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The importance of the transit peptide and the transported protein for protein import into chloroplasts

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Summary. We compared the transport in vitro of fusion proteins of neomycin phosphotransferase II (NPTII) with either the transit peptide of the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase or the transit peptide and the 23 aminoterminal amino acids of the mature small subunit. The results showed that the transit peptide is sufficient for import of NPTII. However, transport of the fusion protein consisting of the transit peptide linked directly to NPTII was very inefficient. In contrast, the fusion protein containing a part of the mature SSU was imported with an efficiency comparable to that of the authentic SSU precursor. We conclude from these results that other features of the precursor protein in addition to the transit peptide are important for transport into chloroplasts. In order to identify functional regions in the transit peptide, we analyzed the transport of mutant fusion proteins. We found that the transport of fusion proteins with large deletions in the aminoterminal, or central part was drastically reduced. In contrast, duplication of a part of the transit peptide led to a marked increase in transport.

Key words: Chloroplast protein transport – Mutants – NPTII fusions – Small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase – transit peptide

Introduction

The plant cell contains several membrane-bound organelles, the most conspicuous of which is the chloroplast. Most of the proteins of the chloroplast are encoded in nuclear DNA and synthesized in the cytoplasm. One example of a nuclear-encoded chloroplast protein is the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase. This chloroplast protein is synthesized in the cytoplasm as a higher molecular weight precursor possessing an aminoterminal extension, termed the transit peptide (Dobberstein et al. 1977). Transport of the SSU precursor (pSSU) is a post-translational (Chua and Schmidt 1978) and an energy-dependent process (Grossman et al. 1980). During or after transport of pSSU into the chloroplast, the transit peptide is removed enzymatically to yield the mature form of SSU (Ellis 1981; Schmidt and Mishkind 1986).

A fusion protein consisting of the transit peptide and the 23 aminoterminal amino acids of the mature SSU fused to neomycin phosphotransferase II (NPTII) is imported by chloroplasts in vivo (Schreier et al. 1985). Van den Broeck et al. (1985) have shown that a fusion of the transit peptide and NPTII is imported by chloroplasts both in vivo and in vitro. From their results, Van den Broeck et al. (1985) suggested that the transit peptide is sufficient for transport of a foreign protein into the chloroplast. However, because the transport efficiency of this fusion protein was not compared to that of either the authentic pSSU or the fusion protein of Schreier et al. (1985) it is still possible that other parts of the precursor play a role in transport.

In order to determine whether parts of mature SSU are important for chloroplast import, we compared the transport of two different NPTII fusion proteins and authentic pSSU into isolated intact chloroplasts. As was done previously by Van den Broeck et al. (1985), we assayed NPTII activity to detect the NPTII fusion proteins. A more quantitative and reliable comparison of transport efficiencies was obtained by using radioactively labeled precursor protein synthesized in vitro. We show that, although the transit peptide alone is sufficient to direct a foreign protein into the chloroplast, the mature SSU, or parts thereof, significantly influence transport.

Processing of pSSU has been studied intensively. Incubation of pSSU with partially purified processing enzyme or with chloroplast stroma fractions results in an intermediate. Based on this result, Robinson and Ellis (1984) proposed that maturation of the pSSU occurs in two steps. *Chlamydomonas* pSSU is imported and partially matured by pea and spinach chloroplasts (Mishkind et al. 1985). Cleavage of the *Chlamydomonas* preprotein occurs within a region of the transit peptide which contains the first processing site. This region is conserved among higher plant and algal SSU precursors. Studies in which specific amino acids of pea pSSU were substituted with amino acid analogs have shown that the presence of at least a single proline, arginine and leucine in the precursor protein is essential for both import into chloroplasts and processing by the purified processing enzyme (Robinson and Ellis 1985).

In order to identify which parts of the transit peptide are required for transport, we introduced mutations into the transit peptide and studied the transport of the mutant proteins in vitro. The use of restriction enzymes rather than exonuclease digestion allowed us to create several overlapping deletions and one insertion. The NPTII fusion protein that has the transit peptide attached directly to NPTII was used in order that the function(s) of the transit peptide

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might be studied in the absence of any complex interactions which may exist between the transit peptide and the mature protein. The results show that, although mutants having large deletions in the aminoterminal halves of the transit peptide are transported, their import is severely impaired. Interestingly, some of these mutants lack portions of the conserved region which is reputed to contain the first processing site (Mishkind and Schmidt 1986). In contrast to the decreased import observed for the transit peptide deletion mutants, transport of a fusion protein with a partial duplication of the aminoterminal region of the transit peptide was improved markedly.

Materials and methods

DNA manipulations. DNA manipulations and gel electrophoresis were performed essentially as described by Maniatis et al. (1982). Small scale and large scale isolation of plasmids from *Escherichia coli* was carried out according to Birnboim and Doly as modified by Ish-Horowitz (Maniatis et al. 1982). DNA sequencing was performed according to the method of Maxam and Gilbert (1980).

