TITLE MITOCHONDRIAL ATPASE: BIOCHEMICAL AND MOLECULAR GENETIC ANALYSIS

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INSTITUTION and DATE University of Warwick 1989

MITOCHONDRIAL ATPASE: BIOCHEMICAL AND MOLECULAR GENETIC ANALYSIS

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A thesis submitted for the degree of Doctor of Philosophy

April 1989

(Corrected June 1989)

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It's all Kevin's and Rolf's fault – they made me do it!

Abstract

S.cerevisiae mutants were isolated showing nuclear-coded resistance to the antibiotic venturicidin, a known F_0ATPase binding antibiotic. Two types of mutant were identified, one of which had cross-resistance to a variety of antibiotics and appeared linked to the leu_1 locus on chromosome VII, and one which was cross-resistant to chloramphenicol only and not linked to leu_1. The level of resistance to venturicidin was not increased in isolated mitochondria, therefore resistance shown in both groups is believed to be due to a decrease in plasma membrane permeability to these antibiotics.

The fluorescence properties of several organotin compounds, derivatives of the substituted flavones 3-hydroxyflavone (hof) and penta-hydroxyflavone (morin), were investigated on incubation with rat liver mitochondria. The compound Bu_2SnBr(of) was found to show fluorescence enhancement when added to mitochondrial preparations, which could be lowered by addition of the non-fluorescent compound Bu_3SnAc. Addition of Bu_2SnBr(of) did not affect mitochondrial membrane potential, and conversely the energetic state of the mitochondrial inner membrane had no effect on Bu_2SnBr(of) fluorescence. This compound was shown to be an inhibitor of mitochondrial ATPase, and is thought to have its binding site on the F_0 moiety of that enzyme complex.

The nuclear gene causing respiratory deficiency in the complementation group G57 was cloned and sequenced. This gene (PET_{57}) encoded a protein of 36 Kdal which did not show significant homology with any known protein. The mutant strain was deficient in mitochondrial ATPase activity, but the major F_1ATPase subunits were detected in mutant mitochondria, although in reduced amounts. Mutant F_1 showed abnormal membrane binding and could not be isolated by standard methods. The protein encoded by the gene PET_{57} is transported into mitochondria and is thought to contribute to processing or assembly of one or more of the cytoplasmic subunits of the F_1F_0ATPase complex.

Acknowledgements

The work described in this thesis was carried out in the Department of Chemistry from October 1985 to September 1988, including a four month period at the Sherman Fairchild centre for the life sciences, Columbia University, New York. I would like to thank Professor M.G.H. Walbridge and Professor A. Tzagoloff for providing research facilities at Warwick and Columbia, and the Science and Engineering Research Council for financial support.

In addition I would like to thank Dr D.E. Griffiths for his interest in this work. I am especially grateful to Professor A. Tzagoloff for introducing me to the field of yeast molecular biology, and to many members of his research group and department for their advice and companionship, especially Sandy Gampel, Sharon Ackerman and Sheila Connolly. I would also like to thank Dr I.F. Connerton for instruction in the techniques of yeast mitochondrial genetics, and Drs M. Collins and D. Judge for assistance with computer sequence analysis in general and database searching in particular.

Where materials have been provided or assays performed by people other than myself this has been indicated in the text, and their contribution is gratefully acknowledged. I also thank Rolf Howarth who assisted with formatting the text of this thesis, and Kevin Plucknett for all his support and encouragement over the past few years.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A	Adenine
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
ATPase	Adenosine-5'-triphosphatase
BSA	Bovine serum albumin
Bu	Butyl group
C	Cytosine
CAP	Chloramphenicol
CCCP	m-Chloro carbonyl cyanide phenylhydrazone
cyt	Cytochrome
cyt.ox	Cytochrome oxidase
DCCD	Dicyclohexyl-carbodiimide
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
Et	Ethyl group
EtBr	Ethidium Bromide
EDTA	Ethylenediamine tetra-acetic acid
ERY	Erythromycin
FAD/$FADH_2$	Flavin adenine dinucleotide oxidised/reduced forms
G	Guanine
HEPES	N-2-Hydroxyethylpiperazine-N'-2 ethane sulphonic acid
Hof	3-Hydroxyflavone
Krpm	1000 revolutions per minute
L	Leucinostatin
Me	Methyl group
Morin	Penta-hydroxyflavone
mtDNA	Mitochondrial deoxyribonucleic acid
NAD/NADH	Nicotinamide adenine dinucleotide oxidised/reduced form
NAM	Nuclear accommodation of mitochondria
oli/Ol	oligomycin
OSCP	Oligomycin sensitivity conferring protein
oss/Os	ossamycin
Ph	Phenyl group
P_i	Inorganic phosphate
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
Tris	Tris (hydroxymethyl)methylamine
Ven	Venturicidin
URF	Unassigned reading frame
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

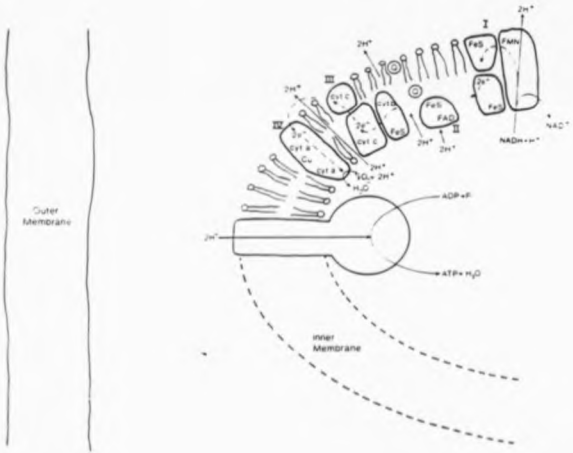
Aerobic organisms derive free energy from the oxidation of fuel molecules such as glucose and fatty acids. Intermediate in the transfer of electrons from these compounds are the carrier molecules nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), which accept electrons released by the degradation reactions of glycolysis and the citric acid cycle, converting them to their reduced forms NADH (and H^+) and $FADH_2$. Cells which can use molecular oxygen as the electron acceptor for the reoxidation of NADH and $FADH_2$ enormously increase their ability to generate ATP, compared with anaerobic cells, which transfer these electrons to an organic metabolite or inorganic oxidant. However, electron transfer from NADH and $FADH_2$ releases enough free energy to drive the synthesis of several ATP molecules. To achieve release of this energy in a controlled, conservable fashion electrons are transferred stepwise through a series of reversible electron carriers, located in the mitochondrial inner membrane (Figure 1.1.a, b).

Mitochondria are centres for respiratory energy metabolism in eukaryotic cells. These organelles have two membrane systems (Figure 1.1.a): the outer membrane, which is freely permeable to small molecules and ions and has few enzymatic activities, and the inner membrane, which is extensively folded into ridges, termed cristae, and intrinsically impermeable (having a different phospholipid composition) with many associated enzyme activities. Hence the mitochondrion contains two compartments; the intermembrane space and the mitochondrial matrix which is totally enclosed by the inner membrane. The matrix houses the soluble enzymes of the citric acid cycle and those for fatty acid oxidation. The complex respiratory assemblies responsible for electron transfer and ATP synthesis span the mitochondrial inner membrane, together with transport systems to carry specific ions and metabolites across the impermeable

a


Matrix
Inter Membrane Space
Outer Membrane
Inner Membrane

b


Outer Membrane
Inner Membrane
ADP+P
ATP+H2O
NADH+H+
NAD+
FeS
FMN
FAD
Cyt b
Cyt c1
Cyt c
Cyt a
Cu
2H+
H2O

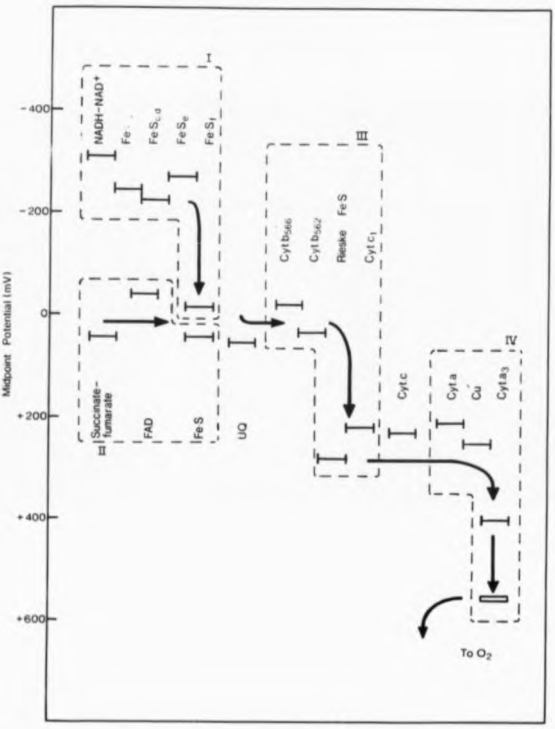


Figure 1.1 (a) The structure of the mitochondrion showing the highly folded inner membrane. (b) The asymmetrical arrangement of respiratory complexes in the inner mitochondrial membrane. (c) The midpoint potential of the respiratory electron carriers, showing the association of carriers of similar midpoint potential to give the four respiratory complexes. The route of respiratory electron transport is indicated with arrows.

membrane bilayer (Tzagoloff, 1982).

The subject of this thesis is the complex multisubunit enzyme which catalyses ATP synthesis, the F_1F_0 ATP synthase (ATPase) common to all respiring organisms. Both genetics and biochemistry of this enzyme can be studied conveniently in the yeast *S.cerevisiae*. Initially we discuss possible mechanisms coupling electron transport to ATP synthesis in the mitochondrial inner membrane, followed by an examination of the respective contributions of the nucleus and the mitochondrial genome to mitochondrial biogenesis. Mechanisms of transport of nuclear-coded proteins to their correct location in the organelle will be considered. A detailed description of the mitochondrial ATPase derived from both biochemical and molecular genetic analysis of a number of species with reference to *S.cerevisiae* is also included.

1.1. Oxidative Phosphorylation

The electrons from NADH and $FADH_2$ have a high transfer potential, releasing a large amount of energy during passage through the mitochondrial electron carriers to molecular oxygen. The mitochondrial electron carriers are arranged in four distinct respiratory assemblies (complex I to IV), each assembly containing numerous centres capable of reversibly accepting electrons. Flavin, iron-sulphur, quinone and heme groups are all utilised to transfer electrons in the direction of increasingly positive redox potential. The tendency of a membrane-bound electron carrier to donate or accept reducing equivalents is usually expressed as its midpoint potential, which is determined with reference to a standard electrode. These carriers can be divided into isopotential pools based on their midpoint potentials (Figure 1.1.c). Transfer of electrons between any of these pools represents a large fall in potential, resulting in a release of energy. At three steps during the transfer of electrons from NADH (two steps for $FADH_2$) the energy released is sufficient to drive the synthesis of ATP. The topology of the respiratory complexes and sites of ATP synthesis are summarised in Figure 1.1.b, c.

The coupling of these energy releasing electron transfer reactions to the energy requiring synthesis of ATP has puzzled biochemists for many decades, and has not yet been resolved (reviewed in Slater, 1987). As passage of electrons through the inner membrane carriers does not directly allow ATP synthesis, a "high energy" mediating factor is necessary. A number of concepts for a coupling mechanism have been proposed, all differing in the nature of this high energy intermediate:

The chemical hypothesis (Slater, 1953) was based on the theory that oxidative phosphorylation had the same basic mechanism as substrate-level phosphorylation, in which energy is conserved in a high energy molecule where inorganic phosphate (P_i) is bound to a carrier molecule. From experimental data, however, it was evident that a non-phosphorylated intermediate was involved as respiration could occur in the complete absence of inorganic phosphate. The simplified reaction proposed for this mechanism is shown below:

$$AH_2 + B + C \quad \rightleftharpoons \quad A \sim C + BH_2$$
$$A \sim C + ADP + P_i \quad \rightleftharpoons \quad A + C + ATP$$

where ~ is a bond in the high energy molecule, A and B represent redox carriers and C is an unknown ligand.

Uncouplers were proposed to act by hydrolysing the A ~ C bond thus dissipating the stored energy. However, compounds causing uncoupling have a diversity in structure which is not consistent with this mode of action. Also, concerted investigation to isolate this chemical intermediate failed to identify the mediating compound, and it is now considered untenable in the form proposed.

The conformational hypothesis (Boyer, 1965), which proposed that the high energy intermediate was a specific conformation of a respiratory carrier. This is a great simplification of the chemical hypothesis as it no longer requires ligand C for formation of the high energy intermediate, thus:

$$AH_2 + B \rightleftharpoons A^- + BH_2$$
$$A^- + ADP + P_i \rightleftharpoons A + ATP$$

The chemiosmotic hypothesis (Mitchell, 1961, 1966, Nicholls, 1982), which has as the intermediate a proton gradient and transmembrane potential ($\Delta\mu_{H^+}$) formed across the mitochondrial inner membrane. Thus the equation for this hypothesis does not associate – with any of the redox carriers;

$$AH_2 + B \rightleftharpoons A + BH_2 + -$$
$$- + ADP + P_i \rightleftharpoons ATP$$

The theory envisages electron transfer through the energy conserving sites resulting in transmitted conformational changes which eject protons from the matrix to the cytoplasmic side of the membrane without compensating movement of counter charges. The circuit is completed by proton transport back into the matrix through the ATP synthase, resulting in synthesis of ATP. This theory proposed that uncouplers catalysed proton translocation across the energy transducing membrane thus dissipating $\Delta\mu_{H^+}$.
Much experimental evidence has been accumulated to support this theory:

1. Translocation of protons from matrix to cytoplasm does take place when electrons are transferred through the respiratory carriers, or when ATP is hydrolysed by mitochondrial ATPase (Mitchell and Moyle, 1965).

2. $\Delta\mu_{H^+}$ can be artificially induced across the inner mitochondrial membrane by a number of different methods, and can drive the synthesis of ATP (Jagendorf and Uribe, 1966, Racker and Stoeckenius, 1974). ATP synthesis by this method can take place as fast, or faster than ATP synthesis driven by respiration (Thayer and Hinkle, 1975).

The mode of action of uncouplers could also be explained by this theory. Known uncouplers are weak acids/bases, and are thought to prevent ATP synthesis by picking up

protons in regions of high proton concentration and releasing them where the proton concentration is low, thus dissipating the proton gradient across the inner mitochondrial membrane.

However the chemiosmotic hypothesis has not been accepted in its entirity, and refinements in the original theory have been made in an attempt to incorporate further experimental evidence. There are discrepancies in the kinetics of this mechanism; from double inhibitor titrations it was evident that the respiratory complexes are not completely isolated from each other, but "direct cross-talk" between them occurs, which is not consistent with having delocalised protons as a pool intermediate (Westerhof *et al*, 1984). Circumstances also exist in which there is no obvious connection between $\Delta\mu_{H^+}$ and oxidative phosphorylation; some alkophilic (Krulwich and Guffanti, 1983) and halophilic bacteria (Helgerson, 1983) have negligible membrane potential but still synthesise ATP.

Some of these discrepancies between predicted and experimental results can be explained by making non-fundamental changes in the chemiosmotic hypothesis, but it is perhaps more fruitful to base investigations on hypotheses which do not need extensive adjustment.

The localised proton hypothesis (Williams, 1961), which differs from the chemiosmotic hypothesis in that it postulates that the protons produced by electron transfer are directly delivered to the ATP synthase, effectively remaining associated with the membrane. To account for generation of the transmembrane potential a second pathway for protons is proposed where a proportion are allowed to leave the membrane.

The collision hypothesis (Boyer, 1984, Slater *et al*, 1985), which combines some aspects of previous theories in proposing energy transfer by random collision of redox complexes with the ATP synthase. The large respiratory complexes are randomly distributed within the plane of the inner mitochondrial membrane, and are free to diffuse in this plane. The frequency of collisions between redox complexes and the ATP synthase is 3 to 5 times the rate of ATP synthesis, and Slater proposes that these collisions are not abortive.

At present none of these hypotheses can be accepted in their entirity and experimentation can only identify areas in which each is correct. The rôle of proton translocation as a critical factor in the coupling of oxidation and phosphorylation is well established, and experimentation has shown that membrane potential can be used to drive ATP synthesis. However, the generation of membrane potential may be of secondary importance, as evidence suggests a more direct route for protons in normal ATP synthesis.

1.2. The Mitochondrial Genome

Mitochondria and chloroplasts are unique among eukaryotic organelles as they contain their own genome encoding a small proportion of their own proteins. Also encoded in the mitochondrial genome are a complete complement of ribosomal RNA (rRNA) and transfer RNA (tRNA) to enable the mitochondrion to translate these proteins using its own protein synthetic apparatus. In most organisms, including *S.cerevisiae*, mitochondrial DNA (mtDNA) exists as a circular superhelix which is not associated with nucleosomes. In *S.cerevisiae* its length ranges from 70-76 Kbp, giving short and long forms due to an increase in non-coding DNA (Prunell *et al*, 1977, Morimoto and Rabinowitz, 1979). Thus the mitochondrion has a degree of autonomy, which can best be defined by describing the proteins for which it codes (for reviews see Grivell, 1983, Tzagoloff and Myers, 1986).

Over 90% of mitochondrial protein is encoded by the nucleus, synthesised on the cytoribosomes and post-translationally imported into the organelle. The mitochondrial genome contributes to 4 enzyme complexes:

1. *Coenzyme QH_2-cytochrome c reductase*: one of the 8 subunits (cytochrome b) is encoded by mitochondrial DNA in *S.cerevisiae* (Weiss and Zigante, 1974, Katan *et al*, 1976).

2. *Cytochrome oxidase*: three of the 7 subunits are mitochondrially-coded in *S.cerevisiae* (Tzagoloff *et al*, 1975d, Eytan, 1975), with all coding regions interrupted by introns.

Some of these introns have been found to code for maturases, whose sole function is to catalyse excision of the intron on which they are encoded (Carignani *et al*, 1983, Hensgens *et al*, 1984).

3. *F_1F_0 ATPase*: the number of mitochondrially coded subunits varies with the organism as summarised in Table 1.1. In *S.cerevisiae* subunits 6, 8 and 9 of mitochondrial ATPase F_0 are encoded on mitochondrial DNA.

4. *Mitochondrial ribosomes*: the *var1* protein of the small ribosomal subunit is mitochondrially coded in *S.cerevisiae*, but its site of synthesis varies in different organisms.

The respiratory complex polypeptides encoded in *S.cerevisiae* mitochondrial DNA are all highly hydrophobic, and thus have much of their amino acid sequence integrated into the membrane.

Chloroplast			Mitochondrion				
Sector	Subunit	Site	Subunit	Maize	*S.cerevisiae*	*N.crassa*	Bovine
	α	O	α	O	C	C	C
	β	O	β	C	C	C	C
F_1	γ	C	γ	C	C	C	C
	δ	C	δ	C	C	C	C
	ε	O	ε	C	C	C	C
			OSCP		C		C
	I	O	6		O	O	O
F_0	II	C	8		O	O	O
	III	O	9	O	O	C	C
			Inhibitor		C		C

Table 1.1 Site of synthesis of subunits for F_1F_0 ATPase in a range of organisms. Subunits encoded in organelle DNA (O) and nuclear DNA (C) are indicated.

Mitochondria also possess a complete biosynthetic system for translation of these proteins. Most of the subunits of the mitoribosomes are imported from the cytoplasm,

but, to date, the dogma that there is no RNA molecule exchange between mitochondrion and cytoplasm still holds true. The rRNA and tRNA required for mitochondrial protein synthesis are coded on mitochondrial DNA. Mitochondrial ribosomes bear a superficial similarity to bacterial ribosomes, but differ substantially on closer examination, having no 5S rRNA and differing numbers of proteins. However, mitochondrial ribosomes are inhibited by chloramphenicol and erythromycin, which inhibit bacterial protein synthesis, but are not affected by cycloheximide and emetine, which inhibit eukaryotic cytoribosomes. This has enabled the two eukaryotic protein synthesis systems to be studied in isolation by selective use of these inhibitors, and was the initial method used for identifying mitochondrially-coded proteins. The mitochondrial genetic code also differs from the universal code (three codons are different in *S.cerevisiae* mitochondria), and is read by a more restricted number of tRNAs (Grivell, 1983).

S.cerevisiae mitochondrial DNA appears to code for 2 rRNAs, 25 tRNAs, 6 known inner membrane proteins and more than 10 reading frames which code for proteins of unknown function (termed unassigned reading frames or URFs). Although the yeast mitochondrial genome does not contain significantly more information than the human genome it is more than 5 times longer (Figure 1.2). Yeast mtDNA coding sequences are separated by long, non-coding regions rich in the bases A and T, and a number of coding regions are interrupted by introns (often encoding maturases). Transcription of the coding regions results in rRNAs with both upstream and downstream sequences which are not translated. In contrast human mtDNA is organised extremely efficiently (Anderson *et al*, 1981), with few if any non-coding nucleotides. Thus there are no signal sequences in non-coding DNA to initiate transcription, so an alternate method of regulation has been suggested by Attardi and colleagues (Montoya *et al*, 1981, Ojala *et al*, 1981) in which one major promoter on each strand is responsible for a full length RNA which is subsequently cleaved. Two of the bovine ATPase genes actually overlap by 40 bases: the ATPase 6 gene is initiated in the coding region of the A6L gene (Fearnley and Walker, 1986).

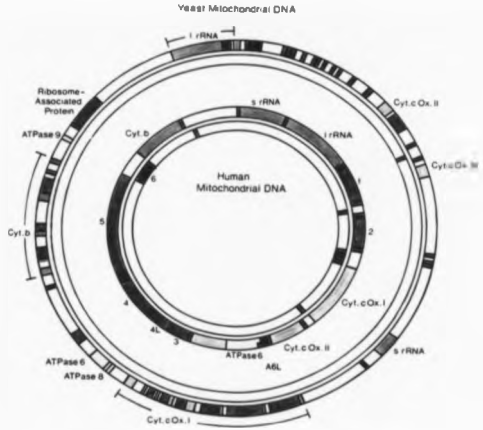


Figure 1.2 Comparison of human and yeast mitochondrial genomes, showing essentially the same set of genes but with different organization. Both strands of each double helix are shown, but the yeast DNA molecule is approximately five times longer than the human DNA, so the scale used is different for each. Coloured regions show genes coding for known proteins in green, yellow and red, for ribosomal RNA in light blue, for transfer RNA in dark blue and for unassigned reading frames in brown (based on Grivell, 1983). Some of the human unassigned reading frames have since been shown to be subunits of NADH dehydrogenase.

Maintaining the mitochondrial genome is a very expensive process in biochemical terms. The nucleus codes for the vast majority of mitochondrial proteins, but also for all the enzymes required for maintenance and replication of mitochondrial DNA, and a number of polypeptides which, together with the mitochondrially-coded rRNAs, tRNAs and 1 ribosomal protein, coordinate to enable the mitochondrial protein synthesis system to function. That the mitochondrial genetic system exists at all suggests that this organelle differs in its origin from that of other organelles. The endosymbiont theory proposes that these organelles have evolved from an ancestral respiring bacterium which was engulfed by a larger primitive eukaryotic cell deriving its energy from the considerably less efficient method of fermentation. However the mitochondrial genome bears little relation to eukaryotic or prokaryotic genetic systems, so the organism from which mitochondria evolved may be no more related to present day prokaryotes than to eukaryotes. However, this can be partially explained by anticipating different evolutionary pressures acting on an endosymbiont.

Initially it was proposed that the mitochondrial genome had been conserved because it encoded hydrophobic inner mitochondrial membrane proteins which needed to be synthesised inside the mitochondrion for their correct integration and assembly. This has been disproved after study of mitochondrial genetic material from a number of organisms:

1. Subunit 9 of mitochondrial ATPase F_0 is mitochondrially-coded in *S.cerevisiae* but nuclear coded in *N.crassa* and higher organisms

2. Most chloroplast ATPase F_1 subunits are synthesised in the organelle

3. One ATPase F_1 subunit is organelle-coded in maize mitochondria

The patterns of gene distribution in different organisms are thought to be due to alternate pathways for "escape" events of genes from the ancestral endosymbiont to the nucleus.

This is consistent with the transitional stage of dual representation of the same gene in nucleus and organelle as demonstrated for subunit 9 of ATPase in *N.crassa* (van den Boogaart *et al*, 1982) and *A.nidulans* (Scazzocchio *et al*, 1983), and the identification of non-functional mitochondrial DNA sequences in the nuclear DNA due to abortive gene translocation (Farrelly and Butow, 1983). Alternative evolutionary pathways by which endosymbiont genes have been transferred to the nucleus are thought to give rise to the organelle genome diversity evident today.

1.3. Mitochondrial Genetics of *S.cerevisiae*

Saccharomyces cerevisiae is an ideal organism on which to base a study of the energy transducing membrane of eukaryotes. Apart from its already well defined genetics, respiratory deficient mutants can be stably propagated on fermentable substrates. However, it is by no means typical as it can survive anaerobically and strains used today have been extensively manipulated and thus differ from those occurring naturally. Consequently the *S.cerevisiae* inner mitochondrial membrane is one of the best characterised membrane systems to date.

Mutations in *S.cerevisiae* mitochondrial DNA are inherited in a non-Mendelian fashion, and can be divided into 4 broad categories:

1. Petite mutations were the first cytoplasmic mutations to be associated with mitochondrial DNA. A petite can lose all of the wild-type (or grande) mitochondrial genome (ρ^0), or retain part of its mitochondrial DNA (ρ^-), which is amplified so that the total amount of DNA approaches that present in normal mitochondria. This provides a small sequence in large quantities useful for analysis, and can be used to locate mutations in other mitochondrial mutants (petite deletion mapping). This mutation leads to a non-revertable respiratory deficient phenotype.

2. *mit*$^-$ mutants, like petites, cannot grow on non-fermentable carbon sources such as glycerol or ethanol, but, in contrast to petites, can still conduct mitochondrial protein synthesis. Because *mit*$^-$ mutations are point mutations or small deletions or insertions, they can often revert to wild-type.

3. *syn*$^-$ mutants are specifically deficient in mitochondrial protein synthesis. These mutations are located in either the rRNA or tRNA genes.

4. Antibiotic or inhibitor resistant mutations confer the ability to grow on non-fermentable substrates in the presence of that antibiotic or inhibitor.

Nuclear-coded genes required for the formation of respiratory-competent mitochondria are grouped under the general category of *PET* genes (Sherman, 1963). Mutations in these genes, *pet* mutations, encompass mutations in genes for structural proteins, or mitochondrial function can be affected because the nucleus encodes enzymes needed for replication and expression of mitochondrial DNA. This effect is usually expressed by pleiotropic deficiency in a number of mitochondrial enzyme activities. Antibiotic resistance can also be mapped to nuclear DNA, where the polypeptides interacting to give the antibiotic binding site are nuclear-coded.

Nuclear Control of Mitochondrial Biogenesis

The involvement of nuclear gene products in regulation of mitochondria has been demonstrated at a number of levels; firstly in the transcription of *S.cerevisiae* mitochondrial DNA, in the processing of the precursor RNA and in the translation of mitochondrial messages. Mutation in the regulatory genes cause respiratory deficiency, and are classified as *pet* mutations. By analysing the phenotype of *pet* mutants, those which carry mutations in genes fulfilling regulatory functions can be tentatively identified. *Pet* mutations analysed fall into three categories when assayed for mitochondrial protein synthesis (Michaelis *et al*, 1982):

1. Complementation groups which have no detectable effect on mitochondrial protein synthesis

2. Groups which block mitochondrial protein synthesis completely

3. Groups which cause absence or alteration of a specific or a restricted number of mitochondrial translation products.

The latter group are of interest as these are candidates for regulation of mitochondrial gene expression by the nucleus. All mutations of this type studied to date have been shown to exert their effects post-transcriptionally (reviewed by Fox, 1986), either by mRNA processing (Dieckmann *et al*, 1984, McGraw and Tzagoloff, 1983, Pillar *et al*, 1983), enhancing translation (at the 5' end) (Roedel, 1986, Fox, 1986) or post translational modification (Pratje *et al*, 1983).

Some nuclear suppressors have been identified which act on specific *mit*$^-$ loci (NAM mutations or nuclear accommodation of mitochondria) (Dujardin *et al*, 1980). These mutations are thought to affect the mitoribosome, causing ambiguity of translation thus allowing pseudoreversion of the *mit*$^-$ mutations they suppress (Zagorski *et al*, 1987). Most of the suppressed mutations are premature ochre terminators in introns or exons of mitochondrial genes, although they also suppresses frame-shift or missense mutations because of their lack of translation fidelity.

Mitochondrial Effect on Nuclear Function

The protein composition of ρ^- mitochondria is essentially identical to that of respiratory competent mitochondria, with the obvious exception of mitochondrially-coded proteins (Schatz *et al*, 1972). The synthesis of *PET* proteins occurs in the absence of an intact mitochondrial genome, and this argues against any regulation of nuclear mRNA by mitochondrial gene products. However, a number of altered cell surface characteristics can be observed in *S.cerevisiae* cells converted to ρ^-, including differing permeability to

a number of metabolites, flocculation behaviour, and degree of partition in a biphasic system (Wilkie and Evans, 1982), but this could be attributed to changes in the cellular balance of the ions and compounds under mitochondrial regulation (Ca^{2+} and ATP for example, Wilkie, 1983).

1.4. Transport

The majority of the mitochondrion's polypeptides are encoded on nuclear DNA and synthesised on cytoribosomes. These proteins must be imported and targeted to their correct subcellular compartment. The mechanism substantiated by recent data involves synthesis of a precursor peptide in the cytoplasm, which is post-translationally targeted to its specific sub-mitochondrial location (reviewed by Reid, 1985). This involves:

1. Recognition of the precursor by a receptor on the mitochondrial outer membrane surface (Henning and Neupert, 1981, Riezman *et al*, 1983).
2. Transfer of the polypeptide into or across one or both mitochondrial membranes.
3. Conversion of the precursor to mature protein (by proteolytic cleavage, conformational change or addition of a prosthetic group for example)
4. Assembly into an active protein complex.

Mechanisms for the growth of mitochondria are based on addition of mitochondrial proteins to an existing mitochondrial template. They take as a premise that receptors for transport are already present and correctly orientated in the membrane (Gillespie, 1987). Receptors in the outer mitochondrial membrane are thought to recognise the subset of cellular proteins destined for mitochondria, and this recognition step is protease-sensitive. Ribosomes associated with the mitochondrial membrane preferentially synthesise mitochondrial proteins (Suissa *et al*, 1984), but their transport is not coupled to synthesis in the manner of endoplasmic reticulum secretary proteins. Some mRNAs fractionate with free ribosomes (Suissa and Schatz, 1982), and recognition by the

membrane receptors can occur at different stages of polypeptide chain completion. Most inner membrane and matrix proteins are synthesised with amino-terminal extensions 20-50 amino acids long (Neupert and Schatz, 1981, Maccecchini *et al*, 1979). These leader sequences tend to have a net positive charge, with the notable exception of coenzyme QH_2-cytochrome c reductase which has a highly acidic N-terminal. The recognition signal can also occur in the secondary or tertiary structure in the absence of a leader sequence, which is generally the rule for proteins in the outer membrane or the inter-membrane space. However, exceptions exist to both of these generalisations about presequences.

Transport across or into the outer mitochondrial membrane occurs in an energy-independent manner, but matrix or inner mitochondrial membrane proteins require an energised inner mitochondrial membrane for transport to occur (Gasser *et al*, 1982, Schleyer *et al*, 1982). It is possible that both mitochondrial membranes are traversed in a single step (Nelson and Schatz, 1979). Once the polypeptide has entered the mitochondrion, a matrix protease then cleaves it to its mature size (Bohni *et al*, 1980, Yaffe *et al*, 1985), with some precursors requiring more than one proteolytic cleavage (for example cytochrome c_1) (Ohashi *et al*, 1982).

Proteins encoded in mitochondrial DNA tend to be hydrophobic integral membrane proteins, but it is evident that in the process of evolution a number of these genes have been transferred to the nucleus. The gene for subunit 9 of mitochondrial ATPase is mitochondrially-coded in *S.cerevisiae* but nuclear-coded in *N.crassa*. This brings into question how this subunit can be integrated into a functional complex from either side of the membrane. However, evidence suggests that transport mechanisms have evolved purely to transfer proteins destined for mitochondria across the two membranes, at which point another process is employed to give the correct topological integration into the membrane. Yeast mitochondrial ribosomes are tightly bound to the inner mitochondrial membrane, and integration of these mainly hydrophobic proteins into this membrane is

thought to occur co-translationally.

1.5. Mitochondrial ATP synthase (ATPase)

The enzyme that catalyses oligomycin sensitive ATP synthesis was first isolated by Kagawa and Racker (1966). Mitochondrial ATPase is a multi-subunit enzyme of dual genetic origin, having two functionally distinct domains (Figure 1.3):

1. F_1 is a water soluble, oligomycin insensitive ATPase comprised of 5 subunits, α, β, γ, δ and ε, which associate to give a stoichiometry of 3α:3β:1γ:1δ:1ε (Futai and Kanazawa, 1980). F_1 houses the active site of ATP synthase, but, when isolated, will only hydrolyse ATP. All 5 subunits are encoded by the nucleus, synthesised on the cytoribosomes, and finally transported into the mitochondrion (Bennet and Butow, 1974).

2. F_0 is membrane-associated and forms a channel across the hydrophobic inner membrane, allowing proton flow through the enzyme back into the matrix. It has the binding sites for oligomycin, tri-ethyl tin, venturicidin etc. (Walter *et al*, 1967, Griffiths and Houghton, 1974, Lardy *et al*, 1975) and confers sensitivity to these compounds on the F_1F_0ATPase.

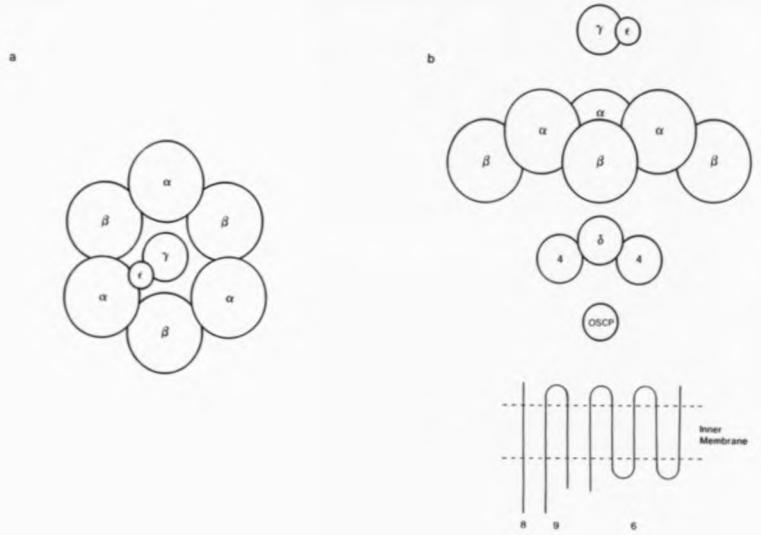


Figure 1.3 Diagrammatic representation of mitochondrial ATPase in the yeast *S.cerevisiae* (a) from matrix (b) side view (based on Todd *et al*, 1980).

