Proc. Nat. Acad. Sci. USA
Vol. 69, No. 12, pp. 3551–3555, December 1972

Mutation of a Cytoplasmic Gene in *Chlamydomonas* Alters Chloroplast Ribosome Function

(chloroplast ribosome/*in vitro* protein synthesis/carbomycin resistance)

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Communicated by Bernard D. Davis, September 20, 1972

ABSTRACT **A mutation, *car*, determining resistance to several macrolide antibiotics, including carbomycin, has been identified in the alga *Chlamydomonas* as cytoplasmic, and mapped in the known cytoplasmic linkage group close to genes determining resistance to other antibiotics, including streptomycin, erythromycin, and spectinomycin. The effect of the *car* mutation on chloroplast ribosome function was demonstrated with an *in vitro* system incorporating amino acids especially developed to assess activity of 70S chloroplast ribosomes. In an S-30 extract containing both 70S chloroplast and 80S cytoplasmic ribosomes, low concentrations of Mg^{++} and spermidine favored 80S ribosome activity, and high concentrations activated 70S ribosomes and reversibly inactivated the 80S component. Under conditions favoring chloroplast ribosome activity, carbomycin inhibited incorporation by an S-30 extract, and by purified 70S ribosomes from wild-type but not from *car* cells. These results show that cytoplasmic genes are directly involved in chloroplast ribosome function and they suggest that the *car* gene product is a ribosomal protein; the results further strengthen the evidence that the cytoplasmic linkage group is located in chloroplast DNA.**

A serious gap in our understanding of cellular organization concerns the precise functional role of cytoplasmic genes (1). All cytoplasmic genes so far examined in the alga *Chlamydomonas reinhardi* show the same pattern of maternal inheritance and map within the same linkage group, long postulated to be located in chloroplast DNA and to code for numerous chloroplast proteins (1–4). Gillham *et al.* (5) have reported that chloroplast ribosomes from some cytoplasmic mutants of *Chlamydomonas* were 66 S rather than 70 S, and Mets and Bogorad (6) found that chloroplast ribosomes from a cytoplasmic erythromycin-resistant mutant were impaired in their erythromycin-binding ability. This paper reports the correlation between a mutation to resistance to the macrolide antibiotic carbomycin, that maps in the cytoplasmic linkage group and an alteration in chloroplast ribosome function, attributed to a change in a ribosomal protein.

We compare the effects of antibiotics on the protein-synthesizing activity of 70S chloroplast ribosomes from wild-type and antibiotic-resistant mutant strains. For this purpose it was necessary to develop an *in vitro* protein-synthesizing system that used chloroplast ribosomes. This paper reports the first demonstration of *in vitro* incorporating activity of chloroplast ribosomes from *Chlamydomonas*. We show that high concentrations of Mg^{++} and spermidine activate 70S chloroplast ribosomes, and low concentrations activate 80S cytoplasmic ribosomes. Both in S-30 extracts and in fractionated systems, the 70S ribosomes of the resistant mutant were resistant to carbomycin, whereas the ribosomes from the wild-type strain were sensitive.

MATERIALS AND METHODS

Cells. Wild-type strain 21gr and tester stocks used in crosses have been described, as have methods of preparation of cells for crosses and analysis of progeny (1, 2). For studies of *in vitro* protein synthesis, cells were grown in minimal medium (7) bubbled with 5% CO_2 in air under continuous light at 25°, with a doubling time of 8 hr, and harvested in log phase (2 to 3×10^6 cells per ml.).

Fractionation. Cells were pelleted and washed in Buffer A (adapted from ref. 14) (25 mM Mg acetate–25 mM KCl–25 mM Tris·HCl, pH 7.8–0.25 M sucrose–6 mM 2-mercaptoethanol) or Buffer B (Buffer A + 10 mM spermidine) or Buffer C (Buffer A + 3 mM spermidine), suspended at 7×10^8 cells per ml, and sonicated (Mullard ultrasonic oscillator) to about 90% cell breakage (3–4 min). The S-30 fraction was prepared as described (8) by centrifugation at 10,000 × *g* for 10 min; the supernatant (S-10) was centrifuged at 30,000 × *g* for 30 min. For preparation of the S-122 fraction, S-30 was centrifuged at 122,000 × *g* for 4 hr, and the top two-thirds of the supernate was retained.

