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A MOLECULAR ANALYSIS OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE SMALL SUBUNIT TRANSIT PEPTIDE FUNCTION

by

Erin Bell

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ABSTRACT

A MOLECULAR ANALYSIS OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE SMALL SUBUNIT TRANSIT PEPTIDE FUNCTION

by

Erin Bell

The small subunit of ribulose 1,5-bisphosphate carboxylase is one of a number of chloroplast proteins which are synthesized in the cytoplasm and post-translationally transported into chloroplasts. Small subunit is synthesized as a precursor with an amino-terminal extension, the "transit peptide", which is required for translocation of the protein. I have developed an *in vitro* assay in order to identify important structural features of the *Zea mays* small subunit transit peptide. Several mutants containing structural alterations at the amino terminus of the small subunit precursor have been constructed and analyzed for transport activity. The alterations in these mutants do affect the efficiency with which they are transported, indicating that this region of the transit peptide has a function in transport of the protein. This function appears to require particular chemical characteristics, rather than a specific sequence or length.

The alterations made at the amino terminus of the precursor do not prevent correct processing of the protein. In contrast, a mutant containing a deletion at the precursor processing site will not be transported correctly. In the absence of correct processing, several intermediate forms of the precursor are seen. The majority of these

are membrane-associated, suggesting that correct processing is required to free the protein from the membrane. All intermediate forms of small subunit seen are inside the chloroplast, indicating that complete translocation of the protein can occur prior to processing.

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CHAPTER 1

INTRODUCTION

LITERATURE REVIEW

The movement of proteins into and through membranes is a necessary event in cells of all types. A great deal of work has been done to characterize membrane and intraprotein components required for protein translocation, although the specific mechanisms involved are as yet poorly understood.

Two mechanisms of protein localization appear to act in eukaryotic cells. One mechanism involves co-translational translocation, and was originally characterized in studies of the movement of secretory proteins in cells. Each translocated protein is encoded as a precursor containing an amino terminal extension, of 16-26 amino acid residues, called a signal sequence. This signal sequence has a polar, basic amino terminus and a central non-polar domain. (89) Following emergence of the amino terminus of a precursor from the ribosome, a signal recognition particle, composed of six polypeptides and a 7S RNA, binds to the polysome complex and halts translation. The entire complex then associates with a "docking" protein in the membrane, releasing the translation block and directing the nascent polypeptide

chain through the membrane. A membrane-associated protease cleaves the signal sequence, leaving the mature form of the protein. (87) This model was extended to the insertion of membrane proteins following the characterization of "stop transfer" sequences within a protein which keep a segment of the protein anchored in the membrane while the rest of the protein is synthesized on the cytoplasmic face. (91) In addition, it has been been proposed that a conformation which will cause the cytoplasmic portion of the protein to fold into the membrane will be assumed where necessary for protein function. There is also evidence that some proteins have a non-cleaved internal signal sequence (7), which can change the point at which the nascent chain starts through the membrane and result in a protein orientation with the amino terminus on the cytoplasmic side of the membrane. Together the possibilities allowed by these sequence signals and insertion mechanisms are thought to account for the majority of membrane protein conformations seen. (89) The cotranslational mechanism used in the translocation of these proteins may be required for allowing a membrane protein to achieve a functional conformation. In addition, it acts to keep the translocation signal available for interaction with the membrane, rather than possibly buried in the tertiary structure of the protein.

The second translocation mechanism used is post-translational transport. There is at least one case of post-translational association of a protein with the ER membrane. (1) In addition, the translocation of proteins into chloroplasts and mitochondria is a post-translational event. This was shown through experiments where proteins produced by <u>in vitro</u> translation of cytoplasmic RNA are

incubated with isolated intact chloroplasts or mitochondria. Even in the absence of translational capacity, transport of proteins into the organelles occurs. (13, 52) While each of these organelles contains its own chromosome, it is estimated that 70-90% of both mitochondrial and chloroplast proteins are nuclear-encoded and cytoplasmically synthesized. (12, 32) Thus, the translocation pathway involved is an essential one, and much work has been devoted to elucidating properties, both of the organelles themselves and of the proteins transported, which function in this process.

When in vitro translation products of cytoplasmic mRNA are compared with organellar proteins by antibody detection it is seen that proteins destined for the organelle are synthesized as larger precursors. (17, 52) DNA and amino acid sequence analyses of some of these precursors indicates that the extension, which has been called a transit peptide, is at the amino terminus. (43, 72) The transit peptides characterized thus far range in size from 20-66 amino acid residues, and have a basic character with a noticable lack of acidic amino acid residues. In addition, they lack the hydrophobic core seen in signal sequences. (41) It has been demonstrated that the transit peptide is required for translocation of a precursor into an organelle, and that the transit peptide is removed at some point during or immediately following this translocation. (23, 59) Exceptions to this are several mitochondrial proteins which are not synthesized as precursors. The majority of these are outer membrane proteins and do not require translocation through the membrane. (25) However, at least one other protein, the inner membrane protein cytochrome c, is not cleaved during translocation. (92)

Studies of the translocation of proteins into organelles have focused on proteins for which a gene has been cloned. Current molecular techniques make possible the construction of essentially any sequence desired, as well as allowing in vitro protein expression from a cloned gene. These techniques have been used to obtain the fusion proteins used in experiments described below. Many of these experiments have been carried out in vitro using isolated organelles, since this permits a quick and through analysis of the results. (3,32) Gene transformation has been used to test translocation in vivo, either in conjunction with in vitro studies or because the complementation of mutants provides a useful assay. (39, 83)

Both the chloroplast and the mitochondrion contain a number of distinct compartments. At least one protein destined for each compartment (except the chloroplast intermembrane space) has been identified and studied. In chloroplasts, both ferredoxin (Fd, 79) and the small subunit (SS) of ribulose 1,5-bisphosphate carboxylase/ oxygenase (RUBISCO, 13) have been examined as proteins which function in the main soluble compartment, the stroma. A component of the photosystem II light harvesting complex (LHCII), located in the thylakoid membranes, has also been analyzed (71), as has plastocyanin (Pc), a thylakoid lumen protein (78). A phosphate translocator of the inner envelope membrane has been identified, as has a 22 kd protein which is the major outer envelope protein. (21) In mitochondria, cytochrome (Cyt) b_2 (24) and Cyt c (92), intermembrane space proteins, porin, an outer membrane protein (22), Cyt c_1, an inner membrane protein (24), and ornithine transcarbamylase (OTc), a matrix protein (35), are some of the proteins which have been examined. Although the

common features mentioned above, general basic character and lack of a hydrophobic core, exist in all the transit peptides, there appear to be no overall sequence homologies. In addition, the work on mitochondrial protein transport suggests that it is not possible to connect a particular translocation strategy with a specific intraorganellar compartment. For example, both Cyt c and Cyt b_2 are inner membrane proteins, but Cyt c is not processed (92) while Cyt b_2 is processed in two steps (24). It is thought that in addition to destination within the organelle, other factors, such as post-translocational association with cofactors, play a role in the mechanisms by which a protein is localized.

SS precursor from one plant species can be transported into and correctly processed in chloroplasts from other species. (15) Thus, functional transport properties are preserved between species, despite poor transit peptide sequence homologies. Furthermore, a recent study indicates that a fusion protein consisting of the SS transit peptide and either a mitochondrial protein (COxIV) or a cytosolic protein (dihydrofolate reductase, DhfR) will be transported into yeast mitochondria both in vitro and in vivo. (41) These fusion proteins are transported less efficiently than either authentic mitochondrial proteins or fusion proteins containing a mitochondrial transit peptide. In addition, they do not appear to be processed to yield a mature protein. Despite this, it is clear that chloroplast and mitochondrial transit peptides share enough essential features to allow some function of chloroplast transit peptides in interaction with mitochondria. This raises the question of how protein sorting occurs in cells containing

both organelle types; this question has not yet been addressed in the literature.

Studies with fusion proteins have been done to define the role that transit peptides play in protein localization. In chloroplasts, two chimeric genes, one containing the coding region of a plant heat shock gene and the other the coding region of the bacterial neomycin phosphotransferase II (NPtII) gene, each fused to the SS transit peptide coding region, have been constructed. (51, 83) Analysis of the resulting chimeric proteins indicates that both are transported into chloroplasts. The product identified within the chloroplast in each case is essentially the same size as the native protein, suggesting that these chimeric proteins are processed correctly. While the chimeric proteins are not transported as efficiently as SS, the results indicate that the transit peptide alone is sufficient for transport. Similar studies in mitochondria, using the transit peptide from either OTc or alcohol dehydrogenase III fused to DhfR, confirm these results. (35, 84)

In the chloroplast experiments described above, NPtII activity is found exclusively in the stromal fraction, the correct compartment for SS. (83) The majority of the heat shock protein is found in the stromal fraction as well, although some is membrane associated. (51) Likewise, the OTc transit peptide directs DhfR to the mitochondrial matrix, the correct location for OTc. (37) These results suggest that the transit peptide has a role in directing a precursor to a specific organelle compartment. This question has been addressed more throughly using two nuclear-encoded chloroplast proteins, Fd (a stromal protein) and Pc (a lumen protein). Chimeric genes encoding the Fd transit

peptide fused to the Pc mature protein and vice versa have been constructed. (77) After transport into isolated chloroplasts, Pc containing a Fd transit peptide is found exclusively in a processed form in the stroma, suggesting that the transit peptide, and not the mature protein, determines the intrachloroplastic location. Fd containing the Pc transit peptide is seen after transport mainly as a protein intermediate in size between its precursor and mature forms. This intermediate is found both in the stroma and on the stromal side of the thylakoid membranes. A minor amount of what appears to be a completely processed form of the protein is seen in the lumen. The authors suggest that here too the transit peptide is directing the localization of the protein, but that something about either Fd itself or its association with stromal components prevents the protein from being transported across the thylakoid membranes. (77) Thus, it appears that although a transit peptide can direct a protein towards a specific compartment, certain characteristics of the mature protein must also exist to allow it to reach this compartment.

The identification of structural features of a transit peptide which are important for its function is a first step in understanding mechanisms of transit peptide-mediated protein translocation. In chloroplasts, this effort has focused on the SS transit peptide, since SS has been most widely characterized in terms of transport. Isolation of a stromal transit peptidase has allowed studies of processing of in vitro-synthesized SS precursor. (66) When SS precursor is incubated with isolated transit peptidase an 18 kd intermediate is seen, suggesting that the precursor is actually processed in two steps. As a corollary to this, a study of the transport of an algal SS precursor

into pea chloroplasts has shown that this algal precursor is processed only to an intermediate size in these chloroplasts. (59) Protein sequence analysis of the algal SS intermediate has identified the cleavage site, which is in a region of the precursor that shows homology to a region near the middle of higher plant SS transit peptides. The authors suggest that this homologous sequence is the site at which the first cleavage of SS precursor occurs in higher plant chloroplasts. (59) A function has also been proposed for sequences adjacent to the final processing site of SS. These sequences are highly conserved, and are thought, due to their location and conservation, to be required for correct processing of the precursor. (44)

Several analyses of specific regions of mitochondrial transit peptides have been done. Deletion analysis of a fusion protein consisting of the COxIV transit peptide fused to DhfR has demonstrated that the 12 amino terminal residues of the transit peptide are sufficient to provide translocation of this protein, although it is not processed. (40) Extensive deletion analysis of the OTc transit peptide has been carried out by Horwich et al. (37) They have found that deletion of an internal 14 amino acid segment completely eliminates translocation of the protein. Deletions near the amino and carboxy termini of the transit peptide affect translocation to varying extents. These researchers have also substituted Gly for Arg residues at one or more locations within the transit peptide and found that one specific Arg residue is essential for translocation, while other appear to play some role but are not required. (37) Analysis of sequences required for translocation has also been done using a non-processed

mitochondrial protein, a 70 kd outer membrane protein. The majority of these non-processed proteins reside in the outer membrane, and it seems possible that movement into rather than through a membrane would involve a different transport mechanism. This appears to be true to some extent, since it has been shown that, unlike other transported proteins, outer membrane proteins do not require energy for translocation. (25) However, mutational analysis of this 70 kd outer membrane protein indicates that it contains a sequence which can function as a transit peptide, as well as a sequence which holds the protein in the outer membrane. Deletion of this second sequence leads to some appearance of the protein in the matrix. When the transit peptide-like sequence is deleted, the protein remains in the cytosol. (31) In addition, the transit-like sequence can direct the import of two fusion proteins containing this sequence and either DhfR or a truncated COxIV. Unlike localization of the 70 kd protein, transport of the fusion proteins requires an energized membrane. (39) It is not known whether an internal sequence which functions like a transit peptide exists in other non-processed mitochondrial proteins as well. If so, it would suggest the existence of a single overall transport mechanism, despite the number of variations seen.

While specific features of proteins are required for translocation to occur, certain organelle features are required as well. Recognition between organelle and protein must occur and be accompanied or followed by association of the precursor with the outer membrane. In mitochondria, evidence for interaction with specific receptors has been obtained for some proteins. Studies with radiolabelled and unlabelled Cyt c indicated competion for and reversible binding to sites on the

mitochondrial surface. (33) In both mitochondria and chloroplasts, binding of precursor to energy-depleted organelles can occur. (14, 64) The physiological significance of the binding is demonstrated by the translocation of these bound precursors (up to 75% in chloroplasts, 14) when ATP or membrane potential is restored. Translocation is not affected by dilution or removal of the reaction mix containing unbound precursors, indicating that it is the bound precursors which are being transported. (14, 64) Treatment of intact organelles with protease prior to incubation with precursors causes a loss of precursor binding and import. A complete block of import following protease treatment can be seen in mitochondrial systems, although the insertion of porin is not affected by this treatment (25, 64) Only a partial inhibition of import by protease pretreatment has been seen in chloroplast systems. Furthermore, two different chloroplast proteins, SS and LHCII, show different degrees of transport inhibition following protease treatment, suggesting that they may not interact with the same membrane protein(s). (14)

As implied above, translocation of proteins requires an energy component. In chloroplasts, it has been shown that this requirement is for ATP. (26) In contrast, work with Neurospora and yeast has shown that mitochondrial protein transport requires an electrochemical membrane potential. (23, 70) A recent study of the mitochondrial proteins Cyt c_1 and ATPase F_1 subunit B (a matrix protein) has examined further this requirement for a membrane potential. (69) At low temperature ($11^{o}C$) partial translocation of each protein occurs, such that the transit peptides are cleaved by a matrix-localized transit peptidase while the bulk of each protein is sensitive to added protease

(i.e., not protected by intact membranes). This partial translocation will not occur without a membrane potential. When the temperature is raised to 25°C the remainder of each protein is translocated, even in the absence of a membrane potential. The authors suggest that a membrane potential functions in translocation of the positively charged transit peptide, and that this event triggers the movement of the protein through the membranes. (69)

Another step in the translocation pathway is processing. In both mitochondria and chloroplasts a soluble, chelator-sensitive protease has been partially purified and shown to be specific for precursor processing. (58, 65) In addition, a second, inner membrane-associated protease has been shown to act on mitochondrial precursors which are processed in two steps. (16) Studies of protein transport using mitochondria incubated with chelator indicate that transport can continue in the absence of processing. (93)

Most of the proteins translocated into organelles do not function alone. For example, LHCII in chloroplasts is part of a complex called photosystem II, and must associate with several pigments and with other proteins to function. Protein associations may occur with other nuclear-encoded proteins, with organelle-encoded proteins, or with both. Experiments with algal mutants deficient in chloroplast translation have demonstrated that synthesis of RUBISCO large subunit (LS) is not required for either SS synthesis or translocation, but that in the absence of LS, SS is rapidly degraded. (73) Likewise, LHCII can be transported and integrated into the thylakoids in the absence of either chloroplast-encoded proteins (12) or chlorophyll b (4), but in the absence of chlorophyll b it becomes sensitive to a specific

protease. (4) Thus, although direct coordination of expression is not necessarily required, proper assembly appears to protect a transported protein from degradation.

In summary, steps involved in the transfer of cytoplasmically synthesized proteins to their sites of function within an organelle include binding to and translocation across the outer membrane(s), movement into or through another set of membranes where necessary, processing to a mature size in most cases and, often, association of the protein with other components of the enzyme or complex in which it functions. The order in which some of these steps occur is not clear, and the study of mitochondrial protein transport indicates that several specific transport pathways exist. It is to be expected that this diversity will be seen in chloroplast protein transport as well when a larger number of proteins are studied.

Recently, a theory concerning the overall mechanism of transit peptide-mediated protein translocation into mitochondria has been formulated. Statistical analysis of the sequences of 23 mitochondrial transit peptides has shown that many are able to form amphiphilic helices with high hydrophobic moments. This trait, combined with a lack of acidic residues up to and through the segment of highest hydrophobic moment, separates the group of transit peptides from a control group of cytosolic proteins. (86) In addition, studies with chemically synthesized peptides containing all or part of the transit peptide from COxIV has shown that these peptides are surface active, inserting spontaneously from aqueous solution into phospholipid monolayers. The peptides are also able to uncouple respiratory control in isolated mitochondria. (67) The model presented is that the transit

peptide forms an amphiphilic helix and inserts its hydrophobic surface into the mitochondrial membrane. The helix is then moved through the membrane in a membrane potential-dependent fashion. (67, 86) This theory is supported by the study discussed earlier, in which it was demonstrated that while translocation of the transit peptide region of a mitochondrial protein precursor requires a membrane potential, translocation of the remainder of the protein does not. (69) The model leaves many questions unanswered, one of which is how the remainder of the protein is translocated. In addition, it is not clear what role the reported receptors and proteinaceous component(s) of the membrane would play. It is especially difficult to imagine how reversible binding, reported for Cyt c (33), could occur if the primary interaction of a protein with a mitochondrion involves spontaneous insertion of a helix into the membrane. However, the potential for these structures in many transit peptides is thought to be significant, and the next step will probably be a direct test of this hypothesis by construction of synthetic peptides or precursor mutants which contain sequences disrupting the formation of an amphiphilic helix.

