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Genes and transcripts for the polypeptides of the cytochrome b6/f complex from spinach thylakoid membranes

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Cytochrome b6/f complex was prepared from washed thylakoid membranes by a procedure involving detergent treatment and centrifugation in sucrose gradients. The complex is composed of at least four polypeptide species, cytochrome f which occurs in two variant forms (mol. wt. 34/33 kd), cytochrome b6 (23 kd), the high-potential Rieske iron-sulfur protein (19 kd) and a fourth subunit (17 kd) of unknown function. Transcripts for the cytochromes f, b6 and subunit 4 were found in plastid RNA, those for the Rieske iron-sulfur protein in cytosolic poly(A)$^+$ RNA. Transcripts for cytochrome b6 and subunit 4 are translated in rabbit reticulocyte lysates into products of correct length. The Rieske iron-sulfur protein and the cytochrome f apoprotein appear to be made as precursors with excess sequences of 7 and 4 kd, respectively. Cytochrome f, cytochrome b6 and subunit 4 are encoded by uninterrupted plastid genes that are located in the large single-copy region of the circular DNA molecule. Each of these genes is present once per chromosome. Their location and direction of transcription have been determined by hybrid-selection mapping and by cell-free transcription/translation of various recombinant DNAs. The genes for cytochrome b6 and for subunit 4 lie near each other, but do not overlap. They are transcribed into a single message. The gene for cytochrome f maps 15 kbp away from this cluster, close to the 3′ end of the gene for the large subunit of ribulosebisphosphate carboxylase/oxygenase, and is transcribed into a separate 4 kb long RNA. All these genes have the same polarities with respect to each other.

Key words: cytochrome genes/thylakoid membrane/plastid DNA/transcripts/spinach

Introduction

Conversion of light into chemical energy in chloroplasts is catalyzed by the coordinated function of lipids and ~40 proteins that constitute the thylakoid membrane. The majority of these proteins are organized into five supramolecular complexes: the reaction center of photosystem I, the reaction center complex of photosystem II, including the proteins for water oxidation; the light-collecting chlorophyll a/b protein complex; the ATP synthase (CF_1-CF_0); and the cytochrome b6/f complex. The functional coordination of these units in electron transport and coupled proton translocation through the membrane are critically dependent on their traverse organization and lateral distribution within the lipid layer of the membrane (for reviews, see Nelson and Hauska, 1979; Anderson, 1981; Nelson, 1982).

The biogenesis of the thylakoid membrane in chloroplasts requires the cooperative expression of genetic information in nucleus and plastids (Bedbrook and Kolodner, 1979; Herrmann and Possingham, 1980). As a first step towards understanding the highly complex gene regulation between the two subcellular compartments and, in wider perspective, structure/function relationships in photosynthesis, we are identifying and physically mapping the genes for polypeptides of this biomembrane (Westhoff *et al.*, 1981, 1983; Herrmann *et al.*, 1982; Alt *et al.*, 1983). Here we describe experiments on the spinach cytochrome b6/f complex which is an integral unit of photosynthetic electron transfer and proton translocation. It acts as a plastoquinol/plastocyanin oxidoreductase between photosystem II and photosystem I in non-cyclic electron transport. It is also involved in the cyclic electron pathway around photosystem I (Bendall, 1982; Hauska *et al.*, 1983). The unit resembles the mitochondrial b/c_1 complex, and it was first isolated from lettuce chloroplasts (Nelson and Neumann, 1972). It recently has been purified to homogeneity in a functional state from spinach chloroplasts and consists of at least four constituent proteins: cytochrome f in two variant forms, cytochrome b6, the high-potential Rieske iron-sulfur protein, and a relatively small protein of an as yet unknown function. The complex also contains 'bound' plastoquinol, and when incorporated into artificial phospholipid vesicles, can function as an electrogenic proton pump (Hurt and Hauska, 1981, 1982a, 1982b; Hurt *et al.*, 1982). Recent evidence suggests that the cytochrome complex is found in both grana and intergrana regions of the thylakoid membrane, unlike photosystem I and II which appear to be spatially separated in non-appressed (stroma-exposed) and appressed regions (Cox and Andersson, 1981; Andersson and Haehnel, 1982).

We have used cell-free translation of compartment-specific RNA fractions, hybrid-selection mapping, and cell-free transcription/translation of recombinant plasmids to identify stable transcripts and genes for all four polypeptides of the cytochrome complex from spinach chloroplasts.

Results

Protein standards and antisera

The cytochrome b6/f complex isolated according to Hurt and Hauska (1981) was resolved into five polypeptides which include the cytochrome f doublet (mol. wt. 33 and 34 kd), cytochrome b6 (mol. wt. 22 kd), the Rieske Fe-S protein (mol. wt. 19 kd) and a component of 17 kd referred to as subunit 4 (Hurt and Hauska, 1982a; Figure 1). Three other low mol. wt. polypeptides (6–15 kd) consistently co-purified with the complex (cf., also Hurt and Hauska, 1982a), especially when the NaBr extraction of the membranes was omitted (Figure 1C). It remains to be established whether these are constituents of the complex. The cytochromes f and b6 can be identified on gels by heme-specific staining with 3,3′,5,5′-tetramethyl-benzidine (TMBZ; Thomas *et al.*, 1976). Their definite assignment as well as that of the Rieske Fe-S protein (Hurt *et al.*, 1981) was accomplished by spectral characterization after selective isolation from the complex. Cytochrome b6 and the Rieske Fe-S protein reverse their positions when urea is included in the gels, as is evident from the