Chloroplast ribosomes were prepared by sonication of cells (4×10^8/ml) in Buffer B (although studies with the S-30 fraction indicate that active ribosomes may be obtained in Buffer A without spermidine). 1.5 ml of the S-10 fraction was layered on 10–30% sucrose gradients in Buffer B and centrifuged for 4 hr at 39,000 rpm (SW40 Ti rotor, Spinco L2-65B). 0.3-ml fractions were collected and diluted. A_{260} was determined, and fractions containing chloroplast ribosomes were pooled. All procedures were performed near 0°, and cell fractions were stored at −70°.

Protein Synthesis. The reaction mixture contained (in 0.3 ml final volume): 13 mM Tris·HCl (pH 7.8), 33 mM KCl, 4 mM phosphoenol pyruvate, 12 μg of phosphoenol pyruvate kinase, 4 mM 2-mercaptoethanol, 887 μM ATP, 44 μM GTP, 12.5 nmol each of a mixture of 19 amino acids excluding phenylalanine, 437 pmol of [^{14}C]phenylalanine (specific activity 472 Ci/mol), 40 μg of *Escherichia coli* transfer RNA, 100 μg of poly(U), and 4.5 A_{260} units of S-30 extract. For 80S ribosome activity, the S-30 fraction was prepared in Buffer A, B, or C, and the reaction mixture was incubated at 10 mM Mg^{++} without spermidine or 8 mM Mg^{++} plus 1 mM spermidine. For 70S ribosome activity in the experiments described, the S-30 fraction was prepared in Buffer B (although activity was

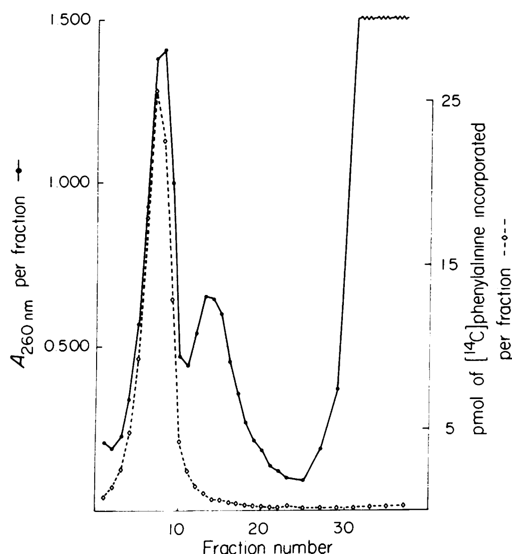

FIG. 1. Sucrose density gradient of S-30 extract from wild-type cells after incorporation by 80S ribosomes (10 mM Mg^{++}, no spermidine). 1.5 ml of reaction mixture (see *Methods*) was incubated at 35° for 25 min, chilled, brought to 25 mM in Mg^{++}, and layered on 10–30% sucrose gradients in Buffer B, 14 ml per tube in a SW40 rotor. 0.3-ml Fractions were collected from the bottom of the tube. A_{260} and hot Cl_3CCOOH-precipitable counts were determined. A_{260} is given per fraction without dilution. Mixture contained 22.5 A_{260} units of S-30 prepared in Buffer A.

also obtained when Buffer A without spermidine was used), and the reaction was incubated at 25 mM Mg^{++} plus 3.3 mM spermidine. In the S-30 system, the mixture was incubated at 35° for 25 min, when the reaction was stopped withl ml of 10% trichloroacetic acid; each tube was heated at 85° for 30 min. Precipitates were harvested on glass fiber filters, washed with 5% trichloroacetic acid and 70% ethanol, and dried. Radioisotope counts were obtained in toluene-based Omnifluor (Nuclear-Chicago liquid scintillation counter, approximate efficiency 87%). For resolution on gradients after incubation, reaction mixtures (1.5 ml) were layered on sucrose gradients prepared as above, fractions were diluted and read at 260 nm, and 200 μg of bovine-serum albumin plus 1 ml of 10% trichloroacetic acid were added to each tube before heating. In the ribosome–S-122 system, the reaction mixtures were incubated at 35° for 60 min, chilled, and 200 μg of bovine-serum albumin plus 1 ml of 10% trichloroacetic acid were added before heating.

