CHARACTERIZATION AND IN VITRO EXPRESSION OF THE CYTOCHROME *b*-559 GENES OF BARLEY. II. IN VITRO TRANSCRIPTION AND TRANSLATION

by

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The two cytochrome *b*-559 apoproteins of 9.4 kD and 4.5 kD molecular weight have been expressed in vitro using DNA templates containing either the two genes *psbE* and *psbF* in tandem or the individual genes. Transcription with *E. coli* RNA-polymerase or SP6 RNA-polymerase has been followed by translation in *E. coli* derived lysates. Simultaneous as well as independent synthesis of the apoproteins is possible. A 9.4 kD in vitro translation product has been identified as apoprotein I by immunoprecipitation with a monoclonal antibody specific for the C-terminal part of the 9.4 kD apoprotein of cytochrome *b*-559. The isolated *psbF* gene directs the synthesis of a translation product with a molecular weight of 4.5 kD corresponding to apoprotein II. Expression of the *psbE* gene requires the presence of endogenous regulatory sequences 5' upstream of *psbE*, while this is not the case for *psbF*. Additional in vitro translation products of 5.7 and 2.4 kD molecular weights are synthesized and probably translated from two reading frames starting with two different out-of-phase ATG codons in the nucleotide sequence of the *psbE* gene.

1. INTRODUCTION

Cytochrome *b*-559 of thylakoid membranes is a 110 kD lipoprotein complex with an apoprotein of 9.4 kD and a second presumptive apoprotein of 4.5 kD molecular weight. In spinach thylakoids both proteins occur in a 1:1 stoichiometry (21). A possible variation in the stoichiometry of the two proteins among plant species of different tissues or developmental stages is, however, not excluded. The genes

coding for the two apoproteins, *psbE* and *psbF*, are bicistronically transcribed into a single mRNA species (9, 11). At present it is not known, if a coordinate synthesis of the corresponding proteins takes place or if a precursor polypeptide is made which subsequently is cleaved into the two polypeptides.

The *psbE* and *psbF* genes of barley chloroplasts were isolated and subcloned into appropriate expression vectors. In the present paper it

Abbreviations: bp = basepairs; DTT = dithiothreitol; kbp = kilobasepairs; kD = kilodalton; mAb = monoclonal antibody; Tris = tris-(hydroxymethyl)-aminomethane.

is shown that the two apoproteins can be synthesized *in vitro* in an *E. coli* derived lysate both when the mRNA is transcribed from a plasmid containing the natural arrangement of the two genes in tandem and when it is transcribed from separate plasmids with inserts covering one or the other gene.

2. MATERIALS AND METHODS

2.1. Plasmids

For *in vitro* expression DNA-fragments were subcloned into pDS6 (17) or pDS6-RBSII (kindly provided by Dr. D. STUEBER, Hoffmann La Roche, Basel). Like all plasmids of the pDS1-family pDS6 is derived from pBR322 and has the strong coliphage promoter T5 (17). It is further specified by a terminator behind a region with multiple cloning sites. The modified vector pDS6-RBSII has a ribosomal binding site (RBS) inserted between the HindIII and BamHI sites of pDS6. This vector is therefore suitable for *in vitro* expression of transcripts without inherent ribosomal binding sites, if procaryotic systems are used for translation. For comparison, the Riboprobe GeminiTM transcription plasmid pGem3 (Promega Biotec) (13), which is commercially available, was used. The plasmid contains the two promoters SP6 and T7 both flanking a pUC18 derived polylinker region.

All expression vectors used contain the *bla*-gene for β -lactamase conferring ampicillin resistance to the bacterial host.

2.2. Strains and media

E. coli HB 101 was the recipient strain for the expression vectors pDS6, pDS6-RBSII and pGem3. The bacteria were prepared for transformation as described in part I (11). Minimal medium (12) supplied with 0.4% casamino acids and ampicillin ($100 \mu\text{g} \times \text{ml}^{-1}$) was used to propagate bacteria with recombinant plasmids.

2.3. Enzymes and chemicals

Riboprobe SP6 RNA- and Riboprobe T7 RNA-polymerase were purchased from Promega Biotec (Madison, Wisconsin, USA). Bal31 exonuclease was obtained from IBI

(Newhaven, Conn., USA). All other enzymes were from Boehringer, Mannheim, FRG. ³⁵S-methionine and α -³⁵S-dATP as radioactive label in the sequencing reactions were obtained from New England Nuclear, USA. ¹⁴C-labelled methylated proteins used as molecular weight markers for autoradiographic characterization of gel-electrophoretically separated translation products were purchased from Amersham, UK.

