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HYBRIDIZATION OF ECORI CHLOROPLAST DNA FRAGMENTS OF EUGLENA TO PULSE LABELED RNA FROM DIFFERENT STAGES OF CHLOROPLAST DEVELOPMENT

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SUMMARY. Transcription of Euglena chloroplast DNA was visualized by autoradiography of hybrids formed between electrophoretically separated EcoRI endonuclease fragments and [^{32}P]-pulse labeled RNA prepared from cells at different stages of chloroplast development. The RNA was hybridized to Southern imprints of EcoRI chloroplast DNA digests. The EcoRI chloroplast DNA fragments were categorized by their ability to hybridize the different pulse labeled RNA's. Some fragments were transcribed: 1. both in the dark and throughout chloroplast development, 2. predominantly in the light, 3. in the dark and for only a brief period of time after transfer of the cells to the light. Other fragments were probably not transcribed.

INTRODUCTION

Chloroplast development in Euglena gracilis requires that the chloroplast DNA be programmed to produce sufficient quantities of RNA at the appropriate time for the translation of the necessary gene products. During chloroplast development the complexity and the abundance of the chloroplast DNA transcripts varies considerably (1, 2). The major transcriptional product of the chloroplast DNA is chloroplast ribosomal RNA (rRNA) (3) and is derived from 10% of the chloroplast DNA (4).

A limit digestion of Euglena chloroplast DNA with the restriction endonuclease EcoRI yields 24 major fragments varying in size from 24 kbp to approximately 0.8 kbp (5, 6, 7). Three of these fragments contain the sequences for the 16S and 23S chloroplast rRNA (4). Since considerably more chloroplast DNA is transcribed than is represented by these rRNA nucleotide sequences, we have attempted to characterize the transcriptional properties of the other EcoRI chloroplast DNA fragments during chloroplast development.

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Euglena cells undergoing chloroplast development were pulse labeled with [^{32}P]-orthophosphate. Total cell RNA was isolated and hybridized to Southern imprints of EcoRI fragments of chloroplast DNA. Autoradiographs of the hybrids reveal that the EcoRI chloroplast DNA fragments may be categorized on the basis of their ability to hybridize pulse labeled RNA's from different stages of chloroplast development. There are four such classes of EcoRI chloroplast DNA fragments. Some fragments are transcribed: 1. both in the dark and throughout chloroplast development, 2. predominantly in the light and little, if any, in the dark, 3. in the dark and for only a brief period of time after transfer of the cells to the light. A few fragments did not appear to be transcribed.

MATERIALS AND METHODS

Cell Growth. Euglena gracilis var. Z cells were grown in a heterotrophic medium (2). Chloroplast development proceeded in cells which were first grown to stationary phase (3-5 x 10 cells/ml) in the dark, then transferred to the light (2500 lux) and maintained with constant shaking for various periods of time. The cell concentration and chlorophyll content of the cells were monitored during chloroplast development (2).

Pulse labeling of RNA. Cells were grown in the heterotrophic medium depleted 20-fold of inorganic phosphate (9). RNA was pulse labeled for 2 hr with [^{32}P]-orthophosphate (20 μCi/ml). The cells were chilled to 4°C by pouring the culture over ice and then collected by centrifugation at 7000 rpm. The cells were stored at -20°C for up to one week.

RNA isolation. [^{32}P]-pulse labeled RNA was isolated by suspending the cells from 200 ml of culture in 25 ml of R buffer (0.1 M Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM MgCl and 0.1 mM EDTA). The suspension was adjusted to 2% (w/v) sodium dodecysulfate with a 20% stock solution plus two volumes of a phenol mixture containing cresol (10% v/v), 8-hydroxyquinoline (0.1% w/v) and saturated with R buffer. The cell lysate was extracted three times with the phenol mixture. The RNA was precipitated with 0.1 volume of 3 M sodium acetate plus 2 volumes of ethanol and suspended in 2 ml of R buffer. DNA was digested with 25 μg of electrophoretically purified DNAase I (Worthington, DPFF 54N332) at 4°C for one hr. The nuclease reaction was stopped by adjusting the mixture to 10 mM EDTA plus 0.5% (w/v) sarkosyl, and the volume of the RNA mixture was increased by the addition of 1 ml of R buffer. One gm of CsCl per ml was added, and the RNA was pelleted in a Spinco SW 50.1 rotor at 30,000 rpm for 20 hr. The RNA pellets were suspended in 5 ml of 10 mM NaCl, 0.1 mM EDTA and 1 mM Tris-HCl (pH 8.0) and dialyzed against the same buffer.

