Respiratory Mutants of *Chlamydomonas*

A thesis submitted for the degree of Doctor of Philosophy

by

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To Mum, Dad and Dan, and all my brothers and sisters
ABSTRACT

The aim of the work described in this thesis was to investigate the role the nuclear genome plays in the biogenesis of the respiratory chain complexes in *C. reinhardtii*. Previous work with the yeast *S. cerevisiae* has demonstrated that its nuclear genome encodes subunits of complexes and also trans-acting factors that aid 1) in the expression of the complex subunits contained within the mitochondrial genome, 2) in the import and biosynthesis of the complex cofactors and 3) the assembly of the complexes.

From a collection of ten *C. reinhardtii* mutants defective in respiratory function, two (M86 and M90) were selected for characterisation. Genetic crosses to a wild type strain confirmed that the respiratory defective phenotype in the mutants has arisen due to a mutation in the nuclear genome. Enzyme activity assays and comparison of growth rate to wild type in the presence and absence of carbon and light showed that the respiratory defective phenotype in the mutants is caused by lack of complex IV function.

Molecular analysis of M90 revealed that the affected gene in the mutant codes for a protein of 105 amino acids, which contains a possible transmembrane spanning domain. Antibodies raised against the recombinant protein indicated that the protein is located in the mitochondria and is a component of complex IV. Analysis of mitochondrial gene expression in M86 demonstrated that the transcript of the only mitochondrial encoded subunit of complex IV in *C. reinhardtii*, COXI, is not present in the mutant.

It was concluded that the affected gene in M90 codes for a subunit of complex IV and the affected gene in M86 codes for a trans-acting factor required for either the transcription of the *COXI* gene or the processing or stabilisation of the *COXI* transcript. This conclusion provides support for a model of respiratory chain biogenesis in *C. reinhardtii* similar to that of *S. cerevisiae*. 
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ABBREVIATIONS

2-D  two-dimensional
ADP  adenosine 5’-diphosphate
AMPS ammonium persulphate
ATP  adenosine 5’-triphosphate
Bistris (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane; 2’bis[2-
hydroxyethyl]amino-2-[hydroxymethyl]-1,3propanediol
BLAST Basic Local Alignment Search Tool
BN  blue native
BSA  bovine serum albumin
cDNA copy deoxyribonucleic acid
dATP  2’deoxyadenosine 5’-triphosphate
dCTP  2’deoxycytidine 5’-triphosphate
DEPC diethylpyrocarbonate
dGTP  2’deoxyguanosine 5’-triphosphate
DNA  deoxyribonucleic acid
DNase deoxyribonuclease
dNTP  2’deoxynucleoside 5’-triphosphate
dTTP  2’deoxythymidine 5’-triphosphate
EDTA ethylenediaminetetraacetic acid (disodium salt)
EST expressed sequence tag
FAD  flavin-adenine dinucleotide, oxidised form
FADH₂ flavin-adenine dinucleotide, reduced form
FMN  flavin mononucleotide
IAA  isoamyl alcohol
MITOP Mitochondrial Proteome database
MOPS 3-[N-Morpholino] propane-sulfonic acid
MPₚ MITOCHONDRIAL PROCESSING PROTEASE
Abbreviations

mRNA  messenger ribonucleic acid
NAD(P)H  β-nicotinamide adenine dinucleotide (phosphate), reduced form
NAD(P)⁺  β-nicotinamide adenine dinucleotide (phosphate), oxidised form
NCBI  National Centre for Biotechnology Information
NMR  nuclear magnetic resonance
Pᵢ  inorganic phosphate
Pfam  Protein Families database of alignments and HMMs
PMSF  phenylmethylsulfonyl fluoride
psi  pounds per square inch
PVP  polyvinyl-pyrrolidone
RFLP  restriction fragment length polymorphism
RNA  ribonucleic acid
RNase  ribonuclease
rRNA  ribosomal ribonucleic acid
SDS  sodium dodecyl sulphate
TEMED  N, N, N', N'-tetramethylethylenediamine
TMPD  tetramethyl-p-phenylenediamine dihydrochloride
tricine  N-tris[hydroxymethyl]methylglycine; N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
tris  tris (hydroxymethyl) aminomethane
tRNA  transfer ribonucleic acid
TIGR  The Institute for Genomic Research
TTC  2, 3, 5-triphenyltetrazolium chloride
U  unit
UV  ultraviolet
v/v  volume for volume
w/v  weight for volume
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CHAPTER ONE
INTRODUCTION

Respiration is essential for the survival of most organisms, providing the energy that fuels cellular processes. It is 'an ATP-generating process in which an inorganic compound (such as O_2) serves as the ultimate electron acceptor. The electron donor can be either an organic compound or an inorganic one' (Stryer, 1981). In eukaryotes, respiration occurs predominantly in the mitochondria and involves the reduction of O_2, the ultimate electron acceptor, to H_2O by the electron donors, NADH and FADH_2 produced during food breakdown, to generate ATP from ADP and P_i.

1.1. MITOCHONDRIA

In eukaryotic cells, mitochondria are present in multiple copies and can make up to 25% of the volume of the cytoplasm although this varies according to the cell function and the organism the cell is from. Mitochondria divide in a similar way to the binary fission mechanism seen in bacterial cell division, with one parent producing two daughter mitochondria. During cell division, the partitioning of the mitochondria to the daughter cells is roughly equal. Each mitochondrion contains multiple copies of its own DNA genome, which encodes components of the organelle. The number of genome copies present per mitochondrion and their mode of transmission to daughter mitochondria is under the control of nuclear genes.

1.1.1. Mitochondrial evolution

Mitochondria are thought to have arisen in eukaryotic cells by the process of endosymbiosis, whereby a nucleus-containing archezoan host cell (lacking mitochondria - amitochondriate) engulfed a bacterial cell capable of respiration. This hypothesis dates back more than a century and is known as the Serial
Endosymbiosis Theory (SET; Taylor, 1974). Mitochondria possess a genome that encodes rRNAs and proteins required for mitochondrial biogenesis (section 1.1.2.). Comparison of some of these genes and their arrangement in the genomes in a range of eukaryotic and prokaryotic organisms (Gray and Spencer, 1996; Andersson et al., 1998) demonstrates their similarity to α–proteobacteria, providing strong support for the SET hypothesis. This has now become the widely accepted explanation for the presence of mitochondria in eukaryotic cells.

From the analysis of mitochondrial genomes of a range of protists (mainly unicellular eukaryotes), it is currently believed that mitochondria arose in eukaryotes due to a single endosymbiotic event. In the last two years, two genomes have been sequenced that are the closest so far to marking the boundaries of this event. The most bacterial-like mitochondrial genome was found in *Reclinomonas americana*, a member of the newly-recognised jakobid group of protists which show similarity to a protozoan group which lack mitochondria (Lang et al., 1997). The most mitochondrial-like eubacterial genome was found in *Rickettsia prowazekii*, an α–proteobacterium (Andersson et al., 1998).

Recently, the SET theory has been challenged. Analysis of protist mitochondrial and nuclear genomes has raised the possibility that mitochondria arose in eukaryotes at the same time as the nucleus instead of as a subsequent event. The support for this comes from two sources: 1) The increasing evidence indicating archezoa are not the most primitive diverging eukaryotes (Edlind et al., 1996; Keeling and Doolittle, 1996; Keeling and McFadden, 1998; Hirt et al., 1999). 2) The discovery of mitochondrial genes in the nuclear genomes of amitochondriate archezoa (Bui et al., 1996; Embley and Hirt, 1998; Germot et al., 1996 and 1997; Hirt et al., 1997; Horner et al., 1996; Roger et al., 1998). This suggests that an amitochondriate eukaryotic cell, a necessary requirement for the SET theory, never existed.
Three models have been suggested to explain the origin of mitochondria in eukaryotes. 1) The Hydrogen Hypothesis (Martin and Müller, 1998), whereby the host cell was dependent on molecular hydrogen supplied by the symbiont. 2) The Syntrophy Hypothesis (Moreira and López-García, 1998), whereby the symbiont utilised methane produced by the host cell. 3) The Ox-tox Hypothesis (Vellai et al., 1998), whereby the symbiont reduced the concentration of otherwise potentially lethal oxygen in the host cell.

1.1.2. The mitochondrial genome

The mitochondrial genome was discovered approximately 50 years ago. Since then, genomes from a wide variety of higher and lower eukaryotes have been sequenced and the resulting analysis of these has indicated all the genomes generally share the same fundamental role i.e. they all contain some of the genes required for mitochondrial biogenesis (Tzagoloff and Myers, 1986). Hydrophobic subunits of the respiratory chain, the site of oxidative phosphorylation in the mitochondria (section 1.1.4.), are encoded by the genome, as are the small and large subunit rRNAs and tRNAs required for the expression of these proteins. In addition, some organisms possess mitochondrial genes for the 5S rRNA and protein components of the organelle’s ribosome. Compared to the animals, the mitochondrial genomes of higher plants possess many more genes, in particular those coding for subunits of the respiratory chain.

Apart from this conserved fundamental role, mitochondrial genomes have evolved to show much diversity in size, conformation, gene content, order and expression (Cummings, 1992). From the analysis of the mitochondrial genomes sequenced so far, it has been demonstrated:

1) They range in size from 6 kbp in Plasmodium falciparum to >200 kbp in plants.
2) They are present either as circular (the majority) or linear molecules. Plant mitochondrial genomes exist as linear, subgenomic molecules (Palmer and Herbon, 1988; Mackenzie and McIntosh, 1999).

3) They replicate by different mechanisms *i.e.* some by the rolling circle mechanism (plants; Mackenzie and McIntosh, 1999) and others by unidirectional replication (animals; Taanman, 1999). The rolling circle mechanism involves the production of a single stranded DNA molecule, which displaces the original parent strand to form a double stranded genome. The displaced parent strand is then used as a template to produce a complementary strand. Thus, two double stranded DNA genomes are produced (Backert *et al.*, 1997). The unidirectional replication method involves the use of two promoters. Replication of one strand from one promoter initiates replication of the complementary strand from the other promoter in the same direction, producing two daughter double stranded genomes (Shadel and Clayton, 1997).

4) Their gene content differs. Some genomes contain a few genes, whereas others contain many (Paquin *et al.*, 1997; Gray *et al.*, 1998) *e.g.* *P. falciparum* encodes only three subunits of the respiratory chain whereas the human mitochondrial genome encodes 13 subunits of the respiratory chain. The size of the genome does not reflect gene content, as the larger genomes have more inter- and intra-genic non-coding regions than the smaller genomes, which are more compact with little non-coding sequence (Leblanc *et al.*, 1997; Mackenzie and McIntosh, 1999).

5) The mode of expression of the genes present on the genome differs. Some genomes use non-standard codon assignments in their genes, others display highly biased codon usage. Some use an altered decoding system which allows 24 tRNAs instead of the minimum 32 tRNAs to read the genetic code. In plants, trypanosomes and some other protists, editing of some RNA transcripts occurs (Odintsova and Yarina, 2000).
The pre-mitochondrial genome contained about 1000 genes (Lang et al., 1999). The analysis of the mitochondrial genomes of protists, animals, plants and fungi has shown this number is severely reduced with the species containing the largest number at 94 (Hibberd, 1975). A large number of genes are missing. It is thought that many of these genes were lost early on in eukaryotic evolution for two reasons: 1) their function no longer being required and 2) by functional replacement by a nuclear gene (Chiu et al., 1975). The remaining missing genes have been transferred to the nucleus and include genes coding for proteins that are required for mitochondrial biogenesis. Evidence for this latter process exists in three forms.

Firstly, by the identification of genes in the mitochondrial genome of one species and the nuclear genome of another (Burger et al., 1996). Secondly, by the presence of protein coding genes in the nuclear genome which are subunits of the respiratory chain (Gray et al., 1998) and thirdly, by the identification of nuclear genes required for mitochondrial biogenesis (Ryan and Jensen, 1995). This transfer process is ongoing (Grabau, 1987; Nugent and Palmer, 1991; Court and Bertrand, 1993) and is thought to occur due to a general momentum of the nuclear genome to incorporate essential genes from an endosymbiont genome. It has been suggested that the retention of genes in the mitochondrial genome occurs due to special properties of the gene product (Heijne, 1986) and/or mode of their regulation (Lang et al., 1999).

