ANALYSIS OF *EUGLENA GRACILIS* CHLOROPLAST DNA

The DNA fragment *Eco*RI · N carries genetic information for a 53 000 M_r polypeptide

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1. Introduction

The literature contains little specific information about *Euglena gracilis* chloroplast protein genes. Although the extent of transciption of protein genes could be in the range of 50 000 base pairs [1,2], no protein genes have been mapped on the circular genome so far. On the other hand, all rRNA genes [3–6] and several of the tRNA genes [5–8] have been mapped.

To obtain some information about loci of protein genes on the chloroplast genome we opted for the following protocol. Highly purified, intact chloroplasts of *Euglena* retain the capacity for light-dependent protein synthesis [9]. We used the same type of chloroplasts as source of RNA to direct protein synthesis in a reticulocyte lysate [10]. Proteins synthesized in the reticulocyte lysate were analytically compared with proteins synthesized within intact chloroplasts. Finally, using the techniques of hybrid arrested [11] and hybrid selected [12] translation it was possible to correlate a given synthesized protein with defined (cloned) DNA fragments of the chloroplast genome. In the following we show that:

(i) The reticulocyte system correctly translates *Euglena* chloroplast mRNAs and
(ii) The restriction fragment *Eco*RI · N of *Euglena* chloroplast DNA specifically interacts with the mRNA of a chloroplast polypeptide of 53 000 M_r.

Abbreviations: SDS, sodiumdodecylsulfate; PIPES, piperazine-*N*-*N*'-bis-[2-ethanesulfonic acid]; RUBPase, ribulose-bisphosphatecarboxylase

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2. Materials and methods

Euglena gracilis (Z-strain, culture collection of Algae, Indiana University) was grown photoheterotrophically on the modified Hutner's medium of [13] with vitamin B_{12} at 50 ng/l.

Chloroplasts were isolated following the basic procedure in [14] and the modifications outlined in [9].

Light-dependent protein synthesis by the isolated chloroplasts was determined as in [9,15] in the presence of 50–60 μCi [^{35}S]methionine (>600 Ci/mmol; Amersham)/ml chloroplasts suspension.

Labelled chloroplasts were lysed in 10 mM Na-pyrophosphate (pH 7.4) and rapidly centrifuged at 12 000 rev./min for 10 min. The supernatant (stromal fraction) was taken up immediately in SDS-sample buffer [16] and heated at 90°C for 1 min. The pellet of thylakoids was washed twice with 10 mM Na-pyrophosphate (pH 7.4) and once with 300 mM sucrose–2 mM tricine–KOH (pH 7.8). The final pellet of thylakoids was resuspended in 100 mM tricine–KOH (pH 7.8) at ~2–2.5 mg chl/ml and stored at −20°C in aliquots of 50–100 μl. Thylakoids were taken up in SDS-sample buffer and heated for 30 s at 90°C just prior to electrophoresis.

SDS–polyacrylamide gel electrophoresis was according to [16] on slab-gels 1–2 mm thick. The running gel (20 cm) consisted of a gradient of 12.5–15% (fig.1) or of 10–15% (fig.2,4) polyacrylamide. The stacking gel was 6% polyacrylamide.

For limited proteolysis, polypeptide bands were cut out from stained gels (period of staining and destaining ≤3 h), equilibrated with distilled water then with upper gel buffer [16] and placed vertically on a second SDS–polyacrylamide gel (1 mm thick)

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which consisted of a 6% stacking gel (1.5 cm) and a 15% running gel (9–10 cm). Gels were overlaid with about 25 μg of chymotrypsin or papain (Boehringer) and run at a constant 5–8 mA such that it took between 4–5 h for the dye front to cross the stacking gel. Once the dye front reached the running gel the current was increased to 15 mA. Radioactivity on gels was detected by fluorography according to [17] following the modifications of [18].

Total RNA was isolated from purified chloroplasts according to [19] or [20].

The rabbit reticulocyte lysate was from Amersham and used for translation experiments as indicated by the supplier. Translation was directed with 1.7 μg total chloroplast RNA.

