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Mutations Altering Chloroplast Ribosome Phenotype in *Chlamydomonas*, I. Non-Mendelian Mutations*

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Abstract. Uniparentally inherited mutations to antibiotic resistance and dependence in *Chlamydomonas reinhardi* exhibit an altered chloroplast ribosome phenotype. Genetic studies demonstrate an absolute correlation between the drug resistance or dependence and the ribosome phenotype in two such mutants.

Two distinct genetic systems exist in the heterothallic, green alga *C. reinhardi*. One is a conventional Mendelian system consisting of 16 linkage groups;[1] the other exhibits uniparental inheritance.[2,3] The uniparental system[3] can be characterized as follows. (1) In the great majority of zygotes (maternal zygotes), genes belonging to the uniparental system are transmitted to all four products of meiosis by the mt^+ (mating type) parent, but to none by the mt^- parent. (2) In every cross a minority class of exceptional zygotes arises in which the uniparental genes are transmitted to the meiotic progeny by both mt^+ and mt^- parents (biparental zygotes) or by the mt^- parent alone (paternal zygotes). (3) Segregation and recombination of uniparental genes occurs among the progeny of the biparental zygotes. (4) Many of the uniparental mutations described to date in Chlamydomonas are resistant to or dependent upon antibiotics. Similar mutations in *Escherichia coli* confer resistance or dependence by altering the structure of the ribosome so it can function in the presence of the antibiotic in question.[4,5]

Ribosomes from wild type cells of *C. reinhardi* and the *y* and *ac-20* mutants can be assigned to five distinct "generic" classes—83S, 70S, 66S, 54S, and 41S—when sedimented in linear sucrose gradients in a buffer containing 25 mM Mg^{2+}.[6] The 70S ribosomes of wild type, which constitute about 40% of the total cell ribosomes[6], are in the chloroplast[6,7] and contain 23S, 16S, and 5S RNA.[6-9] In the *ac-20* mutant the 70S ribosomes are replaced by 66S ribosomes, which also appear to contain 23S and 16S RNA, and are most likely located in the chloroplast.[6] The 83S ribosomes are located in the cytoplasm[6] and contain 25S, 18S, and 5S RNA.[6-8] We suggest that the 54S ribosome particles contain 23S RNA and are the large subunit of the chloroplast ribosome. Surzycki (personal communication) has recently found that the 41S ribosome particles contain only 16S RNA and are presumably the small subunit. In the present paper we show that certain uniparental mutations to antibiotic resistance and dependence in *Chlamydomonas* affect chloroplast ribosomes.

Materials and Methods. Organisms and culture conditions: Strain 137C of *C.*

reinhardi was used as the wild type strain and the following mutants were derived from it. The four streptomycin-resistant uniparental mutants (*sr-2-1, sr-2-60, sr-2-280, sr-2-281*) were isolated from streptomycin-containing medium without mutagenesis;[10] the streptomycin-dependent (*sd-3-18*), erythromycin-resistant (*ery-3-6*), spectinomycin-resistant (*spr-1-1, spr-1-27*), and neamine-resistant (*nr-2-1*) uniparental mutants were all induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.[11] The double mutant *nr-2-1 spr-1-1* was obtained as a recombinant.[12] The acetate-requiring mutant (*ac-20*) was the kind gift of Prof. R. P. Levine in 1968 and a mt^- clone maintained in our laboratory since then has been used for all experiments.[6] Recently this clone was found to contain a second mutation, *cr-1*, which by itself also affects chloroplast ribosome phenotype but has no phenotypic effect on chloroplast ribosomes in combination with *ac-20*.[13] The reversions of the *sd-3-18* mutation (*sd-3-18R19, sd-3-18R20, sd-3-18R35*) were generously provided by Dr. C. G. Arnold in 1970.

Cells were grown as described[6] on the high salt medium of Sueoka,[14] with the addition of 2 g/liter of sodium acetate (HSA).

