

CHARACTERIZATION OF CYTOPLASMIC AND NUCLEAR GENOMES IN THE COLORLESS ALGA *POLYTOMA*

II. General Characterization of Organelle Nucleic Acids

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ABSTRACT

Polytoma obtusum has a main band DNA (α) with a buoyant density in CsCl of $\rho = 1.711$ g/ml and a light DNA satellite (β) with $\rho = 1.682$ g/ml. β -DNA was substantially enriched in a fraction containing small leucoplast fragments and some mitochondria, which was obtained in a pellet sedimenting between 3,000 g and 5,000 g. A crude mitochondrial pellet was also obtained by sedimenting at 12,000 g to recover particulates remaining in the supernate after 10 min at 5,000 g. This fraction contained a third DNA component (γ) with $\rho = 1.714$ g/ml. We have concluded that the leucoplasts of *P. obtusum* contain the β -DNA (1.682) and the mitochondria possess the γ -component (1.714).

Two distinct classes of ribosomes were isolated and separated by sucrose density gradients, a major 79S species and a minor species at 75S. The major species possessed the 25S and 18S ribosomal RNA (rRNA), characteristic of cytoplasmic ribosomes, and these particles co-sedimented in sucrose gradients with the 79S cytoplasmic ribosomes of *Chlamydomonas reinhardtii*. The minor species was present in about 2% of the total ribosomal population but showed an eight-to ninefold enrichment in the leucoplast pellet, suggesting that it was of organelle origin. These 73S particles had RNA components migrating very closely with the 18S and 25S species of the 79S ribosomes, but the base composition of the rRNA from these two classes of ribosomes was significantly different; the rRNA from the 79S ribosomes had a G+C mole ratio of 50.0%, while the rRNA from the 73S class had a ratio of 47.5%. By comparison, chloroplast ribosomes of *C. reinhardtii* were found to sediment at 70S and contain rRNA molecules of 23S and 16S, with a G + C content of 51.0%. These findings support the concept that the *Polytoma* leucoplast possesses characteristic genetic and protein-forming systems.

Our preceding paper (35) described the morphology of *Polytoma* and, in particular, its cytoplasmic organelles. *Polytoma* has a cup-shaped leucoplast which, although often regarded as a degenerate

structure, apparently still possesses an active metabolic role in the synthesis and storage of starch reserves. Both DNA filaments and ribosomal particles are present in the matrix of leucoplasts

and mitochondria, suggesting that both organelles contain DNA genomes and protein synthesis systems. The isolation and characterization of the DNA and ribosomes thus seemed important to an understanding of the organization and expression of the genetic material in both leucoplasts and mitochondria.

In *Chlamydomonas*, the chloroplast DNA was shown to form a DNA satellite with a buoyant density of 1.695 g/ml, constituting about 14% of the whole-cell DNA (6, 7, 26). A light DNA satellite of similar proportion has also been reported in *Polytoma* to have an exceedingly low buoyant density of 1.683 g/ml and rapid renaturability (13). This suggests that this DNA satellite may have an unusually high A + T content and a low complexity, and that it might possibly be of leucoplast origin. In this study, we have investigated the cytological localization of the light satellite DNA and its possible function. Leucoplasts as well as mitochondria from *P. obtusum* have been isolated, and the DNA components of these organelles have been characterized. In addition, two distinct species of ribosomes have also been separately identified, a major (cytoplasmic) and a minor (organelle) fraction. Although we have not specifically localized the minor ribosomal component to the leucoplasts, rather than the mitochondria, this seems its probable location in light of the much greater leucoplast volume. These results indicate that the *Polytoma* leucoplast possesses DNA and probably also ribosomes and thus must retain the ability for protein synthesis.

MATERIALS AND METHODS

Strains and Culture Conditions

The *Polytoma obtusum* strain 1 was kindly supplied by Dr. L. Provasoli, Haskins Laboratories, New Haven, Conn. *Chlamydomonas reinhardtii* strain 137C⁺ mating type, originally obtained through the courtesy of Dr. R. P. Levine of Harvard University, was used in this investigation. *P. obtusum* was grown in the Tris-acetate medium as described previously (35). *C. reinhardtii* was grown in the high salt minimal medium (HSM) as described by Sueoka, Chiang, and Kates (37).

Organelle Fractionation

P. obtusum cells were grown in 12-liter carboys to a concentration of $3-4 \times 10^8$ cells/ml and harvested at late exponential phase. The organelle isolation procedure of Krawiec and Eisenstadt (15) was followed with minor

modifications. Cells were washed twice in a cold solution of 0.25 M sucrose, 0.1 mM EDTA and 0.01 M Tris-HCl (pH 7.6), and then resuspended in the sucrose buffer at a concentration of $1-1.5 \times 10^9$ cells/ml. The cells were broken gently by one passage through a French pressure cell (American Instrument Co., Inc., Silver Springs, Md.) at 750 lb/in². Cell breakage was about 30-40%. The cell lysate was diluted with an equal volume of buffer and shaken gently. The unbroken cells and nuclei were pelleted at 2,000 g for 10 min at 4°C. This step was repeated at least three times until the pellet was negligible. The supernate was centrifuged at 3,000 g for 10 min and the pellet discarded. The supernate was centrifuged at 5,000 g for 15 min to sediment most of the leucoplast fragments. The supernate was finally spun at 12,000 g for 15 min to pellet the mitochondria. Part of the two pellets was used for electron microscopy and the rest for DNA extraction. The crude leucoplast fraction and the mitochondrial fraction could be further purified by sedimentation through a 30-ml linear 0.8-2.0 M sucrose gradient at 18,000 rpm at 4°C for 11 h in an International Model B-60 ultracentrifuge with an SB-110 rotor (International Scientific Instruments, Inc., Mountain View, Calif.)

