs of unlinked nuclear genes: *act* (actidione resistance), *ms* (methionine sulfoximine resistance) and *mt* (mating type) which also identify the zoospore sisters or octospores. The pairs of octospore daughters are recognized by their position on the plates, following local respreading.

Zygote colony analysis: In certain crosses, the results of pedigree analysis were compared with the results of sampling progeny from large zygote colonies, a method used by GILLHAM, BOYNTON and LEE (1974). A zygote suspension was plated at dilution to give about 100 zygote colonies per plate, plates were incubated in the dark as above for pedigree analysis, contaminating vegetative clones were killed by chloroform vapor as above, and zygotes were germinated by transfer of plates to light, where large colonies containing about 10^6 cells formed. Plates were replica-plated to identify exceptional zygotes (biparental plus paternal) by the presence of drug-resistance coming from the *mt* parent. Individual colonies from exceptional zygotes on unselected master plates were transferred to liquid, dispersed to form a suspension of single cells, and then diluted and plated to give about 100 colonies per plate on a complete medium. Colonies were then replica-plated to classify them for all markers segregating in the cross.

UV irradiation: The average frequency of exceptional zygotes from non-irradiated parents in all the crosses listed in Table 2 was 0.46%. For irradiation of the *mt+* parent, a standard dose of 50″ was used in all crosses except Cross 20, in which a series of doses: 15″, 30″ and 50″ were given to aliquot suspensions of the *mt+* parent. The UV dose was calibrated biologically in terms of the yield of exceptional zygotes, which averaged 47% after 50″ irradiation, for the 16 crosses of Table 2.

RESULTS

Origin of mutant genes

All of the mutants used in these studies and listed in Table 1, except *nea* and *spc2*, were recovered after streptomycin mutagenesis (SAGER 1962). The mutant genes: *ac1*, *ac2*, *ac3*, *ac4*, *csd* and *tm1* were isolated from a *mt+* streptomycin strain previously described (SAGER 1962). The other mutant genes were recovered using a modified method, in which the streptomycin-sensitive strain 21gr (wild type, *mt+*) was mutagenized by growth on a complete medium (minimal plus 0.1% acetate, 0.1% yeast extract, and 0.1% casamino acids) with 20 μg/ml streptomycin sulfate (Merck). After incubation for 2–3 weeks in the dark, cells were washed off and replated at suitable dilutions on a series of plates each con-

TABLE 1

Origin and phenotype of chloroplast mutations

Gene	Origin	Mutagen	Phenotype
ac1	5065B	SM	small, flat, light green colonies on minimal agar
ac2	5065B	SM	tiny, high, dark green colonies on minimal agar
ac3	5065B	SM	same as *ac1*
ac4	5065B	SM	same as *ac2*
sm2	21gr	SM	resistant to 500 μg/ml SM on agar resistant to 250 μg/ml SM in liquid
sm3	*sm4* × *sm2* cross	(prob. SM)	resistant to 50 μg/ml SM on agar resistant to 10 μg/ml SM in liquid
sm4	21gr	SM	SM-dependent grows on 50–500 μg/ml on agar; SM-dependent grows on 50–500 μg/ml in liquid
sm5	21gr	SM	same as *sm2*
ery	21gr	SM	resistant to 100 μg/ml on agar
csd	5056B	SM	requires SM for growth at 37° but not at 25°C
car	21gr	SM	resistant to 100 μg/ml carbomycin on agar
cle	21gr	SM	resistant to 100 μg/ml cleocin on agar
spi	21gr	SM	resistant to 100 μg/ml spiromycin on agar
ole	21gr	SM	resistant to 100 μg/ml oleandomycin on agar
spc	21gr	SM	resistant to 100 μg/ml spectinomycin on agar
tml	5065B	SM	grows at 25° but not at 37°C
mr1	21gr	SM	resistant to 50 μg/ml spiromycin, carbomycin, and oleandomycin on agar
mr2	21gr	SM	resistant to 50 μg/ml spiromycin, carbomycin, oleandomycin and erythromycin on agar
nea	GILLHAM	MNNG	resistant to 150 μg/ml neamine on agar
spc2	GILLHAM	MNNG	slow growth and small colony formation on 50 μg/ml spectinomycin

taining a selective drug. The mutations *nea* and *spc2* were recovered after nitrosoguanidine mutagenesis (GILLHAM 1969).

Three spectinomycin-resistant strains have been described, the *spc* mutant from our laboratory, *spr* 1-27-3 (BOYNTON *et al.* 1973) and *spr* 1-6-2 GILLHAM 1969). The *spc* mutant studied by BURTON (1972) is now reported to be identical to *spr* 1–273 (GILLHAM 1974). The physiological and biochemical studies described in BOYNTON *et al.* (1973) were carried out with *spr* 1-27-3, whereas the genetic analyses (GILLHAM, BOYNTON and LEE 1974) were done with *spr* 1–6–2. The mutant *spr* 1–6–2 appears to be identical with our *spc* mutant, giving no *spc*-sensitive recombinants in crosses SAGER and RAMANIS, unpublished) and showing a similar phenotype: high level resistance in liquid culture and on agar, resistance at the 70S ribosome level in poly U directed *in vitro* polypeptide synthesis. The *spr* 1-27-3 mutant, on the contrary, is phenotypically distinguishable on agar, by its low level resistance to the drug, from our *spc* mutant and genetically different as shown by recovery of *spc*-sensitive recombinants (SAGER and RAMANIS, unpublished). CONDE *et al.* (1975) report that *spr* 1-27-3 does not recombine with *spr* 1-6-2; this discrepancy in results of the two laboratories has not yet been resolved.