E.coli	*S.cerevisiae*	Bovine
α	α	α
β	β	β
γ	γ	γ
δ	OSCP	OSCP
ε	δ	δ
	ε	ε
a	6	a
b	ATP4	b
c	9	c
		d
	8	A6L
	inhibitor	inhibitor

Table 1.2 Subunit equivalence of F_1F_0 ATPases

This enzyme can be seen in its simplest form in *E.coli* (reviewed in Senior, 1985), which has the five F_1 subunits and an additional three F_0 subunits, but it increases in complexity in eukaryotes. Increasing subunit number in organisms higher on the evolutionary scale is a common motif among respiratory enzymes. The most complex version yet identified is bovine ATP synthase, which has 13 subunits, a number of which have no equivalent in *E.coli*. Homologous subunits and their site of synthesis are summarised in Table 1.2. All eight subunits of the bacterial ATP synthase complex are encoded on a single operon (Walker *et al*, 1984) which has been completely sequenced. This is termed the *unc* operon as the genes were originally identified by investigation of uncoupled mutants. Genes for the five F_1 subunits are clustered, as are the F_0 subunit genes. The mechanism by which the transcription and translation of these genes from one operon gives the observed stoichiometry of 3α:3β:1γ:1δ:1ε is unknown.

F_1 - The Catalytic Unit of ATP Synthase

The enzymatic activity of ATP synthase is located in its F_1 moiety, which can be removed intact from the membrane giving a soluble homogeneous enzyme preparation capable of high levels of ATP hydrolytic activity. *E.coli* F_1 is easily displaced with

additions of low concentrations of EDTA; equally the complex can be reconstituted by addition of Mg^{2+} ions. *S.cerevisiae* requires more stringent measures to displace F_1. The isolated F_1 presents fewer experimental problems than study of the F_1F_0 complex, and its mechanism of ATP hydrolysis has been investigated with the assumption that it would provide insights into membrane bound ATP synthesis. The applicability of this assumption is discussed by Gautheron *et al* (1984). Isolated F_1 has three enzymatic activities:

1. ATP hydrolysis
2. Exchange between the γ-phosphoryl oxygen of ATP and that of H_2O during hydrolysis of low concentrations of ATP thought to be due to reversals of hydrolysis (Choate *et al*, 1979)
3. Synthesis of [$^{32}\gamma$ ATP] from ADP and ^{32}P (Sakamoto and Tonomura, 1983)

The sites of ATP hydrolysis in F_1 are located on the β subunits, giving three catalytic sites per F_1, with the α subunits possibly contributing to binding of the adenosine moiety. The antibiotic aurovertin binds to the three active sites with different affinities suggesting that these sites are non-identical (Issartel *et al*, 1983). A further ATP binding site occurs on the α subunit, but this is a non-exchangeable site to which ATP binds tightly, and its role is thought to be regulatory in nature. Current experimental approaches suggest that ATP synthesis requires no direct energy input, but that the reaction is driven by the binding of product ATP to a high affinity catalytic site. Energy input is required, however, to displace ATP from this binding site. Cooperativity between the major F_1 subunits is vital for its normal catalytic activity (Ackerman *et al*, 1987). Binding of substrate ADP is thought to result in the release of product ATP from an alternative catalytic site. *E.coli* mutants have been isolated where single base changes in the α subunit result in an F_1 with very low ATPase activity due to loss of α-β intersubunit conformational interaction (Futai and Kanazawa, 1984). A point mutation in the α

subunit can also prevent binding of F_1 to the mitochondrial inner membrane (Maggio *et al*, 1988).

The genes for the α and β subunits have been sequenced in *S.cerevisiae* (Saltzgaber-Muller *et al*, 1983) and also isolated in the yeast *S.pombe* (Boutry *et al*, 1984). The β subunit from *S.cerevisiae* can be integrated into *S.pombe* F_1 resulting in a hybrid complex (Boutry and Douglas, 1983). This indicates that these evolutionary divergent yeasts have a common import and assembly mechanism for cytoplasmically-synthesised mitochondrial proteins.

Structure and Assembly of F_0 (reviewed in Nagley, 1988)

F_1 is a distinct functional unit with a well-defined subunit composition. Thus any subunits not identical with any of the 5 F_1 subunits are usually assigned to F_0 by default, but this does not necessarily mean that they are integral membrane proteins. The mitochondrially-coded subunits all have membrane-spanning helices intimately involved in proton transduction, but the nuclear coded F_0 subunits (sometimes called F_A - associated factors) are thought to be more involved in the coupling of proton translocation and ATP synthesis.

In the yeast *S.cerevisiae* subunits 6, 8 and 9 are all encoded in mitochondrial DNA (Tzagoloff and Meagher, 1972). These subunits are not thought to undergo post-translational cleavage, and are delivered directly to the matrix side of the inner mitochondrial membrane by the mitoribosomes. In contrast, subunit 9 is nuclear-coded in *N.crassa* and bovine cells. Bovine subunit 9 is expressed in a tissue-specific manner as two different species differing in their leader sequences. The *N.crassa* subunit is translated with a positively charged 66 amino acid leader sequence which is cleaved by its matrix protease. Yeast mitochondria with energised membranes can import and correctly process *N.crassa* subunit 9 to its mature form *in vitro* (Schmidt *et al*, 1983). This implies that the import mechanism is not specific for individual mitochondrial

precursors and that these precursors have common signals (Schmidt *et al*, 1983).

Genetic Studies of Oxidative Phosphorylation

Research investigating the interaction of antibiotics which block the proton channel of mitochondrial ATPase has been very productive in locating the genes for the ATPase F_0 subunits. Initially interest was focused on resistant mutants showing cytoplasmic inheritance, with the aim of identifying mitochondrial genes.

Mutants resistant to antibiotics such as oligomycin (Avner and Griffiths, 1973a, b), venturicidin (Griffiths *et al*, 1975, Lancashire and Griffiths, 1975b, Lancashire *et al*, 1974) and triethyl tin (Lancashire and Griffiths, 1975a) were sub-divided into two classes according to their cross-resistance spectrum. Class I mutants showed, in addition to the selective antibiotic, cross-resistance to unrelated inhibitors, uncouplers and/or inhibitors of mitochondrial protein synthesis. Mendelian inheritance was generally the case for this group. These mutations are thought to be due to membrane permeability or some other non-specific effect (Marahiel *et al*, 1977, Rank and Robertson, 1978). Class II mutants were so classified because they possess specific resistance to their selective antibiotic, and are only cross-resistant to related antibiotics (Avner and Griffiths, 1973). Class II mutations expressed oligomycin resistance at mitochondrial and sub-mitochondrial particle level, providing evidence that resistance was due to a specific modification of an inhibitor site in the mitochondrial inner membrane (Griffiths and Houghton, 1974).

Study of Class II mutations enabled identification and mapping of a number of mitochondrial loci. They were inherited mitotically and could be deleted by treatment with ethidium bromide. Initially two unlinked loci were identified with oligomycin resistance markers OLI and OLII, which on later investigation have been found to correspond to mitochondrial ATPase subunits 9 and 6 respectively. A further locus OLIII was mapped, linked to OLI (the gene *Oli1*), and identifiable by its cross resistance to venturicidin, and later OLIV, showing weak oligomycin resistance, was found to be

linked to OLII (the gene *Oli2*).

In addition to these mitochondrial loci, a number of other loci were identified which showed apparent cytoplasmic inheritance, but were not located on mitochondrial DNA. This includes mutants resistant to venturicidin (Griffiths *et al*, 1975, Lancashire and Griffiths, 1975b) and triethyl tin (Lancashire and Griffiths, 1975a), and these markers have not yet been mapped.

From cross-resistance analysis a certain amount of information can be deduced about the binding sites for these antibiotics. Results suggested that oligomycin and triethyl tin bind to completely separate sites, but both of these overlap with the binding site for venturicidin. It also suggests that the oligomycin binding domain is made up of solely mitochondrially-coded polypeptides, whereas that for triethyl tin comprises non-mitochondrial polypeptides.

The genes for the F_0 subunits encoded in mitochondrial DNA, identified in antibiotic mapping studies, have now been cloned and sequenced in *S.cerevisiae* and some other species. From analysis of the protein translation of *oli1*, *oli2* and *aap1* (subunits 9, 6 and 8 respectively) information concerning the secondary and tertiary structure of the mitochondrial F_0 proteins has been deduced (Figure 1.4). Subunit 9 is a protein 76 amino acids long (Macino and Tzagoloff, 1979), having two transmembrane helices joined by a hydrophilic loop which projects into the matrix. It has conserved regions in its glycine-rich N-terminus, its hydrophilic loop region and its C terminal helix, which always has an acidic residue to which DCCD covalently binds. Analysis of mutations mapped in this gene can provide information about functional domains and topology of the protein (Figure 1.5). The antibiotic oligomycin binds to F_0 and prevents proton conduction thus inhibiting ATP synthesis. Point mutations causing resistance to this antibiotic have been located on both helices of subunit 9, the sites on h_2 forming a major domain for oligomycin binding (Ooi *et al*, 1985). Resistant mutations to venturicidin, a functionally

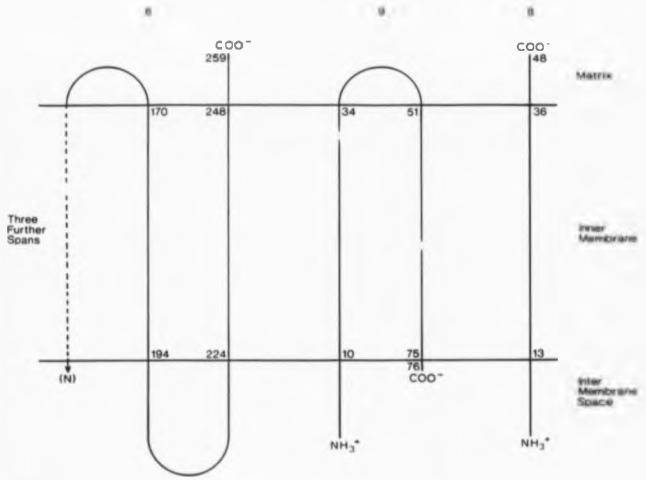


Figure 1.4 The mitochondrially-coded subunits of yeast mitochondrial ATPase F_0, showing their orientation in the mitochondrial inner membrane. Three of the membrane-spanning helices of subunit 6 are not shown, but these are not thought to contribute to the F_0 proton channel. The residues at either end of each transmembrane helix is indicated, as are the amino (N) and carboxy (C) termini of each protein.

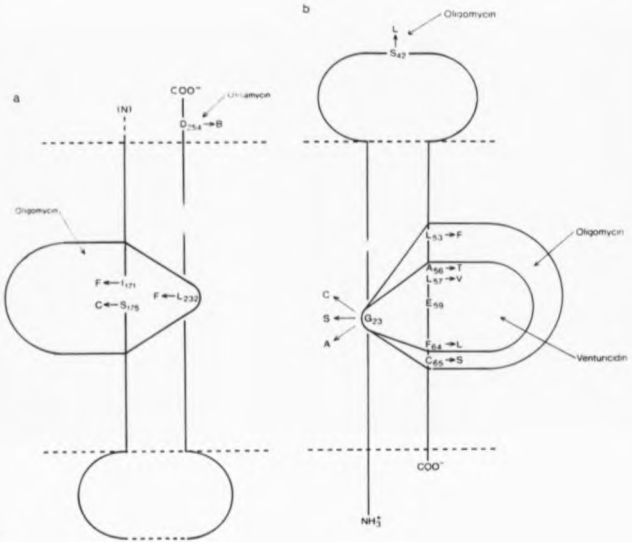


Figure 1.5 Subunits 6 (a) and 9 (b) of ATPase F_0 showing the locations of point mutations which result in expression of resistance to the antibiotics oligomycin, ossamycin and venturicidin. Both the amino acid change and its location are indicated. The domains of resistance to venturicidin and oligomycin are also shown.

similar antibiotic, can also be mapped both on h_1 and h_2 of subunit 9 implying that its two transmembrane stems are directly involved in the proton translocation mechanism (Nagley *et al*, 1986). This is confirmed by analysis of a *mit*$^-$ mutation gly_{23}→asp located on the h_1 helix, which appears to have a defective proton channel.

A *mit*$^-$ mutation in the hydrophilic loop connecting the two helices of subunit 9 has a contrasting phenotype. Mutants where arg_{39}→met are not correctly coupled to F_1. When revertants to this mutation were analysed one was found to be a recessive nuclear-coded suppressor. It is possible that this suppressor mutation could be located in one of the complex's subunits also involved in F_1F_0 interaction.

Subunit 6 has at least 5 transmembrane spans (some sources propose 7, Ray *et al*, 1988), and even in the absence of strong amino acid homology its general structure is conserved between species. Its carboxy-terminal helices h_4 and h_5 are thought to form part of the proton channel, in concert with h_1 and h_2 of subunit 9. Residues involved in oligomycin binding occur in both h_4 and h_5, but no resistance mutants to venturicidin are located in this protein. These α helices are amphiphilic, with their charged residues lying along one face of the helix. The charged amino acids are thought to point inwards to form the proton channel, and to play a vital role in the conduction of protons. The N-terminal region of the protein is needed for the assembly of the complex and does not appear to be directly involved in proton translocation.

Subunit 8 is a proteolipid 48 amino acids in length which is encoded by the mitochondrial *aap1* gene (Velours *et al*, 1984). It has only one transmembrane stem located towards its N-terminal, with a C-terminal charged hydrophilic domain of basic residues involved in F_1F_0 interaction.

Subunit	Phenotype	Locus	Domain	Functional Deficiency	Reference
9(oli1)	O^RV^R	$Gly_{23}\rightarrow Ala$	h_1		[1]
	O^RV^R	$Gly_{23}\rightarrow Cys$	h_1		[2]
	O^RV^R	$Gly_{23}\rightarrow Ser$	h_1		[2]
	O^RV^S	$Leu_{53}\rightarrow Phe$	h_2		[1, 3]
	O^RV^R	$Ala_{54}\rightarrow Thr$	h_2		[2]
	O^RV^R	$Phe_{64}\rightarrow Leu$	h_2		[1, 2]
	mit_1^-	$Gly_{23}\rightarrow Asp$	h_1	Defective proton channel?	[4]
	mit_1^-	$Arg_{39}\rightarrow Met$	loop	Defective coupling to F_1	[4]
	mit_2^-	$Gly_{18}\rightarrow Asp$	h_1	No assembly of 9,8,6	[4]
	mit_2^-	$Ser_{68}\rightarrow stop$	h_2	No assembly of 9,8,6	[4]
	mit_2^-	frameshift (4)*	all	No assembly of 9,8,6	[4]
6(oli2)	O^RV^R	$Ser_{175}\rightarrow Cys$	h_4		[5]
	O^RV^R	$Ile_{171}\rightarrow Phe$	h_4		[6]
	O^RV^S	$Leu_{232}\rightarrow Phe$	h_5		[5]
	Oss^RV^S	$Asp_{254}\rightarrow Asn$	h^5		[6]
	mit_1^-	$Thr_{268}\rightarrow Lys$	h_5	Defective coupling to F_1	[7]
	mit_2^-	$Ser_{256}\rightarrow stop$	C-terminus	Assembly of 8,9, truncated 6	[7]
	mit_2^-	frameshift (34)*		Assembly of 8,9, not truncated 6	[7]
8(aap1)	mit_2^-	frameshift (2)*		Assembly of 9, not truncated 8 or 6	[8,9]

Notes (n)* - all but the first n residues are deleted by the frameshift

mit_1^- - non-functional ATPase but F_0 assembly not grossly impaired

mit_2^- - non-functional ATPase with abnormal F_0 assembly

[1] Ooi *et al*, 1985

[2] Nagley *et al*, 1986

[3] Macino and Tzagoloff, 1979

[4] Nagley and Linnane, 1987

[5] John and Nagley, 1987

[6] Ray *et al*, 1988

[7] John *et al*, 1986

[8] Linnane *et al*, 1985

[9] Macreadie *et al*, 1983

Table 1.3 Location of *mit*$^-$ and antibiotic resistance mutations in mitochondrially-coded ATPase subunits of *S cerevisiae* (Based on Nagley, 1988).

Only one of the nuclear-coded F_0 subunits has been sequenced (Velours *et al*, 1988). ATP subunit 4 is a polypeptide 209 amino acids long, which in its nascent form has a hydrophilic, positively charged leader sequence of 35 amino acids. This protein has a high predicted α helix content, having two distinct domains. The N-terminal domain A is hydrophobic and has few charged amino acids possibly acting as an anchor to the membrane although not long enough to span it, whereas domain B contains a relatively large number of charged residues.

1.6. Objectives of Current Research

The initial aim of this project was to identify nuclear-coded subunits of mitochondrial ATPase F_0, to complement the work on the mitochondrially-coded subunits already in progress. The method chosen was to isolate mutants of the yeast *S.cerevisiae* resistant to antibiotics known to have their active site located within the mitochondrial ATPase F_0 domain, with a view to identifying nuclear-coded resistant mutations. Mutants found to be nuclear-coded would then be subjected to a second selection procedure, which involves determining the degree of resistance to the selective antibiotic at mitochondrial level. Resistant mutations resulting from a structural change in a mitochondrial component could be identified by this strategy.

The antibiotic venturicidin was to be used for selection of resistant mutations, as it was thought to have the greatest potential for generating nuclear mutations showing resistance at mitochondrial level. However, novel ATPase inhibitors were also investigated to determine their usefulness in studies of this nature.

An alternative approach to isolating a nuclear-coded F_0 subunit was undertaken in addition. A *pet* mutant had been isolated by Prof. A. Tzagoloff which was specifically deficient in mitochondrial ATPase activity. This mutant was analysed immunologically and was found to have the major F_1 subunits (this study) so was selected for investigation as a potential F_0 mutant.

CHAPTER 2

INVESTIGATION OF NUCLEAR ANTIBIOTIC RESISTANCE IN *S. CEREVISIAE*

2.1. INTRODUCTION

Oligomycin and other antibiotics which block the proton channel in ATPase F_0 have been useful tools in the study of the energy-transducing membrane. The antibiotics have been employed to elucidate the pathway of oxidative phosphorylation, and drug resistant loci have been vital in mapping mitochondrial DNA and identifying the genes specifying ATPase subunits. By sequencing these mitochondrial genes the location of oligomycin and venturicidin resistance mutations have been pinpointed, and from this aspects of the complex's tertiary structure can be deduced: the polypeptide chain residues which make up the antibiotic binding site can be tentatively identified as being proximal to each other and this can give information about folding of subunits and their spatial organisation with respect to each other. These amino acids are expected to be at or near the proton translocation site of F_0, or cause an allosteric change in that area.

The contribution that the nucleus makes to the F_0 binding site of these antibiotics, if any, has not yet been determined. Nuclear-coded resistance mutants isolated to date have shown complex phenotypic properties. A wide range of cross-resistance to related and unrelated antibiotics has been described (Rank *et al*, 1975, Saunders and Rank, 1982), and cytoplasmic factors causing changes in phenotype further confuse analysis (Cohen and Eaton, 1979, Simmons and Breen, 1983). Nuclear resistance to these antibiotics could conceivably occur at a number of different levels :-

1. Resistance at the plasma membrane level, preventing the antibiotic from entering the cell.

2. Degradation of the antibiotic by modified enzymes in the cytoplasm.

3. Resistance at the mitochondrial membrane level.

4. Degradation of the antibiotic within the mitochondrion.

5. Resistance due to a structural change in the enzyme binding site.

A nuclear contribution to the venturicidin binding site is evident, as demonstrated by Nagley (Nagley *et al*, 1986). The nuclear background in which a particular mitochondrial mutation Phe_{64} to Leu was resident was found to be the determining factor in the expression of venturicidin resistance/sensitivity.

This chapter describes the generation and analysis of nuclear mutations resulting in the expression of venturicidin resistance.

2.2. MATERIALS AND METHODS

Bacteriological peptone, yeast extract, bacto-agar and yeast nitrogen base were purchased from Difco Laboratories, Detroit, Michigan, USA. Adenine sulphate was purchased from BDH Chemicals, Poole, Dorset. Trizmabase, ATP and oligomycin were purchased from Sigma Chemical Co., Poole, Dorset. Venturicidin was a gift from Glaxo UK and Ossamycin a gift from the Bristol-Myers Corporation. All other reagents were of 'AnalaR' grade purity.

Growth Media for Yeast

Two types of medium are used routinely. YPD is used to support growth of respiratory deficient strains and YPG is used to differentiate between respiratory deficient and respiratory competent cells, and for antibiotic containing media.

YPD	YPG
1%(w/v) Yeast extract	1%(w/v) Yeast extract
2%(w/v) Bactopeptone	2%(w/v) Bactopeptone
2%(w/v) Glucose	3%(v/v) Glycerol
2%(w/v) Agar (for plates)	2%(w/v) Agar (for plates)

Glucose is autoclaved separately to prevent caramelisation. The media referred to as N0 and N3 are YPD and YPG respectively, buffered with 50mM Na/K buffer pH 6.25 (10mM Na_2HPO_4/40mM KH_2PO_4)

WO
0.67%(w/v) Bacto Yeast Nitrogen base without amino acids
2%(w/v) Agar (for plates)

If amino acid supplements were required they were added as specified by Sherman *et al*, 1983.

S (sporulation medium)
0.4%(w/v) NaAc
2%(w/v) Agar

Addition of antibiotics

Antibiotics were added to N3 medium in ethanolic solution, with the exception of chloramphenicol which is water soluble. The standard concentrations used for antibiotic-containing media are shown in Table 2.2.1, concentrations differing from these are referred to in the text.

Antibiotic	Media	Concentration
Venturicidin	N3	$2.5\mu gml^{-1}$
Oligomycin	N3	$0.5\mu gml^{-1}$
Ossamycin	N3	$2\mu gml^{-1}$
Leucinostatin	N3	$2\mu gml^{-1}$
Chloramphenicol	N3	$2mgml^{-1}$
Erythromycin	N3	$5mgml^{-1}$

Table 2.2.1 Concentration of antibiotics routinely used in *S.cerevisiae* growth media

Preservation of Strains

Yeast strains were routinely subcultured every 3 months by streaking onto N0 solid medium in bijou bottles, growing up overnight, and storing at 4°C.

Isolation of Mutants: UV Mutagenesis

D273-10B/A1 was grown up overnight in liquid YPD. After spinning down in a bench centrifuge at 5K rpm for 5 mins, cells were re-suspended to a titre of 1×10^8 cells ml^{-1} in phosphate buffer (Sorensons buffer). Aliquots of this suspension were plated onto N3/N3V to check the viability/sensitivity of the strain.

Mutagenesis was carried out under UV set at 1 $Jm^{-2}s^{-1}$. Cells placed in an uncovered Petri dish were irradiated for a total of 2 mins 20 secs with frequent agitation. Mutagenised cells were plated on N3V, YPD and N3 at 5×10^3 cells per plate.

Treatment with EtBr (Slonimski *et al*, 1968)

Haploid *S.cerevisiae* mutants were converted to ρ^- or ρ^0 by treatment with the DNA intercalating agent ethidium bromide (EtBr). This compound is highly carcinogenic and was handled with gloves at all times. EtBr was added to YPD to a final concentration of 50 μgml^{-1} and colonies replicated onto these plates were incubated for three days at 30°C in darkness. After further replication onto a fresh EtBr plate and incubation for three days these haploids were tested for complete conversion to ρ^- by replicating onto

N3. If no growth was observed after three days the culture was assumed to consist of ρ^- cells

Growth Rate Analysis (Avner and Griffiths, 1973a)

A standard inoculum of 1% stationary phase yeast was introduced into various liquid media, and grown at 30°C in an orbital shaker. Turbidity of the cultures was measured using an EEL colorimeter (607nm filter).

Cross Resistance Testing

To test several mutants simultaneously two methods were used :-

1. *Replica-plating*: This well established technique involves taking an impression from a master plate on velveteen cloth and printing onto selective media.

2. *Drop out analysis*: Wells of a microtitre tray were partially filled with 200µl aliquots of sterile distilled water, and each was inoculated with a single mutant strain. Using an aluminium stamper, sterilised by flaming in methanol, a discrete impression from each well could be made on antibiotic media.

Genetic Techniques (Sherman *et al*, 1983)

Mating of strains

Haploid strains of opposite mating types and differing metabolic deficiencies were mixed in equal quantities on an N0 plate and grown overnight at 30°C. Cells were transferred to W0 plates (with amino acid additions if necessary), and incubated at 30°C for 1-2 days.

Complementation Analysis

Diploid colonies were grown on W0 medium at approximately 100 cells per plate, and these master plates were replicated onto the appropriate antibiotic-containing media.

Random Spore Analysis

Diploid cells were incubated on S medium for a minimum of 3 days at 30°C. Optical microscope examination was used to determine whether a sufficient number of tetrads had been formed. When the tetrad count reached approximately 80% cells were scraped off the plate with a sterile loop and incubated in (1%) glusulase solution at 30°C for 30-60 minutes. Cells/spores were then counted, diluted with sterile distilled water, and spread on N0 to give 100-200 colonies per plate. Colonies were identified as certain spores if they had inherited one of the metabolic deficiencies of the parent strains. This was determined by replicating onto W0 medium and picking colonies which did not grow up on W0 to a master plate (N0) for use in further analysis.

Preparation of mitochondria (Connerton, 1986)

A 1%(v/v) inoculum of stationary phase yeast was added to 500ml of YPG liquid medium in a 2l Erlenmyer flask. The culture was harvested at late log. stage in a Sorvall GSA rotor at 2K rpm for 10 mins. The cell pellet was washed once in cold breaking buffer, and resuspended in the same buffer, adding 0.5ml of buffer per gramme wet weight of cells.

Breaking buffer
0.5M Sorbitol
10mM Tris/Cl pH 7.5
1mM EDTA
0.1% BSA (fatty acid free)

The following operations were carried out at 0-4°C. The resuspended yeast cells were transferred to a 250ml screw-capped bottle, together with ½ vol ballotini (0.45-0.5mm diameter, washed in cold TE-sorbitol). Cells were then broken by Lang's hand-shaking method, in three 2 min cycles with 1 min intervals on ice. The cell suspension was then decanted into two 60ml polycarbonate tubes, the beads washed through with 5vols of breaking buffer and the suspensions pooled. The cell debris was pelleted by spinning the

homogenate at 2K rpm for 15 mins in a Sorvall SS34 rotor. The supernatant was re-centrifuged until no pellet was produced (usually twice). The mitochondrial fraction could then be isolated from the supernatant by spinning at 12K rpm for 15 mins. After washing and re-suspending the mitochondrial pellet in TE-Sorbitol the mitochondria were used immediately for ATPase assays or stored at -70°C

Protein Determination

Mitochondrial protein was estimated by the method of Gornall *et al* (1949). Protein was solubilised with an equal volume of 5% deoxycholate for a minimum of 5 minutes before adding Biuret reagent. After a further 20 minutes at room temperature samples were read at 540nm in an SP1800 spectrophotometer against a reagent blank and protein concentration determined from a standard curve (using bovine serum albumin, BSA, as the standard protein) produced under similar conditions.

ATP hydrolysis

Assays were carried out in a volume of 1ml containing approximately 0.1mg of mitochondrial protein, 50mM Tris/Cl pH 8.0, 2mM $MgCl_2$ and 5mM ATP. Mitochondria were pre-incubated in reaction buffer on ice with inhibitors and the reaction initiated by the addition of ATP. Tubes were incubated for 10 mins at 30°C, and the reaction stopped by adding 1ml of 10% TCA. Protein was removed by centrifugation in a bench centrifuge.

Inorganic Phosphate Determination (Heinonen and Lahti, 1981)

This method measures the yellow chromophore of phosphomolybdate when dissolved in acetone to monitor phosphate concentrations.

AAM Solution	
10mM Ammonium Molybdate	1 vol
5M H_2SO_4	1 vol
Acetone	2 vols

AAM solution (4ml) was added to 0.5ml of inorganic phosphate (P_i) in H_2O and the solution vortexed. Citric acid (0.4ml of 1M) was added and after vortexing the optical density of the solution was read immediately against a reagent blank at 355nm with an SP1800 spectrophotometer. The inorganic phosphate present was obtained from a standard curve produced under similar conditions.

2.3. RESULTS

Genotypes of the strains used in this chapter are tabulated below:

Strain	Nuclear Genotype	Mitochondrial Genotype	Source
D273-10B/A1	α *met*$^-$	ρ^+	A. Tzagoloff
CD76	a *leu*$^-$	ρ^+ O^R	I.F. Connerton
D22	a *ade*$^-$	ρ^+	D.Wilkie
D22/A21	a *ade*$^-$	ρ^+O^R	Avner and Griffiths, 1973
114-3			
152-2			
VUV1-280	α *met*$^-$	ρ^+	This study

Table 2.3.1 Nuclear and mitochondrial genotypes of strains used in this chapter

The aim of the research covered in this chapter was to isolate and identify nuclear-coded components of mitochondrial ATPase F_0. The proposed strategy was to generate mutants of *S.cerevisiae* resistant to antibiotics binding to ATPase F_0, which were thought to have a nuclear component. These mutants could then be tested for inheritance and nuclear mutations selected. The nuclear resistant mutants could then be assayed to assess the biochemical resistance of their ATPase to the antibiotic (expressed in terms of pI_{50}

values) with the aim of isolating a mutant resistant in one of the structural components of ATPase. By this resistance marker the gene for this component could then be cloned and its DNA sequence determined.

The criteria for selection of a possible mutation causing an alteration in structure of the ATPase are listed below:-

1. The mutation should show clear nuclear inheritance.

2. The mutation should cause resistance to the selective antibiotic only, or if any cross-resistance is shown it is only to functionally related antibiotics, which could possibly share the same binding site.

3. The mutation should cause some change in the biochemistry of mitochondrial ATPase itself, ideally an increase in the *in vitro* resistance to the selective antibiotic or, failing that, a change in the specific activity of mitochondrial ATPase.

Venturicidin Resistance

Initially mutagenesis was carried out by UV-irradiating cells, as this method has been shown to preferentially produce nuclear-coded mutations. A number of chemical mutagens can also be used which favour nuclear mutations, among then N-methyl N-nitro nitrosoguanidine (see section 4.3) and ethyl methane sulphonate. Alternatively the mutagens ethidium bromide and manganese are used when production of a significant proportion of mitochondrial mutations is desirable, or when it is necessary to delete the mitochondrial genome (conversion to ρ^0). From the initial mutagenesis of D273-10B/A1 a number of venturicidin-resistant mutants were isolated. VUV1-127 were subcultured from medium containing $2.5\mu gml^{-1}$ venturicidin and VUV130-280 were isolated on $1.25\mu gml^{-1}$ venturicidin. Clones were subcultured twice onto venturicidin-containing medium (the selection concentration), and then stored on NO slopes pending further analysis.

The venturicidin-resistant mutants were tested for their degree of tolerance to this antibiotic by drop-out analysis on venturicidin-containing medium, with the venturicidin concentration varied up to 100μgml^{-1}. All of the mutants isolated were resistant even to the highest concentrations of venturicidin, and all clones tested actually grew better on the higher concentrations of the antibiotic (this also applies to the wild-type D273-10B/A1). The explaination for this unusual effect is unknown, but it may be produced by reversal of inhibition due to binding at a low affinity site on mitochondrial ATPase at high concentrations of antibiotic. The V-resistant mutants were also tested for cross-resistance to a variety of functionally related antibiotics, for example oligomycin and ossamycin, but also to the mitochondrial protein synthesis inhibitors erythromycin and chloramphenicol.

Strain	Antibiotic					
	N3V	N3Ol	N3Os	N3CAP	N3ERY	N3L
VUV37	>10	<0.5	1	2	4	1
VUV58	>10	<0.5	1	2	4	1
VUV78	>10	<0.5	1	2	4	1
VUV84	>10	<0.5	1	2	4	1
VUV86	>10	<0.5	1	2	4	1
VUV99	>10	<0.5	1	2	4	1
VUV107	>10	<0.5	1	2	4	1
VUV17	>10	2	1	2	4	1
VUV29	>10	2	1	2	4	1
VUV44	>10	2	1	2	4	1
VUV45	>10	2	1	2	4	1
VUV112	>10	2	1	2	4	1
114-3	>10	<0.5	1	1	4	1
152-2	>10	<0.5	1	1	4	1
D273/10B-A1	0.25	0.25	1	1	4	1

Table 2.3.2 Cross resistance of venturicidin-resistant mutants to related and unrelated antibiotics. Haploid strains were replicated onto plates containing various concentrations of antibiotic and growth was scored after three days incubation at 30°C. Concentration of the inhibitors venturicidin (V), oligomycin (Ol), ossamycin (Os) and leucinostatin (L) are given in μgml^{-1}. Concentrations of chloramphenicol (CAP) and erythromycin (ERY) are given in $mgml^{-1}$. All plates containing inhibitors were of buffered glycerol (N3) medium.

Two different patterns of resistance were evident, a group which were resistant to venturicidin and chloramphenicol only, and a group that showed cross resistance to oligomycin (and sometimes ossamycin) in addition to these two antibiotics (Table 2.3.2).

To determine whether these mutants were located in nuclear or mitochondrial DNA both mitotic and meiotic segregation criteria were used. When two haploid strains are mated the diploid resulting from this cross contains a mixed population of mitochondria. However, during subsequent divisions of this diploid cell one type of mitochondria will be progressively eliminated. Thus if a mitochondrial drug resistant marker was present

in one of the haploids, it would be inherited by some, but not all, of the diploid progeny (mitotic segregation). A mutation in nuclear DNA would be stably inherited in all diploid progeny. On sporulation of a single diploid, the haploid spores would show a 2:2 ratio of resistance to sensitivity for a nuclear mutation (i.e. Mendelian inheritance). However, if the diploid sporulated contained a mitochondrial marker it would be inherited by all haploid spores.

Firstly a number of mutants selected from each cross-resistance group were mated to a standard strain (CD76 or D22). Strain CD76 carries a mitochondrial oligomycin resistant marker, but D22 is a completely sensitive strain. Random diploids were tested for their degree of resistance to concentrations of antibiotics which have been shown to inhibit the sensitive parent strain (Table 2.3.3). Diploids from both groups showed a degree of resistance intermediate between that of the parent sensitive strain and that of the resistant haploids.

Strain	V^R	V^S	O^R	O^S
VUV17	76	0	28	48
VUV29	76	0	28	48
VUV37	76	0	32	44
VUV44	76	0	21	55
VUV45	76	0	26	50
VUV58	76	0	16	60
VUV78	76	0	26	50
UVU84	76	0	25	51
VUV86	76	0	13	65
VUV99	76	0	18	58
VUV107	76	0	19	57
VUV112	76	0	40	36
I14-3	100	0	44	56
I52-2	77	0	33	44
D273/10B-A1	0	76	27	49

Table 2.3.3 Segregation of random diploids from venturicidin-resistant parent strains. Each venturicidin-resistant isolate was independently crossed to strain CD76, which has a mitochondrial oligomycin-resistant marker, and random diploids were replicated onto venturicidin and oligomycin-containing media. Growth was scored after three days incubation at 30°C. Individual diploid clones were scored as venturicidin or oligomycin resistant (V^R or O^R respectively) if growth was observed on plates containing 2.5 μgml^{-1} venturicidin (V) or 0.5 μgml^{-1} oligomycin. If no growth was observed colonies were scored as sensitive to that inhibitor (V^S or O^S). Figures tabulated represent the number of individual diploid clones tested from one cross. The stock inhibitor solutions were added to buffered glycerol medium (N3).