1.1.3. Mitochondrial structure

Transmission Electron Microscopy (TEM) was used to determine a model for the structure of the mitochondrion (Palade, 1952; Sjöstrand, 1956). It is a regular, predominantly cylindrical or spherical shaped organelle and is composed of two distinct membranes (fig 1.1). A smooth, unfolded outer membrane that is permeable to most solutes up to the size of 10 kDa and an extensively folded inner membrane that has a high protein content and is impermeable to most solutes, including protons (von Jagow et al., 1997). The membranes enclose a
dense medium (the matrix), which has since been demonstrated to contain the mitochondrial genome, proteins required for the replication and expression of the genome and enzymes of the intermediate metabolic pathways.

Figure 1.1. Models of mitochondrial membrane structures.
Figure 1.1.a represents the Baffle Model; figure 1.1.b the Crista Junction Model. From Perkins and Frey (2000).

Initially it was believed that the inner membrane protruded into the matrix in a ‘baffle-like’ manner, forming cristae. These protrusions were not thought to form septa i.e. connect with the inner membrane on the opposite side of the matrix (fig 1.1a; Palade, 1952). This theory has become known as the ‘Baffle Model’. However, subsequent experiments to observe mitochondria from a range of eukaryotes (Daems and Wisse, 1966; Hackenbrock, 1966; Lea et al., 1994; Mannella et al., 1994), have proved this theory inaccurate. These experiments instead provide support for the model first suggested by Sjöstrand (1956), that the inner membrane forms independent membrane-bound compartments that form septa that compartmentalise the matrix. The ‘Crista Junction Model’, as it has become known, postulates that these cristae are attached to the inner membrane by crista junctions of 28nm and depending on the source, their conformational state varies from a tubular to a lamella shape (fig 1.1b; Frey and Mannella, 2000).
1.1.4. Mitochondrial function

The main function of the mitochondria is to catalyse the transfer of electrons from the substrates reduced on food breakdown, NADH and FADH$_2$, to molecular oxygen and to generate energy in the form of ATP. This process is called oxidative phosphorylation and involves the function of a series of complexes. These complexes sit in the inner mitochondrial membrane and together form the 'respiratory chain' (fig 1.2). About 90% of the energy utilised by cells is provided by oxidative phosphorylation. Further discussion of the respiratory chain is given in section 1.2.

![Diagram of the plant respiratory chain](image)

**Figure 1.2. The plant respiratory chain.**

The five main components of the respiratory chain are complex I, complex II (SDH), complex III, complex IV and complex V (ATP synthase). There are two mobile electron carriers ubiquinone (UQ) and cytochrome c (Cytc). In addition plants also possess an alternative oxidase (AltOx) and NAD (P) H dehydrogenases (ND). From Rasmusson *et al.* (1998).

Additional functions of the mitochondria include the final stages of glycolysis, the citric acid cycle (CAC), fatty acid β-oxidation and amino acid oxidation. The enzymes which catalyse these processes are located in the mitochondrial matrix and in addition to glycolysis, which occurs in the cytosol, produce the reduced substrates required for ATP generation by oxidative phosphorylation. The
substrates for these processes are imported through the impermeable inner membrane into the matrix by specific transporters functioning within the membrane.

1.2. THE RESPIRATORY CHAIN

The ATP generating respiratory chain is composed of five enzyme complexes and two carrier molecules (fig 1.2). This chain can be sub-divided into two parts: 1) The electron transfer chain, which consists of NADH: ubiquinone oxidoreductase (complex I), succinate: ubiquinone oxidoreductase (complex II), ubiquinol: cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV) and the two carrier molecules, ubiquinone and cytochrome c. 2) The ATP synthase (complex V). The enzyme complexes are thought to exist as individual entities in the inner membrane, although recent data has demonstrated that the enzymes in some organisms interact permanently with one another to form super-complexes (Schägger and Pfeiffer, 2000).

The role of the electron transfer chain is to transfer electrons, via a series of oxidation-reduction reactions, from the reduced substrates NADH and FADH$_2$, to molecular oxygen (Hatefi, 1985). NADH and succinate, which enter the electron transfer chain either directly from the matrix or after import from the cytosol, donate electrons to complex I and complex II respectively. FAD is covalently bound to complex II and is reduced to FADH$_2$ on succinate oxidation. Complex I and II then transfer electrons to ubiquinone, a lipophilic organic molecule free to move in the hydrophobic interior of the inner membrane. Ubiquinone donates the electrons to complex III, which in turn passes them to the carrier molecule, cytochrome c. Cytochrome c is an extrinsic small hydrophilic protein that lies in the intermembrane space. It donates electrons to the final complex of the electron transfer chain, complex IV, which reduces molecular oxygen to water.

The complexes and carrier molecules are able to transfer electrons due to the possession of redox centres such as flavoproteins, iron-sulphur clusters,
cytochromes (include $\alpha$-, $b$- and $c$-type haems), copper and quinones which can be both oxidised and reduced.

In addition, complex I, III and IV couple electron transfer to the pumping of protons ($H^+$), against a concentration and charge gradient, across the inner membrane from the matrix to the intermembrane space. The inner membrane is impermeable to $H^+$ ions and as a result, an electrochemical gradient forms across the membrane. The gradient generates a protonmotive force, $\Delta \mu_{H^+}$, which drives ATP production through complex V (Trumpower, 1990).

Complex V catalyses the production ATP. Protons located in the intermembrane space, diffuse along their concentration and charge gradient, through the complex, providing the energy to drive the condensation of ADP and $P_i$ to ATP in the matrix (Boyer, 1997).

Additional complexes are found in the mitochondrial electron transfer chains of plants and some fungal and protist species. These include some alternative NAD(P)H dehydrogenases, which catalyse the same function as complex I and an alternative oxidase, which accepts electrons from ubiquinol and like complex IV, reduces molecular oxygen to water (fig 1.2; Mackenzie and McIntosh, 1999). Owing to the presence of the alternative oxidase, these organisms possess two respiratory pathways: the 'classical' respiratory pathway and the 'alternative' respiratory pathway. In the classical pathway, electrons flow from the ubiquinone pool through complex III to complex IV where they are used to reduce oxygen to water. In the alternative pathway, electrons from the ubiquinone pool are donated directly to the alternative oxidase where they are used to reduce oxygen to water.
1.2.1. **Complex I, the NADH: ubiquinone oxidoreductase**

Complex I catalyses the transfer of electrons from NADH to ubiquinone (UQ) and also translocates four or five protons (H\(^{+}\)) from the matrix (\(\text{in}\)) into the intermembrane space (\(\text{out}\))(Weiss and Friedrich, 1991).

\[
\text{NADH} + \text{UQ} + \text{H}^{+} + n (\text{H}^{+})_{\text{in}} \leftrightarrow \text{NAD}^{+} + \text{UQH}_{2} + n (\text{H}^{+})_{\text{out}}
\]

The complex has been identified in mammals (Walker 1992), plants (Rasmusson et al., 1998), fungi (Videira 1998) and many bacteria (Yagi et al., 1998).

1.2.1.1. **Composition of complex I**

In eukaryotes, complex I is composed of over 30 subunits encoded by both the mitochondrial and nuclear genome which make the enzyme one of the largest of the respiratory chain, at approximately 900 kDa. The mitochondrial-encoded subunits correspond to the most hydrophobic subunits of the complex. In prokaryotes, the complex is composed of only 13 - 14 subunits, which combine to form the minimal functional unit (Yano et al., 1997). Homologous subunits to all these proteins have been found in the fungi *Neurospora crassa* and mammalian bovine heart complex I, demonstrating conservation of the enzyme (Guénebaut et al., 1998). Seven of the prokaryotic subunits are homologous to mitochondrial encoded subunits and the remainder are homologous to nuclear encoded proteins. Complex I also possesses a FMN prosthetic group and at least six iron-sulphur (FeS) clusters. These are required for electron transfer through the complex.

1.2.1.2. **Structure of complex I**

The low resolution structure of the complex I in eukaryotes was first determined for *N. crassa* using techniques such as electron microscopy (to analyse 2-D
crystals of complex I), protein synthesis inhibition studies and mutational analysis (Nehls et al., 1992; Alves and Videira, 1994; Guénebaut et al., 1997 and 1998). It has since been determined in mammals (bovine heart) and bacteria (Escherichia coli) by use of single-particle image analysis (Grigorieff, 1998; Guénebaut et al., 1997).

The complex is L-shaped with arms of similar size. One arm is embedded in the inner mitochondrial membrane. This is the ‘hydrophobic membrane domain’ and is composed of about 15 subunits that include the mitochondrial-encoded subunits. The other arm, more globular than the hydrophobic membrane domain, projects into the matrix. This ‘hydrophilic matrix domain’ is composed of about 17 nuclear encoded subunits. The orientation of the complex in the membrane has been determined. Three subunits, a 75, 51 and 24 kDa protein, situated at the head of the hydrophilic matrix domain, catalyse the NADH dehydrogenase function of the enzyme. They bind the FMN prosthetic group and four of the FeS clusters (N1a, N1b, N3 and N4). The 51 kDa protein forms the NADH binding site. The hydrophobic membrane domain catalyses the ubiquinone hydrogenase function of the enzyme. It binds one FeS cluster (N2) and one subunit (a 36 kDa mitochondrial encoded protein) has been proposed to form the ubiquinone-binding site. The exact arrangement of the subunits in complex I still needs to be determined as does the location and number of the FeS clusters and ubiquinone binding sites.

1.2.1.3. Function of complex I

The process of electron transfer through complex I starts with the oxidation of NADH by FMN at the NADH binding site. Two electrons and two H+ ions are released. The H+ ions remain in the matrix whilst the electrons are transferred through the complex via the FMN and redox FeS clusters to the ubiquinone binding site where they are donated to ubiquinone. How proton pumping through complex I occurs remains to be determined although it has been suggested to occur by a similar mechanism to complex III (section 1.2.3). The energy for the
process is provided by the increase in redox potential of the pair of electrons as they move through the complex from $-320$ mV at entry to $+50$ mV at exit. The large increase in redox potential results in the release of redox energy, which is used to pump the protons across the membrane (von Jagow et al., 1997).

Little analysis of complex I has been performed in plants due to a number of factors. 1) Difficulties in isolating plant mitochondria. 2) Presence of homologous genes to complex I in the chloroplast genome. 3) Altering function of the respiratory chain in different tissues, species and diverse environmental conditions. 4) Presence of other NADH dehydrogenases in the respiratory chain (Douce and Neuberger, 1989).

1.2.2. The alternative NADH: ubiquinone oxidoreductases

The alternative NADH:ubiquinone oxidoreductases (NADH dehydrogenases) catalyse the transfer of electrons from NADH to ubiquinone, but are unable to pump protons across the inner membrane (Siedow and Umbach, 1995). They therefore do not contribute to the protonmotive force. The enzymes catalyse the same electron transfer reaction as complex I and in some organisms, the enzymes function in addition to complex I (Weiss, 1970).

\[ \text{NADH} + \text{UQ} + \text{H}^+ \rightarrow \text{NAD}^+ + \text{UQH}_2 \]

Alternative NADH dehydrogenases are found in plants, fungi and bacteria. In the eukaryotic respiratory chain, two types of alternative NADH dehydrogenase are present: the ‘internal’ and ‘external’ NADH dehydrogenases. The ‘internal’ NADH dehydrogenases oxidise NAD(P)H located in the matrix and the ‘external’ NADH dehydrogenases oxidise cytoplasmic NADH. The number of copies of these different dehydrogenases present in the respiratory chains varies between species (Kerscher, 2000).
Chapter one

The yeast, *Saccharomyces cerevisiae*, lacks complex I and requires the activity of the alternative NADH dehydrogenases to oxidise NADH during respiratory growth (DeRisi *et al.*, 1997). The function of the alternative NADH dehydrogenases in all other organisms that possess complex I is unknown. Like the alternative oxidase, it has been suggested that in plants, the enzymes are required for thermogenesis (Palmer and Ward, 1985) and the plant stress response (Rychter, 1992).