Electron Microscopy

Samples from both the leucoplast and mitochondrial fractions were fixed in 2% OsO₄ for 2 h at 4°C and embedded in Epon in essentially the same way as described in our previous paper (35).

Preparation of DNA

DNA from the organelle fractions was isolated by first treating the pellet with detergents (2% Triton X-100 and 2% sodium lauryl sulfate [SLS]), followed by pronase and RNase treatments. Details of this procedure have been reported elsewhere (4).

Analytical Density-Gradient Centrifugation

3-5 μg of DNA in 0.1-0.2 ml of SSC (0.15 M sodium chloride, 0.015 M sodium citrate) were mixed with a saturated CsCl solution containing 0.05 M Tris-HCl, pH 8.4, and 1 mM EDTA, to make a final vol of 0.8 ml and an initial density of 1.710 g/ml. Centrifugation in a Spinco Model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) was carried out at 44,000 rpm for 20 h at 25°C. Buoyant densities, band profiles, and approximate amounts of various DNA species were determined from microdensitometer tracings of UV absorption (265 nm) photographs. SPO-1 DNA with a buoyant density of 1.740 g/ml was added to each run as a density marker.

Radioactive Isotope Labeling

[³H]adenine or [¹⁴C]adenine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) was added

to make a final concentration of 0.5 $\mu\text{Ci/ml}$ or 0.2 $\mu\text{Ci/ml}$, respectively, to cultures of $4\text{--}6 \times 10^6$ cells/ml. Cells were allowed to grow to late exponential phase at about 4×10^6 cells/ml, and harvested for ribosome isolation.

For ^{32}P -labeling, cells were grown in 5 mM MES[2-(*N*-morpholino)ethanesulfonic acid]-KOH buffered HSM medium, pH 7.0, in which the phosphate content was reduced 400-fold. 2 $\mu\text{Ci/ml}$ of inorganic- ^{32}P phosphate were added to the culture at 0.5×10^6 cells/ml, and the culture was grown overnight for another two to three generations before harvesting.

Sucrose Density Gradient Analyses of Ribosomes

The method of Hooper and Blobel (12) for the isolation of ribosomes was followed. Cells were harvested at a concentration of $2\text{--}4 \times 10^6$ cells/ml, and were washed twice with an ice-cold buffer of 25 mM Tris-HCl, pH 7.5, 25 mM MgCl_2 , 25 mM KCl (TMK buffer) containing 0.25 M sucrose. The cells were resuspended in this solution at a concentration of $1\text{--}2 \times 10^6$ cells/ml and then broken by one or two passages through a prechilled miniature French pressure cell at 4,000 lb/in². To free the membrane-bound ribosomes, Triton X-100 was added at a final concentration of 2.5% to the S_{10} supernate fraction obtained by spinning the cell lysate at 10,000 g for 10 min. The ribosomes in the S_{10} fraction were subsequently pelleted through 3 ml of 1 M sucrose in TMK buffer at 2°C by centrifuging for 2 h in an A-321 rotor (International B-60 ultracentrifuge) at 55,000 rpm. The ribosomal pellet was resuspended in 0.25 M sucrose in TMK buffer and stored at -70°C for subsequent gel electrophoresis or sucrose gradient centrifugation.

A 12-ml linear sucrose gradient of 0.31–1.1 M sucrose in 5 mM MgCl_2 , 25 mM KCl, 25 mM Tris-HCl (pH 7.5) was used to separate the different ribosomal species. Exponential gradients were also prepared according to Noll (19). Gradients were centrifuged for 3.5 h at 160,000 g_{max} and at 3°C in the International Model B-60 ultracentrifuge equipped with the SB-283 rotor. 0.3-ml fractions were collected at 5°C with an ISCO 640 density gradient fractionator.

Acrylamide Gel Electrophoretic Analysis of rRNA

The S_{10} fraction was made to 2% with SDS and incubated at 37°C for 3 min to dissociate the rRNA from ribosomal proteins. Aliquots were layered onto 2.6% acrylamide gels. Details for the preparation of these gels have been described by Loening (16). The gels were run at 5 mA/gel for 3.5 h at $2\text{--}4^\circ\text{C}$, and then scanned at 260 nm with a slit width of 0.2 mm in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) with a linear transport attachment.

Purification of rRNA

Different species of ribosomes were pooled separately after fractionation on sucrose density gradients, and pelleted at 2°C in an A-321 rotor at 60,000 rpm for 2 h with an International B-60 ultracentrifuge. The ribosomal pellet was suspended in 1–2 ml of ice-chilled Tris-Mg buffer (50 mM Tris-HCl, pH 7.4; 1 mM MgCl_2), which was pretreated in 0.3% diethylpyrocarbonate for 15 min at 37°C to inhibit RNase activity. SLS was added to 2% and the mixture was shaken for 10 min with an equal volume of Tris-Mg buffer-saturated phenol. This phenol extraction step was repeated four to five times until no interphase was visible. The aqueous phase was precipitated with 2–3 vol of ethanol at -20°C . The RNA was pelleted and dissolved in a small volume of Tris-Mg buffer. Residual phenol was removed by repeated extraction with diethyl ether. Ether was removed by bubbling a stream of filtered air or helium through the RNA solution in the cold. The rRNA was further purified by treatment with DNase (electrophoretically purified, Worthington Biochemical Corp., Freehold, N.J.) at a concentration of 10 $\mu\text{g/ml}$ for 30 min at 37°C. DNase was removed by phenol extraction and the above steps were repeated. The purity of the rRNA was analyzed by means of gel electrophoresis as described above. The rRNA was stored frozen in Tris-Mg buffer, and 20 OD units at 260 nm with 1 cm light path was taken to be 1 mg/ml.

