

TARGETING SEQUENCES OF  
RAT UNCOUPLING PROTEIN

RAT BROWN ADIPOSE TISSUE  
UNCOUPLING PROTEIN: IDENTIFICATION OF  
POTENTIAL TARGETING SEQUENCE(S)

By

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                  Identification of Potential Targeting Sequence(s)

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## ABSTRACT

Uncoupling protein, a mitochondrial inner membrane protein found in mammalian brown adipose tissue, functions as an uncoupler of oxidative phosphorylation by serving as a proton carrier when activated, resulting in heat production, the function of the tissue. Unlike most nuclear-encoded mitochondrial proteins, uncoupling protein is not made with a cleavable presequence. With the availability of an uncoupling protein cDNA clone, the region responsible for targeting uncoupling protein to mitochondria was examined using *in vitro* transcription and translation and import into isolated mitochondria. In order to localize the targeting sequence of uncoupling protein, fusion proteins containing portions of uncoupling protein and uncoupling protein modified by site-directed mutagenesis were constructed and analysed for their ability to be imported.

Previously it has been shown that there was a targeting signal within uncoupling protein amino acids 13 to 105 (Liu *et al.*, 1988). However, amino acids 13 to 51 did not target a passenger protein to mitochondria (Liu *et al.*, 1988). Here the role of amino acids 53 to 105 of uncoupling protein in targeting was examined with two new constructs, uncoupling protein amino acids 53 to 105 joined to rat ornithine carbamoyltransferase amino acids 147 to 354 and to mouse dihydrofolate reductase. These two constructs along with uncoupling protein with amino acids 2 to 51 deleted were imported into mitochondria consistent with uncoupling protein amino acids 53 to 105 having a potential

targeting role in uncoupling protein. Further, these three constructs were processed upon import. The major processed forms of all three constructs are approximately 20 amino acids smaller than the initial translation product. Both fusion constructs also have an intermediate-sized processed form approximately 14 amino acids smaller than the initial translation product. Processing suggests that at least the amino terminus of these proteins has reached the mitochondrial matrix.

The location of the proteins was examined using  $\text{Na}_2\text{CO}_3$  extraction. Uncoupling protein and U13-105-OCT (uncoupling protein amino acids 13 to 105 joined to ornithine carbamoyltransferase amino acids 147 to 354) were found in the membrane fraction while the processed forms of Ud2-51 (uncoupling protein with amino acids 2 to 52 deleted) and U53-105-DHFR (uncoupling protein amino acids 53 to 105 joined to dihydrofolate reductase) were found in the aqueous fraction suggesting that uncoupling protein amino acids 2 to 52/53 are involved in membrane localization. Analysis of Ud2-35 (uncoupling protein with amino acids 2 to 35 deleted) revealed that it was associated with both the membrane and aqueous fractions.

Analysis of uncoupling protein amino acids 53 to 105 revealed the potential existence of two positively charged amphipathic  $\alpha$ -helices. Based on the sizes of processed forms and on the helical wheel projection for the first possible sequence, arginine<sub>54</sub>, lysine<sub>56</sub> and lysine<sub>67</sub> were changed to glutamines, individually and in various combinations using oligonucleotide site-directed mutagenesis. All mutant proteins were imported into mitochondria even when all three basic amino acids were replaced. The results suggest that this portion

of uncoupling protein, amino acids 54 to 67, is not a targeting signal in the protein.

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## LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BiP	immunoglobulin heavy chain binding protein
bis-acrylamide	N,N'-methylene bis-acrylamide
BRL	Bethesda Research Laboratories
BSA	bovine serum albumin
C-terminal	carboxy-terminal
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cdNA	complementary DNA
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid
cf	compare
Da, kDa	daltons, kilodaltons
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
DEPC	diethylpyrocarbonate
DHFR	dihydrofolate reductase
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid

dsDNA	double-stranded deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
Fab	fragment (antigen-binding)
FAD	flavin adenine dinucleotide
FCCP	carbonylcyanide-4-trifluoromethoxyphenylhydrazone
F <sub>0</sub> F <sub>1</sub> 9	subunit 9 of the F <sub>0</sub> F <sub>1</sub> ATPase
F <sub>1</sub> β	subunit β of the F <sub>0</sub> F <sub>1</sub> ATPase
GDP	guanosine diphosphate
GIP	general insertion protein
GRP-78	glucose-regulated protein of 78 kDa
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hr	hour
hsp60	heat shock protein of 60 kDa
hsp70	heat shock protein of 70 kDa
IBI	International Biotechnologies, Inc.
IgG	immunoglobulin G
IPTG	iso-propylthio-β-galactoside
LB	Luria-Bertani
m <sup>7</sup> GpppG	7-methyl guanosine triphosphate
MAS1	mitochondrial assembly gene 1
MAS2	mitochondrial assembly gene 2
min	minute
MOPS	3[N-morpholino]propanesulphonic acid

MPP	matrix processing peptidase
N-terminal	amino-terminal
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NEN	New England Nuclear
NTP	nucleoside triphosphate
OCT	ornithine carbamoyltransferase
PEG	polyethylene glycol
PEP	processing enhancing protein
pet	petite
PFU	plaque forming unit
PMSF	<u>para</u> -methylsulphonylfluoride
PPO	2,5-diphenyloxazole
RF	replicative form
Rubisco	ribulose-1,5-bisphosphate carboxylase-oxygenase
SDS	sodium dodecyl sulphate
sec	second
SSA1-4	stress-seventy subgroup family A, genes 1 to 4
Ssa1p/Ssa2p	gene product of SSA1 and SSA2
ssDNA	single-stranded DNA
TBE	Tris-borate-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tes	N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
TES	Tris-EDTA-saline
TFB	standard information buffer
Tris	Tris(hydroxymethyl)aminomethane
U	units

UCP            uncoupling protein

v                volume

## 1. INTRODUCTION

The cell is the basic unit of life. Within the eukaryotic cell, the nucleus controls growth and cell division but mitochondria are the power plants which supply the required energy. Most proteins found within the cell are coded for by nuclear DNA and are synthesized in the cytosol including the majority of mitochondrial proteins. These proteins must by some means be targeted to mitochondria, imported into the organelles and sorted to their appropriate subcompartment (the outer membrane, the intermembrane space, the inner membrane or the matrix). Further they must reach their mature conformation and, finally, be assembled into their functional complexes. This includes the formation of those protein complexes in which some of the subunits are proteins encoded by mitochondrial DNA and synthesized within the organelle.

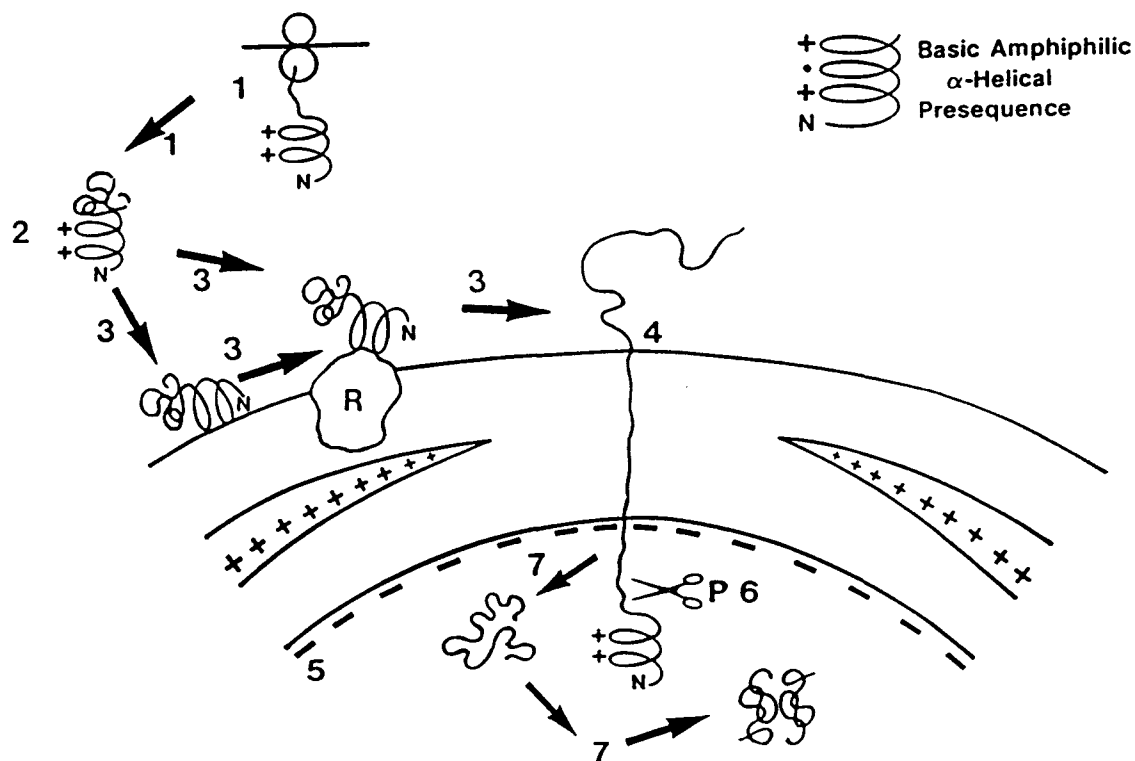
The mechanism by which nuclear-encoded mitochondrial proteins synthesized in the cytosol are targeted to and imported into mitochondria is under investigation by several groups. There are many comprehensive reviews on mitochondrial protein import (Douglas *et al.*, 1986; Hartl *et al.*, 1989; Hay *et al.*, 1984; Nicholson & Neupert, 1988; Pfanner & Neupert, 1987a). Consequently, a comprehensive review is not given here. Rather, the aspects of mitochondrial protein import that will be covered include discussions on precursors, targeting signals, receptors, protein translocation, energy requirements for import, processing of precursors, sorting and assembly of the proteins. Emphasis will be given to the discussion on targeting signals as this



project deals with the targeting of uncoupling protein. The discussion will then turn to uncoupling protein, the structural relationship between uncoupling protein, the ADP/ATP carrier and the phosphate carrier, and finally, the import model of the ADP/ATP carrier as a starting point for a model for uncoupling protein.

### 1.1 MODELS FOR MITOCHONDRIAL PROTEIN IMPORT

How are proteins synthesized in the cytosol targeted to the mitochondria? What is the mechanism by which the proteins are translocated across the membrane(s)? At what steps is energy required and in what form for translocation? Once targeted to mitochondria, the proteins are directed to their correct sub-organellar locations. How is this achieved? Membrane proteins must also achieve a specific orientation and location. What is the mechanism by which the membrane proteins achieve their final conformation? These are the types of questions that are currently being addressed. A general model for targeting and import of a matrix protein with a presequence is shown in Fig. 1. The events which occur have been divided into the following steps: 1) mitochondrial proteins are synthesized in the cytosol on free polysomes. 2) The majority of imported matrix proteins are made with an N-terminal extension or presequence containing most, if not all, of the information needed for targeting the protein to mitochondria. 3) After passage through the cytosol, the targeting sequence reacts directly with the receptor (R), or reacts with the outer membrane and then with the putative receptor. The precursor/receptor complex is then positioned to permit translocation through the membranes. Evidence suggests that the



**Figure 1. Model of Import of Matrix Proteins into Mitochondria.** (Freeman *et al.*, 1989). Details are given in the text. An insert diagram shows the amphipathic  $\alpha$ -helical presequence. Presequences have a high content of basic (+) and hydroxyl (•) amino acids, hydrophobic residues which are not present in a continuous stretch, and lack acidic residues. The arrangement of the residues is such that the hydrophilic amino acids are on one side of the  $\alpha$ -helix and the hydrophobic amino acids on the other side; that is, the helix is amphipathic. P, protease; R, receptor.

precursor must be in an unfolded state for import and that cytosolic factors including ATP or GTP are required to keep the precursor in an import-competent conformation. 4) Translocation of the precursor occurs at contact sites between the outer and inner mitochondrial membranes and the precursor probably crosses the membranes via a proteinaceous pore rather than through the phospholipid bilayer. 5) Translocation is dependent on the membrane potential. 6) For precursors with targeting presequences, the amino terminus enters the matrix ahead of the rest of the protein and the presequence is proteolytically removed by a metal-requiring protease (P). 7) After import, additional processing steps may occur and the protein achieves its final conformation. The mature protein may interact with other proteins to form homo- or hetero-oligomers.

The following discussion will deal with each of the steps in more detail and will include the import of proteins destined to all subcompartments.

### 1.1.1 CYTOSOLIC PRECURSORS

Most mitochondrial proteins are nuclear-encoded, synthesized on cytosolic free polysomes as precursor proteins and are imported by a post-translational mechanism. The evidence supporting a post-translational mechanism for import came first from pulse-chase experiments using intact cells and radioactive amino acids (Haller-mayer *et al.*, 1977; Hartl *et al.*, 1986; Reid & Schatz, 1982; Teintze *et al.*, 1982). Labeled proteins appeared first in the cytosolic pool and then in mitochondria after a lag period. Experiments in which proteins

synthesized in cell-free translation systems were added to and imported by isolated mitochondria helped substantiate a post-translational mechanism and have shown that mitochondrial protein import is independent of concomitant protein synthesis (Korb & Neupert, 1978; Zimmermann & Neupert, 1980). In contrast, there is some evidence from yeast for co-translational import.

Early evidence supporting cotranslational translocation for mitochondrial proteins came from work done by Butow and coworkers (Ades & Butow, 1980; Kellems & Butow, 1972; Kellems *et al.*, 1974). It was shown that cytoplasmic 80 S ribosomes were tightly attached to yeast mitochondria after inhibition of protein elongation with cycloheximide. Ribosomes remaining after washing were released with puromycin indicating that they were coupled to the nascent polypeptide. This indicates that the polypeptides were probably being translocated across the mitochondrial membranes during synthesis. However, in these experiments, it was not shown that incomplete precursors were spanning the mitochondrial membranes. In a later study Suissa and Schatz (1982) showed that mRNA extracted from mitochondrially bound polysomes was enriched in imported mitochondrial protein mRNAs. However, none of the mRNAs for the 12 imported mitochondrial proteins was predominantly associated with mitochondrially bound polysomes (Suissa & Schatz, 1982). Mitochondrially bound polysomes may play a role in protein import but do not appear to be obligatory for the process. It may be that the relative kinetics for protein synthesis and protein binding to mitochondria are responsible for the difference between the two mechanisms (Suissa & Schatz, 1982).

Cytosolic precursors differ from their mature counterparts. The most obvious difference is the precursors' larger size. Other differences include that they are soluble in the cytosol, including integral membrane proteins, are recognized by the mitochondrial import machinery and are in an import competent conformation (Hartl et al., 1989). The larger precursor proteins are synthesized with an amino-terminal extension which likely conveys many of these requirements. For example, subunit 9 of the Neurospora crassa  $F_0F_1$  ATPase ( $F_0F_19$ ), a very hydrophobic protein of 81 amino acids, is synthesized with a very hydrophilic 66 amino acid presequence that could help convey cytosolic solubility (Viebrock et al., 1982). The precursor forms aggregates possibly by the interaction of the hydrophobic portion of the precursor thereby exposing the hydrophilic presequence to the cytosol. For those integral membrane proteins made without presequences, solubility must be conferred by a conformation which differs from that of the mature protein. This would also include the formation of complexes in the cytosol to convey solubility. Such is the case for the ADP/ATP carrier, an inner membrane protein which forms soluble complexes (Zimmermann & Neupert, 1980). Also, a different conformation is assumed as the mature protein in the mitochondrial inner membrane binds carboxyatractyloside and passes through a hydroxyapatite column in the presence of the inhibitor while the precursor does not (Schleyer & Neupert, 1984).

Experiments in which the presequence is removed show that mature proteins are not imported into mitochondria (Gasser et al., 1982) indicating that the presequence is required for recognition by the mitochondrial import machinery. However, there is some evidence that

the mature portion of the protein can contain redundant targeting information as seen with the yeast  $F_1$ -ATPase  $\beta$  subunit ( $F_1\beta$ ) (Bedwell et al., 1987). Deletion of the entire  $F_1\beta$  subunit presequence did not prevent import although the efficiency was decreased. Obviously, for proteins synthesized without presequences, sequences recognized by the import machinery reside within the protein itself (Bedwell et al., 1987).

For either of these two sets of proteins, those with and those without presequences, the precursors must be in an import competent conformation. This is in part accomplished by their solubility in the cytosol. Equally important is that the precursor be unfolded to some degree (Eilers & Schatz, 1988; Zimmermann & Meyer, 1986). Several lines of evidence support this conclusion. Eilers and Schatz (1986) examined the effect of conformation on the import of a fusion protein joining the presequence of cytochrome oxidase subunit IV to dihydrofolate reductase. In the presence of methotrexate, an antifolate drug which binds tightly to dihydrofolate reductase, the fusion protein was not imported into mitochondria suggesting that the protein must be able to unfold for import (Eilers & Schatz, 1986). This led Rothman and Kornberg (1986) to propose the existence of an ATP-dependent "unfoldase" which would keep precursors in an import competent, unfolded conformation. In support of the need for an unfolded conformation for import, Chen and Douglas (1987a) showed that the import of a  $F_1\beta$ -copper metallothionein fusion protein was inhibited in the presence of copper. Verner and Schatz (1987) found that unlike completed chains, incompletely synthesized precursor chains did not require ATP for import and were more sensitive

to protease digestion. Further, Pfanner et al. (1987a) found that precursors with the same presequence but different mature portions required different levels of ATP or GTP for import and that the precursors studied, including the ADP/ATP carrier were more sensitive to protease digestion in the presence than in the absence of ATP or GTP, again suggesting the importance of an unfolded conformation for import competence.

The role of the proposed unfoldase is very similar to the role ascribed to the 70 kDa heat shock proteins by Pelham (1986). These proteins disrupt the quaternary structure or denature protein aggregates. Indeed, it has recently been suggested that the unfoldase activity is a member of the hsp70 gene family (Deshaies et al., 1988a,b; Pelham, 1988) and that the original role of hsp70 be extended to relaxing the tertiary structure of single polypeptides. Convincing results come from studies with yeast both in vitro (Chirico et al., 1988) and in vivo (Deshaies et al., 1988a). In the study by Chirico et al. (1988), translocation of prepro- $\alpha$ -factor, synthesized in a wheat germ translation system, into yeast microsomes required Ssalp/Ssa2p, which are two yeast hsp70 proteins, and an N-ethylmaleimide sensitive activity. In the in vivo study, depletion of Ssalp resulted in an accumulation of prepro- $\alpha$ -factor and F<sub>1</sub> $\beta$  precursor under steady state conditions (Deshaies et al., 1988a). Pulse-labeling experiments and experiments using accessibility to exogenously added protease demonstrated that the accumulation of newly synthesized prepro- $\alpha$ -factor resulted from a block in translocation of the protein across the membrane rather than in the covalent modification which occurs within

the lumen of the endoplasmic reticulum (Deshaies et al., 1988a). These results demonstrated that hsp70s function in post-translational import (Deshaies et al., 1988a).

Chaperones were defined by Ellis (1987) as cellular proteins which assist folding and assembly of oligomeric protein structures but do not form part of the final structure of the protein complex nor do they necessarily contain information specifying assembly. Chaperones were then divided into three groups; nucleoplasmins, the BiP group and chaperonins (Ellis et al., 1989). Nucleoplasmins were first described by Laskey et al. (1978) along with the concept of molecular chaperones. The nucleoplasmins are involved in nucleosome assembly. The BiP group includes the immunoglobulin heavy chain binding protein (BiP or GRP-78) which is found in the lumen of the endoplasmic reticulum, the major hsp70 protein in mammalian cell cytosol and the yeast cytosolic hsp70 proteins, Ssa1p and Ssa2p. Chaperonins are proteins found in mitochondria, chloroplasts and prokaryotes and appear to be involved in the assembly of oligomeric protein complexes. They include the Escherichia coli groEL gene (Ellis et al., 1989), the chloroplast ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) subunit-binding protein (Hemmingsen et al., 1988) and hsp60 (Cheng et al., 1989; Ostermann et al., 1989).

Besides the involvement of hsp70s, other cytosolic factors are suggested to be required for import. These factors are reported present in rabbit reticulocyte lysate (Argan & Shore, 1985; Ono & Tuboi, 1988). Murakami et al. (1988) partially purified an N-ethylmaleimide sensitive component from a yeast post-ribosomal supernatant which acts



synergistically with Ssalp/Ssa2p in the in vitro import of delta<sup>1</sup>-pyrroline-5-carboxylate dehydrogenase into yeast mitochondria. Randall and Shore (1989) reported an N-ethylmaleimide sensitive, import stimulating factor that interacts with the surface of mitochondria. Its activity may be dependent on the structure of the mature portion of the precursor as deleting the carboxy-73 amino acids from preornithine carbamoyltransferase resulted in the lack of stimulation for import by the factor (Randall & Shore, 1989). The exact nature and function these cytosolic factors play in precursor solubility and import competent conformation is unclear, however, studies with purified components should help to elucidate the number and role of these factors.

#### 1.1.2 MITOCHONDRIAL TARGETING SIGNALS

The search for mitochondrial targeting signals began by examining the possible role of presequences of imported proteins in part because the signal targeting proteins to the endoplasmic reticulum is contained within the presequence. The role of the presequence can be studied directly using recombinant DNA technology to join its coding sequence to the coding sequence of a non-mitochondrial passenger protein such as dihydrofolate reductase. The resulting fusion protein can then be synthesized using an in vitro expression system. The first studies with fusion proteins demonstrating the role of mitochondrial presequences were carried out by Hurt et al. (1984) and Horwich et al. (1985). Hurt et al. (1984) showed that a fusion protein joining the N-terminal 53 residues of the yeast cytochrome c oxidase subunit IV precursor to mouse dihydrofolate reductase was imported to the mitochondrial matrix. A

later study showed that only the first 12 amino acids were necessary for signalling import (Hurt et al., 1985). Horwich et al. (1985) found that the presequence of human ornithine carbamoyltransferase fused to dihydrofolate reductase targeted the protein to mitochondria. In a later report they showed that the midportion of the presequence was all that was required for targeting and that arginine<sub>23</sub> was critical for targeting function (Horwich et al., 1986).

Once it had been established that presequences target proteins to mitochondria, their sequences were compared to look for homology but none was found. (A list of presequences can be found in the review by Hartl et al. (1989) and include 2 in the intermembrane space, 28 in the inner membrane including 4 which face the cytosol, 14 which face the matrix and 10 which are intrinsic proteins, and 41 in the matrix.) Although no sequence homology exists, presequences do share some common structural features. These include being rich in basic amino acids (usually arginine), lacking acidic residues, having a high content of hydroxyl group-containing amino acids (usually serine), and having a tendency to fold into an amphipathic  $\alpha$ -helix. An example is the presequence of a mitochondrial matrix protein, rat malate dehydrogenase, given here using the single letter amino acid code: MLSALARPVGAALRRSFSTSAQNN (adapted from Nicholson & Neupert, 1988). Hypothetical roles for these structural features include interaction with receptors, spontaneous penetration into the bilayer due to the amphipathic nature of the signal, and translocation by an electrophoretic mechanism driven by the mitochondrial membrane potential (negative inside) due to the net positive charge of the signal. It is

possible that a combination of these are involved in the targeting signal's function.

The importance of positive charge in the targeting signal has been studied. Substitution of arginine<sub>23</sub> by glycine in the presequence of ornithine carbamoyltransferase resulted in lack of import into mitochondria (Horwich et al., 1986). In a later study, Horwich et al. (1987) showed that as the net positive charge was reduced, there was a corresponding loss in import function. Another study removed the targeting signal from F<sub>1</sub>β and analyzed the spontaneous mutations which resulted in the correct localization of the altered protein (Vassarotti et al., 1987). The modifications observed were the replacement of amino-terminal acidic residues with neutral or basic amino acids thus generating less negative and more positive charge. They suggest that this would result in a less acidic amphipathic helix. In another study, Chu et al. (1987a) replaced arginine<sub>14</sub> with asparagine, glutamine, alanine or glutamic acid in the presequence of rat malate dehydrogenase and found a decrease in the relative level of import again demonstrating the importance of positive charge. Moreover, they also found that replacing leucine<sub>13</sub> with glutamic acid or asparagine abolished import. Asparagine substitution for leucine<sub>13</sub> indicated that the change decreased import by a mechanism which was independent of changes in net charge (Chu et al., 1987b). Substitutions with proline, histidine or arginine severely diminished import while replacement with glutamine, tyrosine, valine or alanine did not affect import suggesting that uncharged residues are also important and may play a role in binding precursors to the mitochondrial surface (Chu et al., 1987b).

The importance of a high hydrophobic moment has been addressed by von Heijne (1986). Analysis of 23 mitochondrial targeting sequences with respect to their potential to form amphipathic helices revealed that most of the sequences could be expected to form helices with a high hydrophobic moment. It was also observed that the segments with maximal hydrophobic moment closely coincided with the critical regions for targeting defined by deletions and point mutations (von Heijne, 1986).

Studies with synthetic peptides emphasize the importance of the amphipathic structure. The chemically synthesized signal peptide of yeast cytochrome *c* oxidase subunit IV is soluble in aqueous buffer and yet spontaneously inserts into phospholipid monolayers (Roise *et al.*, 1986; Tamm, 1986). The effect is enhanced in the presence of anionic phospholipids and incorporation is consistent with the peptide becoming at least partially  $\alpha$ -helical (Roise *et al.*, 1986; Tamm, 1986). Anionic phospholipids also induce an  $\alpha$ -helical structure for a synthetic peptide corresponding to the first 27 residues of preornithine carbamoyltransferase (Epanand *et al.*, 1986). The interaction of this peptide with anionic lipid vesicles is independent of a transbilayer potential (Skerjanc *et al.*, 1987). While an amphipathic  $\alpha$ -helix appears to be a common structural feature for targeting signals, Roise *et al.* (1988) using artificial presequences showed that while all active presequences were amphipathic they were not necessarily  $\alpha$ -helical. Results from a later study by Lemire *et al.* (1989) support the hypothesis that the targeting signal for a matrix protein is usually a positively charged amphipathic  $\alpha$ -helix. Sequence analysis of 89 randomly generated peptide sequences fused to the amino terminus of

mature yeast cytochrome oxidase subunit IV showed that their efficiency as targeting signals correlated with the potential to fold into an amphipathic  $\alpha$ -helix (Lemire et al., 1989). An analysis carried out by von Heijne et al. (1989) on 36 mitochondrial targeting presequences revealed two structurally distinct domains: an amino-terminal domain with amphipathic  $\alpha$ -helical character and a carboxy-terminal domain with different amphipathic properties. From these results and others, it seems that both positive charge and a high hydrophobic moment resulting in an amphipathic  $\alpha$ -helix or another amphipathic structure are important for interaction with anionic phospholipids of the outer membrane.

There is also evidence that regions other than the targeting signal are involved in targeting. In studies joining the presequence of  $F_0F_1$ 9 to dihydrofolate reductase, import rates increased when some of the mature, hydrophobic portion of subunit 9 were included in the fusion protein indicating that hydrophobic "assistant" sequences can increase import efficiency (Pfanner et al., 1987b).

Recent studies have shown that cryptic and artificial sequences can also target passenger proteins to mitochondria. Hurt and Schatz (1987) fused the first 85 amino acids of dihydrofolate reductase to dihydrofolate reductase and found that the resulting protein was imported into mitochondria. Random sequences from the E. coli genome can also function to target passenger proteins to mitochondria (Baker & Schatz, 1987) as can artificial presequences (Allison & Schatz, 1986).

Despite the fact that cryptic and artificial sequences can target proteins to mitochondria, mitochondria maintain a specific protein composition. It has been shown that non-mitochondrial proteins are

targeted to mitochondria, however, this occurs at low rates (Hurt et al., 1986). The number of steps involved in targeting and import contribute to the specificity and the efficiency of import. It could be that some steps may be bypassed but this reflects the obligatory features for translocation (Pfaller et al., 1989).

Not all mitochondrial proteins are synthesized with cleavable presequences and yet, they too are targeted to and imported into mitochondria. These include proteins of the outer membrane and proteins from the other subcompartments. For example, two outer membrane proteins, the yeast 70 kDa protein and porin from yeast and *N. crassa*, are not synthesized with an N-terminal extension. In the case of the 70 kDa protein, the amino terminus resembles presequences of other mitochondrial proteins. Hase et al. (1984) showed that all the information for targeting and sorting is contained within the N-terminal 41 residues and that matrix targeting is mediated by the first 11 amino acids. This was accomplished by a series of deletions and fusions joining different lengths of the N-terminal region to  $\beta$ -galactosidase. When the first 41 amino acids of the protein were deleted, the resulting construct was recovered with the cytosolic fraction indicating that the N-terminal 41 amino acids contained all the information for targeting and anchoring the 70 kDa protein. Fusion studies confirmed this result. Fusing the N-terminal 61 amino acids to  $\beta$ -galactosidase resulted in mitochondrial targeting whereas the N-terminal 21 amino acids failed to efficiently target the fusion protein to mitochondria. Some of the deletion constructs which removed sequences downstream from amino acid 11 completely abolished normal attachment to the mitochondrial outer

membrane but still allowed a fraction of the construct to reach the matrix indicating that the residual targeting was most likely effected by amino acids 1 to 11 (Hase et al., 1984).