In order to eliminate non-specific binding of the [^{32}P]-labeled RNA to nitrocellulose strips during hybridization, the RNA was further purified through Cs_2SO_4 gradients. The [^{32}P]-labeled RNA was layered on top of a 1.0 ml Cs_2SO_4 (ρ = 1.541 g/cc) cushion and centrifuged in a Spinco SW 50.1 rotor at 40,000 rpm at 25 C for 20 hr. The RNA, which formed a pellet, was suspended in water and dialyzed against water. The RNA was stored at -20°C.

Chloroplast DNA isolation. Supercoiled chloroplast DNA was isolated according to Chelm and Hallick (3).

Agarose gel electrophoresis. Chloroplast DNA was digested with the restriction endonuclease EcoRI (Miles Laboratories) and analyzed by electrophoresis in agarose gels (4). The size of the chloroplast DNA fragments was determined using λ-DNA digested with Hind III as a standard (10).

Hybridization of RNA to restriction endonuclease fragments of chloroplast DNA. Restriction endonuclease fragments of chloroplast DNA were eluted from agarose gels onto strips of Millipore filter paper (HA, 0.45 μ) (8). Hybridization of RNA to the Southern imprints was carried out for 72 hr. as previously described (4). The hybrids on the Millipore strips were located by autoradiography (4).

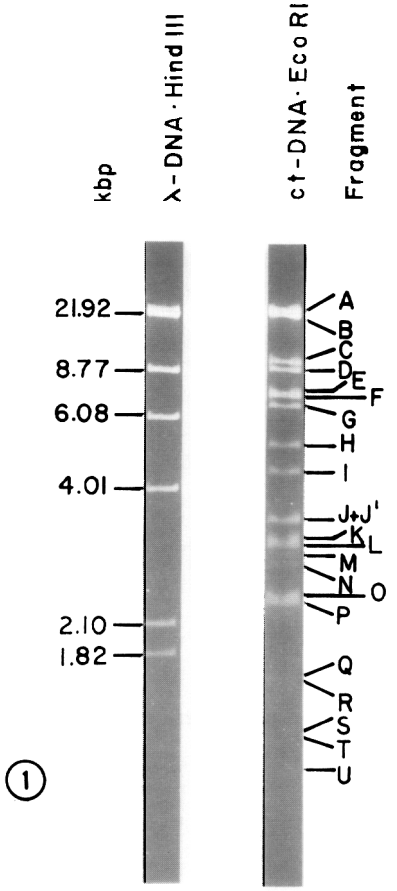
RESULTS AND DISCUSSION

Total cellular RNA was pulse labeled with [^{32}P]-orthophosphate at various times during chloroplast development. Fig. 1 shows the developmental regime during which the cells were pulse labeled. Less than one cell division occurred during the formation of the chloroplast. The cellular chlorophyll content, a measure of chloroplast development, increased from undetectable quantities in the dark grown cells to 5.0 pg/cell. Cells which remained in the light for longer than 73 hr showed no further increase in chlorophyll content. The duration of the [^{32}P]-pulse was two hr or one sixth of the normal time for cell division.

The various [^{32}P]-pulse labeled RNA's were hybridized to Southern imprints of EcoRI chloroplast DNA fragments. Fig. 2 shows the EcoRI limit digestion products of chloroplast DNA electrophoretically separated in agarose gels. The λ-DNA digested with Hind III was run concurrently with the chloroplast DNA and serves as a standard to identify the various EcoRI chloroplast DNA fragments. The chloroplast DNA fragments are referred to as EcoRI-A through U in descending size (6). EcoRI-F, -L and -P fragments contain the nucleotide sequences for the mature chloroplast rRNA's (4).

