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

III. Ribosomal RNA Cistrons of the Nucleus and Leucoplast

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ABSTRACT

The colorless alga *Polytoma obtusum* has been found to possess leucoplasts, and two kinds of ribosomes with sedimentation values of 73S and 79S. The ribosomal RNA (rRNA) of the 73S but not the 79S ribosomes was shown to hybridize with the leucoplast DNA (ρ = 1.682 g/ml). Nuclear DNA of *Polytoma* (ρ = 1.711) showed specific hybridization with rRNA from the 79S ribosomes. Saturation hybridization indicated that only one copy of the rRNA cistrons was present per leucoplast genome, with an average buoyant density of ρ = 1.700. On the other hand, about 750 copies of the cytoplasmic rRNA cistrons were present per nuclear genome with a density of ρ = 1.709. Heterologous hybridization studies with *Chlamydomonas reinhardtii* rRNAs showed an estimated 80% homology between the two cytoplasmic rRNAs, but only a 50% homology between chloroplast and leucoplast rRNAs of the two species. We conclude that the leucoplasts of *Polytoma* derive from chloroplasts of a *Chlamydomonas*-like ancestor, but that the leucoplast rRNA cistrons have diverged in evolution more extensively than the cistrons for cytoplasmic rRNA.

Polytoma obtusum, a colorless chloromonad, has been shown to possess two distinct classes of ribosomes, a major 79S class which constitutes about 98% of the total cellular ribosomes, and a minor 73S class. Fine structural analysis and subcellular fractionation have indicated that the 79S class represents the cytoplasmic ribosomes, while the 73S ribosomes are of organelle origin, derived mainly or entirely from the leucoplasts (19, 20). The ribosomes of the mitochondria have not yet been isolated.

Polytoma was found to contain two major DNA species, α-DNA and β-DNA (10). The latter possesses a very low buoyant density (ρ = 1.682 g/ml), is rich in A+T base pairs, and represents the leucoplast genome (18). Its peculiar properties tend to suggest that the informational content of the leucoplast genome may be very low. Since *Polytoma* lacks the capacity for photosynthesis, the following questions are raised: (*a*) whether the leucoplast genome is transcribed and (*b*) what is the mode of origin of the ribosomal particles

observed in the plastid by electron microscopy (19). If the rRNA of the previously identified 73S ribosomes (20) is indeed coded inside the leucoplast, sequences homologous to this rRNA should exist in the leucoplast genome.

Besides morphological similarities, *Polytoma* and *Chlamydomonas* possess cytoplasmic ribosomes of almost identical physical properties, and the base ratios of their rRNA components were found to be very close (20). Since rRNA cistrons have been shown to be an evolutionarily conservative character, so that cross hybridization between rRNA and DNA of different organisms was extensive (4, 16), the degree of phylogenetic affinity between *Polytoma* and *Chlamydomonas* may be further assessed by a study of the extent of molecular homology in the cytoplasmic and plastid ribosomal cistrons in these two organisms.

In order to resolve these questions, homologous and heterologous DNA-rRNA hybridization experiments between *Polytoma* and *Chlamyodomonas* have been conducted. The results presented in this report show that (*a*) rRNA cistrons are present in the leucoplast genome, (*b*) the leucoplast DNA anneals preferentially with rRNA from the 73S ribosomes but not the cytoplasmic rRNA, and (*c*) about 80% sequence homology is present in the nuclear rRNA cistrons between *Polytoma* and *Chlamydomonas*, but only 50% homology in cistrons for plastid rRNA.

MATERIALS AND METHODS
Cells and Culture Conditions

The *P. obtusum* strain 1 was kindly supplied by Dr. D. L. Provasoli, Haskins Laboratories, New Haven, Conn. *Chlamydomonas reinhardtii* strain 137C+ mating type, originally obtained from Dr. R. P. Levine of Harvard University, was used in this investigation. *P. obtusum* was grown in the Tris-acetate medium as described previously (17). *C. reinhardtii* was grown in the high salt minimal medium (HSM) as described by Sueoka, Chiang, and Kates (21).

Radioactive Isotope Labeling

[³H]adenine or [¹⁴C]adenine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) with a final concentration of 0.5 μCi/ml or 0.2 μCi/ml, respectively, was added to vegetative cultures at a cell concentration of 4-5 × 10⁵ cells/ml. Cells were allowed to grow to late exponential phase at about 4 × 10⁶ cells/ml, and harvested for ribosome isolation.

For ³²P-labeling, cells were grown in 0.2% sodium acetate-supplemented HSM medium with 1/400 of its

original phosphate content, buffered with 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, Calbiochem, San Diego, Calif.) and KOH at pH 7.0. 2 μCi/ml of inorganic [³²P]phosphate were added at 0.5 × 10⁶ cells/ml and the culture was grown overnight for another three-four generations before harvesting.

