CHLOROPLAST RIBOSOME BIOGENESIS IN *CHLAMYDOMONAS*

Selection and Characterization of Mutants Blocked in Ribosome Formation

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ABSTRACT

Chloroplast protein synthesis in *Chlamydomonas reinhardtii* is dispensable when cells are provided acetate as a carbon source. Mutants defective in synthesis, assembly, or function of chloroplast ribosomes are therefore conditionally viable. Positive selection of nonphotosynthetic cells on arsenate has been combined with a simple screening procedure to isolate mutants with a broad spectrum of defects in chloroplast protein synthesis. Eight new mutants deficient in chloroplast ribosomes have been isolated. Three of these have been characterized genetically and phenotypically, and compared with two previously described ribosome mutants, *ac-20* and *cr-1*. A working model of ribosome assembly is proposed which suggests possible biochemical roles for these five Mendelian gene loci.

Protein synthesis is generally regarded as an indispensable function, and mutants defective in ribosome structure, assembly, or function would normally be lethal. Under permissive conditions, however, the protein-synthesizing systems of mitochondria of baker's yeast, *Saccharomyces cerevisiae*, a facultative anaerobe (cf. Mahler, 1973), and of the chloroplast of the green acetate flagellate, *Chlamydomonas reinhardtii* (Boynton et al., 1972, 1973; Goodenough and Levine, 1971) appear to be dispensable. In addition, variegated plastome mutants of barley, cotton, and *Pelargonium* have been described in which the white sectors show a deficiency of chloroplast ribosomes while green sectors are normal and apparently serve to support growth of the chloroplast ribosome-deficient tissue (Börner et al., 1973; Hagemann et al., 1973; Katterman and Endrizzi, 1973).

In *Chlamydomonas*, chloroplast protein synthe-sis appears to be necessary only for photosynthetic growth (Boynton et al., 1973); mutants blocked in chloroplast ribosome formation or function are thus conditionally viable when supplied with acetate (Boynton et al., 1972; Goodenough and Levine, 1971). *Chlamydomonas* therefore offers a unique potential for identification of genes affecting components of a protein-synthesizing system. For such a model system to be useful experimentally, positive techniques are needed for selection and identification of suitable mutants. Genetic control of a limited number of ribosomal components can be studied by using antibiotic resistance mutants, which are readily isolated in *Chlamydomonas* (Boynton et al., 1973; Burton, 1972; Gillham et al., 1970; Mets and Bogorad, 1971, 1972; Schlanger et al., 1972; Schlanger and Sager, 1974), as they are in bacteria. In this paper we report the use of a positive selection method involving preferential killing of photosynthetic

cells by arsenate (Togasaki and Hudock, 1974), and a screening protocol (Harris et al., 1973) which yields a broad spectrum of mutants with defects in chloroplast protein synthesis unrelated to antibiotic resistance. These include mutants blocked in formation of chloroplast ribosomes. Three new Mendelian mutants deficient in chloroplast ribosome monomers are compared phenotypically and genetically to the previously described chloroplast ribosome mutants ac-20 and cr-1 (Boynton et al., 1970, 1972; Goodenough et al., 1971). From our data we have constructed a formal scheme of ribosome assembly which indicates possible functional roles for each of these five nuclear gene loci. This scheme in turn can be used to suggest biochemical approaches to verify the molecular defects in each of the mutants.

MATERIALS AND METHODS

Growth Conditions

Haploid and diploid vegetative clones of C. reinhardtii strain 137c were cultured at 25°C under ~6,000 lx cool white fluorescent light on 1.5% agar plates of Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965), and on TAP or on high salt liquid medium with (HSA) or without (HS) 0.2% acetate (Sueoka, 1960), in 300-ml shake cultures under ~15,000 lx cool white fluorescent light. No significant differences were found in photosynthetic activity or whole cell ribosome profiles between cells grown to midlog phase on TAP and HSA liquid medium.

Mutagenesis and Arsenate Selection of Acetate-Requiring Mutants

Cells grown in TAP liquid medium to midlog phase in the light were harvested and resuspended at a density of 3.8×10^6 cells/ml in 0.02 M sodium citrate, pH 5.0, containing 1 μg/ml N-methyl-N'-nitro-N-nitrosoguanidine (Lee and Jones, 1973). The suspension was shaken in darkness for 30 min at 25°C, centrifuged, and washed once with citrate buffer and twice with TAP medium. After the density had been adjusted to 5×10^5 cells/ml, 1-ml aliquots were dispensed into 100 individual tubes (13×100 mm). These tubes were shaken under 15,000 lx constant illumination for 24 h to allow about four cell doublings. Cells were then plated on to TAP agar containing sodium arsenate and incubated in light. This procedure allowed expression of the mutant phenotype but ensured that mutants isolated from different plates were of independent origin. The concentration of arsenate must be adjusted for light intensity and for strain differences. Under our conditions, 1 mM arsenate for haploid cells and 3 mM for diploids gave a survival rate of approximately 1×10^{-4} in unmutagenized cultures.

Green colonies were picked as they appeared on the TAP-arsenate plates and transferred to TAP-arsenate master plates, which were subsequently replicated to TAP + light (mixotrophic), TAP + dark (heterotrophic), and minimal medium + light (phototrophic). Minimal medium contains K_2HPO_4/KH_2PO_4, 6.8 mM, pH 7.0; NH_4Cl, 7.5 mM; $MgSO_4$, 0.4 mM; $CaCl_2$, 0.3 mM, plus the trace elements of Hutner et al. (1950).

Cells unable to grow on minimal medium (acetate requirers) were selected and grown to midlog phase in 300-ml TAP liquid cultures. Photosystem II (Hill reaction activity) was assayed as photoreduction of dichlorophenol-indophenol (Boynton et al., 1973; Hoober et al., 1969). RuDPCase activity was measured by a modification of the procedure of Boynton et al. (1972).

Cell suspensions (2–4 ml, 5×10^7 cells/ml) in buffer A of Hoober and Blobel (1969) without sucrose were broken at 5,000 lb/in² in a ⅜-in diameter French pressure cell of an Aminco model 5-598A motor-driven press (American Instrument Co., Inc., Travenol Laboratories, Inc., Silver Spring, Md.) and centrifuged at 40,000 g for 20 min at 2°C. A 100-μl aliquot of enzyme (1/1–1/8 dilution of supernate with 0.5 mg/ml bovine serum albumin in buffer A without sucrose) was added to 900 μl of reaction mixture containing 50 μmol Tris-HCl, pH 7.5; 2.5 μmol $MgCl_2$; 1.0 μmol dithiothreitol; 0.1 μmol ribulose diphosphate (RuDP) (Sigma Chemical Corp., St. Louis, Mo.); 40 μmol $KHCO_3$; and 8 μCi $NaH^{14}CO_3$. $NaH^{14}CO_3$ (New England Nuclear, Boston, Mass., 50 mCi/mmol) was mixed with $KHCO_3$ to a final concentration of 200 μCi/mmol HCO_3^-, frozen, and thawed immediately before use. After incubation for 20 min at 25°C, the reaction was stopped with 0.5 ml of glacial acetic acid. Samples were transferred to open scintillation vials and dried at 80–100°C to drive off unfixed $^{14}CO_2$. The residue was dissolved in 0.1 ml water and counted in 10 ml Bray's scintillation fluid in a Beckman LS-233 scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a dpm controller programed to correct for sample quenching and to calculate micromoles CO_2 fixed per hour per reaction tube. Our modified procedure has resulted in about a twofold increase in RuDPCase activity over values reported by Boynton et al. (1972, 1973).

10-ml cell samples for chlorophyll and whole cell protein determinations were pelleted at 12,000 g and frozen. The pellet was resuspended and chlorophyll extracted with 80% acetone in dim light for 20 min. After centrifugation for 10 min at 12,000 g, total chlorophyll was determined in the supernate by the method of Arnon (1949). The pellet was dissolved in NaOH and assayed for protein (Boynton et al., 1972).

Whole cell ribosome profiles of putative mutants grown in TAP or HSA liquid cultures were analyzed on 10–30% sucrose gradients (Tris-HCl, pH 7.5, 25 mM; $MgCl_2$, 25 mM; KCl, 25 mM) without Triton addition (method modified from Bourque et al., 1971). Gradients (17 ml) were centrifuged for 15 h at 22,500 rpm, 4°C, in a

Spinco SW-27.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and scanned at 254 nm using ISCO density gradient fractionating equipment. Optical density traces were made over a spread of approximately 22 cm using a Sargent recorder, to facilitate comparison of peak areas by cutting and weighing Xerox copies of such traces.

Electron Microscopy

Cells grown on TAP liquid medium were harvested by centrifugation, washed once with HSA medium, and fixed for 2 h at room temperature in a equal volume mixture of 4% glutaraldehyde (70%, Ladd Research Industries, Inc., Burlington, Vt.) in 0.004 M Na_2HPO_4-KH_2PO_4, pH 7.0, and HSA culture medium. After three washes in the phosphate-HSA buffer, they were postfixed in 2% OsO_4 in 0.1 M phosphate buffer, pH 7.0, for 2 h at room temperature. After dehydration in ethanol, cells were embedded in a low viscosity epoxy resin (Spurr, 1969). Sections cut with a DuPont diamond knife (E. I. DuPont de Nemours & Co., Wilmington, Del.) on a Cambridge ultramicrotome (Cambridge Instrument Co., Ossining, N.Y.) were mounted on naked 75 × 300 mesh grids and contrasted with uranyl acetate (saturated solution in 70% ethanol) and lead citrate.

Genetic Analysis

Standard techniques of crossing, tetrad analysis, and diploid formation were used (Levine and Ebersold, 1960; Levine and Goodenough, 1970; Gillham, 1963; Ebersold, 1967). Gametes were differentiated overnight in N⁻ liquid medium (HSA without NH_4Cl). Zygotes were matured in light on N⁻ plates containing 4% agar (K. Van Winkle-Swift, manuscript in preparation). Products of crosses were scored for acetate requirement by streak tests on HS plates.

Diploids used to assess complementation between ribosome mutants were isolated on HSA plates with the complementary arginine auxotrophic markers arg-2 and arg-7. Complementation for ribosome function was tested with respect to RuDPCase activity, Hill reaction activity, and ribosome profile as described above.