Plasmid constructions of genes with wild-type transit peptide. To obtain the fusion proteins TPNPTII and TPSSNPTII, the intermediate plasmids described below were used. The gene encoding TPNPTII was constructed by assembling portions of plasmids pTP2, pKM109/15 (Reiss 1982) and ptac12/Hind. Plasmid pTP2 was derived from pTP1 which carries the *Eco*RI-*Sph*I fragment of pPSR6 (Cashmore 1983) that codes for the promoter and transit peptide of SSU in pBR327 (Soberon et al. 1980). pTP1 was linearized by digestion with *Sal*I, the ends of the fragments were filled in using the large fragment of DNA polymerase I and ligated with an equimolar mixture of *Bam*HI (sequence CGGATCCG) and *Sph*I (sequence GGCATGCC) linkers. The ligation products were digested with *Sph*I and the ends of the plasmid were ligated together. The sequence of the resulting polylinker (Fig. 1) was confirmed by DNA sequence analysis. To construct ptac/Hind, plasmid ptac12 (Amann et al. 1983), which contains the hybrid *tac* promoter, was restricted at the unique *Pvu*II site and a *Hin*dIII linker (sequence CAAGCTTG) was attached. pKm109/15 contains the NPTII gene with an upstream *Bam*HI site. To construct pTPK1 carrying the assembled TPNPTII gene, an *Eco*RI-*Bam*HI vector fragment from pKM109/15 was ligated with the *Hin*dIII-*Bam*HI fragment of pTP2 that contains the transit peptide coding sequence and the *Eco*RI-*Hin*dIII fragment of ptac12/Hind that carries the *tac* promoter. The plasmid pSSKM3 which encodes TPSSNPTII consists of a *Sph*I-*Sal*I fragment of pTPK1 as vector, ligated with a *Sph*I-*Sau*3AI fragment which contains the coding sequence of the 23 amino acids from mature SSU from pSS15 (Broglie et al. 1981; Coruzzi et al. 1983) and a *Bam*HI-*Sal*I fragment which contains the NPTII gene from pKM109/9 (Reiss et al. 1984b).

The plasmids ptac/TPNPTII and ptac/TPSSNPTII were derived from pTPK1, pSSKM3, and pOLI. The *Eco*RI-*Eco*RV fragment from pTPK1 or pSSKM3 was exchanged with the analogous fragment from pOLI. The intermediate vector pOLI which introduced an oligonucleotide containing the model ribosome binding site was constructed in the following manner. ptac12 was linearized with *Pvu*II and ligated with two non-phosphorylated complementary synthetic oligonucleotides (sequences 5′ CTGATGGCTTCTATGATATC and 5′ GATATCATAGAAGCCATCAG, provided by the University of Arizona oligonucleotide synthesis facility) in molar excess. Integration of the oligonucleotides was verified by digestion with *Eco*RI and *Eco*RV and with *Eco*RI and *Pvu*II. To verify the structure of the oligonucleotide junction sequences, the nucleotide sequence of this region of ptac/TPNPTII was determined.

Plasmids pSP64/TPNPTII and pSP64/TPSSNPTII were obtained by inserting the *Hin*dIII-*Sal*I fragments containing the hybrid genes from pTPK1 and pSSKM3, respectively, into the polylinker of pSP64 (Melton et al. 1984). Plasmid pSP64/SSU was assembled from different intermediates and in various steps. The gene construction consists of a *Hin*dIII-*Sma*I vector fragment derived from pSP64/TPSSNPTII, the *Hin*dIII-*Sph*I fragment encoding the transit peptide (derived from pPSR6), the *Sph*I-*Kpn*I fragment encoding the aminoterminal part of the SSU from pSS15, and the *Kpn*I-*Hpa*I fragment carrying the carboxyterminal part of the SSU gene from pPSR6. To obtain authentic NPTII from SP6-transcription and translation in wheat germ extracts, the *Bgl*II-*Sal*I fragment which contains the NPTII gene from pKM2 (Beck et al. 1982) was ligated with pSP65 (Melton et al. 1984) which had been digested with *Bam*HI and *Sal*I.

Construction of plasmids with mutant transit peptides. Restriction endonucleases were used to construct mutants in the coding sequence of the transit peptide in the following manner. A *Bst*XI-*Sal*I fragment which contains the transit peptide coding sequences from pTP2 was isolated and subsequently restricted by one of the following enzymes: *Eco*RV, *Hin*fI, *Nla*IV, or *Hae*III. The ends of the fragments generated by digestion with *Hin*fI were filled in using *E. coli* DNA polymerase large fragment. Mixtures of fragments produced by each enzyme were restricted with *Hin*dIII and *Bam*HI. To produce the various mutants, appropriate restriction digests were mixed and ligated to a *Hin*dIII-*Bam*HI vector fragment derived from pTPK1. The ligation mixtures were used to transform cells of *E. coli* (strain 71-18, Messing et al. 1977). Cells carring recombinant plasmids were selected on ampicillin and subsequently tested for their ability to grow on kanamycin. The correct structure of the mutations was confirmed by digestion with restriction endonucleases. Plasmids ptac/PNi6/25 and ptac/PNd26/35 were derived by cloning *Eco*RV-*Bam*HI fragments containing the mutant transit peptide sequences into a vector fragment produced from ptac/TPNPTII by digestion with *Eco*RV and *Bam*HI. Plasmids ptac/PNd6/25 and ptac/PNd6/29 were derived directly from ptac/TPNPTII by ligating the vector which had been digested with *Eco*RV and *Bam*HI with either a *Nla*IV-*Bam*HI or a *Hae*III-*Bam*HI fragment.

