HYBRIDIZATION OF PROKARYOTIC AND EUKARYOTIC 5S rRNA TO *EUGLENA GRACILIS* CHLOROPLAST DNA

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1. Introduction

The evolutionary discontinuity separating prokaryotes from eukaryotes [1] is bridged in an almost unambiguous manner by 5S ribosomal RNA [2,3]. This molecule has been found in the large subunit of all prokaryotic, eukaryotic and chloroplast ribosomes so far examined, though it appears to be absent in mitochondrial ribosomes [4]. 5S rRNA is highly conserved with a rate of base substitution slightly slower than cytochrome *c* messenger RNA or the paired regions of transfer RNA [5,6]. Since all examples may be considered as homologous, 5S rRNA would seem to be an ideal molecule with which to distinguish the direct filiation [7,8] and symbiotic [9] hypotheses for the origin of chloroplasts. If the direct filiation hypothesis is correct, the cytoplasmic and chloroplast 5S rRNAs within a single eukaryotic plant should show a greater degree of homology to each other than to any prokaryote. However, if the symbiotic hypothesis is correct, the cytoplasmic and chloroplast molecules should show little resemblance, but the chloroplast 5S rRNA should show most similarity to the 5S rRNA of some photosynthetic prokaryote.

In this paper the results obtained by hybridization of a number of 5S rRNAs to *Euglena gracilis* chloroplast DNA, are interpreted in the light of the principal hypotheses for the origin of chloroplasts.

2. Materials and methods

2.1. *Organisms and culture conditions*

Euglena gracilis Klebs, strain 1224/5Z Pringsheim, obtained from the Culture Centre for Algae and Protozoa, Cambridge, UK (abbreviated to Cam.), was grown to stationary phase in an autotrophic medium [10] at 25°C. A chloroplast DNA free stain of *Euglena*, $W_{15}ZHL$, (generously provided by Dr H. Lyman, State University of New York at Stony Brook, Stony Brook, New York), was grown in a heterotrophic medium [11] The blue-green algae, *Anabaena variabilis*, Cam. strain 1403/8 Pringsheim, *Anacystis nidulans*, Cam. strain 1405/1 Kratz/Allen, *Gloeocapsa alpicola*, Cam. strain 1430/1 Allen and an endophytic *Nostoc* sp. [12], were cultured at 34°C in Medium C [13] modified to contain 1 g/litre of sodium bicarbonate. *Escherichia coli* MRE 600 was harvested in mid-log phase after growth at 37°C in a rich medium [14]. All organisms were grown in 50 litre batches and after harvesting stored at −20°C. Algal cultures were gassed with 5% CO_2 in air and illuminated at 4000 lux by three 40 W fluorescent lights (Cryselco, warm white).

2.2. *Preparation and labelling of 5S rRNA*

Ribosomes were prepared from *E. coli* and *A. nidulans* [15] and the RNA extracted with phenol [14]. RNA was prepared from other algal cells [16] after their passage through a French Press at 16 000–20 000 p.s.i. in buffers containing polyvinyl sulphate at 20 μg/ml and Macaloid added to a final concentration of 2 mg/ml. The crude RNA fraction obtained was purified by DEAE-cellulose chromatography [17]. The column eluates were concentrated by ultrafiltration (Amicon Diaflo UM-20E filter) before ethanolic precipitation of RNA. 5S RNA was separated from transfer and high mol. wt RNA by two cycles of molecular sieving on a 2.6 × 100 cm column of G-75 Sephadex, equilibrated with 0.8 M NaCl 0.02 M sodium

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phosphate buffer pH 6.5, 1% (v/v) methanol and 0.02% (w/v) sodium azide at 4°C. Final resolution of 5S rRNA was achieved by electrophoresis in a 40 X 14 X 0.3 cm slab of 10% polyacrylamide gel [18]. The 5S rRNA was located by staining with methylene blue [19] and recovered from the gel in buffered phenol as described [20], with the omission of carrier RNA. The aqueous phase was extracted once with ether, adjusted to 0.3 M NaCl, and loaded onto a 0.6 X 2.0 cm column of DEAE-cellulose. The column was washed with 15 ml of 0.3 M NaCl–0.01 M Tris–HCl pH 7.5 and the RNA eluted in 3 ml of 1.0 M NaCl in 7 M urea [18]. 5S rRNA was precipitated with 2.5 vol of ethanol after standing overnight at −20°C, and collected by centrifugation at 17 000 *g* for 30 min (Sorval HB-4 rotor). The RNA was lyophilized after dialysis and dissolved at a concentration of 1 mg/ml in 50 mM sodium acetate buffer (pH 5.0 at 80°C).

Each 5S rRNA was labelled in vitro with 250 μCi of ^{125}I (Radiochemical Centre) by thallium trichloride oxidation at a carrier iodide concentration of 10^{-5} M. Conditions of reaction and removal of unstable intermediates were essentially as described [21].