1.2.2.1. Composition of the alternative NADH dehydrogenases

The alternative NADH dehydrogenases analysed so far are composed of one subunit, encoded by the nuclear genome. Genes encoding alternative NADH dehydrogenases have been identified in *S. cerevisiae* (Luttik *et al.*, 1998), potato (Rasmusson *et al.*, 1999), *Yarrowia lipolytica* (Kerscher *et al.*, 1999) and *E. coli* (Young *et al.*, 1981). A cDNA of a NADH dehydrogenase protein has also been isolated from *N. crassa* (Melo *et al.*, 1999). Comparison of all the gene sequences determined so far has revealed that all the alternative NADH dehydrogenase proteins are homologous. This homology and the fact that some fungi possess only the external form of the alternative NADH dehydrogenase, has led to the suggestion that eukaryotes initially possessed only one external NADH dehydrogenase. The presence of the other external and internal alternative NADH dehydrogenases found in the respiratory chain are thought to have arisen by duplication of the gene encoding the enzyme and the addition of a mitochondrial targeting sequence to the protein (Kerscher, 2000). From the analysis of purified internal NADH dehydrogenase from *S. cerevisiae* (de Vries and Grivell, 1988), it has been suggested that the FAD prosthetic group acts as the redox centre in these enzymes.

1.2.2.2. Structure of the alternative NADH dehydrogenases

The crystal structures of the homologous alternative NADH dehydrogenases have not yet been determined. However, structural analysis of the sequenced
alternative NADH dehydrogenases has indicated various important structural features of the enzymes. All the enzymes possess two dinucleotide binding $\beta$-$\alpha$-$\beta$ fold domains (Wierenga et al., 1985). It has been suggested these bind either the FAD prosthetic group or the substrate NADH, although this has yet to be demonstrated. In *N. crassa* and potato, possible $\text{Ca}^{2+}$ binding domains are present in the alternative NADH dehydrogenases. Proteins with these domains may bind to the inner membrane in a different way to the other alternative NADH dehydrogenases (Rasmusson et al., 1999). The positions of the ubiquinone binding sites have not yet been determined, although it is thought that a conserved tryptophan residue is involved in the process (de Vries et al., 1992). No transmembrane spanning domains are present in the enzymes and it is thought that the enzymes attach themselves to either the matrix facing or intermembrane facing side of the inner membrane via an amphiphilic membrane attachment domain (Mitra et al., 1993).

1.2.2.3. Function of the alternative NADH dehydrogenases

The mechanism of electron transfer through the alternative NADH dehydrogenases is not known. The enzymes are insensitive to most inhibitors including the complex I inhibitor rotenone (Soole et al., 1992).

1.2.3. Complex II, the succinate:ubiquinone oxidoreductase

Complex II is a citric acid cycle enzyme which catalyses the oxidation of succinate to fumarate and is found in all aerobic organisms. It is the only enzyme of the cycle that: 1) is embedded in the inner mitochondrial membrane and 2) acts as a direct electron donor to the respiratory chain. The oxidation of succinate results in the reduction of covalently bound FAD to FADH$_2$, which in turn donates electrons, via a series of redox reactions, to ubiquinone (Hatefi, 1985).

\[
\text{succinate} + \text{UQ} \rightarrow \text{fumarate} + \text{UQH}_2
\]
1.2.3.1. Composition of complex II

Complex II is composed of only four or five protein subunits, which bind four to six redox centres: a FAD prosthetic group, three different FeS clusters (denoted S1 \([2\text{Fe}-2\text{S}]^{2+,1+}\), S2 \([4\text{Fe}-4\text{S}]^{2+,1+}\) and S3 \([3\text{Fe}-4\text{S}]^{1+,0}\) ) and maybe one or two b-haems. The sequences of some eukaryotic mitochondrial genomes have indicated that the protein subunits are encoded by both the mitochondrial and nuclear genome in lower eukaryotes (Burger et al., 1996), but only the nuclear genome in higher eukaryotes. Comparison of the primary sequences of these nuclear encoded subunits has revealed that in different species, the subunits which bind the FAD and FeS clusters are well-conserved, whereas the others are not (Ackrell, 2000).

1.2.3.2. Structure of complex II

Complex II consists of a hydrophilic domain, which projects into the matrix in eukaryotes and the cytosol in prokaryotes, and a hydrophobic domain, which anchors the hydrophilic domain to the inner membrane (Ackrell, 1992; Hägerhäll, 1997). The complex can be easily isolated as separate domains. The hydrophilic domain is composed of two subunits, a 64-79 kDa protein that covalently binds the FAD prosthetic group (flavoprotein – Fp) and a 27-34 kDa protein that contains the three FeS clusters (Iron-sulphur subunit – Ip). The hydrophobic domain is composed of one larger or two smaller subunits, of size 23-30 kDa or 13-18 kDa and 11-16 kDa respectively. The subunits form the ubiquinone binding site and may bind one or two b-haems.

1.2.3.3. Function of complex II

The process of electron transfer through complex II starts with the hydrophilic domain, which carries out the succinate dehydrogenase function of the complex. Succinate donates electrons to the FAD catalytic site, which then reduces the S1 and S3 FeS clusters. These clusters are oxidised by ubiquinone, which binds to
the ubiquinone-binding site in the hydrophobic domain. It has been suggested that the S2 and b-haems are evolutionary relics that are non-functional in complex II (von Jagow et al., 1997). They possess a redox potential much more negative than the complex donor and acceptor, making electron transfer through them thermodynamically unfavourable.

The recent determination of the X-ray structure of the structurally and functionally similar enzyme, quinol:fumarate oxidoreductase, is contributing to the further understanding of the process of electron transfer through the complex (Ackrell, 2000). Complex II does not couple electron transfer to proton pumping across the inner membrane. The redox potential of the electrons as they pass from FADH$_2$ to ubiquinone does not increase (+50 mV), so no redox energy is released to transfer protons across the membrane.

1.2.4. Complex III, the ubiquinol:cytochrome c oxidoreductase

Complex III catalyses the transfer of electrons from ubiquinol to cytochrome c and also translocates four protons across the inner membrane.

\[
\text{UQH}_2 + 2\text{cytc}^{3+} + 2\text{H}^+_\text{in} \leftrightarrow \text{UQ} + 2\text{cyt c}^{2+} + 4\text{H}^+_\text{out}
\]

The complex has been characterised in fungi (Tzagoloff, 1995), mammals (Trumpower and Gennis, 1994) and higher plants (Jänisch et al., 1996).

1.2.4.1. Composition of complex III

Complex III is approximately 248 kDa in size and is composed of 10-11 conserved subunits (Gencic, 1991; Brandt, 1994; Jänisch, 1995; Schägger, 1995) and at least 40 phospholipids (Yue et al., 1991). Only one of the subunits is encoded by the mitochondrial genome, the cytochrome b protein. The other proteins are encoded by the nuclear genome.
Three of the subunits, cytochrome b, cytochrome c1 and the Rieske iron-sulphur protein (subunits III-V respectively), are responsible for the electron transfer function of the complex. They bind the redox centres of the protein, haem bL, bH, and c1 and a FeS cluster [2Fe-2S]. This ‘core’ complex is homologous to the functional three-subunit bacterial complex III in both primary sequence and redox centre composition (Trumpower, 1990).

The other subunits are supernumerary (I, II, VI-XI). None bind prosthetic groups and they are not directly involved in respiratory function. Analysis of these subunits has been performed to establish roles for these proteins. Two of the subunits, known as the core subunits, are the largest subunits of the complex (I and II). They are homologous to the yeast and mammalian matrix located MPP complex that processes the mitochondrial-imported proteins (Xia et al., 1997). Furthermore, in plants, these subunits, along with an additional supernumerary subunit, function as the MPP (Braun and Schmitz, 1995). The binding of supernumerary subunit IX has been suggested to prevent MPP activity in bovine mitochondria (Deng et al., 1998; Shenoy et al., 1998). Roles for the other subunits are little understood, although it has been demonstrated by deletion studies in yeast that the subunits are required for the assembly of the complex.

1.2.4.2. Structure of complex III

The structure of complex III has been determined in detail from electron microscopy and X-ray diffraction studies of complex III crystals from N. crassa, beef and chicken heart mitochondria (Wingfield et al., 1979; Xia et al., 1997; Iwata et al., 1998; Zhang et al., 1998). The complex spans the matrix, inner membrane and intermembrane space and is thought to exist in vivo as a dimer (Xia et al., 1997). In the monomer, the matrix-spanning region constitutes over half the molecular mass of the enzyme. The positions of the four redox centres in the complex have been determined and models of the atomic structures of the core subunits, the cytochrome b subunit, some supernumerary subunits and part
of the Rieske iron-sulphur and cytochrome c1 proteins in the complex, have been constructed.

The three subunits required for the electron transfer function of the complex are located in the inner membrane and the intermembrane space, although both the C-terminus of the cytochrome c1 protein and N-terminus of the Rieske iron-sulphur protein span into the matrix. The cytochrome b protein is located in the inner membrane. It spans the membrane eight times, houses the two \( b \)-haems, \( b_L \) and \( b_H \) and forms two quinone-binding sites, \( Q_i \) and \( Q_0 \). The \( Q_i \) and \( Q_0 \) sites bind the inhibitors antimycin A and MOA-stilbene, respectively. The Rieske iron-sulphur and cytochrome c1 proteins span both the inner membrane, via one transmembrane domain, and the intermembrane space. Each of these proteins binds a redox centre. Cytochrome c1 binds haem c1 and the Rieske iron sulphur protein binds the FeS cluster. These centres are located in the intermembrane space. The supernumerary subunits are located in the matrix, inner membrane and intermembrane space, with the two core subunits I and II forming the bulk of the matrix-spanning domain.

### 1.2.4.3. Function of complex III

Mitchell (1976) first suggested a mechanism for electron transfer through complex III. "The Q-cycle" has since evolved as the most favoured mechanism for electron transfer through complex III (fig 1.3; Trumpower, 1990; Brandt and Trumpower, 1994). It explains observations which include the reduction of cytochrome b by ubiquinol (Erecinska et al., 1972; Wikström and Berden, 1972), the \( 2H^+/e^- \) stoichiometry of the complex, i.e. two protons are pumped through the complex per electron transferred (Brand et al., 1976) and the presence of two ubiquinone binding sites in the complex, as demonstrated from inhibitor binding experiments (von Jagow and Link, 1986).
Ubiquinol (QH$_2$) donates two electrons to the Q$_0$ site located in the cytochrome $b$ protein (cyan). The electrons flow down two pathways. The first electron passes to the FeS centre (Fe$_2$S$_2$) located in the Rieske iron-sulphur protein (yellow), then to the cytochrome $c_1$ of the cytochrome $c_1$ protein (blue) where the electron reduces cytochrome $c$ (red). The second electron passes through the redox centres $b_l$ and $b_{h1}$ to the second ubiquinone binding site, Q$_1$, reducing bound ubiquinone to a semiquinone. Repetition of the process results in the production of ubiquinol and two reduced cytochrome $c$ molecules. From http://arc-gen1.life.uiuc.edu/Bioph354/bc-complex_summary.html.

The Q-cycle is based on the oxidation of the two ubiquinol molecules at the Q$_0$ site and the reduction of one molecule of ubiquinone at the Q$_1$ site. Electron transfer through the complex starts with ubiquinol binding to the Q$_0$ site and the release of two electrons, one at a time. The first one is transferred along a high potential chain, through the FeS cluster to haem$c_1$, which is then oxidised by cytochrome $c$. This binds to the intermembrane spanning soluble domain of cytochrome $c_1$ and accepts one electron only. The other electron is passed along a low potential chain, via haem $b_l$ and haem $b_{h1}$ to the ubiquinone binding site Q$_1$. Here, the electron is used to reduce a ubiquinone molecule to the semiquinone form. The addition of another electron, which is supplied by repetition of the cycle described above and the uptake of two protons further reduce this molecule to ubiquinol from the matrix. The net products of these electron transfer reactions are two reduced cytochrome $c$ molecules, four protons (released from the reduction of two molecules of ubiquinol) and a ubiquinone.
Models have been suggested to explain why the bifurcated electron transfer mechanism exists in complex III (Brandt, 1996; Yu et al., 1998). The observed movement of the functional region of the Rieske iron-sulphur protein is thought to contribute to this (Xia et al., 1997; Tian et al., 1998). It has also been suggested that the electron transfer process is shared between the two monomer components of the dimer complex (Yu et al., 1998).

