

THE IMPORT OF PROTEINS INTO ISOLATED
HIGHER PLANT MITOCHONDRIA

by
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I lift my eyes to the hills -
where does my help come from?
My help comes from the LORD,
The Maker of heaven and earth.

Psalm 121, vs 1-2.

I declare that this thesis was written and composed by myself,
and that the work presented herein is my own,
unless otherwise stated.

TABLE OF CONTENTS

TABLE OF CONTENTS	i
ACKNOWLEDGEMENTS	vii
ABSTRACT	viii
ABBREVIATIONS	x
CHAPTER 1 - INTRODUCTION	1
1.1 General introduction	1
1.1.1 The endomembrane system	1
1.1.2 The organelles	4
1.2 Mitochondria	5
1.2.1 Structure	5
1.2.2 Function	6
1.2.3 The uniqueness of plant mitochondria	7
1.2.3.1 The alternative oxidase pathway	7
1.2.3.2 Exogenous NADH- and NADPH-dehydrogenases	7
1.2.3.3 Endogenous NADH-dehydrogenase	8
1.2.3.4 Glycine decarboxylase and serine hydroxy methyl transferase	8
1.2.3.5 NAD ⁺ -linked malic enzyme	9
1.2.4 Mitochondrial biogenesis	9
1.2.5 Mitochondrial DNA	10
1.2.5.1 Variation in size and gene content	10
1.2.5.2 Variation in organisation	10
1.2.5.3 Variation in structure	12
1.2.6 Plant mitochondrial DNA	12
1.2.6.1 Structure	12
1.2.6.2 Protein coding genes and RNA editing	12
1.2.6.3 Ribosomal RNAs and genes	13
1.2.6.4 Transfer RNAs and genes	14
1.2.6.5 Gene structure	14
1.2.6.6 Promiscuous DNA	15

1.3	Mitochondrial protein import	15
1.3.1	Introduction	15
1.3.2	Synthesis	16
1.3.3	Targeting	16
1.3.4	Receptors	19
1.3.5	Energy requirements	21
1.3.6	Contact sites	22
1.3.7	Role of heat shock proteins	23
1.3.8	Proteolytic processing of precursors	24
1.3.9	The two step maturation of precursor proteins	26
1.3.10	Modification and assembly	27
1.3.11	Mitochondrial proteins synthesised without N-terminal presequences	28
1.3.11.1	Porin	28
1.3.11.2	The adenine nucleotide translocator	29
1.3.11.3	Cytochrome <i>c</i>	32
1.4	Protein import into plant mitochondria	34
1.4.1	Protein import <i>in vivo</i>	34
1.4.2	Protein import <i>in vitro</i>	34
1.4.3	Mechanism of protein import	35
1.4.4	Specificity of targeting	36
1.5	Protein import into chloroplasts	36
1.5.1	Targeting	36
1.5.2	Sorting	37
1.5.3	Processing	38
1.5.4	Receptors	38
1.5.5	Energy requirements	39
1.5.6	Specificity of targeting	40
1.6	Summary	40
CHAPTER 2 - MATERIALS AND METHODS		42
2.1	Biological materials	42
2.1.1	Plant materials	42
2.1.2	<i>Escherichia coli</i> strains and genotypes	42
2.1.3	Bacterial plasmids	42

2.1.4	Adenine nucleotide translocator clones	43
2.1.4.1	<i>ANT-A</i> , a full-length cDNA clone from <i>Zea mays</i>	43
2.1.4.2	<i>ANT-G1</i> , a genomic clone from <i>Zea mays</i>	43
2.1.4.3	<i>PANT-1</i> , a cDNA clone from <i>Solanum tuberosum</i>	43
2.2	Miscellaneous	43
2.2.1	Chemicals	43
2.2.2	Restriction endonucleases and DNA modification enzymes	44
2.2.3	Radioisotopes	44
2.2.4	Autoradiography	44
2.2.5	Bacteriological media	44
2.3	DNA analysis	44
2.3.1	Large scale preparation of plasmid DNA from <i>Escherichia coli</i>	44
2.3.2	Construction of recombinant plasmid DNA molecules	45
2.3.3	Nucleotide sequencing of plasmid DNA	45
2.4	RNA analysis	46
2.4.1	Isolation of total RNA from <i>Zea mays</i> dark-grown coleoptiles	46
2.4.2	Isolation of messenger (poly A ⁺) RNA	47
2.5	<i>In vitro</i> expression of cDNAs and recombinant clones	47
2.5.1	<i>In vitro</i> transcription	47
2.5.2	<i>In vitro</i> translation systems	47
2.5.2.1	Rabbit reticulocyte lysate	47
2.5.2.2	Wheat germ S-30 system	48
2.5.3	Determination of percentage incorporation of L-[³⁵ S]-methionine	48
2.6	Import of radiolabelled proteins into isolated plant mitochondria	49
2.6.1	Isolation of mitochondria	49
2.6.1.1	From <i>Zea mays</i>	49
2.6.1.2	From <i>Solanum tuberosum</i>	49
2.6.2	Determination of protein concentration	50
2.6.3	Import into mitochondria	50
2.6.3.1	Inhibition of the matrix protease	51
2.6.3.2	Inhibition of the membrane potential	51
2.6.3.3	Fractionation of proteins by SDS/polyacrylamide gel electrophoresis	51
2.7	Immunoprecipitation	52
2.8	Computer analysis	53

CHAPTER 3	- CHARACTERISATION OF A <i>ZEA MAYS</i>	
	MITOCHONDRIAL PROTEIN IMPORT SYSTEM	54
3.1	Introduction	54
3.2	Isolation of <i>Zea mays</i> mitochondria	55
3.3	A cDNA encoding the MnSOD from <i>Nicotiana plumbaginifolia</i>	55
3.4	The <i>in vitro</i> expression and import of MnSOD	55
3.5	The import of MnSOD into <i>Zea mays</i> mitochondria isolated from different aged tissue	57
3.6	The effect of total mitochondrial protein on the import of MnSOD	59
3.7	The effect of wheat germ S-30 on the import of MnSOD	61
3.8	The effect of 1,10-phenanthroline on the import of MnSOD	64
3.9	The effect of valinomycin on the import of MnSOD	66
3.10	The effect of time on the import of MnSOD	67
3.11	The effect of ATP on the import of MnSOD	70
3.12	The effect of GTP and UTP on the import of MnSOD	75
3.13	Conclusions	78
CHAPTER 4	- THE <i>IN VITRO</i> IMPORT OF THE <i>ZEA MAYS</i>	
	ADENINE NUCLEOTIDE TRANSLOCATOR	80
4.1	Introduction	80
4.2	A full-length <i>Zea mays</i> adenine nucleotide translocator cDNA, <i>ANT-A</i>	81
4.3	The <i>in vitro</i> expression and import of G1ANT	81
4.4	The processing of the <i>Zea mays</i> adenine nucleotide translocator is inhibited by 1,10-phenanthroline	85
4.5	Determination of the translational start site in <i>ANT-A</i>	87
	4.5.1 Deletion of ATG 3	90
	4.5.2 The <i>in vitro</i> expression of GA2 and GA3	90
	4.5.3 The <i>in vitro</i> import of G1ANT and GA3	94
	4.5.4 The start of translation in <i>ANT-A</i> is at ATG 1	98
4.6	Immunoprecipitation of a poly A ⁺ RNA translation with a <i>Zea mays</i> polyvalent adenine nucleotide translocator antibody	101
4.7	Construction of a fusion protein between the N-terminal end of the <i>Zea mays</i> adenine nucleotide translocator and the mouse cytosolic protein dihydrofolate reductase	103

4.8	The <i>in vitro</i> expression of a fusion protein between the N-terminal end of the <i>Zea mays</i> adenine nucleotide translocator and the mouse cytosolic protein dihydrofolate reductase	105
4.9	Import of the <i>Zea mays</i> adenine nucleotide translocator after removal of the N-terminal end	112
4.10	Resequencing of <i>ANT-A</i>	115
4.11	Resequencing of <i>ANT-G1</i>	121
4.12	N-terminal protein sequence of the <i>Zea mays</i> adenine nucleotide translocator	123
4.13	An adenine nucleotide translocator from <i>Solanum tuberosum</i> is processed upon import into isolated <i>Zea mays</i> mitochondria	123
4.14	The <i>Zea mays</i> and <i>Solanum tuberosum</i> adenine nucleotide translocator proteins are processed upon import into isolated <i>Solanum tuberosum</i> mitochondria	124
4.15	Comparison of the amino acid sequences of adenine nucleotide translocator proteins from different organisms	127
4.16	Conclusions	130
CHAPTER 5 - DISCUSSION AND FUTURE EXPERIMENTS		135
5.1	Introduction	135
5.2	Characterisation of a <i>Zea mays</i> mitochondrial <i>in vitro</i> import system	135
5.3	The <i>in vitro</i> import of the <i>Zea mays</i> adenine nucleotide translocator	137
5.4	Is the <i>Zea mays</i> adenine nucleotide translocator synthesised with an N-terminal extension?	138
5.5	Is <i>MANT-1</i> full-length?	139
5.6	Targeting of the adenine nucleotide translocator to the mitochondrion	142
5.6.1	Does the <i>Zea mays</i> adenine nucleotide translocator contain internal targeting information?	143
5.6.2	Does the N-terminal extension of the <i>Zea mays</i> adenine nucleotide translocator contain mitochondrial targeting information?	143
5.7	Is the <i>Saccharomyces cerevisiae</i> adenine nucleotide translocator processed?	144
5.8	Why does the <i>Zea mays</i> adenine nucleotide translocator contain an N-terminal extension?	145

5.9	Future experiments	150
5.9.1	The <i>in vitro</i> import of the <i>Zea mays</i> adenine nucleotide translocator into chloroplasts -	150
5.9.2	Deletion analysis of the <i>Zea mays</i> adenine nucleotide translocator N-terminal extension	151
5.9.3	Localisation of the <i>Zea mays</i> adenine nucleotide translocator after import into isolated mitochondria	151
5.9.4	The <i>in vivo</i> import of the <i>Zea mays</i> adenine nucleotide translocator	152
	5.9.4.1 Plant transformation	152
	5.9.4.2 Yeast transformation	153
5.9.5	The <i>Zea mays</i> adenine nucleotide translocator processing protease	153
5.10	Summary	154
	REFERENCES	156
	APPENDIX	172

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ABSTRACT

Investigations of plant mitochondrial protein import have been very limited and few reports exist in the literature. The work presented in this thesis describes the development of an *in vitro* *Zea mays* mitochondrial protein import system, which has enabled the import of plant nuclear-encoded mitochondrial proteins to be examined.

This *Z. mays* import system was characterised and partially optimised with the *Nicotiana plumbaginifolia* manganese superoxide dismutase (MnSOD). Results suggest that the energy requirements for protein import into plant mitochondria are similar to those of *Saccharomyces cerevisiae* and *Neurospora crassa*, requiring both an energised inner mitochondrial membrane and ATP. The inclusion of 1,10-phenanthroline inhibited the processing, but not the import of MnSOD and indicated that the processing activity within *Z. mays* mitochondria was dependent upon the presence of metal ions. The plant mitochondrial processing protease may therefore be similar to the characterised *S. cerevisiae* and *N. crassa* matrix processing protease.

The import of the *Z. mays* adenine nucleotide translocator (ANT) protein was then investigated. Unlike the ANT of *S. cerevisiae* and *N. crassa*, this plant ANT was synthesised as a precursor protein, which was processed upon import into mitochondria isolated from both *Z. mays* and *Solanum tuberosum*. The subsequent isolation of an *S. tuberosum* ANT cDNA clone, *PANT-1*, enabled the import of a second plant ANT to be investigated. Results corroborated the findings previously obtained with the *Z. mays* ANT and it was therefore concluded that the import of plant ANT proteins are distinctly different from those of *S. cerevisiae* and *N. crassa*.

The processing site within the *Z. mays* ANT protein was predicted to lie within a region internal to the ANT protein sequences from *S. cerevisiae*, *N. crassa* and *Bos taurus*. The ATG codon (ATG 3), previously predicted to be the start of translation within the *Z. mays* cDNA, *ANT-A*, was therefore questioned. Deletion analysis of the 5' region of *ANT-A* suggested that an in frame ATG codon, ATG 1, 174 bp 5' to ATG 3, encoded the initiating methionine despite the presence of an in frame TGA stop codon 147 bp 3' to ATG 1.

Subsequent resequencing of the 5' region of *ANT-A* revealed a number of sequencing errors. These resulted in the loss of this previously predicted TGA stop codon and the extension of the ORF from 987 bp to 1161 bp. The complete resequencing of the cDNA has now been achieved and this has been renamed *MANT-1*

(Winning *et al.*, 1991). N-terminal sequencing of the isolated *Z. mays* ANT protein and comparison with the deduced amino acid sequence of *MANT-1* predict an N-terminal extension of 77 amino acids.

Mitochondrial targeting information appears to reside within two regions of the *Z. mays* ANT protein, since deletion of the the first 97 amino acid residues does not inhibit import, whilst the same amino acids residues can function to target the mouse cytosolic protein dihydrofolate reductase into isolated *Z. mays* mitochondria. The possible function of this 77 amino acid residue N-terminal extension is discussed.

ABBREVIATIONS

A	adenine
ANT	adenine nucleotide translocator
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin (fraction V)
Butyl-PBD	2-(4'-tert.-Butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole
C	cytosine
°C	degrees centigrade
C-	carboxy-
cDNA	complementary DNA
cpm	counts per minute
CTP	cytosine 5'-triphosphate
DHFR	dihydrofolate reductase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EGTA	ethyleneglycol bis-(b-aminoethyl ether) N,N,N',N'-tetraacetic acid
G	guanine
g	gram
GIP	General Insertion Protein
GTP	guanine 5'-triphosphate
h	hour
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (sodium salt)
hsp	heat shock protein
kbp	kilobase pair
kDa	kilodalton
l	litre
Ltd.	limited
M	molar
MBq	megabequerel
min	minute
ml	millilitre
mM	millimolar
mm	millimeter
mmol	millimole
MnSOD	manganese superoxide dismutase
MPP	matrix processing peptidase
mtDNA	mitochondrial DNA
MW	molecular weight
N-	amino-
nm	nanometer
N-type	normal, fertile
NY	New York
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PEP	processing enhancing protein
pH	negative logarithm of the hydrogen ion concentration
plc.	public limited company
PMSF	phenylmethylsulfonyl fluoride
poly A	polyadenylation
psi	pounds per square inch
RNA	ribonucleic acid
rpm	revolutions per minute
Rubisco	ribulose 1,5-bisphosphate carboxylase/oxygenase
S	Svedberg unit
SDS	sodium dodecyl sulphate
sec	second
SSU	small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase
T	thymine
TBq	terabecquerel
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene-diamine
Tris	tris(hydroxymethyl)methylglycine
TTP	thymine 5'-triphosphate
U	unit
USA	United States of America
μCi	microcurie
μg	microgram
μl	microlitre
μm	micrometer
v/v	volume to volume (given as a percentage)
w/v	weight to volume (given as a percentage)

CHAPTER 1

INTRODUCTION

1.1. General introduction.

The transport of proteins within the eukaryotic cell is a continuous and complex process. Each membrane and membrane-bound compartment is unique in protein composition and signals must therefore exist to ensure the correct routing of individual proteins. This sorting process is mediated by specific targeting signals, which reside within the protein molecule. These function to localise the protein, from the site of synthesis in the cytoplasm, to the desired membrane where it is recognised by specific receptor proteins, which may then mediate the translocation of the protein.

Much work has been aimed at unravelling the nature of these targeting signals and identifying the structural determinants or motifs necessary to locate proteins to specific membranes or membrane-bound compartments (for review see Pugsley, 1989). Table 1.1 summarises this information to date. As can be seen such targeting signals are diverse in structure. They may be situated at the end or in the middle of the protein, whilst others are located on N-terminal extensions, which are then proteolytically removed once the protein has reached its final location.

Proteins synthesised in the cytosol may follow one of two routes depending upon the final location. Those destined for export, the plasma membrane, or the lysosome must first enter the endomembrane system (the endoplasmic reticulum (ER) and the Golgi apparatus) via the ER. As proteins are transported through this membrane network they are subject to a number of sorting processes, which determine their final location. Those destined for the mitochondrion, chloroplast, peroxisome and nucleus, however, are targeted directly to the specific organelle.

1.1.1. The endomembrane system.

In general, proteins directed to the endomembrane system are synthesised with an N-terminal extension, or signal peptide (SP), which is proteolytically cleaved upon import. This includes proteins destined for secretion, as well as the majority of plasma membrane (type 1a) proteins (Singer, 1990). Once the SP

Table 1.1; Summary of defined eukaryotic protein targeting signals.

TARGET	SIGNAL NAME	LOCATION OF SIGNAL	CHARACTERISTICS	
ER ^a	signal peptide	N-terminus	Generally found as extensions of 15 to 35 residues. Characterised by a basic N-terminal region followed by a hydrophobic core and small apolar amino acids at -1 and -3 positions relative to the cleavage site.	
ER lumen	KDEL	C-terminus	The C-terminal amino acids -Lys-Asp-Glu-Leu (KDEL) are thought to act as a retention signal for soluble ER proteins. Similar sequences, RDEL (-Arg-Asp-Glu-Leu) and HDEL (His-Asp-Glu-Leu) are found in mammalian and yeast ER proteins respectively.	
Lysosome	M - 6 - P	N-linked core oligosaccharides	Phosphorylation of mannose residues on the core oligosaccharide (N-linked to Asn residues in the sequence Asn-X-Ser/Thr).	
Mitochondrion	presequence	N-terminus	Generally found as extensions of 16 to 80 residues. Rich in basic and hydroxylated residues, lack acidic residues. Predicted to form an amphiphilic α -helix.	∞
Chloroplast	transit peptide	N-terminus	Similar to mitochondrial presequences, but predicted to form an amphiphilic β -sheet.	
Peroxisome	peroxisome targeting signal	C-terminus or internal	Consensus sequence now proposed (Ser/Ala/Cys)-(Lys/His/Arg)-Leu.	
Nucleus	karyophilic signal	nonspecific	Rich in basic residues, mainly Lys. Turn inducing residues (Pro or Gly) are often found	

^aProteins targeted to the ER include those destined for the Golgi apparatus, the lysosome and storage vesicles, as well as those constitutively secreted by the cell.

emerges from the ribosome, the translation of the protein is arrested through the binding of signal recognition particle (SRP). Translation is not completed until SRP binds to the SRP-receptor, or Docking Protein, located on the ER membrane. The SP therefore ensures that a protein is correctly targeted to the ER.

Like most targeting signals SPs show little primary sequence identity. Instead, a number of general features within the protein sequence are thought to be important for targeting. SPs vary in length from 15 to 35 amino acid residues. They contain a hydrophobic core, which may form an α -helix. The N-terminal region is rich in positively charged amino acids, whilst apolar amino acids are often found at positions -1 and -3 relative to the cleavage site.

Proteins imported into the ER will include both the soluble and membrane proteins of the ER itself. There must therefore be mechanisms to ensure that these proteins remain within the ER. Some soluble ER proteins have been shown to contain a sequence of four amino acid residues, -Lys-Asp-Glu-Leu (KDEL) at the extreme C-terminus. This sequence is thought to function as a retention signal, since alteration of this sequence results in the secretion of the ER protein. Similar C-terminal sequences, -Arg-Asp-Glu-Leu (RDEL) and -His-Asp-Glu-Leu (HDEL), have been found in some mammalian and yeast ER proteins respectively. At the present time it is unclear what sequences are important for the retention of the ER membrane proteins, although 'signal patches' have been proposed.

The transport of proteins through the endomembrane system and to the final location is mediated by membrane-bound transport vesicles. These carry the protein to the next compartment by a process of invagination and coalescence. After leaving the ER, proteins are transported to the Golgi apparatus. This is distinct from the ER and divided into a number of compartments, in which various protein modifications occur. It is within the Trans-Golgi network (TGN) that the major sorting processes occur. This is thought to be mediated by specific receptors, which are clustered at distinct points within the TGN membrane. Proteins are thereby sorted into specific transport vesicles, which represent different branches of the sorting pathway. So far four discrete transport vesicles have been recognised; the lysosomal, regulated, basolateral and apical. These vesicles also differ in composition, as well as in the proteins they transport. Lysosomal and regulated vesicles, for instance, are coated in the protein clathrin. Some secretory proteins, however, are not sorted by specific receptors. Instead, these are continuously exported to the cell surface by the default or constitutive pathway. It is within these transport vesicles that the proteins destined for the plasma membrane are thought to be transported.

With the exception of lysosomal proteins, little is known about the signals required for this sorting process. Lysosomal proteins are distinguished by the addition of mannose-6-phosphate (M-6-P) residues onto N-linked core oligosaccharides. These are recognised by the M-6-P receptor, which then mediates the sorting into lysosomal vesicles. Some lysosomal proteins, however, lack M-6-P residues, but are still localised to the lysosome.

1.1.2. The organelles.

Mitochondria and chloroplasts contain their own DNA, which encodes only a limited number of organellar proteins. Such proteins remain at the site of synthesis or are transported to other regions within the organelle. Little is known, however, about the targeting information contained within these organelle-encoded proteins. These are also the only two organelles which contain more than one membrane system. Proteins synthesised by these organelles, as well as those imported from the cytosol must therefore contain additional information to ensure that the correct suborganellar compartment is reached.

In general, nuclear-encoded mitochondrial and chloroplast proteins are synthesised with N-terminal extensions, termed presequences or transit peptides respectively, which are proteolytically cleaved upon import. It is within these N-terminal extensions, that the specific targeting information resides. They are very similar in structure, although the chloroplast transit peptides appear to be longer (Franzen *et al.*, 1989). Like SPs they show no primary sequence homology, but share a number of general features. This includes a preponderance of basic and hydroxylated amino acids and a lack of negatively charged residues. Structural analyses predict that mitochondrial presequences may form amphiphilic α -helices, whilst chloroplast transit peptides form amphiphilic β -sheets (von Heijne *et al.*, 1989). Specificity may therefore be determined by specific structural motifs within the protein. These may be important for the initial insertion into the membrane, since this appears to be receptor mediated.

The transport of proteins into peroxisomes, or microbodies, is less well understood. It is still unclear whether peroxisomes are derived from the ER or whether this organelle, like mitochondria and chloroplasts, arise from the incorporation of proteins into a pre-existing membrane (Trelease, 1984). The post-translational import of proteins, however, has been demonstrated.

The import of some cytoplasmically synthesised peroxisomal proteins appears to be mediated by a sequence of three amino acids -Ser-Lys-Leu (-SKL) at

the C-terminus. These may be located at the extreme C-terminus or within close proximity to it (Gould *et al.*, 1987; 1988). Amino acid substitutions within this tripeptide has led to the following consensus sequence consisting of Ser/Ala/Cys in position one, Lys/His/Arg in position two and Leu in position three (Gould *et al.*, 1989).

The targeting signals of proteins destined for the nucleus appear to be less well defined in their position within the protein. They may vary from an N-terminal or C-terminal location to an internal one. Such signals tend to be short sequences, between 4 and 17 amino acid residues, which are enriched in positively charged amino acids, mainly lysines. They have also been found to contain at least one turn-inducing residue. Transport is thought to occur through the nuclear pore, since gold particles were shown to cluster at this region when coated with the nuclear protein nucleoplasmin. Like the soluble and membrane proteins of the ER, nuclear proteins are thought to contain retention signals, since disruption of the nuclear membrane resulted in the release of only 10 to 15 nuclear proteins (for review see Dingwall and Laskey, 1986).

The transport of proteins within the eukaryotic cell is therefore dependent upon a variety of targeting signals. These function to sort individual proteins to the correct subcellular location. Such targeting sequences therefore play a major role in maintaining the integrity and function of the cell. The following sections will examine in detail the structure and function of the mitochondrion and the specific targeting mechanisms involved in the biogenesis of this organelle.

1.2. Mitochondria.

Mitochondria are found in most eukaryotic cells. In electron micrographs they typically appear as rod shaped organelles, approximately 0.5 μm x 2 μm in size. Phase-contrast analysis, however, reveals that these organelles are highly dynamic structures, continuously changing shape, fusing and dividing (Douce, 1985). The numbers of mitochondria vary depending upon the cell type and function. Yeast, for example, may contain from 1 to 50 mitochondria, whilst animal cells may contain thousands (Alberts *et al.*, 1989).

1.2.1. Structure.

Mitochondria are bound by two membranes, which are distinct in both lipid and protein content and also in function. The outer membrane is permeable to

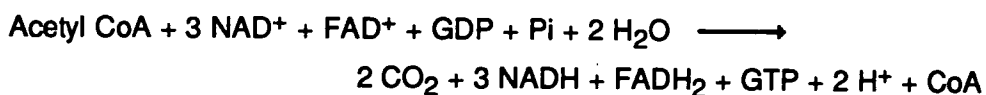
molecules of 10 kDa or less due to the presence of the pore forming protein, porin. In contrast, the inner membrane is impermeable to these small molecules. This is aided by the large amounts of cardiolipin, which is absent from the outer membrane. A number of transport proteins, however, allow the passage of specific molecules across this membrane.

The two membrane systems subdivide the mitochondrion into two distinct compartments, the inter membrane space, lying between the two membranes and an internal matrix compartment bounded by the inner membrane. The matrix frequently contains cristae, which are formed from numerous infoldings of the inner membrane. These increase the surface area and therefore contact with the matrix. This is important, since the enzyme components of the inner membrane and matrix are closely linked (Alberts *et al.*, 1989).

1.2.2. Function.

One of the major functions of the mitochondrion is the synthesis of adenosine 5'-triphosphate (ATP). This is formed from the oxidative breakdown of sugars, fatty acids and occasionally amino acids. Pyruvate generated by cytoplasmic glycolysis enters the mitochondrial matrix and oxidation to CO₂ and H₂O is completed by the combined action of pyruvate dehydrogenase, the tricarboxylic acid (TCA) cycle within the matrix and the respiratory electron transport chain of the inner membrane.

The TCA cycle involves the co-ordinated and sequential interaction of nine enzymes. The overall reaction being illustrated below.



The reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) are then reoxidised by the electron transport chain. This consists of a series of electron carriers the final acceptor being molecular oxygen. As electrons are transferred a proton (H⁺) gradient is generated across the inner membrane. This drives the F₀F₁-ATP synthase, which generates ATP from adenosine 5'-diphosphate (ADP) by a process known as oxidative phosphorylation. The movement of electrons along the transport chain and synthesis of ATP are therefore said to be coupled. The pumping of protons occurs at three sites within

the electron transport chain, at complex I (NADH dehydrogenase), complex III (bc_1 complex or cytochrome *c* reductase) and at complex IV (cytochrome *c* oxidase).

1.2.3. The uniqueness of plant mitochondria.

Although the basic structure and function of mitochondria are similar in both plants and animals, plant mitochondria display a number of distinct differences. The respiration rate of plant mitochondria, for instance, is much higher than in animal cells. Plant mitochondria also show low or undetectable levels of fatty acid oxidation. There are also differences in the nature of the electron transport chain, particularly the presence of additional oxidases and NAD(P)H-dehydrogenases. These features are outlined below. For reviews see Douce (1985) and Douce (1989).

1.2.3.1. The alternative oxidase pathway.

In the presence of cyanide (CN^-) all plant tissues so far studied display a residual respiration, although this activity appears to vary depending upon the tissue. A second oxidase, termed the alternative oxidase, has therefore been proposed, since complex IV is completely inhibited by CN^- . This residual activity can be specifically inhibited by salicylhydroxyamic acid (SHAM). This CN^- -resistant pathway is also found in fungi such as yeast and *Neurospora*. The branch point of electron transport appears to be after NADH dehydrogenase, but before complex III. It is therefore proposed that ubiquinone may feed electrons into this alternative oxidase pathway. Unlike the CN^- -sensitive pathway, however, the alternative oxidase is not coupled to ATP synthesis. It has therefore been suggested that it may be important for the production of heat. This has been proposed, since the pathway is highly active in the spadices of *Arum* lilies, raising the temperature to 15 °C above ambient. As yet, the identity of the constituent proteins remains unclear, although two proteins of 35 kDa and 36 kDa have been implicated (Elthon and McIntosh, 1987).

1.2.3.2. Exogenous NADH- and NADPH-dehydrogenases.

The inner membranes of animal mitochondria are impermeable to NADH and they are therefore unable to oxidise external NADH. Plant mitochondria, however, can oxidise exogenous NADH, as can fungi. This is due to the presence of an

external NADH-dehydrogenase located on the outer surface of the inner membrane. This is distinct from the internal NADH-dehydrogenase (complex I), because it is rotenone resistant. It can also be inhibited by chelating agents such as EGTA, since it is dependent upon the presence of calcium for activity. It is unclear whether electrons are transferred directly to complex III, or if ubiquinone is involved. Transfer to complex III, however, does not occur if endogenous NADH is present. Exogenous NADPH can also be oxidised by plant mitochondria, thus an external NADPH-dehydrogenase has been proposed. This is distinct from the external NADH-dehydrogenase, since it can be inhibited by a lower concentration of chelating agents. As with the alternative oxidase, the function of these dehydrogenases remains unknown.

1.2.3.3. Endogenous NADH-dehydrogenase.

In addition to complex I, the inner membranes of plant mitochondria contain a second NADH-dehydrogenase. These dehydrogenases are distinguished by their sensitivity to the inhibitor rotenone, complex I being sensitive. The second is insensitive and feeds into the electron transport chain via ubiquinone. The oxidation of NADH by this second dehydrogenase is therefore coupled with the synthesis of only two molecules of ATP. This rotenone resistant NADH-dehydrogenase, however, has a much lower affinity for NADH than complex I. Again, the physiological significance is unknown.

1.2.3.4. Glycine decarboxylase and serine hydroxy methyl transferase.

Both of these enzymes are unique to plant mitochondria and are required in the process of photorespiration. This is a complex process involving the chloroplast, peroxisome and mitochondrion (for review see Canvin, 1990). Photorespiration, or photosynthetic carbon oxidation (PCO) is defined as the light dependent oxidation of fixed carbon. It occurs mainly in C₃ plants, which combine carbon from atmospheric CO₂ with ribulose 1,5-bisphosphate (RuBP), producing the C₃ acid 3-phosphoglycerate (3-PGA). This reaction is catalysed by the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). As its name suggests this enzyme can act as a carboxylase or an oxygenase and these activities are dependent upon the concentration of CO₂ and O₂.

PCO occurs when CO_2 is limiting. Rubisco then acts as an oxygenase and converts RuBP into 3-PGA and phosphoglycolate. Phosphoglycolate is then oxidised by PCO to give CO_2 and 3-PGA. 3-PGA then feeds back into the reductive pentose phosphate pathway, the cycle of enzymes within the chloroplast, which result in the fixation of carbon. Although PCO was generally thought to be a wasteful process due to the loss of fixed carbon as CO_2 , a number of useful physiological roles have been proposed. First it acts a salvage pathway, at low CO_2 levels, whereby 75 % of the fixed carbon can be recovered by the plant. It may also function to decompose the glycolate, which is toxic to cells at high concentrations.

1.2.3.5. NAD⁺-linked malic enzyme.

As discussed in the previous section, photosynthetic carbon oxidation occurs mainly in C3 plants. Plants with a C4 dicarboxylic acid pathway of photosynthesis (C4 plants) and those with Crassulacean acid metabolism (CAM) show very little PCO. This is due to various CO_2 concentrating mechanisms, which ensure that Rubisco functions mainly as a carboxylase. In C4 plants, CO_2 is fixed into oxaloacetate in the mesophyll cells by phosphoenolpyruvate carboxylase (PEPcase). This C4 acid is then converted into malate, which is transported to the cells of the bundle sheath. Here, malate is decarboxylated in the mitochondrion by the action of NAD⁺-linked malic enzyme. This results in the release of CO_2 , which enables Rubisco to act as a carboxylase. The pyruvate formed through this malate decarboxylation is transported back to the mesophyll cells, where it is converted into phosphoenol pyruvate by pyruvate Pi dikinase in the chloroplasts. CAM plants have a similar mechanism, except that CO_2 fixation occurs in the dark, whilst the decarboxylation occurs in the light (for review see Leegood and Osmond, 1990).

1.2.4. Mitochondrial biogenesis.

Mitochondria, like chloroplasts, contain their own DNA. This mitochondrial DNA (mtDNA), encodes only a small number of proteins, perhaps less than 10 % of the total mitochondrial protein, but these are essential for both the function and therefore the biogenesis of the mitochondrion. MtDNA also encodes the rRNA and a number of tRNA components of the mitochondrial translation system, which are necessary for the synthesis of these mitochondrially-encoded proteins (for reviews see Tzagoloff and Myers, 1986; Attardi and Schatz, 1988; Gray, 1990). The remaining structural and functional proteins of the mitochondrion are

encoded in the nucleus, synthesised in the cytoplasm and then imported into the organelle. This targeting and import of nuclear-encoded mitochondrial proteins is reviewed in section 1.3.

1.2.5. Mitochondrial DNA.

1.2.5.1. Variation in size and gene content.

The size of the mitochondrial genome ranges from 16 - 20 kbp in animals, up to 200 - 2400 kbp in plants. The larger size of plant mtDNA, however, is not due to an increase in coding capacity, since the number of polypeptides synthesised by isolated plant mitochondria does not appear to change (Forde *et al.*, 1978). Although this coding capacity does not alter, the actual proteins encoded by mtDNA does vary. The NADH-dehydrogenase (complex I) genes, for instance, are completely absent from *S. cerevisiae* mtDNA (Tzagoloff and Myers, 1986), whilst they are found in the mitochondrial genomes of animals and plants. In contrast, the α -subunit of the F₁-ATP synthase is mitochondrially-encoded in plants, where as in fungi and animals it is encoded in the nucleus. Table 1.2 summarises the mitochondrial genes identified to date.

1.2.5.2. Variation in organisation.

The organisation of the mitochondrial genome varies despite the apparent similarity in coding capacity. In animals, genes are separated by just a few nucleotides and so there is virtually no non-coding DNA. Transcription is from the Heavy (H-) strand only and results in a single polycistronic message. The tRNA genes are arranged throughout the genome, punctuating the protein and rRNA genes and it has been proposed that these may act as signals for the subsequent processing of the polycistronic mRNA.

In contrast, yeast mtDNA organisation is less 'compact'. Non-coding regions including introns, are present. The tRNA genes also differ in arrangement, being clustered, rather than scattered as in the animal genome. Transcription appears to be initiated at at least 13 individual sites. These mRNAs, however, are still polycistronic (for review see Attardi and Schatz, 1988).

Table 1.2; Mitochondrial genes identified to date.

GENE	ABBREVIATION	ANIMALS			YEAST			PLANTS		
Ribosomal RNAs	<i>rrn</i>									
Large subunit		16S			21S			26S		
Small subunit		12S			15S			18S		
5S		-			-			+		
Small subunit ribosomal protein	<i>rps</i>	-			VAR1			S12		
								S13		
								S14		
Transfer RNAs		22			25			?a		
NADH-dehydrogenase (complex I)										
subunit 1	<i>nad1</i>	+			-			+ ^b		
subunit 2	<i>nad2</i>	+			-			+ ^b		
subunit 3	<i>nad3</i>	+			-			+		
subunit 4	<i>nad4</i>	+			-					
subunit 4L	<i>nad4L</i>	+			-					
subunit 5	<i>nad5</i>	+			-			+		
subunit 6	<i>nad6</i>	+			-					
Cytochrome <i>c</i> reductase (complex III)										
COB ^c	<i>cob</i>	+			+			+		
Cytochrome <i>c</i> oxidase (complex IV)										
subunit 1	<i>coxI</i>	+			+			+		
subunit 2	<i>coxII</i>	+			+			+		
subunit 3	<i>coxIII</i>	+			+			+		
F ₀ F ₁ -ATP synthase										
subunit 6	<i>atp6</i>	+			+			+		
subunit 8	<i>atp8</i>	+			+			-		
subunit 9	<i>atp9</i>	-			+			+		
α subunit	<i>atpA</i>	-			-			+		

^aThe total number of tRNAs encoded by plant mtDNA remains unclear, although to date approximately 12 tRNA genes have been identified from various plant mtDNAs (Gray, 1990).

^bThe *nad1* and *nad2* genes have been identified, but it is unknown whether these are translated (Newton, 1988; Gray, 1990).

^cCOB; cytochrome *b*.

Adapted from Gray (1990).

1.2.5.3. Variation in structure.

MtDNA exists in a variety of physical forms. Supercoiled, covalently closed circular DNA may be isolated from animal and yeast mitochondria, whilst linear molecules are found in *Paramecium aurelia* and *Tetrahymena pyriformis*. Plant mtDNA is also isolated mainly as linear molecules, but when isolated from cells grown in tissue culture the proportion of circular mtDNA molecules is increased (Leaver and Gray, 1982; Gray, 1990). The physical structure of plant mtDNA appears more complex than in other organisms. This is discussed further in section 1.2.6.1.

1.2.6. Plant mitochondrial DNA.

1.2.6.1. Structure.

Plant mtDNA is thought to exist as a heterogeneous population of sub-genomic circular DNA molecules, which vary in individual stoichiometric amounts. It is proposed that this organisation is due to specific recombination events occurring between repeated sequences within the mitochondrial genome (Lonsdale *et al.*, 1984; Palmer and Shields, 1984). Differences in recombination frequencies and replication rates would therefore result in the presence of submolar and multimolar forms. The plant mitochondrial genome may therefore be represented as a 'master circle' from which these sub-genomic circles arise.

The 218 kbp mtDNA of *Brassica campestris*, for instance, contains one pair of repeats and recombination would generate two sub-genomic circles (Palmer and Shields, 1984). This multipartite structure of plant mtDNA is not obligatory, however, since repeat elements appear to be absent from the *Brassica hirta* mtDNA (Palmer and Herbon, 1987). A recent review, however, suggests that this view may be too simplistic, since the mtDNA of *Brassica* species is now known to contain at least 50 smaller repeat elements. The organisation of mtDNA may therefore be far more complicated than was originally thought (Andre *et al.*, in press).

1.2.6.2. Protein coding genes and RNA editing.

A number of mitochondrially-encoded protein genes have now been isolated (see Table 1.2). When the deduced amino acid sequence of certain proteins was compared with the homologous protein from other organisms, however, a number of

amino acid differences were found. In particular, the CCG codon appeared to code for tryptophan, rather than arginine. This led to the proposal that mtDNA had diverged from the 'universal' genetic code. It now appears, however, that plant mitochondrial RNA transcripts are edited. This process of RNA editing is also known to occur in *Trypanosome*, Paramyxovirus and mammalian mitochondrial transcripts (for review see Mulligan, 1991).

The process of RNA editing in plant mitochondria results in the conservation of protein sequence. Modification of a cytosine nucleotide (C) to a uridine nucleotide (U) appears to be the most common, but U to C has also been noted. In the wheat *coxIII* transcript, for instance, there are 12 C to U modifications, but only 1 U to C (Gualberto *et al.*, 1990). RNA editing is not only confined to coding regions, but also occurs in the untranslated regions of RNA molecules. The leader sequence of the *Oenothera* small subunit ribosomal protein 14 mRNA is one such example (Schuster *et al.*, 1990).

The mechanism of RNA editing remains unclear. Comparisons of editing sites, however, has revealed a variety of sequence homologies (Gualberto *et al.*, 1990). It is proposed that small antisense RNAs, guide RNAs (gRNAs) may bind to these sites and so mediate the RNA editing recognition process (Blum *et al.*, 1990). The presence of such gRNAs, however, has yet to be shown. RNA editing appears to be necessary for the correct expression of certain mitochondrial genes, since without it translation may not occur, or aberrant proteins would be synthesised. Why mitochondrial genes do not encode the 'correct' protein sequence is unclear. A number of suggestions have been proposed to explain the role of RNA editing, such as the control of mitochondrial gene expression (Mulligan, 1991) or that it has evolved to correct mutations within the mtDNA (Gualberto *et al.*, 1990).

1.2.6.3. Ribosomal RNAs and genes.

Plant mitochondrial ribosomes sediment at about 78S. The small subunit contains an 18S rRNA and the large subunit contains a 26S rRNA. Although these rRNAs are much larger than animal mitochondrial rRNAs, the primary structure is more homologous to the bacterial 16S and 23S homologues (Newton, 1988). Plant mitochondrial ribosomes also contain a 5S rRNA component, which is not found in other eukaryotes. All of these rRNAs are encoded by the mtDNA (see Table 1.2 and Leaver and Gray, 1982). The 18S and 5S rRNA genes are closely linked and in the same transcriptional orientation. The 18S rRNA gene, however, is in the opposite orientation (Gray, 1990). The copy number of these rRNA genes appears to vary

between species. In wheat mtDNA, for instance, the rRNA genes are present in two gene copies, whilst in *Z. mays* the mtDNA encodes only one.

1.2.6.4. Transfer RNAs and genes.

Mammalian mtDNA encodes 22 tRNAs, whilst *S. cerevisiae* mtDNA encodes 25 (Tzagloff and Myers, 1986). This is less than the minimum 32 tRNAs required to translate the genetic code, but the presence of a single tRNA species recognising each of the CUN, GUN, UCN, CCN, ACN, GCN four-codon families enables translation to occur (Leaver and Gray, 1982). The situation in plants is still unclear, although approximately 12 tRNA genes have been identified from various plant mtDNAs, including an initiator and an elongator tRNA^{Met} (see Table 1.2). In general, these are thought to be individually transcribed, since they do not appear to be linked to other tRNA or protein genes. In wheat, however, the initiator tRNA^{Met} is linked to the 18S rRNA gene. Comparison of the primary sequences, as with the rRNA genes, indicate that the tRNA genes are more homologous to the bacterial counterparts than to other mitochondrial tRNAs.

1.2.6.5. Gene structure.

The structure of certain genes within plant mtDNA is different from other organisms (for reviews see Leaver and Gray, 1982; Newton, 1988; Gray, 1990). The *Z. mays* cytochrome *c* oxidase subunit II gene (*coxII*), for instance, contains a 794 bp intron, whereas in fungal and animal mtDNAs the gene is not split (Fox and Leaver, 1981). The genes for cytochrome *c* oxidase subunit I (*coxI*) and cytochrome *b* (*cob*) contain multiple introns in yeast, but not in plants.

Gene maps, representing the plant mtDNA 'master circle' (section 1.2.6.1) have now been published for *Z. mays* (see Gray (1990) for an updated version), *Spinacia oleracea* (Stern and Palmer, 1986) and *Brassica campestris* (Makaroff and Palmer, 1987). Genes are distributed on both strands, with little evidence of clustering. The copy number appears to vary depending upon the species. The *Z. mays atpA* gene, the *B. campestris coxI* gene and the wheat rRNA genes, for instance, are all duplicated.

1.2.6.6. Promiscuous DNA.

Plant mtDNA is unusual, because it appears to contain DNA sequences, which show homology to other organellar DNA. *Z. mays* mtDNA, for instance, contains a 12 kbp region encoding the chloroplast 16S rRNA gene, two tRNA genes and part of the large subunit of Rubisco (Stern and Lonsdale, 1982; Lonsdale *et al.*, 1983). Such regions have been termed 'promiscuous DNA' and are thought to have arisen by gene transfer between organelles (Pring and Lonsdale, 1985). The presence of such 'foreign' DNA does not appear to be limited to mitochondria, but has also been observed within the chloroplast genome and plant nuclear DNA. It is unclear how such DNA is transferred. The transport of RNA molecules between the organelles and the nucleus has been proposed. Indirect evidence suggests that this may already occur in plant mitochondria, since these do not appear to encode sufficient tRNAs for mitochondrial protein synthesis (section 1.2.6.4). The reverse transcription of the RNA intermediate would then allow integration of the foreign DNA (for review see Schuster and Brennicke, 1988).

1.3. Mitochondrial protein import.

1.3.1. Introduction.

In the past 15 years, the question of how proteins are imported into mitochondria has been addressed primarily in the yeast *Saccharomyces cerevisiae* and the filamentous fungus *Neurospora crassa* (for reviews see Attardi and Schatz, 1988; Pfanner *et al.*, 1988a; Hartl *et al.*, 1989; Pfanner and Neupert, 1990). A wealth of knowledge has now been obtained and many advances made in understanding the molecular basis of mitochondrial protein import. This has been achieved mainly through the ability of *S. cerevisiae* to grow anaerobically. Non-lethal mutations can therefore be introduced into the nuclear DNA and this, through *in vivo* complementation, has led to the isolation of numerous nuclear genes (*PET* genes) encoding mitochondrial proteins (Tzagoloff and Myers, 1986).