Double mutants were identified among progeny of crosses between two mutant parents by a rapid technique which exploits the observed ability of the five mutants studied here to complement one another in diploids with respect to photosynthetic function (see Results). This quick diploid method has general utility for genotypic identification in complementing mutants without the necessity of backcrossing. In a tetratype tetrad (e.g., 3:1 acetate-requiring, acetate-independent), one acetate-requiring product will complement with each parent (the two opposite parental types) and the third will complement with neither. Gametes from each tetrad product and each parent tester stock were mated for 1 h and the suspensions plated on HS agar at a density of 0.5-1 × 10⁵ cells/plate. Plates were incubated in light for 4 days before scoring under a dissecting microscope for diploid formation. Diploids formed between complementing mutants appear at this time as discrete, bright green, raised colonies and are readily distinguished from the background of ungerminated zygotes and unmated gametes. When one of the mutant strains tested is leaky, i.e., grows appreciably on HS medium, resolution can be improved by using tester stocks carrying an additional auxotrophic marker, e.g. arg-7, to reduce growth of unmated tester gametes.

RESULTS

Selection of Chloroplast Protein Synthesis Mutants

The protocol used to isolate chloroplast ribosome mutants (Fig. 1) relies on preferential killing of photosynthesizing cells by arsenate, leaving nonphotosynthetic (acetate-requiring) cells as survivors. This principle was suggested by Schneyour and Avron (cf. Schiff et al., 1971) for isolation of photosynthetic mutants of Euglena, and has been tested in reconstruction experiments with Chlamydomonas mutants deficient in photosynthetic electron transport and Calvin cycle enzymes by Togasaki and Hudock (1974).

We have confirmed these results and the validity of the method for isolation of chloroplast protein synthesis mutants in reconstruction experiments using ac-20 and cr-1 (Harris et al., 1973). In mutagenesis experiments using 1-5 μg/ml nitrosoguanidine, we find that acetate-requiring colonies comprise about 1-2% of arsenate-tolerant colonies tested (Table I). Among these acetate-requiring colonies (Fig. 1, Class II), mutants with deficiencies in chloroplast protein synthesis can be identified by a syndrome of chloroplast defects including low ribulose diphosphate carboxylase (RuDPCase) activity, and deficiency of Photosystem II electron transport (Hill reaction activity), as well as characteristic alterations in lamellar organization (Boynton et al., 1972, 1973; Goodenough et al., 1971). Mutants specifically deficient in RuDPCase or Photosystem II are also recovered in good yields, as are other acetate-requiring mutants with normal RuDPCase and Hill reaction activities.

More than 95% of the arsenate-tolerant colonies picked after mutagenesis are resistant to arsenate under all three growth conditions employed (Fig. 1, Class I). These may represent either permeability mutants or phenocopies of such mutants. Mutants which grow on minimal medium but die
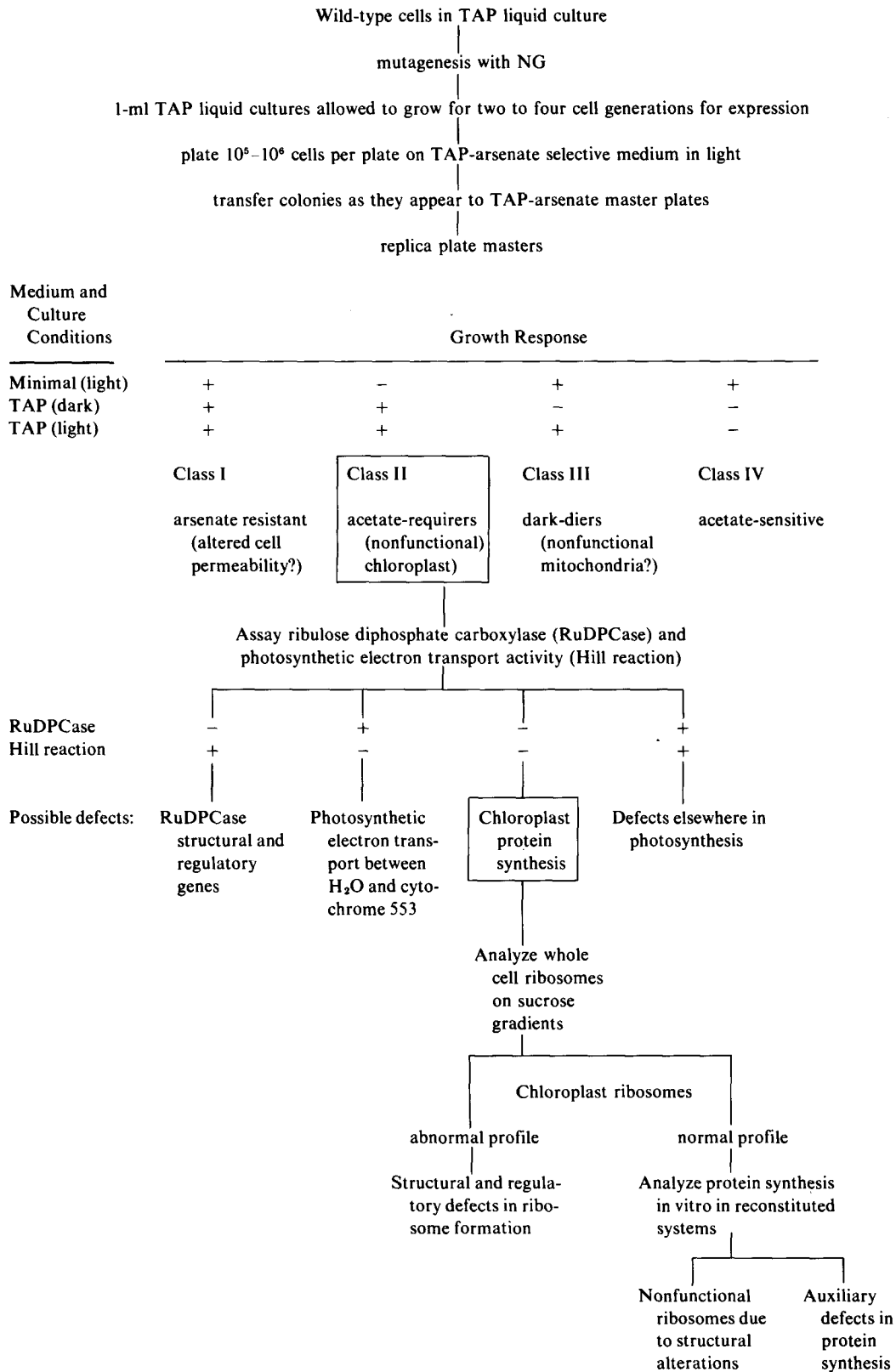
Wild-type cells in TAP liquid culture
|
mutagenesis with NG
|
1-ml TAP liquid cultures allowed to grow for two to four cell generations for expression
|
plate 10^5–10^6 cells per plate on TAP-arsenate selective medium in light
|
transfer colonies as they appear to TAP-arsenate master plates
|
replica plate masters

Medium and
Culture
Conditions

Growth Response

Minimal (light)	+	–	+	+
TAP (dark)	+	+	–	–
TAP (light)	+	+	+	–

Class I

arsenate resistant
(altered cell
permeability?)

Class II

acetate-requirers
(nonfunctional)
chloroplast)

Class III

dark-diers
(nonfunctional
mitochondria?)

Class IV

acetate-sensitive

Assay ribulose diphosphate carboxylase (RuDPCase) and
photosynthetic electron transport activity (Hill reaction)

RuDPCase	–	+	–	+
Hill reaction	+	–	–	+

Possible defects:

RuDPCase
structural and
regulatory
genes

Photosynthetic
electron trans-
port between
H_2O and cyto-
chrome 553

Chloroplast
protein
synthesis

Defects elsewhere in
photosynthesis

Analyze whole
cell ribosomes
on sucrose
gradients

Chloroplast ribosomes

abnormal profile

normal profile

Structural and regula-
tory defects in ribo-
some formation

Analyze protein synthesis
in vitro in reconstituted
systems

Nonfunctional
ribosomes due
to structural
alterations

Auxiliary
defects in
protein
synthesis

FIGURE 1   Protocol for positive selection and identification of mutants with structurally and functionally
defective chloroplast ribosomes. NG, nitrosoguanidine; TAP, Tris-acetate-phosphate medium.

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Recovery of Acetate-Requiring Colonies After Nitrosoguanidine Mutagenesis and Arsenate Selection as in Fig. 1

	Haploid				Diploid		
	Time of plating (h)				Time of plating (h)		
	0	0	24	48	0	0	24
NG mutagenesis							
(1 μg/ml, 30 min, 25°C)	0	+	+	+	0	+	+
Number of doublings	0	0	3.9	6.4	0	0	3.9
Cells plated (× 10^6)	0.22	0.03	0.43	1.2	1.0	0.91	1.4
Plating efficiency	0.53	0.58	—	—	0.52	0.51	0.81
Number of plates	10	20	97	26	9	20	64
Arsenate-tolerant Colonies							
Mean number/plate	15	10	23	34	43	13	78
Frequency (× 10^{-4})	0.68	3.7	0.54	0.29	0.83	0.28	0.63
Acetate-Requiring Colonies							
Number isolated	1	3	23	15	0	0	1 (mixed colony)
Frequency in total cell population (× 10^{-7})	9.3	61	7.7	8.8	—	—	0.77

Wild-type haploid mt^+ and diploid (arg-2 arg-7$^+$/arg-2$^+$ arg-7) stocks were used.
NG, nitrosoguanidine.

on TAP in the dark (Fig. 1, Class III) may have altered mitochondrial functions and presumably survive in light by virtue of normal photosynthetic ability (A. Wiseman, personal communication). Two mutants have been found which grow on minimal medium or on TAP-arsenate, but die on TAP alone (Fig. 1, Class IV).

Togasaki and Hudock (1974) have advanced several possible hypotheses to account for the preferential killing of photosynthetic cells by arsenate, but consider none of them a fully satisfactory explanation. Arsenate is known to be a competitive inhibitor of phosphate in ATP formation both in photosynthesis and in respiration (Avron and Jagendorf, 1959; Crane and Lipmann, 1953). We might account for the observed arsenate tolerance of mutants of classes II, III, and IV by postulating an intracellular pool of inorganic phosphate shared by mitochondria and chloroplast. In wild-type cells, arsenate would prevent both respiratory and photosynthetic ATP formation in light, with the result that cells die.

Cells blocked in photosynthesis may be unable to form ATP photosynthetically, or may be prevented from utilizing ATP by a block in the carbon cycle. Either condition would result in accumulation of ADP and phosphate. Chloroplast phosphate pools may then be made available to mito-chondria, thus reducing arsenate toxicity sufficiently for respiratory ATP formation to occur. The observation by Togasaki and Hudock (1974) that arsenate is less toxic to wild-type cells in darkness than in light is consistent with this hypothesis. Conversely, a block in mitochondrial ATP formation may allow more phosphate and ADP to be utilized in the chloroplast. Acetate-sensitive mutants (class IV) could have mitochondrial alterations which make acetate-stimulated respiration lethal, perhaps by accumulation of some toxic byproduct. These cells could survive either on arsenate or on minimal medium by repressing mitochondrial functions in favor of photosynthesis. It is worth noting that wild-type cells grow normally on HSA plates at concentrations of arsenate which are lethal in TAP medium, probably because of the 14-fold greater phosphate concentration in the HSA medium.