Separation of Ribosomal Subunits

Pelleted ribosomes were dissociated into subunits by resuspending in a solution of 50 mM Tris-HCl (pH 7.5), 25 mM MgCl_2 , 500 mM KCl, and 1 mM dithiothreitol. The subunits were separated in a linear sucrose gradient of 5–20% (wt/vol) in the above buffer, spun at 38,000 rpm at 16°C for 3.5 h in a SB-283 rotor. Fractions of the different subunits were pooled separately and RNA was extracted for further analyses.

Analytical Sucrose Gradient for rRNA

50 μl of rRNA were layered onto a 4-ml linear gradient of 0.31–1.1 M sucrose in 0.1 M sodium acetate and 0.05 M Tris-acetate (pH 7.2) and centrifuged in an SB-405 rotor at 60,000 rpm at 2°C for 5 h for the separation of the various rRNA species dissociated from ribosomes.

Base Composition Analysis

Ribosomal RNA was isolated and purified from ^{32}P -labeled cells, and about 1–5 μg of rRNA containing a minimum of 20,000 cpm were placed in 0.2 ml of 10 mM Tris-acetate (pH 8.5) with 10 mM CaCl_2 , 350 U of micrococcal nuclease (Worthington Biochemical) in 5 μl of 50% glycerol and 10 mM Tris-acetate (pH 8.5) were added, and the mixture was incubated at 37°C for 2 h. The mixture was then titrated with 1% glacial acetic acid

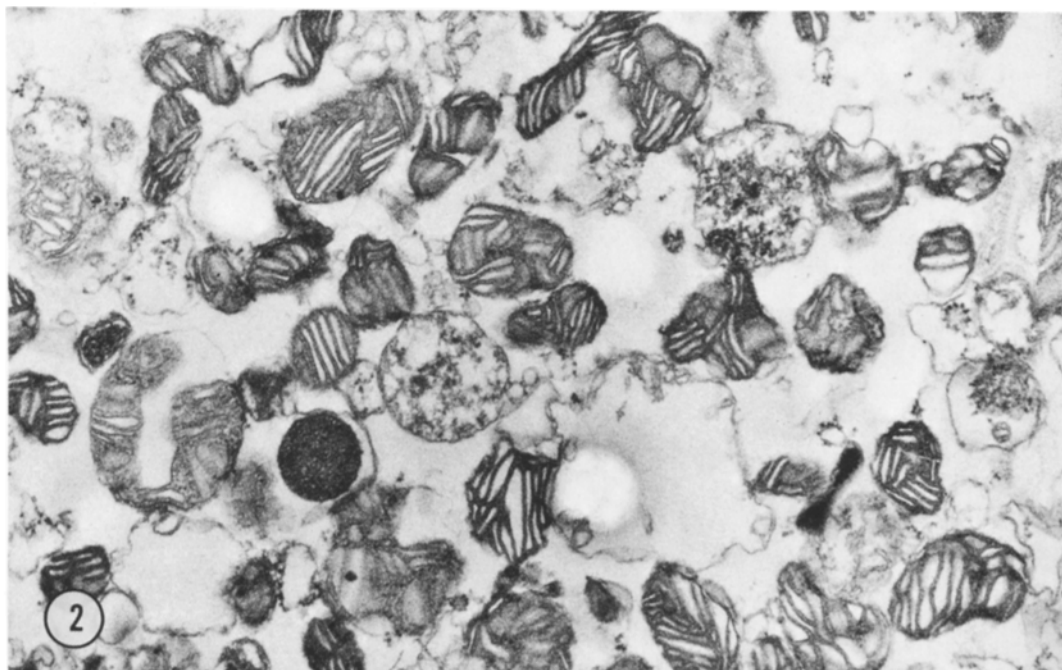
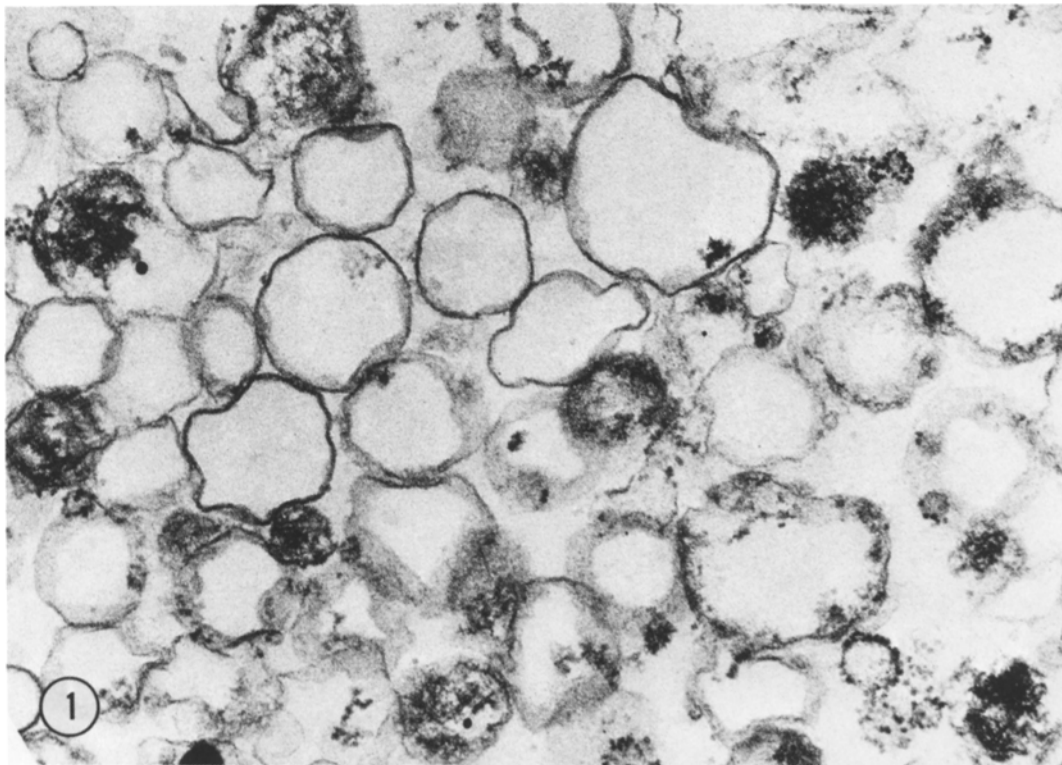


FIGURE 1 Section of the lower half of the leucoplast pellet, showing small vesicles. The pellet was prepared from particulates spun down between 3,000 g and 5,000 g and fixed with 2% OsO₄. Sections were doubly stained with 3% aqueous uranyl acetate and lead citrate. × 40,000.

