IN VITRO TRANSCRIPTION AND TRANSLATION OF THE *psbD* GENE ENCODING THE D-2 PROTEIN OF PHOTOSYSTEM II IN BARLEY

by

EVA M. NEUMANN

Department of Physiology, Carlsberg Laboratory
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

Present address: Department of Biology, Texas A & M University,
College Station, Texas 77843-3258, USA

Keywords: Chloroplast DNA, SP6 polymerase, gemini-vector, quinone binding

The plastoquinone-binding polypeptide D-2 of the reaction center complex of photosystem II in barley has been expressed in vitro using the expression vector pGem3TM containing the *psbD* gene of chloroplast DNA as template. The full length D-2 polypeptide with an apparent mol. weight of 32 kD is a major product when mRNA is transcribed with SP6 RNA polymerase from an insert containing the *psbD* gene with a 36 bp 5' leader sequence and a 3' tail of 99 bp downstream of the stop codon. Translation of the mRNA had to be done in a rabbit reticulocyte lysate. Translation also occurred from the internal AUG codons giving rise to truncated D-2 polypeptides with sizes of 30, 25 and 17 kD. They are immunoprecipitable with antisera either raised against amino acid residues 235 to 241 or against 345 residues of the D-2 polypeptide. A truncated translation product of 12 kD probably initiated at codon 247 is not recognized by either antiserum. An additional immunoprecipitable protein with an apparent molecular weight of 46 kD is observed by SDS-PAGE and is interpreted as a homomeric aggregate of D-2 polypeptides. Upon addition of methanol solubilized 2,6 dichloro-p-benzoquinone or other quinones a preferential translation of the full-length and truncated D-2 polypeptides is observed. The use of in vitro synthesized D-2 polypeptides for studies of binding quinones, other electron carriers and chlorophyll chromophores is discussed.

1. INTRODUCTION

The smallest putative photosystem II reaction center complex isolated by NANBA and SATOH (19) from higher plants contains the polypeptides D-1 (32 kD), D-2 (30 kD) and the 9.2 and 4.3 kD subunits of cytochrome *b-559* (14, 19). In the complex are bound five molecules of chlorophyll *a*, two molecules pheophytin *a*, one molecule of β -carotene and two cytochrome

b-559 heme molecules. The complex exhibits reversible absorbance changes indicative of accumulation of reduced pheophytin (19).

Analyses of the crystallized photosynthetic reaction center of purple bacteria (5, 16, 17, 18) have identified in the L and M subunits the amino acid residues that serve 1) as ligands to the reaction center special pair chlorophyll, 2) as ligands for the non-heme iron and 3) the

Abbreviations: bp = basepairs; cp = chloroplast; kD = kilodalton; SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; Tris = tris-(hydroxymethyl)-aminomethane.

residues forming binding sites to the primary and secondary electron acceptors, the plastoquinones Q_A and Q_B . Plastoquinone Q_A binds to two helices in the subunit M while Q_B is found extended between two helices of subunit L. The primary structure of the D-1 and D-2 polypeptides deduced from the corresponding genes *psbA* (13, 21, 27) and *psbD* (1, 9, 21, 26, 27) in chloroplast DNA has revealed pronounced similarities of the location of certain amino acids and of the distribution of hydrophobic domains to those of the L and M polypeptides (18). It has therefore been suggested that in the reaction center complex of photosystem 2 in higher plants D-1 corresponds functionally to the L subunit and D-2 to the M subunit of the purple bacteria (17, 18, 29, 30) and that they form a heterodimer in the thylakoid membrane.

While experimental evidence is sought for the binding of the special pair reaction center chlorophyll, the pheophytin chromophore, the non-heme iron and the plastoquinones to the D-1 and D-2 polypeptides by studies with particles isolated from thylakoids, one might alternatively approach these questions by starting with the genes for D-1 and D-2, express them in vitro and try to bind the chromophores and electron carriers to the synthesized polypeptides. Towards this end I have cloned the *psbD* and part of the *psbA* gene from barley and determined their nucleotide sequence (21). In the present paper I present the construction of expression plasmids for the *psbD* gene and an analysis of the products obtained by in vitro transcription and translation from them. Due to the presence of nine methionine residues in D-2 of barley several translation products are obtained from *psbD*. The full-length D-2 polypeptide is, however, the major translation product from mRNA transcribed with SP₆ RNA polymerase using the recombinant plasmid pGem 101-7. Surprisingly, under certain conditions translation of the in vitro transcripts of *psbD* is stimulated by addition of quinones.

2. MATERIALS AND METHODS

2.1. Materials

The Riboprobe Gemini system pGem-3TM, SP₆ and T7 RNA polymerases were purchased

from Promega Biotec, Madison, WI, USA. Most of the restriction enzymes, deoxy- and dideoxy-triphosphate-nucleotides, RNase inhibitor and RNase-free DNase were obtained from Boehringer Mannheim. The restriction endonuclease HgiAI was obtained from New England Biolabs, Beverly, MA, USA. Radioactive nucleotides and ³⁵S-methionine were purchased from New England Nuclear. Rabbit reticulocyte lysate (N.90) was obtained from Amersham. 2,5 dimethyl-p-benzoquinone and 2,6 dichloro-p-benzoquinone were purchased from Eastman Kodak, and phenyl-p-quinone and Coenzyme Q10 were from Sigma.

2.2. Transcription and translation of DNA, cloned into pGem 3

The expression vector pGem 3 has a multiple cloning site, which is framed by the T7 and the SP₆ promoter, thus allowing transcription of any gene inserted into the multiple cloning site in both directions either by use of T7 RNA polymerase or SP₆ RNA polymerase. Transcription was performed according to the manufacturer's recommendation, a modification of previously published methods (15).