When stable vegetative diploid cells are subjected to mutagenesis under the same conditions, recessive Mendelian mutations will not be expressed, and most acetate-requiring isolates recovered should be mutants in chloroplast DNA, which segregate vegetatively (Lee et al., 1973). Diploid mutants recovered can be returned to the haploid state by a 2N × N cross (Gillham, 1963). In our experience, such mutants can be isolated

only if cells are allowed several generations for segregation and expression before being challenged with arsenate. Only one diploid acetate-requiring mutant was found in the experiment shown in Table I, and this appeared to be segregating in a mixed colony.

Another possible approach to selection of mutations in chloroplast DNA would be mutagenesis of synchronous haploid cultures during the time of chloroplast DNA replication. However, yields of non-Mendelian (chloroplast) streptomycin-resistant mutants were found by Lee and Jones (1973) to be enhanced only about twofold by this means, and the background of Mendelian mutants was high. The use of diploid parent stocks may thus be preferable (Lee et al., 1973).

Phenotypic Characterization of Chloroplast Ribosome Mutants

In two experiments to test the efficacy of our selection protocol, more than 100 acetate-requiring mutants were recovered. Out of 63 tested, 13 mutants were identified as potentially deficient in chloroplast protein synthesis. Eight mutants have abnormal ribosome profiles characterized by a deficiency of chloroplast ribosome monomers. Three of these are described in detail in the remainder of the paper. Five mutants have normal ribosomal profiles on sucrose gradients but show the syndrome of defects associated with failure of chloroplast protein synthesis. These may make functionally defective ribosomal components which are nevertheless capable of assembly into particles of normal ribosomal size, or they may have defects in auxiliary components of protein synthesis such as tRNAs, activating enzymes, factors, etc.

We have decided to designate all new chloroplast ribosome genes, with the exception of antibiotic resistant mutants, with the symbol cr-, numbered sequentially following the convention established by Boynton et al. (1970). Typical ribosome profiles of cr-2, cr-3, and cr-4, the three new mutants which have been studied in detail, are shown in Fig. 2 together with those of wild type, ac-20, and cr-1. Such ribosome profiles are highly reproducible over a range of 1–15 A_{260} units loaded per gradient, and relative sizes of the various ribosome peaks are absolutely characteristic of an individual mutant (Table II A). Since centrifugation conditions were constant for all gradients, and since the pattern for each genotype

was not altered by varying the amount of ribosomes loaded per gradient, the differences in ribosome profile among the mutants cannot be attributed to pressure or concentration effects (Chua et al., 1973; Infante and Baierlein, 1971).

We have identified ribosome peaks in accordance with the generic classification of Bourque et al. (1971). The major peaks of 83s and 70s represent cytoplasmic and chloroplast ribosome monomers, respectively (Bourque et al., 1971; Chua et al., 1973; Hoober and Blobel, 1969; Surzycki et al., 1970). The 54s particles accumulated by cr-1 were found by Boynton et al. (1970) to contain 23s ribosomal RNA, and are therefore believed to be the large subunit of the chloroplast ribosome. The 54s particles accumulated by cr-2 and cr-3 (Fig. 2, Table II A) are assumed to be similar to those found in cr-1, although RNA analyses have not yet been done. Since deficiency of 41s particles is associated with deficiency of 16s rRNA in cr-1 (Boynton et al., 1970), these particles are thought to be the small chloroplast ribosomal subunit. Bourque et al. (1971) found 66s particles replacing 70s in their ac-20 stock (an ac-20 cr-1 double mutant, Boynton et al., 1970), but did not distinguish 66s particles in wild-type cells. 66s ribosomes are also seen in certain antibiotic-resistant mutants (Gillham et al., 1970), but are not necessarily identical to those seen in ac-20 (Boynton et al., 1970). With increased resolution, Boynton et al. (1972) showed that both 66s and 70s ribosomes could also be distinguished in wild type, ac-20 and cr-1, but considered that both classes represented chloroplast ribosome monomers. In the gradients shown here (Fig. 2), the 66s peak is clearly resolved from the 70s. The finding that the 66s ribosome content of mutants does not vary nearly as much as the 70s (Table II A,B) raises the question of whether these 66s particles are really chloroplast ribosomes after all. Further analysis of RNA content, subunit structure, etc., of purified 66s particles will be required to resolve this issue.

We find that the 70s ribosome content of a given mutant can be correlated with its RuDPCase and Hill reaction activities, but not with chlorophyll (Fig. 3). This supports the contention that chloroplast ribosome function in vivo is specifically required for RuDPCase synthesis and for formation of some photosystem II component, but is not necessary for chlorophyll synthesis (Boynton et al., 1972, 1973; Goodenough and Levine, 1970). It is
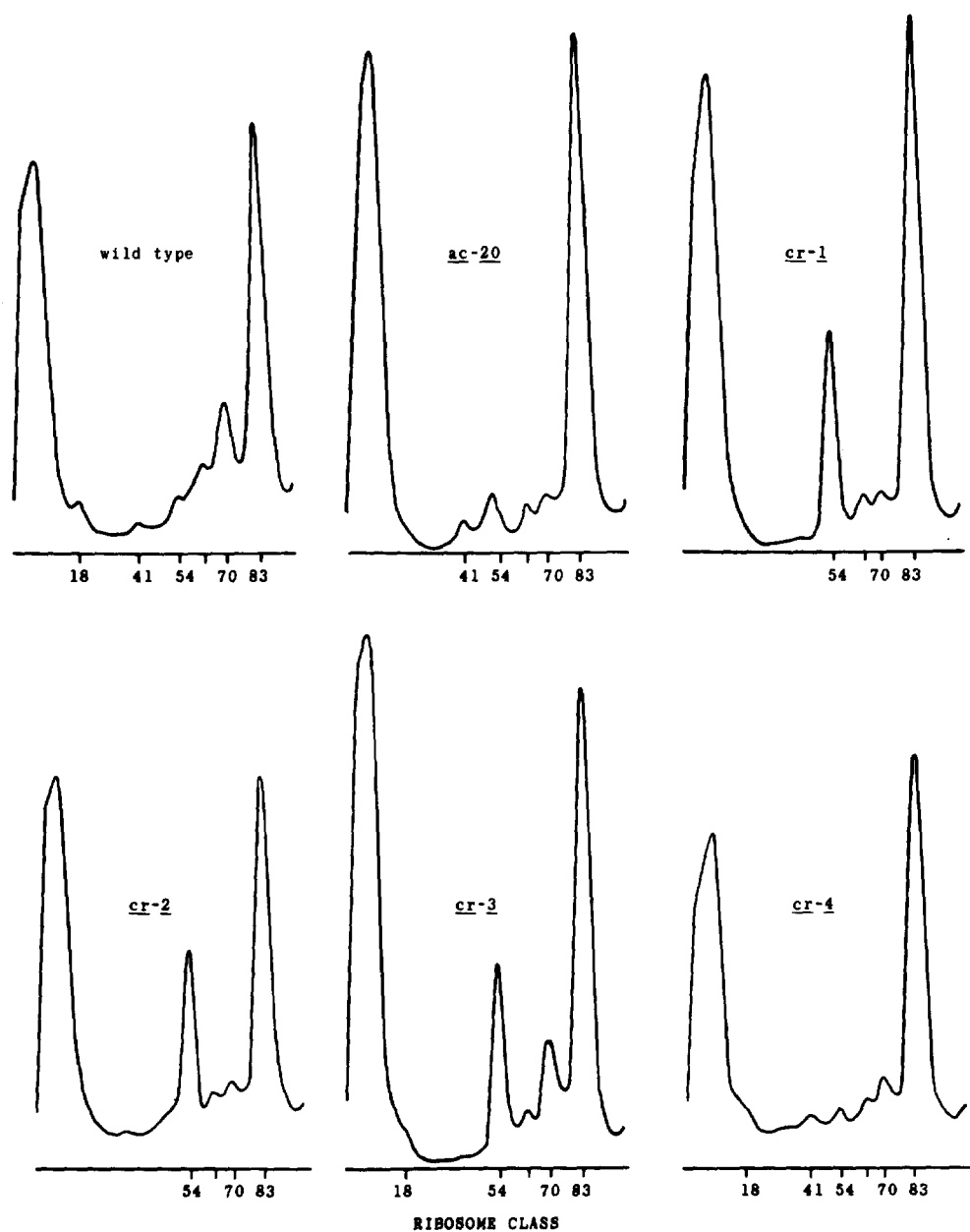
FIGURE 2 Typical ribosome profiles (A_{264}) of wild type and five ribosome-deficient mutants. Ribosome peaks are assigned to generic classes based on approximate sedimentation constant as described by Bourque et al. (1971). 66s peak is indicated between 54s and 70s peaks but is not labeled. 18s peak is attributed to the enzyme aggregate ribulose diphosphate carboxylase (RuDPCase), mol wt > 500,000. This peak is seen only in wild type and in mutants with an appreciable level of RuDPCase activity. Traces are enlarged from 4-in ISCO strip chart recordings, where the 17-ml gradient is displayed over approximately 3 cm spread, with top of gradient to left in each case. Ordinate varies from 0.25 to 2.5 A_{264} full scale setting on chart recorder, depending on the amount of material loaded on individual gradients.

TABLE II A

Mean Distribution of Ribosome Particles After Sucrose Gradient Analysis of Wild Type and Mutants Deficient in Chloroplast Ribosomes

Genotype	Number of determinations	41s	54s	66s	70s	83s
wild type	16	1.1 ± 0.2	3.6 ± 0.3	8.7 ± 0.6	25.4 ± 0.8	61.2 ± 1.2
ac-20	6	4.2 ± 0.8	8.0 ± 0.4	5.0 ± 0.3	6.6 ± 0.9	76.2 ± 2.2
cr-1	12	0.8 ± 0.3	24.6 ± 1.4	4.4 ± 0.3	6.5 ± 0.9	63.7 ± 1.5
cr-2	12	0.6 ± 0.3	23.2 ± 0.8	3.4 ± 0.5	6.7 ± 0.9	66.1 ± 1.8
cr-3	13	0.4 ± 0.2	18.2 ± 1.2	5.5 ± 0.6	17.4 ± 0.9	58.8 ± 2.0
cr-4	8	1.9 ± 0.4	1.4 ± 0.4	5.0 ± 0.7	10.6 ± 1.4	81.2 ± 1.5

Peaks are identified as follows: 41s, small subunit of chloroplast ribosomes; 54s, large subunit of chloroplast ribosomes; 70s and possibly 66s, chloroplast ribosome monomers; 83s, cytoplasmic ribosome monomers (Bourque et al., 1971). In each class, the percentage of the total ribosome area occupied by the peak is given together with the standard error.