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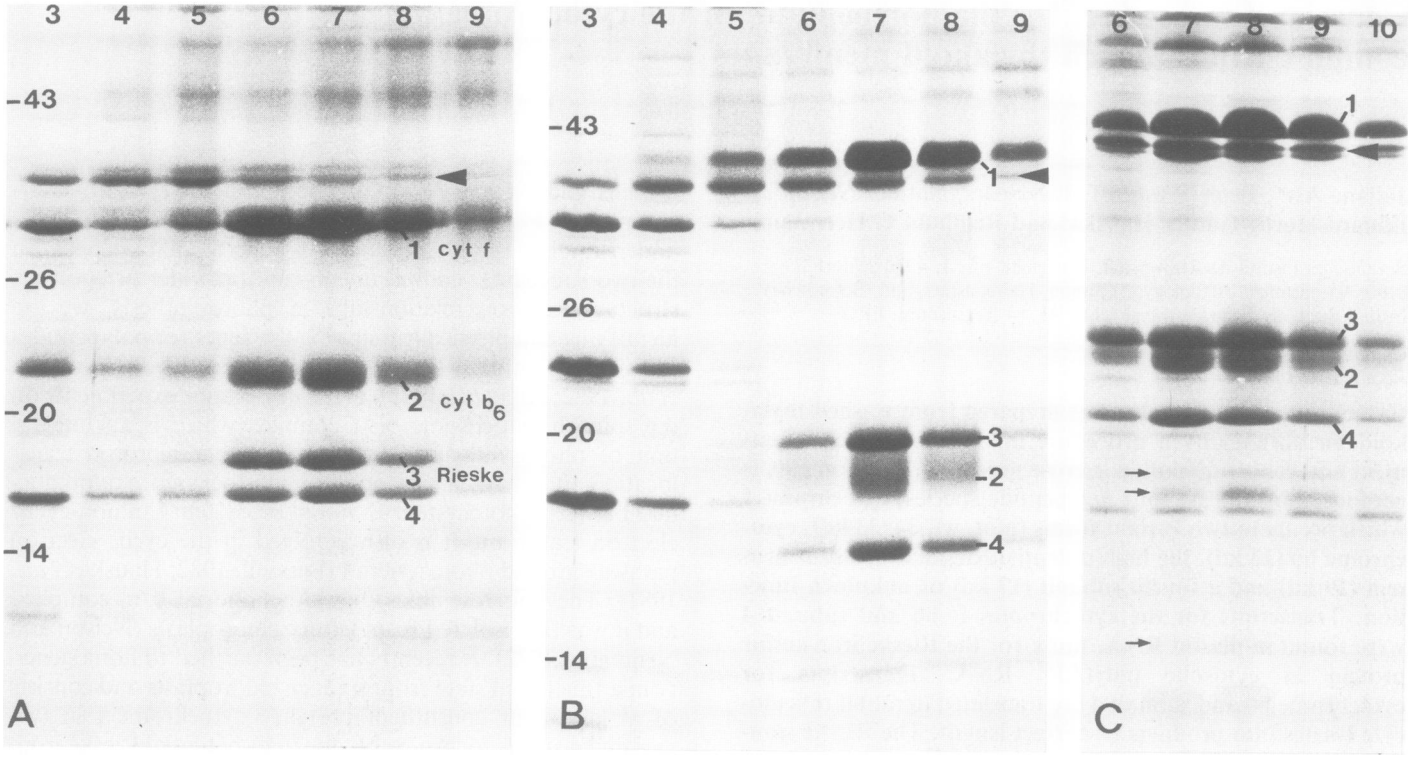

Fig. 1. Purification of cytochrome b6/f complex from spinach thylakoid membranes by zonal centrifugation in continuous sucrose gradients. The gradients were separated into 30 fractions of which aliquots were subjected to electrophoresis in a SDS/12–18% polyacrylamide gradient gel **(panel A)** or a SDS/5 M urea/15% polyacrylamide gel **(panels B** and **C)** and stained with Coomassie Brilliant Blue. **Panel C** illustrates cytochrome complex prepared from thylakoid membranes not washed with NaBr. Low mol. wt. components co-purifying with the complex are marked by an arrow. Fractions from the top of the gradient start on the left. The positions of the four subunits of the complex are indicated in each gel system: cytochrome f (–1; the doublet is not resolved under the chosen conditions), cytochrome b6 (–2), Rieske Fe-S protein (–3), subunit 4 (–4). Mol. wt. standards are ovalbumin (43 kd), chymotrypsinogen (26 kd), soybean trypsin inhibitor (20 kd) and lysozyme (14 kd). The three proteins at 34, 23 and 16 kd in the upper section of the gradient are associated with water splitting activity of photosystem II (Westhoff *et al.*, in preparation). Triangles label an impurity, possibly ferredoxin-NADP$^+$ oxidoreductase.

position of the diffuse zone of cytochrome b6 in Figure 1B. The TMBZ reaction confirmed this conclusion, and it was also shown by electrophoresis of the complex in a broad band horizontal 0–5 M urea/polyacrylamide gradient gel (experiment not shown). Cytochrome f and an impurity, possibly ferredoxin-NADP$^+$ oxidoreductase, behaved similarly (Figure 1).

The antisera against the individual proteins of the complex that were used to identify cell-free translation products were tested for their specificity by immunoblot analysis (Towbin *et al.*, 1979) *versus* both total chloroplast thylakoid proteins and purified cytochrome complex (Figure 2). They were found to be monospecific. The polypeptides of the cytochrome f doublet recognized by f-sera were also separately characterized by partial proteolysis with *Staphylococcus* V8 protease, and found to give indistinguishable oligopeptide patterns (experiment not shown; cf., also Hurt and Hauska, 1982a).

Intracellular origin of transcripts for polypeptides of the cytochrome complex

We have recently shown that all mRNAs studied to date originating in the nucleus are polyadenylated, while those in plastids are characteristically poly(A)$^-$ (Westhoff *et al.*, 1981; Herrmann *et al.*, 1982). Therefore, to analyse the intracellular location of transcripts for components of the cytochrome complex, plastid RNA (ptRNA) and poly(A)$^+$ RNA were translated in an mRNA-dependent cell-free rabbit reticulocyte lysate and the products were compared with the authentic polypeptides by electrophoresis and immunoprecipitation.

When treated with antisera to the Rieske Fe-S polypeptide, the poly(A)$^+$ RNA translation products yield a band of mol. wt. 26 kd (Figure 3), whereas no protein was identified by immunoprecipitation of the translation products from ptRNA, suggesting that this component derives from nuclear genes. The precipitated Fe-S polypeptide is ~7 kd larger than the mature protein. No signal was obtained from poly(A)$^+$ RNA-directed assays with antisera raised against the cytochromes b6, f and subunit 4.

When translation products of ptRNA were fractionated on polyacrylamide gels the fluorograms were dominated by the large subunit of ribulosebisphosphate carboxylase/oxygenase and ATP synthase α- and β-subunits (Westhoff *et al.*, 1981). Immunoprecipitation with cytochrome b6, cytochrome f and subunit 4 antisera gave very faint bands which, although discrete, were too weak to allow unambiguous identification. However, strong evidence that the genes for these three polypeptides are in fact located in the plastome was obtained by translation of hybrid-selected mRNA (Westhoff *et al.*, 1981; Bünemann *et al.*, 1982) in conjunction with transcription-translation in an *Escherichia coli* protein synthesizing system (Alt *et al.*, 1983). The selection of specific mRNAs involved hybridization of total ptRNA to 12 *Sal*I fragments from the entire circular spinach plastid chromosome (Herrmann *et al.*, 1980a) that were bound to Sephacryl S-500. Following their release, the mRNAs were translated in a cell-free system derived from rabbit reticulocytes. Of these, fragments *Sal*I-2/3 (22.3 and 20.5 kbp) which are difficult to