FIGURE 2 Section through the upper half of the leucoplast pellet showing both leucoplast fragments and mitochondria. Fixation and staining were the same as in Fig. 1. × 30,000.

to pH 7.0, and 10 μ l of spleen phosphodiesterase (Worthington, 13 U of the enzyme stored in 2 ml of 50% glycerol) were added and the solution was again incubated at 37°C for 2 h. The mixture was then dried, and the nucleotides were redissolved in 0.2–0.3 ml of methanol. The mixture was spotted on 90-cm 3 mM Whatman chromatographic paper with a 1- μ l micropipette. Electrophoresis was performed with 0.05 M citrate buffer (pH 3.5) at 1,600 V for 4–5 h. 1-cm strips were cut and counted in 5 ml of Toluene-base scintillation fluid.

RESULTS

Organelle Isolation

Two organelle fractions were obtained by means of differential centrifugation. The pellet sedimenting between 3,000 g and 5,000 g was examined under the electron microscope. The lower half of the pellet contained many small leucoplast vesicles. They were probably fragments that were broken off at various constricted regions of the organelle, with the membrane resealed to form vesicles (Fig. 1). The upper half of the pellet was heavily contaminated by mitochondria (Fig. 2). The rough endoplasmic reticulum was another contaminant, but the fraction was essentially free of nuclei and whole cells. The densities of the leucoplast fragments and mitochondria were similar, since further separation by sucrose gradient centrifugation failed to resolve the two organelles and yielded only one band at a density of $\rho = 1.2$. This pellet will be referred to as the leucoplast fraction.

The cell particulates that remained in the supernate at 5,000 g, but were spun down at 12,000 g, consisted mainly of mitochondria, many of which were damaged, possessing stripped outer membranes and leached matrix. This pellet will be referred to as the mitochondrial fraction.

DNA Characterization

DNA was extracted from these two fractions and analyzed by analytical CsCl gradient centrifugation. Whole-cell DNA showed two bands: a main band (α), 86%, with a density of 1.711 g/ml, and a satellite band (β), 14%, with a density of 1.682 g/ml (Fig. 3 a). However, a third DNA band was resolved from the leucoplast fraction. Besides the α - and β -DNA species, a heretofore unidentified DNA species (γ) with a buoyant density of 1.714 g/ml was detected (Fig. 3 c). The α -DNA constituted approximately 5% or less of this DNA preparation, while the rest consisted of β - and γ -DNA of almost equal proportions. Only the

γ -band DNA ($\rho = 1.714$ g/ml) was detectable in the mitochondrial fraction (Fig. 3 b).

When a DNA sample isolated from the leucoplast fraction was heat denatured in 0.1 SSC and then renatured in 4 \times SSC at 65°C for 2 h, both β - and γ -DNA reannealed and returned to densities less than 0.002 g/ml heavier than their native buoyant densities (Fig. 3 d). The renatured peak of γ -DNA in CsCl appeared to be sharper than its native state, probably because of complex formation, while the renatured β -DNA peak was slightly broader than its native state. The small amount of α -DNA present in the leucoplast fraction was not detected in the vicinity of γ -DNA in the renatured sample, since the α -DNA failed to renature under the reannealing conditions that permitted both β - and γ -DNA to renature.

Ribosomal RNA Characterization

RNA from the supernate of cell lysates (contained in the S_{10} preparation), when subjected to electrophoresis on a 2.6% acrylamide gel, showed only two RNA peaks (Fig. 4 b). The proportion of these two RNA species, calculated by weighing the areas under these peaks, as expected was found to be 2:1.

To compare the molecular weights and electro-

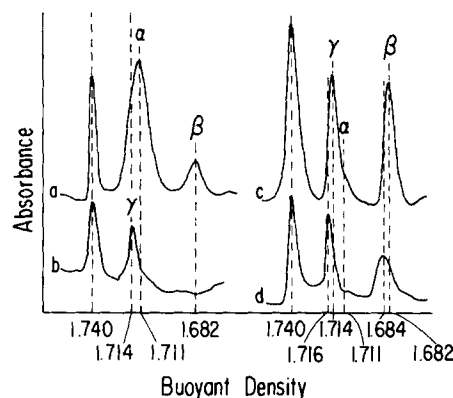


FIGURE 3 Microdensitometer tracings of DNA samples extracted from whole-cell and organelle fractions of *P. obtusum*. (a) Whole-cell DNA with two prominent peaks; the DNA satellite (β) with a density of 1.682 g/ml is presumably the leucoplast DNA; (b) native DNA from the mitochondrial fraction; (c) native DNA obtained from the leucoplast fraction; and (d) DNA from leucoplast fraction, first heat denatured in 0.1 SSC at 100°C for 5 min and then renatured in 4 \times SSC at 60°C for 2 h. Marker DNA was obtained from phage SPO-1 ($\rho = 1.740$ g/ml).

