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Measurement of the Transcription of Nuclear Single-Copy Deoxyribonucleic Acid during Chloroplast Development in *Euglena gracilis*†

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ABSTRACT: The fraction of nuclear single-copy deoxyribonucleic acid (DNA) transcribed at different stages of chloroplast development in *Euglena gracilis* (Z strain) was measured by RNA–DNA hybridization. *Euglena* cells were grown in a heterotrophic medium in the dark to stationary phase and transferred to the light. Total cell RNA was isolated at various stages of chloroplast development and hybridized in a vast excess to ^{125}I-labeled single-copy DNA. The fraction of ^{125}I-labeled single-copy DNA in the form of a duplex was measured by using S1 nuclease. The amount of RNA–DNA hybrid in the duplex mixture was determined by correcting for the contribution of DNA–DNA renaturation. The fraction of single-copy DNA transcribed was calculated by multiplying by 2 the amount of DNA in the form of an RNA–DNA hybrid and correcting for the reactivity of the single-copy DNA probe with total DNA. In dark-grown cells (i.e., prior to the initiation of chloroplast development), the complexity of total cell RNA derived from single-copy DNA was 8.0×10^7 nucleotides. After initiation of chloroplast development, the complexity of the total cell RNA derived from single-copy DNA first increased slightly to 8.9×10^7 nucleotides and then progressively decreased to 7.4×10^7 and 6.4×10^7 nucleotides after 12, 48, and 72 h of exposure to light, respectively. Total cell RNA isolated from cells which had never been cultured in the dark had a complexity of 6.5×10^7 nucleotides.

Chloroplasts are complex organelles which require a multitude of membrane structures, enzymes, and electron-transport constituents to carry out photosynthesis. The development of a functional chloroplast from a proplastid, the progenitor of chloroplasts, presents an interesting example of the need for the coordinate expression and interaction of two distinct genomes within the plant cell. Both the chloroplast and the nuclear DNAs contribute genetic information required for the production of a photosynthetically competent organelle (Schiff,

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1975).

The unicellular green alga *Euglena gracilis* is a simple eucaryotic organism in which chloroplast development can be easily manipulated. *Euglena* can grow either autotrophically in the light or heterotrophically in the dark. Dark-grown *Euglena* cells are devoid of functional chloroplasts, and exposure of these cells to light induces the development of proplastids into photosynthetically competent chloroplasts (i.e., greening). The complexity of the RNA transcribed from the chloroplast DNA during this process has been measured (Rawson & Boerma, 1976; Chelm & Hallick, 1976). If all of the chloroplast RNA transcripts are expressed as polypeptides, then the chloroplast DNA codes for ~75 proteins (Rawson & Boerma, 1976). The involvement of the nuclear DNA must, therefore, be implicated simply by the relatively small coding capacity of the chloroplast genome.

The purpose of the following experiments was to measure the complexity and the abundance of RNA transcripts derived from the nuclear single-copy DNA during chloroplast development in *Euglena*. Total cellular RNA was isolated from cells undergoing chloroplast development and hybridized to radioactive single-copy DNA. The hybridization of stage-specific RNAs to the single-copy DNA was used to estimate the fraction of single-copy DNA transcribed at specific stages of organelle development.

Materials and Methods

Cell Growth. *E. gracilis* Klebs (Z strain, The Culture Collection of Algae at the University of Texas at Austin, No. 753) cells were grown in a heterotrophic medium (Rawson & Boerma, 1976). Chloroplast development proceeded in cells which were first grown to stationary phase [(5–6) × 10^6 cells/mL] in the dark and then transferred to the light (2500 lux) and maintained with constant shaking for various periods of time. The cell concentration and the chlorophyll content of the cells were monitored during chloroplast development (Rawson & Boerma, 1976).

RNA Isolation. Total cell RNA was isolated from cells collected at various stages of chloroplast development according to Rawson & Boerma (1976). Several RNA preparations isolated at the same developmental stage were combined and used for the hybridization experiments. Total cell RNA was also isolated from cells which had not undergone chloroplast development but instead had been cultured in the light for several thousand generations. These cells are referred to as continuously light grown in the text. The different RNA preparations were sized on agarose gels.