The observed partial dominance is typical of the nuclear resistant mutations to these antibiotics isolated to date. Currently all mitochondrial antibiotic resistant mutations that have been isolated are dominant as they are present as single copies. The pattern of resistance of a typical mitochondrial mutation is demonstrated in the previously characterised O^R mutant CD76. This gives a heterogeneous population of diploids, some completely resistant and some completely sensitive depending on whether they had inherited the O^R or O^S mitochondria from the haploid parent strains.

A single diploid resulting from each of the crosses between the V^R resistant mutations and the standard strains was sporulated by the method outlined in section 2.2, and spores analysed for their resistance to the antibiotics. Between 50 and 100 spores were isolated from each cross and the results are shown in Table 2.3.4. The two groups show Mendelian inheritance, giving an equal number of resistant and sensitive haploid offspring. In contrast, the mitochondrial V^R strain produces offspring 100% resistant or 100% sensitive depending on the phenotype of the diploid sporulated, as it is cytoplasmically inherited.

All of the venturicidin resistant mutations produced in this study were in a D273/10B-A1 background, so they all have the metabolic deficiency *met*$^-$, a requirement for the amino acid methionine. Complementation of the metabolic deficiencies of haploid strains was used to isolate diploids from mating crosses, the strain CD76 having the *leu*$_1$ marker. On examining the spores from these diploids it was found that group II (cross resistant to oligomycin) does not segregate with the metabolic deficiency marker *leu*$_1$, as the resistant spores isolated from this cross will only carry the marker *met*$^-$. This suggests that the mutation is on chromosome VII and tightly linked to *leu*$_1$, which indicates it is a member of the pleiotropic drug resistant (*pdr*) linkage group described by Cohen and Eaton (Cohen and Eaton,1979, Cohen,1977) as this maps close to the centromere of chromosome VII adjacent to this *leu* marker. Its phenotype shows many of the characteristics exhibited by this linkage group, including the papillation of diploid colonies thought to be due to gene conversion. Group I mutations segregate independently of the metabolic deficiency markers.

Strain	V^R	V^S	O^R	O^S
VUV17	27	30	11	46
VUV29	30	31	61	0
VUV37	21	29	47	3
VUV44	10	12	22	0
VUV45	37	24	48	3
VUV58	24	21	2	43
VUV78	21	17	1	37
VUV84	25	18	12	31
VUV86	24	22	40	6
VUV99	34	40	16	58
VUV107	28	26	0	52
VUV112	70	86	34	122
D273/10B-A1	4	60	2	62

Table 2.3.4 Random spore analysis of progeny from the venturicidin-resistant parent strains. Venturicidin-resistant isolates were crossed to strain CD76 and a single diploid from each cross was transferred to sporulation medium. After incubation at 30°C for a minimum of 3 days, spores were separated by treatment with glusulase and plated onto NO medium at a titre of 100 cells per plate. Certain spores were identified by replicating this plate onto WO medium and transferring colonies which did not grow to an NO master plate which was then used for antibiotic resistance testing. Antibiotic plates were scored after three days incubation at 30°C. Spores which grew up on plates containing 2.5 $\mu g ml^{-1}$ venturicidin (V) or 0.5 $\mu g ml^{-1}$ oligomycin (O) were classified as resistant to that inhibitor (V^R or O^R respectively). Spores which showed no growth were classified as sensitive (V^S or O_S respectively). Figures tabulated represent the number of spores produced from a single diploid tested. Inhibitor plates were made up in buffered glycerol medium (N3).

Resistant spores of different mating types generated by this sporulation were crossed to each other to determine complementation groups. This was uninformative, with seemingly no correlation between the cross and the degree of resistance shown in the diploids. A single diploid taken from each of a number of crosses was sporulated, and the spores analysed to determine whether Group I and Group II were different linkage groups. Results are shown in Table 2.3.5, and this gives further evidence that Group I and Group II are unlinked, inter-group crosses showing 4:0 V^R:V^S ratios, intra-group crosses being approximately 2:1 V^R:V^S. The latter ratio differs from that expected (3:1),

and this may occur because the doubly-resistant genotype is lethal.

Cross	V^R	V^S
GII × *GII*		
VUV99 × 78	50	0
VUV58 × 99	51	0
VUV78 × 84	63	1
VUV58 × 84	41	0
VUV100 × 37	61	0
GI × *GII*		
VUV86 × 44	114	52
VUV86 × 45	33	18
VUV107 × 29	48	29
VUV99 × 112	48	27
VUV107 × 112	56	30
VUV17 × 78	30	8
GI × *GI*		
VUV17 × 112	67	0

Table 2.3.5 Random Spore analysis of crosses between venturicidin resistant mutants. Mutants are classified as members of Group I if they are linked to leu_1. A single diploid from each cross was sporulated by standard methods as outlined previously, and certain spores tested for their degree of resistance to 0.5 μgml^{-1} oligomycin (O) and 2.5 μgml^{-1} venturicidin after three days incubation at 30°C. Spores were classified as resistant (O^R or V^R) if they grew on medium containing that inhibitor and sensitive (O^S or V^S) if they showed no growth. Figures tabulated represent the number of spores tested from a single diploid produced from one cross. Inhibitor stock solutions were added to buffered glycerol medium (N3).

A number of mutants from each group were analysed biochemically to assess their ATPase activity, together with the degree of resistance *in vitro* of the ATP synthase to a variety of antibiotics. Mitochondria from each mutant were prepared as described in section 2.2, and incubated with a range of antibiotic concentrations. The P_i produced by hydrolysis of ATP present in the assay was measured colorimetrically. Resistance mutations located in the mitochondrial genes coding for ATP synthase subunits confer an increased resistance to the enzyme *in vitro*. This indicates that the change causing resistance is located in one of the structural units of the enzyme, and is not due to

transport or degradation factors. The percentage of residual activity (compared with a control without antibiotic) was plotted against antibiotic concentration allowing the pI_{50} values for that antibiotic to be determined (the concentration of antibiotic needed to cause 50% inhibition of enzyme activity). The pI_{50} of both V^R groups and of wild type mitochondria lies in the region of 0.16 µg of antibiotic per mg of mitochondrial protein. No changes in venturicidin pI_{50} is apparent in the V^R groups compared to the wild type, and this also applies to oligomycin pI_{50} for those strains cross-resistant to that antibiotic. A comparison between both groups of mutant and the wild-type D273/10B-A1 is shown in Figure 2.3.1, and includes an assay on the mitochondrially-coded oligomycin resistant mutation in the strain D22/A21 to indicate the effect of a structural change in the antibiotic binding site on the pI_{50} value. The pI_{50} for oligomycin in this strain is 10.5 $\mu g mg^{-1}$ compared with 3.6 $\mu g mg^{-1}$ for wild-type mitochondria.

Thus these two groups of venturicidin resistant mutations do not fulfill the required criteria for a possible ATPase structural mutation, and any attempt to clone either would be hampered by their partial dominant phenotype. A number of further attempts were made to isolate venturicidin resistant mutants and one was successful in isolating a venturicidin resistant mutant which was not cross-resistant to any of the antibiotics in the study. This mutant was isolated by plating unmutagenised cells on venturicidin medium at a titre of 10^7 cells per plate. Resistant colonies were subcultured and analysed as previously described. Diploids produced from this strain were partially dominant and showed a 2:2 ratio of resistance to sensitivity when sporulated. However, on analysis of its mitochondrial ATPase pI_{50} no difference from the wild-type pI_{50} was observed.

2.4. CONCLUSIONS

Resistant mutants of *S.cerevisiae* have been generated to a number of ATPase inhibiting antibiotics (see Appendix I of this thesis). The best definition of phenotype was observed in venturicidin resistant mutants, and these were investigated in the greatest detail.

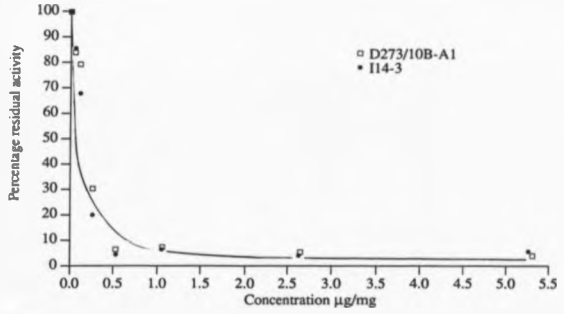


Fig. 2.3.1a Mitochondrial ATPase resistance to the antibiotic venturicidin in wild type (D273/10B-A1) and venturicidin resistant (I14-3) strains.

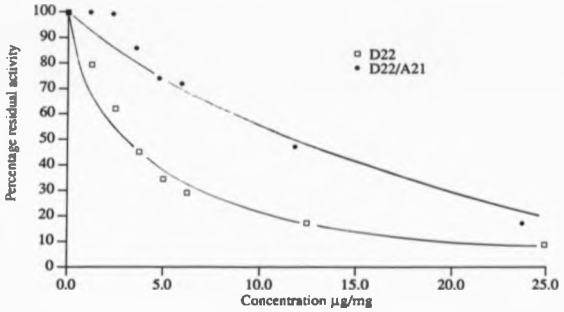


Fig. 2.3.1b Mitochondrial ATPase resistance to the antibiotic oligomycin in wild type (D22) and mitochondrially-coded oligomycin resistant strain (D22/A21).
Methods for ATPase assay, phosphate determination and protein assay are described in section 2.2. Inhibitor concentration is given in mug of inhibitor per mg of mitochondrial protein.

Although numerous attempts to produce mutants were undertaken using a variety of nuclear or mitochondrial DNA specific mutagens, none of the mutants analysed fulfilled all three criteria selected to identify ATPase structural mutations. Two groups of non-allelic mutations were identified, one of which appeared to be a member of the *pdr* linkage group described by Saunders and Rank (1982).

It appears that structural mutations in the nuclear components of ATPase F_0 resulting in V^R are rare, or may not even occur, and by far the most frequent mutant type generated lowers membrane permeability to these antibiotics in a non-specific manner.

CHAPTER 3

BIOCHEMISTRY OF ORGANOTIN DERIVATIVES OF 3-HYDROXYFLAVONE AND MORIN

3.1. INTRODUCTION

Trialkyl tin compounds are known to inhibit oxidative phosphorylation (Aldridge, 1958, Aldridge and Street, 1964), showing different modes of action depending on concentration. At lower concentrations trialkyl tins bind to mitochondrial ATPase, causing loss of both ATP hydrolytic and ATP synthetic activities in an "oligomycin-like" manner. Its binding site has been located on F_0 of ATPase (Sone and Hagihara, 1964, Cain, 1976), and it has been shown to cause inhibition of proton conduction (Cain *et al*, 1977, Cain and Griffiths, 1977).

At higher concentrations these compounds have an uncoupling activity whose mode of action was attributed to a chloride/hydroxyl exchange across the inner mitochondrial membrane (Selwyn *et al*, 1970, Dawson and Selwyn, 1974). However, research performed in this laboratory, has shown that this depolarisation of the mitochondrial membrane potential takes place in minimal halide media, and that addition of exogenous chloride has little or no effect on the rate or steady-state estimates of depolarisation (Connerton, 1986). The third activity of trialkyl tins is that they cause gross swelling of mitochondria when suspended in potassium chloride or isothionite media.

Trialkyl tin binding studies have given complex binding curves which have been interpreted as follows: there are at least two classes of binding site, a high affinity component which corresponds to the inhibition of ATPase activity (Rose and Aldridge, 1972, Cain and Griffiths, 1977), and a low affinity binding site whose function is as yet undetermined. The ATPase binding site is proposed to involve histidine and thiol/dithiol groups, as mono and dithiols can specifically reverse trialkyl tin-mediated ATPase

inhibition.

The spectrum of activity shown by trialkyl tins in rat liver mitochondria ranges between trimethyl tin chloride, which is a poor inhibitor of mitochondrial ATPase (pI_{50}>100) but an effective uncoupler, to tributyl tin chloride which has a lesser effect on mitochondrial membrane potential but with a pI_{50} of 2.0, makes it a good inhibitor of ATPase (Connerton, 1986). However, by far the most effective tin compounds developed to date with respect to their inhibition of ATPase are penta-coordinate compounds, whose tin moiety is internally coordinated to nitrogen (van Koten *et al*, 1975). These compounds have no demonstratable effect on mitochondrial membrane potential.

This chapter describes some properties of a group of recently synthesised tin compounds, including their effects on mitochondrial ATPase and mitochondrial membrane potential. A description of their fluorescence properties is also included with the aim of determining their possible use as a fluorescent probe for mitochondrial ATPase F_0.

3.2. MATERIALS AND METHODS

3-hydroxyflavone was purchased from Aldridge Chemicals and penta-hydroxyflavone (morin) from the Sigma Chemical Co., Poole, Dorset. CCCP was purchased from Sigma and DSMP$^+$ from Gallard-Schlessinger Inc. Organotin derivatives were synthesised in this laboratory by Dr. D. E. Griffiths and Mr. K. Allsop. All other reagents were of standard 'AnalaR' grade purity.

Preparation of Rat Liver mitochondria

Livers were excised from adult male Wistrar rats (200-300g), finely chopped at 4°C and washed in ice-cold homogenisation buffer (250mM sucrose, 10mM Hepes pH 7.5, 1mM EDTA) to remove any blood. The liver was homogenised in homogenisation buffer with 4-5 passes in a glass homogeniser with a teflon pestle. The homogenate was centrifuged

at 2.5K rpm for 10 mins in a Sorvall SS34 rotor, the supernatant decanted and recentrifuged. The supernatant from the second spin was centrifuged at 12K rpm for 15 mins which produced a mitochondrial pellet. This pellet was washed and then resuspended in a minimum volume of mitochondrial resuspension buffer (250mM sucrose, 10mM Hepes pH 7.5).

A similar method was used for the preparation of rat heart mitochondria.

Preparation of submitochondrial particles

Rat liver submitochondrial particles were prepared by sonicating a suspension of rat liver mitochondria in suspension buffer for four 15 second periods with 1 min intervals on ice to cool. The sonication tube was immersed in wet ice to prevent overheating. The sonic suspension was centrifuged at 10K rpm in a Sorvall SS34 rotor for 10 mins at 4°C, the supernatant decanted and centrifuged for 30 mins at 35K rpm in a Beckman 50Ti rotor at 4°C. This produced submitochondrial particles (smps) as an orange translucent pellet, which was resuspended in mitochondrial resuspension buffer (250mM sucrose, 10mM Hepes pH7.5).

ATPase Assays

Estimation of protein, assay for ATP hydrolytic activity and determination of inorganic phosphate was performed as in section 2.2.

Fluorimetric Assays

Using DSMP$^+$

The lipophylic dye DSMP$^+$ was used as an indicator of mitochondrial membrane potential in preparations of rat liver mitochondria, essentially as described by Mewes and Rafael (1981). A Perkin Elmer fluorimeter was used with both the excitation and emission slits set at 10mm. DSMP$^+$ (2 µl of a 1 mM solution in ethanol) was added with

stirring to a four-sided 4 ml plastic cuvette containing mitochondria and buffer. An appropriate volume of mitochondria to give a total of 1 mg protein was added to mitochondrial resuspension buffer (250 mM sucrose, 10 mM HEPES, pH 7.5) to a total volume of 3 ml. Excitation and emission wavelengths were set at 479 and 589nm respectively, and, after addition of $DSMP^+$, the fluorescence was allowed to achieve a maximum value before further additions. Compounds to be tested for their uncoupling activity were added in ethanolic solution (Mewes and Rafael, 1981, Connerton, 1986), ensuring that the ethanol concentration did not exceed 1% of the total volume.

Using Sn derivatives

A Perkin-Elmer fluorimeter was used with slit widths set as described previously. Initially compounds were investigated to identify where excitation and emission wavelengths combined to give a maximum value for fluorescence. A total of 3 ml containing 1 mg of mitochondrial protein in mitochondrial resuspension buffer was added to a four sided, 4 ml plastic cuvette, to which 2 µl of a 10 mM solution of one of the Sn compounds was added. Initially the emission wavelength was set at an arbitrary value (usually 500 nm) and the excitation wavelength was scanned. This was then set to any maxima observed in turn, and used to determine the wavelength required for maximum emission of fluorescence.

In later experiments using $Bu_2SnBr(of)$, excitation and emission wavelengths were set as in Table 3.3.1, using 2 µl of a 1 mM ethanolic solution of the Sn compound per mg of liver mitochondrial protein. Less rat heart and submitochondrial protein was added as it gave a greater enhancement of fluorescence per unit protein, but this will be outlined in the text.

3.3. RESULTS

A series of organotin derivatives of 3-hydroxyflavone (Hof) and penta-hydroxyflavone (morin) (structures shown in Figure 3.3.1a and b respectively) were synthesised by an addition reaction following the method of Blunden and Smith (Blunden and Smith, 1982). Equimolar quantities of the tin di-halide structure of the product is shown in Figure 3.3.1c (after Blunden and Smith, 1982). These compounds are thought to have a penta-coordinated Sn moiety, which is internally coordinated to oxygen on one of the rings of hydroxyflavone.

The di- and tri-phenyl (of) compounds were crystallised by evaporating the solvent. On dissolving these two compounds in ethanol they lost their yellow colour so they were subsequently dissolved in DMF or acetone for use in assays. Other compounds were not recrystallised but used as an ethanolic solution of the oil left after evaporation of solvent, with the molarity calculated assuming the reactions had gone to completion. On attempting to recrystallise $Bu_2SnCl(of)$, white crystals were produced which redissolved in ethanol to give a yellow solution. However, this solution proved inactive when used in fluorescence assays, as it was not displaced by Bu_3SnAc and it was therefore discarded.

A total of eight compounds were tested for their fluorescent properties when bound to mitochondria. Five 3-hydroxyflavone derivatives were produced, and their bound fluorescence is summarised in Table 3.3.1, with the exception of the Ph_3 compound (this immediately lost its colour on addition to the cuvette). These derivative compounds were all yellow in colour. Three morin derivatives were also tested, and generally gave poor results showing a minimal increase in fluorescence on binding to mitochondria which could not be reduced on adding the non-fluorescent Bu_3SnAc. These compounds were green/orange in colour. All assays were carried out using 1mg mitochondria per cuvette and 2µl of a 1mM solution of each of the analogues. A typical recorded trace is shown in Figure 3.3.2.

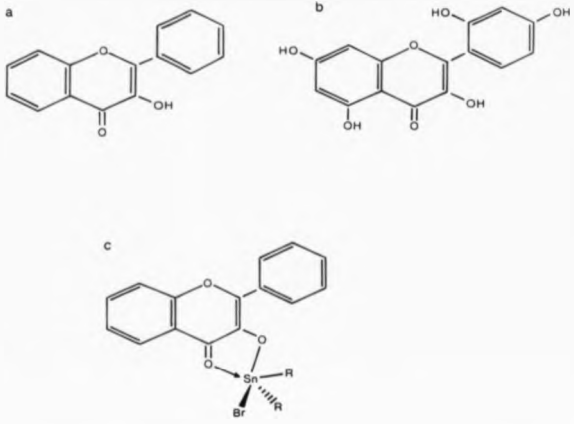


Figure 3.3.1 (a) The chemical structure of 3-hydroxyflavone and (b) Morin. (c) The penta-coordinate organotin derivative of 3-hydroxyflavone where R can be either a methyl, ethyl or butyl group.

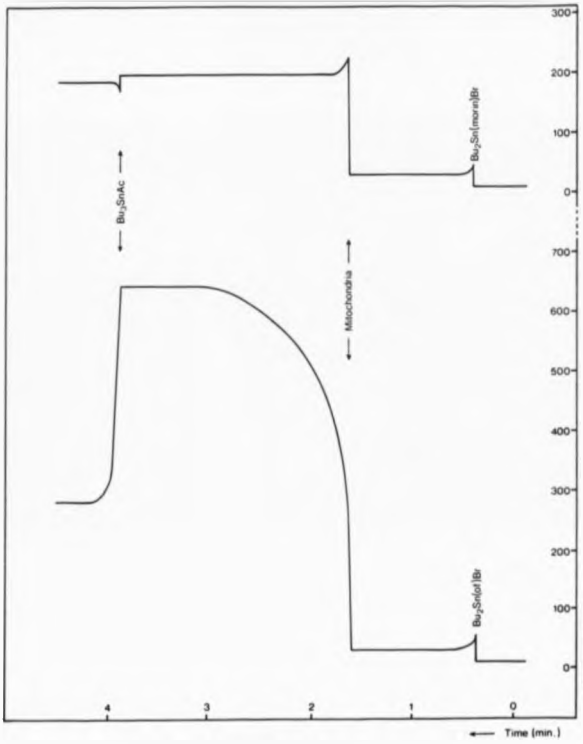


Figure 3.3.2 Fluorescence traces from mitochondria with additions of the butyl derivatives of 3-hydroxyflavone and morin. Mitochondrial resuspension buffer (3 ml) was added to a four-sided fluorescence cuvette in a Perkin Elmer fluorimeter. Excitation and emission wavelengths are given in Table 3.3.1. The fluorescent organotin derivative was added (2 µl of a 1 mM solution in ethanol), followed by a volume of rat liver mitochondria containing 1 mg of mitochondrial protein. The non-fluorescent Bu_2SnAc was added (2 µl of 1 mM in ethanol) after a maximum value for fluorescence had been reached.

Compound	Excitation	Emission	Fluorescence Enhancement	+BuSnAc
Me_2SnBr(of)	392	455	++	to baseline
Et_2SnCl(of)	390	450	+++	to baseline
Bu_2SnBr(of)	400	470	+++	to baseline
Ph_2SnCl(of)	393	473	++	to baseline
Et_2SnClMorin	400	510	+	no effect
Bu_3SnBrMorin	402	497	+	no effect
Ph_3Morin	393	456	+	no effect

Table 3.3.1 Fluorescence properties of organotin derivatives of 3-hydroxyflavone and morin. Excitation and emission wavelengths are those which give a maximum value for the fluorescence of each compound when incubated with rat liver mitochondria in mitochondrial resuspension buffer.

The fluorescence properties of these compounds are proposed to be due to their binding on the F_0 domain of mitochondrial ATPase, at the site where previously analysed Sn compounds have been found to bind. This premise was used as a direction for further research. Enhancement of fluorescence occurs on binding to its site in mitochondria, but the fluorescent compound can be displaced by a non-fluorescent Sn compound which binds the same site with corresponding loss of fluorescence, supporting this theory. This is not the case for morin derivatives (Figure 3.3.2).

Tri-alkyl tin compounds have been found to be potent inhibitors of the mitochondrial ATPase. The newly-synthesised compounds were thus assayed to determine their effectiveness in an ATPase assay (Figure 3.3.3). The morin analogues showed very poor inhibition, their I_{50} being relatively large (Figure 3.3.3c). In contrast, the 3–hydroxyflavone derivatives Bu_2SnBr(of) and Et_2SnCl(of) are good inhibitors, their I_{50} in the range of Bu_3SnAc (Figure 3.3.3a, b). The results are not presented in numerical form for a number of reasons. Firstly, the molarity of the solutions used was estimated assuming that the synthesis reactions had gone to completion, but this may not have been the case. This assumption does not consider the possibility of incomplete or side

reactions and degree of solubility in ethanol or in water. I_{50} values can be obtained from Figure 3.3.3, but these inhibition curves are only useful for comparison, and results are assumed to be very crude.

3-hydroxyflavone is known to be a weak inhibitor of mitochondrial ATPase when binding to F_1. To eliminate this as a possible site for ATPase inhibition, 3–hydroxyflavone compounds were investigated to determine whether F_1 was inhibited. F_1 was prepared from rat liver mitochondria by the chloroform extraction method of Beechey (Beechey *et al*, 1975 and described in section 5.2), and 10µl of a 10mM solution of inhibitor was used in an ATPase assay (far exceeding the range in which the inhibitors are effective in a mitochondrial preparation). No inhibition of the rate of ATPase hydrolysis was observed.

The mitochondrial ATPase assay results correspond well with the data from the earlier fluorescence experiments, as the degree of fluorescence enhancement appears to correlate with the compound's effectiveness as an ATPase inhibitor. Further experiments were performed using one of these compounds as representative of the group to provide a detailed analysis of their properties. The compound $Bu_2SnBr(of)$ was selected for further study because it was a good inhibitor of mitochondrial ATPase, it showed a large fluorescence enhancement on adding to mitochondria, and it was displaced by addition of Bu_3SnAc. The compounds synthesised with morin as starting material were not studied further as they did not show the most promising properties.

Previously studied compounds of this type showed diverse properties. At low concentrations they selectively inhibit mitochondrial ATPase in an oligomycin-like fashion, but at higher concentrations they cause dissipation of the membrane potential. $DSMP^+$ is a fluorescent probe which is an accurate indicator of membrane potential (Mewes and Raphael, 1981). Its fluorescence is enhanced on adding to energised mitochondria due to its import into the relatively lipophilic environment of the

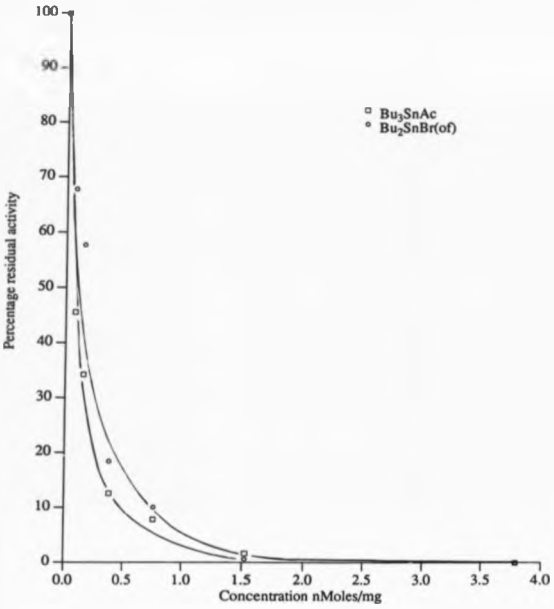


Fig.3.3.3a Resistance of mitochondrial ATPase to the antibiotics Bu_3SnAc and $Bu_2SnBr(of)$. Rat liver mitochondria were prepared by differential centrifugation of homogenised liver tissue as described in section 3.2. ATPase activity, estimation of inorganic phosphate and protein determination are described in section 2.2. Concentration is given in nM inhibitor per mg of mitochondrial protein.

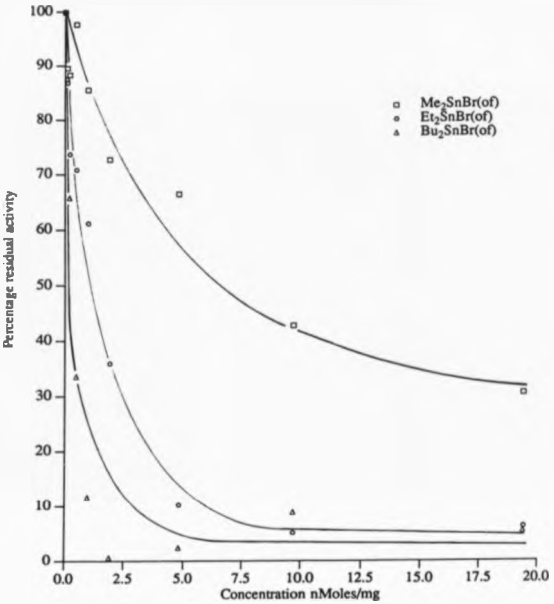


Fig.3.3.3b Resistance of mitochondrial ATPase to organotin derivatives of 3-hydroxyflavone. Rat liver mitochondria were prepared as described in section 3.2, and methods for ATPase assay, inorganic phosphate and protein determination are covered in section 2.2. Concentration of inhibitor is given in nM per mg of mitochondrial protein.

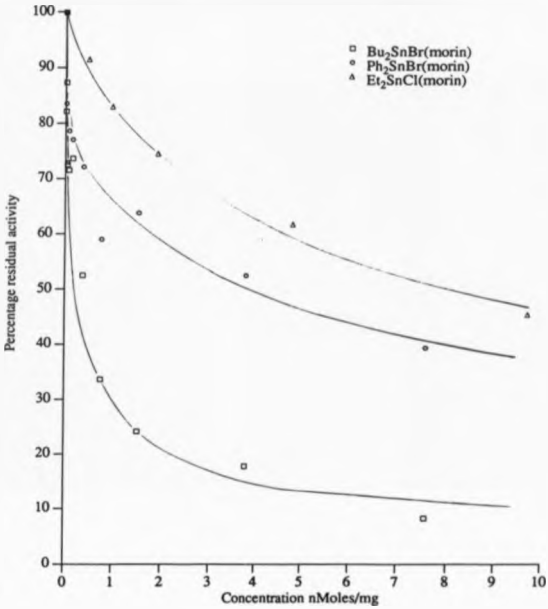


Fig 3.3.3c Resistance of mitochondrial ATPase to organotin derivatives of morin (penta-hydroxyflavone). Rat liver mitochondria were assayed for ATPase activity in the presence of varying concentrations of antibiotic as described in section 2.2. Concentration of inhibitor is given in nM per mg of mitochondrial protein.

mitochondrial matrix. On adding an uncoupler, for example CCCP, a sharp decrease in fluorescence is produced (Figure 3.3.4).

This assay was used to determine whether $Bu_2SnBr(of)$ caused uncoupling of the inner mitochondrial membrane. Recorder traces of this compound, compared with Bu_3SnAc, are shown in Figure 3.3.4. Bu_3SnAc causes uncoupling to take place, but $Bu_2SnBr(of)$ does not significantly affect the mitochondrial membrane potential. As the excitation and emission wavelengths of $DSMP^+$ are 479 and 589 nm respectively, no interference was anticipated due to the fluorescence of $Bu_2SnBr(of)$ (ex.400 em.470 nm). Because of its penta-coordinated Sn, $Bu_2SnBr(of)$ is of the same general structure as the Ve series of compounds described by Aldridge (Aldridge, 1978, Aldridge *et al*, 1981, Emanuel *et al*, 1984), and this is reflected in the properties thus demonstrated. It inhibits mitochondrial ATPase, although it is not as efficient an inhibitor as Ve 2283 (pI_{50} 0.15nmoles mg^{-1}protein) or Ve 2281 (pI_{50} 0.10nmoles mg^{-1} protein). However like the Ve compounds it does not affect mitochondrial membrane potential (Connerton, 1986).

To determine whether the energetic state of the inner mitochondrial membrane affected the fluorescence of bound $Bu_2SnBr(of)$ the uncoupler CCCP was used. $Bu_2SnBr(of)$ was added to rat liver mitochondria and its fluorescence allowed to reach steady-state, after which 2µl of CCCP was added. The recorder trace is shown in Figure 3.3.5, and from this it can be seen that the fluorescence enhancement is unchanged when the mitochondrial membrane is uncoupled.

The non-fluorescent alkyl tin compound Bu_3SnAc can be used to lower the fluorescence of $Bu_2SnBr(of)$ to its baseline in a titratable fashion (Figure 3.3.6). Other ATPase inhibitors can also be titrated against the fluorescence (Figure 3.3.7). The ATPase inhibitor venturicidin causes the greatest fluorescence decrease, followed by leucinostatin. Oligomycin has negligible affect on the fluorescence. This effect is more evident when mitochondria are preincubated with the inhibitors venturicidin and

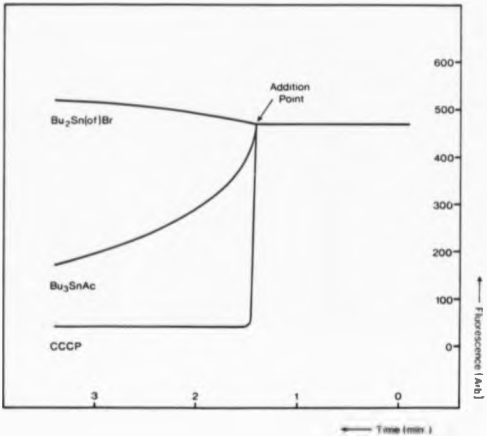


Figure 3.3.4 The compound $Bu_2SnBr(of)$ does not affect mitochondrial membrane potential. Rat liver mitochondria were incubated with the fluorescent probe DiSMP$^+$ (2 µl of a 1 mM solution). After reaching maximum fluorescence 2 µl of a 10mM solution of $Bu_2SnBr(of)$ was added. The uncoupler CCCP (1 µl of a 1mM solution) was used to demonstrate the effect that lowering mitochondrial membrane potential has on the fluorescence of DiSMP$^+$.

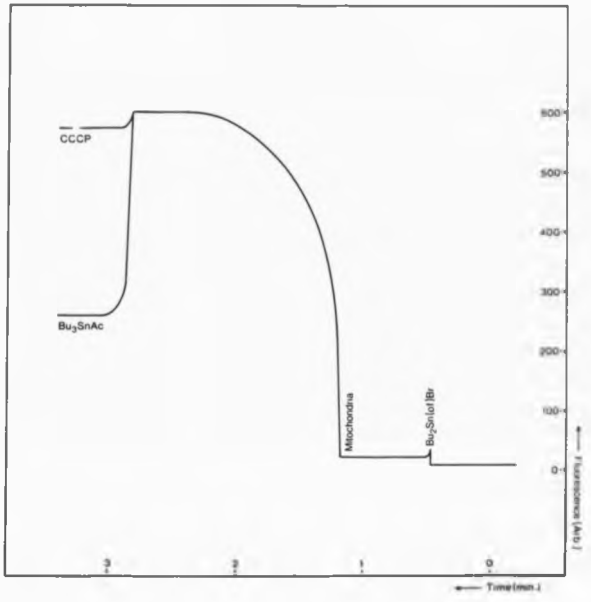


Figure 3.3.5 The uncoupler CCCP does not affect the fluorescence of $Bu_2SnBr(of)$. $Bu_2SnBr(of)$ was added to mitochondria in mitochondrial resuspension buffer, and after reaching maximum fluorescence CCCP was added (2 µl of a 1 mM solution). The experiment was repeated adding 2 µl of 1 mM Bu_3SnAc

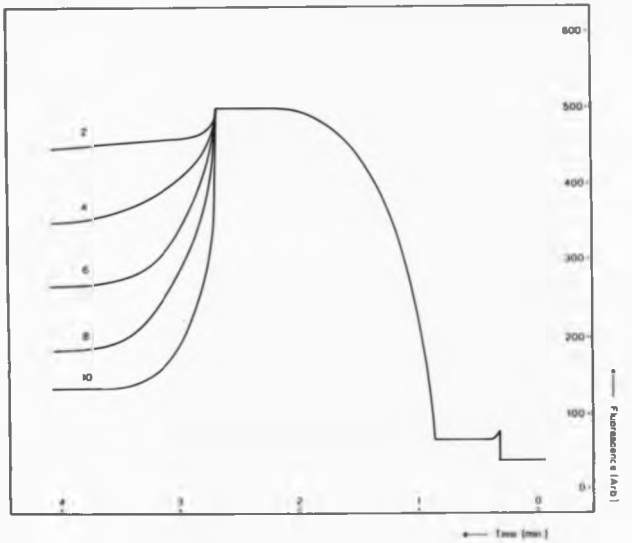


Figure 3.3.6 The non-fluorescent organotin compound Bu_3SnAc can be used to lower the fluorescence of $Bu_2SnBr(of)$ in a titratable fashion. $Bu_2SnBr(of)$ (2 µl of a 1 mM solution) was incubated with rat liver mitochondria in mitochondrial resuspension buffer, and after reaching maximum fluorescence additions of Bu_3SnAc were made (in nmoles added per assay).

