Volume 184, number 1 FEBS 2519 May 1985

The chloroplast 32 kDa protein is synthesized on thylakoid-bound ribosomes in *Chlamydomonas reinhardtii*

David Herrin and Allan Michaels*

Biology Department, University of South Florida, Tampa, FL 33620, USA

Received 13 March 1985

A cloned cpDNA fragment containing a portion of the gene for the 32–36 kDa thylakoid protein of *Chlamydomonas* (polypeptide D-1) was isolated. Hybridization probing of RNA from soluble and membrane fractions of *Chlamydomonas* showed that the mRNA for D-1 is bound to thylakoid membranes. Run-off translation of thylakoid-bound polysomes (rough thylakoids) with [^{35}S]-methionine yields polypeptide D-1 as the major product. Peptide mapping with *S. aureus* V-8 protease of D-1 synthesized (1) in vivo, (2) in vitro by rough thylakoids and (3) in the reticulocyte lysate directed by non-polyadenylated RNA showed that D-1 is synthesized as a precursor in the reticulocyte lysate but as the mature polypeptide by rough thylakoids.

Chlamydomonas *psbA gene* *DNA-RNA hybridization* *Rough thylakoid* *In vitro protein synthesis* *32 kDa precursor*

1. INTRODUCTION

A major product of chloroplast protein synthesis is a thylakoid membrane polypeptide of 32–36 kDa which is believed to be the secondary electron acceptor for PS II, QB. This polypeptide appears to form an essential part of the quinone binding site and is recognized by herbicides such as diuron [1,2]. It was termed polypeptide D-1 in *Chlamydomonas* by Chua and Gillham [3] and a similar polypeptide D-2, which referred to diffusely labeled polypeptides 1 and 2. The gene for polypeptide D-1, psbA, has been isolated and sequenced from several photosynthetic organisms and its deduced amino acid sequence is highly conserved [2].

A precursor, 1.5–2 kDa larger than the mature polypeptide, has been observed in higher plants [4,5]. Proteolytic processing results in removal of amino acids from the carboxyl terminus after the precursor is synthesized and inserted into the thylakoid membrane [5,6]. So far, synthesis and processing of D-1 has been studied in vivo and in organello. Here we show that the mRNA for polypeptide D-1 is associated with thylakoid membranes and that this polypeptide is the major ^{35}S-labeled product of in vitro protein synthesis by rough thylakoids (thylakoids with bound polysomes). Evidence is also presented for a precursor to D-1 in *Chlamydomonas* that is processed by rough thylakoids.

2. MATERIALS AND METHODS

2.1. *Subcellular fractionation and protein synthesis by rough thylakoids*

Synchronous and asynchronous cultures of wild-type *Chlamydomonas reinhardtii* 137c+ were used in all experiments [7]. Subcellular fractionation and rough thylakoid membrane isolation from asynchronous cells were carried out at 0–4°C as follows. Cells, harvested by centrifugation and resuspended in 25 mM Hepes-KOH (pH 7.8), 25 mM $MgCl_2$, 25 mM KCl, 1 mM DTT contain-

*To whom correspondence should be addressed

Abbreviation: cpDNA, chloroplast DNA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30 © 1985 Federation of European Biochemical Societies

ing 0.25 M sucrose, 100 μg/ml chloramphenicol and 1.5 mM hydroxystilbamidine isethionate as a ribonuclease inhibitor [8], were broken with the French press (4000 psi), homogenized further with a hand-held Potter-Elvejheim homogenizer and centrifuged at 30000 × *g* for 15 min. The supernatant (S-30), which contains the chloroplast stroma, was quick-frozen and stored at −70°C.

Thylakoid membranes were isolated from the pellet (P-30) by floating the membranes to the interface between 1.5 and 1.25 M sucrose layers by ultracentrifugation (26000 rpm) for at least 3 h in an SW-27 rotor (Beckman). Rough thylakoid membranes used for in vitro protein synthesis were isolated from synchronous and asynchronous cells by a slightly different protocol as described in [7]. The yield of membranes and the amount of membrane-bound RNA (0.25 mg RNA/mg chlorophyll) were similar to the two procedures but the latter procedure allowed subsequent run-off in vitro of the membrane-bound polysomes. Preparation of the *E. coli* S-100 and conditions for translation of thylakoid-bound polysomes were described in [7].