2.4. Preparation and analysis of recombinant plasmids

DNA was isolated and purified as described in (11). The orientation of fragments inserted into pDS6, pDS6-RBSII and pGem3 was checked by digesting the recombinant plasmid with appropriate restriction enzymes, or by sequencing with the dideoxy chain termination method of SANGER *et al.* (16) using synthetic 15-mer oligonucleotide primers complementary to the binding sites of the *E. coli*, SP6 and T7 RNA-polymerases, respectively. Plasmid DNA was prepared for sequencing as described in (5).

2.5. *In vitro* transcription

In vitro transcription of DNA cloned into pDS6 or pDS6-RBSII was done according to STUEBER *et al.* (18) but without capping of the RNA. 2 μg of supercoiled plasmid DNA were transcribed by 0.3 units of *E. coli* RNA-polymerase in a final volume of 10 μl containing 20 mM-HEPES/KOH, pH 7.5, 10 mM-Mg-acetate, 200 mM-K-acetate, 0.2 mM-spermidine, 5 mM-DTT, 10 units of RNasin (human placental RNase inhibitor, Boehringer) and 0.5 mM each of GTP, CTP, UTP and ATP. After 15 minutes incubation at 37 °C the mixture was put on ice and was either directly used for translation assays or stored at -20 °C.

Transcription of DNA cloned in pGem3 was carried out according to the instructions of Promega Biotec either with SP6 RNA-polymerase or T7 RNA-polymerase using linearized plasmid DNA as templates. Before translation the assay was treated with DNase I (1 u/ μg DNA) for 10 minutes at 37 °C and then extracted with phenol and chloroform. The precipitated RNA was redissolved in water and stored at -20 °C.

2.6. In vitro translation

RNA obtained by in vitro transcription of cloned DNA-fragments was translated in an *E. coli* S30 lysate according to ZUBAY (22), prepared as described in PRITCHARD and HOLLAND (14) using the *E. coli* strain Y1090 or the strain PR7 (2).

Usually, a 50 μ l reaction mixture contained 5 μ l of the pDS6 transcription assay or one fourth of the pGem3 transcription assay in 5 μ l water, 15 μ l S30 extract, and 12.5 μ l 4 $\times$ concentrated low molecular weight mix. To the translation assay was further added 5 mM-Mg-acetate and 25 μ Ci 35 S-methionine. The four times 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-acetate, 30 μ g $\times$ ml⁻¹ folinic acid, 0.1 mg $\times$ ml⁻¹ *E. coli* t-RNA, 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) polyethylenglycol.

2.7. Immunoprecipitation

35 S-methionine-labelled translation products were immunoprecipitated with the monoclonal antibody mAb 6A5 recognizing the C-terminal part of the 9.4 kD polypeptide of cytochrome *b*-559 ((19) and O. VALLON, pers. commun.) and protein A-sepharose (Pharmacia) as described in HØYER-HANSEN et al. (10).

2.8. Polyacrylamide-gel-electrophoresis

In vitro synthesized products before and after immunoprecipitation were separated by electrophoresis on SDS containing 18% (w/v) polyacrylamide gels using the high molarity Tris-buffer system of FLING and GREGERSON (7). After electrophoresis gels were transferred to 50% (v/v) methanol and prepared for fluorography by soaking for 20 minutes in a solution of 20% (w/v) sodium salicylate, 10% (v/v) glycerol and 50% (v/v) methanol (4). The dried gels were exposed to Kodak XAR X-Omat X-ray films at -80 °C using Kodak X-Omatic regular intensifying screens.

3. RESULTS

DNA-fragments containing either both cytochrome *b*-559 genes together or the individual *psbE* and *psbF* were inserted into the multiple cloning sites of the expression vectors pDS6 (18) and pGem3 (Promega Biotec) (Figure 1). Transcripts made from pDS6 and pGem3 were translated in an S30 lysate prepared from *E. coli* cells (14, 22). Two different kinds of *E. coli* cells were used for preparing the lysate. While in the beginning *E. coli* strain Y1090 was used, later experiments were performed with an S30 lysate obtained from the *E. coli* strain PR7 (2).

