Analysis of the *Euglena gracilis* chloroplast genome

Fragment *Eco-I* encodes the gene for the M_r 32000–33000 thylakoid protein of photosystem II reaction center

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The DNA fragment *Eco*-I (4700 kilobasepairs) from the circular chloroplast genome of *Euglena gracilis* is shown to be transcribed in etioplasts at all stages of light-induced plastid development and in fully differentiated chloroplasts. Major stable transcription products are mRNAs of 14S and 17S. Using a rabbit reticulocyte lysate translation system we can show that the fragment *Eco*-I selects a mRNA which directs the synthesis of a M_r 32000–33000 polypeptide. *Eco*-I also hybridizes with a 330 basepair DNA probe cut from within the spinach chloroplast gene encoding the M_r 32000 thylakoid membrane protein of the photosystem II reaction center. We conclude that the fragment *Eco*-I carries the corresponding *Euglena gracilis* chloroplast gene.

Euglena gracilis *Chloroplast genome* *Restriction enzyme* *Thylakoid membrane* *DNA fragment* Eco-*I* *Protein 32000* M_r

1. INTRODUCTION

It was shown that the *Euglena gracilis* chloroplast genome is transcribed both in dark-grown cells containing undifferentiated etioplasts, and in light-grown cells containing fully-developed green chloroplasts. Furthermore, it is known that during light-induced plastid development qualitative and quantitative changes in transcription products occur [1–5]. Among the major transcription products figure the rRNAs and tRNAs but less is known about the quality and the differential expression of mRNAs encoding specific chloroplast proteins.

The transcription of several cloned chloroplast DNA fragments during light-induced chloroplast development has been studied. Among all these fragments, we see that *Eco-I* is transcribed at all stages of plastid differentiation, major transcription products being mRNAs of about 17S and 14S. A 14S transcription product was also observed in chloroplast of spinach and identified as mRNA for an M_r 32000 protein of the photosystem II reaction center [6]. To test this possibility in case of *Euglena* we used a cloned DNA probe from the spinach chloroplast genome carrying an internal part of the *P32*-gene. We hybridized it to Southern blots of *Eco* fragments of total *Euglena* chloroplast DNA. The 4700 basepairs *Eco-I* fragment was the only one to interact with the probe

Abbreviations: DBM, diazobenzyloxymethyl; SDS, sodium dodecylsulfate; PIPES, piperazine-*N*-*N*'-bis-|2-ethanesulfonic acid|; *Eco*-(I) fragments, DNA fragments obtained through digestion with the restriction endonuclease *Eco*RI; *P32*g gene coding for a M_r 32000 thylakoid membrane protein of the photosystem II reaction center

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suggesting that the *P32*-gene is located on this DNA fragment. This observation is in agreement with a preliminary report [7] mentioning that *Eco-I* contains the information for the *P32*-gene.

As second line of evidence, we used an in vitro translation system known to correctly read *Euglena* chloroplast mRNA [8]. Using essentially the same protocol we show that *Eco-I* selects a mRNA mainly directing the synthesis of an M_r 33000 protein in a rabbit reticulocyte lysate. A second protein of M_r ~46000, of unknown function is also synthesized what could be related to the 17 S mRNA interaction with *Eco-I*.

2. MATERIALS AND METHODS

Euglena gracilis (Z strain, culture collection of Algae, Indiana University) was grown heterotrophically in a modified Hutner's medium with vitamin B_{12} at 50 ng/l [9]. Plastids were isolated following the basic procedure in [10] and the modifications outlined in [11]. Total RNA was isolated from purified chloroplasts according to [12]. Total chloroplast RNA was fractionated on an 1.5% agarose 5 M urea gel as in [13] and transfered to DBM paper as in [14].