phoretic properties of the rRNA species from *Polytoma* and *Chlamydomonas*, equal amounts of the S_{10} fraction, prepared from an equal number of cells of the two organisms, were mixed, treated with SLS, and subjected to co-electrophoresis. As was the case with the control *Chlamydomonas* (Fig. 4 a), only four peaks were observed (Fig. 4 c). However, the ratios of the peak areas for 25S/23S and 18S/16S were twice those of *Chlamydomonas* (Fig. 4 a) since cytoplasmic rRNA peaks for the two species overlapped exactly. The results were identical when purified *Polytoma* cytoplasmic ribosomes were used. These results indicated that, in *Polytoma*, only two species of rRNA were separable by gel electrophoresis, and that the two species of cytoplasmic rRNA had the same molecular weight as the 25S and 18S cytoplasmic rRNA of *Chlamydomonas*, i.e., 1.30×10^6 , and 0.69×10^6 , respectively (17). Under these experimental condi-

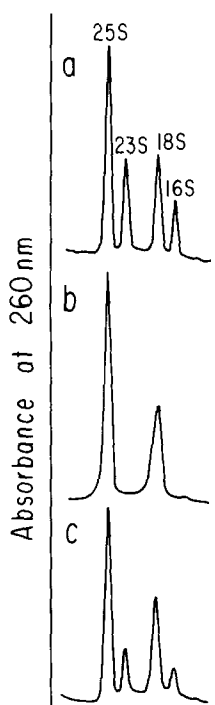


FIGURE 4 Gel electrophoretic patterns of rRNA. The S_{10} fraction was treated with 2% SLS and run in 2.6% acrylamide at 4°C at 5 mA per gel for 3.5 h. The gels were scanned at 260 nm in a Gilford 240 spectrophotometer. (a) Separation of *Chlamydomonas* rRNA into the 25S, 23S, 18S, and 16S species. (b) 25S and 18S rRNA from the S_{10} fraction of *Polytoma* cells. (c) Co-electrophoresis of *Polytoma* and *Chlamydomonas* rRNA.

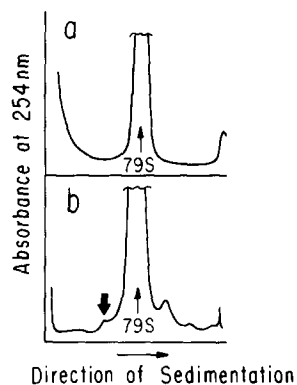


FIGURE 5 Sedimentation profiles of purified ribosomes in sucrose gradient. (a) Ribosomes prepared from the S_{10} fraction. (b) Ribosomes were purified by pelleting through a 3-ml cushion of 1 M sucrose in the same TMK buffer and then 5 OD units of the purified ribosome suspension (0.1 ml) was centrifuged for 3.5 h in a 12-ml linear sucrose gradient (0.31–1.1 M) at 38,000 rpm at 2°C. A dimer peak of the 79S ribosomes is also evident to the right of the main peak.

tions, using the S_{10} fraction, no 23S and 16S rRNA species were detected in *Polytoma*.

Characterization of Two Distinct Classes of Ribosomes

Although the S_{10} fraction prepared from the cell lysate showed only the 79S ribosomal species (Fig. 5 a), after the ribosomes in the S_{10} fraction had been further purified by pelleting through a 3-ml cushion of 1 M sucrose, sucrose gradients showed a small, more slowly sedimenting component in addition to the prominent cytoplasmic ribosomal peak (Fig. 5 b). The smaller ribosomal species represented only about 2% of the total cellular ribosomes.

When equal numbers of ^{14}C -labeled *Polytoma* cells were mixed and harvested with an equal number of ^3H -adenine-labeled *Chlamydomonas* cells, both the ^{14}C -labeled and ^3H -labeled cytoplasmic ribosomes co-sedimented as a single peak in a linear sucrose density gradient (Fig. 6). Thus, *Polytoma* possessed a 79S cytoplasmic ribosomal species similar to that of *Chlamydomonas*. To determine the sedimentation coefficient for the slow-sedimenting ribosomal particles in *Polytoma*, ^{14}C -labeled ribosomes were pooled and then rerun in a second sucrose density gradient with the 70S chloroplast ribosomes from *Chlamydomonas* as marker. The *Polytoma* ribosomal species was found to sediment with a coefficient of 73S (Fig.

7). When ^{32}P -labeled RNA from these 73S ribosomes was extracted and co-sedimented with ^3H -labeled cytoplasmic rRNAs (25S and 18S), the ^{32}P -labeled rRNA peaks were found to sediment closely with the cytoplasmic rRNA species (Fig. 8). The ratio of the two rRNA peaks from the 73S ribosomes was about 1.8:1, comparable to that of the cytoplasmic rRNA.

An eightfold enrichment of the 73S ribosomes, as compared with the whole-cell lysate, was de-

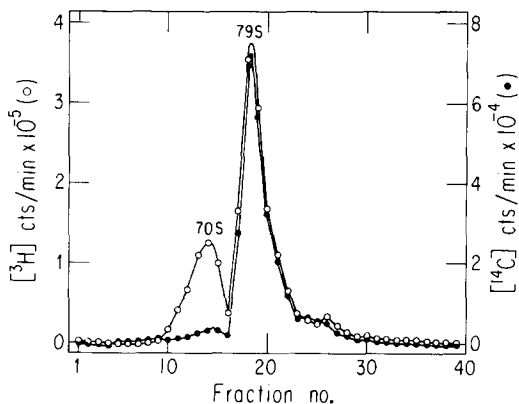


FIGURE 6 Co-sedimentation of ^{14}C -labeled *Polytoma* ribosomes and ^3H -labeled *Chlamydomonas* ribosomes in sucrose density gradient. Conditions were the same as described for Fig. 5. 0.3-ml fractions were collected from the top. The fractions were precipitated in 5% cold TCA after the addition of 70 μg carrier DNA (Herring sperm), and the precipitate was collected on glass fiber disks for counting. \bullet , ^{14}C ; \circ , ^3H .