Table 1 provides a list of the non-outer membrane proteins synthesized without cleavable presequences that are known at present. The proteins are organized according to their location within the mitochondrial subcompartments: 3 in the matrix; 2 in the intermembranespace; and 11 in the inner membrane (cytochrome bc<sub>1</sub> complex subunit VI and the ubiquinone-binding protein are counted as one as are cytochrome c oxidase subunit VIa-c which are also counted as one protein; see Table 1 for an explanation). It should be noted that of those proteins whose sequence is known from several sources, there is no presequence in any case. This includes examples from mammals and fungi.

The targeting information of these proteins must reside within the mature protein itself. Proteins made without a cleavable presequence can be divided into three groups, those whose amino termini resemble presequences, those whose amino termini are similar to presequences but differ by having one or two acidic residues and those whose amino termini do not resemble presequences. Amino termini which resemble presequences are indicated by (++) in Table 1, while those which are similar but differ by having one or two acidic residues are indicated by (+). Amino termini which do not resemble presequences are indicated by (-). Examples from the first two groups include 2-isopropyl malate synthase, 3-oxoacyl-CoA thiolase and cytochrome c oxidase subunit VIc (see Table 1 for references). Amaya et al. (1988) found that amino acids 1 to 61 of rat 3-oxoacyl-CoA thiolase could

TABLE 1 Mitochondrial Proteins without Presequences

Protein	Evidence and Reference	Matrix
<b>Proteins</b>		
2-isopropyl malate synthase (+) (yeast)	protein mobility on gel*, uptake into isolated mitochondria <sup>1</sup> targeting studies using fusion proteins from expression plasmids <sup>2</sup>	
3-oxoacyl-CoA thiolase (++) (rat)	protein mobility on gel <sup>3</sup> cDNA sequence <sup>4</sup>	
cyt-21 mitochondrial ribosomal protein (++) ( <i>N. crassa</i> )	DNA sequence <sup>5</sup>	
<b>Intermembrane Space Proteins</b>		
adenylate kinase (-) (chicken) (bovine)	protein mobility on gel <sup>6</sup> cDNA sequence <sup>7</sup>	
cytochrome c (-) ( <i>N. crassa</i> ) (rat)	relative molecular mass of precursor and mature forms equal <sup>8</sup> protein mobility on gel, amino-terminal amino acid sequence <sup>9</sup> protein mobility on gel, amino acid sequence <sup>10</sup>	
<b>Inner Membrane Proteins</b>		
ADP/ATP carrier (-) (human) ( <i>N. crassa</i> ) (rat) (yeast)	cDNA sequence, comparison to bovine amino acid sequence <sup>11</sup> protein mobility on gel <sup>12</sup> protein mobility on gel <sup>13</sup> cDNA sequence <sup>14</sup>	
ATP synthase subunit d (+) (bovine)	amino acid sequence and cDNA sequence <sup>15</sup>	
cytochrome <u>bc</u> <sub>1</sub> complex:		
subunit VI (-) (bovine <sup>†</sup> ) ( <i>N. crassa</i> ) (yeast)	protein mobility on gel <sup>16</sup> protein mobility on gel <sup>17</sup> DNA sequence and [ <sup>35</sup> S]met at positions 1 and 3 of both precursor and mature forms <sup>18</sup>	
subunit VII (+) (yeast)	DNA sequence <sup>19</sup>	



subunit VIII (-) (yeast)	DNA sequence and amino-terminal amino acid sequence <sup>20</sup>
hinge protein (-) (human)	DNA sequence <sup>21</sup>
ubiquinone-binding protein (++) (rat) (human)	partial cDNA sequence, protein mobility on gel after <u>in vitro</u> translation and immunoprecipitation <sup>16</sup> cDNA sequence <sup>22</sup>
QCR9 (++) (yeast)	DNA sequence and amino-terminal amino acid sequence <sup>23</sup>
cytochrome c oxidase:	
subunit VIIa (-) (yeast <sup>†</sup> )	protein mobility on gel <sup>24</sup> cDNA sequence <sup>25</sup>
subunit VIa <sup>§</sup> (-) (rat) (human)	cDNA sequence <sup>26, 27</sup> cDNA sequence <sup>28</sup>
subunit VIb? (-) (human)	cDNA sequence <sup>29</sup>
subunit VIc (++) (rat) (human)	DNA sequence <sup>30</sup> cDNA sequence <sup>31</sup>
subunit Vc (++) (sweet potato)	protein mobility on gel after <u>in vitro</u> translation and immunoprecipitation <sup>32</sup>
uncoupling protein (hamster) (rat)	amino acid sequence <sup>33</sup> cDNA sequence, amino-terminal amino acid sequence <sup>34, 35</sup>

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(++) N-terminal region resembles presequences.

(+) N-terminal region is similar to presequences but differ by having one or two acidic residues in the region.

(-) N-terminal region does not resemble presequences nor is the nature of the targeting signal obvious.

\*Protein mobility on gel indicated that the precursor has the same mobility as the mature protein on SDS polyacrylamide gels.

<sup>†</sup>It should be noted that in the case of cytochrome bc<sub>1</sub> complex subunit VI, the bovine subunit has been found to be nearly identical to the rat and human ubiquinone-binding protein (Suzuki et al., 1988) indicating that these may be the same protein.

<sup>‡</sup>It should also be noted that yeast cytochrome oxidase subunit VIIa has a C-terminal 4 amino acid extension (Wright et al., 1986).

<sup>§</sup>In the case of cytochrome c oxidase subunits VIa-c, the homology of all these subunits is still unclear so that the origin of the differences is not apparent.

1. Hampsey et al., 1983; 2. Beltzer et al., 1988; 3. Miura et al., 1986;

4. Arakawa et al., 1987; 5. Kuiper et al., 1988; 6. Watanabe & Kubo, 1982; 7. Kishi et al., 1987; 8. Korb & Neupert, 1978; 9. Zimmermann et al., 1979; 10. Matsuura et al., 1981; 11. Battini et al., 1987; 12. Zimmermann & Neupert, 1980; 13. Hatalová & Kolarov, 1983; 14. Adrian et al., 1986; 15. Walker et al., 1987; 16. Nishikimi et al., 1986; 17. Teintze et al., 1982; 18. van Loon et al., 1984; 19. De Haan et al., 1984; 20. Maarse & Grivell, 1987; 21. Ohta et al., 1987; 22. Suzuki et al., 1988; 23. Phillips et al., 1989; 24. Mihara & Blobel, 1980; 25. Wright et al., 1986; 26. Schlerf et al., 1988; 27. Goto et al., 1989; 28. Fabrizi et al., 1989; 29. Taarman et al., 1989; 30. Suske et al., 1987; 31. Otsuka et al., 1988; 32. Nakagawa et al., 1987; 33. Aquila et al., 1985; 34. Ridely et al., 1986; 35. Bouillaud et al., 1986

target mature ornithine carbamoyltransferase to mitochondria indicating that the N-terminal 61 amino acids function as a non-cleavable signal for targeting and import. Examples from the third group include cytochrome c, adenylate kinase, and the ADP/ATP carrier (see Table 1 for references). Current understanding on the targeting and import of the ADP/ATP carrier will be discussed in detail later on as the carrier is homologous to uncoupling protein and can serve as a starting point for the search of the targeting signal in uncoupling protein. However, it should be noted here that Pfanner et al. (1987c) showed that the C-terminal two-thirds of the N. crassa protein could be imported into isolated mitochondria indicating that targeting information can reside in regions distant from the amino terminus.

### 1.1.3 IMPORT RECEPTORS

The binding of mitochondrial proteins to a receptor is suggested to be an early step in the import process. There are several lines of evidence to support the existence of these receptors. One approach to determine the nature of the targeting region and the receptor with which it interacts is the use of synthetic peptides to inhibit the import of precursors. In early competition experiments, a synthetic peptide corresponding to the 27 N-terminal amino acids of rat preornithine carbamoyltransferase blocked the import of the complete precursor, the rat malate dehydrogenase precursor and rat uncoupling protein precursor whereas a synthetic peptide corresponding to amino acids 16 to 27 of the precursor failed to block import (Gillespie et al., 1985). This led Gillespie et al. (1985) to conclude that a common mitochondrial signal

recognition apparatus is employed for the import of both inner membrane and matrix proteins and that the competition observed was for an essential component of the import apparatus, possibly an import receptor. Ono and Tuboi (1988) showed that a 34 amino acid peptide of preornithine aminotransferase inhibited the import of ornithine aminotransferase, a large subunit of succinate dehydrogenase, sulfite oxidase and porin. Chu et al. (1989) showed that a synthetic peptide corresponding to the N-terminal 28 amino acids of rat premalate dehydrogenase inhibited the import of the rat precursors of malate dehydrogenase and ornithine carbamoyltransferase. A matrix enzyme synthesized without a cleavable presequence but having an N-terminus similar to presequences, rat 3-oxoacyl-CoA thiolase, was shown to inhibit the import of bovine ornithine carbamoyltransferase, rat medium-chain acyl-CoA dehydrogenase and rat acetoacetyl-CoA thiolase, three matrix enzymes synthesized as larger precursors (Mori et al., 1985). Results of all these experiments indicated common receptors or shared import components.

Another approach used is to pretreat mitochondria with low concentrations of proteases which leave the outer membrane intact but could still destroy any possible proteinaceous surface receptors. Pretreatment of mitochondria with trypsin inhibited the import of yeast cytochrome  $b_2$ , yeast citrate synthase (Riezman et al., 1983) and rat ornithine carbamoyltransferase (Argan et al., 1983). Pretreatment of mitochondria with low concentrations of either trypsin or proteinase K inhibited the import of the following *N. crassa* proteins: porin, the ADP/ATP carrier,  $F_0F_1$ 9,  $F_1\beta$  and the Fe/S protein of the  $bc_1$ -complex

(Pfaller et al., 1989) but not apocytochrome c (Nicholson et al., 1988). Pretreatment with elastase inhibited the import of the N. crassa proteins porin and the ADP/ATP carrier but not the import of  $F_0F_1$  or  $F_1\beta$  (Zwizinski et al., 1984). Pfaller et al. (1988) later showed that pretreatment with elastase did inhibit the import of  $F_0F_1$  but not  $F_1\beta$ . Taken together these results suggest the existence of at least three types of receptors, one for porin,  $F_0F_1$  and the ADP/ATP carrier, another for  $F_1\beta$  and a third for apocytochrome c. The binding sites for porin (Pfaller & Neupert, 1988) and the ADP/ATP carrier (Hartl et al., 1989) have been titrated separately indicating the existence of a separate receptor for the ADP/ATP carrier bringing the total of separate receptors to 4. Recently, Söllner et al. (1989) report the identification of a N. crassa 19 kDa protein of the outer membrane which they believe is a specific mitochondrial import receptor. Monospecific IgG and Fab fragments against the protein inhibited the import of the N. crassa proteins porin, cytochrome c<sub>1</sub>, the Fe/S protein of the bc<sub>1</sub> complex,  $F_0F_1$  and  $F_1\beta$  at the level of high affinity binding of the precursors to mitochondria. The antibodies and the Fab fragments did not inhibit the import of the ADP/ATP carrier or apocytochrome c (Söllner et al., 1989). Their results indicate the existence of two N. crassa receptors, one for the ADP/ATP carrier and one for the rest of the mitochondrial proteins. Söllner et al. (1989) clarified the observation from previous studies using differential sensitivity to elastase that suggested the existence of separate receptors for porin and  $F_1\beta$  (Zwizinski et al., 1984; Pfaller et al., 1988). Mild elastase treatment generated initially an 18 kDa fragment and subsequently a 17

kDa fragment (Söllner et al., 1989). The import of porin and other precursors required the intact receptor or the 18 kDa fragment whereas import of  $F_1\beta$  was unaffected provided that the 17 kDa fragment was present. Söllner et al. (1989) propose that precursors similar to the ADP/ATP carrier would use the same receptor. This would include precursors which do not carry a signal sequence at the N-terminus of the precursor, and were most likely not present in the prokaryotic ancestors of mitochondria. The import of apocytochrome c represents a third pathway distinct from all other mitochondrial proteins presently known. Translocation into the intermembrane space is coupled to the covalent addition of heme by cytochrome c heme lyase, an intermembrane space enzyme (Nicholson et al., 1988) and import is independent of protease-accessible surface components (Söllner et al., 1989).

Studies with the ADP/ATP carrier have led to a clearer understanding of a possible receptor and other components of the import pathway. Import of the carrier has been studied extensively by Neupert and his coworkers. They have found that there are two steps involved in the binding of the N. crassa protein to mitochondria (Pfaller et al., 1988; Pfanner & Neupert, 1987b; Pfanner et al., 1987a). The first step is the binding to the receptor which can occur at low temperature, low NTP levels or in the absence of the membrane potential. At this point, the protein is still accessible to externally added proteases. The second step requires higher temperatures and NTP levels. Pfaller et al. (1988) extended their studies of binding sites to include the Fe/S protein of the bc<sub>1</sub>-complex,  $F_0F_1$  and  $F_1\beta$ . They showed that the precursors interacted first with distinct protease-accessible binding

sites on the mitochondrial surface. Competition observed after this point suggested that the import pathways converged at a common insertion site in the outer membrane where the precursors became protease-resistant. These results led Pfaller et al. (1988) to suggest that these proteins interact with another mitochondrial protein in the outer membrane after binding to their specific receptors. This protein they termed the general insertion protein (GIP) (Pfaller et al., 1988).

The binding to receptor sites (Hartl et al., 1989) and binding to GIP (Pfaller et al., 1988) have been titrated separately for the ADP/ATP carrier using Scatchard analysis. Results showed that there are approximately 10 times as many GIP sites as specific receptors. In competition experiments, porin competed for binding with the Fe/S protein, the ADP/ATP carrier,  $F_0F_1^9$  and  $F_1\beta$  but not cytochrome  $c$  (Pfaller et al., 1988). The competition between the ADP/ATP carrier and porin was for the GIP. Cytochrome  $c$  does not compete with any protein tested to date, but binding sites have been titrated for this protein as well (Hennig et al., 1983).

#### 1.1.4 PROTEIN TRANSLOCATION

Outer membrane proteins appear to have the simplest import pathway (Hartl et al., 1989). It is proposed that they bind first to specific binding sites and then interact with the GIP before being sorted to the outer membrane where assembly takes place. Import of these proteins is independent of a membrane potential and no proteolytic processing occurs.

Translocation across a membrane does not occur for outer membrane proteins. However, for proteins of the other mitochondrial subcompartments, at least one membrane must be crossed. The lipid portion of the phospholipid bilayer poses a barrier to the hydrophilic portions of proteins and as a result, import models suggest that translocation occurs through a proteinaceous pore (e.g. Singer et al., 1987). In support of this idea, trapped precursors of  $F_1\beta$ , the ADP/ATP carrier and cytochrome  $b_2$  can be extracted from the membranes by protein denaturants such as alkaline pH or urea (Pfanner et al., 1987d).

Given that translocation is via a proteinaceous pore, two possible pathways exist for import into the matrix. The first is for the precursor to cross the outer membrane, the intermembrane space and then the inner membrane, or secondly, to cross at contact sites where outer and inner membranes come together. The evidence is in favour of translocation occurring at contact sites. Schleyer and Neupert (1985) found that the precursors of  $F_1\beta$  and cytochrome  $c_1$  could be trapped in the import pathway by low temperature such that the amino termini were processed by a matrix protease while a portion of the precursors were still accessible to externally added proteases at concentrations that did not degrade the outer membrane. The precursors could also be trapped by first binding to C-terminal directed antibodies before import. These results showed that the inner and outer membranes were close enough to be spanned by the precursors.

A later study by Schwaiger et al. (1987) showed that translocation intermediates were located at contact sites.  $F_1\beta$  was trapped by first prebinding the precursor with antibodies before being



added to mitochondria. Protein A gold was used to react with the antibodies and for visualization by electron microscopic analysis. The gold particles were observed over the contact sites between the outer and inner membranes (Schwaiger et al., 1987).

#### 1.1.5 ENERGY REQUIREMENTS

Early studies by Schleyer et al. (1982) using inhibitors of electron transport (rotenone, antimycin A and potassium cyanide), an inhibitor of  $F_0F_1$ ATPase (oligomycin), protonophores, i.e. uncouplers, (CCCP and FCCP), a  $K^+$  ionophore (valinomycin) and an inhibitor of the ADP/ATP carrier (carboxyatractyloside) showed that protein import requires energized mitochondria. Similar results were obtained by Gasser et al. (1982) and Kolansky et al. (1982). Two components make up the electrochemical potential of energized mitochondria, the membrane potential ( $\Delta\psi$ ) and the chemical potential ( $\Delta pH$ ). Pfanner and Neupert (1985) showed that the ADP/ATP carrier could be imported by a valinomycin-induced potassium diffusion potential which could not be inhibited by the addition of a protonophore indicating that the membrane potential is the energy source. It has since been accepted that the membrane potential is required for the initial interaction of the precursor with the inner membrane (Hartl et al., 1989). Completion of translocation occurs without the membrane potential as has been shown for  $F_1\beta$ , cytochrome  $c_1$  (Schleyer & Neupert, 1985), the Rieske Fe/S protein (Hartl et al., 1986), the ADP/ATP carrier (Pfanner & Neupert, 1987b) and cytochrome  $b_2$  (Pfanner et al., 1987d). The current hypothesis for the role of the membrane potential is that it exerts an

electrophoretic effect on positively charged presequences or domains in precursors synthesized without cleavable presequences to pull the presequences or domains into the mitochondrial inner membrane (Hartl et al., 1989).

Import also requires energy in the form of NTPs (ATP or GTP) and the requirement is independent of that for the membrane potential. Pfanner and Neupert (1986) demonstrated this for  $F_1\beta$  by showing that import of the protein did not occur in the presence of the membrane potential without the addition of ATP or GTP. Import also did not occur without the presence of the membrane potential even if NTPs were present (Pfanner & Neupert, 1986). NTPs were shown to be required for the initial interaction with the mitochondrial surface and higher levels of NTPs were required for the completion of translocation. This has been demonstrated for  $F_1\beta$  (Chen & Douglas, 1987b; Pfanner & Neupert, 1986), the ADP/ATP carrier (Pfanner et al., 1987a), porin (Kleene et al., 1987), a fusion protein joining  $F_0F_1^9$  to dihydrofolate reductase (Pfanner et al., 1987a), and a fusion protein joining the presequence of cytochrome oxidase subunit IV to dihydrofolate reductase (Eilers et al., 1987).

The role of NTPs in the import of precursors into mitochondria appears to be to provide energy to keep the precursors in an import-competent conformation. The evidence includes results from fusion protein studies where the proteins were more sensitive to proteinase K digestion in the presence of NTPs suggesting that the proteins were in an unfolded, import-competent conformation (Pfanner et al., 1987a). Further evidence comes from studies where acid/base treatment (exposure



to trichloroacetic acid, followed by alkali and rapid neutralization) of porin replaced the NTP requirement (Pfanner *et al.*, 1988) and incompletely synthesized precursors required lower NTP levels (Verner & Schatz, 1987). This role for NTPs is in line with the activity of the BiP group of chaperones or more specifically, the hsp70s. That is, hydrolysis of ATP by chaperones could disaggregate cytosolic aggregates and unfold the precursor for import.

Hwang and Schatz (1989) have shown that ATP is required in the matrix to complete translocation of precytochrome  $b_2$  and a fusion protein joining the presequence of cytochrome oxidase subunit IV to dihydrofolate reductase. This requirement was observed after the membrane potential-dependent step. In contrast, the import of porin into the outer membrane did not require ATP in the matrix but only outside the inner membrane, i.e. cytosolic ATP (Hwang & Schatz, 1989).

#### 1.1.6 PROCESSING

Proteolytic processing is obviously necessary for precursors of larger size to reach their mature length and perhaps conformation. Early work with *N. crassa* showed that a matrix peptidase responsible for the cleavage of the presequence of  $F_0F_1$  requires divalent cations for its activity and that it is inhibited by metal chelators, including ortho-phenanthroline (Schmidt *et al.*, 1984). Hawlitschek *et al.* (1988) purified the matrix peptidase and found it was comprised of two components, a 57 kDa matrix processing peptidase (MPP) and a 52 kDa processing enhancing protein (PEP), both of which are required but do not form a tight complex. Corresponding components have been identified



in yeast and are the gene products of MAS1 (Witte *et al.*, 1988; the PEP component) and MAS2 (Pollock *et al.*, 1988; the MPP component). Two sequentially acting matrix proteases have been identified and characterized in rat liver (Kalousek *et al.*, 1988) but how they relate to MPP and PEP is not known. Protease I is insensitive to EDTA chelation and N-ethylmaleimide while protease II is inactivated by both treatments (Kalousek *et al.*, 1988). A preparation enriched in protease II with only a trace amount of contaminating protease I has little or no cleavage activity toward mitochondrial precursors but rapidly and efficiently converts the intermediate forms of malate dehydrogenase and ornithine carbamoyltransferase to their mature size (Kalousek *et al.*, 1988). On the other hand, a preparation of protease I free of protease II is able to process the  $\beta$  subunit of propionyl-CoA carboxylase to its mature size (Kalousek *et al.*, 1988).

Other proteins also require a second cleavage step in order to achieve their final length. These proteins are intermembrane space proteins or proteins embedded in the inner membrane but extending into the intermembrane space and include cytochrome  $b_2$  and cytochrome  $c_1$  (Hartl *et al.*, 1987; Teintze *et al.*, 1982), cytochrome  $c$  peroxidase (Reid *et al.*, 1982) and the Rieske Fe/S protein (Hartl *et al.*, 1986). This second processing step has only been studied in depth in yeast and *N. crassa*. The second step occurs at the outer surface of the inner membrane and is insensitive to treatment with metal chelators (Hartl *et al.*, 1987). Evidence suggests that there are two proteases in the intermembrane space in yeast mitochondria. A temperature-sensitive *pet* mutant was shown to be defective in the second step for the processing



of cytochrome  $b_2$  but not cytochrome  $c$  peroxidase (Pratje & Guiard, 1986; Pratje *et al.*, 1983).

There is no consensus sequence for the cleavage site of the matrix protease, however, analysis of a representative sample of 36 mitochondrial presequences by von Heijne *et al.* (1989) revealed that arginine is frequently found a position -10 or -2 relative to the cleavage site. A more extensive analysis has been performed by Hendrick *et al.* (1989). Of the 50 mitochondrial proteins examined 8 are cleaved in two places by matrix proteases and when analysed, were found to have a highly conserved 3 amino acid motif in common. This included an arginine at position -10, a hydrophobic amino acid at position -8 and serine, threonine or glycine at position -5 relative to the second cleavage site (Hendrick *et al.*, 1989). The first cleavage occurred between -9 and -8 putting the arginine at -10 at position -2 relative to the cleaved bond. Another 14 proteins conformed with this motif, however, 2 step processing has not been confirmed for these proteins. A further 15 proteins were found to have arginine at position -2 meaning that 74% of the matrix processing cleavage sites follow an arginine at position -2 relative to the cleavage suggesting that the first enzyme is responsible for the cleavage (Hendrick *et al.*, 1989). The lack of a strict consensus sequence suggests that a common structure is recognized by the processing apparatus (Nicholson & Neupert, 1988). Also, proteolytic processing is not species specific indicating the existence of a common mechanism (Hartl *et al.*, 1989).

Other types of processing occur besides proteolytic cleavage. This includes the covalent attachment of prosthetic groups such as heme



to cytochrome c and c<sub>1</sub>, and the Fe/S centers on several subunits of respiratory complexes I, II, and III; the non-covalent attachment of coenzymes and cofactors such as NAD<sup>+</sup>, FAD, hemes and metal ions (Hartl et al., 1989). It should also be noted that processing is not needed for translocation of proteins into mitochondria. For example, after inhibition of the processing peptidase, the Fe/S protein of the bc<sub>1</sub> complex was still imported into mitochondria (Hartl et al., 1986).

#### 1.1.7 SORTING AND ASSEMBLY

Outer membrane proteins and proteins of the matrix appear to have the simplest sorting mechanism. It is suggested that outer membrane proteins such as N. crassa porin (Kleene et al., 1987; Pfaller & Neupert, 1987) and the yeast 70 kDa protein (Hase et al., 1984; Hurt et al., 1985) bind first to specific receptors on the mitochondrial surface, interact with the GIP and then are inserted into the outer membrane. Matrix proteins are proposed to follow the route shown in Fig. 1. They bind to their appropriate receptor, interact with a GIP and are translocated at contact sites to the matrix where processing and assembly occurs.

Proteins of the inner membrane and the intermembrane space follow more complicated sorting mechanisms. These proteins could be inserted directly into their appropriate subcompartment (the stop-transfer or stop-transport hypothesis) or they can be translocated to the matrix and then redirected to their final destination (the evolutionary or conservative model). Evidence exists for both mechanisms.



The stop-transfer hypothesis of Blobel (1980) was applied to mitochondrial targeting by Hurt and van Loon (1986). It is based on the linear arrangement of a matrix-targeting domain, a stop-transport domain and cleavage sites. The arrangement of these functional domains would determine the final location of the protein. Matrix and inner membrane proteins would have a matrix-targeting domain followed by a cleavage site. Intermembrane space proteins would have a matrix-targeting domain, a cleavage site, a stop-transfer domain holding the protein in the inner membrane, and a final cleavage site releasing the protein into the intermembrane space. Outer membrane proteins would have a matrix-targeting domain followed directly by a stop-transfer domain.

Evidence in support of this model comes from studies on the yeast 70 kDa protein, cytochrome  $c_1$  and rat pre-ornithine carbamoyltransferase fused to the stop-transfer domain of vesicular stomatitis virus G protein. Hase *et al.*, (1984) report that the 11 amino-terminal residues of the 70 kDa protein is responsible for matrix targeting while residues 9 to 38 anchor the protein in the outer membrane. In an analogous study the presequence of cytochrome  $c_1$  directed dihydrofolate reductase to the intermembrane space, while the first half of the presequence directed the fusion protein to the matrix (van Loon *et al.*, 1986). Kinetic studies showed that a fusion protein of the presequence of cytochrome  $c_1$  fused to cytochrome  $c$  oxidase subunit IV was transported to the intermembrane space but was never found in the matrix (van Loon & Schatz, 1987). Another study of a fusion protein joining the 19 amino acid stop-transfer domain of vesicular stomatitis virus G protein toward the C-terminus of pre-ornithine carbamoyltransferase



anchored the protein in the inner membrane via the stop-transfer sequence with the N-terminus facing the matrix and the short C-terminal tail in the intermembrane space (Nguyen *et al.*, 1988). When the same stop-transfer sequence was placed near the N-terminus of the protein, the protein was inserted in the outer membrane in the opposite orientation (Nguyen *et al.*, 1988).

Despite the above evidence, there are problems with this model. This mechanism of targeting has not been demonstrated for naturally occurring inner membrane proteins synthesized as larger precursors. It is possible that some proteins are sorted via the stop-transfer model, however, it is clear that the model does not apply to all proteins.

The evolutionary model or conservative sorting mechanism was first described by Hartl *et al.* (1986) working with the Fe/S protein of the  $bc_1$  complex of *N. crassa*. They demonstrate that the precursor is first translocated to the matrix where the first 24 amino acids of the presequence are removed. The remaining 8 residue peptide of the intermediate redirects the protein across the inner membrane in the absence of a membrane potential to its final location in the intermembrane space attached to the inner membrane. Hartl *et al.* (1986) suggest that the 24 amino acid presequence was added when the gene was transferred to the nucleus and that the presequence targets the precursor to the matrix where the "ancestral" assembly pathway takes over. This type of sorting has also been demonstrated for *N. crassa* cytochrome  $c_1$  and yeast cytochrome  $b_2$  (Hartl *et al.*, 1987) and for the Fe/S protein and cytochrome  $c$  oxidase subunit IV of yeast (van Loon & Schatz, 1987). At present, the results for the sorting mechanism for



cytochrome  $c_1$  in yeast (van Loon & Schatz, 1987) and in *N. crassa* (Hartl *et al.*, 1987) are contradictory and further work is required to determine which pathway is followed.

The final step for imported proteins includes folding of the protein to its mature conformation and assembly into homo- or hetero-oligomers. This step is poorly understood at the present time, however, chaperonins such as hsp60 appear to be involved (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). The co-ordination of both the nuclear and mitochondrial genomes is also poorly understood. This is especially true for protein complexes whose subunits are derived from both genomes. A review of this area is beyond the scope of the thesis and the review by Hartl *et al.* (1989) can be consulted for an overview and further references.

## 1.2 BROWN ADIPOSE TISSUE AND UNCOUPLING PROTEIN

Brown adipose tissue functions to produce heat in newborn mammals, small mammals in the cold and in animals arousing from hibernation (Nicholls & Locke, 1984). The heat produced by non-shivering thermogenesis is the result of an activated 32 kDa protein unique to brown adipose tissue mitochondria. Most commonly this protein is now termed the uncoupling protein or thermogenin. In mitochondria, oxidative phosphorylation generates an electrochemical gradient as protons are expelled across the inner membrane. The energy stored in the gradient generates ATP as protons flow through the ATP synthase. When uncoupling protein is activated in brown adipocyte mitochondria, the protein functions as a membrane potential driven proton translocator

(Klingenberg & Winkler, 1985) thereby uncoupling oxidative phosphorylation. The ATP synthase is bypassed and the energy is released as heat.

