HYBRIDIZATION OF EUGLENA RNA TO THE HEAVY AND THE LIGHT CHLOROPLAST DNA COMPONENTS SEPARATED IN ALKALINE CsCl GRADIENTS.

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SUMMARY. Chloroplast DNA from Euglena gracilis was separated in an alkaline CsCl density gradient into a heavy and a light component. The heavy and the light DNA components each contain 18 percent self-complementary nucleotide sequences. Equal masses of the heavy and the light components do not contain equal molar complementary nucleotide sequences. The light DNA component contains sequences complementary to the majority of the chloroplast DNA transcripts. The heavy DNA component contains sequences complementary to chloroplast ribosomal RNA.

INTRODUCTION

Chloroplast DNA from Euglena gracilis is a circular molecule (1) with a molecular weight of 92 x 10^7 (2). The chloroplast DNA can be fractionated in an alkaline CsCl equilibrium density gradient into a heavy and a light component (3, 4). Each fraction contains self-complementary and non-complementary nucleotide sequences (4). Chloroplast ribosomal RNA (rRNA) is complementary to sequences in the heavy component (3).

This report attempts to: 1. Quantitate the fraction of self-complementary nucleotide sequences in the heavy and the light chloroplast DNA components. 2. Measure the fraction of each component which is complementary to chloroplast DNA transcripts.

MATERIALS AND METHODS

Isolation and fractionation of chloroplast DNA. Euglena gracilis var. Z cells were grown in either an autotrophic or a heterotrophic medium with constant illumination (5). Chloroplasts were isolated from cells grown in the autotrophic medium. Chloroplast DNA was isolated from chloroplasts purified on renografin gradients (6). The chloroplast DNA was separated into a heavy and a light component in preparative CsCl equilibrium density gradients. Purified chloroplast DNA (175 µg) was denatured in 22.7 ml of 1 x SSC (0.15 M NaCl, 0.05 M sodium citrate) with 1/10 vol of 1 N NaOH, and the density was adjusted to 1.701 g/cc with solid CsCl (31.8 gm). The sample was equally divided into four pollyallomer tubes (8 ml/tube), overlayed with mineral oil and centrifuged in a Beckman Type 65 rotor at 44,000 rpm, at 25°C, for 72 hr. The gradients were monitored using a flow cell attached to a Gilford recording spectrophotometer. The peak fractions of the heavy and light chloroplast DNA components were combined, neutralized with 1/10 vol 1 M NaH_2PO_4 and dialyzed against 0.1 x SSC

and 10^{-3} M EDTA (pH 8.0). Each component was purified on a second alkaline CsCl gradient. The peak fractions were collected, neutralized and dialyzed against 0.1 x SSC and 10^{-3} M EDTA (pH 8.0). The DNA samples were precipitated with 1/10 vol 3 M sodium acetate and 2 vol of ethanol. The precipitate was collected by centrifugation, redissolved in 2.0 ml water and dialyzed overnight against water. The recovery of the heavy and light components was 20 and 17 µg, respectively.

In vitro labeling of the heavy and light chloroplast DNA components. The heavy and light chloroplast DNA components were labeled in vitro with [^{125}I]-iodine (5). At optimal settings on a Packard Tricarb Liquid Scintillation Counter (Model 3320), the specific activities of the heavy and light DNA components were 1.5 x 10^6 and 2.2 x 10^6 cpm/µg, respectively. The single-stranded molecular weight of the [^{125}I]-DNA was 75,000 (230 nucleotides).

Total cell RNA and chloroplast rRNA isolation. Total cell RNA was isolated from a 500 ml culture of Euglena cells growing exponentially (1 x 10^6 cells/ml) in the light in the heterotrophic medium. The RNA was purified by pelleting it through CsCl (7).

