Proc. Nat. Acad. Sci. USA
Vol. 71, No. 5, pp. 1715–1719, May 1974

Localization of Five Antibiotic Resistances at the Subunit Level in Chloroplast Ribosomes of *Chlamydomonas*

(chloroplast genes/ribosomal subunit/*in vitro* polypeptide synthesis)

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Communicated by M. M. Rhoades, January 21, 1974

ABSTRACT **The chloroplast ribosomes from five antibiotic resistant strains of *Chlamydomonas*, each carrying one mutant gene mapping in chloroplast DNA, have been shown to be resistant to the corresponding antibiotic in a poly(U)-directed amino-acid incorporating assay system. The alteration conferring resistance was localized to the 30S subunit in ribosomes from streptomycin, neamine, and spectinomycin resistant strains, and to the 50S subunit in ribosomes from cleocin and carbomycin resistant strains. Spectinomycin resistant ribosomes showed no cross-resistance to any other drugs, but limited cross-resistance was noted with the other mutant ribosomes. The similarity between these findings and results reported by others with bacterial ribosomes supports our hypothesis that at least some chloroplast ribosomal proteins are coded by genes in chloroplast DNA.**

Organelle DNAs with coding potential for many proteins are present in the mitochondria and chloroplasts of all eukaryotic cells (1). In general, chloroplast DNAs with a genomic size of at least 10^8 daltons (2) may code for several hundred proteins, while the 5-μm circular mitochondrial DNAs of animal cells may code for no more than 20 to 30 proteins (3). A well-established function of organelle DNAs is as template for the transcription of organelle ribosomal and transfer RNAs (1); the evidence is accumulating that some proteins associated with organelle function, i.e., photosynthesis and respiration, are determined by organelle genes (4, 5). Are some or all of the organelle ribosomal proteins also coded by the corresponding organelle DNA?

This paper presents evidence that 70S chloroplast ribosomes isolated from each of five antibiotic resistant mutant strains of *Chlamydomonas* are resistant to the corresponding antibiotic, as a result of alterations localized to the level of ribosomal subunits. Mutations conferring resistance to streptomycin (*sm2*), neamine (*nea*), spectinomycin (*spc*), carbomycin (*car*), and cleocin (*cle*) each show non-Mendelian inheritance and map in a single linkage group (1, 6, 7). Results to be reported here provide further evidence that this linkage group is located in chloroplast DNA; 70S ribosomes and reassociated subunits from wild-type and five resistant mutant strains each carrying one of these mutant genes were examined for their antibiotic resistance in a poly(U)-directed phenylalanine incorporating system. Resistances to streptomycin, neamine, and spectinomycin were localized to the 30S subunit, and resistances to cleocin and carbomycin to the 50S subunit. The pattern of subunit localization, mirroring that found in bacterial ribosomes (8) as well as the low levels of cross-resistance between mutants, provide strong, albeit indirect, evidence that each of the mutant genes responsible for resistance codes for a chloroplast ribosomal protein. Several lines of evidence that non-Mendelian mutations to drug resistance in *Chlamydomonas* induce alterations in chloroplast ribosomes have been previously reported (7, 9–13).

MATERIALS AND METHODS

Cells. Wild-Type strain *21 gr*, media, and growing conditions have been previously described (7). Non-Mendelian mutant strains—each resistant to one of the antibiotics streptomycin, spectinomycin, carbomycin, and cleocin—were selected after streptomycin mutagenesis (14) of wild-type strain *21 gr* by growth on antibiotic-containing agar. The neamine resistant strain was obtained from Dr. N. W. Gillham. Growth characteristics of the strains and mapping of the genes *sm2*, *nea*, *car*, *spc*, and *cle* were previously described (1, 6, 7).

Fractionation. The S-30, S-122, and ribosome fractions were prepared as before (7), with the following modifications. Cells were collected by centrifugation, washed in Buffer A (25 mM Mg acetate, 25 mM KCl, 25 mM Tris·HCl at pH 7.8, 0.25 M sucrose, and 6 mM 2-mercaptoethanol) and sonicated 3–4 min to about 90% cell breakage. Ribosomes were prepared by centrifuging either the S-30 fraction or a resuspended ribosomal pellet through a 10–30% sucrose gradient in Buffer A for 4.5 hr at 39,000 rpm (SW40 Ti rotor, Spinco L2-65B). Gradients were fractionated on an Isco Gradient Fractionator and monitored with an Isco UA-4 Absorbance Monitor, single beam optics at 254 nm, 2-mm light path.

