A MEASUREMENT OF THE FRACTION OF CHLOROPLAST DNA TRANSCRIBED IN EUGLENA

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SUMMARY. The fraction of the chloroplast DNA transcribed in the single celled alga Euglena has been determined by RNA-DNA hybridization. A vast excess of total cell RNA from cells which were rapidly dividing in the light was hybridized in liquid to [^{125}I] - chloroplast DNA, and the resulting duplexes separated on hydroxyapatite columns. The contribution of DNA-DNA duplex formation was determined separately and was used to calculate that portion of the duplex which was actually a RNA-DNA hybrid. Sixteen percent of the single stranded chloroplast DNA forms a duplex with this RNA suggesting that 32 percent of the double stranded DNA molecule is being transcribed into RNA under these conditions of cell growth.

INTRODUCTION

The chloroplast DNA from the alga Euglena gracilis is a circular molecule[1] with a molecular weight of approximately 90 x 10^6. Approximately four percent of this molecule has been shown by hybridization experiments to code for ribosomal RNA[2]. Experiments with several mutants lacking chloroplast DNA[3,4] suggest that this molecule also codes for several species of tRNA molecules. Even if it is shown later that the chloroplast DNA codes for one copy of each species of tRNA, the maximum amount of the chloroplast genome necessary to code for these structural RNA molecules would be 7 to 8 percent of the molecule.

This report describes an attempt to measure the fraction of the chloroplast DNA transcribed in rapidly dividing green cells. A vast excess of cold total cell RNA was hybridized to trace amounts of low molecular weight labeled chloroplast DNA, and the resulting duplexes were separated on hydroxyapatite columns. The results were corrected for DNA-DNA hybridization.

MATERIALS AND METHODS

In vitro labeling of chloroplast DNA.

Chloroplast DNA was labeled in vitro with [^{125}I]-iodine. Chloroplasts were purified on renografin gradients and the chloroplast DNA subsequently isolated[5]. Although the chloroplast DNA prepared in this fashion was completely devoid of contaminating nuclear and mitochondrial DNA's, it was banded in CsCl equilibrium density gradients to remove residual protein and RNA[2]. The DNA was sonicated to obtain molecules with a single stranded molecular weight of 150,000 (or 350 nucleotides/single strand). For efficient in vitro labeling, the chloroplast DNA was denatured by boiling at 100°C and immediately cooled to 0°C. The resulting single stranded DNA was iodinated using [^{125}I]-iodine and thallic chloride as the catalyst[6]. A 2.0 ml reaction mix of 10^{-4} M KI, 0.10 M/0.04 M sodium acetate/acetic acid buffer (pH 5.0), 1.0 mC/ml of carrier free [^{125}I]-iodine (New England Nuclear) in the form of NaI, 7.8 µg/ml of chloroplast DNA and 2.3 x 10^{-3}M $TlCl_3$ was incubated at 60°C for 30 minutes and then cooled to 4°C. An unstable side product (probably a 5-iodo-6-hydroxydihydropyrimidine[6]) was removed by adding to the reaction mix 0.10 ml of freshly prepared 0.1 M Na_2SO_3 and then raising the pH to 8.7 with 0.15 ml 1 M ammonium acetate/0.5 M ammonium hydroxide (pH 9.7). The mixture was heated again at 60°C for 30 minutes and quickly cooled. The iodinated DNA was separated from the reactants on a Sephadex G-25 column. At optimal settings on a Packard Liquid Scintillation Counter (Model 3320) the resulting [^{125}I]-DNA had a specific activity of 1.2 x 10^6 cpm/µg. When monitored by Sephadex G-25 chromatography, less than five percent of the [^{125}I]-iodine was released from the DNA after 40 hours of annealing at 60°C in 0.48 M Na phosphate (pH 6.8).

Preparation of RNA

Total cellular RNA was isolated from Euglena gracilis var. Z

growing exponentially in the light in a heterotrophic medium (Euglena Broth, Difco Laboratories). Cells were suspended in 0.15 M NaCl, 0.1 M Tris-HCl (pH 8.0), and 1% SDS (sodium dodecylsulfate). The viscosity of the suspension was decreased by passing the cellular suspension through a French press at 15,000 psi. The cell lysate was extracted once with chloroform-isoamylalcohol (24:1). One gram of CsCl was added to each ml of the aqueous phase which was then layered over 1.2 ml of a 5.7 M CsCl cushion. The RNA was pelleted in a SW 50.1 rotor at 30K rev/min at 25°C for 18 hours[7]. The DNA remained at the interphase between the two CsCl solutions, and protein was forced to the top of the upper CsCl solution. The resulting RNA pellet was resuspended in water, dialyzed against water, and precipitated with 1/10 volume of 3 M Na acetate and 2 volumes of ethanol. The RNA was resuspended in 0.48 M Na phosphate (pH 6.8) containing [^{125}I]-chloroplast DNA.

Hybridization of [^{125}I]-chloroplast DNA to RNA

Hybridization of a vast excess of RNA to the [^{125}I]-chloroplast DNA was carried out in liquid at 60°C in 0.48 M Na phosphate (pH 6.8). Both DNA-DNA duplexes and RNA-DNA duplexes were measured as the fraction of radioactive DNA bound to hydroxyapatite[8] in 0.12 M phosphate buffer and eluted in 0.48 M phosphate buffer at 60°C. The extent of duplex formation was monitored as a function of the product of the initial RNA concentration x time (Rot) in moles nucleotide-liter^{-1}-sec.