Genetic analysis: Standard methods were used for making crosses and for dissection and analysis of tetrads.[11,15]

Ribosomes: Preparation and separation of ribosomes in linear sucrose gradients, as well as the assignment of sedimentation velocities, has been described in detail.[6]

Ribosomal RNA: Preparation of ribosomal RNA from whole cells and separation of the high molecular weight species on 2.5% acrylamide–0.5% agarose gels were performed according to Bourque, Boynton, and Gillham.[6] Recently, we have found that relatively undegraded ribosomal RNA can be prepared from ribosome fractions isolated from sucrose gradients and frozen at −20°C for as long as three months without further treatment. After thawing, the fractions from several gradients of a given ribosome peak were pooled and the resulting ribosome suspension immediately mixed with an equal volume of a solution containing 0.01 M Tris·HCl, pH 7.8–1.2% sodium dodecyl sulfate–10% sucrose, and a small amount of bromophenol blue tracking dye, then placed directly on the gels.

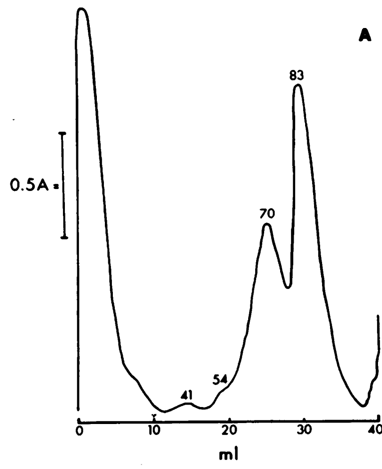
Electron microscopy: Fixation, dehydration, embedding, and sectioning procedures were identical with those described.[6]

Results. Ribosome phenotypes of the different uniparental mutants: The results presented in Table 1 show that certain uniparental mutations to streptomycin and erythromycin resistance contain the same relative proportions of 70S ribosomes as wild type. Among the four streptomycin-resistant mutants, two have 70S ribosomes. These ribosomes are entirely replaced by ribosomes sedimenting at 66S and 54S in the other two mutants. The 66S ribosomes and

TABLE 1. *Ribosome phenotypes of different uniparentally inherited mutations to antibiotic resistance in* C. reinhardi.

Stock	% Ribosomes in each generic class: 83S	70S	66S	54S	70S + 54S	66S + 54S	41S	No. of independent expts.
Wild type	61	37	0	1	38*	—†	1	10
ery-3-6	64	36	0	0	36	—	0	1
sr-2-1	64	35	0	1	36	—	0	2
sr-2-280	61	34	0	5	39	—	0	1
sr-2-60	52	0	30	16	—	46	2	3
sr-2-281	60	0	29	12	—	41	0	2
spr-1-27	62	0	35	2	—	37	tr	2
nr-2-1 spr-1-1	51	0	40	9	—	49	0	2

* Summed in 70S + 54S column.
† Summed in 66S + 54S column.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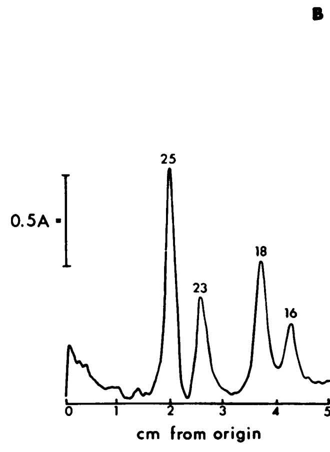
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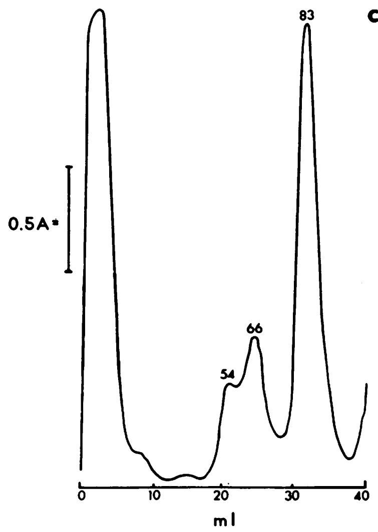
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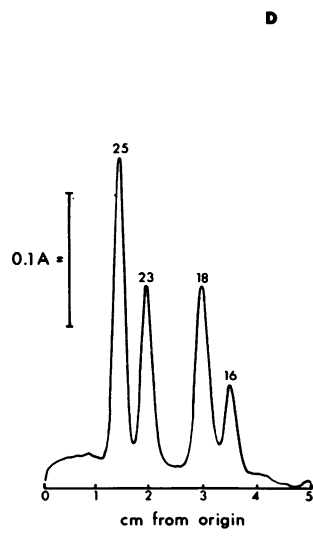
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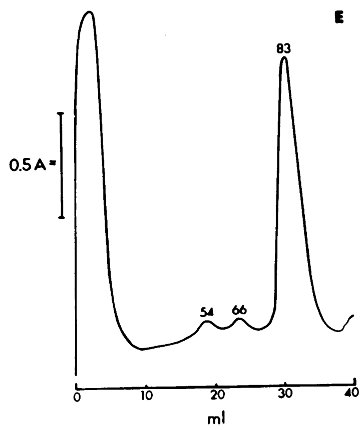
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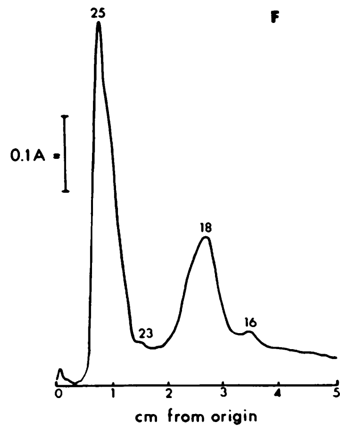
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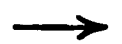