Subunits of chloroplast ribosomes were prepared as adapted from Chua *et al.* (15). Chloroplast ribosomes were sedimented at 40,000 × *g*, 15 hr, 50 Ti rotor, resuspended in 0.1 ml of iced distilled water, and brought to subunit buffer concentration (25 mM Mg acetate, 50 mM Tris·HCl at pH 7.5, 400 KCl, and 6 mM 2-mercaptoethanol). Ribosomes were centrifuged in a 5–20% sucrose gradient in subunit buffer at 20° (3 hr, 39,000 rpm, SW40 Ti, Spinco L2-65B); 0.25-ml fractions were collected and absorbance read at 260 nm (Gilford spectrophotometer).

Protein Synthesis. The reaction mixture was modified from that previously described (7). The final volume was 0.15 ml; purified ribosomes or subunits and the S-122 fraction were used in all experiments. For chloroplast (70S) ribosome activity, 25 mM Mg^{++} was used in experiments with streptomycin, spectinomycin, and neamine, and 18 mM Mg^{++} with 0.5 mM spermidine was used in experiments with carbomycin and cleocin. For cell-sap (80S) ribosome activity, 10 mM Mg^{++} was used in experiments with the first three drugs, and 8 mM Mg^{++} with 0.5 mM spermidine was used in ex-

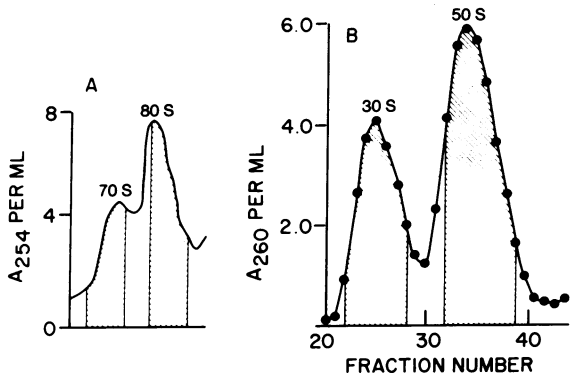

FIG. 1. Preparation of ribosomes. (*A*) Separation of chloroplast (70 S) and cell-sap (80 S) ribosomes on a 10–30% sucrose gradient in 25 mM Mg^{++}. As described in *Materials and Methods*, 45 A_{260} units of S-30 fraction were layered and centrifuged. Only the lower half of the gradient is shown. *Shaded areas* indicate fractions pooled for incorporation studies. (*B*) Preparation of chloroplast ribosome subunits. For this preparation, 20 A_{260} units of chloroplast ribosomes were centrifuged through a 5–20% sucrose gradient at 20° in 400 mM KCl. The 30S and 50S peak fractions were pooled as indicated.