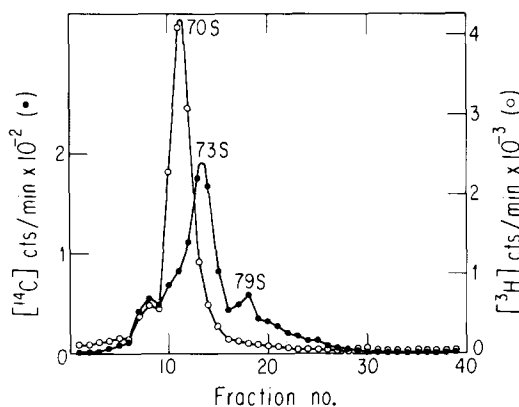


FIGURE 7 Determination of sedimentation rate of the slow-sedimenting ribosome class. ^{14}C -labeled *Polytoma* ribosomes were sedimented in sucrose gradient, and the slow-sedimenting class was pooled and then co-sedimented with ^3H -labeled *Chlamydomonas* 70S ribosomes as previously described. \bullet , ^{14}C ; \circ , ^3H .

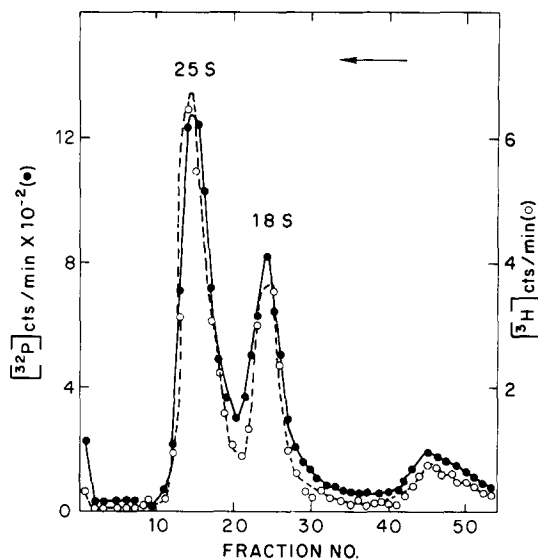


FIGURE 8 Determination of sedimentation rates of rRNA from the 73S ribosomes. ^{32}P -labeled ribosomes were treated with SDS and centrifuged in a 4-ml analytical sucrose density gradient. ^3H -labeled 79S ribosomes were treated similarly and added as markers. \bullet , ^{32}P ; \circ , ^3H .

tected in the leucoplast fraction, thus suggesting that this class of ribosomes probably originated from the plastid (Fig. 9). Ribosomes in the mitochondrial fraction have not been examined because of the low yield of material.

Chemical Composition of rRNA

Although rRNA of the two ribosomal classes of *Polytoma* appeared to be identical in size, they nevertheless showed significant differences in their overall base composition (Table I). Ribosomal RNA from the 73S ribosomes had a G + C mole ratio of 47.5%, slightly lower than that of the cytoplasmic rRNA (50.0%). The difference was significant and reproducible. The rRNA of 73S ribosomes was also considerably higher in adenine, while the rRNA of 79S ribosomes was highest in guanine. The base ratios of the rRNA from the 61S and 41S subunits were also different. The 25S rRNA was higher in both adenine and guanine, which were present in more or less equal amounts, while the 18S rRNA was highest in guanine and lowest in cytosine.

Subunits of Cytoplasmic Ribosomes

The purified cytoplasmic 79S ribosomes of *Polytoma* were dissociated into subunits by sus-

pending in a buffer containing 500 mM KCl. The two subunits were found to co-sediment with those of *Chlamydomonas* cytoplasmic ribosomes (Fig. 10). Therefore, the cytoplasmic ribosomal subunits of *Polytoma* have been assigned the values of 61S and 41S (18). Ribosomal RNA was extracted from each of these two subunits and analyzed in a 4-ml sucrose density gradient. The 25S rRNA species was obtained from the 16S subunit and 18S rRNA from the 41S subunit, with little cross contamination (Fig. 11).

DISCUSSION

Three species of DNA, each with a distinct buoyant density, have been characterized in *P. obtusum*. Since isolated nuclei yielded only the main band DNA (α) (32), the other two DNA species appear to be of cytoplasmic origin. The γ -DNA has been identified as mitochondrial since it was shown to be the sole DNA species in the isolated mitochondrial fraction. It has not been previously identified since it was concealed by the nuclear peak in whole-cell DNA samples in CsCl gradients. The γ -DNA was fast renaturable, suggesting that the complexity of the mitochondrial genome is relatively low. It has a buoyant density of 1.714 g/ml, corresponding to a G + C content of 55%. Recently, Ryan et al. (25) have found that the mitochondrial DNA of *Chlamydomonas* has a buoyant density of 1.706 g/ml and a kinetic complexity of 1.7×10^7 daltons.

Polytoma leucoplasts were large and fragile with many highly constricted regions (35). During isolation, this organelle appeared to break easily and the membrane apparently resealed to form vesicles of various sizes. The compartments containing large starch granules sedimented more rapidly

than whole cells, while the small fragments, which usually contained only the matrix material, remained in the supernate during the initial low-speed centrifugation (2,000 g). It was the latter component that made up the bulk of our leucoplast preparation, in which the β -DNA ($\rho = 1.682$ g/ml) constituted about 50% of the total DNA. It is thus evident that the β -DNA satellite is probably of leucoplast origin. Since many mitochondria co-sedimented with the leucoplast fragments, the mitochondrial DNA (γ) was the major DNA contaminant in this pellet, while nuclear DNA contamination was negligible.