2.2. *Cloning of cpDNA and nucleic acid hybridizations*

Chloroplast DNA (cpDNA) was isolated from the CW-15 strain of *Chlamydomonas* as described by Grant et al. [9]. The DNA was cloned into the *Eco*RI site of phosphatase-treated pBR325 using standard techniques and *E. coli* HB101 as the host [10]. Plasmid DNA was isolated from small (1 ml) and large (500 ml) cultures of *E. coli* using the procedure of Holmes and Quigley [11]. *Eco*RI-restricted cpDNA was electrophoresed and transferred to nitrocellulose as described by Southern [12] after fragmentation of the DNA in situ [10]. The transfer buffer was 10 × SSPE [10].

The extraction and manipulation of RNA by oligo(dt)cellulose chromatography, electrophoresis and blotting onto nitrocellulose (northern blot), and cell-free translation were performed as described [13]. For dot-blot hybridization the RNA fractions were treated with DNase I (20 μg/ml) for 30 min and phenol-extracted. The RNA was denatured with 50% formamide, 6% formaldehyde, SSPE at 50°C, brought to 3.0 M NaCl, 0.1 M NaH_2PO_4, 10 mM EDTA (pH 7.4) and applied to nitrocellulose using a dot-blot manifold (BRL). Hybridization to Northern, Southern, and dot-blots was performed as described [13]. The DNA probes were radioactively labeled by nick-translation using $dCT^{32}P$ (ICN) and a nick-translation kit (BRL). Unincorporated nucleotides were removed by spun-column chromatography on Sephadex G-50 [10].

Hybridization selection and translation was performed as described by Miller et al. [14] in a 50% formamide buffer; 200 μg total cellular RNA were hybridized for 4–6 h at 41°C to 10–20 μg plasmid DNA immobilized on nitrocellulose. After washing, the hybridized RNA was eluted in H_2O and translated in a reticulocyte lysate using $[^{35}S]$methionine (New England Nuclear).

2.3. *SDS-PAGE and peptide mapping*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [15] on 14 or 15% acrylamide gels. Samples were prepared for electrophoresis by incubation at 100°C (1–3 min) in Laemmli's buffer, containing 50 mM DTT. Digestion with *S. aureus* V-8 protease was carried out in situ during re-electrophoresis on a 15% acrylamide gel [16]. Sodium salicylate was used for fluorography [17], after the gels were stained with Coomassie blue. Standard proteins of known molecular mass were used to calibrate the gels.

3. RESULTS

3.1. *Isolation of a cloned DNA probe for D-1*

Evidence has been presented that the *Chlamydomonas* gene for polypeptide D-1, psbA, spans the *Eco*RI site between fragments *Eco*14 and *Eco*24 [18,19]. Four of the five exons of this gene are located on fragment *Eco*14. To obtain a specific probe for D-1 mRNA, cpDNA was cloned into the *E. coli* plasmid pBR325. Clones containing this fragment were initially identified by the size of the cpDNA insert in pBR325. However, since fragments *Eco*14 and *Eco*15 co-migrate, clones containing 14 were further distinguished by the absence of *Bam*HI restriction sites in *Eco*14 and their presence in *Eco*15 [20]. Final confirmation that we had cloned fragment *Eco*14 was obtained by Southern blot hybridization of one of the cloned plasmids, pEC23, to *Eco*RI-restricted cpDNA (fig.1A). The major hybridization signal