All SP6 vector derivatives were obtained by cloning the *Hin*dIII-*Sal*I fragments that contain the entire mutant gene of the intermediate plasmids into pSP64. DNA sequence analysis was used to verify that the SP6 derivatives of the mutants had the correct structure.

Expression in E. coli. Cultures of *E. coli* (strain 71–18, Messing et al. 1977) harboring the various plasmids were grown overnight in Luria-Bertani (LB) medium containing 100 μg/

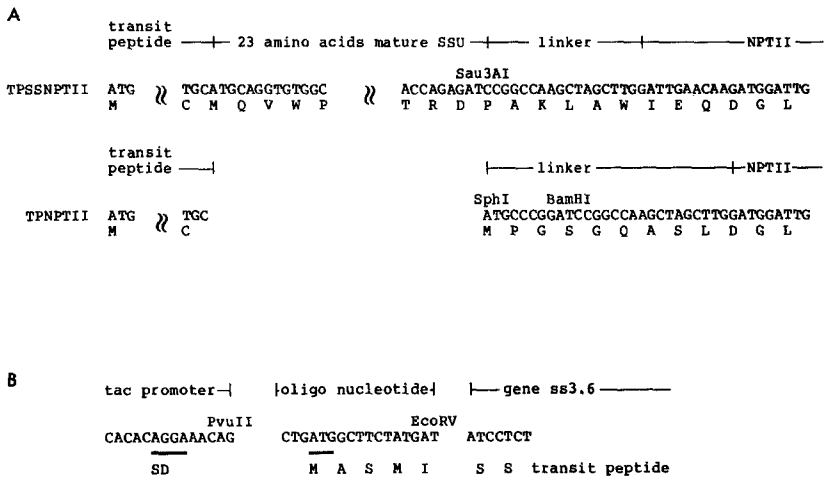

Fig. 1. A Junction sequences of the different gene constructions. **B** Sequence of the model ribosome binding site used for expression in *Escherichia coli*. The derived amino acid sequence is shown below the DNA sequence. Restriction endonuclease recognition sites pertinent for the gene construction are indicated. The gene fragments or synthetic oligonucleotide sequences from which the chimeric genes were derived are identified. The Shine and Dalgarno (SD) sequence (Shine and Dalgarno 1974) and the initiation codon of the transit peptide are *underlined* in **B**

ml ampicillin. For expression of the cloned genes, the cells were diluted 1/100 in LB medium containing ampicillin and the inducer IPTG (2 mM, isopropyl β-D-thiogalactoside) and grown to late mid-log phase. The cells were concentrated 20-fold by centrifugation. Crude lysates were prepared by probe sonication in 50 mM Hepes, pH 8, 110 mM K acetate, 0.8 mM Mg acetate. The cell lysates, cleared of cellular debris by centrifugation at 15,000 g for 1 min, were used directly for uptake.

Expression in vitro. SP6 transcripts were synthesized and capped essentially as described by Krieg and Melton (1984) with the exception that the DNase treatment was omitted. Plasmid DNA purified on CsCl gradients was linearized with *SalI* and the templates were transcribed with SP6 polymerase in the presence of RNasin. The RNA transcripts were capped with vaccinia virus guanylyl transferase in the presence of RNasin. The capped synthetic mRNA was translated in wheat germ extracts (typically 30 µl reaction volumes) in the presence of ^{35}S-methionine as specified by the supplier (Bethesda Research Laboratories, Gaithersburg, Maryland). Pea poly(A)$^+$ RNA was obtained as described by Schreier et al. (1985).

In vitro uptake. The in vitro transport experiments, protease treatment, and fractionation were performed essentially as described by Bartlett et al. (1982). Intact chloroplasts were isolated from 10- to 14-day-old pea plants (Ferry Morse, Progress No. 9) using the Percoll gradient technique. Each incubation mixture for uptake contained either 100 µl of *E. coli* cell lysate or 20 µl of wheat germ extract in a standard reaction as described by Bartlett et al. (1982). The mixture was incubated in the light for 1 h. The chloroplasts were recovered by centrifugation, washed twice, and resuspended in a total volume of 80 µl. Treatment of the chloroplasts with exogenous protease was performed in the following manner. The chloroplasts (40 µl) were incubated with trypsin and chymotrypsin (312 µg/ml each) for 15 min in ice water. The protease digestion was terminated as described.

Protein gels and enzymatic assay. NPTII assays were performed as described by Reiss et al. (1984a). SDS-polyacrylamide gel electrophoresis (15% gels) was performed as described by Laemmli (1970) and fluorography was performed as described by Chamberlain (1979).

Results

Constructions with wild-type transit peptide

Two basic fusion proteins were used in these experiments (Fig. 1A). The first fusion protein, TPNPTII, has the transit peptide sequence from the pea SSU gene ss3.6 (Cashmore 1983) connected to the NPTII gene via a short linker. This linker differs from that used by van den Broeck et al. (1985) to obtain an analogous fusion protein. The second fusion protein, described by Schreier et al. (1985), contained the 23 aminoterminal amino acids of mature SSU between the transit peptide and NPTII. However, the gene construction used by Schreier et al. retained the intron located between the codons specifying the second and third amino acids of mature SSU in the gene ss3.6. To obtain our fusion protein, TPSSNPTII, the intron was removed by combining genomic and cDNA (Broglie et al. 1981; Coruzzi et al. 1983) sequences. Similarly, an authentic SSU precursor lacking intervening sequences was obtained by combining cDNA and genomic sequences (see Materials and methods).