In contrast, studies with plant mitochondria have been very limited and progress is hampered, because mutations in plant nuclear DNA affecting mitochondrial function would be lethal. The isolation of plant nuclear-encoded mitochondrial genes has therefore been much more difficult. The section below therefore reviews mitochondrial protein import in *S. cerevisiae* and *N. crassa*, but

will include reports from mammalian studies where appropriate. The situation in plants is discussed later in section 1.4.

1.3.2. Synthesis.

Protein import into mitochondria is a highly complex process. Distinct steps, however, have now been characterised and these are summarised in Table 1.3. Nuclear-encoded mitochondrial proteins are first synthesised in the cytosol and then imported into the mitochondrion. This has been demonstrated *in vivo* by pulse chase experiments after cytoplasmic ribosome synthesis had been inhibited (Teintze *et al.*, 1982) and also by mitochondrial re-energisation after the addition of uncouplers of oxidative phosphorylation (Reid and Schatz, 1982). This post-translational import of nuclear-encoded mitochondrial proteins has also been confirmed by the numerous cell-free experiments that have been performed. It therefore appears that no obligate coupling between protein synthesis and protein import exists, although Suissa and Schatz (1982) do not rule out the possibility that co-translational import may also occur *in vivo*.

1.3.3. Targeting.

Removal of the N-terminal presequence prevents the targeting of the protein to the mitochondrion (van Loon *et al.*, 1986). Such sequences must therefore contain delivery signals or targeting information, which localise the protein to the mitochondrion. This has been corroborated by experiments with hybrid proteins in which it is demonstrated that mitochondrial presequences can direct the import of non-mitochondrial proteins, such as the mouse cytosolic protein dihydrofolate reductase (DHFR) or the bacterial β -galactosidase (Horwich *et al.*, 1985; Emr *et al.*, 1986).

Presequences vary considerably in length from 16 amino acids (Oudshoorn *et al.*, 1987) up to 80 amino acids (Guiard, 1985). Experiments with hybrid proteins have shown that as little as 9 amino acids can specifically target a passenger protein to the mitochondrion (Keng *et al.*, 1986). What then are the characteristics that define a mitochondrial targeting signal?

Primary sequence comparisons have revealed little homology. This is exemplified by the two isoforms of the bovine F_0 -ATP synthase subunit 9 (F_09), which have an identical mature protein sequence, but considerably different presequences (Gay and Walker, 1985). This lack of specific amino acid sequence

Table 1.3; Distinct steps in mitochondrial protein import.

1. cytoplasmic synthesis of the precursor protein^a.
2. maintenance of the precursor in a translocation-competent state; requires ATP and cytosolic factors (hsp70)^b.
3. targeting and subsequent binding at specific receptor sites on the outer mitochondrial membrane.
4. translocation through contact sites; requires an inner membrane potential ($\Delta\Psi$).
5. processing of the precursor to the mature form by the matrix processing protease^c.
6. re-folding of the mature protein; requires mhsp70^d and mhsp60^e.
7. sorting of the mature protein to the appropriate mitochondrial subcompartment and final assembly.

^asome nuclear-encoded mitochondrial proteins are synthesised without cleavable presequences (N-terminal extensions), but can still be regarded as precursor proteins, since the mature protein displays different properties to that of the precursor.

^bcytoplasmic 70 kDa heat shock protein.

^cin some cases the generation of the mature protein requires a second protease which is located within the mitochondrial inter membrane space (Hartl *et al.*, 1986; 1987).

^dmitochondrial 70 kDa heat shock protein.

^emitochondrial 60 kDa heat shock protein.

Adapted from Pfanner and Neupert (1990).

was confirmed by Allison and Schatz (1986) who showed that artificial sequences, containing only arginine, serine and leucine, plus the initiating methionine, could function as mitochondrial targeting signals.

Comparisons of mitochondrial presequences have, however, revealed a number of general features. There is a preponderance of basic, positively charged amino acids, which tend to be periodically arranged along the presequence, a general lack of acidic residues and a high content of hydroxylated amino acids (Hurt and van Loon, 1986). These overall characteristics indicate that secondary and/or tertiary structures may be important in determining the targeting function of the presequence. Structural prediction analyses and experimental evidence indicate that many mitochondrial presequences may form positive amphiphilic α -helices (Roise *et al.*, 1986; von Heijne, 1986).

Site directed mutagenesis has been used to study this possible requirement for an amphiphilic α -helix, as well as investigating the necessity of individual amino acids within the presequence. Care, however, must be taken in the interpretation of such experiments, since single amino acid changes may have far reaching effects upon the tertiary structure of a protein. It has been postulated, however, that such disruption of a predicted α -helix resulted in a less efficient targeting signal (Horwich *et al.*, 1986; Roise *et al.*, 1988). Vasserotti *et al.* (1987) demonstrated that spontaneous mutations could restore the import of a protein from which the mitochondrial presequence had been removed. Subsequent analysis of the amino acid sequence indicated that modifications had occurred within an N-terminal α -helix, which resulted in a decrease in the net negative charge. It has been shown that an amphiphilic β -sheet can also form an active presequence, so targeting may be due to the amphiphilic nature of presequences rather than the actual formation of an α -helix (Roise *et al.*, 1988).

How amphiphilicity contributes to the targeting function of the presequence is still not clear. It is known that proteins enter the inner mitochondrial membrane in a manner dependent upon the membrane potential ($\Delta\Psi$). The α -helix may therefore allow the initial insertion into the mitochondrial outer membrane. The positive amphiphilic presequence could then be subject to an electrophoretic effect exerted by $\Delta\Psi$ (negative inside, positive outside).

In order to function as a targeting signal it appears that the presequence must be readily exposed on the surface of the protein. This was demonstrated when an internal protein sequence from DHFR could target the complete DHFR protein to the mitochondrion when exposed at the N-terminus (Hurt and Schatz, 1987). This observation is important, since it indicates that potential targeting signals may lie

buried within non-mitochondrial proteins and this has important implications when the evolution of the mitochondrial presequences is considered. It has also been shown that 1 % of completely random DNA fragments from the *Escherichia coli* genome encoded polypeptides that could target DHFR to mitochondria (Baker and Schatz, 1987).

1.3.4. Receptors.

The import of proteins into mitochondria is mediated by distinct protein binding sites located on the surface of the mitochondrial outer membrane. These binding sites or receptors are thought to be proteinaceous in structure, since treatment of mitochondria with low amounts of proteases inhibited the binding and therefore the import of precursor proteins (Zwizinski *et al.*, 1984). Competition studies with naturally occurring precursors (Pfaller and Neupert, 1987) or chemically synthesised presequences (Gillespie *et al.*, 1985; Ono and Tuboi, 1988; Glaser and Cumsky, 1990) could also inhibit import, again suggesting the presence of specific binding sites.

A number of outer membrane proteins from both *S. cerevisiae* and *N. crassa* have now been proposed to function as import receptors (for review see Baker and Schatz, 1991). The isolation and characterisation of these proteins has been achieved mainly through the use of antibodies generated against mitochondrial outer membrane proteins. Ohba and Schatz (1987a) used this technique to implicate a 45 kDa outer membrane protein from yeast. Antibodies generated against this protein, however, were not monospecific and subsequent analysis indicated that a 42 kDa protein rather than the 45 kDa protein was involved in the import pathway. This protein was termed import site protein 42, or ISP42, and was the first component of the transmembrane import machinery to be identified (Vestweber *et al.*, 1989).

Using the same monoclonal antibody technique, two possible receptor proteins of 19 kDa and 72 kDa (termed mitochondrial outer membrane protein 19 and 72, or MOM19 and MOM72 respectively) have been isolated from the *N. crassa* mitochondrial outer membrane (Sollner *et al.*, 1989; Sollner *et al.*, 1990). MOM19 is distributed over the entire mitochondrion and exposed on the outer surface, whilst MOM72 is an integral membrane protein enriched at the contact sites between the outer and inner mitochondrial membranes (see section 1.3.6). They therefore differ in their location and distribution within the mitochondrial outer membrane. They also appear to differ in function, since monoclonal

antibodies raised against MOM19 inhibit the import of porin, the Rieske iron sulphur protein (FeS), F₀9 and the β -subunit of the F₁-ATP synthase (F₁ β), whilst antibodies against MOM72 inhibit only the adenine nucleotide translocator (ANT). A subsequent report, however, suggests that in the absence of MOM72, ANT may be imported via MOM19 (Steger *et al.*, 1990).

Treatment of MOM19 with increasing amounts of elastase generates an 18 kDa and then a 17 kDa protein moiety. The import of porin, FeS and F₀9 correlates with the presence of MOM19 and the 18 kDa moiety, whereas the import of F₁ β requires only the 17 kDa moiety. Distinct functional sites therefore appear to exist on MOM19 (Sollner *et al.*, 1989). These results therefore appear to confirm a previous report which suggested that F₀9 and F₁ β were imported by different receptor proteins (Pfaller *et al.*, 1988).

Both MOM19 and MOM72 are proposed to function only as surface receptors mediating the initial binding and therefore selection of precursor proteins. A subsequent import step has now been identified, which involves the insertion of precursors into a proteinase K resistant site within the outer membrane (Pfaller and Neupert, 1987 and Pfaller *et al.*, 1988). This common insertion site termed general insertion protein (GIP) is distinct from MOM19 and MOM72, since these are both sensitive to proteinase K (Sollner *et al.*, 1989; Sollner *et al.*, 1990).

Competition studies with various precursors have also indicated that the insertion into GIP occurs after the initial MOM19 and MOM72 binding. Porin, for example, can interfere with the import of ANT despite the fact that both utilise different outer surface receptors (Pfaller and Neupert, 1987). The number of binding sites for porin and ANT, however, are very similar, suggesting the existence of a common import receptor (Pfaller and Neupert, 1987; Pfaller *et al.*, 1988). Recently, two further proteins (MOM38 and MOM22) have also been implicated in receptor function. One of which, MOM38, is postulated to be equivalent to GIP or form part of it (Kiebler *et al.*, 1990).

The import of ANT into yeast mitochondria is mediated by MAS70 (mitochondrial assembly protein 70), the major 70 kDa protein of the outer membrane (Hines *et al.*, 1990). This protein had been previously sequenced and is now known to have homology with MOM72, the *N. crassa* homologue (Steger *et al.*, 1990). Antibodies directed against MAS70 inhibit the import of ANT by about 75 % and this correlates well with the comparable experiment with MOM72. Unlike the *N. crassa* protein though, MAS70 also appears to mediate the import of F₁ β . Receptor proteins from yeast and *N. crassa* may therefore vary in specificity.

MAS70 is distinct from the previously identified yeast protein, ISP42, since both differ in sensitivity to trypsin digestion. The resistance of ISP42 to trypsin has suggested that this is the yeast homologue of GIP (MOM38). More significant, however, is the homology between the ISP42 (Baker *et al.*, 1990) and the MOM38 (Kiebler *et al.*, 1990) cDNAs. A third cDNA encoding a mitochondrial receptor has also been isolated (Murakami *et al.*, 1990). This encodes a yeast protein (p32) with a relative molecular mass of 32 kDa and was identified using anti-idiotypic antibodies (Pain *et al.*, 1990). The same technique was used to identify a putative chloroplast receptor protein (Pain *et al.*, 1988). Interestingly, both the p32 cDNA and that of MOM38 show some homology to mammalian mitochondrial phosphate carrier proteins. This may indicate a structure/function relationship, since both are involved in transport (Kiebler *et al.*, 1990). It should also be noted that all three cDNAs appear to encode proteins without an N-terminal extension, but this is not unusual in proteins destined for the outer mitochondrial membrane (see section 1.3.11.1).

1.3.5. Energy requirements.

The insertion of precursor proteins into the inner membrane requires the electrical gradient or membrane potential ($\Delta\Psi$) generated by the proton (H^+) translocating F_0F_1 -ATP synthase. This has been demonstrated by the use of inhibitors of oxidative phosphorylation and uncouplers, such as ionophores, which result in the dissipation of the H^+ gradient (Schleyer *et al.*, 1982). An electrical gradient ($\Delta\Psi$) rather than a pH gradient (ΔpH) is required for the import of precursor proteins, since a potassium ion, K^+ , diffusion gradient was able to mediate import into isolated mitochondria (Pfanner and Neupert, 1985).

Protein import into mitochondria requires not only the $\Delta\Psi$, but also energy in the form of ATP (Pfanner and Neupert, 1986; Chen and Douglas, 1987a). This is thought to be required for the unfolding of the precursor protein, since loosely folded proteins can be imported without the addition of ATP (Verner and Schatz, 1987; Pfanner *et al.*, 1988b). This requirement for an unfolded configuration had previously been indicated, since the import of a chimaeric protein containing DHFR was blocked upon the addition of methotrexate, which binds specifically to DHFR (Eilers and Schatz, 1986). Similarly, point mutations which destabilise the tertiary structure of a precursor protein allowed import to occur at lower temperatures, again demonstrating that an unfolded configuration was required (Vestweber and Schatz, 1988a). It had also been noted that the sensitivity of an *in*

vitro translated mitochondrial precursor protein to proteinase K decreased when nucleotide triphosphates were removed from the rabbit reticulocyte lysate (Pfanner *et al.*, 1987a). This apparent correlation between the unfolding of the precursor and the requirement for ATP led to the hypothesis that an ATP-dependent unfoldase might be located in the cytosol or on the outer membrane of the mitochondrion (Rothman and Kornberg, 1986). Substitution with other nucleotide triphosphates has shown that ATP can be replaced with guanosine 5'-triphosphate (Eilers *et al.*, 1987).

1.3.6. Contact sites.

The import of precursor proteins into the matrix or inner mitochondrial membrane occurs at specific regions within mitochondria. At these points the outer and inner mitochondrial membranes are in close association with each other and as such are termed contact sites. This has been demonstrated by importing precursors at low temperatures or low ATP concentrations. Such conditions generate translocation intermediates, which span both mitochondrial membranes (Schleyer and Neupert, 1985). Similar results are obtained if the precursors are first prebound with antibodies before import. When labeled with protein A-gold, such intermediates can be visualised by electron microscopy and are seen clustered at the contact sites of mitochondria (Schwaiger *et al.*, 1987).

The number of these import sites appears to be limiting, since the accumulation of these translocation intermediates can inhibit the import of other precursor proteins (Vestweber and Schatz, 1988b). Pon *et al.* (1989) isolated a sub-mitochondrial fraction which was enriched in contact sites. This retained the capacity to transport proteins in an ATP-dependent manner and provided further evidence that protein import occurs through contact sites. A common import pathway appears to exist, probably involving GIP (section 1.3.4), since precursor proteins destined to the inner membrane, matrix or inter membrane space can be extracted from these contact sites (Pfanner *et al.*, 1987b). It is still unclear, however, whether or not the import of outer membrane proteins, such as porin, involves these contact sites (see section 1.3.11.1).

Proteinaceous structures may be essential components of contact sites, since translocation intermediates can be extracted under conditions of alkaline pH or with urea (Pfanner *et al.*, 1987b). After removal of the outer mitochondrial membrane the contact sites persist (Schwaiger *et al.*, 1987), confirming the observation by Ohba and Schatz (1987b) that protein import can still occur into

mitoplasts. Indeed, two mitochondrial proteins were found to be enriched in the contact site fraction (Pon *et al.*, 1989), but these are yet to be identified.

1.3.7. Role of heat shock proteins.

A number of reports have indicated that proteinaceous factors localised in the cytosol are required for the import of proteins into mitochondria. Such factors have been found in rabbit reticulocyte lysate (Chen and Douglas, 1987b; Ono and Tuboi, 1988) and a yeast cell lysate (Ohta and Schatz, 1984). These cytosolic factors appear to belong to the 70 kDa heat shock protein (hsp) family, since inhibiting the expression of a plasmid-borne yeast hsp70 gene (*SSA1*) in a *S. cerevisiae* strain disrupted in the chromosomal *SSA1*, *SSA2* and *SSA4* hsp genes, resulted in the cytoplasmic accumulation of unprocessed mitochondrial F₁-ATP synthase β -subunit precursor (Deshaies *et al.*, 1988). It has also been shown that a yeast post-ribosomal supernatant could stimulate mitochondrial protein import. This was subsequently found to consist of at least two distinct factors, one being a 70 kDa heat shock related protein (Murakami *et al.*, 1988). These hsp70-like proteins also appear to be involved in the transport of proteins across the membrane of the endoplasmic reticulum (Chirico *et al.*, 1988; Desahaies *et al.*, 1988). It therefore seems that these hsps may play a role in protein transport in general.

It is proposed that these hsps function as molecular chaperones (Ellis, 1987), since by binding to a protein they are thought to prevent or disrupt inappropriate protein-protein interactions (Pelham, 1986; Pelham, 1988). Such hsp associations with mitochondrial precursor proteins are thought to bring about the loosely folded 'import competent' configuration required for import (Deshaies *et al.*, 1988; Pelham, 1988). ATP hydrolysis is then thought to facilitate the dissociation of this 'import competent' precursor protein from the hsp70.

As well as being found in the cytosol, two hsps have now been located in the mitochondrial matrix. These are thought to be involved in both the re-folding and the assembly of the imported protein. Cheng *et al.* (1989) demonstrated that a *S. cerevisiae* strain carrying a mutation in the constitutively expressed nuclear gene *mif4* (for mitochondrial import function) imported and processed the F₁-ATP synthase β -subunit precursor, but could not assemble the imported protein. This nuclear gene was subsequently found to encode the heat shock protein, hsp60 (Reading *et al.*, 1989). This is structurally related to the groEL protein from *Escherichia coli* (McMullin and Hallberg, 1988) and the Rubisco subunit binding

protein (Hemmingsen *et al.*, 1988), both of which were previously shown to be involved in protein assembly. Similar structurally related hsp60s have also been found in the mitochondria of *Xenopus laevis*, *Zea mays* and *Homo sapiens* (McMullin and Hallberg, 1988) and *Tetrahymena thermophila* (McMullin and Hallberg, 1987). Recent investigations by Cheng *et al.* (1990) indicate that a functional matrix located hsp60 is required for its own assembly.

As with the cytoplasmic hsp70, ATP hydrolysis is required for the release of the imported protein from hsp60, but this occurs subsequent to the re-folding of the imported protein. The hsp60 was therefore proposed to be an ATP-dependent 'folding catalyst' (Ostermann *et al.*, 1989). Interestingly, Pfanner *et al.* (1987a) proposed that mitochondrial protein import required ATP hydrolysis at two sites, one within the mitochondrial matrix and the second at a site external to the inner membrane.

A second matrix located hsp has now been implicated in the *S. cerevisiae* import pathway, since a precursor protein stuck in the mitochondrial membrane was cross-linked to a 70 kDa protein (Scherer *et al.*, 1990). The first 15 N-terminal amino acid residues of this 70 kDa protein were identical to residues 24 to 38 of an hsp70 encoded by the nuclear gene *SSC1* (Craig *et al.*, 1989). It therefore appears that this matrix hsp70 is synthesised with a 23 amino acid presequence (Scherer *et al.*, 1990).

1.3.8. Proteolytic processing of precursors.

The processing or maturation of precursor proteins synthesised with N-terminal extensions occurs in the mitochondrial matrix. Partial purification of the enzyme involved indicated that divalent metal ions, such as Zn^{2+} , Co^{2+} and Mn^{2+} were required for activity (McAda and Douglas, 1982; Bohni *et al.*, 1983). The mitochondrial processing protease has now been isolated from both *S. cerevisiae* (Yang *et al.*, 1988) and *N. crassa* (Hawliitschek *et al.*, 1988). In both organisms, the enzyme appears to consist of two distinct proteins with differing molecular weights and properties. In *N. crassa*, the catalytic activity resides on the larger protein (apparent MW 57 kDa), termed the matrix processing peptidase (MPP). The full processing activity of MPP, however, requires a second protein (apparent MW 52 kDa), termed the processing enhancing protein (PEP). In *N. crassa*, PEP is 15 x more abundant than MPP and is localised within the inner membrane rather than the matrix (Hawliitschek *et al.*, 1988). This is in contrast to *S. cerevisiae*, in

which both MPP and PEP are soluble matrix proteins (Jensen and Yaffe, 1988; Witte *et al.*, 1988).

Interestingly, the PEP from *N. crassa* was found to be identical to subunit I of the cytochrome *bc*₁ complex (Schulte *et al.*, 1989; Weiss *et al.*, 1990). Subsequent analysis of the *S. cerevisiae* and *N. crassa* proteins, MPP, PEP and subunit II of the cytochrome *bc*₁ complex, as well as subunit I of the cytochrome *bc*₁ complex from *S. cerevisiae* has revealed a high degree of homology and has led to the proposal that these proteins may have originated from the same ancestral gene. The divergence of PEP and subunit I in *S. cerevisiae* may reflect the ability of this organism to grow under anaerobic conditions. Such conditions would therefore necessitate the divergence of PEP from subunit I (Schulte *et al.*, 1989).

Two temperature-sensitive *S. cerevisiae* mutants, *mas1* and *mas2* (for mitochondrial assembly), deficient in the activity of the matrix located protease, have previously been described (Yaffe and Schatz, 1984; Yaffe *et al.*, 1985). The subsequent isolation of one of these nuclear genes, *mas1*, and complementation by the PEP cDNA (Hawlitschek *et al.*, 1988) has revealed this to be the *S. cerevisiae* PEP equivalent (Witte *et al.*, 1988), whilst *mas2* (or *mif2* for mitochondrial import functions) encoded MPP (Jensen and Yaffe, 1988; Pollock *et al.*, 1988). The protein, MAS1, appears to be synthesised as a larger precursor, which is processed upon import. This therefore implies that its maturation requires a pre-existing form of the active protein (Witte *et al.*, 1988). Until recently, it remained uncertain whether MAS2 was processed. Isolation of the cDNA and subsequent import experiments with the *in vitro* translated radiolabelled product, however, indicate that, as with PEP, MPP is also processed upon import (Schneider *et al.*, 1990).

As previously discussed (section 1.3.3), the primary sequence of mitochondrial targeting signals show little homology, rather they exhibit a number of general features, such as a preponderance of basic and hydroxylated amino acids and a lack of negatively charged amino acids (Hurt and van Loon, 1986). This lack of sequence homology therefore raises the question as to how these presequences are recognised by MPP and specifically cleaved. It is proposed that the presequence forms a distinct secondary or tertiary structure which is then recognised by MPP. This has been indicated by the fact that a denatured precursor can not be processed (Miura *et al.*, 1986).

Comparisons of a number of presequences and the analysis of the frequency of amino acid residues occurring at specific positions has indicated a prevalence of arginine residues at positions -2 and -10 relative to the processing site (Hendrick

et al., 1989; von Heijne *et al.*, 1989), but it is still unclear just how MPP recognises and specifically cleaves the precursor.

1.3.9. The two step maturation of precursor proteins.

A number of precursor proteins destined for the inner membrane and inter membrane space, are also cleaved by the action of MPP (Gasser *et al.*, 1982a; Reid *et al.*, 1982; Hartl *et al.*, 1986; van Loon *et al.*, 1986; Hartl *et al.*, 1987), but the final maturation depends upon a second protease. Originally such precursor proteins were thought to only partially enter the mitochondrial matrix where they were cleaved to an intermediate by the matrix protease. This processing event was thought to reveal a second import signal, the so called 'stop transfer' signal, which then prevented the remainder of the protein from entering into the matrix (Hurt and van Loon, 1986; van Loon *et al.*, 1986; van Loon and Schatz, 1987; van Loon *et al.*, 1987). It is now thought that such proteins completely enter the matrix, since processing intermediates of the Rieske FeS protein, cytochrome *b*₂ and cytochrome *c*₁ have been shown to accumulate within the matrix (Hartl *et al.*, 1987). A second targeting signal is then revealed and the intermediate is re-exported. The final maturation then occurs on the outer face of the inner membrane by an as yet unidentified protease (Hartl *et al.*, 1987). This method of protein sorting is described as conservative, since upon re-export, the protein is proposed to follow an ancestral pathway, previously developed by the prokaryotic progenitor of the mitochondrion (Hartl *et al.*, 1986; Pfanner *et al.*, 1988a). The precise mechanism, however, remains unknown.

The Rieske FeS protein, an inter membrane space protein, however, is processed entirely within the matrix, the final location being determined by a second targeting signal within the mature protein (Hartl *et al.*, 1987). This does not appear to be unusual, however, since numerous experiments suggest that the targeting or sorting information does not reside entirely within the N-terminal presequence. The deletion of a hydrophobic domain from the mature region of an inner membrane protein, for instance, results in the mis-localisation of the protein to the matrix (Glaser *et al.*, 1990). Also, the binding of a chimaeric protein, consisting of the F₀-ATP synthase subunit 9 presequence fused to DHFR, to mitochondria in the absence of a membrane potential ($\Delta\Psi$) only occurred when part of the mature F₀-ATP synthase subunit 9 protein was also present (Pfanner *et al.*, 1987c).

1.3.10. Modification and assembly.

In addition to the proteolytic removal of the presequence, an imported mitochondrial protein often requires further modification before it becomes fully functional. The covalent attachment of heme, for instance, is an important step in the formation of active cytochromes. Interestingly, such modifications appear to be essential for the import of these proteins. The second proteolytic cleavage of cytochrome c_1 , for example, requires the addition of heme before the maturation can take place (Gasser *et al.*, 1982a; Nicholson *et al.*, 1989). Cytochrome c also requires the addition of heme, but in this case the actual import of the protein is dependent upon this process (Nicholson *et al.*, 1987; Nicholson *et al.*, 1988).

The majority of imported mitochondrial proteins are subunits of larger oligomeric protein complexes, which may include protein subunits encoded by the mitochondrial genome (Table 1.2). In order to form a fully functional complex these subunits must be assembled correctly. It is this final step along the import pathway which is perhaps the least well understood. Many questions still remain unanswered such as 'where and how does protein assembly occur?', 'in what order are the proteins assembled?' or 'what controls or regulates this?'. From our knowledge of heat shock proteins and the role that these play in mitochondrial protein import, it seems reasonable to postulate that these may be involved at some stage, perhaps acting as a scaffold for the ordered assembly. Indeed, as previously discussed (section 1.3.7), a mutation in a nuclear gene encoding a mitochondrial hsp60, resulted in the import and processing, but not the assembly of the mature F_1 ATP- synthase β -subunit (Reading *et al.*, 1989).

Nuclear and mitochondrial mutations which inhibit the synthesis of one or more subunits have, to a certain extent, proved useful in examining the assembly process. In the absence of mitochondrial protein synthesis, for instance, the yeast nuclear-encoded F_1 -ATP synthase was assembled and was enzymatically active (Pfanner and Neupert, 1987a). The assembly of the F_1 -ATP synthase therefore appears to be independent of mitochondrial protein synthesis, at least in yeast.

It is assumed that the biogenesis of functional mitochondria is dependent upon the co-ordinated expression of both mitochondrial and nuclear genomes, but just how this process is regulated remains unknown. Inhibition of the *N. crassa* mitochondrial protein synthesising system did not inhibit the import, processing or assembly of nuclear-encoded subunits. The expression of the nuclear genome therefore does not appear to be dependent upon the presence of mitochondrial translation products (Pfanner and Neupert, 1987a). Rather, the nuclear genome

appears to synthesise proteins essential for the expression of the mitochondrial genome. A *S. cerevisiae* strain containing the nuclear mutation *PET494*, for example, was deficient in the mitochondrially-encoded cytochrome *c* oxidase subunit III, despite the fact that the mRNA was present (Costanzo and Fox, 1986). It is the nucleus, therefore, that is thought to control the expression of the mitochondrial genome (Pfanner and Neupert, 1987a).

1.3.11. Mitochondrial proteins synthesised without N-terminal presequences.

Not all nuclear-encoded mitochondrial proteins are synthesised with N-terminal presequences. Information required to target these proteins must therefore reside within the 'mature' protein molecule. Porin, an outer membrane protein, the adenine nucleotide translocator, an inner membrane protein, and cytochrome *c*, an inter membrane space protein are three such examples. The import pathways of these proteins have been extensively studied and are outlined below.

1.3.11.1 Porin.

The import pathway of this major outer membrane protein is perhaps the most simple, since there is no detour into the mitochondrial matrix (for review see Pfaller *et al.*, 1990). Although porin is not synthesised with an N-terminal extension, targeting information appears to reside at the N-terminal end of the protein, which is predicted to form an α -helix (Kleene *et al.*, 1987). Porin is imported directly into the outer membrane and there is therefore no requirement for an energised inner membrane. The porin α -helix has no net positive charge and it is interesting to speculate that the lack of basic residues within the porin helix may in fact prevent the import of the protein into the inner membrane.

The import pathway of porin does, however, display many features characteristic of other nuclear-encoded proteins. ATP is required for import, presumably for the unfolding and maintenance of a loosely folded configuration (section 1.3.5), whilst the initial binding to the mitochondrion is mediated by specific receptors localised on the outer membrane (Zwizinski *et al.*, 1984; Pfaller *et al.*, 1988). The import pathway then converges upon GIP within the outer membrane (section 1.3.4), which is thought to be equivalent to the previously characterised porin high-affinity binding sites (Pfaller and Neupert, 1987). This

common import site is utilised by both porin and other imported proteins such as the F₁-ATP synthase β -subunit, the Rieske FeS protein and the adenine nucleotide translocator, since porin can inhibit the import of these proteins, whereas digestion of the porin specific receptors with elastase relaxes this inhibition (Pfaller *et al.*, 1988).

The final step in the import pathway is unclear. It is envisaged that once bound to GIP, porin is then transferred to the outer membrane where it is assembled to either the dimeric or trimeric form, but it is as yet unclear as to whether this process involves the mitochondrial contact sites or not. A model of this pathway is illustrated in Figure 1.1.

1.3.11.2. The adenine nucleotide translocator.

The import of the *N. crassa* adenine nucleotide translocator (ANT) has been studied in detail and distinct steps have now been characterised (see Figure 1.2). Translocation into the inner membrane occurs through contact sites and requires a membrane potential. Disruption of this potential results in the generation of an import intermediate, which is protected from exogenous protease. Unlike the fully imported protein, however, the intermediate remains extractable at alkaline pH. This step along the ANT import pathway has been termed stage 3 (Pfanner and Neupert, 1987b). The initial binding of ANT to the mitochondrion is termed stage 2 and is distinguished from stage 3 by its sensitivity to protease. Stage 1 is defined as the cytosolic synthesis of the protein. The import of ANT is mediated by specific receptors, which have been isolated from both *N. crassa* (Sollner *et al.*, 1990) and *S. cerevisiae* (Hines *et al.*, 1990) and are termed MOM72 and MAS70 respectively (section 1.3.4). The binding of ANT at stage 3 is thought to be mediated by GIP, since porin can inhibit the import of a number of proteins including ANT (Pfanner and Neupert, 1987b; Pfaller *et al.*, 1988), whilst the initial binding of ANT, stage 2, is unaffected (Sollner *et al.*, 1989; 1990). Binding to GIP appears to require ATP, but once bound, insertion into the inner membrane, stage 4, requires only the membrane potential ($\Delta\Psi$). The fully assembled 'mature' protein, stage 5, is generated without any requirement for ATP or $\Delta\Psi$ (Pfanner and Neupert, 1987b; Pfanner *et al.*, 1987a). This can be distinguished from the 'precursor' form, since it can bind the inhibitor carboxyatractyloside (Schleyer and Neupert, 1984).

As with porin, targeting information appears to reside at the N-terminal end of the protein. Experiments indicated that the first 111 amino acid residues of the *S. cerevisiae* ANT were sufficient to target a non-mitochondrial protein into

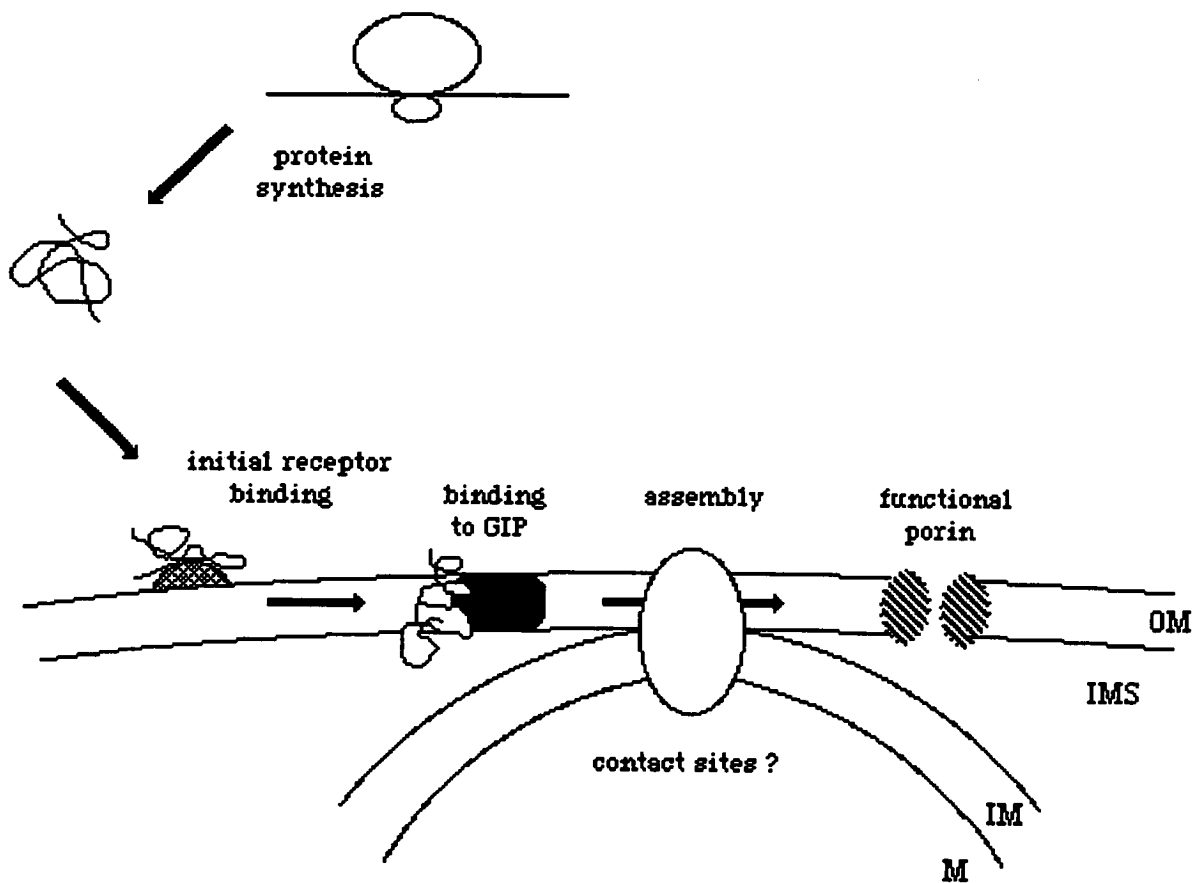


Figure 1.1; Proposed import pathway of porin in *N. crassa*.

OM; outer membrane, IMS; inter membrane space, IM; inner membrane, M; matrix, GIP; general insertion protein. Adapted from Pfaller *et al.* (1990).

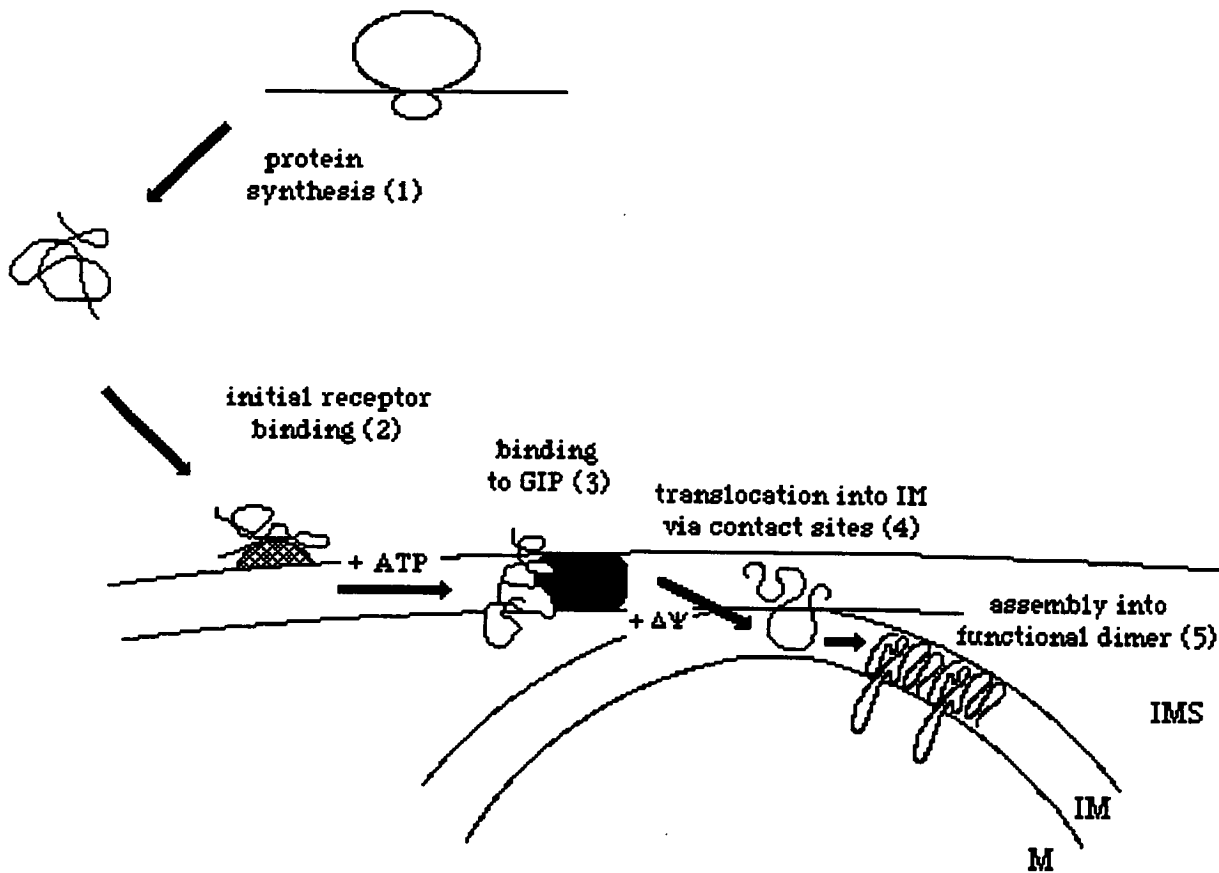


Figure 1.2; Proposed import pathway for the adenine nucleotide translocator in *N. crassa*.

OM; outer membrane, IMS; inter membrane space, IM; inner membrane, M; matrix. Adapted from Pfanner and Neupert (1987b).

mitochondria (Smagula and Douglas, 1988). The import of this fusion protein, however, was incomplete and only appeared to reach stage 3 along the usual import pathway. It was therefore concluded that sequences beyond the initial 111 amino acid residues were also necessary for the import of ANT (Smagula and Douglas, 1988). This was indicated by Pfanner *et al.* (1987d) who demonstrated that the C-terminal two thirds of ANT could also be targeted to the mitochondrion.

It therefore appears that targeting information resides along the whole length of the protein. Examination of the amino acid sequence revealed a tripartite structure consisting of three homologous domains of approximately 100 amino acid residues (Saraste and Walker, 1982). The authors therefore proposed that the ANT has originated from the triplication of an ancestral gene. Each domain is thought to contain two membrane spanning α -helices separated by a hydrophilic region. Such structures have previously been implicated in mitochondrial targeting (section 1.3.3) and so could explain the results obtained by both Smagula and Douglas (1988) and Pfanner *et al.* (1987d).

1.3.11.3. Cytochrome *c*.

The import pathway of cytochrome *c* is distinctly different from all other imported mitochondrial proteins. This inter membrane space protein is imported into mitochondria without a requirement for ATP or an inner membrane potential. Instead, it appears to insert spontaneously into the outer mitochondrial membrane (Nicholson *et al.*, 1987; Nicholson *et al.*, 1988). As mentioned in section 1.3.10, cytochrome *c* must be modified through the addition of heme, before it can become fully functional. Cytochrome *c* is therefore said to exist in two forms, the precursor (apocytochrome *c*) and mature (holocytochrome *c*) form. The covalent attachment of heme by the enzyme cytochrome *c* heme lyase appears to drive the import process (Nicholson *et al.*, 1988). When this addition of heme is inhibited by deuterohemin, import is inhibited, but apocytochrome *c* remains associated with the mitochondria. Specific, high affinity binding sites have therefore been proposed, since the bound apocytochrome can be displaced by excess apoprotein or can be chased into the mitochondrion by the addition of protohemin (Hennig and Neupert, 1981). A chimaeric protein, consisting of the presequence of cytochrome *c*₁ fused to cytochrome *c*, has been utilised to demonstrate this unique import pathway (Stuart *et al.*, 1990), a model of which is illustrated in Figure 1.3.

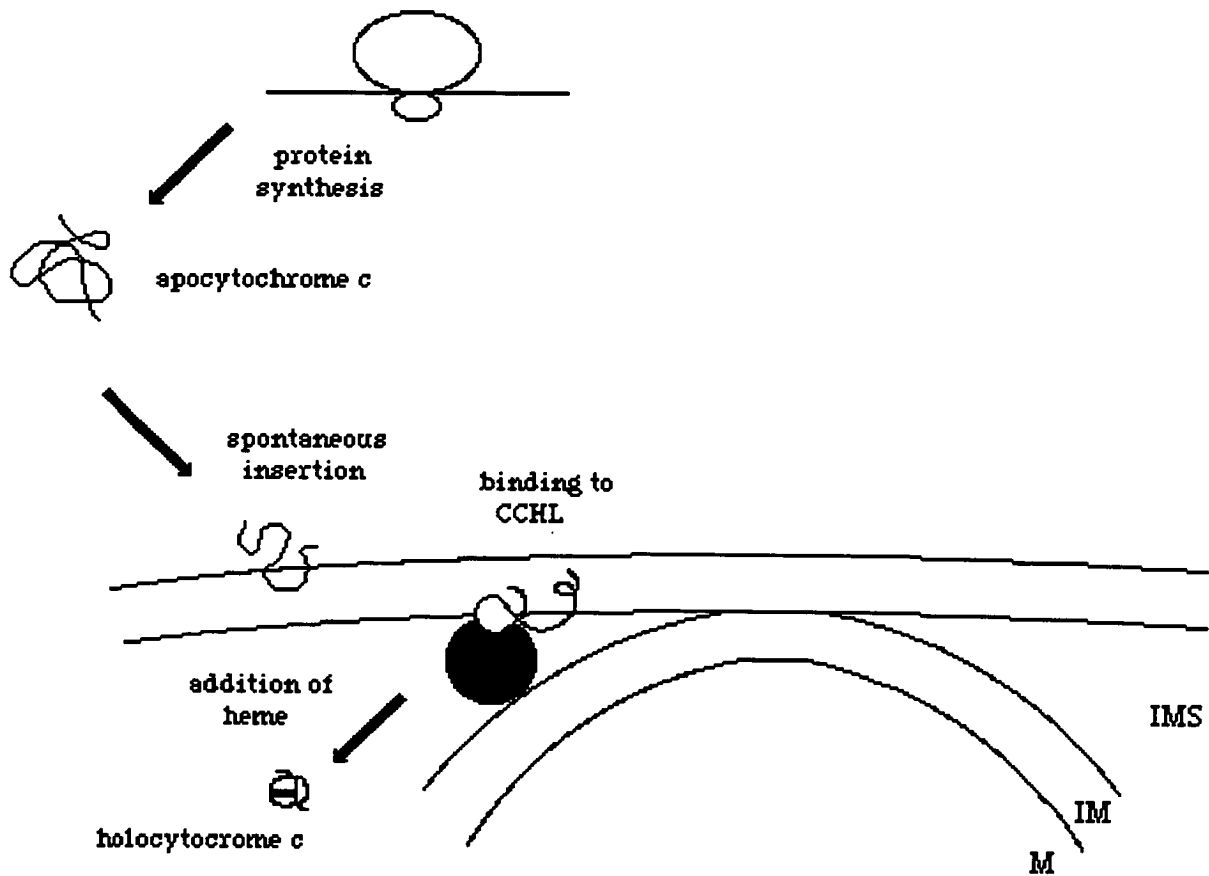


Figure 1.3; Proposed import pathway for cytochrome *c* in *N. crassa*.