Preparation of Radioactive Single-Copy DNA. Single-copy DNA was prepared from DNA 500 nucleotides in length. Samples of DNA in 0.12 M sodium phosphate (pH 6.8) were reassociated at 60 °C to C_0t 87 (molar nucleotides s) while those in 0.48 M sodium phosphate were reassociated at 73 °C and corrected to equivalent C_0t values according to Britten et al. (1974). Single-stranded single-copy DNA was separated from the reassociated DNA by hydroxylapatite chromatography (Rawson, 1975), concentrated with butanol (Stafford & Bieber, 1975), and reassociated a second time to C_0t 87. The purified single-copy DNA had a mean length of 270 nucleotides.

One preparation of single-copy DNA was used in the iodination of two single-copy DNA probes (I and II). The DNA was iodinated in vitro by a modification of the method described by Chan et al. (1976). A 1.5-mL reaction mixture of 10 μM KI, 0.10 M sodium acetate (pH 5.0), 5.8 M $NaClO_4$, 40 μg of single-copy DNA, and 1.0 mCi/mL carrier-free [^{125}I]iodine (New England Nuclear) in the form of NaI and 1 mM $TlCl_3$ was incubated at 60 °C for 20 min and cooled to 25 °C. The reaction mixture was brought to 30 mM sodium phosphate and bound to 0.25 g of hydroxylapatite at 25 °C. The column was washed free of unbound [^{125}I]iodine with the same buffer and followed by a wash with 0.5 M sodium phosphate at 60 °C to elute the iodinated DNA. Iodinated DNA was dialyzed against distilled water overnight. ^{125}I-Labeled single-copy DNAs I and II had specific activities of 2.7 × 10^6 and 5 × 10^5 cpm/μg, respectively. The mean size of the ^{125}I-labeled single-copy DNA was monitored on agarose gels and detected by autoradiography. The size of both single-copy probes (270 nucleotides) was unchanged by iodination.

Hybridization and Renaturation. A vast excess of RNA (5000–10 000:1) from different stages of chloroplast development was hybridized in liquid to ^{125}I-labeled single-copy DNA at 68 °C in either 0.20 or 0.40 M sodium phosphate (pH 6.8). Reaction mixtures of 250–500 μL were prepared in 3-mL glass conical tubes and overlaid with mineral oil to prevent evaporation. The hybridization reaction was started by boiling the mixture for 5 min and immediately transferring it to a 68 °C bath. At various times, 10-μL samples were removed from the reaction mixture and diluted into a final volume of 250 μL of S1 nuclease assay buffer.

The extent of duplex formation was measured as a function of the product of the initial RNA concentration (R_0, molar nucleotides) × time (t, seconds). The R_0t values were reported as equivalent R_0t (R_0t^E) values, indicating the correction for acceleration of the rate of renaturation in other than standard Na^+ concentrations (Britten et al., 1974). The extent of ^{125}I-labeled single-copy DNA self-association during hybridization reactions was determined in parallel experiments using the same hybridization mixtures minus the driver RNA.

Renaturation experiments were performed in the same manner as the hybridization reactions, substituting total DNA for total RNA and expressing the extent of duplex formation in terms of C_0t^E.

Melting Curve. The thermal stability of reassociated DNA samples was measured by thermal elution of radioactive DNA from hydroxylapatite (Rawson & Boerma, 1976).

S1 Nuclease Assay. The fraction of the ^{125}I-labeled single-copy DNA in the form of a double-stranded structure was determined by its resistance to S1 nuclease. The S1 nuclease assay was performed by a modification of the DEAE filter paper technique of Maxwell et al. (1978). Aliquots from hybridization or renaturation reactions were diluted into a final volume of 250 μL of S1 reaction mixture [1 mM $ZnSO_4$, 5 mM 2-mercaptoethanol, 0.20 M NaCl, 25 mM sodium acetate (pH 4.5), and 2 × 10^2 units of S1 enzyme (Sigma)]. A 100-μL sample was immediately spotted onto a DE-81 filter disk (Whatman) to determine the total amount of ^{125}I-labeled single-copy DNA in a given volume. The remaining volume was incubated at 37 °C for 1.5 h, and a second 100-μL aliquot was spotted onto a similar filter disk to assay for ^{125}I-labeled single-copy DNA existing as double-stranded structures. Filters were then washed 3 times for 5 min each in 0.48 M sodium phosphate (pH 6.8) to remove the S1 digestion products. Filters were dried and counted in a Packard liquid scintillation counter (Rawson & Boerma, 1976).