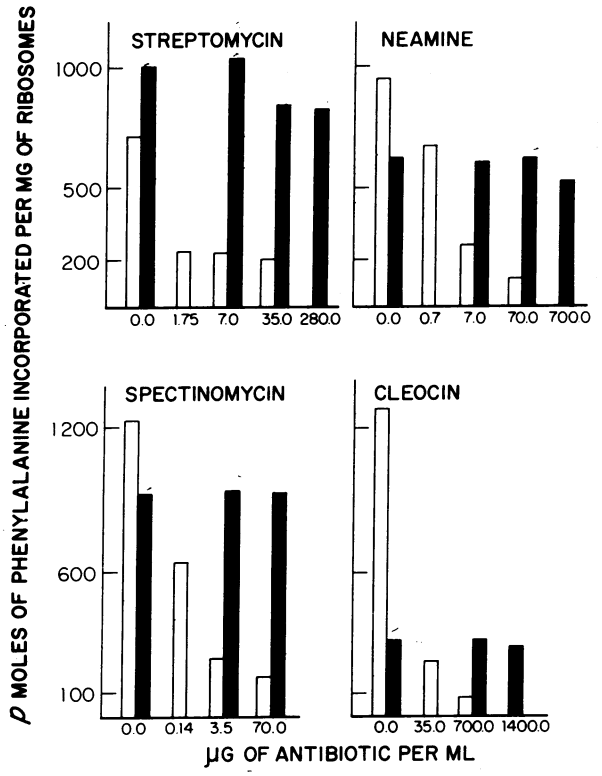

FIG. 2. Effect of antibiotics on phenylalanine incorporation with wild-type and mutant chloroplast ribosomes and homologous S-122 fractions. *White bars*, system containing wild-type 70S ribosomes and S-122 fraction; *black bars*, mutant 70S ribosomes and S-122 fraction. Source of mutant components was *sm2*, *nea*, *spc*, and *cle*, in the streptomycin, neamine, spectinomycin, and cleocin experiments, respectively. The wild-type 70S systems contained 4.5 μg of ribosomes and 0.126 to 0.168 A_{280} units of the S-122 fraction. Mutant systems contained 4.5 μg of *sm2* and *nea* ribosomes, 4.6 μg of *cle* ribosomes, and 6.6 μg of *spc* ribosomes. In these systems, 0.138 to 0.168 A_{280} units of S-122 fraction were employed, except for 0.060 A_{280} units in the *cle* mixture. Incubation time was 1–2 hr in different experiments.

periments with carbomycin and cleocin. For activity of reassociated chloroplast ribosome subunits, 30 mM Mg^{++} was used in experiments with the first three drugs and 25 mM Mg^{++}, with 3.3 mM spermidine was used in experiments with carbomycin and cleocin. Subunits were stored and used in protein synthesis as pooled fractions from the gradient, and the incubation mixture was 70 mM KCl. Ribosomes were the last component added (50 S before 30 S), mixtures were incubated, and counts obtained as previously described (7). Ribosome concentration was calculated from A_{260} by assuming 14.4 A_{260} = 1 mg of ribosomes.

To maximize comparability, results are reported as final yield of trichloroacetic acid precipitable counts after 1 or 2 hr, rather than as incorporation rates. Time course studies (ref. 7, and unpublished material) showed slow continuing incorporation at reduced rates after 30–40 min with no loss by degradation for at least 2 hr in the purified systems used in these experiments.

Materials. Pyruvate kinase was obtained from Boehringer-Mannheim. Carbomycin was a gift from Pfizer, streptomycin sulfate was a gift from Merck, and spectinomycin, cleocin, and neamine were gifts from Upjohn. Other special components were obtained as previously noted (7).

RESULTS

Isolation of Ribosomes. In whole cell extracts of *Chlamydomonas*, two ribosome populations have been demonstrated: 80S particles from the cell-sap and 70S from the chloroplast (9, 16, 17), the latter comprising about 35–40% of the total (7, 18). Separation of the ribosomal species on a 10–30% sucrose gradient is shown in Fig. 1*A*. The shaded areas denote fractions pooled for incorporation studies in this and previous work (7). Fig. 1*B* shows the subunit pattern obtained when chloroplast ribosomes were centrifuged through a sucrose gradient in 400 mM K^+ at 20°; 30S and 50S subunits were pooled as indicated.

Effect of Antibiotics on Activity of Cell-Sap and Chloroplast Ribosomes from Wild-Type and Resistant Mutants. The wild-type cell is sensitive to antibiotics affecting protein synthesis, including spectinomycin, streptomycin, neamine, carbomycin, cleocin, chloramphenicol, and cycloheximide. The sensitivity of 80S ribosomes to cycloheximide and of 70S ribosomes to chloramphenicol and carbomycin was previously described (7). The sensitivity of 70S ribosomes from wild-type cells to a series of antibiotics (spectinomycin, streptomycin, neamine, and cleocin) in the *in vitro* phenylalanine-incorporating system is shown in Fig. 2. The 80S ribosomes were also tested and found resistant to these antibiotics and carbomycin. The magnitude of the difference between cell-sap and chloroplast ribosomes was at least 500-fold for spectinomycin, 400-fold for streptomycin, 100-fold for neamine, 20-fold for cleocin, and 5000-fold for carbomycin.