### 1.2.1 UNCOUPLING PROTEIN FUNCTION

The evidence suggesting that uncoupling protein is responsible for the uncoupling of brown adipose tissue mitochondria is summarized in a review by Nicholls and Locke (1984). Cross-linking analysis has shown that uncoupling protein functions as a dimer in vivo (Klingenberg & Appel, 1989; Lin & Klingenberg, 1982). In hamster, the disulfide bridge is likely to be formed between two cysteines at position 304 (Klingenberg & Appel, 1989). Other studies have shown that uncoupling protein is inhibited by purine nucleoside di- or triphosphates and is activated by fatty acids. Purine nucleotides bind to uncoupling protein at the outer face of the inner membrane thus inhibiting proton transport (Heaton et al., 1978). A direct correlation has been shown for nucleotide affinity and inhibition of proton transport (Heaton et al., 1978; Lin & Klingenberg, 1982) with the decreasing order of affinity for GTP, GDP, ATP and ADP (Lin & Klingenberg, 1982). Although the highest affinity is exhibited for GTP, ATP likely acts in vivo due to its higher concentration (Klingenberg, 1984; Nicholls & Locke, 1984).

Fatty acids increase proton transport and induce uncoupling of brown adipose tissue mitochondria (Locke et al., 1982). In non-energized mitochondria this results from lowering uncoupling protein's affinity for purine nucleotides (Rial et al., 1983). But in energized mitochondria it has been proposed that free fatty acids act by causing a



conformational change and not by competing for the nucleotides (Rial et al., 1983).

Direct proof that uncoupling protein is responsible for the observed effects comes from studies in which purified uncoupling protein was functionally reconstituted into liposomes (Klingenberg & Winkler, 1985; Strielman et al., 1985). Uncoupling protein was studied with respect to proton transport, nucleotide binding and the effect of free fatty acids. Uncoupling protein was shown to be necessary for the proton influx indicated by the increase in pH observed upon the addition of valinomycin to potassium ion loaded liposomes (Klingenberg & Winkler, 1985). From this study, Klingenberg and Winkler (1985) concluded that uncoupling protein's function as a proton translocator is dependent on the membrane potential. It was shown that GDP could inhibit the proton influx (Klingenberg & Winkler, 1985). Strielman et al. (1985) demonstrated that fatty acids increased the proton permeability of liposomes with uncoupling protein present suggesting that free fatty acids activate uncoupling of brown adipose tissue mitochondria and that they act via the uncoupling protein. These results strongly suggest that uncoupling protein is indeed responsible for the uncoupling of brown adipose tissue mitochondria, however, in the reconstitution studies, small amounts of other proteins were present (20% of other protein, Klingenberg & Winkler, 1985; 10% of other protein, Strielman et al., 1985).

### 1.2.2 UNCOUPLING PROTEIN, THE ADP/ATP CARRIER AND THE PHOSPHATE CARRIER: THE STRUCTURAL RELATIONSHIP

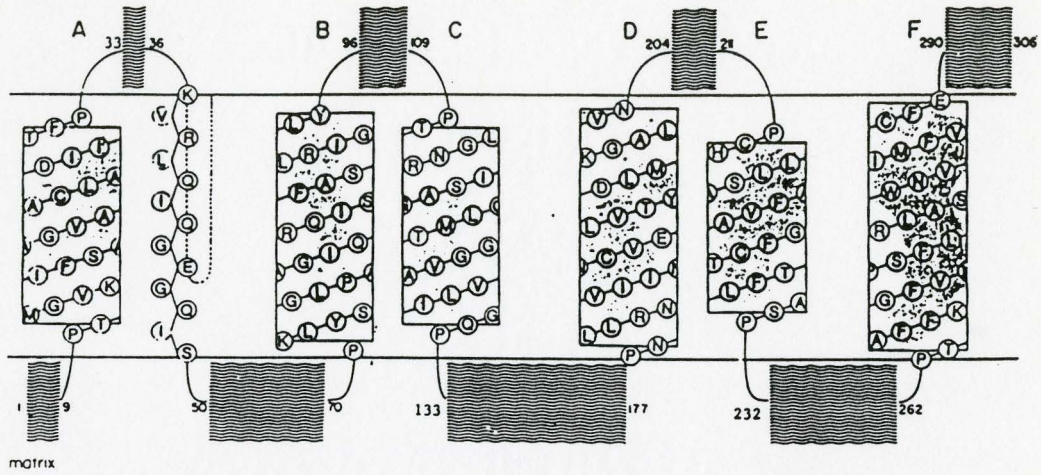
The primary structure for hamster uncoupling protein was determined by direct protein sequencing. The protein has 306 amino acids and a molecular mass of 33,215 Da (Aquila *et al.*, 1985). A full length cDNA clone for the rat protein has been obtained and sequenced (Bouillaud *et al.*, 1986; Ridley *et al.*, 1986a). The predicted amino acid sequence is 91.5% identical to the hamster sequence and consists of 307 amino acids with a molecular mass of 33,084 Da (Bouillaud *et al.*, 1986; Ridley *et al.*, 1986a). The N-terminal 30 amino acids were also determined by direct protein sequencing and are identical with the deduced sequence with the exception of the N-terminal methionine which is removed from the precursor (Ridley *et al.*, 1986a). Cleavage of the initiating methionine is not unusual and has been reported for other proteins (Tsunasawa *et al.*, 1985). The sequencing results confirm previous findings that uncoupling protein is synthesized as a mature length precursor (Freeman *et al.*, 1983; Freeman & Patel, 1984; Freeman *et al.*, 1985; Ricquier *et al.*, 1983).

Once the primary sequence was known, it became possible to predict secondary structure and a membrane disposition model for uncoupling protein. Hydropathy calculations which take sided  $\alpha$ -helices and  $\beta$  strands into account suggest the existence of 6  $\alpha$ -helices and an amphipathic  $\beta$  strand (Aquila *et al.*, 1985, 1987). The membrane disposition model is shown in Fig. 2a. Standard hydropathy calculations (Kyte & Doolittle, 1982) were performed by Runswick *et al.* (1987) and suggest the existence of 6 membrane spanning domains. It should be

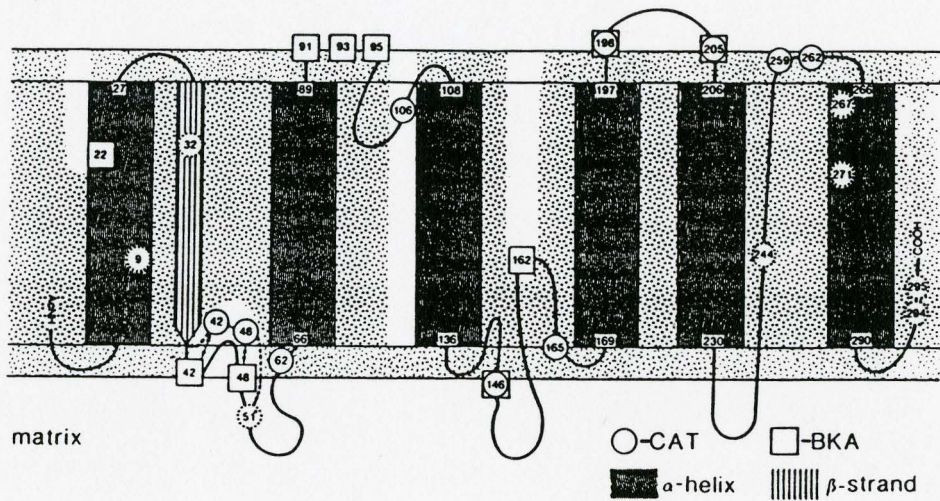


Figure 2. Membrane Disposition Models of Three Mitochondrial Transport Proteins. a, Hamster uncoupling protein as illustrated in Aquila *et al.*, (1985). The six membrane spanning  $\alpha$ -helices are labeled A-F. Areas with residues more hydrophobic than glycine are shaded grey. A possible  $\beta$ -strand is shown between helix A and a stretch of unknown structure as depicted by shaded boxes. The single amino acid code is used. b, Bovine heart ADP/ATP carrier as illustrated in Bogner *et al.*, (1986). Membrane spanning  $\alpha$ -helices and  $\beta$ -strands were derived from hydrophobicity distribution calculations for amphipathic  $\alpha$ - and  $\beta$ -structures. The direction of the segments toward the cytosol or the matrix was based on the availability of lysine residues for chemical derivitization in mitochondria and submitochondrial particles. The position of the lysines in the headgroup region is based on comparisons of incorporation into soluble protein, mitochondria and particles. Inaccessible lysines are marked by a dashed circle. Lysines accessible in a carboxyatractylate-protein complex are marked by a full circle. Those accessible in the bongkrekate-protein complex are marked by a rectangle. Possible assignment of lysines to the translocation path is indicated by omission of background pattern. c, Bovine phosphate carrier as illustrated in Aquila *et al.* (1987). The six membrane spanning  $\alpha$ -helices are labeled A-F. A possible  $\beta$ -strand is shown between helices A and B. The intervening hydrophilic segments are shown as hatched boxes as their structure is unclear. The single amino acid code is used.

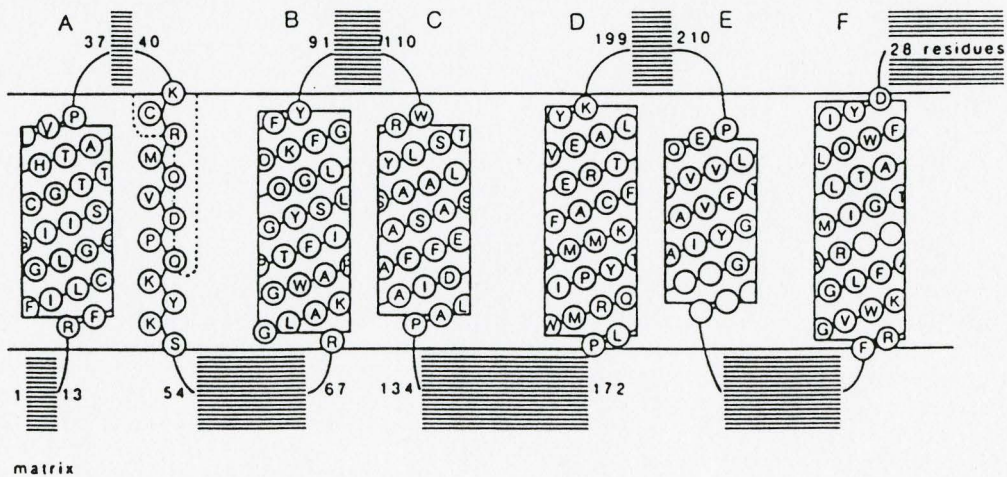
a cytosol



b cytosol



c cytosol



noted that differences between the two models leads to opposite positions of the N-terminus with respect to the C-terminus. There is evidence showing that the C-terminus is located on the cytosolic face of the inner membrane (Eckerskorn & Klingenberg, 1987). Further analysis of uncoupling protein in the membrane is required to determine which basic model is correct.

Analysis of the primary structure revealed that several features are very similar to bovine heart ADP/ATP carrier whose primary structure had been previously determined by Aquila et al. (1982). The ADP/ATP carrier is composed of 297 amino acids with a molecular mass of 32,906 Da. An updated membrane disposition model for the carrier is shown in Fig. 2b and is the result of a study using a lysine-specific labeling reagent with modified mitochondria, sub-mitochondrial particles and solubilized protein (Bogner et al., 1986). Note that in this model the C-terminus is proposed to reside within the membrane. A similar study has not yet been performed on the uncoupling protein.

The primary sequences of 3 mitochondrial carriers, the uncoupling protein (Aquila et al., 1985), the ADP/ATP carrier (Aquila et al., 1982) and the phosphate carrier (Aquila et al., 1987; Runswick et al., 1987) have been compared and are very similar (Aquila et al., 1987; Runswick et al., 1987). All three proteins have a tripartite structure of three 100 amino acid repeats. All nine repeats in the three proteins are related to each other with homology amongst the full sequences and between the repeats (Aquila et al., 1987; Runswick et al., 1987). A membrane disposition model for the phosphate carrier is shown in Fig. 2c as presented by Aquila et al. (1987). The similarity in the amino acid

sequences and in the membrane disposition models for the three proteins has led to the suggestion that the proteins have evolved by gene triplication followed by diversification (Aquila *et al.*, 1987). It has also been proposed that uncoupling protein belongs to a subgroup of proton/anion cotransporters and has evolved by the deletion of the anion binding site making uncoupling protein a degenerated proton/anion cotransporter (Aquila *et al.*, 1985).

Despite the similarity amongst the proteins, there is a major difference in the precursors. Unlike the ADP/ATP carrier and the uncoupling protein, the phosphate carrier is synthesized with a 49 amino acid presequence (Runswick *et al.*, 1987). Consequently, it is possible that the targeting and import of the phosphate carrier is also different from that of the other two proteins.

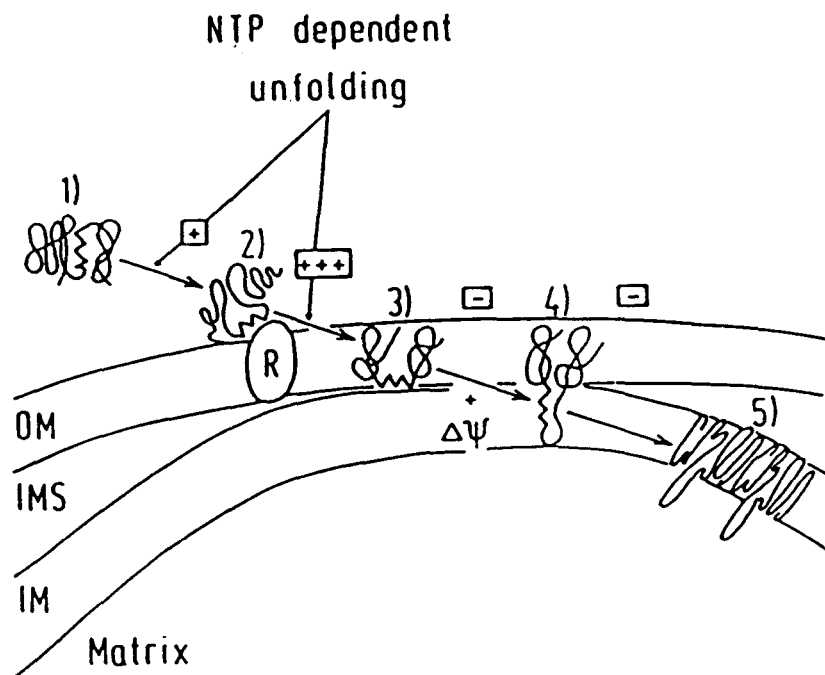
### 1.2.3 THE ADP/ATP CARRIER: A MODEL FOR UNCOUPLING PROTEIN TARGETING

Because the ADP/ATP carrier is structurally similar to the uncoupling protein, it is possible that the uncoupling protein has a similar mechanism for import. Both proteins are synthesized as mature length precursors and would therefore contain the targeting information within the mature sequence. Targeting sequences of proteins synthesized with presequences are rich in basic amino acids. There are several regions rich in basic amino acids within uncoupling protein that could be targeting sequence candidates; amino acids 54 to 67, 73 to 92, and 138 to 153 (Ridley *et al.*, 1986a,b). The current knowledge on import of the ADP/ATP carrier can serve as a starting point for the targeting of uncoupling protein.



A model for the import of the *N. crassa* ADP/ATP carrier has been proposed (Pfanner & Neupert, 1987b; Pfanner *et al.*, 1987a) and is shown in Fig. 3. The steps include: 1) the ADP/ATP carrier is synthesized as a mature length precursor on free polysomes in the cytosol. 2) The protein then binds to a receptor in the outer membrane in an NTP-dependent, membrane potential-independent manner. 3) The protein is then translocated into saturable protease protected sites within the outer membrane in an NTP-dependent process (interaction with GIP). 4) The protein is translocated from the outer membrane to the inner membrane at contact sites being led by a hairpin and this step requires a membrane potential. An intermediate was observed spanning both membranes simultaneously if import occurred at low temperatures (Pfanner *et al.*, 1987d). 5) The completion of translocation into the inner membrane occurs in a membrane potential-independent manner.

One approach used to identify the targeting sequence of a precursor not synthesized with a cleavable presequence is to fuse regions of the precursor to a passenger protein which does not normally reside within mitochondria. This approach is very similar to fusing presequences to passenger proteins and often, the same passenger proteins are used for both types of precursors. In targeting studies on the yeast ADP/ATP carrier, the first 115 amino acids of the protein were fused to *E. coli*  $\beta$ -galactosidase and the resulting fusion protein was imported into mitochondria (Adrian *et al.*, 1986). The possible targeting region has been defined even further by Smagula and Douglas (1988a,b) to be within amino acids 72 and 111. This was based on results of two fusion proteins, amino acids 1 to 111 joined to



**Figure 3. Import of the ADP/ATP Carrier.** The requirement for a membrane potential ( $\Delta\psi$ ) is indicated. The requirements for NTPs are indicated in squares from high requirement (+++) to no requirement (-). OM, outer mitochondrial membrane; IMS, intermembrane space; IM, inner membrane; R, receptor;  $\Delta\psi$ , membrane potential. The steps are explained in the text. As illustrated in Pfanner *et al.* (1987a).

dihydrofolate reductase which was imported into mitochondria, and amino acids 1 to 72 joined to dihydrofolate reductase which was not imported (Smagula & Douglas, 1988a,b). Amino acids 72 to 111 have not been tested directly. More convincing evidence that this region is responsible for targeting the ADP/ATP carrier would come from the lack of import of the precursor in which the suspected region was altered. It is possible that there exists a second targeting sequence within the ADP/ATP carrier. Pfanner et al. (1987c) deleted the first 103 amino acids and found that the *N. crassa* protein was still imported into mitochondria. This is not surprising due to the tripartite nature of the protein. Analysis of each domain reveals that the C-terminal half of each domain contains a positively charged stretch of about 20 amino acids which are predicted to form an  $\alpha$ -helix sharing characteristics with mitochondrial presequences (Pfanner et al., 1987c).

The uncoupling protein parallels the ADP/ATP carrier in that it is possible that two targeting sequences exist within the protein. A fusion protein joining rat uncoupling protein amino acids 13 to 105 to ornithine carbamoyltransferase amino acids 147 to 354 was imported by mitochondria and inserted in the inner membrane (Liu et al., 1988). Further, a fusion protein joining uncoupling protein amino acids 13 to 51 to ornithine carbamoyltransferase was not imported indicating that this segment of uncoupling protein containing amino acids 13 to 51 does not carry sufficient information for targeting although it might be necessary (Liu et al., 1988). Deletion of the first 51 amino acids resulted in a deletion construct which was imported poorly into mitochondria (Liu et al., 1988). The protein, after the first 101 amino acids had been deleted (UCP102-307), was imported by mitochondria but

was not integrated within a membrane (Liu *et al.*, 1988). This indicates that the information to target protein to the inner membrane is contained within the first 101 amino acids of the protein. Import of the UCP102-307 is not surprising in light of the tripartite structure of the protein.

### 1.3 SCOPE AND GOALS OF PROJECT

The aim of this project was to identify the targeting sequence or sequences for rat uncoupling protein. Based on the above information, the search for a targeting sequence would begin within amino acids 13 to 105. For this, uncoupling protein cDNA and the cDNA for uncoupling protein amino acids 13 to 105 joined to ornithine carbamoyltransferase amino acids 147 to 354 (obtained from Dr. G. Shore's laboratory, note that uncoupling protein amino acid 105 and ornithine carbamoyltransferase amino acid 146 are identical with the same serine codon, TCA) were used and changed by oligonucleotide site-directed mutagenesis to produce uncoupling protein lacking amino acids 2 to 35 and uncoupling protein amino acids 53 to 105 fused to ornithine carbamoyltransferase. These were subcloned into an expression vector. A second fusion protein was also constructed to aid in the search, this one joining uncoupling protein amino acids 53 to 105 to dihydrofolate reductase. The import of the fusion proteins was used to narrow the region responsible for targeting uncoupling protein. In order to test potential targeting regions directly, site-directed mutagenesis was used to affect single amino acid substitutions within uncoupling protein itself. The criteria for targeting was based on the successful import of the protein by isolated mitochondria.

## 2. MATERIALS

### 2.1 SOLUTIONS, BUFFERS AND REAGENTS

Bacto-tryptone and bacto-yeast are supplied by Difco, Detroit, Michigan. The 1 kb ladder, the restriction enzyme PstI, 10x restriction enzyme buffers, bacteriophage M13mp18 and M13mp19, *E. coli* JM107 and library efficiency HB101 cells, IPTG, and Blue-gal were supplied by BRL, Gaithersburg, Maryland. The restriction enzymes EcoRI and KpnI, one-phor-all buffer plus: 10x concentration, the vectors pSPT18 and 19, T<sub>4</sub> polynucleotide kinase, the Klenow fragment of DNA polymerase, m<sup>7</sup>GpppG, and T<sub>7</sub> and SP6 RNA polymerases were supplied by Pharmacia, Uppsala, Sweden. IBI, New Haven, Connecticut, supplied 10x ligase reaction buffer and T<sub>4</sub> DNA ligase. *E. coli* BW313 cells were a generous gift from Len Rasile and Dr. H.P. Ghosh. TFB was a gift from Dr. A.B. Futcher's and Dr. C. Harley's laboratories. [ $\alpha$ -<sup>32</sup>P]dATP (1000 to 1500 Ci/mmol), [ $\gamma$ -<sup>32</sup>P]ATP (10mCi/ml), [<sup>14</sup>C]formaldehyde (17 mM, 0.25 mCi) and [<sup>35</sup>S]methionine (9.85 mCi/ml) were supplied by New England Nuclear, Lachine, Quebec. [ $\alpha$ -<sup>35</sup>S]dATPaS (10 mCi/ml) was supplied by Amersham, Arlington Heights, Illinois. Uridine and CCCP were supplied by Sigma, St. Louis, Missouri. The oligonucleotide primers AB268, AB270, AB271, AB484, AB572, AB636-AB640 were supplied by the Central Facility of the Institute for Molecular Biology and Biotechnology at McMaster University. A list of these can be found at the end of this section. The T<sub>7</sub> promoter primer was supplied by Promega Biotech, distributed through Bio/Can Scientific Inc., Mississauga, Ontario.



Bio101, as distributed by Bio/Can Scientific, supplied the NaI solution, the NEW solution and the glass milk in their GeneClean kit. Promega Biotech supplied 5x Transcription Buffer, 100 mM DTT, RNasin at 40 U/ $\mu$ l, 10 mM stocks of the NTPs, T<sub>7</sub> and SP6 RNA polymerases, RNase-free DNase 1 U/ $\mu$ l, nuclease treated lysate and 1 mM amino acid mixture (minus methionine). All other chemicals are reagent grade.

## 2.2 Plasmid Preparation Solutions

LB Medium: 10 g bacto-tryptone

5 g bacto-yeast

10 g sodium chloride

per 1 L, pH 7.5 with NaOH

LB Agar: 15 g agar in 1 L LB medium

Lysozyme Solution: 50 mM glucose (Mr 180.16) 0.9008 g

10 mM EDTA (Mr 346.2) 0.3462 g

25 mM Tris (Mr 121.1) 0.3028 g

per 100 ml, pH 8.0 with HCl

Alkaline SDS: 0.2 M NaOH (Mr 40) 0.8 g

1% (w/v) SDS 1.0 g/100 ml

High Salt Solution: 3 M potassium acetate: 60 ml of 5 M potassium acetate

1.8 M formic acid: 5 ml of 90% formic acid

35 ml sterile water

Acetate-EDTA-MOPS: 0.1 M sodium acetate (Mr 136.08) 1.36 g

0.05 M MOPS (Mr 209.3) 1.0465 g

1 mM EDTA (Mr 346.2) 0.0345 g

per 100 ml, pH 8.0 with NaOH

CDTA-Tris: 1 mM CDTA (Mr 346.2) 0.0346 g  
10 mM Tris (Mr 121.1) 0.1211 g  
100 ml, pH 7.5 with HCl

LiCl Solution: 5 M LiCl (Mr 42.39) 21.20 g  
0.05 M MOPS (Mr 209.3) 1.0465 g  
1 mM CDTA (Mr 346.2) 0.0346 g  
per 100 ml, pH 8.0 with NaOH

Tetracycline: 10 mg tetracycline/ml 95% ethanol

Chloramphenicol: 170 ug chloramphenicol/ml 95% ethanol

### 2.3 Agarose Gel Solutions

1% Agarose Gel: 1.5 g agarose

50 ml 0.5x TBE

Dye Mix: 0.1% (w/v) xylene cyanol

0.1% (w/v) bromophenol blue

10 mM Na<sub>2</sub>EDTA

95% (v/v) deionized formamide

10 x TBE: 1 M Tris (Mr 121.1) 121.1 g

0.89 M boric acid (Mr 61.83 ) 55 g

20 mM Na<sub>2</sub>EDTA (Mr 372.24) 7.4 g

per 1 L, pH 8.3 with boric acid or Tris

Ethidium Bromide Stain: 10 mg ethidium bromide/ml

### 2.4 Restriction Endonuclease Digestion and Ligation

10x Restriction Enzyme Buffer (high salt, supplied):

100 mM MgCl<sub>2</sub>, 500 mM NaCl, 500 mM Tris-HCl pH 8.0

10x Concentration One-phor-all Buffer Plus (supplied)

10x Ligase Reaction Buffer (supplied):

100 mM MgCl<sub>2</sub>, 10 mM DTT, 4 mM ATP, 250 mM Tris-HCl pH 7.8

## 2.5 Cell Transformation Solutions

YT Broth: 8 g peptone

5 g yeast extract

5 g NaCl

per 1 L, pH 7.5 with NaOH

B Top Agar: 10 g peptone

8 g NaCl

6 g agar per 1 L, pH 7.5 with NaOH

B Bottom Agar: 10 g peptone

8 g NaCl

12 g agar per 1 L, pH 7.5 with NaOH

CaCl<sub>2</sub> Solution: 50 mM CaCl<sub>2</sub> (Mr 147.02) 0.735 g

10 mM Tris (Mr 121.1) 0.1211 g

per 100 ml, pH 8.0 with HCl

## 2.6 High Efficiency Competent Cells - Hanahan Solutions

SOB Medium: 20 g Bacto-tryptone

5 g Yeast extract

0.5 g NaCl

per 1 L, pH 7.5 with NaOH

After autoclaving, 20 ml sterile 1 M MgSO<sub>4</sub> was added

SOC Medium: SOB medium plus 20 mM glucose



TFB (supplied): 10 mM K-Tes pH 6.2  
100 mM RbCl  
45 mM  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$   
10 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$   
3 mM hexamine  $\text{CoCl}_3$

## 2.7 ssDNA Purification Solutions

TES Buffer: 20 mM Tris (Mr 121.1)  
10 mM NaCl (Mr 58.44)  
0.1 mM  $\text{Na}_2\text{EDTA}$  (Mr 372.24)  
pH 7.5 with HCL

Chloroform: iso-amyl alcohol; (24:1 v/v)

20% (w/v) PEG, 2.5 M NaCl: PEG 8000 20 g

2.5 M NaCl (Mr 58.44) 14.6 g

per 100 ml

3 M Sodium Acetate: 3 M sodium acetate (Mr 82.03) 24.6 g

per 100 ml, pH 5.0 with acetic acid

## 2.8 dsDNA Purification Solutions for Sequencing

2 M Ammonium Acetate: 2 M ammonium acetate (Mr 77.08) 1.54 g

per 10 ml

4 M NaOH: 4 M NaOH (Mr 40.0) 4 g

per 100 ml

4 mM EDTA: 4 mM  $\text{Na}_2\text{EDTA}$  (Mr 372.24) 0.1489 g

per 100 ml

## 2.9 Urea Sequencing Gel Solutions

Instagel: 40% (w/v) acrylamide:bis-acrylamide (19:1 w/w)

acrylamide 19 g

bis-acrylamide 1 g

50% (w/v) urea 105 g

10x TBE 25 ml per 250 ml

Ammonium Persulphate: 10% (w/v) ammonium persulphate

TEMED

## 2.10 Sequenase Reagents

5x Annealing Buffer: 200 mM Tris-HCl pH 7.5

100 mM MgCl<sub>2</sub>

250 mM NaCl

Labeling Nucleotide Mix: 1.5 μM each of dCTP, dGTP, dTTP

Termination Nucleotide Mix: 50 mM NaCl

80 μM each of dATP, dCTP, dGTP, dTTP

for each of the mixes plus

A Mix: 8 μM ddATP

C Mix: 8 μM ddCTP

G Mix: 8 μM ddGTP

T Mix: 8 μM ddTTP

Stop Solution: 95% (v/v) formamide

20 mM EDTA

0.05% (w/v) Bromophenol Blue

0.05% (w/v) Xylene Cyanol FF

### 2.11 T7 Sequencing Kit Reagents

Annealing Buffer: supplied

Labeling Mix: supplied

A Mix-Short: supplied

C Mix-Short: supplied

G Mix-Short: supplied

T Mix-Short: supplied

Enzyme Dilution Buffer: supplied

Stop Solution: supplied

### 2.12 Oligonucleotide Site-Directed Mutagenesis

5 M NaCl: 5 M NaCl (Mr 58.44) 29.2 g per 100 ml

1 M Tris pH 7.8: 1 M Tris (Mr 121.1) 12.1 g

per 100 ml, pH 7.8 with HCl

100 mM MgCl<sub>2</sub>

100 mM DTT

1 mM ATP

10x Annealing Buffer: 200 mM Tris-HCl pH 7.5: 200 µl of 1 M

Tris-HCl, pH 7.5

100 mM MgCl<sub>2</sub>: 50 µl of 2 M MgCl<sub>2</sub>

500 mM NaCl: 100 µl of 5 M NaCl

10 mM DTT: 20 µl of 500 mM DTT

per 1 ml

Solution B: 200 mM Tris-HCl pH 7.5: 200 µl of 1 M Tris-HCl,

pH 7.5

100 mM MgCl<sub>2</sub>: 50 µl of 2 M MgCl<sub>2</sub>



100 mM DTT: 200  $\mu$ l of 500 mM DTT

per 1 ml

2.5 mM dNTP Mix: 2.5 mM each of dATP, dCTP, dGTP, dTTP

10 mM ATP

### 2.13 GeneClean Solutions from Bio101

NaI solution:  $\text{Na}_2\text{SO}_3$

NaI in 50 ml final volume

NEW: Tris, ethanol, EDTA, NaCl pH 7 - 8.3

### 2.14 DNA Recovery Solutions from Low Melting Agarose

0.2 M Tris: 0.2 M Tris (Mr 121.1) 2.42 g

per 100 ml, pH 8.0 with HCl

3 M Sodium Acetate: 3 M sodium acetate (Mr 82.03) 24.6 g

per 100 ml, pH 5.2 with acetic acid

### 2.15 Transcription In vitro

DEPC-treated Water: 500  $\mu$ l of DEPC per 500 ml water left at 20 °C  
overnight, then autoclaved