TABLE II B

Distribution of Ribosome Particles in Diploids and Double Mutants Determined From Gradients Shown in Fig. 8

Genotype	41s	54s	66s	70s	83s
Diploids					
ac-20/ac-20⁺	2	3	7	24	64
cr-1/cr-1⁺	0	11	5	20	64
cr-2/cr-2⁺	2	5	6	23	64
cr-3/cr-3⁺	1	2	5	23	69
cr-4/cr-4⁺	2	3	5	24	66
ac-20 cr-1⁺/ac-20⁺ cr-1	1	5	4	18	72
ac-20 cr-2⁺/ac-20⁺ cr-2	2	3	6	24	65
ac-20 cr-3⁺/ac-20⁺ cr-3	1	2	8	23	66
ac-20 cr-4⁺/ac-20⁺ cr-4	2	5	6	24	63
cr-1 cr-2⁺/cr-1⁺ cr-2	0	14	4	16	66
cr-1 cr-3⁺/cr-1⁺ cr-3	0	10	3	19	68
cr-1 cr-4⁺/cr-1⁺ cr-4	0	12	5	19	64
cr-2 cr-3⁺/cr-2⁺ cr-3	1	4	6	25	64
cr-2 cr-4⁺/cr-2⁺ cr-4	0	4	5	24	67
cr-3 cr-4⁺/cr-3⁺ cr-4	1	4	6	25	64
Double Mutants					
ac-20 cr-1	1	4	6	5	84
ac-20 cr-2	3	8	5	7	77
ac-20 cr-3	5	9	10	9	67
ac-20 cr-4	3	6	3	4	84
cr-1 cr-2	0	46	6	7	41
cr-1 cr-3	0	28	4	7	61
cr-1 cr-4	2	11	6	7	74
cr-2 cr-3	2	18	3	7	70
cr-2 cr-4	0	15	4	7	74
cr-3 cr-4	0	8	6	17	69

also apparent from Fig. 3 that the 70s ribosomes formed by the relatively leaky mutants cr-3 and cr-4 have normal activity and are thus presumably not defective, whereas those of cr-1 may be partially defective.

Both ac-20 and cr-1 are capable of slow phototrophic growth. Under these conditions, RuDPC-ase and ultrastructure become partially normalized (Boynton et al., 1972; Goodenough and Levine, 1970; Togasaki and Levine, 1970), but chloroplast ribosome deficiency remains unchanged (Boynton et al., 1972). We have repeated these studies with cr-2, cr-3, and cr-4, and have extended our analysis to include Hill reaction activity (Table III). Ribosome phenotypes are not measurably altered in any of the mutants grown under phototrophic conditions. cr-2 and cr-3 resemble ac-20 and cr-1 in showing a substantial increase in RuDPCase activity under phototrophic conditions. However, RuDPCase does not increase appreciably in cr-4. Only ac-20, cr-1, and cr-3 show an increase in Hill reaction activity comparable to the change in RuDPCase when grown phototrophically.

When chloroplast protein synthesis is impaired, either by mutations such as ac-20 and cr-1 (Boynton et al., 1972; Goodenough and Levine, 1970) or by antibiotic inhibition (Boynton et al., 1973; Goodenough, 1971), organization of the chloroplast lamellar system is altered. Chloroplasts of such cells have lamellae which are either unpaired or stacked into giant grana, and in addition lack a pyrenoid. Chloroplasts of cr-2, cr-3, and cr-4 show a similar syndrome of ultrastructural defects (Figs. 4–7), with cr-2 having the most extreme manifestations (Fig. 5) and cr-3 the least (Fig. 6), as compared to wild type (Fig. 4); cr-4 is affected to an intermediate degree (Fig. 7). It is interesting that the severity of the ultrastructural abnormalities directly mirrors the relative deficiencies in 70s ribosomes, RuDPCase, and Hill reaction (Fig. 3).
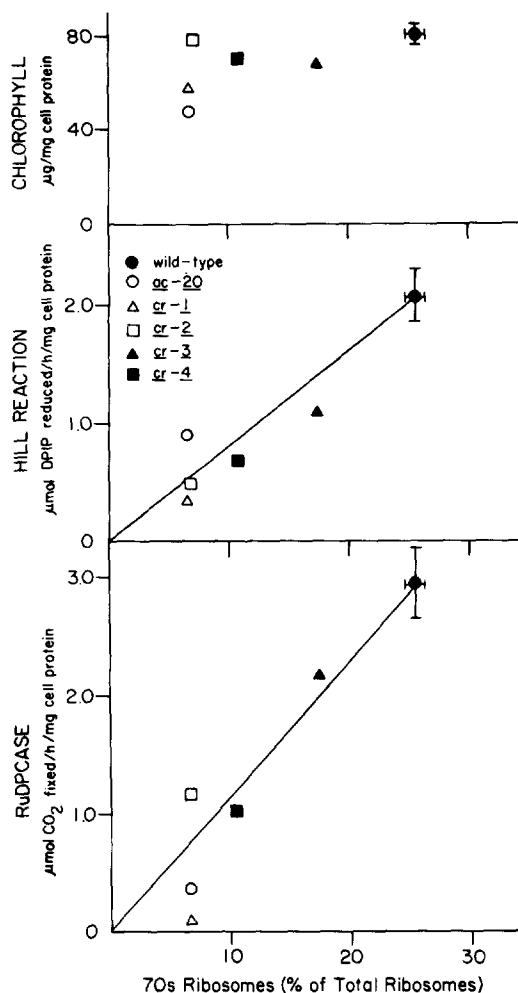
FIGURE 3 RuDPCase activity, Hill reaction activity, and chlorophyll content expressed on a whole cell protein basis, plotted against the 70s ribosome content of wild type and the five chloroplast ribosome-deficient mutants. Individual points are based on the mean of one to five determinations for mutants and 8–10 determinations for wild type. Standard errors are shown for wild type. *DPIP*, dichlorophenol-indophenol.

Genetic Characterization of Chloroplast Ribosome Mutants

ac-20 has previously been mapped to linkage group XIII (Hastings et al., 1965). Although *cr-1* is a Mendelian mutation (Boynton et al., 1970), it has not been mapped. In crosses to wild type, *cr-2*, *cr-3*, and *cr-4* show Mendelian inheritance (Table IV A). Isolates recovered in the minus mating type (*mt⁻*) retain the abnormal ribosome profile char-

acteristic of the plus (*mt⁺*) parent. Each of the five ribosome mutants has been crossed to *arg-2* and *arg-7* and the complementary arginine markers used to isolate diploids of each ribosome mutant with its wild-type allele and with each of the other four ribosome mutants. All five mutants complement one another with respect to photosynthetic activity and ribosome profile. The significance of these results will be considered in the Discussion.

Pairwise crosses in all possible combinations have also been made among the five mutants. Wild-type recombinants and double mutants recovered in each cross further support the conclusion that these mutants represent five distinct loci. Preliminary segregation data (Table IV B) suggest that none of the loci are closely linked. The excess of NPD over PD tetrads observed in two of the crosses may be due to the small sample size, or may be comparable to the cases of excess NPD's between nonlinked genes in *Chlamydomonas* discussed by Gowans (1960). We are now in the process of mapping *cr-1* and the three new mutants. None of the four appears to be linked to *arg-2* or *arg-7* (linkage group I) or to mating type (linkage group VI).

We have repeatedly observed a tendency for stocks of *cr-1* to alter their ribosome phenotype over time, probably by the occurrence of secondary mutations. These changes involve a reduction in 54s subunit accumulation, in some cases accompanied by an increase in 70s ribosomes so that the profile resembles that of wild type, or in other cases with no increase in 70s ribosomes, producing a profile like that of *ac-20*. However, isolates of *cr-1* with the characteristic ribosome phenotype shown in Fig. 2 and Table II A can always be recovered by outcrossing such double mutants. Although *cr-2*, *cr-3*, and *cr-4* have not shown a tendency to revert or accumulate new mutations, our experience with *cr-1* has led us to check frequently the ribosome profiles of all stock cultures, and to verify the ribosome phenotype of all products of at least one tetrad from each cross. All arginine-acetate double mutants were therefore checked for ribosome profile before diploid formation, and all products of at least one tetratype tetrad were checked from all crosses between mutants.

We have compared the individual mutants and all possible double mutants and diploids in terms of amount of chloroplast ribosome monomers, accumulation of large chloroplast ribosomal sub-

units, and function of chloroplast ribosomes in vivo (measured as RuDPCase and Hill reaction activities) (Tables II A,B, and V; Fig. 8). Among the five individual mutants, only cr-1 and cr-2 are indistinguishable with respect to these parameters, and these two mutants show different responses in diploids and double mutants. The discussion will deal in more detail with these comparisons in terms of a model for ribosome assembly.