Additionally, translation of pDS6 transcripts was tried in a commercially available rabbit reticulocyte lysate (Amersham) and in a wheat germ extract (kindly provided by SUSAN MADRID). Though, in these cases transcripts were "capped" by the addition of 7-methylguanosine triphosphate to the transcription mixture (18) no proteins were synthesized in the rabbit reticulocyte lysate and only low amounts of proteins were produced in the wheat germ extract (data not shown). S30 lysates prepared from intact chloroplasts (1) of spinach and barley did not show any protein synthesis due to exogenously added DNA or transcripts derived from pDS6 (data not shown).

3.1. Concomitant expression of the two cytochrome *b*-559 genes

For in vitro expression of *psbE* and *psbF* appropriate DNA-fragments containing both genes were isolated and subcloned into the expression vectors pDS6 and pGem3 (Figure 1, Table I).

3.1.1. 1150 bp *AccI*-*Bam*HI fragment

A 1150 bp DNA-fragment containing both genes and a 138 bp sequence 5' upstream of the *psbE* startcodon and noncoding DNA 3' downstream of *psbF* was isolated by digesting the 4.2 kbp *Bam*HI fragment derived from pHvC 46 (ref. 11, Figure 3) with *AccI* (Figure 1). The fragment was blunt-ended and subcloned into the *Sma*I site of pDS6. The orientation of the fragment with respect to the T5-promoter was determined by sequencing using a 15-mer

Figure 1. DNA-fragments containing either the two cytochrome *b*-559 genes together or the individual genes *psbE* and *psbF*. Endonuclease restriction enzymes and Bal31 exonuclease used in isolation of the various fragments are indicated. EcoRI restriction sites given along the first fragment allow a comparison with the physical map of the pHvC 46 insert (ref. 11, Figure 3) and the nucleotide sequence (Figure 4).

The fragments were inserted into pDS6 in correct (+) or inverse (-) orientation with respect to the T5 promoter and transcribed by *E. coli* RNA polymerase. Fragments inserted into the twin promoter transcription vector pGem3 were transcribed with both SP6 and T7 RNA-polymerase.

oligonucleotide complementary to the *E. coli* polymerase binding site as a primer. A recombinant pDS6 vector containing the insert in the correct orientation was compared to a vector with the insert in inverse orientation. The same fragment was also inserted into pGem3 using the AccI and BamHI sites of the polylinker. SP6 RNA-polymerase had to be used for transcription of the genes. Transcription with T7 RNA-polymerase was done as a control.

In vitro translation of transcripts derived from the recombinant pDS6 with the 1150 bp insert was performed both in a S30 lysate from *E. coli* Y1090 and a S30 lysate from strain PR7. The former gave considerable endogenous

protein synthesis by itself and with transcripts from the vector without insert (Figure 2, two leftmost lanes) while the lysate from PR7 made only a few endogenous proteins with transcripts from the vector without inserts (Figure 2, lane 5). The recombinant pDS6 with the insert in correct orientation yielded with both lysates three polypeptide bands with sizes of 9 kD, ~5 kD and less than 5 kD (Figure 2). If the vector contains the insert with reverse orientation the 9 kD polypeptide is made in higher amounts and only traces of the smaller peptides can be detected. A monoclonal antibody (mAb 6A5) specific for the C-terminal part of the 9.4 kD polypeptide of cytochrome *b*-559 ((19) and pers. com-

Table I. Proteins synthesized by *in vitro* transcription/translation using recombinant pDS6 and pGem3 plasmids as templates. Inserts include either both cytochrome *b*-559 genes or individual *psbE* and *psbF* genes. The DNA fragments have been inserted into pDS6 either in correct (+) or in inverse (-) orientation with respect to the T5 promoter of the plasmid. DNA fragments inserted into pGem3 had to be transcribed with SP6 polymerase. Transcription with T7 polymerase was done as a control.