5x Transcription Buffer (supplied): 200 mM Tris-HCl, pH 7.5

30 mM  $\text{MgCl}_2$

10 mM spermidine

50 mM NaCl

100 mM DTT (supplied)

NTP Mix: 2.5 mM each of ATP, CTP, UTP and 0.5 mM of GTP

10 mM Nucleotide Stocks from Promega

10 M Ammonium Acetate (Mr 77.08) 7.7 g per 10 ml

**2.16 Translation In Vitro: lysate solution and amino acid mixture**  
supplied by Promega

**2.17 Heart Mitochondria Solutions**

Medium B: 10 mM HEPES (Mr 238.3) 1.19 g  
220 mM mannitol (Mr 182.2) 20 g  
70 mM sucrose (Mr 342.3) 11.98 g  
1 mM EGTA (Mr 380.4) 0.190 g  
per 500 ml, pH 7.5 with KOH

Medium A: Medium B plus 2 mg BSA/ml

Resuspension Medium: 0.25 M sucrose (Mr 342.3) 8.5 g  
10 mM HEPES (Mr 238.3) 0.0357 g  
2 mM  $K_2HPO_4$  (Mr 174.18) 0.026 g  
5 mM sodium succinate (Mr 162) 6.0 g  
1 mM DTT (Mr 154.3)  
1 mM ATP (Mr 605.2)  
0.08 mM ADP (Mr 427.2)  
per 100 ml, pH 7.2 with KOH

Cycloheximide: 15 mg/ml solution in 95% ethanol

Chloramphenicol: 40 mg/ml solution in absolute ethanol

### 2.18 Brown Adipose Tissue Mitochondria Solutions

Sucrose-Tes pH 7.2: 0.25 M sucrose (Mr 342.3) 42.79 g

5 mM Tes (Mr 229.3) 0.579 g

per 500 ml, pH 7.2 with KOH

Sucrose-EDTA-Tris: 0.3 M sucrose (Mr 342.3) 25.67 g

10 mM Tris (Mr 121.1) 0.3027 g

2 mM Na<sub>2</sub>EDTA (Mr 372.24) 0.1862 g

per 250 ml, pH 7.2 with HCl

### 2.19 Lowry Reagents

Lowry A: 2% (w/v) Na<sub>2</sub>CO<sub>3</sub> (Mr 105.99) 20 g

0.02% (w/v) Na,K tartrate (Mr 282.231) 0.2 g

0.1 N NaOH (Mr 40) 4 g

per 1000 ml

Lowry B: 0.5% (w/v) CuSO<sub>4</sub>·5H<sub>2</sub>O (Mr 249.69) 0.5 g

per 100 ml

Lowry C: 50 ml Lowry A

1 ml Lowry B

Lowry D: 5 ml Phenol Reagent Solution 2N (Folin-Ciocalteu):

supplied

5 ml water

### 2.20 Mitochondria Import Solutions

1.5 mM CCCP: 50 mM CCCP (Mr 204.6) 0.0102 g in 1 ml 95% ethanol

diluted to 1.5 mM in 95% ethanol

5 mg Proteinase K/ml in 2x PK buffer

2x PK Buffer: 20 mM Tris (Mr 121.1) 0.2422 g  
10 mM Na<sub>2</sub>EDTA (Mr 372.24) 0.3722 g  
1% (w/v) SDS 1 g  
per 100 ml, pH 8.0 with HCL

100 mM PMSF: 100 mM PMSF (Mr 174.2) 0.01742 g in 95% ethanol

0.1 M Na<sub>2</sub>CO<sub>3</sub>: 0.1 M Na<sub>2</sub>CO<sub>3</sub> (Mr 105.99) 1.06 g  
per 100 ml, pH 11.5 with NaOH

### 2.21 Uncoupling Protein Purification Solutions

Standard Medium: 20 mM morpholinopropane sulfonate (Mr 209.3)  
20 mM Na<sub>2</sub>SO<sub>4</sub> (Mr 142.02)  
0.16 mM Na<sub>2</sub>EDTA (Mr 372.24), pH 6.7 with NaOH

0.3 M Sucrose, 10 mM Tris, 2 mM EDTA, pH 7.2:

0.3 M sucrose (Mr 342.3)  
10 mM Tris (Mr 121.1)  
2 mM Na<sub>2</sub>EDTA (Mr 372.24), pH 7.2 with HCL

### 2.22 SDS Polyacrylamide Gel Solutions

30% Acrylamide/Bis Solution: 9 g acrylamide  
0.24 g bis-acrylamide  
22.5 ml water

Separating Gel: 18.75 ml water

15 ml separating gel buffer  
25 ml acrylamide/bis-acrylamide solution  
0.6 ml 10% (w/v) SDS  
0.12 ml 50% (v/v) glycerol

20  $\mu$ l TEMED

0.45 ml 10% (w/v) ammonium persulphate

Separating Gel Buffer: 1.5 M Tris (Mr 121.1) 182 g  
per 1 L, pH 8.8 with HCl

Stacking Gel: 11.8 ml water

5 ml stacking gel buffer

3.8 ml acrylamide/bis-acrylamide solution

40  $\mu$ l 50% (v/v) glycerol

100  $\mu$ l 10% (w/v) SDS

20  $\mu$ l TEMED

125  $\mu$ l 10% (w/v) ammonium persulphate

Stacking Gel Buffer: 0.5 M Tris (Mr 121.1) 61 g  
per 1 L, pH 6.8 with HCl

2X SDS Sample Buffer: 1 ml glycerol

1 ml stacking gel buffer

1 ml  $\beta$ -mercaptoethanol

0.5 ml 0.2% (w/v) bromophenol blue

1.5 ml 20% (w/v) SDS

10x Reservoir Buffer: 2.5 M Tris (Mr 121.1) 30.2 g

2 M glycine (Mr 72) 144 g

per 1 L, pH 8.3 with HCl

Reservoir Buffer: 1x reservoir buffer: 100 ml of 10x buffer

0.1% (w/v) SDS: 10 ml of 10% (w/v) SDS

per 1 L



### 2.23 <sup>14</sup>C-Labeling of Proteins

Borate Buffer: 1 M borate (Mr 61.83)

pH 9.0 with NaOH

Sodium Borohydride: 5 mg sodium borohydride/ml made fresh

<sup>14</sup>C-Formaldehyde: 0.25 mCi diluted to 17 mM with water

### 2.24 Oligonucleotide Primers

AB268: 5'-pCGAGCCAAGATGACCGCCAAA-3'

This was used in site-directed mutagenesis to join the initiating methionine to threonine<sub>36</sub> in UCP. AB268 corresponds to nucleotides -9 to 3 and 106 to 114 of the antisense strand of rat UCP cDNA. The resulting construct was called Ud2-35.

AB269: 5'-pCGAGCCAAGATGATTAGGTAT-3'

This oligonucleotide was not used but could be used to join the initiating methionine to isoleucine<sub>53</sub> in UCP. AB269 corresponds to nucleotides -9 to 3 and 157 to 165 of the antisense strand of rat UCP cDNA.

AB270: 5'-pCAACCCACCATGACCGCCAAA-3'

AB271: 5'-pCAACCCACCATGATTAGGTAT-3'

These were used in site-directed mutagenesis to join methionine<sub>13</sub> to threonine<sub>36</sub> and isoleucine<sub>53</sub> respectively in UCP13-105OCT147-354 (details for this construct are given by Liu *et al.*, 1988). AB270 corresponds to nucleotides 28 to 39 and 106 to 114 while AB271 corresponds to nucleotides 28 to 39 and 157 to 165 of the antisense strand of rat UCP cDNA. The resulting constructs were called U36-105-OCT and U53-105-OCT respectively. The former construct did not yield a

translation product.

AB484: 5'-pGATCGTGCA-3'

This was used as a bridge in the construction of UCP53-105DHFR. The first four nucleotides hybridize to the 5'-overhang of the BglII cut and the last four nucleotides hybridize to the PstI 3'-overhang. The centre nucleotide was needed to keep the construct in frame.

AB572: 5'-CCTTGAGTTCTCTACTG-3'

This is a sequencing primer used to sequence across the bridge joining UCP53-105 to DHFR corresponding to nucleotides of the coding strand of DHFR cDNA.

AB636: 5'-AAGTGC A ACCCACCATG-3'

This is a sequencing primer used in the screening of mutants and corresponds to nucleotides 23 to 39 of the antisense strand of rat UCP cDNA.

AB637: 5'-pCCAGTACTATTTCAGTATAAAGG-3'

AB638: 5'-pCTATTAGGTATCAAGGIGICTTA-3'

AB639: 5'-pCACCCCTGGCCCAGACAGAAGG-3'

AB640: 5'-pCCAGTACTATTTCAGTATCAAGGIGICTTA-3'

These were used in site-directed mutagenesis to create point mutations in UCP in which arginine<sub>54</sub>, lysine<sub>56</sub> and lysine<sub>67</sub> are changed to glutamines. They correspond to nucleotides 149 to 170, 155 to 177, 189 to 209 and 149 to 177 respectively of the antisense strand of rat UCP cDNA.

## 2.25 Plasmids

Schematic diagrams of all constructs used or made are shown below. Inserts are represented by wavy lines. Important restriction sites are indicated.

pSPT18-UCP; UCPd2-35; U-Q54; U-Q56; U-Q67; U-Q54,56; U-Q56,67;

U-Q54,56,67. These vectors are linearized with PstI for transcription.



pSPUCPd2-51: obtained from Dr. G.C. Shore. The parent vector is pSP64.

This vector is linearized with EcoRI for transcription.



pSPUCP13-105OCT: obtained from Dr. G.C. Shore. The parent vector is pSP64. This vector is linearized with EcoRI for transcription.



pSPT19-UCP53-105OCT. This vector is linearized with EcoRI for transcription.









### 3. METHODS

#### 3.1 ISOLATION OF PLASMID DNA

The method used for the isolation of plasmid DNA is a modified version of the method presented by Birnboim (1983). Plates were streaked to yield single colonies which were picked and transferred to 3 ml LB medium and left to incubate at 37°C overnight with shaking at 200 rpm. Ampicillin was added to each tube to a final concentration of 40 ng/ml. LB medium (500 ml) with 40 ng ampicillin/ml was inoculated with 1 ml of the culture and left to incubate until an  $A_{600}$  of 0.8 to 1.0 was reached. Chloramphenicol was added to give a final concentration of 0.7  $\mu\text{g}$  chloramphenicol/ml and incubation was allowed to continue at 37°C for 22 hr. For better yields of plasmid DNA, 1 ml of the overnight culture was used to inoculate 50 ml of YT medium containing 40 ng ampicillin/ml and left to incubate until an  $A_{600}$  of 0.6 was reached. The 50 ml culture was then added to 450 ml of YT medium containing 40 ng ampicillin/ml and left to grow for a further 2.5 hr. At this point, chloramphenicol was added to give a final concentration of 20  $\mu\text{g}$  chloramphenicol/ml and incubation continued overnight.

Cells were harvested by centrifugation in 2, 250 ml polycarbonate bottles at  $6,000 \times g_{\text{max}}$  for 10 min at 5°C. The pellets were resuspended in water (25 ml) and transferred to 40 ml polypropylene Sorvall tubes. The centrifugation was repeated at  $6,000 \times g$  for 10 min at 5°C.

Each pellet was resuspended in 0.5 ml of an ice-cold solution containing 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0 (lysozyme solution) without lysozyme. The cells were lysed by the addition of 4.5 ml of lysozyme solution containing 5 mg lysozyme. The suspensions were well mixed and kept at 0°C for 30 min. Ten millilitres of 0.2 M NaOH and 1% (w/v) SDS (alkaline SDS) at room temperature was added to the mixture which was swirled until homogeneous and clear, and then left at 0°C for 10 min.

High salt solution (3 M potassium acetate, 1.8 M formic acid, 7.5 ml) was added and swirled vigorously for a few min until a coarse white precipitate formed. The mixture was then allowed to stand for 30 min at 0°C. This was then centrifuged at 12,000xg for 10 min at 0°C and the clear lysate was decanted into 4 Sorvall tubes. Two volumes of 95% (v/v) ethanol were added to each tube to precipitate the nucleic acids for 10 min at room temperature.

The precipitate was collected by centrifugation at 16,000xg for 20 min at 0°C. The pellet was dissolved in 2 ml of a solution with 0.1 M acetate, 1 mM EDTA and 50 mM MOPS, pH 8.0 (acetate-EDTA-MOPS buffer) and a 10 µl sample was taken for agarose gel analysis. The tubes were combined and brought to a total volume of 5 ml. The nucleic acids were precipitated with

two volumes of 95% ethanol and left at room temperature for 10 min.

The nucleic acids were collected by centrifugation at 12,000xg for 30 min at 0°C. The pellet was dissolved in 2 ml of a solution containing 1mM EDTA and 10 mM Tris-HCl, pH 7.5 (Tris-EDTA) and a 10 µl sample was taken for agarose gel analysis. The total volume was

measured and an equal volume of a solution containing 5 M LiCl, 1 mM EDTA and 50 mM MOPS, pH 8.0 (LiCl solution) was added. The sample was held at 0°C for 15 min and the heavy precipitate was removed by centrifugation at 12,000xg for 10 min at 0°C.

The supernatant was transferred to a 15 ml Corex tube and then heated for 10 min at 60°C. Any additional precipitate was removed by centrifugation at 12,000xg for 10 min at 0°C. The supernatant was transferred to a new 15 ml Corex tube. Two volumes of 95% ethanol were added to precipitate DNA for 10 min at room temperature.

The precipitate was collected at 12,000xg for 30 min at 0°C and redissolved in 2.5 ml of acetate-EDTA-MOPS solution. A 10 µl sample was taken for gel analysis. The sample was then precipitated with 2 volumes of 95% ethanol at room temperature for 10 min. The precipitate was collected at 12,000xg for 30 min at 0°C and then dried under vacuum. The pellet was then dissolved in 2 ml of Tris-EDTA solution.

The solution containing the plasmid was made up to 4.8 ml with Tris-EDTA solution and 5 g of CsCl was added. The sample was mixed gently by swirling and inversion until the salt dissolved. Eighty microlitres of ethidium bromide (10 mg/ml) was added to the CsCl solution which was then transferred to quick seal type 65 polycarbonate tubes (6.3 ml), the tubes were sealed and centrifuged in a Type 80Ti rotor on a Beckman ultracentrifuge for 22 hr at 48,000 rpm at 17°C.

The lower band containing closed circular plasmid DNA was removed from the tube with a syringe and a 22 gauge 1.5 inch needle. Ethidium bromide was extracted 6 times with water-saturated butanol. The aqueous layer was placed in a 30 ml Corex tube with 2 volumes of

water. Two volumes of 95% ethanol was then added and the DNA left to precipitate for 10 min at room temperature.

The precipitate was collected at 12,000xg for 30 min at 0°C and then dried under vacuum. The pellet was dissolved in a minimum volume of Tris-CDTA solution (200 µl). The absorbance was determined with 20 µl of sample diluted in 1 ml of water. Samples were frozen rapidly with liquid nitrogen and stored at -20°C.

### 3.2 SMALL SCALE PLASMID PREPARATION

An E. coli culture was grown overnight at 37°C and 1.5 ml centrifuged in a microfuge tube for 5 min at room temperature. The pellet was resuspended in 0.1 ml of lysozyme solution containing 1 mg lysozyme/ml. The sample was then left at 0°C for 5 min after which, 0.2 ml of alkaline SDS was added and mixed by gentle inversion. Following a 5 min incubation at 0°C, 0.15 ml of high salt solution was added to the tube, mixed by inversion and left for a further 15 min at 0°C. The tube was then centrifuged for 5 min at room temperature.

The supernatant (0.35 ml) was transferred to a fresh microfuge tube while being careful to avoid the sticky pellet. Ethanol (95%, 0.9 ml) was added to the supernatant and the DNA was left to precipitate at 0°C for 15 min. The pellet was collected by a 5 min centrifugation at room temperature, washed by dissolving in 0.1 ml of acetate-CDTA-MOPS solution and reprecipitating with 0.2 ml of 95% ethanol. The DNA was recovered after 15 min at 0°C, the wash step repeated and the final pellet was dried and dissolved in 30 µl of 0.1x Tris-CDTA solution.



### 3.3 ELECTROPHORESIS OF DNA

The method used for electrophoresis of DNA is that given by Maniatis (1983). Agarose (1.5 g) was added to 150 ml of 0.5x TBE buffer. The mixture was heated until the agarose melted. The solution was poured into a horizontal gel apparatus and combs were inserted so as to make wells without going through the gel. The gel was allowed to set and then equilibrated with 0.5x TBE buffer.

The sample for electrophoresis contained approximately 200 ng of nucleic acid, 4  $\mu$ l of dye mix and water to make a total volume of 20  $\mu$ l. Electrophoresis conditions varied from overnight at 20 V to 15 min at 200 V. The gel was stained in a solution containing 15  $\mu$ l of ethidium bromide (10 mg/ml) in 150 ml of distilled water for 30 min before recording the result by ultraviolet fluorescence photography.

### 3.4 RESTRICTION ENDONUCLEASE DIGESTION AND LIGATION

For the digestion of the desired plasmid, the appropriate amount of the plasmid, 2  $\mu$ l of 10x one-phor-all buffer plus, water and 0.5 to 1  $\mu$ l of the enzyme to 20  $\mu$ l were mixed and incubated for 1 hr at 37°C. Then, a 4  $\mu$ l sample was taken to check the completeness of the reaction by electrophoresis on a 1% agarose gel at 200 V for 15 min. When the reaction was complete, the sample was subjected to electrophoresis on a 1% agarose gel and the appropriate band was isolated using the protocol from the GeneClean kit or the sample was subjected to electrophoresis on a 1% low melting point agarose gel for 3 hr at 100 V at 4°C and the appropriate band was removed after visualization using ethidium bromide



staining and ultraviolet fluorescence, both methods to be described later.

For the ligation reaction, a molar ratio, based on insert to vector length, of 4:1 insert to vector was used. The reaction volume of 20  $\mu$ l consisted of the insert (typically, 200 ng of cDNA insert), the vector (approximately 200 ng of pSPT18 or pSPT19), 2  $\mu$ l of 10x ligase reaction buffer, 0.02 units of T4 DNA ligase (for sticky end ligation) and water to make up the volume. The samples were incubated overnight at 15°C. The reactions were stopped by the addition of 2  $\mu$ l 3 M sodium acetate pH 5.0.

### 3.5 CELL TRANSFORMATION

The protocol for the preparation of competent host cells and host cell transformation were taken from the BRL M13 Cloning/Dideoxy Sequencing instruction manual. YT broth (1 ml) was inoculated with a loop from a single colony of *E. coli* JM 107 cells (maintained on minimal media plates) or 294 host cells (stored at -70°C in 16% (v/v) glycerol). The broth was incubated overnight at 37°C with shaking at 200 rpm.

The culture was diluted 1:100 in YT broth and the incubation was continued at 37°C until the  $A_{550}$  reached 0.4 to 0.5. At this point, the growth flask was placed at 0°C for 20 min. Twenty millilitres of culture was transferred to 2 chilled 30 ml Corex tubes and the cells were pelleted by centrifugation at 2,500 g for 15 min at 4°C. The supernatant was decanted and the cells were resuspended in half the original volume of a sterile, ice-cold solution containing 50

mM CaCl<sub>2</sub> and 10 mM Tris-HCl, pH 8.0 by gentle swirling and kept at 0°C for 30 min.

The cells were centrifuged again at 2,500 g for 15 min at 4°C and the supernatant decanted. The cell pellet was resuspended in one-tenth of the original volume using ice-cold 50 mM CaCl<sub>2</sub>, 10 mM Tris-HCl, pH 8.0 by gentle swirling. Competent *E. coli* JM107 cells were used the same day of preparation while competent *E. coli* 294 cells could be stored at -70°C in 16% (v/v) glycerol, thawed and then used for transformations.

Lawn host cells were prepared from the same overnight culture used to make the competent host cells by transferring 500 µl of the culture to 100 ml sterile YT medium and left to incubate at 37°C for 2 to 3 hr with shaking at 200 rpm.

For the transformation, sterile borosilicate glass tubes were chilled at 0°C. The competent host cells were mixed by gentle swirling and samples of 0.3 ml transferred into each tube. One to forty nanograms of phage DNA was then added to appropriate tubes, mixed gently and left to incubate at 0°C for 30 min before plating.

B top agar was melted and held at 50°C. IPTG (10 µl), and Blue-gal (50 µl) were added to the tubes as necessary. The transformed cells were warmed two tubes at a time at 42°C for 2 min. In the meantime, B top agar (3 ml) was added to the tubes containing IPTG and Blue-gal. After the 2 min, 0.2 ml of lawn cells was added to the competent cells and the two sets of tubes were mixed and quickly poured onto B agar plates. The plates were then rocked and covered. This was repeated until all the samples had been plated. Once the top agar had

solidified, the plates were inverted and left to incubate at 37°C overnight.

### 3.6 PREPARATION OF HIGH EFFICIENCY COMPETENT CELLS

The protocol for the production of high efficiency competent cells is that given by Hanahan (1983). One millilitre of an overnight *E. coli* JM107 culture grown in SOB medium was used to inoculate 100 ml of SOB medium. The culture was left to grow at 37°C until an A<sub>550</sub> of 0.45 to 0.55 is reached for rec<sup>-</sup> strains such as JM107 cells. From this point, only the required volume of culture was used for the remainder of the preparation. For one transformation using 200 µl of competent cells, 2.5 ml of the original culture was required.

The cells were collected in a 15 ml polypropylene tube at 0°C for 10 to 15 min. The cells were then collected using a table top centrifuge at 2500 rpm for 12 min at 4°C. The pellet was resuspended in one-third the original volume in TFB solution and left at 0°C for 10 to 15 min. The cells were collected again using a table top centrifuge at 2500 rpm for 10 min at 4°C. The cells were resuspended in 0.08x the original volume in TFB solution to which was added fresh DMSO (7 µl/200 µl competent cells). The cells were then left at 0°C for 5 min after which a solution containing 2.25 M DTT and 40 mM potassium acetate pH 6.0 (7 µl/200 µl competent cells) was added for a further 10 min incubation at 0°C. Finally, more DMSO was added (7 µl/200 µl competent cells) and the cells were left at 0°C for at least 5 min. The cells were then ready for use.



For transformation, 210  $\mu$ l of the competent cells were added to sterile, chilled borosilicate tubes. The DNA was added in quantities of 10  $\mu$ l or less and the mixture was left at 0°C for 30 min. The cells were warmed for 90 sec at 42°C and then placed at 0°C for 1 to 2 min.

For plating phage, 200  $\mu$ l of lawn cells were added along with 3 ml of B top agar. The sample was gently mixed and plated immediately. For plating vectors, 800  $\mu$ l of SOC solution was added and left to incubate for 1 hr at 37°C with shaking at 225 rpm. Then 100  $\mu$ l or 100  $\mu$ l of a 1:10 dilution was spread on an LB plate containing 40 ng ampicillin/ml.

### 3.7 PURIFICATION OF SINGLE-STRAND BACTERIOPHAGE DNA

The procedure used for the purification of phage DNA was that in the BRL M13 Cloning/Dideoxy Sequencing instruction manual, pp 44-49. Follow the steps from the E. coli overnight culture at the top of Fig. 4 on page 77 until ssDNA template is made for a schematic diagram of the protocol. Note that for sequencing purposes, uridine is not included in the preparation of ssDNA, rather YT medium (2 ml) was inoculated with a single colony of M13 E. coli host JM 107 cells and grown overnight at 37°C with shaking.

The overnight growth (100  $\mu$ l) was diluted into 5 ml of YT medium and each tube was inoculated with a single white plaque. The borosilicate glass culture tubes were incubated at 37°C for 6 hr with vigorous shaking. Approximately 1.5 ml of the cultures were transferred to 1.5 ml polypropylene microcentrifuge tubes. Cells were recovered by centrifugation for 5 min at room temperature. The virus found in the

supernatant was used to prepare the DNA templates for sequencing. The bacterial pellets were stored at 4°C as virus stock.

The viral supernatants were transferred to fresh microcentrifuge tubes and stored at 4°C. Before use, a second 5 min centrifugation was done to ensure the removal of all cells. Culture supernatant (1.2 ml) was transferred again and 300 µl of a solution containing 20% (w/v) PEG 8000 and 2.5 M NaCl was added to it. The solution was mixed well on a vortex mixer and left to stand at room temperature for 15 min. The remaining viral supernatant was also stored at 4°C as stock. The tubes containing the 20% PEG, 2.5 M NaCl solution were then centrifuged for 10 min at room temperature to recover the virus. The supernatant was completely removed with the use of a drawn out glass capillary.

The viral DNA was resuspended in 100 µl of a solution containing 10 mM NaCl, 0.1 mM EDTA and 20 mM Tris-HCl, pH 7.5 (TES solution) and extracted with 50 µl of phenol saturated with a solution containing 1 mM EDTA and 10 mM Tris-HCl, pH 7.5 (TE buffer). The tubes were mixed vigorously using a vortex mixer for 15 to 20 sec and the phases were separated by centrifugations for 2 to 3 min. The lower organic phase was removed and the aqueous phase was extracted with 50 µl of chloroform/iso-amyl alcohol (24:1, v/v). The phases were mixed with a vortex mixer for 15 to 20 sec and then separated by centrifugation for 2 to 3 min. The upper aqueous phase (80 µl) was removed to a 1.5 ml microcentrifuge tube. Care was taken to avoid the interface and lower phase. Nine microlitres of 5 M sodium acetate pH 5.0 and 200 µl of 95% ethanol was added to the aqueous phase. The DNA was precipitated overnight at -20°C.



The precipitate was collected by centrifugation for 10 min at 4°C and the pellet was then washed with 1 ml of cold 80% ethanol. The sample was left at 0°C for 30 min and then centrifuged again for 10 min at 4°C. The ethanol was poured off and the pellet was briefly dried under vacuum. Viral DNA was resuspended in 20 µl of TE buffer. DNA samples were then stored at -20°C. The presence of template DNA was checked by using 2 to 3 µl for visualization on agarose gels.

### 3.8 DENATURATION OF dsDNA FOR SEQUENCING

For sequencing of dsDNA, the plasmid must be free of contaminating RNA. To achieve RNA-free dsDNA from a mini preparation of dsDNA, the preparation was treated with 20 µg of DNase-free RNase A for 3 hr. RNase A was removed with two phenol:chloroform (1:1, v/v) extractions followed by a chloroform extraction. The DNA was precipitated using 2 M NH<sub>4</sub>Ac and ethanol. After centrifugation, the pellet was washed, dried and resuspended in 20 µl of water. A 2 µl sample was taken for visualization on an agarose gel.

The protocol for the preparation of template DNA is given by Zhang *et al.* (1988). To denature the pure DNA sample, 1 µl of 4M NaOH and 1 µl of 4 mM EDTA were added and the solution left to incubate at room temperature for 5 min. The sample was then "neutralized" by quickly adding 2 µl of 2 M NH<sub>4</sub>Ac and 60 µl of 100% ethanol. The DNA was left to precipitate on ice for 30 min. The DNA was then pelleted by a 15 min centrifugation in a microfuge at 4°C. The pellet was washed once with 70% ethanol and following a 5 min centrifugation, was dried for 10 min. The DNA was then ready for immediate sequencing. The DNA was

resuspended in 7  $\mu$ l of water and to this was added 2  $\mu$ l of Sequenase buffer and 1  $\mu$ l (5 ng) of the sequencing primer. The primer was annealed to the DNA by heating at 65°C for 5 min and then allowing the reaction to cool slowly. Sequencing was then carried out as described in the Sequenase protocol.

### 3.9 PREPARATION OF UREA SEQUENCING GELS

The procedure used to prepare the sequencing gel is as described in the BRL M13 Cloning/Dideoxy Sequencing manual. The plates were cleaned with Sparkleen, a non-abrasive detergent, and then rinsed with deionized water and left to dry. Immediately before assembly, the plates were rinsed with ethanol and wiped with lint free Kimwipes.

The gel was prepared by de-gasing 75 ml of the Instagel solution (19 g acrylamide, 1 g bis-acrylamide, 105 g urea, 25 ml 10x TBE solution per 250 ml) under vacuum for 5 min. Ammonium persulphate (10% (w/v), 0.45 ml) and 30  $\mu$ l of TEMED were added to the Instagel. The gel solution was mixed by swirling and was poured within 10 min of adding the ammonium persulphate.