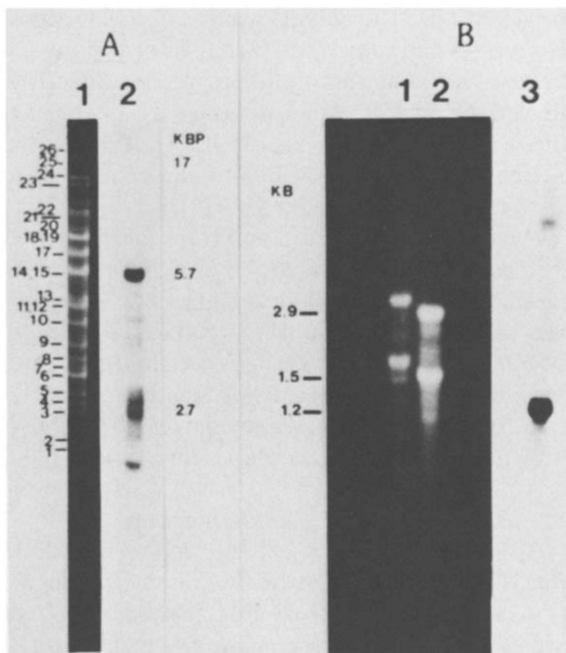

Fig.1. Characterization of recombinant plasmid pEC23 by Southern and Northern blot hybridization. ^{32}P-labeled pEC23 was hybridized to a Southern blot of *Eco*RI-restricted cpDNA (A) and to a Northern blot of total cellular RNA (B). In (A), lane 1 shows the agarose gel of *Eco*RI-restricted cpDNA and lane 2, the autoradiograph after hybridization to pEC23. The restriction fragment nomenclature of Rochaix [18] was used. In (B), lane 1 shows the agarose gel of total *Chlamydomonas* RNA and lane 3, the autoradiograph after hybridization to pEC23. Lane 2 contained *E. coli* rRNA as molecular size markers. KB, kilobases; KBP, kilobase pairs.

co-migrates with fragments 14 and 15. Faint hybridization signals were also observed with fragments 3, 4, 9 and 10. Cross-hybridization of cpDNA fragments was also noted by Rochaix [20].

The mature transcript of the psbA gene is consistently observed at 1.2–1.25 kb in different species of plants and *Chlamydomonas* [18,21]. Hybridization of pEC23 to a Northern blot of total cellular RNA gave a major complementary species of approx. 1.2 kb (fig.1B). Faint hybridization signals of higher M_r are seen in overexposed blots (not shown), but the 1.2-kb species accounts for more than 95% of the hybridizing radioactivity. Furthermore, hybridization selection of total RNA with pEC23 and subsequent translation of the

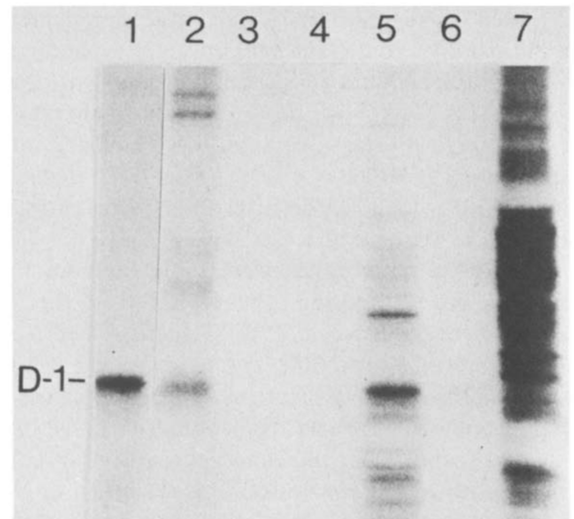

Fig.2. Hybridization-selected translation with plasmid pEC23. Lane 3 shows the endogenous translation products of the reticulocyte lysate; lane 4, the products of calf liver tRNA; lane 5, the hybridization-selected translation products of pEC23 from total RNA; lane 6, the translation products hybrid-selected with pBR325; lane 7, the translation product of control yeast mRNA. Lane 1 contained purified thylakoid membranes labeled in vivo with $^{35}SO_4$ and lane 2, total cellular membranes labeled in the presence of cycloheximide, as markers for polypeptide D-1.

selected RNA gave a prominent translation product of 36 kDa that co-migrated with polypeptide D-1 (fig.2). The results from these experiments are consistent with those of Rochaix's group [18,19], who used hybridization-arrested translation and cross-hybridization with a heterologous spinach probe to localize the psbA gene to *Eco*14. Therefore, we conclude that the gene for polypeptide D-1 is located on *Eco*14 and that we had obtained a specific probe for D-1 mRNA.

3.2. *Subcellular distribution of D-1 and D-2 mRNA*

Having obtained a portion of the *psbA* gene, the distribution of D-1 mRNA in soluble (S-30), total membrane (P-30) and thylakoid membrane (Thyl) fractions of *Chlamydomonas* cells, with and without chloramphenicol pretreatment, was determined by dot-blot hybridization. Pretreatment with chloramphenicol has been shown to increase the amount of ribosomes recovered with thylakoid

the S-30, P-30 and Thyl protein fractions showed no evidence of extensive cross-contamination, with unique polypeptides appearing in each fraction (fig.3A). Strong hybridization was obtained with RNA from the membrane fractions, particularly thylakoids (fig.3B). The subcellular distribution of D-1 mRNA in cells with and without chloramphenicol pretreatment is similar, and shows that the antibiotic did not cause artifactual association of the mRNA with thylakoids (fig.3B). Much stronger hybridization signals were obtained with thylakoid RNA fractions isolated from synchronous cells during the light period, compared to thylakoid RNA from asynchronous cells.