DISCUSSION

Our working model for ribosome assembly is similar to formal schemes used to deduce enzymatic pathways from studies of mutants and suggests possible biochemical sites of action of the five mutations which produce 70s ribosome deficiency. The rationale for this model can be summarized as follows: (a) the phenotype of an individual mutant demonstrates whether the mutation blocks ribosome formation completely or partially and whether formation of one ribosomal subunit or both is affected. For example, a mutant unable to assemble small subunits will accumulate large subunits and be deficient in monomers; (b) the phenotype of diploids containing one mutant and one wild-type allele indicates whether the mutation is dominant, partially dominant, or recessive. In general, a mutant at a locus which codes for a product required in stoichiometric amounts, i.e. a structural component, would be expected to show partial dominance, since 50% of the ribosome component made in the diploid will be defective. Thus the total number of functional ribosomes per cell will be subnormal although greater than the haploid mutant level. A mutant gene recessive to wild type is likely to affect a catalytic function rather than a stoichiometric one, since a diploid heterozygous for an allele specifying an enzymatic step will probably have a sufficient level of enzyme to allow normal ribosome assembly. However, a stoichiometric mutant for which the wild-type allele overcompensates in function could be confused with a catalytic mutant. Absolute dominance may indicate mutation in a regulatory gene; (c) the phenotype of diploids made from two haploid mutant parents indicates whether the mutants can complement one another in function. Such complementation tests in a system where macromolecular assembly is being studied can give information not only about allel-

TABLE III

Ribulose Diphosphate Carboxylase (RuDPCase) Activity, Hill Reaction Activity, and Chlorophyll Content of Wild-Type and the Chloroplast Ribosome-Deficient Mutants

		Hill Reaction		RuDPCase			
		DPIP reduced	DPIP reduced	CO_2 fixed	CO_2 fixed	Chlorophyll	
Genotype	Growth Conditions						
		$\mu mol/min/$ 10^9 cells	$\mu mol/h/mg$ cell protein	$\mu mol/h/$ 10^9 cells	$\mu mol/h/mg$ cell protein	$mg/10^9$ cells	$\mu g/mg$ cell protein
wild type	mixotophic	2.0	2.7	123	2.7	2.6	105
	phototrophic	2.2	3.0	124	4.0	2.6	107
ac-20	mixotrophic	0.5	0.9	19	0.38	2.1	43
	phototrophic	0.8	1.4	80	2.7	1.9	65
cr-1	mixotrophic	0.6	0.5	7.3	0.10	4.1	58
	phototrophic	0.7	1.4	15	0.50	1.9	67
cr-2	mixotrophic	0.6	0.5	27	1.0	4.6	72
	phototrophic	0.4	0.4	80	1.6	5.2	86
cr-3	mixotrophic	0.4	0.8	65	2.2	1.8	62
	phototrophic	1.2	2.2	119	3.6	2.5	79
cr-4	mixotrophic	0.6	0.8	53	1.2	2.8	63
	phototrophic	0.5	1.1	58	2.1	1.9	67

Comparison was made after growth of midlog phase under mixotrophic (HSA liquid, ~ 15,000 lx) and phototrophic (HS liquid, 5% CO_2 in air, ~ 15,000 lx) growth conditions.
Data are from single determinations in which mixotrophic and phototrophic conditions were compared for a given genotype in the same experiment.
DPIP, dichlorophenol-indophenol.
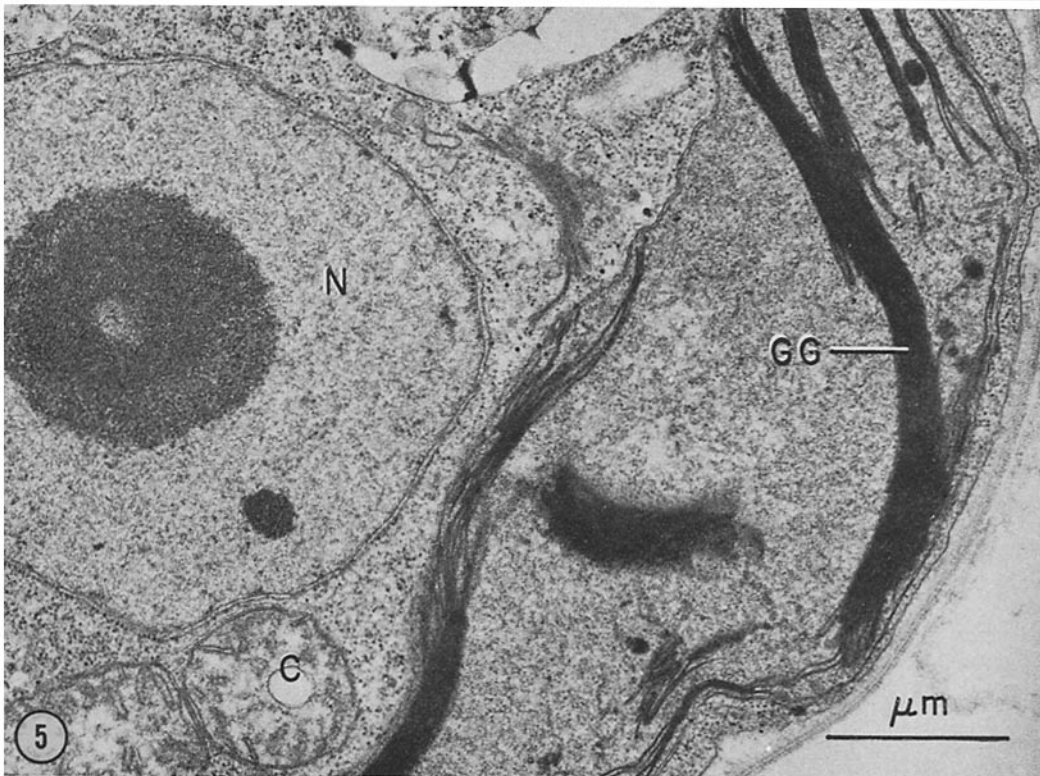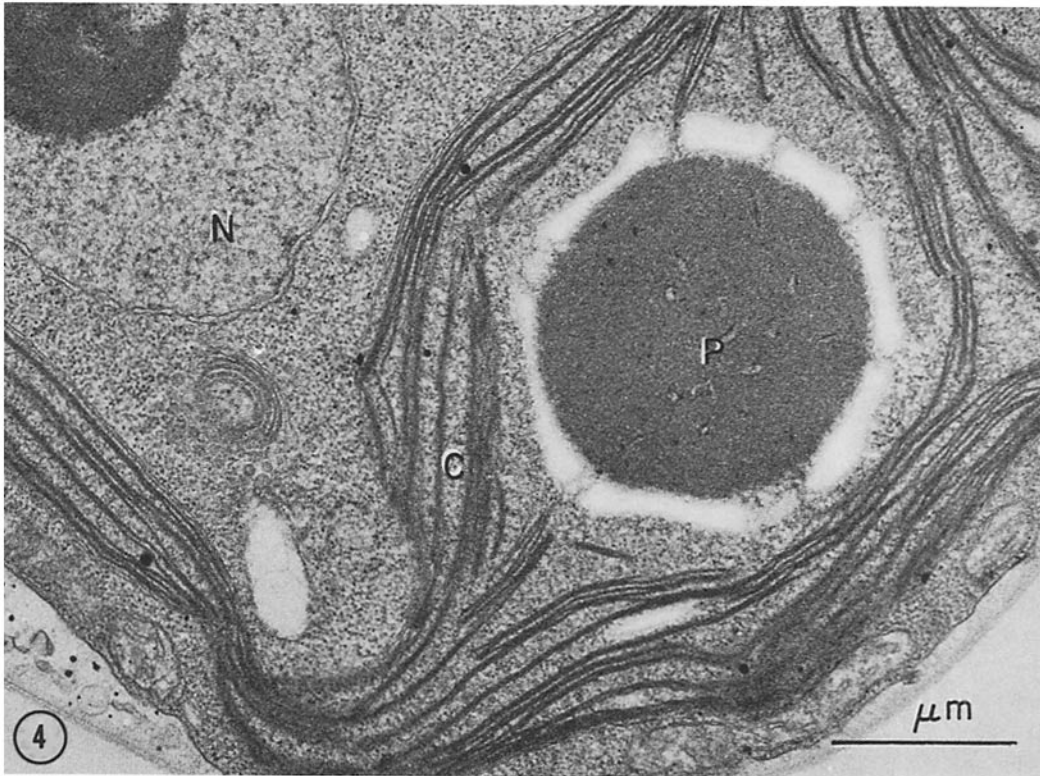
ism, but also about functional relationships in mutants known from recombination frequencies to be nonallelic. Two nonallelic mutants, neither of which is dominant to wild type, presumably involve interrelated functions if they fail to complement one another in a diploid; (d) haploid double mutants can be used in conjunction with data from diploids to order genes in a biosynthetic pathway. In general, a mutation affecting an early step in a pathway will be epistatic to a mutation in a later step. The principal exceptions to this rule should be dominant mutations in regulatory genes, which would be detected by their behavior in diploids.

We have used these criteria to construct the scheme of ribosome assembly shown in Fig. 9. The phenotypes of the five mutants we have studied place them in two groups (cf. Fig. 8, Tables II A, B, and V): ac-20 and cr-4 appear to be involved in synthesis of components common to both the 41s and 54s ribosomal subunits, whereas cr-1, cr-2, and cr-3, all of which accumulate 54s particles, are probably mutations in a pathway specific to 41s subunit formation. cr-1 is partially dominant in diploids with wild type (Fig. 8, Table II B, Table V) and may therefore affect production of a component needed in stoichiometric amounts. ac-20, cr-2, cr-3, and cr-4 are all recessive to wild type and may affect catalytic functions.

All four double mutants carrying the ac-20 allele have the ribosome phenotype of ac-20 (Table II B, Fig. 8). Although neither ac-20 nor cr-4 accumulates 54s subunits, they can be distinguished by the greater percentage of 70s ribosomes in cr-4 (Figs. 2 and 8, Table II A) and correspondingly greater RuDPCase activity (Fig. 3, Table III). cr-4 is partially epistatic to cr-1, cr-2, and cr-3 (Table II B, Fig. 8). We have therefore placed ac-20 and cr-4 earlier in sequence than cr-1, cr-2, and cr-3, generating a forked pathway in which steps common to the synthesis of both subunits precede synthesis of the subunits individually (Fig. 9).

cr-1 and cr-2 are epistatic to cr-3 in double mutants, since none of these double mutants has as great a content of 70s ribosomes as cr-3 (Table II B, Fig. 8), but cannot be ordered relative to each other since their ribosome profiles are indistinguishable. We have placed cr-1 before cr-2 in Fig. 9 with the rationale that the cr-1+ gene may control production of stoichiometric quantities of a ribosome precursor which is then modified by the product of the cr-2+ gene.

We can use this scheme to infer possible biochemical defects in ribosome formation in each of the individual mutants and to suggest ways in which each mutant should be analyzed further: ac-20 has been studied previously and has been found to be deficient in 16s and 23s rRNA (Bourque et al., 1971; Surzycki, cited in Goodenough et al., 1971), and possibly 5s rRNA (Surzycki and Hastings, 1968). The stock of ac-20 used by Bourque et al. (1971) also contained the cr-1 mutation (Boynton et al., 1970). It is not clear whether this is also true of the stock used by Surzycki. Structural genes for chloroplast rRNA are thought to be coded in chloroplast DNA (Surzycki et al., 1970; Surzycki and Rochaix, 1971). Since ac-20 shows Mendelian inheritance, it is probably not a mutation in an rRNA cistron, but is more probably a mutant in some other gene needed for rRNA formation. Goodenough and Levine (1971) have suggested that ac-20 directly affects chloroplast rRNA synthesis. Our data are consistent with this hypothesis, but do not distinguish between a deficiency of chloroplast RNA polymerase and other defects, for example in methylation or cleavage of precursor RNA (Carritt and Eisenstadt, 1973; Kuriyama and Luck, 1973, 1974; Pace, 1973).

cr-4 also appears to be blocked at an early catalytic step in ribosome formation before diversion of the 41s and 54s pathways. Like ac-20, cr-4 may be defective in synthesis or processing of rRNA, but the rRNA content of this mutant has not yet been examined. The slight accumulation of 54s subunits in double mutants of cr-4 with cr-1, cr-2, and cr-3 (Table II B, Fig. 8) is the expected result of combination of a leaky mutant at an early step with a stringent mutant at a later step.

cr-1, cr-2, and cr-3 all accumulate 54s particles and are therefore suspected of being defective in

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FIGURE 4 Median section through mixotrophically grown wild-type cell, showing parts of nucleus (N) with nucleolus, and the cup-shaped chloroplast (C) with a well-developed pyrenoid (P). The chloroplast lamellar system is largely organized into two-disk grana. × 24,000.