Chloroplast transit peptides share general features with mitochondrial transit peptides, and may be able to form similar helical structures, although such an analysis has not been reported. A major difference between chloroplast and mitochondrial protein transport is the requirement for ATP rather than a membrane potential. Since the role that ATP plays has not been determined, it is not clear how important this difference is in the mechanism of protein translocation.

The studies described above have contributed to a basic understanding of the processes involved in protein translocation. It

is known that several components of both the organelle and the translocated protein are required, including the transit peptide, a protein component associated with the outer membrane, an energy component, and a transit peptidase. However, the specific mechanisms involved in the function of most of these components are still not understood. Based on what is known so far, the appropriate direction to take appears to be a continued dissection of the transport system. By identifying features of individual components which are essential to the role they fill, it should be possible to gain some knowledge of specific mechanisms. This in turn will provide a more complete understanding of the translocation process as a whole.

INTRODUCTION TO THESIS

Studies described earlier, using chimeric proteins consisting of a transit peptide fused to a non-chloroplast protein, indicate that the transit peptide alone is sufficient to provide translocation capacity. The transit peptide appears to function in more than one step of translocation, since the chimeric proteins are not only transported but can be directed into the appropriate compartment and correctly processed. (51, 83) An obvious next step in understanding mechanisms of transit peptide function is defining the roles that specific features within a transit peptide play in targeting, translocation, and processing.

The most widely studied nuclear-encoded chloroplast protein is SS of RUBISCO. This protein, once transported, assembles in the chloroplast with chloroplast-encoded LS to form a holoemzyme composed of eight large and eight small subunits. RUBISCO, which functions in the stroma, is the most abundant protein in leaves, comprising over 50% of soluble leaf protein in some species. (46) Genes encoding SS have been sequenced from at least six higher plant species. (44, this work) Comparison of the derived amino acid sequences indicates that less homology between species exists in the transit peptide region than in the "mature" region of the precursor. However, blocks of homology are found at three positions within the transit peptide, and the position of proline and some charged amino acid residues show conservation. (33) As mentioned earlier, SS precursor from one higher plant species can be transported into chloroplasts isolated from another species, indicating that functional characteristics are shared between species. This has

led to the speculation that the conserved sequence features seen have functional properties. (44)

My work has focused on characterizing functional regions within the SS transit peptide, starting with analysis of the regions which show sequence conservation. I have constructed several mutants of a Zea mays SS precursor by making specific alterations in regions of the SS gene encoding some of these conserved features. A report on the effect that these alterations have on transport of the mutant precursors into isolated Zea mays chloroplasts, along with discussion of what these results might mean, is presented here. In addition I present the results of my characterization of the in vitro transport assay used in this study.

CHAPTER 2

CHARACTERIZATION OF TRANSPORT SYSTEM

INTRODUCTION

Transport of nuclear encoded proteins into chloroplasts was originally demonstrated using isolated intact chloroplasts and proteins translated in vitro. (13, 34) This in vitro transport assay was quickly recognized as a powerful tool in understanding how translocation of chloroplast protein precursors occurs, and several studies were done to optimize the assay. (26, 27) The goals in this were both to characterize the system as a whole and to make it possible to examine the transport of precursors less abundant than RUBISCO small subunit (SS). A drawback of the early system was that techniques were not yet available for the isolation of a single mRNA species, making it difficult to examine the transport of a specific chloroplast protein precursor. A later study utilized hybrid selection to obtain a specific mRNA population (14), but we felt that it would be useful to develop an expression system in which specific alterations in the gene would be manifested in the protein. This would permit one to do not only studies on the transport of a single protein, but also on how changes in this protein affect transport properties. Originally work

was done to express the SS gene at high levels in Escherichia coli (see Appendix A), but difficulties with expression of SS, combined with the appearance of a simple and efficient in vitro transcription system, led me to take an in vitro approach to SS expression. The in vitro expression system produces a single labelled protein, making it possible to quantify both the amount of SS precursor added to the transport assay and the amount of SS translocated. This has permitted a study of the efficiency of my in vitro assay system, and allowed me to determine some of the limitations of the system. This chapter reports both my analysis of the in vitro transport assay and the characterization of its components.

MATERIALS AND METHODS

DNA modification

Restriction endonucleases, Bal31 and S-1 exonucleases, and T-4 DNA ligase were from either New England Biolabs (Beverly, MA) or Bethesda Research Laboratories (Gaithersburg, MD). Enzyme reactions were carried out according to manufacturer's recommendations or as described by Maniatis et al. (57)

Oligonucleotide annealing

Oligonucleotides of the desired sequence were synthesized on an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer using phosphoramidite chemistry. (6) For annealing, 0.4-1.0 nmol of each

complementary oligonucleotide was mixed in 100uL of 100 mM Tris-HCl pH 7.5. The mix was heated to 90°C for 1 minute, quick chilled on ice, kept at 70°C for 30 minutes and then slowly cooled, first to room temperature and then to 4°C. 20 pmol of the resulting linker was used in each ligation reaction.

DNA sequence analysis

DNA sequencing was carried out using the chemical cleavage method described by Maxam and Gilbert.(55)

In vitro transcription

In vitro transcription was carried out using the Genescribe system (U. S. Biochemical Corp., Cleveland. OH). All rbcS constructions were inserted into the Genescribe vector pT7-2. For the transcription reaction template DNA was digested with EcoR1 and extracted with phenol. Transcription reactions were in a 50 uL volume and contained 2 ug template DNA, 40 mM Tris-HCl pH 8.0, 15 mM $MgCl_2$, 5 mM DTT, 1 mM each ATP, CTP, GTP, and UTP, 25 ug BSA, and 10-20 units T-7 RNA polymerase (U. S. Biochemical). The reaction mixture was incubated at 37°C for 30 minutes. mRNA was precipitated in 0.3 M sodium acetate and 2.2 volumes absolute ethanol, washed with 80% ethanol, dried, and resuspended in water for use in an in vitro translation reaction.

In vitro translation

Nuclease treated rabbit reticulocyte lysate was obtained from Promega Biotec (Madison, WI). Each reaction included 50% nuclease treated lysate, 25 mM potassium acetate, 500 uM magnesium acetate, 25

uM each amino acid (except methionine), 0.5 mCi ^{35}S methionine/ml (approx. 1200 Ci/mmol, Amersham Corp., Arlington Heights, IL), and mRNA from an in vitro transcription reaction. A control reaction with no added mRNA was done with each set of reactions. Incubation was at 30°C for one hour.

Determination of SS precursor concentration

To analyze the incorporation of amino acids into protein, a 1-2 uL aliquot of the reaction mix was added to 1 mL of 1 N NaOH, 1.5% H_2O_2 and incubated at 37°C for 10 minutes. 4 mL of 25% TCA, 2% casamino acids was then added. After incubation on ice for 30 minutes, precipitates were collected onto a glass fiber filter by suction. The filter was washed with 10 mL 8% TCA, followed by 2 mL acetone. The number of counts bound to the filter was determined using a liquid scintillation counter. For each set of in vitro translation reactions, zero time controls were also analyzed for counts bound to a filter. A final incorporation value for each translation reaction was obtained by averaging three replicate counts and subtracting the zero time counts.

Gel electrophoresis

Proteins were analyzed on 10-17.5% gradient SDS-polyacrylamide gels using the buffer system described by Laemmli. (47) Samples were suspended in SDS sample buffer to give final concentrations of 10% glycerol, 2% SDS, 2% B-mercaptoethanol, and 62 mM Tris-HCl pH 6.8, with bromophenol blue included as a tracking dye. Samples were heated at 90°C for 3.5 minutes and loaded onto 0.8 mm thick slab gels. Gels were run at 2.5 W constant power for seven hours, stained in 7% glacial

acetic acid, 50% methanol, 0.1% Coomassie Brilliant Blue R, and destained in 7% glacial acetic acid, 20% methanol, 4% glycerol. Gels were fluorographed according to Chamberlain (11), and dried at 60^{o}C for 2 hours under vacuum. They were then exposed to x-ray film (XAR, Kodak, Rochester, NY). Radioactive bands were excised from dried gels, cut into slices, and the proteins were extracted using NCS tissue solubulizer (Amersham Corp.) as described by Walter et al. (88) Amounts of radioactivity present were determined by liquid scintillation counting. Based on the comparison of counts obtained by excision of proteins from gels and counts obtained by TCA precipitation of in vitro translation reactions, the efficiency of counting out of gels is approximately 70%. Since all numerical data obtained were from the counting of gel slices, this factor was taken into consideration when determining transport efficiency.

Chloroplast isolation

Zea mays seeds (FR9CMS x FR37, Illinois Foundation Seeds, Champaign, IL) were sown in vermiculite and grown under 16 hours light, 28^{o}C, 8 hours dark, 21^{o}C. Chloroplasts from eight day old maize seedings were isolated according to Leto et al (48) with slight modifications. Leaves were cut into 1 cm segments before grinding, and were ground using a blender modified to hold replacable razor blades. (42) Percoll (Sigma, St. Louis, MO) gradients were 25-80%. Chloroplasts removed from the lower band of the gradient were washed with resuspension buffer (0.3 M sorbitol, 50 mM Hepes-KOH pH 8.2, 2mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 100 uM DTT), spun in a Sorvall SS34 rotor

to 6000 rpm with immediate braking, and resuspended in sorbitol-Hepes buffer (0.33 M sorbitol, 50 mM Hepes-KOH pH 7.7). This preparation has been reported to give 70% intact chloroplasts. (48) Chlorophyll concentration was determined by the method of MacKinney. (53) The resuspension volume used (400 uL) routinely gave chlorophyll concentrations of 2-3 mg chlorophyll/mL. Direct counts of known dilutions of the chloroplast suspension were made to determine the number of chloroplasts per quantity of chlorophyll. Four separate chloroplast preparations were quantified in this manner, with a minimum of 13 hemocytometer squares scanned and 600 chloroplasts counted each time. The average count was 9.35×10^5 chloroplasts per ug chlorophyll.

Preparation of antibody specific against RUBISCO SS

A crude protein preparation enriched in RUBISCO was prepared according to the method of Hall and Tolbert (29) except that corn rather than spinach leaves were used. Protein precipitated by the addition of $MgCl_2$ to the supernatent was analyzed on SDS polyacrylamide gels. The band corresponding to SS was excised and the protein was eluted. Protein determination was done according to the method of Lowry. (50) Approximately 150 ug of protein was suspended in Freunds complete adjuvant (Sigma) and injected into a rabbit. Booster injections with an equal amount of protein suspended in Freunds incomplete adjuvant were done 1, 2, 4, and 8 weeks later; serum was analyzed for antigenic activity at 5 weeks. This serum was found to have high antigenic activity against SS, and was used without further purification.

Immunodetection of proteins from gels

Proteins from SDS polyacrylamide gels were transferred to nitrocellulose membrane (0.45 um, Schleicher & Schuell, Keene, NH) using a Transphor apparatus (Hoefer Scientific Instruments, San Francisco, CA) at 1-1.5 amps for five hours. The transfer buffer was 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. (82) Blots were quenched at room temperature overnight in 20 mM Tris-HCl pH 7.4, 0.9% NaCl (Tris-saline buffer) with 3% BSA, then incubated with specific antiserum at dilutions of 1:500 to 1:2000 in Tris-saline with 1% BSA at 37^{o}C for one hour. Blots were washed three times for 15 minutes each in Tris-saline with 0.1% BSA and 0.1% Triton X-100 before addition of protein A-linked alkaline phosphatase (Sigma) at a concentration of 0.1 ug/mL in the same buffer. After incubation for 1 hour at room temperature, blots were washed three times for 15 minutes each in 100mM Tris-HCL pH 7.5, 100mM NaCl, 2mM $MgCl_2$, 0.05% Triton X-100 and then washed twice in AP 9.5 buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Color detection of the immune complexes was performed using 3.3 mg nitro blue tetrazolium and 1.6 mg 5-bromo-4-chloro-3-indoxylphosphate (in 33 uL dimethylformamide) per 10 mL of AP 9.5 buffer. After 2-10 minutes of color development under a black cloth, the reaction was stopped by removal of development buffer and incubation in 10 mM Tris-HCl pH 7.5, 1 mM EDTA for 5-10 minutes. Blots were then dried and stored in the dark.

Anti-RUBISCO SS was obtained as described above. Anti-LHCII was obtained against isolated pea LHCII, and was provided by Dr. B. Barry. Anti-RUBISCO was obtained against isolated RUBISCO from tomato, and was provided by B. Wilson.

Immunoprecipitation of proteins

15 uL of *in vitro* translation mix was added to 21 uL of water and 4 uL 20% SDS, then heated to 90°C for 2 minutes. 50 uL of 2X immunoprecipitation buffer (100 mM Tris-HCl pH 7.4, 600 mM NaCl, 10 mM EDTA, 2% Triton X-100) and 10 uL of anti-SS antiserum were added, and reaction was kept at 25°C for 45 minutes with occasional mixing. 50 uL of formalin-fixed Staph A cells (Immunoprecipitin, Bethesda Research Labs.) was added and incubation continued at 25°C for 15 minutes with occasional mixing. 1 mL of wash buffer (50 mM Tris-HCl pH 7.4, 300 mM NaCl, 1% Triton X-100, 0.1% SDS) was then added. After mixing, cells were briefly spun to pellet and wash buffer was removed. Following two more washes the cells were suspended in SDS sample buffer, heated to 90°C for 3.5 minutes, and pelleted again. The supernatant was removed and analyzed using SDS polyacrylamide gel electrophoresis.

Transport incubation

Tranport incubation conditions were based on those described by Bartlett *et al*. (3) Incubations were carried out in 185 x 13 mm polypropylene tubes at room temperature in the light. Incubation was for 30 minutes, except where otherwise noted, with gentle agitation every five minutes. Each 200 uL incubation contained 20 uL of translation mix and chloroplasts equalling 40 ug of chlorophyll, with buffer of 50 mM Hepes-KOH pH 8.0, 8.4 mM methionine, and approximately 0.3 M sorbitol (the exact sorbitol concentration depended on the volume of the chloroplast suspension added). Following the incubation period 0.6 mL sorbitol-Hepes buffer was added, and the chloroplasts were

pelleted in a SS34 rotor spun to 6000 rpm with immediate braking. After removal of supernatent, 0.45 mL sorbitol-Hepes buffer was added and chloroplasts were gently resuspended using a micropipetter. Chloroplasts not to be protease treated were pelleted as above and the pellet was kept on ice. Protease treatment was as described by Cline et al. (14) 50 uL of 1 mg/mL thermolysin (Sigma) was added and samples were placed on ice for 30 minutes. 100 uL of sorbitol-Hepes buffer containing 50 mM EDTA was then added and samples were pelleted as described above. For analysis of whole chloroplasts, SDS sample buffer was added to the pellet and the sample was vortexed until resuspension was complete. For analysis of the stromal fraction 20 uL of 5 mM EDTA was added to the chloroplast pellet and sample was vortexed for 30 seconds, frozen in a dry ice-ethanol bath, allowed to thaw at room temperature, and spun in a SS34 rotor at 8000 rpm for 10 minutes. The supernatant, which contained soluble chloroplast proteins, was added to SDS sample buffer. All samples were heated and electrophoresed as described above.

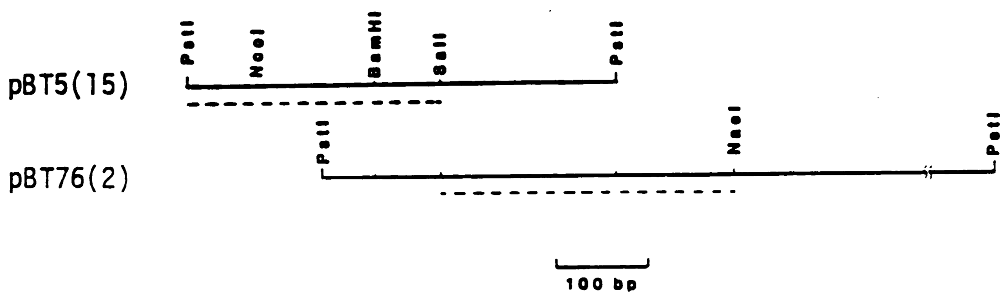
RESULTS

Characterization of a Zea mays small subunit gene

Two overlapping cDNA clones, pBT76(2) and pBT5(15), each encoding a portion of a Zea mays RUBISCO small subunit gene (rbcS), were obtained from Dr. W. Taylor, UC Berkeley. (Figure 1A) Both gene fragments were sequenced (pBT5(15) by E. Bell and pBT76(2) by J.