The polymerized gel was subjected to pre-electrophoresis for 1 hr at 1500 V before application of samples. The 2  $\mu$ l samples were heated at 90 to 100°C for 3 to 5 min then transferred to 0°C. Before application of the samples, the wells were rinsed to remove any urea. Electrophoresis was at 30 to 45 mA to maintain a temperature of about 50°C until the dyes ran off the gel. The gel was then transferred to Whatman 3 MM paper and covered with Resinite plastic wrap. The gel was

then dried before exposure to Kodak X-Omat AR film for appropriate lengths of time at  $-70^{\circ}\text{C}$ .

### 3.10 DIDEOXY SEQUENCING OF SINGLE-STRANDED DNA

The protocol used for sequencing using dideoxy nucleotides is given by United States Biochemicals, Cleveland, Ohio in their Sequenase kit. To anneal the primer to the DNA, 2  $\mu\text{l}$  of the Annealing Buffer, and 1  $\mu\text{l}$  of the primer were added to the DNA. Water was added to a final volume of 10  $\mu\text{l}$ . The sample was heated for 2 min at  $65^{\circ}\text{C}$  and then allowed to cool slowly to less than  $35^{\circ}\text{C}$ .

During that period, 2.5  $\mu\text{l}$  of the Termination Mixes were added to the appropriate microcentrifuge tubes. The Labeling Mix was diluted 1:5 and enough modified T<sub>7</sub> DNA polymerase for the reaction was diluted 1:8 in ice-cold TE solution. The tubes containing the Termination Mixes were prewarmed to  $37^{\circ}\text{C}$ .

For the labeling reaction 1  $\mu\text{l}$  of 0.1 M DTT, 2  $\mu\text{l}$  of dilute Labeling Mix, 0.5  $\mu\text{l}$  [ $\alpha$ - $^{32}\text{P}$ ]dATP (10 mCi/ml) and 2  $\mu\text{l}$  dilute DNA polymerase were added to the annealed DNA mix. The sample was mixed and left to incubate for 5 min at room temperature.

For the termination reaction, 3.5  $\mu\text{l}$  of the labeling reaction was added to each of the four termination tubes. The contents were mixed and the reaction was continued for a further 5 min at room temperature. The reactions were stopped by adding 4  $\mu\text{l}$  of the Stop Solution. Samples were heated for 2 min at  $75^{\circ}\text{C}$  immediately before loading into wells of the sequencing gel.

The protocol used for sequencing UCP mutants using dideoxy nucleotides is given by Pharmacia, Uppsala, Sweden in their T<sub>7</sub> Sequencing kit. To anneal the primer to the DNA, 1.5 to 2 µg of template DNA in 10 µl was mixed with 2 µl of 0.80 µM primer and 2 µl of annealing buffer. The sample was heated for 10 min at 60°C and then left to cool slowly to room temperature.

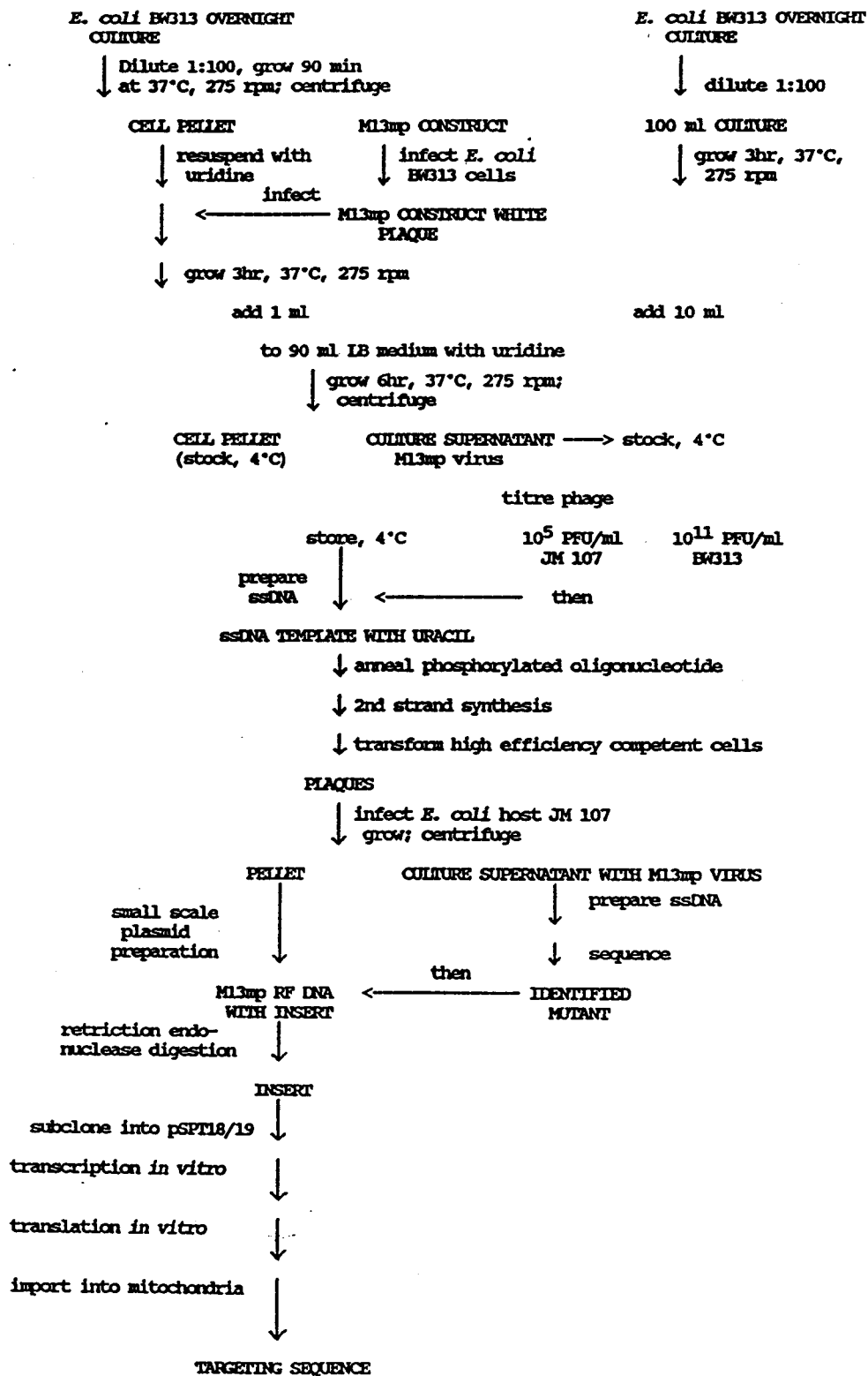
During that period, V-bottom shaped wells of a 96 well plate were labeled A, C, G, and T and 2.5 µl of the A mix-short, C mix-short, G mix-short and T mix-short were added to the corresponding wells. T<sub>7</sub> DNA polymerase was diluted to 1.5 U/µl using cold enzyme dilution buffer.

For the labeling reaction, 3 µl of labeling mix, 1 µl of [ $\alpha$ -<sup>35</sup>S]dATP $\alpha$ S (10 µCi) and 2 µl of diluted T<sub>7</sub> DNA polymerase were added to the annealed template and primer and left to incubate for 5 min at room temperature. The plate containing the sequencing mixes were prewarmed for at least 1 min at 37°C. After the labeling reaction was completed, 4.5 µl of the labeling reaction was transferred into each of the 4 prewarmed sequencing mixes and left to incubate for 5 min at 37°C at which point, 5 µl of the stop solution was added to each well and the samples were collected at the bottom of the wells by a brief spin using a modified salad spinner. Samples were heated at 75 to 80°C for 2 min immediately before loading into wells of the sequencing gel.

### 3.11 OLIGONUCLEOTIDE SITE-DIRECTED MUTAGENESIS

The method used is based on the method developed by Zoller and Smith (1983, 1984) with a modification introduced by Kunkel (1985). See Fig. 4 for a schematic diagram of the procedure.

Figure 4. Schematic Flow Diagram of Oligonucleotide Site-Directed Mutagenesis





E. coli BW 313 cells were infected with the desired bacteriophage and plated to obtain single plaques. LB medium (2 ml) was inoculated with 20  $\mu$ l of a culture of uninfected BW313 cells grown overnight and left to incubate for 90 min at 37°C with shaking at 275 rpm. The cells were recovered by a 5 min centrifugation at 5,000xg. The cells were then resuspended in 2 ml of prewarmed LB medium containing 0.5  $\mu$ g uridine/ml. The cells were infected with an isolated plaque grown on E. coli BW313 cells. At the same time, 10 ml of prewarmed LB medium was inoculated with 100  $\mu$ l of an overnight culture of E. coli BW313 cells and both cultures were grown for 3 hr at 37°C with shaking at 275 rpm.

After the incubation period, 1 ml of the infected cells and 10 ml of uninfected cells were mixed with 90 ml prewarmed LB medium containing 0.5  $\mu$ g uridine/ml. The culture was left to grow for 6 hr at 37°C with shaking at 275 rpm. At the same time E. coli JM107 and BW313 cultures were started to be used as lawn cells.

After the 6 hr incubation, the 100 ml culture was collected by centrifugation at 5,000xg for 5 min to remove the cells. The supernatant was stored at 4°C for use the next day. A sample of the bacteriophage was taken and diluted 1:10<sup>3</sup> and 1:10<sup>7</sup> to titer the bacteriophage using 200  $\mu$ l of lawn JM107 cells (1:10<sup>3</sup> dilution, 10 and 20  $\mu$ l) and 200  $\mu$ l of lawn BW313 cells (1:10<sup>7</sup> dilution, 10 and 20  $\mu$ l). After an overnight incubation at 37°C, if the difference in the order of magnitude in plaque forming units is 10<sup>4</sup> to 10<sup>5</sup> in favor of the BW313 cells, then uracil has been incorporated into the DNA and ssDNA can be prepared.

In order to prepare ssDNA, the supernatant was subjected to another centrifugation at 5,000xg for 5 min at 4°C. A solution containing 19.3 ml of 20% PEG and 2.5 M NaCl was added to the resulting supernatant and left to stand at room temperature for 30 to 45 min. The solution was subjected to a centrifugation for 15 min at 10,000xg and the supernatant was decanted while being careful not to lose the pellet. The pellet was resuspended in 1 ml of TE solution and subjected to centrifugation for 5 min to remove insoluble matter. An equal volume of water-saturated phenol was added to extract the aqueous phase. The aqueous phase was then extracted with an equal volume of phenol:chloroform (1:1, v/v). The aqueous phase was then extracted again with chloroform:iso-amyl alcohol (24:1, v/v) to remove any traces of phenol. To precipitate the ssDNA, 10 µl of 5 M NaCl and 2.5 volumes of 95% ethanol were added and the mixed solution was left at 0°C for 30 min. The ssDNA was recovered by a 10 min centrifugation at 4°C. The pellet was washed once with 70% ethanol, dried and dissolved in 50 µl of TE solution. To visualize the ssDNA, 3 µl of the sample was applied and mobilized on a 1% agarose gel.

Once the ssDNA was visualized on a 1% agarose gel, the oligonucleotide was phosphorylated. Phosphorylated primer is stable for one week only. A solution containing 1.5 µl of 1 M Tris-HCl pH 7.8, 1.5 µl of 100 mM MgCl<sub>2</sub>, 0.75 µl of 100 mM DTT, 1.5 µl of 1 mM ATP and 1.75 µl water was added to 700 ng of primer for phosphorylation. To this mixture, 1 µl of T<sub>4</sub> polynucleotide kinase was added and the sample was incubated at 37°C for 45 min. To stop the reaction, the sample was heated for 10 min at 65°C.

For the best results of annealing the phosphorylated primer to the ssDNA, a molar ratio of 20:1 of primer to template was used as suggested by M. Smith (personal communication to Len Rasile). The annealing reaction was performed in a microcentrifuge tube in 10  $\mu$ l of solution consisting of the uracil-containing template, the phosphorylated primer, 1  $\mu$ l of the annealing buffer and water to make up the volume. The contents were mixed and collected at the bottom of the tube by a quick centrifugation. The sample was heated for 5 min at 90 to 95°C and then left to cool slowly to room temperature. Again, the contents were collected at the bottom of the tube by a quick centrifugation.

To the annealed reaction a solution containing 1  $\mu$ l of 100 mM DTT, 100 mM MgCl<sub>2</sub> and 200 mM Tris-HCl, pH 7.5 (solution B), 4  $\mu$ l of 2.5 mM dNTP mix, 1  $\mu$ l of 10 mM ATP, 2  $\mu$ l (3 U) of T<sub>4</sub> DNA ligase, 0.5  $\mu$ l (3 U) of the Klenow fragment of DNA polymerase and water were mixed together to a total of 20  $\mu$ l. The contents were collected at the bottom of the tube and left overnight at 15°C. The next steps involved transforming high efficiency competent *E. coli* HB101 or 294 cells (10<sup>8</sup> transformants/ $\mu$ g DNA), picking isolated plaques to prepare dsDNA for screening by size and ssDNA from appropriate plaques for sequencing.

### 3.12 ISOLATION OF DNA FROM AGAROSE

The protocol used to isolate DNA from agarose gels is that given by the Bio101 company, La Jolla, California, with their GeneClean kit. The method is given below.

Agarose containing DNA was excised from the ethidium bromide stained agarose gel and weighed. If the agarose had a mass less than 0.4 g, then the steps were carried out in a microcentrifuge tube. Next, 2 to 3 volumes of the NaI stock solution were added and the tube was placed in a 45 to 55°C water bath for 1 to 2 min. The contents of the tube were mixed and the tube was replaced in the water bath for a further 5 min in order for the agarose to become completely dissociated. For DNA of less than 5 µg, 5 µl of glass milk was added, mixed and left at 0°C for 5 min. The sample was pelleted by centrifugation for 5 sec and the NaI supernatant was discarded. The pellet was washed 3 times with 10 to 50 volumes of NEW solution by resuspending the pellet followed by a 5 sec centrifugation. After the third centrifugation, another one was required to remove the last of the liquid. The pellet was resuspended in 10 µl of water, placed in a 45 to 55°C water bath for 2 min followed by a 30 sec centrifugation and the DNA was removed in the supernatant. A second elution was done to obtain an additional 10 to 20% of the DNA.

### 3.13 ISOLATION OF DNA FROM LOW MELTING POINT AGAROSE GELS

DNA samples were run on 1% low melting agarose gels at various voltage readings for various lengths of time at 4°C. After visualization, the appropriate band was cut from the gel, chopped into smaller pieces and placed in an Eppendorf tube to which was added 200 µl of 0.2 M Tris-HCl pH 8.0 and 10 µg of tRNA. The tube was heated for 10 min at 65°C until the gel melted. An equal volume of phenol saturated with 0.2 M Tris-HCl pH 8.0 was added for the first extraction. The phenol

was back-extracted with 200  $\mu$ l of water and the aqueous phases combined. The aqueous sample was then extracted twice with a solution of Tris-HCl-saturated phenol:chloroform (1:1, v/v) and 4% isoamyl alcohol, with each extraction followed by a back-extraction. The combined aqueous sample was then extracted with 500  $\mu$ l of chloroform and then three times with 500  $\mu$ l of water-saturated ether. The DNA in the aqueous phase was precipitated with one tenth the volume of 3 M NaAc, pH 5.2 and one volume of isopropanol and left on ice for 15 min. The DNA was recovered by a 15 min centrifugation and followed by a wash with 70% ethanol. The DNA sample was dried and resuspended in 20  $\mu$ l of water or TE buffer. A 5  $\mu$ l sample was usually taken for visualization on an agarose gel.

### 3.14 TRANSCRIPTION IN VITRO

The procedure followed for in vitro transcription is that given by Promega, Wisconsin, USA, in their kit. The method is outlined below.

The following components were added in order: DEPC-treated water to make up the volume to 50  $\mu$ l, 5x Transcription Buffer (10  $\mu$ l), 100 mM DTT (10  $\mu$ l), RNasin (2  $\mu$ l-80 U), NTP mix containing 2.5 mM of ATP, CTP, UTP and 0.5 mM GTP (10  $\mu$ l), 5 mM  $m^7$ GpppG (1  $\mu$ l), 2  $\mu$ g of linear plasmid and 90 U T<sub>7</sub> or 10 U SP6 RNA polymerase (1  $\mu$ l). The reaction was incubated at 37 to 40°C for 2 hr after which time, 2  $\mu$ l of DNase (2 U) was added for a further 15 min incubation at 37°C. The transcript was extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1, v/v/v) and once with an equal volume of chloroform:iso-amyl alcohol (24:1, v/v). To precipitate the RNA and remove DNA, 0.2x the volume of 10 M NH<sub>4</sub>OAc was added along with 2.5



volumes of 95% ethanol. The transcript was left to precipitate overnight at  $-20^{\circ}\text{C}$  and the pellet was collected by a 15 min centrifugation at  $4^{\circ}\text{C}$ . The RNA was washed once with ice-cold 70% ethanol, dried and resuspended in 20  $\mu\text{l}$  of DEPC-treated water with 40 U of RNasin and stored at  $-70^{\circ}\text{C}$ .

For linearized plasmids with 3'-overhangs (PstI cut), the DNA is first treated with the Klenow fragment of DNA polymerase as suggested by Promega Notes (March 1985) in order to decrease extraneous transcription.

### 3.15 TRANSLATION IN VITRO

The protocol used for in vitro translation is that given by Promega with their nuclease-treated lysate. The protocol is given below.

For a 50  $\mu\text{l}$  reaction, 35  $\mu\text{l}$  of the nuclease-treated lysate and 1  $\mu\text{l}$  of the amino acid mixture minus methionine were mixed. The RNA, usually 3  $\mu\text{l}$ , was diluted with water to 9  $\mu\text{l}$ , heated for 5 min at  $65^{\circ}\text{C}$ , and then added directly to the lysate mixture. Finally, 5  $\mu\text{l}$  of [ $^{35}\text{S}$ ]methionine was added to the translation mixture and the reaction was incubated at  $30^{\circ}\text{C}$  for 20 min. Samples (1  $\mu\text{l}$ ) were taken at time zero and at 20 min to determine acid-insoluble counts.

### 3.16 PREPARATION OF RAT HEART MITOCHONDRIA

The protocol used to prepare rat heart mitochondria is described previously (Argan et al., 1983) and is given here. Hearts from new-born to 2 week-old Sprague-Dawley or Wistar rats were rinsed first in an ice-

cold solution containing 10 mM HEPES, 220 mM mannitol, 70 mM sucrose and 1 mM EGTA, pH 7.5 (medium B) and then homogenized in 10 ml of ice-cold medium A (medium B with 2 mg BSA/ml) for 2 sec in a 40 ml polypropylene tube using an Ultraturrax disintegrator with a rheostat setting of 90. The homogenate was then diluted to 40 ml using ice-cold medium B and the mixture was then subjected to centrifugation for 10 min at 4°C in an SS34 rotor at 500xg. The upper three-quarters of the supernatant was removed for a further centrifugation at 5,910xg at 4°C for 10 min.

The pellet was resuspended in 10 ml of ice-cold medium B and centrifugation was repeated at 500xg for 10 min at 4°C. The supernatant was then subjected to further centrifugation at 5,910xg for 10 min at 4°C to pellet the final mitochondrial fraction. The ring of broken mitochondria was washed from the final pellet which was then resuspended in 1 ml of a solution containing 250 mM sucrose, 5 mM succinate, 10 mM HEPES, 2 mM  $K_2HPO_4$ , 1 mM DTT, 1 mM ATP, and 0.08 mM ADP, pH 7.5 (resuspension buffer). An option is the addition of 15 to 20  $\mu$ g of cycloheximide for the inhibition of protein synthesis and/or the addition of 80  $\mu$ g of chloramphenicol for the inhibition of mitochondrial protein synthesis.

For a quick approximation to determine the mitochondrial protein concentration, 10 and 20  $\mu$ l samples were added to 1 ml of 0.1 M NaOH, mixed using a vortex mixer and the  $A_{280}$  measured. The concentration of mitochondrial protein was then determined using the following equation based on a standard curve with air as the reference: Concentration =  $[(A_{280} - 0.055) \div 0.00225] \div \text{volume } (\mu\text{l})$  where 0.055 is the  $A_{280}$  for 0.1

M NaOH. For more accurate determinations, 5 and 10  $\mu$ l samplers were taken for Lowry protein determinations done at later times.

### 3.17 PREPARATION OF RAT BROWN ADIPOSE TISSUE MITOCHONDRIA

Brown adipose tissue was first rinsed twice in a solution containing 0.25 M sucrose and 5 mM Tes-KOH, pH 7.2. The tissue was then homogenised by 3 sets of 8 strokes in a 40 ml polypropylene tube containing 8 ml of the same solution with 40 mg BSA using an Ultraturrax disintegrator with a maximum reading of 80 (rheostat at 42 to 48). The homogenate was filtered through two layers of cheese cloth into an ice-cold beaker. The filtrate was transferred to a fresh tube and made up to a volume of 30 ml with the sucrose-Tes solution containing 5 mg BSA/ml for a 10 min centrifugation at 8,500xg at 4°C. The fatty layer was discarded along with the supernatant. The sides of the tube were wiped to remove any residual fat. The pellet was resuspended in 1 to 2 ml of the sucrose-Tes, pH 7.2 solution containing 5 mg BSA/ml and then made up to 25 ml using the same solution.

The resuspended pellet was subjected to centrifugation for 10 min at 4°C at 2,500xg and the supernatant was removed carefully to avoid the nuclear fraction pellet. The supernatant containing mitochondria was then subjected to centrifugation for 10 min at 8,500xg at 4°C. The resulting supernatant was then discarded and the mitochondrial pellet was resuspended in 1 to 2 ml of a solution containing 0.3 M sucrose, 2 mM EDTA and 10 mM Tris-HCl, pH 7.2 and then made up to a volume of 25 ml with the same solution. The mitochondrial fraction was pelleted by centrifugation for 10 min at 4°C at 8,500xg. The pellet was resuspended



in the sucrose-EDTA-Tris solution and the wash was repeated twice more. The final pellet was resuspended in 0.5 to 1 ml of the resuspension buffer described for heart mitochondria. A sample was taken for protein determination and then BSA was added to a final concentration of 2 mg BSA/ml.

### 3.18 BIO-RAD PROTEIN DETERMINATION

The protocol used for protein determination is that given by Bio-Rad, Rockville Centre, New York, with their reagent based on the method of Bradford (1976). The standard assay procedure is given below.

Standards of ovalbumin ranging from 0 to 100  $\mu\text{g}$  and appropriately diluted samples in 0.1 ml volumes were placed in clean, dry test tubes to which was added 5 ml of the dilute dye reagent. The contents were mixed using a vortex mixer being careful to avoid excessive foaming. After a 5 min waiting period, the absorbance of the standards and samples were read at 595 nm. An empty cuvette was used as the reference.

### 3.19 LOWRY PROTEIN DETERMINATION

The determination of protein concentration as described by Lowry (Lowry *et al.*, 1951) is given below.

Standards of BSA ranging from 0 to 100  $\mu\text{g}$  and appropriately diluted samples were made up to 1 ml with water in test tubes. To the standards and samples was added 5 ml of reagent C which is composed of 50 ml of reagent A and 1 ml of reagent B. The samples were mixed using a vortex mixer and left to stand at room temperature for 30 min. After

this period, 0.5 ml of diluted Folin reagent (1:1 with water) was added and mixed immediately using the vortex mixer. The samples were left to stand at room temperature for 1 hr before measuring the  $A_{660}$ . An empty cuvette was used as the reference.

### 3.20 PROTEIN IMPORT INTO MITOCHONDRIA

For the import experiments, 75  $\mu$ g of mitochondrial protein in 100  $\mu$ l was used per reaction. To the mitochondria, 50  $\mu$ l of the translation reaction (200 000 to 400 000 cpm of translation diluted with lysate previously prepared in our laboratory) was added. The samples were incubated for 60 min at 30°C. After the incubation period, the mitochondria were collected by a 5 min centrifugation at 4°C. The pellet was washed using the mitochondrial isolation buffer B (150  $\mu$ l) and the centrifugation was repeated. The final mitochondrial pellet was dissolved in 2X SDS sample buffer and diluted 1:1 with water before SDS polyacrylamide gel electrophoresis.

If CCCP was to be used, 1  $\mu$ l of 1.5 mM stock solution was added before the incubation period. If samples were to be treated with proteinase K, 3  $\mu$ l of a 5 mg proteinase K/ml stock was added to cooled tubes after the import incubation period and the sample was further incubated for 30 min at 0°C. To stop the digestion, 3  $\mu$ l of 100 mM PMSF was added and the sample was left at 0°C for a further 10 min.

### 3.21 SUBFRACTIONATION OF MITOCHONDRIA

In order to determine the location of the imported proteins, import incubations with untreated mitochondria and mitochondria treated

with CCCP were washed as above but were not dissolved in SDS sample buffer. Instead, mitochondrial pellets were resuspended in 150  $\mu$ l of cold 0.1 M  $\text{Na}_2\text{CO}_3$  pH 11.5 using a pipette and a vortex mixer. The samples were left on ice for a least 1 hr and then membranes were pelleted by centrifugation in an airfuge rotor at 30 psi for 15 min. The supernatant was removed and saved. The pellet was washed with 100  $\mu$ l of 0.1 M  $\text{Na}_2\text{CO}_3$  and following a brief centrifugation, the wash supernatant was removed and discarded, and the sides of the tube were wiped dry with tissue. The membrane pellet was then dissolved in 2X SDS sample buffer and diluted 1:1 with water before applying a sample for SDS polyacrylamide gel electrophoresis. A sample of the supernatant was also diluted 1:1 with 2X SDS sample buffer before analysis.

### 3.22 UNCOUPLING PROTEIN PURIFICATION

The method used to purify uncoupling protein from mitochondria is as given by Lin and Klingenberg (1982). Mitochondria (100 mg protein) were treated with 3.6 ml of standard medium containing 3.2% (v/v) Lubrol WX and left for 30 min at 0°C. The sample was then centrifuged at 100,000xg for 30 min. The pellet was resuspended in a solution containing 0.3 M sucrose, 2 mM EDTA, 10 mM Tris-HCl, pH 7.2 and centrifuged again for 30 min at 100,000xg. The pellet was resuspended in 3.2 ml of standard medium containing 5% (v/v) Triton X-100 and left at 0°C for 30 min. The sample was then centrifuged for 30 min at 100,000xg and the resulting supernatant applied to a hydroxylapatite mini-column (blue pipette tip) equilibrated with standard medium. Uncoupling protein was eluted at room temperature with standard medium.



### 3.23 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

For the analysis of protein uptake or location by mini gel electrophoresis, a 12.5% (w/v) polyacrylamide separating gel was used based on the method of Laemmli (1970). The separating gel was allowed to polymerize for approximately 1 hr before the stacking gel was poured. Once the stacking gel had been poured, samples were applied to the gel within 30 min. After electrophoresis, the gel was treated with PPO in DMSO for fluorography and then dried for fluorography.

### 3.24 $^{14}\text{C}$ -LABELING OF PROTEINS

The method for labeling proteins with [ $^{14}\text{C}$ ]formaldehyde is that given by Rice and Means (1971). The protocol is given below.

Borate buffer (1 M, pH 9.0) was added to 400  $\mu\text{g}$  of uncoupling protein in a volume of 700  $\mu\text{l}$  to a final borate concentration of 0.2 M and the solution was cooled on ice. Formaldehyde (94  $\mu\text{l}$ , NEN, 17mM, 0.25mCi) was then added and mixed on a vortex mixer. After 30 sec, 8  $\mu\text{l}$  of 5 mg sodium borohydride/ml was added four times, capping and shaking after each addition. After 1 min, 40  $\mu\text{l}$  of the borohydride solution was added and mixed by shaking. Unreacted formaldehyde was removed by dialysis against 4, 2 L volumes of isolation buffer at 4°C. Five microlitres of the sample were used to determine acid-precipitable counts.

## 4. RESULTS

### 4.1 SUBCLONING INTO AN IN VITRO EXPRESSION VECTOR

Several vectors are now available which allow large scale production of mRNA and subsequent capping for translation. These vectors contain the promoter for one or more of bacteriophage SP6, T<sub>3</sub> or T<sub>7</sub> RNA polymerase. A multiple cloning site is present in the vectors beside the promoters to allow for ease of insertion of sequences for transcription. The rat uncoupling protein cDNA insert was first subcloned from the PstI site of pUCP15 (Ridley *et al.*, 1986a) into the PstI site of pTZ18U which was then linearized with BglI. Subsequent transcription and translation failed to yield any uncoupling protein.

It was not realized at this point that 3'-overhangs produced by restriction enzymes such as BglI or PstI used to linearize plasmids for transcription could result in RNA being transcribed from the opposite strand of that desired (Promega Notes, March 1985) possibly resulting in complementary RNA hybridizing to the mRNA thus preventing translation. The transcription reaction procedure was then modified slightly to remove the 3'-overhangs by digesting with the Klenow fragment of DNA polymerase before transcription. Again, translation of the transcript failed to yield uncoupling protein.

A further modification was made to the construct to eliminate the 5'-oligo(dG)-oligo(dC) extension of the UCP cDNA insert without modifying the 3'-end. It was thought that the transcript could be forming secondary structures using G-C pairing which would explain the



block in the translation. In order to accomplish this, the pTZ18U-UCP was digested with KpnI and then religated. This eliminated the 5'-PstI site so that the plasmid could be linearized by PstI. Still, translation of the transcript failed to produce uncoupling protein.