Isolation and Fractionation of DNA

Cells at late exponential phase were harvested and lysed by one passage through a French pressure cell (American Instrument Co., Inc., Silver Springs, Md.) at 2,000 lb/in² and subsequent detergent treatment. DNA was isolated by pronase plus RNase digestion, followed by chloroformisoamyl alcohol extraction as detailed previously (7, 17).

Whole-cell DNA thus obtained was further purified by preparative CsCl density gradient centrifugation. *Polytoma* leucoplast DNA and *Chlamydomonas* nuclear DNA were isolated either by two consecutive CsCl density gradients or by hydroxyapatite column chromatography (18). All DNA samples were dialyzed against several changes of SSC (or 0.15 M NaCl plus 0.015 M Na₃ citrate, pH 7.0) and the purity was then checked with analytical CsCl isopycnic centrifugation. Microdensitometer tracings of *Polytoma* whole cell DNA, leucoplast DNA, and *Chlamydomonas* nuclear DNA are shown in Fig. 1. No contamination by other DNA species in the latter two samples could be detected.
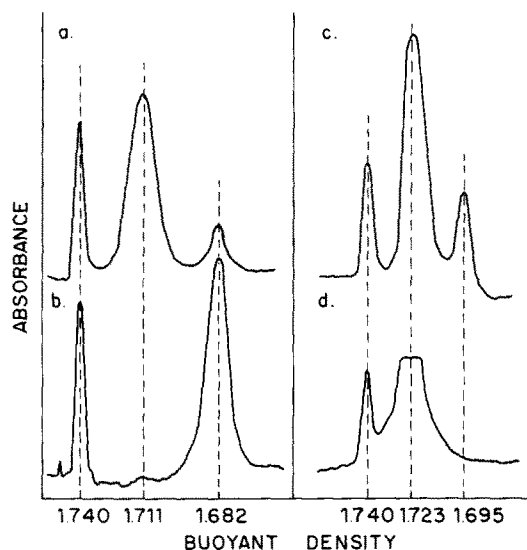

FIGURE 1 Microdensitometer tracings of purified DNA samples centrifuged in neutral CsCl density gradients at 44,000 rpm at 25°C for 20 h. (*a*) 6 μg of *P. obtusum* whole-cell DNA plus 1.5 μg phage SPO-1 DNA (ρ = 1.740 g/ml) as density marker; (*b*) purified *Polytoma* leucoplast DNA, 4 μg input; (*c*) 7 μg of *C. reinhardtii* whole-cell DNA; and (*d*) 5 μg of purified *Chlamydomonas* nuclear DNA.

Isolation of Ribosomes

Different classes of ribosomes from *Polytoma* and *Chlamydomonas* were isolated and fractionated in sucrose density gradients as previously described (9, 20).

Purification of Ribosomal RNA

Different species of ribosomes were pooled separately from gradient fractions and pelleted at 280,000 g_{max} for 2 h at 2°C. Ribosomes were suspended in a Tris-Mg buffer (1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4) and 2% sodium dodecyl sulfate was added to dissociate the rRNA from the proteins, which were removed by several extractions with phenol in the cold. RNA was precipitated with ethanol at $-20°C$ and then redissolved in Tris-Mg buffer. Residual phenol was removed by repeated extraction with diethyl ether, and ether was removed by bubbling a stream of dry filtered air through the RNA solution. The rRNA was further purified by DNase treatment (electrophoretically purified, Worthington Biochemical Corp., Freehold, N. J.), and the DNase was then removed with phenol.

The purity of rRNA was examined by gel electrophoresis and alkaline digestion (0.3 N KOH at 37°C for 16 h) to insure that over 99% of the radioactive counts were alkali labile. Purified rRNA was stored frozen in Tris-Mg buffer. 20 OD units of 260 nm with 1-cm light path were taken as the equivalent of 1 mg/ml.

DNA-RNA Hybridization

The filter hybridization technique of Gillespie and Spiegelman (8) was followed. Formamide was used to lower the optimal temperature for the formation of molecular hybrids (5, 13). DNA samples were denatured by adding three parts of 1 M NaOH to 10 parts of DNA solution in SSC and were allowed to stand at room temperature for 15–30 min for complete denaturation. The DNA samples were neutralized with three parts of 2 M NaH_2PO_4 in the cold, and 20× SSC was added to give a final cation concentration equivalent to 4× SSC. DNA samples were loaded onto nitrocellulose filters (B-6, Schleicher and Schuell) under gravity, and each filter was washed with 30 ml of 4× SSC under suction. Filters were then dried at room temperature for 4 h before being put into a 65°C incubator for another 2–6 h. Duplicate blank and DNA filters were transferred to new scintillation vials and incubated in the hybridization mixture of 0.5 ml of 4× SSC and formamide with different concentrations of rRNA for the prescribed length of time. When the reaction was terminated, the filters were extensively washed and treated with pancreatic RNase (20 μg/ml) and T_1 RNase (25 U/ml) for 1 h at 37°C. The filters were again washed on both sides with 100 ml of 4× SSC and air dried. They were counted in 5 ml of toluene-base scintillation fluid (3.8 liters, toluene; 15.12 g, PPO; 0.375 g, POPOP) in a Packard Tricarb liquid scintillation

spectrometer (Packard Instrument Co., Downers Grove, Ill.).