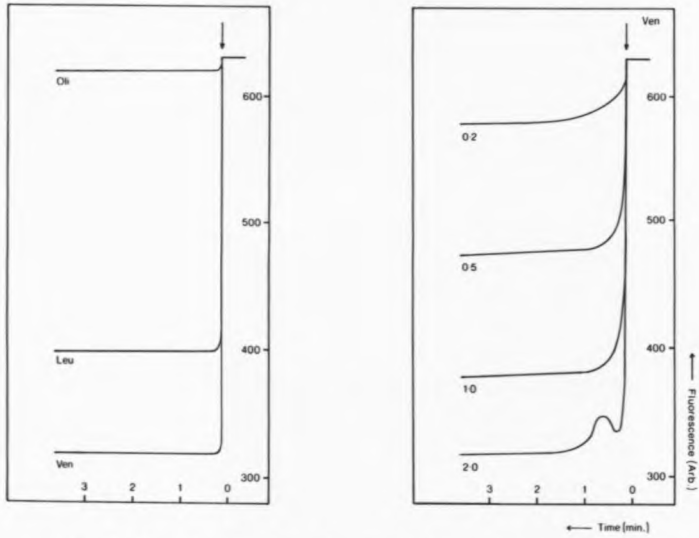


Figure 3.3.7 Some ATPase inhibitors lower the fluorescence of $Bu_2SnBr(of)$ (a) The mitochondrial ATPase inhibitors oligomycin (oli), venturicidin (ven) and leucinostatin (leu) were added to mitochondria incubated with $Bu_2SnBr(of)$ (2 µl of a 1 mM solution) (b) Titration of varying concentra-

oligomycin prior to addition of Bu_2SnBr(of). This provides further evidence that venturicidin and oligomycin have differing binding sites, and supports research done by other authors (Cain, 1976, Kiehl and Hatefi, 1980).

On adding Bu_2SnBr(of) to smps fluorescence enhancement is observed, which is titratable with Bu_3SnAc. The increase in fluorescence is greater (per mg protein) in smp preparations and is presumed to be due to the increase in the ATP synthase per unit protein in the smp preparation (which consists only of mitochondrial inner membrane protein, Figure 3.3.8). An increase in fluorescence enhancement per mg of mitochondria can also be demonstrated in rat heart preparations, in comparison with rat liver mitochondria. Heart mitochondria are enriched in ATPase because of the increased requirement for ATP in that tissue.

3.4. CONCLUSIONS

Derivatives of 3-hydroxyflavone and morin have been investigated to determine their possible use as extrinsic fluorescent probes for the mitochondrial inner membrane protein ATP synthase. The Sn compounds derived from reaction with 3-hydroxyflavone were generally found to give a greater fluorescence enhancement and be more effective as ATPase inhibitors than the corresponding morin derivatives. Thus a 3-hydroxyflavone derivative was chosen for further study (Bu_2SnBr(of)). This compound reflected the properties of the previously studied group of penta-coordinate Sn compounds (Aldridge, 1978, Aldridge *et al*, 1981, Emanuel *et al*, 1984), although it was less effective as an ATPase inhibitor. When added to energised mitochondria Bu_2SnBr(of) did not affect the membrane potential, and conversely uncoupling agents had no effect on its fluorescence. Enhancement of fluorescence was observed in submitochondrial particle preparations which were produced with no membrane potential. The enhancement of fluorescence has been ascribed to a binding and not a transport phenomenon.

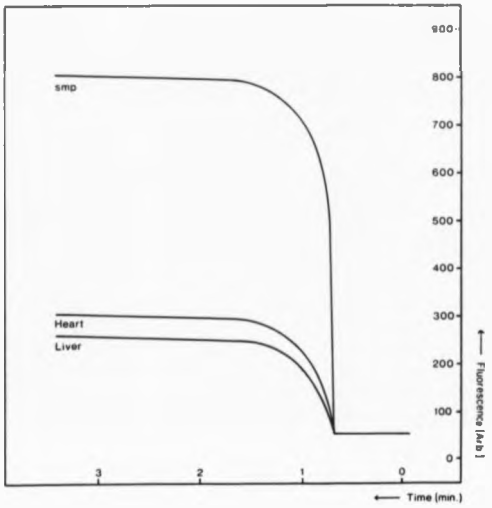


Figure 3.3.8 The fluorescence enhancement of $Bu_2SnBr(of)$ is greater (per mg of protein) in submitochondrial particles than in mitochondrial preparations. $Bu_2SnBr(of)$ (2 µl of a 10 mM solution) was incubated in mitochondrial resuspension buffer with 0.2 mg of protein from submitochondrial particles (smp) rat heart and rat liver preparations. Incubation was continued until a maximum value for fluorescence was reached.

The binding site of these compounds has been tentatively identified as identical with the tri-alkyl tin binding site on the ATP synthase F_0 domain as a result of the following evidence:

1. The fluorescence enhancement observed is due to binding of the compound to a site or sites within the mitochondrion, and not to a transport mechanism as for $DSMP^+$.

2. The fluorescence enhancement increases per mg protein with increasing proportions of the ATP synthase enzyme (and other inner membrane proteins) in the sample (rat heart and smp preparations).

3. Mitochondrial ATPase is inhibited by these compounds but ATPase F_1 is not affected.

4. The fluorescence of the compound is lowered by, and can be titrated against, additions of a non-fluorescent tri-alkyl tin solution, which is consistent with the compounds competing for the same site.

The binding site is also competed for by venturicidin and to a lesser extent leucinostatin, but not by oligomycin, providing further evidence that the venturicidin and oligomycin sites are non identical.

The possible use of these compounds as fluorescent probes for mitochondrial ATPase F_0 is discussed in Chapter 6.

CHAPTER 4

ISOLATION AND SEQUENCE ANALYSIS OF THE GENE *PET*$_{57}$

4.1. INTRODUCTION

As *S.cerevisiae* is a facultative anaerobe it can stably propagate mutations on fermentable media which cause respiratory-deficient mitochondria. Nuclear-coded mutations conferring this phenotype are termed *pet* mutations. The nuclear-coded gene products are fundamentally important in the biogenesis of mitochondria, being both directly and indirectly involved in mitochondrial respiration/oxidative phosphorylation. The genetic material required for assembly of such a complex organelle must comprise a significant proportion of the cell's genetic information.

Pet mutants can be produced both as conditional and non-conditional mutants. Anticipating that some *PET* products may be shared by the rest of the cell, conditional mutants have been generated (Michaelis *et al*, 1982, Edwards *et al*, 1983). However this method has the disadvantage that the mutant phenotype in not always well differentiated under selective conditions. Although a more limited assortment of non-conditional mutants can be generated, they are considerably easier to clone by complementation with a wild-type recombinant plasmid library (Dieckmann and Tzagoloff, 1983).

All of the mutants produced by A. Tzagoloff *et al* (1975a) were of the non-conditional type, selected for their inability to grow on glycerol, a non-fermentable substrate. Initially five phenotypic classes were defined depending on which of three mitochondrial enzyme activities were affected.

This chapter describes the cloning and sequencing of one of these mutants, which was deficient in ATP hydrolytic activity.

4.2. MATERIALS AND METHODS

The following reagents were purchased from Sigma: DTT, β-mercaptoethanol, RNAase A, Ampicillin, BSA. Glusulase was purchased from DuPont and dialysis tubing from Spectropore. All other reagents were of Anal-R grade purity, with the exception of those used for culture media.

Media (supplementary)

In addition to YPD and YPG, Galactose containing medium was also used, primarily in growing yeast for mitochondrial preparation.

YPGal
1% (w/v) Yeast extract
2% (w/v) Bactopeptone
2% (w/v) Galactose
2% (w/v) Agar for plates

Transformation of *S.cerevisiae* (Beggs, 1978)

Transformation medium

This medium is used for growing cells from spheroplasts, hence it has an inclusion of 1.2M sorbitol to prevent lysis of spheroplasts.

T	TGly
1.2M Sorbitol	1.2M Sorbitol
0.67% N-base w/o amino acids	0.67% N-base w/o amino acids
2% (w/v) Glucose	0.05% (w/v) Glucose
	3% (v/v) Glycerol
2% (w/v) Agar for plates	2% (w/v) Agar for plates

All operations were sterile, and at room temperature unless stated. An overnight inoculum in 10ml YPGal was grown to early stationary phase (approx. 10^8 cells). The culture was centrifuged for 5 mins at 2K rpm, and the cell pellet washed with 5ml dH_2O.

Cells were re-suspended in 3ml DTT solution and shaken for 10 mins at 30°C. They were then centrifuged for 5 mins at 2K rpm and washed twice with 1.2M sorbitol.

DTT solution	Glusulase solution
1.2M sorbitol	1.2M sorbitol
25mM EDTA pH 7.5	100mM Na citrate pH 6.0
7.7 $mgml^{-1}$DTT	10mM EDTA
	2% glusulase

Both solutions were sterilised by filtration

Cells were re-suspended in 1ml glusulase solution and shaken at 30°C for 30 mins. 1.2M sorbitol (5ml) was added and the suspension was centrifuged and washed once with 1.2M sorbitol, and once with 1.2M sorbitol, 10mM $CaCl_2$.

After re-suspending in 0.1ml 1.2M sorbitol, 10mM $CaCl_2$, cells were transferred to an Eppendorf tube. DNA (5-10µl) was added, and the cells incubated at 30°C for 15 mins. To this 0.9ml of 20% PEG, 10mM Tris pH 8, 10mM $CaCl_2$ was added, mixed by inversion, and incubated for 10 mins at room temperature. The tube was then centrifuged for 5 mins at 2K rpm and the supernatant removed with a sterile pasteur pipette.

Cells were re-suspended in 150µl of 1.2M sorbitol, 10mM $CaCl_2$, plus 50µl YPD in 1.2M sorbitol and shaken at 30°C for 20 mins, then plated on selective medium containing 1.2M sorbitol (TGly plates) and non-selective medium to check the efficiency of transformation (T plates). Transformant colonies could be seen under magnification after two days incubation at 30°C.

Transformation of *E.coli* (Cohen *et al*, 1972)

E.coli RR1 was inoculated into 50ml L-broth and grown at 37°C to 100 Klett units. All further steps were carried out between 0-4°C unless stated. Cells were centrifuged in a Sorvall centrifuge with SS34 rotor at 3.5K rpm for 10 mins, washed with 10ml 10mM $CaCl_2$ and re-suspended in 10ml 50mM $CaCl_2$. After 30 mins on ice cells were

centrifuged and re-suspended in 5ml 50mM $CaCl_2$, and 200µl aliquots transferred to sterile Eppendorfs.

DNA (5-10µl) was added to competent cells, which were incubated on ice for 30 mins. Cells were heat shocked at 42°C for two minutes and added to 3ml of L-broth. After shaking at 37°C for 45 mins, 0.2ml was spread on a selective plate (containing ampicillin, amp). Remaining cells were spun down, re-suspended in a small volume, and spread on a second plate.

After growing at 37°C overnight transformant colonies were picked and patched out for DNA minipreps.

E.coli plasmid preparation

Large scale

An overnight culture grown up in 500ml L-broth plus ampicillin was spun down in an International centrifuge at 8K rpm for 10 mins. Cells were washed with 100ml 10mM tris/Cl pH 7.5 and re-suspended in 25ml lysozyme mix.

Lysozyme mix

50µl RNase ($10mgml^{-1}$ stock)
4mg lysozyme
50mM glucose
10mM Tris
1mM EDTA
dH_2O to 100ml

After incubation on ice for 45 mins, 50ml 0.2N NaOH, 0.1% SDS was added and the suspension was stirred gently. 7M NaOAc (36ml) was added with thorough mixing, followed by centrifugation in a Sorvall centrifuge at 12K rpm for 15 mins.

To the supernatant from this spin three volumes of 100% ethanol was added, the suspension spun at 4K rpm for 10 mins and the supernatant discarded. The pellet was

dissolved in 10ml NH_4OAc, re-precipitated with ethanol and re-dissolved in 10ml TE buffer. This solution was pipetted into dialysis tubing and dialysed twice against 2l of TE to remove any ethanol.

A caesium chloride gradient was made up by adding 6.5g CsCl per 6g DNA solution. Ethidium bromide (5mg ml^{-1}, 130µl) was added per tube, which were centrifuged in a Beckman ultracentrifuge 50Ti rotor at 39K rpm for 40hrs (at 20°C). The plasmid band runs lower than the nuclear DNA band and was taken from the gradient using a bent syringe. It was then extracted three times with isoamyl alcohol to remove EtBr and twice with ether. The DNA was dialysed twice against 2l of 10mM Tris/Cl pH 7.5, 0.1mM EDTA to remove CsCl, dispensed into Eppendorfs, and stored at −70°C.

Medium Scale (Triton Procedure)

Cells were scraped with a sterile spatula from a confluent *E.coli* plate spread the previous day. These were transferred to 0.5ml 5% sucrose, 20mM Tris/Cl pH 8.0 and vortexed until well suspended. Lysozyme solution (0.8ml) was added and cells incubated on ice for 30 mins.

Lysozyme solution	Lysis solution
2ml lysozyme (5$mgml^{-1}$ stock)	3ml of 10% Triton X-100
1ml of 0.125M EDTA	75 ml of 250mM EDTA
in Tris/Cl pH 8.0	15ml of 1M Tris/Cl pH 8.0
0.1ml RNase A (10$mgml^{-1}$ stock)	dH_2O to 100 ml

Lysis solution (0.8ml) was added, the suspension was mixed by inversion and centrifuged at 40K rpm in a Beckman ultracentrifuge 50Ti rotor for 15 mins at 4°C. The supernatant from this spin was transferred to a 15ml corex tube, followed by an equal volume of H_2O-saturated phenol. After mixing the phases were separated at 7K rpm for 10 mins in a Sorvall centrifuge (SS34 rotor), and the upper phase washed three times with ether. The DNA was then precipitated by making 0.3M NaCl, adding three volumes of 100% ethanol and spinning down at 7K rpm for 10 mins. The oily layer left by this

precipitation contains DNA together with some Triton. The oil was dissolved in 2M NH_4OAc and the DNA reprecipitated with 3ml of ethanol. After one further reprecipitation the DNA was washed with 80% ethanol and dried in a speed-vac. The pellet was dissolved in 200-500µl H_2O, and stored at -70°C.

Small Scale (Birnbaum Preparation, Mini-prep)

Cells were taken with sterile toothpicks from patches grown up overnight, and suspended in lysozyme mix (100µl). After a 5 min incubation on ice 100µl 2N NaOH, 0.1M SDS was added with mixing, followed by 75µl 7M NaOAc. The eppendorf tubes were centrifuged for 5 mins in a microfuge and the supernatant transferred to new Eppendorf tubes.

DNA was precipitated with 100% ethanol, re-dissolved in 200µl of 2M NH_4OAc, 0.2M EDTA, re-precipitated, washed and dried in a speedvac. The DNA was then dissolved in 30µl H_2O, and 5µl was used in restriction enzyme digests.

***S.cerevisiae* plasmid preparation**

Transformed *S.cerevisiae* was grown up overnight in 10ml of WO with amino acid supplements to allow growth. Cells were spun down at 2K rpm for 5 mins, re-suspended in 1ml 1.2M sorbitol, and transferred to an Eppendorf tube. After centrifuging, cells were re-suspended in zymolase mix, and incubated at 30° for 1 hour.

Zymolase mix	RNase Solution
3ml of 2M sorbitol	0.5ml of 0.5M glucose
0.5ml of 1M Na citrate pH 7.0	0.125ml of 1M Tris/Cl pH 8.0
0.6ml of 0.5M EDTA	0.1ml of 0.5M EDTA
50µl β-mercaptoethanol	0.125ml of 10mgml^{-1} RNase A
25mg zymolase 20000	4.1ml H_2O
1ml H_2O	

The spheroplasts thus produced were completely re-suspended in 100µl RNase solution and 200µl 0.2N NaOH, 1% SDS was added immediately. The suspension was mixed by

inversion and incubated on ice for 30 mins. Tubes were centrifuged in a microfuge for two minutes, the supernatant collected and transferred to a fresh Eppendorf tube. The DNA was precipitated with 100% ethanol twice, washed in 80% ethanol, and dried in a speedvac.

The DNA pellet was then dissolved in 100µl of sterile 10mM Tris/Cl pH 7.5, 30mM $CaCl_2$ and used to transform *E.coli*.

DNA manipulation

Restriction Enzyme Digestion and Gel Electrophoresis

Standard procedures were used for restriction endonuclease digestion, according to manufacturer's instructions. An appropriate volume of Ficol stop mix (FSM) was added to the digest, which could then be loaded on 1% vertical agarose gels made with TBE buffer. Gels were run at 100mA for 1hr with TBE buffer in both upper and lower tanks, stained with EtBr and photographed using a transilluminator.

For large-scale preparation of DNA fragments a flat bed gel was used, with EtBr added to both gel and running buffer at 1µl to 10 ml (from 5mgml^{-1} stock).

Treatment with CIP

Calf intestinal phosphatase (CIP) is used to remove the 5' phosphate from DNA to prevent self-ligation. When restriction enzyme digests were carried out at 37°C, CIP was added before starting the incubation. If digests required incubation at a higher temperature, CIP was added after the digest incubation, and the DNA incubated a further 30 minutes at 37°C.

Preparative Isolation of DNA

DNA was isolated preparatively by electroelution. Bands were sliced from agarose gels

and inserted into dialysis tubing containing 1/10 TBE buffer. The tubing was clipped at both ends after removing any bubbles and excess buffer. It was placed in an electroelution tank containing 1/10 TBE buffer, and left for 5-10 mins at 600V.

The agarose slice was removed after checking that the DNA had eluted out of the agarose on a transilluminator. After agitating the remaining liquid in the dialysis tubing to displace DNA from the membrane, the liquid was transferred to an Eppendorf tube. This was extracted once with phenol, three times with ether, ethanol precipitated, ethanol washed, and dried. The pellet was dissolved in H_2O and 1µl run on a check analytical gel.

Ligation of DNA

Vector DNA used in ligation reactions was treated with calf intestinal phosphatase (CIP) to prevent self-ligation. Two volumes of ligase buffer were made up using equal proportions of the following solutions, mixed in the stated order.

2× ligase buffer
0.4M Tris/Cl pH 7.5
0.1M $MgCl_2$
10mM ATP
0.1M DTT
0.5mg ml^{-1} BSA

DNA fragments to be ligated were added in equal proportions established by comparison on an analytical gel. The ligation was set up as follows :-

2× ligase buffer	10µl
H_2O + DNA(s)	10µl
T4 DNA ligase	1µl

Incubations were carried out either at 37°C for 2-3hrs or at room temperature overnight.

Treatment with Klenow

DNAs were treated with Klenow (DNA polymerase I, large fragment) in a reaction mix made up as follows :-

nick translation buffer	10µl
each dNTP (0.8M)	10µl
Klenow fragment	1 (5U)
H_2O + DNA	to 100µl

The reaction was incubated at room temperature for 30 mins and stopped by the addition of 4µl 0.5M EDTA.

DNA Sequencing (Maxam and Gilbert, 1977)

DNA was cut to lengths ranging between 100-600 bases with a restriction endonuclease. The fragments generated were treated with CIP to remove 5' phosphates. When the digestion had gone to completion the reaction mix was extracted with phenol (once) and ether (three times), and the DNA ethanol precipitated, washed and dried.

5' End Labelling

The DNA precipitate was dissolved in 20µl H_2O in an Eppendorf tube, and heated at 90°C for 5 mins to separate the two DNA strands. The Eppendorf was cooled immediately on wet ice to prevent the DNA strands from re-annealing. The following reagents were added in stated order to the side of the tube :-

kinase buffer	3µl
spermidine (0.1M)	1µl
γ^{32}P-ATP	2µl
polynucleotide kinase	2µl

The reagents were mixed and incubated at 37°C for 1hr. 2M NH_4OAc, 0.2mM EDTA (70µl) was then added and the DNA ethanol precipitated, washed and dried.

The DNA was then dissolved in 30µl DMSO dye mix, heated at 90°C for 7 mins and quickly cooled on wet ice. It was then loaded on a thick 6% polyacrylamide gel (strand separation gel) and run at 250V overnight.

DMSO dye mix	
30% DMSO, 1mM EDTA	100µl
xylene cyanol/bromophenol blue	5µl

Gel Elution

The strand separation gel was removed from the gel tank, one glass plate removed and the gel covered with saran wrap. The gel was then exposed to X-ray film without an intensifying screen, usually for one minute (but dependent on the radioactivity of the gel), and the position of the gel very carefully marked onto the X-ray film.

The position of each DNA strand was determined by carefully aligning gel and developed film. Bands were cut from the gel with a sharp scalpel blade and counted for radioactivity. Each separate excised band was inserted into a 1000µl pipette tip sealed at its point with a haemostat and plugged with glass wool.

The gel slices were ground to a gritty consistency with a metal rod, and 0.4ml elution buffer added. Each pipette tip was covered tightly with parafilm, placed in a 5ml glass tube, and incubated at 45°C for 1hr. After incubation the sealed ends were cut open, parafilm removed, and the liquid drained into the glass tubes. To extract the remaining liquid the pipette tips and tubes were spun at 2K rpm for 5 mins. Carrier RNA (10µl) was added to each glass tube, followed by 1ml 100% ethanol. The suspension was transferred to etched Eppendorf tubes, frozen on dry ice for 5 mins and the DNA spun down in a microfuge. DNA was re-suspended in 300µl of 2M NH_4OAc, 0.2mM EDTA, 0.02N HAc, re-precipitated, washed, dried and counted for radioactivity.

Sequencing Reactions

Depending on the radioactivity of the pellet, the DNA was dissolved in >40µl (minimum) H_2O. The solution was distributed between four etched Eppendorf tubes (marked with the number of the strand and G, A, T, C) for each of the sequencing reactions (6µl for G and C, 10µl for A and T). Reaction times depended on the length of the DNA fragment (larger fragments have more reaction sites) as follows :-

Reaction	No. of bases			
	600-400	400-300	300-200	<200
G	30 sec	30 sec	30 sec	30 sec
A	6 min	8 min	10 min	12-15 min
T	4-5 min	7 min	8-9 min	10 min
C	4-5 min	7 min	8-9 min	10 min

Table 4.2.1 Incubation time for sequencing reactions

Samples were re-suspended in a volume of formamide loading buffer based on their radioactivity after the piperidine wash. The minimum volume used was 12-13µl, to give enough solution to enable two or more gels to be run.

Formamide loading buffer	
formamide	100µl
xylene cyanol and bromophenol blue	10µl
0.5M EDTA	1µl

Samples were heated at 90°C for 5 mins, cooled quickly on wet ice, and loaded on a sequencing gel. These gels were thin polyacrylamide/urea slabs, 7% polyacrylamide used for 1st and 2nd channels, 6% for 3rd channels and 20% used to determine the order of bases proximal to the 5' label.

Gels were exposed overnight to Kodak X-omat film with a Lightning intensifying screen at -70°C.

G	A	T	C
DNA 6µl add 200µl G-buffer 1µl DMS, mix, spin (*) secs at r.t. 50µl DMS-stop then 5µl ctRNA, mix 1ml 100% EtOH, mix freeze 3', cfuge 5' rinse 1× w/ 80% EtOH add 300µl 2M NH_4OAc/ EDTA/HOAc mix 0.9ml 100% EtOH freeze 5', cfuge 5' wash 2× 80% EtOH/EDTA 1× 80% EtOH dry	DNA 10µl add 8µl citrate pH 4.0 mix, spin (*) mins at 80°C to stop put on ice add 300µl 2M NH_4OAc/EDTA/HOAc then 5µl ctRNA, mix 0.9ml 100% EtOH, mix freeze 5', cfuge 5' wash 2× 80% EtOH/EDTA 1× 80% EtOH dry	DNA 10µl add 20µl ddH_2O mix, spin 30µl hydrazine mix, spin (*) mins at r.t.	DNA 6µl add 24µl 5M NaCl mix, spin 30µl hydrazine mix, spin (*) mins at r.t.
		add 300µl 2M NH_4OAc/EDTA/HOAc 5µl carrier tRNA, mix 0.9ml 100% EtOH, mix freeze 3', cfuge 5' rinse 1× with 80% EtOH add 300µl 2M NH_4OAc/EDTA/HOAc, mix 0.9ml 100% EtOH, mix Freeze 3', cfuge 5' wash 2× 80% EtOH/EDTA 1× 80% EtOH dry	

Piperidine wash:

add 40µl 1.5% piperidine (150µl piperidine:850µl cold H_2O)
mix, spin
incubate 30 mins at 90°C, cover
immediately into speedvac for 30 mins
wash 2× with 12µl hot H_2O (ddH_2O at 90°C)
20 mins for each wash while centrifuging in the speedvac
dry

* for times see table 4.2.1

Table 4.2.2 Maxam and Gilbert DNA sequencing reactions

Miscellaneous

Nick Translation

The reaction mix was as follows, incubation was at 30°C for 1hr.

DNA	4µl
10× nick translation buffer	2µl
3× cold NTPs	3× 3µl
1× labelled NTP (TTP)	3µl
DNase (0.01mg ml^{-1})	1µl
DNA polymerase	1µl

During the incubation a G50 column was prepared. A 1ml syringe (with plunger removed) was packed to capacity with G50 swollen in TE. This was placed in a test tube and aligned so that it drained into an Eppendorf tube. The column was spun at 2K rpm for 4 mins and washed through with two volumes of 100µl of STE.

STE
100mM NaCl
10mM Tris/Cl pH7.5
1mM EDTA

The reaction was stopped with 80µl of 50mM EDTA, and loaded onto the column (total 100µl). The column was centrifuged at 2K rpm for 4 mins and drained into a thick-walled Eppendorf. This solution was used as a probe in hybridisation proceedures.

Colony Hybridisation

An ampicillin plate of *E.coli* transformant colonies was grown up overnight and replicated onto a second ampicillin plate. This replica was left at 37°C for 4hrs and stored at 4°C. A sterile Whatman #40 filter paper, notched for orientation, was carefully lowered onto the master plate ensuring no air bubbles were trapped between plate and filter. The top of the filter was labelled with pencil - label side up is colony side down. The plates were turned over and left for 15 mins at room temperature. The filter was

removed with tweezers and floated colony side down in a tray of 0.5M NaOH (250ml) for 15 mins. After three 300ml washes in neutralising solution, the filters were placed colony side up on a piece of Whatman #3 paper and dried at 80°C for 1hr.

Neutralising solution	20× SSC
1M Tris/Cl pH 8.0	3M NaCl
3M NaCl	0.3M Na citrate

Filters were rinsed in 6× SSC and bagged colony side out, 2 filters per bag. Probe DNA (10µl from nick translation) was added to 0.5ml cDNA, heated at 90°C for 5 mins and cooled rapidly on wet ice. Bags were filled with 5ml hybridisation buffer and injected with the probe. Sealed bags were incubated at 65°C overnight.

Hybridisation buffer
6× SSC
1% SLS

The filters were washed twice for 15 mins in 300ml of 2× SSC, 0.1% SDS, then four times for 5 mins in 250ml of 5mM Tris/Cl pH 8.0. After drying on a paper towel colony side up, filters were exposed for 5hrs or overnight depending on their radioactivity.

***S.cerevisiae* Chromosomal DNA Preparation**

A 1% inocculum into 500ml of YPD was grown up overnight and harvested at 5K rpm for 10 mins in a Sorvall GSA rotor. Cells were pooled and washed in 1.2M sorbitol. The pellet was re-suspended in 3ml zymolase mix per gram wet weight of cells, and incubated at 37°C for 1hr. After optical microscope examination to ensure spheroplast formation had occurred, 100ml of 1.2M sorbitol was added and the spheroplasts were pelleted at 5K rpm for 5 mins. After 1 further wash in 1.2M sorbitol, cells were re-suspended in 3ml of lysis solution per gram of cells.

Lysis solution	PCI
1.5% SDS	250ml $CHCl_3$
20mM Tris pH 7.5	250ml H_2O sat. phenol
1mM EDTA	10ml isoamyl alcohol
0.2M NaCl	

The suspension was mixed by rolling gently with a glass rod to minimise breakage of the DNA. An equal volume of PCI was added, gently mixed and centrifuged at 5K rpm for 10 mins. The supernatant was collected with a wide mouth pipette, an equal volume of PCI added to it, centrifuged, and the supernatant further extracted with an equal volume of $CHCl_3$. The aqueous phase was transferred to a corex tube and two volumes of 100% ethanol added. DNA was pelleted at 2K rpm for 5 mins, washed twice with 80% ethanol and inverted to dry. The pellet was dissolved in 6ml of TE, and dialysed against 2l of TE for 2hrs. CsCl gradients were set up as described previously, adding only 40µl EtBr to avoid breakage of the DNA. The centre prominent band was taken from the gradient and treated as in section 4.2.2.

5µl of the DNA solution was used as a test digest.

Southern Blotting

Total chromosomal DNA was digested with a restriction endonuclease and the digest run on a 1% agarose gel to produce a smear of DNA. The EtBr-stained gel was photographed and notched. The gel was gently shaken twice for 20 mins in 500ml of 0.4N NaOH, 1.5M NaCl to denature, and neutralised for three periods of 10 mins in 350ml of 1M Tris/Cl pH 8.0, 3M NaCl. The DNA was transferred overnight to nitrocellulose in 20× SSC.

The nitrocellulose was rinsed in 2× SSC for 5 mins and dried between Whatman #1 papers. It was then baked for 2hrs at 80°C. The nitrocellulose was wetted in 6× SSC and sealed in a bag with 5ml of hybridisation mix. The heat-denatured probe was injected

and the sealed bag incubated at 65°C overnight.

The nitrocellulose was washed twice for 15 mins in 200ml of 2× SSC, 0.1% SDS, then for four 5 min periods in 5mM Tris/Cl pH 8.0. Excess liquid was blotted off and the gel exposed overnight at –70°C.

4.3. RESULTS

Generation and Classification of *pet* Mutants

The strategy employed by Prof. A. Tzagoloff and colleagues to produce and classify *pet* mutants is briefly described in this section, and is discussed in detail in Tzagoloff *et al*, 1975a. Yeast strain D273-10B/A1 was mutated by treatment with N-methyl N-nitro nitrosoguanidine or ethyl methane sulphonate, which are mutagens shown to specifically affect nuclear DNA, and mutants selected for their inability to grow on glycerol containing medium. These respiratory-deficient mutants produced were screened for their ability to carry out mitochondrial protein synthesis by differential inhibition of nuclear protein synthesis with cycloheximide, and mutants deficient in mitochondrial protein synthesis were stored for future analysis, but were not investigated in this study. This step eliminates the most probable mitochondrial mutation, a mit$^-$ mutation in the large rRNA-coding gene. It also eliminates nuclear mutations which cause deficiencies in mitochondrial protein synthesis. Nuclear-coded mutants were selected on their ability to complement a ρ^0 tester strain derived from *S.carlsbergensis* by ethidium bromide mutagenesis (CB11 ρ^0). Crosses which gave rise to diploids showing no growth on EG after 2-3 days were classified as cytoplasmic.

Mutants were further characterised according to phenotype by preliminary enzymatic analysis. Assays for ATPase, cytochrome oxidase and cytochrome QH_2 reductase were carried out and mutants grouped according to which enzyme activity, or combination of enzyme activities were affected (Tzagoloff *et al*, 1975a,b,c). Five general groups were

defined, three of which describe specific deficiencies in the enzyme complexes assayed, one in which pleiotropic deficiency in more than one of the enzyme complexes was observed, and one in which none of the complexes in the assay were affected. These three enzymes are unique in that they are under dual genetic control and thus of much interest in terms of biosynthetic control, transport and assembly of the subunits of the complex.

Each mutant was crossed to CB11 and respiratory deficient spores were isolated to generate haploids of different mating type. These were crossed to the original mutants, and those producing diploids which did not grow on EG after 2-3 days were assigned the same complementation group. This experiment classifies mutants together that affect the same activity, thus one cannot compensate for the mutation in another.

Preliminary Genetic and Biochemical Analysis of Complementation Group G57

The genotypes of strains used in this study are show in Table 4.3.1. A complementation group, shown to have deficient ATP hydrolase activity in the initial selection carried out by Prof. Tzagoloff and colleagues, was chosen for analysis. Firstly experiments were carried out to confirm the nuclear location of the mutation, and the ATPase deficient phenotype that had been evident in preliminary enzymatic analysis. Complementation group G57 is comprised of 8 independent isolates which have recessive mutations producing a respiratory deficient phenotype. Mutants are complemented by crossing to the haploid ρ^0 strain CB11 ρ^0, indicating that the defect is located in a nuclear gene as the ρ^0 strain has no mitochondrial genetic material and thus cannot complement any mitochondrial mutation. Mutants are not complemented by inter-group crosses, confirming that they are in the same linkage group. The mutation has a stable genotype having a low rate of conversion to cytoplasmic petites when grown under non-selective conditions.

Strain	Nuclear Genotype	Mitochondrial Genotype
CB11	a	ρ^+
CB11ρ^0	a	ρ^0
D273/10B-A1	α *met*$^-$	ρ^+
D273/10B-A1ρ^0	α *met*$^-$	ρ^0
KL14ρ^o	α	ρ^0
W303	a/α *his*$^-$*ura*$^-$*trp*$^-$*ade*$^-$	ρ^+
B264	a pet_{57}	ρ^+
B264/U1-U4	pet_{57} *ura*$^-$	ρ^+
W150	a pet_{57}	ρ^+
C80	α pet_{57}	ρ^+
N540	α pet_{57}	ρ^+
E54	α pet_{57}	ρ^+
E113	α pet_{57}	ρ^+
E394	α pet_{57}	ρ^+
E695	α pet_{57}	ρ^+
E822	α pet_{57}	ρ^+

Table 4.3.1 Nuclear and Mitochondrial genotype of *S.cerevisiae* strains used in this chapter

Mitochondria were prepared from all of the strains in the linkage group and assayed for ATPase activity according to the method outlined in the following chapter. The results are shown in Table 4.3.2, and from these it is evident that all members of the complementation group G57 show deficiency in mitochondrial ATPase activity, generally showing <10% of the activity of the wild-type.