Comparison of transport of the fusion proteins TPNPTII and TPSSNPTII by enzymatic activity

TPNPTII and TPSSNPTII were expressed in *E. coli* cells under the control of the inducible *tac* promoter (Amann et al. 1983). A synthetic oligonucleotide was used to provide a model ribosome binding site (Fig. 1B). Precursor proteins and NPTII were obtained as crude lysates of the *E. coli* cells. Authentic NPTII was obtained from cells carrying pKM2 (Beck et al. 1982).

Transport experiments were performed by incubating isolated intact chloroplasts with the preproteins (Bartlett et al. 1982). After the incubation period, the chloroplasts were recovered and the extracts assayed for NPTII activity. For this purpose, the proteins were separated on a nondenaturing polyacrylamide gel and NPTII enzymatic activity was detected by phosphorylation of kanamycin in situ (Reiss et al. 1984a). Radioactive kanamycin phosphate was visualized by autoradiography (Fig. 2). The enzymatic activities obtained with the precursor proteins in the *E. coli* extracts are presented in lanes 1 to 3. The transport experiment is presented in lanes 4 to 9. Whereas TPSSNPTII activity appeared as a single relatively weak spot, TPNPTII showed a spot of high activity that migrated slightly faster

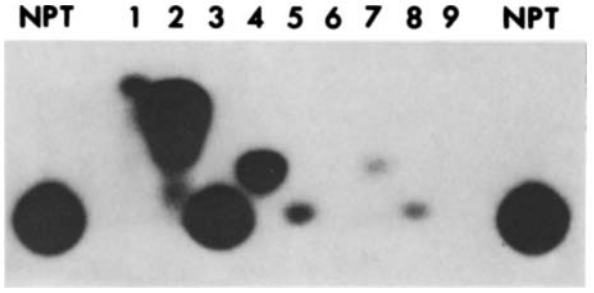

Fig. 2. Protein transport analyzed by assaying neomycin phosphotransferase II (NPTII) activity. Protein extracts were separated on non-denaturing polyacrylamide gels and NPTII enzymatic activity was determined in situ. Extracts of precursor proteins: TPSSNPTII (lane 1), TPNPTII (lane 2), NPTII (lane 3). Chloroplasts before (lanes 4, 5, 6) and after (lanes 7, 8, 9) protease treatment: TPSSNPTII (lanes 4, 7), TPNPTII (lanes 5, 8) and NPTII (lanes 6, 9). NPTII standards (NPT) were derived from pKM2 extracts

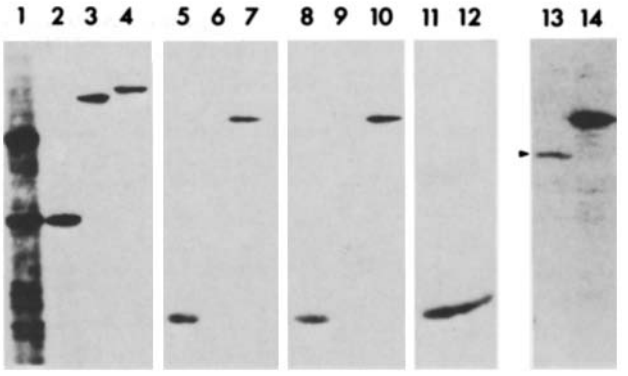

Fig. 3. Transport of labeled precursor proteins. Precursors obtained in vitro from pea poly(A)$^+$ RNA (lane 1), pSP64/SSU (lane 2), pSP64/TPNPTII (lane 3) and pSP64/TPSSNPTII (lane 4). Chloroplasts before (lanes 5, 6, 7, 11) and after (lanes 8, 9, 10, 12, 13, 14) protease treatment: SSU, lanes 5, 8; TPNPTII, lanes 6, 9; TPSSNPTII, lanes 7, 10. Transport of precursor obtained from pea poly (A)$^+$ RNA-primed wheat germ extracts, lanes 11 and 12. Lanes 13 and 14 are identical to lanes 9 and 10, with the exception that a longer exposure is shown

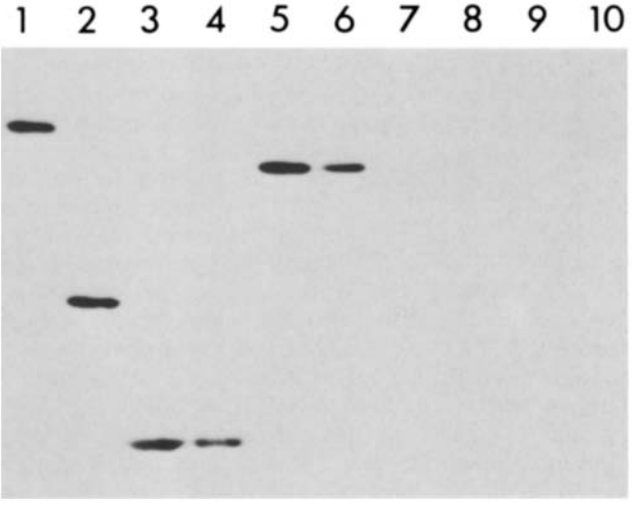

Fig. 4. Fractionation of chloroplasts after uptake of labeled precursors. Precursors: TPSSNPTII (lane 1) and SSU (lane 2). Stroma fractions of chloroplasts incubated with pSSU: chloroplasts without (lane 3) and with protease treatment (lane 4). Stroma fractions of chloroplasts incubated with TPSSNPTII: chloroplasts before (lane 5) and after (lane 6) protease treatment. Membrane fractions from chloroplasts incubated with SSU precursor: chloroplasts without (lane 7) and with (lane 8) protease treatment. Membrane fractions of chloroplasts incubated with TPSSNPTII: chloroplasts without (lane 9) and with (lane 10) protease treatment