Properties of mutant strains (see Table 1)

The mutations in genes: *ac1*, *ac2*, *sm2*, *sm3*, *sm4*, *nea*, *ery*, and *csd* were described in previous papers (SAGER and RAMANIS 1963, 1965, 1970). As previously noted, the *ac1* and *ac2* mutant alleles confer reduced ability to grow photosynthetically. Mutants carrying *ac1* form small, flat, light green colonies, *ac2* mutants form tiny normal green colonies on minimal agar and the two mutants can be easily distinguished; but grow normally to form large, dark green colonies on acetate-agar. Strains carrying the *ac3* mutation resemble *ac1*, and *ac4* resembles *ac2*.

The mutations to drug resistance: *car*, *cle*, and *spc*, were described in previous biochemical studies (SCHLANGER, SAGER and RAMANIS 1972; SCHLANGER and SAGER 1974; BLAMIRE, FLECHTNER and SAGER 1974). The additional mutations introduced in this paper are: resistance to oleandomycin (*ole*); to spiromycin (*spi*); temperature sensitivity (*tm1*); and two mutations conferring multiple drug resistance: *mr1*, resistant to spiromycin, carbomycin and oleandomycin; and *mr2*, resistant to the same three plus erythromycin. All other drug-resistant strains listed show no interfering cross-resistance.

Construction of stocks for crosses to this study

All stocks used in this work are descendants of a single pair of mating strains, 21gr and 4Y, which are clonal isolates of the strains received from G. M. Smith. Smith's strains came initially from a single zygote isolated from nature, so we may assume our starting strains were minimally F_1's from the same zygote, and probably progeny of additional rounds of intercrossing by Smith. The wild-type strains have shown a high efficiency of mating and no lethality of zygotes or zoo-

spores at any time. All mutants have been selected either in *mt*+ strain 21gr or in an *mt*+ F_2 from 21gr × 4Y (5065B).

The crosses to be considered in this and subsequent papers of this series are listed in Table 2. Stock construction has followed the principle of choosing progeny on a single line of descent as parental strains, and of back-crossing mutants to the same *mt* tester stock whenever possible. With the exception of newly induced or spontaneous mutations, we consider our stocks to be relatively isogenic, most of them being the products of at least six generations of intercrossing of progeny from 21gr and 4Y.

TABLE 2

*Crosses used in these studies**

Cross	P_1 (*mt*+)		P_2 (*mt*—)	Number of zygotes	Number of zoospores
1	*ac1 ac2*+ *sm2-s sm3-s csd-s* 6978a	×	*ac1*+ *ac2 sm2-r sm3-r csd-r* 7018g	159	571
2	*ac2*+ *sm2-s ery-r* 11,108–5	×	*ac2 sm2-r ery-s* 7018g	62	219
3	*ac1 ac2*+ *sm4-d sm2-s ery-s* 229–3–4	×	*ac1*+ *ac2 sm4-s sm2-r ery-r* 11,209–5	42	140
4‡	*ac1 sm4-d nea-s sm2-s* 229–3–4	×	*ac1*+ *sm4-s nea-r sm2-r* 12–6–2 [from S × (G × S)]	42	145
5‡	*ac2*+ *sm2-s ery-s nea-r* 12–4 [from S × (G × S)]	×	*ac2 sm2-r ery-r nea-s* 11,154–4	53	186
6	*ac1*+ *ac2 sm2-s ery-s spc-r tm-r* 11,554–4	×	*ac1 ac2*+ *sm2-r ery-r spc-s tm-s* 11,358–3	47	170
7	*ac1 ac2*+ *sm2-s ery-s spc-r car-s tm-r* 11,932–4	×	*ac1*+ *ac2 sm2-r ery-r spc-s car-r tm-s* 11,927–1	7	35
8	*ac1 ac2*+ *sm2-s ery-s cle-s spc-r tm-r* 11,932–4	×	*ac1*+ *ac2 sm2-r ery-r cle-r spc-s tm-s* 11,907–3	16	63
9	*ac1*+ *ac2 sm2-r ery-r ole-r spc-s tm-s* 11,968–6	×	*ac1 ac2*+ *sm2-s ery-s ole-s spc-r tm-r* 11,909–3	12	43
10	*ac1*+ *ac2 sm2-r ery-r spi-r spc-s tm-s* 11,978–3	×	*ac1 ac2*+ *sm2-s ery-s spi-s spc-r tm-r* 11,909–3	15	57
11	*ac1 ac2*+ *sm2-r ery-r car-r spc-s tm-s* 11,925–5	×	*ac1*+ *ac2 sm2-s ery-s car-s spc-r tm-r* 11,927–2	41	163
12	*ac1*+ *ac2 sm2-r ery-s spc-s tm-s* 13,363–4	×	*ac1 ac2*+ *sm2-s ery-r spc-r tm-r* 13,363–7	63	242
13	*ac2*+ *sm2-s spc-s mr1-r tm-r* 12,632–6	×	*ac2 sm2-r spc-r mr1-s tm-s* 13,181–6	32	125
14	*ac2*+ *sm2-s spc-s mr2-r tm-r mt*+ 12,674–1	×	*ac2 sm2-r spc-r mr2-s tm-s mt-* 13,181–6	55	211
15†	*ac2*+ *ac3 sm2-s ery-s spc-s tm-r* 7788–12	×	*ac2 ac3*+ *sm2-r ery-r spc-r tm-s* 13,181–6	—	—
16†	*ac4 sm2-r ery-s spc-s tm-s* 7860–19	×	*ac4*+ *sm2-s ery-r spc-r tm-r* 11,932–4	—	—
17†	*ac1 sm2-r ery-r kan-s tm-s* 11,925–5	×	*ac1*+ *sm2-s ery-s kan-r tm-r* 11,482–4	—	—

