EVIDENCE FOR THE DIRECTION OF CHLOROPLAST RIBOSOMAL RNA SYNTHESIS BY CHLOROPLAST DNA

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Although the existence of DNA in chloroplasts has been firmly established (for reviews see Schiff and Epstein, 1965, and Kirk, 1966), the informational role of chloroplast DNA is not known. Evidence is presented here that chloroplast DNA codes for the RNA of chloroplast ribosomes.

Chloroplast DNA has been found in Euglena gracilis, but not in cytoplasmic mutants of Euglena which are unable to form chloroplasts (Ray and Hanawalt, 1965; Edelman et al., 1965). The chloroplasts of Euglena also contain a species of ribosomal RNA which differs from that found in the cytoplasm (Brawerman and Eisenstadt, 1964) and an enzymic mechanism for RNA synthesis (Shah and Lyman, 1966). To test if the synthesis of chloroplast ribosomal RNA is directed by chloroplast DNA rather than by the DNA of the nucleus, the formation of specific chloroplast DNA-RNA hybrids was investigated, since these could provide evidence of DNA-directed RNA synthesis (Hall and Spiegelman, 1961).

METHODS

Chloroplasts were prepared according to Brawerman and Eisenstadt (1964) using cultures of Euglena gracilis, strain Z, which were grown autotrophically (Smillie, 1963). After freezing to -15°C, the chloroplasts were suspended in 2 volumes of 0.005 M $MgSO_4$ and lysed by addition of 0.25 volume of 5% (w/v)

sodium deoxycholate. The suspension was then brought to 0.1 M with respect to tris-HCl buffer, pH. 9.0, and extracted with 80:20 (v/v) water-phenol. The aqueous layer was collected after centrifuging at 10,000 x g for 10 min and dialysed against water for 1 hour followed by dialysis overnight against a solution containing 0.015 M NaCl and 0.15 M sodium citrate, pH 7.0, at 37°, in a continuous flow system. After digestion with ribonuclease (Marmur, 1961) and then pronase (0.5 mg/ml), followed by further dialysis, the DNA was concentrated by evaporation of the solvent. The DNA fibres were removed after the addition of 2 volumes of ethanol. Chloroplast DNA (ρ=1.686) was obtained free of nuclear DNA (ρ=1.707) by successive density gradient fractionations in CsCl. Ribosomal RNA was prepared as described by Brawerman and Eisenstadt (1964). Ribosomal ^{32}P-RNA was prepared from cells which had been grown in a medium of low phosphorus level containing ^{32}P (Ray and Hanawalt, 1965), except that KH_2PO_4 (1.2 g/l) was added to the medium 4 hours before harvest. The ^{32}P-RNA was treated with electrophoretically purified deoxyribonuclease (Worthington Biochemical Corpn.) and reprecipitated with ethanol, after which the alkali-stable counts were around 0.1 to 0.2%. The ^{32}P-RNA prepared from chloroplasts had a specific activity of 3,200 counts/min/μg, and its sedimentation behaviour on sucrose gradients was the same as that reported by Brawerman and Eisenstadt (1964) for unlabelled ribosomal RNA from Euglena chloroplasts.

The annealing of DNA and ribosomal ^{32}P-RNA was carried out as described by Gillespie and Spiegelman (1965), except that a longer incubation period (48 hours) was found necessary. In some experiments, an excess of unlabelled ribosomal RNA was included in the incubation medium in order to assess its effect on the formation of the DNA-RNA hybrid.

The experimental results are shown in Table I. Experiment No. 1 demonstrated the formation of a ribonuclease-resistant hybrid from chloroplast DNA and chloroplast ribosomal ^{32}P-RNA. The amount of RNA used was sufficiently high to saturate all binding sites on the chloroplast DNA available to

RESULTS AND DISCUSSION

Table I. Hybridization of Ribosomal ^{32}P-RNA with Chloroplast DNA

Experiment No.	Source of ^{32}P-RNA (29 μg)	Source of unlabelled RNA (100 μg)	Counts/100 min in hybrid	Percentage related to No. 1
1	chloroplasts	-	9,594 ± 3.2%	100
2	"	autotrophic cells	7,157 ± 4.8%	75
3	"	chloroplasts	5,906 ± 6.8%	62
4	"	dark-grown cells	9,082 ± 2.4%	95
5	"	ZUV-1 cells	10,566 ± 5.0%	110
6	dark-grown cells	-	133 ± 80%	1

Chloroplast DNA (3 μg) was annealed with 29 μg of ribosomal ^{32}P-RNA from chloroplasts in experiments Nos. 1 to 5 and from dark-grown Euglena cells in experiment No. 6. In experiments Nos. 2 to 5 inclusive, an excess of unlabelled ribosomal RNA (100 μg) from different sources was included in the annealing mixtures. The amount of hybrid formed is expressed in the last column as a percentage of the amount of hybrid formed between chloroplast DNA and RNA in experiment No. 1.

ribosomal RNA. At saturation, it was calculated that about 1% of the total available sites on the chloroplast DNA were occupied by the ribosomal RNA.