than TPSSNPTII. We assume that the difference in the activities observed reflects differences in the expression of the two fusion proteins in *E. coli*. In addition, two minor spots of activity that migrated faster than the major TPNPTII spot are visible. These minor spots correspond to proteins that were also visible in autoradiograms of extracts of *E. coli* cells labeled with ^{35}S-methionine (data not shown). We conclude that these minor spots are unspecific degradation products of the precursor protein produced in *E. coli*.

Extracts from chloroplasts incubated with TPSSNPTII showed a strong signal at a position intermediate between authentic NPTII and TPSSNPTII. A weaker signal at the position of authentic NPTII was observed in extracts of chloroplasts which had been incubated with TPNPTII. This result shows that both fusion proteins were successfully transported. Authentic NPTII offered to chloroplasts in amounts similar to TPNPTII was not imported.

To confirm transport of TPNPTII and TPSSNPTII, the chloroplasts were treated with protease after uptake (Bartlett et al. 1982). Both proteins were inside the chloroplast, as shown by their resistance to protease digestion. However, some decrease in NPTII activity was observed after the protease treatment. This decrease, which is most obvious in Fig. 2 with TPSSNPTII, was observed only when uptake was analyzed under the non-denaturing conditions necessary for NPTII assays. The reduction in activity results from protease digestion during sample processing under native conditions. Consistently, the effect was more pronounced on TPSSNPTII, possibly because this fusion protein is a suitable target for the protease. Therefore, the results obtained before protease treatment more accurately reflect the transport efficiencies of these proteins. Both TPNPTII and TPSSNPTII were transported into chloroplasts while NPTII, which lacks a transit peptide, was not imported by chloroplasts. However, the two fusion proteins appeared to be transported with drastically different efficiencies. Because the specific activity of NPTII fusion proteins depends on the nature of the aminoterminal extension (Reiss et al. 1984b), the differences in NPTII activity which we observed could reflect the differences in sequence between TPNPTII and TPSSNPTII. To resolve this point, we measured import of radioactively labeled precursor proteins, a method which allows a more accurate quantitation of transport since it does not depend on enzymatic activities.

Comparison of transport of pSSU, TPNPTII and TPSSNPTII using radioactively labeled preprotein

In order to obtain radioactively labeled preproteins, the chimeric genes coding for TPNPTII, TPSSNPTII and pSSU were modified to allow transcription by SP6 polymerase. The modified plasmids pSP64/TPNPTII, pSP64/TPSSNPTII and pSP64/SSU were transcribed in vitro, the synthetic mRNA was capped with guanylyl transferase (Krieg and Melton 1984), and translated in wheat germ

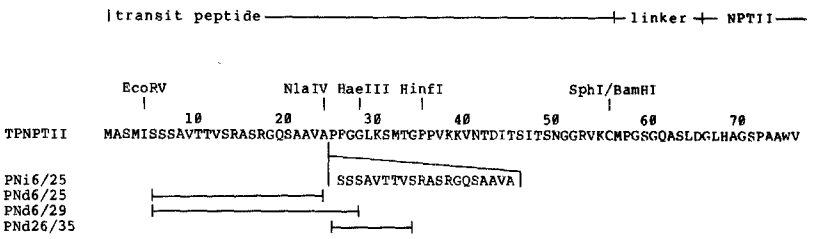

Fig. 5. Schematic representation of mutations in the transit peptide of TPNPTII. The derived amino acid sequence of the transit peptide and the junction between the transit peptide and neomycin phosphotransferase II (NPTII) are shown. The locations of the restriction endonuclease sites referred to in the text are indicated. Deletions are shown as *bars*. The sequence of the partial duplication in the transit peptide is shown

extracts (Bartlett et al. 1982) in the presence of ^{35}S-methionine. Translation of pSP64/SSU RNA yielded a polypeptide identical to that obtained from translation of pea leaf poly(A)$^+$ RNA.

The radioactive precursors were incubated with isolated intact chloroplasts and extracts of chloroplasts analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. The TPSSNPTII protein was transported in amounts comparable to the authentic pSSU (Fig. 3). Import of TPNPTII, which lacks the 23 amino acids from the mature SSU, was detectable only after long exposure times (compare Fig. 3, lanes 9 and 13). Transport of pSSU translated from SP6 RNA was identical to that translated from pea poly(A)$^+$ RNA. NPTII synthesized in vitro was not imported by chloroplasts (data not shown). Resistance of the labeled proteins to externally added protease confirmed that these proteins were inside the chloroplast.