Chloroplast rRNA was isolated from chloroplast ribosomes (8). Chloroplast ribosomes (2700 µg) were diluted to 10 ml with 1 x SSC. The solution was adjusted to 2% SDS (sodium dodecylsulfate) and extracted first with an equal volume of phenol, cresol (10% v/v) and 8-hydroxquinoline (0.1% w/v) saturated with 1 x SSC and then with chloroform-isoamylalcohol (24:1, v/v). The RNA was precipitated with 1/10 vol of 3 M sodium acetate and 2 vol of ethanol and resuspended in 8 ml 1 x SSC. Solid CsCl was added (1.0 gm/ml) to adjust the density to 1.55 g/cc, and the RNA was pelleted through CsCl (7).

Hybridization and reassociation of the chloroplast DNA components. Hybridization of a vast excess of total cell RNA or chloroplast rRNA to the two [^{125}I]-DNA components was carried out in liquid at 62°C in 0.48 M PB (sodium phosphate buffer, pH 6.8) (7). The reassociation of the [^{125}I]-heavy and the [^{125}I]-light chloroplast DNA components was followed individually and together using conditions similar to those for hybridization. The formation of duplex structures was monitored as a function of the product of either the initial RNA concentration (Ro, M-nucleotide) or the initial DNA concentration (Co, M-nucleotide) x time (sec) (9).

The best fit of the experimentally observed points of the hybridization and reassociation reactions was determined by a non-linear least squares computer program (10). The RNA-DNA hybridization data were analyzed assuming psuedo-first order kinetics, and the reassociation data were analyzed by assuming single component second order kinetics. The computer program permits the insertion of the appropriate equation describing the reaction and evaluates the experimental data accordingly.

Melting curves of renatured [^{125}I]-DNA. The T_m of the reassociated [^{125}I]-heavy plus [^{125}I]-light chloroplast DNA components was determined by melting the duplex off hydroxylapatite (7).

Analytical CsCl equilibrium density gradients. Analytical CsCl gradients were run in a Spinco Model E ultracentrifuge at 44,000 rpm for 36 hr. at 25°C. Buoyant densities in alkaline CsCl gradients were determined from the limiting isoconcentration distance and the initial density of the solution (11). Buoyant densities were determined in neutral CsCl gradients relative to the phage SP01 DNA (ρ=1.742 g/cc).

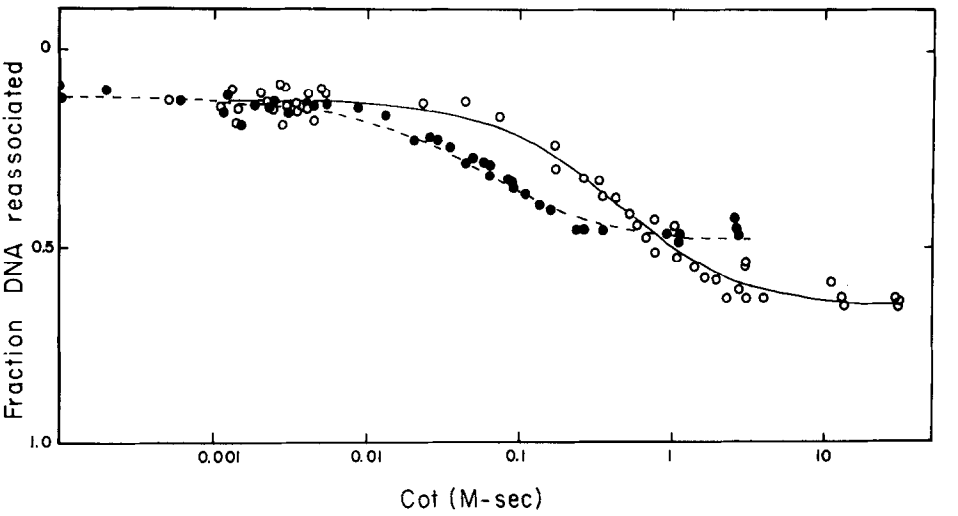

Figure 1. Reassociation kinetics of the [^{125}I]-heavy, [^{125}I]-light and [^{125}I]-heavy plus [^{125}I]-light chloroplast DNA components. Samples of varying concentrations of [^{125}I]-heavy (●), [^{125}I]-light (O), and equal molar quantities of [^{125}I]-heavy plus [^{125}I]-light (□) chloroplast DNA components were reassociated in 0.48 M PB at 62°C for varying periods of time. The Cot values were corrected for the relative increase in the rate of reassociation in 0.48 M PB to that in 0.12 M PB. The curves drawn through the experimental points represent a least squares fit for a single second order rate reaction.