2.3. *Preparation of chloroplast DNA*

DNA was isolated from *Euglena* chloroplast [22], obtained free of nuclear and mitochondrial contamination by flotation on gradients of Urografin (Schering Chemicals Ltd.) [23]. Chloroplast DNA of mean density 1.685 g/cm^3 was collected after preparative CsCl density gradient centrifugation [24] neglecting the DNA shoulder of density 1.700 g/cm^3, although this is known to result in the preferential loss of some rRNA cistrons [25]. The purity of the chloroplast DNA was measured by analytical CsCl density gradient centrifugation against a *Micrococcus luteus* DNA standard, of density 1.731 g/cm^3.

2.4. *DNA–RNA hybridization*

Chloroplast DNA was denatured with alkali, and after neutralization immobilized on 25 mm membrane filters (Schleicher and Schuell BA85) [26]. The hybridization reaction was carried out at 45°C for 15 h in 1 ml of 4 X SSC, 50% formamide, containing an appropriate quantity of RNA. Filters were washed and treated with ribonuclease as described [27]. The RNA input was taken as the TCA-precipitable material remaining after incubation. Blank filters retained less than 0.015% of input RNA.

For thermal analysis of the DNA-RNA hybrids, filters containing approx. 2000 cpm of hybridized 5S rRNA, were incubated for 10 min in 2 ml of 1 X SSC at 5°C intervals from 50°C to 95°C. The radioactivity eluted at each temperature was counted following the additon of 15 ml of Aquasol (New England Nuclear). Counts eluting from appropriate blank filters were subtracted.

3. Results and discussion

Each 5S rRNA was purified to the same degree by preparative electrophoresis in a slab of 10% polyacrylamide gel. Chloroplast DNA was also highly pure as judged by CsCl density gradient centrifugation. The degree of hybridization expected precluded the use of in vivo ^{32}P-labelled RNA since specific activities in excess of 10^6 cpm/μg cannot regularly be obtained from the majority of blue-green algae. Under the conditions of the reaction employed in vitro labelling of the 5S rRNAs with ^{125}I yielded specific activities in the range $1-2 \times 10^7$ cpm/μg. This corresponding to a 1% incorporation of iodine.

No resolution of 5S rRNA from autotrophically grown *Euglena* into chloroplast and cytoplasmic species could be detected by electrophoresis in either 10% or 12.5% polyacrylamide gels (data not shown). However, the 5S rRNA from autotrophic *Euglena* was found to contain a fraction which readily hybridized to chloroplast DNA [28]. This fraction must have been transcribed from chloroplast DNA since it was lacking in the equivalent 5S rRNA from *Euglena* $W_{15}ZHL$, and has therefore been concluded to be chloroplast 5S rRNA. The percentage of chloroplast DNA capable of annealing with this RNA could not be determined by hybridization saturation, since neither the exact proportion nor the specific activity of the chloroplast 5S rRNA within the mixture was known. The other 5S rRNAs examined showed no tendency to saturate chloroplast DNA even at high RNA inputs (fig.1.), this fact reflecting the low efficiency of heterologous hybridization between distantly related nucleic acids [29], (G. H. Pigott, personal communication). In order that a direct comparison may be made of the homology of different 5S rRNAs, their hybridizations have been

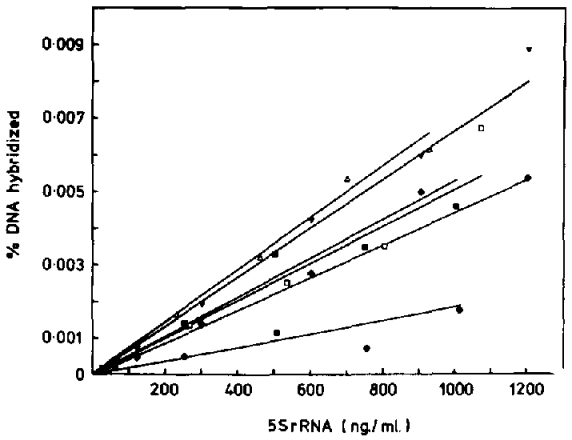

Fig.1. Hybridization of *Euglena* chloroplast DNA with 5S rRNA. Filters containing 5 μg *Euglena* chloroplast DNA were hybridized with increasing concentrations of ^{125}I-labelled 5S rRNAs from *Gloeocapsa alpicola* (△), *Anacystis nidulans* (▼), *Escherichia coli* (■), *Nostoc* sp. (□), *Anabaena variabilis* (♦), and *Euglena* W_{15}ZHL (●) as described in Materials and methods.

Table 1
Relative hybridization of 5S rRNA to *Euglena* chloroplast DNA

Sources of 5S rRNA	% DNA hybridized ± standard deviation(s) at DNA/RNA ratio of 50/1 (× 10^5)
Gloeocapsa alpicola	72 ± 4.2
Anacystis nidulans	68 ± 4.2
Nostoc sp.	52 ± 6.8
Anabaena variabilis	46 ± 6.3
Escherichia coli	54 ± 7.8
Euglena W_{15}ZHL (cytoplasm)	19 ± 5.3

compared at an arbitary DNA/RNA ratio of 50/1 (table 1).