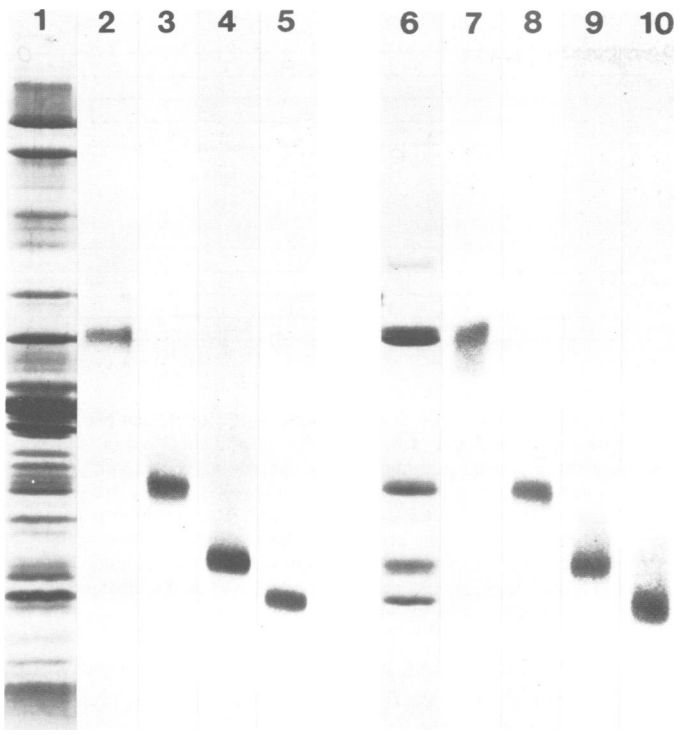

Fig. 2. Specificity test of rabbit antisera directed against constituent polypeptides of spinach cytochrome b6/f complex. Thylakoid membrane proteins or purified cytochrome complex were separated on a SDS/12 – 18% polyacrylamide gradient gel, electrophoretically transferred to nitrocellulose sheets, incubated with individual antisera, followed by radioiodinated *S. aureus* Protein A, and then subjected to autoradiography on Kodak X-Omat AR X-ray film. **Lanes 1** and **6**: Coomassie Brilliant Blue staining of thylakoid membranes and purified cytochrome complex, respectively. Autoradiographs obtained with anti-cytochrome f (**lanes 2** and **7**), anti-cytochrome b6 (**3** and **8**), anti-iron/sulfur protein (**4** and **9**) anti-subunit 4 (**5** and **10**).

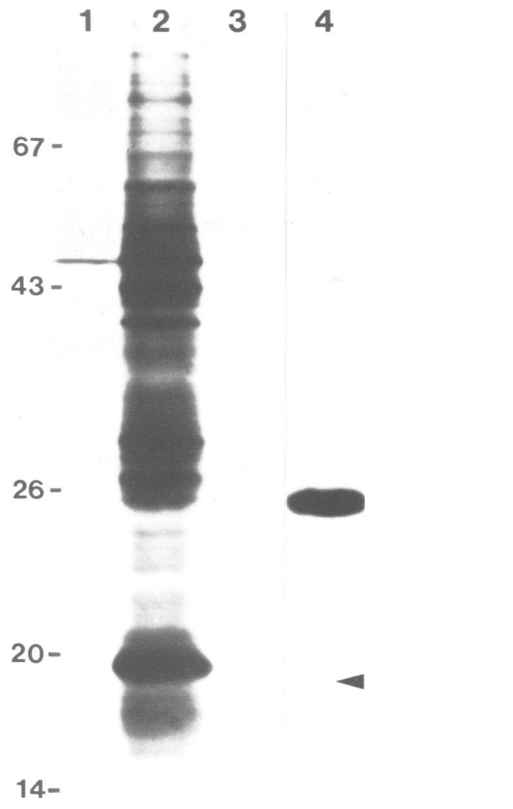

Fig. 3. Analysis of [^{35}S]methionine-labelled products synthesized *in vitro*. A rabbit reticulocyte lysate was primed by total cellular poly(A)$^+$ RNA from spinach leaflets. The labelled products were electrophoretically fractionated on a SDS/10 – 15% polyacrylamide gradient gel and detected by fluorography. **(1)**: background incorporation without added RNA; **(2)** pattern derived from total poly(A)$^+$ RNA. Immunoprecipitates with **(3)** non-immune serum and **(4)** anti-Rieske Fe-S protein. The position of the protein is marked by an arrow. Reference proteins included albumin (67 kd) in addition to those listed in the legend of Figure 1.

resolve electrophoretically, and therefore were combined, selected transcripts for cytochrome f (data not shown). The 5.2-kbp fragment *Sal*I-8 and, on long exposures, the adjacent 5.8-kbp *Sal*I-7, selected transcripts for both cytochrome b6 and subunit 4 (Figure 4, lanes 5 and 8).

Location of the genes for cytochrome b6 and subunit 4

We determined the positions of the genes for cytochrome b6 and polypeptide 4 on *Sal*I-7/*Sal*I-8 by mapping restriction sites for the endonucleases *Xho*I, *Bam*HI and *Eco*RI, subcloning the *Eco*RI and *Bam*HI terminal and partial fragments and using them in hybrid-selection and cell-free translation. The resulting products were fractionated by gel electrophoresis and immunoprecipitation. Figure 5a shows the fine map, arrangement and transcription polarity of the two genes on *Sal*I-8.

Digestion of excised *Sal*I-8 with *Bam*HI generated three fragments of 2.84, 2.06 and 0.3 kbp of which the latter is an internal fragment (identical with primary fragment *Bam*HI-27). The two larger fragments lie at either end of *Sal*I-8 since the plasmid pWHsp 208 DNA digested with this enzyme gave two new fragments of 6.7 and 2.3 kbp instead of the 2.84-and 2.06-kbp fragments. Their arrangement can be deduced from reciprocal digestion of *Bam*HI-15a (2.44 kbp) and *Sal*I-11 (0.6 kbp) obtained from the chimeric DNAs pWHsp 415a and pWHsp 211. Upon digestion with *Sal*I, the former fragment yields the 2.06-kbp fragment derived from *Sal*I-8 in addition to a small fragment of ~200 bp. This small fragment is also obtained upon cleavage of *Sal*I-11 with *Bam*HI. Consequently, the 2.06-kbp *Bam*HI subfragment of *Sal*I-8 is adjacent to *Sal*I-11 and hence the 2.84-kbp *Bam*HI subfragment abutts fragment *Sal*I-7. A similar approach was used to locate *Xho*I and *Eco*RI cleavage sites. *Xho*I cuts *Sal*I-8 into segments of 1300 (identical with fragment *Xho*I-10), 1170, 800, 630 (*Xho*I-16) and 300 (*Xho*I-17) bp, *Eco*RI produces four secondary fragments of 2.38, 2.26, 0.36 and 0.2 kbp (Figure 5a) all of which can be resolved on 1.3% agarose gels.