Strain	S.A.	S.A. + rutamycin
D273/10B-A1	4.304	1.192
B264	0.198	0.167
N540	0.317	0.304
E54	0.315	0.213
E113	1.610	0.476
E394	0.346	0.346
E695	0.565	0.377
E822	0.189	0.169

Table 4.3.2 Specific activity (in $\mu M mg^{-1} min^{-1}$)of mitochondrial ATPase from members of the G37 complementation group. Mitochondria were prepared by the zymolase procedure (described in Chapter 5) from an overnight culture of each mutant strain and tested for ATPase activity. Mitochondrial protein (0.1 mg per assay) was incubated with ATP and inorganic phosphate generated was measured colourimetrically. Specific activity was calculated for each sample, with wild-type mitochondria assayed as a control. Rutamycin was added to a further incubation (5 µl of 1 $mgml^{-1}$) to determine whether the residual ATPase activity was rutamycin-sensitive.

Isolation of the PET_{37} Gene by Complementation

One of the groups 8 members, B264, was crossed to W303 and a number of *PETura*$^-$ spores were collected. The purpose of this is to generate spores with a requirement for uracil, as this is the marker used for the plasmid library, but also to produce a variety of spores for transformation, as different spores from the same cross show much variation in their efficiencies for transformation. Spores B264/U1-B264/U10 were generated from the B264 cross and were used for transformation. These spores were transformed with a wild type library constructed from a SauIIIa partial digest inserted into the BamHI site of shuttle vector Yep24 (Figure 4.3.1). Transformants were selected on TGly plates, which provide selection both for the wild type copy of the *PET* gene and for the *ura*$^+$ plasmid marker. Transformant colonies were picked onto WO after 3 days, and subsequently replicated onto EG as a check on retention of their respiratory competent phenotype. After growing up overnight on non-selective medium (YPD) cells were spread for single colonies on YPD. These plates were grown up overnight, and then replicated onto WO

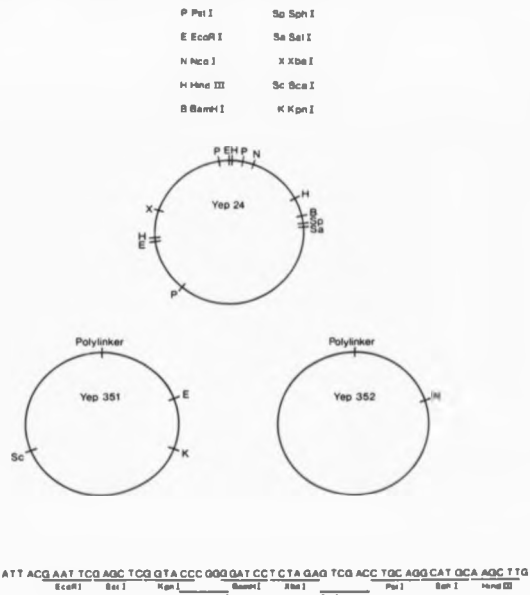


Figure 4.3.1 Plasmid vectors used in this study. The plasmids Yep 351 and Yep 352 were constructed by Hill *et al*, 1986. All three plasmids are shuttle vectors containing the *E.coli* gene specifying ampicillin resistance. Yep 24 and Yep 352 have the *S cerevisiae ura* gene, and Yep 351 carries the *leu* marker.

and EG to check for co-retention/co-deletion of the markers *PET*/*ura*$^+$.

The purpose of this analysis is to check that the *PET* gene is present on the transforming plasmid as there are several other possibilities that would allow the transformed yeast to grow on the selective medium:

1. The complete plasmid containing *ura*$^+$ and *PET* markers could be stably integrated into the chromosomal material of the cell by a recombination event, as its sequence has homology with yeast chromosomal DNA. Any progeny would be *PETura*$^+$ as the integrated DNA could not be lost from the cell.

2. The yeast can spontaneously mutate to *PET* by either reversion of the original mutation or by a suppressor mutation because of the selective pressure imposed. After being transformed by *ura*$^+$ coding plasmids the resulting transformant colonies would have a *PETura*$^+$ phenotype. Plasmids are spontaneously lost by cells and need to be maintained by exerting selective pressure. Thus all progeny would be *PET* but would be either *ura*$^+$ or *ura*$^-$ depending on whether they contain the *ura*$^+$ plasmid.

3. When transforming yeast there is a high probability of more than one type of plasmid entering one cell. This gives a heterogeneous population of plasmids. Progeny from such a transformant having a *ura*$^+$*PET* plasmid allowing it to grow on selective medium, and also a *ura*$^+$ plasmid could have any one of the following phenotypes: *ura*$^+$*PET*, *ura*$^+$*pet* or *ura*$^-$*pet*.

4. The transformant has only the *ura*$^+$*PET* plasmid so the markers are either both retained or both lost in any progeny ie. coretained or codeleted.

The *ura*$^+$*PET* plasmid could be prepared from a type 4 colony, or from a type 3 colony after several rounds of selection for a *ura*$^+$*PET*/*ura*$^-$*pet* segregating colony.

A number of transformant colonies were examined in this way and four which showed good co-retention/co-deletion of markers were selected; B264/U1#1B, B264/U1#2A, B264/U4#1A and B264/U4#2B. The nomenclature used for these strains refers firstly to the spore used in that transformation (e.g. B264/U1), then to the number of the independent transformation (i.e. 1 or 2 as two independent transformations were performed on each spore), and finally to a letter which was sequentially assigned to each transformant taken from a particular plate. Plasmid DNA prepared from these four colonies by the method outlined in section 4.2.1 was used to independently transform *E.coli* RRI. DNA from mini-preps of these *E.coli* transformants was analysed by restriction enzyme digestion with EcoRI. In three of the transformants non-identical but overlapping plasmid inserts were present (Figure 4.3.2), each of these having an identical 0.8 Kbase piece excised from within the insert, and several non-identical fragments. This provides evidence that the correct insert had been isolated, and suggests that the coding region is adjacent to or within the 0.8 Kbase EcoRI fragment.

Subcloning of the PET_{57} gene

The plasmid from B264/U4#2B was selected for further analysis as it was incorrectly thought to have the smallest insert (in fact it had an EcoRI doublet), and was mapped by restriction enzyme digestion (Figure 4.3.3). Using this map restriction sites were chosen to sub-clone smaller fragments of the insert into the shuttle vector Yep 352 which also uses the *ura*$^+$ gene as a selectable marker for the plasmid. Back-transforming plasmid constructs into the spore B264/U4 allowed determination of the region of DNA in which the gene lay.

Firstly pieces ST1 and ST2 were produced by digestion with Sst/Sph and Xba/Sst respectively and inserted into the plasmid Yep 352 to give the plasmid constructs pG57/ST1 and pG57/ST2 respectively. Together with the parent plasmid pG57/T1 these constructs were used to transform B264/U4. The transformant colonies were picked onto YPD plates after 3-4 days. After growing at 30°C for 1-2 days colonies were replicated

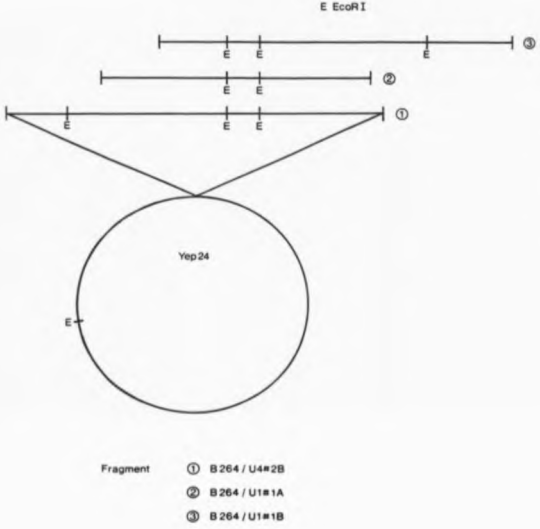


Figure 4.3.2 The DNA fragments isolated which transform strain B264/U4 to respiratory competence. A wild type library made by inserting 5-15 Kbases of DNA from a Sau IIIa partial digest into plasmid vector Yep 24 was used to transform B264 to respiratory competence. DNA isolated from yeast transformants was transferred to *E.coli* and plasmid DNA prepared by the method of Birnbaum. Digestion of DNA from 3 independent yeast transformants with EcoRI showed the plasmid inserts were non-identical but overlapping.

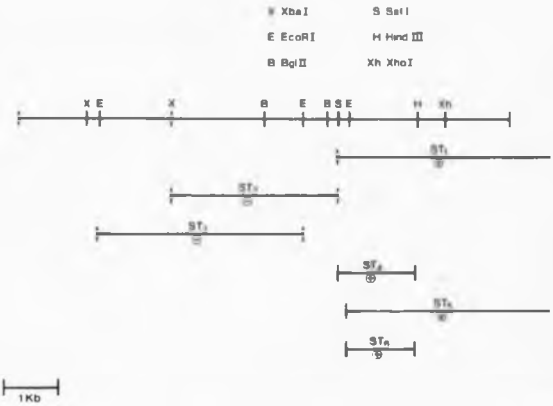


Figure 4.3.3 Restriction map of the plasmid insert B264/U4#2B, together with fragments used for back transformation. Fragments ST_1 to ST_6 were excised from the parent plasmid pQ57/T1 and inserted into the plasmid vector Yep 352. These constructs were used to back-transform into B264/U4. Fragments which transformed this strain to respiratory competence are marked (+), those which failed to do so (−).

onto YPG to determine whether the plasmid insert had transformed them to respiratory competence. On comparison, colonies from certain transformations grow at a faster rate, and these are usually found to be respiratory competent when replicated onto YPG, and thus contain a functional copy of the gene of interest.

In this transformation pG57/ST1 and pG57/T1 gave rise to respiratory competent yeast transformants. The plasmid pG57/ST1 was further digested with EcoRI to remove a 0.2Kbase piece, and self-ligated (pG57/ST3). This procedure was repeated digesting pG57/ST1 with HinDIII to remove a 1.2Kbase piece (pG57/ST4). Transformants produced from both of these plasmids grew up when replicated onto YPG, thus locating the gene between the EcoRI and HinDIII sites as this is common to both plasmids. To verify this location a construct was made by digesting pG57/ST4 with EcoRI, self-ligating and transforming B264/U4 (pG57/ST6). As expected, respiratory competent colonies were produced. Thus the smallest fragment transforming B264/U4 to respiratory competence was a 1.2Kbase EcoRI HinDIII piece (Figure 4.3.3).

Sequencing Strategy

This fragment was completely sequenced by the Maxam and Gilbert chemical method. Firstly digestions were made with a number of enzymes recognising sites 4 bases in length. The enzymes HinF and TaqI were chosen for this sequence as they gave well separated fragments of a reasonable size (preferably 100-600 bases). By sequencing DNA from these digestions the complete order of bases could be derived from overlaps. A further double digestion was needed with TaqI and BstN to derive the sequence of the largest fragment. An open reading frame was found which extended farther than the EcoRI terminal site. The gene was being transcribed from an in frame start codon in the vector (Figure 4.3.4). The 5' end sequence of the open reading frame was determined by taking an SstI HinDIII fragment (ST4) 1.4 Kbases long and sequencing strands from a HinF digest, which gave the sequence of the complete reading frame. It was discovered that the 12 N-terminal codons had been deleted. The complete open reading frame was

approximately 1Kbase long coding for a protein with 325 amino acids with a predicted molecular weight of 36 Kdal. The sequencing strategy is shown in Figure 4.3.5, and the complete sequence and its translation in Figure 4.3.6.

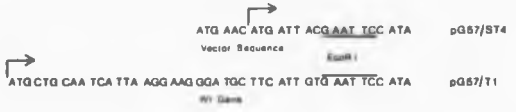


Figure 4.3.4 The gene for *pet57* is transcribed from an in frame start codon in the vector Yep 352 in construct pG57/ST4.

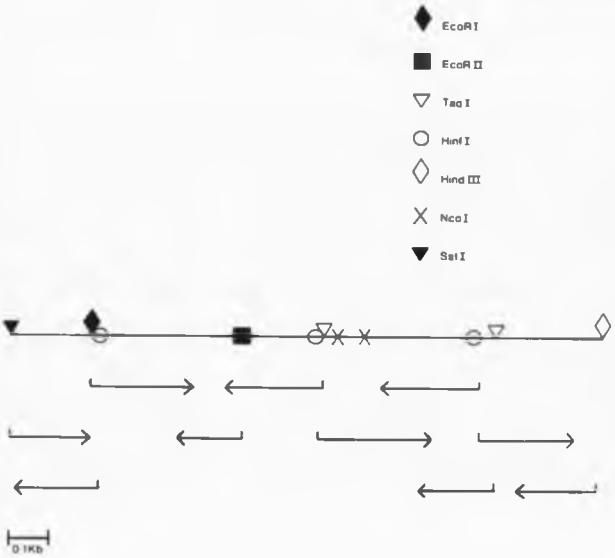


Figure 4.3.5 The experimental strategy used to determine the complete sequence of the PET_{77} gene. DNA was cut into fragments 100-600 bp in length using the restriction enzymes indicated, and the 5' phosphate of each strand labelled with ^{32}P. Strands were separated by polyacrylamide gel electrophoresis, and preparatively isolated. The Maxam and Gilbert chemical method was used to determine the sequence of each strand. The complete DNA sequence was derived from overlapping sequences produced by cutting the DNA with different restriction enzymes.

```
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TCA TCT GAG AAA TTT CTT CAC TGT TAC TTT TTA ATT GCT ATA TCT TTA AAC TCA TTC ATT
          10                  20                  30                  40                  50                  60

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TTC TTC AGA GAA AAG AAA AAA ATT AAG GTA AAG GAA AAG GCT AAA TAA GAA CAA AAC ACT
          70                  80                  90                 100                 110                 120

--- --- --- --- --- --- --- --- --- --- --- --- Met Leu Pro Ser Leu Arg Lys Gly
TGA AGG CAA ACG AAA CCC TAC AAA AGA GAA TAC GGA ATG CTG CCA TCA TTA AGG AAG GGA
          130                 140                 150                 160                 170                 180

Cys Phe Ile Val Asn Ser Ile Arg Leu Lys Leu Pro Arg Phe Tyr Ser Leu Asn Ala Gln
TGC TTC ATT GTG AAT TCC ATA AGA TTG AAA CTG CCC CGA TTC TAC TCA TTA AAT GCC CAG
          190                 200                 210                 220                 230                 240

Pro Leu Gly Thr Asp Asn Thr Ile Glu Asn Asn Thr Pro Thr Glu Thr Asn Arg Leu Asn
CCA CTG GGG ACA GAC AAC ACG ATT GAG AAT AAC ACT CCT ACG GAA ACA AAT AGA TTG AAC
          250                 260                 270                 280                 290                 300

Lys Thr Ser Gln Lys Phe Trp Glu Lys Val Ser Ser Asn Arg Asp Val Glu Lys Gly Lys
AAA ACT TCA CAG AAG TTT TGG GAA AAG GTG TCA TCA AAT AGG GAT GTT GAG AAA GGA AAG
          310                 320                 330                 340                 350                 360

Ile Ala Leu Gln Leu Asp Gly Arg Thr Ile Lys Thr Pro Leu Gly Asn Gly Ile Ile Val
ATT GCT CTA CAA TTA GAT GGC AGG ACT ATA AAA ACT CCT CTA GGA AAT GGA ATT ATA GTT
          370                 380                 390                 400                 410                 420

Asp Asn Ala Lys Ser Leu Leu Ala Tyr Leu Leu Lys Leu Glu Trp Ser Ser Leu Ser Ser
GAT AAT GCA AAG TCT CTC TTA GCA TAC CTA TTA AAA CTG GAG TGG TCG TCC CTA TCC AGT
          430                 440                 450                 460                 470                 480

Leu Ser Ile Lys Thr His Ser Leu Pro Leu Thr Ser Leu Val Ala Arg Cys Ile Asp Leu
CTT TCC ATC AAA ACT CAC TCT TTG CCA CTA ACT TCA TTA GTG GCA AGA TGC ATA GAT TTA
          490                 500                 510                 520                 530                 540

Gln Met Thr Asn Glu Pro Gly Cys Asp Pro Gln Leu Val Ala Lys Ile Gly Gly Asn Ser
CAA ATG ACA AAT GAG CCT GGC TGT GAC CCT CAA TTA GTT GCA AAG ATT GGA GGC AAC AGT
          550                 560                 570                 580                 590                 600

Asp Val Ile Lys Asn Gln Leu Leu Arg Tyr Leu Asp Thr Asp Thr Leu Leu Val Phe Ser
GAT GTT ATA AAA AAT CAG TTG TTA AGA TAT TTA GAT ACC GAT ACT TTA TTG GTC TTT TCC
          610                 620                 630                 640                 650                 660

Pro Met Asn Glu Phe Glu Gly Arg Leu Arg Asn Ala Gln Asn Glu Leu Tyr Ile Pro Ile
CCT ATG AAT GAG TTT GAA GGA AGA TTA CGC AAT GCG CAA AAT GAG TTA TAT ATA CCC ATC
          670                 680                 690                 700                 710                 720

Ile Lys Gly Met Glu Glu Phe Leu Arg Asn Phe Ser Ser Glu Ser Asn Ile Arg Leu Gln
ATC AAA GGA ATG GAA GAG TTT TTA CGC AAC TTT TCA TCC GAG TCT AAT ATT CGA CTA CAA
          730                 740                 750                 760                 770                 780

Ile Leu Asp Ala Asp Ile His Gly Leu Arg Gly Asn Gln Gln Ser Asp Ile Val Lys Asn
ATT TTA GAT GCC GAC ATC CAT GGG TTA CGA GGC AAT CAG CAG TCG GAT ATC GTT AAG AAT
          790                 800                 810                 820                 830                 840

Ala Ala Lys Lys Tyr Met Ser Ser Leu Ser Pro Trp Asp Leu Ala Ile Leu Glu Lys Thr
GCA GCA AAA AAA TAT ATG AGC AGC TTA TCA CCA TGG GAT CTT GCA ATT CTT GAA AAA ACT
          850                 860                 870                 880                 890                 900
```

```
Val Leu Thr Thr Lys Ser Phe Ile Cys Gly Val Leu Leu Leu Glu Asn Lys Lys Asp Thr
GTA TTA ACC ACA AAG TCC TTC ATT TGC GGC GTG CTA TTA TTA GAA AAT AAA AAA GAT ACT
          910         920         930         940         950         960

Ala Asn Leu Ile Pro Ala Leu Lys Thr Asp Met Asp Asn Ile Val Arg Ala Ala Thr Leu
GCG AAC TTA ATT CCC GCC TTG AAA ACT GAT ATG GAT AAT ATT GTA CGT GCC GCC ACC TTA
          970         980         990        1000        1010        1020

Glu Thr Ile Phe Gln Val Glu Lys Trp Gly Glu Val Glu Asp Thr His Asp Val Asp Lys
GAA ACA ATC TTC CAA GTT GAA AAG TGG GGA GAG GTT GAA GAT ACT CAT GAC GTT GAC AAA
         1030        1040        1050        1060        1070        1080

Arg Asp Ile Arg Arg Lys Ile His Thr Ala Ala Ile Ala Ala Phe Lys Gln --- --- ---
AGA GAC ATC AGA AGA AAA ATT CAT ACT GCT GCG ATT GCT GCT TTT AAG CAA TAA TAT GGT
         1090        1100        1110        1120        1130        1140

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TGA TTC AAT TGT TGT TCT ATT ACT ACT TTT CTA TGA TAT ATA TAC AAG CAT ATA TTC GAA
         1150        1160        1170        1180        1190        1200

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
AAT TTC TGT AAA TAA ACA TGA TTA TAT TTT TTG ACG AAT ATT ACT GAT TAG GTC TGA TTT
         1210        1220        1230        1240        1250        1260

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TGA ACC GAT GAC GTC CTG CGT CAA CTC TTG GGG CAT TAT TGT AGA GGA ACA TTT CTG TAA
         1270        1280        1290        1300        1310        1320

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TGA ATG CTT GAG ATC CAT TGC TGA TGA AAC TGA CGA AAG TGG ACT TGC AGT AGC ATC TTG
         1330        1340        1350        1360        1370        1380

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ATT GTT TGA GTG CGA TAC AGC TTC CGT GTG GTC AGT GGT ATA AAA GCT TAT TGT TGC AGT
         1390        1400        1410        1420        1430        1440

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
TGA GCA ACG TGT TAT AAA AAA TCA GTT GTT AAG ATA TTT AGA TAC CGA TAC TTT ATT GGT
         1450        1460        1470        1480        1490        1500

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
CTT TTC CCC TAT GAA TGA GTT TGA AGG AAG ATT ACG CAA TGC GCA AAA TGA GTT ATA TAT
         1510        1520        1530        1540        1550        1560

--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
ACC CAT CAT CAA AGG AAT GGA AGA GTT TTT ACG CAA GTT TCA TCC GAG
         1570        1580        1590        1600
```

Figure 4.3.6 The complete sequence of fragment ST4, with the protein translation of the open reading frame.

Computer analysis of the PET_{57} sequence

The Staden DNA and protein sequence analysis programs (ANALYSEQ and ANALYSEP respectively), available on the University of Warwick UNIX system 'Daisy', were used for manipulation of the DNA sequence. When direct comparison with predetermined sequences was required these were transferred from the EMBL DNA database stored on the IBM system 'Sky'. An extensive search was made with the protein translation of PET_{57} on the system at Edinburgh University with the assistance of Dr M. Collins, the database probed being the most complete available at the time. This search looks for regions of amino acid homology within the sequences compared, so is preferable to the type of search which determines the overall degree of homology of two sequences as it also finds regions of good homology in sequences which are dissimilar overall. The local searches did not show homology with any known ATPase subunit, and the more extensive search did not show any other significant homologies. The computer analysis is shown in detail in Appendix II.

Phe	TTT	6	Ser	TCT	3	Cys	TAT	3	Cys	TGT	1
Phe	TTC	4	Ser	TCC	7	Cys	TAC	2	Cys	TGC	3
Leu	TTA	22	Ser	TCA	8	*	TAA	1	*	TGA	0
Leu	TTG	6	Ser	TCG	2	*	TAG	0	Trp	TGG	4
Leu	CTT	3	Pro	CCT	5	His	CAT	3	Arg	CGT	1
Leu	CTC	1	Pro	CCC	3	His	CAC	1	Arg	CGC	2
Leu	CTA	7	Pro	CCA	4	Gln	CAA	7	Arg	CGA	3
Leu	CTG	4	Pro	CCG	0	Gln	CAG	5	Arg	CGG	0
Ile	ATT	13	Thr	ACT	12	Asn	AAT	17	Ser	GGT	0
Ile	ATC	7	Thr	ACC	3	Asn	AAC	6	Ser	AGC	2
Ile	ATA	6	Thr	ACA	5	Lys	AAA	16	Arg	AGA	8
Met	ATG	6	Thr	ACG	2	Lys	AAG	10	Arg	AGG	3
Val	GTT	8	Ala	GCT	4	Asp	GAT	14	Gly	GGT	0
Val	GTC	1	Ala	GCC	5	Asp	GAC	6	Gly	GGC	5
Val	GTA	2	Ala	GCA	7	Glu	GAA	9	Gly	GGA	8
Val	GTG	4	Ala	GCG	3	Glu	GAG	9	Gly	GGG	2

Table 4.3.3 Codon usage in the PET_{57} open reading frame

The open reading frame has a possible signal sequence, having 5 basic residues and 4 hydroxyl groups. Thus the N-terminus has a net positive charge, which is a common feature of leader sequences directing proteins to the mitochondrial inner membrane or matrix. This is discussed further in Appendix II. Codon usage of the PET_{57} protein translation is shown in Table 4.3.3.

Construction of a disrupted PET_{57} Reading Frame

To determine whether this reading frame coded for the *PET* phenotype a disruption of the reading frame was constructed. This involves deleting a fragment of the gene of interest and inserting a piece of DNA containing a known marker gene into the reading frame thus inactivating the gene. The only usable restriction sites within the reading frame were 2 NcoI sites cutting out a fragment 73 bases in length. Because Yep 352 has 1 NcoI site the fragment ST4 was transferred to plasmid Yep 351, which is not cut by NcoI. This construct was digested with NcoI to remove the small fragment, treated with

Klenow to make blunt ended, and then with CIP to prevent self-ligation.

The yeast *his*III gene was cut out of the plasmid Puc18 by digesting with BamHI, treating with Klenow, and then redigesting with SmaI to excise a 2.4 Kbase fragment containing the complete sequence for the *hisIII*. This gives a blunt ended piece because the overhang left by BamHI is filled in with Klenow and digestion with SmaI leaves a blunt end. The *his*III fragment was blunt-end ligated into Yep 351/ST4 and transformed into *E.coli* RR1. Colonies with the correct construct were identified by colony hybridisation using a *his*III probe because blunt end ligation has a very low efficiency. A control ligation was also carried out with vector but no insert to determine the amount of vector self-ligation. Colonies showing positive in the hybridisation were checked by restriction enzyme digestion of mini-preps, and one with the *his*III insert was selected. A restriction map of the construct is shown in Figure 4.3.7.

DNA prepared from this positive clone was used to produce an integrating vector, in which the *his*III insert is flanked by sequences of the disrupted G57 reading frame. The Yep 351/ST4/*his*III construct was digested with ScaI and HpaI to produce a linear piece containing the disrupted gene. This was used to transform yeast strains aW303, αW303 and a/αW303. Transformant colonies were selected on T plus ura/ade/thr amino acid additions and colonies produced were picked onto YPD. This plate was replicated onto EG to determine whether transformants had acquired a respiratory deficient phenotype. Over 50% of the transformant colonies did not grow on glycerol medium. On mating these colonies separately to ρ^0 tester strains CB11ρ^0 and KL14ρ^0, diploids produced grew on glycerol-containing medium showing that respiratory deficiency in these strains was not due to ρ^- formation (Rayco and Goursot, 1985). The ATPase activity of mitochondria prepared from these deleted strains is shown in Table 4.3.4 together with the ATPase activity of strain B264/U4 transformed to respiratory competence with plasmids pG57/t1 and pG57/ST6. Chromosomal DNA was prepared from respiratory deficient colonies (judged from their inability to grow on glycerol), from transformed

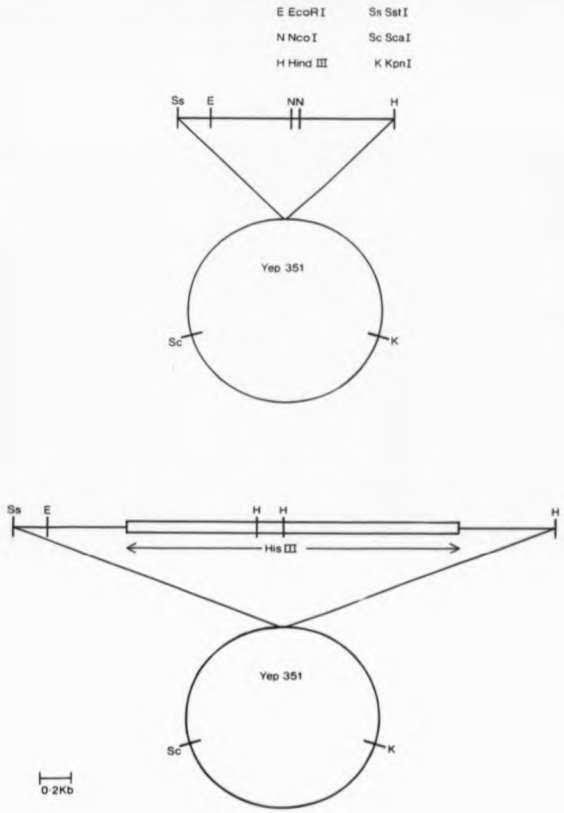


Figure 4.3.7 The predicted restriction map of the disruption construct, compared with that of the wild type gene. The fragment ST4 was transferred to plasmid Yep 351, a 73 bp fragment removed with NcoI, and the NcoI sticky ends filled by treatment with Klenow. The *S.cerevisiae* gene *his*III was blunt-end ligated into this gap. Also shown are the restriction sites used to linerise the construct to produce an integrating vector.

strains aW303 and αW303 called aW303∇G57 and αW303∇G57 respectively.

Strain	S.A. – Rutamycin	S.A. + Rutamycin
aW303	5.24	0.96
aW303G57	0.255	0.221
αW303G57	0.211	0.190
B264/U4	0.310	0.255
B264/U4/pG57/T1	5.14	0.68
B264/U4/pG57/ST6	4.97	0.91

Table 4.3.4 Specific activity (in $\mu M mg^{-1} min^{-1}$) of mitochondrial ATPase from strains transformed to respiratory competence and respiratory deficiency. Strain B264/U4 was transformed to respiratory competence by the plasmids pG57/T1 and pG57/ST6. Strain W303 (both a and α mating types) was transformed to respiratory deficiency by the integration of PET_{57} disrupted with *his III*. Methods for preparation of mitochondria by the zymolase procedure and for assay of ATPase activity are described in detail in Chapter 5.

This DNA was digested with restriction enzymes EcoRI, HinDIII and SatI, and this gel blotted onto nitrocellulose. Nick translation labelled ST4 was used as a probe. If aW303∇G57 and αW303∇G57 have been transformed to respiratory deficiency because the disrupted gene has integrated into their chromosomal DNA thus destroying the wild-type gene, the restriction map of the region will have altered. Fragments produced by enzymes which do not cut *his III* will be shifted to a higher molecular weight because of the disruption, for example EcoRI and SatI. Enzymes which cut within *his III* will also produce fragments of anomalous molecular weight, for example HinDIII. By examining the restriction maps of wild-type and disrupted genes, we can determine whether transformation to respiratory deficiency is due to incorporation of the disrupted gene. The autoradiograph produced from the Southern blot of wild type and disrupted DNA is shown in Figure 4.3.8, together with the expected restriction map with and without the gene disruption. From this it can be seen that the bands from the restriction digests in both aW303∇G57 and αW303∇G57 are identical, and that this is consistent with the restriction map proposed for the disrupted gene.

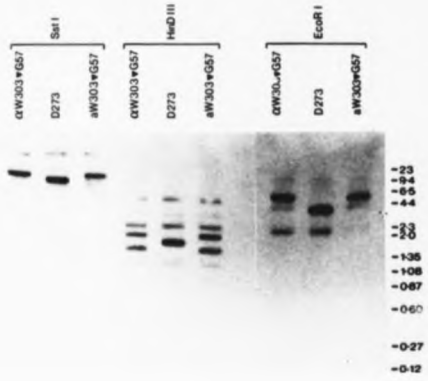


Figure 4.3.8 The experimentally determined restriction map of DNA prepared from disrupted and wild-type strains. Total chromosomal DNA was prepared from strain D273-10B/A1 and from strains aW303ΨG57 and αW303ΨG57 which had been transformed to respiratory deficiency by the disruption construct. DNA from each strain was digested with EcoRI, HindIII and SalI and run on an agarose gel. The gel was transferred to nitrocellulose by Southern blotting, and probed with fragment ST4 labelled by nick translation. Bands which hybridised to ST4 were visualised by autoradiography and size standards added by comparison with the original gel.

4.4. DISCUSSION

The gene causing respiratory deficiency in the *pet* mutant B264 has been cloned by complementation with a wild-type library, and its DNA sequence determined. Analysis of the sequence revealed an open reading frame 1 Kbase in length. A disrupted copy of this reading frame provided evidence that it encoded the gene responsible for the respiratory deficient phenotype as wild-type cells could be made respiratory deficient by replacing the wild type copy of this reading frame with a copy disrupted by insertion of the *hisIII* gene. The predicted protein sequence coded for by this gene is a 36 Kdal species which has no homology with the protein sequence of any of the subunits of the mitochondrial ATPase previously sequenced in other species, and is not thought to be a subunit of the enzyme.

The N-terminus of this protein has a net positive charge and also a number of hydroxyl groups, but no acidic amino acid side chains. Analysis of the N-terminal region of nuclear-coded mitochondrial proteins targeted to the inner membrane or matrix has shown that most mitochondrial targeting sequences are significantly enriched in the amino acids Arg, Leu and Ser (but not Lys), but have few Asp, Glu, Val and Ile residues (von Heijne, 1986). This amino acid bias is evident in the N-terminal region of the PET_{57} predicted protein sequence so it could be a protein destined for the inner membrane of the mitochondrion, or the mitochondrial matrix. A hydrophobicity plot of the protein shows that it does not contain the high percentage of hydrophobic sequences common to the mitochondrially-coded F_0 polypeptides so the protein is not an integral membrane protein.

However the 12 N-terminal residues of the protein do not affect its function as they can be completely deleted. The attenuated protein produced can still compensate for the respiratory defficiency in mutant strains, thus is transcribed, translated and transported to its correct site in fully functional form.

CHAPTER 5

IMMUNOLOGICAL CHARACTERISATION OF COMPLEMENTATION GROUP G57

5.1. INTRODUCTION

This chapter describes some phenotypic characteristics of the mutant group G57 which were obtained by a combination of biochemical and immunological analysis. The immune replica technique (Cabral *et al*, 1978, Todd *et al*, 1979) was used, which involves antisera recognising antigenic determinants already resolved by polyacrylamide gel electrophoresis, without the need for immune precipitation. The major subunits of ATPase F_1 are dissociated and separated by electrophoresis, and detected by their reaction with anti-F_1 antibody (which is labelled by binding ^{125}I-Protein A to the F_c portion of the antibody). This immunological technique can be used to detect a specific protein, or group of associated proteins, in a complex mixture of protein, for example F_1 subunits in a mitochondrial preparation.

5.2. MATERIALS AND METHODS

Zymolase 20000 was purchased from Miles.

Preparation of *S.cerevisiae* Mitochondria

All procedures were at 0-4°C unless stated.

Enzymatic Method

A 1% (vol/vol) inoculum into 500ml of YPGal was grown up overnight at 30°C (usually 16-17 hrs). Cells were harvested in an International centrifuge at 2K rpm for 7 mins, and washed with 100ml 1.2M sorbitol. The pellet was weighed and suspended in 3ml

zymolase mix per gram wet weight of cells.

Zymolase mix
30ml of 2M sorbitol
3.75ml of 1M KPO_4 pH 7.5
0.1ml of 0.5M EDTA
0.5ml of β-mercaptoethanol
25mg zymolase 20000
H_2O to 50ml

After incubating at 37°C for one hour spheroplast formation was monitored under the microscope, and the zymolase digestion continued if incomplete.

If spheroplast formation was satisfactory, 1.2M sorbitol (150ml) was added and the spheroplasts spun down in a Sorvall GSA rotor at 5K rpm for 10 mins. After washing with an additional 150ml 1.2M sorbitol, spheroplasts were re-suspended in 40ml 0.5M sorbitol, 20mM Tris/Cl pH 7.5, 0.1mM EDTA (isolation buffer) and blended in a Waring blender for 30 secs. The suspension was spun at 2K rpm for 10 mins and the supernatant taken and re-centrifuged. The supernatant was collected and centrifuged at 15K rpm for 15 mins in a Sorvall SS34 rotor. The mitochondrial pellet thus produced was washed three times with 30ml of isolation buffer, re-suspended in a minimal volume of isolation buffer or TE, and stored at -70°C.