Further evidence that the chloroplast ribosome is the target of spectinomycin, streptomycin, neamine, cleocin, and carbomycin in the wild-type cell was obtained from *in vitro* studies employing cell fractions derived from resistant mutants. These mutants show non-Mendelian inheritance, and map in a single linkage group located in chloroplast DNA (1, 6).

Each mutant strain tested carried a single marker: *spc*, *sm2*, *nea*, *cle*, or *car*, determining resistance to spectinomycin, streptomycin, neamine, cleocin, or carbomycin, respectively.

TABLE 1. *Cross-resistance study**

Antibiotic	Source of ribosomes and the S-122 fraction					
	spc	*sm2*	*nea*	*cle*	*car*	wild-type
Spectinomycin						
3.5 μg/ml	[5.1]	1.0	1.7	1.06	1.29	1.0
70.0 μg/ml	[7.2]	1.11	2.1	1.3	1.56	1.0
Streptomycin						
35 μg/ml	0.89	[2.9]	1.09	0.96	1.27	1.0
Neamine						
7 μg/ml	1.06	3.88	[3.56]	N.D.†	1.88	1.0
70 μg/ml	1.0	2.58	[8.25]	0.83	1.25	1.0
Cleocin						
140 μg/ml	0.95	0.90	0.79	[8.4]	0.47	1.0
700 μg/ml	0.71	2.1	0.57	[16.67]	1.1	1.0
Carbomycin						
35 μg/ml	0.81	1.25	0.56	2.75	[6.94]	1.0

* Phenylalanine incorporation by wild-type and each of the five mutant 70S S-122 systems was tested in the absence and presence of five antibiotics at the concentrations shown. In each experiment, the ratio of incorporation in the presence of the antibiotic to incorporation in its absence was calculated for each concentration employed and divided by the ratio obtained in the wild-type system. This second ratio is given in the Table; a value of 1.0 indicates the mutant is as sensitive as the wild-type. Ratios obtained for the homologous mutant shown in boxes were obtained from data of Fig. 3.

† Not done.

The carbomycin resistance of chloroplast ribosomes from the *car* mutant was previously reported (7). The effect of antibiotics on amino-acid incorporating systems employing chloroplast ribosomes and supernatant factors from the other four resistant mutants was compared with the effect on the chloroplast ribosome system from wild-type (Fig. 2).

In the spectinomycin study, the wild-type 70S system was partially inhibited by 0.14 μg of spectinomycin per ml, whereas the spectinomycin-resistant mutant system was insensitive to 70 μg/ml, 500 times the concentration which inhibited the wild-type system. A similar contrast was seen between wild-type and each of the other mutant strains when tested with the corresponding antibiotic. The 80 S, S-122 systems from each of the resistant mutants gave the same resistance as the wild-type 80S system. Time course experiments (unpublished) showed that the antibiotic inhibited both the rate and extent of phenylalanine incorporation in wild-type, but not in the respective mutant.

Chloroplast ribosomes and S-122 fractions from wild-type and each of the mutants were mixed in homologous and heterologous combinations and assayed for incorporating activity, in the absence and presence of the corresponding antibiotic. In each of the mutant-derived chloroplast systems, it was the ribosome and not the S-122 fraction which determined resistance.

Subunit Exchange Experiments. In order to localize the genetic lesion in each of the resistant ribosomes, 30S and 50S subunits (Fig. 1*B*) from wild-type and from each of the mutants were reassociated in four combinations as shown in Fig. 3. The ability of these reassociated ribosomes to synthesize polyphenylalanine was tested in the absence and presence of the antibiotics corresponding to the mutant employed.