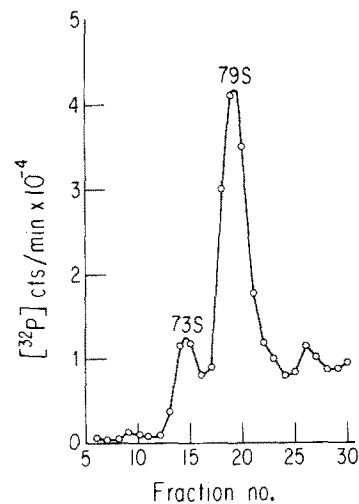


FIGURE 9 Sedimentation profile of ribosomes isolated from the leucoplast fraction. Leucoplasts were isolated from ^{32}P -labeled cells, and ribosomes were purified by pelleting through 1 M sucrose before analysis on sucrose gradient. The 73S species constituted about 17% of the total amount of counts under the two peaks.

TABLE I
Chemical Composition of *Polytoma* rRNA

Nucleotide	Cytoplasmic ribosomes (79S)			Organelle ribosomes (73S)
	25S	18S	Total rRNA	Total rRNA
	$\bar{x} \pm SD (n = 6)$	$\bar{x} \pm SD (n = 6)$	$\bar{x} \pm SD (n = 6)$	$\bar{x} \pm SD (n = 4)$
C	21.86 \pm 0.41	22.00 \pm 1.21	21.97 \pm 0.67	21.39 \pm 0.29
A	27.85 \pm 0.76	25.84 \pm 1.24	26.33 \pm 0.56	29.33 \pm 0.82
G	27.35 \pm 0.65	27.22 \pm 1.02	28.02 \pm 0.86	25.71 \pm 0.84
U	22.89 \pm 0.54	24.90 \pm 0.71	23.68 \pm 0.60	23.55 \pm 0.48
G + C	49.21 \pm 0.67	49.24 \pm 1.78	49.98 \pm 1.08	47.47 \pm 0.36

The difference in G + C content between rRNAs from 73S particles and those from cytoplasmic ribosomes (79S) is statistically significant: $t = 4.396$ and $P < 0.003$.

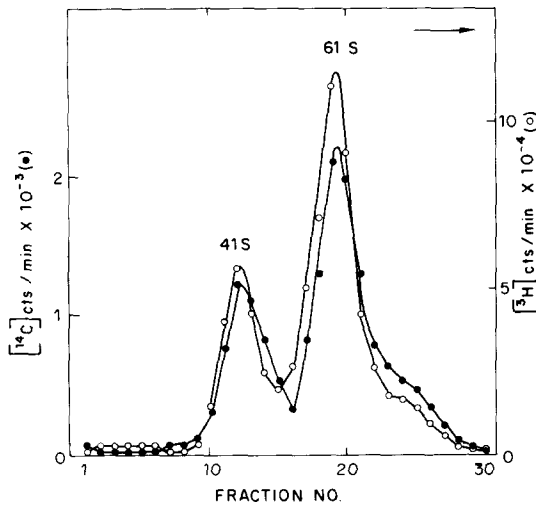


FIGURE 10 Fractionation of subunits from 79S ribosomes in sucrose gradient. 1 OD unit of purified cytoplasmic ribosomes was dissociated in 0.2 ml of a buffer containing 500 mM KCl and then centrifuged for 3.5 h in a 5–20% (wt/vol) linear sucrose gradient at 38,000 rpm at 16°C. ^3H -labeled *Chlamydomonas* cytoplasmic ribosomal subunits were co-sedimented as markers. ●, ^3H ; ○, ^{14}C .

The present study confirmed certain peculiar characteristics of the DNA satellite in *Polytoma* first reported by Kieras and Chiang (13) and extended by our subsequent studies (34). First, it has an exceedingly low buoyant density (1.682 g/ml), the lowest thus far reported for plastids of any kind. Kirk (14), after a survey of the studies on plastid DNAs, concluded that their buoyant densities fall into a range of 1.692–1.698 g/ml and thus a G + C content of 33–39%. The only exception is *Euglena*, whose chloroplast DNA was shown to have the distinctly lower density of 1.685 g/ml and a G + C content of 25% (2, 8, 21). By comparison, *Polytoma* leucoplast DNA has a buoyant density so low that the corresponding G + C content could no longer be estimated from the empirical equation of Schildkraut, Marmur, and Doty (28). The exceedingly rapid renaturation of β -DNA indicates that the leucoplast genome may consist of highly repetitive simple sequences. Finally, β -DNA represented approximately 16% of the whole-cell DNA. This is roughly comparable in amount to the 14% of chloroplast DNA in vegetative cells of *C. reinhardtii* (6), as well as the irreversibly bleached mutants of *Chlamydomonas*, *U3A*, and *U3N* (39). This is in striking contrast to the situation in *Euglena*, where at least some of the

irreversibly bleached mutants have reportedly lost their plastid DNA entirely (9, 22). It is thus implied that the expression of the leucoplast genome may still be necessary to the normal growth of *Polytoma* cells. This difference in the interdependence of nuclear and plastid DNA among different organisms has also been postulated by Chiang (5).

