HYBRIDIZATION STUDIES WITH RNA AND DNA ISOLATED FROM
EUGLENA GRACILIS CHLOROPLASTS AND MITOCHONDRIA

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1. Introduction

Euglena gracilis when grown in light contains chloroplasts and mitochondria. Both types of organelles contain their own set of duplex DNA [1—3] which replicate at a different pace than the nuclear DNA [4,5] does. These organelles also are equipped with functional 70 S ribosomes containing 23 S, 16 S rRNA [6].

Organellar rRNA is considered to be a transcript of the respective organellar DNA. *Euglena* chloroplast DNA (chDNA) was shown to code for 23 S, 16 S rRNA [7—9]. The number of cistrons per chromosome, however, is still in doubt and could be between one and three [9—11]. We have reported that the hybridization capacity of chDNA for 23 S, 16 S chloroplast RNA varies with the amount of a 'heavy' chDNA component, isolated from highly purified chloroplasts. This 'heavy' component, depending on the average fragment size, has a buoyant density of 1.692 to 1.701 g/cc (peak densities) [12]. Since *Euglena* mitochondrial DNA (mtDNA) was reported to have densities in the range of 1.690 to 1.692 g/cc [1—3], the 'heavy' chDNA might be, at least partly, of mitochondrial origin. In order to clarify this point, we found it necessary to isolate and characterize mtDNA from *Euglena gracilis* and in particu-

lar to measure the affinity of chloroplast rRNA for this DNA. Further, it seemed necessary to measure as well the hybridization capacity of mtDNA towards its own major RNA components since such an experiment was still lacking.

Mitochondrial rRNA hybridizes with mtDNA to 3.7%, while only to 0.16% with chDNA under identical incubation conditions. Chloroplast rRNA hybridizes with total chDNA, containing 30% of 1.692 g/cc DNA to 2.9%, while only to 0.12% with mtDNA. We conclude that the 1.692 g/cc DNA found in chloroplast DNA preparations is not of mitochondrial origin.

2. Materials and methods

2.1. *Buffers*

Buffer I: 0.05 M Tris—HCl (pH 7.9), 0.1 M KCl, 0.01 M $MgCl_2$, 5 mM 2-mercaptoethanol. Buffer II: 0.01 M Tris—HCl (pH 7.9), 0.1 M KCl, 0.01 M $MgCl_2$. Buffer III: 0.1 M Tris—HCl (pH 7.9), 2.5% sodium do decyl sulfate, 0.01 MEDTA. Buffer IV: 0.1 M NaCl, 0.05 M Na_2HPO_4, 0.05 M NaH_2PO_4, 0.1 mM EDTA (pH 6.8). Buffer V: 0.04 M Tris—acetate (pH 7.9), 0.1 M sodium acetate, 1 mM magnesium acetate.

2.2. *Isolation and purification of mitochondrial RNA*

For the isolation of mitochondria, we used the aplastidic strain W_3BUL (a gift from J. A. Schiff, Brandeis University). Cells were grown in a heterotrophic medium [13] modified by using the trace element combination of Cramer and Meyers [14]. W_3BUL

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does not contain chDNA [2] yielding, therefore, un-contaminated mtDNA.

Mitochondria were isolated from freshly harvested cells (batches of 150 g, wet weight) by grinding with a mortar, pestle and glass beads [6]. The mitochondria were purified by differential centrifugation and flotation in a Renografin (Squibb and Sons, Inc., Princeton) step gradient [15].

Purified mitochondria were lysed in 5% Triton X-100 (Buffer I). Membranous material was removed by centrifugation (Sorvall SS-34, 48 200 g, 10 min). The supernatant was adjusted to 2% sodium dodecyl sulfate (SDS) and shaken for 5 min with a half volume of phenol/m-cresol/8-hydroxyquinoline (PCQ, 90/10/ 0.1, v/v/w). A volume of chloroform/isoamyl alcohol (24/1, v/v) equal to the PCQ volume was added and shaking was continued for 5 more min. The aqueous phase was treated with deoxyribonuclease (Bovine Pancreas, DN-EP, Sigma) for 1 hr. After further extraction with chloroform/isoamyl alcohol, the RNA was precipitated with 1.5 vol of ethanol ($-20°$C). The precipitate, resuspended in Buffer II, was reprecipitated with ethanol.