inserted DNA-fragment	genes	pDS6		pGem3	
		orientation	molecular weight of proteins (kD)	polymerase	molecular weight of proteins (kD)
1150 bp <i>AccI</i> - <i>Bam</i> HI	<i>psbE</i> + <i>F</i>	+	9, 5, 4, 2	SP6	9, 5, 4, (2)
"	"	-	9	T7	-
400 bp <i>Bal</i> 31	<i>psbE</i> + <i>F</i>	+	4	SP6	5, 4, 2
"	"	-	-	T7	-
400 bp <i>Bal</i> 31+36 bp 5' extension	"	+	4		
"	"	-	-		
270 bp <i>Bal</i> 31- <i>Hae</i> III	<i>psbE</i>	+	-	SP6	-
"	"			T7	-
370 bp <i>AccI</i> - <i>Hae</i> III	"	+	9	SP6	9
"	"			T7	-
350 bp <i>Eco</i> RI	<i>psbF</i>	+	4	SP6	4
"	"	-	-	T7	-
200 bp <i>Hae</i> III- <i>Hin</i> FI	"	+	4	SP6	-
"	"			T7	-

mun.) was used for immunoprecipitation of the translation products. As seen in Figure 2, it recognized the 9 kD polypeptide obtained by *in vitro* expression of the recombinant pDS6 clones with the insert in both orientations. Whereas immunoprecipitation of translation products obtained in the S30 extract of strain Y1090 included also several proteins due to endogenous protein synthesis when the insert was in the correct orientation, there is no comparable background with the insert in reverse orientation or if translates obtained in the *E. coli* PR7 lysate were immunoprecipitated (Figure 2). The fact that the 9 kD immunoprecipitable cytochrome *b*-559 translate is obtained by inverse orientation of the insert indicates that transcription is from the chloroplast DNA promoter, included in the 1150 bp *AccI*-*Bam*HI fragment (11).

Transcripts derived from pGem3 containing the 1150 bp fragment in an orientation appropriate for the SP6 polymerase gave with this enzyme rise to the same three polypeptide bands as obtained with pDS6 (Figure 3). If instead of

SP6 RNA-polymerase T7 RNA-polymerase was used for transcription no vector transcript directed protein synthesis was observed in the two different *E. coli* S30 lysates used for translation (Figure 3). The monoclonal antibody (mAb 6A5) recognized also in this case only the 9 kD polypeptide.

3.1.2. 400 bp *Bal*31 fragment

Partial digestion of the 1150 bp *AccI*-*Bam*HI fragment with *Hin*FI releases a 470 bp fragment containing both genes and additional 40 bp 5' upstream of the *psbE* startcodon and 60 bp 3' downstream of the *psbF* stopcodon (Figure 4). The fragment was further treated with *Bal*31 exonuclease to reduce the number of noncoding nucleotides at both ends of the fragment. Various fragments obtained by this treatment were inserted into the *Sma*I site of pUC13 and sequenced. A 400 bp fragment starting 12 nucleotides 5' upstream of the ATG-startcodon and including the putative ribosomal binding site, and ending 13 nucleotides beyond the *psbF*

Figure 2. In vitro expression of *psbE* and *psbF* using recombinant pDS6 as templates. The 1150 bp *AccI*-*Bam*HI fragment was inserted into pDS6 in correct (+) or inverse (-) orientation with respect to the T5 promoter of the vector.

Transcripts obtained from recombinant pDS6 with *E. coli* RNA-polymerase were translated in two different S30 lysates: The first one was prepared from the *E. coli* strain Y1090 and the second one was prepared from *E. coli* strain PR7 (2). The Y1090 S30 lysate shows considerable endogenous protein synthesis by itself and with the transcripts from the vector without insert (lanes 1, 2 from left). The PR7 lysate shows no (lane 5) and with vector derived RNA little endogenous protein synthesis (lane 6). 35 S-methionine labelled translation products were autoradiographed after separation on 18% (w/v) acrylamide gels (7).

In vitro translation products derived from recombinant pDS6 were immunoprecipitated with a monoclonal antibody (mAb 6A5) specific for the C-terminal part of the 9.4 kD polypeptide of cytochrome *b*-559 (α -Cyt. *b*-559). In case of recombinant pDS6 with the insert in correct orientation immunoprecipitation of translates obtained in the Y1090 lysate is compared to immunoprecipitation of translates obtained in the PR7 lysate. The 9.4 kD apoprotein recognized by the antibody is indicated by an arrow. 14 C-labelled methylated molecular weight markers comprised carbonic anhydrase (30 kD), soybean trypsin inhibitor (21.5 kD), cytochrome *c* (12.5 kD) and aprotinin (6.5 kD).