54S ribosome particles, present in about a 2:1 ratio in both mutants, together constitute over 40% of the total ribosomes of the cell. The double mutant (*nr-2-1 spr-1-1*), resistant to both neamine and spectinomycin, shows an essentially similar ribosome phenotype. In wild type, the 54S and 66S classes constitute less than 1% of the total. In a second mutation to spectinomycin resistance (*spr-1-27*) the 70S ribosomes are replaced by 66S ribosomes, but the proportion of 54S ribosome particles remains about the same as it is in wild type.

Analysis of the inheritance of ribosome phenotype: Reciprocal crosses were made between a uniparental mutation resistant to streptomycin having an altered ribosomal phenotype (*sr-2-60*) and wild type (*ss*) cells to determine whether streptomycin resistance and altered ribosome phenotype are inherited together. In the *sr-2-60* mutant the 70S ribosomes are entirely replaced by 66S ribosomes and large amounts of 54S ribosome particles are accumulated (compare Figs. 1*A*, 1*C;* Table 1). From the cross *ss* mt^+ × *sr-2-60* mt^-, 29 complete tetrads were obtained and, upon analysis, 27 of these contained four *ss* progeny; the other two were exceptional and contained four *sr-2-60* progeny (Table 2). Analysis of clones derived from each of the four meiotic products from five of the *ss* tetrads revealed that every clone contained 70S ribosomes (Table 2). On the other hand, clones derived from the *sr-2-60* progeny completely lacked 70S ribosomes and contained 66S and 54S ribosomes (Table 2) in a 2:1 ratio (Table 3) as did the *sr-2-60* parental stock (Table 1). From the reciprocal cross, 26 complete

TABLE 2. *Inheritance of streptomycin resistance and chloroplast ribosome phenotype in reciprocal crosses between a streptomycin-sensitive wild type stock* (ss) *and a uniparentally inherited mutation to streptomycin resistance* (sr-2-60).

Cross	Segregation pattern of the four meiotic products of each tetrad	No. of tetrads analyzed On streptomycin	In centrifuge
ss mt^+ × *sr-2-60* mt^-	4 *ss* (70 S): 0 *sr-2-60* (66S + 54S)	27	5
	0 *ss* (70 S): 4 *sr-2-60* (66S + 54S)	2	2
sr-2-60 mt^+ × *ss* mt^-	0 *ss* (70 S): 4 *sr-2-60* (66S + 54S)	26	6

TABLE 3. *Ribosome phenotypes of streptomycin-sensitive wild type* (ss) *and -resistant* (sr-2-60) *isolates from reciprocal crosses of* sr-2-60 × ss *and from the double mutant* ac-20 sr-2-60.