2.2.1. Linearization of the plasmid

If not indicated otherwise, CsCl purified plasmid DNA was linearized with HindIII (in case of pGem 101-6, pGem 101-8, transcribed with T7 RNA polymerase), XbaI (in case of pGem 101-7, transcribed with T7 RNA polymerase), SacI (in case of pGem 101-7, pGem 101-9, transcribed with SP₆ RNA polymerase), extracted with phenol:chloroform:isoamylalcohol (25:24:1) and recovered by NaCl-ethanol precipitation. The linearized plasmid was incubated with T4 DNA polymerase in the presence of deoxy-triphosphate-nucleotides to produce blunt DNA ends.

2.2.2. Transcription

2 µg of linearized plasmid DNA were incubated in a transcription mix consisting of 5x transcription buffer (200 mM Tris-HCl pH 7.5; 30 mM MgCl₂; 10 mM spermidine; 50 mM NaCl),

10 µl 0.1 M DTT, 5 U RNase inhibitor, 2.5 mM each of ATP, GTP, UTP, CTP, in a total volume of 50 µl. RNA synthesis was started by adding 10 U of either SP6 RNA polymerase or T7 RNA polymerase. The samples were incubated at 40 °C. To boost RNA synthesis an additional 10 U of enzyme were added after 60 minutes and the RNA polymerase reaction was allowed to continue for another hour.

2.2.3. Removal of DNA template

2 U of DNase were added to the transcription mix. After incubation for 15 min at 37 °C the mixture was extracted with phenol:chloroform:isoamylalcohol (25:24:1) and with chloroform:isoamylalcohol (24:1). RNA was recovered by sodium acetate ethanol precipitation. The dried RNA pellet was solubilized in 50 µl H₂O. Measurements of the optical density at 260 nm revealed an average RNA yield of about 40-50 µg.

2.2.4. Translation in reticulocyte lysates

4 µl of purified RNA were incubated with 18 µl of the following translation mix: 80 µl of rabbit reticulocyte lysate and 20 µl ³⁵S-methionine (final concentration of 2 µM corresponding to about 55 µCi per translation assay). The endogenous methionine pool in the rabbit reticulocyte lysate system is 18 µM. The samples were incubated at 30 °C for 1 h and then kept at -20 °C until use.

2.2.5. Translation in an E. coli S30 lysate

The E. coli lysate was prepared according to ZUBAY (33) as described in PRITCHARD and HOLLAND (25), using the E. coli strain PR7 (3). This strain was a kind gift from J. GRAY, Cambridge.

Translation was performed by incubating 2-5 µl of purified mRNA in 30 µl S30 extract, 25 µl 4x concentrated low molecular weight mix, 5 mM Mg-acetate and 50 µCi ³⁵S-methionine in 50 µl. The 4x concentrated low molecular weight mix adjusted the reaction mixture to 44 mM Tris-acetate, pH 8.2, 55 mM K-acetate, 7.4 mM Ca-acetate, 27 mM NH₄-acetate, 5 mM Mg-ac-

etate, 30 µg/ml folinic acid, 0.1 mg/ml E. coli tRNA, 2.2 mM ATP, 0.55 mM each of GTP, UTP and CTP, 0.25 mM of the 20 amino acids minus methionine, 1.4 mM DTT, 21 mM phosphoenolpyruvate and 20% (w/v) polyethylene-glycol.

2.2.6. Translation in a wheat germ extract

pGem 101-7 was used as template for synthesis of uncapped and capped (by addition of 7-methyl-guanosine triphosphate to the transcription mixture) mRNA according to manufacturer's recommendations (Promega). Translation assays of 2 µl capped or uncapped mRNA were then performed in 25 µl, containing 15 µl nuclease-treated wheat germ extract from Amersham (code RPN.1), 1 µl unlabelled amino acid mixture (minus methionine) from Amersham (code N.133), 30 µCi ³⁵S-methionine and K-acetate concentration between 100 and 200 mM.

Alternatively, translation experiments were performed in a wheat germ extract, kindly provided by SUSAN MADRID (Copenhagen).

2.3. Polyacrylamide gel electrophoresis (PAGE)

TCA was added to 10 µl of each translation assay to a final concentration of 10%. Proteins were allowed to precipitate on ice for 20 min. The protein precipitates were neutralized with 1 M Tris and solubilized in loading buffer (63 mM Tris-HCl pH 6.8; 2.3% SDS; 8% glycerol; 5% β-mercaptoethanol; bromphenol blue). The samples were boiled for 5 min and loaded on a 12.5% polyacrylamide gel. The gels were prepared by overlaying a separation gel (12.5% acrylamide:bisacrylamide 37:1; 750 mM Tris-HCl pH 8.85; 2.5% SDS; 16 µl TEMED and 140 µl 10% Ammoniumpersulfate (APS) in 40 ml) with a stacking gel (5% acrylamide:bisacrylamide 37:1; 125 mM Tris-HCl pH 6.8; 0.1% SDS; 15 µl TEMED; 200 µl 10% APS in 20 ml). Electrophoresis was performed overnight at room temperature using as running buffer: 0.05 M Tris, 0.19 M glycine with 0.1% SDS added to the upper buffer. The apparent molecular weights of synthesized protein products were

Figure 1. Location of the psbD gene and part of the psbC gene on a 2.23 kb EcoRI/BamHI fragment of barley cpDNA as determined by nucleotide sequence analysis (21). The position of relevant recognition sites for restriction enzymes are shown. Thick bars represent the coding region of psbD and psbC, respectively. Arrows indicate the first ATGs in the coding region and the translation termination signal of psbD.

estimated by co-electrophoresis with ^{14}C -methylated molecular weight standards (Amersham).