A similar analysis of mRNA distribution using a cloned gene for polypeptide D-2 (psbD) obtained from Dr Rochaix gave essentially the same results (fig.3B), although the *psbD* gene is not as highly expressed as the *psbA* gene. D-2 is a thylakoid polypeptide that has many properties in common with D-1. We have previously shown that D-2 is synthesized by rough thylakoids isolated from synchronous cells during the light period [7].

3.3. *In vitro synthesis of D-1 by rough thylakoids and in the reticulocyte lysate*

To determine whether the thylakoid-associated D-1 mRNA was actively being translated, rough thylakoids were incubated with an *E. coli* S-100 under conditions that allow protein synthesis [7]. Using [^{35}S]methionine as radioactive label, the major product of rough thylakoids co-migrates with

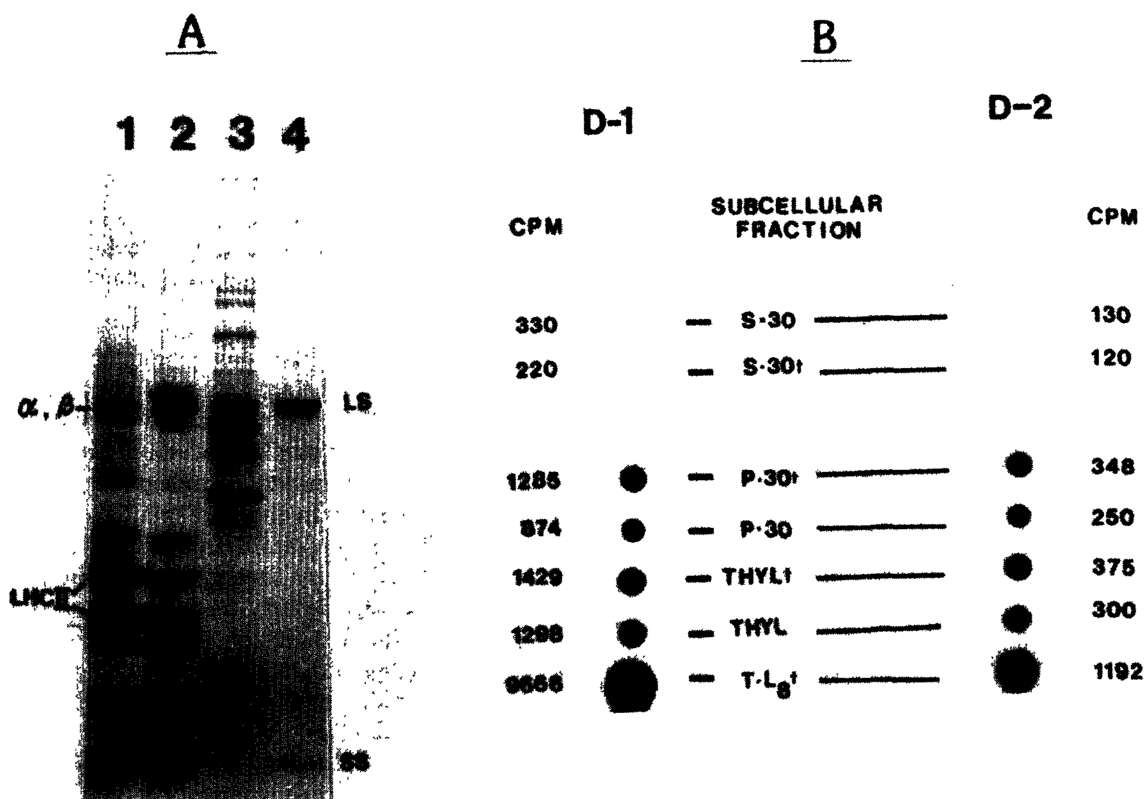

Fig.3. Distribution of D-1 and D-2 mRNA among soluble and membrane fractions as determined by dot-blot hybridization. S-30, P-30 and thylakoid membrane (Thyl) fractions from asynchronous cells, and thylakoid membranes from synchronous cells at 8 h into the light period (T-L8), were prepared as described in section 2. (+) indicates that the cells were pretreated with 100 μg/ml chloramphenicol for 10 min. In (A), the fractions were analyzed by SDS-PAGE: lane 1, Thyl; lane 2, P-30; lane 3, S-30; lane 4, purified ribulose-1,5-bisphosphate carboxylase from *Chlamydomonas*. (B) shows the dot-blot hybridization analysis of 10 μg RNA from each subcellular fraction probed with ^{32}P-labeled pEC23 (D-1) and pCP55 (D-2).