The concentration of formamide in all hybridization experiments was determined by testing the efficiency of DNA-RNA hybrid formation at 37°C after 20 h of incubation. The optimal formamide concentration for cytoplasmic rRNA annealing to nuclear DNA was 40–50%, while 30–40% was optimal for plastid rRNA. An incubation period of 12 h was found to be sufficient for maximum hybrid formation in all cases. Most experiments were performed with a hybridization time of 15–18 h at 37° C in an incubation mixture containing 4× SSC—35% formamide for plastid DNA and 4× SSC—40% formamide for nuclear DNA, such that the rRNA-DNA reassociation was carried out at a temperature approximately equivalent to Tm $-30°C$ of the corresponding DNA species used.

Competition Hybridization

In all competition hybridization experiments, DNA filters were first hybridized with varying amounts of cold rRNA as described. After RNase treatment, the filters were washed and incubated in 0.3% diethylpyrocarbonate at 37°C for 10–15 min to inactivate the residual RNase. The filters were then challenged by a fixed amount of the labeled rRNA and processed as before.

Melting of DNA-RNA Hybrids

DNA-RNA hybrids were thermally dissociated by raising the temperature at 5°C intervals. The filters containing hybrids with over 300 cpm were air dried and washed in 0.1 SSC. The radioactive counts released in the wash were not taken into consideration. The filters were then immersed in 1 ml of 0.1 SSC and incubated in a water bath for 10 min for equilibrium at 25°C. Subsequently, the filters were transferred to another vial with 1 ml of 0.1 SSC and incubated at a temperature 5°C higher. This basic process was repeated for every 5°C increase in temperature to 100°C. The radioactive RNA released into the solution was counted in 10 ml of Triton-toluene scintillation fluid (333 ml, Triton X-100; 667 ml toluene; 5 g, PPO; 0.1 g, POPOP).

RESULTS

Origin of the rRNA of the 73S Ribosomes

Small (73S) ribosomes were found to occur in *Polytoma* in minute amounts. Since complete separation of mitochondria and leucoplasts was not obtained, the origin of the 73S ribosomes, whether mitochondrial or leucoplastic, could not be concluded merely by organelle fractionation studies. To ascertain that a significant portion of the rRNA isolated from this 73S class of ribosomes are encoded in the leucoplast genome,

molecular hybridization experiments with different combinations of rRNA and DNA were carried out. CsCl profiles of the preparations used are shown in Fig. 1; Fig. 2 shows that the leucoplast DNA hybridized with the rRNA of 73S ribosomes to a saturation level of 0.47%. Since each *Polytoma* cell contains 1.82×10^{10} daltons of leucoplast DNA, there were about 43 sets of the leucoplast ribosomal genes per cell. On the other hand, the leucoplast DNA did not anneal significantly with *Polytoma* cytoplasmic 25S and 18S rRNA (<0.03% hybridization). As a control, *Clostridium perfringens* DNA, which is well-known for its low G+C content (25%), was used to anneal with rRNA of the 73S ribosomes from *Polytoma*. The control hybridization level did not exceed 0.02% (Fig. 2).

To obtain enough counts in the hybrid for melting analysis, ^{32}P-labeled rRNA of the 73S ribosomes and leucoplast DNA were hybridized at an rRNA/DNA ratio of 1/500, for 5 h at 60°C to Cot 3 (where Cot equals the initial single-stranded DNA concentration X reassociation time) (6). Under our experimental conditions, more than 50% of the ^{32}P-labeled rRNA molecules hybridized with the leucoplast DNA, which also reannealed with itself to over 80%. When the rRNA-DNA hybrid was melted, the profile was highly cooperative, though biphasic, as was evident from
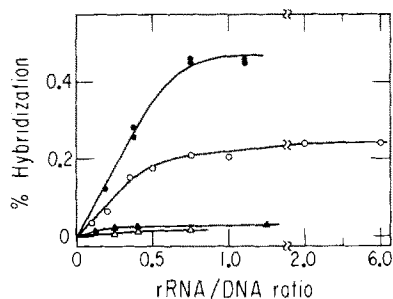