OM; outer membrane, IMS; inter membrane space, IM; inner membrane, M; matrix, CCHL; cytochrome *c* heme lyase. Adapted from Stuart *et al.* (1990).

1.4. Protein import into plant mitochondria.

So far there have been few reports in the literature investigating protein import into plant mitochondria. Such studies have been hampered primarily by the lack of plant nuclear-encoded mitochondrial genes and cDNA clones. The few reports that do exist, however, indicate that the mechanism of protein import into plant mitochondria is similar to that of *S. cerevisiae* and *N. crassa*.

Cells from green plant tissue contain both chloroplasts and mitochondria. The biogenesis of these two organelles is very similar, since both contain their own DNA. In both cases, this encodes only a limited number of organellar proteins. The remainder must therefore be imported from the cytosol. It is envisaged that protein targeting is specific ~~but it is still unclear if this is indeed the case~~. The section below will therefore attempt to summarise the progress made to date concerning protein import into plant mitochondria.

1.4.1. Protein import *in vivo*.

Only four reports have demonstrated protein import into plant mitochondria *in vivo* (Boutry *et al.*, 1987; Schmitz and Lonsdale, 1989; Hemon *et al.*, 1990; Huang *et al.*, 1990). These investigations, however, have examined the import of chimaeric proteins, rather than authentic nuclear-encoded mitochondrial proteins. In two cases, the presequence of the β -subunit of the F₁-ATP synthase from *Nicotiana plumbaginifolia* (Boutry and Chua, 1985) was fused to a non-mitochondrial passenger protein (Boutry *et al.*, 1987; Hemon, 1990). Schmitz and Lonsdale (1989) used the N-terminal presequence from a yeast nuclear-encoded mitochondrial protein. These preliminary investigations do suggest that targeting is specific, since no mis-routing of these passenger proteins occurs. Huang *et al.* (1990), however, have since demonstrated that the presequence of cytochrome *c* oxidase subunit Va from yeast could target chloramphenicol acetyltransferase into both mitochondria and chloroplasts.

1.4.2. Protein import *in vitro*.

A limited number of experiments have examined protein import *in vitro*. Plant mitochondria have been isolated from a variety of species including *Zea mays* (White and Scandalios, 1987), *Vicia faba* (Whelan *et al.*, 1988), and *Spinacia oleracea* (Whelan *et al.*, 1990). When available, proteins have been synthesised

directly from the cDNA clone. Otherwise antibodies have been used, after the import reaction, to immunoprecipitate the desired protein from poly A⁺ RNA translation products. These experiments have indicated that import into plant mitochondria is dependent on the presence of ATP and an energised inner mitochondrial membrane. The mechanism of protein import into plant mitochondria therefore appears to be similar to that in *S. cerevisiae* and *N. crassa*.

Experiments have also been performed to address the question of specificity. Mitochondria and chloroplasts have been isolated from *S. oleracea* leaf tissue and attempts made to import a variety of mitochondrial proteins. Results suggest that targeting is specific, since the β -subunit of the F₁-ATP synthase from both *N. plumbaginifolia* and *N. crassa* were imported only into isolated *S. oleracea* leaf mitochondria and not into the intact chloroplasts (Whelan *et al.*, 1990). Subsequent experiments, however, indicate that a chloroplast extract can process both mitochondrial and chloroplast precursors (Whelan *et al.*, 1991). The significance of this remains unclear.

1.4.3. Mechanism of protein import.

As indicated in section 1.4.2, the mechanism of protein import into plant mitochondria appears to be similar to that in *S. cerevisiae* and *N. crassa*. The results by Schmitz and Lonsdale (1989) suggested that the mechanism is highly conserved between plants and yeast, since a yeast presequence can target a non-mitochondrial protein into plant mitochondria *in vivo*. Huang *et al.* (1990), however, demonstrated that another yeast presequence targeted the passenger protein chloramphenicol acetyltransferase to the chloroplast, as well as to mitochondria. It therefore remains unclear whether yeast presequences are specific in targeting proteins to plant mitochondria. Results from Bowler *et al.* (1989a), however, indicate that the manganese superoxide dismutase from *N. plumbaginifolia* was imported into yeast mitochondria *in vivo*. This suggests that the mitochondrial import mechanisms of yeast and plants are similar.

The energy requirements for protein import into plant mitochondria appear similar to that in *S. cerevisiae* and *N. crassa*, since dissipation of the mitochondrial inner membrane potential by the addition of the potassium ionophore valinomycin and removal of ATP from the import reaction by apyrase inhibit import *in vitro* (Whelan *et al.*, 1988). Both of these requirements are necessary for mitochondrial protein import in *S. cerevisiae* and *N. crassa* (see section 1.3.5).

1.4.4. Specificity of targeting.

From the limited results described above it appears that proteins are specifically imported into mitochondria and chloroplasts. It should be noted, however, that the transit peptide of the Rubisco small subunit (SSU) from *Chlamydomonas reinhardtii*, could direct a passenger protein into isolated yeast mitochondria (Hurt *et al.*, 1986). The complete SSU from *Pisum sativum* can also be imported into isolated *N. crassa* mitochondria (Pfaller *et al.*, 1989). Import of these two nuclear-encoded chloroplast proteins, however, occurred at low efficiency. To confirm that protein import is specific, further investigations will be required, both *in vivo* and *in vitro*, with a wide range of mitochondrial and chloroplast proteins.

As with *S. cerevisiae* and *N. crassa*, plant nuclear-encoded proteins are synthesised with N-terminal extensions, or presequences, which are cleaved upon import. Deletion analysis of the *Z. mays* MnSOD presequence indicates that the targeting information required for mitochondrial import resides within the presequence (White and Scandalios, 1989). Some plant proteins, however, such as the cytochrome *c* oxidase Vc subunit from *Ipomoea batatas* are synthesised without an N-terminal extension and there is therefore no processing upon import (Nakagawa *et al.*, 1987). Interestingly, the N-terminal region of this protein is predicted to form an amphiphilic α -helix. Such structures are thought to exist in the presequences of *S. cerevisiae* and *N. crassa* mitochondrial proteins (section 1.3.3). The lack of cDNA clones and genes of imported plant mitochondrial proteins, however, has prevented similar analyses. The existence of such secondary and tertiary structures within plant mitochondrial presequences still remains unknown.

1.5. Protein import into chloroplasts.

1.5.1. Targeting.

As mentioned in section 1.4, chloroplasts are also dependent upon the import of proteins from the cytosol (for reviews see Schmidt and Mishkind, 1986; Keegstra and Bauerle, 1988; Keegstra *et al.*, 1989; Smeekens *et al.*, 1990). As with mitochondria, these proteins are synthesised as larger precursor proteins with N-terminal extensions, termed transit peptides, which are processed upon import. These function to target the protein to the chloroplast, since removing the

transit peptide abolishes import (Mishkind *et al.*, 1985). Gene fusion experiments also confirm the targeting function of chloroplast transit peptides, since they can direct foreign proteins into the organelle both *in vitro* and *in vivo* (Schreier *et al.*, 1985; Van den Broek *et al.*, 1985; Smeekens *et al.*, 1987; Meadows *et al.*, 1989).

As with mitochondrial presequences, chloroplast transit peptides lack any primary sequence homology. It is therefore assumed that secondary and/or tertiary structures are important for targeting. Comparisons of the primary sequences, however, indicate that transit peptides share a number of common features. They are rich in the hydroxylated amino acids, serine and threonine, which form 20 - 35 % of the total, whilst negatively charged residues are generally absent. Transit peptides appear to have a net positive charge (Keegstra *et al.*, 1989; von Heijne *et al.*, 1989).

Early comparisons of chloroplast transit peptides indicated three blocks of amino acid homology. These were thought to form a structural framework, which was important in the targeting, translocation or processing of the transit peptide (Karlín-Neumann and Tobin, 1986). This comparison, however, was based on a limited number of transit peptides. A more recent analysis indicates that such homology blocks do not exist (von Heijne *et al.*, 1989). As discussed previously, mitochondrial presequences have been predicted to form amphiphilic α -helices (section 1.3.3). Such structures are thought to be absent from chloroplast transit peptides, but some are predicted to form amphiphilic β -sheets (von Heijne *et al.*, 1989).

1.5.2. **Sorting.**

Unlike the mitochondrion, the chloroplast contains a third membrane-bound compartment, the thylakoid lumen. Proteins destined for the chloroplast must therefore be sorted between the outer and inner envelopes, the thylakoid membrane, and the three compartments, the inter membrane space, the stroma and the thylakoid lumen (for review see Keegstra, 1989). Most work investigating the sorting of proteins, or intra-organellar targeting, has examined those destined for the stroma and the thylakoid lumen. The information for the sorting of proteins appears to reside upon the transit peptide, since exchanging the transit peptide of ferredoxin, a stromal protein, with that of plastocyanin, a thylakoid protein, resulted in the mis-localisation of plastocyanin to the stroma (Smeekens *et al.*, 1986).

The transit peptide of thylakoid lumen proteins appears to be divided into two functionally distinct domains (Hageman *et al.*, 1990). The N-terminal region shows many characteristics of the transit peptides of stromal proteins, whilst the C-terminal half resembles bacterial signal peptides (Smeekens and Weisbeek, 1988). It is proposed that this N-terminal domain functions to target thylakoid proteins to the stroma where it is processed by the stromal processing peptidase (Smeekens *et al.*, 1986). The remaining C-terminal domain of the transit peptide then targets the intermediate into the thylakoid lumen, where it is then processed by a second protease to yield the mature protein (Kirwin *et al.*, 1988).

The targeting of proteins to the chloroplast envelope is less understood. ~~When the transit peptide of the light harvesting chlorophyll *a/b* binding protein (CAB) is replaced with that from SSU, CAB is still imported and correctly located to the outer envelope. The information required for the sorting of this protein, therefore appears to reside within the mature protein (Smeekens *et al.*, 1990).~~

1.5.3. Processing.

As indicated above, imported chloroplast proteins are processed by the stromal processing peptidase. This chelator sensitive stromal peptidase was first described by Robinson and Ellis (1984). The maturation of thylakoid lumen proteins, however, requires the action of a second peptidase (Hageman *et al.*, 1986). This appears to be associated with the lumen side of the thylakoid membrane (Kirwin *et al.*, 1988) and unlike the stromal processing peptidase, its activity is not dependent upon metal ions (Kirwin *et al.*, 1987). It is unclear whether this is the only thylakoid processing peptidase, since the activity described by Kirwin *et al.* (1988) is specific only for plastocyanin.

1.5.4. Receptors.

How proteins are distinguished by the chloroplast remains unclear. Proteinaceous structures on the outer envelope membrane are thought to be involved, since treatment of the chloroplast with proteinase inhibits import (Cline *et al.*, 1985). By decreasing the levels of ATP or temperature the translocation of proteins across the outer envelope can be inhibited. This has enabled the number of chloroplast receptor binding sites to be estimated. Investigations with SSU indicate that these may vary from 1500 to 3500 per chloroplast (Friedman and Keegstra, 1989).

So far, three proteins have been implicated as putative chloroplast receptor proteins. A 66 kDa envelope protein was identified through cross-linking experiments, which bound the SSU precursor (Cornwell and Keegstra, 1987). A 30 kDa protein was identified through the binding of anti-idiotypic antibodies. These were generated from antibodies recognising the SSU transit peptide (Pain *et al.*, 1988). Lastly, a 51 kDa protein, P51, has been isolated, the phosphorylation of which correlated with the import of the SSU precursor (Hinz and Flugge, 1988).

Recently, Schnell *et al.* (1990), used the same anti-idiotypic antibodies as Pain *et al.* (1988), to purify the chloroplast import receptor protein and the corresponding cDNA from *P. sativum*. The nucleotide sequence encoded a protein, p36, with a calculated molecular weight of 36 kDa and was found to have homology with the phosphate translocator. The authors propose that p36 is unlikely to be the phosphate translocator, since this would be located within the inner envelope membrane. Such a dual functioning of a protein, however, is not unknown and has in fact been found for PEP, the mitochondrial matrix processing enhancing peptidase (section 1.3.8). Interestingly, a 30 kDa inner envelope protein was photolabelled with the wheat SSU transit peptide. This was proposed to be the phosphate translocator, since it was the major protein of the inner membrane (Kaderbhai *et al.*, 1988).

1.5.5. Energy requirements.

Protein import into chloroplasts requires only ATP (Theg *et al.*, 1989). This is in contrast to mitochondrial protein import, which requires both ATP and an energised inner membrane (section 1.3.5). Unlike protein import into mitochondria, this requirement for ATP is specific, since it can not be replaced by CTP, GTP or UTP. The binding of precursors to the chloroplast envelope also requires ATP, although at much lower concentrations and, unlike the requirement for import, ATP can be substituted by other nucleotide triphosphates (Olsen *et al.*, 1989). The site of ATP utilisation for precursor binding and import appears to be within the chloroplast (Olsen *et al.*, 1989; Theg *et al.*, 1989).

The role of ATP in chloroplast protein import is unknown. Mitochondrial precursors must be in an unfolded configuration before import can occur and ATP is thought to mediate this process (section 1.3.5). It seems unlikely that the chloroplast requirement for ATP is prior to import, since the site of ATP utilisation is within the chloroplast. One hypothesis proposes that ATP is utilised to phosphorylate components of the translocation machinery or the precursor itself

(Keegstra *et al.*, 1989). The correlation between the import of the SSU precursor and the phosphorylation of the putative receptor P51 provides evidence for this hypothesis (Hinz and Flugge, 1988).

1.5.6. Specificity of targeting.

It is still unclear how chloroplast and mitochondrial nuclear-encoded proteins are distinguished by these two organelles (see section 1.4.4). Only one group has attempted to address this question *in vivo*, the results of which indicate that targeting is specific (Boutry *et al.*, 1987). Similar results have been obtained *in vitro*, but again, this is based on a single report (Whelan *et al.*, 1990).

A number of mechanisms may exist to confer organellar specificity to chloroplast and mitochondrial precursor proteins. The presence of specific receptor proteins on the outer membranes of the chloroplast and mitochondria could determine which proteins bind to the organelle. Such receptors would therefore act as a stringent control at a very early step along the import pathway. The presence of such receptor proteins on both mitochondrial and chloroplast membranes has already been discussed (sections 1.3.4 and 1.5.4).

Another important observation is that the energy requirements for chloroplast and mitochondrial protein import are distinctly different (sections 1.3.5 and 1.5.5). This may be important in controlling which precursors may enter the organelle. Structural predictions of both chloroplast transit peptides and mitochondrial presequences are also different. Rather than the predicted amphiphilic α -helices of mitochondrial presequences, some chloroplast transit peptides may contain amphiphilic β -sheets (section 1.5.1). It has also been noted that chloroplast transit peptides are generally longer than mitochondrial presequences (Franzen *et al.*, 1990).

The importance of these differences and the role each may play in determining specificity remains unclear. As discussed in section 1.4.4, further investigations are required to determine whether or not protein import into chloroplasts and mitochondria is specific and if so how this specificity is brought about.

1.6. Summary.

This chapter has examined in detail the structure, function and biogenesis of the mitochondrion. The importance of protein import was discussed and our

knowledge of the mechanisms involved was reviewed (section 1.3). This, however, is limited primarily to *S. cerevisiae* and *N. crassa* in which numerous nuclear-encoded genes and cDNAs encoding mitochondrial proteins have been isolated. In contrast, similar studies with plant mitochondria have been very limited, but the few reports that do exist were discussed in section 1.4. The remaining chapters describe the development, characterisation and partial optimisation of a plant mitochondrial *in vitro* import system, which has enabled the import of plant nuclear-encoded mitochondrial proteins to be investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1. Biological materials.

2.1.1. Plant materials.

Zea mays L. seed, genotype 3377, fertile (N-type) cytoplasm, was supplied by Pioneer Hybrid International Inc., Plant Breeding Division, Johnston, Iowa, USA.

Solanum tuberosum L. var. Desiree, was supplied by the Department of Agriculture and Fisheries for Scotland, East Craigs Official Seed Testing Station, Edinburgh.

2.1.2. *Escherichia coli* strains and genotypes.

JM101 *supE thi Δ(lac-proAB)*

F' [*traD36 proAB⁺ lacI^q lacZΔM15*]

HB101 *supE44 hsdS20(r_B⁻m_B⁻) recA13 ara-14*

proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 (Sambrook *et al.*, 1989)

2.1.3. Bacterial plasmids.

pGEM 1 and pGEM 3 Promega Corporation.

pBS M13+ Stratagene Cloning Systems.

pSOD Gift from C. Bowler, Gent (Bowler *et al.*, 1989a)

pDS₇DHFR Gift from G.A. Reid, Edinburgh.

2.1.4. Adenine nucleotide translocator clones.

2.1.4.1. *ANT-A*, a full-length cDNA clone from *Zea mays*.

A 1136 bp cDNA, pANT-1, encoding a *Z. mays* ANT had been previously isolated (Baker and Leaver, 1985). This encodes an ORF of 954 bp, but was thought to be incomplete, since the cDNA terminated 2 nucleotides 5' to the putative ATG codon. In addition, transcriptional analysis suggested that the *Z. mays* ANT mRNA may be as long as 1600 bp. Subsequent screening of a *Z. mays* (line MUTIND-FR7205034) cDNA library prepared from 3 day old dark-grown coleoptiles with pANT-1 resulted in the isolation of a full-length cDNA, *ANT-A* (Bathgate pers. comm.). This encodes an ORF of 987 bp.

2.1.4.2. *ANT-G1*, a genomic clone from *Zea mays*.

ANT-G1 was isolated from a library prepared from 3 day old dark-grown *Z. mays* (genotype B37, fertile (N-type) cytoplasm) on an 8 kbp *Eco* RI fragment (Bathgate *et al.*, 1989). The full-length cDNA *ANT-A* is homologous to this genomic clone (Bathgate pers. comm.).

2.1.4.3. *PANT-1*, a cDNA clone from *Solanum tuberosum*.

A 1453 bp cDNA, *PANT-1*, was isolated by screening a potato tuber library with *ANT-A* (Winning pers. comm.). This encodes a 1158 bp ORF, but the cDNA is thought to be incomplete, since the ORF is contiguous to the 5' end.

2.2. Miscellaneous.

2.2.1. Chemicals.

These were purchased from BDH Chemicals Ltd. (BDH), SIGMA Chemical Company Ltd. (SIGMA), Boehringer Mannheim GmbH (BM), Pharmacia LKB Biotechnology (Pharmacia) and Difco Laboratories (DIFCO) unless otherwise stated.

2.2.2. Restriction endonucleases and DNA modification enzymes.

All restriction endonucleases were purchased from Bethesda Research Laboratories Life Technologies Inc. (BRL). DNA modification enzymes were purchased from BM, Amersham and Northumbria Biologicals Ltd. (NBL) unless otherwise stated.

2.2.3. Radioisotopes.

[$\alpha^{35}\text{S}$]-dATP, specific activity >37 TBq (>1000 Ci)/mmol, [$\alpha^{32}\text{P}$]-dCTP, specific activity 110 TBq (3000 Ci)/mmol and L-[^{35}S]-methionine, specific activity 39.2 TBq (1060.7 Ci)/mmol were purchased from Amersham and New England Nuclear Research Products (DuPont).

2.2.4. Autoradiography.

Cronex 4 X-ray film was exposed at -70 °C and developed in an Agfa-Gevaert Gevamatc 60 automated developer. The developer and fixer used were Agfa-Gevaert G153 and G353 respectively.

2.2.5. Bacteriological media.

E. coli bacterial and recombinant strains were inoculated into and grown in LB Broth; 10 g/l DIFCO Bactotryptone, 5 g/l DIFCO Bacto Yeast Extract, 10 g/l sodium chloride, pH 7.5 or on LB Agar; LB Broth made 1.5 % (w/v) agar with DIFCO Bacto Agar. Both LB Broth and LB Agar contained 100 µg/ml ampicillin.

2.3. DNA analysis.

2.3.1. Large scale preparation of plasmid DNA from *Escherichia coli*.

Plasmid DNA was isolated from *E. coli* using standard methods (Maniatis *et al.*, 1982). The concentration of DNA in aqueous solution was determined by

measuring the OD at 260 nm. For DNA 1 OD unit is equivalent to 50 µg DNA.

2.3.2. Construction of recombinant plasmid DNA molecules.

Recombinant plasmid DNA molecules and the generation of subclones were constructed using standard molecular biology techniques (Maniatis *et al.*, 1982). In general, the *in vitro* expression vectors pGEM 1 and pGEM 3 were used for the construction of all clones for *in vitro* transcription analysis (see section 2.5.1). The vector pBS M13+, however, was used for the *in vitro* transcription analysis of pFUSION and pINTERNAL (section 4.7 and 4.9 respectively), as well as for nucleotide sequencing (see section 2.3.3). Restriction endonuclease digestion of the DNA was used to determine whether the required recombinant plasmid had been obtained. DNA was fractionated on 0.8 - 2.0 % (w/v) agarose gels.

2.3.3. Nucleotide sequencing of plasmid DNA.

A Sequenase^R Version 2.0 DNA Sequencing Kit was purchased from United States Biochemical Corporation. Plasmid sequencing was performed with 10 µg of caesium chloride purified DNA (Maniatis *et al.*, 1982) and followed the procedure for dideoxynucleotide chain termination (Sanger *et al.*, 1977) as recommended by the manufacturer.

Sequencing reactions contained 5 µCi [$\alpha^{35}\text{S}$]-dATP. Depending on the nucleotide sequence under analysis the DNA was primed with the M13 -20 universal primer; 5' -GTAAAACGACGGCCAGT- 3' or the synthetic oligonucleotides; 5' -GGAAGTACTTGAAC TCG- 3' or 5' -GCACAGAGCGGGGTCTG- 3'. These were purchased from University of Edinburgh Oswell DNA Service.

The sequencing reaction products were separated on 4 mm thick 6 % (w/v) polyacrylamide gels (Maniatis *et al.*, 1982) in 8 M urea and were polymerised with 0.6 % (w/v) ammonium persulphate and 0.078 % (v/v) TEMED. Gels were dried under vacuum onto 3MM Whatman chromatography paper and exposed for 1 - 2 days as described in section 2.2.4.

2.4. RNA analysis.

2.4.1. Isolation of total RNA from *Zea mays* dark-grown coleoptiles.

All manipulations described were performed on ice. Where necessary solutions were sterilised by autoclaving at 15 psi for 20 min. All containers were sterilised by baking at 150 °C overnight.

Z. mays was grown at 28 °C in the dark for 36 h. Coleoptiles were then harvested into liquid nitrogen. The tissue was transferred to a mortar and ground with a pestle until a fine powder was obtained. Liquid nitrogen was added throughout to keep the tissue frozen. Grinding buffer; 6 % (w/v) 4-aminosalicylate, 1 % (w/v) triisopropyl naphthalene sulphonate, 6 % (v/v) phenol equilibrated in 100 mM Tris-HCl pH 7.4, 50 mM Tris-HCl pH 8.4 was then added dropwise and the grinding and addition of liquid nitrogen continued. The ground tissue was then transferred to a 250 ml flask and 1 x volume of phenol/chloroform; 50 % (v/v) phenol equilibrated in 100 mM Tris-HCl pH 7.4, 48 % (v/v) chloroform, 2 % (v/v) isoamyl alcohol, 0.1 % (w/v) 8-hydroxyquinoline was added. This was then allowed to thaw on ice during which time it was mixed occasionally.

When thawed the mixture was transferred to 30 ml Corex tubes, mixed and the phases separated by centrifugation at 5000 rpm for 10 min at 4 °C. The aqueous phase was removed and the phenol/chloroform extraction repeated 3 times. A single extraction with chloroform was then performed. The nucleic acids were recovered by precipitation with 2.5 x volume of ethanol and 0.2 M sodium acetate pH 6.0 at -20 °C overnight followed by centrifugation at 10000 rpm for 10 min at 4 °C. The pellet was then washed in 70 % (v/v) ethanol and dissolved in water (0.5 ml/g starting tissue maximum). The RNA was separated from the DNA by precipitation with 3.0 M sodium acetate pH 6.0 for 2 - 3 h on ice. The RNA was then recovered by centrifugation at 10000 rpm for 10 min at 4 °C and the pellet dissolved in 0.2 - 1 ml of water and reprecipitated with 2.5 x volume of ethanol and 0.2 M sodium acetate pH 6.0. The pellet was then finally dissolved in 0.2 ml of water and the concentration of RNA determined by measuring the OD at 260 nm. For RNA 1 OD unit is equivalent to 40 µg RNA (Maniatis *et al.*, 1982).

2.4.2. Isolation of messenger (poly A⁺) RNA.

Poly A⁺ was selected from total RNA using an oligo(dT)-cellulose column (Maniatis *et al.*, 1982)

2.5. *In vitro* expression of cDNAs and recombinant clones.

2.5.1. *In vitro* transcription.

In general, the expression vectors pGEM 1 and pGEM 3 were used for the transcription of all cloned cDNAs or generated constructs and subclones. The vector pBS M13+, however, was used for the *in vitro* transcription of pFUSION and pINTERNAL (see section 2.3.2). All constructs were orientated to allow transcription from the T7 promoter. The plasmid pSOD, however, required SP6 RNA polymerase for transcription. All plasmids were linearised with an appropriate restriction endonuclease to enable run off transcription.

Transcription reactions were performed with 2 µg linearised DNA template in transcription buffer; 0.01 % (w/v) BSA, 40 mM HEPES pH 7.5, 10 mM DTT, 6 mM magnesium acetate, 2 mM spermidine, 0.5 mM ATP, CTP and TTP and 0.1 mM GTP, to which 250 µM CAP (m⁷G(5')ppp(5')G), 20 U RNAGuard (RNase inhibitor) and 20 U RNA polymerase (T7 or SP6) were added. Reactions were performed in volumes of 20 µl, incubated at 40 °C for 30 min, after which 1 µl of 10 mM GTP was added and the incubation continued for a further 30 min. The products of the *in vitro* transcription reaction were then used immediately or stored at -70 °C until required.

2.5.2. *In vitro* translation systems.

2.5.2.1. Rabbit reticulocyte lysate.

A nuclease treated/message dependent rabbit reticulocyte lysate was purchased from Amersham. Translations were performed in 15 µl volumes containing 80 % (v/v) lysate, 6.7 % (v/v) *in vitro* transcription reaction (see section 2.5.1), 0.37 MBq L-[³⁵S]-methionine at 30 °C for 1 h.

2.5.2.2. Wheat germ S-30 system.

A cell-free wheat germ *in vitro* translation system was prepared according to Weir (1980). The dialysis, however, was against HEPES buffer; 120 mM potassium acetate, 20 mM HEPES pH 7.6, 5 mM magnesium acetate, 1 mM DTT, rather than grinding buffer. Translations were performed in 50 μ l volumes containing 20 % (v/v) wheat germ S-30, 0.37 MBq L-[³⁵S]-methionine, 1 μ g. poly A⁺ RNA, 20 U RNAGuard (RNase inhibitor) and wheat germ premix; 100 mM potassium acetate, 20 mM HEPES pH 7.6, 8 mM creatine phosphate, 1.8 mM DTT, 1.25 mM magnesium acetate, 1 mM ATP, 250 μ M spermidine, 50 μ M GTP, 25 μ M all amino acids except L-methionine, 20 μ g/ml creatine phosphokinase (in 50 % glycerol). The translation reaction had been optimised for potassium and magnesium ion concentrations (the final concentrations of which were 124 mM and 2.25 mM respectively). Incubations were at 25 °C for 1 h.

2.5.3. Determination of percentage incorporation of L-[³⁵S]-methionine.

The incorporation of L-[³⁵S]-methionine was estimated from trichloroacetic acid (TCA) precipitable protein. Duplicate 1 μ l aliquots of the translation reaction was spotted onto 3MM Whatmann chromatography paper and air dried. The unincorporated L-[³⁵S]-methionine was then removed by washing in cold 10 % (w/v) TCA for 30 min, followed by 5 % (w/v) TCA at 90 °C for 15 min and then 4 washes in cold 5 % (w/v) TCA for 5 min. Finally the filter was washed in cold ethanol for 5 min, air dried and counted in 5 ml scintillation fluid (4 g/l Butyl-PBD in toluene). The % incorporation was then calculated as follows;

(Average cpm_{translation} after washing) - (Average cpm_{background} after washing)

divided by (Average cpm translation without washing).

The cpm_{background} was estimated from a control translation reaction containing no

The $\text{cpm}_{\text{background}}$ was estimated from a control translation reaction containing no RNA.

2.6. Import of radiolabelled proteins into isolated plant mitochondria.

2.6.1. Isolation of mitochondria.

2.6.1.1. From *Zea mays*.

Z. mays was grown in the dark at 28 °C for 72 h unless otherwise stated. Mitochondria were isolated from coleoptiles according to standard laboratory methods (Leaver *et al.*, 1983; Purdue, 1988). A 26 % (v/v) continuous Percoll gradient (in resuspension buffer; 0.4 M mannitol, 10 mM tricine pH 7.2, 1 mM EGTA pH 7.2), was used to separate mitochondria from the contaminating plastids, by centrifugation at 18000 rpm for 90 min at 4 °C. The lower mitochondrial band was removed and the volume made up to 60 ml with resuspension buffer. The purified mitochondria were then recovered by centrifugation at 10000 rpm for 10 min at 4 °C, after which the supernatant was removed by suction through a sterile pasteur pipette attached to a water vacuum line. This wash process was repeated and the final mitochondrial pellet resuspended in 200 - 500 μl resuspension buffer.

2.6.1.2. From *Solanum tuberosum*.

Mitochondria were isolated from tubers which were peeled, washed and homogenised using a Moulinex juice extractor (all manipulations being performed rapidly and at 4 °C. The juice was collected into grinding buffer; 0.4 M mannitol, 25 mM MOPS, 8 mM L-cysteine, 1 mM EGTA, 0.1 % BSA, pH 10 rather than pH 7.8, (5 ml/g starting tissue) which was stirred continuously. After homogenisation was complete the pH was checked and if necessary brought to pH 7.2 with potassium hydroxide. The homogenised tissue was then filtered through 8 x layers of muslin and the standard procedure for the isolation of mitochondria then followed (see section 2.6.1.1).

2.6.2. Determination of protein concentration.

Total mitochondrial protein was quantified by a method based on Bradford (1976). Protein solutions to be assayed were made up to a volume of 0.1 ml with water and 5 ml of reagent; 10 % (v/v) phosphoric acid, 5 % (v/v) ethanol, 0.01 % (w/v) Coomassie brilliant blue G-250 added. After 2 min, the OD at 595 nm was measured. BSA (0 - 50 µg) standards were used to determine a calibration curve.

2.6.3. Import into mitochondria.

Generally 2 x import reactions were performed with 100 µg total mitochondrial protein (estimated according to section 2.6.2) in volumes of 500 µl. Mitochondria were incubated in import buffer; 181 mM mannitol, 90 mM potassium chloride, 10 mM tricine pH 7.2, 9.8 mM magnesium chloride, 4.8 mM potassium phosphate pH 7.2, 0.8 mM EGTA pH 7.2 (final pH 7.2), with 10 mM ATP, 2.5 mM L-methionine, 2 mM DTT, 1 mM GTP and 2 - 4 % (v/v) translation products (see section 2.5.2.1) at 25 °C for 30 min.

Import was characterised by the protection of the internalised protein to exogenous proteinase K. The reaction was therefore divided into two and one half treated with 100 µg/ml proteinase K for 30 min on ice. PMSF (dissolved in ethanol) was then added to 1 mM and the incubation continued for another 10 min. To the second 250 µl volume PMSF was immediately added to 1 mM and incubated on ice for 10 min.

To confirm that import had occurred, a completed 1 x import reaction was incubated with 0.1 % (v/v) Triton X-100 (purchased from Intertechnique Ltd.) and 100 µg/ml proteinase K for 30 min on ice. PMSF was then added to 1 mM and the incubation continued on ice for another 10 min. The addition of Triton X-100 solubilises the mitochondrial membranes, therefore any imported protein would now be susceptible to digestion by proteinase K.

Mitochondria were then recovered by centrifugation through a 20 % (w/v) sucrose, 10 mM Tris-HCl pH 7.2 cushion (in 1.5 ml Eppendorf tubes) for 5 min at 4 °C. The pellet was resuspended in 4 x sample buffer; 240 mM Tris-HCl pH 6.8, 30 % (w/v) glycerol, 4 % (w/v) SDS, 0.02 % (w/v) bromophenol blue, which had

been diluted 1 : 4 with resuspension buffer and DTT was added to 10 mM.

As a further control, a volume of translation products equivalent to that added to a 1 x import reaction was incubated with 100 µg/ml proteinase K. This was performed in 40 µl volumes, the final volume made up with import buffer. After 30 min on ice PMSF was added to 1 mM and the incubation continued for another 10 min. A 1 : 4 dilution of sample buffer was then added followed by DTT to 10 mM. In the absence of mitochondria the radiolabelled precursor should be completely digested. This control should confirm that the presence of a proteinase K resistant protein in an import reaction is due to import, since mitochondria had been incubated with an equivalent amount of proteinase K.

Samples were then fractionated by SDS/PAGE (see section 2.6.3.3). 10 % of the translation products added to the import reaction was also run as a control.

2.6.3.1. Inhibition of the matrix protease.

The mitochondrial matrix protease was inhibited by the metal ion chelator 1,10-phenanthroline. Import reactions were performed according to section 2.6.3, but included up to 40 mM 1,10-phenanthroline (dissolved in DMSO).

2.6.3.2. Inhibition of the membrane potential.

The mitochondrial membrane potential ($\Delta\Psi$) was dissipated by the addition of the potassium ion ionophore valinomycin. Import reactions were performed according to section 2.6.3, but included up to 90 µM valinomycin (dissolved in ethanol).

2.6.3.3. Fractionation of proteins by SDS/polyacrylamide gel electrophoresis.

Proteins were fractionated by electrophoresis on 14 % (w/v) SDS/polyacrylamide (PAGE) gels (standard laboratory methods). The apparent MW of the radiolabelled proteins was estimated by electrophoresis of the following marker proteins; BSA (66 kDa), catalase (57.4 kDa), aldolase (39 kDa), carbonic anhydrase



(29 kDa), trypsin inhibitor (20 kDa), myoglobin (17.1 kDa) and cytochrome *c* (12.4 kDa). Before loading, proteins were denatured by incubating at 100 °C for 3 min (DTT was previously added to 10 mM). After staining gels with 45 % (v/v) methanol, 8 % (v/v) glacial acetic acid, 0.2 % (w/v) Coomassie brilliant blue R250 and destaining in the same solution (without the addition of Coomassie brilliant blue R250), the gels were dried onto 3MM Whatman chromatography paper and exposed for 2 - 4 weeks as described in section 2.2.4.

2.7. Immunoprecipitation.

Poly A⁺ RNA was used to programme the wheat germ S-30 system (2.5.2.2). After determining the incorporation of L-[³⁵S]-methionine (2.5.3) the required amount of translation was made up to 125 µl with wheat germ premix. SDS was then added to 3 % (w/v) and any complexes were dissociated by boiling for 5 min. The reaction was then transferred to a Beckman thick walled polycarbonate tube (13 x 51 mm), diluted with 2.4 ml of TNET; 1 % (v/v) Triton X-100, 150 mM sodium chloride, 50 mM Tris-HCl pH 7.4, 5 mM EDTA pH 8.0, 2 mM PMSF and centrifuged at 100000 rpm in a Beckman TL-100 Tabletop ultracentrifuge for 10 min at 4 °C.

The supernatant was then removed and mixed gently for 12 - 16 h at 4 °C with the required amount of antiserum. To collect the precipitate a 0.1 mg/ml suspension of Protein A sepharose was swollen in TNET by mixing at 4 °C for 1 h. A 50 µl aliquot was then added to the precipitation and mixing was continued. After 1 h the precipitate was recovered by centrifugation for 30 sec in a microcentrifuge. The pellet was washed 5 x in TNET and resuspended in sample loading buffer; 50 mM sodium carbonate, 12 % (w/v) sucrose, 2 % (w/v) SDS, 0.04 % (w/v) bromophenol blue. The sample was then incubated at 37 °C for 5 min, after which DTT was added to 10 mM. The Protein A sepharose was then removed by centrifugation in a microfuge for 10 sec and the supernatant was fractionated by SDS/PAGE (see section 2.6.3.4).

2.8. Computer analysis.

The University of Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Software Package Version 7.0, was used to determine the calculated molecular weight and predict the tertiary structure of amino acid sequences using the programmes of Devereux *et al.* (1984)

CHAPTER 3

CHARACTERISATION OF A ZEA-MAYS MITOCHONDRIAL PROTEIN IMPORT SYSTEM

3.1. Introduction.

Mitochondrial protein import has been studied extensively in the yeast *Saccharomyces cerevisiae* and the filamentous fungus *Neurospora crassa* and over the last 15 years the mechanisms involved have been well characterised (see section 1.3). Several studies with animal mitochondria have also been reported. In contrast, very little is understood about protein import into plant mitochondria and the processes involved. Such investigations have been hampered by the lack of isolated cDNAs and genes for nuclear-encoded mitochondrial proteins.

In 1987, only two such cDNAs had been isolated and characterised; the β -subunit of the F_0F_1 -ATP synthase from *Nicotiana plumbaginifolia* (Boutry and Chua, 1985) and a putative full-length cDNA clone encoding the adenine nucleotide translocator (ANT) from *Zea mays* (Baker and Leaver, 1985). This list has now grown and includes the manganese superoxide dismutase (MnSOD) from *N. plumbaginifolia* (Bowler *et al.*, 1989b) and *Z. mays* (White and Scandalios, 1988), the δ -subunit of the F_0F_1 -ATP synthase from *Ipomoea batatas* (Kimura *et al.*, 1990) and the β -subunit of the F_0F_1 -ATP synthase from *Z. mays* (Winning *et al.*, 1990) to name a few.

With the development of plant mitochondrial *in vitro* protein import systems, this ever increasing number of cDNAs and genes should enable the mechanisms of plant mitochondrial protein import to be elucidated and characterised. The establishment of such systems will also allow important questions to be addressed, such as how proteins are sorted between different cellular compartments. This is of particular interest, since the cells from green plant tissue contain chloroplasts, which as described in section 1.5.1, are also dependent upon the import of cytoplasmically synthesised precursor proteins for their biogenesis.

Preliminary studies by Purdue (1988) and Whelan (1988) have demonstrated that protein import into isolated higher plant mitochondria is possible. The work presented in this chapter follows on from that of Purdue (1988) and undertakes to characterise a *Z. mays* mitochondrial *in vitro* protein

import system. This system is then utilised to investigate the import of the *Z. mays* adenine nucleotide translocator (Chapter 4).

3.2. Isolation of *Zea mays* mitochondria.

Mitochondria were isolated from dark-grown *Z. mays* coleoptiles according to Purdue (1988) using a 26 % (v/v) Percoll gradient (section 2.6.1.1). Mitochondria isolated in this way were suitable for import studies. They were typically 95 % intact as measured by CN⁻-sensitive oxygen consumption after addition of exogenous reduced cytochrome *c* (Douce, 1985). They were also coupled, with an estimated P/O ratio of 2.3 using the respiratory substrate malate, the theoretical maximum being 3.0 (Purdue, 1988).

3.3. A cDNA encoding the MnSOD from *Nicotiana plumbaginifolia*.

This was kindly provided by C. Bowler, Gent. The 930 bp *Hpa* I - *Pst* I fragment from the 966 bp cDNA (Bowler *et al.*, 1989a and Figure 3.1A) had been isolated and cloned into the *in vitro* expression vector pGEM2 digested with *Sma* I and *Pst* I. This allowed transcription of the cDNA from the SP6 promoter and the resulting construct had been called pSOD (Bowler pers. comm. and Figure 3.1B). To enable run-off transcription pSOD was linearised at the unique *Pst* I restriction endonuclease recognition site.

3.4. The *in vitro* expression and import of MnSOD.

The majority of nuclear-encoded mitochondrial proteins are synthesised as precursor proteins with N-terminal extensions, or presequences, which are processed on or during import by the mitochondrial matrix processing protease. The *N. plumbaginifolia* MnSOD (MnSOD) is one such protein and contains a presequence of 24 amino acid residues (Bowler *et al.*, 1989b and Figure 3.1A). MnSOD was used, since it was readily labelled in the rabbit reticulocyte lysate cell-free translation system employed. The processing of this protein to one with lower molecular weight and the resistance of this mature form to exogenous protease allowed import to be readily characterised. All eukaryotic MnSODs are located in the matrix, however, the sub-mitochondrial location of this plant MnSOD still remains uncertain (Bowler *et al.*, 1989b).

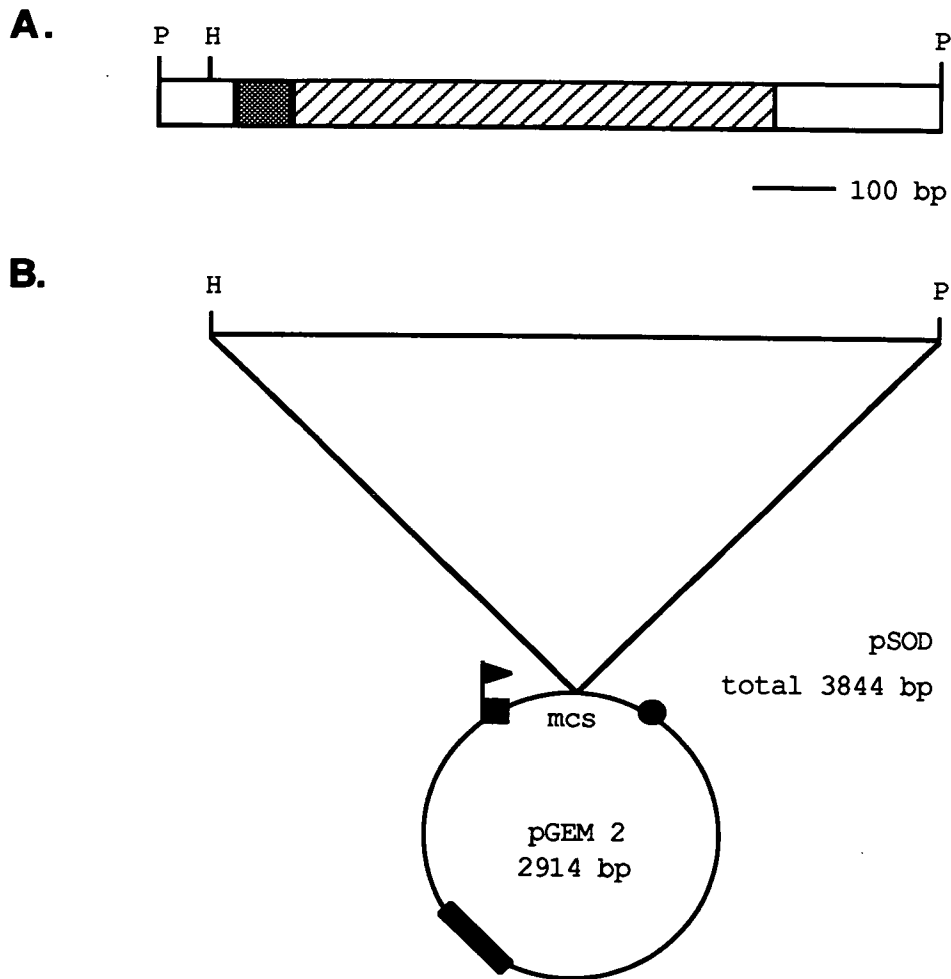


Figure 3.1; The physical map of the 966 bp cDNA encoding the MnSOD from *N. plumbaginifolia* (A) and the construction of pSOD (B).

A. The 5' and 3' non-coding regions are represented by the white rectangles and the sequences encoding the presequence and the mature protein are represented by the stippled and the hatched rectangles respectively. The restriction endonuclease recognition sites are marked H; *Hpa*I and P; *Pst*I.