membranes and has been used when isolating rough thylakoids [7,22]. SDS-PAGE analysis of polypeptide D-1 synthesized in vivo (fig.4). The identity of the in vitro product was confirmed by peptide mapping with *S. aureus* V-8 protease (fig.5, lanes 9–15). No substantial differences were noted between the peptide maps of D-1 synthesized in vivo, and by rough thylakoids.

When non-polyadenylated RNA from *Chlamydomonas* 137c is translated in a reticulocyte lysate in the presence of [^{35}S]methionine, the major product migrates on SDS gels of about 36 kDa, similar to D-1 [13]. Peptide mapping of the putative D-1 synthesized in the reticulocyte lysate, compared to D-1 synthesized in vivo showed the polypeptides to be similar but not identical (fig.5, lanes 1–8). An additional peptide of slightly higher molecular mass than the two smaller ones found with the in vivo synthesized polypeptide was consistently observed. At the higher protease concentrations this peptide may be converted to the two peptides of lower molecular mass observed in the in vivo and rough thylakoid product (fig.5). These results are similar to those reported for the precursor to the 32-kDa protein from Spirodela and maize [4,5]. The size difference between the precursor and mature polypeptide, however, does not appear to be as great as in the higher plants and we cannot consistently separate the two forms by one-dimensional SDS-PAGE. The absence of the additional peptide after V-8 digestion of D-1 synthesized by rough thylakoids suggests that correct processing of the precursor has occurred in this system.

Fig.4. ^{35}S-labeled products of protein synthesis by rough thylakoids. Rough thylakoids, isolated from asynchronous cells, were supplemented with an *E. coli* S-100 and incubated for protein synthesis in the presence of [^{35}S]methionine (lane 3). Thylakoid membranes from cells labeled in vivo with $^{35}SO_4$ in the absence (lane 1) and presence (lane 2) of 10 μg/ml cycloheximide were included as a marker for D-1. The gap in lane 3 (indicated by arrow) results from the gel being cut before it was fluorographed.

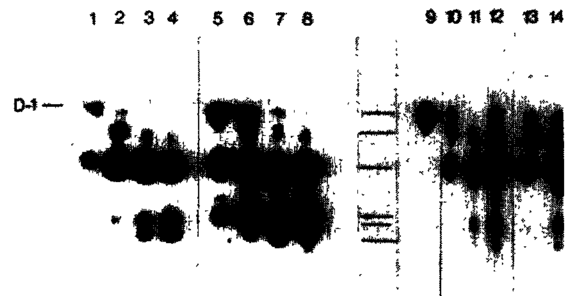

Fig.5. Peptide mapping of D-1 synthesized in vivo and in vitro. Lanes 1–4, 13 and 14 contained D-1 synthesized in vivo in the presence of cycloheximide and $^{35}SO_4$. Lanes 5–8 contained the 36-kDa polypeptide synthesized in the reticulocyte lysate and lanes 9–12 received the 36 kDa translation product of rough thylakoids. [^{35}S]Methionine was used for in vitro translations. Lane 1, 5 and 9 received no protease; 0.1 μg V-8 protease was added to lanes 2, 6, 10 and 13; 0.5 μg to lanes 3 and 7; 0.7 μg to lane 12 and 1 μg to lanes 4, 8, 11 and 14. Undigested D-1 migrated to the position indicated to the left. Common peptides are indicated by the lines between lanes 8 and 9, and an arrow designates the peptide unique to the polypeptide synthesized in the reticulocyte system. The peptides occurring in lanes 1 and 5 result from cross-diffusion of the protease in the adjacent lanes during electrophoresis.