The four protons released during ubiquinol oxidation are transferred to the intermembrane space and contribute to the proton motive force. The energy for the process is provided by the increase in the redox potential, from +50 mV to +250 mV, of the electrons as they are transferred from ubiquinol to cytochrome c. The mechanism of how this occurs is unknown. It is thought that the protons must be pumped through the transmembrane helices of cytochrome b, the Rieske iron-sulphur protein and cytochrome c1 as bacterial enzymes, composed of only these subunits, are able to translocate protons (Miki et al., 1990; Link and Iwata, 1996).

1.2.5. Complex IV, the cytochrome c oxidase

Complex IV catalyses the transfer of electrons from cytochrome c to molecular oxygen and also pumps protons across the inner membrane from the matrix to the intermembrane space.

\[ 4\text{cytc}^{2+} + \text{O}_2 + 8\text{H}^+_{(\text{in})} \rightarrow 4\text{cytc}^{3+} + 2\text{H}_2\text{O} + 4\text{H}^+_{(\text{out})} \]

The complex has been studied in mammals, plants, yeasts, protists and prokaryotes using biochemical, spectroscopic and genetic techniques.
1.2.5.1. Composition of complex IV

In eukaryotes, the subunit composition of complex IV increases as the evolutionary complexity of the organism increases, from seven in the lower eukaryote Dictyostelium discoideum to ten (plants), twelve (yeast) and thirteen (mammals) in the higher eukaryotes (Jänsch et al., 1996; Grossman and Lomax, 1997). There are three conserved subunits: COXI, II and III. In most organisms, these subunits are encoded by the mitochondrial genome (Nugent and Palmer, 1993). The COXI, II and III subunits are also present in most prokaryotes (Grossman and Lomax, 1997). As some prokaryotes require only these subunits for complex IV function, they are considered to be the respiratory functional subunits.

The additional subunits found in eukaryotes are encoded by the nuclear genome and are conserved between species. The role of the subunits in complex IV function is not clear, although it has been suggested they play a role in the assembly of the enzyme and its regulation (Kadenbach, 1986). Some of the subunits are present as isoforms, which are differentially expressed according to the environment in and around the cell (Bonne et al., 1993).

The redox centres haem \( a \), haem \( a_3 \), Cu\(_A\) and Cu\(_B\) are found in complex IV. These are required for respiratory function. One zinc and one magnesium ion have been detected in the mammalian complex, as have certain phospholipids (Tsukihara et al., 1996). The roles of the molecules in complex function are unclear, although a role for magnesium in regulation has been suggested (Rich and Moody, 1997).

1.2.5.2. Structure of complex IV

The determination of the high resolution crystal structure of complex IV from the bacterium Paracoccus denitrificans (Iwata et al., 1995) and mammalian
mitochondria from bovine heart (Tsukihara et al., 1996) contributed significantly to the understanding the basic structure of complex IV.

Complex IV is a highly asymmetric enzyme that is thought to exist in situ as a dimer. It spans the inner membrane and possesses a large domain, which projects into the intermembrane space. The arrangement of the conserved subunits of the complex, COXI, II and III are nearly identical in bovine heart and P. denitrificans (fig 1.4). It is assumed the arrangement is conserved in other eukaryotic and prokaryotic species.

**Figure 1.4. Structure of the three core subunits of complex IV.**

COXI (orange) spans the membrane 12 times and binds the CuB (green), haem a and haem a₃ (red) redox centres. COXII (blue) spans the membrane two times, forms a globular domain in the intermembrane space and binds the Cu₅ redox centre (green). COXIII (green) spans the membrane seven times. From Lehninger (2000).

COXI spans the inner membrane twelve times and binds the redox centres haem a, haem a₃ and CuB. Mutational analysis has demonstrated that six conserved histidine residues ligand these centres (Hosler et al., 1993). The redox centres are located towards the intermembrane space side of the membrane and are positioned at the same latitude within the membrane. Haem a₃ and CuB form the oxygen-binding binuclear centre. The complex IV inhibitor cyanide binds to this site.
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COXII spans the inner membrane twice and possesses a large hydrophilic C-terminus that projects into the intermembrane space, above COXI. This domain contains a copper binding motif and ligands the Cuₐ redox centre (Adman, 1991). This is composed of a copper pair (Cu²⁺ - Cu¹⁺; Blackburn et al., 1994), which ligate to COXII by two cysteine residues, one histidine residue and either a methionine or glutamate residue.

COXIII spans the inner membrane seven times. The first and second transmembrane domains are divided from the rest (domains III to VII) by a V-shaped cleft. Some of the lipid components are thought to bind this site. Although COXIII is conserved, its functional role is unknown. COXIII is not required for electron transfer as it does not bind any redox centres, nor is it needed for proton translocation through the complex (Hendler et al., 1991). It has been suggested that the subunit forms an entrance for the oxygen channel leading to the active site (Riistama et al., 1996) or is involved in complex IV assembly (Haltia et al., 1989).

The arrangement of the nuclear encoded subunits of the bovine heart complex IV (COXIV, Va, Vb, VIa–c, VIIa-c and VIII) has also been determined. Subunits COXIV, VIa, VIc, VIIa, VIIb, VIIc and VIII span the membrane once interacting with each other and the core subunits, COXI, II and III. COXVIa is thought to cause dimerisation of the complex (Tsukihara et al., 1996). COX subunits Va, Vb and VIb are globular proteins which do not span the membrane but are attached to the core subunits of the complex, projecting into either the matrix (Va and Vb) or intermembrane space (VIb). COXVIb, along with COXI, bind the zinc ion. As most of these subunits appear to be conserved in the eukaryotic species so far analysed, it is assumed that the nuclear encoded subunits from different eukaryotic organisms are arranged in a similar way.
1.2.5.3. Function of complex IV

Complex IV function can be divided into three processes: electron transfer, oxygen reduction and proton translocation from the matrix to the intermembrane space. Oxygen reduction and proton translocation both contribute to the protonmotive force by removing protons from the matrix.

The pathway of electron transfer through complex IV has been determined. Reduced cytochrome $c$ binds to complex IV and donates an electron to the $\text{Cu}_A$ redox centre (Hill, 1991). A possible binding site for the carrier molecule is a corner formed by the flat intermembrane facing region of COXI and the globular domain of COXII (Iwata et al., 1995; Witt and Ludwig, 1997). The binding of cytochrome $c$ to this site is thought to be dependent on electrostatic interactions between the ring of lysine residues located at the edge of the haem group in cytochrome $c$ and the ten acidic residues located in the predicted binding site (Reider and Bosshard, 1980). $\text{Cu}_A$ then donates electrons to haem $a$, which in turn reduces the haem $a_3 / \text{Cu}_B$ binuclear centre (Winkler et al., 1995). At the binuclear centre, the electrons are used to reduce molecular oxygen to water.

The mechanism by which oxygen is reduced to water is known as the catalytic cycle (fig 1.5; Wikström et al., 1997). This cycle can be divided into four stages. Each stage requires the addition of one electron and one proton to the binuclear centre (to preserve electroneutrality).

**Stage one** - The oxidised binuclear centre (O-state) is converted to the one electron reduced state (E-state).

**Stage two** - Oxygen binds to the binuclear centre and is reduced to a peroxy intermediate (P-state). There are two possible pathways for this step (Zaslavsky and Gennis, 2000). In the first pathway, the second electron and proton bind to the binuclear centre first to form the partially reduced state (R-state). Oxygen then binds and is reduced to the peroxy intermediate (P-state). In the second
pathway, the oxygen molecule binds first to form the OXY-state. This is then reduced by the subsequent addition of the electron and proton.

Stage three - The peroxy intermediate is further reduced to a ferryl intermediate (F-state).

Stage four - The ferryl intermediate is reduced to two molecules of water and the binuclear centre returns to the O-state.

Figure 1.5. A model for the catalytic cycle.
Oxygen is reduced to water at the binuclear centre in four stages (Ox→E, E→P, P→F and F→Ox). Each stage requires the addition of one proton and one electron. Two possible pathways for the oxygen reduction reaction in stage 2 have been suggested. From Zaslavsky and Gennis (2000).

The protons required for oxygen reduction are thought to enter the binuclear site from the matrix by one of two channels: the K- and D-channel (Iwata et al., 1995). The protons utilised in the first two stages of the catalytic cycle pass through the K-channel (Vygodina et al., 1998) and it has been suggested that protons utilised in the final two stages of the catalytic cycle pass through the D-channel, although this model remains under intense investigation (Konstantinov et al., 1997). The protons are likely to be conducted through the channels by ‘hopping’ along hydrogen bonds formed by amino acid side chains and internal water molecules, the latter of which are thought to be critical components of proton conducting channels. Possible pathways used by oxygen to enter the binuclear site and water to exit have also been suggested (Riistama et al., 1996; Tsukihara et al., 1996). Four protons are pumped across the inner membrane from the matrix to the intermembrane space for each molecule of oxygen reduced to water. The process is coupled to stages three and four of oxygen reduction. It
is generally agreed that two protons are pumped across the inner membrane at each stage.

The basic mechanism linking oxygen reduction to proton translocation is reasonably well understood (Rich et al., 1998). 1) The presence of electrons in the binuclear centre causes the uptake of protons from the matrix to the binuclear centre to counterbalance the negative charges of the electrons. 2) The final two stages of oxygen reduction result in the uptake of protons (so-called substrate protons) from the matrix. The protons already in the complex are physically separated from the oxygen-reduction chemistry and therefore can not be used in the oxygen reduction. 3) The substrate protons electrostatically repel the protons already present in the complex, which are consequently transferred into the intermembrane space.

The pathways that the protons use to enter and exit the binuclear centre are unclear. These are presently under intense investigation, as are the ‘gating’ processes which result in proton expulsion into the intermembrane space and substrate proton uptake from the matrix during oxygen reduction (Mills et al, 2000; Wikström et al., 2000; Zaslavsky and Gennis, 2000).

The redox potential of the electrons increases from $+250 \text{ mV}$ to $-1.2 \text{ mV}$ as they pass from cytochrome $c$ to oxygen. The greatest increase occurs in the final two stages of oxygen reduction and this provides the energy for proton translocation: i.e. the P-state transition to the F-state transition and the F-state transition to the O-state transition (Wikström, 1989; Verhovsky et al., 1996).

1.2.6. Complex V, the ATP synthase

Complex V catalyses ATP synthesis from ADP and $P_i$. Energy is provided for the process by the protonmotive force driving the movement of protons, along their concentration and charge gradient, through the complex from the intermembrane space ($_{\text{out}}$) to the matrix ($_{\text{in}}$).
ADP + P_i + nH_{out}^+ \rightarrow ATP + H_2O + nH_{in}^+

To differing extents, analysis has been performed to characterise the complex in bacteria, mammals, yeast and plants.

### 1.2.6.1. Composition of complex V

The number of different subunits present in complex V varies between organisms. The bovine heart complex is composed of sixteen different subunits (Walker et al., 1995) whereas yeast and plants contain at least thirteen (Jänsch et al., 1996; Spannagel et al., 1997). A few of these subunits are encoded by the mitochondrial genome. The rest are nuclear encoded. All possess homology in sequence and number to the ‘minimal’ eight subunits found in the prokaryotic ATP synthase, which are present in a different stoichiometric amount and are essential for ATP synthase activity (commonly termed $\alpha_3, \beta_3, \delta, \varepsilon, \gamma, a, b_2, c_{9-12}$; Deckers-Hebestreit and Altendorf, 1996). Homology exists between some of the additional subunits in bovine and yeast. Possible functions for the additional subunits of yeast complex V, such as a role in the assembly and dimerisation of the complex, have been suggested as a result of mutational analysis (Devenish et al., 2000).

ATP synthases are found in bacteria and the chloroplasts and mitochondria of eukaryotes. They are structurally and functionally conserved among the species (Boyer, 1997; Fillingame, 1997). The determination of the structure and function of this enzyme has resulted from the combination of analysis of the complex in the different systems using techniques such as electron microscopy, cross-linking studies, NMR and X-ray crystallography (Nakamoto et al., 1999; Cross, 2000).