FIGURE 2 Saturation hybridization of *Polytoma* leucoplast DNA with increasing concentrations of various rRNA species. ●, ^{32}P-labeled rRNA from *Polytoma* 73S ribosomes (12,907 cpm/µg) annealed with leucoplast DNA (1 or 2 µg); ○, *Chlamydomonas* chloroplast rRNA (^{3}H-labeled, 4,692 cpm/µg) annealed with leucoplast DNA (4 µg/filter); ▲, *Polytoma* cytoplasmic rRNA (^{32}P-labeled, 16,769 cmp/µg) annealed with leucoplast DNA (4 µg/filter); △, *Clostridium perfringens* DNA (2 µg/filter) annealed with *Polytoma* ^{32}P-labeled rRNA from 73S ribosomes. Percent hybridization was expressed in terms of (µg of rRNA bound)/(µg of DNA on filter).
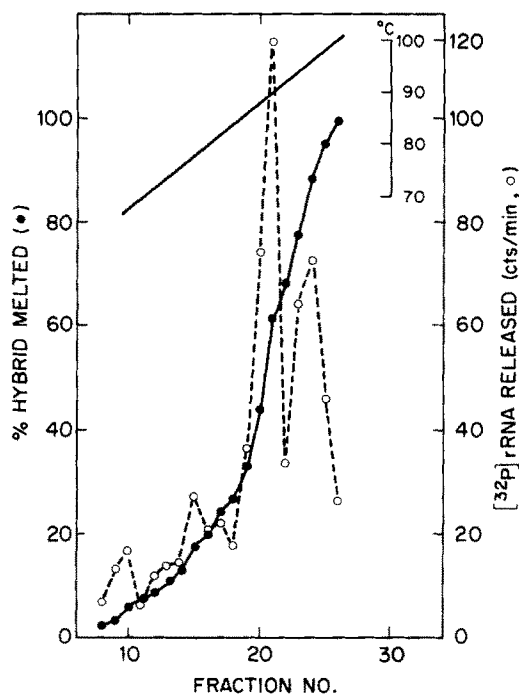

FIGURE 3 Melting of rRNA-DNA hybrids on hydroxyapatite column. Ribosomal RNA from 73S ribosomes was hybridized with leucoplast DNA to a Cot of 3 in 0.14 M phosphate buffer at 60°C. The reaction mixture was diluted fivefold with 0.14 M PB and loaded onto a water-jacketed column pre-equilibrated at 60°C, and single-stranded material was first eluted with 16 ml of 0.14 M PB. Temperature was raised linearly at 18°C/h and the column was eluted with 0.14 M PB (i.e., 0.21 M Na$^+$) at a rate of 36 ml/h. 4-ml fractions were collected and counted directly in 10 ml of triton-toluene scintillation fluid. ○, [^{32}P]rRNA melted; ●, cumulative percent hybrid melted.

the differential plot. The overall Tm of the hybrids was 91°C in 0.14 M sodium phosphate buffer, i.e., 0.21 M Na$^+$ (Fig. 3). This Tm is equivalent to 74°C at a Na$^+$ concentration of 0.0195 M (Na$^+$ concentration of 0.1 SSC) (12). The melting of other DNA-rRNA hybrids on filters was also routinely performed in 0.1 SSC.

Chlamydomonas chloroplast rRNA was also hybridized with *Polytoma* leucoplast DNA on filters to assess the extent of homology in the plastid ribosomal genes of these two organisms. A saturation level of 0.24% was reached at an RNA/DNA ratio of 2 (Fig. 2). When subjected to melting analysis of 0.1 SSC (0.0195 M Na$^+$), approximately 60% of the heterologous hybrid

melted cooperatively with a Tm of 68°C, while the rest melted broadly at low temperature (Fig. 4).

Localization of Leucoplast Ribosomal Cistrons in CsCl Gradient

To determine the buoyant density of the ribosomal cistrons, purified leucoplast DNA was banded in a CsCl density gradient and each fraction was hybridized with a constant amount of ^{3}H-labeled Chlamydomonas chloroplast rRNA, owing to its ready availability and sequence homology with the leucoplast rRNA cistrons. DNA sequences complementary to the chloroplast rRNA were mainly localized at a region with an average density of 1.685 g/ml and slightly heavier than the average peak density of leucoplast DNA (Fig. 5 a).