Figure 1. Construction and analysis of pSS19.
A. Restriction maps of rbcS region from two clones, pBT5(15) and pBT76(2). The dashed line under each clone indicates the gene fragment used in the construction of pSS19.
B. DNA and derived amino acid sequences of rbcS in the clone pSS19. The underlined "MET" indicates the position of the initiation codon. The arrow indicates the site at which the precursor is processed to the mature protein

A



B

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                  AUG AUU ACG CCA AGC UUG CAU GCC UGC AGG GGG GGG GGG GGG GGG GGA GCA

AGC AAG CAA GCA GCG AGC ACA UAC AUA CUA GGC AGC CAG GCA GCC AUG GCG CCC ACC GUG
                                                           |MET Ala Pro Thr Val
                                    151                             181
AUG AUG GCC UCG UCG GCC ACC GCC GUC GCU CCG UUC CAG GGG CUC AAG UCC ACC GCC AGC
MET MET Ala Ser Ser Ala Thr Ala Val Ala Pro Phe Gln Gly Leu Lys Ser Thr Ala Ser
                                    211                             241
CUC CCC GUC GCC CGC CGC UCC UCC AGA AGC CUC GGC AAC GUC AGC AAC GGC GGA AGG AUC
Leu Pro Val Ala Arg Arg Ser Ser Arg Ser Leu Gly Asn Val Ser Asn Gly Gly Arg Ile
                                    271                             301
CGG UGC AUG CAG GUG UGG CCG GCC UAC GGC AAC AAG AAG UUC GAG ACG CUG UCG UAC CUG
Arg Cys MET Gln Val Trp Pro Ala Tyr Gly Asn Lys Lys Phe Glu Thr Leu Ser Tyr Leu
       ↑
                                    331                             361
CCG CCG CUG UCG ACG GAC GAC CUG CUG AAG CAG GUG GAC UAC CUG CUG CGC AAC GGC UGG
Pro Pro Leu Ser Thr Asp Asp Leu Leu Lys Gln Val Asp Tyr Leu Leu Arg Asn Gly Trp
                                    391                             421
AUA CCC UGC CUC GAG UUC AGC AAG GUC GGC UUC GUG UAC CGC GAG AAC UCC ACC UCC CCG
Ile Pro Cys Leu Glu Phe Ser Lys Val Gly Phe Val Tyr Arg Glu Asn Ser Thr Ser Pro
                                    451                             481
UGC UAC UAC GAC GGC CGC UAC UGG ACC AUG UGG AAG CUG CCC AUG UUC GGC UGC AAC GAC
Cys Tyr Tyr Asp Gly Arg Tyr Trp Thr MET Trp Lys Leu Pro MET Phe Gly Cys Asn Asp
                                    511                             541
GCC ACC CAG GUG UAC AAG GAG CUG CAG GAG GCC AUC AAA UCC UAC CCG GAC GCC UUC CAC
Ala Thr Gln Val Tyr Lys Glu Leu Gln Glu Ala Ile Lys Ser Tyr Pro Asp Ala Phe His
                                    571                             601
CGC GUC AUC GGC UUC GAC AAC AUC AAG CAG ACG CAG UGC GUC AGC UUC AUC GCC UAC AAG
Arg Val Ile Gly Phe Asp Asn Ile Lys Gln Thr Gln Cys Val Ser Phe Ile Ala Tyr Lys
                                    631
CCC CCG GGC AGC GAC UAG ACC GCG CCC GCC GGG GCA CCG AGC UCG AAU UCA CUG GCC
Pro Pro Gly Ser Asp *2*
```

Figure 1.

Fitchen) and found to be completely homologous in the region in which they overlapped (paper in preparation), suggesting that both cDNA clones were derived from a single gene. Together the two fragments spanned the entire coding region of rbcS. In order to construct an intact gene, the 276 bp PstI-SalI fragment from pBT5(15) was isolated and ligated to the 319 bp SalI-NaeI fragment isolated from pBT72(2). (Figure 1A) The resulting fragment, containing the coding sequence as well as 72 bp of 5' non-coding sequence, was inserted into the PstI and SmaI sites of pUC19. This clone, called pSS19, was the "parent" for all following SS gene constructions.

The sequence of the rbcS region of pSS19 is shown in Figure 1B. The derived amino acid sequence is that encoded by the largest open reading frame found, and is 78% homologous to the amino acid sequence of SS from wheat. (9) The site at which the precursor is processed to the mature protein (indicated by arrow on figure) was determined by comparison of this derived sequence with the protein sequence of mature small subunit from several species. (54, 60, 81) Based on this placement of the processing site, the transit peptide is 47 amino acids long.

Characterization of chloroplast type

Zea mays is a C-4 species, meaning that it uses a spatial separation of different photosynthetic activities to increase photosynthetic efficiency. Since RUBISCO has competing carboxylase and oxygenase activities and the carboxylase activity is more productive for the plant, it is advantageous to have RUBISCO in a high CO_2 environment. This is achieved in C-4 plants by having two distinct

photosynthetic cell types, one of which, the mesophyll cells, surrounds the other, the bundle sheath cells. Primary carboxylation occurs via PEP carboxylase in mesophyll cells. A product (malate in the case of Zea mays) is then transported into bundle sheath cells where it is decarboxylated to provide high local CO_2 levels which are used by RUBISCO. At the same time, photosystem II (PSII), which evolves oxygen, is active almost (or completely) exclusively in mesophyll chloroplasts.

Analysis of the two cell types has demonstrated that RUBISCO is localized exclusively in bundle sheath chloroplasts. (8) Components of PSII are mainly (or exclusively) localized in mosophyll chloroplasts. (75) This information was used to determine whether my chloroplast preparation contained one or both chloroplast types. SDS polyacrylamide gels were used to analyze either chloroplast membrane proteins or chloroplast soluble proteins. The proteins were transferred to nitrocellulose filters and incubated first with a specific antiserum and then with a colorometric detection system, as described in Methods. Filters containing chloroplast membrane proteins were incubated with antibody against a group of thylakoid membrane proteins which make up the light harvesting complex of photosystem II (LHCII). (Figure 2A) The presence of significant amounts of LHCII in the proteins from these chloroplasts, as determined by antibody reaction, indicates that the chloroplast preparation routinely used for my experiments contains mesophyll chloroplasts. Filters containing soluble chloroplast proteins were reacted with antibody against either SS or RUBISCO holoenzyme. (Figure 2B) Reaction of these antibodies

Figure 2. Immunoblot analysis of Zea mays chloroplast preparations. A. Chloroplast membrane proteins analyzed by SDS polyacrylamide gel electrophoresis, blotted, and reacted with antiserum against pea LHCII. Chloroplasts equivalent to 10 ug of chlorophyll were analyzed per lane. B. Chloroplast soluble proteins analyzed as above and reacted with antiserum against (1) tomato RUBISCO holoenzyme or (2) corn RUBISCO SS. For (1) the equivalent 15 ug chlorophyll was loaded per lane; for (2) the equivalent of 10 ug chlorophyll was loaded per lane.

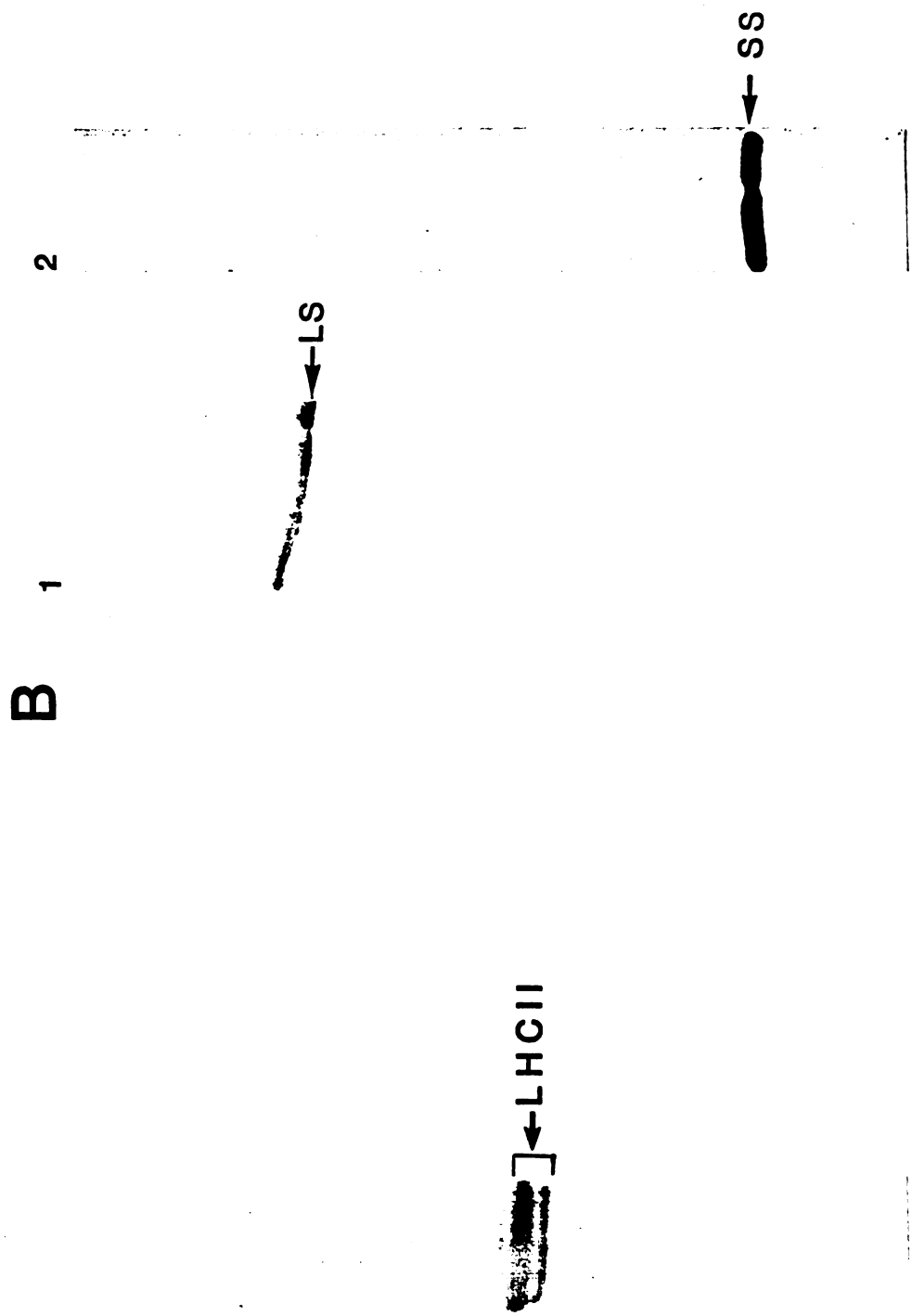


Figure 2.

with specific proteins indicates that bundle sheath chloroplasts are present in my preparations as well.

Expression and transport of small subunit precursor

For reasons described in Appendix A, the construction of a vector to direct the synthesis of wild type SS mRNA involved the use of an oligonucleotide linker. This double stranded linker contained an NcoI cohesive end (but did not regenerate a NcoI site), a HindIII site 19 bp upstream of this sticky end, and a blunt end. pSS19 (Figure 3A) was prepared for linker insertion by digestion with HindIII, treatment with the nuclease Bal31 for 1.5-6 minutes, and finally with NcoI digestion. Following linker insertion, the HindIII-EcoRI gene fragment was isolated from this clone and inserted using the same restriction sites into the Genescribe transcription vector pT7-2 (Figure 3B). The final clone, called pTEB2, gave wild type SS precursor when transcribed and translated as described in Methods. Based on the sequence determined, SS precursor is approximately 19 kd. Figure 4A shows the single labelled protein of appropriate size obtained using this *in vitro* expression system. This protein can be immunoprecipitated by antiserum raised against *Zea mays* RUBISCO small subunit. (Figure 4A)

Transport experiments were carried out with a control incubation to which translation mix containing no SS precursor was added. No labelled band was seen in the control lane of stromal proteins analyzed on SDS gels, indicating that the labelled protein seen in the chloroplast stromal fraction in other lanes is the result of the addition of labelled SS precursor. (Figure 4B) The labelled band seen comigrates with stained SS on these gels (data not shown), which is a

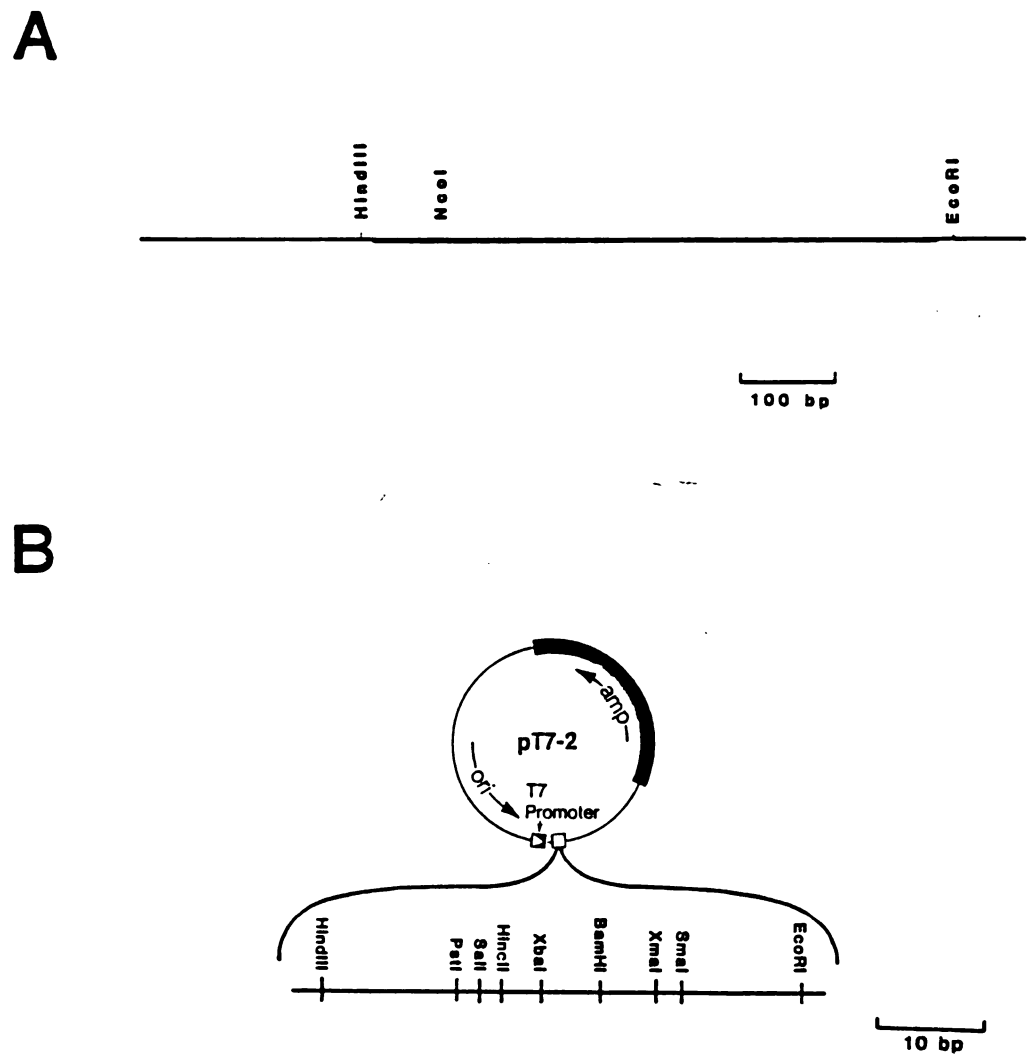


Figure 3. The structure of DNAs used in pTEB2 construction.
A. Partial restriction map of pSS19. The heavier line indicates the rbcS region.
B. Map of the Genescribe vector pT7-2. The poly-linker region is shown in detail

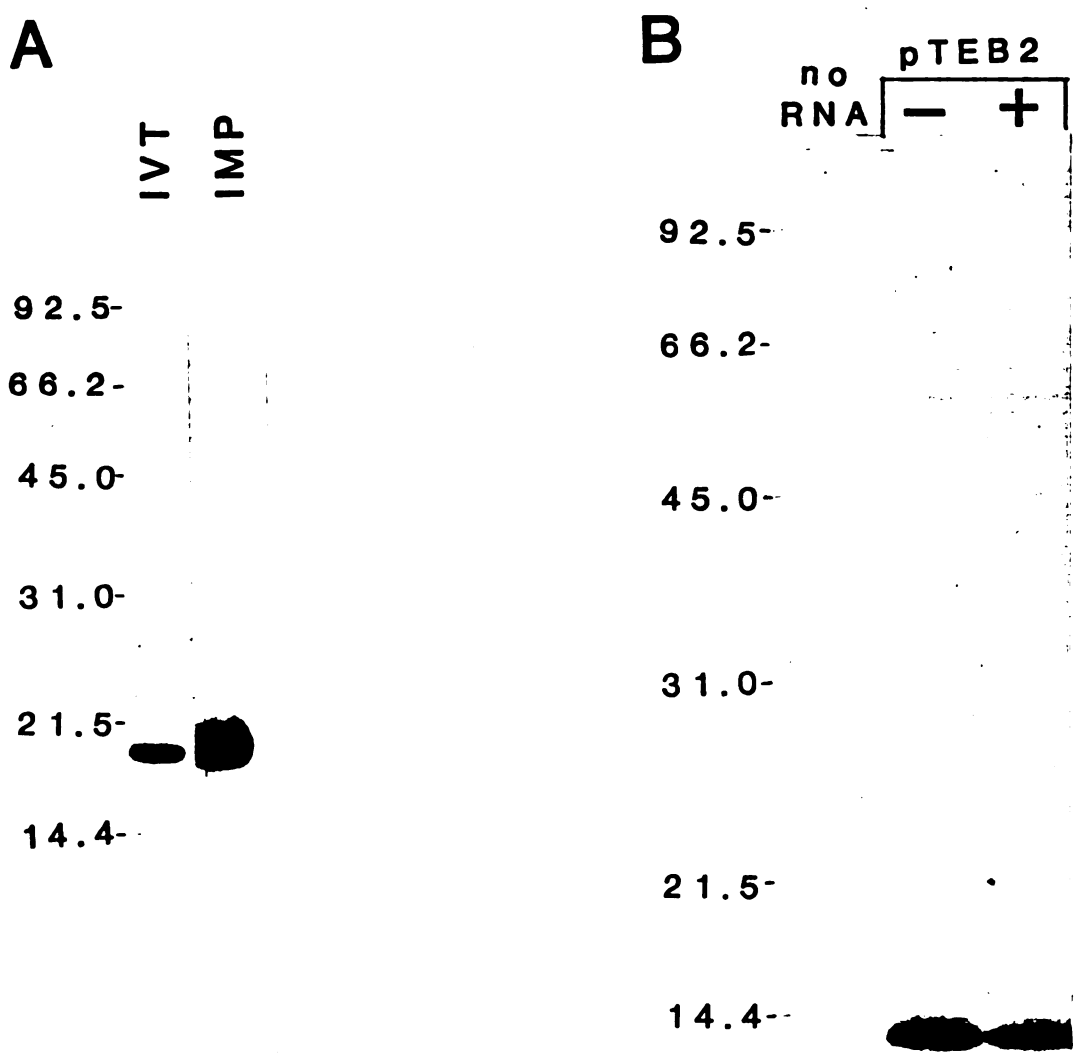


Figure 4. Analysis of pTEB2 expression products.
A. Fluorogram of SDS gel analysis of 2 uL of <u>in vitro</u> translation products (IVT) or of immunoprecipitation using 15 uL of the same translation reaction (IMP) and anti-SS antiserum.
B. Fluorogram of SDS gel analysis of the chloroplast soluble fraction from translocation assay which included either a control translation reaction (no RNA) or a translation reaction containing pTEB2 expression products (pTEB2). (+) and (-) indicate the presence or absence of a protease treatment following the incubation period. Molecular weights are in kilodaltons.

strong indication that the precursor is being correctly processed in this system. In addition, little or no loss of mature SS is seen when the chloroplasts are incubated with protease as described in Methods (Figure 4B), indicating that the processed protein is protected by intact chloroplast membranes.

Efficiency of *in vitro* RUBISCO SS transport

The distribution of radiolabelled protein into various compartments of the transport system following a transport incubation is shown in Table 1. All values were obtained from counting labelled proteins following SDS gel analysis, compared to the total amount of precursor added to the incubation. The incubation supernatant fraction was obtained by pelleting chloroplasts out of the incubation mix without the addition of wash buffer.

The transport efficiency, the percentage of added SS precursor which is transported and processed, is about 23%, as compared to 30% efficiency reported for pea SS transport. (51) Protein found in the various fractions totalled to 57% of the protein added, leaving 43% of the precursor added unaccounted for. (Table 1) Part of this protein may be degraded during the course of the incubation, although no degradation products are apparent on the gels. In addition, some protein may be loosely associated with the membranes and be lost during the wash steps. One surprising aspect of this analysis was the difference between the total amount of processed SS seen and the amount seen when only the soluble fraction was analyzed. One explanation for this is incomplete lysis of the chloroplasts under the normal lysis conditions used. However, when the chloroplasts were lysed in 0.5 mL 5

Table 1. Localization of added protein in incubation fractions. The percentage of added SS precursor found in different fractions of a transport incubation following the incubation period is shown below. Incubation conditions were as described in Methods except that the first centrifugation was done without the addition of wash buffer; this supernatent was the "incubation supernatent". Each value shown is an average of values from two separate experiments.

Incubation fraction analyzed	SS form seen	Percent of added protein seen
chloroplast, soluble	processed	8.1 + 0.6%
chloroplast, membrane	precursor	7.8 + 1.9%
chloroplast, total	processed	22.7 + 1.9%
incubation supernatent	precursor	26.5 + 5.5%

mM EDTA (as opposed to the normal lysis volume of 20uL), 15.9% of the total added protein was found in a processed form in the membrane fraction, which is essentially the same proportion seen in this fraction under normal lysis conditions. This suggests that some association of processed SS with the membranes is occuring.

The SS precursors associated with the chloroplast pellet are located on the outer surface of the chloroplasts, as demonstrated by their succeptibility to protease treatment. (Figure 5) The presence of SS precursors both on the chloroplast surface and remaining in the incubation mix after transport suggests that there are factors other than precursor availability limiting SS uptake in this system. An experiment was done to determine whether it is chloroplast factors or transport buffer factors which are limiting protein translocation. This experiment is outlined in Figure 6A. Two side by side incubations were done, and were stopped after 10 minutes by pelleting of chloroplasts and removal of the supernatent. In one incubation, the supernatant (i.e. used transport buffer) was added to fresh chloroplasts, while in the other fresh incubation buffer was added to the previously incubated chloroplasts. Again incubation was carried out under normal conditions for 10 minutes, after which the incubations were stopped as described above and analyzed in the usual manner. As a control, an incubation for 10 minutes under normal conditions was carried out. The results indicate that in chloroplasts to which fresh incubation buffer was added for the second incubation (sample B) approximately 50% more labelled protein was found in the stromal fraction than in chloroplasts from the control incubation. (Figure 6B) The fact that only 50% rather than 100% more was found may be due to a decline in chloroplast

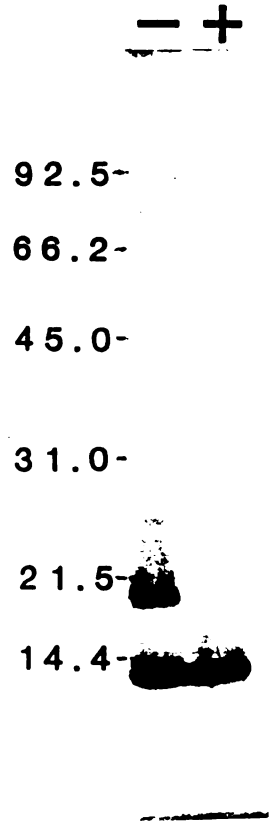
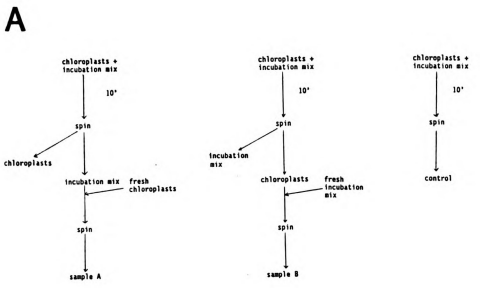


Figure 5. SS precursor succeptibility to added protease. Fluorogram of SDS gel analysis of the total chloroplast fraction from translocation assays containing pTEB2 expression products. (+) and (-) indicate the presence or absence of a protease treatment following the incubation period.



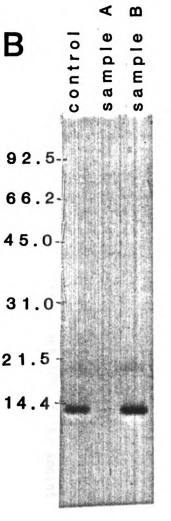


Figure 6. Mixing of new and used transport incubation components. A. Outline of mixing experiments. All incubations were for ten minutes, otherwise the conditions were as described in Methods. Completed incubations were centrifuged as described in Methods except that wash buffer was not added to the incubation before the spin. Samples A, B, and "control" were the end products, as shown above. B. Fluorogram of SDS gel analysis of the chloroplast soluble fractions obtained in the experiments outlined above. Samples A, B, and "control" correspond to the end products shown in panel A.

function over the course of the experiment. Another possibility is that some sites on the envelope membrane are occupied by non-transportable precursors and are thus not available to the added precursors.