When *Xho*I, *Bam*HI or *Eco*RI secondary fragments were immobilized on Sephacryl S-500 and used to select mRNAs, all secondary fragments of *Sal*I-8 and the adjacent 1.4-kbp *Sal*I/*Eco*RI secondary fragment of *Sal*I-7 yielded transcripts which could be translated into cytochrome b6 and subunit 4. This finding precluded the assignment of the genes within this stretch of DNA but indicated the existence of a large polycistronic transcript. The order and position of the two genes on *Sal*I-8 were determined by transcription-translation of the parental and derivative chimeric DNAs in the *E. coli* cell-free system (Figure 6). Translation of these DNAs resulted in several polypeptide products (Figure 6, lanes 1, 5, 8 and 11). The parental clones pWHsp 208 gave both proteins in correct size (Figure 6, lanes 3 and 4). Only cytochrome b6 was found with subclone pWHsp 208/B1 (insert the 2.84-

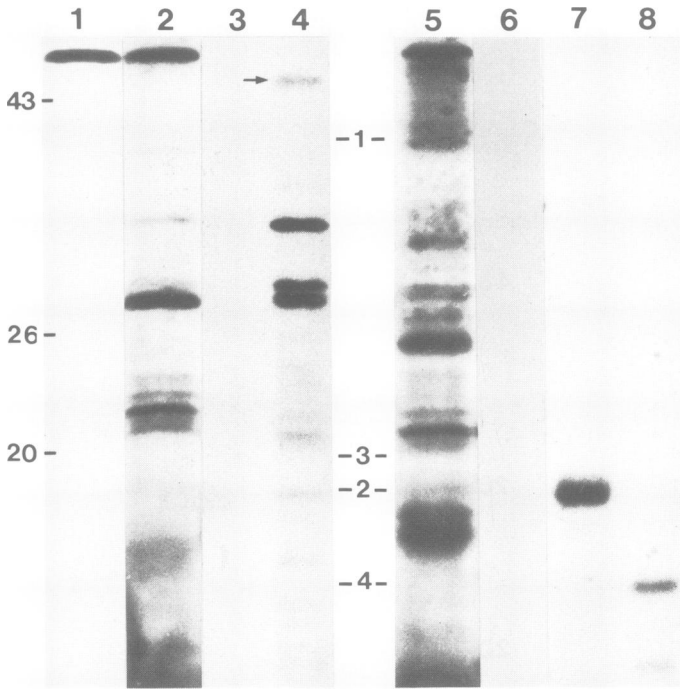

Fig. 4. Selection of transcripts for cytochrome f, cytochrome b6 and subunit 4 by their complementarity to spinach ptDNA. Total ptRNA was hybridized to immobilized fragments *Bam*HI-7 or *Sal*I-8. The [^{35}S]-methionine-labelled products generated by cell-free translation of the released RNA fractions were analysed by immunoprecipitation, electrophoresis in SDS/urea/polyacrylamide gels and fluorography. **(1)** No message control; **(2)** products of RNA selected by *Bam*HI-7 and immunoprecipitates with **(3)** non-immune serum and **(4)** anti-cytochrome f. **(5)** Products of RNA selected by *Sal*I-8 and immunoprecipitates with **(6)** non-immune serum, **(7)** anti-cytochrome b6 and **(8)** anti-subunit 4. The positions of authentic cytochrome f (-1-), cytochrome b6 (-2-), Rieske Fe-S protein (-3-) and subunit 4 (-4-) are given between **lanes 4** and **5**, those of the size standards in kd on the left (cf. Figure 1). The position of the pre-cytochrome f apoprotein is marked by arrow in **lane 4**.

kbp overlap between *Bam*HI-8a and *Sal*I-8; Figure 5; Figure 6, lane 9), and a truncated product of this protein of 10 kd with pWHsp 208/B1 + 3 DNA, that includes the 2.84-plus 0.3-kbp secondary fragments in correct orientation (Figure 6, lane 7). Corresponding data were obtained with the *Eco*RI derivative clones (Figure 5a; data not shown). All bands represent proteins derived from translation of the insert; none of the compounds was produced by the vector alone. Therefore, the DNAs pWHsp 208/B1 + 3 and pWHsp 207/E1 + 4 still contain the intact 5′ end of the gene for subunit 4. The sizes of the truncated products and overlap of the two small internal *Eco*RI and *Bam*HI primary fragments define the boundary of the structural gene and the polarity of its transcription. The small *Eco*RI and *Bam*HI fragments contain ~100 and 280 bp of the 600-bp coding sequence for the protein. (For transcription polarity of the b6 gene see Discussion.)

Location of the gene for cytochrome f

A similar approach was used to map the gene for cytochrome f. The fragments *Sal*I-2/*Sal*I-3, which selected cytochrome f transcripts, cover >40 kbp of DNA. The location of the 1.0-kbp cytochrome gene was further investigated using smaller cloned *Pst*I and *Bam*HI fragments which covered this region (Herrmann *et al.*, 1982). Of these, only the 5.3-kbp fragment *Bam*HI-7 selected cytochrome f message

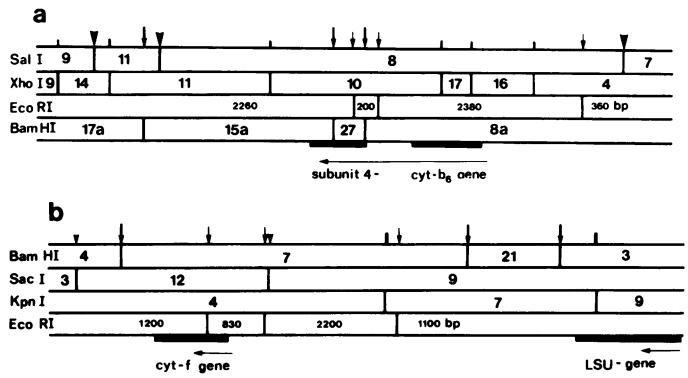

Fig. 5. Restriction fine maps of those segments of the spinach plastid chromosomes bearing genes for cytochrome b6 and subunit 4 **(a)** and cytochrome f **(b)**. Fragments are numbered in descending order (*Sal*I, *Xho*I, *Sac*I, *Kpn*I and *Bam*HI) or designated in bp (*Eco*RI). *Eco*RI cuts are only given for fragments *Sal*I-8 **(a)** and *Bam*HI-7 **(b)**. The positions of the three genes and the location of the gene for the large subunit of ribulosebisphosphate carboxylase/oxygenase **(b;** Herrmann and Possingham, 1980; Whitfeld and Bottomley, 1980) are indicated as well as the direction of their transcription (arrows, cf. Figures 6 and 7).