Mitochondrial RNA (Buffer II) was further purified in a 12 ml convex sucrose gradient in Buffer II (Spinco SW40 rotor, 40 krpm, 16.5 hr). The peak fractions corresponding to 14 S, 11 S, 9 S RNA were combined, dialysed against 0.1 × SSC (standard saline citrate = 1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), concentrated in vacuo and reextracted with phenol/ chloroform/isoamyl alcohol (25/24/1; v/v/v). The aqueous phase was passed through Sephadex G-50, fine grade column (1.5 cm × 30 cm). The fractions containing RNA were combined and the RNA again precipitated with ethanol. From approximately 700 g cells (wet weight), we obtained 1 mg of mitochondrial RNA. The A_{260}/A_{280} and A_{260}/A_{240} ratios were approximately 2.0.

2.3. *Isolation and purification of chloroplast rRNA*

The isolation and purification of 23 S, 16 S rRNA from *Euglena gracilis* Klebs (z-strain) chloroplasts has been described in detail [16].

2.4. *Labeling of mitochondrial and chloroplast RNA*

One mg of each were labeled in vitro with 5 mCi [^{3}H] dimethylsulfate (New England Nuclear, 385 mCi/mM) according to Smith et al. [17], and as modi-

fied by Rawson and Haselkorn [9]. The labeled RNA again was passed through the Sephadex column to remove low molecular weight labeled components and then filtered 3 times through nitrocellulose filters (Schleicher and Schuell, B6) to remove residual basic proteins and poly-(A) RNA fragments.

2.5. *Isolation of mitochondrial DNA*

Purified mitochondria were lysed in Buffer III and the DNA was isolated using PCQ, and chloroform/iso-amyl alcohol as protein denaturing agents. RNA was digested with ribonuclease (bovine pancreas, type VII, Sigma). The reextracted aqueous phases were dialysed against Buffer IV and further purified by passing through a methylated bovine serum albumin coated Kieselguhr column (MAK). Fractions containing nucleic acids were combined and further purified via a preparative CsCl density gradient. Final yield from several batches was 1.25 mg DNA/1.8 kg of cells (wet weight). This mtDNA had a buoyant density of 1.689 g/cc.

2.6. *Isolation of chloroplast DNA*

This was done as reported earlier [12]. This chDNA preparation contained a major DNA component of 1.685 g/cc density (approx. 70%) and a 'heavy' DNA component of 1.692 g/cc. Average molecular weight was 2×10^7

3. Results and discussion

3.1 *Characterization of mitochondrial RNA*

Controversial reports concerning the molecular weight of *Euglena gracilis* mitochondrial RNA makes it necessary to shortly characterize the mitochondrial RNA used in the following experiments. Krawiec and Eisenstadt [18] obtained 14 S and 11 S RNA as the two major mitochondrial RNA components. On the other hand, Avadhani and Buetow (6) reported 23 S and 16 S RNA to be the RNA components found in the mitochondrial 70 S ribosomes. These latter authors inferred that the 14 S, 11 S RNA though of ribosomal origin could be degradation products of the 23 S, 16 S RNA. This assumption is supported by the fact, that the base composition reported for both RNA preparations [6,18] match within the limits of analytical error.

Although we followed the procedure of Avadhani and Buetow (see paragraph 2.2.), we routinely found

before sucrose gradient purification only 14 S, 11 S,
9 S RNA along with a considerable amount of transfer
RNA (fig. 1a). The S-values are assigned relative to
23 S, 16 S rRNA from *E. coli* ribosomal subunits
(fig. 1b). The 14 S, 11 S, 9 S RNAs were separated in
preparative sucrose gradients from tRNA and used in
the following hybridization experiments. We consider
these RNAs also as degraded but nevertheless repre-
sentative mitochondrial rRNA. However, this isolation
procedure does not exclude contamination by messen-
ger RNA (mRNA).