Cloned chloroplast restriction DNA fragments were labelled by nick translation using d[α-^{32}P]-CTP (> 350 Ci/mmol, Radiochemical Center, Amersham) as in [15] and hybridized to fractionated chloroplast RNA for 48 h at 42°C in the following mixture: 5 × NaCl/Cit (1 × NaCl/Cit: 0.15 M NaCl and 0.015 M sodium citrate) 50% formamide, 0.02% of each bovine serum albumin, ficoll and polyvinylpyrrolidone and 500 μg sonicated denatured calf thymus DNA/ml. After hybridization, the DBM filters were washed 2 times at 65°C in 2 × NaCl/Cit dried and autoradiographed using Typon X-ray films. The heterologous hybridization of the *Eco*RI DNA pattern with the 330 basepair spinach probe prepared from the pSoc B5.11 clone was done at 42°C for 12 h in the following reaction mixture: 5 × NaCl/Cit, 15% formamide, 0.075 M Na_2HPO_4, 200 μg sonicated calf thymus DNA/ml and 0.02% of each bovine serum albumin, ficoll and polyvinylpyrrolidone. After hybridization, the filters were washed at 65°C in 2 × NaCl/Cit, dried and autoradiographed.

Translation of the chloroplast RNA was performed in the rabbit reticulocyte lysate from Amersham according to specifications of the supplier.

The hybrid selected translation experiments were according to [16]. Total chloroplast RNA (30 μg) was hybridized with 2 μg cloned chloroplast DNA fragment fixed on nitrocellulose filters. Hybridization was at 50°C for 2 h in 65% deionized formamide, 10 mM Pipes (pH 6.4), 400 mM NaCl. After hybridization, the filters were washed 10 times in 1 × NaCl/Cit containing 0.5% SDS and 3 times in 2 mM Na_2EDTA (pH 7.9). The RNA was released in 1 mM Na_2EDTA (pH 7.9) by heating the filters at 100°C for 60 s and rapid cooling. The RNA was ethanol precipitated, dried and used for the translation system. After translation, the proteins were electrophoresed on a SDS–polyacrylamide slab gel according to [17]. The running gel (20 cm) consisted of a gradient of 10–15% polyacrylamide. The stacking gel was 6% polyacrylamide. Radioactivity on gels was detected by fluorography according to [18] following the modifications of [19].

3. RESULTS AND DISCUSSION

We isolated total RNA from purified etioplasts from dark grown cells (fig. 1A), and from developing plastids isolated from cells exposed for 4 h (B), 8 h (C) and 12 h (D) to light, and from fully differentiated chloroplasts (72 h, E). The RNA was electrophoresed, fixed on DBM filters according to [14] and hybridized to nick translated *Eco-I* DNA fragments, cloned in pBR322. In fig. 1 the stained RNA patterns (left) of each sample are aligned with the corresponding autoradiograph (right). The results can be summarized as follows:

(1) The fragment *Eco-I* is transcribed at all stages tested, contrary, e.g., to *Eco-O* (2700 basepairs) which is not transcribed at all (not shown);
(2) Two major transcription products are always discernible migrating equivalent to RNAs of about 14 S and 17 S, respectively;
(3) The relative intensity of these two bands remains about the same for etioplasts (A) and the plastids from various developing stages (B–D), the 14 S band being always the stronger one. In fully differentiated chloroplasts (E) the two bands reach about equal intensity;