Mechanical Method

Cells were harvested as before, washed and re-suspended in MTE buffer so that each 30ml of cell suspension contained 5 grams wet weight of cells. An equal volume (30ml each) of cells and glass beads were poured into a shaker bottle, which was shaken for one minute in a Bronwill shaker. The broken cells were decanted and the cell debris pelleted twice at 2.5K rpm for 10 mins in a Sorvall GSA rotor. Mitochondria were isolated by centrifuging the supernatant at 15K rpm for 15 mins in a Sorvall SS34 rotor, washed three times with MTE buffer and re-suspended in MTE or TE buffer.

Preparation of Submitochondrial Particles (smps)

Mitochondria were sonicated for a single 5 sec burst in a Braunsonic 1510 sonicator at full power. The suspension was centrifuged in a Beckman 50Ti rotor at 40K rpm for 20 mins at 4°C. The smps appeared as a translucent pellet, and were re-suspended in TE buffer. The supernatant from this spin was saved and is referred to as sonic supernatant.

Preparation of *S.cerevisiae* F_1

By Sonication (Tzagoloff, 1969)

Submitochondrial particles were subjected to sonication, up to 3 mins in 30 sec bursts at room temperature, with one minute intervals at room temperature to prevent overheating. The suspension was centrifuged in a Beckman 50Ti rotor at 40K rpm for 20 mins at 25°C. The supernatant from this spin contained F_1 in wild type strains

By Extraction with Chloroform (Beechey et al, 1975)

Submitochondrial particles were suspended in 250mM sucrose, 10mM Tris/SO_4 pH 7.6, 1mM EDTA. 1 vol of AR chloroform was added and the tube was vortexed for 30 secs, centrifuged in a microfuge for one minute and the aqueous layer removed to a fresh Eppendorf. This was centrifuged for a further 10 mins in a microfuge and the supernatant taken as the F_1 fraction.

Preparation of the F_1F_0 Complex (Tzagoloff and Meagher, 1971)

Submitochondrial particles were suspended in 250mM sucrose, 10mM Tris/Ac pH 7.5 at 25mg ml^{-1}. 4mM Tris/Ac pH 7.5 (2.9 vols) and 0.1vol 10%(w/v) solution of Triton X-100 (to 0.25% final) was added to 1 vol of smp suspension, and ATP was added to a concentration of 2mM and EDTA to 1mM. The suspension was centrifuged at 40K rpm in a Beckman 50Ti rotor for 30 mins at 4°C. The supernatant was collected and 0.4ml layered on a 5ml linear gradient of sucrose (7-20% w/v) containing 5mM Tris/Ac pH 7.5,

0.1% Triton X-100, 2mM ATP, 1mM EDTA and centrifuged at 60K rpm in a Beckman 65Ti rotor for three hrs at 4°C. Fractions were collected by puncturing the bottom of the tube.

Protein Estimation

Protein concentrations were determined by the method of Lowry (1951).

Determination of ATPase Activity

ATPase activity was assayed in a 1ml reaction containing 50mM Tris/SO_4 pH 8.0, 4mM $MgSO_4$, 0.1mg mitochondrial protein and 10mM ATP. The reaction was timed after addition of ATP and stopped with 0.2ml 50% TCA after 12 mins. Inorganic phosphate produced was determined by adding 0.2ml of assay solution to 5ml 0.5% TCA. 5% ammonium molybdate (0.5ml) and ANS reagent (0.15ml) were added, mixing well after each addition. After incubating for 10 mins at room temperature tubes were read spectroscopically at 660nm. The phosphate liberated was determined by comparison with a standard curve constructed under similar conditions.

Protein Analysis

Proteins were separated on 12% polyacrylamide gels by the method of Laemmli (Laemmli, 1970). Gels to be subjected to immunological analysis were electrophoretically transferred to nitrocellulose sheets in a vertical tank at 100V for one hour or 50V for four hrs. The lanes on which protein standards were run were cut away before transferring, and stained with bromophenol blue by standard methods. The gel to be transferred was notched at one corner for orientation, and plug gel removed with a sharp scalpel. The gel and nitrocellulose were sandwiched between 2 sheets of Whatman #3 and 4 Scotch-brite pads ensuring that no air bubbles were trapped between gel and nitrocellulose. This was placed in a plastic holder which was slotted into a tank containing western transfer buffer. Care was taken to ensure the nitrocellulose was

positive of the gel when a voltage was applied.

Transfer Buffer
192mM glycine
25mM Tris/Cl
20% MeOH

Rinse Buffer	Rinse Buffer + NaCl
10mM Tris/Cl pH 8.0	10mM Tris/Cl pH 8.0
1mM EDTA	1mM EDTA
150mM NaCl	1M NaCl
0.1% Triton X-100	0.1% Triton X-100

The nitrocellulose was sealed in a seal-and-save bag and vibrated for 45 mins in 10-15ml of rinse buffer plus 1% whole milk powder. Antibody was added at a 100-400 dilution depending on its strength, injected into the bag together with 0.5ml of rinse buffer plus milk. After incubation at room temperature for a minimum of one hour with agitation, the nitrocellulose was removed and rinsed three times for 15 mins in 250ml of rinse buffer. After re-bagging with 10-15ml of RB and milk, 10-15µl of ^{125}I-labelled protein A in 0.5ml of RB and milk was added, and the sealed bag incubated at room temperature for a minimum of one hour with agitation. The nitrocellulose was removed and washed three times with 250ml RB plus 1M NaCl, blotted dry, and exposed overnight at -70°C.

Total RNA preparation from *S.cerevisiae*

All solutions used were made RNase free by autoclaving and gloves were worn throughout. A 50ml overnight culture of the yeast strain in YPD was centrifuged at 5K rpm for 10 mins in a Sorvall GSA rotor and the pellet re-suspended in 5ml LETS solution.

LETS	PCI
100mM LiCl	250ml $CHCl_3$
10mM EDTA	250ml H_2O sat. phenol
10mM Tris/Cl pH 7.4	10ml isoamyl alcohol
1% SDS	

To this ½ vol of glass beads was added, followed by 1 vol of PCI. After vortexing for one minute, tubes were centrifuged at 5K rpm for 5 mins (Sorvall SS34 rotor), the aqueous layer removed and the PCI extraction repeated until there was no interface between the two layers. The aqueous fraction was then extracted once with $CHCl_3$, LiCl added to a final concentration of 0.2M, and finally 2 vols ethanol were added. After leaving at -20°C for 4 hrs the RNA was centrifuged out at 10K rpm for 10 mins, ethanol washed and then dried under vacuum. RNA was dissolved in 300µl H_2O and the debris removed by centrifugation. The RNA was stored at -70°C.

Northern Blotting

All steps prior to transfer were RNase free. An RNase free 1% agarose gel (TBE buffer) was prepared using autoclaved agarose and gel plates. RNA samples were loaded and the gel run, stained and photographed as for a DNA gel. After notching for orientation the gel was soaked twice for 20 mins in 200ml 200mM NaAc buffer pH 4.0.

Meanwhile NBM paper was treated for blotting by floating in 100ml of 20%(w/v) sodium dithionite at 65°C for 30 mins. After pouring off the dithionite the paper was washed thoroughly with dH_2O (rinsed 10 times). The paper was rinsed in 100ml 30% acetic acid, then washed thoroughly with dH_2O. 100ml of cold 10% HCl was used to float the paper, to which 3ml of a 3% solution of sodium nitrite was added. The paper was left to soak at a temperature between 0-4°C for a minimum of 30 mins.

Paper was then rinsed twice with dH_2O, twice with 200mM NaAc, and put immediately onto the gel to transfer by the same method as that used in Southern blotting described in

the previous chapter. The RNA was transferred overnight to NBM paper.

After tracing the outline of the gel in pencil the paper was sealed into a bag with 7ml of blocking buffer.

Blocking buffer	Hybridisation buffer
50% formamide	30-50% formamide
5× SSC	5× SSC
5× Denharts solution	5× Denharts solution
50mM $NaPO_4$ pH 6.5	25mM $NaPO_4$ pH 6.5
1%(w/v) glycine	0.1mgml^{-1} cDNA (heat denatured)
0.1mgml^{-1} cDNA (heat denatured)	probe (heat denatured)

After leaving at 45°C overnight the paper was removed, rinsed in 5× SSC and rebagged. Hybridisation mix was added at 1ml per lane on the gel, and the sealed bag was left at 45°C overnight. The paper was pre-washed three times for 10 mins in 2× SSC, 0.1% SDS, washed twice for 15 mins in 0.5× SSC, 0.1% SDS and air dried for 10 mins. It was then exposed at −70°C.

If DBM paper was to be re-used it was washed for one hour at 45°C in 95% formamide, 0.1% SDS, 10mM EDTA, 50mM tris/Cl pH 7.5. Then paper was washed at 45°C twice for 15 mins in 2× SSC, 0.1%SDS, and twice for 15 mins in 0.1% SSC, 0.1% SDS before rehybridising.

Fusion Protein preparation

Protein Production

The *E.coli* expression vectors pATH 3 and pATH 22 were used for the production of a chimeric protein. The 313 carboxy terminal residues of PET_{57} was ligated into the two vectors so that it was in frame with the *trp E* gene. This construct was used to transform *E.coli*, and cells were selected on LB/Amp/Trp plates. The amino acid tryptophan was added to prevent the transformants from expressing the hybrid protein, as this would

exert selective pressure to eliminate the hybrid gene. Cells were taken from an LB/Amp/Trp plate and used to inocculate 10 ml of M9+CA+W+Amp (omitting the Amp for the RR1 control).

10× M9+CA	
$Na_2HPO_4.H_2O$	6g
KH_2PO_4	3g
NaCl	0.5g
NH_4Cl	1g
Casamino acids	5g
H_2O	to 100ml

After growing to mid-log phase (2-4 hours) cultures were dumped into 2l flasks containing 100ml M9+CA+Amp (no Amp in RR1 control) and shaken well for 1-2hrs. 3β-indoleacrylic acid was added to 20μgml^{-1} (from a 2$mgml^{-1}$ stock in ethanol) and cells grown for a further 4hrs. The flasks were left at between 0-4°C overnight. After pelleting the cells at 5Krpm for 10 mins and washing in 20mM Tris/Cl pH 7.5, they were re-suspended in cell lysis solution (20ml per 100ml culture).

Cell Lysis solution
50mM tris/Cl pH 7.5
5mM EDTA
3$mgml^{-1}$ lysozyme

After allowing to lyse on ice for one hour, 5M NaCl was added (1.4ml), and the suspension mixed. The detergent NP-40 (1.5ml of a 10% solution) was added, and the viscous suspension allowed to stand on ice for 30 mins. To shear the DNA the suspension was sonicated for 10 secs, taking care that lipids were not allowed to foam due to excessive sonication. The protein was then pelleted in a Sorvall SS34 rotor at 10Krpm for 10 mins, washed once in 20ml 1M NaCl, 10mM Tris/Cl pH 7.5, and once in 10mM Tris/Cl pH 7.5. The pellets were re-suspended in 10mM Tris/Cl pH 7.5 and checked for the presence of a high molecular weight protein species on an analytical Laemmli gel.

Protein purification

Proteins produced in this way were purified by separation on a Bio-Gel A-0.5 column equilibrated in wash buffer. Firstly the column was washed through overnight with fresh wash buffer.

Wash Buffer
10mM Tris/Cl pH 7.5
0.1% SDS
5mM β-mercaptoethanol

The fusion protein was pooled from all preparations which showed a large amount of a protein species at the estimated molecular weight of the fusion product. It was solubilised in 1% SDS, 5mM β-mercaptoethanol with heating at 90°C for 1-2 mins. After allowing to cool, glycerol was added to 20%(v/v) and the sample loaded onto the column. Fractions were collected over a period of 18hrs, and their protein content estimated by reading at 280nm in quartz cuvettes against a buffer blank. Larger proteins elute from the column first, and as the fusion product is generally the largest protein present the first samples in which protein was detected were run on an analytical gel. Samples with high proportions of the fusion protein were pooled. To this 1/20th volume of 5M NaCl was added, followed by four volumes of ice-cold acetone to precipitate the protein. The suspension was either allowed to stand on ice for one hour, or left overnight at -20°C. The protein precipitate was centrifuged out at 8Krpm for 10 mins in a Sorvall SS34 rotor, washed with H_2O, and redissolved in a minimum volume of 1% SDS. This solution was checked on an analytical gel and its protein concentration determined.

Raising Antibodies

Antibodies were raised in rabbits, using two rabbits for the *trpE/PET*$_{57}$ antigen. A test bleed was taken before injection of the antigen to give a zero time control. The initial injection was of 1-1.5ml (2-3mg protein), mixed with Freund's adjuvent. A second test bleed was taken 3 weeks later, immediately prior to a booster injection of 0.5-1ml (1-

2mg) of antigen. Plasma from these bleeds was used as antibody in western blotting.

5.3. RESULTS AND DISCUSSION

As sequence analysis did not prove effective in identifying the source of the respiratory deficiency in the complementation group G57, a course of biochemical analysis in conjunction with immunological characterisation was embarked upon. Two different antibodies were used as probes. The first section describes western blots in which an anti-F_1 antibody was used. This antibody had been prepared by injecting a preparation of F_1 into rabbits, and was kindly provided by Dr S.H. Ackerman. The second section describes in detail the raising of antibodies to a fusion protein made by ligating the C-terminal end of the *PET*$_{57}$ reading frame into a bacterial expression vector and results derived from use of that antibody in western blotting.

Anti F_1 antibody

Mitochondria prepared from each independent isolate in the complementation group had already shown that most had less than 10% ATPase activity. To check that these mutants had all the major subunits of mitochondrial ATPase F_1, 10μg of total mitochondrial protein was prepared from each strain by Zymolase digestion and was run on a Laemmli gel followed by western blotting onto nitrocellulose paper. This paper was probed with anti-F_1 antibody as described in section 5.2. From the autoradiograph shown in Figure 5.3.1 it can be seen that all of the mutants have the major subunits α, β and γ, which are the same size as the wild type.

Strain B264, the parent strain of the spores used in the gene cloning, was examined in detail.

Firstly experiments were carried out to determine whether the mutants F_1 was bound to the inner mitochondrial membrane. In ρ^- mitochondria, which are lacking the

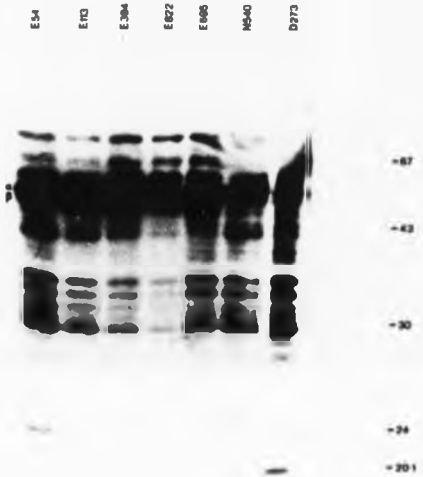


Figure 5.3.1 Comparison of ATPase F_1 subunits in complementation group G57 and wild type D273/10B-A1. Mitochondria were prepared from all strains by the zymolase method. 10µg of total mitochondrial protein from each strain was solubalised by heating in Laemmli loading buffer at 90°C for 3-5 minutes and proteins were seperated overnight by running on a Laemmli gel at 70V. The proteins were transferred to nitrocellulose by Western blotting, and incubated sequentially with anti-F_1 antibody (raised in rabbits) and ^{125}I protein A. The proteins which were recognised by the antibody are detected by autoradiography.

mitochondrially-coded F_0 subunits 6, 8 and 9, F_1 subunits are still synthesised and imported into the mitochondrion. However, because of the F_0 subunit deficiencies the assembled F_1 is not bound to the mitochondrial membrane. By determining whether the mutant F_1 was membrane-bound it is possible to examine whether any F_1F_0 interactions were affected by the mutation in group G57.

The method used to determine whether F_1 is membrane-bound depends on the method used for preparation of the mitochondria. Preparation of mitochondria by breaking cells with glass beads will damage the mitochondrion such that the integrity of the mitochondrial membrane is lost, and its matrix contents released. Thus matrix enzymes will be lost into the supernatant when the mitochondria are pelleted. Preparation of mitochondria by the enzymatic method using zymolase leaves mitochondria intact, although the yield of mitochondria per gram wet weight of cells is smaller. Thus if the F_1 subunits are imported into the mitochondrion but not bound to the membrane they will appear in the post-mitochondrial supernatant in glass bead prepared mitochondria. However in zymolase prepared mitochondria the F_1 subunits will be present in the mitochondrial preparation and will only be released into the supernatant when the mitochondria are disrupted by short sonication and the submitochondrial particles thus produced spun down.

All mitochondria used for this experiment were produced by the enzymatic method. Sonication was carried out at 0-4°C for 5 sec and submitochondrial particles resuspended to their original volume in TE buffer. Equal volumes of mitochondria, sub-mitochondrial particles and sonic supernatant were loaded onto a Laemmli gel (based on a volume containing 10 μg of mitochondrial protein), western blotted and probed with anti-F_1. The results are shown in Figure 5.3.2a. The mutant's F_1 appears in the submitochondrial particle fraction and thus is still associated with the membrane, as is the wild-type control.

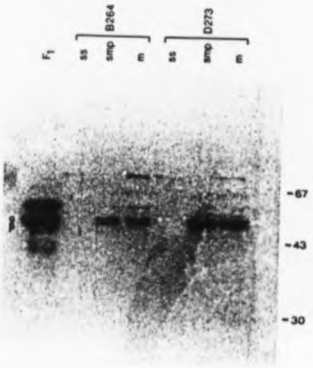
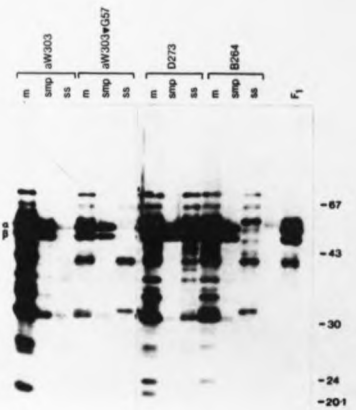


Figure 5.3.2 (a) The ATPase subunits of mutant B264 are membrane bound. Mitochondria (m) from B264 and D273/10B-A1 were sonicated for 5 seconds, and then centrifuged for 20 minutes at 100 000*g*. The supernatant (ss) was decanted and the submitochondrial pellet (smp) resuspended to its original volume. A volume equivalent to 10 mg ml^{-1} mitochondria of each sample was loaded on a Laemmli gel. Anti-F_1 was used as probe.



(b) Disruption mitochondria show the same protein profile as mutant mitochondria on sonication.

However a number of differences can be observed when comparing the equivalent mutant and wild-type lanes on the autoradiogram produced from this experiment, and these differences are consistent throughout all other preparations. In the sonic supernatant from the mutant strain a clear band is present at a molecular weight of 42 Kdal, which is much less evident in the wild-type sonic supernatant. Additionally, other bands are missing in the mutant fractions. However, an equivalent band occurs in the lane in which a preparation of F_1 is run. In addition a 33 Kdal species is released on sonication of the mutant strain, but not released from the wild-type D273/10B-A1. These bands are also evident in the sonic supernatant from disruption mitochondria (Figure 5.3.2b).

Prolonged sonication will remove wild-type F_1 from the inner mitochondrial membrane, and free F_1 can be detected in the sonic supernatant. F_1 is cold-labile and thus all steps were carried out at room temperature to preserve its activity. To determine whether mutant F_1 could be activated when removed from the membrane, mutant and wild-type zymolase mitochondrial preparations were sonicated for a total of 3 mins each with intervals to prevent overheating. The suspensions were seen to clarify and become more translucent during sonication. Pelleted submitochondrial particles were resuspended in the starting volume of TE buffer, and mitochondria, smp's and sonic supernatant were assayed for ATPase activity (Table 5.3.1). When removed from the mitochondrial membrane F_1 is insensitive to oligomycin and rutamycin.

Fraction	S.A	S.A.+ Rutamycin
D273/10B-A1 mitochondria	3.44	0.314
D273/10B-A1 smp	2.28	0.56
D273/10B-A1 3 min sonic pellet	0.772	0.456
D273/10B-A1 3 min sonic sup.	16.67	15.0
B264 mitochondria	0.738	0.676
B264 smp	0.173	0.224
B264 3 min sonic pellet	0.738	0.676
B264 3 min sonic sup.	1.778	1.50

Table 5.3.1 ATPase activity of fractions from a prolonged sonication of mutant and wild type submitochondrial particles. Submitochondrial particles prepared from a five second sonication of mitochondria as described in section 5.2 were subjected to a further sonication for a total of three minutes at room temperature (with intervals for cooling). After centrifugation at 100 000g for 30 minutes at 20°C the supernatant and pellet produced were tested for ATPase activity, and for sensitivity of ATPase activity to Rutamycin. Specific activity is given in $\mu Mmg^{-1}min^{-1}$.

Thus active F_1 ATPase activity is present in the sonic supernatant from the wild-type, but no significant ATPase activity is seen with the mutant. The wild type F_1 is also more active when released from the mitochondrial membrane as it is freed from regulating factors, and this accounts for the high specific activity shown. Fractions were analysed immunologically to determine whether mutant F_1 was released from the membrane, volumes loaded based on that containing 10µg mitochondrial protein. Figure 5.3.3 shows that no F_1 subunits are detectable in the sonic supernatant from the mutant mitochondria.

Wild-type F_1 can be released specifically by extraction of the membranes with chloroform according to the method of Beechey *et al* (1975). A small-scale preparation of F_1 from mutant and wild type was assayed for ATPase activity. In the wild-type the expected high specific ATPase activity was present, which was insensitive to oligomycin. The mutant F_1 ATPase activity was not detectable, and mutant F_1 was only detectable in low quantity compared to wild-type in a western blot (Figure 5.3.4).

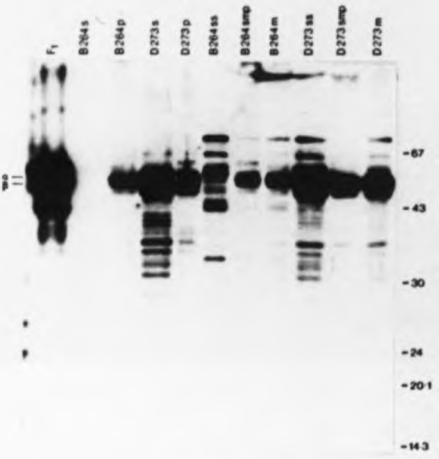


Figure 5.3.3. F_1 is not detectable in the supernatant fraction on prolonged sonication of B264. Submitochondrial particles (smp) produced from B264 and D273/10B-A1 mitochondria were sonicated for a total of 3 minutes in 30 second bursts at room temperature. The resulting suspension was centrifuged at 195000*g* for 20 minutes at room temperature, the supernatant (s) decanted and the pellet (p) resuspended to its original volume. A volume equivalent to 10 mgml^{-1} of each sample was loaded onto a Laemmli gel. Anti-F_1 was used as probe. The mitochondrial fraction (m) and sonic supernatant (ss) from the submitochondrial preparation are also shown.

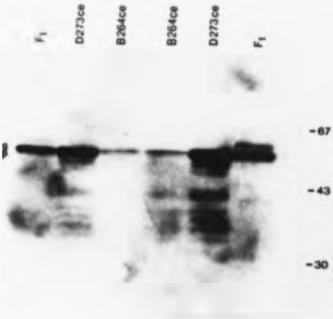


Figure 5.3.4 F_1 is not detectable in the supernatant fraction (ce) on extraction of B264 mitochondria with chloroform. Mitochondria from B264 and D273/10B-A1 were treated with chloroform as described by Beechey *et al* (1975). Concentrated samples of supernatant were loaded on a Laemmli gel, and probed with anti-F_1 antibody.

Fraction	S.A. – Oligomycin	S.A. + Oligomycin
D273 mitochondria	3.46	0.61
B264 mitochondria	0.812	0.75
D273 chloroform extract	14.1	14.3
B264 chloroform extract	1.9	1.95

Table 5.3.2 Chloroform extraction of wild-type and mutant submitochondrial particles by the method of Beechey (Beechey *et al*, 1975). Submitochondrial particles were extracted with AR chloroform at room temperature. After centrifugation the aqueous layer (chloroform extract) was tested for ATPase activity.

To attempt to assign the mutant to F_1 or F_0 a reconstitution experiment was performed, which involved treating submitochondrial particles with urea to degrade F_1. After washing, an F_1 preparation was added which would bind to wild-type F_0 still present in the membranes. The ATPase activities of the reconstituted complex are shown in Table 5.3.3, together with ATPase activity of the submitochondrial particles before treatment with urea. The bound activity on the mutant membranes is significant, especially when compared to the unbound activity (i.e. the mutant membranes can bind F_1 such that it remains active). This activity is also sensitive to oligomycin and is comparable to that of the reconstituted complex using wild-type membranes. This provides evidence that the mutants of group G57 have a defective F_1 moiety. The previous experiment showed that the mutant's F_1 is only detectable when associated with mitochondrial membrane fractions. Investigations were now made to find out whether the mutant's F_1 is assembled into a complex with F_0, and if so, whether this complex shows any gross differences to the wild type complex. The detergent Triton disrupts the mitochondrial membrane and will release the wild-type F_1F_0 complex intact. The complex will be present in the supernatant fraction after spinning the Triton extraction at 100Kg for 30 mins.

	S.A.–Oligomycin	S.A.+Oligomycin
D273 submitochondrial particles	9.17	3.33
B264 submitochondrial particles	1.1	0.76
D273 reconstituted complex	3.02	1.6
B264 reconstituted complex	2.57	1.48

Table 5.3.3 Reconstitution of wild type and mutant membranes with wild type F_1. Submitochondrial particles were treated with urea and then washed thoroughly with buffer. Wild type F_1 was added to the stripped membranes and incubated at room temperature for 30 minutes before re-cooling to 4°C and washing to remove unbound F_1. ATPase activity of samples was estimated by the method outlined in section 5.2. Activity was not observed in any other samples, including the stripped membrane samples.

The Triton extract thus produced was loaded onto a continuous gradient of 7-20% sucrose to determine whether the mutant and wild type complex run to the same level in the gradient. A total of 30 fractions (0.2ml) were drawn off from the bottom of the gradient, fractions being labelled 1-30 in order of decreasing density. Alternate fractions were treated with 4× Laemmli loading buffer and 20µl loaded on a Laemmli gel. The two gradient cross sections are compared in Figure 5.3.5. The wild-type F_1F_0 ATPase peaks in fractions 16-20 whereas the mutant's F_1 is not detectable in the Triton extract.

This experiment was repeated using the detergents Triton, deoxycholic acid and cholic acid, with varying concentrations of KCl (to 1M). This should result in the release of all F_1 from the wild-type membrane. Supernatant from each of these reactions was compared with an equal volume of pelleted material resuspended to its original volume. As can be seen from Figure 5.3.6 the major subunits of F_1 in the mutant preparation are barely detectable in the pelleted material and undetectable in the supernatant in all detergent plus salt preparations. In the wild-type F_1 is clearly present in the supernatant from the detergent extracts.

This exposes the possibility that the mutant F_1 may be more susceptible to protease digestion than the wild-type when removed from the membrane. To investigate this a

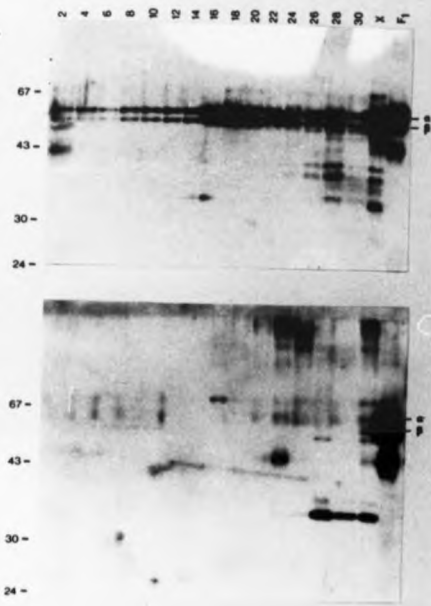


Figure 5.3.5 Sucrose gradients of the Triton extracts from mutant and wild-type. Submitochondrial particles from B264 and D273/10B-A1 were extracted with Triton X-100 (0.25%), centrifuged at 100000g for 3 hours at 4°C, and 0.4ml of the supernatant layered onto a 5ml linear gradient of 7-20%(w/v) sucrose. After centrifuging the gradients at 120000g for 30 minutes at 4°C fractions were taken from the bottom of the gradient (fraction 1 represents the highest density). Identical volumes of alternate fractions were loaded onto a Laemmli gel and probed with anti-F_1 antibody.

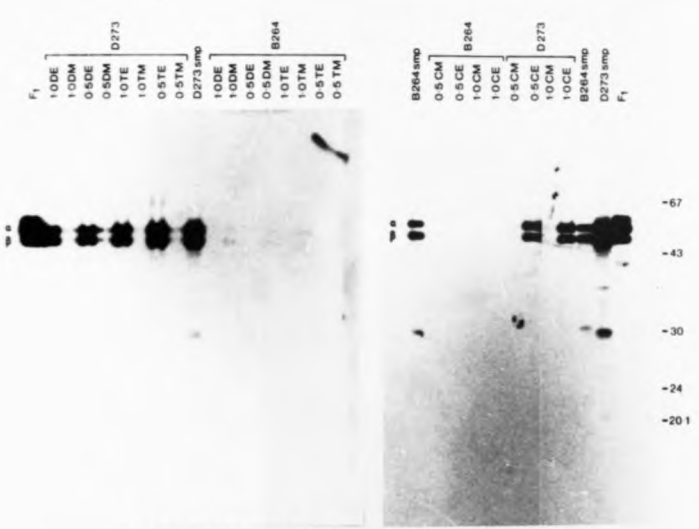


Figure 5.3.6 Extraction of mutant and wild-type submitochondrial particles with detergent and KCl. Detergents used were Triton (T), deoxycholic acid (D) and cholic acid (C), samples are labelled either detergent extract (E) or pelleted membrane (M), and the salt concentration is given (1.0% or 0.5%). Pelleted membrane fractions were resuspended to the original volume, and a volume equivalent to that containing 10mgml^{-1} submitochondrial particle protein was loaded. Anti-F_1 was used as probe.

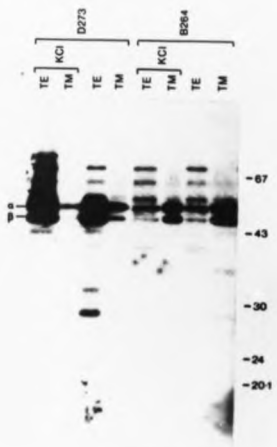


Figure 5.3.7 Extraction of mutant and wild-type submitochondrial particles with Triton X-100 (T) and KCl in the presence of the protease inhibitor PTFB. The detergent extraction was repeated adding 1% PTFB at every stage. Supernatant (E) and pelleted membrane (M) fractions are shown.

protease inhibitor PTFB was added at 20μgml^{-1} (1:100 dilution of stock solution in ethanol) to the mitochondrial preparation, and its concentration kept constant throughout preparation of submitochondrial particles and further Triton extraction. Figure 5.3.7 shows that the majority of F_1 is still in the pellet fraction in the mutant, with small but detectable amounts, especially of the α subunit in the supernatant. In the wild-type control, F_1 has been released into the supernatant as expected.

Also there is consistently less F_1 detectable in mutant fractions of mitochondria per unit protein when compared with wild-type fractions. This could be due to gradual degradation of the enzyme on the membrane, if it is indeed more susceptible to protease digestion than wild type, but it could also be explained by differing rates of transcription or instability of the mutant RNAs. Thus a northern blot was used to compare quantities of wild type and mutant F_1 RNA. Firstly total RNA was prepared from mutant and wild type strains by the method outlined in section 5.2. This RNA was run on a normal agarose gel together with size standards (Figure 5.3.8) and two lanes of each sample blotted onto NBM paper.

Two different DNA probes were used, both cut from plasmid vectors provided by Dr S. H. Ackerman. Sequences used were from the F_1 α and β subunits as these are the only F_1 subunits that have yet been cloned (Saltzgaber-Muller *et al*, 1983). The complete α gene was used as probe (with short vector sequences at either end), and in addition an 830 bp region of the gene for the β subunit was used in a second hybridisation. These fragments were labeled by nick translation and used in separate hybridisations, each being used to probe both mutant and wild-type RNA.

Unfortunately any signal produced with the α DNA hybridisation was indistinguishable from the background signal resulting from hybridisation to rRNA, which is abundant in total RNA preparations. An attempt was made to wash off the probe, and to re-hybridise under more stringent conditions (i.e. a higher concentration of formamide) but with no

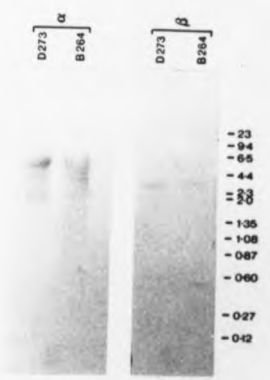


Figure 5.3.8 Northern blotting of total RNA from mutant and wild-type cells. RNA extracts from B264 and D273/10B-A1 were probed with fragments of DNA from the genes encoding the α and β subunits of mitochondrial ATPase.

greater success. However a clear band is distinguishable with the β DNA probe which, when read against the molecular weight markers run on the original gel, corresponds in size to β mRNA.

On comparing the quantities of β mRNA from wild-type and mutant no significant difference can be detected. The wild-type mRNA is slightly more abundant than the mutant mRNA but this difference can be explained when referring back to the UV photograph of the gel, because the mutant lane used in this hybridisation has been subject to a small amount of degradation. Differences between the mutant and wild-type at transcriptional level are not thought to be the cause of differences in the level of the F_1 subunits.

Preparation and use of fusion protein antibody

To gain more information concerning the nature of the protein for which the cloned gene codes, a fusion between this gene and *trp E* was constructed using the bacterial expression vectors pATH 3 and pATH 22. The fragment ST6, encoding the 313 carboxy-terminal residues of PET_{57}, was inserted into these vectors in frame with the *trp E* gene (Figure 5.3.9a). The calculated molecular weight of the fusion product was 65K daltons. After making the initial construct, transformation into *E.coli* RR1 was conducted under conditions in which the fusion product would not be expressed. The gene is repressed by tryptophan, so this amino acid was added to media until the final growth stage. The fusion gene product was prepared as in section 5.2, and Figure 5.3.9b shows a comparison between pellet and supernatant in the clones expressing the fusion product, compared with the wild-type RR1 pellet.