B. The 930 bp *Hpa*I - *Pst*I fragment was cloned into pGEM 2 allowing transcription from the SP6 promoter, represented by the black square (the direction of transcription by the arrow). The origin of replication is represented by the black circle, the ampicillin gene by the black rectangle and the multiple cloning site by mcs (Bowler *et al.*, 1989a and Bowler pers. comm.).

To investigate the import of MnSOD, RNA generated by the *in vitro* transcription of pSOD was used to programme a rabbit reticulocyte lysate cell-free translation system (section 2.5.2.1). The pSOD translation products were then incubated with *Z. mays* mitochondria isolated from 3 day old dark-grown coleoptiles at 25 °C for 30 min. A 2 x import reaction was performed with 100 µg total mitochondrial protein as described in section 2.6.3, the protein concentration being estimated according to section 2.6.2. After the incubation was complete the import reaction was divided and one half treated with 100 µg/ml proteinase K.

As a control, a volume of pSOD translation products equivalent to that added to a single (1 x) import reaction was also treated with 100 µg/ml proteinase K. This should completely digest the translation products and would confirm that any proteinase K resistant protein associated with mitochondria was due to import. As a further control, a 1 x import reaction was performed and then treated with proteinase K and 0.1 % (v/v) Triton X-100. This should result in the complete digestion of the previously protected protein due to the disruption of mitochondria by the detergent. Proteins were then fractionated by SDS/PAGE and pSOD translation products equivalent to 10 % of that added to a 1 x import reaction were run as a control. The autoradiograph of the gel is shown in Figure 3.2.

Results show that the *in vitro* translation of MnSOD generates a precursor protein with an estimated molecular weight of 26 kDa (lane 1), which is processed upon import into isolated *Z. mays* mitochondria (lane 3). The processed protein runs with an estimated molecular weight of 24.5 kDa, which compares well with that of 24 kDa previously estimated by Bauw *et al.* (1987) and the calculated molecular weight of 22.8 kDa (Bowler *et al.*, 1989b). The mature MnSOD is imported, since it remains resistant to exogenous proteinase K (lane 4) and on treatment with proteinase K and Triton X-100 the protein is completely digested confirming that import has occurred (lane 5). A volume of pSOD translation products equivalent to that added to a 1 x import reaction is also completely digested by the same amount of proteinase K again indicating that MnSOD has been imported into isolated *Z. mays* mitochondria (lane 2).

3.5. The import of MnSOD into *Zea mays* mitochondria isolated from different aged tissue.

The results from Figure 3.2 demonstrated that mitochondria could be successfully isolated from *Z. mays*, which were capable of importing a nuclear-

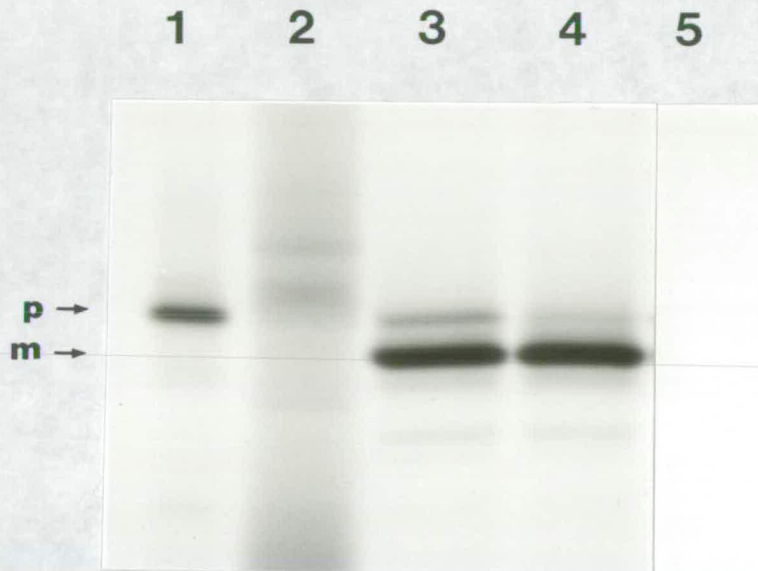


Figure 3.2; Autoradiograph, after SDS/PAGE, showing the *in vitro* import of MnSOD into *Z. mays* mitochondria.

A 2 x import reaction was performed according to section 2.6.3 with 100 μg total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. The incubation was at 25 $^{\circ}\text{C}$ for 30 min with 10 μl of pSOD rabbit reticulocyte lysate translation products. The reaction was then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 2; pSOD translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 3; pSOD translation products associated with mitochondria.
- Lane 4; pSOD translation products associated with mitochondria after treatment with proteinase K.
- Lane 5; pSOD translation products associated with mitochondria after treatment with proteinase K and Triton X-100.

encoded mitochondrial precursor protein. Characterisation of this plant mitochondrial *in vitro* protein import system could therefore take place. Mitochondria isolated from tissue of different ages may vary in their ability to import precursor proteins. This was therefore investigated by examining the import of MnSOD into mitochondria isolated from 2, 3, and 4 day old dark-grown *Z. mays* coleoptiles. By day 4 the dark-grown *Z. mays* coleoptiles had approximately doubled in length compared to day 2.

pSOD rabbit reticulocyte translation products were incubated at 25 °C for 30 min with *Z. mays* mitochondria isolated from 2, 3 or 4 day old dark-grown coleoptiles. 2 x import reactions were performed with 100 µg total mitochondrial protein as described in section 2.6.3. After the incubation was complete each reaction was divided and one half was treated with 100 µg/ml proteinase K. A volume of pSOD translation products equivalent to that added to a 1 x import reaction was treated with 100 µg/ml proteinase K to confirm the import of MnSOD. Proteins were then fractionated by SDS/PAGE and a volume of pSOD translation products equivalent to 10 % of that added to a 1 x import reaction were run as a control. The autoradiograph of the gel is shown in Figure 3.3.

Results show that MnSOD is processed upon import into *Z. mays* mitochondria isolated from 2, 3 and 4 day old tissue (compare lane 2 with lanes 4, 5 and 6) and the mature MnSOD remains resistant to exogenous proteinase K (lanes 7, 8 and 9). An volume of pSOD translation products equivalent to that added to a 1 x import reaction, however, was completely digested by the same amount of proteinase K indicating that import has occurred (lane 1). There appears to be little difference in the level of import of MnSOD into mitochondria isolated from either 2, 3 or 4 day old dark-grown coleoptiles (compare lanes 7, 8 and 9). Mitochondria isolated from 3 day old dark-grown coleoptiles were therefore used in all subsequent import reactions. These were chosen to simplify harvesting.

3.6. The effect of total mitochondrial protein on the import of MnSOD.

The effect of total mitochondrial protein upon the import of MnSOD was investigated. pSOD translation products were incubated at 25 °C for 30 min with *Z. mays* mitochondria isolated from 3 day old dark-grown coleoptiles. 2 x import reactions were performed with 50, 100, 200 and 400 µg total mitochondrial protein as described in section 2.6.3. After the incubation was complete each



Figure 3.3; Autoradiograph, after SDS/PAGE, showing the *in vitro* import of MnSOD into *Z. mays* mitochondria isolated from 2, 3 and 4 day old dark-grown coleoptiles.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein isolated from 2, 3 and 4 day old dark-grown coleoptiles. Incubations were at 25 °C for 30 min with 10 µl pSOD rabbit reticulocyte lysate translation products. The reactions were then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 2; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 3; blank.
- Lane 4; pSOD translation products associated with mitochondria isolated from 2 day old tissue.
- Lane 5; pSOD translation products associated with mitochondria isolated from 3 day old tissue.
- Lane 6; pSOD translation products associated with mitochondria isolated from 4 day old tissue.
- Lane 7; pSOD translation products associated with mitochondria isolated from 2 day old tissue after treatment with proteinase K.
- Lane 8; pSOD translation products associated with mitochondria isolated from 3 day old tissue after treatment with proteinase K.
- Lane 9; pSOD translation products associated with mitochondria isolated from 4 day old tissue after treatment with proteinase K.

import reaction was divided and one half was treated with 100 µg/ml proteinase K and the proteins fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 3.4.

Results show that with each amount of total *Z. mays* mitochondrial protein added to the reactions MnSOD is processed upon import (compare lane 2 with lanes 3 - 6). In each case, the mature MnSOD remains resistant to exogenous proteinase K (lanes 8 - 11). Comparison of lanes 3 - 6 indicates that import of MnSOD appears to be saturated at 50 µg total mitochondrial protein/1 x import reaction (lane 4). This amount of protein was therefore used in all subsequent import reactions.

3.7. The effect of a wheat germ S-30 on the import of MnSOD.

Import studies with *S. cerevisiae* and *N. crassa* isolated mitochondria have indicated that the import of precursor proteins is not possible when these are synthesised in a wheat germ cell-free translation system (Whelan *et al.*, 1988). Using such a system, however, Whelan *et al.* (1988) managed to achieve protein import into mitochondria isolated from *Vicia faba*. In contrast, precursors synthesised in a wheat germ cell-free translation system could not be imported into isolated *Z. mays* mitochondria under the conditions described here (not shown). The effect of increasing amounts of a wheat germ S-30 on the import of pSOD rabbit reticulocyte translation products was therefore investigated.

pSOD translation products were incubated with *Z. mays* mitochondria at 25 °C for 30 min. 2 x import reactions were performed with 100 µg total mitochondrial protein as described in section 2.6.3 and 0, 50, 100 or 150 µl of a wheat germ S-30 (prepared according to section 2.5.2.2). After the incubation was complete each import reaction was divided and one half was treated with 100 µg/ml proteinase K and the proteins fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 3.5.

Results show that in the absence of the wheat germ S-30, MnSOD is processed upon import (compare lanes 1 and 2) and the mature form remains resistant to exogenous proteinase K (lane 3). When wheat germ S-30 is included in the reaction, however, the processing of MnSOD is inhibited (compare lane 2 and lane 4). This inhibition increases with the amount of wheat germ S-30 included in the reaction (compare lane 2 with lanes 4, 6 and 8). With 150 µl of wheatgerm S-

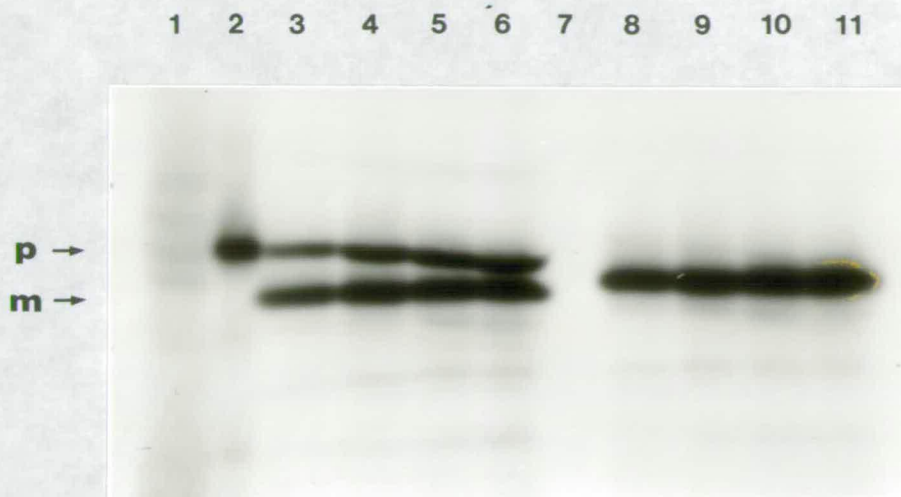


Figure 3.4; Autoradiograph, after SDS/PAGE, showing the effect of total mitochondrial protein on the *in vitro* import of MnSOD.

2 x import reactions were performed according to section 2.6.3 with 50, 100, 200 or 400 μg total *Z. mays* mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were at 25 $^{\circ}\text{C}$ for 30 min with 10 μl of pSOD rabbit reticulocyte lysate translation products. The reactions were then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 2; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 3; pSOD translation products associated with 25 μg total mitochondrial protein.
- Lane 4; pSOD translation products associated with 50 μg total mitochondrial protein.
- Lane 5; pSOD translation products associated with 100 μg total mitochondrial protein.
- Lane 6; pSOD translation products associated with 200 μg total mitochondrial protein.
- Lane 7; blank.
- Lane 8; pSOD translation products associated with 25 μg total mitochondrial protein after treatment with proteinase K.
- Lane 9; pSOD translation products associated with 50 μg total mitochondrial protein after treatment with proteinase K.
- Lane 10; pSOD translation products associated with 100 μg total mitochondrial protein after treatment with proteinase K.
- Lane 11; pSOD translation products associated with 200 μg total mitochondrial protein after treatment with proteinase K.



Figure 3.5; Autoradiograph, after SDS/PAGE, showing the effect of wheat germ S-30 on the *in vitro* import of MnSOD into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were at 25 °C for 30 min with 10 µl of pSOD rabbit reticulocyte lysate translation products. 0, 50, 100 or 150 µl of wheat germ S-30 were included in the import reactions. The reactions were then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 2; pSOD translation products associated with mitochondria.
- Lane 3; pSOD translation products associated with mitochondria after treatment with proteinase K.
- Lane 4; pSOD translation products associated with mitochondria incubated with 25 µl of wheat germ S-30.
- Lane 5; pSOD translation products associated with mitochondria incubated with 25 µl of wheat germ S-30 after treatment with proteinase K.
- Lane 6; pSOD translation products associated with mitochondria incubated with 50 µl of wheat germ S-30.
- Lane 7; pSOD translation products associated with mitochondria incubated with 50 µl of wheat germ S-30 after treatment with proteinase K.
- Lane 8; pSOD translation products associated with mitochondria incubated with 75 µl of wheat germ S-30.
- Lane 9; pSOD translation products associated with mitochondria incubated with 75 µl of wheat germ S-30 after treatment with proteinase K.

30 included in a 2 x import reaction, little of the unprocessed MnSOD remains resistant to proteinase K. This indicates that the inclusion of the wheat germ S-30 is inhibiting the import of MnSOD and not just the processing (compare lanes 4, 6 and 8 with lanes 5, 7 and 9). The rabbit reticulocyte lysate cell-free translation system was therefore used in all subsequent import experiments rather than the more homologous wheat germ S-30 system.

3.8. The effect of 1,10-phenanthroline on the import of MnSOD.

MnSOD is synthesised as a precursor with an N-terminal extension, or presequence. Upon import into isolated *Z. mays* mitochondria this is processed to give a mature protein with an estimated molecular weight of 24.5 kDa. The enzyme responsible for the processing of such presequences has been shown to be located in the mitochondrial matrix and the processing activity was partially purified from *S. cerevisiae* (McAda and Douglas, 1982). The mitochondrial processing protease was found to be a metalloprotease, since the processing activity was dependent upon heavy metal ions such as Mn^{2+} , Co^{2+} and Zn^{2+} and could be inhibited by 1,10-phenanthroline, a metal ion chelator (McAda and Douglas, 1982; Bohni *et al.*, 1983). The effect of 1,10-phenanthroline on the import of MnSOD was therefore investigated. This would determine whether the activity of the *Z. mays* mitochondrial processing protease was also dependent upon metal ions.

pSOD translation products were incubated with *Z. mays* mitochondria at 25 °C for 30 min. 2 x import reactions were performed with 100 µg total mitochondrial protein as described in section 2.6.3 and 2, 10, 20 and 40 mM 1,10-phenanthroline. 1,10-phenanthroline was dissolved in DMSO, therefore to confirm that this did not interfere with the import of MnSOD a 2 x import reaction, equivalent to that with 20 mM 1,10-phenanthroline, was also performed, but included 4 % (v/v) DMSO. After the incubation was complete each import reaction was divided and one half was treated with 100 µg/ml proteinase K. Proteins were then fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 3.6.

Results show that when DMSO is included in the reaction there is no inhibition of the import or the processing of MnSOD (compare lanes 2 and 3). The mature protein remains resistant to exogenous proteinase K (lane 4), whereas an equivalent volume of pSOD translation products alone are completely digested by the same amount of proteinase K (lane 1) indicating that import has occurred. With



Figure 3.6; Autoradiograph, after SDS/PAGE, showing the effect of 1,10-phenanthroline on the *in vitro* import of MnSOD into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 μ g total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were at 25 $^{\circ}$ C for 30 min with 10 μ l of pSOD rabbit reticulocyte lysate translation products. 2, 10, 20 or 40 mM 1,10-phenanthroline were included in the import reactions. A 2 x import reaction was also performed with 4 % (v/v) DMSO. The reactions were then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 2; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 3; pSOD translation products associated with mitochondria incubated with 4 % (v/v) DMSO.
- Lane 4; pSOD translation products associated with mitochondria incubated with 4 % (v/v) DMSO after treatment with proteinase K.
- Lane 5; pSOD translation products associated with mitochondria incubated with 2 mM 1,10-phenanthroline.
- Lane 6; pSOD translation products associated with mitochondria incubated with 2 mM 1,10-phenanthroline after treatment with proteinase K.
- Lane 7; pSOD translation products associated with mitochondria incubated with 10 mM 1,10-phenanthroline.
- Lane 8; pSOD translation products associated with mitochondria incubated with 10 mM 1,10-phenanthroline after treatment with proteinase K.
- Lane 9; pSOD translation products associated with mitochondria incubated with 20 mM 1,10-phenanthroline.
- Lane 10; pSOD translation products associated with mitochondria incubated with 20 mM 1,10-phenanthroline after treatment with proteinase K.
- Lane 11; pSOD translation products associated with mitochondria incubated with 40 mM 1,10-phenanthroline.
- Lane 12; pSOD translation products associated with mitochondria incubated with 40 mM 1,10-phenanthroline after treatment with proteinase K.

1,10-phenanthroline included in the import reaction, however, the processing of MnSOD is inhibited. This inhibition increases with the concentration of 1,10-phenanthroline (compare lane 3 with lanes 5, 7, 9 and 11).

The inclusion of 1,10-phenanthroline does not effect the import of MnSOD, since the unprocessed precursor remains resistant to exogenous proteinase K (compare lanes 7 and 8, lanes 9 and 10 and lanes 11 and 12). The results therefore show that, as in *S. cerevisiae* and *N. crassa*, the import of precursor proteins into plant mitochondria is independent of the processing.

The processing of MnSOD is completely inhibited by the inclusion of 40 mM 1,10-phenanthroline, but the amount of unprocessed, proteinase K resistant precursor is severely reduced compared to the import reaction containing 20 mM 1,10-phenanthroline (compare lanes 10 and 12). Similar experiments with *N. crassa* mitochondria have indicated that a concentration of 4 - 6 mM 1,10-phenanthroline inhibited precursor processing, but upon treatment with proteinase K, little of the precursor remained associated with mitochondria (Zwizinski and Neupert, 1983). In contrast, when 20 mM 1,10-phenanthroline is included in the incubation with *Z. mays* mitochondria, the unprocessed MnSOD precursor remains associated with mitochondria and resistant to exogenous proteinase K (compare lanes 9 and 10). This concentration of 1,10-phenanthroline was therefore used in all subsequent experiments in which the inhibition of the *Z. mays* processing activity was investigated. The inhibition of the processing activity by 1,10-phenanthroline suggests that the *Z. mays* mitochondrial protease is also metal ion dependent. This activity was tentatively located to the matrix, since MnSOD could be processed to the mature protein upon incubation with a crude *Z. mays* mitochondrial soluble fraction (not shown).

3.9. The effect of valinomycin on the import of MnSOD.

In *S. cerevisiae* and *N. crassa* it is known that the membrane potential ($\Delta\Psi$), generated by the pumping of hydrogen ions across the inner mitochondrial membrane, is required for the import of precursor proteins into mitochondria (see section 1.3.5). Disruption of this membrane potential can be achieved by the addition of ionophores such as valinomycin. The effect of valinomycin (a potassium ion ionophore) on this *Z. mays* mitochondrial *in vitro* import system was therefore investigated.

pSOD translation products were incubated with isolated *Z. mays* mitochondria at 25 °C for 30 min. 2 x import reactions were performed with 100

μg total mitochondrial protein as described in section 2.6.3 and 9, 18 and 90 μM valinomycin. Valinomycin was dissolved in ethanol, therefore to confirm that this did not interfere with the import of MnSOD a 2 x import reaction, equivalent to that with 90 μM valinomycin, was also performed, but included 10 % (v/v) ethanol. After the incubation was complete each import reaction was divided and one half treated with 100 $\mu\text{g/ml}$ proteinase K. Proteins were then fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 3.7.

Results show that when ethanol is included in the reaction there is no inhibition in the import or the processing of MnSOD (compare lanes 1 and 2). The mature protein remains resistant to exogenous proteinase K indicating that import has occurred (lane 3). The presence of valinomycin in the import reaction, however, inhibits the processing of MnSOD (compare lane 2 with lanes 4, 6 and 8). The import of MnSOD, however, is not completely inhibited until 90 μM valinomycin is included in the reaction. This is indicated by the sensitivity of the unprocessed MnSOD to exogenous proteinase K (compare lanes 3 with lanes 5, 7 and 9). ~~The inclusion of 90 μM valinomycin in the reaction therefore abolishes the import of MnSOD and indicates that import is dependent upon an energised inner mitochondrial membrane.~~

It should be noted that the concentration of valinomycin required to inhibit the import of MnSOD is very much higher in this system than in others previously reported. Whelan *et al.* (1988), for instance, found that 9 μM valinomycin was sufficient to inhibit the import of ANT into *Vicia faba* mitochondria. Purdue (1988), however, found that 18 μM valinomycin could inhibit the import of the cytochrome *c* oxidase subunit IV from yeast into *Z. mays* mitochondria, but only after the preincubation of mitochondria with valinomycin for 5 min prior to import. It therefore remains unclear whether plant mitochondria require a membrane potential for protein import, since such high concentrations of valinomycin must apparently be used to inhibit import.

3.10. The effect of time on the import of MnSOD.

The import of MnSOD into isolated *Z. mays* mitochondria was investigated with respect to time. pSOD translation products were incubated with isolated *Z. mays* mitochondria at 25 $^{\circ}\text{C}$ as described in section 2.6.3. A 12 x reaction was performed and after 0, 1, 2.5, 5, 10 and 20 min, 500 μl aliquots were removed and one half treated with 100 $\mu\text{g/ml}$ proteinase K. Proteins were then fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 3.8.

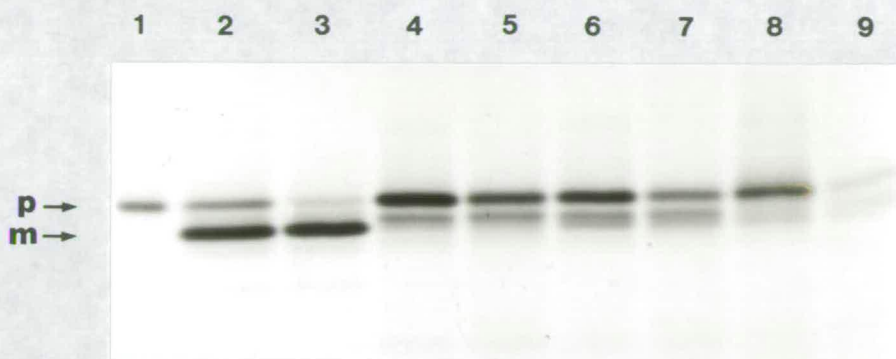


Figure 3.7; Autoradiograph, after SDS/PAGE, showing the effect of valinomycin on the *in vitro* import of MnSOD into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 μg total mitochondrial protein isolated from 3 day old dark grown coleoptiles. Incubations were at 25 $^{\circ}\text{C}$ for 30 min with 10 μl of pSOD rabbit reticulocyte lysate translation products. 9, 18 or 90 μM valinomycin were included in the import reactions. A 2 x import reaction was also performed with 10 % (v/v) ethanol. The reactions were then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 2; pSOD translation products associated with mitochondria incubated with 10 % (v/v) ethanol.
- Lane 3; pSOD translation products associated with mitochondria incubated with 10 % (v/v) ethanol after treatment with proteinase K.
- Lane 4; pSOD translation products associated with mitochondria incubated with 9 μM valinomycin.
- Lane 5; pSOD translation products associated with mitochondria incubated with 9 μM valinomycin after treatment with proteinase K.
- Lane 6; pSOD translation products associated with mitochondria incubated with 18 μM valinomycin.
- Lane 7; pSOD translation products associated with mitochondria incubated with 18 μM valinomycin after treatment with proteinase K.
- Lane 8; pSOD translation products associated with mitochondria incubated with 90 μM valinomycin.
- Lane 9; pSOD translation products associated with mitochondria incubated with 90 μM valinomycin after treatment with proteinase K.



Figure 3.8; Autoradiograph, after SDS/PAGE, showing the effect of time on the *in vitro* import of MnSOD into *Z. mays* mitochondria.

A 12 x import reaction was performed according to section 2.6.3 with 600 μg total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. The incubation was at 25 $^{\circ}\text{C}$ with 60 μl of pSOD rabbit reticulocyte lysate translation products. 500 μl were removed at 0, 1, 2.5, 5, 10 and 20 min and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 2; pSOD translation products associated with mitochondria incubated for 0 min.
- Lane 3; pSOD translation products associated with mitochondria incubated for 0 min after treatment with proteinase K.
- Lane 4; pSOD translation products associated with mitochondria incubated for 1 min.
- Lane 5; pSOD translation products associated with mitochondria incubated for 1 min after treatment with proteinase K.
- Lane 6; pSOD translation products associated with mitochondria incubated for 2.5 min.
- Lane 7; pSOD translation products associated with mitochondria incubated for 2.5 min after treatment with proteinase K.
- Lane 8; pSOD translation products associated with mitochondria incubated for 5 min.
- Lane 9; pSOD translation products associated with mitochondria incubated for 5 min after treatment with proteinase K.
- Lane 10; pSOD translation products associated with mitochondria incubated for 10 min.
- Lane 11; pSOD translation products associated with mitochondria incubated for 10 min after treatment with proteinase K.
- Lane 12; pSOD translation products associated with mitochondria incubated for 20 min.
- Lane 13; pSOD translation products associated with mitochondria incubated for 20 min after treatment with proteinase K.

Results show that MnSOD is rapidly imported into isolated *Z. mays* mitochondria, since after 1 min a small amount of the processed MnSOD remains resistant to exogenous proteinase K (lane 5). As the incubation continues the amount of MnSOD imported gradually increases. This is indicated by the resistance of the processed MnSOD to exogenous proteinase K (compare lane 5 with lanes 7, 9, 11 and 13). After 20 min the import of the MnSOD appears to have reached a maximum (compare lanes 3, 5, 7, 9, 11 and 13).

3.11. The effect of ATP on the import of MnSOD.

In addition to an energised inner membrane the import of proteins into *S. cerevisiae* and *N. crassa* mitochondria is dependent upon the presence of ATP (see section 1.3.5). The requirement of ATP for the import of MnSOD into the *Z. mays* mitochondrial *in vitro* import system was therefore investigated.

pSOD translation products were incubated at 25 °C for 30 min with *Z. mays* mitochondria isolated from 3 or 4 day old dark-grown coleoptiles. 2 x import reactions were performed as described in section 2.6.3 with 100 µg total mitochondrial protein and 2, 5, 10 or 20 mM ATP. After the incubation was complete each import reaction was divided and one half was treated with 100 µg/ml proteinase K. Proteins were then fractionated by SDS/PAGE. The autoradiographs of the gels are shown in Figures 3.9 and 3.10.

Results show that ATP is required for the complete import of MnSOD. With no additional ATP in the import reaction the majority of MnSOD remains in the precursor form (Figure 3.9, compare lane 1 with lanes 3 and 4). The import of the MnSOD, however, is not prevented, since the unprocessed precursor remains resistant to exogenous proteinase K (Figure 3.9, lanes 5 and 6), whereas an equivalent volume of the pSOD translation products alone are digested by the same amount of proteinase K (Figure 3.9, lane 2).

These import reactions are not totally devoid of ATP, however, since the rabbit reticulocyte lysate cell-free translation system used contains endogenous ATP (Pfanner and Neupert, 1986). Rabbit reticulocyte lysate has also been shown to contain cytosolic components, which are required for import. These are now thought to be heat shock proteins (hsps), which function to maintain the mitochondrial precursor in an 'import-competent' configuration (see section 1.3.7). One or a combination of these factors may therefore have resulted in the import of the MnSOD precursor to a proteinase K resistant location.

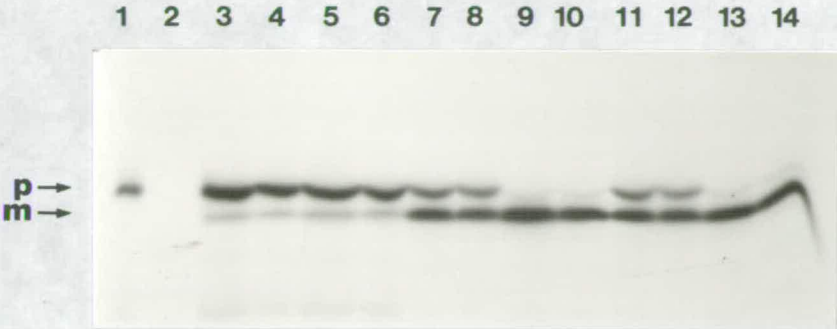
Figure 3.9; Autoradiograph, after SDS/PAGE, showing the effect of 2 and 5 mM exogenous ATP on the *in vitro* import of MnSOD into *Z. mays* mitochondria isolated from 3 or 4 day old dark-grown coleoptiles.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein. Incubations were at 25 °C for 30 min with 10 µl of pSOD rabbit reticulocyte lysate translation products. 0, 2 and 5 mM exogenous ATP was included in the import reactions. The reactions were then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 2; pSOD translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 3; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with no additional ATP.
- Lane 4; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with no additional ATP.
- Lane 5; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with no additional ATP after treatment with proteinase K.
- Lane 6; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with no additional ATP after treatment with proteinase K.
- Lane 7; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with 2 mM ATP.
- Lane 8; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with 2 mM ATP.
- Lane 9; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with 2 mM ATP after treatment with proteinase K.
- Lane 10; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with 2 mM ATP after treatment with proteinase K.
- Lane 11; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with 5 mM ATP.
- Lane 12; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with 5 mM ATP.
- Lane 13; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with 5 mM ATP after treatment with proteinase K.
- Lane 14; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with 5 mM ATP after treatment with proteinase K.

Figure 3.9.



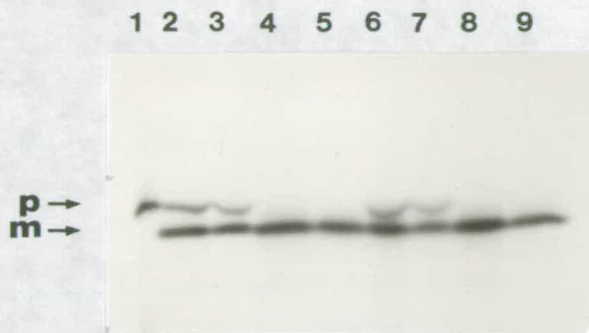


Figure 3.10; Autoradiograph, after SDS/PAGE, showing the effect of 10 and 20 mM exogenous ATP on the *in vitro* import of MnSOD into *Z. mays* mitochondria isolated from 3 or 4 day old dark-grown coleoptiles.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein. Incubations were at 25 °C for 30 min with 10 µl of pSOD rabbit reticulocyte lysate translation products. 10 and 20 mM exogenous ATP were included in the import reactions. The reactions were then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

- Lane 1; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 2; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with 10 mM ATP.
- Lane 3; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with 10 mM ATP.
- Lane 4; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with 10 mM ATP after treatment with proteinase K.
- Lane 5; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with 10 mM ATP after treatment with proteinase K.
- Lane 6; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with 20 mM ATP.
- Lane 7; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with 20 mM ATP.
- Lane 8; pSOD translation products associated with mitochondria isolated from 3 day old tissue incubated with 20 mM ATP after treatment with proteinase K.
- Lane 9; pSOD translation products associated with mitochondria isolated from 4 day old tissue incubated with 20 mM ATP after treatment with proteinase K.

An important point to mention here is that exogenous ATP is the only form of energy added to the import reactions described so far. No respiratory substrates have been supplied and therefore there will be no synthesis of ATP by oxidative phosphorylation. This is not unusual, and previous reports have shown that ATP alone can drive import (Nelson and Schatz, 1979; Schleyer *et al.*, 1982). An energised inner membrane is thought to be generated by the transport of external ATP into the mitochondrial matrix by the adenine nucleotide translocator (Chen and Douglas, 1987a). This is proposed to occur *in vivo*, since mitochondrial protein import into *S. cerevisiae rho⁻* mutants still occurs. These lack an active electron transport chain and therefore ~~the F_0F_1 ATP synthase will be uncoupled and~~ unable to generate a membrane potential (Nelson and Schatz, 1979).

The combined energy requirements of ATP and an energised inner membrane can, however, be distinguished. The membrane potential is required for the insertion of the precursor into the inner membrane (section 1.3.5). The complete translocation of the precursor, however, requires additional ATP (Schleyer and Neupert, 1985; Pfanner *et al.*, 1987a). The imported, but unprocessed MnSOD precursor may therefore be inserted into the inner membrane, but without higher concentrations of ATP it is unable to be fully translocated into the mitochondrial matrix.

When 2 mM ATP is included in the reaction MnSOD is processed upon import into the isolated *Z. mays* mitochondria (Figure 3.9, lanes 7 and 8) and the mature form remains resistant to exogenous proteinase K (Figure 3.9, lanes 9 and 10). The addition of 5, 10 and 20 mM ATP appears to have no additional effect upon the import of MnSOD (compare lanes 7 - 10 with lanes 11 - 14 in Figure 3.9 and lanes 2 - 9 in Figure 3.10). Mitochondria isolated from tissues of different ages does not appear to effect the import of MnSOD confirming earlier results (section 3.5 and Figure 3.3) ,

ATP therefore appears to be required for the complete import of MnSOD. From this a number of hypotheses can be made. Firstly, that low concentrations of ATP may trap the MnSOD precursor along the import pathway in such a way that processing is prevented. Secondly, that the *Z. mays* processing protease may require ATP as a cofactor. The lack of ATP would therefore prevent processing of imported precursor proteins. Thirdly, ATP may be required for the release of the protein from a mitochondrial hsp. The existence of such proteins have been previously demonstrated (see section 1.3.7). Further experiments will therefore be necessary to determine the precise role of ATP in this *Z. mays* mitochondrial protein import system.

3.1.2. The effect of GTP and UTP on the import of MnSOD.

The effect of GTP and UTP upon the import of MnSOD into *Z. mays* mitochondria was investigated in order to determine whether or not these could supplement for the requirement of ATP. pSOD translation products were incubated at 25 °C for 30 min with *Z. mays* mitochondria. 2 x import reactions were performed as described in section 2.6.3 with 100 µg total mitochondrial protein and 2, 5, 10 mM ATP, GTP or UTP. After the incubations were complete the reactions were divided and one half was treated with 100 µg/ml proteinase K. As a control a 1 x import reaction was performed with 10 mM ATP, which was subsequently treated with proteinase K and Triton X-100. This should result in the complete digestion of the previously protected protein due to the disruption of mitochondria by the detergent. Proteins were then fractionated by SDS/PAGE. The autoradiographs of the gels are shown in Figures 3.11A and 3.11B.

Results show, as in Figure 3.9 and Figure 3.10, that at low ATP concentrations MnSOD is imported to a proteinase K resistant location, but not processed (compare lanes 3 in Figure 3.11A and Figure 3.11B). On the addition of ATP, however, the MnSOD precursor is processed to a mature form (Figure 3.11A, lanes 4 - 6), which is resistant to exogenous proteinase K (Figure 3.11B, lane 4 - 6). After treatment with proteinase K and Triton X-100, however, the imported MnSOD is completely digested (Figure 3.11B, lane 14). Import is also indicated by the complete digestion of a volume of pSOD translation products, equivalent to that added to a 1 x import reaction, by the same amount of proteinase K (lane 1 in Figure 3.11A and Figure 3.11B).

GTP or UTP can supplement for the requirement of ATP, since when these are included in the import reaction MnSOD is imported. A mature form of MnSOD is observed (Figure 3.11A, lanes 7 - 10), which remains resistant to proteinase K (Figure 3.11B, lanes 7 - 10). The import, however, is not complete, since on treatment with proteinase K the majority of the imported protein remains in the unprocessed form (Figure 3.11B, compare lanes 4 - 6 with lanes 7 - 10) In fact, increasing the amount of GTP or UTP appears to inhibit the processing, but not the import of MnSOD (Figure 3.11B, compare lanes 4 - 6 with lanes 7 - 12). Interestingly, a similar inhibition by GTP was observed by Gasser *et al.* (1982b), whilst investigating the ability of a mitochondrial extract to process precursor proteins.

Figure 3.11; Autoradiographs, after SDS/PAGE, showing the effect of ATP, GTP and UTP on the *in vitro* import of MnSOD into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were at 25 °C for 30 min with 10 µl of pSOD rabbit reticulocyte lysate translation products. 0, 2, 5 and 10 mM exogenous ATP, GTP or UTP were included in the import reactions. The reactions were then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.

A.

- Lane 1; pSOD translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 2; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 3; pSOD translation products associated with mitochondria incubated with no additional ATP, UTP or GTP.
- Lane 4; pSOD translation products associated with mitochondria incubated with 2 mM ATP.
- Lane 5; pSOD translation products associated with mitochondria incubated with 5 mM ATP.
- Lane 6; pSOD translation products associated with mitochondria incubated with 10 mM ATP.
- Lane 7; pSOD translation products associated with mitochondria incubated with 2 mM UTP.
- Lane 8; pSOD translation products associated with mitochondria incubated with 5 mM UTP.
- Lane 9; pSOD translation products associated with mitochondria incubated with 10 mM UTP.
- Lane 10; pSOD translation products associated with mitochondria incubated with 2 mM GTP.
- Lane 11; pSOD translation products associated with mitochondria incubated with 5 mM GTP.
- Lane 12; pSOD translation products associated with mitochondria incubated with 10 mM GTP.

B.

- Lane 1; pSOD translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 2; pSOD translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lanes 3-12; as in (A) except mitochondria were treated with proteinase K after import.
- Lane 13; blank.
- Lane 14; pSOD translation products associated with mitochondria incubated with 10 mM ATP after treatment with proteinase K and Triton X-100.

Figure 3.11.

A.



B.



3.13. Conclusions.

The results presented above indicate that mitochondria isolated from *Z. mays* dark-grown coleoptiles can, under suitable conditions, import a plant nuclear-encoded mitochondrial precursor protein. The *N. plumbaginifolia* MnSOD (MnSOD) was used to characterise and to a certain degree optimise this plant mitochondrial *in vitro* protein import system. MnSOD was synthesised as a precursor protein with an estimated molecular weight of 26 kDa, which was processed upon import to the mature protein with an estimated molecular weight of 24.5 kDa. Import was characterised by the resistance of this processed protein to exogenous proteinase K. A volume of pSOD translation products, equivalent to that added to a single import reaction, however, was completely digested by the same amount of proteinase K. As a further control an import reaction treated with proteinase K and Triton X-100 resulted in the complete digestion of MnSOD (section 3.4).

The import of MnSOD into this plant mitochondrial protein import system only occurred when the precursor was synthesised in a rabbit reticulocyte lysate cell-free translation system. Import of the same precursor synthesised by a wheat germ S-30 translation system was not possible. In fact, inclusion of the wheat germ S-30 in the import reaction inhibited protein import of the MnSOD synthesised in the rabbit reticulocyte lysate. The rabbit reticulocyte lysate cell-free translation system therefore had to be used throughout rather than the more homologous wheat germ S-30 system (section 3.7).

The import of MnSOD into isolated *Z. mays* mitochondria displayed a number of characteristics similar to those already demonstrated in *S. cerevisiae* and *N. crassa*. Firstly, the processing of the precursor could be inhibited by the addition of 1,10-phenanthroline (section 3.8). This is a known inhibitor of the mitochondrial matrix processing protease from *S. cerevisiae* and *N. crassa* (McAda and Douglas, 1982; Bohni *et al.*, 1983). Secondly, valinomycin, a potassium ionophore, which destroys the mitochondrial inner membrane potential, inhibited protein import (section 3.9). Thirdly, the complete import of MnSOD was dependent upon the presence of exogenous ATP, a concentration of 2 mM being sufficient (section 3.11). The precise role of ATP in this *Z. mays* mitochondrial protein import system, however, is yet to be fully elucidated. Low concentrations of GTP and UTP could supplement, to a limited extent, the requirement for ATP, but higher concentrations appeared to inhibit processing (section 3.12).

Protein import of MnSOD was rapid and occurred after only 1 min (section 3.10). Mitochondria isolated from dark-grown coleoptiles of different ages showed no obvious difference in the ability to import proteins, therefore 3 day old coleoptiles were used as the source of mitochondria (section 3.5). The amount of total mitochondrial protein included in the import reaction, however, did affect the amount of MnSOD precursor imported. 100 μ g total mitochondrial protein/2 x import reactions was therefore used throughout (section 3.6).

Results therefore show that mitochondria can be successfully isolated from *Z. mays*, which are capable of importing a nuclear-encoded mitochondrial precursor protein. Investigation of this plant mitochondrial protein import system indicates that it displays many features similar to those of *S. cerevisiae* and *N. crassa*. This partially characterised system was subsequently employed to investigate the import of a second plant nuclear-encoded protein the adenine nucleotide translocator (ANT) from *Z. mays* (Chapter 4).

CHAPTER 4

THE *IN VITRO* IMPORT OF THE *ZEA MAYS* ADENINE NUCLEOTIDE TRANSLOCATOR

4.1. Introduction.

The adenine nucleotide translocator (ANT) is the most abundant protein of the mitochondrial inner membrane. It is an integral membrane protein and functions as a homodimer mediating the exchange of adenine nucleotides between the matrix and the cytosol (Klingenberg, 1985). It is encoded in the nucleus and must therefore be imported into the mitochondrion after synthesis in the cytosol. The import pathway of this protein has been extensively studied in *Saccharomyces cerevisiae* and *Neurospora crassa* and distinct steps have now been characterised. Unlike the majority of other nuclear-encoded mitochondrial proteins, ANT is synthesised without an N-terminal extension or presequence and therefore, is not processed upon import (see section 1.3.11.2). Although there is no change in the apparent molecular weight, the precursor and mature forms can be distinguished by the specific binding of the inhibitor carboxyatractyloside. This only binds to the assembled and functionally active protein and as such, has been used as a parameter for import (Schleyer and Neupert, 1984).

The availability of a full-length cDNA clone encoding the *Zea mays* ANT (Bathgate pers. comm.) and the development of a homologous mitochondrial *in vitro* import system (Chapter 3) has enabled the import of this plant nuclear-encoded mitochondrial protein to be investigated. Earlier work by Purdue (1988), indicated that the import of ANT may be distinctly different to that in fungal systems, since the protein appeared to be processed upon import into mitochondria. Work was hampered, however, by the lack of a full-length cDNA. This chapter will describe the results obtained from the *in vitro* import of the *Z. mays* ANT and present evidence that these earlier observations were correct. The conclusions which are drawn are discussed in Chapter 5.

4.2. A full-length *Zea mays* adenine nucleotide translocator cDNA, *ANT-A*.

A 1136 bp cDNA, pANT-1, encoding the *Z. mays* ANT had previously been isolated (Baker and Leaver, 1985). This contained a 954 bp ORF, but it was unclear whether this was full-length, since the cDNA terminated 2 nucleotides 5' to the putative ATG codon. In addition, transcript analysis suggested that the *Z. mays* ANT mRNA was approximately 1600 bp long (section 2.1.4.1). Subsequent screening of a *Z. mays* cDNA library with pANT-1 resulted in the isolation of a 1575 bp full-length ANT cDNA, *ANT-A*. This was contained on an *Eco* RI fragment and encoded a 987 bp ORF. The nucleotide sequence and the deduced amino acid sequence of which is shown in Figure 4.1 (Bathgate pers. comm.).