Materials. Carbomycin was the generous gift of Chas. Pfizer & Co.; phosphoenol pyruvate, pyruvate kinase, and cycloheximide ("Actidione") were purchased from Calbiochem; Omnifluor and [14C]phenylalanine from New England Nuclear; puromycin dihydrochloride, bovine-serum albumin, and spermidine trihydrochloride, from Sigma Chem. Co.; chloramphenicol, *E. coli B* tRNA, and sucrose (special density gradient grade) from Schwarz/Mann; ATP and GTP from P-L Biochemicals; and poly(U) from Miles Laboratories.

Carbomycin solutions were freshly prepared for each experiment.

RESULTS

Genetic characterization of the *car* mutation

A *car* mutation was recovered after streptomycin mutagenesis (9). Wild-type cells of plus mating type (*mt*+) were incubated 6 days on agar medium containing 20 μg/ml of streptomycin, washed off, and replated on agar medium containing 50 μg/ml of carbomycin. One of the mutant colonies growing on carbomycin–agar, called *car*-1 and chosen for further study, was subcultured in the absence of the drug and tested at each subculture for maintenance of resistance. Cells remained uniformly resistant to 150 μg/ml of carbomycin after many doublings in the absence of the drug, and the mutant was therefore considered genetically resistant (*car-r*).

The mutant strain (*mt*+ *car-r*) was crossed with a tester stock carrying three nuclear and three cytoplasmic markers (2), and one of the resulting F_1 progeny (*mt*−) was backcrossed to an *mt*+ tester, with the same sets of nuclear and cytoplasmic marker genes. In both crosses, the *car* gene showed typical maternal inheritance: transmission to all progeny of the allele from the *mt*+ parent, except for rare instances of spontaneous biparental transmission. In the F_1 backcross (*mt*+ *car-s* x *mt*− *car-r*) all progeny (scored as zygote colonies) were sensitive to carbomycin except for a

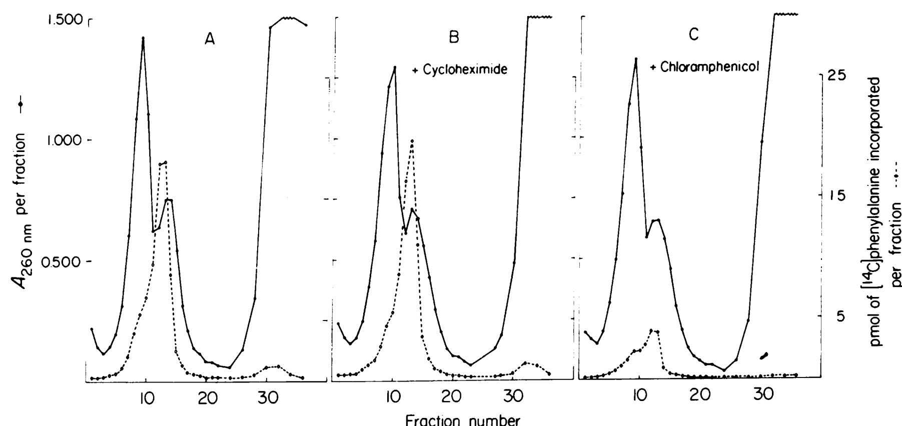

FIG. 2. Sucrose density gradients of S-30 extract from wild-type cells after incorporation by 70S ribosomes (25 mM Mg^{++}, 3.3 mM spermidine). Reaction mixtures containing 22.5 A_{260} units of S-30 prepared in Buffer B were as in Fig. 1 (except as noted below), and were incubated, layered on gradients, centrifuged, and analyzed as in Fig. 1. (*A*) Incubated without antibiotics; (*B*) 0.95 mM cycloheximide added; (*C*) 1.6 mM chloramphenicol added.

residual 0.6%, which grew on carbomycin-agar and represented examples of spontaneous biparental transmission.