The pellets in the expressing clones show a high molecular weight species at 65K daltons, that expected for the fusion protein. This protein is over-expressed and, in addition, there are lower molecular weight species which are probably degradation products. The high molecular weight protein was purified by passing through a Bio-gel

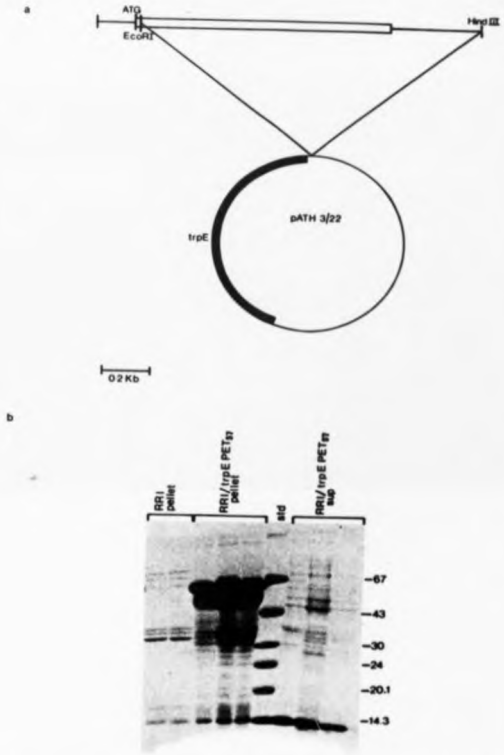


Figure 5.3.9 A *trpEPET*$_{sp}$ fusion protein of molecular weight 65 000 daltons is produced from bacterial expression vectors pATH 3/22 (a). the plasmid construct, (b). comparison of protein content of RR1/*trpEPET*$_{sp}$ pellet and supernatant fractions, and RR1 without the overexpressing plasmid.

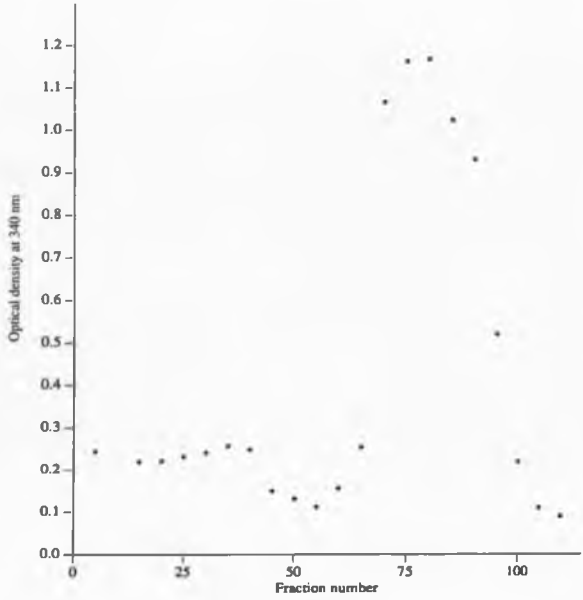


Figure 5.3.16 Protein profile of fusion product in Bio-gel eluate. The fragment ST4 was cloned into the EcoR1 site of bacterial expression vectors pATH 3 and 22 in frame with the *trpE* gene. The protein product from this construct was prepared from RR1 cells transformed with this plasmid. It was further purified by seperation on a Bio-Gel A-0.5 column, fractions were collected over an 18 hour period and their protein content estimated from optical density at 280 nm.

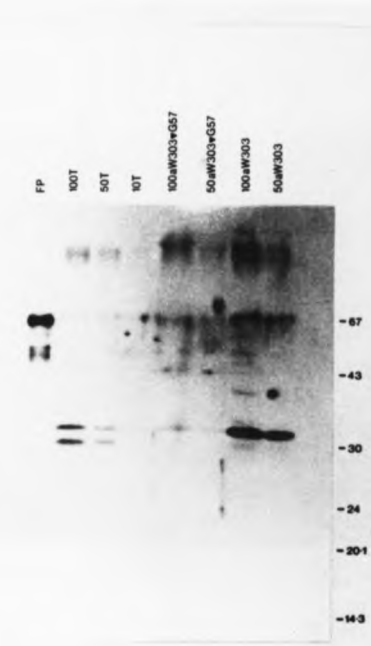


Figure 5.3.11 Mitochondrial samples probed with antibody to the *rpsEPET*$_{FP}$ fusion product. Antibody used was from the first bleed at a 1:100 dilution in rinse buffer + milk. Transformants (T), disruption (aW303ΔG57) and wild-type (D273) mitochondrial protein was loaded at protein concentrations of 50 and 100μg per lane. 1μg of fusion protein was loaded for comparison.

column, the elution profile obtained is shown in Figure 5.3.10. Fractions 68 to 78 were pooled and precipitated as described in section 5.2.

Antibodies raised in rabbits to this fusion protein were used to identify the pet_{57} gene product. Mitochondria from a transformant strain, a disrupted strain and wild-type were compared by western blotting and probing with this antibody at a 1:100 dilution in rinse buffer and milk. Varying concentrations of mitochondrial protein were loaded, up to 100µg per lane. The major species present in the wild-type is of an apparent molecular weight of 34K daltons (Figure 5.3.11). This species is also present in the deleted strain and the transformant (in reduced amounts). This is not the translation product of the reading frame for the PET_{57} protein as it is detectable in the deleted strain.

A second protein can be seen in the wild-type at an apparent molecular weight of 32K daltons. This protein is not detectable in the disrupted strain, but is expressed in the transformant over 10 times more than in the wild-type as would be expected from a multicopy plasmid. This is thought to be the G57 translation product, and its molecular weight of 32K daltons is consistent with the gene having a cleavable leader sequence. This indicates that G57 expresses a protein which is imported into mitochondria. It is unlikely to be a structural protein as it is not present in significant quantity in mitochondrial fractions.

5.4. CONCLUSIONS

A mutant phenotype has been described, which shows a number of unusual features which differ from any previously analysed mutants. The selectable phenotype used to clone the gene was its respiratory deficiency, thus allowing selection of transformants on the non-fermentable substrate glycerol. This was found to be due to a mutant ATPase activity. Thus investigation focused on this enzyme, which was identified by its reaction with anti-F_1 antibody.

Firstly the major subunits of F_1-ATPase were present in all the members of the complementation group. No differences could be observed in size of these subunits in mitochondrial protein samples. If the mutation was located in one of the genes coding for an F_1 subunit, the pattern expected when analysing its complementation group in this way would show a number of group members which had premature stop signals causing an attenuated transcript. This would be either degraded, and hence not observed on blotting, or the shortened protein would be detectable at a lower molecular weight than its wild-type equivalent. This is not the case for complementation group G57 so we must conclude that the cloned gene does encode an F_1 subunit.

The subunits of F_1 were membrane associated in the mutant, but inactive with respect to their ATPase activity. This highlights the question as to whether the F_1 domain is itself inactive, or whether it is incorrectly coupled to the membrane, either due to some factor in F_0 or because it is binding to something other than F_0 (i.e. a different membrane component). Mutants of *E.coli* with proton permeable membranes and low ATPase activity have been investigated, where F_1 shows abnormal binding characteristics. In contrast, this resulted in an F_1 which was easier to remove from the membrane, and when stripped from the membrane F_1 had normal ATPase activity (Maggio *et al*, 1988). This was found to result from a point mutation in the α subunit.

When considering the lack of ATPase activity shown in the mutant strain, it is useful to know that when reconstituted from its constituent subunits only α, β and γ are needed to produce a functional ATP hydrolysing unit. These subunits are present in the mitochondrial fractions of the mutant strains. The other subunits δ and ε are needed for integration into the membrane to produce a functional enzyme capable of ATP synthetic activity.

The fact that F_1 subunits cannot be detected when attempts have been made to displace them from the membrane could be attributed to two differing phenomena :-

1. The F_1 domain of the enzyme could be exhibiting abnormal membrane binding characteristics as discussed previously. This would explain why displacement from the membrane was not detectable after using a variety of methods which released the wild-type F_1 (or even the complete wild-type complex F_1F_0). These include prolonged sonication, extraction with choloroform and extraction with a number of detergents which normally isolate the complex. Detergent extraction with KCl was also attempted, which will remove all F_1 from the wild type membrane.

2. The F_1 subunits could be degraded as soon as they were removed from the membrane, which would explain why no α and β subunits could be detected in either the pellet or supernatant in the detergent and salt extracts. This would also explain certain discrepancies when comparing the quantities of wild-type and mutant subunit in further preparations. The major protease mitochondrial and submitochondrial particle activity present in mitochondria is that of a serine protease. A protease inhibitor PTFB was used to investigate this possibility, in all steps from production of mitochondria through sonication and isolation of smps to the final extraction with triton and KCl. However, this experiment did not provide any evidence to support the theory of degradation, and any protection from protease digestion (not a significant amount) only affected the membrane bound subunits.

Thus neither of these possibilities provides a satisfactory explanation of the mutant F_1.

Although it appeared from examining all mutant strains that α and β subunits were present in approximately equal quantities, when comparing later preparations of B264 and D273/10B-A1 mitochondrial and submitochondrial particle preparations the mutant strain was found to have consistently less α and β subunit. This was not found to be due to transcriptional factors, as analysed by preparation of total RNA from mutant and wild type and Northern blotting with α and β probes. The quantities of RNA in the mutant and wild-type strains was almost identical, and any difference was not sufficient to

explain the different subunit stoichiometries observed on western blotting.

The mutant F_1F_0 complex could not be isolated by the usual method of detergent extraction, so no information could be derived about the size or gradient migration properties of the complex. However, the reconstitution experiment shows that the F_0 of the mutant strain is capable of binding wild-type F_1 and integrating it into a functionally active complex. This experiment adds to the evidence that the F_1 domain of the ATPase enzyme is mutant and causes the respiratory deficiency in B264.

The protein coded for by the gene that is mutated in B264 is not one of the structural units of ATPase F_1, as although it is a similar size to the γ subunit it had little homology with the sequence of the γ protein in other species. Therefore the mutation must be in a component which controls synthesis or assembly of F_1.

Analysis of mitochondrial protein samples with fusion protein antibody shows that the protein coded for by *PET*$_{57}$ is present in mitochondria, but not in the quantities expected for a structural protein.

CHAPTER 6

GENERAL CONCLUSIONS AND DISCUSSION

6.1. Antibiotic Resistance

The nuclear-coded genes for the α and β subunits of F_1 (genes ATP 1 and ATP 2 respectively) and the gene for ATPase subunit 4 (ATP 4) have been isolated in *S.cerevisiae* by other laboratories (Saltzgaber-Muller *et al*, 1983, Velours *et al*, 1988). The genes ATP 1 and ATP 2 were cloned by genetic complementation of *pet* mutants specifically lacking the α and β subunits respectively with a wild-type plasmid library. The gene ATP 4 was isolated from a *S.cerevisiae* genomic library by hybridisation with an oligonucleotide probe, constructed by back-translation of the amino-terminal sequence of the isolated protein. The genes for the α and β subunits of mitochondrial ATPase in the fission yeast *S.pombe* were also cloned by genetic complementation of mutants lacking either the α or β subunits (Boutry *et al*, 1984). Thus the method of choice for cloning nuclear subunits of mitochondrial ATPase is not by expression of antibiotic resistance, even though antibiotics binding the F_1 moiety of the enzyme are available (for example aurovertin).

In this study the majority of mutants isolated showed cross-resistance to other antibiotics affecting mitochondrial function (Groups I and II), including the antibiotics chloramphenicol and erythromycin. This indicates that the site of resistance is not located on ATPase F_0 as chloramphenicol and erythromycin inhibit mitochondrial protein synthesis, but suggests resistance at the mitochondrial membrane or plasma membrane level resulting in lowered permeability to a variety of antibiotics. This type of cross-resistance is a feature of nuclear-coded mutants isolated in previous studies (Avner and Griffiths, 1973a, b, Griffiths *et al*, 1975, Lancashire and Griffiths, 1975a, b). Some of these cross-resistant mutations were ambiguous in their mode of inheritance, showing both nuclear and cytoplasmic factors (Cohen, 1977, Cohen and Eaton, 1979), which led

to the proposal of an additional cytoplasmic element with which expression of resistance is associated (Guerineau *et al*, 1974). However, most sources believe that the cytoplasmic loci involved are located on the mitochondrial genome (Cohen and Eaton, 1979, Simmons and Breen, 1983).

Group I mutants isolated in this study are thought to be alleles of the *pdr* linkage group on *S.cerevisiae* chromosome VII described by Saunders and Rank (1982), based on linkage to leu_1 and phenotypic properties. As well as multiple antibiotic resistance, diploid crosses of haploids from this group with the sensitive strain D22 give isolates with a semi-dominant background but having resistant papillae identical to the *pdr* allele *ant1* characterised by Cohen and Eaton (1979). Group II mutants ($Ven^R Cap^R$) are probably controlled by a different pleiotropic chromosomal gene. Mutants of similar phenotype, non-allelic to *pdr* (Saunders and Rank, 1982), have been described by Guerineau *et al* (1974).

Crosses between all venturicidin mutants isolated and a sensitive wild-type strain gave rise to diploids of intermediate resistance. Complementation tests were of little use in defining associated groups of mutants because of ambiguity of resistance in diploids. Mutations of this phenotype would be extremely difficult to clone, and if this was ever attempted it would be on the assumption that a gene dosage effect would mean that a partially dominant gene could appear dominant if expressed in sufficient quantity. This would be achieved by transformation of a sensitive strain with a high copy number plasmid containing a genomic library prepared from a resistant strain.

None of the mutants generated in this study showed increased resistance to the selective antibiotic at mitochondrial ATPase level. In comparison, the mitochondrial ATPase of *S.cerevisiae* strains having mitochondrially-coded antibiotic resistance invariably shows an increase in I_{50} for that antibiotic (Connerton, 1986, Ray *et al*, 1988). Unless this could be demonstrated in a nuclear mutant it would be uneconomic to attempt to identify the

mutant gene by cloning because of the problems associated, as it is unlikely to be a structural component of the complex. As the nuclear components of F_0 have already been identified in *S.cerevisiae* (Velours *et al*, 1988) a more direct method of cloning the genes is now available, and has been used successfully to clone ATP4, the structural gene for ATP subunit 4 (Velours *et al*, 1988). This would involve limited protein sequencing and probing a wild-type genomic library with an oligonucleotide probe derived from this sequence, thus identifying the gene for a known ATPase F_0 subunit.

From sequence analysis of mitochondrial DNA from oligomycin and venturicidin resistant mutants having mutations in *oli1* and *oli1l* the amino acids responsible for resistance in subunits 9 and 6 respectively have been defined (Ooi *et al*, 1985, Nagley *et al*, 1986, John *et al*, 1986, Ray *et al*, 1988). From this domains involved in antibiotic binding and, by inference, domains important in proton translocation have been identified. This type of analysis may prove difficult with the nuclear-coded subunits, as by far the most frequent mutation to antibiotic resistance appears to be in a non-specific membrane factor (this study, Avner and Griffiths, 1973a,b, Guerineau *et al*, 1974, Griffiths *et al*, 1975, Lancashire and Griffiths, 1975a,b). The only nuclear-coded antibiotic resistant mutation resulting in an alteration of a structural component of ATPase was isolated by Willson and Nagley (1987) when they identified a nuclear suppressor to a *mit*$^-$ mutation which also conferred venturicidin resistance to the complex.

6.2. Organotin/Hydroxyflavone Complexes

At present studies are at a very early stage, and experiments are in progress with other 3-hydroxyflavone derivatives to determine which have the most desirable characteristics, and to further identify their binding site. However, the potential of these compounds as fluorescent probes of mitochondrial ATPase F_0 is already evident. A number of possible applications can be envisaged. Trialkyl tin compounds are known to bind F_0, but no

resistant loci have been mapped to the mitochondrial genome, suggesting that the binding site is composed of nuclear-coded subunits only, in contrast to oligomycin and venturicidin. The fluorescent compounds could conceivably be used as a simple method of screening groups of *pet* mutants for the presence of this binding site. Unknown compounds could be screened for F_0 binding at this site, and known ATPase inhibitors investigated to determine whether they share the organotin binding site. It may also be possible to monitor F_0 in biochemical preparations. All are potentially interesting directions for future research.

6.3. The *PET*$_{57}$ mutant

This thesis describes a *S.cerevisiae PET* mutant which lacks functional F_1F_0 ATPase activity. The DNA encoding the *PET*$_{57}$ gene has been completely sequenced, and was found to encode a putative protein product 36 Kdal in size. This protein has a region at its N-terminus which shows a number of features evident in known mitochondrial signal sequences which are post-translationally cleaved. Antibodies to a *trpE/PET*$_{57}$ fusion protein detect a protein species of molecular weight 31 Kdal in mitochondrial preparations, which is not observed in mitochondria from a strain where *PET*$_{57}$ was disrupted with *his III*, but is overexpressed (in comparison with wild-type) in mitochondria prepared from strains transformed with a complete copy of *PET*$_{57}$ in a plasmid vector. So from this evidence it would appear that the *PET*$_{57}$ protein product is transported into mitochondria.

The mutant mitochondrial F_1F_0 ATPase from the mutant parent strain (B264) shows many abnormal characteristics. The level of the F_1 α and β subunits in mitochondrial preparations appears significantly lower in the mutant that in the wild-type. The F_1 or the F_1F_0 complex has not been successfully isolated from B264, but whether this is due to abnormal membrane binding characteristics or to increased susceptibility to protease digestion is unclear at present. It is also evident that the complex can be reconstituted

into functional, oligomycin-sensitive form by adding back wild-type F_1 to stripped mutant membranes. The protein product of PET_{57} would appear to exert its effects post-transcriptionally, as comparison of RNA levels for the β subunit of F_1 ATPase between mutant and wild-type does not show a significant reduction in the mutant strain which could account for the lowered level of the β subunit in the mutant mitochondria.

The biochemistry of the pet_{57} mutant phenotype shows a number of similarities with the mutant pet_{936} isolated by Todd (Todd *et al*, 1979). The author describes a mutant which lacks functional F_1F_0 ATPase activity and shows a similar repression of the mitochondrial enzyme complex cytochrome oxidase. Investigations were focused on the mitochondrial ATPase. On immunoprecipitation of this complex with antisera to a Triton extract (i.e. to the complete F_1F_0 complex) only 18% of the wild-type level of ATPase determinants were precipitated in the mutant. The amount of translatable mRNA was compatible in mutant and wild type, although this was not quantified in terms of F_1 α and β mRNA as the respective genes ATP1 and ATP2 had not been cloned at that time. The author also identifies two additional protein species present in immunoprecipitates from the mutant strain, and changes in the relative stoichiometry of other ATPase subunits. The conclusions drawn to explain the phenotype of this mutant suggests that the nuclear gene product from PET_{936} enhances translation of mRNA encoding nuclear ATPase and cytochrome oxidase subunits, and by this common translation mechanism the biogenesis of the two complexes are coordinated at the molecular level. Thus the PET_{936} gene product is expected to exert its effect outside the mitochondrion, so mitochondrial import would not be necessary. A similar mutant has been observed in the fission yeast *S.pombe* (Goffeau *et al*, 1974) in which a single nuclear mutation results in deficiencies in mitochondrial ATPase and cytochrome oxidase. However, mitochondrial ATPase activity is present in this mutant, although sensitivity to oligomycin is lost, and there is evidence that the mutation is in a structural subunit, so this mutant group may be unrelated to that described previously (current

study, Todd *et al*, 1979).

Cheng and collegues (1989) describe a conditional recessive *pet* mutant, *mif4*, which is essential in conferring a conformation on imported mitochondrial proteins making them competent for further assembly or sorting. The *mif4* gene encodes heat shock protein hsp60, which is one of a group of proteins termed "chaperonins" as they chaperone oligomeric protein assembly. No mature F_1ATPase subunit could be detected in the supernatant after extraction of mutant mitochondria with chloroform, and the authors conclude that the β subunit fails to assemble into the ATPase complex. The mutant also fails to assemble cytochrome b_2 and the Rieske Fe/S protein of complex III, and these proteins accumulate in the mitochondrial matrix as incompletely processed intermediates. Therefore the phenotype of this mutant also has similarities to the PET_{57} mutant phenotype.

However, when the PET_{57} DNA and protein translation were used in DNA and protein database searches, no homology with chaperonin proteins was discovered. The EMBL nucleotide sequence library was searched (release 19, May 1989) which contains the *E.coli* and wheat chaperonin sequences. The Genebank DNA sequences (March, 1989) and the PIR and Swiss-Prot protein sequence databases (both March, 1989) were also searched. A more specific analysis, comparing the PET_{57} protein translation with the ten known chaperonin proteins including that investigated by Cheng (Cheng *et al*, 1989), did not show significant homology with alignments of the chaperonin sequences. In addition there was no apparent ATP binding site, and chaperonin proteins are thought to have some ATPase activity. So the PET_{57} protein is not a member of the group of chaperonins reported to date.

Many *PET* genes have been isolated and for some their protein products identified. A specific function can be attributed to some protein products, others have been identified tentatively. *PET* genes and their function are summarised in Table 6.1. The known *PET*

products fulfill the following requirements: in addition to structural proteins and tRNA synthases, factors exist which process mitochondrial mRNA and enhance its translation.

Predicted Product	Gene Name	Reference
α subunit of F_1	*ATP1*	
β subunit of F_1	*ATP2*	Saltzgaber-Muller *et al*, 1983
ATPase subunit 4	*ATP4*	Velours *et al*, 1988
Subunit 4 of cyt.ox	*COX4*	Maarse *et al*, 1984
Subunit 5 of cyt.ox	*COX5*	Koerner *et al*, 1985
Subunit 6 of cyt.ox	*COX6*	Wright *et al*, 1984
ADP/ATP translocator	*OP1*	O'Malley *et al*, 1982
Ribosomal protein	*MRP1*	
tRNA synthetases	*MSY, MST1, MSD*	Pape *et al*, 1985
	MSF1,MSW	Myers and Tzagoloff, 1985
Cyt.b maturase	*CBP2*	McGraw and Tzagoloff, 1983
Cyt.b 5'end processing	*CBP1*	Dieckmann *et al*, 1984
Cyt.b translation factor	*CBP1, CBP6*	Dieckmann and Tzagoloff, 1985
	MK2	Roedel *et al*,1985
Cyt.ox sub 1 pre-mRNA splicing	*MSS116, MSS51*	Faye and Simon, 1983a, b
Cyt.ox sub 2 translation factor	*PET111*	Poutre and Fox, 1987
Cyt.ox sub 3 translation factor	*PET494, PET54*	Mueller *et al*, 1984
Mitochondrial protein assembly	*mif4*	Cheng *et al*, 1989
Cytoplasmic RNA translation	*PET936*	Todd *et al*, 1979

Table 6.1 *PET* genes and their predicted protein products

The protein encoded by PET_{57} does not fall under any of these categories as it appears to affect the products of nuclear genes. Evidence has been presented in this thesis that this protein is transported into mitochondria, and this argues against it exerting its effects on the mitochondrial F_1 ATPase subunits at the level of mRNA. The requirement of nuclear proteins to enhance the translation of mitochondrial mRNA appears a common mechanism in the nuclear control of mitochondrial biogenesis, and it is not unreasonable to propose a similar mechanism for the control of cytoplasmically-derived mitochondrial subunits. It is possible (but unlikely) that the PET_{57} protein product could enhance the

translation of both nuclear and mitochondrial mRNA in their respective cellular compartments, which would allow for transport of the protein into mitochondria. However, the simplest explanation is that the PET_{57} protein product is involved in processing or assembly of subunits imported from the cytoplasm, such that its absence causes aberrant integration into the ATPase complex.

Future investigations into the pet_{57} mutation are initially directed at determining whether the mutation is allelic to the pet_{936} mutation described by Todd (Todd *et al*, 1979). The first step will be to determine whether pet_{57} and pet_{936} are in the same complementation group by mating the two strains and observing whether respiratory competent progeny result. The biochemistry of the mutant parent strain B264 can also be further investigated which would give an indication as to whether the two mutations had identical phenotypes. Firstly B264 mitochondria could be assayed for cytochrome oxidase, as Todd reports a pleiotropic deficiency in this enzyme in pet_{936} mitochondria. Use of the antibody to $trpE/PET_{57}$ should be able to answer questions about the location of the PET_{57} protein; whether the protein is membrane-bound or soluble, and whether it is detectable in any significant quantity in the cytosol. The PET_{57} protein is an essential factor in the biogenesis of mitochondrial F_1F_0 ATPase, an enzyme of dual biosynthetic origin, and further investigations may clarify factors coordinating nuclear and mitochondrial protein synthesis systems.

APPENDIX I

Preliminary Analysis of *S.cerevisiae* Resistance to other ATPase Inhibitors

This appendix covers results generated from the use of other antibiotics known to inhibit mitochondrial ATPase by binding to the F_0 domain of the enzyme complex. Each antibiotic was used in preliminary studies to determine whether it inhibited oxidative growth of *S.cerevisiae*, and if it could be used to generate resistant mutants.

Materials and Methods

All methods used in this chapter have been covered previously in Chapter 2, in which suppliers of chemicals for *S.cerevisiae* media and antibiotics have also been identified.

Results

Initially antibiotics were added to solid media to give plates containing increasing concentrations of antibiotic. The sensitive strain D273/10B-A1 was then streaked on each concentration and its growth scored after 3 days incubation at 30°C. Antibiotic plates were used within 24 hours of their preparation. Table Ia shows the extent of growth on antibiotic-containing media using four different antibiotics. The 3-hydroxyflavone derivative and the morin derivative were described in this study, leucinostatin was investigated by Lardy (Lardy *et al*, 1975) and diethylstilbesterol by McEnery and Pederson (1986).

The fluorescent Sn compounds did not inhibit *S.cerevisiae* growth in either solid or liquid media (results not shown), which was thought to be due to either a permeability barrier at plasma membrane level or to reaction with the media chemicals. Diethylstilbesterol inhibited *S.cerevisiae* growth at high concentration, but was unstable in the medium – plates containing this antibiotic had to be used immediately so it was difficult to achieve

Antibiotic	Antibiotic Concentration in Media (μgml^{-1})							
	0.5	1	2	4	5	10	15	20
Diethylstilbesterol	+++	+++	+++	+++		++	–	–
Leucinostatin	+++	++	–		–	–		
Bu_3Sn(of)Br	+++	+++	+++			+++		
Bu_3Sn(morin)Br	+++	+++	+++			+++		

Table Ia Resistance of D273/10B-A1 to the antibiotics diethylstilbesterol, leucinostatin and organotin compounds. The sensitive strain D273/10B-A1 was replicated onto N3 (buffered glycerol) medium with antibiotic additions. Growth was scored after three days incubation at 30°C with (+++) representing uninhibited growth and (–) used for concentrations which inhibited growth completely.

good levels of inhibition. Leucinostatin inhibited growth at a lower concentration, there was good definition between inhibition and growth, and plates could be kept for more than two weeks without the antibiotic losing its effectiveness. Therefore this antibiotic was selected for further study.

Leucinostatin resistance

This antibiotic has a mode of action similar to that of the tri-alkyl tin compounds which have been previously investigated in this laboratory. At low concentrations leucinostatin causes a loss of ATPase activity, but when the concentration is increased the compound causes dissipation of the mitochondrial membrane potential. This can be observed easily in rat liver mitochondria (Figure Ia) where addition of the compound causes a fall in fluorescence of the extrinsic fluorescent probe $DSMP^+$ (this experiment is described in detail in section 3.2). *S.cerevisiae* mitochondria are poorly coupled and thus this experiment cannot be applied.

The effect of this compound on mitochondrial ATPase is of interest because the leucinostatin and venturicidin binding sites are thought to overlap (Lardy *et al*, 1975). Its dual mode of action also suggests comparison with the tin compounds may be of interest.

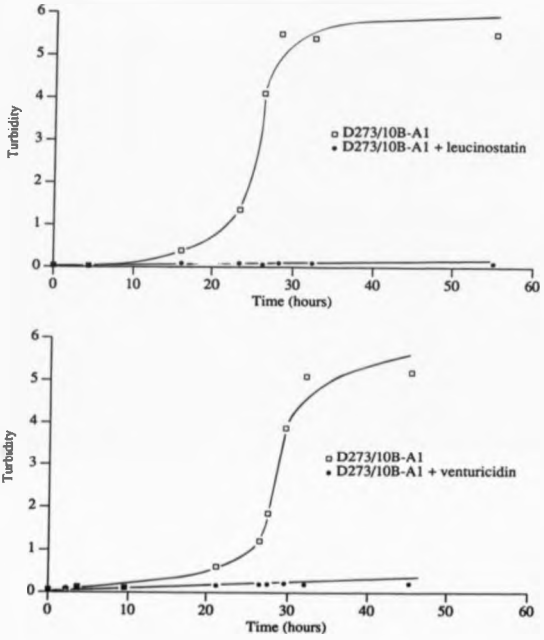


Figure 1a Turbidity of D273/10B-A1 in liquid N3 with addition of (a) 5 μgml^{-1} leucinostatin and (b) 5 μgml^{-1} venturicidin. Turbidity was measured on an EEL colourimeter using a 607nm filter as described in Chapter 2.

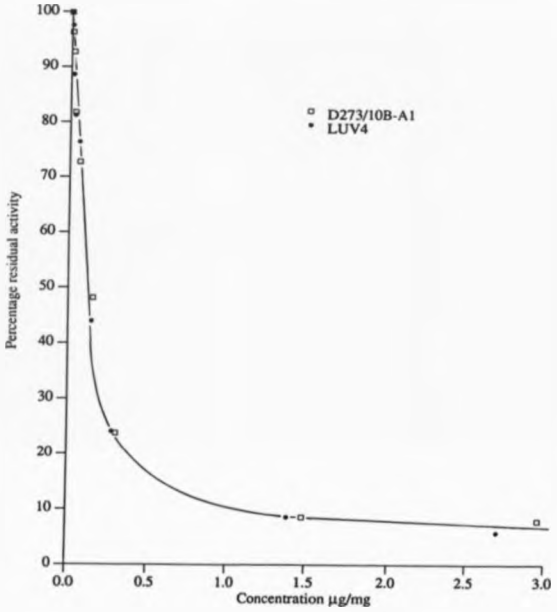


Figure 1b Mitochondrial ATPase resistance to the antibiotic leucinostatin from wild-type (D273/10B-A1) and leucinostatin resistant (LUV4) strains. Methods for ATPase assay, phosphate determination and protein assay are described in section 2.2. Concentration of inhibitor is given in µg per mg of mitochondrial protein.

Firstly strain D273/10B-A1 was grown in liquid culture, with or without leucinostatin, to determine whether this antibiotic solely affected the aerobic growth. Four cultures were inoculated, NO with 5 μgml^{-1} leucinostatin, N3 with 5 μgml^{-1} leucinostatin and controls without the antibiotic. Turbidity of these cultures was measured at varying time intervals and is shown in Figure Ib, together with a similar experiment using venturicidin as inhibitor. From this it can be seen that leucinostatin has similar effects on D273/10B-A1 growth as the previously characterised antibiotic venturicidin. Leucinostatin inhibited aerobic growth in the culture medium (at 30°C for 50 hours.

Leucinostatin was added to solid medium from an ethanolic stock solution, using a variety of concentrations to determine over which limits the antibiotic was effective. A small amount of growth of the wild-type (sensitive) strain D273/10B-A1 was observed on solid N3 medium containing 1 μgml^{-1} leucinostatin, but 2 μgml^{-1} was completely effective in inhibiting growth.

D273/10B-A1 was mutated using UV irradiation by the method described in Chapter 2 and resistant mutants were selected on N3 medium containing 2 and 5 μgml^{-1} leucinostatin. L^R mutants were isolated at 2 μgml^{-1}, but not at the higher concentration. Further UV mutation of these L^R mutants could generate resistance to 5 μgml^{-1}, but this level of resistance was never achieved in a single step mutation. Cross resistance of the mutants isolated is shown in Table Ib.

To determine whether any of the L^R mutations were mitochondrially-coded, the selection procedure described by Lancashire and Mattoon (1979) was employed. This is based on the fact that a mitochondrial mutation will show dominant resistance in diploid if mated to a ρ^o tester strain, but the diploids produced from converting mitochondrial resistant mutations to ρ^o with EtBr and mating with a ρ^+ tester strain will be sensitive. To convert mutant strains to ρ^o, colonies were grown on $EtBr^-$ containing medium for three days and then replicated once on to a fresh EtBr plate. It was discovered that a proportion of

Strain	N3L	N3Ol	N3V	N3CAP	N3ERY	Mating
LUV4	2	<0.5	1	1	4	–
LUV42	2	<0.5	1	1	4	–
LUV7	2	<0.5	1	1	4	+
LUV85	2	<0.5	1	1	4	+
DLUV4a	5	<0.5	0.5	1	4	–
DLUV42a	5	>5	1	4	10	–
DLUV7a	5	>5	1	4	10	+
DLUV85a	5	<0.5	1	1	4	+
D273-10B	1	<0.5	0.25	1	4	+

Table Ib Cross-resistance of leucinostatin-resistant mutants. Strains with the prefix D have been mutated twice and their singly-mutated parent strains are also shown. Growth on antibiotic containing media was scored after three days at 30°C. Concentrations of the inhibitors leucinostatin (L), oligomycin (Ol) and venturicidin (V) are given in $\mu g ml^{-1}$. Concentrations of chloramphenicol (CAP) and erythromycin (ERY) are given in $mg ml^{-1}$. Antibiotic plates were made up in buffered glycerol medium (N3).

the L^R mutants were sensitive to ethidium bromide, and also that they would not mate to tester strains. This was retained in doubly-mutated strains originating from the deficient mutants.

Mutant mitochondria were tested for ATPase activity and degree of resistance to leucinostatin compared to the parent strain. Methods used are described in chapter 2.

Mutant mitochondria showed no observable increase in I_{50} for leucinostatin, and from this we must conclude that a structural change in an ATPase subunit is unlikely. Inhibition curves are shown in Figure Ib, and I_{50} for D273-10B/A1 was 0.12 µg/mg protein.

APPENDIX II

Guidelines on the Use of Computers in DNA Sequencing Projects

This appendix describes programs available on the UNIX system at Warwick for the analysis of DNA and protein sequence data. In addition it covers programs available at other U.K. universities, specifically Cambridge and Edinburgh. All programs described should be readily available to researchers at Warwick in the near future if they obtain user codes on the SERC machine at Daresbury. Where analyses have been performed on the DNA sequence resulting from this project, or its protein translation, the output has been included for illustration. Programs not used in this project have been described in outline.

Input of Data from Gels

Information can be taken directly from photographs or autoradiograms of gels by using a digitiser, a lightpen and a lightbox. This method is used for

1. Restriction mapping of DNA

2. Generation of a consensus sequence from a number of sequencing gels.

Firstly the computer can be used to estimate the lengths of fragments produced by restriction enzyme digests by comparison with size standards of known length run on the same gel (`FRAGSIZE`). The most probable restriction maps of the restriction sites for the enzymes used can then be generated (`RESTRICT`), together with an associated error value. The error value is used to differentiate between a "good" map and a "poor" map. These programs are not yet available at Warwick, and their accuracy is questionable at present as they are in a basic form, with problems known to occur when analysing enzymes producing small fragments.

DNA sequence data can be analysed on the UNIX system at Warwick. The method available, termed the "DB System" is normally applied to fragments generated by the Sanger dideoxy sequencing method, but is probably transferable to Maxam and Gilbert gels. The user defines the four areas containing the sequencing lanes using pen and digitiser, and then inputs the sequence into the computer by touching the pen to successive bands on the gel (GELIN). The data from each gel is stored in a separate file. Vector sequences can be identified by the SCREENV program, and subsequently removed from the contaminated files. The program DBAUTO is then implemented to compare each gel with all others in the sequence project database, and to arrange gels which overlap into contigs. A consensus sequence is calculated from each contig and used as a basis for further analysis (comparison with a DNA database, determination of putative open reading frames etc.).