### 1.2.6.2. Structure of complex V

Complex V consists of two domains, one located in the matrix ‘F_1’ and one in the inner membrane ‘F_0’. A central rotor stalk and a stator stalk connect the domains, which are composed of both F_0 and F_1 subunits (fig 1.6).
The α, β, δ, γ and ε subunits make up the F₁ domain and the α, b and c subunits form the F₀ domain. From Lehninger (2000).

The determination of the crystal structure at 2.8Å of the bovine heart F₁ domain (Abrahams et al., 1994) contributed significantly to understanding the structure of the domain in mitochondrial complex V. The F₁ domain is composed of the α, β, ε, δ and γ subunits and is spherically shaped. The three α subunits (grey; fig 1.6) and three β subunits (purple; fig 1.6) of the domain extend from the top to the bottom of the sphere and are arranged alternately. The β subunits exist in three conformational states. These have been designated ‘loose’, ‘tight’ and ‘open’ according to their binding affinities for different substrates.

The loose conformation binds ADP + Pᵢ, the tight conformation binds ATP and the open conformation binds nothing. The N- and C-terminal ends of the γ subunit (green; fig 1.6) fill the core of this sphere and interact with the β subunit in the open conformation. The γ subunit rotates in 120° steps, connecting each β subunit in turn, forcing it to change from the tight confirmation to the open confirmation (Noji et al., 1997).
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The rotor stalk is composed of the central region of the \( \gamma \) subunit and the \( \varepsilon \) subunit (blue; fig 1.6; Bulygin et al., 1998). These subunits rotate and connect the \( F_1 \) domain to the central region of the \( F_0 \) domain. The stator stalk is a stationary structure and is composed of the two \( b \) subunits and the \( \delta \) subunit (McLachlin et al., 1998). These subunits connect the \( \alpha \) and \( \beta \) subunits to the outer surface of the \( F_0 \) domain. The role of this stalk is to make sure conformational changes in the \( \beta \) subunit occur relative to rotation in the \( \gamma \) subunit.

The \( F_0 \) domain is composed of the \( a \), \( b \) and \( c \) subunits. The majority of this domain consists of the multiple copies of the \( c \) subunit. Each \( c \) subunit consists of two transmembrane domains, which fold like a hairpin, and a polar loop, which connects the domains. A conserved carboxyl group is present in one of the transmembrane domains. The \( c \) subunits are arranged perpendicular to the plane of the membrane and form two concentric circles, which can rotate. The N-terminal transmembrane domain faces inwards and the C-terminal domain outwards. The subunits of the rotor stalk bind to some of the polar loops of these \( c \) subunits. The \( a \) subunit is attached to the outer ring of the \( c \) subunits. This binds to the components of the stator stalk (Singh et al., 1996). The \( F_0 \) domain is sensitive to the inhibitor oligomycin.

1.2.6.3. Function of complex V

The mechanism of ATP synthesis is understood and is known as the 'binding change mechanism' (Boyer, 1993 and 1997). The production of ATP occurs in the catalytic sites of the \( \beta \) subunits of the \( F_1 \) domain. As stated before, the three \( \beta \) subunits cycle through three conformational states termed loose, tight and open; each subunit existing in a different confirmation at any given time. ATP production occurs during this cycle as follows (fig 1.7). ADP and \( P_i \) bind to a \( \beta \) subunit in its loose state. The conversion of the \( \beta \) subunit to the tight state results
in the condensation of ADP and $P_i$ to ATP. The $\beta$ subunit tightly binds and stabilises the ATP molecule.

![Figure 1.7. Mechanism of ATP synthesis](image)

**Figure 1.7. Mechanism of ATP synthesis**

$\beta$ subunits sequentially cycle through three different conformational states Open (O), Loose (L) and Tight (T), during which ATP is synthesised. ATP release is powered by the rotation of the $\gamma$ subunit, three molecules of ATP being produced per complete rotation. From Cross (2000).

Clockwise rotation (120°) of the $\gamma$ subunit in the core of the $F_1$ domain provides the energy required for the final step, *i.e.* release of ATP. It forces the tight domain to convert to the open state, which results in the simultaneous release of ATP and conformational changes in the other $\beta$ subunits (from open and loose to loose and tight, respectively). This is the only energy-requiring step in the ATP synthase cycle. The cycle is continuous and for each full turn (360°) of the $\gamma$ subunit, three molecules of ATP are produced.

The energy for the rotation of the $\gamma$ subunit is provided by the protonmotive force. Protons move along their concentration and charge gradient through the $F_0$ domain, causing the clockwise rotation of the $c$ subunits that in turn rotate the attached $\gamma$ and $\varepsilon$ subunit (fig 1.6).

A mechanism which couples proton translocation to ATP synthesis has been suggested (Boyer, 1999). It is thought the $c$ subunits rotate in a step-wise process.
(fig 1.6). Rotation starts with the binding of a proton, from the intermembrane space, to the conserved carboxylic residue exposed on a c subunit when facing the a subunit. The binding of the proton causes the ring of c subunits to rotate by one subunit (30°) relative to the a subunit. Repetition of this process results in the generation of 'torque'. This causes the rotation of the γ subunit in 120° steps. Recently, possible structural changes in the c subunits which result in the rotation of the γ and ε subunits have been suggested (Rastogi and Girvin, 1999; Dmitriev et al., 1999). The protons remain attached to the c subunits for a full revolution and are thought to be released into the matrix through a pore in the a subunit when they return to the a/c subunit interface.

The transfer of twelve protons through the F₀ complex results in a complete revolution of both the c subunits and the γ subunit and the production of three molecules of ATP (Cross, 2000).

1.2.7. The Alternative Oxidase

The alternative oxidase catalyses the transfer of electrons from ubiquinol to molecular oxygen, which results in the uptake of protons from the mitochondrial matrix and the production of water (Huq and Palmer, 1978). No protons are transferred across the inner membrane to contribute to the protonmotive force. 

\[ 2\text{UQH}_2 + \text{O}_2 \rightarrow 2\text{UQ} + 2\text{H}_2\text{O} \]

The alternative oxidase is found in plants, fungi and some protists (Moore and Siedow, 1991). Apart from a role in the thermogenesis of the flower Araceae (Meeuse, 1975), the function of the alternative oxidase is not fully understood, as it does not contribute to protonmotive force. The enzyme may play a role in reducing the level of reactive oxygen species (ROS) in conditions which result in their increased production i.e. chilling, pathogen attack, ageing and inhibition of the main respiratory pathway (Maxwell et al., 1999).
1.2.7.1. Composition of the alternative oxidase

The alternative oxidase is composed of only one subunit, which is encoded by the nuclear genome (Elthon and McIntosh, 1987). The subunit is encoded by the highly conserved Aox1 gene (Li et al., 1996). In some plants, a small family of Aox1 genes are present in the nuclear genome. These are differentially expressed in a developmental and tissue specific manner. The significance of the presence of these different subunits is unknown (Whelan et al., 1996). A di-iron catalytic centre is proposed to be present in the enzyme (Siedow et al., 1995).

1.2.7.2. Structure of the alternative oxidase

The alternative oxidase has yet to be isolated as a purified enzyme owing to its strong association with the mitochondrial inner membrane. As a result, the structure of the enzyme has not been determined. However, on the basis of results from biochemical analysis of the enzyme when in a partially purified form or in a mitochondrial membrane fraction (Minagawa et al., 1990; Berthold and Siedow, 1993) and sequence and structural analysis, some structural predictions for the protein have been made (Siedow et al., 1995; Andersson and Nordlund, 1999).

It is thought that the alternative oxidase is orientated towards the matrix side of the inner membrane (Moore and Siedow, 1991) and exists as a dimer (Umbach and Siedow, 1993). Recently, it has been suggested that the protein is an interfacial membrane protein lacking transmembrane spanning domains and a possible ubiquinol binding site has been indicated in the protein (Andersson and Nordlund, 1999). The C-terminal region of the protein is predicted to contain four small α-helices. Two of these possess a conserved motif: Glu-X-X-His. It has been suggested that these residues (the carboxylate and the histidine residue) bind a di-iron centre, which forms the active redox site of the protein (Siedow et al., 1995).
The alternative oxidase is thought to belong to the di-iron carboxylate family of proteins. These have recently been recognised as redox-active iron proteins that catalyse mainly dioxygen dependent redox reactions. The active site of these enzymes also contains a di-iron centre that is co-ordinated to the protein by two histidines and four carboxylate residues (Sjoberg, 1997; Yoshizawa, 1998). Similar to the alternative oxidase, the motif Glu-X-X-His is conserved in these proteins on two α-helices and these are involved in the binding of the Fe centres.

1.2.7.3. Function of the alternative oxidase

The alternative oxidase reduces oxygen in a single four electron transfer step (Vanlerberghe and McIntosh, 1997). The mechanism of this electron transfer process is thought to be similar to the di-iron carboxylate proteins. In these complexes, a molecule of ubiquinol donates two electrons to the di-iron centres, which become reduced. Molecular oxygen then binds to the centres to form a diferric-peroxide intermediate. The subsequent addition of two electrons to the site by ubiquinol results in the reoxidation of the Fe centres and the production of two molecules of water (Nordlund and Eklund, 1995). The function of the enzyme is inhibited by salicylhydroxamaic acid (SHAM) and n-propyl gallate (Schonbaum et al., 1971; Siedow and Bickett, 1981).

1.3. BIOGENESIS OF THE RESPIRATORY CHAIN

The biogenesis of the respiratory chain in eukaryotes requires genetic contribution from both the nuclear and mitochondrial genomes as both encode the subunits of the respiratory complexes. The subunits encoded by the mitochondrial genome are synthesised in the mitochondrial matrix and used directly. The subunits encoded by the nuclear genome are synthesised in the cytosol and are imported into the mitochondria to their site of action. To be imported, most of these proteins possess a targeting sequence at their N-terminal end (Whelan and Glaser, 1997). Processing enzymes, located in the
mitochondrial matrix and intermembrane space, remove these targeting sequences to produce the mature protein (Sjöling and Glaser, 1998; Braun and Schmitz, 1999).

The expression of the nuclear and mitochondrial genes is co-ordinated. Firstly, to ensure that the correct ratio of subunits required for assembly into respiratory complexes is available. Secondly, to ensure that the number of respiratory complexes assembled is regulated, allowing respiratory activities to meet the demands of the cell. The co-ordination of gene expression is achieved by two mechanisms: 1) Regulation of mitochondrial gene expression. This function is performed by nuclear encoded proteins (Grivell, 1995). 2) Regulation of nuclear gene expression by external factors and the mitochondrion (Scarpulla, 1997). This latter type of regulation is known as global regulation. It not only controls the synthesis of the nuclear encoded subunits of the respiratory complexes, but also those nuclear gene products required for the expression of the mitochondrial genes. Mutational analysis, predominantly in S. cerevisiae has been performed to identify the proteins involved in these processes (Tzagoloff and Dieckmann, 1990).

1.3.1. Regulation of mitochondrial gene expression

A number of nuclear encoded proteins have been identified that regulate mitochondrial gene expression at all levels i.e. transcription, processing and stabilisation of precursor and mature mRNAs, and translation of the mRNAs. These proteins are not part of the basic transcriptional and translational machinery of the mitochondrial genome and have been found to regulate the expression of individual mitochondrial genes. For example, at least 18 nuclear encoded proteins have been identified as factors required for the expression of the coxl gene in S. cerevisiae (Pel et al., 1992).
1.3.2. Global regulation

Global regulation controls the long-term expression directly of the nuclear genes and indirectly of the mitochondrial genes. External factors and the mitochondrion regulate gene expression from the nuclear genome by activating signal transduction pathways that act to upregulate or suppress the expression of nuclear genes. Many proteins required for these signalling pathways have been identified (Scarpulla, 1997).

A number of external factors have been determined that activate signal transduction in eukaryotic organisms. These include changes in oxygen levels and carbon source in yeast (de Winde and Grivell, 1993) and hormones and muscle activity in mammals. Changes in light intensity and colour, the oxygen level and the absence or presence of a carbon source have all been suggested as possible stimuli that regulate nuclear gene expression in photosynthetic organisms, although this has yet to be been demonstrated (Mackenzie and McIntosh, 1999).