Comparison of *ANT-A* with pANT-1 indicates that *ANT-A* extends 286 bp beyond the 5' terminus and 153 bp beyond the 3' terminus of pANT-1. *ANT-A* was not entirely identical with the putative genomic clone, *ANT-G1* (Bathgate *et al.*, 1989), since the 3' end of the cDNA extended 153 bp beyond the 3' terminus of *ANT-G1*. In addition, the 5' end of the cDNA contained 60 bp of unknown origin (see Figure 4.1). These differences may be due to the fact that *ANT-A* and *ANT-G1* were isolated from two different lines of *Z. mays* (sections 2.1.4.1 and 2.1.4.2).

The *Eco* RI fragment containing *ANT-A* was cloned into the expression vector pGEM 1 allowing *in vitro* transcription from the T7 promoter. The resulting construct was termed pG1ANT (Figure 4.2). The restriction endonuclease *Bgl* II was used to linearise pG1ANT at the unique recognition site within the 3' non-coding region thereby allowing run-off transcription.

4.3. The *in vitro* expression and import of G1ANT.

The RNA generated by the *in vitro* transcription of pG1ANT was used to programme a rabbit reticulocyte lysate cell-free translation system. The pG1ANT translation products were then incubated with isolated *Z. mays* mitochondria at 25 °C for 30 min. A 2 x import reaction was performed with 100 µg total mitochondrial protein as described in section 2.6.3. After the incubation was complete the reaction was divided and one half treated with 100 µg/ml proteinase K.

As a control, a volume of the pG1ANT translation products equivalent to that added to a single (1 x) import reaction was also treated with 100 µg/ml proteinase K. This should completely digest the translation products and confirm that any proteinase K resistant protein associated with mitochondria was due to import. As a

Figure 4.1.

1	<u>GCGCCTCTGAAAGTTTCGGAGCCGCCGGCCCGTCCGCTCCCTCTCGCC</u>	48
49	<u>TCCGTCCCCAAGGCCAATAGTTTTTCAGGTGGCAATGGCGGACCAGGCT</u>	96
97	<i>Bal I</i> AACCAACCCACTGTCCTTCATAAGCTCGGTGGCCAGTCCACCTGGCCT	144
145	CGATCATCTCTGAAGGTGTACGGGCCCGTAACATCTGCCCATCTGTCT	192
193	<u>CATGTTATGAAAGGAGATTTGCCACAAGGAACTACATGACCCAGAGCC</u>	240
241	<u>TTTGGGGCCCTTGCAATGTCTGTAGCGGTGGCATCAATGTCCCAGTG</u> M S V S G G I N V P V	288
289	ATGCAGACCCCGCTCTGTGCTAATGCTCCTGCTGAGAAAGGAGGCAAG M Q T P L C A N A P A E K G G K	336
337	<i>Pvu II</i> AACTTCATGATTGATTTTCATGATGGGCGGTGTTTCAGCTGCTGTTTCA N F M I D F M M G G V S A A V S	384
385	AAGACTGCTGCTGCTCCCATCGAGCGTGTGAAGCTGCTTATTCAGAAC K T A A A P I E R V K L L I Q N	432
433	CAAGATGAGATGATTAAGTCTGGTAGGCTATCAGAGCCGTACAAGGGT Q D E M I K S G R L S E P Y K G	480
481	ATTGTTGACTGCTTCAAACGTACCATTAAGGATGAAGGTTTCTCTTCC I V D C F K R T I K D E G F S S	528
529	TTGTGGAGGGGATACACTGCTAATGTTATTTCGTTACTTCCTACTCAG L W R G Y T A N V I R Y F P T Q	576
577	GCTTTGAACTTTGCATTTAAGGACTACTTCAAGAGGTTGTTCAACTTC A L N F A F K D Y F K R L F N F	624
625	AAGAAGGATAGGGATGGCTATTGGAAGTGGTTTGCTGGCAACCTGGCC K K D R D G Y W K W F A G N L A	672
673	TCTGGTGGTGCTGCTGGTGCTTCCTCTTTGTTTTTGTGTACTCCCTG S G G A A G A S S L F F V Y S L	720
721	GACTACGCGAGAACAAGGTTGGCTAATGACCGAAGGCTGCCAAGGGA D Y A R T R L A N D A K A A K G	768
769	GGAGGTGAAAGGCAGTTCAATGGGCTTGTCGATGTCTACCGCAAGACA G G E R Q F N G L V D V Y R K T	816
817	CTCAAGTCTGATGGTATTGCTGGGCTTTACCGTGGATTTAACATCTCC L K S D G I A G L Y R G F N I S	864
865	TGTGTTGGAATCATTGTTTATCGTGGTCTGTACTTTGGACTCTATGAT C V G I I V Y R G L Y F G L Y D	912

Figure 4.1 continued.

913	TCCATCAAGCCAGTTGTCCTTACTGGCAACCTCCAGGACAATTTCTTT	960
	S I K P V V L T G N L Q D N F F	
961	GCCAGTTTCGCTCTGGGTTGGCTGATCACTAATGGTGCTGGTCTTGCA	1008
	A S F A L G W L I T N G A G L A	
1009	TCTTACCCCATCGATAACCGTCCGCAGAAGGATGATGATGACATCTGGT	1056
	S Y P I D T V R R R M M M T S G	
1057	GAGGCTGTCAAGTACAAGAGCTCCTTGGACGCGTTCAGCAGATTCTT	1104
	E A V K Y K S S L D A F Q Q I L	
1105	AAGAAGGAAGGGCCCAAGTCCCTGTTCAAGGGTGCTGGTGCTAACATT	1152
	K K E G P K S L F K G A G A N I	
1153	CTTCGTGCCATTGCTGGTGCTGGTGTGCTTTCTGGCTATGACCAGCTC	1200
	L R A I A G A G V L S G Y D Q L	
		*
1201	CAGATCCTCTTCTTCGGAAAAGAAGTACGGCTCCGGCGGTGCTTAAATG	1248
	Q I L F F G K K Y G S G G A	
		*
1249	GAGAAATAATGTAGACGAACAAGAGCAGTGTGTGTTCCCGGTCCTTTC	1296
1297	CAATCAGGATCTGGTGAAGTTTTTGCCTTTCATTTTCAAGAAATTAAT	1344
1345	AATTCATGTAGAGGGAGGATTCCTCCAACAATTTGTTTTGGATGGAGA	1392
	<i>Bgl</i> II	
1393	CTTAGATCTCCAAACACTCGTAACTATCCTACTAGTGTGACATCGGTC	1440
1441	CCCAAAAACATTTATGGTCATAGAGTCTGCCCTTCGTTTGTGATCGG	1488
1489	GCAATTTTTCTTGCATACAATGCCTTCGTTTAAGTATCGGATGAGTTG	1536
1537	CTGCGACTAATTATTTGTTTGTCTTTACCTTTTGCAAAA	

Figure 4.1; The nucleotide and deduced amino acid sequence of the *Z. mays* ANT 1575 bp cDNA *ANT-A*.

A 987 bp ORF begins at 256 bp (ATG 3) and ends at 1243 bp. Homology to the genomic clone *ANT-G1* begins at 61 bp and ends at 1423 bp (Bathgate *et al.*, 1989). The first 60 bp are of unknown origin and are underlined. Homology to the 1136 bp cDNA pANT-1 begins at 287 bp and ends at 1423 bp (Baker and Leaver, 1985). The first three in frame ATG codons are marked 1, 2 and 3 and underlined. The stop codons are marked with an asterisk and underlined and the unique restriction endonuclease recognition sites *Bal* I, *Bgl* I, *Pvu* II and *Bgl* II are marked and underlined. The terminal *Eco* RI sites are not shown.

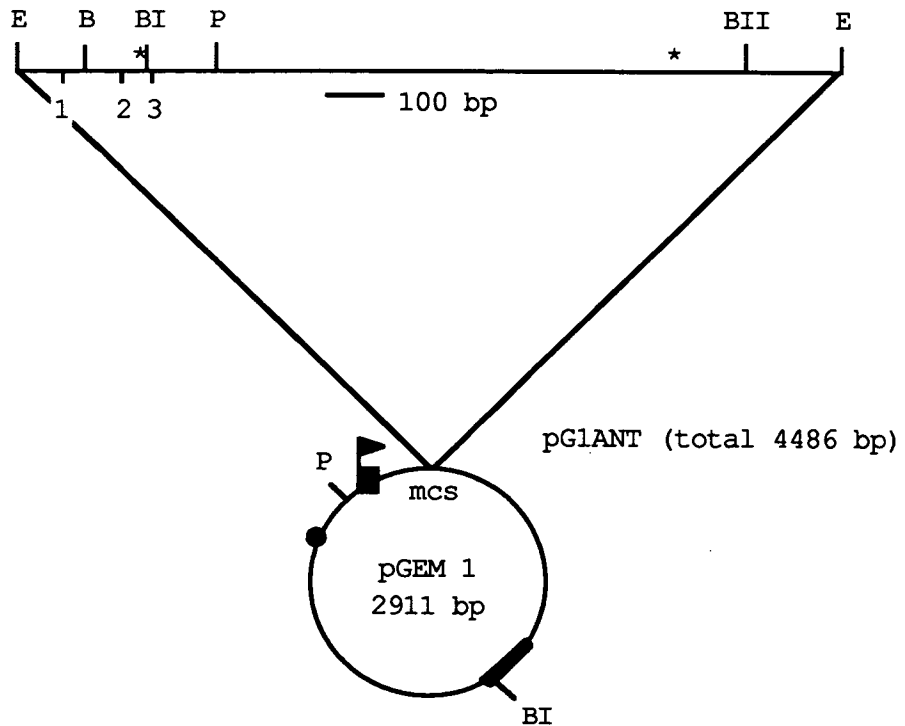


Figure 4.2; The construction of pG1ANT.

The 1575 bp *Z. mays* ANT cDNA ANT-A was cloned into the expression vector pGEM 1 allowing transcription from the T7 promoter, represented by the black square (the direction of transcription by the arrow). The restriction endonuclease recognition sites are marked; E; *Eco* RI, B; *Bal* I, BI; *Bgl* I, P; *Pvu* II and BII; *Bgl* II. The first three in frame ATG codons are represented as 1, 2 and 3 and the stop codons are marked with an asterisk. The origin of replication is represented by the black circle, the ampicillin gene by the black rectangle and the multiple cloning site by mcs. The orientation of the cDNA was determined through restriction endonuclease digestion (not shown).

further control, a 1 x import reaction was performed and then treated with proteinase K and 0.1 % (v/v) Triton X-100. This should result in the complete digestion of the previously protected protein due to the disruption of mitochondria by the detergent. Proteins were then fractionated by SDS/PAGE and pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction were run as a control. The autoradiograph of the gel is shown in Figure 4.3.

Results show that the *in vitro* translation of the *Z. mays* ANT generates a precursor protein, termed G1ANT, with an estimated molecular weight of 37 kDa (lane 1), which is processed upon import into isolated *Z. mays* mitochondria (lane 3). The processed protein runs with an estimated molecular weight of 30 kDa. The processed protein is imported, since it remains resistant to exogenous proteinase K (lane 4). On treatment with proteinase K and Triton X-100, however, the protein is completely digested (lane 5). The results from Figure 4.3 indicate that the import of this plant ANT is distinctly different to that of *S. cerevisiae* or *N. crassa* in that it appears to be synthesised as a larger precursor protein, which is processed upon import into isolated mitochondria. The *in vitro* translation of pG1ANT also generates a proteinase K resistant protein, labelled mbp, with an estimated molecular weight of 48 kDa (lanes 1 and 2). This has been postulated to be a ³⁵S-methionine binding protein (mbp) and was seen consistently in all rabbit reticulocyte translations performed, even those programmed with a mock transcription reaction, containing no DNA (see section 4.5.2 and Figure 4.8).

4.4. The processing of the *Zea mays* adenine nucleotide translocator is inhibited by 1,10-phenanthroline.

To determine whether the processing event that accompanies the import of G1ANT into isolated *Z. mays* mitochondria (Figure 4.3) is due to the action of the mitochondrial matrix protease, 1,10-phenanthroline was included in the import reaction. This is a metal ion chelator and a known inhibitor of the mitochondrial matrix protease from *S. cerevisiae* and *N. crassa* (section 3.8).

pG1ANT rabbit reticulocyte translation products were incubated with isolated *Z. mays* mitochondria at 25 °C for 30 min. 2 x import reactions were performed (+/- 20 mM 1,10-phenanthroline) with 100 µg total mitochondrial protein as described in section 2.6.3. After the incubation was complete each reaction was divided and one half was treated with 100 µg/ml proteinase K. A volume of pG1ANT translation products equivalent to that added to a 1 x import

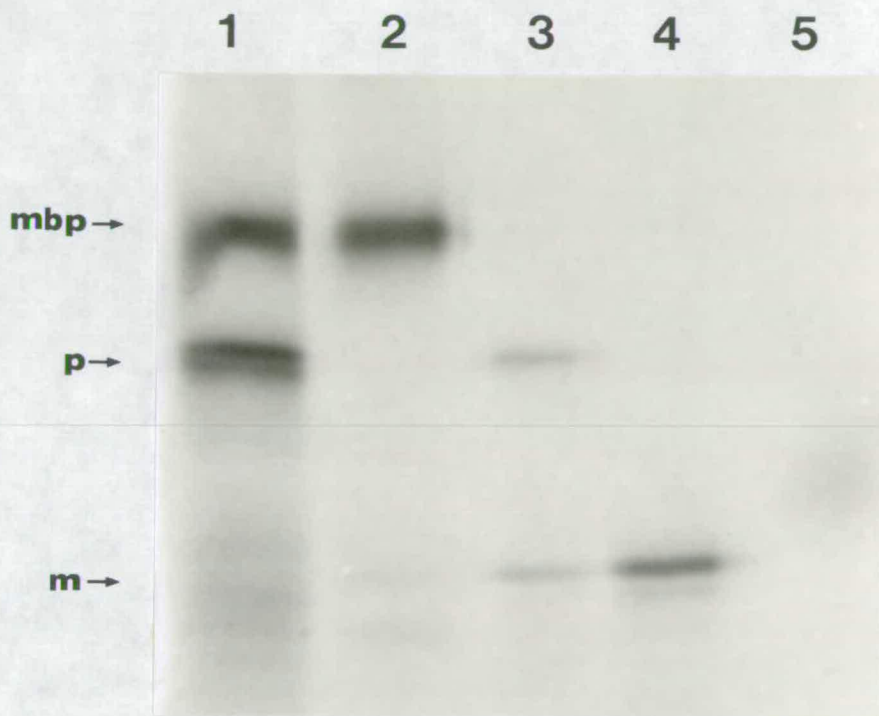


Figure 4.3; Autoradiograph, after SDS/PAGE, showing the *in vitro* import of G1ANT into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. The incubation was performed at 25 °C for 30 min with 20 µl of pG1ANT rabbit reticulocyte lysate translation products. The reaction was then divided and one half treated with proteinase K.

p; precursor protein. m; mature protein.
mbp; methionine binding protein

- Lane 1; pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 2; pG1ANT translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 3; pG1ANT translation products associated with mitochondria.
- Lane 4; pG1ANT translation products associated with mitochondria after treatment with proteinase K.
- Lane 5; pG1ANT translation products associated with mitochondria after treatment with proteinase K and Triton X-100.

reaction was treated with 100 µg/ml proteinase K. Proteins were then fractionated by SDS/PAGE and a volume of the pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction was run as a control. The autoradiograph of the gel is shown in Figure 4.4.

Results show that when 20 mM 1,10-phenanthroline is included in the import reaction the processing of G1ANT is inhibited (compare lane 3 and lane 5). Without 1,10-phenanthroline in the reaction there is complete protection of the processed G1ANT from exogenous proteinase K and little of the precursor remains (lane 4). With 1,10-phenanthroline included in the reaction, however, the G1ANT precursor remains resistant to the exogenous protease indicating that this has been imported into mitochondria, but not processed (compare lanes 6 and 4). The processing of G1ANT, however, is not completely inhibited by the inclusion of 1,10-phenanthroline in the import reaction (lanes 5 and 6).

4.5. Determination of the translational start site in *ANT-A*.

ATG 3 is presumed to be the start of translation within *ANT-A*, since there is an in frame TGA stop codon 27 bp upstream (Figure 4.1). Assuming this ATG is used as the start of translation, a protein with a calculated molecular weight of 35.9 kDa is predicted from the deduced amino acid sequence. The results from Figures 4.3 and 4.4 indicate that the *Z. mays* ANT is synthesised as a precursor protein with an estimated molecular weight of 37 kDa, which is processed upon import into mitochondria to a protein of 30 kDa. The *Z. mays* ANT therefore appears to be synthesised with an extension of approximately 7 kDa (as estimated from SDS/PAGE). Subsequent analysis of the deduced amino acid sequence indicated that the first 67 amino acid residues encoded by *ANT-A* would have a calculated molecular weight of 6.95 kDa. Assuming that the processing of G1ANT is N-terminal, the predicted position of the processing site lies between the glycine (G) and arginine (R) amino acid residues indicated in Figure 4.5. Comparison of the N-terminal *Z. mays* ANT amino acid sequence with those from *S. cerevisiae*, *N. crassa* and *Bos taurus*, however, indicates that such a processing event would occur within a region internal to the other ANT protein sequences. This seemed very unlikely and put into question whether ATG 3 was being used as the start of translation in the *in vitro* rabbit reticulocyte lysate system.

Interestingly, when the DNA sequence around the ATG codons is compared to the Kozak (CCACCATGG) consensus sequence for translational initiation (Kozak,

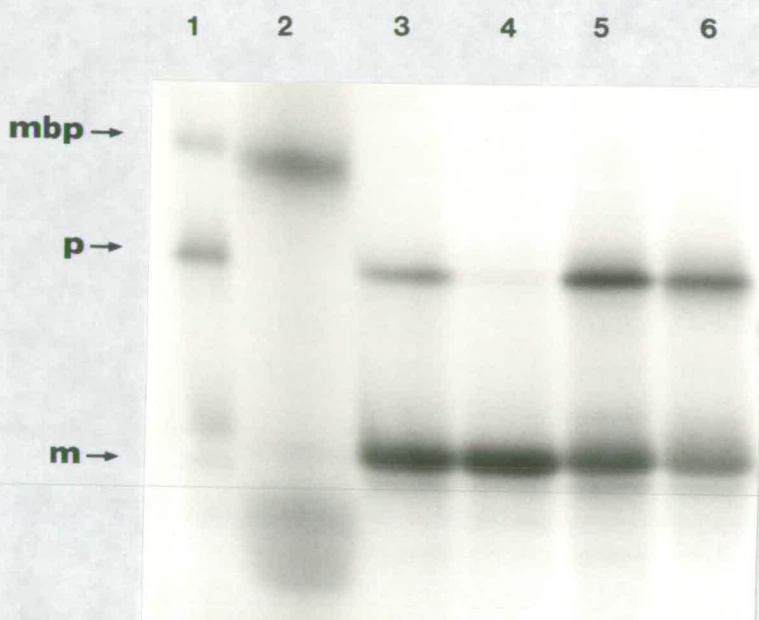


Figure 4.4; Autoradiograph, after SDS/PAGE, showing the effect of 1,10-phenanthroline on the *in vitro* import of G1ANT into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 μ g total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were performed at 25 $^{\circ}$ C for 30 min with 20 μ l of pG1ANT rabbit reticulocyte lysate translation products. Where appropriate 20 mM 1,10-phenanthroline was included in the reaction.

p; precursor protein. m; mature protein.
mbp; methionine binding protein.

- Lane 1; pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 2; pG1ANT translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 3; pG1ANT translation products associated with mitochondria.
- Lane 4; pG1ANT translation products associated with mitochondria after treatment with proteinase K.
- Lane 5; pG1ANT translation products associated with mitochondria incubated with 1,10-phenanthroline.
- Lane 6; pG1ANT translation products associated with mitochondria incubated with 1,10-phenanthroline after treatment with proteinase K.

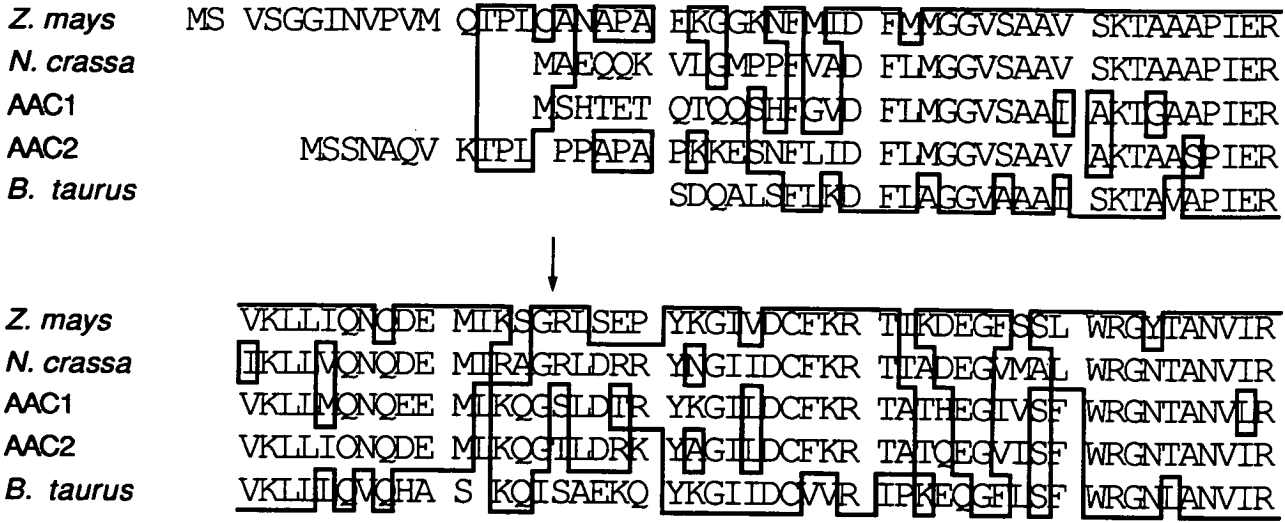


Figure 4.5; A comparison of the N-terminal amino acid sequence of the ANT proteins from *Z. mays*, *N. crassa*, *S. cerevisiae* and *B. taurus*. Sequences are aligned to maximise homology.

The *Z. mays* (Bathgate pers. comm.), *N. crassa* (Arends and Sebald, 1984) and the *S. cerevisiae* AAC1 and AAC2 (Lawson and Douglas, 1988) sequences are deduced from the nucleotide sequences. The *B. taurus* sequence (Aquila *et al.*, 1982) was determined directly. The arrow marks the position of the predicted processing site within the *Z. mays* ANT protein.

1984), ^{the sequence surrounding} ATG 1 is more homologous than ATG 3 having 3/6 bp identical, compared to the 1/6 bp found for ATG 3. When the sequences are compared to the longer ^{phat} consensus sequence (TAAACAATGGCT) produced by Joshi (Joshi, 1987), however, both have identical homologies (4/9 bp). The sequence around ATG 2 shows no homology with the Kozak sequence and has only 1/9 bp homology to the Joshi sequence.

4.5.1. Deletion of ATG 3.

To determine whether ATG 3 was used as the start of translation *in vitro*, the 5' region of *ANT-A* was deleted. Initially, however, a construct was made to confirm that the 3' non-coding DNA was not interfering with the *in vitro* expression of G1ANT. pG1ANT was therefore digested with the restriction endonucleases *Bgl* II and *Bam* HI, the recognition sites of which are unique to pG1ANT. *Bgl* II digests within the 3' non-coding region of *ANT-A* (see Figures 4.1 and 4.2), whilst *Bam* HI digests within the multiple cloning site of the vector (Figure 4.6). Isolation of the large fragment and subsequent religation would result in the loss of 179 bp from the *ANT-A* 3' non-coding region (Figure 4.6). The construct generated was termed pGA2 (Figure 4.6).

In order to determine whether ATG 3 was being used as the start of translation, 243 bp from the 5' end of *ANT-A* were deleted. This was achieved by digesting pGA2 with the restriction endonuclease *Bgl* I, the recognition site of which is unique to *ANT-A* (Figure 4.7). Deletion of this 5' region of *ANT-A* would result in the removal of the in frame ATG codons ATG 1 and ATG 2, as well as the TGA stop codon and would leave only 12 bp 5' to ATG 3 (Figure 4.1). The 3' overhangs left from the digestion of pGA2 with *Bgl* I were removed through the action of T4 DNA polymerase and the *Bgl* I (blunt-ended) - *Hind* III fragment was then subcloned into pGEM 3 digested with *Sma* I and *Hind* III. The resulting construct was termed pGA3 (Figure 4.7).

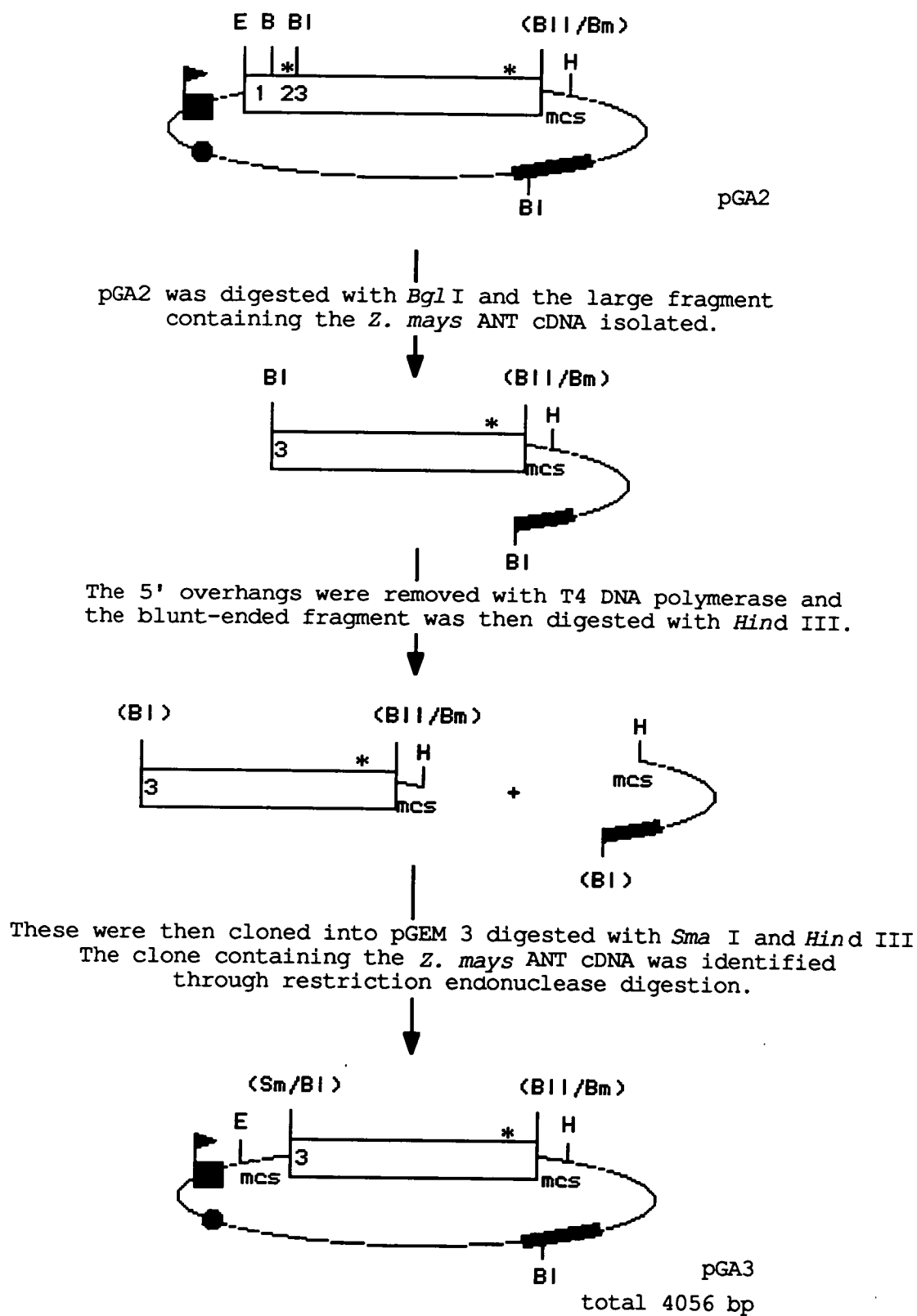
4.5.2. The *in vitro* expression of GA2 and GA3.

Both pGA2 and pGA3 were linearised with *Hind* III to allow run-off transcription and the RNA generated from these clones and from pG1ANT was used to programme a rabbit reticulocyte lysate translation system (see section 2.5.1 and 2.5.2.1). Incubations were performed at 30 °C for 1 h, after which an aliquot of each translation was fractionated by SDS/PAGE. As a control, a mock transcription

Figure 4.7; The construction of pGA3.

This cloning procedure removed 243 bp from the 5' end of *ANT-A* and allowed transcription from the T7 promoter, represented by the black square (the direction of transcription being indicated by the arrow). The restriction endonuclease sites are marked E; *Eco* RI, B; *Bal* I, BI; *Bgl* I, BII; *Bgl* II, Bm; *Bam* HI, H; *Hind* III and Sm; *Sma* I (those marked within brackets have been lost). The first three in frame ATG codons are marked 1, 2 and 3 and the stop codons are marked with an asterisk. The origin of replication is represented by the black circle, the ampicillin gene by the black rectangle and the multiple cloning sites by mcs.

Figure 4.7.



reaction (containing no DNA) was used to programme the rabbit reticulocyte lysate and an equivalent amount was also fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 4.8.

The expression of pGA3 results in the synthesis of a number of protein products. The major product ran with an estimated molecular weight of 31 kDa, 6 kDa smaller than G1ANT (compare lanes 3 and 5). This change in size must be due to the alterations made at the 5' end of *ANT-A*, since removal of the 3' non-coding region had no effect on the size of the protein generated after transcription from the T7 promoter (compare lanes 3 and 4). The rabbit reticulocyte lysate translation programmed with the mock transcription generated a protein with an estimated molecular weight of 48 kDa (lane 2). This was seen consistently in all translations and has been postulated to be a ³⁵S-methionine binding protein (mbp).

4.5.3. The *in vitro* import of G1ANT and GA3.

The import into isolated *Z. mays* mitochondria of the pGA3 translation products were compared with those of pG1ANT. 2 x import reactions were performed with 100 µg total mitochondrial protein at 25 °C for 30 min as described in section 2.6.3. The reactions were then divided and one half was treated with 100 µg/ml proteinase K. A volume of pG1ANT and pGA3 translation products equivalent to that added to a 1 x import reaction were incubated with 100 µg/ml proteinase K to confirm that import had occurred. As a further control, a 1 x import reaction was performed with a rabbit reticulocyte lysate programmed with a mock transcription reaction (containing no DNA). After the incubation was complete, the reaction was then treated with 100 µg/ml proteinase K. Proteins were then fractionated by SDS/PAGE. The Coomassie blue stained gel is shown in Figure 4.9A and the corresponding autoradiograph in Figure 4.9B. The Coomassie blue stained *Z. mays* ANT protein is indicated with the arrow (Hawkesford pers. comm.).

Upon import into isolated *Z. mays* mitochondria, GA3 is processed to the same molecular weight as G1ANT (Figure 4.9B, compare lanes 4 and 7). Comparison of the Coomassie blue stained gel and the corresponding autoradiograph indicates that both processed forms run with an identical molecular weight to the Coomassie blue stained *Z. mays* ANT protein (compare Figure 4.9A and Figure 4.9B). The processed forms are imported into mitochondria, since these are

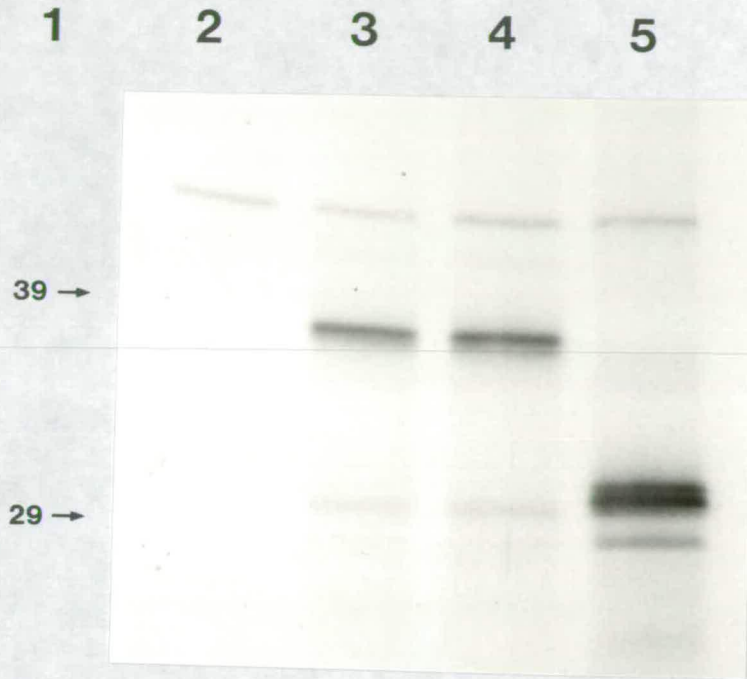


Figure 4.8; Autoradiograph, after SDS/PAGE, showing the pG1ANT, pGA2 and pGA3 rabbit reticulocyte lysate translation products.

A rabbit reticulocyte lysate was programmed with RNA generated from the pG1ANT, pGA2 and pGA3 *in vitro* transcription reactions according to section 2.5.2.1. Incubations were at 30 °C for 1 h, after which 5 µl aliquots were fractionated by SDS/PAGE. An equivalent volume of a rabbit reticulocyte lysate programmed with a mock transcription reaction (containing no DNA) was run as a control.

- Lane 1; molecular weight markers (kDa).
- Lane 2; translation products from a mock transcription reaction.
- Lane 3; pG1ANT translation products.
- Lane 4; pGA2 translation products.
- Lane 5; pGA3 translation products

Figure 4.9; The Coomassie blue stained gel (A) and autoradiograph (B), after SDS/PAGE, showing the *in vitro* import of G1ANT and GA3 into isolated *Z. mays* mitochondria.

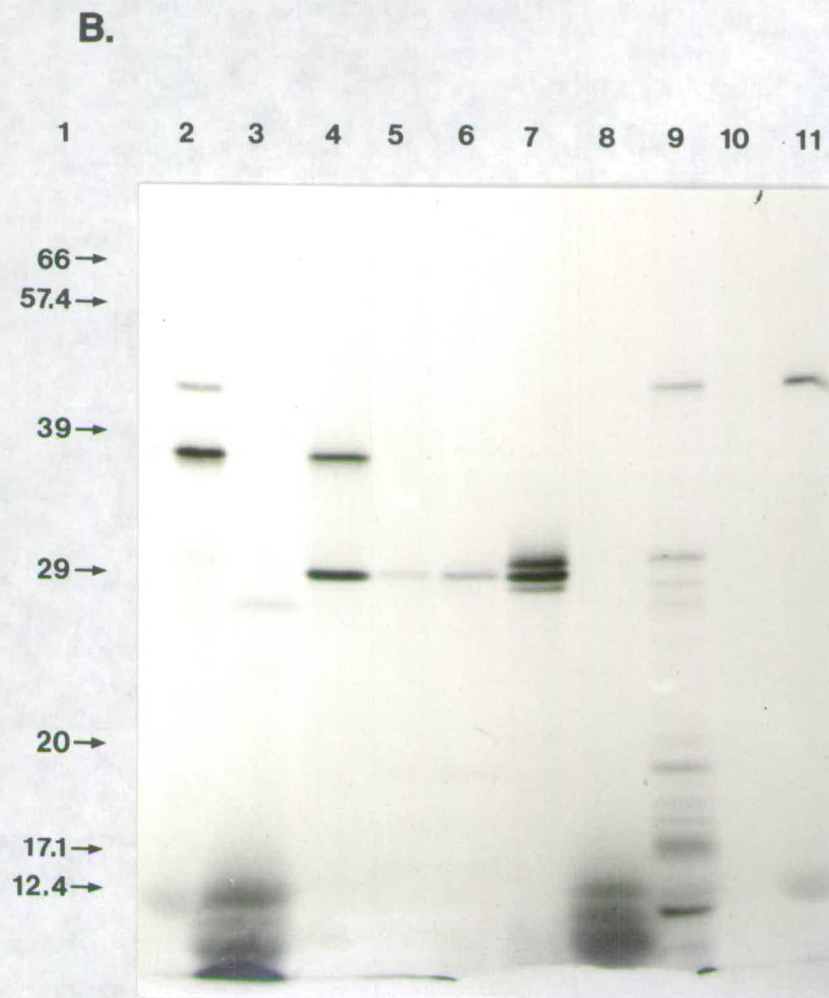
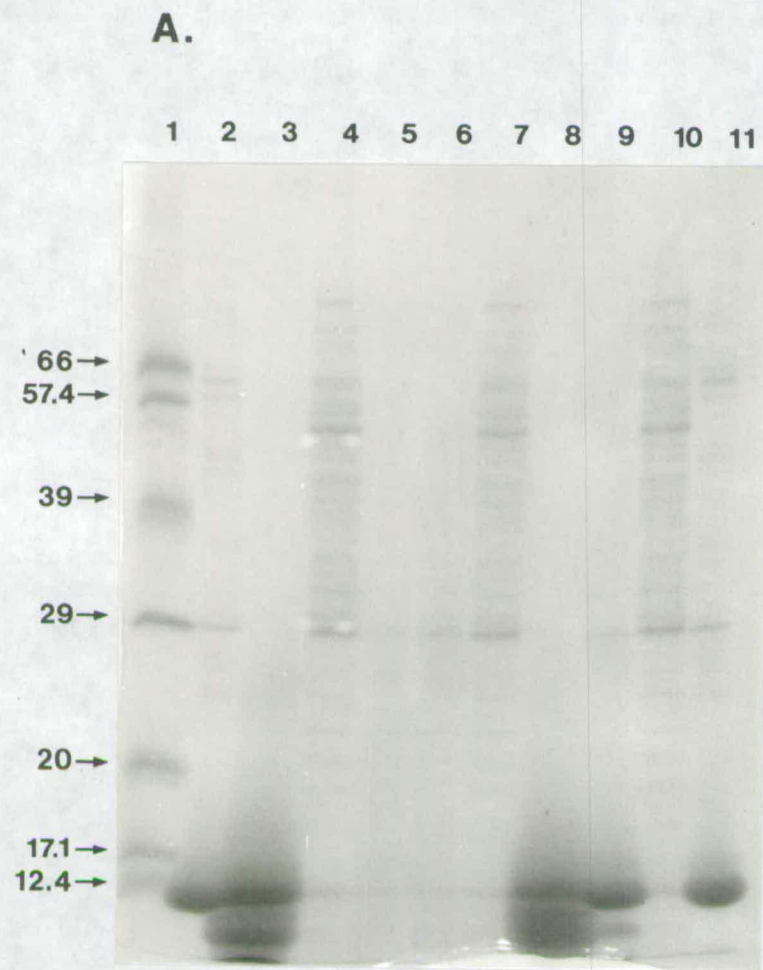
2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were at 25 °C for 30 min with 20 µl of pG1ANT and pGA3 rabbit reticulocyte lysate translation products. As a control a 1 x import reaction was performed with 10 µl of a rabbit reticulocyte lysate programmed with a mock transcription reaction (containing no DNA). The arrow marks the position of the Coomassie blue stained *Z. mays* ANT protein (Hawkesford pers. comm.).

A.

- Lane 1; molecular weight markers (kDa).
- Lane 2; pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 3; pG1ANT translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 4; pG1ANT translation products associated with mitochondria.
- Lane 5; pG1ANT translation products associated with mitochondria after treatment with proteinase K
- Lane 6; pGA3 translation products associated with mitochondria after treatment with proteinase K.
- Lane 7; pGA3 translation products associated with mitochondria.
- Lane 8; pGA3 translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 9; pGA3 translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 10; translation products from a mock transcription reaction associated with mitochondria.
- Lane 11; translation products from a mock transcription reaction equivalent to 10 % of that added to a 1 x import reaction.

B. as in A above.

Figure 4.9.



protected from exogenous proteinase K (Figure 4.9B, lanes 5 and 6). The same amount of proteinase K, however, completely digests a volume of pG1ANT and pGA3 translation products equivalent to that added to a 1 x import reaction (Figure 4.9B, lanes 3 and 8). The putative ³⁵S-methionine binding protein synthesised when the rabbit reticulocyte lysate is programmed with a mock transcription reaction (Figure 4.9B, lane 11) does not associate with or import into the isolated *Z. mays* mitochondria (Figure 4.9B, lane 10).

4.5.4. The start of translation in *ANT-A* is at ATG 1.

The translation of pGA3 generated a much smaller protein compared to pG1ANT (Figure 4.8). These results indicate that the extension predicted for G1ANT must be N-terminal, since only alterations at the 5' end of *ANT-A* result in a change in the molecular weight (Figure 4.7). The synthesis of this smaller protein occurred even though pGA3 retained ATG 3, the putative start of translation. It was therefore postulated that either ATG 1 or ATG 2 were used as the start of translation in G1ANT, rather than ATG 3.

To try and determine which ATG codon was being used to initiate the translation of G1ANT, a third construct termed pGA4 was made (Figure 4.10). Utilising the unique *Bal* I endonuclease recognition site in *ANT-A* 128 bp from the 5' end of *ANT-A*, including ATG 1, could be deleted (Figure 4.1). The *Bal* I - *Hind* III fragment from pGA2 was therefore isolated and subcloned into pGEM 3 digested with *Sma* I and *Hind* III. This cloning procedure would then allow run-off transcription to be performed from the T7 promoter after linearising pGA4 with *Hind* III.

RNA generated from the *in vitro* transcription of pGA4 was then used to programme a rabbit reticulocyte lysate translation system. Similar reactions were performed with pG1ANT and pGA3. An aliquot of each translation was then fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 4.11.

As with pGA3 (section 4.5.2), the expression of pGA4 generates a number of protein products, the major product having an estimated molecular weight of 30.5 kDa. These translation products are all smaller than G1ANT (compare lanes 2 and 4) and this is therefore consistent with the hypothesis that the start of translation is not at ATG 3. The construction of pGA4 removed only ATG 1 from *ANT-A* and it was therefore postulated that this ATG codon was being used as the start of translation *in vitro*.

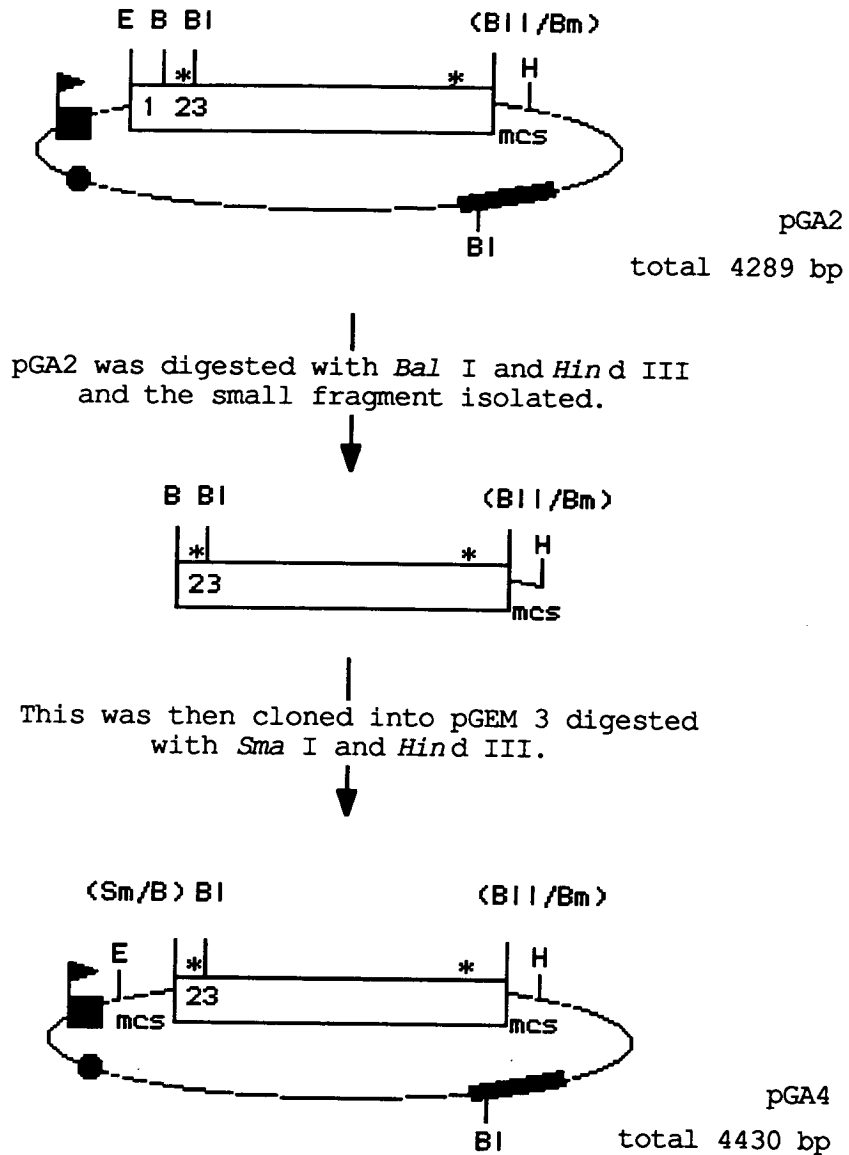


Figure 4.10; The construction pGA4.