In contrast, the fresh chloroplasts which had used incubation buffer added (sample A) contained only about 10% of the amount of labelled mature SS protein as that seen in the control incubation. (Figure 6B) These results suggest that the factors limiting uptake are a property of the incubation mix and not the chloroplasts, since chloroplasts can take up additional precursors when presented in new incubation mix. From gels, the precursors seen remaining in the incubation mix or bound to chloroplast outer membranes after incubation appear to be intact. However, there could be something about them which prevents their transport, or it could be that another component of the incubation mix which is somehow required for transport becomes depleted. This question was addressed by carrying out incubations using a smaller number of precursors. The amount of precursor-containing translation mix added was decreased, while a standard volume of translation mix was maintained by adding that which contained no SS precursors. Thus, the concentration of "transport factors" other than SS precursor, if they exist, was held constant. It was found that despite the precursor concentration change, a constant percentage of the precursors added were being transported. (Figure 7) This suggests that it is something about the precursors themselves in this system which prevents the majority of them from being transported. The limitation could involve the aggregation of SS precursor into an inaccessible form, or perhaps the presence of a detrimental

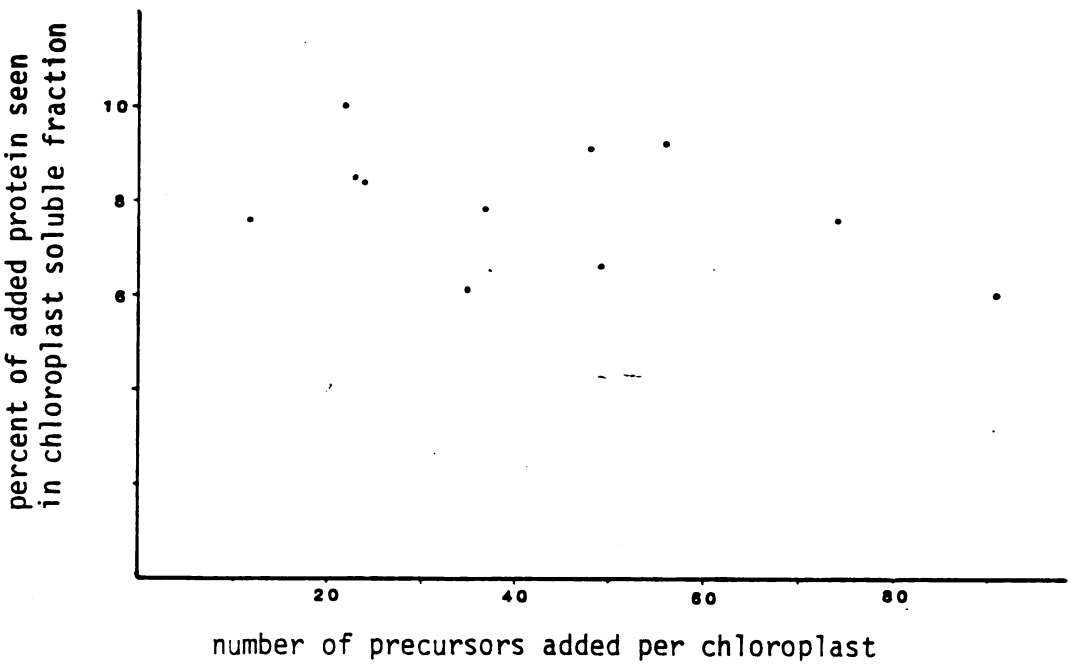


Figure 7. Percent of added SS precursors found in the chloroplast soluble fraction following incubation. Transport incubations and quantification of proteins translocated were performed as described in Methods. Each point is the result of a single experiment.

modification or the absence of a beneficial modification of the precursor. In addition, since we are unable to account for all the precursor added when totalling up the amount seen in various fraction after transport, it appears that some precursor breakdown occurs over the course of the incubation.

Time course of import of RUBISCO SS

Equivalent transport incubations were started at the same time and stopped at specific time points using the wash conditions described in methods. Final pellets were kept on ice until all incubations were done, then lysed and analyzed as described in methods. As shown in Figure 8, the relative number of percursors transported increased sharply in the first ten minutes, more slowly over the next five minutes, and then levelled off or declined slightly during the last twenty-five minutes. Similar results have been published recently for the transport of pea SS into pea chloroplasts. (51)

It is not known why the uptake of SS slows so dramatically after the first ten minutes of the incubation. One possibility is that a majority of the transportable precursors are transported within the first ten minutes. In addition, however, the chloroplasts appear to lose their capacity to import precursor over a forty minute incubation. If an incubation minus translation mix is started and the SS precursors are added after ten minutes (i.e., for the last thirty minutes of the incubation) 48% of the amount normally transported in forty minutes is transported. This declines to 25% when the translation mix is added for the last fifteen minutes of the incubation, and to 18% when it is added for the last five minutes. Thus, there is a decline in

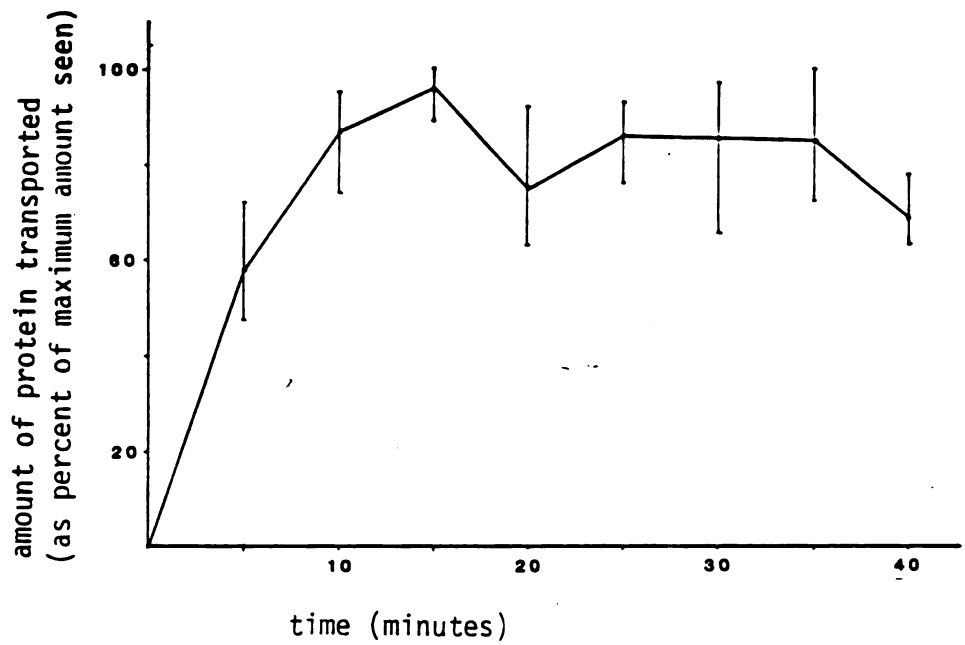


Figure 8. Time course of protein translocation.
Eight equivalent transport incubations were started at 0 minutes, with one stopped at each of the time points indicated. The level of protein translocation is shown as a percentage of the maximum amount of translocated protein seen in the experiment. Each point is an average of three separate experiments; error bars indicate the range seen between experiments.

chloroplast uptake capacity over time. However, considering that the decline in SS precursor uptake rate occurs so dramatically after only ten minutes, and that 96% of the precursors to be imported are imported after fifteen minutes (Figure 8), it appears that the limitation in protein uptake seen is due mainly to lack of transportable precursors.

A couple of explanations are possible for the slow decline in the amount of SS in the soluble fraction after twenty minutes. First, it is to be expected that some chloroplast lysis will occur over the course of the incubation. This would mean that processed SS is released to the incubation medium, and would be removed during the wash steps following the incubation. It is also possible that the protein is being degraded during the course of the incubation. As demonstrated above, the chloroplast preparations used contain both mesophyll and bundle sheath chloroplasts. Since neither LS or SS are expressed in mesophyll cells (8, 49) it is not known whether SS can be transported into mesophyll chloroplasts, although one preliminary report suggests that it may be possible. (2) If any SS is transported into mesophyll chloroplasts, then this portion of the total processed SS would be expected to turn over at a noticable rate, based on reports that when there is no LS available for assembly into holoenzyme, processed SS is selectively degraded. (73) Since all incubations are analyzed at the same time, this potential turnover could only be a factor in the apparent decline seen in uptake if turnover occurs more rapidly at 25^{o}C in the light than on ice. These two or three factors, a decrease in transport accompanied or followed by an increase in degradation of chloroplasts and/or protein, can probably account for the decline in SS levels seen at the later points of the time course.

Saturation of the transport response

The number of precursors per chloroplast needed to saturate the transport response was determined through a number of sequential experiments. Unfortunately, the constraints of the *in vitro* expression system limited the number of SS precursors available for addition to an incubation. Therefore, instead of drastically altering this factor, the number of chloroplasts included (as measured by the amount of chlorophyll added) was decreased until a point was reached where an increase in the number of SS precursors added to an incubation did not result in an increase in the amount of labelled SS found in the stroma. The last stage of this experiment is shown in Figure 9. The results indicate that the approximate number of SS precursors per chloroplast needed to saturate the transport response is 74. At this level of precursor available, and based on the transport efficiency reported earlier, 17 mature SS proteins are seen per chloroplast. This is in comparison to the figure of up to 1800 SS per chloroplast reported for transport into pea chloroplasts. (14)

A number of factors need to be taken into consideration when comparing these numbers. First, it has been reported that the levels of RUBISCO in *Zea mays* leaves are 5-6 times lower as percent of total soluble protein than those seen in the C-3 species tobacco and potato (pea is a C-3 species). This is due to a smaller amount of RUBISCO present rather than a greater amount of total soluble protein in these plants. (46) Thus, it is reasonable to think that lower levels of SS might need to be transported in corn. In addition, it is possible that SS precursor cannot be transported into mesophyll chloroplasts, in which case a portion of the chloroplast population would be serving

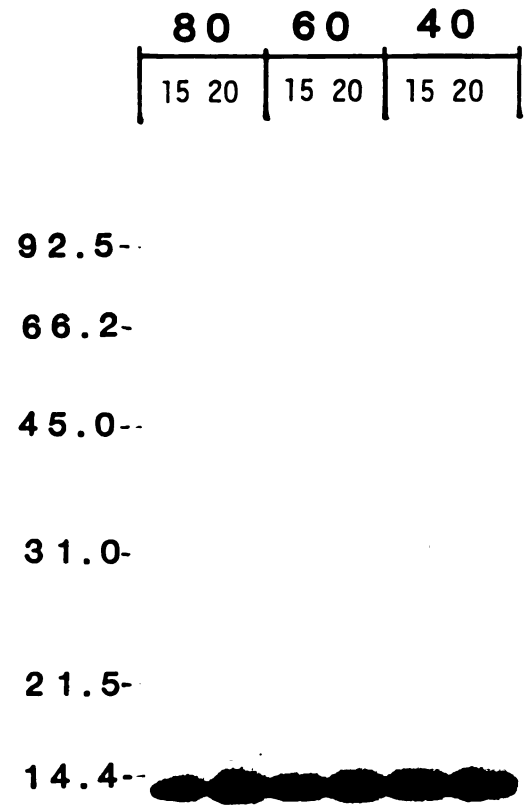


Figure 9. A test for saturation of the transport response. Fluorogram of SDS gel analysis of chloroplast soluble fractions. Transport incubations were as described in Methods, except that different precursor and chloroplast concentrations were tested to determine conditions under which the transport response was saturated. Either 20 or 15 uL of precursor-containing translation mix was added, with 5 uL of control translation mix added to the 15 uL to maintain constant volume. Three different chloroplast concentrations (equivalent to 80, 60, or 40 ug of chlorophyll) were tested.

only to dilute the active component. However, even with these factors taken into consideration it is clear that the numbers obtained for saturation of the transport response are extremely low, and are unlikely to be a reflection of the magnitude of SS transport in vivo.

DISCUSSION

A system for the transport of proteins into chloroplasts in vitro has been developed as a tool for studying factors involved in this transport. I have utilized this system for studying the transport of Zea mays RUBISCO small subunit into Zea mays chloroplasts. My study of some of the characteristics of this transport assay indicates that limitations do exist. First, only about 25% of the precursors added to the assay are capable of being transported, regardless of the concentration in which they are added. This suggests that some property of the precursors themselves is limiting transport. Some possible explanations might be aggregation of these precursors, either with other precursors or with components of the in vitro translation mix, breakdown of the precursor in a way which is not detectable on SDS polyacrylamide gels, or the presence or absence of a modification. Another limitation is a loss over time in the ability of the chloroplasts to transport precursor, with approximately 50% of transport ability lost after ten minutes of incubation in the light. This factor may well play a role in determining the low value obtained for saturation of the transport response.

Despite these problems with the in vitro transport assay, it does have several positive characteristics. First, SS precursor is transported into intact chloroplasts and processed to the correct size. Based on this we feel that it is being processed correctly, especially in light of earlier reports that SS processed in an in vitro assay is capable of being assembled into RUBISCO holoenzyme. (13) A second asset of the system is that it uses a single labelled precursor, making it possible to do the type of efficiency tests discussed above. This is especially important for studies like those described in the next chapter, where comparisons between the transport of wild type and mutant SS precursors are done. In addition, the in vitro expression system makes in possible to study the transport of any protein for which the gene is available, and to direct the synthesis of chimeric proteins where desired. Given these possibilities, it is clear that the system described is a useful one for analyzing some components of chloroplast protein transport.

CHAPTER 3

ANALYSIS OF SMALL SUBUNIT MUTANTS

INTRODUCTION

Several common features exist between SS transit peptides of higher plant species examined thus far. These include conservation of the positions of some proline and charged amino acid residues, as well as sequence conservation blocks found at the beginning, middle, and end of the transit peptide (regions I, II, and III, see Figure 10). Both regions II and III, which are highly conserved between species, are proposed to be required for specific processing events. (44, 59) Within the framework of conserved sequences region I is the least conserved, and, indeed, conservation of sequences at the amino terminus of the protein could be represented in ways other than that shown in Figure 10. It is generally felt, however, that some significant similarities do exist in this region. (44) If nothing else, the preponderance of methionine, alanine, and serine residues is noticable; at least seven of the first ten residues in each species is one of these three. One author has proposed that overall amino acid composition rather than specific sequence is important for function of this region, which perhaps acts just in keeping the amino terminus free

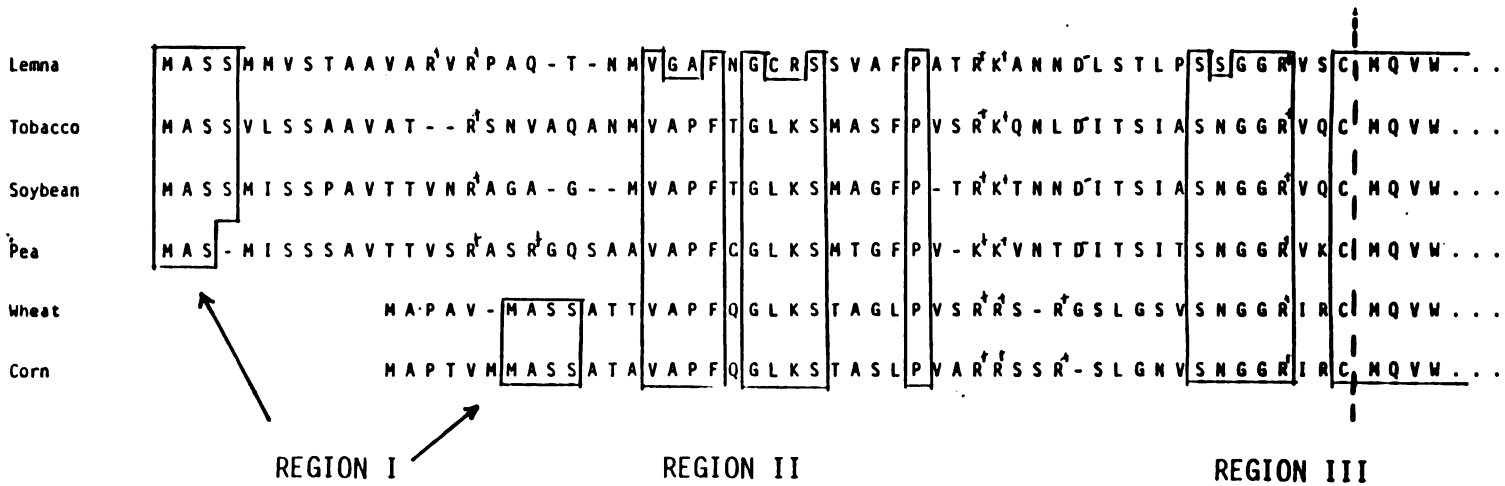


Figure 10. RUBISCO SS transit peptide sequence comparisons. Comparison of SS transit peptide sequences from Lemna (80), tobacco (56), soybean (5), pea (10), wheat (8), and corn. Regions of high amino acid sequence homology are boxed. The dashed line is the site at which the precursor is processed to give the mature protein.

for interaction with the chloroplast envelope. (74) Other than this speculation, no specific function for the amino-terminal region of the SS transit peptide has been proposed. A major difference between the Zea mays (corn) SS precursor and the SS precursor most commonly used in transport studies, that from pea, is the length of the amino-terminal region. This fact, combined with the lack of knowledge generally about the function of the amino terminus of SS precursors, makes this region an attractive one for study in a system using Zea mays SS precursor and chloroplasts.

Several Zea mays rbcS mutants have been constructed by making alterations in region of the gene which encodes the amino terminus of the precursor. These mutants, along with a wild type SS gene, have been expressed using the in vitro system described in the previous chapter. Each mutant has been assayed in vitro for transport efficiency relative to wild type SS precursor.

MATERIALS AND METHODS

DNA sequence analysis

Plasmid DNA was prepared for sequence analysis as described by Haltiner et al. (30) Sequencing reagents for dideoxy chain termination reactions were from New England Biolabs and were used as described by the manufacturer, based on the method of Sanger et al. (68) Genescribe sequencing primer was from U. S. Biochemical Corp., and was used at a concentration of 2.7 pmol per microgram DNA. Sequencing by the

chemical cleavage method, where necessary, was done according to Maxam and Gilbert. (55)

Gel electrophoresis

Analysis of protein samples was carried out as described in Chapter 2. DNA fragments were analyzed on 8% polyacrylamide gels, with gel and running buffers of 89 mM Tris, 89 mM borate, 2.5 mM EDTA, pH 8.3. Samples were suspended in the same buffer plus 5% glycerol, with xylene cyanol and bromophenol blue as tracking dyes.

Other methods

DNA modification, quantitation of precursor, chloroplast isolation, and transport incubations were as described in Chapter 2.

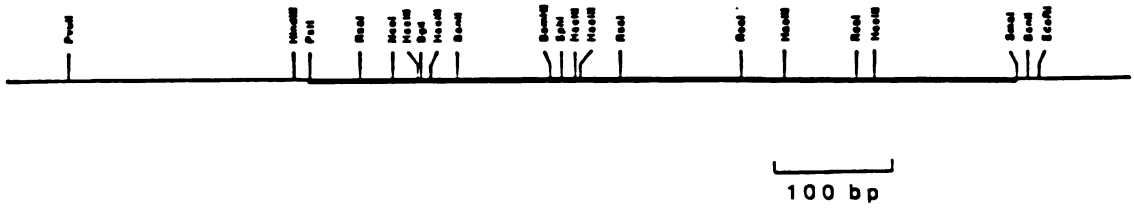
RESULTS

Construction of mutants

The clone pSS19, described in the previous chapter, was the clone from which all rbcS gene constructions, both mutant and wild type, were derived. A restriction map of the rbcS gene in pSS19 is shown in Figure 11A.

My original approach to obtaining high levels of SS expression was to express the protein in E. coli. DNA manipulations of pSS19, as described in Appendix A, gave the clone pZS13. This clone expressed at high levels a fusion protein with the same amino acid structure as that

A



B

Ile Pro Met Met Ala Ser Ser

5′. . . ATT CCA **ATG ATG GCC TCG T**CG G. . .

3′. . . TAA GGT **TAC TAC CGG A**GC AGC C. . .

Figure 11. DNAs used in construction of rbcS mutants.
A. Restriction map of pSS19. The heavier line indicates the rbcS region.