The double-strand and single-strand activity of the enzyme was monitored throughout each experiment. Samples used to determine these activities were digested under the same conditions as those in the hybridization experiments. Double-stranded nuclease activity was determined by digesting supercoiled ^{3}H-labeled pBR313 plasmid DNA. No double-

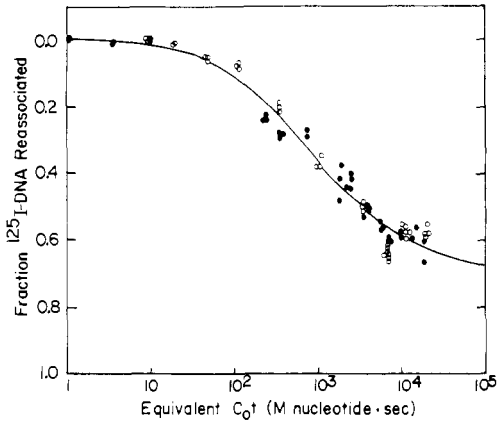

FIGURE 1: Reassociation of ^{125}I-labeled single-copy DNA probes with total cell DNA. ^{125}I-Labeled single-copy DNA 260 nucleotides in length was annealed with an excess (5000–10000:1) of total cell DNA 300 nucleotides long. The fraction of ^{125}I-labeled single-copy DNA renatured was assayed by resistance to S1 nuclease. (●) ^{125}I-Labeled single-copy DNA I; (○) ^{125}I-labeled single-copy DNA II. The curve drawn through the data represents the best least-squares fit of a single second-order reaction for the reassociation of ^{125}I-labeled single-copy DNA I. The computer fit for ^{125}I-labeled single-copy DNA II is nearly identical and not included.

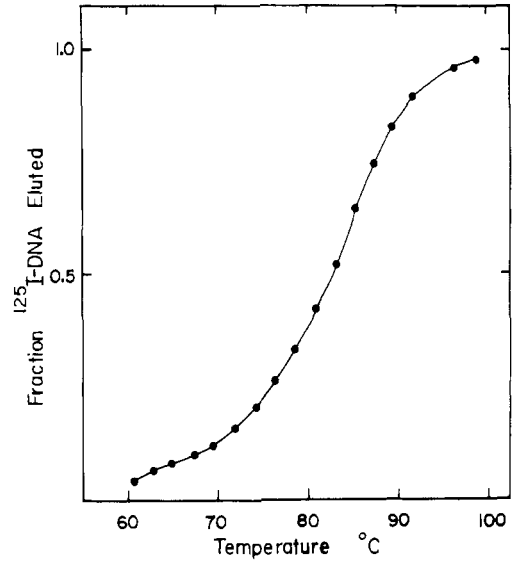

FIGURE 2: Thermal elution profile of renatured ^{125}I-labeled single-copy DNA I. Renatured DNA–DNA duplexes (C_0t 20000) were adsorbed on hydroxylapatite in 0.12 M sodium phosphate (pH 6.8) at 60 °C and washed extensively with 0.12 M sodium phosphate (pH 6.8) at 60 °C. The temperature of the column was then raised in increments, and the column was equilibrated at each temperature for 5 min and washed with 0.12 M sodium phosphate buffer (pH 6.8). After the final elution with 0.12 M sodium phosphate (pH 6.8), at greater than 97 °C, the columns were washed with 0.48 M sodium phosphate (pH 6.8) to assure that all labeled DNA had been melted from the column in 0.12 M sodium phosphate buffer. The T_m of the reassociated ^{125}I-labeled single-copy DNA duplex was 82.5 °C.

strand activity was ever detected. The single-strand S1 nuclease activity was determined by boiling radioactive DNA samples and then immediately digesting with the enzyme. The degree of single-strand activity varied with the enzyme preparation and phosphate concentrations of the samples but was equal to or greater than 94%. The data from each experiment were corrected for the single-strand activity of the enzyme by dividing the percent single-strand DNA from each sample by the fraction of single-strand DNA digested by the enzyme in control experiments.