RESULTS AND DISCUSSION

The heavy and the light chloroplast DNA components were characterized in neutral and alkaline CsCl equilibrium density gradients. The buoyant densities of each component were identical to those reported in the literature (3, 4).

Fig. 1 shows the reassociation kinetics of the [^{125}I]-heavy, [^{125}I]-light, and [^{125}I]-heavy plus [^{125}I]-light chloroplast DNA components. Eighteen percent of the nucleotide sequences in both the heavy and the light components are self-complementary. When equal molar quantities of the heavy and light components are reassociated together, the reaction goes to 55 percent completion. The rate constant for this reaction is 2.7 M^{-1}-sec^{-1} in contrast to 3.6 M^{-1}-sec^{-1} for the reassociation of unfractionated [^{125}I]-chloroplast DNA (5). The duplex formed by the reassociation of the heavy plus light components melts in a co-operative fashion with a

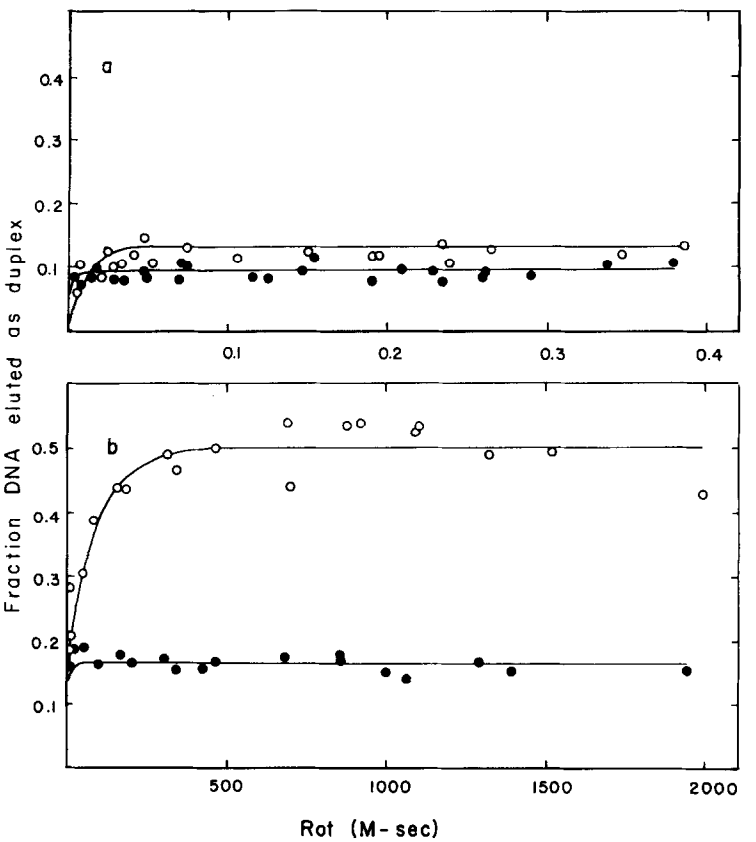

Figure 2. Hybridization of chloroplast rRNA and total cell RNA to [^{125}I]-heavy [^{125}I]-light chloroplast DNA components. Fifty µl samples of the [^{125}I]-heavy (0.0398 µg/ml) or the [^{125}I]-light (0.0315 µg/ml) chloroplast DNA component and either chloroplast rRNA (1.37 µg/ml) or total cell RNA (7.48 mg/ml) were hybridized in 0.48 M PB at 62°C. The Rot values were calculated from the initial total cell RNA concentrations and corrected for the increased rate of reassociation in 0.48 M PB to that in 0.12 M PB. The curves drawn through the experimental points represent a least squares fit for a single component pseudo-first order reaction. a) Hybridization of chloroplast rRNA to ([^{125}I]-heavy chloroplast DNA component (O) and [^{125}I]-light chloroplast DNA component (●). b) Hybridization of total cell RNA to [^{125}I]-heavy chloroplast DNA component (●) and [^{125}I]-light chloroplast DNA component (O).

T_m of 73.5°C, which is 2.5° below the T_m of reassociated [^{125}I]-chloroplast DNA. The inability of the mixture of the heavy and the light components to completely reassociate is not due to degradation, because when either the [^{125}I]-heavy or [^{125}I]-light component is reassociated in the presence of cold chloroplast DNA, the reaction goes to 80% completion. Instead, the

Table 1. Fraction of Heavy and Light Chloroplast DNA Components Complementary to Total Cell RNA and Chloroplast rRNA.[1]

Chloroplast DNA Component	RNA	Fraction [^{125}I]-DNA in Form of Duplex (RNA:DNA and DNA:DNA)	Fraction of [^{125}I]-DNA Duplex as DNA:DNA	Fraction of [^{125}I]-DNA Complementary to RNA
Heavy	rRNA	0.133	0.070	0.063
	Total Cell RNA	0.165	0.070	0.095