Fig. 3 shows several representative autoradiographs of the hybrids formed between the [^{32}P]-pulse labeled RNA and the Southern imprints of EcoRI chloroplast DNA fragments. The EcoRI chloroplast DNA fragments coding for chloroplast rRNA are continually transcribed in the dark and the light. Other chloroplast DNA fragments appear to vary in their ability to

hybridize to the different pulse labeled RNA's. Table I summarizes the different classes of EcoRI chloroplast DNA fragments as defined by their hybridization to RNA from cells which were undergoing chloroplast development.



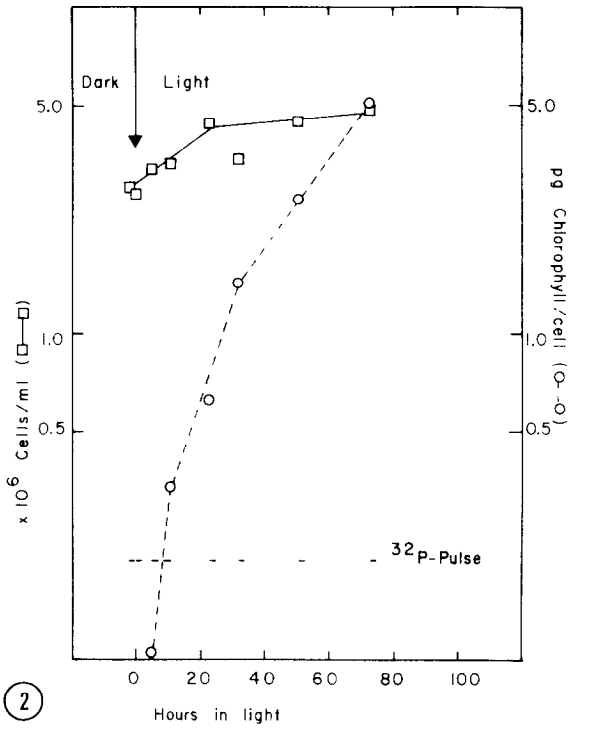


Figure 1. EcoRI restriction endonuclease cleavage of chloroplast DNA. Chloroplast DNA (0.5 μg) was digested with EcoRI, analyzed by electrophoresis in 0.8% (w/v) agarose gels and visualized by fluorescence of ethidium bromide bound to the DNA. λ-DNA (0.2 μg), digested with Hind III, was run concurrently as a molecular weight marker. The EcoRI chloroplast DNA fragments range in size from 24 to 0.86 kilobase pairs and are referred to as EcoRI-A to U in descending size.

Figure 2. Growth curve of E. gracilis during chloroplast development. Five hundred ml flasks containing 200 ml heterotrophic medium depleted 95% of phosphate were grown in the dark with constant shaking. The cells were transferred into the light just prior to reaching stationary phase. [^{32}P]-orthophosphate (20 μCi/ml) was added at different times during chloroplast development. The cells were allowed to synthesize RNA for 2 hr, chilled and collected by centrifugation. The dashes indicate the time and duration of each [^{32}P]-pulse. The chlorophyll content per cell for each flask was measured immediately before the [^{32}P]-pulse.