TABLE 2—Continued

Cross	(P_1 (*mt*+)		P_2 (*mt*—)		Number of zygotes	Number of zoospores
18‡	*ac2*+ *sm2-s spc1-s spc2-r tm-r spc-r* 1–27–3 (G)	×	*ac2 sm2-r spc1-r spc2-s tm-s* 13,181–6		20	71
20	*ac1 ac2*+ *sm2-s ery-r spc-r mr1-s tm-r* 14,678–1	×	*ac1*+ *ac2 sm2-r ery-s spc-s mr1-r tm-s* 14,689–1	15″ UV	22	88
				30″ UV	35	139
				50″ UV	31	123
21	*ac2*+ *spc2-r ery-r sm2-s mr1-s tm-r* 14,704–1 (F_1 from cross 18)	×	*ac2 spc2-s ery-s sm2-r mr1-r tm-s* 14,689–1		24	87
22‡	*ac1 sm2-r spc-r ery-r nea-s tm-s* 11,925–1	×	*ac1*+ *sm2-s spc-s ery-s nea-r tm-r* 13,971–4 (F_1 from G × S)		54	214
23‡	*ac2 sm2-r spc-r ery-r mr1-r nea-s tm-s* 14,761–2	×	*ac2*+ *sm2-s spc-s ery-s mr1-s nea-r tm-r* 13,971–4 (F_1 from G × S)		27	101
24	*ac2*+ *sm5-r ery-s spc-s tm-s* sr 643	×	*ac2 sm5-s ery-r spc-r tm-r* 14,681–1		19	72
25	*ac2 sm3-r ery-s spc-s tm-s* 11,054–1 (F_1 from G × S)	×	*ac2*+ *sm3-s ery-r spc-r tm-r* 14,679–1		17	65

* Crosses 1–5 were previously described (SAGER and RAMANIS 1970).
† Liquid culture sampling only. See SINGER, SAGER and RAMANIS 1976.
‡ Crosses include *spc2-r* and *nea-r* markers from GILLHAM. (SAGER and RAMANIS, in prep.)
(M) From a maternal zygote. All other stocks are progeny from biparental zygotes.
G: stock from GILLHAM.
S: stock from SAGER.

Pedigree analysis

The method we have developed to organize raw pedigree data into a workable form will be presented here to aid the reader in visualizing how the data in subsequent tables were compiled. Let us consider a zygote from Cross 6. When all progeny have been classified for all segregating markers, the results from the original notebooks are transcribed into the form shown in Table 3. This form permits the rapid enumeration of segregations occurring either in the zygote or at the first or second post-meiotic mitotic doubling. Zygotic events are recognized by the presence of parental alleles at the level of the zoospore. If the zoospore is still heterozygous for all or some of the markers in the cross, then the opportunity exists for segregations to occur at subsequent doublings.

The allelic segregation patterns exhibited by chloroplast gene pairs in zoospore clones have previously been described (SAGER and RAMANIS 1968; SAGER 1972). Three patterns of allelic distribution occur, as shown in Figure 1. In Type I, both daughter cells are still heterozygous (*het*) for all three markers, whereas in Type II, one is a *het* for *sm* and the other is homozygous and in Type II both progeny are homozygous for *sm*, each carrying one of the parental alleles. We have interpreted the Type III pattern to be the result of a reciprocal recombinational event, and the Type II pattern as the result of a gene conversion event (SAGER 1972). It is important to stress here, although the detailed evidence will be presented in later papers, that different gene-pairs can and often do undergo both Type II

TABLE 3

A typical zygote pedigree

	Cross 11				Zygote no. 12,408															
	Clone 1				Clone 2				Clone 3				Clone 4				Zoospores*			
Genes	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	A	B	C	D
ac1	0	3	0	3	0	0	1	2	0	0	0	0	0	0	1	1	0	0	0	0
ac2	0	3	0	3	0	0	1	2	0	0	0	0	0	0	1	1	0	0	0	0
sm2	0	3	2	3	0	1	1	0	0	2	0	0	2	0	0	0	0	0	0	0
ery	0	3	2	3	1	1	1	1	1	2	2	0	2	1	0	0	0	1	0	0
car	0	3	2	3	1	1	1	1	1	2	0	1	0	0	0	0	0	1	0	0
spc	2	3	2	3	2	0	0	1	2	2	2	1	0	0	2	0	2	0	0	0
tm1	2	3	2	3	0	0	0	0	0	0	0	1	0	0	2	0	2	0	0	0

Code
0 = *het*
1 = P_1 parental allele
2 = P_2 parental allele
3 = missing

* A, B, C, D are the genotypes of the four zoospores inferred from the clonal data.

	Segregation pattern			
Zygotic	*spc, tm1*	*ery, car*		
Type II				
1st d.	*sm2, ery, car*		*spc*	*ac1, ac2*
2nd d.		*sm2, sm2, spc, spc*	*sm2, ery, car, tm1*	*sm2, spc, tm1*
Type III				
1st d.				
2nd d.		*ac1, ac2*	*ery, car, spc*	*ery*

and Type III events in the same cell at the doubling, in addition to Types I and II or I and III. According to the interpretation in Figure 1, only Type II events can contribute to deviations from 1 : 1 segregation. If Type II events are the result of gene conversion, the deviations from 1:1 might be anticipated if particular mutant alleles had a different probability of conversion than did the corresponding wild-type (FOGEL and MORTIMER 1971).

In addition to providing for an unequivocal distinction betwen zygotic and post-zygotic events, pedigree analysis makes possible the distinction between reciprocal and nonreciprocal segregational events. For example, in clone 1, at the first mitotic doubling, the genes *sm2*, *ery*, and *car* showed a Type II segregation pattern, and in clone 2, the gene *sm2* showed Type II segregations in each pair of progeny at the second doubling, and so did *spc*. In clone 3, *ery* and *car* showed Type II segregations at the second doubling of one pair of daughter cells, and Type II events at the second doubling in the other pair. However, the Type II events occurred on different strands and involved different parental alleles.