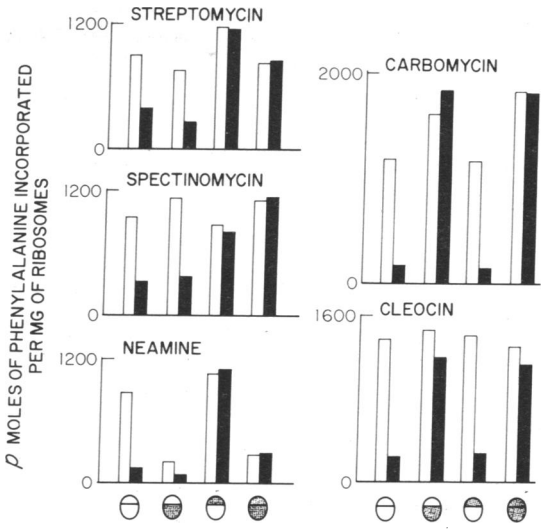

FIG. 3. Effect of antibiotics on incorporating activity by recombined 30S and 50S ribosome subunits from wild-type and resistant mutants. Subunits were reassociated in the four combinations shown. Wild-type subunits are *white;* mutant subunits are *dotted,* and were obtained from the mutant corresponding to the antibiotic indicated in each experiment. Phenylalanine-incorporating activity of reassociated ribosomes was examined in the absence (*white bars*) and presence (*black bars*) of the antibiotic corresponding to the mutant ribosomes. All incubations contained 0.140 A_{280} units of the wild-type S-122 fraction. Where indicated, mixtures contained 0.028 A_{260} units of wild-type 30S subunits and 0.050 A_{260} units of wild-type 50S subunits; 0.025 A_{260} units of mutant 30S particles, except for 0.030 A_{260} units from the *car* mutant; and 0.044 A_{260} units of 50S particles from *cle,* 0.046 from *nea,* 0.050 from *sm2* and *spc,* and 0.060 from *car.* Incubation was for 2 hr in the cleocin experiment, and 3 hr in the others.

Control experiments showed that these systems were dependent on the presence of both subunits. The S-122 fraction from wild-type cells was used throughout.

As shown in Fig. 3, the alteration in each of the mutant chloroplast ribosomes was subunit specific: resistances to streptomycin, spectinomycin, and neamine were each determined by the 30S subunit; resistances to cleocin and carbomycin were each determined by the 50S subunit.

Studies of Cross-Resistance. Incorporating systems (chloroplast ribosomes and the S-122 fraction) from each of the five mutants were assayed for their resistance to each of the antibiotics used in this study. The resistance levels of the homologous systems (e.g., ribosomes and S-122 from the streptomycin resistant mutant tested with streptomycin) were compared with the resistance levels of each of the heterologous systems. In order to standardize the results, the ratio of incorporation in the presence of the antibiotic to the incorporation in its absence was determined. This ratio was then divided by the corresponding ratio of incorporation obtained for the wild-type with the same antibiotic, to give the numbers shown in Table 1.

No significant cross-resistance was seen, except that the *sm2* system showed moderate resistance to neamine, *nea* showed slight resistance to spectinomycin, *cle* showed moderate resistance to carbomycin, and *car* showed slight resistance to streptomycin and neamine and borderline resistance to spectinomycin. Resistance to these antibiotics by the *car*

mutant may be caused by pleiotropic interaction between the subunits. The *spc* system gave no cross-resistance. Additional experiments, with spiramycin and kanamycin, showed that the ribosome system for the *nea* strain was strongly resistant to kanamycin, *car* was strongly resistant to spiramycin, *cle* was moderately resistant to spiramycin, and *sm2* was moderately resistant to kanamycin.

DISCUSSION

This paper has described the antibiotic resistance of chloroplast ribosomes of *Chlamydomonas* extracted from a series of non-Mendelian mutant strains, each resistant to one of the following antibiotics: streptomycin, neamine, spectinomycin, cleocin, and carbomycin. Resistance was assayed by poly(U)-directed phenylalanine incorporation in an *in vitro* system containing purified chloroplast ribosomes and an S-122 fraction derived from each of the strains. The chloroplast ribosomes from each mutant were shown to be resistant to the corresponding antibiotic, with limited cross-resistance towards the other antibiotics. In subunit exchange experiments, the resistances to streptomycin, spectinomycin, and neamine were each localized to the 30S subunit, and resistances to cleocin and carbomycin were localized to the 50S subunit.