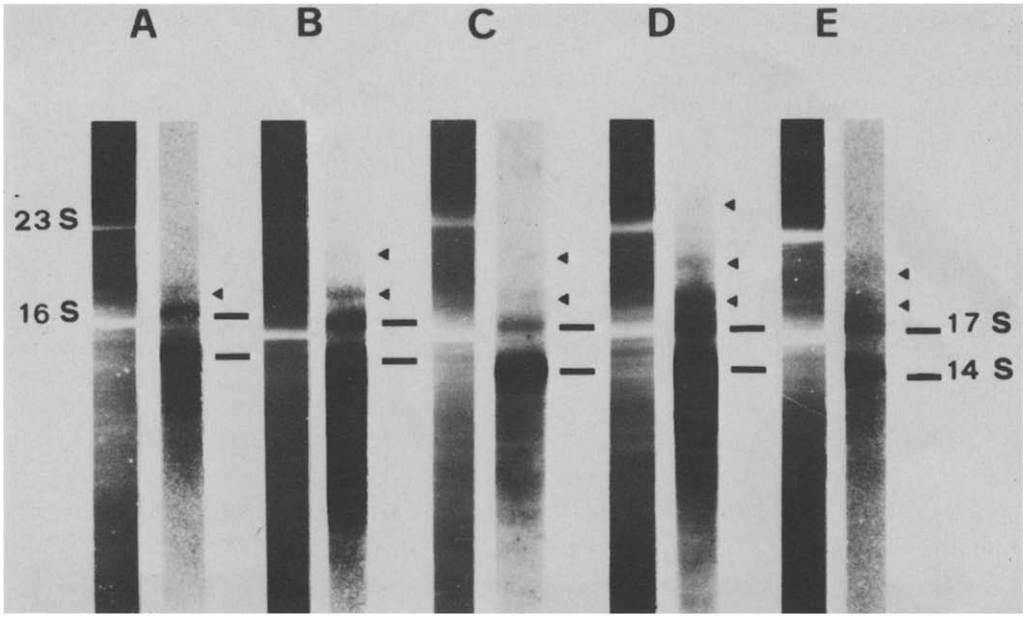

Fig. 1. Hybridization of the ^{32}P-labelled DNA fragment *Eco-I* with total RNA isolated from *Euglena gracilis* from different developmental stages: (A) etioplasts; (B) 4 h light; (C) 8 h light; (D) 12 h light; (E) 72 h light. In each panel, we show the ethidium bromide stained gel (left) aligned with the corresponding autoradiograph (right). The 23 S and 16 S rRNAs show up as the most prominent bands. The 5 S and 4 S RNAs migrated to the bottom of the gels (not seen). Arrows point towards bands discussed in the text.

(4) One or more faint band(s) can be seen above the 17 S band, especially in the 4 h (B) and 12 h (D) pattern. These RNAs may be instable primary transcription products, becoming apparent under our experimental conditions at stages of intensified transcription but they are less visible, e.g., in etioplasts pattern (A) and in the pattern of fully differentiated chloroplasts (E).

(5) In all autoradiographs, we see below the 14 S RNA band broad streaks, especially prominent in patterns B and D. We believe it to be degradation products of the 17 S and/or 14 S mRNAs.

(6) We conclude from these observations that *Eco-I* is already transcribed in etioplasts and very extensively during chloroplast development. Major stable transcription products are most likely to occur.

At this point, we may add that two other cloned chloroplasts DNA fragments, *Eco-H* (5400 basepairs) and *Eco-K* (3300 basepairs) interact with two and one type of mRNA, respectively, which are synthesized at all stages of the chloroplast development. These mRNAs migrate between the 23 S and 16 S rRNA, on an 1.5% agarose 5 M urea gel.

It was shown that a mRNA of about 14 S from spinach chloroplasts encodes the *P32*-gene [6]. We used a 330 basepair fragment cut from within the spinach chloroplast *P32*-gene and hybridized it to the *Eco*RI pattern of total *Euglena gracilis* chloroplast DNA (fig. 2). By this measure, we exclude any non-gene-related interaction between the labelled probe and the chloroplast DNA fragments. Only *Eco-I* yields a strong radioactive signal what indicates that this fragment indeed carries sequences homologous or partly homologous to the spinach *P32*-gene. This result is in line with a report showing that *Eco-I* carries the *P32*-gene [7]. This result also proves that structural parts of protein genes remain rather conserved during evolution. Therefore, cross-hybridization experiments using DNA probes of known genetic function allow to easily retrieve analogous genes in genomes also of distantely related organisms.