The transported proteins were smaller than the precursors. The apparent molecular weights observed for the mature forms of pSSU and TPNPTII were as expected for the correctly processed proteins. As molecular weight markers NPTII made in bacteria, and the precursor and processed forms of SSU synthesized from pea poly(A)$^+$ RNA were used (data not included). However, the product of processed TPSSNPTII migrated with an apparent molecular weight which was higher than would be expected if 23 amino acids were added to NPTII. Although a gel artifact cannot be excluded, this aberrant migration seems to reflect incorrect processing. The main argument for incorrect processing at present is that only the processed TPSSNPTII behaves abnormally. Concerned that this abnormality might indicate failure of this protein to reach its expected destination, the chloroplast stroma, we separated post-uptake chloroplasts into membranes and stroma. The processed form of TPSSNPTII was found only in the stroma fraction (Fig. 4). From this result we conclude that the TPSSNPTII cleavage product reaches the destination expected of a protein with an SSU transit peptide. Occasionally, additional polypeptides which migrated faster than the TPSSNPTII cleavage product were observed in chloroplasts incubated with TPSSNPTII (see for example Fig. 7B, lanes 6 and 9). Because the appearance of these proteins was independent of treatment with external protease, we conclude that these proteins are either unspecific degradation products of the imported protein or processing intermediates.

In order to exclude the possibility that the inefficient transport of TPNPTII was due to degradation of the preprotein in the uptake mixture, we examined the incubation medium after removal of the chloroplasts. Whereas very little pSSU or TPSSNPTII remained in the medium, TPNPTII was present at nearly its initial concentration (data not shown). We found no evidence that degradation of the precursor occurred during the incubation with the chloroplasts.

Based on data obtained from several experiments we estimated the uptake efficiencies of the various preproteins. Approximately 50% of the offered pSSU and TPSSNPTII molecules were routinely recovered with the chloroplasts. However, based on the small amount of precursor remaining in the incubation mixture after transport, the actual transport efficiencies may be higher. The difference between the two estimates is probably due to the loss of chloroplasts during the treatment after the uptake. In contrast to the efficient import of TPSSNPTII, the transport of TPNPTII was very inefficient. We estimate that the transport efficiency of TPNPTII was 10%, or less, of that of pSSU or TPSSNPTII.

Construction of mutants in the transit peptide of TPNPTII

Mutants in the transit peptide were constructed using restriction endonuclease sites within the coding region of the transit peptide of TPNPTII. Three deletion mutants and one insertion mutant were obtained by combining restriction fragments to create changes within the transit peptide. The derived amino acid sequences of these mutants together with a map of the restriction endonuclease sites used in their construction are shown in Fig. 5.

Analysis of transit peptide mutants using enzymatic activity

Precursor protein was obtained from *E. coli* cells transformed with plasmids, ptac/PNd6/25, ptac/PNd6/29, ptac/PNd26/35, or ptac/PNi6/25, which have the mutant genes under control of an expression unit consisting of the *tac* promoter and the oligonucleotide ribosome binding site described earlier. Mutant precursor proteins were incubated with isolated intact chloroplasts and transport was monitored by assaying NPTII activity (Fig. 6). The NPTII activity migrating at the position of the precursor protein was sensitive to treatment with externally added protease and is probably precursor bound to the outer surface of the chloroplasts. The second activity migrated indistinguishably from authentic NPTII, the product expected for the imported and processed form of TPNPTII. This faster-migrating NPTII-like activity was resistant to protease and thus represents protein residing within the chloroplast. Taking the NPTII-like activities as a measure of transport, uptake of the insertion mutant PNi6/26 appears comparable to that of the wild-type preprotein, TPNPTII. However, shorter exposures than those presented reveal that this insertion actually improves transport. Compared to TPNPTII (Fig. 2), the transport of the deletion mutants PNd6/29 and PNd26/35 was reduced drastically. Uptake of mutant PNd6/25 was not detectable.

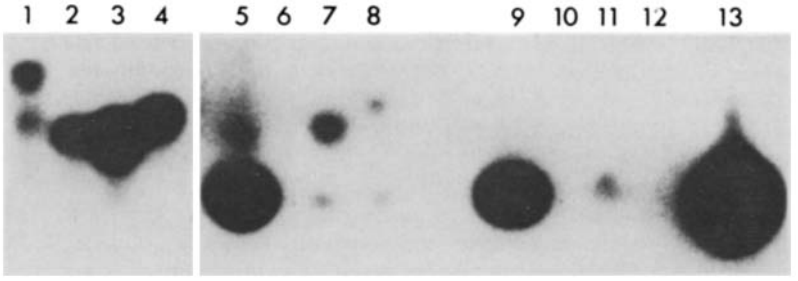

Fig. 6. Transport of precursors with mutant transit peptides. Proteins present in extracts of chloroplasts and cells of *Escherichia coli* were separated on non-denaturing polyacrylamide gels and neomycin phosphotransferase II (NPTII) enzymatic activity was determined in situ. The activities of the precursors are shown in lanes 1 (PNi6/25), 2 (PNd6/25), 3 (PNd6/29) and 4 (PNd26/35). Chloroplasts before (lanes 5, 6, 7, 8) and after (lanes 9, 10, 11, 12) protease treatment: PNi6/25 (lanes 5, 9), PNd6/25 (lanes 6, 10), PNd6/29 (lanes 7, 11) and PNd26/35 (lanes 8, 12). An NPTII standard derived from pKM2 is shown in lane 13