This cloning procedure removed 128 bp from the 5' end of *ANT-A* and allowed transcription from the T7 promoter, represented by the black square (the direction of transcription being indicated by the arrow). The restriction endonuclease recognition sites are marked; E; *Eco* RI, B; *Bal* I, B I; *Bgl* I, B II; *Bgl* II, B m; *Bam* HI, H; *Hind* III and Sm; *Sma* I (those marked within brackets have been lost). The first three in frame ATG codons are represented as 1, 2 and 3 and the stop codons are marked with an asterisk. The origin of replication is represented as the black circle, the ampicillin gene as the black rectangle and the multiple cloning sites as mcs.

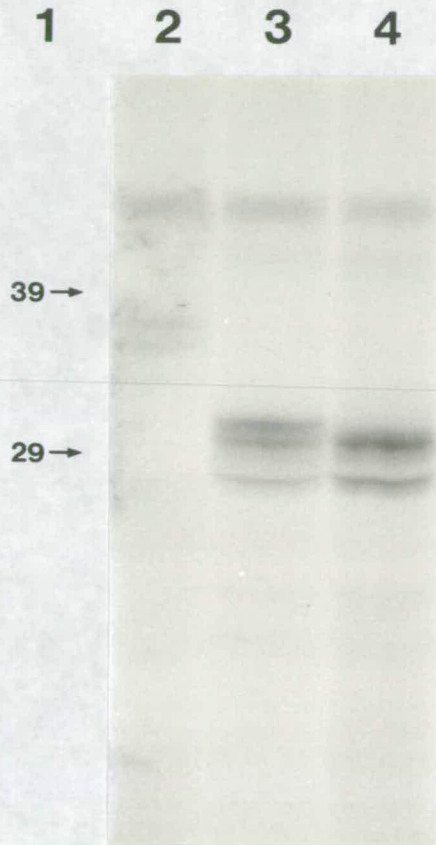


Figure 4.11; Autoradiograph, after SDS/PAGE, showing the pG1ANT, pGA3 and pGA4 rabbit reticulocyte lysate translation products.

A rabbit reticulocyte lysate was programmed with RNA generated from the pG1ANT, pGA3 and pGA4 *in vitro* transcription reactions according to section 2.5.2.1. Incubations were at 30 °C for 1 h, after which 5 μ l aliquots were fractionated by SDS/PAGE.

- Lane 1; molecular weight markers (kDa).
- Lane 2; pG1ANT translation products.
- Lane 3; pGA3 translation products.
- Lane 4; pGA4 translation products.

4.6. Immunoprecipitation of a poly A⁺ RNA translation with a *Zea mays* polyvalent adenine nucleotide translocator antibody.

The results presented above indicate that the *in vitro* expression of *ANT-A* synthesises a larger precursor protein, which is processed upon import into isolated *Z. mays* mitochondria. This processed form has the same mobility on SDS/PAGE as the Coomassie blue stained *Z. mays* ANT protein. The availability of an antibody recognising the *Z. mays* ANT made it possible to determine whether *Z. mays* poly A⁺ RNA encodes a larger ANT protein than the Coomassie blue stained mitochondrial protein observed after SDS/PAGE (Figure 4.9A).

Z. mays poly A⁺ RNA was isolated from 36 h dark-grown coleoptiles and used to programme a wheat germ S-30 *in vitro* translation system. This was used in preference to the rabbit reticulocyte lysate translation system, because the incorporation of ³⁵S-methionine was much higher. After programming with the *Z. mays* poly A⁺ RNA, 5 x 10⁶ cpm TCA precipitable protein were incubated with 10 µl or 30 µl of the *Z. mays* ANT antibody at 4 °C overnight (see section 2.7). The immunoprecipitated products were then fractionated by SDS/PAGE and 2 x 10⁵ cpm TCA precipitable protein from the wheat germ S-30 translation programmed with the *Z. mays* poly A⁺ RNA was run as a control. The autoradiograph of the gel is shown in Figure 4.12.

The results indicate that the *Z. mays* ANT antibody recognises two proteins with estimated molecular weights of 38 kDa and 37 kDa (lanes 3 and 4). These are comparable to the estimated molecular weight of the G1ANT protein synthesised after the *in vitro* expression of *ANT-A* (Figure 4.3). A minor protein with an estimated molecular weight of 30 kDa, however, is also observed (lanes 3 and 4). This may be due to a processing activity within the wheat germ S-30. The results of the immunoprecipitation therefore indicate that the *Z. mays* poly A⁺ RNA can encode two ANT proteins that are of greater molecular weight than the Coomassie blue stained protein found within isolated *Z. mays* mitochondria.

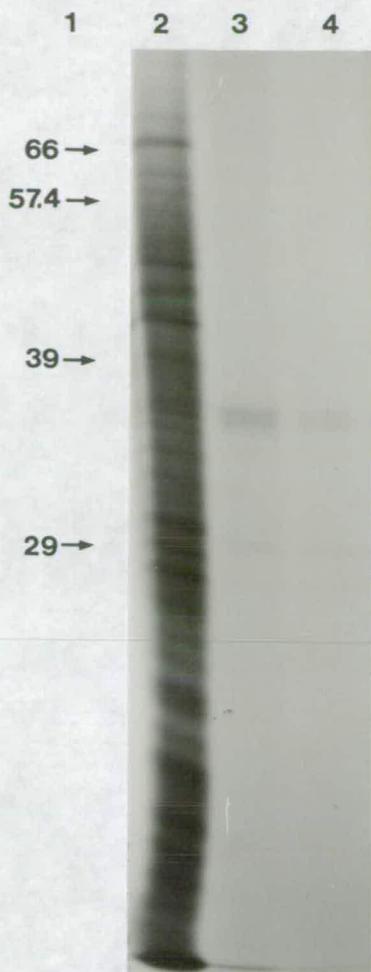


Figure 4.12; Autoradiograph, after SDS/PAGE, showing the immunoprecipitation of a wheat germ translation programmed with *Z. mays* poly A⁺ RNA with an antibody recognising the *Z. mays* ANT protein.

Poly A⁺ RNA was isolated from 36 h dark-grown coleoptiles and used to programme a wheat germ S-30 system according to section 2.5.2.2. The % incorporation of ³⁵S-methionine was estimated from TCA precipitable protein (section 2.5.3) and 5 x 10⁶ cpm were then incubated with a polyvalent antibody recognising the *Z. mays* ANT protein. The immunoprecipitated products were fractionated by SDS/PAGE and an aliquot of the poly A⁺ RNA wheat germ translation containing 2 x 10⁵ cpm was run as a control.

- Lane 1; molecular weight markers (kDa).
- Lane 2; 2 x 10⁵ cpm *Z. mays* poly A⁺ RNA wheat germ translation products.
- Lane 3; 5 x 10⁶ cpm *Z. mays* poly A⁺ RNA wheat germ translation products after immunoprecipitation with 30 µl of the *Z. mays* ANT antibody.
- Lane 4; 5 x 10⁶ cpm *Z. mays* poly A⁺ RNA wheat germ translation products after immunoprecipitation with 10 µl of the *Z. mays* ANT antibody.

4.7. Construction of a fusion protein between the N-terminal end of the *Zea mays* adenine nucleotide translocator and the mouse cytosolic protein dihydrofolate reductase.

From the *in vitro* experiments outlined so far, the *Z. mays* ANT appears to be synthesised as a larger precursor, which is processed upon import (Figure 4.3). Deletion analysis of *ANT-A* indicated that ATG 1, rather than ATG 3, is used for the start of translation *in vitro* (section 4.5 and Figure 4.11). ^{Using computer predictions} analysis of the deduced amino acid sequence ^{and the change in MW as estimated from SDS/PAGE} now suggests that the processing site will lie between the first proline (P) and the third valine (V) amino acid residues in the *Z. mays* ANT protein illustrated in Figure 4.5 and not at the position previously marked with the arrow. Such a processing event would generate a mature *Z. mays* ANT protein with an N-terminus comparable to the ANT proteins from *S. cerevisiae*, *N. crassa* and *B. taurus* (Figure 4.5).

The postulated N-terminal extension on the *Z. mays* ANT protein may therefore function as other mitochondrial presequences and target the protein into mitochondria (section 1.3.3). To investigate this, an in frame fusion protein between the N-terminal end of *ANT-A* and the complete mouse cytosolic protein dihydrofolate reductase (DHFR) was constructed.

The complete coding sequence of the DHFR gene was contained on the plasmid pDS₇DHFR. pDS₇DHFR was therefore digested at the unique *Bam* HI restriction endonuclease site. This lay 11 bp upstream from the DHFR ATG codon. The recessed 3' termini were then filled in by the 5' to 3' polymerase activity of the Klenow fragment of DNA polymerase I. The linearised pDS₇DHFR was then digested with *Eco* RI and the large fragment used as the cloning vehicle for the insertion of the 373 bp *Eco* RI - *Pvu* II *ANT-A* fragment (Figure 4.1). This cloning procedure generated an in frame fusion with the N-terminal end of *ANT-A* and the mouse DHFR gene. The resulting construct was termed pCS90.1 (Figure 4.13). Assuming the ATG codon ATG 1, encoded by *ANT-A*, was used as the start of translation *in vitro*, this fusion protein should contain 97 amino acid residues from the N-terminal end of the *Z. mays* ANT protein.

The construction of the in frame fusion regenerated a *Bam* HI restriction endonuclease recognition site (Figure 4.13). This was utilised to identify the pCS90.1 clone, but to confirm the nucleotide sequence at the fusion junction the *Eco* RI - *Hind* III fragment from pCS90.1 was cloned into pBS M13+. This construct was termed pFUSION and would allow sequencing of the sense strand from the M13 -

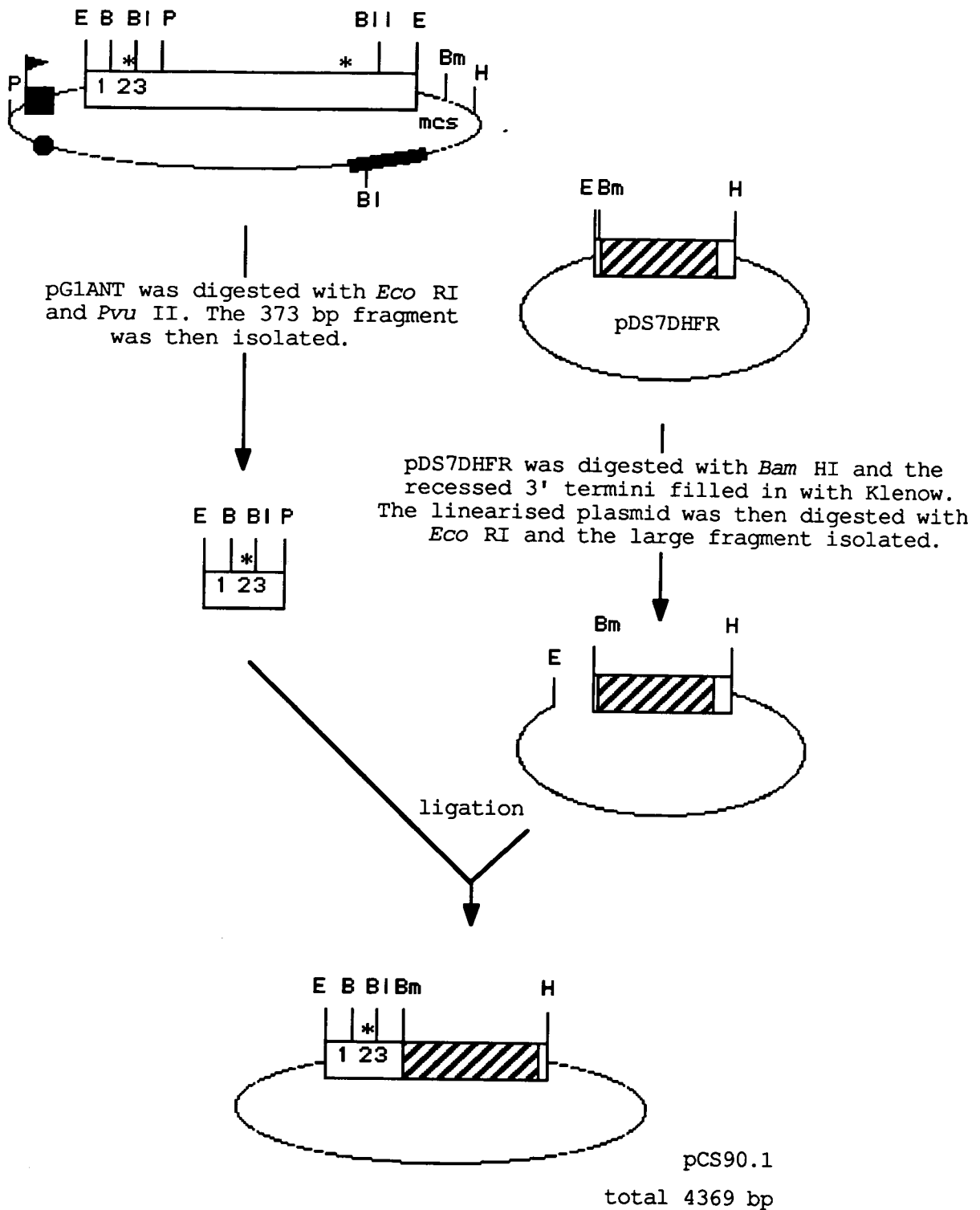


Figure 4.13; The construction pCS90.1.

This cloning procedure generated an in frame fusion between the 373 bp *Eco* RI - *Pvu* II fragment from *ANT-A* (represented by the white rectangle) and the mouse DHFR coding sequence (represented by the hatched rectangle). The restriction endonuclease recognition sites are marked; E; *Eco* RI, B; *Bal* I, BI; *Bgl* I, P; *Pvu* II, BII; *Bgl* II, Bm; *Bam* HI and H; *Hind* III. The first three in frame ATG codons of *ANT-A* are represented as 1, 2 and 3 and the stop codons are marked with an asterisk. The origin of replication is represented as the black circle, the ampicillin gene as the black rectangle and the multiple cloning site as mcs.

20 universal primer (5' -GTAAAACGACGGCCAGT- 3'), as well as *in vitro* transcription from the T7 promoter (Figure 4.14).

Attempts to sequence the fusion junction between the 5' end of *ANT-A* and the mouse DHFR gene from the M13 primer, however, were unsuccessful, since the site of interest was too far from the M13 primer. A synthetic oligonucleotide (5' -GGAAGTACTTGAAC TCG- 3') was therefore used. This hybridises to the sense strand of the DHFR gene from 106 bp to 90 bp downstream of the DHFR ATG initiation codon and would allow sequencing through the fusion site into the *ANT-A* sequence. The results of this sequencing is shown in Figure 4.15A. The nucleotide sequence of the sense strand at the fusion junction and the deduced amino acid sequence is shown in Figure 4.15B. The four amino acid residues numbered I, II, III and IV have no origin from either *ANT-A* or the DHFR coding sequence, but are generated through the construction of pCS90.1. The amino acid residues underlined originate from the *Z. mays* ANT. The first adenine nucleotide (A) is marked 355 and is equivalent to the A at position 355 bp within *ANT-A* (Figure 4.1).

These results therefore confirmed that the sequence around the fusion junction in pFUSION was as expected. An in frame fusion had therefore been constructed between the N-terminal end of *ANT-A* and the complete mouse DHFR gene.

4.8. The *in vitro* expression and import of a fusion protein between the N-terminal end of the *Zea mays* adenine nucleotide translocator and the mouse cytosolic protein dihydrofolate reductase.

To allow run-off transcription, pFUSION was linearised at the unique *Hind* III restriction endonuclease site and the RNA generated was used to programme a rabbit reticulocyte lysate translation system. The pFUSION translation products were then incubated with isolated *Z. mays* mitochondria at 25 °C for 30 min. 2 x import reactions were performed with 100 µg total mitochondrial protein as described in section 2.6.3. After the incubation was complete the reaction was divided and one half treated with 100 µg/ml proteinase K.

A volume of pFUSION translation products equivalent to that added to a 1 x import reaction was incubated with the same amount of proteinase K. This would confirm that any proteinase K resistant protein associated with mitochondria was due to import. As a further control, a 1 x import reaction was performed and then

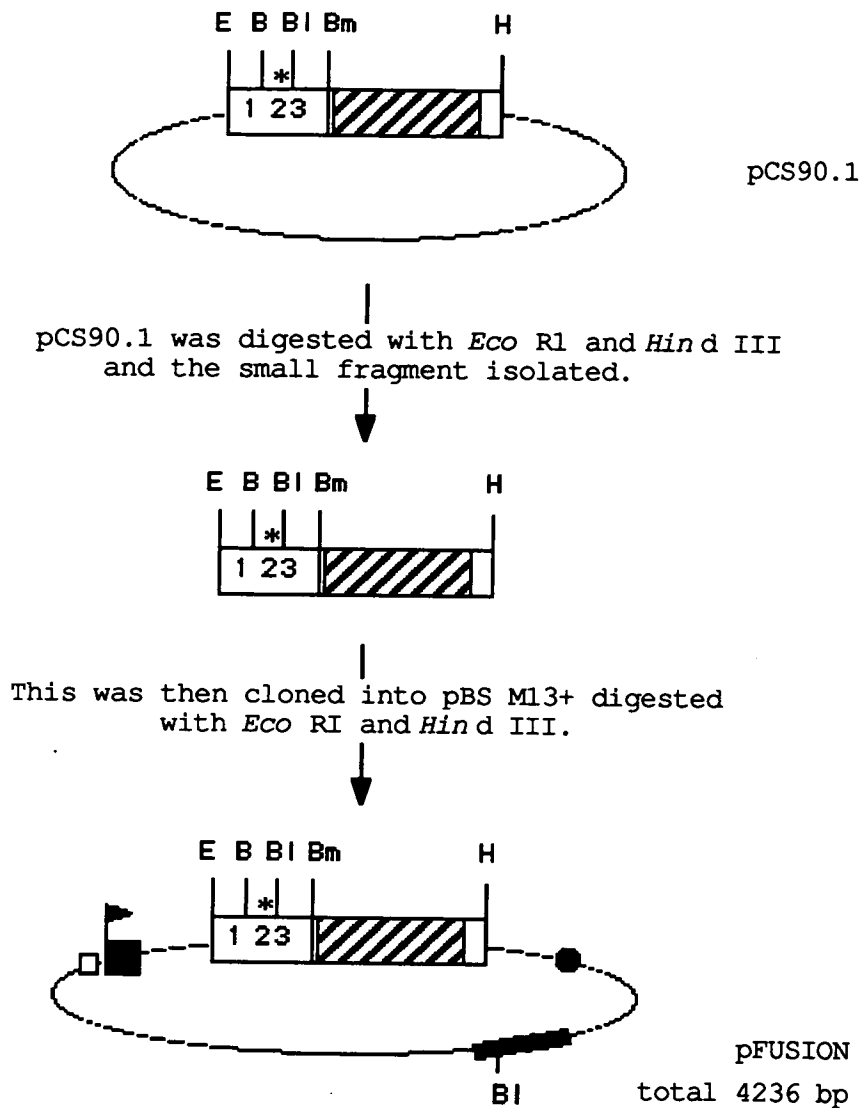


Figure 4.14; The construction of pFUSION.

In an attempt to sequence the fusion junction between the 373 bp *Eco* RI - *Pvu* II fragment from *ANT-A* (represented by the white rectangle) and the mouse DHFR coding sequence (represented by the hatched rectangle) the 1036 bp *Eco* RI - *Hind* III fragment from pCS90.1 was cloned into pBS M13+. This would allow sequencing of the sense strand using the M13 -20 universal primer (the binding site of which is represented by the open square). The orientation also allowed transcription from the T7 promoter, represented by the black square (the direction of transcription being indicated by the arrow). The restriction endonuclease recognition sites are marked; E; *Eco* RI, B; *Bal* I, B I; *Bgl* I, Bm; *Bam* HI and H; *Hind* III. The first three in frame ATG codons of *ANT-A* are represented as 1, 2 and 3 and the stop codons are marked with an asterisk. The origin of replication is represented as the black circle, the ampicillin gene as the black rectangle and the multiple cloning site as mcs.

Figure 4.15; The nucleotide sequence (A) and deduced amino acid sequence (B) of the fusion junction between the 373 bp *Eco* RI - *Pvu* II fragment from *ANT-A* and the complete mouse DHFR coding sequence.

A. The autoradiograph of the polyacrylamide gel after sequencing of the fusion junction using the synthetic oligonucleotide 5' -GGAAGTACTTGAAC TCG- 3'. This allowed sequencing of the non-sense strand. The corresponding sense strand is shown with the regenerated *Bam* HI site boxed. The DHFR ATG translation initiation codon is also boxed and marked with an asterisk.

B. The sense strand and deduced amino acid sequence of the fusion junction. The regenerated *Bam* HI site is underlined and the ATG encoding the translational start of the DHFR protein is marked with an asterisk. The amino acid residues originating from *ANT-A* are underlined, whilst those generated from the cloning procedure are numbered I - IV. The position of the first adenine nucleotide (A) is marked 355 and is equivalent to that at 355 bp within *ANT-A* (Figure 4.1).

treated with proteinase K and Triton X-100. This should result in the complete digestion of any previously protected protein due to the disruption of mitochondria by the detergent.

The import of the cytosolic protein DHFR was also investigated. The gene had been isolated from pDS₇DHFR on an *Eco* RI - *Hind* III fragment and cloned into pGEM 3 (not shown). The resulting construct was termed pDHFR and allowed run-off transcription from the T7 promoter after linearisation with *Hind* III. G1ANT was also imported into the isolated *Z. mays* mitochondria. The proteins were then fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 4.16.

Results show that the *in vitro* expression of pFUSION generates a protein with an estimated molecular weight of 31 kDa (lane 7). This is 10 kDa larger than the DHFR protein (lane 12), the calculated molecular weight of which is 21.6 kDa (Hurt *et al.*, 1984). These results are consistent with the hypothesis that ATG 1 is the start of translation in *ANT-A*, since a calculated increase of only 4 kDa would be expected if the initiation of the fusion protein was at ATG 3.

The fusion protein is imported into mitochondria (lane 8), since the protein remains resistant to exogenous proteinase K (lane 9). When an import reaction is treated with proteinase K and Triton X-100, however, the fusion protein is completely digested (lane 2), although a small amount of a lower molecular weight protein is still present. A volume of pFUSION translation products equivalent to that added to a single import reaction, however, is completely digested by the same amount of proteinase K (lane 14)

The cytosolic protein DHFR does not associate with or import into mitochondria (lanes 10 and 11). The addition of the N-terminal 373 bp from *ANT-A* onto the coding sequence of DHFR therefore results in the import of this usually cytosolic protein into isolated *Z. mays* mitochondria. Mitochondrial targeting information therefore appears to reside within the N-terminal region of the *Z. mays* ANT protein.

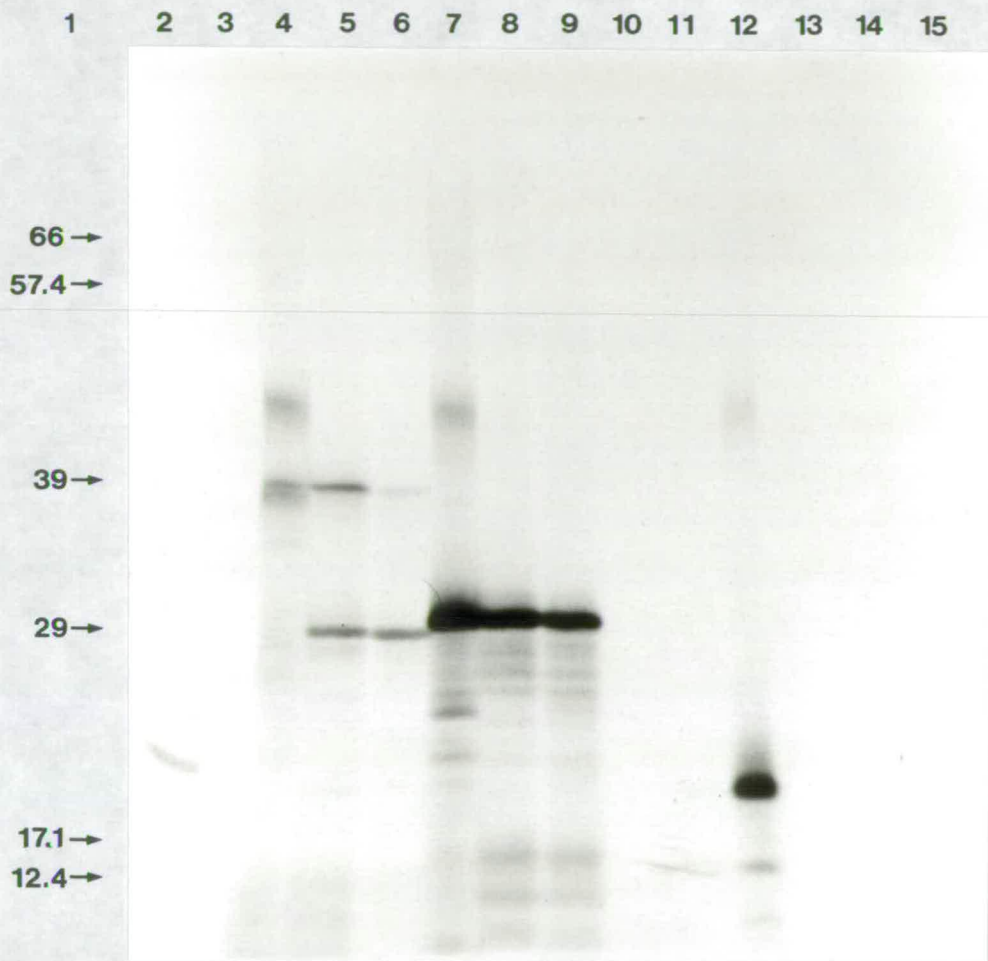
The fusion protein, unlike G1ANT, is not processed upon import (compare lanes 8 and 9 with lanes 5 and 6). This was unexpected since the 373 bp *Eco* RI - *Pvu* II fragment from *ANT-A* is predicted to contain the processing site. The construction of the chimaeric protein, however, may have resulted in the alteration of the tertiary structure. The processing site of the *Z. mays* ANT may, therefore, no longer be recognised by the *Z. mays* mitochondrial processing enzyme.

Figure 4.16; Autoradiograph, after SDS/PAGE, showing the *in vitro* import of G1ANT, FUSION and DHFR into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were at 25 °C for 30 min with 40 µl of pG1ANT and pFUSION rabbit reticulocyte lysate translation products and 20 µl of pDHFR rabbit reticulocyte lysate translation products.

- Lane 1; molecular weight markers (kDa).
- Lane 2; pFUSION translation products associated with mitochondria after treatment with proteinase K and Triton X-100.
- Lane 3; blank.
- Lane 4; pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 5; pG1ANT translation products associated with mitochondria.
- Lane 6; pG1ANT translation products associated with mitochondria after treatment with proteinase K.
- Lane 7; pFUSION translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 8; pFUSION translation products associated with mitochondria.
- Lane 9; pFUSION translation products associated with mitochondria after treatment with proteinase K.
- Lane 10; pDHFR translation products associated with mitochondria after treatment with proteinase K.
- Lane 11; pDHFR translation products associated with mitochondria.
- Lane 12; pDHFR translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 13; pDHFR translation products equivalent to that added to a 1 x import reaction treated with proteinase K.
- Lane 14; pFUSION translation products equivalent to that added to a 1 x import reaction treated with proteinase K.
- Lane 15; pG1ANT translation products equivalent to that added to a 1 x import reaction treated with proteinase K.

Figure 4.16.



4.9. Import of the *Zea mays* adenine nucleotide translocator after removal of the N-terminal end.

The results from Figure 4.16 indicate that the N-terminal region of the *Z. mays* ANT contains information allowing the targeting of DHFR into isolated mitochondria. ANT proteins from *S. cerevisiae* and *N. crassa*, however, must contain internal targeting information, since these proteins are not processed upon import into isolated mitochondria (Smagula and Douglas, 1988; Pfanner and Neupert, 1987b).

To determine whether the *Z. mays* ANT protein contains internal targeting information a fifth construct was made, termed pINTERNAL. The 1053 bp *Pvu* II - *Hind* III fragment was isolated from pGA3 and cloned into pBS M13+ digested with *Sma* I and *Hind* III (Figure 4.17). This would contain 289 amino acid residues out of the 387 amino acids encoded by *ANT-A* (assuming ATG 1 is used as the start of translation).

To allow run-off transcription, pINTERNAL was linearised at the unique *Hind* III restriction endonuclease site and the RNA generated was used to programme a rabbit reticulocyte lysate translation system. The pINTERNAL translation products were then incubated with isolated *Z. mays* mitochondria (\pm 20 mM 1,10-phenanthroline) at 25 °C for 30 min. 2 x import reactions were performed with 100 μ g total mitochondrial protein as described in section 2.6.3. To determine that import had occurred the reactions were then divided and one half treated with 100 μ g/ml proteinase K. As a control, G1ANT was also imported into the isolated *Z. mays* mitochondria. Proteins were then fractionated by SDS/PAGE and the autoradiograph of the gel is shown in Figure 4.18.

Results show that the *in vitro* expression of pINTERNAL generates a major protein with an estimated molecular weight of 27 kDa (lane 11). This is smaller than the processed G1ANT protein (lanes 5 and 6) and therefore indicates that the processing site lies within the first 373 bp of *ANT-A*. This smaller, internal ANT protein, however, is still imported into mitochondria (lane 8) and remains resistant to exogenous proteinase K (lane 7). When an import reaction is treated with proteinase K and Triton X-100, however, the internal *Z. mays* ANT protein is completely digested (lane 14).

It is unclear, however, from the results shown in Figure 4.18 whether the proteinase K has completely digested the pINTERNAL translation products (lane 13), since the molecular weight of this protein coincides with a smear of

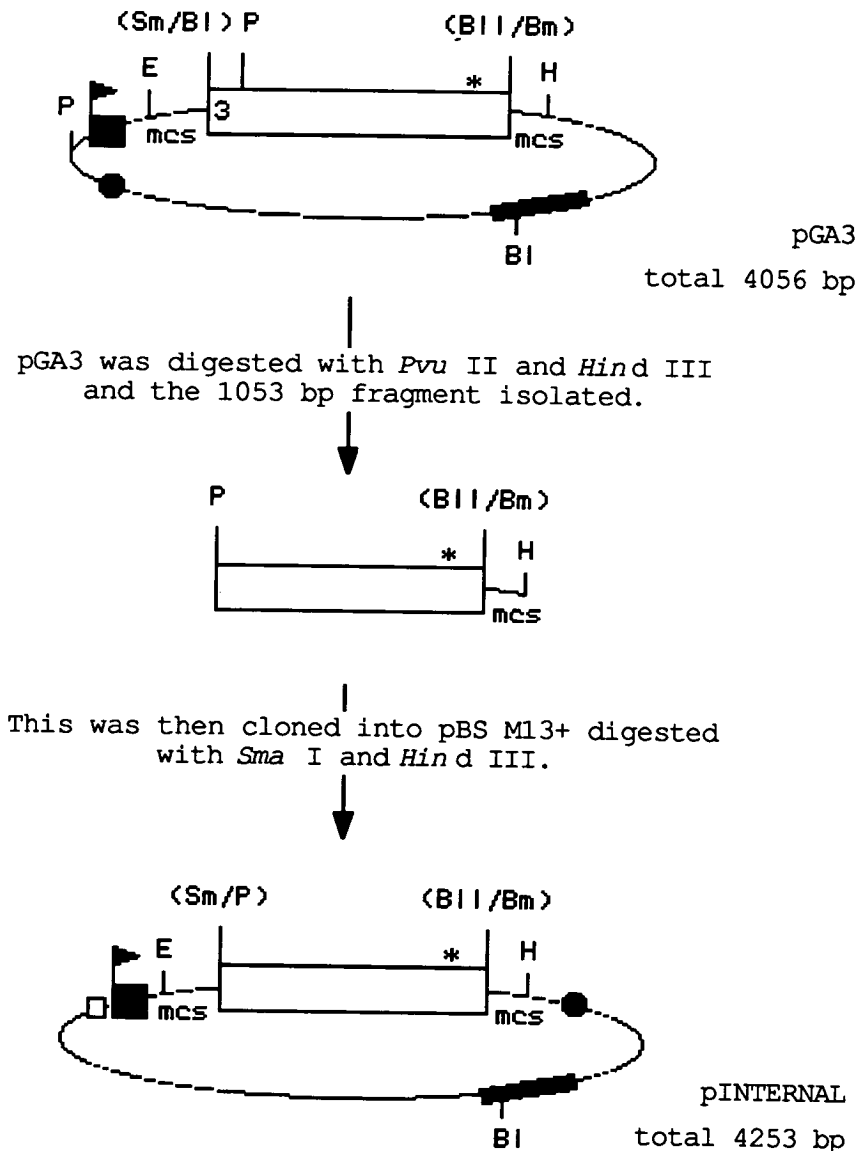


Figure 4.17; The construction of pINTERNAL.

The 1053 bp *Pvu* II - *Hin* d III *ANT-A* fragment from pGA3 was cloned into pBS M13+. This cloning procedure allowed transcription from the T7 promoter, represented by the black square (the direction of transcription being indicated by the arrow). The restriction endonuclease recognition sites are marked; Sm; *Sma* I, B1; *Bgl* I, P; *Pvu* II, B11; *Bgl* II, Bm; *Bam* HI, H; *Hin* dIII and *Eco* RI (those marked within brackets have been lost). The third in frame ATG codon of *ANT-A* (ATG 3) is represented as 3 and the stop codon is marked with an asterisk. The origin of replication is represented as the black circle, the ampicillin gene as the black rectangle and the multiple cloning sites as mcs. The binding site of the M13 -20 universal primer is represented as the open box.



Figure 4.18; Autoradiograph, after SDS/PAGE, showing the *in vitro* import of pG1ANT and pINTERNAL rabbit reticulocyte translation products into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 μ g total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were at 25 $^{\circ}$ C for 30 min with 40 μ l of pG1ANT and pINTERNAL rabbit reticulocyte lysate translation products. Where appropriate 20 mM 1,10-phenanthroline was included in the reaction.

- Lane 1; molecular weight markers (kDa)
- Lane 2; pG1ANT translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 3; blank.
- Lane 4; pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 5; pG1ANT translation products associated with mitochondria.
- Lane 6; pG1ANT translation products associated with mitochondria after treatment with proteinase K.
- Lane 7; pINTERNAL translation products associated with mitochondria after treatment with proteinase K.
- Lane 8; pINTERNAL translation products associated with mitochondria.
- Lane 9; pINTERNAL translation products associated with mitochondria incubated with 1,10-phenanthroline after treatment with proteinase K.
- Lane 10; pINTERNAL translation products associated with mitochondria incubated with 1,10-phenanthroline.
- Lane 11; pINTERNAL translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 12; blank.
- Lane 13; pINTERNAL translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 14; pINTERNAL translation products associated with mitochondria after treatment with proteinase K and Triton X-100.

radiolabelled proteins, which are presumably produced through the action of the proteinase K (compare lane 11 and 13). This is not unusual, and such a smear of radiolabelled proteins is seen each time this control is performed (compare lane 2 and 13).

The results suggest, however, that the *Z. mays* ANT, like those from *S. cerevisiae* and *N. crassa* contains internal targeting information and as expected, the internal *Z. mays* ANT protein is not processed unlike G1ANT (compare lanes 8 and 7 with lanes 5 and 6). The presence of 20 mM 1,10-phenanthroline, however, appeared to decrease the amount of the internal ANT protein associated with the isolated mitochondria (compare lanes 7 and 9).

4.10. Resequencing of *ANT-A*.

Although attempts to sequence the fusion junction between the 5' region of *ANT-A* and the complete mouse DHFR gene from the M13 primer were unsuccessful (section 4.7), a number of nucleotide differences were discovered within the first 268 bp of *ANT-A*. A synthetic oligonucleotide (5' -GCACAGAGCGGGGTCTG- 3') which hybridises to the sense *ANT-A* strand at 308 bp to 292 bp (Figure 4.1) was used to confirm the sequence obtained with the M13 -20 primer. Table 4.1 summarises these sequencing errors and the effect that these have on the 5' region of *ANT-A* is illustrated in Figure 4.19A.

Table 4.1 shows that resequencing the 5' region of *ANT-A* revealed 10 nucleotide sequencing errors within the first 268 bp. The presence of the T nucleotide and the absence of the G nucleotide (errors 5 and 10 respectively) were perhaps the most significant. The presence of the T nucleotide between 133 and 134 bp resulted in a frame shift and the TGA stop codon at 229 bp was lost. The original ORF, however, was regained due to the absence of the G nucleotide at 253 bp.

The corrected 5' nucleotide and deduced amino acid sequence of this 5' region of *ANT-A* is illustrated in Figure 4.19A. Analysis of the sequence shows that ATG 1 remains unchanged and is marked ATG 1 as before. This, however, is now predicted to be the start of translation, since the in frame TGA stop codon between ATG 1 and ATG 3 is no longer present. ATG 2 is lost, but a second ATG (ATG 2') occurs 147 bp 3' to ATG 1. In fact, ATG 2' contains the T and G nucleotides of the TGA stop codon previously thought to exist. Like ATG 1, ATG 3 also remains unchanged, since the original ORF has been regained through the absence of the G nucleotide (error 10). An ORF of 1161 bp encoding a protein of 387 amino acid

Figure 4.19; The nucleotide and deduced amino acid sequence of the 5' region of *ANT-A* after resequencing (**A**) and the original *ANT-A* sequence (**B**).

Sequencing errors are underlined and numbered 1-10. The first three in frame ATG codons are marked 1, 2' and 3 and underlined. The new 1161 bp ORF begins at ATG 1. The TGA stop codon (underlined and marked with an asterisk in **B**) is now out of frame due to the presence of the T nucleotide (error 5) between 136 bp and 138 bp. The absence of the G nucleotide (error 10) between 256 bp and 257 bp brings the original ORF lost through the presence of the T nucleotide back into frame. All nucleotide positions (bp) are given with respect to the new nucleotide sequence. The unique restriction endonuclease recognition sites *Bal* I and *Bgl* I are marked and underlined.

Figure 4.19.

A.

```

1      1
      CGGCCTCTGAAAGTTCGGAGCCGCCGGCCCGTCCGCCTCCCTCT      45

46     2      3      4      1
      CCGCCTCCCGTCCCCAAGGCCAATAGTTTTCAAGTGGCAATGGCGGAC      93
                                     M A D

94     CAGGCTAACCAACCCACTGTCCTTCATAAGCTCGGTGGCCAGTTCCAC      141
      Q A N Q P T V L H K L G G Q F H
                                     Bal I 5

142    67      8
      CTGCGCTCGATCATCTCTGAAGGTGTACGGGCCCGTAACATATGCCCA      189
      L R S I I S E G V R A R N I C P

190    9      2'
      TCTGTCTCATCTTATGAAAGGAGATTTGCCACAAGGAACTACATGACC      237
      S V S S Y E R R F A T R N Y M T

10
      G
      Bgl I      Δ 3
238    CAGAGCCTTTGGGGCCCTTCAATGTCTGTT
      Q S L W G P S M S V
  
```

B.

```

1      GCGCCTCTGAAAGTTCGGAGCCGCCGGCCCGTCCGCCTCCCTCTCGCC      48

49     1
      TCCGTCCCAAGGCCAATAGTTTTCAGGTGGCAATGGCGGACCAGGCT      96
                                     Bal I

97     AACCAACCCACTGTCCTTCATAAGCTCGGTGGCCAGTCCACCTGGCCT      144

145    CGATCATCTCTGAAGGTGTACGGGCCCGTAACATCTGCCCATCTGTCT      192

193    2      *
      CATGTTATGAAAGGAGATTTGCCACAAGGAACTACATGACCCAGAGCC      240

241    Bgl I      3
      TTTGGGGCCCTTGCATGTCTGTT
      M S V
  
```

residues is now predicted. The calculated molecular weight of this protein is 42.4 kDa.

Table 4.1; The nucleotide sequencing errors found in the 5' region of *ANT-A*.

NUMBER	ERROR	POSITION (bp)^a
1	presence of a C	- 1
2	presence of a C	between 45 and 46
3	presence of a C	between 51 and 52
4	presence of an A not a G	75
5	presence of a T	between 133 and 134
6	presence of a C not a G	141
7	presence of a G not a C	142
8	presence of a A not a C	179
9	presence of a C not a G	196
10	absence of a G	253

^aThe position of the sequencing errors are given in relation to the original *ANT-A* sequence (see Figure 4.1)

The complete resequencing of *ANT-A* has now been achieved and the cDNA has been renamed *MANT-1* (Winning *et al.*, 1991 and Figure 4.20). A number of additional errors were also found within the nucleotide sequence and these are summarised in Table 4.2. One error (error 3 in Table 4.2) resulted in a change in the deduced amino acid sequence from a tyrosine residue (Y) to an asparagine residue (N). Interestingly, this amino acid change increases the homology of the *Z. mays* ANT protein with those from other organisms (section 4.15). The absence of the A nucleotide at position 1257 (error 4) resulted in the loss of the second TAA stop codon in the original *ANT-A* sequence (compare Figure 4.1 and Figure 4.20).

Figure 4.20 continued.

814	AAGACACTCAAGTCTGATGGTATTGCTGGGCTTTACCGTGGATTTAAC	861
	K T L K S D G I A G L Y R G F N	
862	ATCTCCTGTGTTGGAATCATTGTTTATCGTGGTCTGTACTTTGGGCTC	909
	I S C V G I I V Y R G L Y F G L	
910	TATGATTCCATCAAGCCAGTTGTCCTTACTGGCAACCTCCAGGACAAT	957
	Y D S I K P V V L T G N L Q D N	
958	TTCTTTGCCAGTTTCGCTCTGGGTGGCTGATCACTAATGGTGCTGGT	1005
	F F A S F A L G W L I T N G A G	
1006	CTTGCATCTTACCCCATCGATACCGTCCGCAGAAGGATGATGATGACA	1053
	L A S Y P I D T V R R R M M M T	
1054	TCTGGTGAGGCTGTCAAGTACAAGAGCTCCTTGGACGCGTTCCAGCAG	1101
	S G E A V K Y K S S L D A F Q Q	
1102	ATTCTTAAGAAGGAAGGGCCCAAGTCCCTGTTCAAGGGTGTGGTGTCT	1149
	I L K K E G P K S L F K G A G A	
1150	AACATTCTTCGTGCCATTGCTGGTGCTGGTGTGCTTTCTGGCTATGAC	1197
	N I L R A I A G A G V L S G Y D	
1198	CAGCTCCAGATCCTCTTCTTCGAAAGAAGTACGGCTCCGGCGGTGCT	1245
	Q L Q I L F F G K K Y G S G G A	
	*	
1246	<u>TAAATGGAGAAATATGATGACGAACAAGAGCAGTGTGTGTTCCCGGTC</u>	1293
1294	CTTTCCAATCAGGATCTGGTGAAGTTTTGCCTTTCATATCGAAGAAA	1341
1342	TTAATAATTCATGTAGAGGGAGGATTCTTCCAACAATTTGTTTTGGAT	1389
	<i>Bgl</i> II	
1390	GGAGACTT <u>AGATCT</u> CCAACACTCGTAACTATCCTACTAGTGTGACAT	1437
1438	CGGTCCCCAAAAACATTTATGGTCATAGAGTCTGCCCTTCGTTTGTCA	1485
1486	GTCGGCAATTTTCTTGCATACAATGCCTTCGTTTAAAGTATCGGATG	1533
1534	AGTTGCTGCGACTAATTATTTGTTTGTCTTTACCTTTTGCAAAA	

Figure 4.20; The complete nucleotide and deduced amino acid sequences of *MANT-1* (Winning *et al.*, 1991).