Isolation and Renaturation of Escherichia coli DNA. *E. coli* DNA was isolated according to Rawson (1975) and sonicated to a mean length of 300 nucleotides. *E. coli* DNA was iodinated as previously described for single-copy *Euglena* DNA. The specific activity was 4.8×10^6 cpm/μg. Renaturation of ^{125}I-labeled *E. coli* DNA to an excess of unlabeled *E. coli* was assayed by S1 nuclease, using the Renaturation and assay conditions described for *Euglena* single-copy DNA.

Analysis of Data. The data were fit to the appropriate curve by using a nonlinear least-squares regression (Pearson et al., 1978). The DNA–DNA renaturation reaction was described by the expression $C = C_0/(1 + k_2C_0t)^{0.44}$ (Smith et al., 1975), where C is the transient single-stranded DNA concentration at time t, k_2 is the rate constant for the renaturation reaction, and C_0 is the initial single-stranded DNA concentration. The hybridization reactions were assumed to follow pseudo-first-order kinetics (Galau et al., 1974).

Results

Characterization of ^{125}I-Labeled Single-Copy DNA. Two radioactive single-copy DNA probes (^{125}I-labeled single-copy DNAs I and II) were prepared by iodinating single-copy DNA in vitro. Trace amounts of the ^{125}I-labeled single-copy DNAs were annealed with an excess of total DNA 300 nucleotides long, and the extent of renaturation was assayed by S1 nuclease (Figure 1). The solid line through the data of Figure 1 represents the best fit for the renaturation of ^{125}I-labeled single-copy DNA I with total cell DNA. When the two ^{125}I-labeled single-copy DNA preparations were renatured with total DNA, both were driven to 71% completion and showed nearly identical renaturation kinetics. The computed rate constants for ^{125}I-labeled single-copy DNAs I and II were 6.17 $\times 10^{-3}$ and 5.84×10^{-3} M^{-1} s^{-1}, respectively. These values are in close agreement with 6.53×10^{-3} M^{-1} s^{-1}, the second-order rate constant which best describes the reassociation of the single-copy component in total DNA 300 nucleotides in length (Rawson et al., 1979). Attempts to resolve these data (^{125}I-labeled single-copy DNAs I and II) into two second-order components were unsuccessful, suggesting the absence of detectable contaminating repetitive sequences.

Duplexes formed by the renaturation of ^{125}I-labeled single-copy DNA I with total DNA were melted on hydroxylapatite in 0.12 M sodium phosphate (Figure 2). The predicted melting temperature (T_m) of native DNA (47% G+C) in this buffer and for DNA of this size is 86.1 °C (Schildkraut et al., 1962; Britten et al., 1974). The actual T_m determined for duplexes formed with ^{125}I-labeled single-copy DNA I was 82.5 °C, a difference of −3.6 °C from the predicted T_m of native DNA.

Hybridization of RNA from Various Stages of Chloroplast Development. *Euglena* cells were grown in the dark to stationary phase and then transferred to the light. The extent of chloroplast development was monitored by determining the amount of chlorophyll per cell at various times after exposure to light (Figure 3).

Total cell RNA was isolated from dark-grown cells and cells containing chloroplasts at various stages of development and hybridized to trace amounts of ^{125}I-labeled single-copy DNA. The extent of hybrid formation as a function of R_0t was assayed by using S1 nuclease. The fraction of ^{125}I-labeled single-copy DNA self-association which occurred during the hybridization reactions was determined by incubating trace amounts of ^{125}I-labeled single-copy DNA in the same buffer in the absence of RNA for times comparable to the longest hybridization reactions (final C_0t of 8). No DNA–DNA duplexes were detected in these experiments. This is consistent with the observation that ^{125}I-labeled single-copy DNA does

Table I: Hybridization of ^{125}I-Labeled Single-Copy DNA to Total Cell RNA

origin of RNA prepn (length of light exposure)	obsd k_2 (M^{-1} s^{-1})[a]	fraction of single-copy DNA transcribed[b]	complexity of RNA derived from single-copy DNA (nucleotides)[c]	fraction of total cellular RNA driving the reaction[d]	no. of diverse RNA transcripts per cell[e]
0 h	1.0×10^{-4}	0.48	8.0×10^{7}	0.0027	0.9
12 h	7.6×10^{-5}	0.53	8.9×10^{7}	0.0023	0.7
48 h	9.1×10^{-5}	0.42	7.0×10^{7}	0.0022	0.9
72 h	1.2×10^{-4}	0.38	6.4×10^{7}	0.0030	1.2
continuously light grown	1.5×10^{-4}	0.39	6.5×10^{7}	0.0034	1.4