In this paper, pedigree data are being used to count up the frequencies and allelic ratios of segregations of individual genes seen in the table as 1's and 2's. The further analysis of these data will be deferred to later papers of this series.

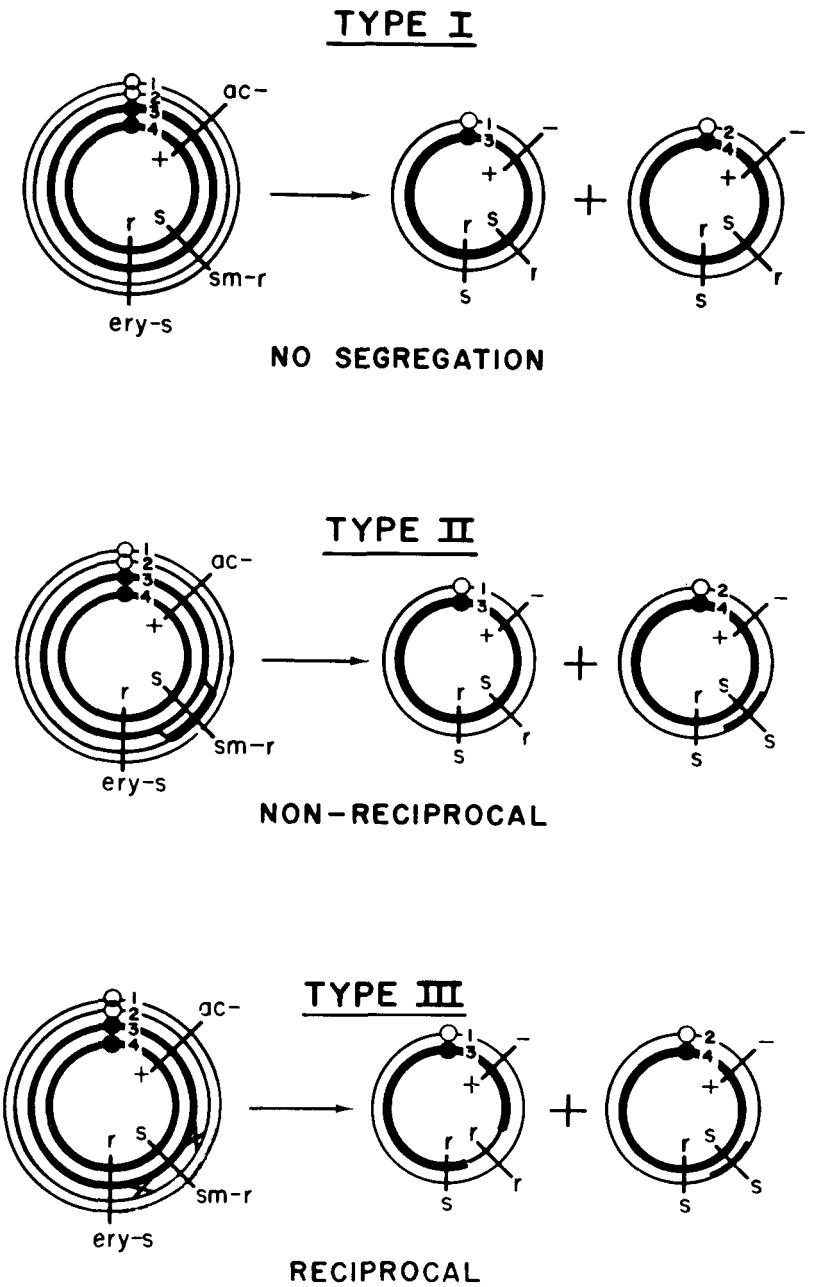

FIGURE 1.—The chloroplast genome is shown on the left in circular form (SINGER, SAGER and RAMANIS 1976) at a four-strand stage after replication and before cell division; and at the right after cell division. Each line represents a double-stranded DNA. Type I, the commonest pattern, resembles mitotic segregation of nuclear chromosomes. All three markers shown remain heterozygous (het). Type II depicts a gene conversion-like event at the *sm* locus, produces one *het* daughter and one homozygous *sm-s*. Type III depicts a reciprocal event giving rise to one pure *sm-r* and one pure *sm-s* daughter.

Clone 1 contained only two daughters which are considered sister products of the first mitotic doubling. The second doubling does not always occur synchronously and we purposely time the respreading procedure to obtain fewer rather than excess progeny.

Segregational events occurring in zygotes

Table 4 itemizes the frequencies of segregation of individual genes occurring in many of the crosses listed in Table 2. The markers *ac1*, *ac2*, *sm2*, *ery*, *spc*, and *tm1* provide the backbone of the genetic evidence, both because of their presence in most of the crosses, and because they are well distributed in different sectors of the genetic map (SAGER and RAMANIS 1976; SINGER, SAGER and RAMANIS 1976). In the tables presented in this paper, *ac1* and *ac2* are considered as one gene, because they are closely linked, and no significant differences in their