These results provide strong, albeit indirect, evidence that each of the non-Mendelian genes conferring drug resistance does so by coding for an altered chloroplast ribosomal protein. Direct evidence in support of this view comes from our current studies in which differentially labeled proteins from the 30S subunit of wild-type and mutant strains are compared by co-chromatography on carboxymethylcellulose. An altered protein of the 30S subunit has been detected in mutant strains carrying the *sm2* gene (Ohta, Inouye, and Sager, in preparation). Further supporting evidence comes from studies in which a non-Mendelian erythromycin resistant strain was shown to contain chloroplast ribosomes with reduced erythromycin binding capacity (10) and with an altered two-dimensional gel electrophoretic pattern as compared with wild type (12). In a non-Mendelian spectinomycin resistant strain, chloroplast ribosomes were reported to have lost spectinomycin-binding ability (11), and changes in sedimentation constants have been reported for chloroplast ribosomes from non-Mendelian strains resistant to streptomycin, spectinomycin, and to neamine (9).

These results are remarkably similar to findings previously described for bacterial ribosomes. Both chloroplast and bacterial ribosomes from wild-type cells exhibit sensitivity to the same set of antibiotics; similar mutations conferring antibiotic resistance and dependence have been described in both systems; and resistances have been localized to the same subunit in *Chlamydomonas* and bacteria (8). Subunit homology between bacterial and chloroplast ribosomes has been shown to be high and hybrid ribosomes are active in polypeptide synthesis (20). Even the map positions of the mutant genes are similar in *Chlamydomonas* (1), and in *Bacillus subtilis* (19), with the genes for streptomycin and neamine resistances on one side, and the gene for spectinomycin resistance on the other side of a cluster of genes for resistance to erythromycin, cleocin, carbomycin, and other antibiotics affecting the 50S subunit. Resistances to numerous drugs have been localized to the ribosome level in bacterial mutants (8), and in a few of them, further identified as a peptide change or amino-acid substitution in a particular ribosomal protein (21–23).

In yeast, mutants resistant to inhibitors of mitochondrial protein synthesis, such as chloramphenicol, erythromycin, and paromomycin, have been mapped in mitochondrial DNA (24). Evidence that the ribosomes themselves are altered by mutation has been obtained for one chloramphenicol and two erythromycin loci by assaying for peptidyl transferase activity in the presence and absence of antibiotics (25). No structural differences in proteins of the large ribosomal subunit were detected by acrylamide gel electrophoresis, but further examination by other methods might provide evidence of whether the ribosomes have been mutationally altered at the protein level.

The fact that *Chlamydomonas* contains both chloroplast and mitochondrial ribosomes raises a problem in the interpretation of drug resistance data. How do mutations in chloroplast DNA that confer resistance upon chloroplast ribosomes to antibiotics such as chloramphenicol, erythromycin, neamine, and carbomycin affect mitochondrial protein synthesis? In other organisms, mitochondrial ribosomes are sensitive to some or all of these drugs. At least three possibilities may be considered: (*i*) mitochondria are impermeable to antibiotics to which the chloroplast is sensitive, (*ii*) mitochondrial ribosomes of wild-type cells are resistant to these antibiotics, or (*iii*) chloroplast genes code for both chloroplast and mitochondrial ribosomal proteins. In the absence of *in vitro* studies of mitochondrial protein synthesis or identification of mitochondrial genes in *Chlamydomonas*, one cannot yet distinguish critically among these possibilities. Mammalian mitochondria, for example, are relatively impermeable to some antibiotics, e.g. erythromycin (25).

This paper provides strong evidence that several cytoplasmic genes presumably located in chloroplast DNA (1)—*sm2*, *spc*, *nea*, *cle*, and *car*—code for proteins of the chloroplast ribosome, and raises the possibility that other genes, such as *sm3*, *sm4*, *spi*, and *ery*, may code for additional proteins. Other work recently reported with *Chlamydomonas* (10, 12), *Acetabularia* (26), and tobacco (4) suggests that some chloroplast ribosomal proteins may be coded by nuclear genes. This interesting possibility would represent yet another instance, like that proposed for RUdP carboxylase (27), mitochondrial F_1-ATPase (3, 5, 24), and cytochrome oxidase (3, 5, 24), of close interdigitation of components coded by nuclear and organelle genomes in cellular coordination.

We are grateful to Dr. Nam-Hai Chua of The Rockefeller University for describing to us his method for preparation of chloroplast ribosome subunits prior to publication (15). This work was supported by grants from the National Institutes of Health (GM-13970), the American Cancer Society, and The City University of New York.

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