The mitochondrion regulates gene expression from the nuclear genome in two ways (Poyton and McEwen, 1996). By the retrograde signalling pathway, which up-regulates gene expression, and by the intergenomic signalling pathway, which down-regulates nuclear gene expression. The retrograde signalling pathway is probably activated by the production of reactive oxygen species (ROS) in the mitochondria. These are produced by the respiratory chain and unless reduced to water, are potentially lethal to the cell. They cause the increased production of the respiratory complexes. The intergenomic signalling pathway involves the regulation of nuclear genes by the mitochondrial genes. So far, this pathway has only been shown to exist in mutants lacking mitochondrial genes and the mechanism by which it occurs is not understood.
1.3.3. Assembly

Nuclear encoded proteins also regulate the assembly of the respiratory enzyme subunits and cofactors into complexes. Many of these proteins have been identified (Grivell et al., 1999). Each complex appears to have its own set of assembly factors. For example, nine proteins have been identified that are required for the assembly of complex IV. Mutants lacking these proteins possess all the nuclear and mitochondrial encoded subunits but lack complex IV activity. The subunits are sometimes present at reduced levels or absent due to the increased turnover of the subunits in the absence/incomplete assembly of complex IV.

1.3.4. Defects in the biogenesis pathway

Some serious human organ specific diseases are caused by defects in the proteins required for the biogenesis of the respiratory chain (Vedel et al., 1999). These diseases include Leigh Syndrome (defective in complex IV), Leber’s hereditary optic neuropathy (LHON - defective in complex I and III) and possibly Alzheimer disease (Complex IV). As a consequence, this area of research is of high medical significance and there has been much importance attached to identifying the key proteins involved in biogenesis of the complexes, the genes involved and their function.

A few key organisms have been used to study the respiratory chain pathway, predominantly mammal tissue and yeast. At present, the respiratory chain has not been studied in detail in plants, where the situation is complicated by the presence of the chloroplast and photosynthetic processes. *Chlamydomonas reinhardtii* has been used as a model for eukaryotic photosynthesis for many years and is an ideal model to use to study respiration in photosynthetic organisms.
1.4. **CHLAMYDOMONAS REINHARDTII**

*C. reinhardtii* is a green unicellular alga. It is a member of the genus *Chlamydomonas*, one of 33 members of the *Chlamydomonadaceae* family. The organism has been found living in a wide variety of habitats *i.e.* freshwater lakes, ponds, soil, forests and deserts. The standard strain used worldwide for experimental analysis was derived from a zygospore isolated from a soil sample collected near Amherst, Massachusetts by G. M. Smith, in 1945 (Hoshaw, 1965).

1.4.1. Cell Architecture

*C. reinhardtii* cells are either spherically or ovaly shaped (fig 1.8; Harris, 1998). The cells range in length from 8 to 22 μm. The main structural features of each cell include 1) Two flagella (F). These are typically 1.5 to 2x the length of cell body. 2) A single cup shaped chloroplast (C), which partially surrounds the nucleus. The chloroplast constitutes up to 40% of the total cell volume. An eyespot (E) and a pyrenoid (P) structure are embedded in this organelle. 3) A single nucleus (N) with a fenestrated membrane. 4) Several small mitochondria (M) that are scattered throughout the cytoplasm of the cell. These exist as either small oval or elongated structures. The elongated structures sometimes branch to form an interconnecting network throughout the cell. The mitochondria constitute 1-3% of the total cell volume. 5) A cell wall. This is composed of glycoproteins with a high hydroxyproline content.
1.4.2. Life Cycle

*C. reinhardtii* has a simple life cycle and is capable of both asexual (vegetative) and sexual reproduction (Harris 1998).

1.4.2.1. Vegetative reproduction

Vegetative *C. reinhardtii* cells are haploid and reproduce by mitosis. Under ideal conditions, wild type cells reproduce mitotically every eight hours, *i.e.* in liquid

Figure 1.8. The ultrastructure of a *C. reinhardtii* cell.

A drawing of an electron micrograph demonstrating the presence and arrangement of the Chloroplast (C), Eyespot (E), Endoplasmic Reticulum (ER), Flagella (F), Golgi apparatus (G), Mitochondria (M), Nucleus (N), Pyrenoid (P) and Vacuole (V) in a *C. reinhardtii* cell. From a drawing by Keith Roberts.
medium containing inorganic salts and a carbon source with light as an energy source (mixotrophic conditions). They stop dividing when the cell density reaches $1-2 \times 10^7$ cells/ml (stationary phase).

Vegetative cells are able to reproduce in/on both liquid and solid media. Although they grow best under mixotrophic conditions, the cells will grow in the absence of a carbon source (phototrophic) and in the absence of the energy source light (heterotrophic), although they reproduce at a slower rate.

### 1.4.2.2. Sexual reproduction

Sexual reproduction in *C. reinhardtii* is only induced in adverse conditions. Vegetative cells form gametes, which then fuse to form zygotes. These then meiotically divide to form four haploid offspring, the ‘tetrad’ (fig 1.9).

**Figure 1.9. Sexual reproduction in *C. reinhardtii*.**

Gametes of opposite mating type ($m^-$ and $m^+$) fuse by flagellar pairing to form zygotes. Most of these undergo a period of maturation and divide meiotically to form four offspring, the tetrad. Some zygotes do not meiotically divide. Instead they mitotically divide to form diploid cells. From Harris (1989).
In the laboratory, the removal of nitrogen from the growth medium initiates sexual reproduction (Beck and Haring, 1996). It causes vegetative cells of each mating type to differentiate into the corresponding gamete. A mating type minus gamete (mt⁻) or a mating type plus gamete (mt⁺). Differentiation is controlled by a complex locus located in the nuclear genome (Ferris and Goodenough, 1997). Gametes of opposite mating type are compatible to mate, their fusion being initiated by the pairing of mt⁻ and mt⁺ gamete flagella. This triggers a cascade of events, such as cell wall breakdown by lysin and contact of mating type specific structures in the apex of the cell, which lead to the cytoplasmic fusion of the gametes to form a zygote (Buchanan et al., 1989; Wilson et al., 1997). Fusion occurs in about three and a half hours.

Before the zygote meiotically divides, it undergoes a period of maturation. This usually lasts for four to seven days and occurs in the dark. In the first 24 hours of the maturation period the flagella are re-absorbed into the cell, the gamete chloroplasts and nuclei fuse and the zygote develops a thick, protective wall (Woessner and Goodenough, 1992). The size of the zygote doubles during this period. Transferring the zygote to a source of nitrogen, moisture and light induces germination. The zygote meiotically divides to produce four progeny, which are released when the zygote wall bursts. 1-10% of zygotes do not produce haploid progeny. These instead divide by mitosis to form diploid cells (Remacle and Matagne, 1998).

1.4.3. The three genomes and their inheritance

Genomes are found in each of the three major organelles of C. reinhardtii cells. The nucleus contains a large genome (1.0 to 1.6 x 10⁸ bp). Seventeen linkage groups have been mapped to this genome (Harris, 1989). The chloroplast contains a circular genome of 196 kbp. Around 80 copies of the genome are present per chloroplast (Chiang and Sueoka, 1967). The mitochondrion contains a small (15.8 kbp) linear genome. About 50 copies of the genome are present per
cell (Ryan et al., 1978). These are divided amongst the dozen mitochondria dispersed throughout the cytoplasm.

The pattern of inheritance of the genes with each genome was determined by analysing the phenotype of offspring arising from reciprocal sexual crosses of gametes possessing mutations in their nuclear, mitochondrial or chloroplast genomes (fig 1.10).

![Figure 1.10. The inheritance patterns of genes in C. reinhardtii.](image)

Analysis of the mode of transmission of nuclear (left), chloroplast (middle) and mitochondrial (right) mutations allow the determination of the pattern of gene segregation during sexual reproduction. From Harris (1989).

Nuclear genes are inherited in a Mendelian fashion and segregate 2:2 in the tetrad progeny. Chloroplast genes are mostly inherited from the mt\(^+\) parent and mitochondrial genes from the mt\(^-\) parent, *i.e.* the chloroplast and mitochondrial genes are uniparentally inherited, whereas the nuclear genes are inherited biparentally.

The molecular basis for the uniparental inheritance of chloroplast and mitochondrial genes has been determined. The mt\(^-\) chloroplast genome and the mt\(^+\) mitochondrial genomes are degraded during the zygote maturation period (Beckers et al., 1991). They are therefore not available for inheritance during germination. The chloroplast genome is degraded within the first few hours of
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the zygote maturation period by a nuclease. The mt\textsuperscript{+} genome is methylated and it is thought this protects it from degradation. The mitochondrial genome is degraded slowly during zygote maturation and is only fully eliminated at the onset of germination. The elimination of this genome has been suggested to occur due to the elimination of the mt\textsuperscript{+} mitochondria from the zygote (Beckers et al., 1991).

1.4.4. A model organism

C. reinhardtii is used as a model system to study many cellular pathways and mechanisms including photosynthesis, cell motility, cell wall biosynthesis and respiration. It was selected as a model for a number of reasons.

1) The organism is cheap to maintain. It has few requirements for growth (a carbon source, light, inorganic salts and incubation at 18-33°C). Experiments are therefore feasible with it.

2) The organism grows quickly. The cells double every eight hours therefore analysis of cell phenotype can be performed quickly.

3) Genetic analysis is feasible in the organism. Techniques are available to induce gamete formation in C. reinhardtii cells, identify the opposite mating types, mate the gametes and analyse the tetrad offspring. The origin of any mutation whether in nuclear, mitochondrial or chloroplast genome can be established. Linkage between genes can also be determined. Genetic maps of both the nuclear genome (Harris, 1998) and the chloroplast genome (Harris et al., 1989) have been constructed by recombinational analysis with characterised mutants. Crosses with these known mutants can establish the location of newly characterised genes. The construction of a genetic map for the mitochondrial genome is still in progress (Remacle et al., 1995).
4) The organism is able to dispense with photosynthesis, respiration, cell motility and a cell wall. Mutants defective in these processes can be isolated and characterised. In fact, collections of mutants defective in many cell metabolic processes are available for characterisation (Chlamydomonas Genetics Center, Duke University. E-mail: chlamy@duke.edu).

5) The organism has a haploid vegetative growth cycle. Therefore, any mutant phenotype is expressed immediately and can be selected for further analysis e.g. respiratory mutants are selected by their inability to grow in the dark.

1.4.5. Recent developments

*C. reinhardtii* has been used since the 1950s as a model to study metabolic pathways on a biochemical and physiological level. However, in the past decade, recent advances in molecular biology have completely transformed *C. reinhardtii* research. It is now possible to generate new interesting mutants, clone and characterise nuclear genes and perform detailed functional analysis with known genes. The development of an effective transformation system for the nuclear, mitochondrial and chloroplast genome contributed significantly to this. Transformation requires a selectable marker and a suitable system to deliver the marker into the cell genomes.

1.4.5.1. Nuclear transformation

The first markers used for nuclear transformation were the cloned *C. reinhardtii* genes *NIT1* (Fernandez et al., 1989) and *ARG7* (Debuchy et al., 1989). These genes were chosen as selectable markers because when transformed into a recipient cell lacking the corresponding gene, they produced a selectable phenotype that allowed the identification of transformants. The *NIT1* gene codes for a nitrate reductase, which allows cells to grow on nitrate as a sole nitrogen source. *ARG7* codes for argininosuccinate lyase, an enzyme required for arginine biosynthesis. Rescued cells are able to grow in the absence of arginine.
Initially, these markers were transformed into the nuclear genome by the technique of biolistics. In this method, cells are bombarded at high velocity with gold or tungsten particles coated in the marker DNA (Sanford et al., 1993). However, the glass bead DNA delivery system developed by Kindle (1990) has proved to be a simpler, more effective method for delivering DNA to the nuclear genome and is now used routinely for nuclear transformations. In this method, a suspension of cells lacking a cell wall are agitated with DNA and glass beads.