stopcodon (Figure 4) was chosen for in vitro expression. The fragment was isolated from recombinant pUC13 by digestion with *Bam*HI

and *Sac*I, blunt-ended by T4 DNA-polymerase and inserted into the *Sma*I sites of pDS6 and pGem3. In the case of recombinant pSD6 both

Figure 3. In vitro expression of the *psbE* and *psbF* genes included in the 1150 bp *AccI*-*Bam*HI fragment in the twin promoter system pGem3. Transcription was done with SP6 RNA-polymerase and as a control with T7 RNA-polymerase. For translation an S30 lysate from *E. coli* strain Y1090 (left) and from *E. coli* strain PR7 were used.

³⁵S-methionine labelled translation products were autoradiographed after separation on 18% (w/v) acrylamide gels (7). Lanes 1 and 4 from reaction mixtures without vector derived RNA. Molecular weight markers indicated on the left were as in Figure 2.

orientations of the insert with respect to the T5 promoter were compared. In the case of recombinant pGem3 transcription with SP6 RNA-polymerase was compared to transcription with T7 RNA-polymerase.

pDS6 with the 400 bp *Bal*31 fragment in correct orientation gave rise to the synthesis of a protein with a molecular weight of about 4 kD (Figure 5). If the vector contained the same DNA-fragment in inverse orientation no in vitro

product could be detected (Figure 5).

With the insert of the 1150 bp *AccI*-*Bam*HI fragment in pDS6 (section 3.1.1) the distance between the T5 promoter and the startcodon of *psbE* is about 150 bp but it is only 40 bp in the case of the inserted 400 bp *Bal*31 fragment. In order to examine whether a longer distance between promoter and the ATG-startcodon is required for expressing the genes and may reestablish the synthesis of the larger protein

Figure 4. Nucleotide sequence of the chloroplast DNA region containing the *psbE* and *psbF* genes. Relevant restriction sites are indicated and the derived amino acid sequences for the two genes given. Numbering starts at the first base of the *psbE* startcodon. Putative ribosomal binding sites are boxed. Amino acids recognized by the monoclonal antibody (mAb 6A5) are underlined by a wavy line. Double-lined arrows indicate the positions up to which non-coding sequences adjacent to the genes were degraded by Bal31 exonuclease treatment. Two-out-of-phase ATG codons within the *psbE* nucleotide sequence are indicated by horizontal brackets and their corresponding reading frames are written underneath the normal amino acid sequence of the *psbE* product.

having the molecular weight of about 9 kD, the 400 bp Bal31 fragment was elongated 5' upstream of the ATG-codon of *psbE*. For this purpose a 36 bp fragment was released from the polylinker region of pUC18 (20), blunt-ended and ligated to the 400 bp Bal31 fragment. The

increase in the distance between promoter and startcodon of *psbE* hardly altered the pattern of translation products but it stimulated the production of the about 4 kD protein dramatically (Figure 5).

If the appropriate pGem3 vector was used for

Figure 5. In vitro expression of psbE and psbF from a 400 bp Bal31 fragment inserted into pGem3 and pDS6. From pGem3 the insert was transcribed by SP6 polymerase when placed in correct orientation (+). In pDS6 the 400 bp Bal31 fragment was inserted in correct (+) and inverse (-) orientation with respect to the T5 promoter and was transcribed by *E. coli* RNA-polymerase. The unmodified insert started 12 nucleotides upstream of the psbE startcodon (Figure 4). A modified insert with a 5' extension of 36 bp had 48 nucleotides upstream of the ATG-startcodon. ³⁵S-labelled translation products were obtained in an S30 lysate from Y1090 *E. coli* cells. Controls without vector derived RNA are given in the leftmost and rightmost lanes. The proteins were separated on an 18% (w/v) polyacrylamide gel (7). Molecular weight markers indicated on the right of the pertinent autoradiogram were as in Figure 2 and included the B-chain of insulin (3.4 kD).

in vitro expression of the 400 bp Bal31 fragment, a 5 kD molecular weight product was prominent and less of the 4 kD polypeptide was obtained (Figure 5). Additionally a smaller polypeptide of about 2-3 kD molecular weight was synthesized (Figure 5). No proteins were synthesized if T7 polymerase instead of SP6 polymerase was used (Figure 5). A translation product of 9 kD molecular weight was not

obtained with these constructs. None of the translation products obtained from the 400 bp Bal31 fragment was immunoprecipitable with the monoclonal antibody mAb 6A5 (data not shown).