The 1577 bp cDNA contains a 1161 bp ORF beginning at 85 bp and ending at 1246 bp (the ATG codon ATG 1 is marked 1 and underlined). Homology to the genomic clone *ANT-G1* begins at 64 bp and ends at 1425 bp (Bathgate *et al.*, 1989). The first 63 bp are of unknown origin and are underlined. Homology to the 1136 bp cDNA pANT-1 begins at 290 bp and ends at 1425 bp (Baker and Leaver, 1985). The stop codon is marked with an asterisk and underlined. The N-terminal sequence obtained from the isolated *Z. mays* ANT protein (section 4.12) is indicated below the deduced amino acid sequence (Liddell pers. comm.) and the predicted processing site is marked with an arrow. The unique restriction endonuclease recognition sites *Bal* I, *Bgl* I *Pvu* II and *Bgl* II are marked and underlined.

Table 4.2; Additional nucleotide sequencing errors found in *MANT-1*.

NUMBER	ERROR	POSITION (bp) ^a
1	presence of a T not an A	540
2	presence of an A not a T	541
3	presence of a G not an A	903
4	absence of an A	1257
5	presence of an A not a T	1260
6	presence of a T not an A	1261
7	presence of an A not a T	1330

^aThe position of the sequencing errors are given in relation to the original *ANT-A* sequence (see Figure 4.1)

Winning pers. comm.

4.11. Resequencing of *ANT-G1*.

To confirm these sequencing errors both strands of the 5' region of the genomic clone, *ANT-G1*, were resequenced. *ANT-G1* is predicted to be the *Z. mays* ANT genomic clone corresponding to the cDNA *ANT-A* (Bathgate *et al.*, 1989). The same errors were therefore expected to be found. It should be noted, however, that the genomic clone and the cDNA were isolated from different lines of *Z. mays* (section 2.1.4.1 and 2.1.4.2). It can not, therefore, be said for certain that these two clones are identical.

In order to resequence the 5' region of *ANT-G1*, the 1645 bp *Bgl* II restriction endonuclease fragment was isolated and cloned into pBS M13+, which had been digested with *Bam* HI. The *Sac* I recognition sites within the gene and the multiple cloning site were then used to determine the orientation and a clone which would allow sequencing of the 5' end from the M13 -20 universal primer was retained and termed pCS90.8 (Figure 4.21). In order to sequence the non-sense strand, the same synthetic oligonucleotide (5'- GCACAGAGCGGGTCTG -3') was used as before (section 4.10).

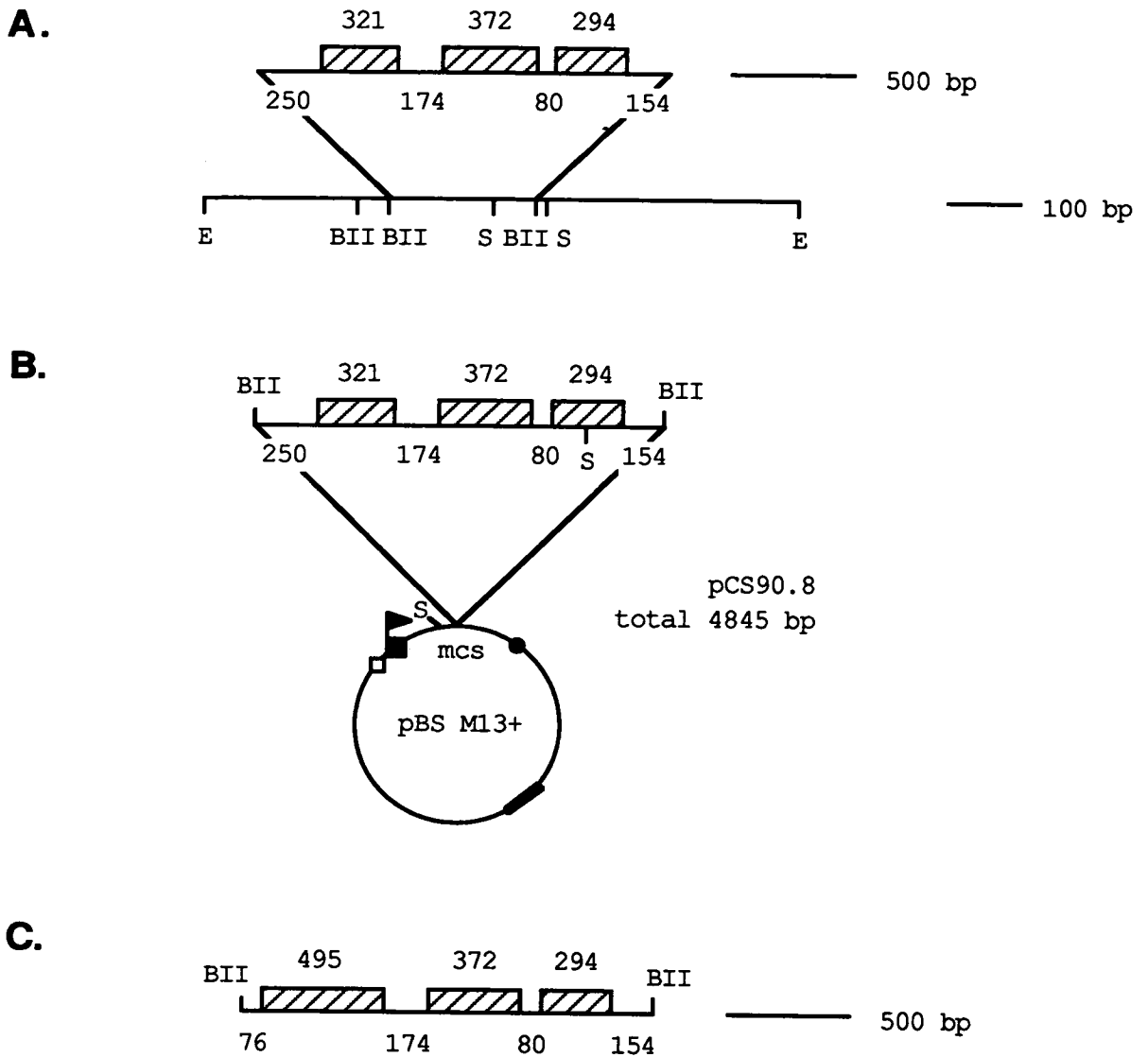


Figure 4.21; A diagrammatic representation of the *Z. mays* *ANT* genomic clone *ANT-G1* (adapted from Bathgate *et al.*, 1989).

A. The 8 kbp *Eco* RI fragment containing *ANT-G1*.

The restriction endonuclease recognition sites are marked as E; *Eco* RI, BII; *Bgl* II and S; *Sac* I. The enlarged region shows the length of the three exons (represented by the hatched rectangles) and the 5' and 3' non-coding DNA and introns (represented by the single lines). The length of the exons and non-coding DNA is marked above and below respectively (bp).

B. The cloning of the 1645 bp *Bgl* II fragment into pBS M13+.

This cloning procedure would allow sequencing of the 5' end of *ANT-G1* from the M13 -20 primer (the binding site of which is represented by the open square). The orientation of the fragment was determined through digestion with the restriction endonuclease *Sac* I (S).

C. The corrected sequence of *ANT-G1* after resequencing the 5' region.

The length of the exons (represented by the hatched rectangles) and the 5' and 3' non-coding DNA (represented by the single lines) are marked as in A. The first exon is now extended 174 bp upstream due to the sequencing errors discovered in the 5' region.

Results indicated that the same errors found in *ANT-A* were duplicated in *ANT-G1* (not shown). Preliminary sequencing of the 5' region of *ANT-G2*, a second *Z. mays* ANT clone (Bathgate *et al.*, 1989) has also revealed the addition of the T nucleotide and the absence of the G nucleotide (Day pers. comm.). It therefore appears that both of the *Z. mays* ANT genes contain a much larger ORF than was originally predicted.

4.12. N-terminal protein sequence of the *Zea mays* adenine nucleotide translocator.

Previous attempts to sequence the N-terminus of the *Z. mays* ANT protein had been unsuccessful, but because of the data obtained from the *in vitro* import studies renewed efforts were made by A. Liddell. The results in Figure 4.20 show the N-terminal protein sequence obtained (Liddell pers. comm.) in relation to the nucleotide and deduced amino acid sequence of *MANT-1*.

The N-terminal protein sequencing data indicates that the processing site lies between the asparagine residue (N) and the alanine residue (A) which are 77 and 78 amino acids away from the methionine encoded by ATG 1. A presequence of 77 amino acids is therefore predicted for the *Z. mays* ANT, which has a calculated molecular weight of 8.5 kDa. This extension is only 3 amino acids shorter than that of the yeast protein cytochrome *b₂*, which is the longest mitochondrial presequence known to date (Guiard, 1985).

4.13. An adenine nucleotide translocator from *Solanum tuberosum* is processed upon import into isolated *Zea mays* mitochondria.

The experiments outlined in this section and the next were performed in collaboration with Dr. B.M. Winning. The experimental evidence presented above indicates that the *Z. mays* ANT, unlike its fungal counterparts, is synthesised with an N-terminal presequence, which is processed upon import into isolated mitochondria (Figure 4.3). The import of the plant ANT protein therefore appears to be distinctly different to that of *S. cerevisiae* or *N. crassa*. The availability of a *Solanum tuberosum* ANT cDNA clone, *PANT-1*, although not full-length (Winning pers. comm.), enabled the *in vitro* import of a second plant ANT to be investigated.

This cDNA had been cloned into an *in vitro* expression vector and termed pPOTANT (not shown). RNA generated by the *in vitro* transcription of pG1ANT and

pPOTANT was used to programme a rabbit reticulocyte lysate translation system. The translation products were then incubated with isolated *Z. mays* mitochondria at 25 °C for 30 min. 2 x import reactions were performed (+/- 20 mM 1,10-phenanthroline) with 100 µg total mitochondrial protein as described in section 2.6.3. After the incubation was complete the reactions were divided and one half was treated with 100 µg/ml proteinase K. Proteins were then fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 4.22.

Results show that the *in vitro* translation of the *S. tuberosum* ANT synthesises a precursor protein (lanes 13), which is processed upon import into isolated *Z. mays* mitochondria (lane 11). The *S. tuberosum* ANT runs with an estimated molecular weight of 36 kDa and is processed to a mature protein of 30 kDa. The *S. tuberosum* ANT is imported, since the processed protein remains resistant to exogenous proteinase K (lane 10). A volume of pPOTANT translation products equivalent to that added to a 1 x import reaction are also completely digested by the same amount of proteinase K (lane 12).

When 20 mM 1,10-phenanthroline is included in the import reaction the processing of the *S. tuberosum* ANT is inhibited (compare lanes 9 and 11). Without 1,10-phenanthroline in the reaction there is complete protection of the processed *S. tuberosum* ANT from the exogenous proteinase K and little of the precursor remains (lane 10). With the inclusion of 1,10-phenanthroline, however, the *S. tuberosum* ANT precursor remains resistant to the exogenous protease indicating that this has been imported, but not processed (compare lanes 8 and 10). The processing of the *S. tuberosum* ANT, however, is not completely inhibited by the inclusion of 1,10-phenanthroline in the import reaction (lanes 8 and 9).

It should be noted that in this experiment the *Z. mays* ANT precursor and the mature protein, ran with an estimated molecular weight of 38 kDa and 31 k Da respectively. This is 1 kDa larger than that previously noted.

4.14. The *Zea mays* and *Solanum tuberosum* adenine nucleotide translocator proteins are processed upon import into isolated *Solanum tuberosum* mitochondria.

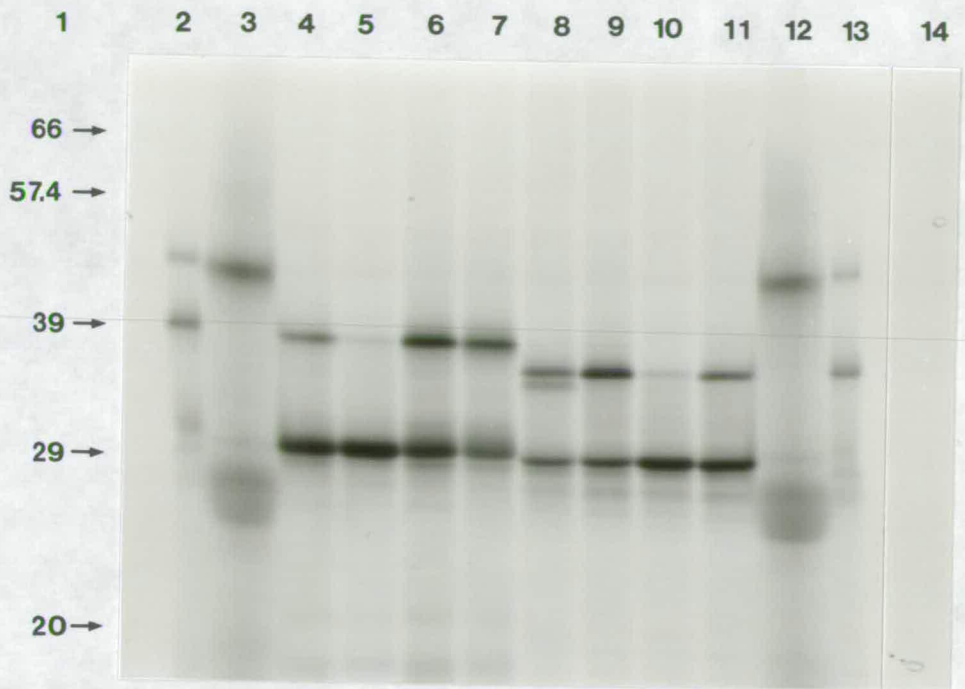
The *S. tuberosum* ANT was synthesised as a precursor protein, which was processed upon import into isolated *Z. mays* mitochondria. To determine whether a similar processing event would occur upon import into mitochondria isolated from a

Figure 4.22; Autoradiograph, after SDS/PAGE, showing the *in vitro* import of pG1ANT and pPOTANT translation products into *Z. mays* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein isolated from 3 day old dark-grown coleoptiles. Incubations were at 25 °C for 30 min with 20 µl of pG1ANT and pPOTANT rabbit reticulocyte lysate translation products. Where appropriate 20 mM 1,10-phenanthroline was included in the reaction.

- Lane 1; molecular weight markers (kDa).
- Lane 2; pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 3; pG1ANT translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 4; pG1ANT translation products associated with mitochondria.
- Lane 5; pG1ANT translation products associated with mitochondria after treatment with proteinase K.
- Lane 6; pG1ANT translation products associated with mitochondria incubated with 1,10-phenanthroline.
- Lane 7; pG1ANT translation products associated with mitochondria incubated with 1,10-phenanthroline after treatment with proteinase K.
- Lane 8; pPOTANT translation products associated with mitochondria incubated with 1,10-phenanthroline after treatment with proteinase K.
- Lane 9; pPOTANT translation products associated with mitochondria incubated with 1,10-phenanthroline.
- Lane 10; pPOTANT translation products associated with mitochondria after treatment with proteinase K.
- Lane 11; pPOTANT translation products associated with mitochondria.
- Lane 12; pPOTANT translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 13; pPOTANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 14; pPOTANT translation products associated with mitochondria after treatment with proteinase K and Triton X-100.

Figure 4.22.



different plant species, the import of the *Z. mays* and *S. tuberosum* ANT proteins into mitochondria isolated from *S. tuberosum* was investigated.

RNA generated by the *in vitro* transcription of pG1ANT and pPOTANT was used to programme a rabbit reticulocyte lysate translation system. The translation products were then incubated at 25 °C for 30 min with *S. tuberosum* mitochondria isolated from tubers according to section 2.6.1.2. 2 x import reactions were performed (+/- 20 mM 1,10-phenanthroline) with 100 µg total mitochondrial protein as described in section 2.6.3. After the incubation was complete the reaction was then divided and one half was treated with 100 µg/ml proteinase K. Proteins were then fractionated by SDS/PAGE. The autoradiograph of the gel is shown in Figure 4.23.

Results show that both the *Z. mays* and *S. tuberosum* ANT precursor proteins are processed upon import into isolated *S. tuberosum* mitochondria (lanes 4 and 11). These processed proteins are imported, since they remain resistant to exogenous proteinase K (lanes 5 and 10). A volume of pG1ANT and pPOTANT translation products equivalent to that added to a 1 x import reaction, however, are completely digested with the same amount of proteinase K (lanes 3 and 12).

When 20 mM 1,10-phenanthroline is included in the import reaction the processing of the *Z. mays* and *S. tuberosum* ANT proteins is inhibited (lanes 6 and 9). The unprocessed ANT precursor proteins remain resistant to the exogenous protease (lanes 7 and 8), but to a lesser extent than when the same experiment was performed with isolated *Z. mays* mitochondria (Figure 4.22). The estimated molecular weights of the processed *Z. mays* and *S. tuberosum* ANT proteins are identical to those obtained upon import into isolated *Z. mays* mitochondria (section 4.13).

The availability of this *S. tuberosum* cDNA has therefore enabled the import of a second plant ANT to be investigated. The results have corroborated the findings obtained from the *in vitro* import of the *Z. mays* ANT, in that plant ANT proteins appear to be synthesised as precursors with N-terminal extensions, which are processed upon import into mitochondria.

4.15. Comparison of the amino acid sequences of adenine nucleotide translocator proteins from different organisms.

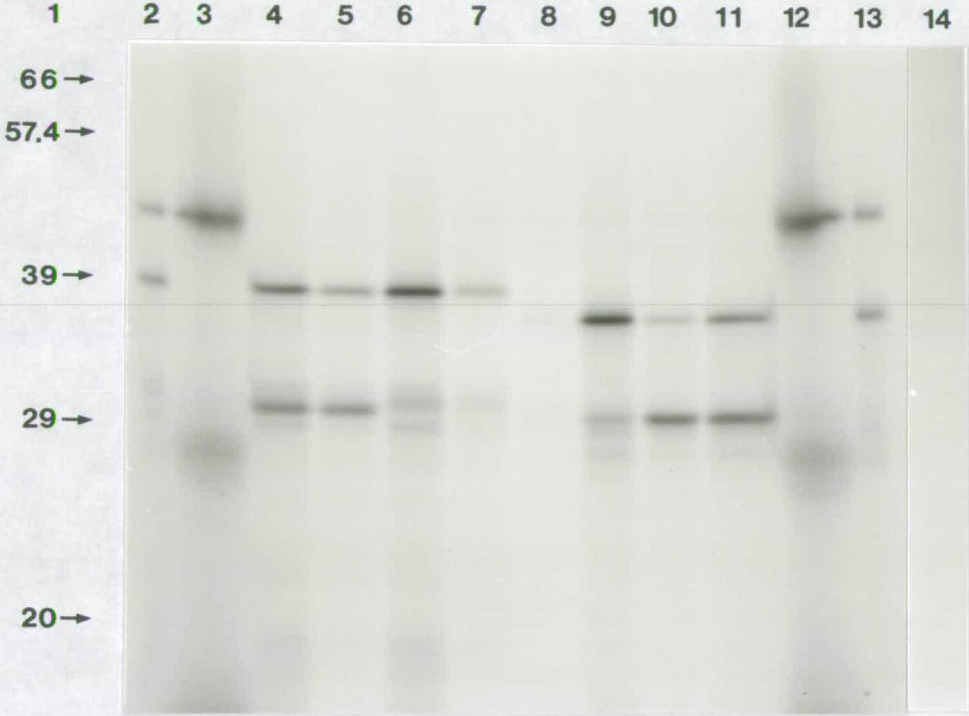
Alignment of the deduced ANT amino acid sequences from the *Z. mays* MANT-1 and *S. tuberosum* PANT-1 cDNAs, indicates that these two plant ANT proteins are

Figure 4.23; Autoradiograph, after SDS/PAGE, showing the *in vitro* import of pG1ANT and pPOTANT translation products into *S. tuberosum* mitochondria.

2 x import reactions were performed according to section 2.6.3 with 100 µg total mitochondrial protein isolated from *S. tuberosum* tubers. Incubations were at 25 °C for 30 min with 20 µl of pG1ANT and pPOTANT rabbit reticulocyte lysate translation products. Where appropriate 20 mM 1,10-phenanthroline was included in the reaction.

- Lane 1; molecular weight markers (kDa).
- Lane 2; pG1ANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 3; pG1ANT translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 4; pG1ANT translation products associated with mitochondria.
- Lane 5; pG1ANT translation products associated with mitochondria after treatment with proteinase K.
- Lane 6; pG1ANT translation products associated with mitochondria incubated with 1,10-phenanthroline.
- Lane 7; pG1ANT translation products associated with mitochondria incubated with 1,10-phenanthroline after treatment with proteinase K.
- Lane 8; pPOTANT translation products associated with mitochondria incubated with 1,10-phenanthroline after treatment with proteinase K.
- Lane 9; pPOTANT translation products associated with mitochondria incubated with 1,10-phenanthroline.
- Lane 10; pPOTANT translation products associated with mitochondria after treatment with proteinase K.
- Lane 11; pPOTANT translation products associated with mitochondria.
- Lane 12; pPOTANT translation products equivalent to that added to a 1 x import reaction after treatment with proteinase K.
- Lane 13; pPOTANT translation products equivalent to 10 % of that added to a 1 x import reaction.
- Lane 14; pPOTANT translation products associated with mitochondria after treatment with proteinase K and Triton X-100.

Figure 4.23.



much larger than those from *N. crassa*, *S. cerevisiae* or *B. taurus* (Figure 4.24). Both plant ANT protein sequences extend beyond the N-termini of these other proteins. This is consistent with the hypothesis that plant ANT proteins are synthesised as larger precursors with N-terminal extensions, which are processed upon import into mitochondria. The homology between the *Z. mays* and *S. tuberosum* ANT N-terminal extensions is only 21 % compared to 89 % found within the mature proteins. This is not unusual, since it is known that mitochondrial targeting presequences show little primary sequence homology (section 1.3.3).

Resequencing of the entire *Z. mays* ANT cDNA resulted in the discovery of an additional 7 nucleotide errors (section 4.10). One of these errors (error 3 in Table 4.2) resulted in the change of a tyrosine residue (Y) to an asparagine residue (N). This alteration in the deduced *Z. mays* ANT protein sequence is marked with an asterisk in Figure 4.24 and results in the increased homology of the *Z. mays* ANT with those from *S. tuberosum*, *N. crassa*, *S. cerevisiae* and *B. taurus*.

4.16. Conclusions.

The availability of a full-length *Z. mays* ANT cDNA, *ANT-A*, enabled the import of this protein to be investigated *in vitro*. Results presented above indicate that the *in vitro* expression of *ANT-A* synthesised a protein (G1ANT) with an estimated molecular weight of 37 kDa. Upon import into isolated mitochondria this was processed to a mature protein with an estimated molecular weight of 30 kDa (Figure 4.3). This processing event occurred upon incubation with mitochondria isolated from both *Z. mays* coleoptiles and *S. tuberosum* tubers (section 4.13 and 4.14). The processed *Z. mays* ANT protein remains resistant to proteinase K, but when Triton X-100 was included in this reaction the protein was completely degraded, indicating that import had occurred. The processing of the *Z. mays* ANT could be inhibited by the inclusion of 20 mM 1,10-phenanthroline in the reaction (Figure 4.4). These results indicate that the import of the *Z. mays* ANT is distinctly different to that of *S. cerevisiae* and *N. crassa*, which unlike the majority of nuclear-encoded mitochondrial proteins are synthesised without an N-terminal extension, or presequence, and are therefore not processed upon import into mitochondria.

The import of the *Z. mays* ANT was therefore investigated in more detail. The results described above indicated that the *Z. mays* ANT was synthesised with an extension of 7 kDa. Comparison of the deduced N-terminal amino acid sequence with those from *S. cerevisiae*, *N. crassa* and *Bos taurus*, however, suggested that such a

Figure 4.24.

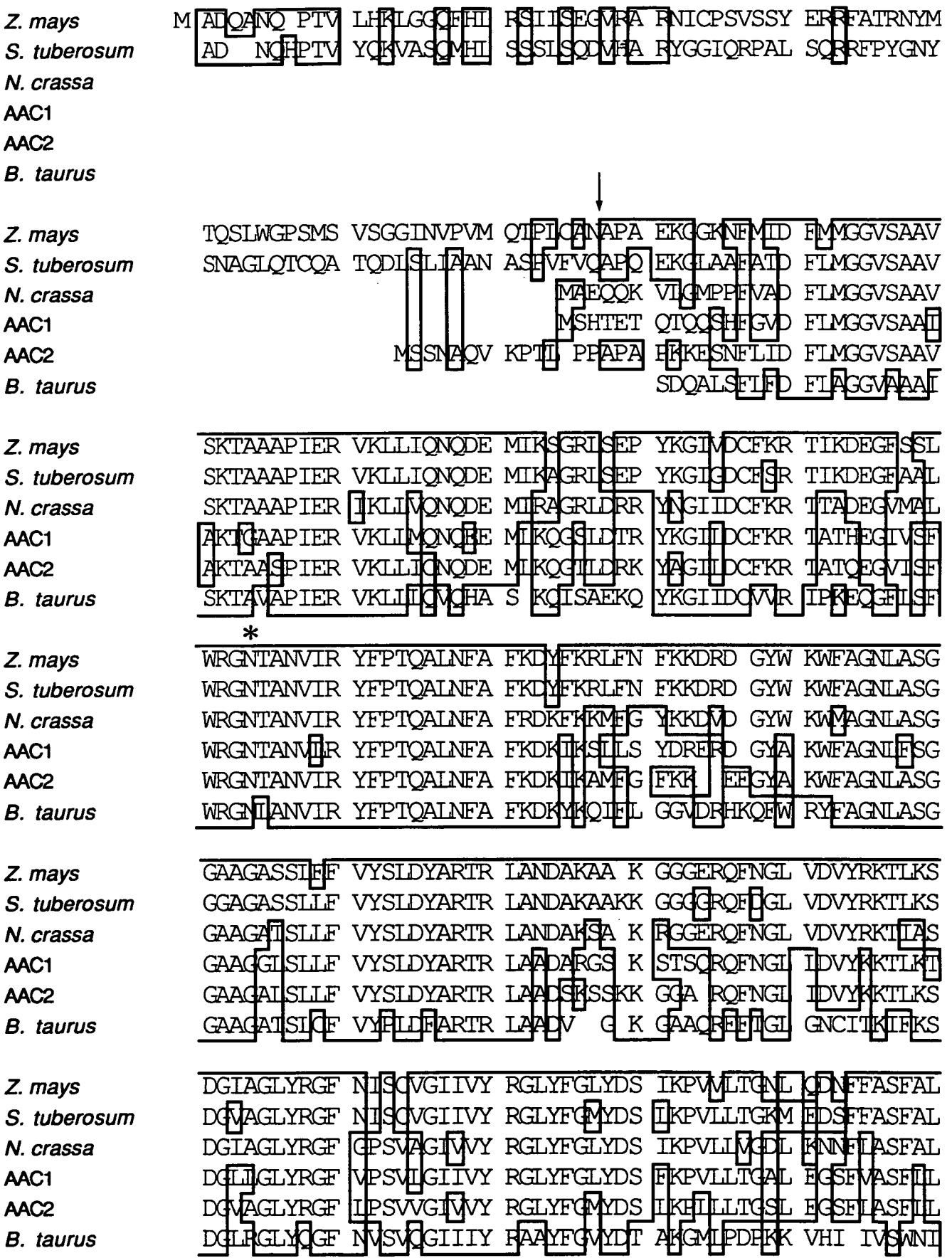


Figure 4.24 continued.

<i>Z. mays</i>	GWLIITNGAGL	ASYPIDTVRR	RMMMTS	G	EAVKYKSSLD	AFDQILKKEG
<i>S. tuberosum</i>	GWLIITNGAGL	ASYPIDTVRR	RMMMTS	G	EAVKYKSSFED	RENQILKNEG
<i>N. crassa</i>	GWCVITTAAGL	ASYPEDTIRR	RMMMTS	G	EAVKYKSSFED	AASQIVAKEG
AAC1	GWLIITNGAST	ASYPIDTVRR	RMMMTS	G	QTIKYDGAID	CLRKIVQKEG
AAC2	GWVITIGAST	CSYPIDTVRR	RMMMTS	G	QAVKYDGAID	CLRKIVAAEG
<i>B. taurus</i>	AQTVIIVAVAGL	VSYFEDTVRR	RMMMSGGKG	ADIMYTGTVD	QWRKIIVKDEG	
<i>Z. mays</i>	PKSLFKGAGA	NILRAIAGAG	VLSGYDQLQI	LEFGKKYGS	GGGA	
<i>S. tuberosum</i>	PKSLFKGAGA	NILRAVAGAG	VLVGYDKLQV	IVFGKKYGS	GGGG	
<i>N. crassa</i>	VKSLFKGAGA	NILRGVAGAG	VLSTIYDQLQV	LIIFGKAFK	GGSG	
AAC1	AYSLFKGCGA	NILRGVAAG	VLSLYDQLQL	IMFGKKFK		
AAC2	VGSLFKGCGA	NILRGVAGAG	VLSMYDQLQM	ILFGKKFK		
<i>B. taurus</i>	PKAFKGAWS	NILRGMGAF	VLVLYDEIKK	FM		

Figure 4.24; A comparison of the amino acid sequence of the ANT proteins from *Z. mays*, *S. tuberosum*, *N. crassa*, *S. cerevisiae* and *B. taurus*. Sequences are aligned to maximise homology.

The *Z. mays*, *S. tuberosum* (Winning pers. comm.), *N. crassa* (Arends and Sebald, 1984) and *S. cerevisiae* AAC1 and AAC2 (Lawson and Douglas, 1988) sequences are deduced from the nucleotide sequences. The *B. taurus* sequence was determined directly (Aquila *et al.*, 1982). The arrow marks the predicted processing site within the *Z. mays* ANT protein. This was determined from N-terminal sequencing of the isolated *Z. mays* ANT protein (Liddell pers. comm.). The asterisk marks amino acid change after resequencing of the *Z. mays* cDNA, ANT-A (section 4.10).

processing event would occur within a region internal to the other ANT protein sequences (Figure 4.5). This therefore put into question whether ATG 3, the predicted start of translation (see Figure 4.1), was used in the rabbit reticulocyte lysate translation system.

A construct (pGA3) was made in which the 5' end of *ANT-A* was deleted (section 4.5.1). Despite the fact that ATG 3 was maintained, the *in vitro* expression of this clone synthesised a much smaller protein with an estimated molecular weight of 31 kDa (Figure 4.8). This was the first indication that ATG 3 was not used as the start of translation. Subsequent experiments, such as the deletion of the in frame ATG codon, ATG 1 (section 4.5.4) and the generation of a fusion protein between the 5' region of *ANT-A* and the complete DHFR coding sequence (section 4.7) indicated that the start of translation used in the rabbit reticulocyte lysate translation system was at ATG 1 rather than ATG 3.

It is now known that the initial nucleotide sequence of *ANT-A* contained a number of sequencing errors (section 4.10). This corrected sequence no longer contains the in frame TGA stop codon, which lay 27 bp upstream from ATG 3, but the ORF continues for an additional 174 bp from ATG 3 to ATG 1 (Figure 4.19). The complete ORF is therefore 1161 bp long and encodes a protein of 387 amino acids (Figure 4.20), which is much larger than the ANT proteins from *S. cerevisiae*, *N. crassa* or *B. taurus* (Figure 4.24). These results therefore corroborated the findings obtained from the *in vitro* expression of *ANT-A*. The complete resequencing of *ANT-A* has now been achieved and the cDNA has been renamed *MANT-1* (Winning *et al.*, 1991).

The protein sequence obtained after N-terminal sequencing of the isolated *Z. mays* ANT protein corresponded to the deduced amino acid sequence of the *Z. mays* ANT cDNA. The start of homology was at the alanine residue, 78 amino acids away from the methionine encoded by ATG 1 (Figure 4.20). This therefore indicates that the *Z. mays* ANT is synthesised with an N-terminal extension of 77 amino acids.

Targeting information appears to reside in both the internal region of the protein, as well as in the N-terminal extension. The fusion of the first 373 bp of 5' DNA from the *Z. mays* ANT cDNA to the complete coding sequence of DHFR resulted in the targeting of DHFR into isolated mitochondria (section 4.7 and Figure 4.16). This region of the *Z. mays* ANT cDNA, however, encoded 97 amino acid residues and would therefore contain 20 amino acid residues of the mature ANT protein sequence. This is discussed further in section 5.6.2. A construct, pINTERNAL, was also made in which the same 373 bp were deleted from the *Z. mays* ANT cDNA (section 4.9). Upon incubation with mitochondria, this shortened *Z. mays* ANT protein was also

imported (Figure 4.18). This, however, is not unexpected, since the processing site was predicted to lie within the 97 amino acid residues deleted from the ANT protein.

From the results presented in this chapter, it is therefore proposed that the *Z. mays* ANT is synthesised as a precursor protein with an N-terminal extension of 77 amino acids, which is processed upon import into mitochondria. As already mentioned, this is in contrast to the ANT proteins from *S.cerevisiae* and *N. crassa*, which are synthesised without N-terminal extensions. The import of this plant ANT therefore appears to be distinctly different to that described in these other organisms. Results with a second plant ANT protein indicate that this phenomenon is not unique to the *Z. mays* ANT, since the *S. tuberosum* ANT is synthesised as a larger protein, which is also processed upon import (Figures 4.22 and 4.23).

CHAPTER 5

DISCUSSION AND FUTURE EXPERIMENTS

5.1. Introduction.

Investigations of protein import into plant mitochondria have been very limited and few reports exist in the literature. The mechanisms of protein targeting and the import pathways involved have therefore yet to be fully elucidated. Work has been hampered primarily through the lack of isolated cDNAs and nuclear genes encoding plant mitochondrial proteins. Another major difficulty is the inability to introduce mutations affecting mitochondrial function, since these would be lethal to the plant. This, however, is not true of the yeast *Saacharomyces cerevisiae*, in which numerous genes have been isolated through *in vivo* complementation and the ability of this organism to grow anaerobically (Tzagoloff and Myers, 1986). This has allowed the mechanisms of fungal mitochondrial protein import be analysed in detail (see section 1.3). Preliminary studies have, however, demonstrated that protein import into isolated higher plant mitochondria is possible (White and Scandalios, 1987; Whelan *et al.*, 1988; Purdue, 1988).

5.2. Characterisation of a *Zea mays* mitochondrial *in vitro* import system.

The results presented in Chapter 3 describe a system allowing protein import into isolated *Zea mays* mitochondria. This was characterised and partially optimised with the nuclear-encoded mitochondrial protein manganese superoxide dismutase (MnSOD) from *Nicotiana plumbaginifolia* (Bowler *et al.*, 1989b). The import of this protein into isolated *Z. mays* mitochondria displayed many features characteristic of the *S. cerevisiae* and *Neurospora crassa* mitochondrial protein import systems.

MnSOD was synthesised as a precursor protein with an estimated molecular weight of 26.5 kDa. Upon import this was processed to a mature form with an estimated molecular weight of 24.5 kDa, which was comparable to that of 24 kDa previously estimated by Bauw *et al.* (1987). Protein import was characterised by the protection of the processed protein to exogenous proteinase K and the complete

digestion of an equivalent volume of translation products by the same amount of proteinase K. As a further control, an import reaction was performed and then treated with proteinase K and Triton X-100. This resulted in the complete digestion of the previously protected protein due to the disruption of mitochondria by the detergent (Figure 3.2).

The import of MnSOD was dependent upon exogenous ATP (Figure 3.9 and Figure 3.10) and an energised inner mitochondrial membrane (Figure 3.7). This suggests that the energy requirements for mitochondrial protein import in this plant system are similar to those of *S. cerevisiae* and *N. crassa*. The processing event which accompanied the import of MnSOD could be inhibited by the inclusion of 1,10-phenanthroline within the import reaction (Figure 3.6). This is a metal ion chelator and a known inhibitor of the mitochondrial matrix processing protease of *S. cerevisiae* and *N. crassa*. (McAda and Douglas, 1982; Bohni *et al.*, 1983). The inhibition of the *Z. mays* mitochondrial processing activity by 1,10-phenanthroline suggests that this is also dependent upon the presence of metal ions. This activity may therefore be similar to that of *S. cerevisiae* and *N. crassa*.

The import of MnSOD into isolated *Z. mays* mitochondria was only possible when the precursor was synthesised in a rabbit reticulocyte lysate translation system. In contrast, when a wheat germ S-30 system was used the MnSOD precursor could not be imported (section 3.7). It is known that rabbit reticulocyte lysate contains proteinaceous factors, which are required for the import of proteins into isolated *S. cerevisiae* mitochondria (Chen and Douglas, 1987b; Ono and Tuboi, 1988). Cytoplasmic factors from *S. cerevisiae* cell lysates have also been shown to stimulate the mitochondrial import of precursor proteins (Ohta and Schatz, 1984). These are thought to be heat shock proteins (hsps) and been give the name molecular chaperones, since by binding to a protein they prevent or disrupt inappropriate protein-protein interactions and maintain the precursor in an 'import-competent' configuration (see section 1.3.7). Import into this *Z. mays* mitochondrial protein import system may therefore be dependent on such factors present in the rabbit reticulocyte lysate translation system. When the wheat germ S-30 was included in the import reaction, the import of MnSOD synthesised in the rabbit reticulocyte lysate was inhibited (Figure 3.5). It remains unclear why this is so, but it could be speculated that the inclusion of the wheat germ S-30 may, somehow, prevent the MnSOD precursor from forming an 'import-competent' configuration.

The results presented in Chapter 3 demonstrate that the import of MnSOD into isolated *Z. mays* mitochondria displays many features characteristic of the *S.*

cerevisiae and *N. crassa* mitochondrial protein import systems. After the initial characterisation and optimisation of this *in vitro* protein import system, the import of a homologous protein, the *Z. mays*-adenine nucleotide translocator was investigated.

5.3. The *in vitro* import of the *Zea mays* adenine nucleotide translocator.

The adenine nucleotide translocator (ANT) is the most abundant protein of the inner mitochondrial membrane. It is an integral membrane protein and functions as a homodimer mediating the exchange of adenine nucleotides between the mitochondrial matrix and the cytosol (Klingenberg, 1985). It is encoded in the nucleus and must therefore be synthesised in the cytosol and imported into the mitochondrion. The import of ANT has been well characterised in both *S. cerevisiae* and *N. crassa*. and it has been found that ANT, unlike the majority of nuclear-encoded mitochondrial proteins, is synthesised without an N-terminal presequence and therefore is not processed upon import (Smagula and Douglas, 1988; Pfanner and Neupert, 1987b).

Although there is no change in the size of the protein, the precursor and mature forms can be distinguished by the specific binding of the inhibitor carboxyatractyloside. This only recognises the fully assembled and functionally active protein and binds noncompetitively and with high affinity. The binding of carboxyatractyloside to ANT has therefore been used as a parameter for import (Schleyer and Neupert, 1984).

The results presented in Chapter 4 suggest that the import of the *Z. mays* ANT is distinctly different from that of *S. cerevisiae* and *N. crassa*, since it is synthesised as a larger precursor protein (G1ANT) with an estimated molecular weight of 37 kDa, which is then processed upon import. The processed protein having an estimated molecular weight of 30 kDa (Figure 4.3). This processing event occurred not only upon import into isolated *Z. mays* mitochondria, but also into mitochondria isolated from *Solanum tuberosum* (Figure 4.23). In both cases, processing could be inhibited by the inclusion of 1,10-phenanthroline in the import reaction. This suggests that the *Z. mays* mitochondrial processing activity is dependent upon metal ions and therefore similar to that in *S. cerevisiae* and *N. crassa* (section 3.8). The same concentration of 1,10-phenanthroline inhibited the processing of MnSOD (Figure 3.6), but a soluble *Z. mays* mitochondrial fraction would only process MnSOD (not shown). It therefore remains unclear

whether the same enzyme is responsible for the processing of MnSOD and G1ANT. This is discussed further in section 5.9.5.

5.4 Is the *Zea mays* adenine nucleotide translocator synthesised with an N-terminal extension?

Originally the *Z. mays* ANT cDNA, *ANT-A*, was predicted to contain an ORF of only 987 bp (Figure 4.1). The ATG codon (ATG 3) was proposed to be the start of translation, since an TGA stop codon lay 27 bp upstream (Bathgate, pers. comm.). The deduced *Z. mays* ANT protein sequence was therefore only 17 amino acid residues longer than the *S. cerevisiae* AAC1 protein (Figure 4.5). From the import of the *Z. mays* ANT, an N-terminal extension of 6.95 kDa was predicted (section 4.5). Comparison of the N-terminal *Z. mays* ANT amino acid sequence with those of *S. cerevisiae*, *N. crassa* and *Bos taurus* ANT, however, indicated that the processing site would lie within a region internal to the other ANT protein sequences (Figure 4.5). These results, as well as the deletion analysis of the 5' region of *ANT-A* (section 4.5), put into question whether ATG 3 was used as the start of translation in the rabbit reticulocyte lysate system.

After resequencing the 5' region of *ANT-A*, a number of sequencing errors were discovered (Table 4.1). These resulted in the loss of the previously predicted TGA stop codon and the extension of the original ORF from ATG 3 to ATG 1 (Figure 4.19). An ORF of 1161 bp encoding a protein of 387 amino acid residues was therefore predicted. The complete resequencing of *ANT-A* has now been achieved and the cDNA has been renamed *MANT-1* (Winning *et al.*, 1991). Additional errors were discovered (Table 4.2), one of which resulted in a change in the deduced amino acid sequence from a tyrosine (Y) amino acid residue to an asparagine (N) residue. Interestingly, this increased the homology between the *Z. mays* ANT protein and those from *S. cerevisiae*, *N. crassa* and *B. taurus* (Figure 4.24). Comparison of the *Z. mays* ANT amino acid sequence with those from *S. cerevisiae*, *N. crassa* and *B. taurus* now indicates that the *Z. mays* ANT protein extends beyond the N-termini of these other ANT proteins (Figure 4.24). This is consistent with the hypothesis that the *Z. mays* ANT is synthesised as a larger precursor protein with an N-terminal extension, which is processed upon import into isolated higher plant mitochondria.

The isolation of a *S. tuberosum* ANT cDNA, *PANT-1* (Winning, pers. comm.), although not full-length, enabled the import of a second plant ANT to be investigated. This was synthesised as a larger precursor protein, which was

processed upon import into mitochondria isolated from both *Z. mays* and *S. tuberosum* (Figure 4.22 and 4.23). These results therefore corroborated the evidence previously obtained from the *in vitro* import experiments with the *Z. mays* ANT. Comparison of the *S. tuberosum* ANT amino acid sequence with those from *S. cerevisiae*, *N. crassa* and *B. taurus* indicates that the amino acid sequence of this second plant ANT also extends beyond the N-termini of these other ANT proteins (Figure 4.24).

N-terminal sequencing of the isolated *S. tuberosum* ANT protein has enabled the position of the processing site to be predicted. The amino acid sequence obtained is shown in Figure 5.1A. This was not a single sequence, but a mixture and this is thought to be due to a contaminating protein visible after the sample was fractionated by SDS/PAGE (Winning pers. comm.). Comparison with the deduced amino acid sequence from *PANT-1* indicates that the start of homology is at the alanine residue, 76 amino acid residues away from the first amino acid residue encoded by the cDNA. Alignment of the N-terminal deduced amino acid sequence of the ANT from *Z. mays* and *S. tuberosum* indicate that the position of the predicted processing site is identical (Figure 5.1B).