Further evidence of the maternal inheritance pattern of the *car* gene is based on the results of UV-irradiation of the *mt+* parent just before mating, a procedure that increases the frequency of biparental transmission of cytoplasmic genes (10). When the *mt+* parent in the F_1 backcross (*mt+ car-s*) was irradiated, the frequency of zygote colonies growing on carbomycin agar increased from 0.6 to 80% of the total zygote colonies growing in the absence of the drug. Thus, the *car* mutation was shown to be cytoplasmic both by its pattern of maternal transmission in unirradiated controls and by its biparental transmission pattern after UV irradiation of the *mt+* parent.

Genetic linkage of the *car* gene was determined in crosses involving the linked cytoplasmic genes *ery* (erythromycin resistance), *spc* (spectinomycin resistance), and *sm2* (streptomycin resistance), by methods developed in this laboratory (1, 2, 10). The *car* gene was found to lie between *spc* and *sm2* and to be tightly linked to *ery* (1). In separate crosses, the genes *spi* and *ole*, conferring resistance to the related macrolide antibiotics spiramycin and oleandomycin, were also found to be tightly linked to *ery* (1). The four genes, *ery*, *car*, *spi*, and *ole*, may actually represent different mutations of the same gene (Sager & Ramanis, unpublished). Many macrolide antibiotics inhibit protein synthesis in bacterial ribosomes (11) and bind to the 50S ribosomal subunit (12).

Protein synthesis on cytoplasmic and chloroplast ribosomes of wild-type *Chlamydomonas*

An *in vitro* system was developed to examine the effect of carbomycin on *Chlamydomonas* protein synthesis. Two classes of ribosomes have been identified in extracts of *Chlamydomonas:* cytoplasmic ribosomes sedimenting at 80 S and chloroplast ribosomes sedimenting at 70 S (13, 14).

Incorporation activity of an S-30 extract stimulated by poly(U) has been described (8). Chloroplast ribosomes are inactive in this system. Fig. 1 shows that when an S-30 extract was incubated (8) and then centrifuged through a 10–30% sucrose gradient two absorbance peaks were seen, corresponding to the two ribosome classes (see ref. 14), but amino-acid-incorporating activity was associated only with the 80S peak. [The absence of counts at the top of the gradient indicated that, as in other systems (15), peptide chains were not released from the active ribosome complex when poly(U) was used as messenger.]

TABLE 1. *Effect of antibiotics on incorporation by 80S ribosomes in a wild-type S-30 extract (10 mM Mg^{++}, no spermidine)*

Conditions	pmol Phenylalanine incorporated/mg of 80S ribosomes†
Complete system*	131
− poly(U)	4
+ Cycloheximide (0.95 mM)	19
+ Chloramphenicol (1.6 mM)	124
+ Carbomycin (0.1 mM)	119

* See legend to Fig. 1 and *Methods*.

† Amount of 80S ribosomes was estimated by measurement of areas under 80S and 70S peaks in sucrose gradient preparations of several incubated S-30 extracts. 80S ribosomes represented about 65% and 70S ribosomes about 35% of the absorbance at 260 nm. If we assume the 4.5 A_{260} units of S-30 used per 0.3-ml incubation mixture are ribosomal, about 3 A_{260} units come from 80S ribosomes. If 24 A_{260} units = 1 mg RNA, and these 80S ribosomes are 40% RNA (Ishida and Sager, unpublished), then each 0.3 ml of incubation mixture contains about 0.3 mg of 80S ribosomes.