FIGURE 5 Median section through mixotrophically grown cr-2 cell, showing parts of nucleus (N) and chloroplast (C). The pyrenoid is absent, and the chloroplast lamellae are stacked into giant grana (GG). × 24,000.

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TABLE IV
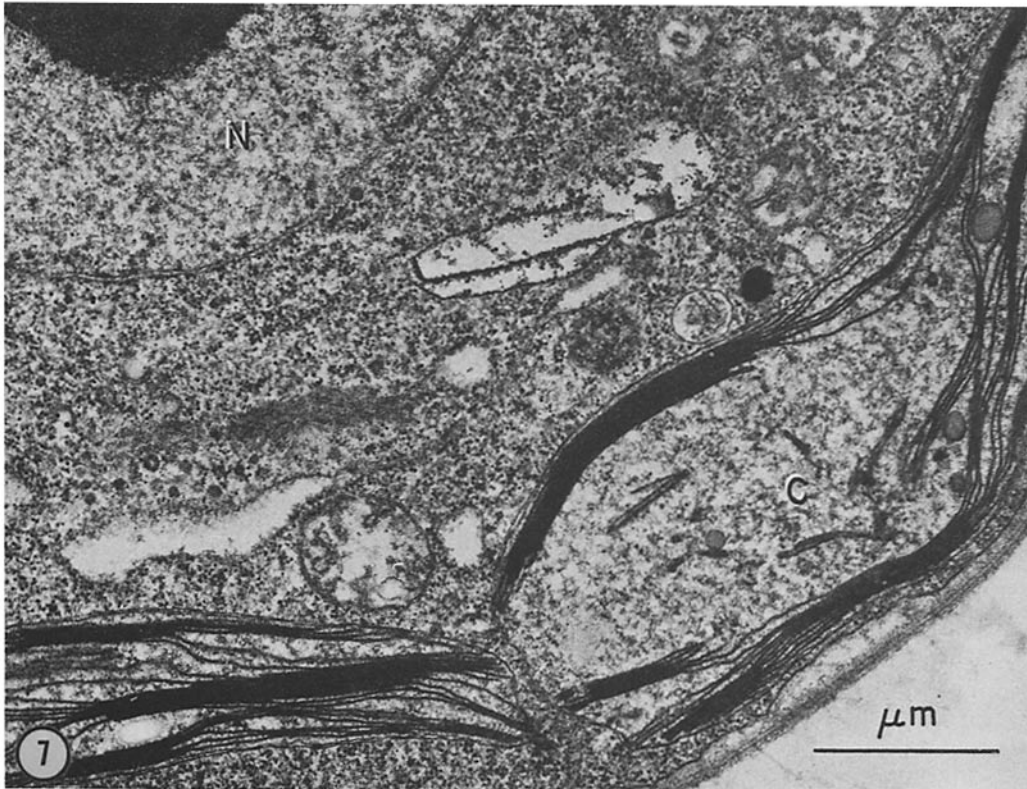
Segregation Patterns of Chloroplast Ribosome Mutations

A. Cross	Tetrads showing 2:2 segregation for acetate requirement	Aberrant tetrads	Total tetrads scored
cr-2 mt+ × cr-2+ mt-	23	2	25
cr-3 mt+ × cr-3+ mt-	11	1	12
cr-4 mt+ × cr-4+ mt-	23	0	23

B. Cross	PD	NPD	T	Total tetrads scored
ac-20+ cr-2 mt+ × ac-20 cr-2+ mt-	1	3	7	11
ac-20+ cr-3 mt+ × ac-20 cr-3+ mt-	1	2	5	8
ac-20+ cr-4 mt+ × ac-20 cr-4+ mt-	1	0	2	3
cr-1+ cr-2 mt+ × cr-1 cr-2+ mt-	0	1	4	5
cr-1+ cr-3 mt+ × cr-1 cr-3+ mt-	5	10	15	30
cr-1 cr-4+ mt+ × cr-1+ cr-4 mt-	1	1	2	4
cr-2 cr-3+ mt+ × cr-2+ cr-3 mt-	2	7	9	18
cr-2 cr-4+ mt+ × cr-2+ cr-4 mt-	5	2	6	13
cr-3 cr-4+ mt+ × cr-3+ cr-4 mt-	8	9	10	27

A. Segregation of acetate-requiring progeny in initial crosses of cr-2, cr-3, and cr-4 to wild type. Aberrant segregations may be attributed to product lethality and extra mitotic divisions of surviving products. Nitrosoguanidine is known to cause product lethality (N.W. Gillham, unpublished observations).

B. Segregation of chloroplast ribosome genes in preliminary crosses made to produce double mutants.

PD, parental ditype (four acetate-requiring: none acetate-independent); NPD, nonparental ditype (two acetate-requiring: two acetate-independent); T, tetratype (three acetate-requiring: one acetate-independent).

synthesis of 41s ribosomal subunits. They can be distinguished by comparing their ribosomal phenotypes as haploids, diploids, and double mutants. Each shows partial functional complementation with the others, and all three are capable of recombination to give wild-type progeny. Thus three distinct loci with related functions in formation of the small chloroplast ribosomal subunit are now identified.

cr-1 appears to have a stoichiometric defect in 41s subunit synthesis, since diploids of cr-1 with its wild-type allele still accumulate some 54s subunits and are slightly deficient in 70S ribosomes (Fig. 8, Table II B). The 54s particles accumulated by cr-1 contain only 23s rRNA, and whole cell preparations are deficient in 16s rRNA (Boynton et al., 1970). Since cr-1 shows Mendelian inheritance, it is presumably not a mutation in a structural gene for 16s rRNA. cr-1 might, however, be defective in a core protein which is essential for assembly of the proteins of the 41s subunit on 16s rRNA and without which 16s rRNA is degraded.

cr-2 and cr-3 are more likely to be defective in catalytic steps in 41s subunit synthesis, since both these mutations are recessive to wild type. Information on the course of ribosome formation in bacteria indicates there may be certain assembly factors which are not themselves part of the mature ribosome structure (Bryant and Sypherd, 1974); cr-2 and cr-3 could be defective in proteins of this type. Another possibility is that one or both of these mutants is deficient in methylation of rRNA, since they are phenotypically remarkably similar to the "poky" strain of

FIGURE 6   Median section through mixotrophically grown cr-3 cell, showing parts of nucleus (N) and chloroplast (C). A deranged pyrenoid (P) can be seen, and chloroplast lamellae are abnormally stacked. × 24,000.