The presence of ribosomal particles in the leucoplast has been suggested by our fine structure studies (35). With the biochemical characterization of a distinct class of organelle ribosomes (73S) in the present study, it is evident that the leucoplasts indeed contain their own ribosomes. We cannot rule out the possibility that the 73S ribosomes come in part from the mitochondria, but several factors indicate that they derive largely from the leucoplast. First, the volume of the leucoplast within the cell is much larger; second, RNA sequences complementary to highly purified leucoplast DNA were found within this fraction, as shown by our hybridization studies (33); and third,

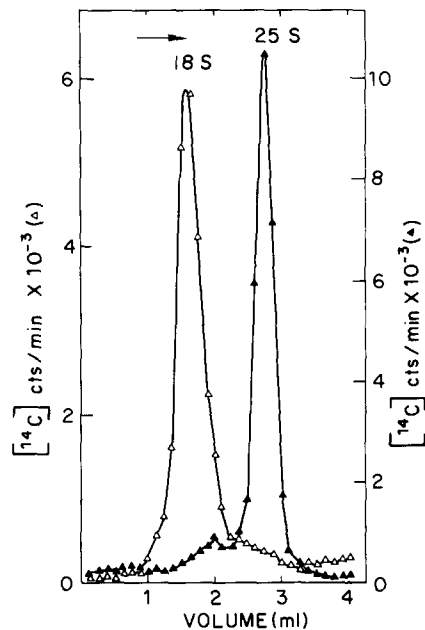


FIGURE 11 Analytical sucrose density gradient of rRNA extracted from 61S and 41S ribosomal subunits. Purified subunits were pooled from sucrose gradient fractions and pelleted. rRNA was purified with SLS and phenol extraction as described under Methods. About 1 μg of RNA was centrifuged in a 4-ml sucrose gradient at 60,000 rpm at 2°C for 5 h. ▲, 25S rRNA from the 61S subunit; △, 18S rRNA from the 41S subunit. Arrow indicates the direction of sedimentation.

our leucoplast fraction showed an eightfold enrichment of 73S ribosomes.

The 73S ribosomes were found to be different from the cytoplasmic ribosomes (79S) with respect to their sedimentation coefficients and the G + C content of their constituting rRNA, but the sedimentation patterns of the two component rRNA species of the 73S and 79S ribosomes were very similar. That the 73S particles represent a distinctive class of ribosomes, and not just a degradative product of 79S particles, was borne out by the fact that: (a) the relative proportion of the two major component rRNA species of the 73S particles was close to that of the normally expected 2:1 ratio for intact ribosomes, and (b) the overall G + C content of the rRNA of the 73S ribosomes was significantly lower than that of the cytoplasmic rRNA. Other evidence in support of this conclusion includes the preferential enrichment of the 73S ribosomes in the leucoplast fraction and our rRNA-DNA hybridization studies which will be reported separately (33).

In *Chlamydomonas*, chloroplast ribosomes constituted about one-third of the total cellular ribosomes and had a sedimentation coefficient of 70S (1, 12, 29). These ribosomes contained 23S and 16S rRNA as opposed to the 25S and 18S rRNA found in organelles of *Polytoma*. Contrary to the relatively low G + C content of mitochondrial rRNA in lower eukaryotes, 35% in *Neurospora* (24) and 25% in yeast (10), plastid rRNA usually has a G + C content of around 50% in spite of the size differences (23, 30, 36). The G + C content of rRNA from *Polytoma* 73S ribosomes is similar to

that of *Chlamydomonas* chloroplast rRNA (N. C. Martin and K.-S. Chiang, unpublished observation), being 47.5% and 51.0%, respectively.

Although the cytoplasmic ribosomes of *Polytoma* and *Chlamydomonas* possess very similar properties, as summarized in Table II, the plastid ribosomes appeared to be quite different in their sedimentation properties. The faster sedimentation rate of the *Polytoma* leucoplast ribosomes is probably due to the larger size of their two component rRNA species (Table II). The data therefore suggest that the plastid ribosomes have diverged in evolution to a greater extent than the cytoplasmic ribosomes. A further investigation into the sequence homology of the nuclear and plastid cistrons between *Polytoma* and *Chlamydomonas* has been undertaken and the results will be reported elsewhere.

Chloroplast ribosomes have been shown to be active in protein synthesis (27, 38). The presence of ribosomes in the *Polytoma* leucoplast strongly suggests that parts of the plastid genome are being transcribed, and the presence of a leucoplast protein synthetic system is implicated. As in other organelle protein synthesis, the translation of genetic information is doubtless also dependent upon the presence of a full complement of leucoplast tRNAs and translational factors. In the mitochondria of some petite yeast mutants, protein synthesis does not occur although the presence of ribosomes in them has been detected both morphologically and biochemically (Todd and Swift, unpublished observation; 11). Such non-functional mitochondria are rare in cell systems,

TABLE II
Comparison of Ribosomal and rRNA Properties in *P. obtusum* and *C. reinhardtii*

	<i>P. obtusum</i>	<i>C. reinhardtii</i>
Cytoplasmic ribosomes		
Sedimentation coefficient	79S	79S
Subunits	61S and 41S	61S and 41S
rRNA species	25S and 18S	25S and 18S
Average G + C content of rRNA	50.0%	50.3%
Plastid ribosomes		
Sedimentation coefficient	73S	70S
Subunits	—	52S and 37S‡
rRNA species	25S and 18S	23S and 16S
Average G + C content of rRNA	47.5%	51.0%*

* N. C. Martin, D. A. Goodenough and K.-S. Chiang, unpublished data.

‡ See reference 18.

since few other cells possess the efficient anaerobic metabolism of the yeast cell. Also, cytoplasmic petites are unstable. One would expect that the nonfunctional genomes characteristic of petite mitochondria would not survive in evolution. Contrary to the situation in petite yeast mitochondria, which are essentially nonfunctional and often have large deletions in their genomes (3, 11, 20), the leucoplast in *Polytoma* not only possesses a unique genome with a kinetic complexity comparable to that in other plastids (31) but also probably performs certain important metabolic functions as discussed previously (35). We favor the view that the ribosomes in the leucoplast are parts of an essential protein-forming system, possibly with a role in the remaining functions of the plastid, such as the storage and reutilization of polysaccharides, even though the photosynthetic properties of the plastid have long ago been lost.

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