Recently, a second *S. tuberosum* ANT cDNA, *PANT-2*, has been independently isolated (Schmitz pers. comm.). Comparison of the deduced amino acid sequence of this second ANT protein with those of *S. cerevisiae*, *N. crassa* and *B. taurus* indicates that this too extends beyond the N-termini of these other ANT proteins (Figure 5.2). When the predicted amino acid sequence of *PANT-1* is compared with *PANT-2*, a homology of 88 % is found. Over one third of the amino acid differences (17 of the 46 amino acid residues) occur within the first 66 amino acid residues of the protein. This is not unexpected, since mitochondrial presequences show little primary sequence homology (section 1.3.3). A second *Z. mays* ANT cDNA, *MANT-2*, has also been isolated (Winning *et al.*, 1991) and this also displays a similar N-terminal extension (Figure 5.2)

5.5. Is *MANT-1* full-length?

The sequencing errors discovered in the *Z. mays* ANT cDNA resulted in the loss of the previously predicted TGA stop codon and the extension of the original *Z. mays* ANT ORF 174 bp upstream from ATG 3 to ATG 1. Analysis of the corrected nucleotide sequence (Figure 4.20), however, indicates that there are no translational stop codons between the initiating methionine encoded by ATG 1 and the

A.

Z. mays - (D) AEKGGKNFMIDFMMG-VSA-VS-TA-

S. tuberosum ---EKG-ANMYIDQIMG
 ---IVLFIEFAVPFLSA

B.

Z. mays M ADQANQ.PTV LHKLGQFHL RSIISEGVRA RN.IC...PS
S. tuberosum AD..NQHPTV YQKVASQML SSSLSQDVHA RY.GGIQRPA

Z. mays VSSYERREFAT RNYMIQSLWG PSMSVSGGIN VPVMQTPLCA
S. tuberosum LS..QRREFPY GNYSNAGL.Q TCQATQDLSL IANASPVFV

↓

Z. mays -DAEKGGKN FMIDFMMG-V SA-VS-TA-
 NAPAEEKGGKN FMIDFMMGGV SAAVSKTAAA PIERVKLLIQ
S. tuberosum QAPQEKGLAA FATDFLMGGV SAAVSKTAAA PIERVKLLIQ
 ---EKG-AN MYIDQIMG
 ---IVLFIE FAVPFLSA

Figure 5.1; The amino acid sequence obtained after N-terminal sequencing of the isolated *Z. mays* and *S. tuberosum* ANT proteins (A) and alignment with the deduced N-terminal amino acid sequences (B).

Sequencing of the *S. tuberosum* protein did not yield a single sequence, but a mixture (A). When compared with the deduced amino sequence of the *S. tuberosum* ANT (B), homology was found (Winning pers. comm.). The predicted position of the processing site was identical to that in the *Z. mays* ANT protein (indicated by the arrow). - ; amino acid could not be identified, a gap inserted to increase alignment identities.

MANT-1	M	ADQANQ.PTV	LHKLGGQFHL	RSIISEGVRA	RN.IC...PS
MANT-2	M	ADQANQ.PTV	LHKLGGQFHL	SSSFSEGVRA	RN.IC...PS
PANT-1		AD..NQHPTV	YQKVASQMHL	SSSLSQDVHA	RY.GGIQRP
PANT-2	M	AD.MNQHPTV	FQKAANQLSD	LRSSLSQDVH	ARYGGVQ.PA

N. crassa
AAC1
AAC2
B. taurus

MANT-1		VSSYERRFAT	RNYMTQSLWG	PSMSVSGGIN	VPVMQTPPCA
MANT-2		FSPYERRFAT	RNYMTQSLWG	PSMSVSGGIN	VPVMPTPLFA
PANT-1		LS..QRFPY	GNYSNAGL.Q	TCQATQDLSL	IAANASPVFV
PANT-2		IY..QRHFAY	GNYSNAGL.Q	RGQATQDLSL	ITSNASPVFV

N. crassa
AAC1
AAC2
B. taurus

MA
MS
MSSNAQVK TPL.....P

MANT-1		NAPAEKGGKN	FMIDFMMGGV	SAAVSKTAAA	PIERVKLLIQ
MANT-2		NAPAEKGGKN	FMIDFMMGGV	SAAVSKTAAA	PIERVKLLIQ
PANT-1		QAPQEKGLAA	FATDFLMGGV	SAAVSKTAAA	PIERVKLLIQ
PANT-2		QAPQEKGFAA	FATDFLMGGV	SAAVSKTAAA	PIERVKLLIQ

N. crassa
AAC1
AAC2
B. taurus

EQQVLGMPP FVADFLMGGV SAAVSKTAAA PIERIKLLVQ
HTETQTQOSH FGVDFLMGGV SAAIAKTGAA PIERVKLLMQ
PAPAPKESN FLIDFLMGGV SAAVAKTAAS PIERVKLLIQ
SDQALS FLFDFLAGGV AAISKTAVA. PIERVKLLIQ

Figure 5.2; A comparison of the N-terminal amino acid sequence of the ANT proteins from *Z. mays*, *S. tuberosum*, *N. crassa*, *S. cerevisiae* and *B. taurus*.

The *Z. mays* MANT-1 and MANT-2 (Winning *et al.*, 1991), *S. tuberosum* PANT-1 (Winning pers. comm.) and PANT-2 (Schmitz pers. comm), *N. crassa* and *S. cerevisiae* AAC1 and AAC2 (Lawson and Douglas, 1988) sequences were deduced from the nucleotide sequence. The *B. taurus* sequence (Aquila *et al.*, 1982) was determined directly. Sequences are aligned to maximise homology.

5' end of the cDNA. It is therefore possible that the *Z. mays* ANT cDNA clone, *MANT-1*, is not full-length.

It was originally thought that homology to the genomic clone, *ANT-G1*, began 64 bp into the *MANT-1* sequence (Figure 4.20). The origin of the additional 63 bp at the 5' end of the *MANT-1* cDNA sequence was therefore unclear. The same sequence of 63 nucleotides, however, has now been found 1064 bp 5' to the 495 bp exon in *ANT-G1* (Figure 4.21C). *ANT-G1* may therefore contain a fourth exon (Day pers. comm.). It is still uncertain whether ATG 1 encodes the initiating methionine of the ANT protein, since examination of this 63 bp nucleotide sequence reveals no translational stop codons.

5.6. Targeting of the adenine nucleotide translocator to the mitochondrion.

The ANT from *S. cerevisiae* and *N. crassa* is synthesised without an N-terminal extension, therefore the information required to target the protein to the mitochondrion must lie within the protein. Experiments with isolated *S. cerevisiae* mitochondria, indicated that the first 111 amino acid residues of the *S. cerevisiae* ANT could target the mouse cytoplasmic protein dihydrofolate reductase (DHFR) into mitochondria. The first 72 amino acid residues of the *S. cerevisiae* ANT, however, could not target DHFR to mitochondria (Smagula and Douglas, 1988). The imported fusion protein, was extractable under conditions of high pH, even though it remained resistant to exogenous proteinase K. It was therefore proposed that an intermediate stage along the usual ANT import pathway had been reached (Pfanner and Neupert, 1987b).

The *S. cerevisiae* ANT therefore appears to contain targeting information at the extreme N-terminal end, but for complete protein import, sequences beyond these first 111 amino acid residues are required (Smagula and Douglas, 1988). Deletion analysis of the *N. crassa* ANT has shown that a protein lacking the first 103 amino acid residues is still targeted and imported into mitochondria (Pfanner *et al.*, 1987d). At first glance these results seem to contradict those described above, but to explain this the structure of the ANT must be considered.

Saraste and Walker (1982) propose that the ANT may have originated from the triplication of an ancestral gene, since a tripartite structure consisting of three homologous domains of approximately 100 amino acid residues is observed in the *B. taurus* protein. Each domain consists of two membrane spanning α -helices, separated by a hydrophilic region. Similar α -helices are predicted to exist within

the *S. cerevisiae* ANT protein. The first lying between amino acid residues 71 and 97 (Smagula and Douglas, 1988). Each third of the ANT protein sequence may therefore contain targeting information, since such structures have been implicated in the targeting function of mitochondrial presequences (section 1.3.3). This tripartite structure of the ANT could therefore explain the results obtained by Smagula and Douglas (1988) and Pfanner *et al.* (1987d).

5.6.1. Does the *Zea mays* adenine nucleotide translocator contain internal targeting sequences?

To determine whether the *Z. mays* ANT could be imported in the absence of the N-terminal extension, a construct termed pINTERNAL was generated in which the first 373 bp of the *Z. mays* ANT cDNA were removed (section 4.9). The major 27 kDa protein synthesised in the rabbit reticulocyte lysate translation system was imported into isolated *Z. mays* mitochondria and protected from exogenous proteinase K (Figure 4.18). There was therefore sufficient targeting information remaining in this N-terminally deleted *Z. mays* ANT protein to allow import to occur. This was not unexpected, since the N-terminally deleted *Z. mays* ANT protein will contain at least two of the three 100 amino acid domains, which had previously been shown to be sufficient for import (Pfanner *et al.*, 1987d).

5.6.2. Does the N-terminal extension of the *Zea mays* adenine nucleotide translocator contain mitochondrial targeting information?

To determine whether the *Z. mays* ANT N-terminal extension contained mitochondrial targeting information, an in frame fusion protein was generated between the first 373 bp of the *Z. mays* ANT cDNA and the complete coding sequence of the mouse cytosolic protein DHFR (section 4.7). DHFR was chosen as the passenger protein, because previous reports have shown that, when it is fused to known mitochondrial targeting presequences, DHFR can be imported into mitochondria (Hurt *et al.*, 1984; Hurt *et al.*, 1985; Horwich *et al.*, 1985).

As with the N-terminally deleted ANT, the fusion protein was imported into isolated *Z. mays* mitochondria and protected from exogenous proteinase K (Figure 4.16). The *Z. mays* ANT N-terminal extension therefore appears to contain sufficient mitochondrial targeting information to allow import of the mouse DHFR. N-terminal sequencing of the isolated *Z. mays* ANT protein, however, indicated that

the *Z. mays* ANT is synthesised with an N-terminal extension of 77 amino acids (Figure 4.20). The 373 bp of the *Z. mays* ANT cDNA used in the generation of this fusion protein will therefore encode the first 20 amino acid residues of the 'mature' *Z. mays* ANT protein, as well as the 77 amino acid N-terminal extension. It could therefore be argued that the targeting information originates from the 'mature' *Z. mays* ANT sequence rather than from the N-terminal extension.

These 20 amino acid residues from the 'mature' *Z. mays* ANT protein sequence, however, correspond to residues 4 to 24 within the *S. cerevisiae* AAC1 protein (Figure 4.24). As mentioned previously (section 5.6) the first 111 amino acid residues of the *S. cerevisiae* AAC1 protein contained sufficient targeting information allowing the import of an ANT-DHFR fusion protein, whilst a similar construct containing the first 72 amino acids could not target DHFR to mitochondria (Smagula and Douglas, 1988). It therefore seems unlikely that the first 20 amino acid residues of the 'mature' *Z. mays* ANT would contain sufficient information to target DHFR into mitochondria. A more precise analysis of the function of the *Z. mays* ANT N-terminal extension must be performed, however, to confirm the function of the N-terminal extension. This should now be possible, since the sequence of the 'mature' protein is known (see section 5.9.1).

It should be noted that random DNA fragments from the *Escherichia coli* genome encoded polypeptides that were able to target DHFR into mitochondria (Baker and Schatz, 1987). Similarly, an internal protein sequence within DHFR, could target DHFR into mitochondria, when fused to the N-terminal end (Hurt and Schatz, 1987). Potential targeting signals may therefore lie buried within non-mitochondrial proteins or be encoded by completely random DNA fragments. It could therefore be argued that the N-terminal extension of the *Z. mays* ANT may fortuitously contain mitochondrial targeting information, but this seems unlikely, since the import and the processing of the *Z. mays* ANT shows many features displayed by those mitochondrial proteins containing N-terminal extensions or presequences (section 5.3). In addition, the *S. tuberosum* ANT protein is also synthesised as a larger precursor, which is processed upon import (Figure 4.22 and 4.23). This phenomenon is therefore not unique to the *Z. mays* ANT protein.

5.7. Is the *Saccharomyces cerevisiae* adenine nucleotide translocator processed?

Analysis of the *S. cerevisiae* AAC1 protein has shown that it is synthesised without an N-terminal extension and therefore is not processed upon import into

mitochondria (Smagula and Douglas, 1988). Two further genes from *S. cerevisiae*, termed AAC2 and AAC3, encoding an ANT protein have now been isolated (Lawson and Douglas, 1988; Kolarov *et al.*, 1990), but as yet no results on the import of these two proteins have been published. It has, however, been reported that the protein encoded by AAC2 may be processed upon import into isolated *S. cerevisiae* mitochondria (Douglas pers. comm.). A comparison of the proteins encoded by AAC1 and AAC2 indicates that AAC2 is 10 amino acids longer at the N-terminus (Figure 4.24). It is therefore possible that this is a short mitochondrial presequence.

Interestingly, when the hydrophobicity plots of ANT proteins from different organisms are compared, the ANT encoded by AAC2 and the *Z. mays* pANT-1 both contain a distinct hydrophilic peak, which is absent from all other ANT proteins (Lawson and Douglas, 1988). This is located near the N-terminus and it is interesting to speculate that, once imported into mitochondria, this may somehow act as a signal for the processing of these two proteins.

ANT is proposed to belong to a family of mitochondrial inner membrane carrier proteins including uncoupling protein and the phosphate carrier (Aquila *et al.*, 1987). These share the similar tripartite structure proposed to exist within the ANT protein (section 5.6). Recent isolation of the rat and bovine phosphate translocator cDNAs, however, has revealed that these proteins are synthesised with a presequence of 44 and 49 amino acid residues respectively (Pratt *et al.*, 1991). The import of the rat phosphate translocator into mitochondria isolated from both rat and *S. cerevisiae* has indicated that this presequence is both processed upon import and is necessary for the import of the protein (Pratt *et al.*, 1991). It therefore appears that the import pathway of this protein has diverged from that of the related ANT and uncoupling proteins. It may be possible, therefore, that a similar divergence has occurred in the ANT proteins from higher plants.

5.8. Why does the *Zea mays* adenine nucleotide translocator contain an N-terminal extension?

The presence of chloroplasts and other plastids is the major difference between plant cells and those from either *S. cerevisiae* or *N. crassa*. As discussed in section 1.5.1, the biogenesis of the chloroplast is very similar to that of the mitochondrion. Both are semi-autonomous containing their own circular DNA, which encodes only a minority of the organellar proteins. The remainder are encoded by the nucleus, synthesised in the cytosol and then imported. These nuclear-encoded chloroplast proteins are synthesised as larger precursor proteins

with N-terminal extensions, termed transit peptides, which function to target the protein to the organelle (Schmidt and Mishkind, 1986; Keegstra and Bauerle, 1988; Keegstra, 1989; Smeekens, *et al.*, 1990). It could be speculated then, that the N-terminal extension of the *Z. mays* ANT may be necessary for the specific targeting of the protein to the mitochondrion and not to the chloroplast.

Only a few experiments have investigated the specificity of protein targeting (section 1.4.1 and 1.4.2). The presequence of the β -subunit of the F_1 -ATP synthase, for instance, has been used to specifically target non-mitochondrial proteins into mitochondria (Boutry *et al.*, 1987; Hemon *et al.*, 1990), whilst a yeast presequence was able to target the bacterial protein β -glucuronidase to mitochondria (Schmitz and Lonsdale, 1989). These experiments were performed *in vivo* and indicated that targeting was specific. Similar investigations have been performed *in vitro*. Whelan *et al.* (1990) demonstrated that the β -subunit of the F_1 -ATP synthase from *N. plumbaginifolia* and *N. crassa* could be imported into mitochondria, but not chloroplasts. The question of specificity, however, still remains unclear, since Huang *et al.* (1990) demonstrated that the presequence from the cytochrome *c* oxidase subunit Va from yeast could target chloramphenicol acetyltransferase into both mitochondria and chloroplasts *in vivo*.

It has also been postulated that mitochondrial protein import may be less specific than was previously thought, since a chloroplast transit peptide from *Chlamydomonas reinhardtii* could target DHFR into isolated *S. cerevisiae* mitochondria (Hurt *et al.*, 1986). The small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase from *Pisum sativum* could also be imported into isolated *N. crassa* mitochondria (Pfaller *et al.*, 1989). A mitochondrial bypass import pathway has therefore been proposed. This displays all the characteristics of the usual import pathway, such as a requirement for ATP and an energised inner membrane, but this appears to occur with a much lower efficiency (Pfanner *et al.*, 1988c; Pfaller *et al.*, 1989). Whether such a bypass import pathway occurs in plant mitochondria remains to be seen and further investigations are needed before it can be said with certainty that the mis-targeting of mitochondrial and chloroplast proteins does not occur.

Just how mitochondrial presequences and chloroplast transit peptides are distinguished by the two organelles is still unclear. Receptor proteins are thought to mediate the initial binding of both mitochondrial and chloroplast imported proteins (sections 1.3.4 and 1.5.4 respectively). Such receptors in plant mitochondria are, however, yet to be identified and characterised, but it can be envisaged that these may function to determine which precursors bind to the

organelle and as such, would act as a stringent control at an early stage along the import pathway. The energy requirements of mitochondrial and chloroplast protein import are also different (sections 1.3.5 and 1.5.5). This too may have a controlling effect upon which precursor is imported (section 1.5.6).

Mitochondrial presequences, like chloroplast transit peptides, display little primary sequence homology. The secondary and/or tertiary structure of the presequence are therefore thought to be important in the targeting function (Hurt and van Loon, 1986; Smeekens *et al.*, 1990). Comparisons of mitochondrial presequences has revealed a number of general features including a preponderance of basic, positively charged amino acids, which appear to be periodically arranged. Hydroxylated amino acids are also abundant, whereas negatively charged residues appear to be absent (section 1.3.3).

Structural predictions indicate that mitochondrial presequences may form amphiphilic α -helices (von Heijne, 1986). Such structures are thought to be absent from chloroplast transit peptides, although the C-terminal region may form an amphiphilic β -sheet (von Heijne *et al.*, 1989). Structural analysis of the *Z. mays* ANT 77 amino acid N-terminal extension, however, predicts three β -sheets, rather than α -helices (Figure 5.3).

These statistical analyses and structural predictions have been made by comparing *S. cerevisiae* and *N. crassa* mitochondrial presequences. One such analysis, for instance, contained only one plant mitochondrial presequence amongst the 37 examined (von Heijne *et al.*, 1989). It is therefore possible that plant mitochondrial presequences may contain alternative structural features necessary for targeting.

A simple analysis has noted that chloroplast transit peptides are generally longer than mitochondrial presequences (Franzen *et al.*, 1990). The β -subunit of the F_1 -ATP synthase from *N. plumbaginifolia*, however, is thought to have a presequence of 90 amino acid residues (Boutry *et al.*, 1987) and the 77 amino acid N-terminal extension of the *Z. mays* ANT indicates that this may not necessarily be the case with plant nuclear-encoded mitochondrial proteins.

The incidence of the amino acid residues at or near the processing site of mitochondrial presequences has also been examined and arginine residues appear to be more frequent at positions -2 and -10 relative to the processing site (von Heijne *et al.*, 1989; Hendrick *et al.*, 1989). Analysis of the amino acid sequence surrounding the predicted processing site of the *Z. mays* ANT and the *S. tuberosum* ANT, however shows no homology to this proposed consensus sequence (Figure 5.3).

Figure 5.3. The predicted *secondary* structure of the first 77 amino acid residues from the deduced amino acid sequence of *MANT-1*.

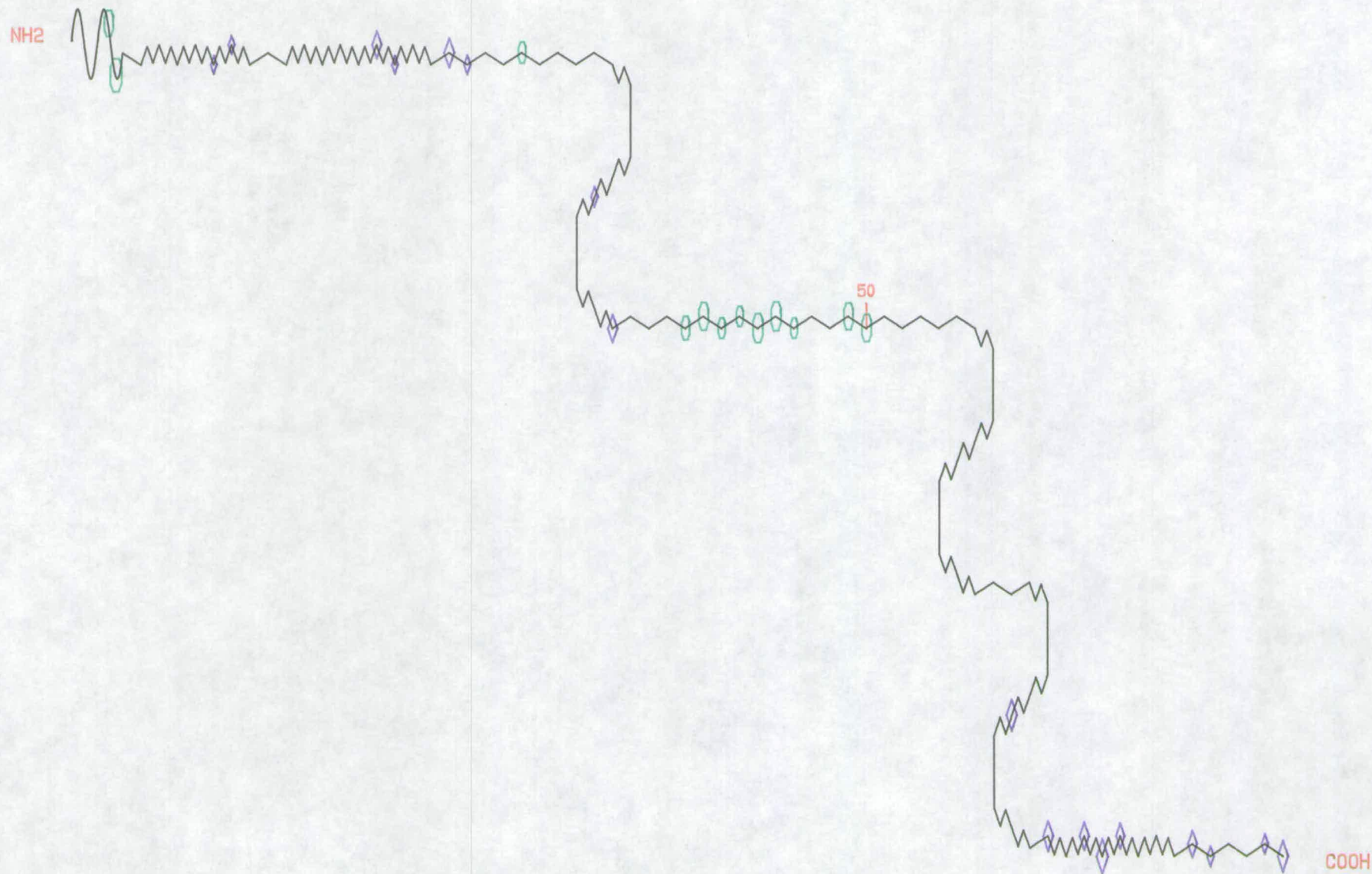
The UWGCG Sequence Analysis Software package Version 7.0 (section 2.8) was used to generate a two-dimensional plot, representing predicted *secondary* structures. Helices are shown with a sine wave, β -sheets are shown with a sharp saw tooth wave, turns with 180 degree turns and coils with a dull saw tooth wave. Hydrophilicity and hydrophobicity are superimposed over the wave in green and blue respectively (the size of the symbol being proportional to the hydrophilic or hydrophobic value). The N-terminus of the deduced amino acid sequence is marked NH₂ and the C-terminus is marked COOH.

PLOTSTRUCTURE of: FIG5.TMP; ck: 7057

TRANSLATE of: bantcdna.seq check: 3275 from: 86 to: 1249

Chou-Fasman Prediction
September 11, 1991 15:46

○ KD Hydrophilicity ≥ 1.3
◇ KD Hydrophobicity ≥ 1.3



5.9. Future Experiments.

5.9.1. The *in vitro* import of the *Zea mays* adenine nucleotide translocator into chloroplasts.

The evidence presented in this thesis indicates that the *Z. mays* ANT, in contrast to that of *S. cerevisiae* and *N. crassa*, is synthesised with an N-terminal extension, which is processed upon import into isolated mitochondria. The *in vitro* import of the *S. tuberosum* ANT appears to confirm these findings. In addition, similar N-terminal extensions are predicted when the deduced amino acid sequence of an independently isolated *S. tuberosum* ANT cDNA, *PANT-1* (Schmitz pers. comm.) and a second *Z. mays* ANT cDNA, *MANT-2* (Winning *et al.*, 1991) are compared to the N-terminal amino acid sequence of the *S. cerevisiae*, *N. crassa* and *B. taurus* ANT proteins (Figure 5.2). To determine whether the *Z. mays* N-terminal extension contains specific mitochondrial targeting information, an *in vitro* chloroplast protein import system could be utilised.

The results presented in Chapter 4 have shown that DHFR can be imported into isolated *Z. mays* mitochondria, when attached to the first 97 amino acid residues of the *Z. mays* ANT (section 4.7). The N-terminal sequencing of the isolated *Z. mays* ANT protein now allows the function of the N-terminal extension to be analysed more specifically. A chimaeric protein containing the first 77 amino acid residues of the *Z. mays* ANT, the predicted N-terminal extension, could therefore be generated and the import of this protein into both chloroplasts and mitochondria investigated.

Preliminary experiments with isolated chloroplasts are currently in progress examining the import of the full-length *Z. mays* ANT, the N-terminally deleted protein and the fusion protein described in Chapter 4 (Robinson pers. comm.). Such investigations should determine whether or not the N-terminal extension of the *Z. mays* ANT functions to specify organellar targeting. Care should be taken with the interpretation of these results, however, since the experiments are *in vitro*. In addition, the N-terminal extension may function to prevent import into chloroplasts, rather than targeting the ANT specifically to the mitochondrion.

5.9.2. Deletion analysis of the *Zea mays* adenine nucleotide translocator N-terminal extension.

Progressive deletions of the *Z. mays* ANT N-terminal extension and subsequent import into mitochondria of the shortened ANT protein could be performed. This would determine whether the N-terminal extension is necessary for import and which regions mediate the targeting of the protein. Similarly, experiments could be performed with varying lengths of the *Z. mays* ANT N-terminal extension fused in frame to a non-mitochondrial protein. Again, the interpretation of such experiments must be made with care, since an inhibition of mitochondrial import may not be due entirely to the deletion, but may have resulted from an alteration in the secondary and/or tertiary structure of the extension.

5.9.3. Localisation of the *Zea mays* adenine nucleotide translocator after import into isolated mitochondria.

It could be postulated that the *Z. mays* N-terminal extension may act in concert with the internal ANT targeting sequence(s) to correctly locate and assemble the protein within the inner mitochondrial membrane. The experiments described in Chapter 4 indicate that the removal of the N-terminal extension does not prevent the import of the *Z. mays* ANT into isolated mitochondria. There is, however, no evidence to suggest that this N-terminally deleted ANT or the full-length ANT are correctly assembled, or whether they are imported to the same location.

Fractionation of mitochondria after import should allow the localisation of each protein to be determined. Examination of a simple membrane and soluble fraction may be sufficient, but if not, separation into outer membrane, inter membrane space, inner membrane and matrix could be performed. In each case, the purity of the fractions could be determined by assaying for specific mitochondrial marker enzymes.

To determine whether the imported protein is correctly assembled, the binding of ANT to carboxyatractyloside (CAT) could be investigated. This binds specifically to the assembled, functionally active ANT and is thought to stabilise the protein, since a CAT-ANT complex can be isolated after solubilisation of mitochondrial membranes with detergent and subsequent passage over hydroxylapatite. Under the same conditions an incorrectly assembled ANT protein would bind to the hydroxylapatite (Schleyer and Neupert, 1984).

5.9.4. The *in vivo* import of the *Zea mays* adenine nucleotide translocator.

5.9.4.1. Plant transformation.

The import of the *Z. mays* ANT appears to be distinctly different to that of *S. cerevisiae* and *N. crassa*, but so far this has only been determined from experiments performed *in vitro*. It is now important to examine whether this *Z. mays* ANT protein is synthesised as a larger precursor protein *in vivo* and if so, which ATG codon is utilised as the start of translation. Plant transformation techniques should be able to establish this, but such experiments would be limited to *N. plumbaginifolia* or *S. tuberosum*, since *Z. mays* is not yet amenable to such genetic manipulations.

The results described in Chapter 4 indicate that the N-terminal extension of the *Z. mays* ANT can function to target a normally cytosolic protein into isolated mitochondria. It should therefore be possible to perform a similar experiment *in vivo*. The subcellular localisation of the passenger protein would then determine whether the N-terminal extension of the *Z. mays* ANT can function to target the protein into mitochondria *in vivo*. Such an experiment would also allow the question of mis-targeting to be addressed.

The experiment described above, however, will give no indication to whether the *Z. mays* ANT is synthesised as a larger precursor protein. To address this question, the *Z. mays* ANT cDNA could be mutated at the predicted processing site. Assuming ATG 1 is utilised as the start of translation *in vivo*, a protein with a higher molecular weight than the 'mature' ANT should then be generated. Isolation of mitochondria from these genetic transformants and subsequent Western blot analysis with the *Z. mays* ANT antibody described in section 4.6, should allow the size of this protein to be determined. This should be readily distinguished from the endogenous ANT protein, if it is synthesised as a larger protein. The *S. tuberosum* ANT, for instance, runs with a similar molecular weight as the *Z. mays* ANT, as estimated by SDS/PAGE (Purcell pers. comm.).

As a preliminary experiment, the import of the mutated protein into isolated mitochondria could be investigated. This would determine whether the mutation introduced into the *Z. mays* ANT protein had any deleterious effect upon mitochondrial import, if not, it would be reasonable to assume that the import *in vivo* would be unaffected.

If such a mutation in the predicted processing site does result in the generation of a larger *Z. mays* ANT protein, attempts could then be made to isolate the protein and examine the N-terminal sequence. This would help to identify the translational start site utilised *in vivo* and would confirm that the protein is synthesised as a larger protein.

5.9.4.2. Yeast transformation.

In addition to plant genetic transformation, the ability of the *Z. mays* ANT protein to complement a yeast mutant, deficient in ANT, could be investigated. One such mutant has already been described and is termed *op*₁, or *PET9*. This was previously utilised to isolate the *AAC1* gene (O'Malley *et al.*, 1982). The ability of *S. cerevisiae* to process the *Z. mays* ANT could also be determined. If it is found that the plant protein is processed, it would be interesting to speculate that the *Z. mays* ANT may be utilising a different import pathway from the endogenous *S. cerevisiae* protein, which is imported without processing.

5.9.5. The *Zea mays* adenine nucleotide translocator processing protease.

Preliminary experiments indicated that a *Z. mays* mitochondrial soluble fraction could not process the full-length *Z. mays* ANT protein, whereas the same fraction processed the *N. plumbaginifolia* MnSOD precursor (not shown). This was unexpected, since upon import into isolated *Z. mays* mitochondria, the processing of the *Z. mays* ANT could be inhibited by the inclusion of 1,10-phenanthroline. A number of possibilities may explain this result. Firstly, the enzyme activity processing the *Z. mays* ANT protein may not be localised within the mitochondrial soluble fraction. There may therefore be a second enzyme responsible for the processing of the *Z. mays* ANT. Secondly, a different enzyme may be located in the soluble fraction of *Z. mays* mitochondria, but this may have a different metal ion requirement for activity. This would therefore account for the lack of processing when the *Z. mays* ANT was incubated with the soluble fraction. Further experiments will therefore be necessary to distinguish between these possibilities.

5.10. Summary.

This thesis has described the characterisation and partial optimisation of a plant mitochondrial *in vitro* import system, which has been utilised to investigate the import of the *Z. mays* ANT. The results presented in Chapter 4 suggest that the import of this protein is distinctly different from that of *S. cerevisiae* and *N. crassa*, since it is synthesised as a larger precursor protein, which is processed upon import. This observation, however, is based entirely from experiments carried out *in vitro* and this must be taken into account when considering these results.

The import of a second plant ANT protein from *S. tuberosum*, however, appears to corroborate the evidence obtained for the *Z. mays* ANT indicating that this phenomenon is not unique to the *Z. mays* ANT protein. In addition, the isolation of two further ANT cDNA clones from *S. tuberosum* (Schmitz pers. comm.) and *Z. mays* (Winning *et al.*, 1991) substantiate these results. Future experiments must therefore concentrate upon the import of the *Z. mays* ANT *in vivo*. Such experiments would confirm these *in vitro* results and to this end preliminary experiments have been described which should help to address this important question.

The development of this plant mitochondrial *in vitro* import system has many potential uses. As discussed in section 5.9.4.1, it could be utilised as a 'test system' to investigate whether a particular protein can be imported into mitochondria. This would be of considerable advantage to the genetic engineer, since such an *in vitro* mitochondrial import system would enable the feasibility of a particular project or experiment to be investigated.

Experiments can be envisaged in which the mitochondrial protein content of the plant is modified, perhaps by the over expression of a particular nuclear-encoded mitochondrial protein, or alternatively by the incorporation of a mutated protein. The effect of these changes upon mitochondrial metabolism could then be investigated, as could any phenotypic effects. Such alterations, may prove useful, since they may result in an increase in the yield or the nutritional value of the plant. The import into plant mitochondria of 'variant polypeptides', such as the 13 kDa protein synthesised by mitochondria of certain *Z. mays* plants (Leaver and Gray, 1982) could also be investigated. Such proteins are thought to be responsible for the male sterile phenotype displayed by these plants. The generation of such 'cytoplasmic male sterile' plants would therefore be of considerable value to plant breeders, since they would be useful for the production of hybrid seed. In all

such experiments, the development of a plant mitochondrial *in vitro* import system is invaluable.

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APPENDIX

Update section

Sequence

Nucleotide sequence of two cDNAs encoding the adenine nucleotide translocator from *Zea mays* L.

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The adenine nucleotide translocator (ANT), an inner mitochondrial membrane carrier protein, plays a central role in cellular energy metabolism, mediating the exchange of adenine nucleotides between the mitochondrial matrix and the cytosol. We have isolated two ANT cDNAs from maize (*Zea mays* L.), containing the entire coding sequences, with a view to gaining an understanding of the expression and biogenesis of this protein in higher plants. ANT cDNAs isolated previously in this laboratory were not full-length. One of these cDNAs, the insert from pANT1 [2], was used as a probe to screen a maize λ gt10 cDNA library. This library was constructed using cDNA synthesised from poly(A)⁺ RNA isolated from 3-day-old dark-grown seedlings. Two of the cDNAs isolated were found to correspond to the known ANT genes in maize, *G1* and *G2* [3]. It was found that the open reading frames of these cDNAs, designated MANT1 and MANT2, did not agree with the corresponding genomic clones. Therefore the genomic clones were resequenced

and it was found that sequencing errors had caused frameshifts; once these were corrected the open reading frames of the cDNAs and the genomic sequences were found to correspond exactly. Figure 1 shows a comparison of the nucleotide and deduced amino acid sequences of MANT1 and MANT2. The sequence analysis was carried out using the GAP computer program [4]. The predicted 387 amino acid proteins encoded by MANT1 and MANT2 show 98% similarity and are homologous to ANT proteins from fungal and mammalian sources [1, 5, 6] except that they contain N-terminal extensions. The significance of this will be discussed elsewhere (B. Winning, C.J. Sarah, C.D. Day and C.J. Leaver, in preparation).

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X57556 (pMANT1) and X59086 (pMANT2).

Winning

M A D

1 CGCGCCTCTGAAAGTTCGGAGCCGCCGCGCCGCTCCGCTCCC...TCTCCGCCTCCCGTCCCA....AGGCCAATAGTTTCAAGTGGCAATGGCGGAC 93
 1GTCCGAGCCGCCGCCAGACCCTCCCTTCCCGCTCCCGTCCCAAGGCCAGCCAAACAGTTTGAAGTGGTAATGGCGGAC 86

Q A N Q P T V L H K L G G Q F H L R S I I S E G V R A R N I C P S
 94 CAGGCTAACCAACCCACTGTCTTCATAAGCTCGGTGGCCAGTTCACCTGGCTCGATCATCTCTGAAGGTGACGGGCCGTAACATATGCCATCTG 193
 87 CAGGCTAACCAACCCACTGTCTTCATAAGCTAGGTGGCCAGTTCACCTGAGCTCCAGCTTCTCTGAAGGTGACGGGCCGTAACATCTGCCCTCTT 186

S S F

V S S Y E R R F A T R N Y M T Q S L W G P S M S V S G G I N V P V M
 194 TCTCATCTTATGAAAGGAGATTGGCCACAAGGAACACTACATGACCCAGAGCCTTTGGGGCCCTCAATGTCTGTAGCGGTGGCATCAATGCCAGTGAT 293
 187 TCTCACCTTATGAAAGGAGATTGGCCACGAGGAACACTACATGACCCAGAGCCTTTGGGGCCCTCAATGTCTGTAGCGGTGGCATCAATGTTCCAGTGAT 286

P

Q T P L C A N A P A E K G G K N F M I D F M M G G V S A A V S K T
 294 GCAGACCCCGCTCTGTCTAATGCTCCTGCTGAGAAAGGAGGCAAGAACTTCATGATTGATTTTCATGATGGGCGGTGTTTCAGCTGCTTTTCAAAGACT 393
 287 GCCGACTCCGCTTTTGGCAATGCTCCAGCCGAGAAAGTGGCAAAAATTCATGATTGATTTTCATGATGGGCGGTGTTTCAGCTGCTTTTCAAAGACT 386

P F

A A A P I E R V K L L I Q N Q D E M I K S G R L S E P Y K G I V D
 394 GCTGCTCTCCATCGAGCGGTGTAAGCTGCTTATTGAGAACCAAGATGAGATGATTAAGTCTGGTAGGCTATCAGAGCCGTACAAGGGTATTGTTGACT 493
 387 GCAGCTGCTCCATTGAAGGAGTGAAGCTGCTTATTGAGAACCAAGATGAGATGATTAAGTCTGGTAGGCTATCAGAGCCGTACAAGGGTATTGTTGACT 486

A

C F K R T I K D E G F S S L W R G N T A N V I R Y F P T Q A L N F A
 494 GCTTCAAACGTACCATTAGAGTGAAGGTTTCTCTTCTTGTGGAGGGGTAACACTGCTAATGTTATTCGTTACTTCCCTACTCAGGCTTTGAACTTTGC 593
 487 GCTTCAAACGTACCATTAGAGTGAAGGTTTCTCTTCTTGTGGAGGGGTAACACTGCTAATGTTATTCGTTACTTCCCTACTCAGGCTTTGAACTTTGC 586

F K D Y F K R L F N F K K D R D G Y W K W F A G N L A S G G A A G
 594 ATTTAAGGACTACTTCAAGAGGTTGTTCAACTTCAAGAAGGATAGGGATGGCTATTGGAAGTGGTTTGTCTGGCAACCTGGCCTCTGGTGGTCTGCTGCT 693
 587 GTTTAAGGACTACTTCAAGAGGCTGTTCAACTTCAAGAAGGACAGGGATGGTTACTGGAAGTGGTTTGTCTGGCAACCTGGCCTCTGGTGGTCTGCTGCT 686

A S S L F F V Y S L D Y A R T R L A N D A K A A K G G G E R O F N
 694 GCTTCTCTTTGTTTTGTTGTTACTCCCTGACTACCGCAGAACAGGTTGGCTAATGACGCAAGGCTGCCAAGGGAGGAGGTAAGGCAAGTTCATG 793
 687 GCTTCTCTTTGTTTTGTTGTTACTCCCTGGATTATGCGAGGACAGGTTGGCCAAATGATGCCAAGGCTGCCAAGGGAGGAGGCAATAGACAATTCATG 786

D

G L V D V Y R K T L K S D G I A G L Y R G F N I S C V G I I V Y R G
 794 GGCTTGTGATGCTACCCGCAAGACACTCAAGCTCTGATGGTATTGCTGGGCTTACCCTGGATTAAACATCTCCTGTGTGGAATCATGTTTATCGTGG 893
 787 GTCTTGTGGATGCTACCCGCAAGACACTCAAACTGATGGTATTGCTGGGCTTACCCTGGATTAAACATCTCTGTGTTGGAATATTGTTTATCGTGG 886

L Y F G L Y D S I K P V V L T G N L Q D N F F A S F A L G W L I T
 894 TCTGTACTTTGGGCTCTATGATTCATCAAGCCAGTTGCTCTTACTGGCAACCTCCAGGCAATTTCTTTGGCAGTTTCGCTCTGGGTTGGCTGATCACT 993
 887 CCTGTATTTCCGACTTTACGATTCTATCAAGCCAGTTGCTCTCACTGGCAGCTCCAGGCAACTTTCTTGGCAGTTTGTCTGGTGGCTGATCACC 986

S

N G A G L A S Y P I D T V R R R M M M T S G E A V K Y K S S L D A
 994 AATGGTGTGGTCTTGCATCTTACCCCATCGATACCCGTCGCGAGAAGGATGATGATGACATCTGGTAGGCTGTCAAGTACAAGAGCTCCTTGGACCGGT 1093
 987 AACGGTGTGGTCTTGCATCTTACCCCATGATGACTGTCGCGAGAAGGATGATGATGACTTCTGGTAGGCTGTCAAGTACAAGAGCTCATTGGACGCAT 1086

F Q Q I L K K E G P K S L F K G A G A N I L R A I A G A G V L S G Y
 1094 TCCAGCAGATTCTTAAGAAGGAAGGCCCAAGTCCCTGTTCAAGGGTGTGGTGTAAACATTTCTCGTGCCATTGCTGGTGTGGTGTGCTTTCTGGCTA 1193
 1087 TCCAGCAGATCCTTAAGAAGGAAGGCCCAAGTCCCTGTTCAAGGGTGTGGTGTAAACATTTCTCGTGCCATTGCTGGCCTGGTGTGCTTTCTGGCTA 1186

D Q L Q I L F F G K K Y G S G G A *
 1194 TGACCAGCTCCAGATCTCTTCTTCGAAAGAAGTACGGCTCCGCGGTGCTTAAATGGAGAAATATGATGACGAACAGAGCAGTGTGTCCCGGCT 1293
 1187 CGACCAGCTCCAGATCTCTTCTTCGAAAGAAGTACGGCTCCGCGGTGCTTAAATGGAGAAATATGATGACGAACAGAGCA...GTTGCTCCCGGCT 1284

1294 CTTTCCAATCAGGATCTGGTGAAGTTTTGCCTTTCATATCGAAGAAATTAATAATTCATGTAGAGGGAGGATTCTTCCAACAATTTGTTTGGATGGAG 1393
 1285 CAGCCAGCTCAGGATCTGGTCAAGTTTTGCCTTTCATATCGAAGAAATTAATAATTTATGTA...GGAGAATTTTCCAAC.ATTCGTTTGGGCGGAG 1380

1394 ACTTAGATCTCCAACACTCGTAACATCTCTACTAGGTGCTGACATCGGTCCCAAAA...ACATTTATGGTCATAGAGTCTGCCCTTCGTTTGTGACT 1489
 1381 ACATTGATCTCCAACACTCGTAACATGCTCTACTAGGTGGTGGCATGGTCCCAAAAACATGGTCATAGGGTGTGCTAGTCTGCCCTTCGTT...GCAGC 1478

1490 CGGGCAATTTTCTTGCATACAATGCCTTCGTTAAGATATCGGATGAGTTGCTGCGACTAATTATTGTTGTCTTTACCTTTTGCAAAA 1579
 1479 CGGGCAATTTTCTTACATATATTCCTTTAAT 1511

Fig. 1. Nucleotide and deduced amino acid sequence comparison of MANT1 and MANT2 cDNAs from *Zea mays* L. MANT1 is the upper sequence and the corresponding predicted amino acid sequence is shown above the nucleotide sequence. MANT2 is the lower sequence; in this case only the amino acid differences to the MANT1 primary sequence are shown below the nucleotide sequence. A putative polyadenylation signal, present in both cDNAs, is underlined.

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Advances in Autotrophic Microbiology and One-Carbon Metabolism

Volume I

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