Since the plastid rRNAs of Polytoma and Chlamydomonas have a base composition of 47.5% and 51%, respectively (20), the leucoplast ribosomal cistrons would be expected to have a similar average G+C content. Therefore, if leucoplast DNA were sheared to gene-size fragments, the ribosomal genes should band at a buoyant density much higher than that of the bulk DNA. This prediction was borne out in the experiment shown in Fig. 5 b. In a CsCl gradient of sheared leucoplast DNA with an average mol wt of 1 × 10^6 daltons, the ribosomal genes banded at a buoyant density of 1.700 g/ml. The peak of hybridization was asymmetrical, skewed to the heavier side,
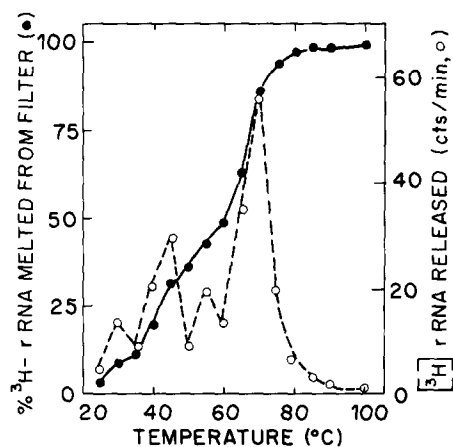

FIGURE 5 Hybridization of C. reinhardtii chloroplast rRNA to P. obtusum leucoplast DNA banded in preparative CsCl density gradient. 75 μg leucoplast DNA was centrifuged to equilibrium and 0.1-ml fractions were collected. 0.2 ml of 0.1 SSC was added to each fraction for absorbance reading at 260 nm(A_{260}). DNA from each fraction was hybridized with 4 μg of ^{3}H-labeled Chlamydomonas chloroplast rRNA (10,579 cpm/μg) in 30% formamide at 37°C. Ribosomal RNA was hybridized with (a) unsheared leucoplast DNA with an average double-stranded mol wt of 10.9 × 10^6 daltons, and (b) leucoplast DNA sheared to an average size of 1.2 × 10^6 daltons. ● A_{260}; △, [^{3}H]rRNA hybridized.

suggesting that the bulk of the ribosomal cistrons may have a buoyant density higher than 1.700 g/ml.

Nuclear Ribosomal Cistrons

Whole-cell DNA of P. obtusum was banded in CsCl density gradients, and each of the DNA fractions was hybridized with a constant amount of ^{32}P-labeled cytoplasmic rRNA (25S and 18S). The peak of hybridization was slightly skewed to the light side of the nuclear DNA band. Since saturation amounts of rRNA were used in the experiment, a cpm/A_{260} plot for the fractions showed that the nuclear rRNA cistrons had an average buoyant density of 1.709 g/ml (Fig. 6). The nuclear ribosomal cistrons thus appeared to be clustered and had an average G+C content only slightly lower than that of the nuclear DNA (ρ = 1.711 g/ml). Whole-cell DNA, rather than just the main band DNA, was used for subsequent hybridization experiments with cytoplasmic rRNA to avoid possible loss of material during DNA frac-

FIGURE 4 Melting curve of heterologous hybrids formed between Polytoma leucoplast DNA and Chlamydomonas chloroplast rRNA. Ribosomal RNA was melted from hybrids on nitrocellulose filter in 0.1 SSC or 0.0195 M Na$^+$ as described in Methods.
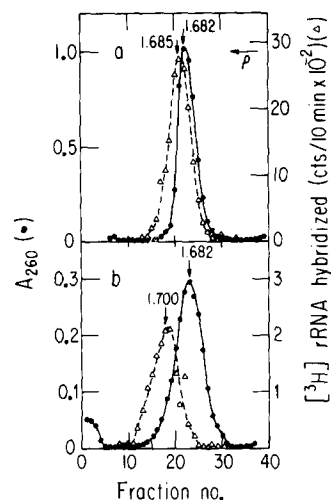
tionation, since the rRNA cistrons were clustered at the light side of the main band DNA and, in agreement with our above observation, cross hybridization between leucoplast DNA and cytoplas-
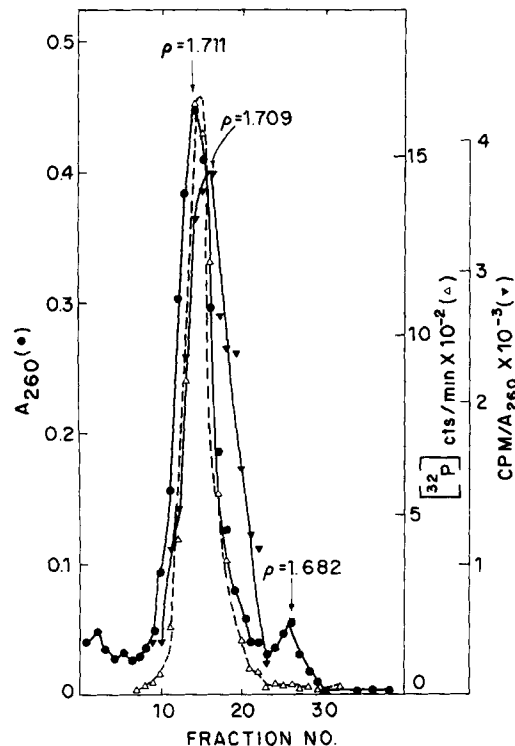
mic rRNA was essentially negligible (see Figs. 2 and 6).