Dominant selectable markers have also been developed for use in nuclear transformation, the CRY1 marker (Nelson et al., 1994) and the ble marker (Stevens et al., 1996). These markers confer antibiotic resistance to cells, so transformants can be isolated simply. As opposed to the ARG7 and NIT1 markers, these markers can be used to transform any strain of C. reinhardtii. The CRY1 marker, which consists of a mutated copy of a C. reinhardtii ribosomal protein gene and the promoter of the highly expressed C. reinhardtii RBCS2 gene, confers resistance to cryptopleurine and emetine. The ble marker is derived from a bacterial gene and confers resistance to zeomycin and phleomycin.

The ble gene is ideal to use as marker for nuclear transformation. The gene is small, any strain of the organism can be transformed and unlike the CRY1 marker, selection of transformants is simple. The ble cassette used for transformation is composed of a slightly modified ble gene and the 5’ and 3’ regulatory regions of the RBCS2 gene (pSP108; fig 1.11). Transformation rates using this marker were initially low due to poor expression of ble. Modifications to the cassette, such as the addition of RBCS2 introns to the ble gene and deletions to the 5’ regulatory region of the cassette (fig 1.11), have increased the number of transformants produced with the marker significantly. The currently favoured form of the marker (pSP124) produces transformation rates comparable to the ARG7 and NIT1 markers.
The addition of intron(s) to the pSP108 marker and deletion of some of its promoter region increased its transformation efficiency 28.4 (pSP115) and 30 fold (pSP124) relative to pSP108. Adapted from Lumbreras et al. (1998).

The ability to transform the nuclear genome has been exploited as a tool for molecular-genetic studies.

1) Markers have been used as insertional mutagens to generate random mutants disrupted in functionally important genes. They are ideal for this technique for three reasons. a) They integrate non-homologously into the nuclear genome enabling the generation of random mutants. b) They usually delete large regions of the genome when they integrate. Therefore mutants generated are usually stable. c) They physically tag the gene disrupted. This allows the isolation of the disrupted gene. There are many examples of use of the technique in C. reinhardtii research to isolate functionally important genes required for various metabolic processes. The technique has been used to isolate genes required for photosynthesis (Gumpel et al., 1995), motility (Tam and Lefebvre, 1993), phototaxis (Parzour et al., 1995), chemotaxis (ErmiLOva et al., 2000), cadmium tolerance (McHugh and Spanier, 1994), salt tolerance (Prieto et al., 1996) and sulphur metabolism (Davies et al., 1994).
2) Structural and functional analysis of nuclear gene products is now possible (eg. Hippler et al., 1998). Although homologous recombination occurs only very rarely during nuclear transformation, if a null mutant exists, it is possible to introduce site-directed variants of the gene into random loci of the nuclear genome and analyse the resultant transformants.

3) Mutated genes are being cloned by genomic complementation using pools of genomic clones. Two wild type genomic libraries of *Chlamydomonas reinhardtii* have been created for this purpose (Purton and Rochaix, 1994; Funke et al., 1997).

4) Genes tagged with ‘reporter molecules’ can be transformed into the genome. This has been exploited to look at gene expression and protein localisation in *Chlamydomonas reinhardtii* cells (Stevens and Purton, 1997).

### 1.4.5.2. Organelle transformation

Transformation of both the chloroplast and mitochondrial genome is possible using biolistics. In the original work performed to develop a transformation system for the chloroplast genome, the cloned chloroplast *Chlamydomonas reinhardtii* gene *atpB* was used as a marker for transformation, and mutants lacking the gene as the recipients (Erickson, 1996). The marker complements the mutants, allowing them to grow in the absence of a reduced carbon source. The dominant marker, the *aadA* cassette, which confers resistance to spectinomycin is now routinely used as the selectable marker for chloroplast transformation (Goldschmidt-Clermont, 1991).

Transformation of the mitochondrial genome has only been demonstrated once, when a mutant disrupted in the *cob* gene (section 1.5.1) was rescued to respiratory competency by transformation with the entire mitochondrial genome of *Chlamydomonas reinhardtii*. As yet, no practical selectable marker for transformation of the genome has been developed, although possible endogenous genes (*cob*) carrying
dominant mutations to the respiratory inhibitors myxothiazol and mucidin are available which could be exploited as markers (Remacle and Matagne, 1998).

In contrast to the nucleus, DNA integrates by homologous recombination into the chloroplast and mitochondrial genomes. Consequently, gene targetting is feasible, allowing the disruption or modification of individual organellar genes. The ability to manipulate the chloroplast genome has been actively exploited by a number of groups interested in studying photosynthesis and chloroplast gene expression (reviewed in Goldschmidt-Clermont, 1998).

1.4.6. Genomics

Two of the most recent advances in *C. reinhardtii* genomics are the development of the expressed sequence tag (EST) database and progress in the physical mapping of the nuclear genome (Davies and Grossman, 1998; Silflow, 1998). These are paving the way to a complete understanding of the *C. reinhardtii* genetic system.

The EST database is comprised of sequences of cDNA clones derived from mRNA. The goal of the EST database is to provide a source that allows the identification and analysis of all the protein coding genes of *C. reinhardtii*. Currently, over 35,000 ESTs have been generated and deposited in a public database (http://www.kazusa.or.jp/en/plant/chlamy/EST/). Homology-based searches using the BLAST algorithm have been carried out on the ESTs, and in many cases a possible function has been identified. Some have subsequently been used to isolate the corresponding gene for analysis.

The nuclear genome has been genetically mapped using known mutants. Work is underway to link this to a physical map of the genome for two reasons: 1) the mutants used to create a genetic map of the genome can be cloned and analysed. 2) Sequencing of the genome can begin. The sequence of the mitochondrial
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Genome is known (Vahrenholz et al., 1993) and the sequencing of the chloroplast genome is almost finished.

1.5. MITOCHONDRIA OF C. REINHARDTII

The basic structure and function of the mitochondria of C. reinhardtii is identical to those found in other eukaryotic species (section 1.1). As revealed by electron microscopy, the organelle is composed of two distinct membranes (Sager and Palade, 1957). The inner membrane projects into a centrally located matrix. The organelle is able to respire due to the possession of a respiratory chain, located in the inner mitochondrial membrane. It also contains a genome that encodes components of the chain.

1.5.1. The mitochondrial genome

Ryan et al. (1978) first demonstrated the presence of a genome in the mitochondria of C. reinhardtii. Mitochondria were isolated as a DNase impermeable fraction from cell wall less cells and viewed under an electron microscope. Further analysis by the same group demonstrated the genome was linear, double-stranded, was present in about 50 copies per cell and possessed a G + C content of 47.5%. Grant and Chiang (1980) provided confirmation of the linearity of the genome, when the genome was mapped by restriction enzyme analysis. This analysis also demonstrated the genome possessed no inter- or intra-molecular heterogeneity.

The genome performs the same function as the mitochondrial genomes from other eukaryotic organisms. It contains some of the genes required for mitochondrial biogenesis. This function was first determined by Boer et al. (1985) who by the use of hybridisation and sequencing analysis, showed that the \textit{cox1, cob} and small and large subunit rRNA genes were present in the genome and demonstrated that the \textit{cox1} gene was expressed and therefore active. The complete contribution made by the mitochondrial genome to mitochondrial
biogenesis has now been established as the genome has been fully sequenced (fig 1.12; accession number U03843; Vahrenholz et al., 1993) and all its genes characterised.

![Diagram of the mitochondrial genome of C. reinhardtii.](image)

**Figure 1.12. The mitochondrial genome of C. reinhardtii.**

The genome contains eight protein-coding genes (cob, nd4, nd5, cox1, nd2, nd6, nd1 and ril), the small (S1 - 4) and large (L1 - 7) rRNA genes and three tRNA genes (W, tryptophan-tRNA: Q, glutamine-tRNA and M, methionine-tRNA). The flags and small arrows indicate the identical terminal repeats located at either end of the genome and their orientation respectively. The large arrows demonstrate the position of transcription start and the direction by which transcription proceeds. Adapted from Remacle and Matagne (1998).

The genome contains 13 genes, all of which lack introns. Seven of the genes code for subunits of the respiratory chain enzymes. These include genes for five subunits of complex I (nd1, 2, 4, 5 and 6), one subunit of complex III (cob) and one subunit of complex IV (cox1). Two of the genes code for the small and large rRNA ribosomal subunits. In *C. reinhardtii*, these subunits are fragmented and arranged in a discontinuous manner in the genome. It is thought these fragments interact by intermolecular pairing to form the conventional small and large subunits (Remacle and Matagne, 1998). The remaining three genes code for the three tRNAs: tryptophan-tRNA (W), glutamine-tRNA (Q) and methionine-tRNA (M). The rRNAs and tRNAs are required for the translation of the mitochondrial-encoded proteins. The genome also encodes a reverse transcriptase like (RTL) protein. Its function is unknown, although it may play a role in replication of the genome (Vahrenholz et al., 1993).
1.5.1.1. Genome replication

The mechanism of mitochondrial genome replication in *C. reinhardtii* is unknown. Two regions of sequence have been implicated in the process (Vahrenholz *et al.*, 1993). 1) The identical inverted repeat sequences found at either end of the linear genome (fig 1.12). 2) An internal sequence located at the 3' end of the L2b gene, which is identical to the outermost 86bp of the inverted repeat structures.

1.5.1.2. Gene expression

Gene expression from the mitochondrial genome in *C. reinhardtii* has been examined. The mitochondrial genes of *C. reinhardtii* are transcribed as two large precursor transcriptional units. The *cob, nd4* and *nd5* genes are transcribed in the leftward direction from one DNA strand. The remaining genes (*cox1, nd2, nd6, nd1* and *rtl*, the tRNAs and the small and large subunit rRNAs) are transcribed in the rightward direction from the other DNA strand (fig 1.12). A promoter for transcriptional start has not yet been identified, although it is thought that it must be located either in or between the *nd5* and *cox1* genes (Gray and Boer, 1988).

The large precursor transcripts are then processed by endonucleolytic cleavage to produce mature RNA molecules. This processing event is little understood. Conserved sequences have been identified within the genome that may act as cleavage sites for the endonuclease, which has yet to be isolated. The *cox1, nd5* and *cob* genes possess a conserved sequence at their 3' terminal ends (Pratje *et al.*, 1984; Boer and Gray, 1986) and the intergenic regions between the tRNA and rRNA gene cluster possess a short, repeated sequence (ACAA; Boer and Gray, 1991).

The mature mRNAs are then translated into protein. The mitochondrial ribosome of *C. reinhardtii* is composed of protein subunits, which are encoded by the nuclear genome and the mitochondrial encoded rRNA molecules (small and large
subunit). The number of protein subunits present in the ribosome is not known. A ribosome binding site (5’ AAAUUUAU), located three to seven nucleotides upstream from the AUG start site, has been defined in the protein coding genes of the genome (Colleaux et al., 1990). Twenty-three tRNAs, instead of the minimum 32 predicted from the universal genetic code, are required for the translation of the eight protein coding genes of *C. reinhardtii* (Boer and Gray, 1988). Although the genes use standard codon assignments, they show highly biased codon usage, i.e. nine codons are not used at all and four very rarely (Michaelis et al., 1990). The mitochondrial genome codes for only three of these molecules. It is thought the remaining tRNAs are imported from the nucleus (Remacle and Matagne, 1998).

1.5.2. The respiratory chain

Very little analysis of the *C. reinhardtii* respiratory chain has been performed. It is assumed that the chain is arranged and functions in a similar way to the respiratory chains found in other eukaryotes.

The respiratory chain of *C. reinhardtii* is composed of the five enzyme complexes (complex I, II, III, IV and V) and two carrier molecules (ubiquinone and cytochrome c) required for oxidative phosphorylation. In addition, it contains an alternative oxidase and some additional alternative NADH dehydrogenases. The presence of these enzymes and carrier proteins in the chain was determined by assaying for the activity of each complex, by immunoblotting and by haem staining (Alexander et al., 1974; Wiseman et al., 1977; Atteia et al., 1992 and 1994; Dorthu et al., 1992; Derzaph and Weger, 1996; Nurani and Franzén, 1996).