Hybrid arrested translation was according to [11]. Total chloroplast RNA (5 μg) were hybridized with 2 μg DNA (*Eco*RI · N) in a 25 μl reaction volume containing 80% deionized formamide, 10 mM PIPES (pH 6.4), and 400 mM NaCl at 48°C, 2 h. The reaction was stopped by adding 200 μl ice cold water. One half of the sample was heated at 100°C, for 60 s and rapidly cooled to release the RNA. RNA was precipitated with ethanol at −70°C, the precipitates were dried and used for the reticulocyte lysate translation system.

The hybrid-selected translation experiments were according to [12]. Total chloroplast RNA (30 μg) were hybridized with 2 μg chloroplast DNA (*Eco*RI · N) fixed on nitrocellulose filters [21]. Hybridization was at 50°C, 2 h, in 65% deionized formamide, 10 mM PIPES (pH 6.4), 400 mM NaCl. After hybridization the filters were washed 10 times in NaCl/Cit (0.15 M NaCl and 0.015 M sodium citrate), 0.5% SDS and 3 times in 2 mM Na_2-EDTA (pH 7.9). The RNA was released in 1 mM Na_2-EDTA (pH 7.9) by heating the filters at 100°C, 60 s and rapid cooling. The RNA was ethanol precipitated, dried and used for the translation system.

3. Results and discussion

Highly purified intact chloroplasts from photoheterotrophically grown cells were incubated for 40 min in the light in the presence of [^{35}S]methionine. The thylakoid and stroma proteins were electrophoresed separately under denaturing conditions in polyacrylamide gels and autoradiographed (fig.1). Stained profiles (a,d) are aligned with the respective autoradiographs (b,c). From profile b it is evident that numerous thylakoid proteins are synthesized within the chloroplasts as expected from previous results with *E. gracilis* [9,22] and higher plants (cf. review [23]). The profile of labelled stroma proteins (lane c) is less complex though it should be stressed that polypeptides of chloroplast genetic origin may go undetected due to small turnover rates or to a lack of methionine in the molecule. Most conspicuous in lane c are a broad band in the range of 55 000–59 000 M_r, which contains a sharp band of 56 000 M_r, and two other strong bands of ~53 000 M_r and 46 000 M_r. Each of these strongly labelled bands has a corresponding band in the stained profile (lane d),

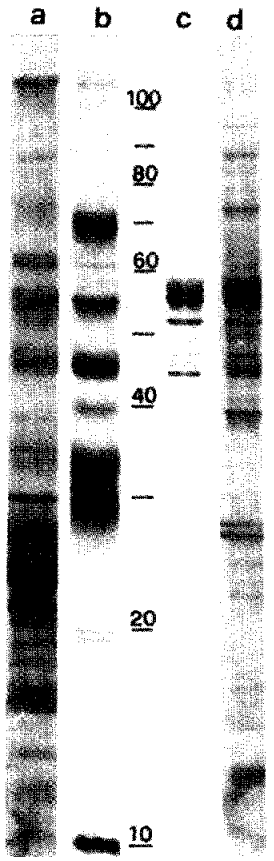

Fig.1. Electrophoretic separation of thylakoid (a,b) and stroma (c,d) polypeptides and autoradiographic detection of major polypeptides synthesized by isolated *Euglena gracilis* chloroplasts in the light (b,c). Films were exposed for 7 days at room temperature. M_r-Values are $\times 10^{-3}$. Electrophoresis conditions: stacking, 10 mA; running, 20 mA, room temperature.

but the relative staining intensities do not parallel the intensities of the radioactive signal, suggesting that the turnover rates may vary considerably for these polypeptides. We may conclude from these results that the de novo synthesized polypeptides are correctly synthesized and are not artefacts of random proteosynthetic processes.