As a control for both transcription and translation, a proven vector was chosen. Rat ornithine carbamoyltransferase precursor cDNA inserted into pSP64 served as a control for transcription, translation and later, for import into isolated mitochondria. Translation of this transcript yielded a unique protein corresponding in size to the ornithine carbamoyltransferase precursor (Mr 39 000), indicating that the procedure worked.

As another control, Dr. Gordon Shore's group sent a construct, pSPUCP, this one containing the uncoupling protein insert cut with TaqI to remove the 5' extension and inserted back into the PstI site of pSP64 with the aid of an oligonucleotide (Liu et al., 1988). When this construct was linearized with EcoRI to yield 5'-overhangs, transcribed, translated and the product immunoprecipitated, uncoupling protein was detected in the translation product and in the immunoprecipitated product (results not shown).

A second construct, pSPUCPd1-12, again in pSP64, was sent by Shore's group in which the first 12 amino acids were deleted (Liu et al., 1988). The plasmid was linearized with EcoRI and transcribed. The mRNA was translated and the shorter uncoupling protein was observed after electrophoretic analysis.

When the translation products of the two constructs were compared directly, one band was seen for the UCPd1-12 construct while two bands



of approximately equal intensity were seen for the construct deleting most of the 5' extension, one corresponding to the uncoupling protein with a molecular mass of 32 000 Da and the second band of higher mobility corresponding to the UCPd1-12 product. This result likely explains why two products for uncoupling protein are sometimes seen in immunoprecipitations from translations of brown adipose tissue polysomes (Freeman *et al.*, 1983). The smaller product probably corresponds to the uncoupling protein initiating at methionine<sub>13</sub>, however, direct protein sequencing is required for proof and this has not been done.

Because the pTZ18U-UCP construct never yielded a transcript which resulted in synthesis of uncoupling protein, another vector was tried. G. Pacepavicius and J. Ramchatesingh, working in our laboratory as Biochemistry 4P3 project students, subcloned the KpnI-PstI uncoupling protein cDNA into the vectors, pSPT18 and pSPT19 respectively. Transcription of the pSPT18-UCP vector made use of T<sub>7</sub> RNA polymerase and pSPT19-UCP construct made use of the SP6 RNA polymerase. In both cases uncoupling protein was observed after transcription and translation but more uncoupling protein was made using the T<sub>7</sub> RNA polymerase and the pSPT18 vector (results not shown).

#### 4.1.1 IMPROVING TRANSCRIPTION AND TRANSLATION

One attempt to improve the quality of the transcript was to compare transcript purified by phenol extraction versus transcript purified by spun column dialysis. The transcripts were radiolabeled with [ $\alpha$ -<sup>32</sup>P]UTP and compared by analysis on a 1% agarose-formaldehyde gel. Recovery was marginally greater when transcript was purified using



spun column dialysis, however, there was no difference in the amount of translation product. Phenol extraction was simpler and was used in subsequent reactions (results not shown).

Another factor which affected the amount of translation product was the concentration of  $m^7GpppG$  used in the transcription reaction. With early transcription reactions, it appeared that high concentrations of the cap (0.5 mM cf 0.1 mM) reduced the amount of the translation product. A final concentration of 0.1 mM  $m^7GpppG$  yielded transcript producing more uncoupling protein than did the transcripts using 0.5 mM  $m^7GpppG$  in the transcription reaction (results not shown). All four reactions maintained the same final concentration of 0.02 mM GTP. The final transcription reaction used 0.1 mM  $m^7GpppG$  and 0.1 mM GTP.

Two bands were sometimes seen in the translation of pSPT18-UCP and pSPT19-UCP transcripts corresponding to the two bands always seen with the translation products observed from pSPUCP. However, the second band of higher mobility probably corresponding to initiation at the second methionine was made in much smaller quantities compared to the full-length protein. This result may reflect the length of the 5'-nontranslated portion of the cDNA. In both pSPT18 and pSPT19, there are 66 nucleotides preceding the initial methionine whereas in pSPUCP, there are 9 nucleotides preceding the initial methionine, 45 preceding methionine<sub>13</sub> and 40 nucleotides preceding methionine<sub>13</sub> in pSPUCPd1-12.

In summary the transcription-translation system was improved by removing the 5' extension thus eliminating the possibility of G-C pairing, removing 3'-overhangs from linear DNA transcription templates to reduce extraneous transcription (Promega Notes, March 1985), using

lower concentrations of  $m^7GpppG$  during transcription and using a high quality fresh lysate for translation.

#### 4.2 IMPORT INTO MITOCHONDRIA

The functioning transcription/translation system was now used to set up the system for import of uncoupling protein into isolated rat heart mitochondria. The analysis of import into mitochondria is based on the association of uncoupling protein with mitochondria incubated with and without CCCP, an uncoupler of oxidative phosphorylation, compared to the same incubations after proteinase K treatment. If uncoupling protein is associated with mitochondria treated with proteinase K but not with mitochondria treated with both CCCP and proteinase K, then uncoupling protein requires a membrane potential for import as do other matrix and inner membrane proteins. Protection from proteinase K digestion can be used as the criteria for import and internalization into mitochondria. As a more direct control in initial experiments, the processing of preornithine carbamoyltransferase to its mature size and its protection from proteinase K digestion was included but not shown.

##### 4.2.1 IMPORT BY RAT HEART MITOCHONDRIA

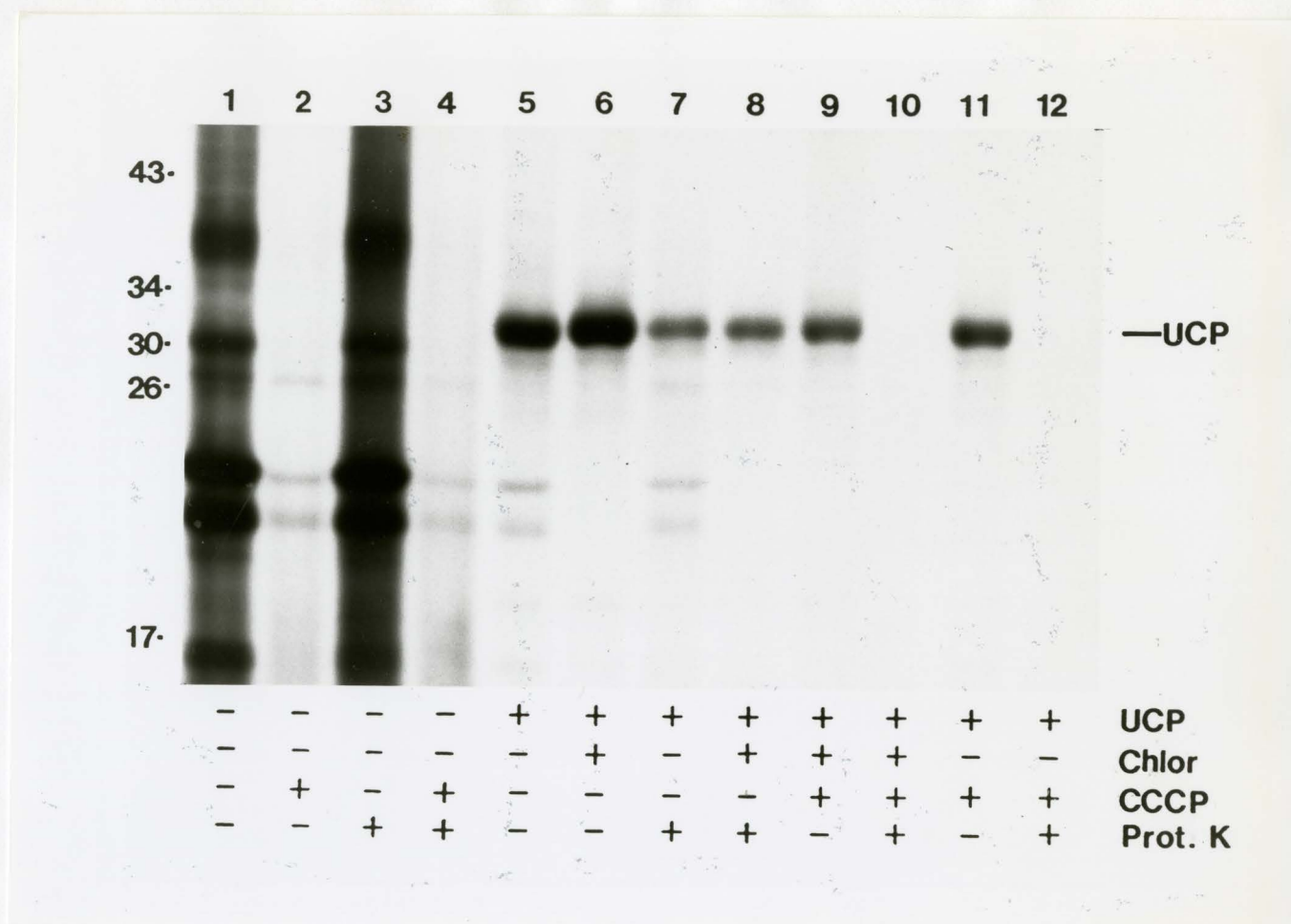
The first several uptake experiments were largely unsuccessful. This was probably due to insufficient mitochondria, too little of the translation being used and the order of preparation of mitochondria and translations. It was noted by Dr. Shore's group that uptake of uncoupling protein was optimal for freshly synthesized protein, *i.e.*



protein that had been translated for 20 minutes and used directly for import (Liu et al., 1988). During the course of these early experiments I found that frozen mitochondria did not support uptake of exogenously added protein, frozen samples of uncoupling protein were not imported into mitochondria and mitochondria from young animals (less than 3 weeks old) worked best for import experiments.

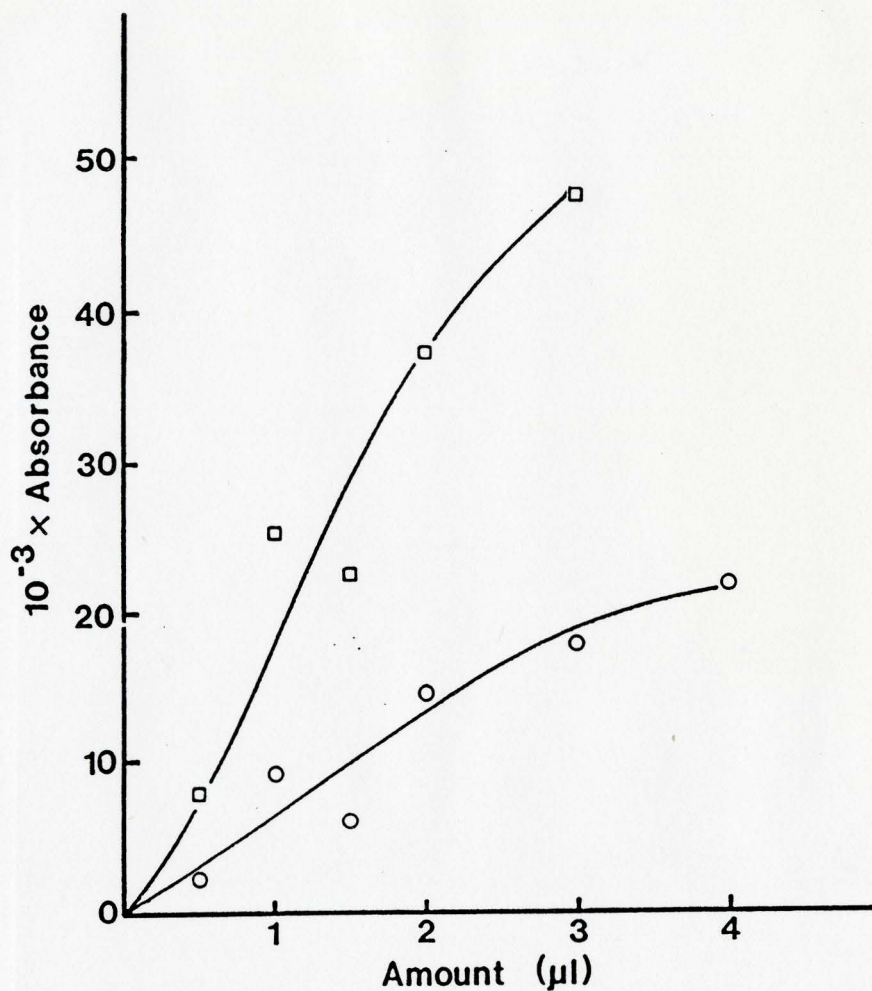
Fig. 5 shows the import of uncoupling protein under various conditions. Lanes 1 to 4 do not have any uncoupling protein present yet radioactively labeled bands are detected. These correspond to mitochondrially synthesized proteins and can be eliminated with the addition of chloramphenicol to the import reaction (Fig. 5, lanes 6, 8-10). The addition of CCCP decreased the amount of mitochondrial proteins synthesized (Fig. 5, lanes 2 and 4) and blocked import of uncoupling protein (lanes 10 and 12), but did not prevent association of uncoupling protein with mitochondria (lanes 9 and 11). Addition of chloramphenicol did not affect the import of uncoupling protein (cf lanes 7 and 8) and was included in all subsequent import reactions unless otherwise stated. Treatment with proteinase K had no effect on mitochondrially synthesized proteins (Fig. 5, cf lanes 1 and 3) or on uncoupling protein imported into mitochondria (cf lanes 8 and 10; lanes 7 and 12).

In order to determine whether or not there was a limit to the amount of uncoupling protein which could be taken up by mitochondria, increasing amounts of freshly translated uncoupling protein were added to import reactions. Fig. 6 shows that for the amounts of translation used in these experiments, a limit was not reached for those reactions



**Figure 5. Import Conditions for Uncoupling Protein.** Concentrations of all components are as described in the Methods section. Mitochondria were prepared from 3 day old rats. Import reactions were carried out for 60 min. Where indicated, CCCP and chloramphenicol were added to a final concentration of 0.1  $\mu$ M and 80  $\mu$ g/ml respectively. Proteinase K digestion where indicated occurred for 30 min. All lanes are mitochondrial pellets. Lanes 1 to 4 have no translated uncoupling protein added but the incubation was carried out in the presence of 100  $\mu$ Ci of [ $^{35}$ S]methionine and 40  $\mu$ l of lysate. Molecular mass markers are indicated on the left in kDa and uncoupling protein marker is indicated on the right. The fluorogram was exposed for 63 hr. Abbreviations: Chlor - chloramphenicol.





**Figure 6. Import of Uncoupling Protein.** Import conditions are as described in the Methods section except that the amount of uncoupling protein translation was varied as indicated with extra lysate added to maintain a volume of 50  $\mu\text{l}$ .  $\square$ , Untreated mitochondria;  $\circ$ , mitochondria digested with 0.15 mg proteinase K/ml for 30 min after import. Each lane of the fluorogram was scanned twice using a Hoefer Scientific densitometer. The plot of absorbance (arbitrary units) versus amount represents the average of the scans.

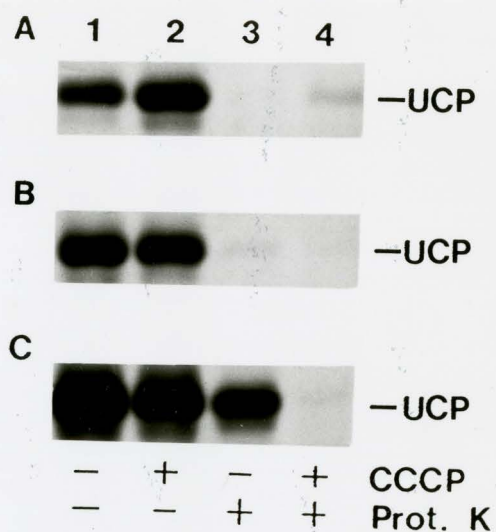
treated with proteinase K (Fig. 6, open circles) and that the relationship between the amount of uncoupling protein added and that imported is roughly linear.

The precursor of malate dehydrogenase can be internalized directly into mitochondria from a prebound state (Chien *et al.*, 1984). But as shown in Fig. 7, this was not observed for uncoupling protein. Panel A serves as a control on the amount of uncoupling protein bound to mitochondria at 0°C in 60 minutes and panel C is a control for standard import conditions. In the actual experiment (Fig. 7, panel B), uncoupling protein was allowed to bind to mitochondria at 0°C for 60 minutes, mitochondria were then collected and resuspended in fresh import medium and left to incubate at 30°C for 60 minutes. The results show that the bound form of uncoupling protein is poorly imported into mitochondria. However, it is possible that in these experiments, malate dehydrogenase would not have been imported either.

The time courses and relative efficiency of import of uncoupling protein (Fig. 8, open circles) and preornithine carbamoyltransferase (Fig. 8, open squares) were also examined. Both proteins were imported equally with import reaching a maximum level at 60 minutes. The decline seen after 60 minutes may reflect protein or mitochondrial degradation.

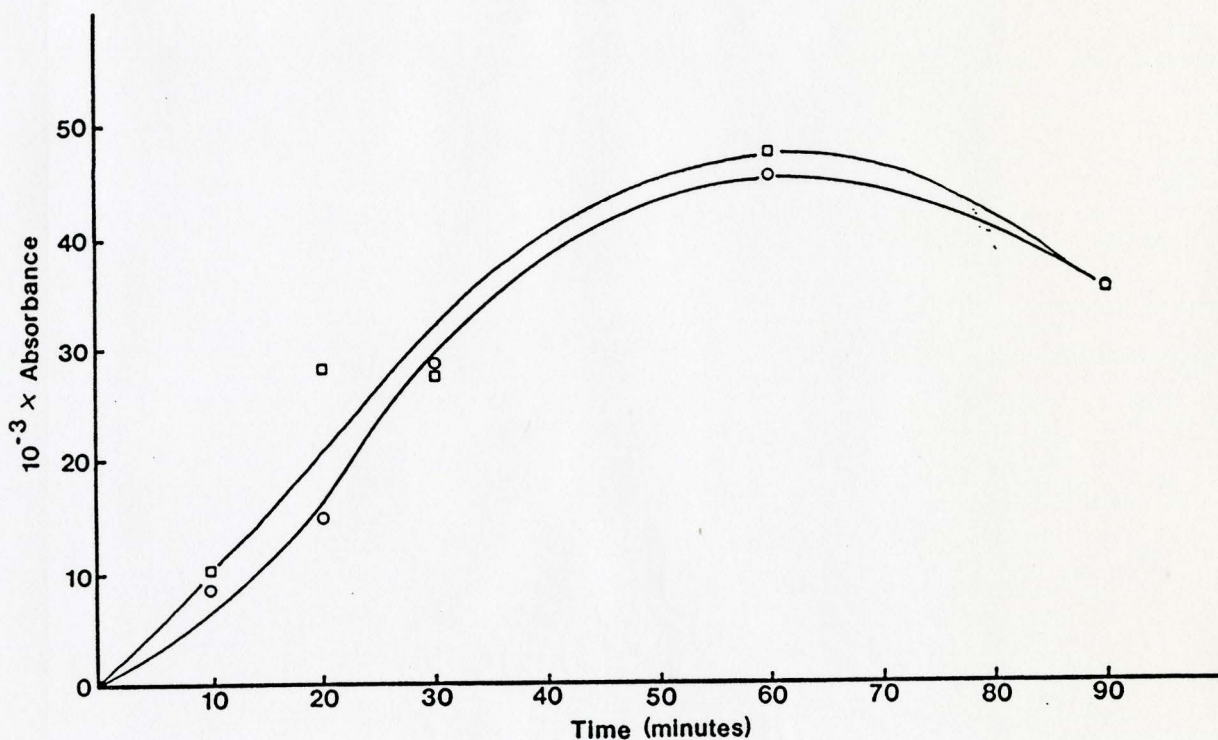
Of special interest in these import experiments is the possibility that the imported protein is able to function in mitochondria from different tissues. A simple, though not definitive answer can be achieved by purifying imported uncoupling protein using the same technique used to purify the protein from brown adipose tissue. Fig. 9 shows that imported uncoupling protein could be purified from





**Figure 7. Import of Bound Uncoupling Protein.** Import conditions are as described in the Methods section with the following exceptions. Panel A: Import was carried out on ice for 60 min. Panel B: Import was carried out on ice for 60 min; the mitochondrial pellet was then resuspended in fresh import buffer and incubation continued at 30°C for 60 min. Panel C: Standard import conditions. Lanes 1 to 4 are mitochondrial pellets. Uncoupling protein marker is indicated on the right.





**Figure 8. Time Course for Import of Uncoupling Protein and Ornithine Carbamoyltransferase.** Import conditions are as described in the Methods section except that standard volumes were increased 9 fold and 150  $\mu$ l samples were removed after 10, 20, 30, 60 and 90 min. Both uncoupling protein (o) and ornithine carbamoyltransferase ( $\square$ ) were added together in duplicate import reactions. The samples were treated with proteinase K and subjected to SDS polyacrylamide gel electrophoresis. Each lane of the flourogram was scanned twice using a Hoefer Scientific densitometer. The plot of absorbance (arbitrary units) versus time represents the average of the integrated values.



**Figure 9. Purification of Imported Uncoupling Protein from Heart Mitochondria.** Uncoupling protein synthesized *in vitro* was imported into heart mitochondria as described in the Methods section. The mitochondrial pellet was then dissolved in Triton X-100 and applied to a hydroxylapatite column to purify uncoupling protein as described in the Methods section. Lanes 1 to 8, collected fractions 1 to 8; lane 9: uncoupling protein marker.

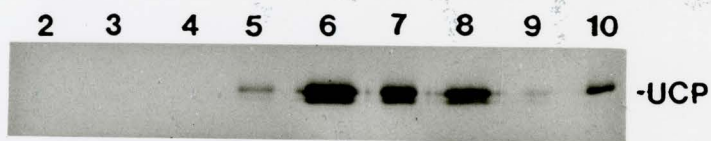


heart mitochondria as it is collected in the flow through fractions (Fig. 9, lanes 5 and 6) as is uncoupling protein purified from mitochondria of its source tissue (Lin & Klingenberg, 1982). This indicates that the imported protein in the membrane is indistinguishable from the natural protein with respect to purification and provides some indication that uncoupling protein imported into heart mitochondria has the potential to uncouple oxidative phosphorylation when activated.

As an aside, it was of interest to determine if isolated mitochondria from brown adipose tissue could import uncoupling protein in vitro, since they do so in vivo. Isolated brown adipose tissue mitochondria did import uncoupling protein although those prepared from rats left at 6°C for 4 days did not import as well as mitochondria prepared from rats left at 23°C (results not shown). The imported uncoupling protein could also be purified from the brown adipose tissue mitochondria as seen in Fig. 10, lanes 6 to 8. Experiments done by C. McCallum and H.V. Patel showed that the double band seen in Fig. 10 was the result of digestion with proteinase K since only a single band was seen with trypsin treatment.

#### 4.3 UNCOUPLING PROTEIN CONSTRUCTS

The design of the first constructs was based on earlier results (Liu et al., 1988). The first 12 amino acids are not required for import of uncoupling protein or the uncoupling protein-ornithine carbamoyltransferase fusion protein, U13-105-OCT. This suggests that a targeting sequence lies within amino acids 13 to 105 as this region joined to ornithine carbamoyltransferase produced as fusion protein



**Figure 10.** Purification of Imported Uncoupling Protein from Brown Adipose Tissue Mitochondria. Uncoupling protein synthesized *in vitro* was imported into brown adipose tissue mitochondria as described in the Methods section. Uncoupling protein was then purified from the mitochondrial pellet as described in the Methods section. Lanes 2 to 9, fractions 2 to 9; lane 10, uncoupling protein marker.



which is imported by mitochondria. Further, a fusion protein of amino acids 13 to 51 of uncoupling protein joined to ornithine carbamoyltransferase was not imported. To complete the examination of this series of constructs, amino acids 2 to 52 needed to be deleted from the hybrid construct in order to test amino acids 53 to 105 for targeting. The basis for the other constructs whose import was examined initially was to simply delete the first 35 amino acids proposed to form the first membrane-spanning region of uncoupling protein (Fig 2a).

In order to create the constructs the uncoupling protein insert was removed from the pSPT18 vector using EcoRI and PstI to be subcloned into the EcoRI-PstI site of M13mp19. At the same time, the UCP-OCT hybrid joining amino acids 13-105 of uncoupling protein to mature ornithine carbamoyltransferase from amino acids 147 to the end was removed from the pSP64 vector using the same enzymes and subcloned into M13mp18. In these orientations, the sequence that would be read from the ssDNA would be the coding sequence in both cases. Once the inserts were subcloned, the phage were used to infect *E. coli* BW313 cells. The cells were then grown in the presence of uridine in order to incorporate uracil into the ssDNA. ssDNA was prepared from the phage after the titre results showed a difference of six orders of magnitude in the survival of phages with uracil in their DNA in BW313 cells over wild type cells. The ssDNA was then used for oligonucleotide site-directed mutagenesis (Fig. 11 for constructs). Once the reaction was complete and dsDNA was made, competent *E. coli* JM107 cells, prepared by Hanahan's method (Hanahan, 1983) and commercially prepared HB101 cells were used for the transformation. It was noted that the purchased HB101 library



A

Val<sub>2</sub>Ser<sub>3</sub>Ser<sub>4</sub>....Pro<sub>33</sub>Leu<sub>34</sub>Asp<sub>35</sub>

```

.....GCC CTC CGA GCC AAG ATG ACC  GCC  AAA  GTC  .....
          CGA GCC AAG ATG ACC  GCC  AAA

```

Met<sub>1</sub>Thr<sub>36</sub>Ala<sub>37</sub>Lys<sub>38</sub>Val<sub>39</sub>

B

Gly<sub>14</sub>Val<sub>15</sub>Lys<sub>16</sub>....Pro<sub>33</sub>Leu<sub>34</sub>Asp<sub>35</sub>

```

..GAA GTG CAA  CCC  ACC  ATG  ACC  GCC  AAA  GTC  ...TCA  GAT
      CAA  CCC  ACC  ATG  ACC  GCC  AAA

```

Glu<sub>8</sub>Val<sub>9</sub>Gln<sub>10</sub>Pro<sub>11</sub>Thr<sub>12</sub>Met<sub>13</sub>Thr<sub>36</sub>Ala<sub>37</sub>Lys<sub>38</sub>Val<sub>39</sub>...Ser<sub>105</sub>Asp<sub>147</sub>

start

OCT...

C

Gly<sub>14</sub>Val<sub>15</sub>Lys<sub>16</sub>....Ser<sub>50</sub>Ser<sub>51</sub>Thr<sub>52</sub>

```

..GAA GTG CAA  CCC  ACC  ATG  ATT  AGG  TAT  AAA  ...TCA  GAT
      CAA  CCC  ACC  ATG  ATT  AGG  TAT

```

Val<sub>9</sub>Gln<sub>10</sub>Pro<sub>11</sub>Thr<sub>12</sub>Met<sub>13</sub>Ile<sub>53</sub>Arg<sub>54</sub>Tyr<sub>55</sub>Lys<sub>56</sub>...Ser<sub>105</sub>Asp<sub>147</sub>

start

OCT....

**Figure 11. Oligonucleotides Used for Creating Deletion Constructs.** a, AB268 used for Ud2-35; b, AB270 used for Ud2-35-OCT; c, AB271 used for U53-105-OCT.

efficiency competent cells were more efficient in transformation than the competent JM107 cells prepared by Hanahan's method.

Eight of about 40 plaques from each of the constructs were picked at random for an initial screening. This consisted of preparing dsDNA from the phage and digesting the DNA with EcoRI and PstI to remove the insert. The deletions removed 102 (methionine<sub>1</sub> to threonine<sub>36</sub>), 66 (methionine<sub>13</sub> to threonine<sub>36</sub>) and 117 (methionine<sub>13</sub> to isoleucine<sub>53</sub>) base pairs, differences which can be observed when compared to the original inserts. The digestion experiments showed that 2 of the 8 plaques for the construct labeled Ud2-35, 1 of the 8 for U36-105-OCT and 3 of the 8 for U53-105-OCT were likely the desired constructs. The ssDNA was then prepared from these and from uncoupling protein controls for M13 dideoxy-sequencing. The sequencing results confirmed those obtained from the digests.

The next stage was to remove the inserts from M13mp18 and mp19 dsDNA using EcoRI and PstI and force subclone them into pSPT18 (Ud2-35) and pSPT19 (U36-105-OCT and U53-105-OCT) in order to transcribe mRNA using the T<sub>7</sub> promoter. Once this had been accomplished, large scale plasmid preparations were done in order to obtain purified plasmid for in vitro transcription/translation reactions. Translation of Ud2-35 and U53-105-OCT resulted in polypeptides of the anticipated size while a U36-105-OCT translation product was not observed. The reason for the lack of a translation product for U36-105-OCT was not pursued.