2.4. Fluorography

Gels were incubated twice for 1 hour in 50% methanol and then for 20 min in 80 g Na-salicylate solubilized in 500 ml 50% methanol and 50 ml glycerol. Fluorograms were obtained by exposure of dried gels to Kodak XAR 5 X-OMAT film at -80°C , using an intensifying plus screen (Kodak).

2.5. Immunoprecipitation

Immunoprecipitation was performed by a modification of the method of HØYER-HANSEN (9): A fraction of the translation assay was mixed with SDS to a final concentration of 2%. After boiling of the samples for 3 minutes they were diluted to a final concentration of 0.2% SDS with dilution buffer (300 mM NaCl; 1% Triton X-100; 50 mM Tris-HCl pH 7.4). Antiserum was added and the samples were incubated overnight at 4°C . Antibody-protein complexes were allowed to bind to protein A-sepharose for 1 hour at room temperature. The samples were pelleted and washed three times with the dilution buffer after adding SDS to a final concentration of 0.1%, followed by three washes with 125 mM Tris-HCl pH 6.8. The dried pellets were solubilized in 80 μl gel loading buffer and boiled for 3 minutes to separate the antibody-protein complex from the column material. Following centrifugation the supernatants were loaded on a polyacrylamide gel.

2.6. Other methods

DNA sequence determination by the dideoxy chain termination method, plasmid DNA preparations, DNA insert preparations, sub-cloning and transformation of cells were performed as described (21).

3. RESULTS

A 2.3 kb EcoRI/BamHI fragment (2, 21) of the barley chloroplast genome contains the entire open reading frame of the psbD gene, consisting of 1062 bp and coding for 353 amino acids. The nucleic acid sequence of the EcoRI/BamHI fragment has been determined and published (21). In addition, 238 bp of the untranslated 5' leader sequence of the psbD gene as well as 985 bp of the 5' end of the psbC gene are located on this fragment. The open reading frame of psbC starts 50 bp upstream from the psbD stop codon. psbC is transcribed in the same direction as the psbD gene but read in a frame shifted by one nucleotide. The organization of the psbD and psbC genes and a map of relevant restriction enzyme sites are presented in Figure 1. To investigate the expression of psbD in an in vitro transcription and translation system, fragments of the EcoRI/BamHI fragment were subcloned into the multiple cloning site of the expression vector pGem 3.

3.1. Construction of expression plasmids for in vitro transcription of the barley psbD gene

a) pGem 101-6

The gene construct pGem 101-6 contained the entire psbD gene sequence including 238 bp

Figure 2. Construction of psbD expression plasmids. First line: Location of the psbD gene on the EcoRI/HindIII fragment of barley cpDNA. Sites for restriction enzymes that were used for construction of the expression plasmids are indicated. Lines 2 to 5 show, aligned to the gene map, the DNA fragments that were inserted into the multiple cloning site of pGem 3 to construct pGem 101-6, 101-7, 101-8 and 101-9. The nucleotide sequences across the junction (indicated by vertical bars) between the inserted part of the psbD region and the multiple cloning site of the vector are given. The structure of the plasmid pGem 3 is shown in the lower part of the figure. Note that pGem 101-7 and pGem 101-9 are drawn in the right orientation with respect to the multiple cloning site, while pGem 101-6 and pGem 101-8 have been inserted in the opposite orientation. More details about construction of the recombinant expression plasmids are given in the text.

of the untranslated 5' leader sequence and 806 bp of the psbC gene. pGem 101-6 was obtained by digesting the EcoRI/BamHI fragment with HindIII followed by ligation of the fragment into the EcoRI/HindIII site of pGem 3 (Fig. 2, pGem 101-6).

b) pGem 101-7
In pGem 101-7 the untranslated 5' leader sequence of psbD was reduced to 36 bp but included the Shine-Dalgarno sequence in position -11 to -6. The 3' sequence downstream from

the TAA stop codon of psbD was limited to 99 bp. This construct was obtained by digesting pGem 101-6 with the restriction enzyme HgiAI, followed by isolation of the 1197 bp fragment. 3'-protruding ends were digested using T4 DNA polymerase and the fragment was ligated into the SmaI site of pGem 3. All recombinant clones revealed the same orientation of the psbD gene with its 5' end near the SP6 promoter (Fig. 2, pGem 101-7).

c) pGem 101-8

pGem 101-8 was constructed to transcribe psbD using T7 DNA polymerase. The insert of pGem 101-7 was cut out by the restriction enzymes XbaI and SacI. Protruding ends of the isolated and purified insert were blunt-ended with T4 DNA polymerase and the DNA was ligated back into the SmaI site of pGem 3. Sequencing of the ends of the insert confirmed the opposite orientation of the insert in pGem 101-8 in comparison to pGem 101-7 (Fig. 2, pGem 101-8).

d) pGem 101-9

pGem 101-9 was constructed to gain information about the translation initiation site of psbD transcripts synthesized in vitro. pGem 101-7 was digested with the restriction enzyme AccI cutting at position 178 in the psbD gene and in the multiple cloning site of the vector, deleting 60 codons of the 5' end of the psbD gene. Protruding ends of the linearized plasmid were filled in with T4 DNA polymerase followed by intramolecular ligation (Fig. 2, pGem 101-9). Thus, translation products of pGem 101-9 transcripts represent a truncated form of D-2, which should exhibit a distinct difference in mobility on PAGE in comparison to full-length psbD-derived translation products as they are expected to be synthesized from pGem 101-7 and pGem 101-8 transcripts.

The orientation of each construct was verified by sequencing the junctions of insert and vector using oligonucleotides complementary to the T7 and the SP6 promoter region as primers for sequencing reactions by the dideoxy chain-termination procedure. Organization of the DNA cloned into the multiple cloning site of pGem 3 as well as the nucleic acid sequence over the borders of vector and insert are shown in Figure 2.