Isolates	Phenotype	% Ribosomes in each generic class 83S	70S	66S	54S	70S + 54S	66S + 54S	41S	No. of isolates tested
ss	Streptomycin-sensitive	55	41	0	4	45	—	1	16
sr-2-60	Streptomycin-resistant	62	0	24	13	—	37	trace	25
ac-20 sr-2-60	Streptomycin-resistant	86	0	7	6	—	13	1	7

FIG. 1. Ribosomal (*left*) and ribosomal RNA (*right*) absorbance profiles from streptomycin-sensitive, *ss* (*A,B*); streptomycin-resistant, *sr-2-60*-(*C,D*) and *ac-20 sr-2-60* (*E,F*) cells. Ribosomal RNA species were separated on agarose–acrylamide gels and their positions identified by scanning the gels for absorbance, *A*, at 260 nm. Ribosomes were separated by sucrose gradient centrifugation and the different species were identified by monitoring their absorbance at 254 nm. All isolates were grown on HSA medium.

tetrads were obtained and all of the progeny in these tetrads exhibited the *sr-2-60* phenotype. Clones derived from the four meiotic products of each of six tetrads were analyzed for ribosome phenotype and every one of the progeny clones contained 66S and 54S ribosomes (Table 2).

Relative amounts of 70S ribosomes (41 ± 4%) in 16 *ss* isolates examined from the cross of *ss* mt^+ × *sr-2-60* mt^- (Table 3) do not differ in a statistically significant fashion from relative amounts observed in our standard wild type clone (37 ± 2%). The 25 clones of *sr-2-60* progeny from the cross of *sr-2-60* mt^+ × *ss* mt^- show a similarly small variation in relative amounts of 66S ribosomes (24 ± 4%) (Table 3) which constitute only about two thirds of the amount of 70S ribosomes made by the *ss* isolates.

Extraction of ribosomal RNA from the 66S ribosomes shows that these ribosomes, like the 70S ribosomes of wild type, contain both 23S and 16S RNA whereas the 54S ribosome particles contain mostly 23S RNA, and some 16S RNA (Table 4). We believe that the 54S ribosome particles of *sr-2-60* contain

TABLE 4. *Properties of ribosomal RNA extracted from whole cells of wild type,* sr-2-60, *and the double mutant* ac-20 sr-2-60 *and from ribosomes of wild type and* sr-2-60.*

Stock	Ribosomal RNA extracted from	Ratios of high to low molecular weight rRNA* 25S/18S (1.94 expected)	23S/16S (1.88 expected)	23S + 16S, per cent of total Observed	Expected
Wild type	Whole cells	1.42	1.51	35.0	36.8
	83S ribosomes†	1.41	1.12‡	5.5	0.0
	70S ribosomes†	2.28‡	2.49	86.0	100.0
sr-2-60	Whole cells†	1.75	2.53	34.0	45.5
	83S ribosomes	1.39	trace‡	0.0	0.0
	66S ribosomes†	1.16‡	3.32	90.0	100.0
	54S ribosomes†	trace‡	4.85	100.0	100.0
ac-20 sr-2-60	Whole cells	1.89	1.59	4.4	12.9

* The expected absorbance ratios were calculated from the molecular weight ratios of 25S/18S and 23S/16S ribosomal RNA.[6] Expected values for relative amounts of 23S + 16S ribosomal RNA are derived from the observed relative amounts of 70S + 54S or 66S + 54S ribosomes of the different genotypes given in Tables 1 and 3. Ratios which are less than expected probably reflect preferential degradation of the larger ribosomal RNA species.

† Two or more gels scanned.

‡ Expected ratios are not applicable.

only 23S RNA, but that our preparations are contaminated with 66S ribosomes which contain both species of RNA, since the 54S and 66S ribosomes do not separate cleanly on our gradients (Fig. 1*C*). Cross contamination between 54S and 66S ribosomes is also reflected by the fact that the ratio of 23S to 16S RNA in the 66S ribosomes of *sr-2-60* is higher than in the 70S ribosomes of wild type which contains very few 54S ribosome particles (Fig. 1 A). The 54S ribosome particles accumulated by the mutant *cr-1* contain only 23S RNA.[13]

Assuming the 54S ribosome particles to be large subunits of the chloroplast ribosome, we have summed 70S and 54S ribosomes to estimate the total proportion of chloroplast ribosomes in wild type and 66S and 54S ribosomes in *sr-2-60*. The *sr-2-60* isolates appear to have nearly the same amounts of chloroplast ribosome "particles" as the *ss* isolates, but the relative proportion of *intact* chloroplast ribosomes is nearly twice as great in the *ss* isolates (Table 3).