3.2. Expression of psbE

In order to express psbE independently from psbF, a 270 bp fragment obtained by partial

Figure 6. Separate in vitro expression of *psbE* and *psbF* using recombinant pDS6 plasmids in which the DNA-fragments were inserted in correct (+) or inverse (-) orientation with respect to the T5 promoter. The 5' end of the inserted DNA-fragments relative to the startcodon of *psbE* is given according to the numbering of the nucleotide sequence (Figure 4). The ATG codon for *psbF* is at +263. ^{35}S -methionine labelled translation products obtained in PR7 lysates were autoradiographed after separation on 18% (w/v) polyacrylamide gels (7). Translation products from *psbE* were immunoprecipitated with a monoclonal antibody specific for cytochrome *b*-559 (α -Cyt. *b*-559). The protein recognized by the antibody is indicated by an arrow. Molecular weight markers indicated on the left were as in Figures 2 and 5. Lanes 3 and 9 are controls from translation assays with RNA derived from vectors without inserts.

digestion of the 400 bp *Bal*31 fragment with *Hae*III was inserted into the *Sma*I sites of pDS6 and pGem3 (270 bp *Bal*31-*Hae*III) (Figures 1 and 4, Table I). Neither the recombinant pDS6 with the insert in correct orientation nor the recombinant pDS6 with the insert in inverse orientation gave rise to any polypeptide in the S30 lysate (Figure 5). No expression was observed by using the pertinent pGem3 either

(data not shown).

In order to test whether possible regulatory sequences 5' upstream of the *psbE* gene are necessary for expression another fragment was used instead. It included an additional 135 bp sequence 5' upstream of the *psbE* startcodon and was obtained by ligating a 300 bp *Acc*I-*Eco*RI fragment to the 70 bp *Eco*RI-*Hae*III fragment (Figures 1 and 4). The first fragment

[illegible]

Figure 7. Nucleotide sequence of a region preceding the *psbE* gene and containing the putative cytochrome *b-559* promoter, compared to the nucleotide sequences of the coliphage T5 promoter serving for *E. coli* RNA-polymerase in the pDS6 expression system (18) and the promoters for T7 RNA-polymerase (6) and SP6 RNA-polymerase (13) used in the twin promoter system pGem3. Hexameric sequences resembling the “-10” and “-35” consensus sequences of bacterial promoters (15) are underlined. A broken line indicates an additional possible regulatory sequence in the nucleotide sequence preceding the *psbE* gene (see also Figure 4). Asterisks indicate the nucleotides at which the RNA chains begin.

was released by digestion of the 1.5 kbp EcoRI fragment (see ref. 11, Figure 3) with *AccI*. The latter fragment containing the terminal part of *psbE* was obtained by digestion of the 350 bp EcoRI fragment (see ref. 11, Figure 3) with *HaeIII*. The 370 bp ligation product was subcloned into pGem3 cut with *AccI* and *SmaI*, whereafter the fragment was reisolated from recombinant pGem3 by digestion with *PstI* and *PvuII*. This larger segment of 460 bp in length was stepwise inserted into pDS6 linearized by *PstI* and *HindIII*: After ligation of the *PstI* ends the remaining *HindIII* tail of the vector was blunt-ended and ligated to the blunt-end of the insert. The resulting plasmid contained the *psbE* gene in correct orientation with respect to the T5 promoter (Figure 1). This recombinant pDS6 vector as well as the recombinant pGem3 (data not shown) gave rise to the synthesis of a 9 kD protein which is immunoprecipitated by the monoclonal antibody specific for cytochrome *b*-559 (Figure 6).

3.3. Expression of psbF

For expression of *psbF* alone the 350 bp *EcoRI* fragment (ref. 11, Figure 3) was inserted into the *EcoRI* cloning sites of pDS6 and pGem3 (Figure 1, Table I). The recombinant pDS6 with the correctly inserted fragment gave rise to the synthesis of a 4 kD polypeptide (Figure 6). No proteins were obtained from

recombinant pDS6 with the fragment in inverse orientation (Figure 6). If the recombinant pGem3 was transcribed with SP6 RNA-polymerase also a 4 kD polypeptide was synthesized (data not shown).