(Figure 4). This fragment has been shown to be located within fragment *Sal*I-3 between the nearer inverted repeat region and the 3′ end of the gene for the large subunit of ribulosebisphosphate carboxylase/oxygenase (Figure 5b). Transcription/translation of pWHsp 407 DNA suggested that the entire cytochrome f gene is contained within *Bam*HI-7. Immunoprecipitation of the translation products yielded, besides several low mol. wt. peptides, a polypeptide of a higher mol. wt. than both authentic cytochrome f and its apoprotein, suggesting the existence of a precursor form for this protein (Figures 4 and 7).

A restriction map was subsequently constructed for fragment *Bam*HI-7 using *Eco*RI in addition to *Sac*I, *Puv*I, *Xho*I and *Kpn*I, which have been shown to cut in this DNA region (Crouse *et al.*, 1978). The arrangement of these fragments is shown in Figure 5b. Double digestion with *Sac*I plus *Kpn*I gives rise to three well resolvable secondary fragments of 2.1 (*Bam*HI/*Sac*I), 1.9 (*Sac*I/*Kpn*I) and 1.3 kbp (*Kpn*I/*Bam*HI) which were isolated from preparative agarose gels and bound to Sephacryl S-500. All three fragments selected RNA which could be translated into the pre-cytochrome f apoprotein again indicating a transcript of considerable size. The *Eco*RI fragments of 2200, 1200, 1100 and 830 bp of plasmid pWHsp 407 DNA were subcloned by insertion into the *Eco*RI site of pBR325. The new templates, pWHsp/E1, 2, 3 and 4, pWHsp/E1 + 3, pWHsp/E1 + 4 were used in cell-free translation. Neither the first three nor the fifth clone gave a cytochrome signal. However, a truncated product of 29 kd immunoprecipitable with anti-cytochrome f was obtained with pWHsp 407/E4 and pWHsp 407/E1 + 4 DNA (Figure 7, lane 9). This suggests that the structural gene contains one *Eco*RI cleavage site and that transcription proceeds from subfragment E4 (830 bp) towards E2 (1200 bp; Figure 5b). However, the truncated product includes probably translated vector sequences at its 3′ end since its size exceeds the coding potential of fragment E4. The transcription polarity and precise position of the gene have been confirmed by nucleotide sequence analysis (Alt, in preparation). The small 830-bp *Eco*RI fragment contains ~300 bp of the 1.1-kbp pre-cytochrome gene and upstream flanking sequences including a potential RNA polymerase binding site, the adja-

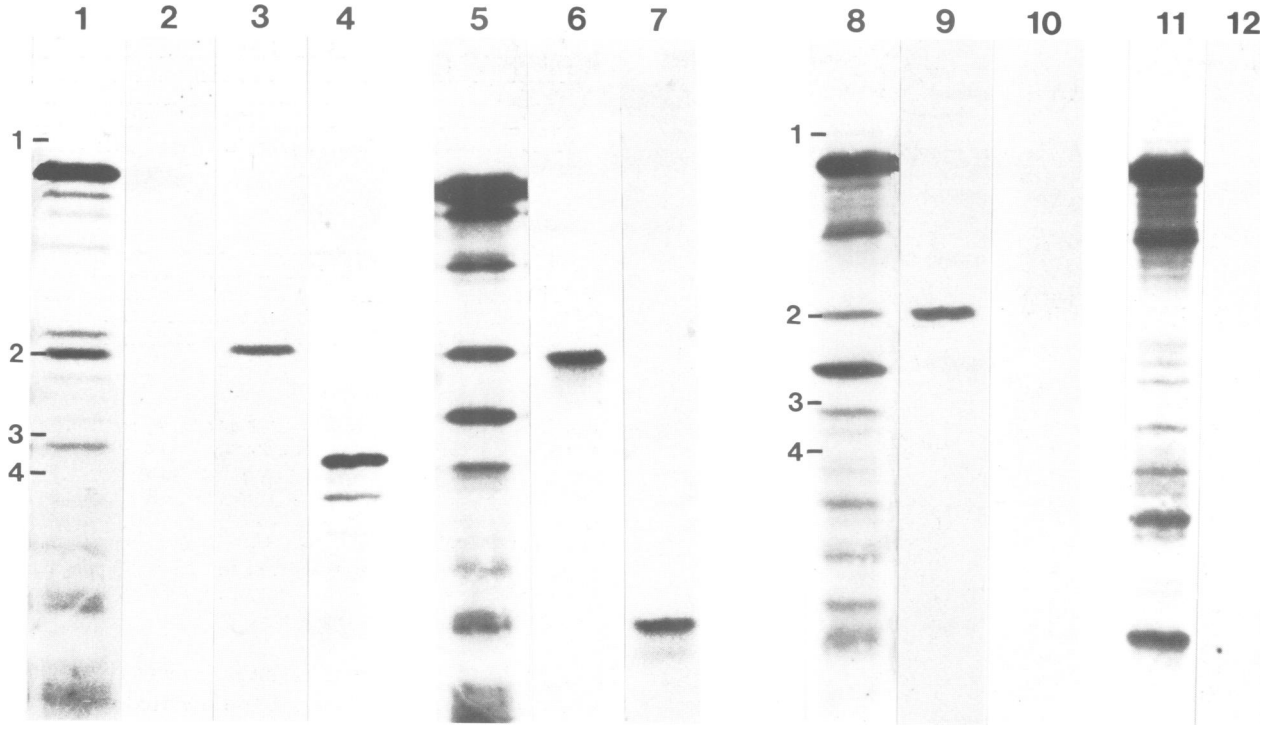

Fig. 6. Fine mapping of the genes for cytochrome b6 and subunit 4 by cell-free coupled transcription/translation of recombinant DNAs in an *E. coli* PR 7 extract. A 2.5 μl aliquot of each 25 μl assay was mixed with 25 μl of 0.125 M Tris/HCl, 10% 2-mercaptoethanol, 2.5% SDS (pH 6.8) and heated at 70°C for 30 s before electrophoresis on a 12–18% polyacrylamide gradient gel. The remaining assay was used for immunoprecipitation with various cytochrome complex antisera. Fluorography of the [^{35}S]methionine-labelled products synthesized *in vitro* by **(lane 1)** plasmid pWHsp 208 and its derivatives **(5)** pWHsp 208/B1 + 3, **(8)** pWHsp 208/B 1 and **(11)** pWHsp 208/B 3. Immunoprecipitates with **(2)** non-immune serum, **(3, 6** and **9)** anti-cytochrome b6, **(4, 7, 10** and **12)** anti-subunit 4. The position of authentic subunits of the cytochrome complex are indicated left of **lanes 1** and **8** (cf. Figure 1).

cent 1.2-kbp *Eco*RI fragment ~800 bp of the 3′ end of the gene.