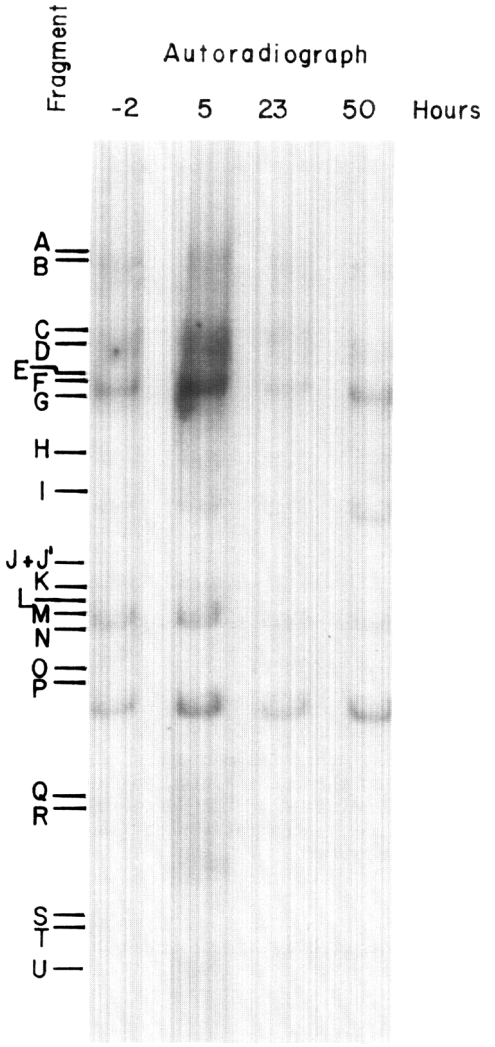


Figure 3. Autoradiographs of hybrids formed between EcoRI chloroplast DNA fragments and [^{32}P]-pulse labeled RNA. EcoRI chloroplast DNA fragments (.3 μg) were separated by electrophoresis in 0.8% (w/v) agarose gels. The DNA fragments were denatured and transferred to Millipore filters. [^{32}P]-labeled RNA from cells pulse labeled 2 hrs prior to transfer of all the flasks to the light (-2 hr) and from cells which had been exposed to light for 5, 23 and 50 hr was hybridized for 72 hr to the Southern imprints. The hybrids were detected by autoradiography.

Four different classes of EcoRI chloroplast DNA fragments exist; those which are: 1. continually transcribed in the dark and in the light, 2. transcribed only in the light and very little, or not at all in the dark, 3. transcribed in the dark and less in the light, 4. not transcribed (i.e. no detectable transcription). Some of the smaller EcoRI chloroplast DNA

Table 1. Transcriptional Properties of EcoRI Chloroplast DNA Fragments.

Transcriptional Characteristics	EcoRI Chloroplast-DNA Fragments[a]
Continually transcribed (dark and light)	A,B,D,F,G,H,I,L,M,P,Q,(or R)
Transcribed in the light and very little, or not at all in the dark	C
Transcribed in the dark and less in the light	J and/or J', N
No detectable transcription	K,O
Uncertain[b]	E,R(or Q),S,T,U

[a]The EcoRI restriction endonuclease fragments of chloroplast DNA are referred to as EcoRI-A through U in descending size. EcoRI-J and J' are [b]different nucleotide sequences (Hallick, personal communication).
[b]EcoRI-E is quite difficult to separate from EcoRI-F. It is, therefore, not certain whether the fogging of the autoradiograph in this region is due to transcription of EcoRI-F alone or transcription of EcoRI-E, also. Some of these other fragments are relatively small (less than 1.7 kilobase pairs), and the uncertainty indicated here may be due to their not being efficiently retained on the Southern imprints because of size, the presence of very low concentrations of RNA in a hybrid, or they simply may not be transcribed.

fragments are difficult to characterize, and their transcriptional properties are uncertain.

The observations made in these studies as to which EcoRI chloroplast DNA fragments are transcribed are strictly qualitative. Hybridization of total cellular RNA, rather than relatively homogeneous RNA transcripts, to DNA immobilized on filters precludes the possibility of saturating all the DNA sequences with their complementary RNA sequences. However, we can expect all the different transcribed chloroplast DNA sequences to have hybridized to some degree with their complementary RNA sequences.

Selective transcription of various regions of the chloroplast DNA during chloroplast development as defined by restriction endonucleases is compatible with the concept of differential gene expression and is in good

agreement with observations made earlier concerning the fraction of the chloroplast DNA transcribed and the abundance of these transcripts during chloroplast development (1, 2, 3). The next step in developing a complete transcriptional map of Euglena chloroplast DNA during chloroplast development will be to follow the hybridization kinetics of individual restriction endonuclease fragments to RNA derived from cells at various stages of chloroplast development. This should permit quantitative statements regarding the transcription of specific regions of the chloroplast DNA.

ACKNOWLEDGEMENTS

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