When *Polytoma* cytoplasmic rRNA was hybridized with whole-cell DNA, a saturation level of 1.30% was reached at an RNA/DNA ratio of less than one. Similar values were obtained whether 5 or 10 μg of DNA were immobilized on the filter (Fig. 7). Since the analytical complexity of the whole-cell genome is 11.4 $\times$ 10^{10} daltons, the number of the nuclear ribosomal cistrons was estimated to be about 750 copies per cell (Table I). Since we have concluded that our vegetative cells are diploid (17), this means that there are approximately 375 rRNA cistrons per genome.

Hybridization with Heterologous Cytoplasmic rRNA and Competitive Studies

The results from the hybridization of increasing amounts of *Chlamydomonas* cytoplasmic rRNA to a fixed amount of *Polytoma* DNA are shown in Fig. 7. Saturation was attained at an RNA/DNA ratio of 1.0 and the saturation level was 1.0%, about 23% less efficient than the homologous *Polytoma* cytoplasmic rRNA (Fig. 7).

Competition studies between labeled cytoplasmic rRNA and cold homologous or heterologous cytoplasmic rRNA were performed to test whether these RNAs were actually competing for the same sites on the *Polytoma* nuclear DNA. Nuclear DNA filters were hybridized with increasing amounts of either cold homologous rRNA or cold heterologous rRNA, and then each was challenged with a fixed amount of labeled homologous rRNA. Labeled *Polytoma* cytoplasmic rRNA hybridized with *Polytoma* nuclear DNA competed with cold homologous cytoplasmic rRNA down to a 5% level, while competition with heterologous *Chlamydomonas* cold rRNA only reached a level of about 20% (Fig. 8 a). When *Chlamydomonas* DNA was used, the competition between homolo-

FIGURE 6 Hybridization of *Polytoma* cytoplasmic rRNA to whole-cell DNA banded in preparative CsCl density gradient. 150 μg of *Polytoma* DNA was centrifuged to equilibrium at 50,000 rpm for 40 h at 25°C. 10-drop fractions (about 0.65 ml) were collected and 0.3 ml of 0.1 SSC was added to each fraction and the absorbance was measured at 260 nm. DNA from each fraction was denatured, fixed to nitrocellulose filter, and annealed with ^{32}P-labeled cytoplasmic rRNA (96,000 cpm/μg) at a concentration of 10 μg/ml in 4$\times$ SSC-40% formamide for 15 h at 37°C. $\bullet$, A$_{260}$; $\triangle$, [^{32}P]rRNA hybridized; $\blacktriangledown$, cpm/A$_{260}$.

TABLE I

Number of Nuclear and Leucoplast Ribosomal RNA Cistrons Determined by DNA-rRNA Hybridization

DNA source	Analytical complexity in daltons*	rRNA source	Mol wt of rRNA‡	Hybridization	Daltons hybridized	No. of cistrons per cell
			daltons	%		
P. obtusum						
Total DNA	11.4 $\times$ 10^{10}	79S ribosomes	1.99 $\times$ 10^6	1.30	1.48 $\times$ 10^9	748
Leucoplast DNA	1.82 $\times$ 10^{10}	73S ribosomes	1.99 $\times$ 10^6	0.47	8.6 $\times$ 10^7	43

* Reference 17.
‡ Molecular weight determinations according to Loening (11).
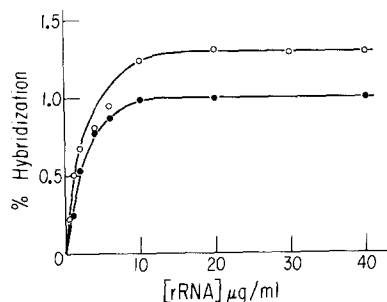
FIGURE 7 Saturation hybridization of *Polytoma* whole-cell DNA with cytoplasmic rRNA. Homologous cytoplasmic rRNA (^{3}H-labeled, 6,045 cpm/μg) was hybridized with 10 μg of *Polytoma* DNA (●), and *Chlamydomonas* cytoplasmic rRNA (^{3}H-labeled, 23,550 cpm/μg) was annealed with 10 μg of *Polytoma* DNA (O). Percent hybridization was expressed in terms of (μg of rRNA bound)/(μg of DNA on filter).

gous cytoplasmic rRNAs was down to a level of about 8%, while the competition level for heterologous rRNAs was 25% (Fig. 8 *b*). The data therefore indicate that heterologous cytoplasmic rRNA competed for 75–80% of the sites bound by the homologous rRNA whether nuclear DNA of *Polytoma* or *Chlamydomonas* was tested.