DNA Analysis by Computer

The restriction map and sequence of PET_{57} were determined without the use of a computer, but the DNA and protein sequence analysis programs were used extensively. ANALYSEQ is R. Staden's collection of sequence analysis programs, which have programs subdivided into groups performing similar functions, and accessible through different menus. The menu number and the instruction number to implement the program discussed is shown in brackets.

Identification of Coding regions

To determine the most likely reading frame of a sequence a variety of methods are available in ANALYSEQ, which can be accessed by entering the "gene search by content menu" (-3). It is usually necessary to analyse all 6 reading frames of a DNA sequence so the complement of the DNA as stored must be generated. To reverse and complement a sequence in ANALYSEQ the general menu (-1) must be accessed, and the reverse and complement option selected (13). Two methods are discussed in detail as

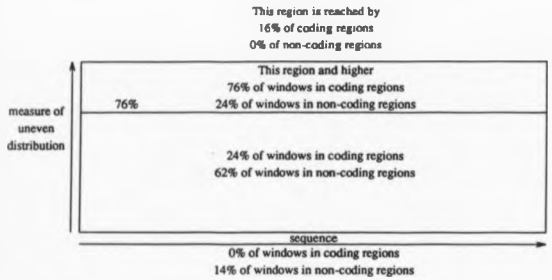


Figure IIa Interpretation of graphical display produced using the method of uneven positional base frequencies (Staden, 1984a).

Figure IIb Output generated by applying the method of uneven positional base frequencies to the coding strand of PET_{57}.

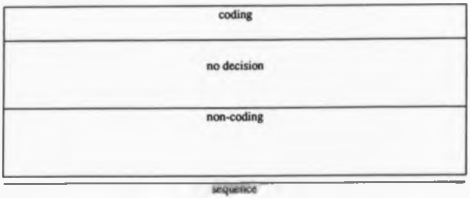


Figure IIc Interpretation of the graphical display using the method of Fickett (1982).

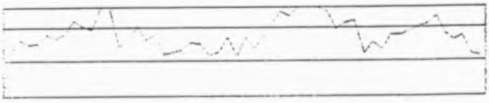


Figure IId Output generated by applying Fickett's method to the coding strand of PET_{57}.

illustration. The first, uneven positional base frequencies (54), relies on the assumptions that :-

1. A non coding region of DNA has a random distribution of nucleotides in all three codon positions, for all three reading frames.

2. A coding region of DNA has some nucleotides overrepresented in certain codon positions, for all three reading frames (Staden, 1984a).

The output generated by this program is graphical, and is interpreted as shown in Figure IIa. Computer analysis of the PET_{37} by this method does not identify it as likely to encode a protein (Figure IIb).

The method of Fickett (21, 1982) was developed from this basic strategy. This method differs from the uneven positional base frequency method as it weights the parameters generated according to their ability to differentiate between coding and non-coding regions of DNA (the weightings were generated by analysis of a library of known proteins). The output from this program classifies windows into three categories as shown in Figure IIc.

The output generated by the PET_{37} protein is shown in Figure IId.

The second method discussed relies on the uneven use of amino acids by proteins. The positional base preference method (58, Staden, 1984a) determines the frequency of each base in each of the 3 codon positions. In a coding region G is frequently found in position 1 and A in position 2. This analysis is repeated for each reading frame, and the method is probably most useful in determining the coding frame of a protein-coding DNA. The results are presented in the form of three graphs per strand, each representing one reading frame (Figure IIe).

Of the two types of option available in ANALYSEQ to determine the probability of a

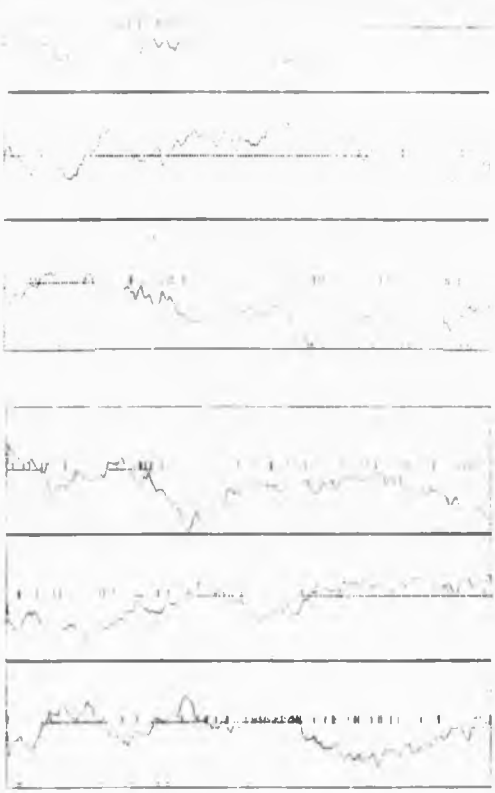


Figure IIa Both the coding (a) and non-coding (b) strands of PET_{21} were analysed by the positional base preference method (Staden, 1984a). The graphical display also shows stop signals as short vertical bars.

DNA sequence coding, the gene search by content menu (-3) contains the most powerful methods available to date. The alternatives, in the gene search by signals menu (-4), include programs to identify start (24) and stop (25) codons, mapping the longest open reading frames (16), and options to mark possible ribosome binding sites (3, 38) or polyadenylation sites (39). These options are self-explanatory so will not be covered in this appendix (see Staden, 1984b).

DNA Database Searching

The initial step towards identifying the gene under investigation is to compare the DNA sequence with sequences of known function stored in a database (such as EMBL, stored on the IBM system SKY at Warwick). The sequences in the database most closely related to the probe sequence can be identified and extracted. For the gene PET_{57} a search of this nature was carried out on the Cambridge University Molecular Biology VAX 8350. The EMBL database was searched with the complete 1.6 Kb sequence of the coding strand PET_{57} using the program FASTN. The best scores were saved and listed with an initial score and an optimised score. The former gives an indication of the degree of homology of the first significant alignment found in a particular sequence, the latter refers to the score when considering the two sequences as a whole. The PET_{57} DNA did not produce any good alignments with any of the entries of the database (Figure IIf). Of the best scores produced, 25% of the database entries were located on yeast mitochondrial DNA. This may be significant, as current theories propose that mitochondrial genes have been gradually transferred to the nucleus, and synthesis of the PET_{57} protein is a requirement for biogenesis of functional mitochondria. However, this was not helpful in directly identifying the PET_{57} gene.

From the analysis of PET_{57} DNA the function of the open reading frame could not be identified. Now we will discuss what information can be generated from computer analysis of the putative protein product of the open reading frame. Again, programs not

GS7.DNA, 1609 NT vs hNBRT library

```
  0      1 :=
  8      0 :
 12      0 :
 16      0 :
 20      0 :
 24    191 :=========================================================
 28   1031 :=========================================================
 32   2434 :=========================================================
 36   2917 :=========================================================
 40   2464 :=========================================================
 44   2197 :=========================================================
 48   2270 :=========================================================
 52   2260 :=========================================================
 56   1690 :=========================================================
 60   1172 :=========================================================
 64    813 :=========================================================
 68    445 :=========================================================
 72    272 :=========================================================
 76    155 :=========================================================
 80    113 :=========================================================
 84     82 :=========================================
 88     55 :===========================
 92     28 :==============
 96     19 :==========
100     15 :========
104      6 :===
108     13 :=======
112      4 :==
116      2 :=
120      0 :
124      3 :==
128      1 :=
132      1 :=
136      0 :
140      0 :
144      0 :
148      0 :
152      0 :
156      0 :
160      0 :
>160     0 :
24201968 residues in 20703 sequences, mean score:  44.5 (10.54)
 1806 scores better than 60 saved, ktup: 6, fact: 6 scan time:  0:40:45.99
The best scores are:                                      init,  opt
>DL;MVNIFH      :  Methanococcus voltae gene homologous to nif 132,  342
>DL;HSAFPCP     :  Human alpha-fetoprotein gene, complete cds  126,  556
>DL;M16711      :  Figure 1. The nucleotide sequence of segmen 124,  566
>DL;M13C26      :  Yeast mitochondrial DNA with tRNA-Asp gene  122,  324
>DL;MISCTRND    :  Yeast mitochondrial gene for transfer RNA-A 122,  308
>DL;CHMPXX      :  Liverwort Marchantia polymorpha chloroplast 114,  510
>DL;CHMPXX-1    :  Liverwort Marchantia polymorpha chloroplast 114,  510
>DL;SCCDC28     :  Yeast CDC28 (cell division control) gene    112,  154
>DL;GMGL02      :  Glycine max leghemoglobin gene (gene Lbc)   112,  348
>DL;MAPRPH29    :  Golden Syrian Hamster PRP gene H29, encodin 112,  398
>DL;MISCTRNA    :  Yeast mitochondrial tRNA genes for tRNA-Ala 110,  582
>DL;DMYP3G      :  Drosophila yolk polypeptide gene YP3        108,  294
>DL;MISC14      :  Yeast mitochondrial tRNA genes (several, ma 108,  613
>DL;GGFERH      :  Chicken ferritin H-subunit gene, complete c 108,  332
>DL;DMYP3       :  D.melanogaster yp3 gene encoding yolk prote 108,  306
>DL;M14751      :  Figure 6. Nucleotide sequences of 5' NTS fr 106,  146
>DL;MISCAAP1    :  Yeast mitochondrial aap1 gene for ATPase su 106,  548
>DL;GMGL05      :  Glycine max leghemoglobin gene (gene II) fr 106,  298
>DL;MICF12S9    :  Crithidia fasciculata mitochondrial genes f 106,  478
>DL;MITGTRN3    :  Torulopsis glabrata mitochondrial genes tRN 106,  254
```

```
DL;MVNIFH    :  Methanococcus voltae gene homologous to nif I32,  342
46.3% identity in 753 NT overlap

                 850        860        870        880        890        900
G57.DNA     TGCAGCAAAAAAATAT-TGAAGAGCTTATCACCATGGGATCTTGCAATTCTTGAAAAAAC
                 ::  :: ::  :    : :: :    :  :::     ::    : ::::
MVNIFH           GACGAAGCATTTAGCAAATACATAALGGATTATGATAGGATAAGAGATTAAAACTA
                        10         20         30         40         50

                 910        920        930        940        950        960
G57.DNA     TGTATTAACCACAAAGTCTTCATTTGCGGCGTGCTATTATTAQAAAATAAAAAGATAC
            :    ::::: ::::      :: :         :  :: ::    : : :  ::   : :
MVNIFH      TCGGAAAACCAAAAAG----ACAGTGTAAAAGGAALTTTTGAGTAAGATTTTAACGAAAAA
              60         70         80         90        100        110

                 970        980        990       1000       1010
G57.DNA     TGCGAACTTAATTCCCGCCTTGAAAACTGATATGGA-TAATATTGTACGTGCCGCCACCT
            :: : :  ::  :   :  :  :: :  :::  : :  ::   :  :  ::  :  :
MVNIFH      TGAGGA--TAGAT-GGGAATATAATATGGATTCGAAGGAAGCATTAATTTGGTGGTAATA
                 120        130        140        150        160

           1020       1030       1040       1050       1060       1070
G57.DNA     TAGAAACAATCTTCCAAGTTGAAAAGTGGGGAGAGGTTGAAGATACTCATGACGTTGACA
            ::  ::  :: ::   : :    :: :    : : :   : ::: : : :  :: : :
MVNIFH      TACTAATTAT-TTAGTATTAATTAACTAATAATAAATAATAAATAATAAAATA-ATTAATA
          170        180        190        200        210        220

           1080       1090       1100       1110       1120       1130
G57.DNA     AAAGAGACAT-CAGAAGAAAAATTCATACTGCTGCGATTGCTGCTTTTAAGCAATAATAT
            : : : :  :    :: : :: :: : ::   :: : ::  :::    : :::::
MVNIFH      ATAAATAATTATTTTTTAATTAATCCTATTCCTCTAATCGATGACTTTTTTTAGTAATAG
            230        240        250        260        270        280

           1140       1150       1160       1170       1180       1190
G57.DNA     GGTT-GATTCAATTGTTGTTCTATTACTACTTTTCTATGATATATATACAAGCATATATT
              ::   ::: :   :    :XXXXX   :::: ::: :::: :: :::: ::::
MVNIFH      ATTTACCTTCCAAAATAAAATTATTACCTATTTTTTATACTATATCTAAAAGCTAATATA
            290        300        310        320        330        340

           1200       1210       1220       1230       1240       1250
G57.DNA     CGAAAATTTCTGTAAATA-AACATGATTATATTTTTTG--ACGAATATTACTGATTAGGT
            :  ::::::   :::::X :  : :: ::: ::   :  ::  :::::: :: :::
MVNIFH      CTTAAATTTAAATAAATATATTTTTATAATAATTAAAGTAACTTTTATTAATG-TTAATA
            350        360        370        380        390        400

              1260       1270       1280       1290       1300       1310
G57.DNA     CTGATTTTGAACCGATGACGTCCTGCGTCA-ACTCTT-GGGGCATTATTGTAGAGGAACA
            : :: :: :    :: :  :  :  : :: ::: ::    :   ::::: :   ::: :
MVNIFH      ATAATATTAATAATATTATATAATATGGCATACTATTAATTGTGATATTGAAATAGAATA
            410        420        430        440        450        460
```

Figure 1| Sample output from a database search, comparing the coding strand of PET_{77} with the EMBL DNA database by use of the program FASTN. The best scores are displayed in order, and part of a sequence alignment is included.

available at Warwick are described in outline, and, where appropriate, the results generated from the protein translation of PET_{57} will be included.

Analysis of Protein Sequences (reviewed by Hodgeman, 1986)

Protein Database Searching

R. Staden's collection of protein sequence analysis programs available at Warwick are accessed by selecting ANALYSEP. The simplest options can be used to calculate the molecular weight of the protein product and its amino acid composition (menu -5, option 19) and its net charge (-5, 20). However, the most successful method currently available to ascribe a function to an unknown open reading frame is by demonstrating substantial homology to a protein of known function. By scanning a protein database such as SWISSPROT (available on SKY), the PROTSEQ database (available at Cambridge University), or the CLAVCUT database (available at Edinburgh University), the proteins giving the best scores in search and alignment programs are selected and listed. Searches for homologous sequences to the PET_{57} protein product were conducted on the latter two databases, and the results generated from each will be considered in turn.

The search conducted by the Biocomputing Research Unit in Molecular Biology at Edinburgh University (E-mail: 'ebmo02@uk.ac.edinburgh.emas-a') was carried out by Dr M. Collins. The output of this search is included in Figure IIg and can be interpreted as follows; although biological significance does not always correlate with the score of alignments, the output lists alignments in order of decreasing score. The most significant information is presented as two different parameters :-

Expected: the expected score is the number of times a collection of unrelated proteins the size of this database would generate this score. Therefore scores less than 1 are of interest.

Loc.Exp.: the local expected score refers to the number of times a collection of unrelated

```
 sharen.seq              USED AS QUERY SEQUENCE, LENGTH   325
USING PROTEIN SEQUENCE DATABASE clavcut
USING PROTEIN SEQUENCE DATABASE local
  Proteins 8216 Residues 2168041
 EDINBURGH AMT 510-4 DAP VERSION OF 26/02/88 (LC)
 TOTAL RESULTS  15991
 THRESHOLD VALUE SET INITIALLY WAS   10
 FINAL THRESHOLD VALUE IS   47
 MAXIMUM SCORE   2783
      Score   Predicted     Observed
         47      2247.         2384
         48      1928.         1975
         49      1654.         1679
         50      1419.         1433
         51      1218.         1267
         52      1045.         1019
         53      896.6          929
         54      769.3          787
         55      660.1          589
         56      566.4          568
         57      486.0          453
         58      417.0          427
         59      357.8          319
         60      307.0          310
         61      263.4          260
         62      226.0          218
         63      193.9          214
         64      166.4          163
         65      142.8          152
         66      122.5          104
         67      105.1          106
         68      90.19           83
         69      77.38           86
         70      66.40           71
         71      56.97           64
         72      48.88           46
         73      41.94           39
         74      35.99           55
         75      30.88           15
         76      26.49           26
         77      22.73           23
         78      19.51           18
         79      16.74           23
         80      14.36           17
         81      12.32            7
         82      10.57            4
         83      9.072            6
         84      7.784            8
         85      6.679            2
         86      5.731            6
         87      4.917           12
         88      4.219            5
         89      3.620            1
         90      3.106            2
         91      2.665            1
         92      2.287            4
         93      1.962            2
         94      1.684            3
         96      1.239            2
         97      1.063            1
         98     0.9125            1
         99     0.7830            1
       2783   0.0000E+00          1
```

```
>MUSKTEPIAP1 EPIDERMAL KERATIN TYPE I (AA AT 1) MOUSE (SQUAMOUS CELL CARCINOMA
 14.377  0.988 -0.203  0.018  1.210    61   705  95
No.   2  Score=   99  Quality    44.395      Loc. Exp.    0.3388E-02 SD    5.758
HITS=  21 MISMATCHES=   3 INDELS=   3   EXPECTED NO.    0.7830E+00 SD    2.186
          .  *  .  .  *  .    .  ***  ***

     79  DDF RTKFETEQSLRMSV EADINGLR        103
    193  EEFLR NFSSESNIRLQIIDADINGLR        218


>ECONSDSBP1  ECOR ENZYME SPECIFICITY SUBUNIT (HSDS) ESCHERICHIA COLI B (STRAIN
 13.969  0.224 -0.151  0.004  0.489    72  6810  98
No.   3  Score=   98  Quality     8.612      Loc. Exp.    0.4201E+00 SD    3.874
HITS=  73 MISMATCHES=  53 INDELS=  17   EXPECTED NO.    0.9125E+00 SD    0.818
          *  *  *  .  *  *  .  .  *  *  ****  .*  *  .  .  .  *  .  .  .  *  .

    133  LSA INANTSSVTVKHLSSRTLQ DTLLPLPPLAE QKIIAEKLDTL LAQVDSTKARLE        186
    140  LVAKIGGN SDVIKMQLL RYLDTDTLLVFSPMNEFEGRLRNAQN LYIPIIKGMEKFLR        197


                  *  .  *  .  .  *  .  .  .  .  *  .  .  *  .  *  .  .  *  .  .  .

    189  QIPQILKRFRQAVLAAAVTG RLTKEDKDFITKKVELDNYKI LIPEDWSETILNNIINT        246
    198  NFSSE SNIRLQILDADINGLR GNQQSD IVKNAA KKYMSSLSP  WDLAILEKTVLT        251


                .  *  **.     .  **   .**

    247  QRP LCYGVVQPGDDIKDGIELI        268
    252  TKSFIC GVLLL EWKKDTANLI        272


>PPA11P1      E1 PEPTIDE (AA AT 1) FPV-1 DNA FROM CHAFFINCH EPITHELIAL WARTS.
 15.334  0.856 -0.205  0.015  3.880    60  1558  98
No.   4  Score=   97  Quality    24.745      Loc. Exp.    0.1038E-01 SD    5.335
HITS=  32 MISMATCHES=  15 INDELS=   4   EXPECTED NO.    0.1063E+01
          *  **  *  .  .  .  *  *  .  **  .* *  *  .  *  **   .  *  .*  * ***

     36  LNGRPKKSCITIAGVPDSGKSMWAYSLINFLNGSVLSFANSKSHFWLQPLT         86
     73  LDGRTIKTPLGNGIIVTNAKSLLAY LLK LKWSSLSSLSIKTS SL PLT        119


>P1KRROVI     KERATIN, 54K TYPE I CYTOSKELETAL - BOVINECYTOKERATIN VIB\ 54-KDA 1
 13.778  0.663 -0.171  0.011  3.719    71  2165  98
No.   5  Score=   96  Quality    20.468      Loc. Exp.    0.7454E-01 SD    3.917
HITS=  34 MISMATCHES=  18 INDELS=   5   EXPECTED NO.    0.1239E+01
          .* *  .  .*  .  .  .  .***.***    .  .  *  .  .  .  **.  .** * *

    213  DDFRLK YEWKVTLR QSVKADINGLRRVLDELTLTRTDLEMQIESLTE ELAYLKK        266
    193  EEF LRNFSSESNIRLQILDADINGLR GMQQSDIVKNAAKKYMSSLSPWDLAILEK        247


>SVGENSTOREV GENOME POLYPROTEIN (CONTAINS: PROBABLE RNA-DEPENDENT RNA
 14.458  0.627 -0.184  0.011  2.449    69  2298  98
No.   6  Score=   96  Quality    21.719      Loc. Exp.    0.4761E-01 SD    4.859
HITS=  30 MISMATCHES=  17 INDELS=   4   EXPECTED NO.    0.1239E+01
          **  .* * * *  .  .  *  .  *  .  .  .  .  .  *  .  .  *  .  .* .  .*

   1141  VFKILNKFKGILSSTERKI I VTQSLDDVVTTFDDMMTIMLELMMD ELS       1188
    166  VFSPMMEFEGRLRNAQNELYIPIIKGMEKFLRMVFSSESNIRLQI LDADIN        215
```

Figure 11g Sample output from a database search comparing the protein translation of PET_{57} with the CLAVCUT database.

proteins the size of this database would produce an alignment with the specific piece of test sequence shown in the alignment. This is also of interest if less than 1.

The CLAVCUT database used contains 8216 proteins giving a total of 2 168 041 residues, and the maximum score possible (by comparing the protein sequence with itself) is calculated at 2783.

The PROTSEQ library was also searched on the Cambridge University Digital Equipment Corporation VAX/VMS (V 4.7). This database contains 2 224 790 residues in 7724 protein sequences. The results are interpreted as follows; the initial score describes the degree of homology in the first significant alignment and the optimised score the degree of homology considering both sequences in their entirety (Figure IIh).

The results generated are different as the two methods of searching rely on different criteria, but neither search could pinpoint a protein sequence with substantial homology to the PET_{57} protein product. At present database searching and alignment programs are under investigation to attempt to identify more subtle and distant relationships between sequences without generating false positives.

The dot matrix graphics program DIAGON is available at Warwick, and is used to identify regions of conserved sequence for both DNA and protein sequences. It is most widely used for the latter; for nucleic acid sequences chance matches occur for 25% of the plot so it is difficult to identify significant homologies from background. The two sequences to be compared are arranged as the two sides of a 2-dimensional plot, one reading horizontally (x axis) and one reading vertically (y axis). The 2-dimensional plot is generated by assigning a score to each coordinate and joining points over a certain value. Thus regions of homology appear as diagonal lines, and parallel diagonal lines show homologous sequences interrupted by introns. For protein sequences two options are available: perfect (identity) matching and proportional matching. For perfect matching the score at a particular point is the sum of the identities found when looking

```
357.PEP, 325 amino acids vs PROTSEQ library

  1      5 :==
  4      7 :=.
  6      9 :=
  8      9 :=====
 10     55 :=========================
 12    109 :==================================================
 14    219 :==================================================
 16    405 :==================================================
 18    501 :==================================================
 20   1040 :==================================================
 22   1044 :==================================================
 24   1007 :==================================================
 26    748 :==================================================
 28    648 :==================================================
 30    334 :==================================================
 32    275 :==================================================
 34    200 :==================================================
 36    151 :==================================================
 38     84 :=======================================
 40     60 :============================
 42     33 :===============
 44     30 :==============
 46      8 :====
 48     32 :===============
 50      4 :==
 52      2 :=
 54      2 :=
 56      1 :
 58      2 :=
 60      1 :
 62      0 :
 64      1 :
 66      0 :
 68      1 :
 70      0 :
 72      1 :
 74      0 :
 76      0 :
 78      0 :
 80      0 :
 82      0 :
2224790 residues in  4724 sequences, mean score:  23.2 (s.19)
 1000 scores better than 28 saved, stop: 2, fact: 0  scan time:  0:04:14.21
The best scores are:                                      init,   opt
>DL;PYD2BOG   : DIHIDROOROTAMIDE ACETYLTRANSFERASE COMPONENT  53,   60
>DL;CP2B8HA   : CYTOCHROME P450IIB1 (PHENOBARBITAL-INDUCIBL  50,   57
 DL;ENVBMILV  : Env POLYPROTEIN PRECURSOR (CONTAINS: COAT P  49,   59
>DL;DCP8ASP   : OROT..TINE 5'-PHOSPHATE DECARBOXYLASE (EC 4.  49,   60
 DL;GUAC8TBI  : ENDOGLUCANASE II PRECURSOR (EC 3.2.1.91) (EN  48,   62
>DL;COLI8PIG  : CORTICOTROPIN-LIPOTROPIN PRECURSOR (PRO-OPI  48,   60
>DL;CP118HUM  : CYTOCHROME P450IA1 (P450-P1) (P-450 6) (TCD  48,   72
 DL;POL8MIVI  : POL POLYPROTEIN (PROTEASE; REVERSE TRANSCRI  47,   47
 DL;POL8MIVB  : Env ISOPENTENYL TRANSFERASE (EC 2.5.1.8) (   47,   52
 DL;COLI8SAT  : CORTICOTROPIN-LIPOTROPIN PRECURSOR (PRO-OPI  47,   59
>DL;CP128HUM  : CYTOCHROME P450IA1 (ISOZYME 6) (TCDD-INDUCI  47,   51
 DL;POL8MAVI  : POL POLYPROTEIN (PROTEASE; REVERSE TRANSCRI  47,   47
>DL;POL8MIVA  : POL POLYPROTEIN (PROTEASE; REVERSE TRANSCRI  47,   47
>DL;POL8MIVA  : POL POLYPROTEIN (PROTEASE; REVERSE TRANSCRI  47,   47
 DL;POL8MIVI  : POL POLYPROTEIN (PROTEASE; REVERSE TRANSCRI  47,   47
 DL;POL8MIV2  : POL POLYPROTEIN (PROTEASE; REVERSE TRANSCRI  47,   47
 DL;POL8MIVA  : POL POLYPROTEIN (PROTEASE; REVERSE TRANSCRI  47,   47
 DL;COLI8SAT  : CORTICOTROPIN-LIPOTROPIN PRECURSOR (PRO-OPI  47,   61
 DL;POL8MIVI  : POL POLYPROTEIN (PROTEASE; REVERSE TRANSCRI  47,   47
>DL;COLI8MUM  : CORTICOTROPIN-LIPOTROPIN                     47,   57
 DL;COLI8MOU  : CORTICOTROPIN-LIPOTROPIN PRECURSOR (PRO-OPI  47,   55
```

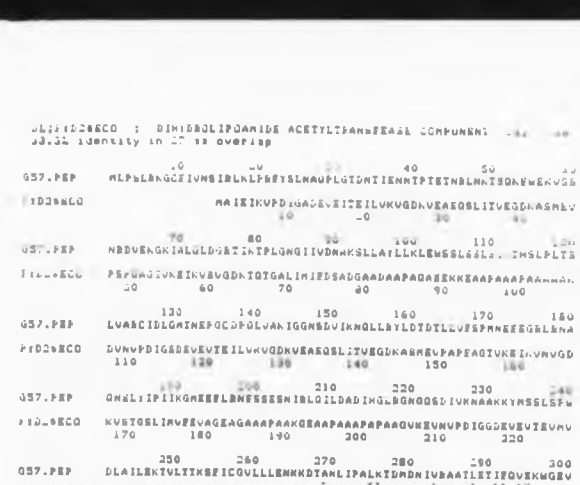


Figure IIA Sample output from a database search comparing the protein translation of PET_{27} with the PROTSEQ database.

back along the diagonal, and when a mismatch is found the score is reset to zero. The method of proportional matching is generally more useful, as many significant protein sequence alignments result from comparison of chemically and structurally similar amino acids and contain few identities. The program calculates a score from comparison of a window (or span) of residues along one diagonal. The resultant score is attributed to the central residue of the span (thus the span is always set to an odd no. of residues), for every region of the sequence, and scores over a certain cut-off value are show on the plot.

Once the graphics plot is displayed a number of options are available to help interpretation. Probably the most useful command for orienting oneself within the sequence is the cross hair option (6); the user can align the cross hair on a specific point using directional keys, read the coordinates of that point (in residues) from the display, and convert the graphical information to a textual display of the sequences at the cross hair (11). Regions of homology can be enlarged on the display by redefining the length of sequence analysed for the plot (14, 15), and a scale added by selecting the "draw ruler or scale" option. The matrix of scores can also be displayed (20). Figure III shows a Diagon plot of the PET_{57} protein compared with itself using proportional matching.

Motif Analysis

Regions which have been conserved in related sequences generally serve a particular structural or functional purpose. Such conserved regions are termed motifs and can give some indication of the function of an unknown protein. However, the use of motifs in analysing protein function is at a very early stage of development, and is at present a last resort in protein analysis. A motif is often so complex that it cannot be described as a simple consensus because the characteristic distribution of amino acids in the length of sequence is so diverse. If a number of separate motifs are present within one protein a "pattern" results, and there may be rules governing the motif separation or allowing the substitution of alternative motifs. The highest level of analysis by this method defines "templates" which describe patterns produced by known protein structures.

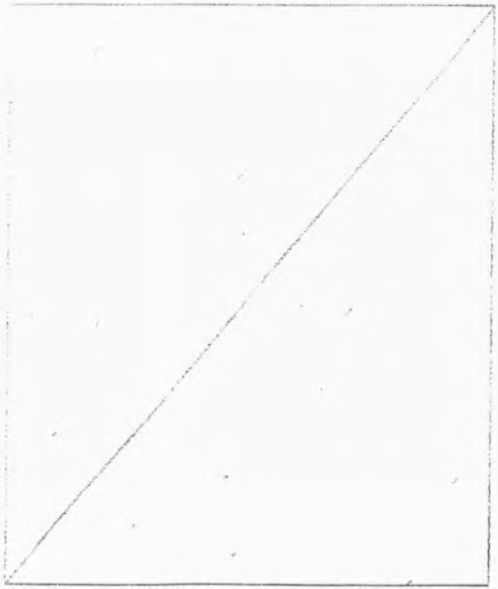


Figure III A DIAGON plot produced by comparing the PET_{31} protein with itself (hence complete identity) using proportional matching. This comparison is useful as it will detect repeated amino acid motifs in a protein sequence.

Improvements to motif analysis are continuing, and in future it may become a very powerful technique for identification of protein functional domains.

By far the most common type of motif used is based on sequence similarity between regions of associated function. To search for the characteristic sequence associated with a particular motif a weight matrix must be generated by programs such as `WEIGHTS` available on the Cambridge University CSBS VAX 8350. This takes sequence alignments of known motifs and calculates the amino acid frequencies in each position as well as giving some measure of the sequence variability. This method is demonstrated in Von Heijne (1986a) when attempting to identify signal sequence cleavage sites. Many examples of a particular motif are required to give a statistically convincing result. When comparing an unknown sequence with a motif scores are generated whose magnitude is related to the degree of homology of a particular sequence window with the characteristic motif sequence.

Prediction of Protein Structure

Information in the primary sequence of a protein can be used to determine its manner of folding. Each amino acid residue is thought to bear information which determined the favoured conformation of that and surrounding residues. These methods rely on data from known crystal structures or from stereochemical and biophysical considerations. The statistical methods of Chou and Fasman (1978) and Robson (Garnier *et al*, 1978) assign probability values to windows of residues, but to date only 50-60% of residues are assigned to their correct structure. However, meaningful results can still be obtained, as this overall figure arises because some regions are more difficult to predict than others.

The Robson algorithm, available in the "Staden Plus" program package, produces output which shows the probability of four different structures; alpha helix, beta sheet, beta turn and "random" coil. However, this type of output should not be overinterpreted as several factors must be taken into account. Firstly, the parameters incorporated into the

Robson program were derived from soluble proteins. Ray (1985) attempted to adapt the Chou and Fasman method to enable analysis of membrane proteins, specifically the ATPase F_0 subunits 6 and 9. The prediction is also based on summed values of surrounding residues and, without thoughtful analysis of output, it is possible to overlook obvious mistakes, such as the inclusion of proline residues in α-helical regions. Short helices and sheets have low predictive values and are difficult to identify. Finally, the effects of post-translational modification on the secondary structure of a protein has not been incorporated into either of these structure prediction programs.

Amino acid hydrophobicity is a useful parameter with which to predict whether a protein is integral to a membrane, anchored to the membrane, or free within the cytosol. On analysis of subunits 6 and 9 of mitochondrial ATPase, a hydrophobicity plot indicates that both polypeptides are highly hydrophobic, and membrane-spanning helices can be distinguished, with extra-membrane loops shown as areas of relative hydrophilicity (Ray, 1985). Analysis of PET_{57} gives a hydrophobicity plot which indicates that it is not an integral membrane protein as it does not have long regions of hydrophobicity (Figure IIj).

The program used to generate hydrophobicity plots again uses a sliding window having an odd number of residues with the mean hydrophobicity of the window being positioned at the central residue of that window. Different information about a protein can be obtained by varying the window length as described by Eisenberg (1984). Evidence exists that a high percentage of mitochondrial targeting sequences may form ampiphilic helices with a high hydrophobic moment (Von Heijne, 1986b). Hydrophilicity (-1 × hydrophobicity) plots can be used in conjunction with secondary structure predictions to determine likely antigenic sites, with a view to raising antibodies to synthetic oligopeptides to aid identification of the parent protein.

Analysis of DNA by computer is presently in a developmental stage, but can still be used productively in DNA and protein sequencing projects. With the rapid pace of nucleotide

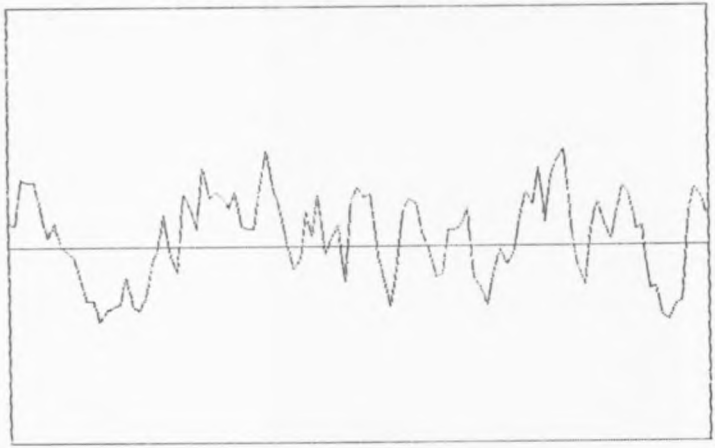


Figure II) A hydrophobicity plot of the PET_{57} protein produced with the HYDROPLOT program available on the Warwick University UNIX system.

sequence analysis resulting in putative protein products of unknown function, computing methods are likely to become increasingly necessary, especially in projects generating a large amount of sequence data. The field of computer analysis will therefore be of increasing importance to molecular biologists, however the limitations of present and future programs must be considered seriously by the researcher when interpreting their output.

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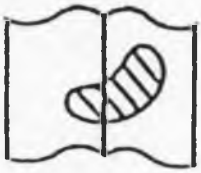

TITLE MITOCHONDRIAL ATPASE: BIOCHEMICAL AND MOLECULAR GENETIC ANALYSIS

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INSTITUTION and DATE University of Warwick 1989