Total RNA isolated from such chloroplasts was used to direct protein synthesis in a commercial rabbit reticulocyte lysate. In fig.2 we compare the proteosynthetic capacity of the reticulocyte lysate with that of isolated chloroplasts. Comparing lane a (no RNA) with b (added RNA) we see that total chloroplast RNA directs the synthesis of a number of discrete polypeptides. We observe in particular 3 strongly labelled polypeptides of $M_r \sim 46\,000$, ~53 000 and ~61 000. Two of them seem to have the same electrophoretic mobility as two polypeptides synthesized by the isolated chloroplast (in organello) (lane c), i.e., the 46 000 and 53 000 M_r polypeptides of the stroma. The 61 000 M_r polypeptide seen in lane b is not present in lane c (stroma polypeptides) meaning that it either is a membrane polypeptide or a precursor form of a stroma polypeptide. We could argue, e.g., that it is a precursor of the strongly labelled 59 000 M_r polypeptide observed in lane c, which comigrates with the large subunit of the purified *Euglena gracilis* RUBPase (not shown). *Euglena* RUBPase large subunit is a 59 000 M_r polypeptide [24], which is synthesized in the chloroplast [22] and was shown to be synthesized in a chloroplast RNA directed wheat germ system [20]. A precursor form of the spinach RUBPase large subunit polypeptide was described in [25].

To test the possible identity of the 46 000 and 53 000 M_r polypeptides synthesized in vitro (reticulocyte) and in organello, we compared the proteolytic fragments generated following digestion with papain and chymotrypsin (fig.3). The excellent coincidence of the numerous spots in both cases (lanes A,B, r *vs* o)

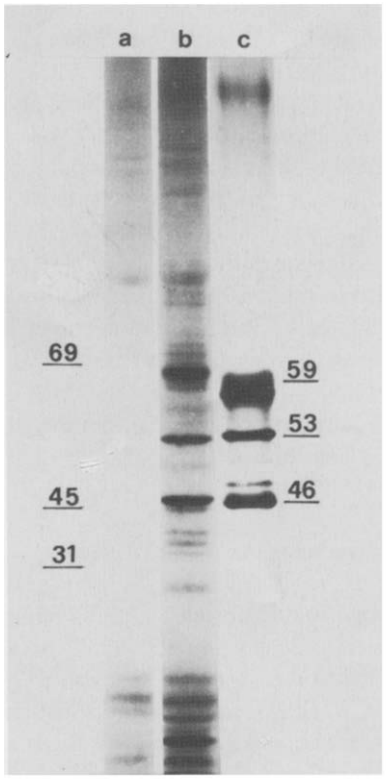

Fig.2. Fluorograph of electrophoretically separated polypeptides synthesized in a rabbit reticulocyte lysate without (a) and with chloroplast RNA (b) and of stroma polypeptides synthesized by isolated chloroplasts in light (c). Films were exposed for ~10 days at −70°C. M_r-Values are $\times 10^{-3}$. Size standards (Boehringer): bovine serum albumin, 69 000; ovalbumin, 45 000; DNase I, 31 000.

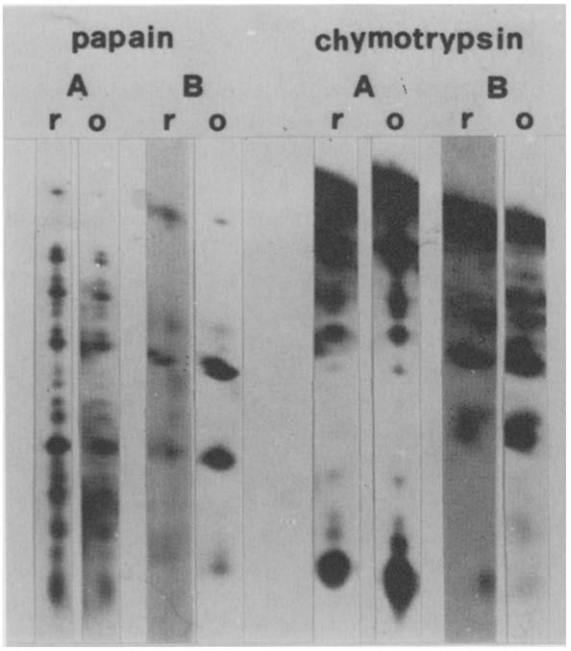

Fig.3. Fluorograph of electrophoretically separated proteolytic fragments obtained following digestion of the 53 000 M_r (A) and of the 46 000 M_r (B) polypeptides: (r) synthesized by the rabbit reticulocyte lysate directed with total chloroplast RNA; (o) synthesized in intact illuminated chloroplasts. Film exposure was for 4–6 weeks at −70°C.

is indicative of identical polypeptides. These results strongly suggest that the reticulocyte lysate correctly translates *Euglena* chloroplast mRNAs recognizing the same reading frame as the chloroplast translation system does.