A smaller 200 bp fragment was released from the 350 bp EcoRI fragment by digestion with HaeIII and HinfI (Figures 1 and 4). Since this fragment did not contain a complete ribosomal binding site it had to be subcloned into the modified vector pDS6-RBSII. To begin with it was inserted into the SmaI site of pGem3 and subsequently released again with BamHI and EcoRI. The BamHI end was ligated to pDS6-RBSII linearized with BamHI and PstI. After blunt-ending the EcoRI and PstI tails the ends were joined. The resulting plasmid contained the insert automatically in the correct orientation with respect to the T5 promoter. The recombinant plasmid gave rise to the production of a single 4 kD polypeptide during translation (Figure 6). No proteins were obtained if the appropriate pGem3 vector without a ribosomal binding site was used instead (data not shown).

Neither the 4 kD polypeptide translated from the transcript of the 350 bp EcoRI fragment nor the corresponding polypeptide obtained with the smaller psbF fragment were recognized by the monoclonal antibody specific for the C-terminal end of the cytochrome *b*-559, 9.4 kD polypeptide (data not shown).

The results on the in vitro expression of the

various DNA-fragments containing *psbE* and *psbF* either in the same or separate plasmids are summarized in Table I.

4. DISCUSSION

In vitro expression of the two cytochrome *b*-559 genes of barley employed to begin with a 1150 bp *AccI*-*Bam*HI fragment containing the two genes in tandem with 138 nucleotides 5' upstream and a 3' extension of about 470 nucleotides. Transcription by *E. coli* RNA-polymerase and phage SP6 RNA-polymerase and translation in an *E. coli* lysate yielded in addition to the expected 9.4 kD and 4.5 kD apoproteins of cytochrome *b*-559 two additional polypeptides with molecular weights of about 5 and less than 4 kD (Figures 2 and 3). Only the 9.4 kD in vitro translation product is recognized by the monoclonal antibody mAb 6A5. By investigating the reaction of this antibody (19) with cytochrome *b*-559 apoproteins from different plant species having slightly different primary structure and with a synthetic tredecapetide corresponding to residues 69-81 of the barley apoprotein I, the residues 80-81 (Ser-Arg) in the C-terminus of the apoprotein were identified as major constituents of the epitope recognized by mAb 6A5 (O. VALLON, personal communication). Immunoprecipitation with this antibody identifies the 9.4 kD polypeptide obtained by in vitro translation as product of the *psbE* gene.

If the 1150 bp *AccI*-*Bam*HI fragment is inserted in pDS6 in inverse orientation with respect to the T5 promoter, unexpectedly also a translation product of about 9 kD is obtained (Figure 2). Its identity with apoprotein I could be shown by immunoprecipitation with the monoclonal antibody mAb 6A5 (Figure 2). If the analogous pGem3 plasmid is transcribed with T7 RNA-polymerase instead of SP6 RNA-polymerase, however, no in vitro translation product has been observed (Figure 3). Actually, there is no open reading frame which might code for a protein of this size on the opposite strand of the 1150 bp *AccI*-*Bam*HI fragment containing the *psbE* and *psbF* genes. Most likely cytochrome *b*-559 promoter sequences (cf. 11) located 5' upstream of *psbE* (Figures 4 and 7) serve as promoter for the *E. coli* RNA-polymerase but

not for the phage T7 RNA-polymerase. Both the T7 and SP6 phage RNA-polymerases used for transcription of DNA inserted in pGem3 have highly specific promoter sequences which do not resemble those recognized by the *E. coli* RNA-polymerase (3, 6). As shown in Figure 7 there is little similarity between the nucleotide sequences of the SP6 (3) or T7 (6) phage promoters and the consensus sequences of bacterial promoters. On the other hand, the T5 promoter of pDS6 serving for *E. coli* RNA-polymerase and the putative cytochrome *b*-559 promoter have typical bacterial "-35" and "-10" sequences (15).

None of the other fragments tested for in vitro expression of *psbE* and *psbF* when inserted into pDS6 in inverse orientation seem to be transcribed (Table I).