The results of these hybridization experiments

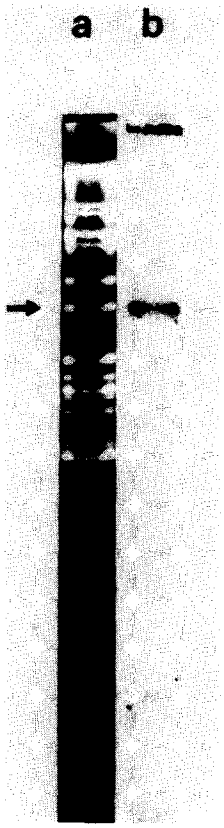

Fig. 2. Hybridization of the 330 basepair spinach chloroplast DNA fragment encoding part of the *P32*-gene to the *Eco*RI fragment pattern of total *Euglena gracilis* chloroplast DNA: (a) stained *Eco* DNA fragments; (b) autoradiographs. Electrophoresis conditions: 1.5% agarose, 18 mA, 20 h. The arrow points towards the *Eco-I* fragment.

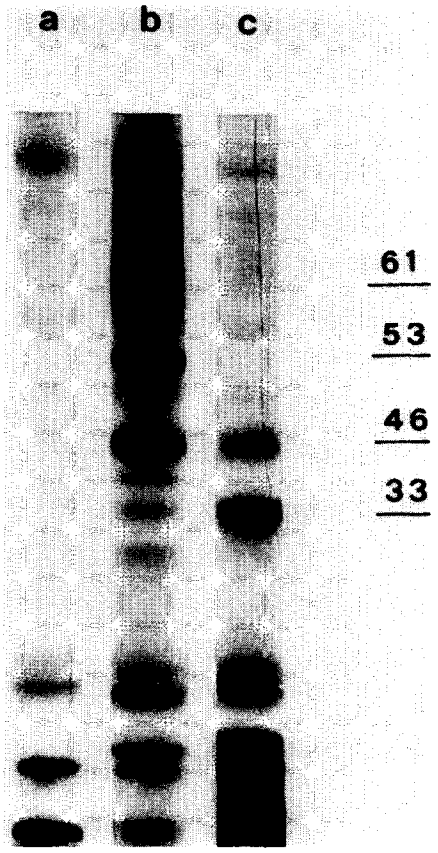

Fig. 3. Fluorograph of electrophoretically separated polypeptides synthesized in a rabbit reticulocyte lysate: (a) without chloroplast RNA; (b) with total chloroplast RNA (1.7 μg); (c) with chloroplast RNA selected by *Eco-I*. M_r-Values are $\times 10^{-3}$. The M_r of the polypeptides synthesized in vitro were estimated using the following standard proteins (Boehringer M_r): bovine serum albumin 69000, ovalbumin 45000 and DNase I 31000 (not shown).

was confirmed by hybrid selected translation experiments. We had shown [8] that the rabbit reticulocyte lysate can correctly translate *Euglena* chloroplast mRNA. In fig. 3. we show the data of a hybrid selected translation experiment. We used filter bound *Eco-I* to retrieve mRNAs which subsequently were used to direct the in vitro protein synthesis. In fig. 3B, we show the electrophoretically separated proteins synthesized in the presence of total chloroplast mRNA and in pattern C those which are synthesized in the presence of mRNA selected by *Eco-I*. The most prominent protein synthesized has an M_r ~33000 what is about equal in size to the precursor protein of the M_r 32000 thylakoid membrane protein of the photosystem II reaction center [20,21]. A second protein (M_r ~46000) of yet unknown function is also synthesized suggesting that *Eco-I* accomodate the corresponding gene. According to [8], this M_r 46000 protein is in the stroma fraction. In pattern B (total RNA), it appears as a prominent band, more so than the M_r 33000 protein. The opposite is seen in pattern C (*Eco-I*). This difference in relative amounts of translation products could be explained by the assumption that the gene for the M_r

46000 protein is only partly located on *Eco-I* and a relative loss of mRNA occurs during the selection process. In [22], the gene for the large subunit of the ribulose 1,5-bisphosphate carboxylase was located on *Eco-A*. This gene contains introns. It will be of great interest to see whether other *Euglena* chloroplast genes do contain introns, since up to now, no introns were found in chloroplast protein genes from higher plants [23–25].

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