We asked now the question whether any of the cloned chloroplast DNA fragments specifically interacts with the synthesis of any of the 3 major polypeptides shown in fig.2. To this end we made hybrid-arrested and hybrid-selected translation experiments using several DNA fragments as probes and in particular the mapped fragments *Eco*RI · I and N (for fragment nomenclature, size and mapping, see [26]). An unequivocal result was obtained with *Eco*RI · N and the 53 000 M_r protein (fig.4). We observed that

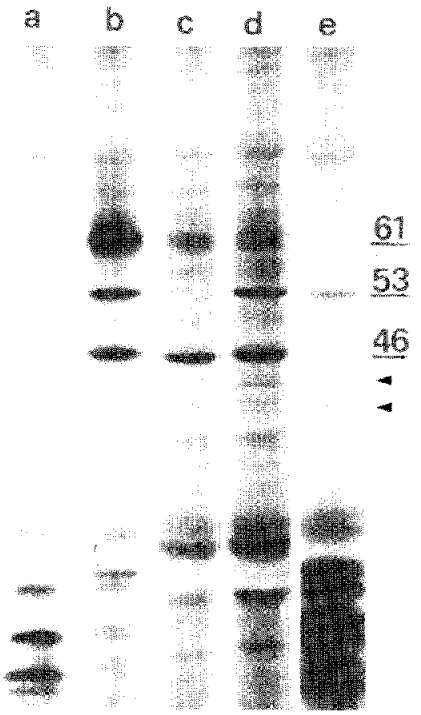

Fig.4. Fluorograph of electrophoreticaly separated polypeptides synthesized in a rabbit reticulocyte lysate: (a) no chloroplast RNA; (b) with total chloroplast RNA; (c) with chloroplast RNA hybridized in liquid with *Eco*RI · N (hybrid-arrested translation; (d) with chloroplast RNA hybridized in liquid with *Eco*RI · N and melted (hybrid-released translation); (e) chloroplast RNA hybridized to and released from *Eco*RI · N fragments fixed on nitrocellulose filter (hybrid-selected translation). Films were exposed for 5 days at −70° C. M_r-Values are $\times 10^{-3}$. Arrows point towards minor bands (see text).

*Eco*RI · N suppresses the synthesis of the 53 000 M_r polypeptide under hybrid arrested conditions (lane c). The corresponding band is present however under hybrid released (lane d) and hybrid selected (lane c) conditions. The specificity of the DNA–RNA interaction is demonstrated by the fact that, e.g., the 46 000 M_r band is present in lanes c and d, but it is absent in lane e. The same holds true for the 61 000 M_r polypeptide, whose synthesis seems not to interfere with *Eco*RI · N and it is consequently absent from lane e, but present, though proportionately less in lanes c and d. Two other minor bands (arrows) can be observed under hybrid selected conditions (lane e) which disappear (hybrid arrested, lane c) and appear (hybrid released, lane d) in parallel with the 53 000 M_r polypeptide. This could mean that *Eco*RI · N interacts with more than one kind of mRNA, something which seems possible in view of its length (2900 base pairs), or that the smaller polypeptides are shortened versions of the 53 000 M_r polypeptide (e.g., premature termination). As controls we include the fluorographs of the reticulocyte system alone (lane a) and with added total chloroplast RNA (lane b). As a preliminary observation we may add at this point that the fragment *Eco*RI · I interacts with the in vitro synthesis of two polypeptides of 46 000 and 33 000 M_r.

We consider the outlined experimental protocol a feasible one to retrieve mRNA and map structural genes on the chloroplast genome. We show that the fragment *Eco*RI · N specifically interacts with mRNA coding for a 53 000 M_r polypeptide which is found under our experimental conditions in the stroma fraction of *Euglena* chloroplasts.

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