B. Structure of the linker used in the construction of pTEB13 is shown in bold face. Surrounding DNA sequences in the clone are shown as well. The corresponding amino acid sequence is shown above the DNA sequence.

encoded by pTEB1. To obtain *in vitro* expression, the upstream PvuII site and the EcoR1 site of pZS13 were used to remove *rbc*S from the clone. This gene fragment was inserted into the HincII and EcoR1 sites of the transcription vector pT7-2, giving the clone pTEB1.

The construction of the clone expressing a wild type SS, pTEB2, is described in Chapter 2. The wild type protein expressed by pTEB2 was used as a standard in the study of the transport of mutant SS precursors.

The clone pTEB6 was constructed from pTEB2. The internal HindIII-BamHI fragment was isolated and digested with HaeIII, which has two sites within the fragment. The HindIII-HaeIII fragment and HaeIII-BamHI fragment were ligated together and then back into the large HindIII-BamHI pTEB2 fragment, with the net effect being deletion of a 9 bp HaeIII-HaeIII piece.

It is thought that in eukaryotic systems translation generally initiates by movement of a ribosome down an mRNA until an initiation codon (ATG) is encountered. (45) Based on this, the first ATG in *rbc*S was removed so that translation would initiate at the next ATG and the protein structure desired for pTEB9 would be obtained. (Figure 12) Conveniently, the first ATG in *rbc*S is contained in the only NcoI site in pSS19, and was easily deleted using the single-strand specific nuclease S-1 to blunt the cohesive ends obtained by digestion with NcoI. Since pTEB2 does not contain an intact NcoI recognition sequence (see Chapter 2), it was necessary to go back to pSS19 for the construction of pTEB9. A clone containing the HindIII-EcoRI *rbc*S fragment inserted into the same sites of pT7-2 was treated with NcoI and S-1 as described, with the resulting blunt ends ligated together to

give the clone pTEB5. Unfortunately, expression of pTEB5 gave low levels of altered SS as compared to the levels obtained from expression of pTEB2. pTEB5 had a larger region upstream of the rbcS coding sequence than pTEB2, so a portion of this was deleted to see if the level of protein expression could be improved. For this deletion the gene fragments HindIII-BamHI and BamHI-EcoRI were isolated from pTEB5. The HindIII-BamHI piece was digested with RsaI, and the larger fragment (RsaI-BamHI) was isolated and ligated to the BamHI-EcoRI gene fragment. This reconstructed gene was ligated into HincII-EcoRI digested pT7-2 to give the clone pTEB9, which gave expression of altered SS at a level comparable to the expression of wild type SS from pTEB2.

pTEB10, a pTEB9 derivative, and pTEB12, a pTEB1 derivative, were each construced using the same strategy used to construct pTEB6.

pTEB13 was constructed using an oligonucleotide linker (Figure 11B) to obtain the desired sequence. The BglI-EcoRI rbcS fragment was isolated and the linker, which contained one blunt end and one BglI cohesive end, was ligated to it. The resulting fragment was inserted into pUC19 which had been digested with HindIII, treated with S-1 nuclease to blunt the HindIII cohesive end, and then digested with EcoRI. From this clone the PvuII-EcoRI gene fragment was isolated; this fragment was then ligated into the HincII and EcoRI sites of pT7-2 to give pTEB13.

Analysis of mutant structure

The derived amino-terminal amino acid structure of each mutant is shown in Figure 12. All clones were sequenced through the region where alterations were made to be sure that the construction scheme yielded

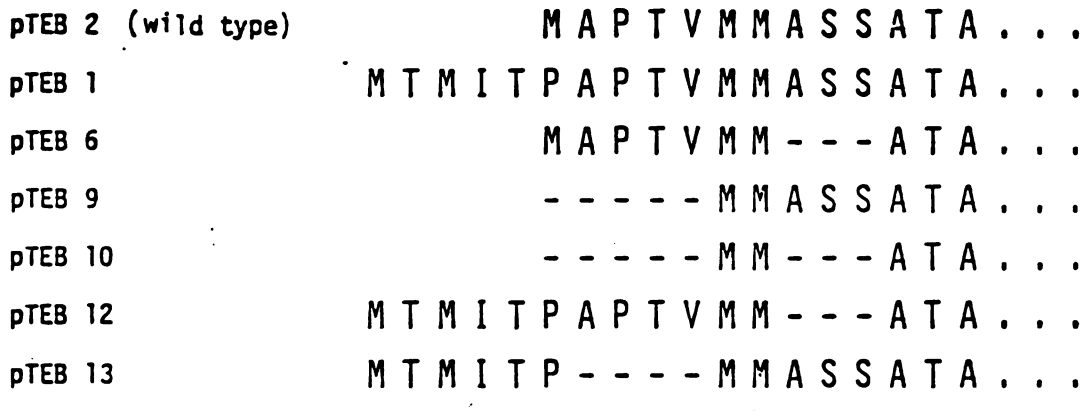


Figure 12. Amino acid sequences of RUBISCO SS mutants. Derived amino acid sequences of the pTEB clones constructed as described in the text. Only the amino-terminal region of the transit peptide is shown in each case. Dashes indicate deleted amino acid residues.

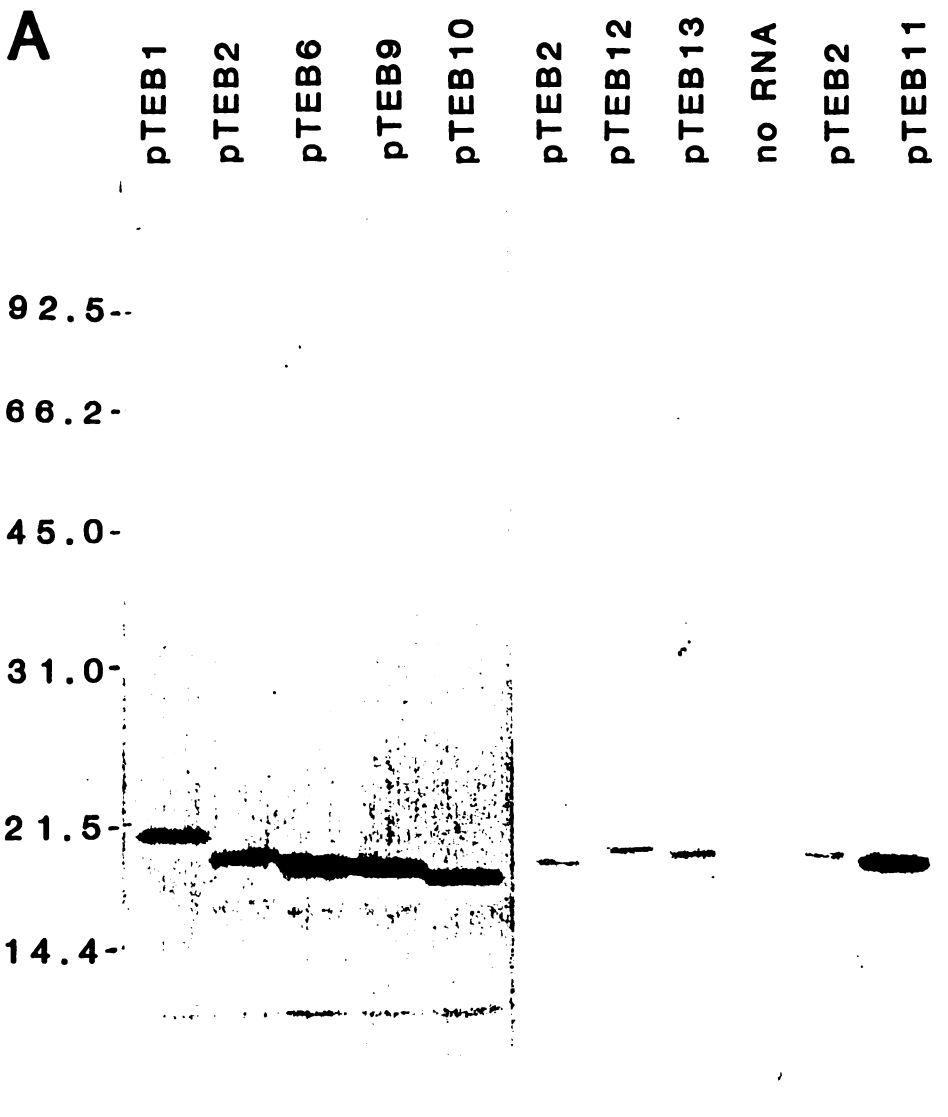
the desired structure. In addition, the HindIII-EcoRI gene fragment from each clone was analyzed on a polyacrylamide gel and found to migrate correctly according to the size expected. The migration pattern of the proteins obtained by expression of these clones is shown in Figure 13. As discussed in Chapter 2, the size of the pTEB2-derived protein appears to be correct, and Figure 13 indicates that the sizes of the altered SS precursors relative to the wild type and to each other appear to be correct as well. The protein encoded by pTEB1 migrated slightly slower than would be expected from the protein sequence. However, the lack of an upstream initiation codon in pTEB1, the ability of anti-SS antiserum to immunoprecipitate pTEB1-derived protein (Figure 13), and the fact that this protein was transported and processed by isolated chloroplasts (Figure 14) all suggest that pTEB1 encodes an altered SS protein of the expected structure.

The remainder of the text describes analysis of the proteins obtained from *in vitro* expression of these clones using T-7 RNA polymerase and rabbit reticulocyte lysate as described in Methods. For convenience, the proteins obtained by expression of the various *rbc*S constructions will be referred to by the name of the clone they are encoded by (i.e. pTEB1, etc.)

Transport incubations

Determination of the number of SS precursors per microliter of translation mix was done as described in Methods. Based on this information a certain volume of precursor-containing translation mix was added to each transport incubation such that an equal number of precursors were added to each. Where necessary the translation mix

Figure 13. In vitro translation products of pTEB mutants.
A. Fluorogram of pTEB in vitro translation products analyzed on SDS gels.
B. Fluorogram of SDS gel analysis of 2 uL pTEB1 translation products (IVT) or of immunoprecipitation using 15 uL of the same reaction (IMP) and anti-SS antiserum.


A
pTEB1
pTEB2
pTEB6
pTEB9
pTEB10
pTEB2
pTEB12
pTEB13
no RNA
pTEB2
pTEB11
92.5
66.2
45.0
31.0
21.5
14.4


B
IVT
IMP

Figure 13.

volume was supplemented with translation mix containing no precursors, so that each incubation contained an equal volume of translation mix. Additional calculations were needed to determine the concentration of pTEB13 in some translation mixes; these calculations are explained in detail in Appendix B.

Incubation of precursors with isolated chloroplasts and analysis of protein translocation were carried out as described in Methods. Figure 14 shows autoradiograms of the transport of the mutant SS precursors in comparison to wild type (pTEB2). As can be seen, all the mutants were processed to a protein which comigrated with wild type processed SS, indicating that the mutants were correctly processed. In addition, Figure 14 shows that treatment of the transport incubations with protease does not significantly diminish the amount of radiolabelled protein in the soluble fraction, indicating that processing of these mutant precursors occurs in conjunction with translocation of the protein into intact organelles.

Relative transport efficiency

Within each experiment, the number of SS precursors added to each incubation was equalized as described above. This meant that it was possible to compare directly the number of precursors transported in each incubation in order to determine a transport efficiency for each mutant relative to wild type. These relative transport efficiencies are shown in Table 2. For each mutant both an average value and a range of values are shown. The different steps involved in the transport reaction itself and in quantitation both before and after the transport reaction provide several opportunities for small errors to be

Figure 14. Analysis of transport incubations containing pTEB mutants. Fluorogram of SDS gel analysis of the chloroplast soluble fraction of translocation assays containing the pTEB mutant indicated or pTEB2 (wild type). Assays and analysis were carried out as described in Methods. Precursor concentrations within an experiment were equalized as described in text, with pTEB2 always included in the experiment as an internal reference. (+) and (-) indicate the presence or absence of a protease treatment following the transport incubation.

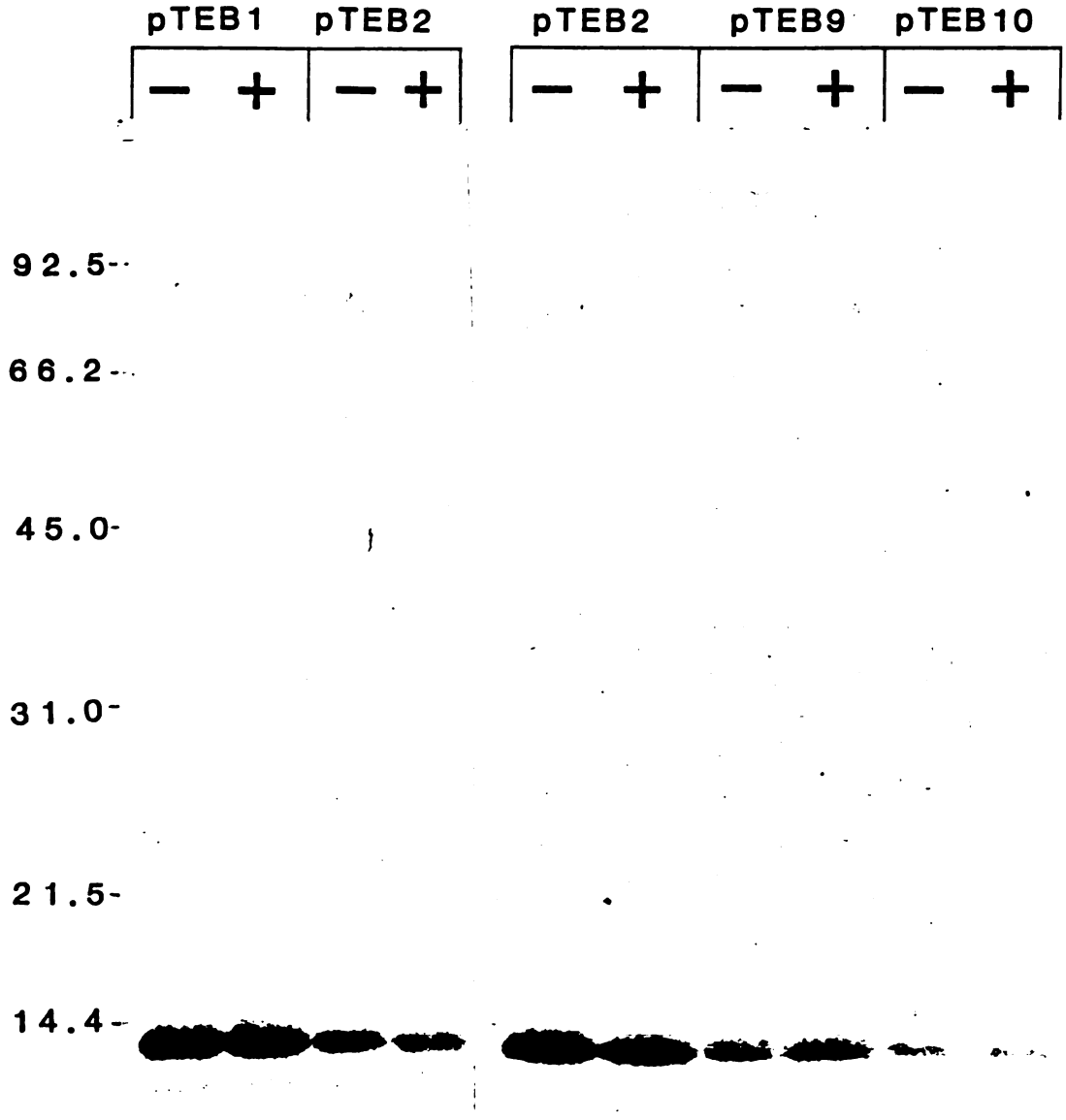


Figure 14.

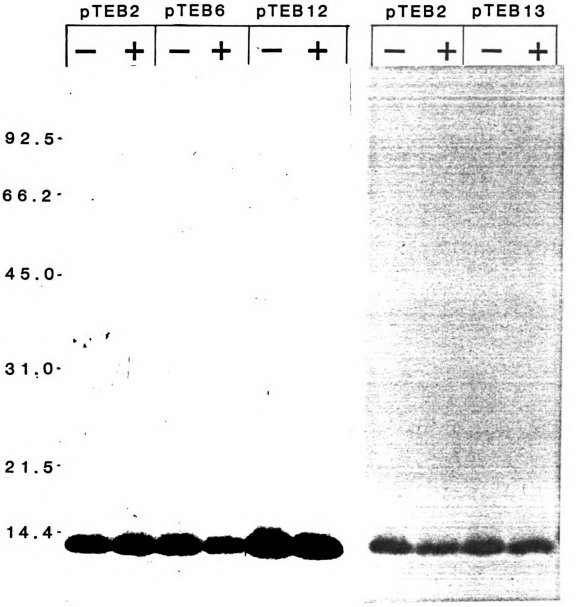


Figure 14 (cont.).

Table 2. Relative transport efficiencies of each mutant as compared to pTEB2. The method of determining relative transport efficiency is described in text. Also shown is the number of precursors per chloroplast (precursor concentration) needed to saturate the transport of each mutant, as well as the relative efficiencies at the highest and lowest precursor concentrations used.

mutant	average relative efficiency	number of experiments	range of rel. efficiencies	precursor concentration at saturation	rel. efficiency at saturation	lowest precursor conc. used	rel. efficiency at lowest precursor conc. used
pTEB1 :	209%	4	192-225%	67	192%	31	225%
pTEB6	98%	4	73-112%	65	73%	24	102%
pTEB9	37%	4	22-49%	97	22%	32	40%
pTEB10	32%	4	24-38%	66	29%	31	24%
pTEB12	172%	5	130-239%	72	nd	27	198%
pTEB13$_a$	124%	3	112-146%	nd	nd	34	114%

a: see Appendix B
nd: not determined

introduced, and this is thought to be the cause of the range of values seen for each mutant. However, in each case the average value shown is an accurate reflection of the data obtained, and as such is useful in comparing the transport efficiencies of the mutants to each other and to wild type.

Saturation of transport response

Determination of the number of precursors needed to saturate the transport response was done as described in Chapter 2. Each mutant was characterized in this manner; the results are shown in Table 2. The tests of saturation were done because it was felt that the changes made in the mutants might alter the way in which precursor concentration affected transport efficiency, giving a curve shaped differently than that seen for wild type. Were this the case, the value obtained for relative transport efficiency of each mutant could be highly dependent on the precursor concentration used. A test for relative efficiency under saturating conditions would remove this factor.

As mentioned in Chapter 2, the constraints of the in vitro expression system limited the number of precursors produced. For each precursor type, the value obtained for precursor concentration (number of precursors added per chloroplast) necessary to saturate the transport response was close to the limits of precursor concentration available. In fact, the concentration needed was often above the concentration available in a particular experiment, and most of the transport incubations were carried out under non-saturating conditions. For pTEB12 saturation conditions were identified but were never available for use in a determination of relative transport efficiency.

Despite a number of attempts, saturating conditions for pTEB13 were not determined.

As Table 2 shows, the relative efficiencies under saturating conditions were always at or near the bottom of the range of relative efficiencies seen. In contrast, the relative efficiencies under the lowest precursor concentrations used were, except in the case of pTEB10, at or near the top of this range. Although a precise correlation is not seen these results suggest that, were all incubations carried out under saturating conditions, the average relative efficiencies seen would be somewhat lower than those seen here. However, in no case, except possibly with pTEB12, would such a shift in the values obtained dramatically alter the conclusions which can be drawn regarding the effect that the alterations made have on transport of the precursor.

DISCUSSION

The SS precursor encoded by pTEB1 has six amino acids derived from the E. coli protein B-galactosidase as the amino terminal structure, and is transported with a relative efficiency of approximately twice that of the wild type precursor. The deletion of the first five amino acids of the transit peptide, the structure encoded by pTEB9, also has a dramatic effect on transport, reducing it by more than 50%. On the other hand, the deletion of an internal sequence which is most of homology block I (A-S-S, see Figure 10), as encoded by pTEB6, has essentially no effect on transport efficiency.

These results indicate that alteration of sequence at the amino terminus of a transit peptide can have a dramatic effect on the efficiency with which a SS precursor is translocated. This has not been demonstrated before for chloroplast protein transport. For mitochondria, an examination of how the deletion of residues 2-7 of the OTc precursor affects transport of this protein indicates that the amino-terminal region is important for efficient translocation here as well. (36)

Three possibilities exist for the way in which these alterations are affecting transport. One is that it is the overall length of the transit peptide which is important. A second is that the specific sequence either added or deleted is important. A third is that it is not specific sequences but rather general chemical characteristics which are important.

Although transport efficiency declines with decreasing length, the mutants containing A-S-S deletions seem to argue against length alone being an important factor in this efficiency. As mentioned above, pTEB6 is transported with essentially the same efficiency as pTEB2. The relative lack of effect of this deletion on transport efficiency is seen again both in pTEB10 as compared to pTEB9 and in pTEB12 as compared to pTEB1.

The unexpected increase in transport efficiency seen for pTEB1 seems to negate the possibility that specific sequences at the amino terminus are required for function, since the sequence added is totally unrelated to SS transit peptide structure. If a specific sequence were involved, the addition of an unrelated sequence would be more likely to

reduce transport by affecting recognition of this sequence than to enhance it.

The remaining possibility is that it is the general chemical character of the amino terminus which is affecting transport efficiency in these mutants. If this is so, the important character seems to be concentrated at the amino terminus, since the M-A-P-T-V deletion has a large effect while the A-S-S deletion has essentially no effect. The sequence added to the amino terminus in pTEB1, M-T-M-I-T-P, has chemical characteristics similar to the wild type amino-terminal sequence, M-A-P- T-V-M. Four of the six amino acid residues (M, M, P, T) are the same, although scrambled in position. Three other residues seen, A, V, and I, are all nonpolar with alphatic side chains. One main difference between the amino-terminal sequences, aside from the order of amino acid residues, is the presence of two rather than one polar residues (T) in pTEB1. In addition, pTEB1 and pTEB 12 have two proline (P) residues within the first 10 amino acids, while the rest of the precursors have one or none.