3.2. *DNA/RNA hybridization*

In fig. 2, we show the saturation curve obtained
when hybridizing increasing amounts of mitochon-

Fig. 2. Hybridization of mitochondrial RNA with mtDNA.
Mitochondrial DNA (5.3 μg DNA/filter) was annealed using
in essence the methods of Gillespie and Spiegelman (22) with
variable amounts of mitochondrial RNA. Each point repre-
sents the average of two values which were corrected for un-
specific RNA binding to the filters. Incubation conditions:
4–8 filters, including blanks, per vial with 2 ml of 2 × SSC,
0.5% SDS, 59°C, 16 hr. Counting: toluene counting solution
(Omnifluor, New England Nuclear), Packard Tricarb Model
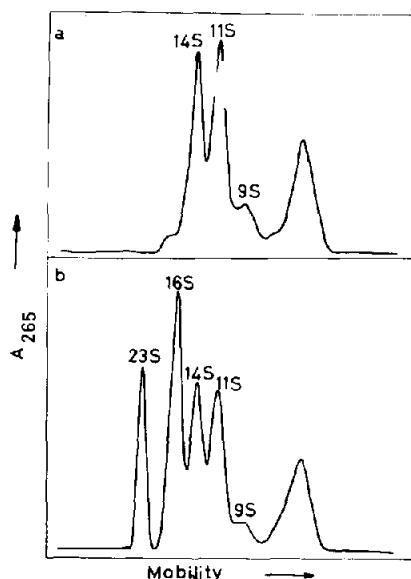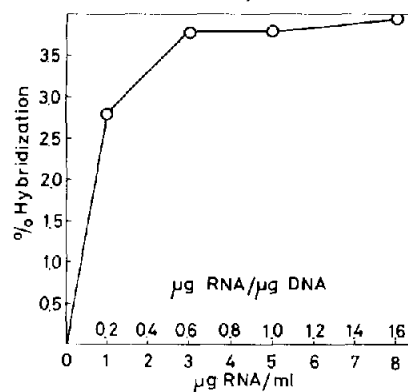3375 Liquid Scintillation Counter.

Fig. 1. Disc gel electrophoretic analysis of *Euglena* mitochon-
drial RNA. 30 μg of mitochondrial RNA (0.2 M sucrose-Buf-
fer V) were layered on precooled 2.4%, 6 cm long polyacryla-
mide gels [21] in Buffer V. The RNA band was first concen-
trated on top of the gel by a 15 min run (5 mA/gel tube).
Subsequent electrophoresis conditions were, 10 mA/gel tube
(16 V/cm), 2 hr, 4°C, Buffer V. The gels (soaked in Buffer
V, 15 min) were scanned at 265 nm with a Gilford 2000
Spectrophotometer equipped with a Model 2410 Linear Trans-
port System. a) mitochondrial RNA; b) mitochondrial plus
E. coli RNA. The ratio of 23 S to 16 S rRNA from *E. coli* re-
presents an arbitrary mixture of the two markers isolated from
purified subunits (a gift from G. Van Dieijen and H. Noll,
Northwestern University).

drial RNA with mtDNA. A plateau is reached at a
μgRNA/μgDNA ratio of 0.6. This experiment was
repeated with several different mtDNA preparations
and we routinely obtained hybridization values be-
tween 3.6 to 3.8%. At higher μgRNA/μgDNA ratios,
the % hybridization slightly and steadily increases, most
likely due to mRNA contamination. Nevertheless, we
consider the average 3.7% hybridization value to re-
flect the percentual amount of ribosomal DNA present
within the mitochondrial genome.

In table 1, the data from homologous and heterolo-
gous hybridization experiments are compiled. These
experiments demonstrate that mtDNA hybridizes to
3.7% with its own rRNA but much less with chloro-
plast rRNA (0.12% .)The reciprocal annealing experi-
ment using total chDNA and either mitochondrial or
chloroplast rRNA shows relatively small affinity between
chDNA and mitochondrial rRNA (0.16%). The 2.9%
hybridization value (chDNA/chloroplast rRNA) cor-
responds to 1.5 copies of the 23 S, 16 S rRNA cistron
per circular chloroplast chromosome [19].

The total chDNA used for these experiments con-
tained about 30% of 1.692 g/cc DNA. If the 1.692 g/cc
DNA would be mtDNA as recently postulated [20],

Table 1

Hybridization of *Euglena* chloroplast and mitochondrial DNA with homologous or heterologous rRNA

Source of DNA (density; g/cc)	Source of RNA	μg DNA filter	$\frac{\mu g \text{ RNA}}{\mu g \text{ DNA}}$	cpm* corrected	Hybridization (%)
mitochondria (1.689)	mitochondria	5.3	1	693	3.7
mitochondria (1.689)	chloroplasts	4.8	1.5	34	0.12
chloroplasts (1.685 + 1.692)	mitochondria	5.4	1	31	0.16
chloroplasts (1.685 + 1.692)	chloroplasts	3.0	1.5	503	2.9

*Average of two filters.