3.2. Transcription of psbD in vitro

The presence of SP6 and T7 promoters in the expression vector pGem 3 provides a method for synthesizing mRNA from genes cloned downstream of one of the bacteriophage derived promoters by use of the appropriate, highly specific RNA polymerases (11, 15). Transcription of the psbD gene constructs in pGem 3 was performed as described in 2.2.2.

3.3. Translation of in vitro-synthesized psbD mRNA in a cell-free E. coli S30 and wheat germ extracts

Since the protein expression machinery in chloroplasts resembles the bacterial one more than the eukaryotic one it was assumed that translation of psbD-derived mRNA should be more efficient in a cell-free E. coli lysate than in an eukaryotic translation system. mRNA, obtained by transcription of pGem 101-7 with SP6 RNA polymerase, was added to an S30 E. coli lysate (12, 25) in the presence of ³⁵S-methionine, but no D-2-related polypeptide could be detected (not shown). This negative result is in contrast to the successful translation of transcripts from psbE and psbF genes encoding the cytochrome *b-559* polypeptides in the same S30 E. coli lysate (12) as used in the present work. Transcripts of the latter genes could not be translated in a rabbit reticulocyte lysate or in a wheat germ extract (12).

In addition, it was tried to translate capped and uncapped psbD mRNAs in two different wheat germ extract preparations. In the commercially available wheat germ system from Amersham the translation of psbD mRNA was investigated in the presence of different K⁺ concentrations (between 100 and 200 mM). The second preparation was made in this laboratory and kindly provided by SUSAN MADRID. In both cases, control experiments with other genes revealed the capability of the extracts to perform in vitro translation of added mRNAs. However, no translation products could be obtained from pGem 101-7 or pGem 101-6 derived mRNAs (not shown).

3.4. Translation of psbD-derived transcripts in a rabbit reticulocyte lysate system

In contrast to the cell free E. coli lysate, the rabbit reticulocyte lysate system translated the psbD transcripts. When pGem 101-7-derived mRNA was used as template, six ³⁵S-methionine containing translation products with apparent molecular weights of 46 kD, 32 kD, 30 kD, 25 kD, 17 kD and 12 kD were synthesized (Fig. 3, "pGem 101-7"). Most prominently labelled were the double band in the 30 kD molecular weight range and the 17 kD and 12 kD polypep-

Figure 3. Fluorogram of proteins obtained by translation of *psbD* mRNA in a rabbit reticulocyte lysate in the presence of ^{35}S -methionine. In vitro transcription, translation and preparation for PAGE were performed as described under 2.3. For analysis of products 5 μl of each translation mix were precipitated with TCA, and half of the solubilized precipitate was loaded on the gel. In the control ("RNA") 4 μl of water was added to the translation mix instead of exogenous mRNA. Lanes 2, 3 and 4 show the protein products obtained by translation of mRNA derived from the expression plasmids pGem 101-7, pGem 101-8 and pGem 101-9, respectively. The sizes of protein standards are indicated.

tide bands. A similar protein pattern appeared when pGem 101-8 (Fig. 3, "pGem 101-8") and pGem 101-6 transcripts (not shown) were translated. Notable differences, using the two latter constructs, were the absence of a 46 kD band and much less incorporation of ^{35}S -methionine into the proteins in the 30 kD range. Upon translation of pGem 101-9-derived mRNA, which lacks 60 amino acid codons of the 5' end of the *psbD* gene, neither the 46 kD nor the 32 kD band appeared (Fig. 3, "pGem 101-9"). It should be noted that in the control assay endogenous mRNA of the rabbit reticulocyte lysate coded for a protein in the 30 kD range.

3.5. Identification of *psbD*-derived translation products by immunoprecipitation with D-2-directed antisera

To identify *psbD* translation products two antisera recognizing D-2 were used in an immunoprecipitation assay. Antiserum "282-t" (kindly provided by the research group of A. TREBST, Ruhr-Universität Bochum) has been raised against the synthetic oligopeptide sequence -AlaPheAsnProThrGlnAla_{COOH}- which constitutes the amino acid sequence from residue 235 to 241 of D-2 (6). Antiserum no. 2 (kindly provided by PETER NIXON, Cambridge) has been raised against a truncated D-2 polypeptide, lacking the first 8 amino acids, fused to β -galactosidase (personal communication and ref. 21). Comparison of the patterns of immunoprecipitated translation products with either of the antisera revealed no differences in antibody specificity (Fig. 4 and Fig. 5). Non-specific immunoprecipitation was absent with both antisera when antisense mRNA transcribed with T7 polymerase from the insert in pGem 101-7 was used in the translation assay (Fig. 4, "pGem 101-7 T7" and Fig. 5, "pGem 101-7 T7"). When translation products of pGem 101-7 were immunoprecipitated the most prominent band was the 32 kD protein, followed by the 46 kD, 30 kD, 17 kD and 25 kD proteins, respectively. Immunoprecipitation of pGem 101-8-derived translation products revealed that the most abundant protein was the 17 kD polypeptide although the 32 kD, 30 kD and 25 kD proteins were also synthesized and

Figure 4. Immunodetection of D-2-related protein translation products with antiserum "282-t" recognizing the sequence -AlaPheAsnProThrGlnAla_{COOH}- in position 235 to 241 of D-2 (7). Transcription, translation and immunoprecipitation were performed as outlined under 2.2 and 2.5. Antisense RNA, derived from pGem 101-7, linearized with Xba I and transcribed with T7 RNA polymerase, was used as a control (lane "pGem 101-7 T7"). Each lane is designated with the expression plasmid and corresponding RNA polymerase used for the experiment. The sizes of protein standards are indicated.