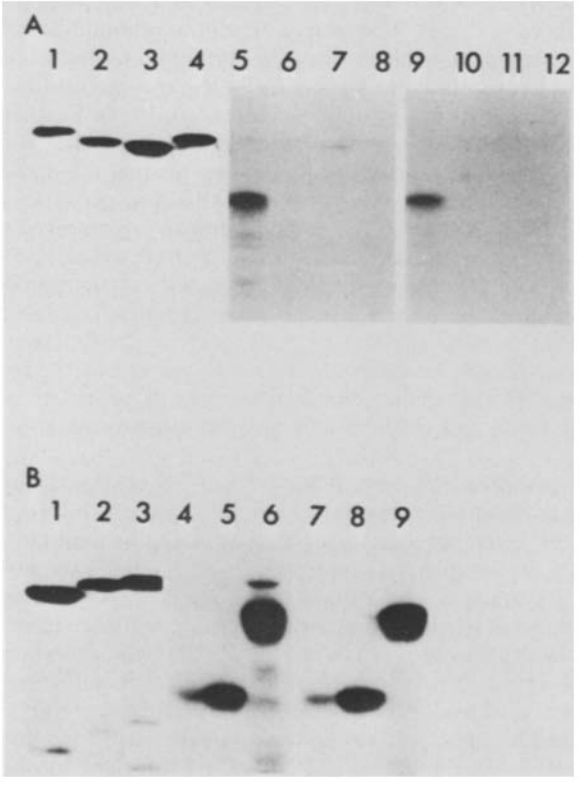

Fig. 7A, B. Transport of radioactively labeled precursors with mutant transit peptides. **A** Transport of deletion mutants. Precursors: TPNPTII (lane 1), PNd6/25 (lane 2), PNd6/29 (lane 3) and PNd26/35 (lane 4). Chloroplasts before (lanes 5–8) and after (lanes 9–12) protease treatment: TPNPTII (lanes 5, 9), PNd6/25 (lanes 6, 10), PNd6/29 (lanes 7, 11) and PNd26/35 (lanes 8, 12). **B** Comparison of the transport of the insertion mutants, TPNPTII and TPSSNPTII. Precursors: TPNPTII (lane 1), PNi26/35 (lane 2) and TPSSNPTII (lane 3). Chloroplasts before (lanes 4–6) and after (lanes 7–9) protease treatment: TPNPTII (lanes 4, 7), PNi6/25 (lanes 5, 8) and TPSSNPTII (lanes 6, 9)

Analysis of transit peptide deletion mutants using labeled preprotein

In order to examine uptake of the mutants in a more quantitative manner, the mutants were recloned into SP6 vectors (Melton et al. 1984) to yield plasmids pSP64/PNd6/25, pSP64/PNd6/29, pSP64/PNd26/35 and pSP64/PNi6/25. Radioactively labeled preprotein was synthesized in vitro and incubated with isolated intact chloroplasts. In contrast to the results obtained with the NPTII assay, transport of labeled precursor proteins with truncated transit peptides was not detectable (Fig. 7A). However, the enhanced import of the precursor protein containing the insertion (mutant PNi6/25) was clearly evident (Fig. 7B). Transport of PNi6/25 was considerably more efficient than that of TPNPTII, but less efficient than that of TPSSNPTII. In contrast to TPSSNPTII, the PNi6/25 preprotein appeared to be processed correctly upon import into chloroplasts.

Discussion

We compared the transport by intact chloroplasts of two transit peptide-NPTII fusion proteins which differed by 23 amino acids of mature SSU sequence. Two methods for detecting the proteins in chloroplast extracts were used to determine the relative transport efficiencies of these proteins. The enzymatic activity of the NPTII fusion proteins synthesized in *E. coli* was assayed to provide a sensitive method for comparison of TPNPTII and TPSSNPTII. This method had been used in previous studies (Van den Broeck et al. 1985; Schreier et al. 1985) and thus allowed a comparison of our results with those obtained previously. In order to obtain more quantitative data on the import of the TPNPTII and TPSSNPTII proteins, we used radioactively labeled precursors synthesized in vitro. This method also enabled us to compare the import of the NPTII fusion proteins with that of authentic pSSU. The results obtained with both methods agreed and showed that, although the transit peptide alone was sufficient to direct NPTII into chloroplasts, transport was strongly influenced by the additional 23 amino acids present in the TPSSNPTII protein. Protein TPSSNPTII was imported with an efficiency comparable to that of authentic pSSU while the TPNPTII polypeptide was imported with a relatively low efficiency.

To examine the transit peptide in more detail, mutations were introduced into the transit peptide of TPNPTII. All of the deletion mutations examined were located in the aminoterminal half of the transit peptide. Two of the mutants (PNd6/25 and PNd6/29) have overlapping deletions. One of these, PNd6/29, and deletion mutant PNd26/35 were imported into chloroplasts with drastically reduced efficiencies. Transport of mutant PNd6/25 was not detectable. An insertion mutation, containing a partial duplication of the transit peptide, increased the transport of the TPNPTII fusion protein.