Discussion

We have relied on two criteria to differentiate between polypeptides derived from the plastid and nuclear genetic compartment: *in vitro* translation of ptRNA and poly(A)$^+$ RNA followed by immunoprecipitation, and on the complementarity of transcripts to ptDNA as in our previous work (Westhoff *et al.*, 1981; Herrmann *et al.*, 1982; Alt *et al.*, 1983). We have used immobilized ptDNA fragments to select discrete mRNAs for *in vitro* translation, in addition to cell-free transcription/translation of recombinant DNA molecules, to locate and characterize the plastid genes for constituent polypeptides of the thylakoid cytochrome complex. The translation products were identified by electrophoretic and immunological criteria, and partial proteolytic digests of these polypeptides.

Immunoprecipitation of poly(A)$^+$ RNA translation products with antisera to the individual cytochrome complex components yields a reacting polypeptide only in the case of the Rieske iron-sulfur protein. The *in vitro* product is a polypeptide which is ~7 kd larger than the mature protein, suggesting that this component originates in the nucleus and is made as a precursor on cytosolic ribosomes as other nuclear-coded chloroplast polypeptides (e.g., Chua and Schmidt, 1979). The observation that this transcript does not hybridize to ptDNA, and also that it programs the synthesis of an *in vitro* product which is processed to the authentic protein during or after transport into isolated chloroplasts reinforces this assumption (unpublished data).

Unequivocal identification of the site of synthesis of the other cytochrome complex polypeptides required an enrichment of their mRNA; poor incorporation, and complex translation patterns in the desired mol. wt. range precluded the assignment initially. This was accomplished by hybrid-selection of mRNA species to immobilized total ptDNA or cloned ptDNA fragments. In like manner, cytochrome f, known to be a product of chloroplast protein synthesis (Doherty and Gray, 1979), cytochrome b6 and subunit 4 were clearly detectable among the translation products of pt-mRNA and poly(A)$^-$RNA (Figure 4). Moreover, all three proteins could be identified among the products of *in vitro* transcription/translation of cloned ptDNA (Figures 6 and 7). The low efficiencies with which transcripts for the cytochrome complex are translated from total ptRNA when compared with the synthesis of other thylakoid polypeptides or of the large subunit of ribulosebisphosphate carboxylase/oxygenase (Westhoff *et al.*, 1981) is most likely due to messenger competition. Northern blots and nucleotide sequencing analysis suggest that cytochrome transcripts are not significantly reduced in quantity nor are they degraded (Bisanz, personal communication), and that each protein contains more than half a dozen methionine residues which permit extensive labelling *in vitro* (Heinemeyer, personal communication).

Cytochrome b6 and subunit 4, as other thylakoid proteins produced by plastid genes, appear to be made as full-length moieties judged by the coincidence of electrophoretic mobilities of the *in vitro* products with those of the authentic protein in several gel systems. These results are in contrast to those for cytochrome f; precipitation with anti-cytochrome f yields a component ~4 kd larger than that of the holoenzyme or of its apoprotein. This suggests that cytochrome f

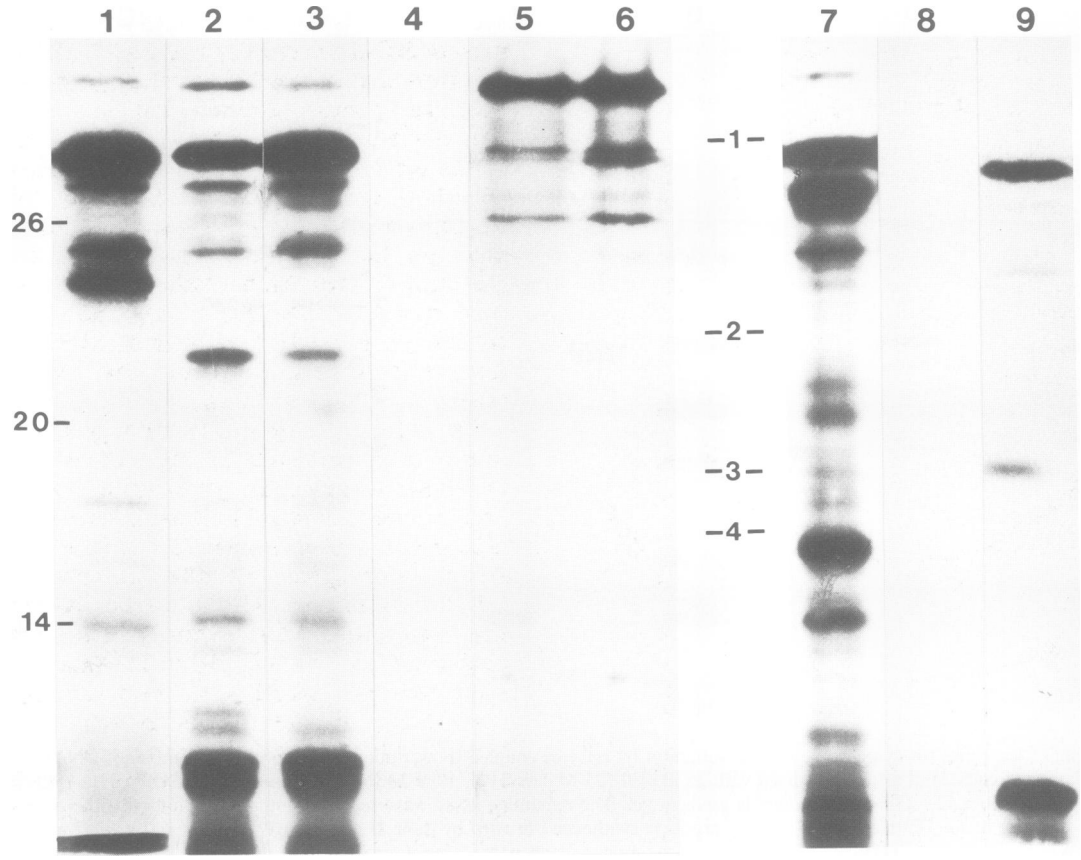

Fig. 7. Physical mapping and polarity of transcription of the pre-cytochrome f apoprotein gene by cell-free coupled transcription/translation of recombinant DNAs in *E. coli* lysates. The sample preparation was identical to Figure 6. Fluorograph of a SDS/12–18% polyacrylamide gradient gel. Lanes correspond to translation products from **(1)** pBR325, **(2** and **3)** pWHsp 407 in two different insert orientations; immunoprecipitates with **(4)** non-immune serum, **(5** and **6)** anti-cytochrome f obtained with the translation patterns shown in **lanes 2** and **3**; **(7)** pWHsp 407/E 1 + 4 corresponding immunoprecipitates with **(8)** non-immune serum and **(9)** anti-cytochrome f. The position of authentic cytochrome f is indicated by -1- between **lanes 6** and **7**. Left: mol. wt. of calibration proteins in kd (cf. Figure 1).

is made as a precursor of higher mol. wt. prior to insertion into the membrane. To date, only one other thylakoid protein originating in plastids, the herbicide-binding '32 kd' protein associated with photosystem II appears to be made as a precursor (Grebanier *et al.*, 1978). The identity, however, of a pre-cytochrome f apoprotein must be confirmed by independent evidence. In spite of the observation that other thylakoid proteins are made as full-length chains and that a pre-cytochrome of the same size is synthesized in both DNA- and RNA-programmed translation assays, the possibility that translational and/or transcriptional initiation and termination signals are not properly recognized in the heterologous systems cannot be ruled out.