There are at least three possible explanations for the effect seen with the structure of pTEB1. One is that the amino terminus now has a similar but more effective character for carrying out the function of this region of the transit peptide. Another possibility is that a repetition of desired features enhances the functional characteristics of this region. A third possibility is that the effect of this addition is completely unrelated to the role that the wild type amino terminus plays but does not interfere with that role. The construction and analysis of the mutant pTEB13 ruled out the first of these possibilities, since if a superior substitution was occuring in pTEB1

the relative transport efficiencies for pTEB1 and pTEB13 should be similar, and they are not. Unfortunately, the results obtained with pTEB13 do not distinguish between the later two possibilities. If enhancement was occuring with the pTEB1 structure, then the M-T-M-I-T-P sequence could be subsitituting for the deleted A-P-T-V sequence in pTEB13. On the other hand it is possible that each of these sequences has an independent effect on transport efficiency. In this case the effect in pTEB13 could be an additive one where the increased efficiency in the step enhanced by the M-T-M-I-T-P sequence (as seen in pTEB1) compensates for the loss of efficiency at the step affected by deletion of the M-A-P-T-V sequence (as seen in pTEB9). The construction of mutants which contain a homologous repeat at the amino terminus (i.e. M- A-P-T-V-M-A-P-T-V or M-T-M-I-T-P-M-T-M-I-T-P) may be able to distinguish between these two possibilities. Based on the sequence similarities seen, however, it seems likely that it is an enhancement/substitution which the M-T-M-I-T-P sequence provides. Naturally, it is important to remember that the mechanisms involved in determining how well a precursor is transported are very unlikely to be as clear cut as the possibilities discussed here. Deletion of the A-S-S sequence does have some effect in pTEB12, for example, and this and other effects of the alterations made probably cannot be explained in any one specific way.

From the results described above, it is clear that no matter how the alterations tested are affecting transport and no matter how one change may be compensating for another, no one of these sequences is absolutely required for transport. The deletion giving the largest decrease in transport efficiency reduces it by 60-70%, but this still

leaves a significant portion of the precursors added being transported. This would suggest that whatever the role that specific regions within the transit peptide are playing, there is flexibility which allows existing sequences to compensate for missing ones, at least to some degree. These results do demonstrate, however, that the amino terminus of the transit peptide does play a role in protein translocation, and that this role is probably dependent on the overall chemical character of this region, rather that on the length of the precursor or the presence of a specific sequence. The function in the translocation process which is affected by the altered transit peptide sequences has not yet been determined. An attempt was made to separate binding from translocation using the conditions described by Cline et al (14) in order to determine whether discrimination between SS precursor types occurs as early as the binding step. Unfortunately, these conditions did not prevent protein translocation in my system, and thus were not useful. A possible alternative might be to examine binding of precursors to isolated envelope membranes, as described by Pfisterer et al. (63) As they become available other methods of dissecting the translocation process may also be useful in identifying a specific step affected.

CHAPTER 4

PRELIMINARY ANALYSIS OF A PROCESSING SITE MUTANT

INTRODUCTION

The sequence around the site where SS precursor is processed to the mature size (region III, see Figure 10) is highly conserved within both the transit peptide and the mature protein. It has been postulated that all or part of this specific sequence is required for correct processing of the precursor. (44) A study in which the SS transit peptide was fused to a foreign protein (NPtII) has ruled out the requirement for this exact sequence on the mature side of the precursor. The fusion construction contains all of the pea SS transit peptide plus the first residue (methionine) of the mature protein, fused to NPtII. (83) Thus, while the actual cleavage site is preserved, the rest of the sequence homology seen on the mature side of the precursor is not. In this case processing of the fusion protein to a product close in size to NPtII is seen and it has been suggested that cleavage at the preserved processing site occurs, although sequence analysis of the processed fusion protein has not been done to verify this. (83) Another study, using isolated transit peptidase, has looked at the effect of modification of the site on processing. (66) If pea

SS precursor is treated with iodoacetate, processing to the mature size does not occur and the accumulation of an intermediate is seen instead. The authors propose that this is due to the carboxymethylation of the cysteine residue at the processing site, but whether this involves non-recognition of this cysteine residue or a more general disruption of the secondary structure is not known. (66) In order to examine the fate of a Zea mays SS precursor which has no processing site, I have constructed such a mutant and analyzed it for transport and processing in the in vitro assay system described earlier.

MATERIALS AND METHODS

Immunodetection of SS expression in E. coli

Single bacterial colonies transformed with the plasmid DNA of interest were picked onto a nitrocellulose filter (0.45 um, Schleicher and Schuell) placed on LB agar plates (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% bactoagar) containing 50ug/mL ampicillin as a selection agent. After growth at 37^{0}C overnight, the filter was removed from the plate and suspended over chloroform vapors for 45 minutes. The filter was then placed in a solution of Tris-saline (see Chapter 2) with 3% BSA, 0.1% Tween 20, 2 ug/mL DNase, and 40 ug/mL lysozyme and allowed to sit at room temperature for 45 minutes. After gentle shaking in the same solution for 90 minutes, this solution was removed and the filter was washed four times for ten minutes each time in Tris-saline with 1% BSA. Incubation with antiserum and color detection of immune complexes was as described in chapter 2. A 1:1000

dilution of antiserum containing antibodies against Zea mays SS was used.

Other methods

All other methods were as described in previous chapters. The chemical cleavage reactions (55) were used for DNA sequence analysis of the mutant.

RESULTS

Construction of a mutant containing a processing site deletion

The SphI site of rbcS contains the sequence encoding the first residue of mature SS (methionine, see Figure 1). Deletions in this region of the gene were made by digestion at the SphI site followed by Bal31 treatment for 2-12 minutes. The DNA was then ligated and transformed into E. coli. The clone pZS13 (see Chapter 3) was used for this construction because pZS13 expresses SS precursor in E coli, allowing screening of the transformants with antibody against SS. This antibody was raised against SS isolated from leaves (see Chapter 2), and thus reacts only with the mature portion of the SS precursor. Therefore, only those clones in which the coding sequence for the mature portion of the protein was in frame with the coding sequence for the transit peptide reacted with the antibody. The colonies selected by immunodetection were further screened by restriction analysis for small deletions, which led to the selection of a single clone, pZS26. This clone and pTEB2 were digested with BanII (see Figure 11), and the

isolated BanII gene fragment from pZS26 was ligated into the BanII vector fragment from pTEB2. This provided a wild type amino-terminal coding region. The resulting clone, pTEB11, was sequenced through the region of the gene which encodes the processing site to determine its structure, the relevant portion of which is shown in Figure 15. As can be seen, deletion has occured on both sides of the processing site.

Analysis of transport and processing of pTEB11

Transport of pTEB11 was assayed using the same conditions described in previous chapters. Membrane, soluble, and total chloroplast fractions were analyzed by SDS polyacrylamide gel electrophoresis. As shown in Figure 16, at least three bands smaller than the precursor, of approximately 14, 16, and 17.5 kd, were seen in the analysis of membrane and total chloroplast fractions. The highest of these was also seen in the analysis of total chloroplasts incubated with pTEB2 (Figure 16). The background seen in these gels is thought to be due to association of the labelled methionine in the assay with the chloroplast membranes, and this 17.5 kd band may be due to the same thing. Alternatively, it may be a membrane-associated SS processing intermediate; 17.5 kd is approximately the size of the intermediate one would expect to see if cleavage at the internal site identified by Mishkind et al (59) was occuring. The two lower molecular weight bands were unique to transport of pTEB11. From the migration pattern one appeared to be slightly larger than mature SS, while the other was the same size or slightly smaller than mature SS. Treatment of the chloroplasts with protease removed the majority of the precursor-sized protein (Figure 16). Some remained, however, suggesting that a

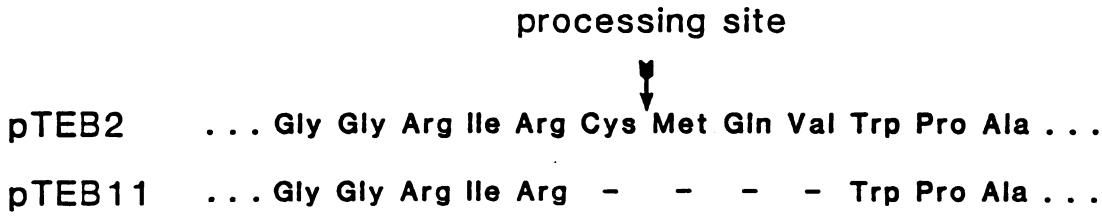


Figure 15. pTEB2 and pTEB11 sequence comparison. Comparison of the amino acid sequence in wild type pTEB2 and in the mutant pTEB11. Only the sequence surrounding the processing site is shown. Dashes indicate amino acid deletions. The processing site is as indicated.

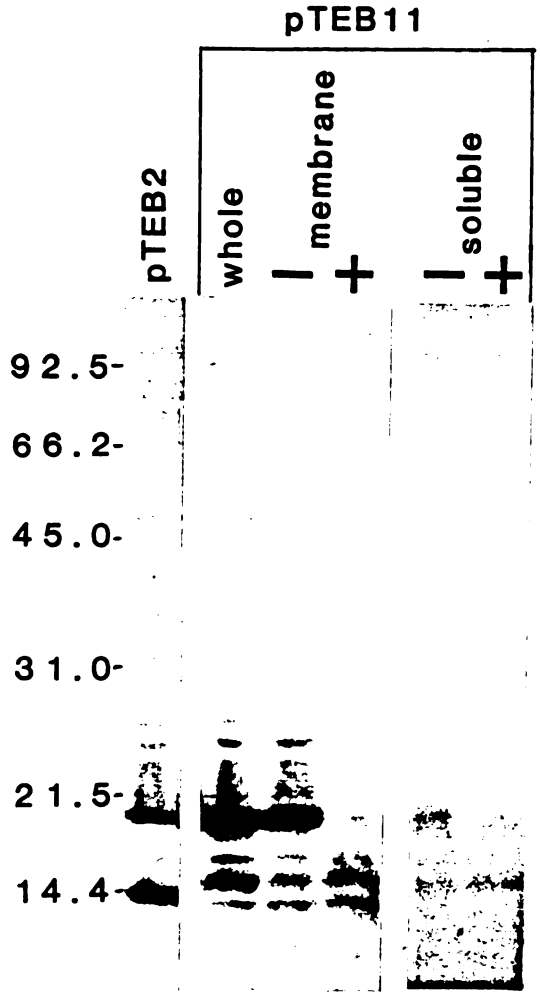


Figure 16. Transport and processing of pTEB11. Fluorogram of SDS gel analysis following transport incubations. The total chloroplast pellet of the pTEB2 incubation was analyzed. pTEB11 incubations were fractionated as indicated. (+) and (-) indicate the presence or absence of a protease treatment following incubation.

fraction of the precursor may be inside the chloroplast. Analysis of the soluble fraction showed two major labelled proteins, one slightly smaller than the precursor (about 18.5 kd) and one slightly larger than mature SS (about 15 kd). (Figure 16) The amount of labelled protein seen in the soluble fraction was small; this portion of the autoradiogram was exposed 2.3 times longer than the portion showing the membrane fraction.

DISCUSSION

I have constructed a SS precursor mutant in which four amino acid residues around the site where the precursor is processed to the mature protein have been deleted. Incubation of this mutant with isolated chloroplasts yields several discrete products, most of them intermediate in size between the precursor and the mature forms of the protein. As indicated by the fact that the intermediate forms of the protein are resistant to added protease (Figure 16), the protein can be translocated even if normal processing does not occur. The protease experiment also suggests that the deletion made does not seriously affect binding of the altered precursor to the chloroplast, since significantly more SS is found attached to the chloroplast in a protease-sensitive location than is found within the chloroplast. It is not clear from these experiments how the deletion made affects the rate of protein translocation.

My results indicate that in the absence of the site at which the precursor is processed, normal cleavage cannot occur. This is not

unexpected, but it is interesting that the majority of the abnormally-processed proteins seen are associated with the membrane fraction. This suggests that a specific processing step may be required to free the protein from the inner envelope membrane. One model is that the transit peptide stays embedded in the inner envelope membrane while the rest of the protein moves into the stroma and stays tethered until cleaved by the transit peptidase. If this is occuring it would suggest that the intermediate products seen in the transport of pTEB11 are the result of carboxy-terminal cleavages, since a transit peptide embedded in the membrane would not be accessible to protease. Alternatively it may be that a small region of the transit peptide near the processing site associates with the membrane and that both transit peptide and mature regions are accessible in the stroma. The pathway for the intermediates seen in the stromal fraction is unclear, but they may simply be proteins dissociated from the membrane by the fractionation procedure.

The study discussed earlier, an examination of the transport and processing of a pea SS precursor treated with iodoacetate (66), has results which are relevant to the results presented above. As mentioned, incubation of iodoacetate-treated precursor with isolated transit peptidase casues accumulation of a single, 18 kd processing intermediate. This intermediate is thought to be the same as that seen transiently in processing of unmodified pea SS precursor. (66) However, when the modified precursor is incubated with isolated chloroplasts, up to eight radiolabelled products are seen in the stromal fraction. The largest of these is approximately 18 kd, the smallest is smaller than processed SS, and one about the size of

processed SS is seen as well. (66) An analysis of the membrane fraction is not shown, so it is not known what, if any, radiolabelled protein is associated with this fraction. Without this information it is difficult to say how much these results differ from mine, although based on what is presented it would appear that the primary cleavage by the transit peptidase occurs normally, and that the intermediate produced is selectively degraded in the stroma by the action of other proteases. The lack of soluble intermediates in my analysis may be an indication that the intermediate cleavage demonstrated for pea SS processing does not occur in Zea mays, or that it occurs in such a way that the intermediate remains membrane-associated. In both studies it is clear that something is preventing the normal processing of the SS precursor. With the mutant pTEB11 this effect can be attributed to the deletion made, and it seems likely that the primary cause is the loss of a needed site. With iodoacetate treatment all the cysteine residues in the protein (there are three) might be affected, but here too it seems likely that the effect seen is due directly to modification of the processing site.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Two general approaches are being taken to increase our understanding of the mechanisms by which proteins are translocated in cells. One involves characterization of components of the translocation system other than the translocated protein itself. This approach has been especially successful in the identification of components in the cytosol and the membrane which are part of the translation arrest/ membrane binding/release of arrest system seen in the initiation of the movement of proteins into and through the ER. (89) With the transport of proteins into organelles, there has been some success in identifying cellular components involved in protein translocation. There have been reports that both cytoplasmic protein (62) and RNA factors (20) are required for the association of precursors with mitochondria *in vitro*, and partial purification of a protein factor has been achieved. The factors were found in rabbit reticulocyte lysates, and the protein factor was found in yeast lysates as well. (62) The role that these factors play, assuming that they function *in vivo*, has not been determined. One study in which proteins to be transported into chloroplasts were synthesized in *E. coli* (83) suggests that either these factors are not required for translocation