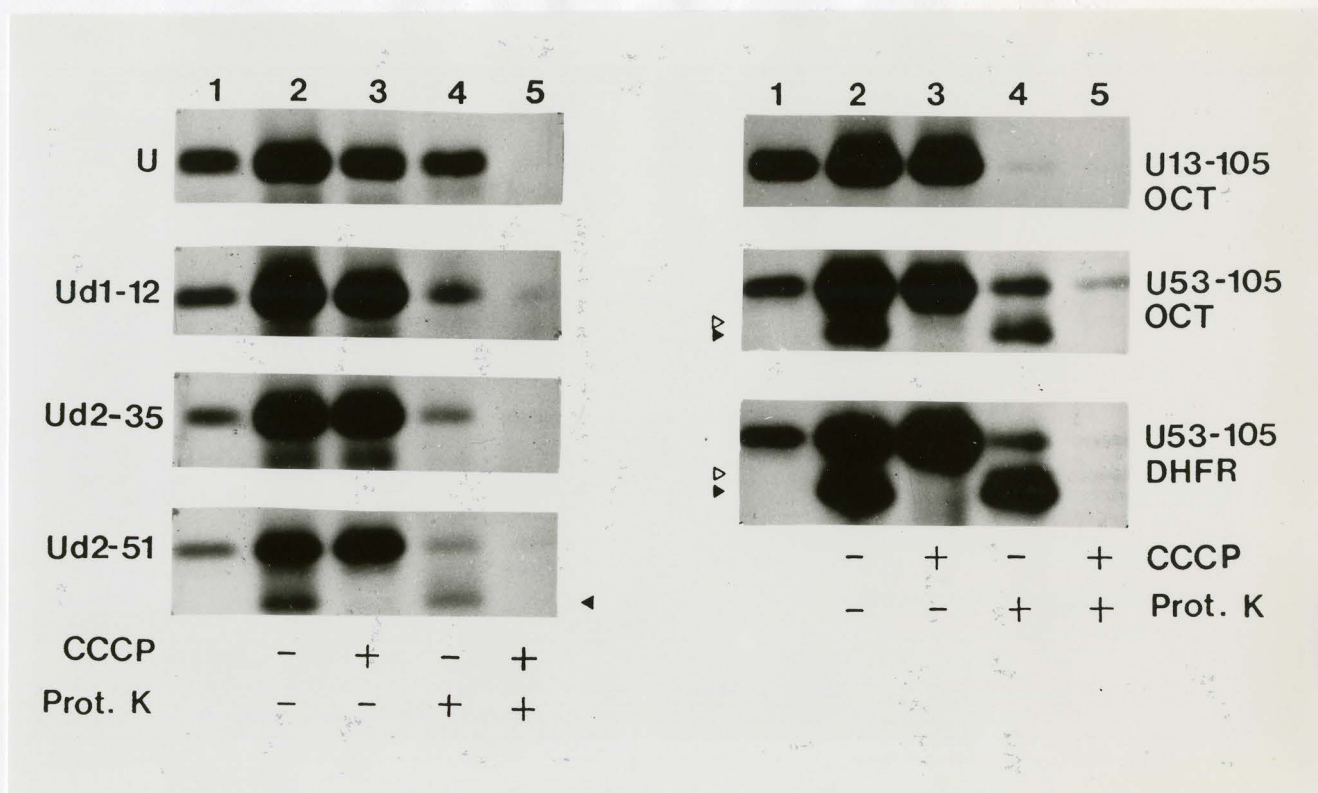
One other construct was made joining uncoupling protein amino acids 53 to 105 to mouse dihydrofolate reductase (U53-105-DHFR). This



was achieved by restriction endonuclease digestion and ligation as described in the Materials section using U53-105-OCT and pSP6DHFRd1-3.

All of these constructs, Ud2-35, U53-105-OCT and U53-105-DHFR, plus those previously reported (Ud1-12, Ud2-51 and U13-105-OCT) were imported into heart mitochondria (Fig. 12, lane 4). The import of the three constructs (Ud2-51, U53-105-OCT and U53-105-DHFR) with uncoupling protein amino acids 53 to 105 at the amino terminus lends support to the hypothesis that these amino acids have a potential targeting function in uncoupling protein itself. Of these three constructs, U53-105-DHFR was imported with the greatest efficiency followed by U53-105-OCT and then Ud2-51 (Fig. 12, lane 4). It was also noted that these three constructs were processed upon import ( $\blacktriangleright$ ) and that both the precursors and the processed forms were resistant to proteinase K digestion. This is unlike the case for OCT in which the precursor is not protected by proteinase K digestion (results not shown). Analysis revealed that the major processed forms of Ud2-51, U53-105-OCT and U53-105-DHFR are approximately 20 amino acids smaller. Although not seen clearly in Fig. 12, U53-105-DHFR and U53-105-OCT also had intermediate-sized forms ( $\blacktriangleright$ ) which are approximately 14 amino acids smaller than the initial translation product. It should be noted that the differences in sizes could not be very accurately determined since results depend very much on how the line is drawn on the semi-log plots of size versus mobility. However, processing suggests that at least the amino termini of the proteins had reached the mitochondrial matrix.

The location of the proteins was then investigated using  $\text{Na}_2\text{CO}_3$  extraction to distinguish proteins which are not extracted and therefore



**Figure 12. Import of Uncoupling Protein and Its Constructs.** Import conditions are described in Methods section. Lane 1, Translation sample representing 2000 cpm (1% of input); lanes 2 to 5, mitochondrial pellets with the conditions shown. ▶, ▷, Major and minor processed forms of the various constructs.



are likely to be in a hydrophobic membrane and those which are extracted and are therefore likely to be soluble or loosely bound (Fujiki *et al.*, 1982). Preliminary results on the final location of the various constructs are shown in Figs. 13 and 14. Comparing lanes 4 to 5 and 4 to 6 for uncoupling protein itself suggests that the protein was in a membrane. The same analysis for Ud2-35 does not lead to a clear interpretation. Ud2-35 appears to be associated with both membrane and aqueous compartments of mitochondria. Analysis of U13-105-OCT shows that the fusion protein was in the membrane (compare lanes 6 to 7). Precursors of Ud2-51 (Fig. 13, compare lanes 6 and 7) and U53-105-DHFR (Fig. 14, compare lanes 6 and 7) were found associated with both the membrane and the soluble fractions while processed forms of Ud2-51 (Fig. 13, compare lanes 6 to 7) and U53-105-DHFR (Fig. 14, compare lanes 6 to 7) were in an aqueous compartment, presumably in the matrix.

Both the import and processing of U53-105-DHFR were further analyzed. The import of the OCT-DHFR construct was included as a control. Figures 15 and 16 show that the processing of U53-105-DHFR and OCT-DHFR respectively can be blocked by preincubation of the fusion proteins with methotrexate as seen from the decrease in the amount of the processed form (compare lanes 3 and 7). For U53-105-DHFR in the presence of methotrexate, some of the complete protein is protected from proteinase K digestion (see Fig. 15, lane 7). This could be protein bound to the outer membrane and protected from digestion due to a tertiary conformation being maintained by methotrexate as seen earlier by Eilers and Schatz (1986) although if this were the case it should

Figure 13. Import and Localization by  $\text{Na}_2\text{CO}_3$  Extraction of Uncoupling Protein and Its Constructs. Import conditions and  $\text{Na}_2\text{CO}_3$  extraction are described in the Methods section. Lane 1 represents 2000 cpm of the translation. All other treatments are indicated. For uncoupling protein (U) and Ud2-35, lanes 2 and 3 are mitochondrial pellets; lanes 4 and 6 are the insoluble membrane fraction while lanes 5 and 7 are the soluble fraction after subfractionation of the mitochondrial pellet. For Ud2-51 and U13-105-OCT, lanes 2 to 5 are mitochondrial pellets; lanes 6 and 8 are the membrane fraction while lanes 7 and 9 are the supernatant fraction after subfractionation of the mitochondrial pellet.



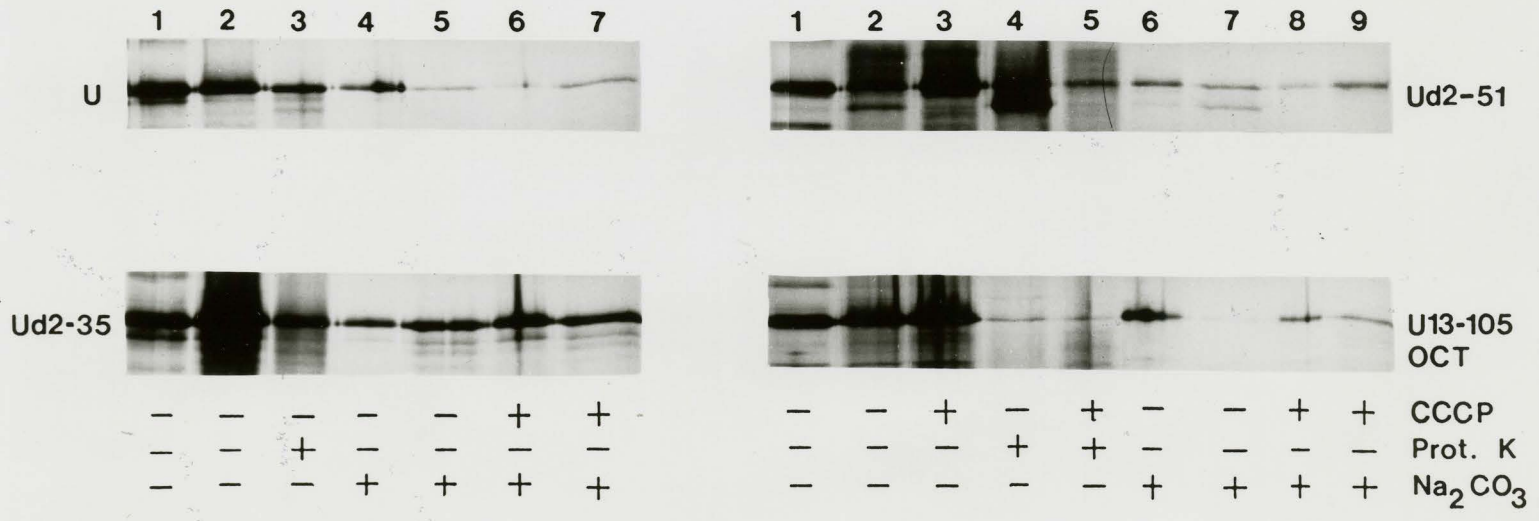


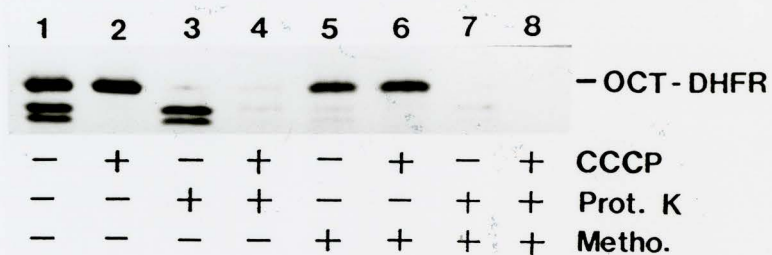


Figure 14. Import and Localization by  $\text{Na}_2\text{CO}_3$  extraction of U53-105-DHFR. Lane 1 represents 2000 cpm of the translation. All other treatments are indicated. Lanes 2 to 5 are mitochondrial pellets; lanes 6 and 8 are the membrane fraction while lanes 7 and 9 are the supernatant fraction after mitochondrial subfractionation.  $\blacktriangleright$ ,  $\blacktriangleright$ , Major and minor processed forms.





**Figure 15. Effect of Methotrexate on the Import of U53-105-DHFR.** Translations were first incubated for 1 min in the presence or absence of 7.5  $\mu$ M methotrexate which was diluted to 150 nM methotrexate in the final import reaction. All lanes are mitochondrial pellets. Lanes 1 to 4 are import reactions in the absence of methotrexate. Lanes 5 to 8 are import reactions in the presence of methotrexate. ▷,▷, Major and minor processed forms.



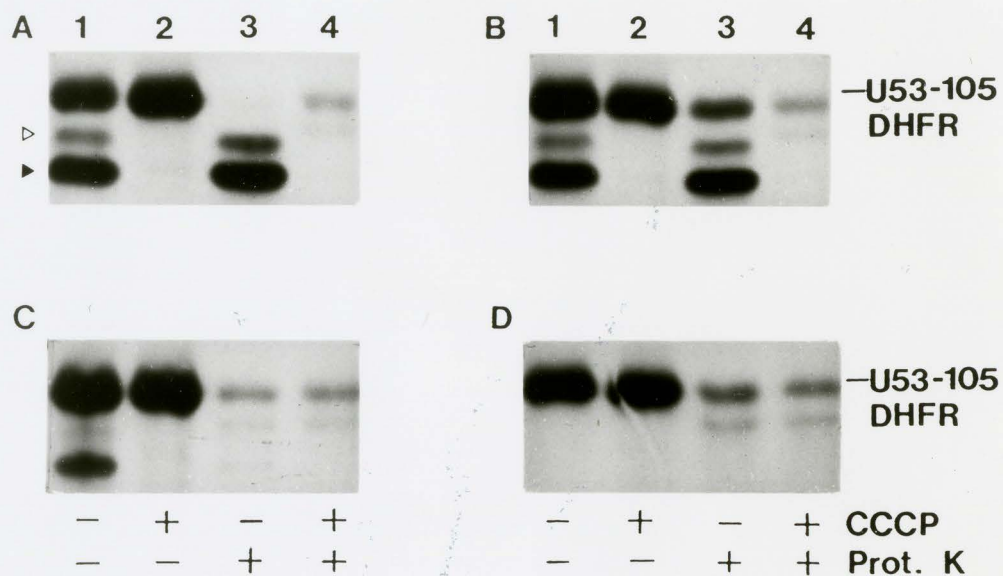
**Figure 16. Effect of Methotrexate on the Import of OCT-DHFR.** Translations were first incubated for 1 min in the presence or absence of 7.5  $\mu$ M methotrexate which was diluted to 150 nM methotrexate in the final import reaction. All lanes are mitochondrial pellets. Lanes 1 to 4 are import reactions in the absence of methotrexate. Lanes 5 to 8 are import reactions in the presence of methotrexate.



have also been seen in the presence of uncoupler and perhaps for OCT-DHFR.

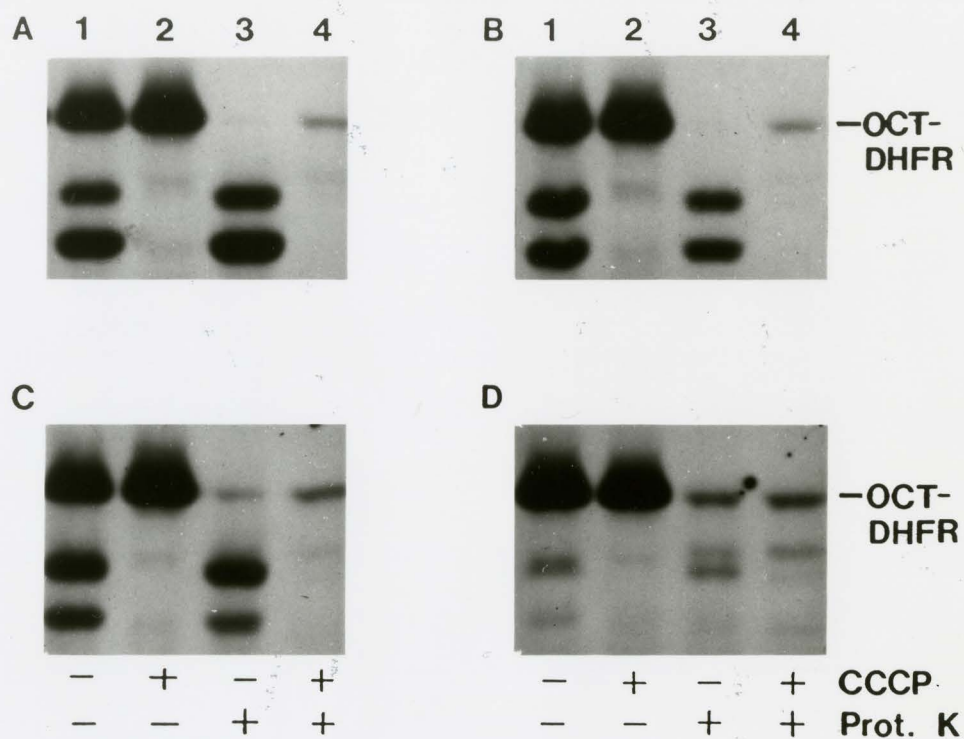
In order to determine whether or not two matrix proteases were involved in the processing, as with other matrix proteins (Sztul *et al.*, 1988; Kalousek *et al.*, 1988), increasing concentrations of *o*-phenanthroline, a membrane-permeable chelator, were tested for both U53-105-DHFR (Fig. 17) and OCT-DHFR (Fig. 18). Processing to both sizes appeared to be inhibited but at higher concentrations of the chelator, some of the unprocessed form was not removed by proteinase K digestion (B-D, lanes 3 and 4). This was the result of inhibition of proteinase K by *o*-phenanthroline as found in control experiments with the U53-105-DHFR translation product (Fig. 19, compare lanes 3 and 5 to 4 and 6).

These results with the fusion proteins and with Ud2-51 suggest that a targeting sequence is contained within uncoupling protein amino acids 53 to 105. This region was analyzed for potential positively charged amphipathic  $\alpha$ -helical structures which are believed to be important for targeting to mitochondria (Harlt *et al.*, 1989). The helical wheel projection for the first two possible sequences are shown in Figs. 20 and 21. Based on the sizes of the processed forms and on the helical wheel projection for the first possible sequence, arginine<sub>54</sub>, lysine<sub>56</sub> and lysine<sub>67</sub> were changed to glutamines, individually and in various combinations using oligonucleotide site-directed mutagenesis. Screening for possible mutants was accomplished by sequencing single-stranded phage DNA prepared from randomly selected plaques. A section of a sequencing gel comparing both uncoupling protein and the triple mutant, U-Q54,56,67 is shown in Fig. 22. The

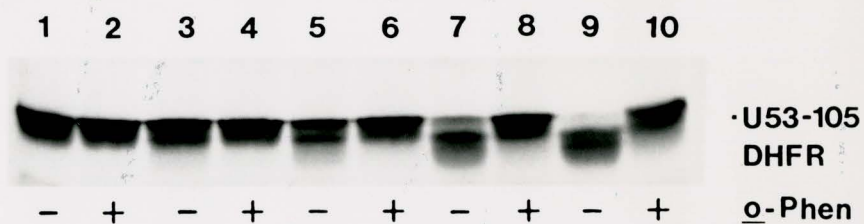


**Figure 17. Effect of *o*-Phenanthroline on the Import of U53-105-DHFR.** Import reactions were carried in the absence of *o*-phenanthroline (A), or in the presence of 0.1 mM (B), 1.0 mM (C) and 5.0 mM (D) *o*-phenanthroline. Lanes 1 to 4 are mitochondrial pellets.  $\blacktriangleright, \blacktriangleright$ , Major and minor processed forms.





**Figure 18.** Effect of *o*-Phenanthroline on the Import of OCT-DHFR. Import reactions were carried in the absence of *o*-phenanthroline (A), or in the presence of 0.1 mM (B), 1.0 mM (C) and 5.0 mM (D) *o*-phenanthroline. Lanes 1 to 4 are mitochondrial pellets.



**Figure 19.** Effect of o-Phenanthroline on Proteinase K Digestion of U53-105-DHFR. Lanes 1 and 2: no proteinase K digestion. Samples were digested with 2 ng proteinase K/ $\mu$ l of reaction for 5 min (lanes 3 and 4), 10 min (lanes 5 and 6), 30 min (lanes 7 and 8) and 60 min (lanes 9 and 10). The presence or absence of o-phenanthroline is indicated.

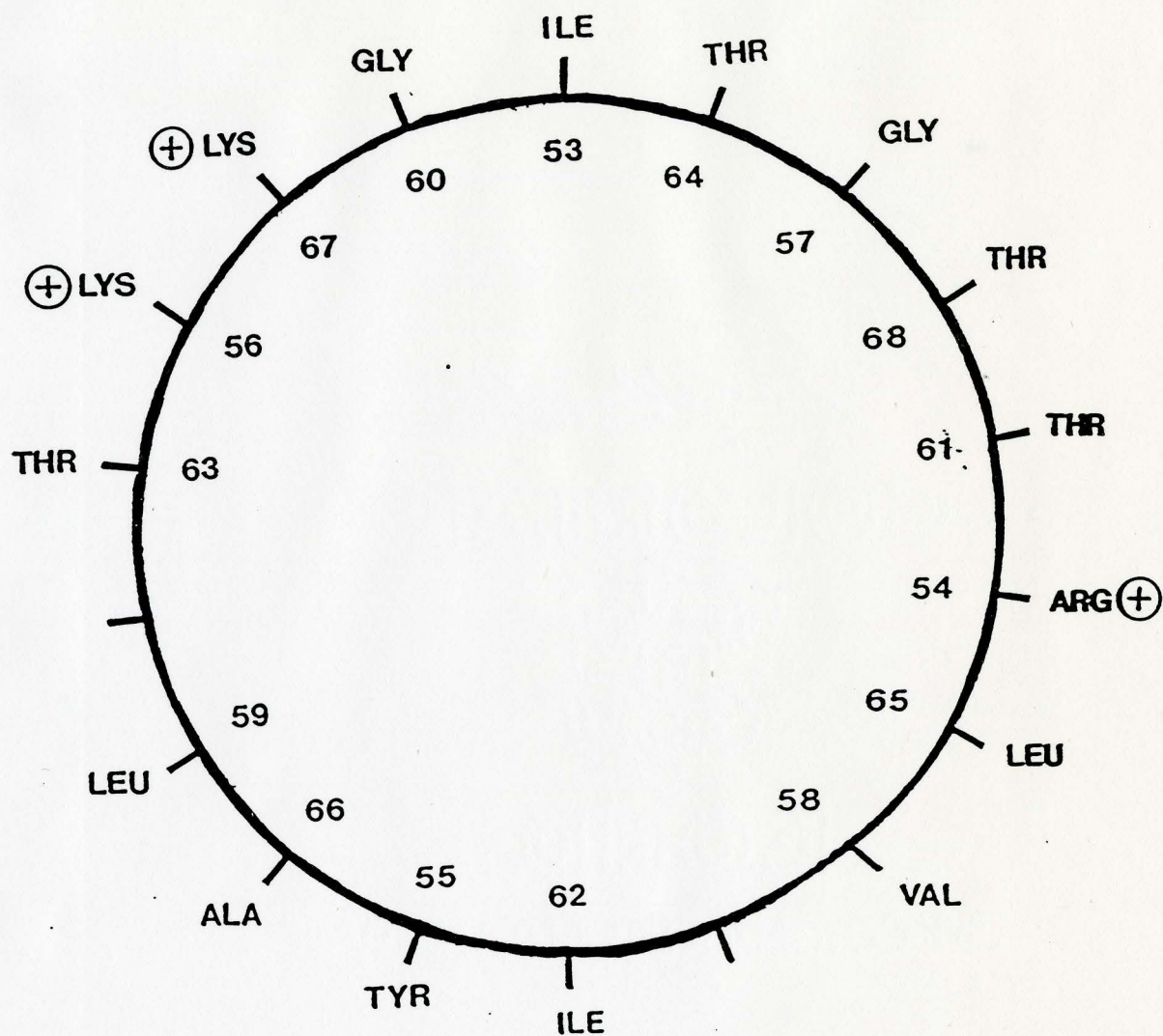


Figure 20. Helical Wheel Projection for a Possible Uncoupling Protein Targeting Sequence. This region corresponds to uncoupling protein amino acids 53 to 68. Basic residues are indicated by +.



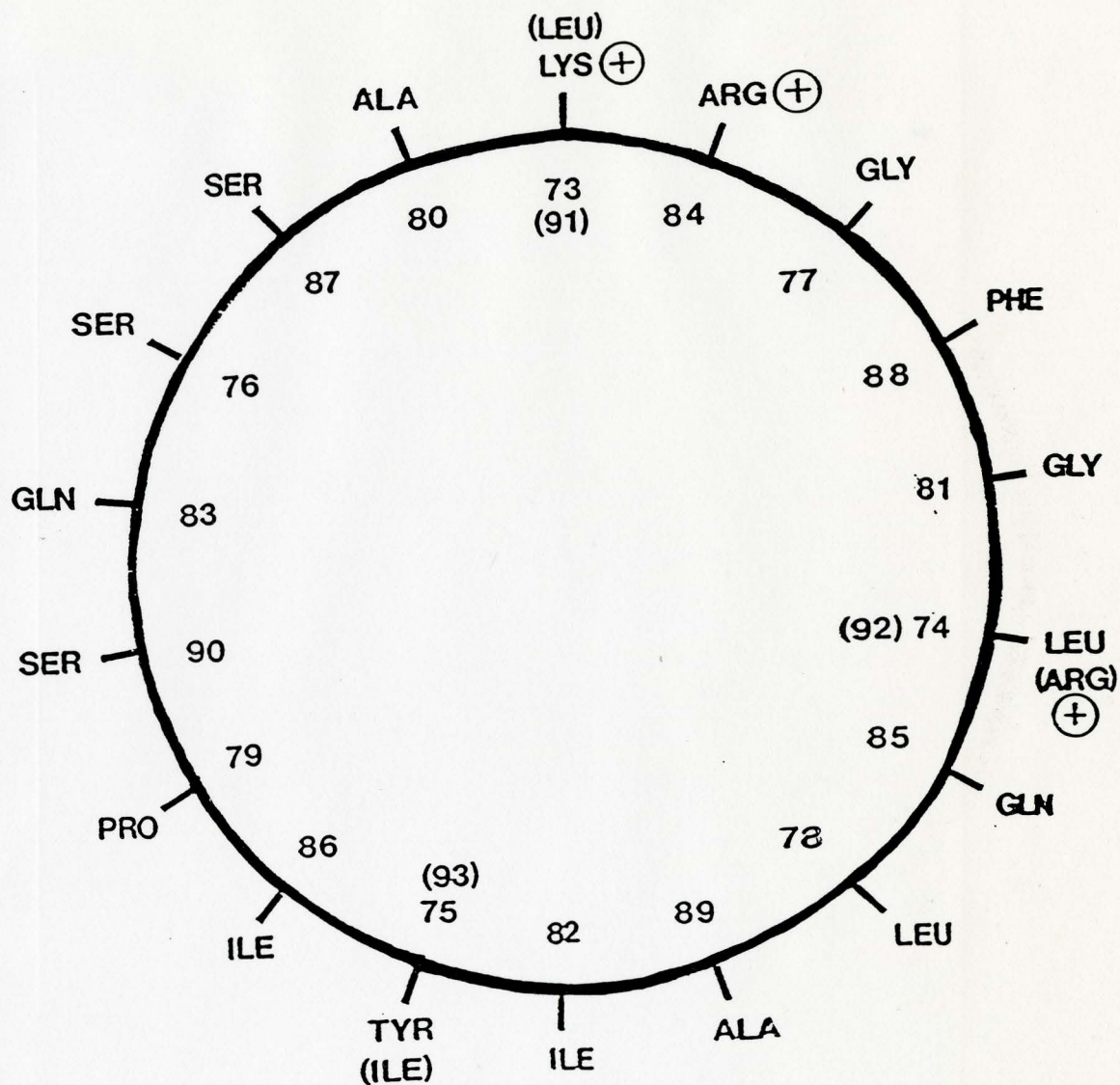


Figure 21. Helical Wheel Projection for a Second Possible Uncoupling Protein Targeting Sequence. This region corresponds to uncoupling protein amino acids 73 to 93. Basic residues are indicated by +. Amino acids in brackets indicate the second turn of the plot.



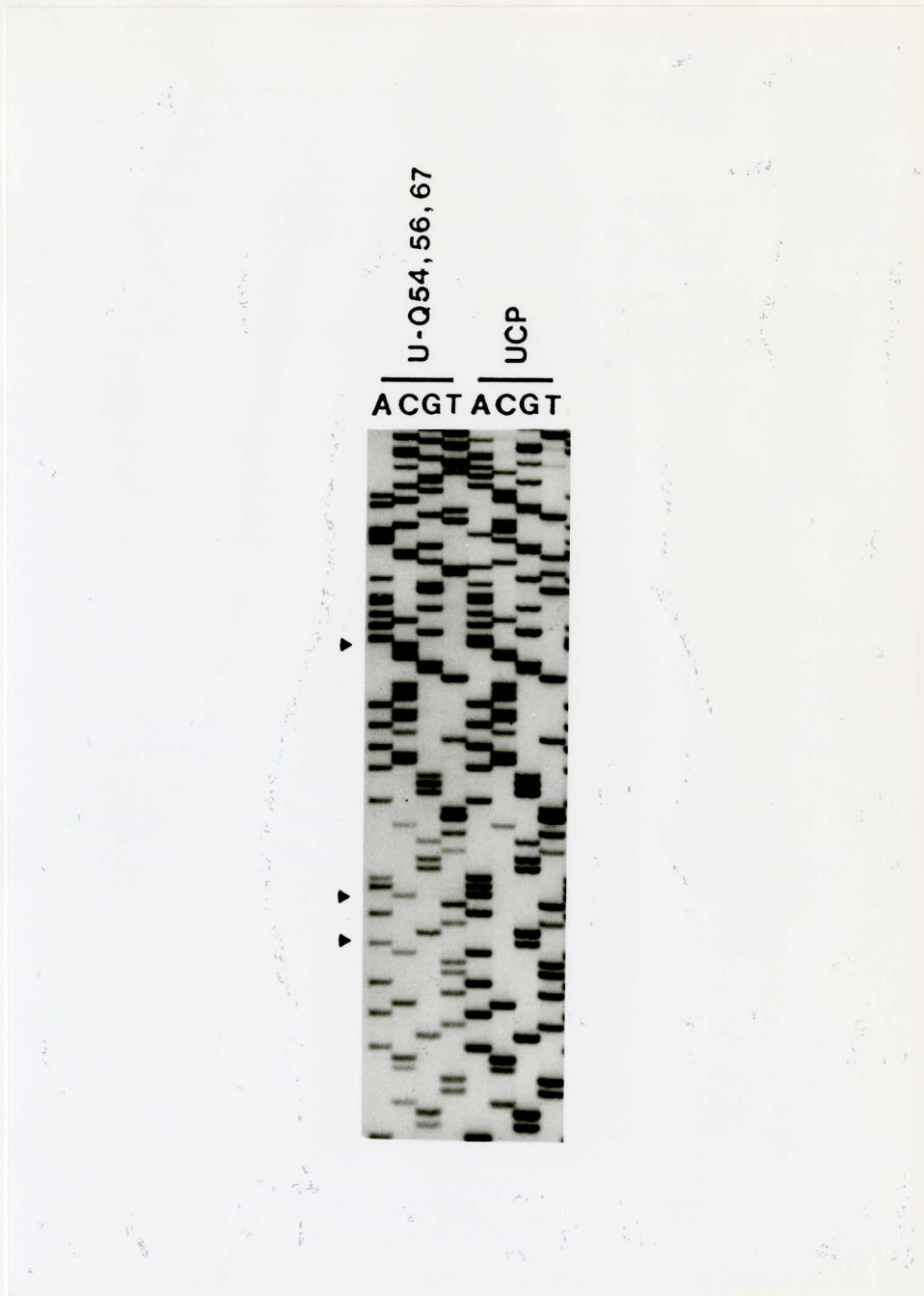


Figure 22. Sequence of UCP and U-Q54,56,67. Sequencing was performed as described in the Methods section using the protocol supplied by Pharmacia with their T7 Sequencing Kit. ▶, Point changes in the mutant.

point mutations are indicated by the arrows. The import results for all the mutants are shown in Fig. 23 along with wild type uncoupling protein for comparison. All the mutants are imported into mitochondria (Fig. 23, lane 3) even when all three of the charges are replaced as seen for U-Q54,56,67.