TABLE 4

Gene segregation frequencies in zygotes*

Cross	No. zoospores	*ac*	*sm2*	*ery*	*spc*	*tm1*	*nea*	Other
2	219	0.46	6.85	12.8	—	—	—	
3	140	4.3	9.3	13.6	—	—	—	
								sm4
4	145	3.5	2.1	—	—	—	7.0	2.1
5	186	0	10.7	12.5	—	—	19.4	
								car
6bc	170	0.12	10.6	21.2	14.7	7.1	—	0.12
7	35	0	—	25.7	14.3	10.0	—	
								cle
8	63	4.8	20.7	30.2	14.3	1.6	—	28.7
								ole
9	43	6.9	14.0	34.9	27.9	21.0	—	9.3
								spi
10	57	0	10.5	21.0	22.8	7.0	—	10.5
								car
11	163	2.5	11.9	14.4	36.2	11.3	—	9.4
12A	144	4.86	13.2	23.6	23.6	7.6	—	
B	98	9.2	16.3	23.5	24.5	10.2	—	
								mr1
13	125	8.8	20.8	—	28.0	12.0	—	24.8
								mr2
14	151	1.3	19.2	—	39.7	10.0	—	16.6
20 (Pedigrees)								*mr1*
15″	87	2.3	11.5	11.5	10.3	1.1	—	2.3
30″	139	6.5	16.5	15.8	17.3	10.8	—	15.1
50″	123	7.3	17.1	15.4	17.1	9.8	—	13.0
								sm5
24	72	2.8	—	36.1	37.5	15.3	—	16.7
								sm3
25	69	10.1	—	33.3	29.0	14.5	—	14.5
Total	2229	3.74†	12.6†	19.2†	24.6†	10.0†		

* Number segregated zoospores/total zoospores.
† Weighted mean frequencies (excluding cross 20–15″ sample).

segregation frequencies have been detected. Recombination frequencies between them will be considered in a later paper.

The principal point to be noted from Table 4 is the significantly different segregation frequencies exhibited by the five markers *ac*, *sm2*, *ery*, *spc*, and *tm1*. In the majority of instances, markers segregated individually and consequently most zoospores were heterozygous for most or all markers. As shown in Table 5, in those zoospores in which segregation occurred, the vast majority contained only one or two homozygous markers, not an entire haploid set. When two markers simultaneously segregated, they were usually adjacent (SAGER and RAMANIS 1976). The results of Tables 4 and 5 show that zygotic segregation involves limited segments of the map, rather than the genome as a whole. This finding is one point in refutation of the multi-copy model of GILLHAM, BOYNTON and LEE (1974) to be discussed below.

The allelic segregation ratios for the zygotic segregation events listed in Table 4 are presented in Table 6. As in Table 4, the results are different for each of the markers. The *ac* marker shows 1:1 segregation, as does *tm1*, whereas *sm2* and *ery* show approximately 2:1 segregation, and *spc* almost 3:1 with excess of the P_1 (i.e. *mt*+) parent. The significance of these results, and their comparison with segregation patterns in zoospores will be considered below. Here we merely point out that the noted variation from gene to gene, whatever its explanation, does not support the multi-copy model proposed by GILLHAM, BOYNTON and LEE (1974).

TABLE 5

Extent of gene segregation in zygotes

Cross	Frequency of homozygous genes per zoospore* None	One	Total (one or none)
2	58.1	23.7	81.8
5	51.6	27.7	79.3
6	63.5	13.5	77.0
7	54.3	18.9	73.2
8	55.6	6.4	62.0
9	34.9	18.6	53.5
10	66.7	10.5	77.2
11	50.9	21.5	72.4
12	61.6	14.9	76.5
13	52.8	16.0	68.8
14	34.6	19.0	53.6
20–15″	74.7	14.9	89.6
20–30″	51.8	24.5	76.3
20–50″	60.2	17.9	78.1
24	39.7	16.4	56.1
25	48.5	21.2	69.7
	av. 53.7	17.9	av. 71.6

* Computed as the number of zoospores with zero or one segregated genes per total zoospores analyzed.

TABLE 6

Allelic segregation ratios in zygotes

Cross	No. zoospores	*ac* P_1	*ac* P_2	*sm2* P_1	*sm2* P_2	*ery* P_1	*ery* P_2	*spc* P_1	*spc* P_2	*tm1* P_1	*tm1* P_2	Other P_1	Other P_2	
2	219	1	0	9	6	24	4	—		—				
3	140	3	3	7	6	9	10	—		—		3^{sm4}	3	
4	145	3	2	3	0	—		—		—		3^{sm4}	0	9^{nea} 1
5	186	0	0	16	4	17	6	—		—				34^{nea} 2
6	170	1	1	14	4	23	3	21	4	8	4			
7	35	0	0	—		7	2	3	2	3	0	1^{car}	1	
8	63	2	1	11	2	13	6	7	2	1	0	14^{cle}	4	
9	43	2	1	4	2	7	8	7	5	7	2	1^{ole}	3	
10	57	0	0	3	3	7	5	10	3	2	2	3^{spi}	3	
11	163	1	3	5	14	17	6	48	10	10	8	11^{car}	4	
12	242	10	5	22	13	33	24	35	23	10	11			
13	125	4	7	16	10	—		22	13	11	4	24^{mr1}	7	
14	151	2	0	25	4	—		50	10	10	5	23^{mr2}	2	
20–15″	87	2	0	8	2	7	3	8	1	1	0	1^{mr1}	1	
30″	139	4	5	16	7	16	7	19	5	8	8	11^{mr1}	10	
50″	123	5	6	12	9	9	11	12	9	3	9	3^{mr1}	12	
24	72	2	0	—		14	12	17	10	2	9	11^{sm5}	1	
25	69	2	5	—		14	6	20	3	6	4	5^{sm3}	5	
Total	2229	44	39	171	86	217	113	279	100	82	66			
	$\%P_1$	53.0		66.5		65.8		73.6		55.4				

Allelic ratios in zoospore clones

The allelic ratios of all gene-pairs scored in zoospore clones of crosses listed in Table 2 are shown in Table 7. The acetate alleles and *tm1* show the closest fit of all to a 1:1 ratio in both the first and second mitotic doublings, whereas *sm2*, *ery*, and *spc* show an excess of segregation by alleles in the P_1 parent, on the average. The deviations from 1:1 are much less extreme than in the zygotes (Table 6), being on the average about 1.5:1. In Table 8, the data of Table 7 have been rearranged in order to show the effects of specific alleles upon segregation frequency. Each of the mutant alleles conferring resistance to streptomycin, erythromycin, and spectinomycin respectively, exhibits a higher frequency of gene conversion when in the P_1 parent than does the corresponding wild-type allele. In the case of *tm1*, the mutant allele, which confers temperature sensitivity, shows less conversion than does the wild-type allele when present in the P_1 parent.