of proteins into chloroplasts, or that E. coli extracts contain the factors or adequate substitutes. An alternative could be that these factors are tightly associated with the organelle in this instance, although they were not in the studies which reported their requirement.

Reports on the presence of receptors in organelle outer membranes have demonstrated that precursor binding involves a protein component, since this binding is at least somewhat succeptible to protease treatment of the organelle prior to incubation with the precursor. (14, 64) However, there appears to have been little progress made in purifying the membrane component(s) involved in this binding. In contrast, one group of cellular components of protein translocation, proteases specific for processing of some precursors to their mature forms, have been isolated from both chloroplasts and mitochondria. (58, 65) Characterization of these proteases has included identifying their location within an organelle as well as some general properties based on the effects that various protease inhibitors have. Transit peptidase preparations have allowed studies of processing and of the specificities of the proteases for different translocated precursors, and will continue to be useful in analysis of the processing of translocated proteins.

The other approach being taken in dissecting mechanisms of protein translocation is the identification of features of the precursors to translocated proteins which act in this process. In organelles, studies with fusion proteins have narrowed down the region required for successful translocation to the transit peptide itself. (35, 83) My work is among the first, particularly in chloroplast protein transport, which analyzes transit peptide function by identifying structural

features which have a role in protein translocation and processing. This study has been done with one particular translocated protein, the SS of RUBISCO from Zea mays. It is not yet clear how applicable this will be to identifying functional characteristics of other transit peptides, either those from SS of other species or those from other translocated chloroplast proteins. One report has attempted to identify common structural features between all the chloroplast protein transit peptide sequences known thus far. (44) However, a comparison of my results with the mutant pTEB11 to the results obtained with iodoacetate-treated pea SS precursor (66) suggests that at least some mechanisms may not be conserved between species.

Several conclusions may be drawn from my work. First, DNA sequence analysis of a cDNA clone encoding part of the Zea mays SS precursor has shown that this SS transit peptide has features similar to those of other SS transit peptides for which the sequence is known. It is most homologous to the SS transit peptide from wheat, another monocot cereal species. (9) This homology could be due to the evolutionary relationship between these species, or could be due to a difference in the function of this region between these and other species. Second, analysis of the mutant pTEB11, which contains a processing site deletion, has shown that this site is required for correct maturation of the precursor. In the absence of this site several incompletely processed forms of the protein are seen, the majority of which are membrane associated. One possible mechanism of translocation is that processing frees the precursor from association with the membrane, leaving the transit peptide embedded. This work would seem to support that mechanism, although minor amounts of two

size classes of pTEB11-derived protein are seen in the stromal fraction. Work with iodoacetate-treated precursor, where processing is blocked as well, appears to confirm the requirement for an unmodified processing site, although in this case the distribution of intermediately-sized proteins is somewhat different than that seen in my work with pTEB11. (66) Upon further analysis this may prove to be a clue to possible differences between SS processing mechanisms in different species. One additional important aspect of the pTEB11 analysis is the indication that translocation occurs prior to processing, as indicated by the fact that the incorrectly processed intermediates are protected from degradation by added protease. (Figure 16)

The majority of my effort has been concentrated on the analysis of several mutant SS precursors which have alterations at the amino terminus of the transit peptide. This work has demonstrated that the amino terminus of the transit peptide plays an important role in the translocation of SS. This role can be enhanced or substituted for by the addition or substitution of an unrelated sequence with similar chemical characteristics, suggesting that it is the nature of this region, rather than the specific sequence, which is important for function. Several general criteria are discussed by von Heijne in his proposal concerning the role of amphiphilic helices in the transport of mitochondrial proteins. (86) Based on these criteria, the amino terminal segments (amino acids 1-18) of both the wild type and the mutant SS precursors appear to have hydrophobic moments as high or higher than those of any other 18 amino acid segment (including residues 2-19, etc.) in the transit peptide. However, this region does

not appear to contain a hydrophobic face sufficient to form a membrane binding domain. Instead, a general mix of polar and hydrophobic residues is seen. If von Heijne's criteria are correct, this would suggest that the amino terminal region of the SS transit peptide is involved in a function other than spontaneous association with the membrane, and in fact raises the question of whether the spontaneous insertion model is applicable to this protein at all. In addition, since all the mutants tested have their region (or, in the case of pTEB9, one of the two regions) of highest hydrophobic moment at the amino terminus, it seems unlikely that this factor alone determines the effectiveness of the amino-terminal structure.

Protein translocation involves several steps, including association of the precursor with the membrane, movement through membranes, and cleavage of the precursor to the mature size. Since the exact stage at which the changes made in these mutants are affecting translocation has not been identified, it is difficult to draw specific models about the effect that these mutations are having. Some general possibilities can be proposed, however. Although based on my results it is unlikely that a specific sequence of the amino terminus is interacting with chloroplast membranes or membrane-associated components, it may be that the overall structure of the amino terminus affects the conformation of the precursor such that another segment of the transit peptide stays available for a required interaction. With the deletion of amino-terminal sequences, as in pTEB9 and pTEB10, the conformation would shift so that the necessary sequence was less accessible to the components with which it interacts. The addition of the unrelated sequence would somehow maintain or enhance the required

conformation. Another possibility is that the amino-terminal sequence is involved in the actual movement of the precursor through the membrane. Unfortunately, essentially nothing is known about this step of the process, and so it is difficult to postulate how the alterations found in the mutants might affect this. Finally, it is known that the changes made in these mutants do not affect correct processing, and it does not seem likely that they are affecting the rate of processing. Although it is possible to imagine a situation where the wild type and altered precursors are translocated at the same rate and then processed at different rates, the data obtained provide no support for this model. If this were occuring, one would expect to see significant quantities of precursor or intermediate forms of the protein within the chloroplast (and therefore protected from degradation by added protease), and this is not seen (Figure 5). One could suggest that processing occurs with the majority of the protein outside the membrane and therefore accessible to protease. However, the results with pTEB11 indicate that even incorrectly processed forms of the protein are protected from protease treatment, suggesting that processing occurs following translocation. Thus, it does not appear that the sole effect of the alterations made is on the rate of processing.

Within the range tested here, the length of the precursor does not appear to have a large effect on the efficiency with which a protein is translocated. Presumably, however, there is a minimum length required to contain the functional characteristics of a transit peptide. It is interesting to note that some mitochondrial transit peptides are significantly shorter than the chloroplast transit peptides which have been analyzed so far. The shortest mitochondrial transit peptide known

is 20 amino acids (CytOxV from yeast, 76), while the shortest chloroplast transit peptide characterized thus far is 33 amino acids (LHCII from petunia, 18). More chloroplast transit peptides will need to be characterized before it is known whether this is due to different structural requirements or is seen simply because the structures of so few chloroplast protein precursors are known.

The results of this study provide information about specific features of the Zea mays SS transit peptide, and are an example of the power that manipulation of protein sequences can have in identifying features of importance within a transit peptide. In order to achieve a more complete understanding of how these features function it is important that the isolation and characterization of cellular components of transport proceeds as well, since this will allow more detailed analysis of the effects that alterations within the transit peptide are having. These efforts have progressed further in mitochondrial studies than in chloroplast studies, and the mitochondrial work provides information upon which to build hypotheses. In doing so, however, it is important to remember the differences between the organelles. Chloroplasts contain an additional membrane and thus two additional compartments, the thlyakoids and the lumen, into which some proteins, such as LHCII and Pc must be moved. The only chloroplast outer envelope membrane protein characterized thus far is synthesized as a precursor (21), unlike mitochondrial outer membrane proteins which may contain transit peptide-like sequences (31) but which do not require processing (25). In addition, a requirement for ATP rather than a membrane potential has been demonstrated in chloroplasts (26), while the opposite has been demonstrated in

mitochondria (23, 70). Besides these differences, there is the important question of how proteins are localized to the correct organelle in tissues which contain both chloroplasts and mitochondria. The work cited earlier, which demonstrated that a chloroplast transit peptide can mediate inefficient translocation of proteins into mitochondria (41), indicates that absolute discrimination is not achieved by the transit peptide alone. Along with the determination of common mechanisms involved in the translocation of proteins into chloroplasts and mitochondria, studies determining the differences, as well as the factors involved in organelle specificity, will make an important contribution to our understanding of mechanisms of protein translocation.

APPENDIX A

EXPRESSION OF RUBISCO SS IN *E. COLI*

INTRODUCTION

For the purposes of the work described in this dissertation, it was important to have a system where SS could be expressed from a cloned gene, since this would permit me to make the alterations desired and assay the effects of these alterations on translocation of the SS precursor. The first system tried was the expression of *rbc*S in *E. coli*. Many researchers have successfully expressed eukaryotic genes in *E. coli* by placing a bacterial sequences adjacent to the gene of interest to provide recognizable transcription and translation factors. (19, for example) Using the same approach, as described below, I obtained expression of a fusion *rbc*S gene in *E. coli*.

MATERIALS AND METHODS

Growth conditions

In most cases plasmid DNA was transformed into the *E. coli* strain JM83 (85). Tests for induction of expression of the gene of interest

were done in the E. coli strain JM105. JM105 cultures were grown for several hours, IPTG to 1mM was added, and growth was continued for an additional two hours. For color selection the chromogenic substrate X-gal was used at a concentration of 12ug/mL in LB/ampicillin plates. A blue color in colonies grown on these plates indicates expression the lacZ' gene fragment, which is contained in the vector used, pUC19 (61). Cells were analyzed by suspension in SDS sample buffer and electrophoresis as described previously. For fractionation, a cell suspension was sonicated with a Sonifier Cell Disruptor (Plainview, N. Y.) using a microprobe and the highest setting three times for 20 seconds each time.

Colony hybridizations

Screening of bacterial colonies by DNA hybridization was done according to the method of Grunstein and Hogness (28). 20 pmol of primer was radiolabelled in a 30 uL reaction containing 100 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 100 uCi ^{32}P ATP, and 5 units polynucleotide kinase. This reaction was incubated at $37^{o}C$ for 45 minutes.

Other methods

All other methods were as described previously.

RESULTS

Construction of a fusion rbcS