In order to ascertain any difference in import ability between wild type uncoupling protein and U-Q54,56,67, their time courses of import was determined as shown in Fig. 24. Not surprisingly, the import of the mutant U-Q54,56,67 was 50% of that of wild type uncoupling protein but the rate of import was similar.

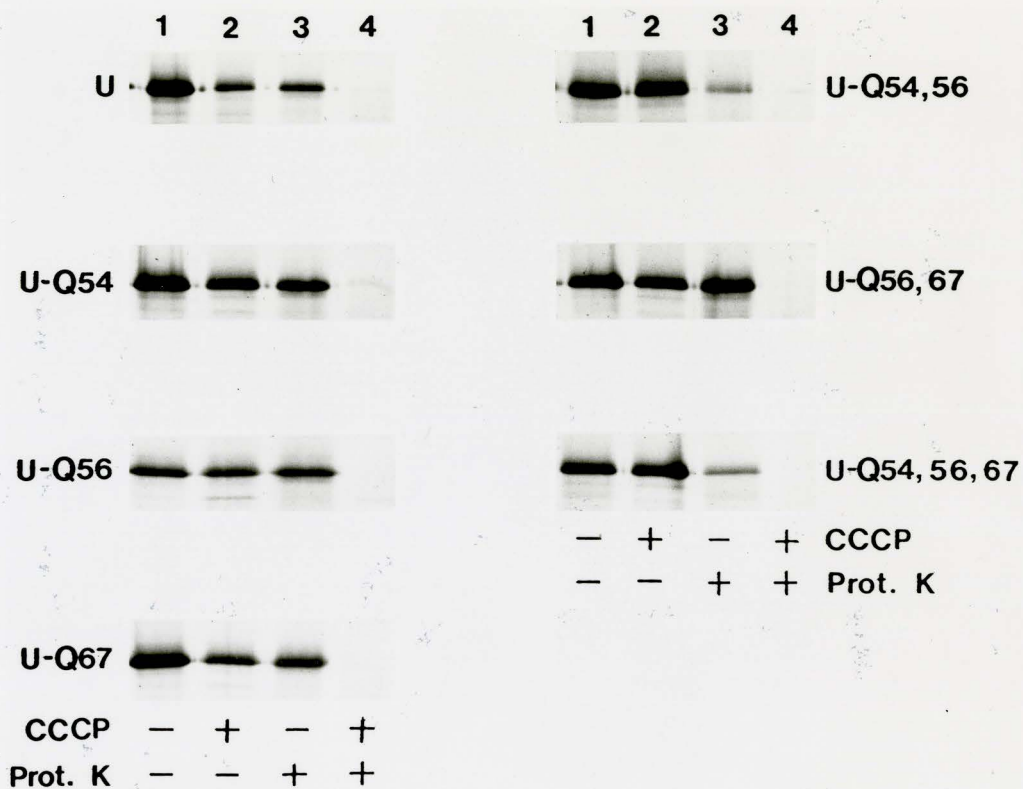


Figure 23. Import of Uncoupling Protein and Its Mutants. Import conditions are as described in the Methods section. Lanes 1 to 4 are mitochondrial pellets. The amount applied to the gel in each case was adjusted to reflect equal amounts of precursor addition to the import reaction. Q represents the glutamine(s) at the indicated amino acid position(s) in the mutant uncoupling protein constructs.



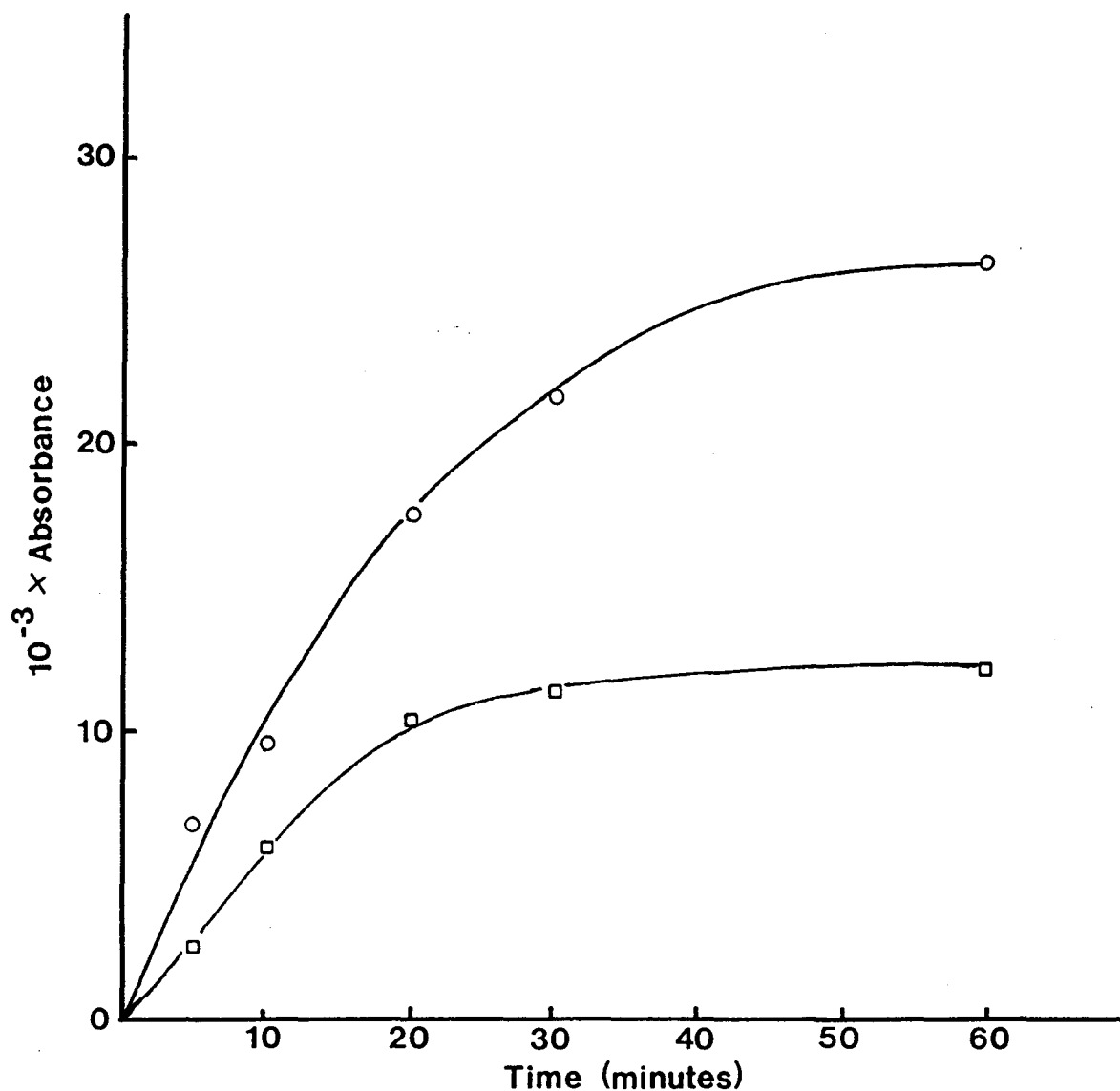


Figure 24. Time Course for Import of Uncoupling Protein and U-Q54,56,67. Import conditions are as described in the Methods except that volumes were increased 6-fold and 150  $\mu\text{l}$  duplicate samples were removed after 5, 10, 20, 30 and 60 min incubations. The samples were treated with proteinase K and subjected to SDS polyacrylamide electrophoresis. Each lane of the fluorograms was scanned twice using a Hoefer Scientific densitometer with absorbance represented by arbitrary units. The plot represents the average from duplicate gels.  $\circ$ , Uncoupling protein;  $\square$ , the mutant, U-Q54,56,67.



## 5. DISCUSSION

### 5.1 THE IMPORT SYSTEM

In order to establish the import system, several factors required standardization. This included the in vitro transcription system and the subsequent translation of the transcript. The import protocol itself required standardization in terms of concentrations of various components like chloramphenicol, CCCP and proteinase K. Once established, the import system could be used to look at some basic questions dealing with imported uncoupling protein including import from a prebound state, conformation and location within mitochondria. Each of these aspects are discussed in more detail below.

#### 5.1.1 TRANSCRIPTION AND TRANSLATION

There appear to be several important factors which affect both in vitro transcription and translation. Among these is the presence of a 5'-oligo(dG)-oligo(dC) extension in the transcription template DNA. It was observed that the removal of most of the 5'-extension by inserting a KpnI/PstI cDNA fragment into expression vectors was sufficient to allow for the in vitro synthesis of uncoupling protein. It was also noted that linear plasmids with 3'-overhangs were inferior templates for RNA polymerases compared to linear plasmids with 5'-overhangs (Promega Notes, March 1985). The 3'-overhang allows the polymerase to use the anti-sense strand as a template resulting in extraneous transcripts which may have complementary regions able to hybridize to the mRNA.

These 3'-overhangs are easily removed by treating the linear DNA with the Klenow fragment of DNA polymerase before using the template in the transcription reaction. Another finding was that not all vectors were equally effective. When the same KpnI/PstI uncoupling protein cDNA insert, which lacks the 5'-oligo(dG)-oligo(dC) extension, was inserted into pSPT18, pSPT19 and pTZ18U there were different degrees of efficiency in the production of utilizable uncoupling protein mRNA by T<sub>7</sub> RNA polymerase. The transcription product from pTZ18U was never translated into uncoupling protein. The reason for this was not pursued. The transcription products from pSPT18 and pSPT19 yielded approximately the same amount of uncoupling protein when translated.

#### 5.1.2 IMPORT OF UNCOUPLING PROTEIN AND ITS CONSTRUCTS

Several factors appear to be important for the import of uncoupling protein into mitochondria. Import of uncoupling protein into isolated heart mitochondria was found to depend on the age of the animals from which mitochondria are prepared. Mitochondria prepared from newborn rats were more efficient at import than mitochondria prepared from 3 week old rats. Mitochondria prepared from rats older than 3 weeks were not efficient at rapidly importing exogenously added protein.

The final standard import conditions achieved included the use of chloramphenicol to prevent mitochondrial protein synthesis which occurs rapidly in mitochondria prepared from rats 3 weeks old and younger, appropriate concentrations of CCCP and proteinase K, the amount of uncoupling protein translation and the length of incubation. However,

once this study was completed, it was learned that proteinase K digestion is a too drastic procedure and that trypsin is the enzyme of choice (G.C. Shore, personal communication).

Once standard import conditions were established, it was possible to study various aspects of uncoupling protein import, particularly, the import of prebound uncoupling protein and the effect of conformation on import. It was observed that uncoupling protein bound to mitochondria at a low temperature was not imported into mitochondria after raising the temperature to 30°C. This was an unexpected result for two reasons. First, the precursor of malate dehydrogenase can be imported into mitochondria after first being bound at 0°C and then raising the temperature to 30°C (Chien *et al.*, 1984). Secondly, the ADP/ATP carrier can be imported into mitochondria after incubation at a low temperature (2°C), then warming to 25°C and adding valinomycin to inhibit the import of free precursor (Pfanner & Neupert, 1987b). Perhaps the import of prebound uncoupling protein could be achieved under different conditions. On the other hand, prebound uncoupling protein may no longer be in an import-competent conformation. It may be of interest to examine the binding of uncoupling protein to de-energized mitochondria and observing if import occurs after re-energization as was seen for the ADP/ATP carrier (Zwizinski *et al.*, 1983; Pfanner & Neupert, 1985).

Although uncoupling protein can be imported into mitochondria, it has not yet been determined whether or not it is functional. In order for the protein to function, it must first assume the same conformation as the native protein. One approach which addresses this question is to purify the imported protein in the same manner as the native protein.

Uncoupling protein imported into mitochondria from both heart and brown adipose tissue mitochondria could be purified in the same manner as native uncoupling protein is purified. Further, uncoupling protein which has been purified after being treated with proteinase K yielded a band of slightly faster mobility. This band may be similar to the T-1 fragment generated by limited tryptic digestion of both isolated uncoupling protein and isolated brown adipose tissue mitochondria which were frozen and thawed (Eckerskorn & Klingenberg, 1987). Eckerskorn and Klingenberg (1987) showed that the cleavage site is located near the C-terminus at lysine<sub>292</sub> and indicated that the 10 residue long carboxy peptide is directed to the cytosolic side of the inner membrane. Similar C-terminal sequencing would be required to demonstrate that the proteinase K cleavage site is near the C-terminus which would indicate that the membrane disposition of imported uncoupling protein is similar to the native protein. No experiments were done at this time to determine whether or not imported uncoupling protein formed dimers. The results from purification and proteinase K treatment provide evidence that the isolated system mimics the *in vivo* system and that imported uncoupling protein is potentially functional. Since uncoupling protein is imported into rat heart mitochondria, it is conceivable that the protein can be imported by mitochondria from other sources including yeast. This has been observed for human ornithine carbamoyltransferase which was imported into yeast mitochondria *in vivo* (Cheng et al., 1987). If this is possible for the uncoupling protein, this could lead to the development of an *in vivo* expression system to study structure/function relationships.



### 5.1.3 LOCALIZATION OF UNCOUPLING PROTEIN AND ITS CONSTRUCTS

Once the standard import conditions were clarified and certain aspects of uncoupling protein import were examined, the localization of the various constructs in mitochondria was investigated. In order to determine the location of the various constructs,  $\text{Na}_2\text{CO}_3$  extraction was used to separate integral membrane proteins from peripheral membrane proteins and proteins of the matrix and intermembrane space. The results were relatively unambiguous for uncoupling protein and U13-105-OCT which were found associated with the membrane fraction, and for the processed forms of Ud2-51 and U53-105-DHFR which were in the soluble fraction. The precursors of Ud2-51 and U53-105-DHFR were found associated with both the membrane and the soluble fraction indicating that some of the precursor is held in the membrane and some is translocated through to the matrix where it is protected from proteinase K digestion. Most strikingly, the precursor of U53-105-DHFR, in the presence of CCCP appears to be located within a membrane. To a lesser extent, the other precursors are also found associated with the membrane fraction in the presence of CCCP. This may simply reflect a problem encountered with the method, it may be an artifact or it may indicate that the protein is inserted into a membrane in the absence of a membrane potential. If the last case is true, it may reflect a stage in the import process, that is, the binding of the precursor to a receptor in the outer membrane and/or interaction with a GIP again in the outer membrane. Both steps occur in the absence of a membrane potential as observed for the import of the ADP/ATP carrier (Pfanner *et al.*, 1987a).



Ud2-35 was found associated with both the membrane and soluble fraction in the presence and absence of CCCP. This result is the most difficult to interpret and may simply reflect incomplete washing. On the other hand, the results may indicate that the construct is only partially inserted in the membrane, hence allowing the construct to partition with both the particulate and soluble fractions.

This problem case may provide some additional information on regions important for membrane insertion. When uncoupling protein amino acids 13 to 105 are present in a construct, the protein is found in the membrane fraction. Deletion of the first 101 amino acids of uncoupling protein yielded a construct, UCP102-307, which was found in the soluble fraction (Liu *et al.*, 1988). This indicates that the information for membrane insertion is contained within amino acids 13 to 105. Proteins with uncoupling protein amino acids 52/53 to 105 are found in the soluble fraction but also in the membrane fraction indicating that this region has sufficient information to retain the protein within the membrane albeit at low efficiency. When uncoupling protein amino acids 36 to 105 are present, the construct appears to be retained within the membrane with slightly greater efficiency. It appears that for complete retention within the membrane, uncoupling protein amino acids 13 to 105 are required.

However, when the presequence for ornithine carbamoyltransferase was placed in front of uncoupling protein, the membrane insertion information contained within uncoupling protein was ignored and the construct, pO-UCP, was targeted to the matrix where it was processed (Liu *et al.*, 1990). It is possible that uncoupling protein and the pO-

UCP construct use different receptors for import. Conceivably, pO-UCP uses the mammalian counterpart to the *N. crassa* receptor, MOM19, identified by Söllner *et al.*, (1989) while uncoupling protein uses the same receptor as the ADP/ATP carrier. A receptor for the *N. crassa* ADP/ATP carrier has been identified as MOM72 by Neupert and coworkers (Hartl & Neupert, 1990). If this is the case, it is possible that only the uncoupling protein receptor is capable of presenting the protein to the inner membrane in such a manner as to be inserted into the membrane (Liu *et al.*, 1990). Continuing with the possibility that uncoupling protein uses the same receptor as the ADP/ATP carrier, the mode of import may also be similar. That is, uncoupling protein may be inserted into the inner membrane by paired amphipathic helices led by internal matrix targeting domains (Liu *et al.*, 1988; 1990) in a manner similar to that depicted for the ADP/ATP carrier as shown in Fig. 3 (Pfanner *et al.*, 1987a).

## 5.2 UNCOUPLING PROTEIN TARGETING SEQUENCE(S)

The approaches that have been used in identifying possible mitochondrial targeting sequences include deleting regions within the precursor and/or constructing fusion proteins joining the suspected sequence to a non-mitochondrial passenger protein. These two approaches were used in an earlier study of uncoupling protein. It was found that the amino-terminal amino acids 13 to 105 of uncoupling protein could target a passenger protein, a portion of mature ornithine carbamoyltransferase, to mitochondria but amino acids 13 to 51 could not (Liu *et al.*, 1988). Constructs were made deleting amino acids 2 to 51



and 2 to 101 of uncoupling protein (Liu *et al.*, 1988). Deletion of amino acids 2 to 51 was found to "severely retard UCP import" and subsequent localization was not performed (Liu *et al.*, 1988). In the case of UCP102-307, import was relatively modest, attaining levels of approximately one-fifth of those observed for UCPd1-12 (Liu *et al.*, 1988). In this study, fusion proteins were constructed by joining uncoupling protein amino acids 53 to 105 to ornithine carbamoyltransferase and dihydrofolate reductase. The present results demonstrate that amino acids 52/53 to 105 (preceded by methionine) of uncoupling protein could target either amino acids 106 to 307 of uncoupling protein (Ud2-51) or two soluble passenger proteins to mitochondria. The efficiency by which uncoupling protein amino acids 52/53 to 105 function in import appears to depend on the passenger proteins with the decreasing order of efficiency observed as U53-105-DHFR, then U53-105-OCT followed by Ud2-51. The difference in efficiency may reflect a difference in the solubility of the passenger (both DHFR and OCT are soluble protein) or the proportion of protein in an import-competent conformation. This is not unlike the findings of Pfanner *et al.* (1987a) where precursors with the same presequence but different mature portions required different levels of ATP or GTP for import.

The processing of Ud2-51, U53-105-OCT and U53-105-DHFR, inhibition of processing by ortho-phenanthroline and the presence of the processed forms of the first and last of these at least in the soluble phase after Na<sub>2</sub>CO<sub>3</sub> extraction suggests that these proteins have been targeted in toto to the matrix. This is supported by the observation that the sizes of the fragments removed indicates that processing is



occurring in a region of the protein (amino acids 71 to 95) that is suggested to be in the membrane (Aquila *et. al.*, 1985).

The evidence suggests that uncoupling protein amino acids 52/53 to 105 have targeting information based on the import of Ud2-51, U53-105-OCT and U53-105-DHFR. However, if this region contains the targeting signal it does not appear to be functioning as it does in uncoupling protein itself. This is based on the processing observed for the three constructs as well as the localization of the processed forms within the matrix. Uncoupling protein is neither processed nor located in the matrix. It is possible that placing uncoupling protein amino acids 52/53 to 105 at the N-terminus is revealing a cryptic targeting sequence which now acts as a targeting presequence. This is not unlike findings of Hurt and Schatz (1987) in which the first 85 amino acids of dihydrofolate reductase fused to complete dihydrofolate reductase targeted the construct into mitochondria or in which random sequences from the *E. coli* genome targeted passenger proteins to mitochondria (Baker & Schatz, 1987). Consequently, the use of fusion proteins is limited in identifying targeting sequences. This indicates that changes should be made in uncoupling protein itself remembering that conformational changes could also occur and make interpretation of results difficult.

The approach used in this study to create changes within uncoupling protein itself was oligonucleotide site-directed mutagenesis. The likely candidate for change was a region of concentrated positive charge. Positive charge is necessary for import of proteins with presequences (Hartl *et al.*, 1989) being important at least for the



membrane potential dependent movement into the inner membrane. This is sensitive to uncouplers and since the import of uncoupling protein is also sensitive at least one of the three areas of positive charge should be important for import although it might not be necessary for interaction with the putative receptor. An observed lack of effect would indicate that the region is not the targeting signal or that positive charge is not required for interaction with the putative receptor. If on the other hand, an effect is observed, it is not possible to determine if the positive charge was required for interaction with the receptor or if the charge prevented translocation into the inner membrane. Amino acids 52/53 to 105 of uncoupling protein contains two areas with a number of positively charged residues which when plotted on helical wheels, show potential amphipathic  $\alpha$ -helices. The first of these from amino acids 54 to 67 is part of a domain suggested to be in the matrix in the topological model of uncoupling protein shown in Fig. 25 (Aquila *et. al.*, 1985). It was expected that as the number of positive charges in this region were replaced by glutamines, the efficiency of import would decrease to the point where replacement of all three positively charged residues would prevent import. However, changing all three of the positively charged residues in the region to glutamines did not prevent the targeting of uncoupling to mitochondria, although, the proportion imported decreased most noticeably for U-Q54,56,67. The import of the mutant U-Q54,56,67 was unexpected. This result indicates that this portion of uncoupling protein is not the sole targeting signal in uncoupling protein or may not be part of the signal at all. Thus, the positively charged areas 73

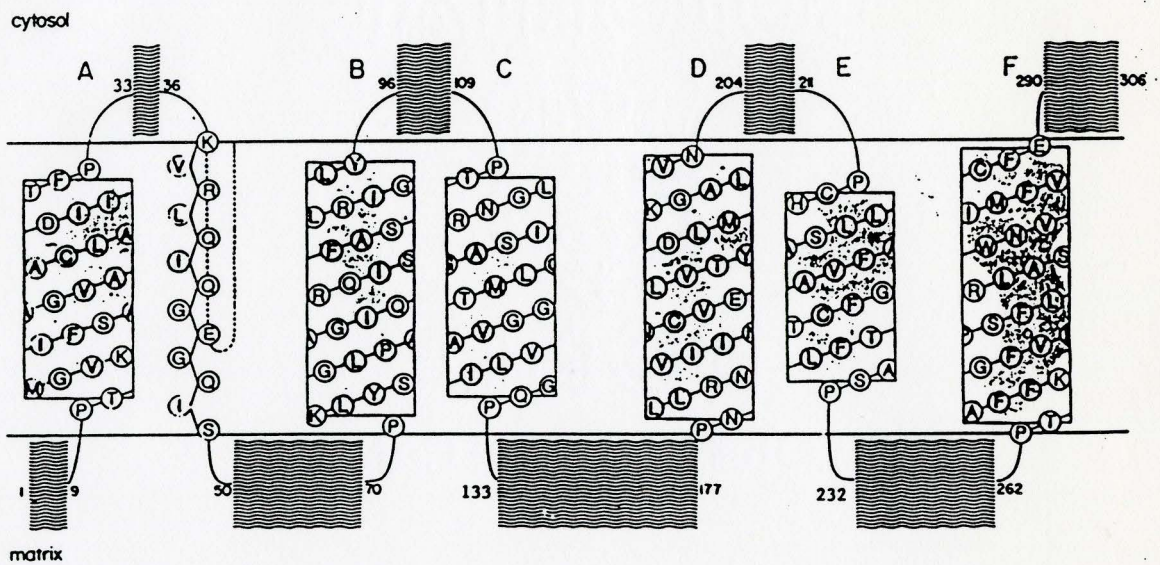


Figure 25. Membrane Disposition Model of Uncoupling Protein. Hamster uncoupling protein as illustrated in Aquila *et al.*, (1985). The six membrane spanning  $\alpha$ -helices are labeled A-F. Areas with residues more hydrophobic than glycine are shaded grey. A possible  $\beta$ -strand is shown between helix A and a stretch of unknown structure as depicted by shaded boxes. The single amino acid code is used.



to 92 and/or 138 to 154 could serve a targeting function. Recent results using fusion proteins with segments of the ADP/ATP carrier suggest that the targeting sequence lies between amino acids 72 to 111 (Smagula & Douglas, 1988a,b). However, these results were based on two fusion proteins, one joining the ADP/ATP carrier amino acids 1 to 111 to dihydrofolate reductase and the second joining amino acids 1 to 72 to the same passenger protein. The former was imported into mitochondria while the latter construct was not (Smagula & Douglas, 1988a,b). Amino acids 72 to 111 were not tested directly and the results should therefore be treated with considerable caution. Consequently, their results would only weakly support the role of uncoupling protein amino acids 73 to 92 in targeting. On the other hand, as shown previously, amino acids 102 to 307 do contain targeting information (Liu *et. al.*, 1988), supporting a targeting role for amino acids 138 to 154. Modification of these areas within uncoupling protein itself should clarify whether either of these areas are the prime targeting signal of uncoupling protein.

### 5.3 FUTURE PLANS

At present, evidence indicates that amino acids 54 to 67 does not target uncoupling protein to mitochondria. Based on the model and the targeting function of uncoupling protein amino acids 13 to 105, constructs should test amino acids 73 to 92 in uncoupling protein. This region has 3 basic amino acids separated by 10 and 7 residues which is in line with the structure of most mitochondrial targeting sequences, making this a good candidate for the targeting sequence. This region,



projected on a helical wheel does display the potential for an amphipathic  $\alpha$ -helix. This does not rule out the possibility that it forms another type of amphipathic structure. To test the region 73 to 92, the same approach used in this study could be used again, that is, to change lysine<sub>73</sub>, arginine<sub>84</sub> and arginine<sub>92</sub> to glutamines in both uncoupling protein and U-Q54,56,67. This would test the region itself and the possibility of the involvement of a number of regions for targeting.

Amino acids 138-154 in the second third of the protein should also be tested for their ability to target uncoupling protein in order to explain the import seen for the last two thirds of the protein reported by Liu *et al.* (1988). Again, the same approach can be used to replace the five basic amino acids in this region. Other possible approaches include deleting the region or introducing acidic amino acids to break up the concentration of positive charge.

Future efforts should be directed first at amino acids 138 to 154 as the major targeting signal based on three arguments. First, this region has a concentration of five positive charges with no interrupting negative charges similar to targeting presequences of proteins synthesized as larger precursors. This region could lead a set of paired amphipathic helices toward the matrix, thus resembling the insertion of a hairpin. Secondly, this region is predicted to reside in the matrix as shown in Fig. 25 (Aquila *et al.*, 1985). This is thought to be important since presequences enter the matrix where they are cleaved for proteins made as larger precursors. Uncoupling protein itself is not cleaved. One possible explanation for this includes interaction with a different

receptor than that used by proteins synthesized as larger precursors. In this case, it is possible that the peptidases are located near the receptor for proteins synthesized as larger precursors and not located near the putative receptor for uncoupling protein. Another possibility includes the lack of a sequence or conformation which is recognized by a matrix peptidase. Finally, uncoupling protein amino acids 102 to 307 is imported into isolated mitochondria (Liu *et al.*, 1988). It is possible that targeting of uncoupling protein may be a function of a number of regions including amino acids 54 to 67, 73 to 92 and 138 to 154. On the other hand, the targeting sequence for uncoupling protein may not resemble any targeting sequences known at present. It is possible that uncoupling protein has a unique receptor for import or it may use the receptor for the ADP/ATP carrier (MOM72 in *N. crassa*, Hartl & Neupert, 1990) which is distinct from the receptor identified by Söllner *et al.* (1989) for precursors such as porin and  $F_1\beta$  (MOM19 in *N. crassa*). If this is true, the targeting sequence and perhaps even the mechanism for uncoupling protein import may be completely different. It is anticipated that further work searching for the targeting signal will reveal the region responsible. This would then clear the way for discovering other structure/function relationships likely using *in vivo* expression systems.

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