RESULTS AND DISCUSSION

Under the conditions used for hybridization most of the hybridized DNA fragments will have had an opportunity for multiple collisions with complementary RNA molecules. Since the average size of the DNA used is considerably smaller than the average size of a structural gene, it is

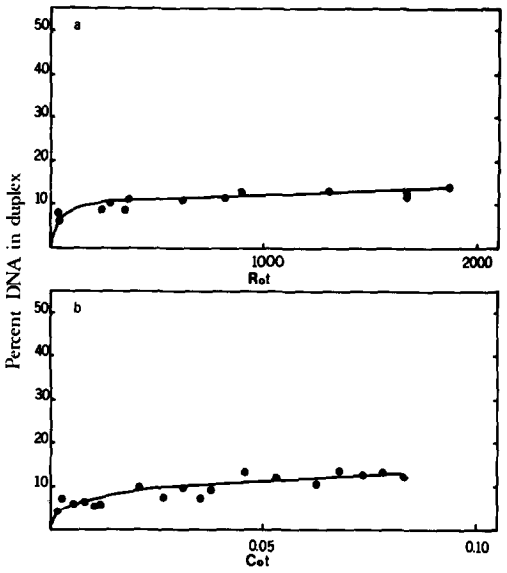

Figure 1. Reassociation of [^{125}I]-chloroplast DNA in the presence and absence of total cell RNA from the heat bleached mutant - ZHB. The reassociation of [^{125}I]-chloroplast DNA in 0.48 M Na phosphate (pH 6.8) was followed by monitoring the fraction of the total TCA (trichloroacetic acid) precipitable counts eluting as a duplex structure from a hydroxyapatite column. Each point represents the analysis of a 50 µl reaction mix containing 0.175 µg/ml of [^{125}I]-chloroplast DNA with a specific activity of 6.56×10^5 cpm/µg. a) Reassociation of [^{125}I]-chloroplast DNA in the presence of total cell RNA (13.3 mg/ml) from the heat bleached mutant (ZHB) which lacks chloroplast DNA. b) Reassociation of [^{125}I]-chloroplast DNA alone for various periods of time comparable to those used in RNA-DNA the hybridizations in a).

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to be expected that the chloroplast DNA in the form of a RNA-DNA hybrid will be nearly completely covered with RNA[8]. The fraction of labeled DNA in a RNA-DNA hybrid will be the difference between the binding of the total duplex and that attributable to DNA-DNA duplex formation. Assuming there is no symmetrical transcription of the chloroplast DNA, then the percent [^{125}I]-chloroplast DNA in RNA-DNA hybrids represents

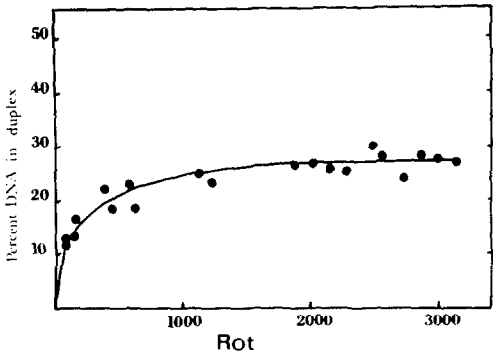

Figure 2. Hybridization of [^{125}I]-chloroplast DNA to total cell RNA from cells growing exponentially in the light. Fifty µl of [^{125}I]-chloroplast DNA (0.175 µg/ml) with a specific activity of 6.56 x 10^5 cpm/µg was hybridized with 9.89 mg/ml of total cell RNA from Euglena gracilis var. Z cells which were growing exponentially in the light. The fraction of [^{125}I]-chloroplast DNA in the form of a duplex was determined as in Figure 1.

half the fraction of chloroplast DNA transcribed into RNA.

Our lack of knowledge of the cellular location of chloroplast DNA transcripts dictates that total cell RNA be used for these experiments. Hybridization of chloroplast DNA to RNA other than that transcribed from chloroplast DNA can be ruled out by hybridizing total cell RNA from a heat bleached mutant (ZHB) lacking chloroplast DNA[9]. Figure 1a shows the fraction of duplex formation of total cell RNA from ZHB cells with labeled chloroplast DNA. The reassociation of [^{125}I]-chloroplast DNA alone under conditions similar to those used for the hybridization studies is shown in Figure 1b. The formation of a chloroplast DNA duplex as a function of the Rot of RNA from the heat bleached mutant closely parallels the renaturation of chloroplast DNA alone. Thus it appears there is no RNA from the heat bleached mutant which hybridizes to the chloroplast DNA. One would thus conclude that any formation of duplexes in excess of that due to DNA-DNA renaturation should be due to hybridization of the DNA with chloroplast RNA transcripts.

When total cell RNA from cells growing rapidly in the light in a heterotrophic medium is hybridized to chloroplast DNA, 27 percent of the radioactive chloroplast DNA is retained as a duplex on hydroxyapatite (Fig. 2). When the [^{125}I]-chloroplast DNA is incubated for a similar period of time and in the same molarity of Na phosphate, only 11 percent of the DNA is retained as a duplex. The fraction of labeled chloroplast DNA in the form of a RNA-DNA hybrid is 16 percent. If there is only asymetrical transcription of the DNA in vivo, then when the cells are rapidly dividing in the light 32 percent of the chloroplast DNA molecule is being transcribed into RNA.

Iodination of the chloroplast DNA does not seem to interfere with the reassociation reaction of the DNA. The half Cot of the reassociation reaction of the [^{125}I]-chloroplast DNA is 0.54, which is similar to that expected from optical reassociation kinetics[10]. The DNA-DNA reassociation reaction has been followed to the point where over 85 percent of the DNA has renatured[9].

The measurement of the fraction of chloroplast DNA transcribed under these growth conditions represents a minimal estimate. It is conceivable that some transcripts may be present in such low quantities that to achieve high enough concentrations of RNA for hybridization of such sequences would require considerably higher Rot values.

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