FIGURE 7   Median section through mixotrophically grown cr-4 cell showing parts of nucleus (N) and chloroplast (C). The pyrenoid is absent and chloroplast lamellae are either unpaired or stacked into giant grana. The abnormalities seen in this mutant are more extreme than in cr-3 (Fig. 6), but less extreme than in cr-2 (Fig. 5). The ultrastructural phenotype is very similar to that described previously for ac-20 and cr-1 (Boynton et al., 1972). × 24,000.
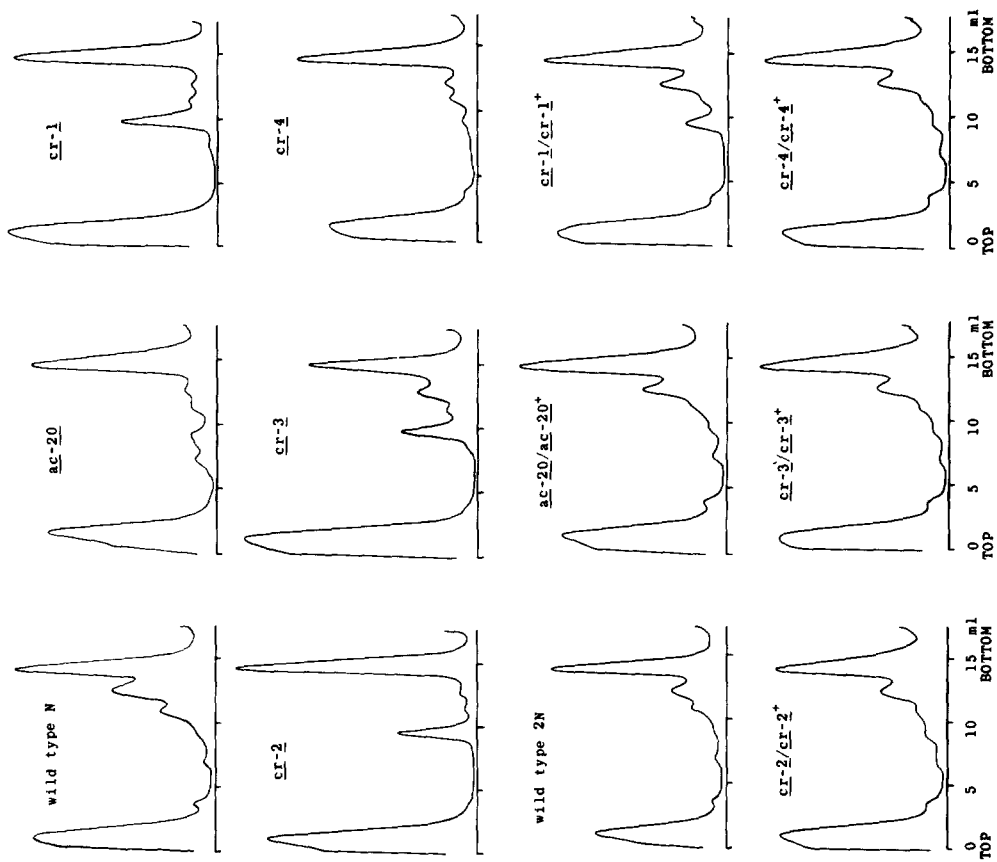
FIGURE 8 Ribosome profiles (A_{254}) of wild-type, the individual ribosome-deficient mutants, and all possible double mutants and diploids. Traces of 17-ml gradients were displayed over 22-cm spread to facilitate quantitation of individual peaks by cutting and weighing. Ordinates vary from 0.25 to 2.5 A_{254} full scale setting on chart recorder, depending on amount of material loaded on individual gradients.
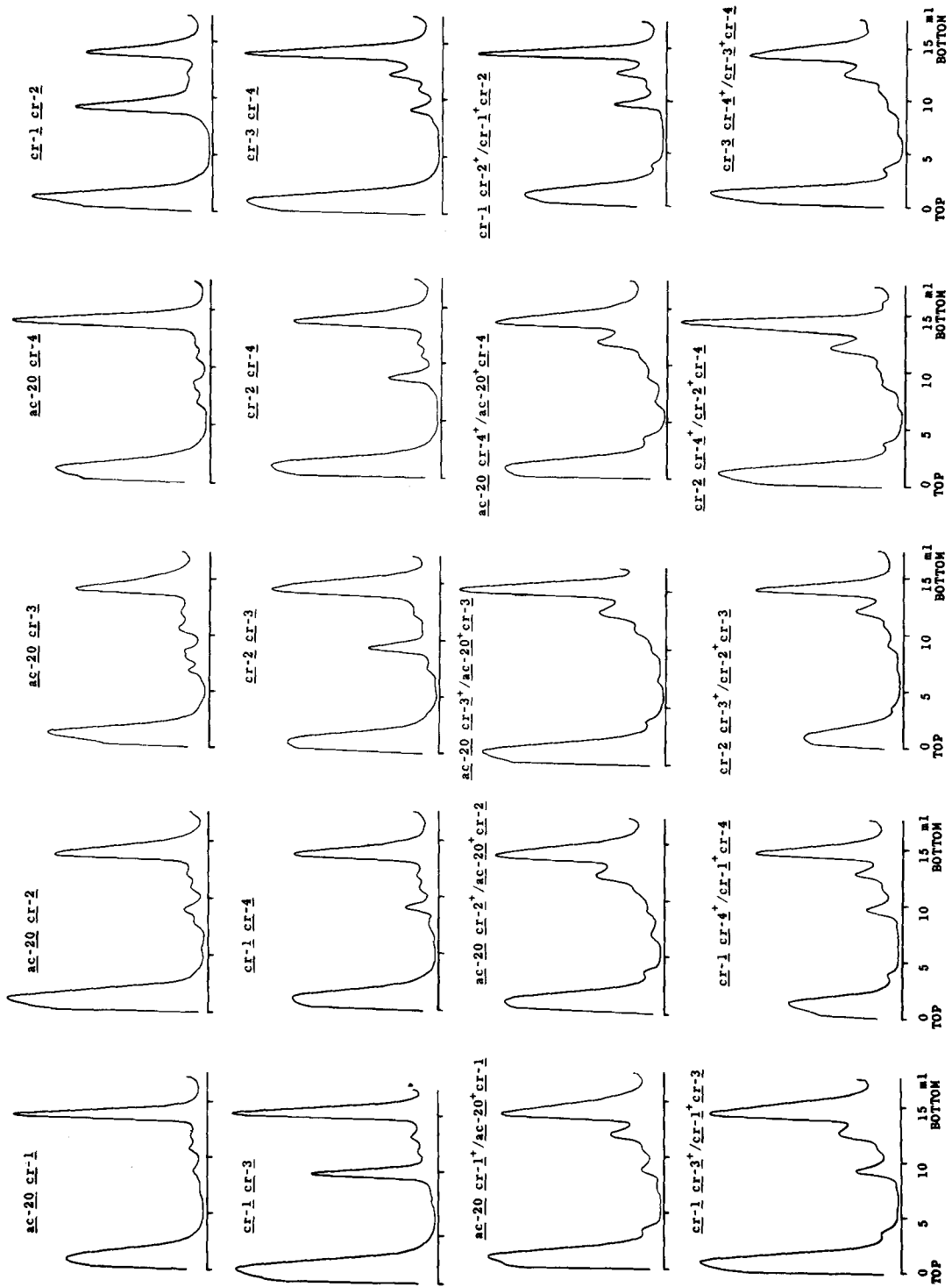
ac-20 cr-1

cr-1 cr-3

ac-20 cr-1⁺/ac-20⁺cr-1

cr-1 cr-3⁺/cr-1⁺cr-3

ac-20 cr-2

cr-1 cr-4

ac-20 cr-2⁺/ac-20⁺ cr-2

cr-1 cr-4⁺/cr-1⁺ cr-4

ac-20 cr-3

cr-2 cr-3

ac-20 cr-3⁺/ac-20⁺ cr-3

cr-2 cr-3⁺/cr-2⁺ cr-3

ac-20 cr-4

cr-2 cr-4

ac-20 cr-4⁺/ac-20⁺ cr-4

cr-2 cr-4⁺/cr-2⁺ cr-4

cr-1 cr-2

cr-3 cr-4

cr-1 cr-2⁺/cr-1⁺ cr-2

cr-3 cr-4⁺/cr-3⁺ cr-4

TOP 0 5 10 15 ml BOTTOM

TABLE V

Summary of Phenotypes of Ribosome-Deficient Mutants Individually, in Diploids, and in Double Mutants

Genotype	Parameter	Haploid pheno-type	Diploid phenotype						Double mutant phenotype of haploid recombinants			
			wild type	ac-20	cr-1	cr-2	cr-3	cr-4	cr-1	cr-2	cr-3	cr-4
wild type	Chloroplast ribosome monomers*	+	+	+	+/−	+	+	+				
	54s subunits accumulated‡	−	−	−	+/−	−	−	−				
	RuDPCase§	+	+	+	+	+	+	+				
	Hill reaction§	+	+	+	+	+	+	+				
ac-20	Chloroplast ribosome monomers	−			+/−	+	+	+	−	−	−	−
	54s subunits accumulated	−			−	−	−	−	−	−	−	−
	RuDPCase	−			+/−	+	+	+	−	−	+/−	−
	Hill reaction	+/−			+	+	+	+	−	−	+/−	−
cr-1	Chloroplast ribosome monomers	−				+/−	+/−	+/−		−	−	−
	54s subunits accumulated	+				+/−	+/−	+/−		+	+	+/−
	RuDPCase	−				+/−	+	+		−	−	−
	Hill reaction	−				+/−	+/−	+/−		−	−	−
cr-2	Chloroplast ribosome monomers	−					+	+			−	−
	54s subunits accumulated	+					−	−			+	+/−
	RuDPCase	+/−					+/−	+			−	−
	Hill reaction	−					+/−	+			−	−
cr-3	Chloroplast ribosome monomers	+/−						+				+/−
	54s subunits accumulated	+						−				~
	RuDPCase	+/−						+				+/−
	Hill reaction	+/−						+				+/−
cr-4	Chloroplast ribosome monomers	+/−										
	54s subunits accumulated	−										
	RuDPCase	+/−										
	Hill reaction	+/~										

* Chloroplast 70s ribosome monomers: + = >20% of total ribosomes; +/− = 10-20% of total ribosomes; − = <10% of total ribosomes.

‡ 54s subunits: + = >15% of total ribosomes; +/− = 10-15% of total ribosomes; − = <10% of total ribosomes.

§ RuDPCase and Hill reaction activity/whole cell protein: + = >75% of wild-type control; +/− = 25-75% of wild-type control; − = <25% of wild-type control.

Chloroplast ribosome profiles of all genotypes are shown in Fig. 8, and quantitation of ribosomes from haploid single mutants and wild type is presented in Table II. Ribulose diphosphate carboxylase and Hill reaction activities of wild-type mixotrophically grown cells are given in Fig. 3.

Neurospora. Kuriyama and Luck (1974) have found that poky is deficient in methylation of mitochondrial rRNA, and that this undermethylation prevents assembly of the small mitochondrial ribosomal subunit. The large subunit can be assembled even though its rRNA is undermethyl-

ated. Given sufficient time, enough small subunits are assembled to permit formation of ribosome monomers, and these undermethylated monomers are functional.

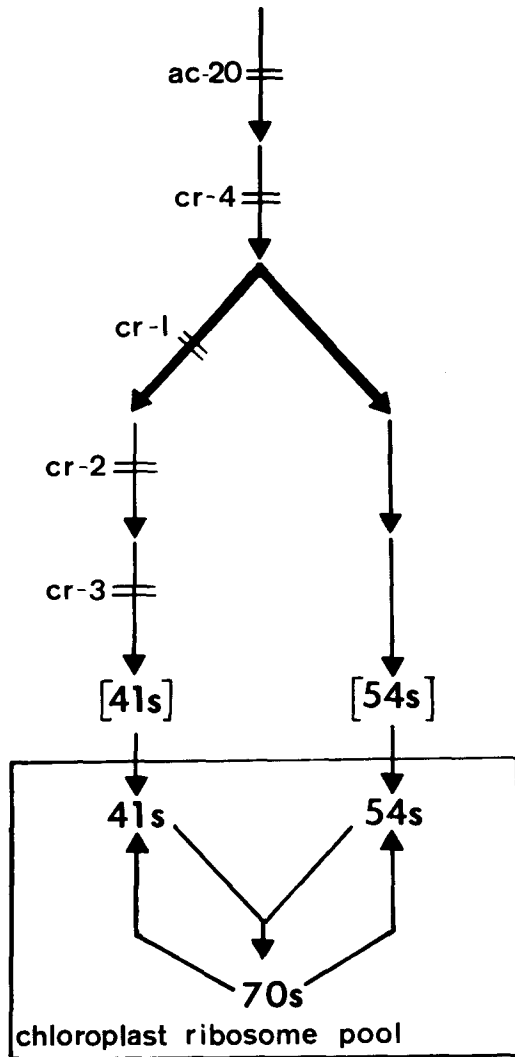In the last 3 yr, seven Mendelian genes, including two loci conferring erythromycin resistance

FIGURE 9 Working model for chloroplast ribosome assembly in *Chlamydomonas*, based on genetic and phenotypic analysis of five nonallelic mutants deficient in chloroplast ribosomes. *ac-20* and *cr-4* appear to be blocked early in ribosome assembly. *cr-1*, *cr-2*, and *cr-3* all accumulate 54s subunits and are therefore thought to be blocked in 41s subunit formation.

described by Mets and Bogorad (1971) and five ribosome loci described here, and at least six non-Mendelian gene loci governing antibiotic resistance (Boynton et al., 1973; Burton, 1972; Gillham et al., 1970; Mets and Bogorad, 1971, 1972; Schlanger et al., 1972; Schlanger and Sager, 1974), have been implicated in the biogenesis of chloroplast ribosomes. Future studies of ribosome-deficient mutants of the type described in

this paper together with antibiotic-resistant mutants should reveal the extent of nucleocytoplasmic interaction in organelle ribosome formation.

REFERENCES

ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris. Plant Physiol.* **24**:1–15.