Evidence that the 54S and 66S ribosomes of *sr-2-60* are in the chloroplast: The Mendelian mutant *ac-20* forms greatly reduced numbers of chloroplast ribosomes[6,7] and these sediment at 66 S rather than 70 S.[6] To determine whether the 66S and 54S ribosomes of *sr-2-60* were of chloroplast origin, we constructed the double mutant *ac-20 sr-2-60* and compared the ribosomes and ribosomal RNA from the double mutant with those of *sr-2-60* and wild type. The double mutant, like *ac-20* alone, contains greatly reduced amounts of 54S and 66S ribosomes as well as 23S and 16S RNA (Figs. 1*E,F;* Table 3). When observed by electron microscopy, chloroplast ribosomes are greatly reduced in number in the double mutant (Fig. 3) as compared to *sr-2-60* (Fig. 2). We conclude that the 66S ribosomes of *sr-2-60* are of chloroplast origin and that the same is probably true of

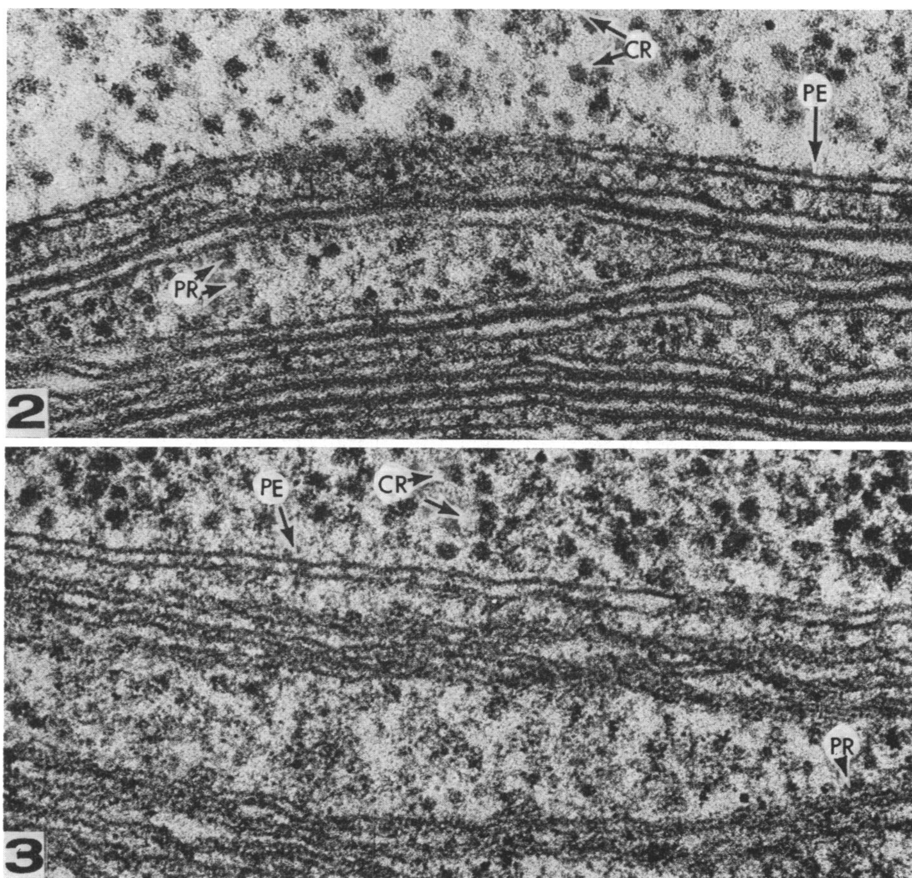

FIG. 2. (*Upper panel*) Section through part of a typical cell of the mutant *sr-2-60* grown on HSA medium. Organization of the chloroplast is indistinguishable from that of a wild type cell. Many chloroplast ribosomes (*PR*) and cytoplasmic ribosomes (*CR*) can be seen on either side of the chloroplast envelope (*PE*). ×120,000.

FIG. 3. (*Lower panel*) Section through part of a typical cell of the double mutant *ac-20 sr-2-60* grown on HSA medium. The chloroplast lamellae are largely unpaired and few chloroplast ribosomes (*PR*) are evident although many cytoplasmic ribosomes (*CR*) can be seen. ×120,000.

the 54S ribosome particles. Like *sr-2-60* alone, the *ac-20 sr-2-60* double mutant will grow on concentrations of streptomycin as high as 1 mg/ml, suggesting that the 66S ribosomes made by the double mutant are similar to the 66S ribosomes of *sr-2-60*.