The presence of excess unlabelled ribosomal RNA from autotrophically grown cells, which contain a large amount of chloroplast RNA, inhibited formation of the chloroplast DNA-^{32}P-RNA hybrid. The effect was even more pronounced with a preparation enriched for chloroplast ribosomal RNA. On the other hand, RNA from dark-grown Euglena that have not formed chloroplasts did not significantly reduce the formation of the hybrid. This shows the specificity of the binding of ribosomal ^{32}P-RNA from chloroplasts to the chloroplast DNA. It can also be seen that, at the same concentrations of nucleic acids, ribosomal ^{32}P-RNA from dark-grown cells did not form a hybrid with chloroplast DNA.

The inhibition of hybrid formation (25%) observed in experiment 2 in the presence of unlabelled ribosomal RNA from autotrophic Euglena is of the same order as might be expected, following the estimations of Smillie et al. (1963) that 17-29% of the cellular RNA is in the chloroplasts. In experiment 3, one would expect an inhibition of annealing of 75%, rather than the observed 38%. However, it should be pointed out that chloroplast ribosomal RNA preparations are not pure, but are merely enriched for chloroplast ribosomal RNA, and that the unlabelled preparation was probably more heavily contaminated with cytoplasmic RNA than the ^{32}P-preparation. The contamination of chloroplast ribosomal RNA with cytoplasmic ribosomal RNA cannot be estimated directly; the only available criterion was microscopic examination of the chloroplast preparations before isolation of ribosomal RNA. These remarks do not apply to the preparation of chloroplast DNA which was prepared free of nuclear DNA.

It is interesting that ribosomal ^{32}P-RNA from dark-grown cells did not anneal with chloroplast DNA. Possibly, had more of the RNA been used, a small amount of hybridization might have been detected. In any case, the inference is that chloroplast DNA codes for little of the ribosomal RNA of dark-grown cells. Accordingly, the considerable net increase in RNA observed when dark-grown cells of Euglena are exposed to light (Brawerman et al., 1962; Smillie et al., 1963) may well occur consequent to transcription from the chloroplast DNA.

The possibility exists that some of the counts bound to chloroplast DNA represent annealing between chloroplast DNA and chloroplast messenger RNA. For several reasons, interference from this type of hybrid is unlikely to be significant. The amount of messenger RNA in similar preparations of ribosomal RNA from Euglena is small (Brawerman and Eisenstadt, 1964), probably less than 5%. Further, in many organisms messenger RNA turns over quickly compared with ribosomal RNA (for discussion see Singer and Leder, 1966) and for this reason, the pulse of unlabelled phosphate given to the

cells four hours before harvesting, should have greatly reduced the specific activity of any messenger RNA. Also the saturation curve obtained in these experiments was smooth and monophasic, indicating that only one species of RNA was annealing, rather than a heterogeneous population of messenger RNA.

The amount of annealing that has occurred (1% of the DNA occupied by ribosomal RNA) is about what could be expected on the basis of similar studies in other organisms (Yankofsky and Spiegelman, 1963; Ritossa and Spiegelman, 1965), and is much lower than would be expected for messenger RNA. A chloroplast from an autotrophic Euglena cell contains about 0.7 - 1.8 x 10^{-14} gm DNA (Kirk, 1966) and hence it can be calculated that each chloroplast has from 20 to 45 cistrons for ribosomal RNA, assuming that only one strand of DNA codes.

The discovery of DNA in the chloroplast is in keeping with the considerable evidence for the control of chloroplast heredity by genetic determinants located outside the nucleus (Rhoades, 1955). The results presented here support the hypothesis that chloroplast DNA functions in the formation of a protein-synthesizing system within the chloroplast by coding for the RNA of chloroplast ribosomes. The role of these ribosomes in protein synthesis in differentiating chloroplasts is dealt with in a subsequent paper (Smillie et al., 1967).

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