[a] The observed k_2 for the hybridization data was analyzed by assuming pseudo-first-order kinetics. [b] Fraction single-copy DNA transcribed = 2 × (fraction of DNA in RNA–DNA hybrid/reactivity of single-copy DNA). [c] Complexity of RNA = complexity of single-copy DNA (1.68×10^8 nucleotide pairs) × fraction of single-copy DNA transcribed. [d] Fraction of RNA driving the reaction = 0.5 × (observed k_2)/(pure k_2). [e] Number of diverse RNA transcripts per cell = (2.1×10^{10} nucleotide RNA/cell) × (fraction of RNA driving reaction/complexity of RNA transcribed).

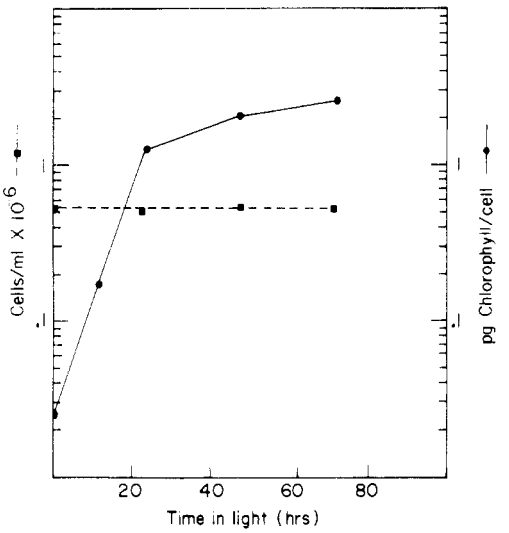

FIGURE 3: Growth curve of *E. gracilis* cells during chloroplast development. Cells were grown on heterotrophic medium in the dark. When the cells reached stationary phase [(5–6) × 10^6 cells/mL], they were illuminated for various periods of time (0, 12, 48, and 72 h), collected by centrifugation, and frozen at −20 °C until RNA extraction. Immediately prior to harvesting the cells, a sample was removed for a cell count and chlorophyll determination.

not renature with total cell DNA prior to C_0t 10 (Figure 2). Thus, DNA–DNA renaturation does not appreciably contribute to the fraction of ^{125}I-labeled single-copy DNA scored as duplex molecules in the hybridization experiments.

Figure 4 represents the best least-squares fit for the hybridization data, assuming a single pseudo-first-order reaction. The rate constants for these reactions and the fraction of single-copy DNA transcribed at different stages of chloroplast development are summarized in Table I. The fraction of single-copy DNA transcribed was calculated by correcting the percent DNA in the form of a hybrid for reactivity of the single-copy probe and multiplying by 2 to account for asymmetrical transcription (Galau et al., 1974). The complexity of the RNA derived from single-copy DNA was calculated by multiplying the fraction of single-copy DNA transcribed by the total complexity (1.68×10^8 nucleotide pairs) of single-copy DNA in the cell (Rawson et al., 1979).

The complexity of the RNA derived from single-copy DNA varied significantly during chloroplast development. The complexity of the RNA is greatest 12 h after initiation of chloroplast development and progressively decreases at later stages of organelle development. There is little difference in the amount of single-copy DNA represented as RNA transcripts in cells which were continuously light grown and in cells which had undergone 72 h of chloroplast development.