Reversion of antibiotic dependence and aberrant chloroplast ribosome phenotype in a single mutational step: The streptomycin-dependent mutant *sd*-3-18, grown in liquid culture on HSA medium with 10 μg/ml of streptomycin, contains reduced proportions of 70S ribosomes and accumulates 54S ribosome particles to a greater extent than HSA-grown wild type (Tables 1, 5). When the mutant is

TABLE 5. *Ribosome phenotypes of a streptomycin-dependent mutant* (sd-3-18) *and strepto mycin-independent reversions of this mutant.*

Genotype	Phenotype	Presence (+) or absence (−) of streptomycin	% Ribosomes in each generic class: 83S	70S	66S	54S	70S + 54S	41S
sd-3-18	Streptomycin-dependent	+	68	25	0	7	32	0
		−	71	17	0	12	29	0
sd-3-18R19	Streptomycin-sensitive	−	49	48	0	2	50	1
sd-3-18R35	Streptomycin-sensitive	−	50	46	0	3	49	1
sd-3-18R20	Streptomycin-resistant	−	50	34	0	16	50	0

transferred to HSA medium, it will continue to grow for several generations before it dies. Under these conditions the proportion of 70S ribosomes is reduced even further and additional 54S ribosome particles accumulate (Table 5).

We have analyzed two reversions of *sd-3-18* to streptomycin sensitivity (*sd-3-18R19, sd-3-18R35*) as well as a "reversion" of *sd-3-18* from streptomycin dependence to streptomycin resistance (*sd-3-18R20*). The two sensitive reversions contain slightly higher proportions of 70S ribosomes and about the same proportion of 54S ribosome particles as wild type (Tables 1, 5). The streptomycin-resistant "reversion" (*sd-3-18R20*) has somewhat less 70S ribosomes than wild type and accumulates 54S ribosome particles. A streptomycin-sensitive isolate, supplied by Dr. Arnold from a cross of *ss* mt^+ × *sd-3-18R20* mt^-, makes normal proportions of 54S and 70S ribosomes. This isolate is phenotypically similar to the great majority of the progeny from the cross *ss* mt^+ × *sr-2-60* mt^- in that neither resistance nor altered chloroplast ribosomal phenotype is observed.

Discussion. Although the cellular location of the uniparental genes in *C. reinhardi* is not known[16,17] our results link streptomycin resistance, dependence, and (possibly) other antibiotic uniparental mutations to changes in chloroplast ribosome phenotype. *A priori*, we had no reason to expect the antibiotic-resistant mutants of this alga to have chloroplast ribosomes with altered sedimentation velocities for, as far as we know, such changes have not been reported for the ribosomes from similar antibiotic-resistant and -dependent mutants of the bacterium *E. coli*. However, changes in sedimentation velocity might well go un-

detected in *E. coli* since an internal standard, such as our 83S ribosomes, is lacking.

In *E. coli*, streptomycin resistance and dependence are alleles of a single gene which controls the production of a protein in the 30S subunit of the 70S ribosome.[18] The gene for spectinomycin resistance controls the production of a different 30S ribosomal protein.[19] Direct effects of antibiotics on ribosomes from sensitive, but not resistant, cells of *E. coli* have been demonstrated in protein synthesis systems *in vitro;* binding to ribosomes from sensitive, but not resistant, bacteria has also been demonstrated with labeled antibiotics.[5,19] We have not yet done similar experiments with chloroplast ribosomes of *Chlamydomonas*. However, since the genetic correlations between altered chloroplast ribosome phenotype and inheritance of *sr-2-60* and reversion of *sd-3-18* are absolutely specific, we conclude that these mutations (and probably others) directly or indirectly affect the amounts, structure, and possibly function of chloroplast ribosomes. We suspect the effect in *Chlamydomonas* is direct since it is in bacteria[4,5] whose ribosomes are complex structures in which the protein and RNA components must be assembled in a very specific sequence.[20] Thus, any mutation affecting ribosome structure might alter the sedimentation velocity either by changing the conformation of the ribosome or by interfering with the attachment of specific ribosomal proteins.

In another paper[13] we present a hypothesis to explain the accumulation of 54S ribosome particles, which we think are large subunits of the chloroplast ribosome, by several Mendelian and uniparental mutations.

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