immunoprecipitated. No protein in the range of 30 kD immunoprecipitated with pGem 101-9-derived translation products, confirming that the 30 kD translation product observed with this plasmid construction (Fig. 3, "pGem 101-9") originated from endogenous rabbit reticulocyte mRNA. It demonstrates further that the immunoprecipitable 32 and 30 kD polypeptides

Figure 5. Immunodetection of D-2-related protein translation products with antiserum no. 2, recognizing a truncated D-2 polypeptide lacking the first 8 amino acids. The experiment is analogous to that described in the legend to Figure 4 except that antiserum no. 2 was used.

are made from psbD message translated from the first and second methionine codons, respectively. As these have been removed from the psbD gene during the construction of pGem 101-9 only the observed truncated D-2 polypeptides initiated from methionine codons in positions 127 (25 kD), 199 and 200 (17 kD) were expected to be present. Immunoprecipitation of the proteins of 32, 30 kD and 17 kD occurred also after translation of pGem 101-6-derived mRNA. However, the overall translation effi-

Figure 6. Immunodetection of D-2-related proteins, synthesized in vitro in the presence of quinones. pGem 101-7 derived mRNA were translated in the presence of ^{35}S -methionine in a rabbit reticulocyte lysate. The entire assay was immunoprecipitated with antiserum "282-t", recognizing the amino acid residues 235 to 241 of D-2, as described under 2.5. Translation was performed in the presence of: lane 1, 5% methanol; lane 2, 0.8 μM ubiquinone 50 (Coenzyme Q_{10}); lane 3, 0.8 μM phenyl-p-quinone; lane 4, 0.8 μM 2,5 dimethyl-p-benzoquinone; lane 5, 0.8 μM 2,6 dichloro-p-benzoquinone. (All translation reaction mixtures contained 5% methanol). The sizes of protein standards are indicated.

ciency was highly reduced. Although pGem 101-6 contained, in addition to the psbD gene, the 5' end of psbC, there was no evidence for synthesis of an additional protein originating from psbC. No immunoprecipitation of the 12 kD protein could be detected in either of the assays. This is expected with the "282-t" antiserum, since the initiator methionine for the 12 kD protein is in position 247, i.e. downstream of the recognized oligopeptide (positions 235 to 241).

3.6. In vitro translation of the psbD gene in the presence of quinones

The D-2 protein of PSII is proposed to bind the primary electron acceptor Q_A , a plastoquinone molecule (5, 29, 30, 31). Thus, it is of particular interest to investigate whether the presence of quinones influences translation of psbD-derived transcripts. To address this question the following experimental approach was used: pGem 101-7-derived mRNA was translated in a rabbit reticulocyte lysate in the presence of ^{35}S -methionine and selected quinones were added to the translation assay to a final concentration of 0.8 μM . All quinones were solubilized in 50% methanol, resulting in a final alcohol concentration of 5% in the translation assay. The results of this experiment are shown in the fluorograph of Figure 6. The presence of methanol alone inhibited the translation of mRNA so that even after a week of exposure no

incorporation of ^{35}S -methionine into any protein could be detected (Fig. 6, lane designated "methanol"). The presence of quinones in the translation assay ameliorated translation to such an extent that bands of three immunoprecipitated proteins with apparent molecular weights of 46 kD, 32 kD and 17 kD could be detected as

Figure 7. In vitro translation of cpRNA and polyA⁺ RNA in the presence of quinones. cpRNA and polyA⁺ RNA, respectively, were translated in the presence of ³⁵S-methionine in a rabbit reticulocyte lysate by use of the translation assay described under 2.2.4. A fluorogram of newly synthesized protein products is shown: lane 1, translation of endogenous mRNA; lane 2-4, translation products of cpRNA in the presence of water (lane 2), 7% ethanol (lane 3), 0.8 μM dichloro-p-benzoquinone and 7% ethanol (lane 4); lanes 5-7: translation products of polyA⁺ mRNA in the presence of water (lane 5), 7% ethanol (lane 6), 0.8 μM dichloro-p-benzoquinone and 7% ethanol (lane 7). Sizes of protein markers are indicated.

newly synthesized products. The most pronounced effect on translation occurred in the presence of 2,6 dichloro-p-benzoquinone. This compound is known to be a potent inhibitor of photosynthetic activity by competing with plastoquinone on the reducing site of PSII (23). To test whether the effect of quinones on translation efficiency was specific for expression of D-2, cpRNA and polyA⁺ RNA from barley were translated in the rabbit reticulocyte lysate system. In these assays ethanol was used as a solvent and, as is shown in Fig. 7, the presence of alcohol again showed a strong inhibitory effect on overall translation reactions. No stimulation of translation efficiency upon quinone addition was observed when polyA⁺ RNA was used. However, the presence of quinones seemed to enhance protein synthesis of two chloroplast encoded polypeptides of about 50 kD and, to a much smaller degree, of about 32-34 kD. These proteins have not been identified yet but their apparent molecular weight suggests that they may be part of the PSII core complex as will be discussed below.

4. DISCUSSION

With the experiments reported in this paper the D-2 polypeptide of the reaction center complex of photosystem II in barley was synthesized in vitro from the *psbD* gene in two steps: mRNA was generated by cloning *psbD* behind the T7 or the SP6 bacteriophage promoter in the expression vector pGem 3, followed by transcription of the gene with T7 or SP6 RNA polymerases. Isolated RNA was then used to prime in vitro translation in a rabbit reticulocyte lysate. The best yield was obtained with the *psbD* gene construct pGem 101-7, which contained only 36 bp of the untranslated 5' leader sequence and 99 bp downstream of the stop codon. The leader sequence included the Shine-Dalgarno sequence -GGAGGA- in position -11 to -6 from the initiation codon (21).