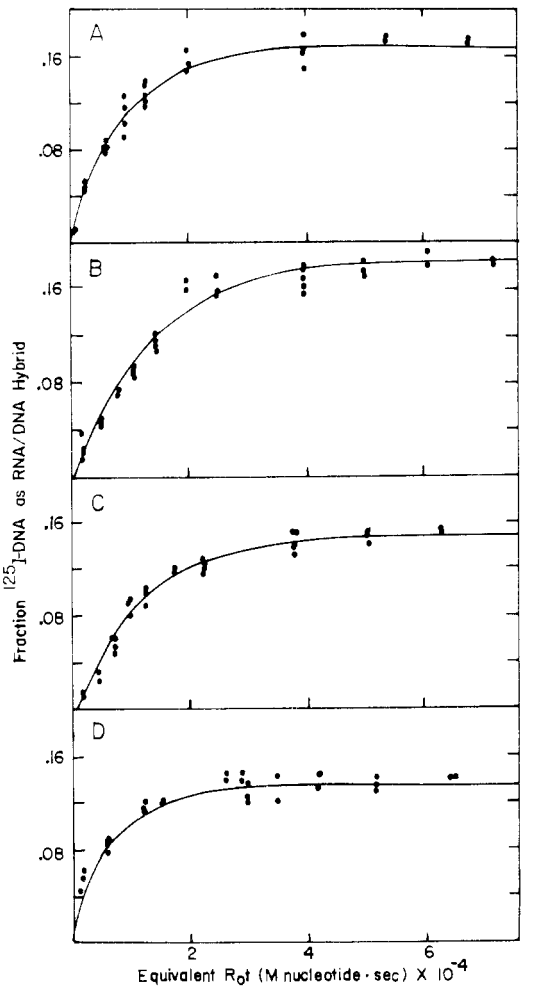

FIGURE 4: Hybridization of ^{125}I-labeled single-copy DNA to total cell RNA from *Euglena* cells at different stages of chloroplast development. Curves A–D represent the hybridization to ^{125}I-labeled single-copy DNA of an excess (5000–10 000:1) of total cell RNA isolated from cells illuminated for 0, 12, 48, and 72 h, respectively. ^{125}I-Labeled single-copy DNA I was used in curves A–C, and ^{125}I-labeled single-copy DNA II was the probe used in curve D.

Fraction of RNA and Number of RNA Molecules Driving the Hybridization Reactions. The fraction of the total mass of RNA which drives a hybridization reaction can be calculated by using the observed rate constant for the hybridization reaction and *E. coli* DNA as a kinetic standard (Galau et al., 1974). In our hands, the rate constant for the reassociation of *E. coli* DNA was 0.20 M^{-1} s^{-1} using the renaturation and

assay procedures described earlier. The calculated values for the fraction of RNA driving each hybridization reaction [fraction = $0.5k_2$(observed)/k_2(expected); Galau et al., 1974] are given in Table I. Corrections for the size of the *E. coli* DNA and total RNA relative to the single-copy DNA size (Hough et al., 1975) were not included since all three have similar molecular weights.

The average number of transcripts of the diverse but complex sequences which drive the hybridization reactions can be calculated by dividing the complexity of the RNA transcribed into the product of the cellular RNA content (12 pg or 2.1×10^{10} nucleotides; Parenti et al., 1969) and the fraction of RNA driving the reaction (Galau et al., 1974). These values are also summarized for each stage of chloroplast development in Table I.

Discussion

Some interesting conclusions can be drawn from the hybridization of single-copy DNA to total cellular RNA isolated from cells containing chloroplasts at different stages of development. A comparison of the fraction of single-copy DNA transcribed from dark- and light-grown cells indicates that the complexity of the RNA derived from single-copy DNA is greater in a dark-grown heterotrophic cell (8.0×10^7 nucleotides) than in a cell with mature chloroplasts (6.5×10^7 nucleotides). The greatest fraction of nuclear single-copy DNA represented as RNA transcripts is seen 12 h after initiation of chloroplast development. At later times during chloroplast development the complexity of the RNA derived from single-copy DNA decreases to a value similar to that of the RNA in cells grown continuously in the light.

In view of these results, it is interesting to consider the biology of chloroplast development as described by Schiff (1975). Upon illumination of dark-grown cells, there is a developmental lag of 12–14 h during which there is little membrane and chlorophyll synthesis. Fifteen hours after the initation of development there is a rapid increase in chlorophyll biosynthesis, a proliferation of membrane material, and a dramatic increase in photosynthetic activity. Development of the organelle is complete by 72–96 h. The developing chloroplast does not depend upon its own photosynthesis for development. It has been suggested that during the first 12 h of development the cytoplasm is the major provider of energy and constituents to the developing organelle, while after 12 h the major synthetic activities occur within the chloroplast itself (Cohen & Schiff, 1976).