[^{3}H]RNA from mitochondria: 3645 cpm/μgRNA; 2% standard deviation.

[^{3}H]RNA from chloroplasts: 5686 cpm/μgRNA; 2% standard deviation.

then this chloroplast DNA preparation should hybridize with mitochondrial RNA to approximately 1.2%. The 0.16% obtained in this experiment (about 13% of the due value) would be the upper limit of a possible mtDNA contamination. We consider, however, the residual affinity in the heterologous hybridization experiments not to be due to a mtDNA contaminant but rather to be the result of base sequence similarities between the two types of organellar rRNA. Since no melting studies of the DNA/RNA hybrids were made, so far, the quality of the hybrids is unknown. As previously reported [12], chDNA enriched in the 'heavy' component (1.692 g/cc) hybridizes with chloroplast rRNA up to 6.9%, which further supports the argument that the 1.692 g/cc chDNA is not of mitochondrial origin, a problem already raised by Ray and Hanawalt [1].

These are the first hybridization studies with *Euglena* mtDNA and mitochondrial RNA. The 3.7% hybridization can tentatively serve to estimate the mitochondrial genome size: Assuming that *Euglena* mitochondria contain, per full complement of genes (genome), only one cistron coding for 23 S and 16 S RNA (equivalent to a molecular weight of approximately 1.6×10^6), the genome size would be equivalent to 4×10^7. Should this genome be accommodated on one DNA molecule as is the case for several fungal and animal mtDNAs, then the *Euglena* mtDNA molecule would have a total length of approximately 20 μm. This would bring *Euglena* mtDNA into a size range comparable with the mtDNA of other protists [23].

References

[1] Ray, D. S. and Hanawalt, P. C. (1965) J. Mol. Biol. 11, 760–768.
[2] Edelman, M., Schiff, J. A. and Epstein, H. T. (1965) J. Mol. Biol. 11, 769–774.
[3] Edelman, M., Epstein, H. T. and Schiff, J. A. (1966) J. Mol. Biol. 17, 463–469.
[4] Manning, J. E. and Richards, O. C. (1972) Biochemistry 11, 2036–2043.
[5] Richards, O. C. and Ryan, R. S. (1974) J. Mol. Biol. 82, 57–75.
[6] Avadhani, N. G. and Buetow, D. E. (1972) Biochem. J. 128, 353–365.
[7] Scott, N. S. and Smillie, R. N. (1967) Biochem. Biophys. Res. Commun. 28, 598–603.
[8] Stutz, E. and Rawson, J. R. (1970) Biochim. Biophys. Acta 209, 16–23.
[9] Rawson, J. R. and Haselkorn, R. (1973) J. Mol. Biol. 77, 125–132.
[10] Stutz, E. and Vandrey, J. P. (1971) FEBS Letters 17, 277–280.
[11] Scott, N. S. (1973) J. Mol. Biol. 81, 327–336.
[12] Vandrey, J. P. and Stutz, E. (1973) FEBS Letters 37, 174–177.

[13] Greenblatt, C. L. and Schiff, J. A. (1959) J. Protozool. 6, 23–28.

[14] Cramer, M. and Meyers, J. (1952) Arch. Mikrobiol. 17, 384–402.

[15] Krawiec, S. and Eisenstadt, J. M. (1970) Biochim. Biophys. Acta 259, 120–131.

[16] Rawson, J. R. and Stutz, E. (1969) Biochim. Biophys. Acta 190, 368–380.

[17] Smith, K. D., Armstrong, G. L. and McCarthy, B. J. (1967) Biochim.Biophys. Acta 142, 323–330.

[18] Krawiec, S. and Eisenstadt, J. M. (1970) Biochim. Biophys. Acta 217, 132–141.

[19] Manning, J. E. and Richards, O. C. (1972) Biochim. Biophys. Acta 259, 285–296.

[20] Stolarsky, L., Slavik, N. and Hershberger, C. L. (1974) Biochim. Biophys. Acta 335, 30–34.

[21] Loening, U. E. (1969) Biochem. J. 113, 131–138.

[22] Gillespie, D. and Spiegelman, S. (1965) J. Mol. Biol. 12, 829–842.

[23] Borst, P. (1972) Annual Rev. Biochem. 41, 333–376.