Translation of the mRNA was not possible with the *E. coli* S30 cell lysate which has been successfully used to translate in vitro-generated mRNA for the *psbE* and *psbF* chloroplast DNA genes from barley (12). Translation of the *psbE* gene encoding the 9.2 kD apoprotein of cy-

tochrome *b-559* requires the presence of endogenous regulatory sequences present in the 138 nucleotides preceding the initiation codon of this gene. Such endogenous regulatory sequences are not necessary for translation of the 2.4 kD apoprotein subunit of cytochrome *b-559*, encoded in the *psbF* gene, where a DNA leader sequence of only 6 nucleotides is sufficient to obtain a translation product from *psbF* mRNA. In the case presented here of the barley *psbD* gene, however, a 5' leader sequence of 238 nucleotides (cf. ref. 21) did not help to obtain translation products of *psbD* mRNA in the *E. coli* lysate. On the other hand, in contrast to the *psbD* gene *psbE* and *psbF* mRNA could not be translated efficiently in the rabbit reticulocyte lysate.

A number of factors could be responsible for the diverse translation efficiency of mRNA transcribed from different chloroplast genes:

a) Translation initiation is highly sensitive to variations in the secondary structure of mRNA with variations in ΔG 's of only -1 to -4 kcal/mole being capable of generating a 1000 fold difference in translation initiation rates (8, 28).
b) Nucleotide distributions in positions -20 to +13 of the initiation codon determine translation efficiency in *E. coli* (8).
c) The 5' leader sequence of the *psbD* gene in barley, spinach, tobacco, liverwort and pea contains a conserved TAA and TAG stop codon in position -21 from the initiation codon (21), that may prevent translation in the *E. coli* lysate. Such a stop codon is also observed in the 5' leader sequence of the *psbA* gene of pea (23) and tobacco (33) at positions -18 and -21, respectively, and may be responsible for the fact that translation of in vitro-generated *psbA* mRNA in the *E. coli* lysate is initiated only at the second methionine codon (4) and not at the first as is the case in the chloroplast (6).

When in vitro-synthesized *psbD* mRNA was added to a rabbit reticulocyte lysate, six protein bands ranging from 46 kD to 12 kD were detected by fluorography. With the exception of the smallest protein these could be immunoprecipitated with antisera recognizing the D-2 polypeptide. The *psbD* gene from barley (21) contains besides the N-terminal methionine codon the eight internal in frame ATG codons

Table I. Positions of internal in frame methionine codons in the psbD gene of barley and expected as well as observed molecular weights of polypeptides initiated from these codons by in vitro translation.

Position of internal AUGs	No. of amino acids from internal AUG to COOH-end	Deduced molecular weight of peptide	Observed molecular weight
1	353	39,562 kD	32 kD
19	335	37,531 kD	30 kD
127	227	25,570 kD	25 kD
199	155	17,269 kD	17 kD
200	154	17,137 kD	17 kD
247	107	12,323 kD	12 kD
272	82	9,251 kD	
282	72	8,106 kD	
330	24	2,690 kD	

listed in Table I. The number of amino acids and the expected molecular weights for polypeptides initiated at these eight internal AUGs are also given in the table. The apparent molecular weights of the observed psbD translation products match the calculated molecular weights, if the following points are considered. The observed abnormal mobilities of the 39.6 and 37.5 kD polypeptides which have been initiated at AUG codon 1 and 19 correspond to the abnormal mobility of the related thylakoid polypeptides D1 (33). Both polypeptides derived from psbD mRNA were recognized by the "282-t" antiserum specific for the sequence ²³⁵-AlaPheAsnProThrGlnAla-²⁴¹ (7) and must thus have been translated in frame. In addition, the 25 kD and the 17 kD polypeptides were recognized by this antibody and they have the expected apparent molecular weight. The prominent 12 kD translation product is not recognized by the "282-t" antiserum in agreement with the anticipation that it is initiated at codon 247, i.e. downstream of the antigenic site. This polypeptide is also not immunoprecipitated by the second antiserum used, which is raised against a fusion protein (22) lacking the first 8 amino acids of D-2. Therefore it is assumed that this antibody does not recognize a domain between amino acid 247 and the carboxy terminus. Initiation at the internal AUGs in positions 272, 282 and 330 would yield smaller polypeptides not recognized by either of the antisera. Thus,

these proteins might well be present among the in vitro translation products (cf. Fig. 3).

In vitro translation products probably arising from out-of-phase ATG codons have been observed by in vitro expression of the psbE gene (12). The psbD gene contains eight out-of-phase ATG codons in positions 38, 59, 96, 185, 333, 633, 683 and 890 (21) giving rise to small reading frames ranging from 12 to 54 amino acids. This would lead to polypeptides with calculated molecular weights between 1200 and 6200 Da, thus not interfering with the interpretation of the pattern of translation products of Figure 3. Likewise initiation at the ATG codon for the psbC gene at position 1010 would have yielded a peptide of only 50 amino acids (5000 kD).

The interpretation that initiation of translation takes place from in-phase internal AUGs is supported by analysis of translation products obtained from pGem 101-9 derived mRNA. Only the 25 and 17 kD immunoprecipitable polypeptides and the 12 kD non-precipitable protein could be detected but no polypeptide in the range of 32 kD was synthesized. This result was expected since the psbD insert of this construct lacks the 5' terminal 178 bp encoding the 60 N-terminal amino acids of D-2.

Particularly interesting is the appearance of a protein band with the apparent molecular weight of 46 kD, which also immunoprecipitated with the antisera recognizing D-2 polypeptides. This band probably consists of sponta-

neously aggregated D-2-related translation products. Such dimer or multimer formation may be due to the very hydrophobic nature of the five membrane spanning α -helices in D-2. The polypeptides D-1 and D-2 of higher plants and cyanobacteria are considered to form a heterodimer in the photosystem II reaction center complex. D-1/D-2 55 kD aggregates possibly arising in connection with Triton X-100 solubilization of thylakoid membranes have been found in SDS-PAGE analysis by MARDER et al. (14). In view of the arrangement of the α -helices of the M subunits in *Rhodospseudomonas* (5, 18) it seems feasible that D-2 polypeptides in vitro form homodimers. In this respect it may be recalled that 53% identity of amino acid residues are found for some of the α -helical regions in D-1 and D-2 (1, 21, 27).