Although a single radioactive signal was obtained with cytochrome b6, additional truncated products were observed in the patterns for cytochrome f (Figures 4 and 7) and, to a lesser extent, subunit 4 (Figure 6, but see also Figure 4). Similar shortened products have been previously noted using the rabbit reticulocyte system for the translation of ptRNA (Westhoff *et al.*, 1981; Herrmann *et al.*, 1982). They appear to be caused by premature termination in the heterologous systems perhaps due to inherent properties of the mRNA species. Their appearance is protein-specific, repeatable and independent of the size of the gene product (Westhoff *et al.*, 1981, 1983; Herrmann *et al.*, 1982).

Fine-mapping of the genes for the cytochrome complex on ptDNA by hybrid-selection translation has been limited by the large size of the transcripts. To determine more precisely the position, gene structure, dosage and direction of transcription, we have isolated various recombinant plasmids. The genes for cytochrome f, cytochrome b6 and subunit 4 are obviously expressed in cell-free *E. coli* lysates, suggesting that they are not interrupted by non-coding sequences; the heterologous translation system is not likely to process such transcripts. The transcription polarity of subunit 4, inferred from the size of the truncated products, apparently is not subject to read-through across the vector/insert junction because the same bands could be detected with templates carrying the same insert in opposite orientations. Digestion of pWHsp 207 DNA with *Bam*HI and *Eco*RI permits translation of an incomplete subunit 4 but leaves cytochrome b6 unaffected (Figure 6). Thus, the location of the subunit 4 gene could be determined and the positioning of the cytochrome b6 gene is a consequence of the placement. Nucleotide sequence analysis has disclosed that both genes are separated by 850 bp and derived from the same DNA strand (Heinemeyer, personal communication). Similarly, cell-free synthesis of *Eco*RI-derivative clones of pWHsp 407 DNA indicates that the *Eco*RI junction must separate the 5′ and 3′ regions of the structural gene (Figure 7) whose polarity has been confirmed by nucleotide sequence analysis.

The three genes of the cytochrome complex are located in the large single-copy region of the spinach plastid chromosome (Figure 8). As with other plastome-coded genes for

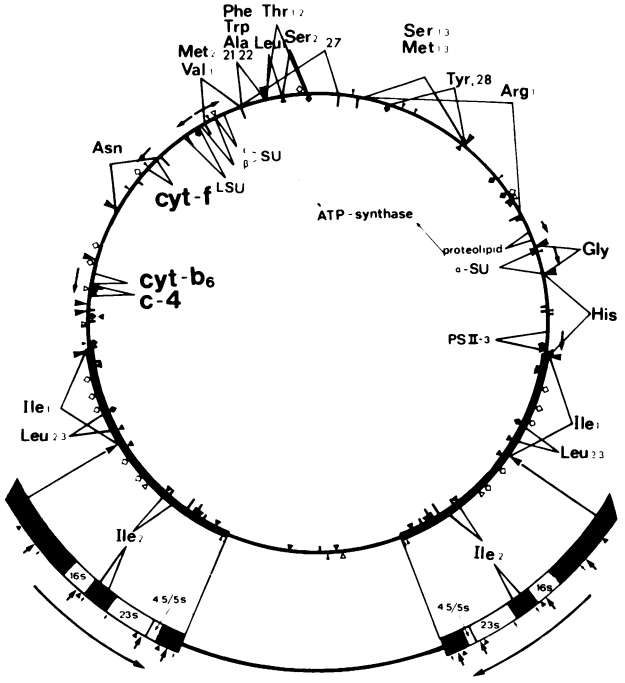

Fig. 8. Positions of the genes for the cytochromes f, b6 and subunit 4 (c-4) on the spinach plastid chromosome. The map has been adapted from Crouse *et al.* (1978), Herrmann and Possingham (1980) and Herrmann *et al.* (1980a). The inverted duplication is indicated by thicker lines and the extended part. The positions of the genes for four ATP synthase subunits (Westhoff *et al.*, 1981; Alt *et al.*, 1983), for the large subunit of ribulose-bisphosphate carboxylase/oxygenase (Herrmann and Possingham, 1980; Whitfeld and Bottomley, 1980), the 32-kd polypeptide associated with photosystem II (PS II-3; Driesel *et al.*, 1980), the two rDNA operons (Whitfeld *et al.*, 1980) and genes for tRNA species (Driesel *et al.*, 1979) are included. Transcription polarities are indicated by arrows.

thylakoid proteins (Herrmann *et al.*, 1982) they are not all clustered. The gene for cytochrome f is ~4.8 kbp from the 3′ end of the gene for the large subunit of ribulosebisphosphate carboxylase/oxygenase (Herrmann and Possingham, 1980; Whitfeld and Bottomley, 1980) and ~15 kbp from the 5′ end of the clustered cytochrome b6/subunit 4 genes. These latter genes are, in turn, 7 kbp from that copy of the inverted repeat that is opposite to the gene for the '32 kd' protein of photosystem II (Driesel *et al.*, 1980; Figure 8). Our data establish that each gene of the cytochrome complex is present in only one copy and is encoded by the same strand of the spinach plastid chromosome. The three genes are transcribed into two RNA species in the same direction as are the genes for the large subunit of ribulosebisphosphate carboxylase/oxygenase, the P_{700} chlorophyll *a* apoprotein of photosystem I reaction center, and for the 51 and 44 kd chlorophyll *a*-conjugated photosystem II proteins. The three genes, however, are of opposite polarity to the genes for the '32 kd' protein associated with photosystem II, and for the ATP synthase subunits α, β, ϵ and proteolipid (Figure 8; Herrmann *et al.*, 1982; Alt *et al.*, 1983).

The detection of a polycistronic transcript for cytochrome b6 and subunit 4 is interesting. Transcripts for both proteins were always obtained even when relatively small fragments were used for hybrid-selection. This co-selection could not be due to cross-contamination since the recombinant DNAs used were isolated from cultures originally obtained from individual colonies. The two polypeptides are probably synthesized, therefore, from a single polycistronic mRNA species which is transcribed from a region of DNA covering most of *Sal*I-8 and running into *Sal*I-7. This organization differs from that of the ATP synthase proteolipid and α genes which also map in the vicinity but apparently are transcribed into discrete RNA species (Alt *et al.*, 1983). However, it recalls the organization of the ATP synthase β and ϵ genes (Westhoff *et al.*, 1981) which overlap by 4 bp, are co-transcribed into a single RNA species (Zurawski *et al.*, 1982) and must, therefore, be decoded in different reading frames. The clustered cytochrome b6/subunit 4 genes fall into yet a third expression class for adjacent genes belonging to one complex; it is the first example of dicistronic transcription from non-overlapping genes. The significance of this organization is, as yet, unclear.