The plasmid pBT(5)15 (see chapter 2) was digested with SalI and the large fragment was ligated to itself. This placed the rbcS initiation codon in frame with the lacZ' coding region which was downstream of rbcS. This clone was then cut with NcoI, treated with S-1 nuclease to blunt the cohesive end, cut with KpnI, and the rbcS gene fragment was isolated. This fragment was ligated into pUC19 which had been cut with HindIII, treated with Bal31 nuclease for 2-5 minutes, and cut with KpnI. The resulting clones were screened on X-gal plates and blue colonies were selected. In theory, the clones giving blue colonies would have the majority of the lacZ' coding sequence, and thus the rbcS gene fragment, in the correct reading frame with the lacZ' initiation codon. Plasmid DNA from the clones giving blue colonies was isolated as a mixed population and digested with SalI and EcoRI. The large fragment was ligated to the SalI-EcoRI gene fragment from pSS19, inserting the remainder of the rbcS coding sequence into the "in-frame" configuration. These final clones were screened by SDS polyacrylamide gel electrophoresis, and a clone which showed the appearance of a unique stained band in these gels at approximately the size expected for SS precursor (19 kd) was identified. The presence of this band was dependent on the presence of IPTG in the medium for a cell strain which requires inducer for lacZ' expression (Figure 17), suggesting that the protein was transcribed from this promoter. DNA sequence analysis of the region around the lacZ' initiation codon showed that this clone did have the lacZ' initiation codon in frame with the rbcS coding region.

Figure 17. Induction of expression product in JM105 cells. *E. coli* strain JM105 cells were transformed with either pUC19 or pZS13. Cells were grown under inducing conditions as described in Methods. (+) and (-) indicate the presence or absence of IPTG. The arrow indicates the protein of interest.

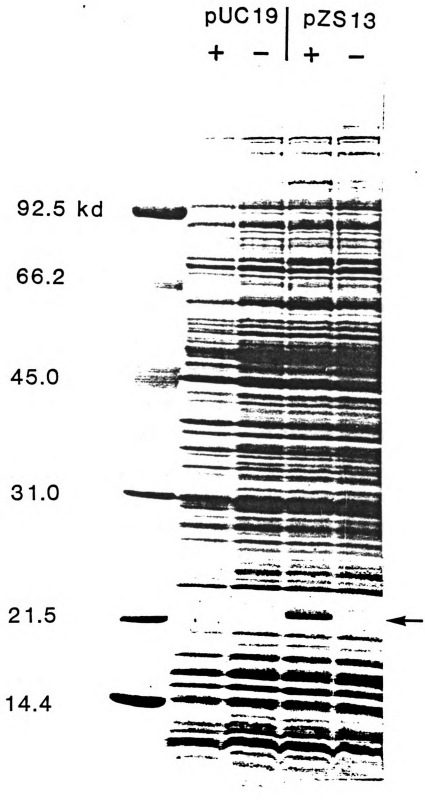


Figure 17.

The derived amino acid sequence of this clone was the same as that shown for pTEB1. (see Figure 12) An antibody against SS was used when it became available to confirm that the induced protein seen was antigenically related to SS. (Figure 18) The clone, designated pZS13, was used in further studies, as well as for the construction of pTEB1 (see Chapter 3).

Construction of a non-fusion sequence for SS expression

For my experiments in determining the relative transport efficiency of SS precursors containing deletions in the transit peptide, it was essential to have a wild type SS precursor. Rather than repeating the random procedure described above in hopes of getting the perfect alignment of regulatory and coding sequences, an oligonucleotide linker was used to insert the desired sequences. This linker (shown in Figure 19), contained several features, including an E. coli consensus ribosome binding site to promote translation initiation (45), a stop codon to prevent translation of any products which started upstream of the desired initiation site, a HindIII site, since the existing one was deleted in the construction scheme, and a NcoI-compatable cohesive end to insure insertion of the linker in the desired orientation. In addition, based on a report of the effect of the three base pairs preceding the initiation codon on translation efficiency (38), the sequence preceding the ATG in the linker was altered as much as possible to give a desirable sequence while still retaining a cohesive end complementary to the NcoI cohesive end. This manipulation meant that the NcoI recognition sequence was not regenerated in the final clone.

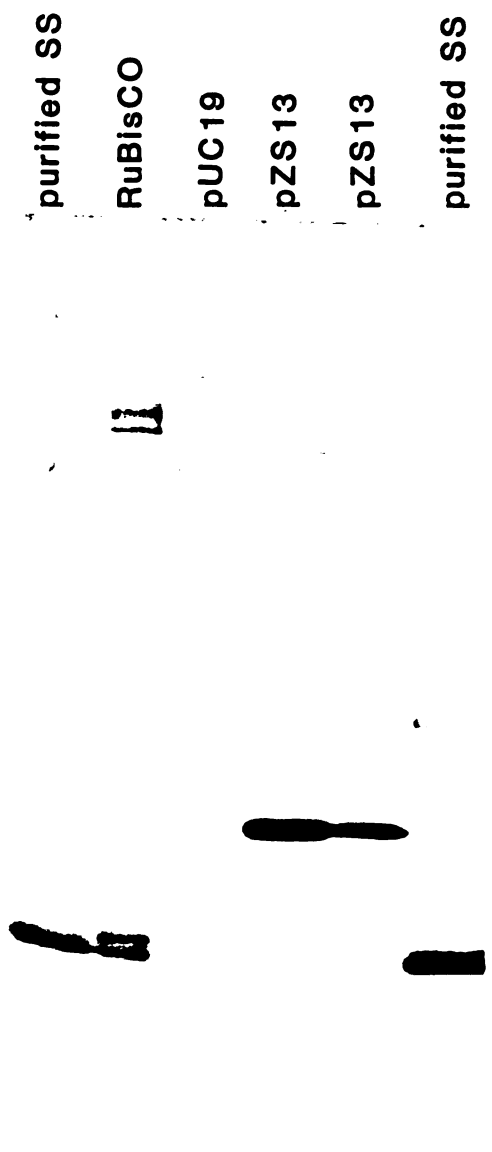


Figure 18. Immunoblot analysis of pZS13 expression products. Cells containing pZS13 were grown as usual, analyzed on SDS gels, blotted, and reacted with anti-SS antiserum as described in Methods. Cells containing pUC19 were included as a control. Also included were a crude RUBISCO prep and purified SS.

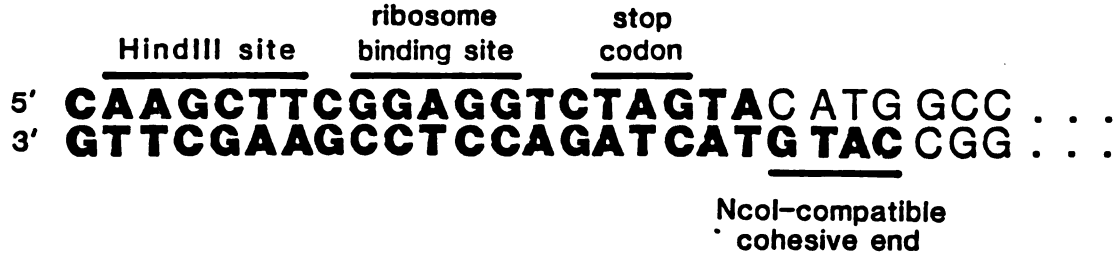


Figure 19. Linker used in the construction of a gene encoding a non-fusion SS precursor.
The sequence of the linker used in pLZS construction is shown in bold face. A portion of the rbcS gene sequence adjoining the linker is also shown. Important features are as indicated and as discussed in the text.

pSS19 was digested with HindIII, followed by Bal31 nuclease treatment for 1.5-6 minutes. The DNA was then digested with NcoI and the linker was ligated in. Resulting clones were screened by colony hybridization, using one of the oligonucleotides from the linker construction as a probe. Two positive colonies were screened by DNA sequence analysis and were found to have the linker sequence inserted as expected. One clone had 4 bp removed by the Bal31 treatment, while the other had 24 bp removed. These clones were designated pLZS18 and pLZS17 respectively.

Despite the presence of supposedly desired features upstream of the rbcS coding region, these clones failed to express high levels of SS precursor. No new protein band was seen on stained SDS gels; immunoblotting detected low levels of SS present in the cells containing these gene constructions.

Other results

Due to the failure to express the non-fusion protein, a scheme for purifying SS precursor from E. coli was not developed. A preliminary fractionation of pZS13-containing cells into soluble and insoluble fractions indicated that fusion SS precursor remained in the insoluble fraction. Further analysis to determine if the protein was associating with the membrane or aggregating with itself or other proteins was not done. There are reports, however, of aggregation of foreign proteins expressed in E. coli (90, for example), and this may have been what was occuring in this instance.

DISCUSSION

This work shows that it is possible to express SS in *E. coli*, at least as a fusion protein. It is not known why the level of SS expression was so low in the clone encoding a non-fusion protein. One possibility is that the reiteration of translation signals (those in the clone, which were not deleted, as well as the ribosome binding site in the linker) was deleterious to expression. In addition, it is possible that the protein was being synthesized but was degraded in the cells. Were this the case, it would appear that the small amino terminal fusion sequence encoded by pZS13 serves to protect the protein in *E. coli*.

This effort for bacterial expression of SS precursor was not prusued beyond the point described here because just as expression dificulties with the non-fusion product were appearing I became aware of the *in vitro* transcription system described earlier (see Chapter 2). This appeared to be a more direct route to obtaining radiolabelled SS precursor in the absence of other radiolabelled proteins.

APPENDIX B

ANALYSIS OF pTEB13 EXPRESSION PRODUCTS

INTRODUCTION

As discussed in Chapter 3, analysis of the incorporation of radiolabelled methionine into protein in in vitro translation reactions was used to determine how much of each reaction mixture to add so that equal numbers of the SS precursors to be compared would be added. The success of this method was dependent on the protein of interest being the only labelled protein in the translation mixture. This condition was met for all the different SS precursors, as shown in Figure 13. Unfortunately, later reactions for the synthesis of the mutant pTEB13 showed a number of unidentified labelled proteins in the reaction as well. This appendix explains the approach taken to overcome this problem.

MATERIALS AND METHODS

Gel scanning

Autoradiograms from SDS polyacrylamide gels of pTEB2 and pTEB13 translation products were scanned using a Gilford "Response" spectrophotometer (Gilford, Oberlin, OH) at a wavelength of 650 nm on the high resolution setting. Integration of peak areas was performed by the spectrophotometer.

Other methods

All other methods were as described previously.

RESULTS

The problem with the pTEB13 translation mixture is shown in Figure 20. One translation reaction had only a single labelled band, which was of the size expected for the mutant pTEB13. The next translation reaction done contained several additional labelled proteins. The reason for this was unknown, since the solutions used for setting up the transcription and translation reactions were the same each time. Some difference in the proportions of the extra labelled proteins relative to the protein of interest was seen if a different T-7 RNA polymerase preparation was used. (Figure 20) The problem was unique to pTEB13; none of the other specific mRNAs being transcribed and translated at the same time showed these extra bands.

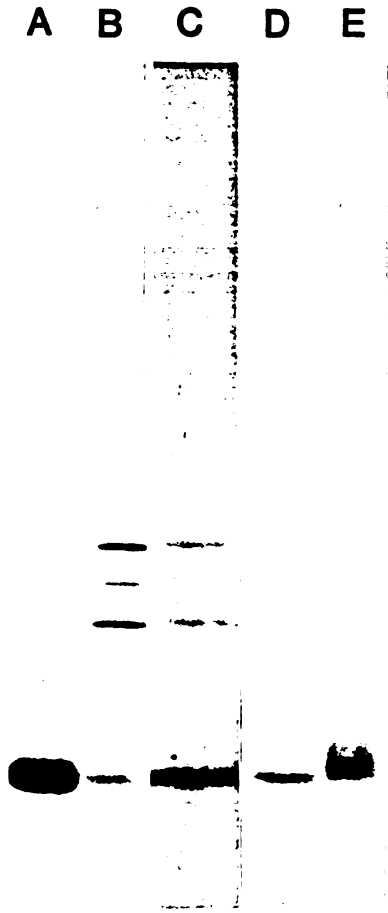


Figure 20. Analysis of pTEB13 expression products.
Fluorogram of SDS gel analysis of proteins produced from the expression of pTEB2 (lane A) or pTEB13 (lanesB-D). Lanes B and C show translation products obtained using two different T-7 RNA polymerase preparations. Lane D shows an "uncontaminated" pTEB13 reaction. Lane E shows immunoprecipitation of the in vitro translation reaction shown in lane C, using anti-SS antiserum. 7.5 times more volume was used in the immunoprecipitation than was loaded in lane C.

Immunoprecipitation of proteins out of the pTEB13 translation reaction using antibody raised against SS gave only the pTEB13 SS precursor band, indicating that the extra bands seen were not derived from or related to SS. (Figure 20) Based on this I felt that if a determination could be made of how much of the labelled protein was SS precursor, these preparations could still be used in my experiments.

A fraction of each in vitro translation mixture was analyzed on SDS gels, fluorographed, and autoradiographed as described previously. The intensity of individual bands in each lane of the autoradiogram was determined using a gel scanner, which also calculated the area under each peak. This information was used to determine how much of the pTEB13 translation mixture to add to a transport incubation. The areas of the gel corresponding to the bands seen on the autoradiogram were excised, and the number of counts in each gel slice was determined as described previously. The comparison of these results to those obtained by scanning the autoradiogram is shown in Table 3. Each number is the average of data obtained from two side by side gel lanes. Also shown in Table 3 are the relative transport efficiencies of pTEB13 as compared to pTEB2 in the three experiments where these methods for quantifying pTEB13 were used.

Three transport experiments were done prior to the appearance of the unidentified bands in pTEB13 translation products. The translation mix from the last of these three is shown in Figure 20. Assuming that the previous two looked the same as the third, i.e. with no additional labelled bands, the results from these three experiments can be compared to the results given here. For the first three experiments the range of transport efficiencies obtained was large: 69, 97, and

Table 3. Comparison of methods for quantifying radiolabelled translation products.
Determination of the percent of the total labelled protein which was the protein of interest. Two different techniques were used, as described in the text. Each value shown is the average from two side-by-side gel lanes. The transport efficiency of pTEB13 relative to pTEB2 for each experiment done with these translation reactions is also shown.

trial number	pTEB13 as percent of total		relative transport efficiency
	gel scans	gel counts	
1	41.3% ± 2.2%	35.3% ± 0.2%	146%
2	46.2% ± 3.2%	42.7% ± 0.3%	112%
3	70.3% ± 5.8%	52.5% ± 0.5%	114%

166%. The average is 111%, which is close to the average of 124% for the data given in Table 3. This agreement suggests that the data presented here is an accurate reflection of the relative transport efficiency of the mutant pTEB13.

One transport experiment was performed with pTEB13 translation mix containing additional labelled bands before the problem was apparent. The gel from this experiment demonstrates that even in the presence of these extra bands, processed SS is the only labelled protein seen in the chloroplast soluble fraction following transport. (Figure 21) In this case the factor of the additional labelled proteins was not taken into account, and thus the amount of pTEB13 transported versus that seen for pTEB2 is low.

DISCUSSION

The factors leading to the appearance of extra labelled proteins in translation mixtures containing pTEB13 have not been determined. It is possible that there is contaminating DNA which has promoters recognizible by T-7 RNA polymerase, although earlier experiments using the same DNA preparation did not contain these contaminating labelled proteins.

Since the additional proteins seen appear not to be derived from or related to SS, the assumption was made that their presence would not influence the translocation of pTEB13. With this assumption, an attempt was made to identify what proportion of the labelled protein was the protein of interest. This was done using two methods, as

Figure 21. Analysis of transport incubation containing "contaminated" pTEB13.
Fluorogram of SDS gel analysis of chloroplast soluble proteins from transport incubations containing either pTEB2 or pTEB13. Gel analysis of the pTEB13 translation products gave a profile similar to that seen in Figure 20, lane C, but only one product was seen in the transport incubation.

described above. In two of the three separate trials done there was close agreement between these two methods, but less agreement was seen in the third trial. Presumably the level of agreement is related to the range of linear response for X-ray film, as well as the level of background in the lane. Some attempt was made to correct for the background by counting a gel slice which was in a region not darkened on the film, but this did not reconcile the differences seen in the third trial.

The value used to calculate the volume of precursor-containing translation mix was that obtained from scanning the autoradiogram, since determining the counts in gel slices took an additional day. Since counting the gel is expected to give a more accurate estimate of the actual situation, this meant that the amount of pTEB13 added to the transport incubation in the third trial was less than it should have been. Therefore, the value given for transport efficiency in trial three is lower that it would have been had a more correct amount of precursor been added. However, on the whole these numbers compare closely to those obtained in earlier pTEB13 transport experiments. This fact, along with the fact that the only labelled protein seen following a translation incubation with a "contaminated" translation mix is processed SS, suggests that the methods used here to determine pTEB13 levels allow a reasonable estimation of pTEB13 relative transport efficiency.

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