Since the primary electron acceptor Q_A is assumed to bind to D-2 it was investigated whether the addition of quinones affects in vitro translation of the *psbD* transcript as a preliminary to studies of binding of quinones to newly synthesized D-2 polypeptides. The quinones had to be solubilized in methanol or ethanol before they were added to the translation assay. While methanol severely depressed the translation activity, the addition of quinones permitted the preferential translation of D-2 polypeptides and D-2-related polypeptides. Most effective was the addition of 2,6 dichloro-p-benzoquinone. This compound is known to act as a potent electron acceptor on the reducing side of photosystem II when used in cell-free photosynthetic reactions (24). The preferential enhancement of D-2 translation could be due to binding of the quinone to the mRNA or to the natural binding site on the newly formed polypeptide. An important binding site of plastoquinone Q_A in *Rhodospseudomonas* is the Trp₂₅₀ residue in the M subunit (18). This tryptophan is conserved in position 255 of the D-2 polypeptide but replaced in D-1 by a phenylalanine residue as is the case in the L subunit of the photosynthetic bacterium (21). All of the D-2-related polypeptides stimulated by 2,6 dichloro-p-benzoquinone contained this residue suggesting that its involvement in binding quinone is possible.

In a control experiment total chloroplast

RNA and polyA⁺ RNA were used to prime translation assay in the presence of 2,6 dichloro-p-benzoquinone. Again, the depression of translation due to ethanol was observed with both templates. Quinone was unable to elicit a preferential translation from polyA⁺ RNA species but a preferential translation of 34 kD and 50 kD polypeptides was observed in the assays using total chloroplast RNA. Further analysis has to reveal if these polypeptides are the D-1 polypeptide and the 47-50 kD chlorophyll binding protein of photosystem II. Interestingly, highly purified photosystem II particles bind a plastoquinone-azide photoaffinity label to a 50 kD polypeptide (20).

In conclusion the in vitro expression of the *psbD* gene was shown to yield D-2 polypeptides. For further experiments of binding plastoquinones, other electron carriers and the chlorophyll chromophores to the D-2 polypeptide it would be desirable to obtain plasmid constructions that produce by in vitro expression only full length D-2 polypeptides. Elimination of the internal AUG codons by site-directed mutagenesis should be feasible especially since several methionines in D-1 are replaced in D-2 by isoleucine residues and vice versa.

ACKNOWLEDGEMENTS

I like to thank Professor DITER VON WETTSTEIN for suggesting this project and encouragement during my stay at the Carlsberg Laboratory with a fellowship from the Commission of the European Community. Dr. SANDY BERRY-LOWE is thanked for instructing me in molecular techniques, Dr. GUNILLA HØYER-HANSEN for performing the in vitro translations of the total plastid RNA and the polyA⁺ RNA after my move to Texas, and INGE SOMMER, NINA RASMUSSEN and ANN-SOFI STEINHOLTZ for help in the preparation of the manuscript. I am indebted to Dr. UDO JOHANNINGMEIER and Dr. PETER NIXON for the antisera and Dr. SUSAN GOLDEN and Dr. JOHN MULLET for a review of the manuscript.