The question arises as to whether specific sequences in the mature part of the SSU precursor are necessary to obtain optimal transport or if other factors are involved. Considering that the majority of the proteins found inside the

chloroplast are imported from the cytoplasm, the presence of a common specific sequence required for transport into the stroma appears unlikely. The amino acid sequences of the precursors of two stromal proteins, ferredoxin (Smeekens et al. 1985) and SSU (Cashmore 1983), are available. Surprisingly, the ferredoxin transit peptide shares homology with a portion of the first 23 amino acids of mature SSU. The homologous residues in ferredoxin are the three charged residues K-Q-Q and the sequence S-S-L-P. These amino acids correspond to the sequence K-K-K and S-Y-L-P in the mature SSU. Whether this homology is fortuitous or whether it influences the targeting of a protein towards different chloroplast compartments remains to be studied. In view of this homology, the requirement of a specific sequence within the 23 amino acids cannot be excluded. However, it appears more probable that other information contained in the 23 amino acids, e.g., charge distribution, is necessary for transport. Alternatively, the three-dimensional structures of the precursors may be important. It is conceivable that the precursor molecule must assume a particular conformation in order to be recognized by the putative receptor complex in the chloroplast envelope. Two different conformations for the transit peptide-NPTII fusion protein can be envisioned. Either the transit peptide and parts of NPTII form a single domain or the transit peptide and the NPTII portions form separate domains. In a single domain model, the correct folding of the NPTII portion might impose an aberrant conformation on the transit peptide. This could interfere with its recognition by the receptor complex. In a two domain model, the transit peptide part might be concealed by the NPTII domain leading to inefficient interaction between the precursor and the receptor. In contrast to the TPNPTII protein, the transit peptide of TPSSNPTII is separated from NPTII by an additional 23 amino acids. Possibly, this separation of the transit peptide from the NPTII moiety allows the transit peptide to assume its native conformation or renders it more accessible to the receptor. The increase in transport observed with the insertion mutant is consistent with the hypothesis that a conformational distortion leads to inefficient recognition by the receptor. The effect of the insertion in the transit peptide may be similar to the effect of the 23 amino acids of mature SSU. That is, the insertion results in an altered conformation which improves recognition by the receptor.

The imported form of the TPSSNPTII polypeptide had an apparent molecular weight that appears larger than expected. It is possible that in this protein some part of the transit peptide which influences maturation is folded incorrectly and cannot be recognized by the processing enzyme. Robinson and Ellis (1984) have shown that processing of pSSU proceeds via an intermediate in vitro. The cleavage that yields the mature SSU is not required for transport into the stroma (Robinson and Ellis 1984). Thus, incorrect processing at this point would not be expected to interfere with transport. We assume that the maturation cleavage of the TPSSNPTII polypeptide is inhibited. Therefore, the imported protein could be the product of the first processing step. The incorrect processing of the TPSSNPTII protein appears to be inconsistent with the hypothesis that a conformational distortion interferes with transport. However, as transport and maturation are independent processes (Robinson and Ellis 1984), they may be affected differently by a particular conformation.

Our deletion mutations are in the region of the transit peptide containing the putative first processing site and, therefore, at least one of these mutants lacks this site. Two of these mutants, PNd6/29 and PNd26/35, are transported into the chloroplast, although with greatly reduced efficiencies. Perhaps, the sequence surrounding the first processing site is not essential for the first cleavage or, alternatively, the first cleavage is not a required step in transport. The processing enzyme may recognize sequences outside of the deleted region. Alternatively, the processing enzyme may measure from the aminoterminus of the transit peptide. Robinson and Ellis (1984) invoked a similar hypothesis to explain the maturation of a modified precursor.

Protein transport into mitochondria has been studied in detail (Hurt et al. 1984, 1985; Schatz and Butow 1983; Horwich et al. 1985). The transit peptide of subunit IV of yeast cytochrome oxidase fused to the aminoterminus of mouse dihydrofolate reductase directs the enzyme into the matrix of isolated mitochondria (Hurt et al. 1984). Further analysis of deletions of the presequence showed that the 12 aminoterminal residues of the transit peptide are sufficient for transport. In this paper we show that precursors which lack portions of the aminoterminal region of the transit peptide from the SSU are transported with drastically reduced efficiency. Although transport is not abolished by all of the mutations, the aminoterminal region of the transit peptide appears to be important for function. From our results we conclude that with respect to the requirement of the aminotermini of the transit peptides the mitochondrial and chloroplast transport systems are similar.

Transport of only two, PNd6/29 and PNd26/35, of the three truncated polypeptides could be detected. Interestingly, the sequence that is deleted in the third mutant, PNd6/25, is completely overlapped by the other two mutants. It cannot be determined from our results whether the deletion in PNd6/25 removed necessary sequence information which was restored in mutant PNd6/29 by upstream sequences or if these effects result from different conformations of the transit peptide. The insertion mutant PNi6/25 has an exact duplication of the region of the transit peptide that was deleted in mutant PNd6/25. Transport of the PNi6/25 polypeptide was nearly as efficient as transport of the TPSSNPTII polypeptide. Therefore we conclude that the information encoded in the transit peptide can be separated without interfering with its function. Insertion mutants of this type might be useful for improving transport of foreign proteins into the chloroplast.

In summary, our results show that the pSSU transit peptide can direct transport of a foreign protein, NPTII, into the chloroplast stroma. However, we also suggest from our results that the mature SSU may play an important role in transport. Based on the results obtained with mutations in the transit peptide, we suggest that the aminoterminal region of the transit peptide is important for function.

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