In vitro expression of the two cytochrome *b*-559 genes *psbE* and *psbF* together was further studied with a 400 bp *Bal*31 fragment in which the 5' upstream region was truncated to position -12 from the *psbE* startcodon and the 3' downstream region to 10 nucleotides from the stopcodon of the *psbF* gene. With this fragment in vitro synthesis of the 9.4 kD apoprotein was no longer possible despite the presence of a GGAG ribosomal binding site in front of the *psbE* gene (Figures 4 and 5). In vitro expression of the recombinant pDS6 plasmid yielded only a polypeptide of about 4 kD molecular weight (Figure 5) which is considered to be apoprotein II. Since STUEBER et al. (18) had reported that the distance between the T5 promoter and the startcodon of inserted genes affects the efficiency of the system, the distance between the startcodon of the *psbE* gene and the T5 promoter was extended by 36 bp. This modification dramatically increased the synthesis of the 4 kD polypeptide but did not elicit the expression of the *psbE* gene (Figure 5). This result suggests that specific sequences preceding the *psbE* gene rather than a certain distance between T5 promoter and startcodon of *psbE* are necessary for expression of the gene.

Unlike transcription by *E. coli* RNA-polymerase the use of SP6 RNA-polymerase permitted the synthesis of two additional polypeptides of 5 kD and less than 4 kD, respectively (Figure 5). These two additional polypeptides are likely

to be translated from the out-of-phase ATG codons in position +131 and +230 (Figure 4). The first of these reading frames starting with position +131 ends with a TAA stopcodon at position +194 and would yield a polypeptide of 21 amino acids which calculates to a molecular weight of 2.4 kD. The out-of-phase reading frame starting with the ATG at nucleotide +230 would extend the N-terminus of apoprotein II with a presequence of 11 additional amino acid residues and result in a polypeptide with a molecular weight of 5.7 kD. This chimaeric protein would not contain the Ser-Arg dipeptide recognized by the monoclonal antibody mAb 6A5 and explains why the antibody did not precipitate this protein, whether SP6 RNA-polymerase is used on the 400 bp Bal31 fragment in the twin promoter system (cf. Figure 5) or *E. coli* RNA-polymerase on the 1150 bp AccI-BamHI fragment in the pDS6 vector (cf. Figure 2). Expression vectors containing the *psbE* gene alone with a 138 nucleotides long "leader sequence" in front of the *psbE* startcodon provided an immunoprecipitable in vitro translation product of 9.4 kD molecular weight (Figure 6). No protein was synthesized in vitro if the 5' region was truncated to position -12 in front of the startcodon (Figure 6). This result is consistent with the absence of *psbE* expression in the case of the 400 bp Bal31 fragment containing both genes and truncated to position -12 in front of the *psbE* startcodon (Table I). As concluded from results obtained by the 36 bp 5' extension of the 400 bp Bal31 fragment specific sequences preceding the *psbE* gene are necessary for expression of the gene.

For expression of the *psbF* gene alone two different fragments have been employed, the 350 bp EcoRI fragment containing in front of *psbF* the terminal part of the *psbE* gene and its smaller 200 bp HaeIII-HinfI derivative with only 6 nucleotides in front of the *psbF* startcodon. Both yielded an in vitro translation product of 4.5 kD molecular weight. Since *psbF* alone can direct the synthesis of this protein it identifies it as cytochrome *b*-559 apoprotein II. This protein is not recognized by the monoclonal antibody mAb 6A5.

In conclusion, the two apoproteins of cytochrome *b*-559 with molecular weights of 9.4

kD and 4.5 kD have been identified among the in vitro translation products obtained by expression of the 1150 bp AccI-BamHI fragment containing both genes and by independent expression of the separated *psbE* and *psbF* genes.

Differences between chloroplast RNA-polymerase and *E. coli* RNA-polymerase (8) or special features of the chloroplast translation system will have to be explored in order to understand why translation products using internal out-of-phase initiator codons have been obtained in the present experiment.

The smallest heme containing subunit of cytochrome *b*-559 is considered to be a heterodimer of the two apoproteins in which heme is ligated by a histidine residue of each chain. For heme binding studies and in vitro assembly of the polypeptides into subunits the production of the apoproteins is recommended from the recombinant pDS6 plasmid with the *psbE* gene contained in the 370 bp AccI-HaeIII fragment and from the recombinant pDS6-RBS II plasmid with the *psbF* gene contained in the 200 bp HaeIII-HinfI fragment.

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