These results show that deviations from 1:1 allelic segregation ratios in zoospore clones are lower than in zygotes of the same crosses, and that a pronounced allele-specific bias is present in zoospore clones but not in zygotes. As will be discussed in a subsequent paper (SAGER and RAMANIS, in preparation) we postulate that restriction enzyme cleavages occurring in zygotes but not in zoospore clones are responsible for the differences in segregation patterns seen in the two

TABLE 7

Non-reciprocal segregation events in zoospore clones

Cross	*ac* 1st P_1	*ac* 1st P_2	*ac* 2nd P_1	*ac* 2nd P_2	*sm2* 1st P_1	*sm2* 1st P_2	*sm2* 2nd P_1	*sm2* 2nd P_2	*ery* 1st P_1	*ery* 1st P_2	*ery* 2nd P_1	*ery* 2nd P_2
2	29	31	15	30	36	20	30	18	36	9	37	12
3	19	9	17	12	19	15	27	9	9	34	9	18
4	24	12	19	22	17	12	17	13	—		—	
5	22	18	34	46	34	35	22	23	38	24	26	15
6	30	21	29	30	32	30	16	29	29	24	14	27
7	3	4	15	8	—		—		3	9	3	6
8	8	7	10	18	12	14	2	11	15	15	5	9
9	8	6	8	10	9	5	13	3	10	3	7	8
10	10	7	14	19	20	6	30	12	19	6	27	6
11	23	21	48	52	43	22	66	20	57	14	73	20
12	39	32	10	8	59	25	20	7	51	52	15	9
13	25	14	18	20	23	17	25	11	—		—	
14	50	24	23	19	40	16	26	20	—		—	
20–15″	15	13	12	12	18	15	11	15	25	18	10	10
30″	25	18	10	17	29	23	12	12	33	24	13	11
50″	21	20	18	12	26	21	25	10	32	20	20	15
24	19	16	5	10	—		—		15	9	3	5
25	11	14	10	6	—		—		14	11	6	3
TOTALS	381	287	315	351	417	276	342	213	386	272	268	174

Cross	*spc* 1st P_1	*spc* 1st P_2	*spc* 2nd P_1	*spc* 2nd P_2	*tr* 1st P_1	*tr* 1st P_2	*tr* 2nd P_1	*tr* 2nd P_2	Other 1st P_1	Other 1st P_2	Other 2nd P_1	Other 2nd P_2
2	—		—		—		—					
3	—		—		—		—					
4	—		—		—		—		19	16^{nea}	17	14
5	—		—		—		—		48	12^{nea}	34	5
6	50	16	41	5	36	22	28	21				
7	6	6	11	3	8	1	6	7	7	10^{car}	12	7
8	18	13	18	4	20	5	16	14	14	16^{cle}	5	6
9	6	8	6	9	7	8	7	7	14	4^{ole}	8	10
10	10	10	8	14	7	8	11	24	19	7^{spi}	21	11
11	23	31	21	35	23	39	27	58	54	10^{car}	69	25
12	48	49	8	10	48	47	14	14				
13	23	23	15	18	36	15	25	16	37	15^{mr1}	15	10
14	29	19	14	12	42	24	23	24	72	3^{mr2}	32	7
20–15″	25	14	12	8	15	29	13	13	17	23^{mr1}	10	10
30″	38	15	21	11	39	17	24	11	29	22^{mr1}	16	18
50″	34	17	22	21	33	30	23	15	32	11^{mr1}	22	24
24	12	15	3	7	17	17	3	7	18	8^{sm5}	11	3
25	14	13	5	5	15	13	10	4	16	17^{sm3}	4	7
TOTALS	336	249	205	162	346	275	230	235				

TABLE 8

Non-reciprocal segregation events in zoospore clones: a summary

	1st doubling			2nd doubling		
	P_1	P_2	% P_1	P_1	P_2	% P_1
ac	381	287	57.0	315	351	47.3
sm2	417	276	60.2	342	213	61.1
*sm2-r**	131	58	69.3	129	42	75.4
*sm2-s**	286	218	56.8	213	171	55.5
ery	386	272	58.7	268	174	60.6
*ery-r**	212	94	69.3	187	82	69.5
*ery-s**	174	178	49.4	81	92	46.8
spc	336	249	57.4	205	162	55.9
*spc-r**	171	81	67.9	125	52	70.6
*spc-s**	165	168	49.6	80	110	42.1
tm1	346	275	55.7	230	235	49.5
*tm-r**	229	143	61.6	158	121	56.6
*tm-s**	117	132	47.0	72	114	38.7

* Lists only crosses in which the *-r* or *-s* allele was carried by the P_1 (*mt*+) parent.

TABLE 9

*Effect of type III segregations on allelic transmission ratios in zoospore clones**

		1st doubling			2nd doubling		
Gene	Type	P_1	P_2	% P_1	P_1	P_2	% P_1
ac							
	II	381	287		315	351	
	III	59	59		142	142	
		440	346	56.0	457	493	48.11
sm2							
	II	417	276		342	213	
	III	126	126		181	181	
		543	402	57.46	523	394	57.04
ery							
	II	386	272		268	174	
	III	136	136		205	205	
		522	408	56.13	473	379	55.5
spc1							
	II	336	249		205	162	
	III	130	130		161	161	
		466	379	55.15	366	323	53.12
tm1							
	II	346	275		230	235	
	III	73	73		93	93	
		419	348	54.63	323	328	49.6

* Type II data are from Table 8.