REFERENCES

1. ALT, J., J. MORRIS, P. WESTHOFF & R.G. HERRMANN: Nucleotide sequence of the clustered genes for the 44 kd chlorophyll *a* apoprotein and the "32 kd"-like protein of the photosystem II reaction center in the spinach plastid chromosome. *Curr. Genet.* 8, 597-606 (1984)
2. BERENDS, T., P.E. GAMBLE & J.E. MULLET: Characterization of the barley chloroplast transcription units containing *psaA-psaB* and *psbD-psbC*. *Nucleic Acids Res.* 15, 5217-5240 (1987)
3. BOTTOMLEY, W. & W.R. WHITFIELD: Cell-free transcription and translation of total spinach chloroplast DNA. *Eur. J. Biochem.* 93, 31-39 (1979)
4. COHEN, B.N., T.A. COLEMAN, J.J. SCHMITT & H. WEISSBACH: *In vitro* expression and characterization of the translation start site of the *psbA* gene product (Q_B protein) from higher plants. *Nucleic Acids Res.* 12, 6221-6230 (1984)
5. DEISENHOFER, J., O. EPP, K. MIKI, R. HUBER & H. MICHEL: Structure of the protein subunits in the photosynthetic reaction centre of *Rhodospseudomonas viridis* at 3Å resolution. *Nature* 318, 618-623 (1985)
6. EYAL, Y., P. GOLOUBINOFF & M. EDELMAN: The amino terminal region delimited by Met₁ and Met₃₇ is an integral part of the 32 kDa herbicide binding protein. *Plant Molec. Biol.* 8, 337-343 (1987)
7. GEIGER, R., R.J. BERZBORN, B. DEPKA, W. OETTMEIER & A. TREBST: Site directed antisera to the D-2 polypeptide subunit of photosystem II. *Z. Naturforsch.* 42c, 117-124 (1987)
8. GOLD, L.: Post-transcriptional regulatory mechanisms in *E. coli*. *Ann. Rev. Biochem.* 57, 199-233 (1988)
9. HOLSCHUH, K., W. BOTTOMLEY & P.R. WHITFIELD: Structure of the spinach chloroplast genes for the D2 and 44 kd reaction-centre proteins of photosystem II and for tRNA^{Ser} (UGA). *Nucleic Acids Res.* 12, 8819-8834 (1984)
10. HØYER-HANSEN, G., L.S. HØNBERG & P.B. HØJ: Probing in vitro translation products with monoclonal antibodies to a 15.2 kD polypeptide subunit of photosystem I. *Carlsberg Res. Commun.* 50, 211-221 (1985)
11. KRIEG, P.A. & D.A. MELTON: Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucl. Acids Res.* 12, 7057-7070 (1984)
12. KRUPINSKA, K.: Characterization and in vitro expression of the cytochrome *b-559* genes in barley. II. In vitro transcription and translation. *Carlsberg Res. Commun.* 53, 233-246 (1988)
13. KYLE, D.J.: The 32 000 dalton Q_B protein of photosystem II. *Photochem. Photobiol.* 41, 107-116 (1985)
14. MARDER, J.B., D.J. CHAPMAN, A. TELFER, P.J. NIXON & J. BARBER: Identification of *psbA* and *psbD* gene products, D1 and D2, as reaction centre proteins of photosystem 2. *Plant Molec. Biol.* 9, 325-333 (1987)
15. MELTON, D.A., P.A. KRIEG, M.R. REBAGLIATI, T. MANIATIS, K. ZINN & M.R. GREEN: Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* 12, 7035-7056 (1984)
16. MICHEL, H.: Three-dimensional crystals of a membrane protein complex. *J. Mol. Biol.* 158, 567-572 (1982)
17. MICHEL, H., O. EPP & J. DEISENHOFER: Pigment-protein interactions in the photosynthetic reaction centre from *Rhodospseudomonas viridis*. *EMBO J.* 5, 2445-2451 (1986)
18. MICHEL, H. & J. DEISENHOFER: Photosynthetic reaction center from the purple bacterium *Rhodospseudomonas viridis* and its relevance to photosystem II. In: von Wettstein D., Chua N.-H. (eds.) *Plant Molecular Biology*. NATO-ASI, Series A, Vol 140, pp 597-607. Plenum Press, New York (1987)
19. NANBA, O. & K. SATOH: Isolation of a photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome *b-559*. *Proc. Natl. Acad. Sci. USA* 84: 109-112 (1987).
20. NEUMANN, E.M.: Struktur und Funktion von Membranproteinen auf der Akzeptorseite von Photosystem II. Dissertation zur Erlangung eines Doktors der Naturwissenschaften der Abteilung für Biologie an der Ruhr-Universität Bochum, Bochum 1985
21. NEUMANN, E.M.: Primary structure of barley genes encoding quinone and chlorophyll *a* binding proteins of photosystem II. *Carlsberg Res. Commun.* 53, 259-275 (1988)
22. NIXON, P.J., T.A. DYER, J. BARBER & C.N. HUNTER: Immunological evidence for the presence of the D1 and D2 proteins in PSII cores of higher plants. *FEBS Lett.* 209, 83-86 (1986)
23. OISHI, K.K., D.R. SHAPIRO & K.K. TEWARI: Sequence organization of a pea chloroplast DNA gene coding for a 34,500-dalton protein. *Mol. Cell. Biol.* 4, 2556-2563 (1984)
24. OGILVIE, P.D., L.B. RESCHL & S.P. BERG: Electron acceptance at photosystem II in an oxygen-evolving photosystem II preparation: Clear discrimination of two sites of electron acceptance for quinones and quinonediimines associated with a photosystem II preparation. *Arch. Biochem. Biophys.* 220, 451-458 (1983)

25. PRITCHARD, R.H. & I.B. HOLLAND: Preparation of the components of an *E. coli* in vitro transcription/translation system. C IV. In: Pritchard RH, Holland IB (eds) Basic Clonic Techniques pp. 137-142. Blackwell Scientific Publications, Oxford (1985)
26. RASMUSSEN, O.F., G. BOOKJANS, B.M. STUMMANN & K.W. HENNINGSEN: Localization and nucleotide sequence of the gene for the membrane polypeptide D2 from pea chloroplast DNA. *Plant Molec. Biol.* 3, 191-199 (1984)
27. ROCHAIX, J.-D., M. DRON, M. RAHIRE & P. MALNOE: Sequence homology between the 32K dalton and the D2 chloroplast membrane polypeptides of *Chlamydomonas reinhardtii*. *Plant Molec. Biol.* 3, 363-370 (1984)
28. STORMO, G.D.: Translation initiation. In: Reznikoff W, Gold L (eds.) Maximizing Gene Expression, pp. 195-224. Butterworths, Boston, London (1986)
29. TREBST, A.: The topology of the plastoquinone and herbicide binding peptides of photosystem II in the thylakoid membrane. *Z. Naturforsch.* 41c, 240-245 (1986)
30. TREBST, A.: The three-dimensional structure of the herbicide binding niche on the reaction center polypeptides of photosystem II. *Z. Naturforsch.* 42c, 742-750 (1987)
31. WORLAND, S.T., A. YAMAGISHI, S. ISAACS, K. SAUER & J.E. HEARST: Labeling quinone-binding sites in photosynthetic reaction centers: A 38-kilodalton protein associated with the acceptor side of photosystem II. *Proc. Natl. Acad. Sci. USA* 84, 1774-1778 (1987)
32. ZUBAY, G.: In vitro synthesis of protein in microbial systems. *Ann. Rev. Gen.* 7, 267-288 (1973)
33. ZURAWSKI, G., H.J. BOHNERT, P.R. WHITFIELD & W. BOTTOMLEY: Nucleotide sequence of the gene for the *M*, 32,000 thylakoid membrane protein from *Spinacia oleracea* and *Nicotiana debneyi* predicts a totally conserved primary translation product of *M*, 38,950. *Proc. Natl. Acad. Sci.* 79, 7699-7703 (1982)

Accepted by H. KLENOW