Light	rRNA	0.096	0.078	0.018
	Total Cell RNA	0.502	0.070	0.432

[1] The fraction of heavy and light chloroplast DNA components complementary to various RNA preparations was measured by RNA-DNA hybridization (Figure 2). The extent of DNA-DNA reassociation for comparable Cot values for the DNA in the reaction mixes was calculated from Figure 1. The fraction of heavy and light chloroplast DNA components complementary to the different RNA preparations is equal to (fraction [^{125}I]-DNA in the form of a duplex) - (fraction [^{125}I]-DNA reassociated to similar Cot's).

reaction mixture probably does not contain equal molar quantities of all the complementary sequences in the chloroplast DNA. These observations suggest that some DNA sequences have been lost from one or both DNA components during isolation.

Fig. 2a and 2b show the hybridization of chloroplast rRNA and total cell RNA, respectively, to the [^{125}I]-heavy and the [^{125}I]-light chloroplast DNA components. The fraction of [^{125}I]-DNA eluted from hydroxylapatite columns as double-stranded structures represents both RNA-DNA and DNA-DNA duplexes. The extent of DNA-DNA reassociation in these reactions was determined by renaturing the individual [^{125}I]-DNA components in the absence of RNA to Cot values comparable to those employed in the hybridization reactions (see Fig. 1). Table 1 summarizes the information from Figures 1 and 2 by showing the RNA and the chloroplast DNA components used in the hybridization, the fraction of the different DNA's in the form of a DNA-DNA duplex, and the fraction of the heavy and light DNA components complementary to the different RNA preparations. Forty-three percent of the light DNA component and 9.5 percent of the heavy DNA component are complementary to nucleotide sequences in total cell RNA. In agreement with earlier work (3), chloroplast rRNA is complementary to 6.3 percent and 1.8 percent of the heavy and the light DNA components, respectively.

The specificity of hybridization of only chloroplast DNA transcripts to chloroplast DNA has been previously demonstrated by the inability of total cell RNA from a mutant (ZHB), which completely lacks chloroplast DNA, to hybridize to chloroplast DNA (12). The low complexity of chloroplast DNA (1.36×10^5 nucleotide pairs) and the relatively high Rot values for the hybridization of both chloroplast rRNA and total cell RNA assures that all of the DNA fragments had ample opportunity for multiple collisions with complementary RNA molecules, and that the hybridization reactions had gone to completion.

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