AVRON, M., and A. T. JAGENDORF. 1959. Evidence concerning the mechanism of adenosine triphosphate formation by spinach chloroplasts. *J. Biol. Chem.* **234**:967–972.

BÖRNER, TH., F. HERRMANN, and R. HAGEMANN. 1973. Plastid ribosome deficient mutants of *Pelargonium zonale. FEBS (Fed. Eur. Biochem. Soc.) Lett.* **37**:117–119.

BOURQUE, D. P., J. E. BOYNTON, and N. W. GILLHAM. 1971. Studies on the structure and cellular location of various ribosome and ribosomal RNA species in the green alga *Chlamydomonas reinhardi. J. Cell Sci.* **8**:153–183.

BOYNTON, J. E., W. G. BURTON, N. W. GILLHAM, and E. H. HARRIS. 1973. Can a non-Mendelian mutation affect both chloroplast and mitochondrial ribosomes? *Proc. Natl. Acad. Sci. U. S. A.* **70**:3463–3467.

BOYNTON, J. E., N. W. GILLHAM, and B. BURKHOLDER. 1970. Mutations altering chloroplast ribosome phenotype in *Chlamydomonas.* 11. A new Mendelian mutation. *Proc. Natl. Acad. Sci. U. S. A.* **67**:1505–1512.

BOYNTON, J. E., N. W. GILLHAM, and J. F. CHABOT. 1972. Chloroplast ribosome deficient mutants in the green alga *Chlamydomonas reinhardi* and the question of chloroplast ribosome function. *J. Cell Sci.* **10**:267–305.

BRYANT, R. E., and P. S. SYPHERD. 1974. Genetic analysis of cold-sensitive ribosome maturation mutants of *Escherichia coli. J. Bacteriol.* **117**:1082–1092.

BURTON, W. G. 1972. Dihydrospectinomycin binding to chloroplast ribosomes from antibiotic-sensitive and resistant strains of *Chlamydomonas reinhardtii. Biochim. Biophys. Acta.* **272**:305–311.

CARRITT, B., and J. M. EISENSTADT. 1973. RNA synthesis in isolated chloroplasts: characterisation of the

newly synthesised RNA. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **36**:116-120.

CHUA, N.-H., G. BLOBEL, and P. SIEKEVITZ. 1973. Isolation of cytoplasmic and chloroplast ribosomes and their dissociation into active subunits from *Chlamydomonas reinhardtii. J. Cell Biol.* **57**:798-814.

CRANE, R. K., and F. LIPMANN. 1953. The effect of arsenate on aerobic phosphorylation. *J. Biol. Chem.* **201**:235-243.

EBERSOLD, W. T. 1967. *Chlamydomonas reinhardi:* heterozygous diploid strains. *Science (Wash. D.C.).* **157**:447-449.

GILLHAM, N. W. 1963. Transmission and segregation of a nonchromosomal factor controlling streptomycin resistance in diploid *Chlamydomonas. Nature (Lond.).* **200**:294.

GILLHAM, N. W., J. E. BOYNTON, and B. BURKHOLDER. 1970. Mutations altering chloroplast ribosome phenotype in *Chlamydomonas.* I. Non-Mendelian mutations. *Proc. Natl. Acad. Sci. U. S. A.* **67**:1026-1033.

GOODENOUGH, U. W. 1971. The effects of inhibitors of RNA and protein synthesis on chloroplast structure and function in wild-type *Chlamydomonas reinhardi. J. Cell Biol.* **50**:35-49.

GOODENOUGH, U. W., and R. P. LEVINE. 1970. Chloroplast structure and function in *ac-20,* a mutant strain of *Chlamydomonas reinhardi.* III. Chloroplast ribosomes and membrane organization. *J. Cell Biol.* **44**:547-562.

GOODENOUGH, U. W., and R. P. LEVINE. 1971. The effects of inhibitors of RNA and protein synthesis on the recovery of chloroplast ribosomes, membrane organization, and photosynthetic electron transport in the *ac-20* strain of *Chlamydomonas reinhardi. J. Cell Biol.* **50**:50-62.

GOODENOUGH, U. W., R. K. TOGASAKI, A. PASZEWSKI, and R. P. LEVINE. 1971. Inhibition of chloroplast ribosome formation by gene mutation in *Chlamydomonas reinhardi. In* Autonomy and Biogenesis of Mitochondria and Chloroplasts. N. K. Boardman, A. W. Linnane, and R. M. Smillie, editors. North Holland Publishing Co., Amsterdam. 224-234.

GORMAN, D. S., and R. P. LEVINE. 1965. Cytochrome *f* and plastocyanin: their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi. Proc. Natl. Acad. Sci. U. S. A.* **54**:1665-1669.

GOWANS, C. S. 1960. Some genetic investigations on *Chlamydomonas eugametos. Z. Vererbungsl.* **91**:63-73.

HAGEMANN, R., T. BÖRNER, and R. KNOTH. 1973. Plastid ribosome deficiency in plastid mutants of *Pelargonium* and *Hordeum. Genetics.* **74**:S103-104.

HARRIS, E. H., J. E. BOYNTON, N. W. GILLHAM, and R. TOGASAKI. 1973. Positive selection for mutants in *Chlamydomonas reinhardtii* with defects in chloroplast protein synthesis. *Genetics.* **74**:S109-110.

HASTINGS, P. J., E. E. LEVINE, E. COSBEY, M. O. HUDOCK, N. W. GILLHAM, S. J. SURZYCKI, R.

LOPPES, and R. P. LEVINE. 1965. The linkage groups of *Chlamydomonas reinhardi. Microb. Genet. Bull.* **23**:17-19.

HOOBER, J. K., and G. BLOBEL. 1969. Characterization of the chloroplast and cytoplasmic ribosomes of *Chlamydomonas reinhardi. J. Mol. Biol.* **41**:121-138.

HOOBER, J. K., P. SIEKEVITZ, and G. E. PALADE. 1969. Formation of chloroplast membranes in *Chlamydomonas reinhardi y-1.* Effects of inhibitors of protein synthesis. *J. Biol. Chem.* **244**:2621-2631.

HUTNER, S. H., L. PROVASOLI, A. SCHATZ, and C. P. HASKINS. 1950. Some approaches to the study of the role of metals in the metabolism of microorganisms. *Proc. Am. Philos. Soc.* **94**:152-170.

INFANTE, A. A., and R. BAIERLEIN. 1971. Pressure-induced dissociation of sedimenting ribosomes: effect on sedimentation patterns. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1780-1785.

KATTERMAN, F. R. H., and J. E. ENDRIZZI. 1973. Studies on the 70s ribosomal content of a plastid mutant in *Gossypium hirsutum. Plant Physiol.* **51**:1138-1139.

KURIYAMA, Y., and D. J. L. LUCK. 1973. Ribosomal RNA synthesis in mitochondria of *Neurospora crassa. J. Mol. Biol.* **73**:425-437.

KURIYAMA, Y., and D. J. L. LUCK. 1974. Methylation and processing of mitochondrial ribosomal RNA's in *"poky"* and wild type *Neurospora crassa. J. Mol. Biol.* **83**:253-266.

LEE, R. W., N. W. GILLHAM, K. P. VAN WINKLE, and J. E. BOYNTON. 1973. Preferential recovery of uniparental streptomycin resistant mutants from diploid *Chlamydomonas reinhardtii. Mol. Gen. Genet.* **121**:109-116.

LEE, R. W., and R. F. JONES. 1973. Induction of Mendelian and non-Mendelian streptomycin resistant mutants during the synchronous cell cycle of *Chlamydomonas reinhardtii. Mol. Gen. Genet.* **121**:99-108.

LEVINE, R. P., and W. T. EBERSOLD. 1960. The genetics and cytology of *Chlamydomonas. Annu. Rev. Microbiol.* **14**:197-216.

LEVINE, R. P., and U. W. GOODENOUGH. 1970. The genetics of photosynthesis and of the chloroplast in *Chlamydomonas reinhardi. Annu. Rev. Genet.* **4**:397-408.

MAHLER, H. 1973. Biogenetic autonomy of mitochondria. *Critical Reviews in Biochemistry.* **1**:381-460.

METS, L. J., and L. BOGORAD. 1971. Mendelian and uniparental alterations in erythromycin binding by plastid ribosomes. *Science (Wash. D.C.).* **174**:707-709.

METS, L., and L. BOGORAD. 1972. Altered chloroplast ribosomal proteins associated with erythromycin-resistant mutants in two genetic systems of *Chlamydomonas reinhardi. Proc. Natl. Acad. Sci. U. S. A.* **69**:3779-3783.

PACE, N. R. 1973. Structure and synthesis of the

ribosomal ribonucleic acid of prokaryotes. *Bacteriol. Rev.* **37**:562-603.

SCHIFF, J. A., H. LYMAN, and G. K. RUSSELL. 1971. Isolation of mutants from *Euglena gracilis*. *Methods Enzymol.* **23** A:143-162.

SCHLANGER, G., and R. SAGER. 1974. Localization of five antibiotic resistances at the subunit level in chloroplast ribosomes of *Chlamydomonas*. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1715-1719.

SCHLANGER, G., R. SAGER, and Z. RAMANIS. 1972. Mutation of a cytoplasmic gene in *Chlamydomonas* alters chloroplast ribosome function. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3551-3555.

SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**:31-43.

SUEOKA, N. 1960. Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardi*. *Proc. Natl. Acad. Sci. U. S. A.* **46**:83-91.

SURZYCKI, S. J., U. W. GOODENOUGH, R. P. LEVINE, and J. J. ARMSTRONG. 1970. Nuclear and chloroplast control of chloroplast structure and function in *Chlamydomonas reinhardi*. *Symp. Soc. Exp. Biol.* **24**:13-27.

SURZYCKI, S., and P. J. HASTINGS. 1968. Control of chloroplast RNA synthesis in *Chlamydomonas reinhardi*. *Nature (Lond.).* **220**:786-787.

SURZYCKI, S. J., and J. D. ROCHAIX. 1971. Transcription mapping of ribosomal RNA genes of the chloroplast and nucleus of *Chlamydomonas reinhardi*. *J. Mol. Biol.* **62**:89-109.

TOGASAKI, R. K., and M. O. HUDOCK. 1974. Effect of inorganic arsenate on photosynthetic algae. I. Response of wild type and four photosynthetic mutant strains of *Chlamydomonas reinhardi* to inorganic arsenate. *Plant Physiol*. In press.

TOGASAKI, R. K., and R. P. LEVINE. 1970. Chloroplast structure and function in *ac-20*, a mutant strain of *Chlamydomonas reinhardi*. I. CO_2 fixation and ribulose-1,5-diphosphate carboxylase synthesis. *J. Cell Biol.* **44**:531-539.