TABLE 10

Effect of UV irradiation of mt+ *parent upon P_1:P_2 ratios of progeny sampled from large zygote colonies from Cross 20*

	P_1:P_2 ratios (expressed as % P_1)				
	No UV	15″ UV	30″ UV	50″ UV	Average
ac	43.6 ± 6.5	46.0 ± 4.8	47.1 ± 4.4	44.0 ± 4.2	45.2 ± 5.0
tm1	48.9 ± 8.0	50.4 ± 3.7	51.3 ± 5.7	52.4 ± 8.7	50.8 ± 6.5
sm2	49.5 ± 13.5	47.0 ± 8.6	52.5 ± 16.7	44.5 ± 20.2	48.4 ± 14.75
spc1	44.8 ± 12.2	55.7 ± 8.7	60.6 ± 9.9	50.6 ± 21.5	52.9 ± 13.1
mr1	48.8 ± 12.7	48.8 ± 6.0	50.8 ± 8.8	47.0 ± 14.2	48.9 ± 10.4
ery	38.9 ± 9.3	52.0 ± 7.1	48.1 ± 9.5	42.6 ± 13.8	45.4 ± 9.9

stages of the life cycle. The data in Tables 7 and 8 are presented to emphasize the deviations from 1:1 by listing only Type II segregations and omitting the reciprocal Type III segregations. If one examines clones in which this distinction is not possible, as in Table 10 and in the data of GILLHAM, BOYNTON and LEE (1974), both types of segregation contribute to the overall ratios seen. For purposes of comparison, therefore, we have added the observed Type III segregations to those of Type II and presented the pooled data in Table 9. As can be seen, addition of Type III data shifts the average values towards 1:1. Thus, the data of Table 9 are more directly comparable with those of Table 10 and with the data of GILLHAM, BOYNTON and LEE (1974) than are the data in the preceding tables of this paper.

Effect of UV irradiation upon P_1:P_2 ratios.

Cross 20 was set up to evaluate the effect of UV irradiation of the *mt+* parent upon each of the parameters being utilized in genetic analysis of the chloroplast genome. Here we examine the effect of this irradiation upon the P_1:P_2 ratios of progeny sampled from large zygote colonies, following the method used by GILLHAM, BOYNTON and LEE (1974).

The results, summarized in Table 10, show that UV irradiation of the *mt+* parent, which we do routinely to enrich for biparental zygotes, does not alter the P_1:P_2 ratios. Our usual UV dose 50″ gives a yield of about 50% exceptional zygotes. In cross 20, 15″ irradiation gave 8.7% exceptional zygotes, 30″ UV gave 25% and 50″ gave 56% exceptional zygotes, results which are in line with those of the other crosses described in this paper.

Table 10 also shows the occurrence of variability in segregation ratios from dose to dose of UV, and from one gene to another. In general, the genes *ac* and *tm1*, which show the closest fidelity to 1:1 also show the smallest variability from one zygote to another. The greatest variability is shown by *sm2* and *spc*, whereas *mr1* and *ery* are intermediate.

These results, delineating the negligible effect of UV irradiation of the *mt+* parent upon allelic segregation ratios, are of great importance in showing that our UV method of enriching for biparental zygotes does not bias allelic segregation data.

DISCUSSION

This is the first of a series of papers, presenting the data from many crosses performed in our laboratory over the last several years, in support of our previously proposed (SAGER 1972) genetically circular, diploid model of the Chlamydomonas chloroplast genome. This paper concerns the segregation ratios of a set of chloroplast alleles observed in the progeny from a series of 16 crosses. These segregation ratios are important because our diploid model assumes equal input and predicts equal output of alleles from the two parental strains. If the output ratios deviate from 1:1, the basis of the deviation needs to be explained.

The results in this paper show that deviations from the expected 1:1 ratios do occur, but that they differ in extent and in time of occurrence from one linked gene to another. Usually, only one or two genes are involved in individual segregation events (Tables 3 and 5; SAGER and RAMANIS 1976). We found further that segregation events occurring in zygotes and those occurring post-meiotically need to be examined separately because their segregation patterns are different. The segregation events occurring in zygotes have been distinguished from those occurring in zoospore clones by means of pedigree analysis. As demonstrated in Table 3, the genotype of each of the four zoospores can be inferred from the phenotypes of the progeny assayed after one or two doublings of each zoospore.

In zygotes, allelic segregation ratios for the genes *sm2*, *ery*, and *spc1* show a greater deviation from 1:1 than for the genes *ac* and *tm1* (Table 6); and the frequency of zygotic segregation is higher for the genes *sm2*, *ery*, and *spc1* than for *ac* and *tm1* (Table 4). In the zoospore clones, (Table 7, 8) however, the deviations from 1:1 are allele-specific, occurring principally when the *resistant* allele is of maternal origin, an effect not visible in the zygotic data. In addition, the frequencies of Type II segregation in zoospore clones are very similar for all genes observed, (Table 7) and do not show the gene-specific differences in frequency that are found in the zygotic segregations of Table 4.