The chloroplast cytochrome complex shares dual genetic origin analogous to that of the corresponding mitochondrial membrane complex (Tzagaloff *et al.*, 1979). Only information for one of the eight polypeptide species of the mitochondrial b/c_1 complex, namely cytochrome b, however, is in a mitochondrial gene. All other components have nuclear origin and are translated on cytosolic ribosomes as precursors which are processed to mature forms upon entry into the organelle (Teintze *et al.*, 1982). The analysis of higher plant cytochrome b/c_1 complex combined with import studies using isolated organelles and nucleotide sequence analysis should allow a more detailed comparison which may reflect the evolutionary relationships of the two organelles, and also aid in the delineation of intracellular routes ensuring that gene products reach their correct destination.

Materials and methods

Etiolation and growth of spinach plants, preparation and restriction of ptDNA, restriction site mapping in agarose sols, isolation of ptRNA, poly(A)$^+$ and poly(A)$^-$ RNA, RNA-programmed cell-free translation in rabbit reticulocyte lysates, DNA-programmed cell-free translation in *E. coli* PR 7 lysates, electrophoresis in agarose or polyacrylamide gels, gel autoradiography and fluorography were carried out as described previously (Herrmann *et al.*, 1980a; Westhoff *et al.*, 1981; Alt *et al.*, 1983). The spinach plants used as a source for RNA were a mixture of etiolated seedlings which had been exposed to 14 h light plus 3–4 week old greenhouse-grown plants. This material should contain substantial amounts of transcripts for thylakoid polypeptides since it was actively synthesizing photosynthetic membranes (Westhoff *et al.*, 1981).

The restriction endonuclease *Xho*I was a generous gift from Dr.P.Seyer (Grenoble). Other enzymes were either prepared according to the protocol of Arrand *et al.* (1978) or obtained from commercial sources (New England Biolabs/Boehringer). Restriction fragments were numbered in the order of decreasing sizes.

Antibodies and immunoassays

Antibodies to the cytochrome f doublet (subunit 1a,b), cytochrome b6 (subunit 2), the Rieske Fe-S protein (subunit 3) and subunit 4 were raised in rabbits from material purified by chromatography (Hurt *et al.*, 1981), by diffusion or by electroelution from ground polyacrylamide gels. Antibody specificity was checked by immunoblot analysis (Towbin *et al.*, 1979).

Recovery of antigen/antibody complexes from immunoprecipitation assays with *Staphylococcus aureus* cells was performed as described previously (Westhoff and Zetsche, 1981) except that the same translation assay was subjected to consecutive precipitations with a series of immune sera. To ensure that no residual antigen/antibody complexes from the preceding precipitation remained after removal of the staphylococcal antibody/antigen complex by centrifugation, the supernatant was shaken for 30 min with 10 μl of a 10% suspension of cells per 0.6 ml of solution and centrifuged. The next antibody was then added to the supernatant and the reaction continued.

Hybrid-selection of ptRNA

The DNA fragments to be used in the hybrid-selection of transcripts were immobilized on Sephacryl S-500 (Bünemann *et al.*, 1982). The diazophenyl thioether derivative of this macroporus material was superior to the previously used Sephadex support both with regard to binding efficiency and selectivity of hybridization.

Preparation of the cytochrome b6/f complex

The thylakoid complex was isolated by the procedure of Hurt and Hauska (1980) generally including treatment with 2 M NaBr in order to remove the ATP synthase (Nelson and Eytan, 1979). Membranes from 1 kg spinach leaves were extracted with octyl-β-D-glucopyranoside and sodium cholate. The complex was recovered from the yellowish 300 000 *g* supernatant by precipitation with $(NH_4)_2SO_4$ (45 – 55%). The precipitate was dissolved in ~3 ml 30 mM Tris-succinate buffer containing 0.2% Triton-X 100, 0.5 mM EDTA, 0.1% soybean asolectin (pH 6.5) and 1.5 ml was applied to a 7 – 30% linear sucrose gradient in 30 mM Tris-succinate, 0.2% Triton X-100 0.5 mM EDTA, 0.1% soybean asolectin, pH 6.5. The gradients were centrifuged at 205 000 *g* for 18 h in a Beckman SW-40 Ti rotor, fractionated and analysed on dissociating polyacrylamide gels. The gels were stained for peroxidase activity with 3,3′,5,5′-tetramethyl-benzidine (to detect cytochromes) employing the procedure described by Thomas *et al.* (1976). Cytochrome f apoprotein was prepared according to Henning and Neupert (1983).

Clones

The plasmids employed in this analysis were selected from a library containing shotgun recombinant DNAs as well as cloned DNAs obtained by ligation of vector with individual fragments as described (Herrmann *et al.*, 1980b, 1982; Alt *et al.*, 1983). The collection spans the entire spinach plastid chromosome and includes overlapping fragments obtained with the enzymes *Sal*I, *Pst*I, *Xho*I and *Bam*HI. The clone nomenclature has been detailed (Herrmann *et al.*, 1982; Alt *et al.*, 1983).

*Eco*RI fragments of the hybrid DNAs pWHsp 208 (insert *Sal*I-8; 5.2 kbp) and pWHsp 407 (insert *Bam*HI-7; 5.3 kbp) which carry genes for polypeptides of the cytochrome complex were subcloned into pBR325 and the desired recombinants selected from amp^r, tet^r, cap^s transformants by colony hybridization (Grunstein and Wallis, 1979) and sizing of the excised insert in agarose gels.

*Bam*HI secondary framents of pWHsp 208 DNA were subcloned in pBR322 in the same manner. Partial digestion products to be used for cloning were purified from gels by electroelution, phenol extraction and ethanol precipitation. Derivative clones were designated on the basis of the parental plasmid including an abbreviation for the consecutive enzyme plus the number of the secondary fragment(s) in order of decreasing size (e.g., pWHsp 208/B1 for the largest secondary fragment obtained after digestion of pWHsp 208 DNA with *Bam*HI). If possible, such clones were denoted according to the primary fragment of the consecutive enzyme. The orientations of the inserts were determined on the basis of known restriction sites in the vector and ptDNA.

After amplification of transformed bacteria, plasmids were purified from cleared lysates by centrifugation in CsCl gradients containing ethidium bromide (Katz *et al.*, 1973).

Experiments involving recombinant DNA were carried out under P2/L2 conditions as specified in the German 'Richtlinien zum Schutz vor Gefahren durch *in vitro* neukombinierte Nucleinsäuren' issue July 1980.

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