Melting of Hybrids

DNA-rRNA hybrids were melted to compare the fidelity of base pairing between homologous and heterologous complexes. Homologous *Polytoma* hybrids (containing nuclear DNA and cytoplasmic rRNA) had a Tm of 80°C, while the heterologous hybrids (formed with *Polytoma* nuclear DNA and *Chlamydomonas* cytoplasmic rRNA) had a Tm of 72°C (Fig. 9 *a*). When *Chlamydomonas* nuclear DNA was used, homologous and heterologous hybrids (with *Chlamydomonas* or *Polytoma* cytoplasmic rRNA) had Tm's of 81°C and 73°C, respectively (Fig. 9 *b*). The depression in Tm was 8°C in both cases, and the heterologous hybrids had a cooperative transition in melting profile similar to that of the homologous hybrids, suggesting that rather extensive well-matched regions were present between the DNA and heterologous rRNA.

DISCUSSION

It is evident from the above results that the leucoplast genome of *P. obtusum* contains ribosomal cistrons, which are also transcribed to provide rRNA for the assembly of the 73S ribosomes. Saturation hybridization indicated the presence of 43 sets of leucoplast rRNA genes per

cell (Table I). Although slight underestimation is likely, owing to the possible loss of material during DNA fractionation, this comes close to one set of rRNA cistrons per single copy of the leucoplast "chromosome" since *P. obtusum* has been shown to possess 55-65 copies of the leucoplast "chromosome" per cell. It is of significance to note that similar conclusions based on hybridization studies have been made in other algal species. *Chlamydomonas* has been shown to possess 40-50 copies of its chloroplast genome (2, 3, 22), each
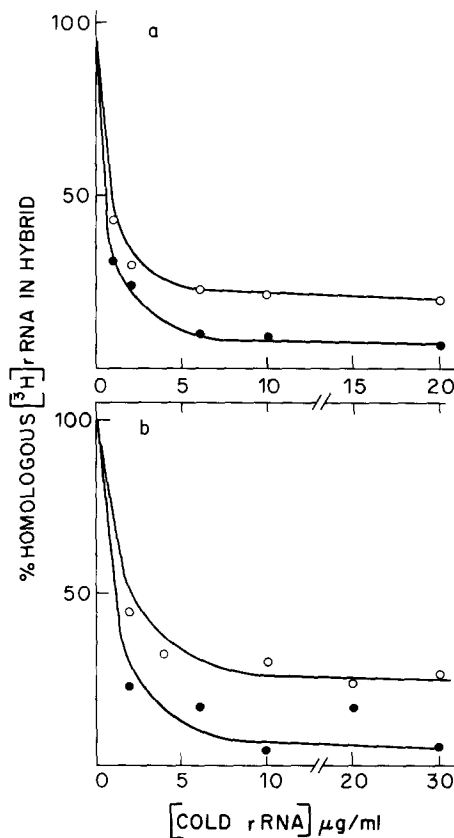

FIGURE 8 Competition hybridization studies of *Polytoma* and *Chlamydomonas* cytoplasmic rRNA. (*a*) Filters of *Polytoma* DNA (5 μg each) were first annealed with increasing concentrations of unlabeled homologous cytoplasmic rRNA or unlabeled *Chlamydomonas* cytoplasmic rRNA. After RNase treatment and extensive washing, each filter was hybridized with 10 μg of ^{3}H-labeled *Polytoma* cytoplasmic rRNA for another 15 h at 37°C. ●, homologous competition; O, heterologous competition. (*b*) Similar experiment as described in (*a*) was repeated using *Chlamydomonas* nuclear DNA and *Chlamydomonas* ^{3}H-labeled rRNA to compete with either unlabeled *Polytoma* rRNA. ●, homologous competition; O, heterologous competition.
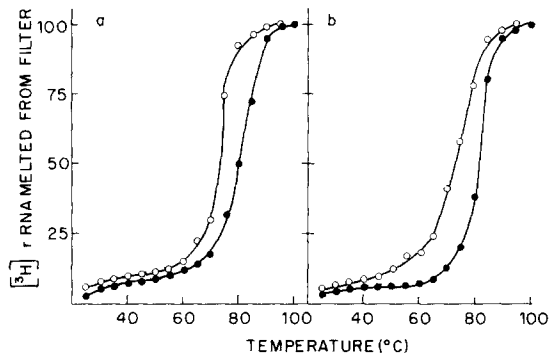
FIGURE 9 Melting curves of DNA-rRNA hybrids. ³H-labeled rRNA was melted from hybrids on nitrocellulose filters in 0.1 SSC or 0.0195 M Na⁺. (a) Hybrids of *Polytoma* DNA annealed with homologous cytoplasmic rRNA (●) or with *Chlamydomonas* cytoplasmic rRNA (○) melted in 0.1 SSC; and (b) melting of hybrids formed by *Chlamydomonas* nuclear DNA and homologous rRNA (●) or *Polytoma* rRNA (○).

containing only one set of the chloroplast rRNA cistrons (1). Rawson and Haselkorn (15) have also reported similar findings for *Euglena*.