These observations need to be assessed in relation to the genetic map. The detailed evidence in support of the gene order and circularity described previously (SAGER 1972) will be published in subsequent papers of this series (SAGER and RAMANIS 1976; SINGER, SAGER and RAMANIS 1976). For purposes of this discussion, let us accept *pro tem* a circular map of the chloroplast genome with *ac* and *tm1* on opposite sides of the attachment point, *ap*, and with *ery* at 180° from *ap*, flanked by *spc* on the side linked to *tm1* and *sm2* on the other side linked to *ac*. (See Figure 3, SINGER, SAGER and RAMANIS 1976)

At the time of zygote formation, each parent contributes a complete chloroplast genome. In maternal zygotes, the copy from the male (*mt*−) parent is degraded and little or no marker rescue occurs. In biparental zygotes recovered after blocking maternal inheritance by UV irradiation of the female parent before mating (SAGER and RAMANIS 1967) the complete genome of the male parent usually remains intact, as shown by the fact that the zoospores are heterozygous for most or all of the markers segregating in the crosses described in this paper. (Somewhat different results have been obtained in crosses with strains from the Gillham-Boynton laboratory, to be discussed in a subsequent paper).

We propose that the higher frequencies of zygotic segregation seen for the genes *spc*, *ery*, and *sm2* result from their map location far from the attachment point, a region that may serve to protect the genes *ac* and *tm1*. Segregation events in zygotes may result from preferential sites of restriction enzyme attack on chloroplast DNA from the male parent in the unprotected region, leading to frequent loss of markers that come from the male parent. In zoospore clones, however, segregation events may be initiated by a different enzyme, attacking at random around the genome; and the bias in 1:1 ratios at this stage may reflect preferential heteroduplex repair, as seen in gene conversion in other organisms (FOGEL and MORTIMER 1971).

GILLHAM, BOYNTON and LEE (1974) suggested that the 1:1 ratios we have reported (SAGER 1972) for parental alleles in progeny of biparental zygotes may be the result of our method of UV irradiation of maternal alleles before mating, a procedure we developed (SAGER and RAMANIS 1967) to increase the yield of biparental zygotes. They examined the effects of UV irradiation upon allelic transmission ratios of mutant alleles conferring resistance to erythromycin, streptomycin, and spectinomycin. (The *spc* mutation they used gives no recombination with our *spc1* and may be the same allele, but we do not know the relation of their other markers to ours.) They found an excess of alleles from the maternal parent transmitted in unirradiated controls, as previously reported for their strains (GILLHAM 1969); and they found that this excess of maternal alleles decreased as a function of UV irradiation.

In this paper we have reported segegation ratios of chloroplast alleles in the progeny of cross 20 after 0, 15″, 30″, and 50″ irradiation (Tables 4,5,6,7,10). No effect of irradiation upon allelic ratios of the sort described by GILLHAM, BOYNTON and LEE (1974) was found. The method used by GILLHAM, BOYNTON and LEE (1974) was the scoring of samples taken from zygote colonies consisting of about 10^6 cells and containing all the clonal descendants from single zygotes.

We examined the effects of increasing doses of UV irradiation in cross 20, both in pedigrees (Tables 4,5,6,7) and in zygote colonies (Table 10). Both methods gave concurrent results: the absence of a UV effect on allelic segregation ratios.

It is noteworthy that the cross 20 data showed deviations from 1:1 for some genes in pedigrees but not in zygote colonies. The reason for this difference is shown in Table 9. When miscopying events (Type II) are singled out, the deviations from 1:1 appear more extreme than when all the segregation events, Type II and Type III in zoospore clones as well as in zygotes, are combined as they are in the data obtained by the zygote colony sampling method.

Thus, the deviations from 1:1 presented in this paper would not have been detected by the method of GILLHAM, BOYNTON and LEE (1974). The pronounced deviations from 1:1 seen in their crosses are of a higher order of magnitude than ours. To examine this difference, we made a set of crosses between the stocks of the two laboratories. The results of those studies, to be presented in a subsequent paper of this series, provide the experimental basis for our proposal that differences in restriction enzyme activity in zygotes may account for the differences in results. On this hypothesis the results reported from both laboratories would be fully consistent with the diploidy of the chloroplast genome.

In summary, the results presented in Tables 4–10 show unambiguously that factors other than the number of genomic copies determine allelic segregation ratios. In the crosses reported in this paper, allelic transmission ratios are predominantly 1:1. Where deviations from 1:1 occur, they are gene specific and follow particular patterns: in zygotes during zoospore formation, the genes *ac* and *tm1* show closer fidelity to 1:1 than other genes linked to them; and in zoospore clones, the deviations show a strong allele-specific bias. These results are consistent with the diploid model of the chloroplast genome previously proposed (SAGER 1972); and they will be further supported by additional lines of evidence in the succeeding papers of this series.

The diploid genetic model appears to be inconsistent with the physical data based on the reannealing kinetic analysis of chloroplast DNA (WELLS and SAGER 1971; BASTIA *et al.* 1971). In that analysis the major component had a genomic size of about 1×10^8 daltons, whereas the Chlamydomonas chloroplast contains about 4×10^9 daltons of DNA (SAGER 1972). However, the presence of 20% or more of the total DNA as a single copy fraction would have been missed in the reported studies, since special precautions (i.e. pre-fractionation) would have been necessary to identify a single-copy component. Thus, the possibility remains open that the 1×10^8 dalton component is a reiterated fraction of the total genome and that a non-reiterated component which could be as large as 8×10^8 daltons, carries all the genes detected by mutation and described in this and other studies (SAGER and RAMANIS 1976; GILLHAM 1974). This interpretation not only reconciles the reannealing data with the genetic data but also is supported by the cytochemical evidence of RIS and PLAUT (1962), who described two DNA bodies in the chloroplast. Their methods could have detected molecules in the size range of $2–4 \times 10^9$ daltons but not molecules one-tenth this size or smaller.

This work was supported by grants from the National Institute of Health (U.S. Public Health Service #GM-13970), from the American Cancer Society, and from the City University of New York.

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Corresponding editor: N. W. GILLHAM