There appear to be significant correlations between the transcriptional events from single-copy DNA in the nucleus and the changes observed in the structure and activity of the developing chloroplast. The complexity of the RNA derived from single-copy DNA is greatest just prior to the surge in development seen at 15 h. By 72 h, the complexity of the RNA transcribed from nuclear single-copy DNA has decreased to the level of continuously light-grown cells. It should be stressed that these hybridization experiments only demonstrate a correlation between chloroplast development and changing RNA populations derived from single-copy DNA. They do not demonstrate that the new sequences are specifically involved in chloroplast formation.

Estimates of the fraction of RNA driving each hybridization reaction indicate that only a small portion of the total mass of the RNA at each stage of development contributes to the hybridization reaction. Since the majority of total RNA is composed of stable RNAs (rRNA and tRNAs) which are not complementary to single-copy DNA, this result is not unexpected. The number of diverse transcripts derived from single-copy DNA is ~1/cell at each stage of development. These values are very similar to the numbers calculated for sea urchin embryos using hnRNA (690 copies/600-cell embryo; Hough et al., 1975) and are somewhat higher than those calculated for *Chlamydomonas* with total RNA (1 copy/3 cells; Howell & Walker, 1977). The techniques used in these studies are biased toward the detection of RNAs of high complexity (and usually low abundance) and are insensitive in the detection of sequences of low complexity and high abundance. Thus, the low values obtained for the number of copies of RNA driving the reaction per cell are not unexpected.

Recently, experiments have been reported (Verdier, 1979) in which the complexity of poly(A)-containing RNA was measured by its hybridization to cDNA during chloroplast development. Two interesting and somewhat surprising observations were made. One, the overall complexity of the total poly(A)-containing RNA in *Euglena* is comparable to the genomic complexity of the organism (i.e., all the nDNA is represented as poly(A)-containing RNA). Two, the most complex fraction of the poly(A)-containing RNA is approximately 15-fold more complex than the total RNA transcripts from single-copy DNA. Verdier's (1979) experiments are not compatible with our estimates of only a fraction (0.39–0.53) of the single-copy DNA being transcribed at any particular stage of chloroplast development.

In summary, *Euglena* is a simple eucaryote whose nuclear single-copy DNA is transcribed extensively. The greatest complexity of RNA derived from single-copy DNA at any one stage of organelle development is equivalent to $\sim 9 \times 10^4$ diverse nucleotide sequences of 1000 bases each and similar to the complexity present in the total RNA of mouse liver (Brown & Church, 1972; Grouse et al., 1972) and the hnRNA of *Xenopus* oocytes (Davidson & Hough, 1971). Although in animal systems a large fraction of the total RNA complexity is restricted to the nucleus, at present there is no information as to the relationship between nuclear and polysomal RNA in *Euglena*. It is expected that only a small portion of the observed complexity of total RNA will be represented as mRNA transcripts on cytoplasmic polysomes.

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Mitochondrial Adenine Nucleotide Carrier. Investigation of Principal Structural, Steric, and Contact Requirements for Substrate Binding and Transport by Means of Ribose-Modified Substrate Analogues†

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ABSTRACT: A selected series of fourteen ribose-modified adenine nucleotide analogues was prepared and characterized as their α-^{32}P- or U-^{14}C-labeled compounds. The capacity of rat liver mitochondria for adenine nucleotide carrier-linked (specific) binding and carrier-mediated transfer across the inner mitochondrial membrane as well as the amount of noncarrier-linked (unspecific) binding of these analogues was determined at 5 °C by means of an inhibitor (atractyloside) stop method and compared with the natural substrates ADP and ATP. Kinetic data of carrier-specific bound analogues were evaluated from Dixon plots and indicate these analogues as competitive inhibitors for mitochondrial [U-^{14}C]AD(T)P uptake. The findings confirm the distinct substrate specificity of the carrier system. By means of the analogues, an experimental proof of the two-step nature of mitochondrial adenine nucleotide translocation, i.e., carrier-specific binding (recognition) and transport, was obtained. Furthermore, the findings provide a detailed description of the basic steric, contact, and structural elements which are prerequisite for carrier-specific binding (A) and additionally for subsequent transport (B): (A) (1) an anti- or syn-positioned β-*N*-glycosyl-linked purine base; (2) a S- or N-type sugar pucker; (3) a cis disposition of the C(4′)–C(5′) bond with respect to the heterocycle; (B) (1) a nonfixed anti-positioned purine base with a *N*-glycosyl torsion angle of approximately −20°; (2) a S-type sugar pucker; (3) a gauche–gauche orientation of the exocyclic C(5′)–O(5′) group; (4) a trans-positioned, i.e., C(2′) ribo hydroxyl, group, which presumably "triggers" the induction of carrier-mediated transport.