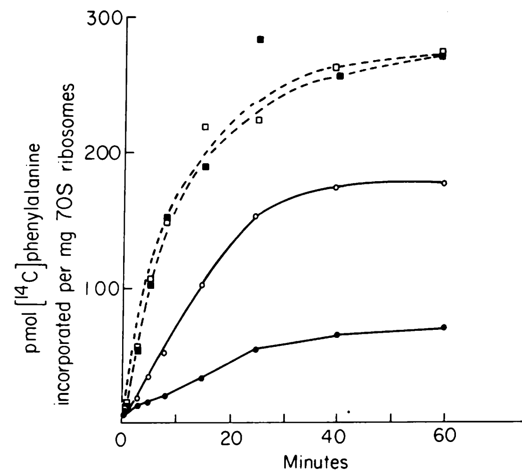

FIG. 3. Effect of carbomycin on phenylalanine incorporation by 70S ribosomes from wild type (○, ●) and *car* (□, ■) mutant. S-30 extracts of wild type and *car* mutant were prepared in Buffer B (as in Fig. 2). Reaction mixtures were prepared and processed as described in *Methods*, and incubated at 35° with 25 mM Mg^{++} and 3.3 mM spermidine for times indicated in the absence (○, □) or presence (●, ■) of 20 μM carbomycin. Wild-type and *car* extracts were examined in separate experiments.

Table 1 shows the results of incubation of an S-30 extract under the same conditions as in Fig. 1, in the presence of cycloheximide, chloramphenicol, or carbomycin. Cycloheximide inhibited protein synthesis 85%, whereas chloramphenicol and carbomycin had little or no effect. These findings are in accord with the sensitivity of cytoplasmic ribosomes from other eukaryotes to cycloheximide and not to chloramphenicol (see ref. 14).

In contrast, incorporation on chloroplast ribosomes was obtained when the S-30 system was incubated at 25 mM Mg^{++} and 3.3 mM spermidine (Fig. 2*A*). Essentially no activity was associated with the 80S ribosomal peak. In contrast with cytoplasmic ribosomes, the activity associated with the 70S peak was resistant to cycloheximide (Fig. 2*B*) and sensitive to chloramphenicol (Fig. 2*C*). In an experiment without gradients, chloroplast ribosomes from the wild-type strain were sensitive to carbomycin (8 μM gave 70% inhibition). Activity on chloroplast ribosomes was obtained in a range of Mg^{++} concentrations from 25–50 mM in the absence of added spermidine. However, a combination of 3.3 mM spermidine with 25 mM Mg^{++} gave twice the activity seen with Mg^{++} alone, and was used in all subsequent experiments.

Thus, wild-type cytoplasmic ribosomes were active in an S-30 system containing 10 mM Mg^{++} but inactive in 25 mM Mg^{++} plus 3.3 mM spermidine, sensitive to cycloheximide, and resistant to chloramphenicol and carbomycin. On the other hand, wild-type chloroplast ribosomes were inactive at 10 mM Mg^{++} but functional at the higher magnesium and spermidine concentrations, resistant to cycloheximide, and sensitive to chloramphenicol and carbomycin.

TABLE 2. *Influence of carbomycin on incorporation by cytoplasmic and chloroplast ribosomes of wild-type cells and car mutants in S-30 extracts*

Conditions	pmol of Phenylalanine incorporated	
	Wild type	*car*
	per mg of 80S ribosomes‡	
Complete system (for 80S activity)*	417	495
+ Cycloheximide (1.2 mM)	29	54
+ Carbomycin (20 μM)	381	460
	per mg of 70S ribosomes§	
Complete system (for 70S activity)†	149	199
+ Cycloheximide (1.2 mM)	139	223
+ Carbomycin (20 μM)	44	242
+ Carbomycin (600 μM)	N	204

* Incubation mixture contained 8 mM Mg^{++} and 1 mM spermidine. Other components as in Fig. 1, except that each tube contained 1.5 A_{260} units of S-30 extract. Wild-type S-30 was prepared in Buffer C and the *car* extract in Buffer B.

† Incubation mixture contained 25 mM Mg^{++} and 3.3 mM spermidine. Other conditions as in Fig. 2. Wild-type and *car* extracts were prepared in Buffer B.

‡ Calculated as described in Table 1.