To ensure that the results obtained were due to genuine hybridization and not non-specific interaction, the thermal stability of the DNA–RNA hybrids was investigated. Since the filters had been treated with ribonuclease prior to melting analysis, 20–40% of the bound radioactivity was removed by 50°C because of the low temperature melting of short oligoribonucleotides [30]. Around 55°C a plateau was obtained and counts released above this temperature have been plotted in fig.2. The profile of *Euglena* cytoplasmic 5S rRNA is characteristic of the melting of a number of rather short sections of both true and non-specific hybrid, in contrast to the thermal stability of the

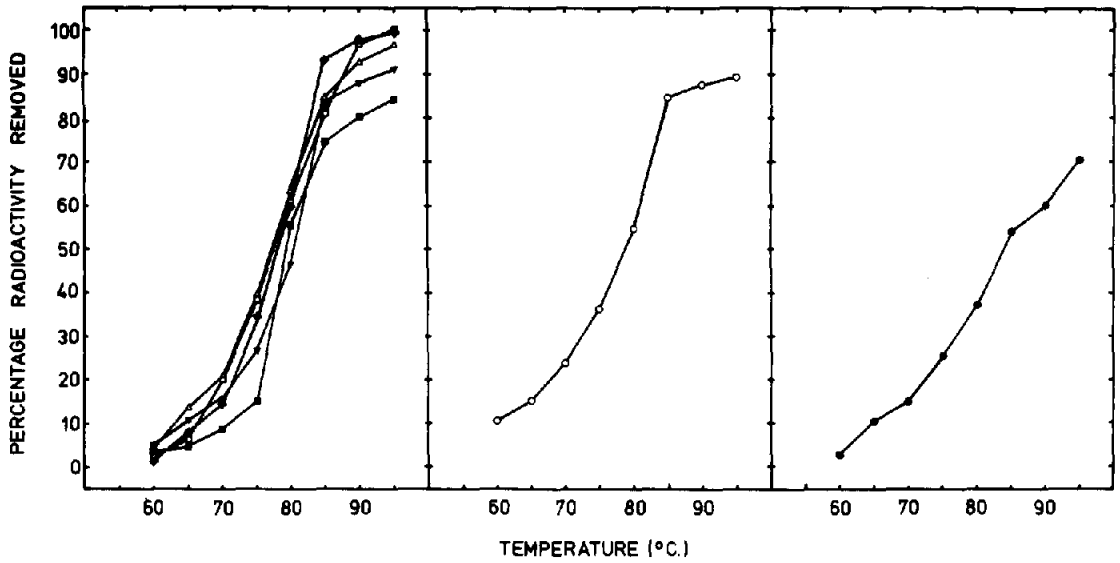

Fig.2. Thermal stability of *Euglena* chloroplast DNA–5S rRNA hybrids. Hybrids were formed and melted as described in Materials and methods. Sources of 5S rRNA; *Escherichia coli* (■), *Anacystis nidulans* (▼), *Gloeocapsa alpicola* (△), *Anabaena variabilis* (♦), *Nostoc* sp. (□), *Euglena gracilis* (○) and *Euglena* W_{15}ZHL (●).

rapidly annealing fraction (chloroplast 5S rRNA) present in the equivalent material from autotrophic *Euglena*. The narrow range of melting obtained with prokaryotic 5S rRNAs, in particular those from *E. coli* and *A. nidulans*, may reflect the melting of a single oligoribonucleotide specifically annealed to *Euglena* chloroplast DNA.

From a comparison of the known 5S rRNA sequences of *E. coli* and *A. nidulans* [31] it is apparent that only two base differences exist between residues 26 and 51. The possibility arises that within this region an oligonucleotide common to *E. coli* and *A. nidulans* is also shared by *Euglena* chloroplast 5S rRNA. Since this region of homology has a G + C content of approximately 62%, the mean hybrid melting temperature (Tm) of 79.5°C for *E. coli* and 80.5°C for an *A. nidulans*, allowing for a 2°C lowering due to iodination, corresponds to the melting of 12–14 nucleotides in the former and 14–16 nucleotides in the latter case [32]. The other thermal profiles may indicate the melting of one or more somewhat shorter sections of hybrid or result from the melting of a single oligoribonucleotide of lower G + C content.

Although DNA–RNA hybridization provides an underestimate of true sequence homology [6], the results indicate that among the organisms examined, *Euglena* cytoplasmic 5S rRNA shares the least homology and *Gloeocapsa alpicola* the greatest homology with *Euglena* chloroplast DNA [33]. This result is in agreement with the predictions of the symbiotic hypothesis for the origin of chloroplasts.

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