Since rRNA of the 73S ribosomes in *P. obtusum* has a G+C content of 47.5%, the rRNA cistrons in the leucoplast genome would be expected to have a similar average G+C content. However, the DNA molecules possessing sequences complementary to the rRNA were banded at a region of extremely low buoyant density of 1.685 g/ml rather than 1.706 g/ml as predicted from the G+C content. When leucoplast DNA was sheared to an average mol wt of 1 × 10⁶ daltons, the rRNA genes were found to band at a buoyant density close to the predicted value (Fig. 5 b). Therefore, it is evident that the rRNA cistrons in the leucoplast DNA are covalently linked with long stretches of very AT-rich regions. This also indicates that rRNA cistrons are not clustered, a finding which agrees with our estimate of only one cistron per leucoplast genome. By the same token, the leucoplast genome may still retain a number of structural genes with relatively high G+C content despite the fact that the overall G+C ratio of the plastid DNA is only 17-18% (18). These G+C-rich sequences are doubtless also separated by long AT-rich base sequences, so that they band with the bulk of the unsheared leucoplast DNA.

The leucoplast DNA did not hybridize with *Polytoma* cytoplasmic rRNA to any significant extent, suggesting that the rRNA genes of plastid and nuclear genomes are very different in base

sequence, although the base ratios of the cytoplasmic rRNA (50% GC) and leucoplast rRNA (47.5%) were fairly close (18). On the other hand, *Chlamydomonas* chloroplast rRNA annealed with the *Polytoma* leucoplast ribosomal cistrons extensively, and about 50% homology existed between them as shown by saturation hybridization. The melting curve of the heterologous hybrids showed that about 50% had a cooperative transition indicating extensive well-matched sequences, while the rest melted broadly at low temperatures. The broad lower portion is doubtlessly due to mismatching in base pairs. In case of the leucoplast homologous hybrids, there was very little early melting material and the Tm was high. The biphasic melting curve of these hybrids suggests that the two leucoplast rRNA species (25S and 18S) may have a slightly different G+C content (see Fig. 3).

Pigott and Carr (14) in their studies on the *Euglena* chloroplast ribosomal genes showed that the homology between *Euglena* chloroplast DNA and rRNA from various species of blue-green algae ranged from 10 to over 40%. Our findings of a significant degree of homology in the plastid ribosomal cistrons between *Polytoma* and *Chlamydomonas*, together with their observations, reflects the conservative character of ribosomal genes among green and blue-green algae.

The nuclear ribosomal genes of *P. obtusum* were repetitive, as saturation hybridization studies showed that there were about 750 rRNA cistrons per cell or 375 copies per haploid genome. These ribosomal genes were probably clustered since they banded as a sharp peak in the CsCl density gradient at a density of 1.709 g/ml, which also suggests that the ribosomal cistrons had a G+C content of about 50%. This is in close agreement with the base ratio of cytoplasmic rRNA which has been found to be 49.98% (18). It is not known whether spacers exist between cistrons, but if they do, the average G+C content of the spacers should also be close to 50%.

Chlamydomonas cytoplasmic rRNA not only has a G+C content (50.4%) very close to that of *Polytoma* cytoplasmic rRNA (50.0%), but also has 80% homology in base sequences with the *Polytoma* nuclear ribosomal genes. Both saturation hybridization and competition hybridization studies confirmed that an estimated 75-80% homology existed in the ribosomal cistrons between *Polytoma* and *Chlamydomonas*. Since only about 50% of sequence homology was found in the

plastid ribosomal cistrons of *Polytoma* and *Chlamydomonas*, the plastid ribosomal cistrons appear to have diverged in evolution more extensively than the nuclear ribosomal cistrons.

When homologous and heterologous hybrids were subjected to melting analyses, both of them gave a high Tm and a cooperative transition profile. The difference in Tm between the homologous and heterologous hybrids was 8° C in both cases, indicating that only a low percentage of mis-matching was present in the heterologous hybrids, while most of the sequences were well-matched. Although few generalizations can be made on the basis of studies on only two species, such good fidelity in heterologous hybrids and the high degree of sequence homology in the ribosomal cistrons suggest that the two chloromonads are phylogenetic relatives. This also supports our previous conclusion drawn from fine structure and ribosome studies (17, 18), namely that the species are clearly related but that the rRNA cistrons of the plastid have diverged in evolution more than those of the nucleus.

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