§ Amount of 70S ribosomes was estimated as in Table 1, with 35% instead of 65% of S-30 absorbance, and assuming that chloroplast ribosomes, like bacterial ones, contain 60% RNA. Fach 0.3 ml of incubation mixture contains about 0.1 mg of 70S ribosomes.

N = not done.

Other experiments showed that S-30 fractions prepared in 25 mM Mg^{++}, either with or without spermidine, may be used to demonstrate protein synthesis on either 70S or 80S ribosomes. Activity on both 70S and 80S ribosomes was obtained in a range of Mg^{++} and spermidine concentrations, the combined Mg^{++} and spermidine requirement always being higher for 70S than for 80S ribosomes. The particular concentrations used in the S-30 experiments described in this paper allowed examination of only chloroplast or only cytoplasmic ribosome activity in whole-cell extracts.

Effect of carbomycin on protein synthesis by S-30 extracts from wild-type and carbomycin-resistant strains

The effect of carbomycin on protein synthesis under conditions of high concentrations of Mg^{++} and spermidine, with S-30 extracts from the wild-type and mutant strains, is shown in Fig. 3. Carbomycin had no effect on incorporation with the mutant S-30 preparation but decreased both the rate and extent of phenylalanine incorporation with the wild-type extract.

A further comparison is given in Table 2. Here, S-30 extracts from wild type and mutant were tested under conditions promoting 80S ribosome activity (8 mM Mg^{++} plus 1 mM spermidine) and 70S ribosome activity (25 mM Mg^{++} plus 3.3 mM spermidine). The 80S ribosomes from both wild-type and mutant strains were sensitive to cycloheximide and resistant to carbomycin. In contrast, under conditions permitting 70S ribosome activity both preparations were resistant to cycloheximide, but the wild-type extract was sensitive to carbomycin at 20 μM while the mutant extract was resistant even to 600 μM.

The altered component in the *car* mutant

For determination of whether the chloroplast ribosomes themselves or some other component of the S-30 extract was altered in the mutant, purified chloroplast ribosomes and S-122 fractions were prepared from the wild type and from the mutant, and the effect of carbomycin on protein synthesis was examined in reconstituted homologous and heterologous systems.

Chloroplast ribosomes were prepared by pooling the 70S peak fractions from S-30 extracts centrifuged through sucrose gradients. In the purified recombined system, activities were 10-times higher than those usually found with S-30 extracts incubated under the same conditions (25 mM Mg^{++} and 3.3 mM spermidine). Perhaps an inhibitor was removed from the ribosomes in the gradient. As shown in Table 3, the homologous system from the wild-type components incorporated 1292 pmol of phenylalanine per mg ribosomes, and the homologous *car* system incorporated 1725 pmol.

With the heterologous systems, carbomycin inhibited incorporation with wild-type ribosomes, but not with ribosomes from the *car* mutant. Thus, the chloroplast ribosome itself (or some tightly bound component) was altered by the cytoplasmic mutation to carbomycin resistance.

DISCUSSION

This report has described a cytoplasmic mutation, *car*, to carbomycin resistance, located in the previously described (1–3) cytoplasmic linkage group of *Chlamydomonas*, and has shown that this mutation alters chloroplast ribosome function. An *in vitro* system incorporating amino acids was developed to examine the effect of the *car* mutation on chloro-

TABLE 3. *Effect of carbomycin on incorporating activity by combinations of chloroplast ribosomes and S-122 fractions from wild type and car mutant*

Source		pmol Phenylalanine incorporated per mg of ribosomes‡	
Chloroplast ribosomes*	S-122†		
		− Carbomycin	*+ Carbomycin (80 μM)*
Wild type	Wild type	1292	108
Wild type	*car*	723	54
car	*car*	1725	1400
car	Wild type	2542	2950
		+ Puromycin (1 mM)	
Wild type	Wild type	85	
car	*car*	192	
		−poly(U)	
Wild type	Wild type	8	
car	*car*	0	

* Amount of 70S ribosomes per incubation: 13 μg from wild type and 12 μg from *car* mutant.

† Amount of S-122 fraction: 0.37 A_{280} units from wild type and 0.25 A_{280} units from *car* mutant.