4. DISCUSSION

The results presented here demonstrate that a major product of chloroplast protein synthesis in *Chlamydomonas*, thylakoid polypeptide D-1, is synthesized primarily on polysomes bound to thylakoid membranes. These results differ from those of Leu et al. [23], who find D-1 mRNA in the soluble and membrane fractions of chloroplasts isolated from the CW-15 mutant of *Chlamydomonas*.

These results also show that polypeptide D-1 from *Chlamydomonas* is synthesized in precursor form, similar to higher plants, and appears to be processed by a thylakoid-bound enzyme. Evidence for a precursor to D-1 in *Chlamydomonas* has also been obtained by Jensen and Schmidt [24].

The synthesis of polypeptide D-1 on the thylakoid membrane may be necessary for the protein to attain the correct orientation in the membrane and to prevent synthesis of this hydrophobic polypeptide in the aqueous stroma. Cohen et al. [25] have recently shown that the translation start site for this polypeptide from higher plants is an internal methionine, at position 37. The initiating methionine is followed by a highly hydrophobic stretch of about 21 amino acids at the amino terminus [21]. We propose that this polypeptide is cotranslationally inserted into thylakoid membranes via this sequence.

ACKNOWLEDGEMENTS

We would like to thank Dr Jean-David Rochaix for plasmid pCP55 containing the D-2 gene. We would also like to thank Chuck Helling and Anna-Lisa Paul for technical assistance. This research was supported by NSF grant PCM-822647 to A.M.

REFERENCES

[1] Ohad, I., Kyle, D.J. and Arntzen, C.J. (1984) J. Cell Biol. 99, 481–485.
[2] Edelman, M., Goloubinoff, P., Marder, J.B., Fromm, H., Devic, M., Fluhr, R. and Mattoo, A.K. (1984) in: Molecular Form and Function of the Plant Genome (Van Vloting-Doting, L., Groot, G.S.P. and Hall, T.C. eds) pp.1–10, Plenum, New York.
[3] Chua, N.H. and Gillham, N. (1977) J. Cell Biol. 74, 441–452.
[4] Grebanier, A.E., Coen, D.M., Rich, A. and Bogorad, L. (1978) J. Cell Biol. 78, 734–746.
[5] Reisfeld, A., Mattoo, A.K. and Edelman, M. (1982) Eur. J. Biochem. 124, 125–129.
[6] Marder, J.B., Goloubinoff, P. and Edelman, M. (1984) J. Biol. Chem. 259, 3900–3908.
[7] Herrin, D., Michaels, A. and Hickey, E. (1981) Biochim. Biophys. Acta 655, 136–145.
[8] Lizardi, P.M. (1980) J. Cell Biol. 87, 292–296.
[9] Grant, D.M., Gillham, N. and Boynton, J.E. (1980) Proc. Natl. Acad. Sci. USA 77, 6067–6071.
[10] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, New York.
[11] Holmes, D.S. and Quigley, M. (1981) Anal. Biochem. 114, 193–197.
[12] Southern, E.M. (1975) J. Mol. Biol. 98, 503–517.
[13] Herrin, D. and Michaels, A. (1984) Plant Mol. Biol. Rep. 2, 24–28.
[14] Miller, J.S., Paterson, B.M., Ricciardi, R.P., Cohen, L. and Roberts, B.E. (1983) Methods Enzymol. 101, 650–675.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[17] Chamberlain, J.P. (1979) Anal. Biochem. 98, 132–135.
[18] Rochaix, J.D. (1981) Experientia 37, 323–332.
[19] Erickson, J.M., Rahire, M., Bennoun, P., Delepelaire, P., Diner, B. and Rochaix, J.D. (1984) Proc. Natl. Acad. Sci. USA 81, 3617–3621.
[20] Rochaix, J.D. (1978) J. Mol. Biol. 126, 597–617.
[21] Zurawski, G., Bohnert, H.J., Whitfield, P.R. and Bottomley, W. (1982) Proc. Natl. Acad. Sci. USA 79, 7699–7703.
[22] Margulies, M.M. and Michaels, A. (1974) J. Cell Biol. 60, 65–77.
[23] Leu, S., Bolli, R., Mendiola-Morgenthaler, L. and Boschetti, A. (1984) Planta 160, 204–211.
[24] Jensen, K.H. and Schmidt, G.W. (1984) Plant Physiol. 75 (suppl.), 173a.
[25] Cohen, B.N., Coleman, T.A., Schmitt, J.J. and Weissbach, H. (1984) Nucleic Acids Res. 12, 6221–6230.