The transfer of ADP and ATP across the inner mitochondrial membrane represents a key process of energy supply in aerobic eucaryotic cells. This transfer is catalyzed by a membrane integral lipoprotein, i.e., the ANP[1] carrier. This protein recently has been isolated from mammalian (Brandolin et al., 1974; Riccio et al., 1975; Shertzer & Racker, 1976; Bojanovski et al., 1976) and yeast mitochondria (Boulay et al., 1979) as well as partially characterized and reconstituted.

However, an understanding in which way substrate and transporter protein interact at the molecular level is still pending. One approach is the use of substrate analogues, which proved to be of exceptional utility in the investigation of ligand–receptor interactions, especially in the field of enzyme-catalyzed reactions (Yount, 1975).

Translocation experiments carried out with a selected series of substrate analogues should provide a detailed description of chemical and structural features, which are of potential importance to mitochondrial transport. Moreover, it should be possible to prove experimentally the two-step nature of the process by means of analogues, which are bound to the carrier but inappropriate for transportation.

We decided to investigate ribose-modified adenine nucleotide analogues, as slight modifications at the ribose moiety, which represents the central molecule part, affect the overall nucleotide structure in a decisive manner.

Recently, on the basis of our findings (Boos et al., 1976a,b) on the binding characteristics, photoaffinity labeling of the carrier–substrate binding region with substrate analogues was achieved (Schäfer et al., 1977).

Experimental Procedures

Materials. [U-^{14}C]ATP (196 mCi/mmol), [U-^{14}C]2′dATP (**2d**) (450 mCi/mmol), and [^{32}P]phosphorus oxychloride (1–50 mCi/mmol) were purchased from Amersham, England. Yeast hexokinase (EC 2.7.1.1), orthophosphoric monoester phosphohydrolase (EC 3.1.3.1), and atractyloside were from Boehringer, Germany; 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5) and 3′dAdo (**3a**) were from Sigma Chemicals, Ger-

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[1] Abbreviations used: ANP, adenine nucleotide (ADP, ATP); 2′dAdo, 9-(β-D-2′-deoxyribofuranosyl)adenine (**2a**); 3′dAdo, 9-(β-D-3′-deoxyribofuranosyl)adenine (**3a**); ara-Ado, 9-(β-D-arabinofuranosyl)adenine (**4a**); xylo-Ado, 9-(β-D-xylofuranosyl)adenine (**5a**); rox-Ado, ribose-oxidized adenosine, 2,2′-[1′-(9-adenyl)-1′-(hydroxymethyl)]dioxodiethyl ether (**6a**); rro-Ado, ribose-ring-opened adenosine, 2,2′-[1′-(9-adenyl)-1′-(hydroxymethyl)]dihydroxydiethyl ether (**7a**); 2′,3′-methoxy-Ado, 2′,3′-*O*-methoxymethylidene-9-(β-D-ribofuranosyl)adenine (**8a**); 2′,3′-isoprop-Ado, 2′,3′-isopropylidene-9-(β-D-ribofuranosyl)adenine (**9a**); 2′,3′-ddAdo, 9-(β-D-2′,3′-dideoxyribofuranosyl)adenine (**10a**); 3′-*O*-methyl-Ado, 3′-*O*-methyl-9-(β-D-ribofuranosyl)adenine (**11a**); lyxo-Ado, 9-(α-L-lyxofuranosyl)adenine (**12a**); αAdo, 9-(α-D-ribofuranosyl)adenine (**13a**); 8,2′-*O*-cyclo-Ado, 8,2′-anhydro-8-oxy-9-(β-D-arabinofuranosyl)adenine (**14a**); 8,3′-*O*-cyclo-Ado, 8,3′-anhydro-8-oxy-9-(β-D-xylofuranosyl)-adenine (**15a**); $(Et)_3NH^+HCO_3^-$, triethylammonium bicarbonate.

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