‡ Reaction mixtures were incubated in 25 mM Mg^{++} and 3.3 mM spermidine, precipitated, and counted. Purified 70S ribosomes and S-122 fractions were substituted for S-30 extract.

plast ribosome activity. With this system, chloroplast ribosomes from the mutant were shown to be resistant to inhibition by carbomycin, whereas chloroplast ribosomes from the wild type were sensitive.

These findings demonstrate definitively that the cytoplasmic gene *car*, and therefore the linkage group of which it is a part, functions in the formation or activation of chloroplast ribosomes. The location of this linkage group in chloroplast DNA, strongly indicated by many lines of evidence (1), is further supported by the correlation between gene mutation and chloroplast ribosome alteration reported here.

The development of a highly active *in vitro* system that incorporates amino acids using chloroplast ribosomes, after numerous failures (Sager, unpublished), resulted from the use of high concentrations of Mg^{++} and spermidine. Although added spermidine is not essential, it appears to act as in other systems (16) to enhance activity. In *Chlamydomonas*, 70S chloroplast ribosomes have a much higher Mg^{++} requirement for structural integrity (13, 14) than do 80S ribosomes, and a higher Mg^{++} optimum for activity. At 25 mM Mg^{++} spermidine doubles the activity of the chloroplast ribosomes and inactivates the 80S cytoplasmic ribosomes, providing conditions for examining 70S ribosome activity in S-30 extracts without fractionation.

The higher Mg^{++} optimum for chloroplast than for 80S ribosomes observed in *Chlamydomonas* parallels the higher Mg^{++} requirements generally observed for amino-acid incorporation and monomer stability of 70S bacterial ribosomes than of 80S eukaryotic ribosomes. In addition, the Mg^{++} optimum for incorporation on ribosomes isolated from yeast mitochondria (21) or from tobacco leaf chloroplasts (22) was higher than that of their cytoplasmic ribosomes.

The activities observed (1292 and 1725 pmol of phenylalanine per mg ribosomes) with the chloroplast ribosome–S-122 system from *Chlamydomonas* approach those found with *E. coli*. Other preparations with ribosomes derived from organelles require addition of *E. coli* supernatant factors for comparable high amounts of incorporation (23, 24).

Numerous similarities between bacterial and chloroplast protein-synthesizing systems have been described (1). In particular, bacteria and chloroplasts are sensitive to certain classes of antibiotics, including the macrolides, aminoglycosides, spectinomycin, and chloramphenicol, all known to inhibit protein synthesis (12). As in bacteria, mutations have been recovered in *Chlamydomonas* conferring resistance to many of these antibiotics (1). Some of the mutations are nuclear, and their effects at the molecular level are largely unknown. The cytoplasmic mutations, presumably located in chloroplast DNA, have been examined with the view that they may resemble the bacterial ones that alter ribosomal proteins.

Our findings support this hypothesis. The *car* mutant cells are resistant to carbomycin and also to the related macrolides, spiramycin and oleandomycin, and their chloroplast ribosomes show parallel resistance to carbomycin and spiramycin in protein synthesis *in vitro*.

A further similarity between bacterial and chloroplast systems lies in the linkage relations of genes conferring resistance to antibiotics and presumably (in some instances, known to be) coding for ribosomal proteins. The gene order *str-nea-kan-ery-spc* reported in *Bacillus subtilis* (17) closely resembles the gene order *sm2-nea-(ery,car,spi,ole)-spc* that we have found in *Chlamydomonas* (1).

Although the existence of chloroplast DNA has been known since 1963 (18, 19) and cytoplasmic genes affecting chloroplast development were first described in 1909 (1), as yet not a single protein has been definitely identified as a chloroplast gene protein. The evidence that erythromycin-resistant strains of *E. coli* have an altered protein, 50-8, of the 50S ribosomal subunit (20), increases the likelihood that the *car* gene product is a protein of the chloroplast 50S ribosomal subunit.

Supported by NIH Grant GM-13970 and American Cancer Society Grant VC-32A.

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