Little analysis has been performed in *C. reinhardtii* to determine the composition of each of the complexes of the chain. The number of subunits present in each complex, with the exception of complex V, has not yet been determined. It is presumed the core subunits required for respiratory function in other eukaryotic
species are required to perform the same function in _C. reinhardtii_ respiratory enzymes. The demonstration of the presence of some core subunits of complex III (the Rieske iron-sulphur protein and the cytochrome c1 protein; Atteia _et al._, 1992 and 1994), complex IV (the COXI protein; Bennoun _et al._, 1995) and complex V (the α and β proteins; Nurani and Franzén, 1996) in mitochondrial protein preparations support this. It is also presumed, as is the case in other eukaryotic organisms, that the respiratory complexes of _C. reinhardtii_ contain additional subunits, which are required for the regulation of the complex activity and the assembly of the complex, _i.e._ supernumerary subunits. The demonstration that the _C. reinhardtii_ complex V is composed of 14 different subunits, instead of the eight core subunits, provides support for this (Nurani and Franzén, 1996).

In yeast and mammals, the core and supernumerary subunits of the respiratory complexes are encoded by both the nuclear and mitochondrial genome. This is also thought to be the case in _C. reinhardtii_. The mitochondrial genome of this organism has been fully sequenced and from comparison to characterised genomes from other organisms, shown to encode the COXI protein of complex IV, the cytochrome b protein of complex III and subunits 1, 2, 4, 5 and 6 of complex I. Although it has not been demonstrated directly that these proteins function as subunits in the respiratory chain, support for the role is provided by the following: 1) In all other eukaryotes, the proteins encoded by the mitochondrial genome function as subunits in the respiratory chain. 2) Mutations in the genes coding for the proteins in _C. reinhardtii_ results in loss of respiratory function (Dorthu _et al._, 1992; Colin _et al._, 1995).

The nuclear genome is thought to encode the remaining subunits of the respiratory complexes. To date, gene sequences have been determined for the following: the alternative oxidase (Dinant _et al._, unpublished, Genebank accession number AF047832), the Rieske iron-sulphur protein (Atteia and Franzén, 1996), the COXIII protein (Perez-Martinez _et al._, 2000), the cyochrome c carrier protein.
(Atteia and Franzén, 1996) and the $\alpha$ subunit (Franzén and Falk, 1992) and $\beta$ subunit (Nurani and Franzén, 1996).

1.5.3. Biogenesis of the respiratory chain

The biogenesis of the respiratory chain of *C. reinhardtii*, like other eukaryotes, requires genetic contribution from both the nuclear and mitochondrial genome. It has been demonstrated that both the genomes encode the subunits of the respiratory complexes. The subunits encoded by the mitochondrial genome have all been identified whereas only a few of the nuclear encoded subunits have been characterised. The nuclear genome is also thought to code for proteins that aid in the synthesis of the nuclear and mitochondrial respiratory subunits and the assembly of the respiratory complexes. So far though, none of these proteins have been identified in *C. reinhardtii*.

*C. reinhardtii* is an ideal model to use to identify proteins required for respiratory chain biogenesis. Respiration is dispensable in the organism so mutants defective in respiratory chain biogenesis can be isolated and the genes (and hence the proteins) characterised. Respiratory mutants die in the dark (heterotrophic conditions), but grow in the light (phototrophic conditions), using photosynthesis to produce the ATP required by the organism.

Some work has been performed with *C. reinhardtii* to identify and characterise the proteins required for respiratory chain biogenesis. Mutants that lack respiratory function (and therefore are defective in respiratory chain biogenesis) have been isolated. The mutants were created by growing haploid wild type cells in the presence of the mutagenic chemicals acriflavine and MNNG. Genetic analysis demonstrated these mutants are disrupted in either a nuclear or mitochondrial gene.
1.5.3.1. The mitochondrial mutants

Thirteen mitochondrial mutants lacking respiratory function have been isolated (table 1.1). The mutants are called *dum* mutants (dark uniparental minus) due to their inability to grow in the dark and the mode of transmission of the mutations to meiotic progeny in reciprocal crosses with wild type cells (Matagne *et al.*, 1989). Biochemical analysis has shown the mutants lack classical respiration and more specifically, most possess little or no complex III or complex IV activity. The *dum* 1, 2, 3, 4, 11, 14, 15, and 16 mutants are defective in complex III activity (Dorthu *et al.*, 1992) and the *dum*18 and 19 mutants are defective in complex IV activity (Matagne *et al.*, 1989; Colin *et al.*, 1995). In addition, the *dum*20 and 24 mutants lack complex I activity, a complex previously thought to be essential for the survival of *C. reinhardtii* cells (Duby and Matagne, 1999). No clear defect for the *dum*6 mutant has been established. It seems to have a leaky phenotype as it grows slowly under heterotrophic conditions, as does the *dum*20 mutant (Remacle *et al.*, 1995).

The molecular basis of these defects has been determined partially or fully for most of the mutants (table 1.1.). The *dum*1, 2, 3, 4, 14 and 16 mutants have deletions ranging from 1.3 to 1.7 kb at the left end of their mitochondrial genomes in regions spanning the *cob* gene (Matagne *et al.*, 1989; Dorthu *et al.*, 1992). The *dum*15, 18 and 19 mutants have either a substitution (*dum*15) or frameshift mutation (*dum*18 and 19) in their *cob* or *cox1* genes (Colin *et al.*, 1995). The *dum*11 and 24 mutants possess two types of mitochondrial genomes which lack the *cob* gene and in the case of *dum*24, the *nd4* gene, *nd5* gene and 5' end of the *cox1* gene as well (Dorthu *et al.*, 1992; Colin *et al.*, 1995; Duby and Matagne, 1999).
Table 1.1. Respiratory mutants of *C. reinhardtii*.

Mitochondrial (M) and nuclear (N) respiratory chain mutants isolated in *C. reinhardtii*. See section 1.5.3. for details and references.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Location</th>
<th>Defective Complex</th>
<th>Functional Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dum1</em>, 2, 3, 4, 6, 11, 14 and 16</td>
<td>M</td>
<td>III</td>
<td>complex III subunit - deletion <em>cob</em> gene</td>
</tr>
<tr>
<td><em>dum15</em></td>
<td>M</td>
<td>III</td>
<td>complex III subunit – double bp substitution at codon 140 of <em>cob</em> gene ser-tyr</td>
</tr>
<tr>
<td><em>dum18</em></td>
<td>M</td>
<td>IV</td>
<td>complex IV subunit – addition of T at codon 145 of <em>cox1</em></td>
</tr>
<tr>
<td><em>dum19</em></td>
<td>M</td>
<td>IV</td>
<td>complex IV subunit – deletion of T at codon 152 of <em>cox1</em></td>
</tr>
<tr>
<td><em>dum20</em></td>
<td>M</td>
<td>I</td>
<td>complex I subunit – unknown mutation</td>
</tr>
<tr>
<td><em>dum24</em></td>
<td>M</td>
<td>I</td>
<td>complex I subunit – deletion of <em>cob</em> and <em>nd4</em> genes</td>
</tr>
<tr>
<td><em>dk32</em>, 43 and 76</td>
<td>N</td>
<td>I, III and IV</td>
<td>unknown – possible involvement in mitochondrial protein synthesis</td>
</tr>
<tr>
<td><em>dk52</em>, 80 and 148</td>
<td>N</td>
<td>IV</td>
<td>unknown – possible involvement in inner mitochondrial membrane organisation</td>
</tr>
<tr>
<td><em>dk97</em>, 105 and 110</td>
<td>N</td>
<td>IV</td>
<td>unknown – possible subunit of complex IV</td>
</tr>
</tbody>
</table>
Chapter one

The *dum* mutants with deletions in their mitochondrial genomes possess some interesting characteristic features. Firstly, the mutants all possess a heterogeneous population of molecules in their mitochondria. These consist of deleted genomes present in either a monomer or dimer form, the latter of which result from the head to head fusion of the deleted ends of the monomers (Dorthu *et al.*, 1992; Randolph-Anderson *et al.*, 1993; Duby and Matagne, 1999). It has been suggested that the monomers dimerise to produce a molecule with a stable left and right terminal end. Genomes with a terminal end deleted are thought to be unstable (Remacle and Matagne, 1998). Secondly, during mitotic division, about 2-10% of the mutants cells form lethal minute colonies. These are thought to arise due to the instability of the deleted mitochondrial genome.

An additional 16 mitochondrial mutants have been identified but these are not defective in respiratory function (Remacle and Matagne, 1998). They were isolated as diploids (as opposed to the haploid cells used for all other respiratory mutagenic work) and selected for their resistance to the complex III inhibitors myxothiazol and mucidin. Analysis of some of them has revealed they possess alterations in the *cob* gene (Di Rago *et al.*, 1989; Daldal *et al.*, 1989).

1.5.3.2. Nuclear mutants

There has been only one attempt so far to identify and characterise the nuclear encoded proteins required for the biogenesis of the respiratory chain in *C. reinhardtii*. Wiseman *et al.* (1977) generated a large number of respiratory defective nuclear mutants and named the mutants *dk* mutants (*dark* dier). Nine of these were characterised further using biochemical analysis, electron microscopy and genetic analysis (*dk*32, 34, 52, 76, 80, 97, 105, 110 and 148). The *dk*32, 34 and 76 mutants demonstrate abnormal mitochondrial ultrastructure and are defective in complex I, III and IV activity. These were suggested to lack genes coding for proteins required directly or indirectly for mitochondrial protein synthesis. The *dk*52, 80 and 148 mutants demonstrate structural abnormalities in the inner mitochondrial membrane and lack complex IV function. These were
suggested to lack inner mitochondrial membrane proteins, which organise the membrane and its enzymatic components. The $dk97$, 105 and 110 mutants, which lack complex IV activity only, were suggested to lack genes coding for subunits of complex IV. From complementation analysis, with the exception of $dk32$ and 34, each mutant was suggested to be disrupted in a different gene. No attempts to clone the genes disrupted in these mutants have been made.

The identification and characterisation of the nuclear encoded proteins required for respiratory chain biogenesis is of importance for two main reasons. 1) It would allow investigation into chloroplast, mitochondrial and nuclear genome interaction. All three genomes must communicate in order to provide optimal conditions for cell function. 2) Many serious organ specific diseases in humans are caused by defects in these proteins (section 1.3.4.). The characterisation of such proteins would provide invaluable insight in to the mechanisms of the diseases and may even lead to a possible treatment for the disorder.

*Chlorella reinhardtii* is an ideal model to use to study the biogenesis of the respiratory chain as in addition to the general characteristics that make the alga an ideal model (Section 1.4.4 and 1.4.5), the organism is able to dispense with respiration.

Recently, in our laboratory, a set of nuclear mutants defective in respiratory function were generated by insertional mutagenesis using the *ble* marker as the insertional mutagen (Section 1.4.5.1; Turner *et al.*, 1996). The mutants were identified as respiratory defective due to their inability to both reduce the chemical TTC and grow in the dark. Subsequent experiments with two of the mutants, M86 and M90 ($M =$ mitochondrial) demonstrated that they lack classical respiratory function (Section 1.2). A genomic cosmid clone that complemented the M90 mutant was isolated.
1.6. Aims of this research

The aims of the research detailed in this thesis were to:

1. Characterise the M86 and M90 mutants genetically to confirm firstly that the mutants were disrupted in a nuclear gene and secondly, the mutation had arisen due to the insertion of the ble marker.

2. Characterise the M86 and M90 mutants using biochemical analysis to determine the exact origin of the respiratory defect. This would involve the assay of individual respiratory enzyme activity and haem spectra analysis.

3. Characterise the M90 mutant on a molecular level. This would involve the cloning and sequencing of the M90 gene from the cosmid clone and the subsequent analysis of the gene product.

4. Analyse mitochondrial gene expression in the M86 and M90 mutants.
CHAPTER TWO
MATERIALS AND METHODS

2.1. GROWTH MEDIA

*C. reinhardtii* cells were cultured in tris-acetate phosphate medium (TAP; Gorman and Levine, 1965) or high salt minimal medium (HSM; Sueoka et al., 1967). TAP consists of 20 mM tris (pH 7.0 - glacial acetic acid), 2.5% (v/v) 4 x B-salts (0.3 M NH₄Cl, 14 mM CaCl₂ · 2H₂O and 16 mM MgSO₄ · 7H₂O), 0.01% (v/v) trace elements, 0.01% (v/v) 1 M KPO₄ pH 7.0 (consisting of 1 M K₂HPO₄ and 1 M KH₂PO₄). HSM consists of 2.5% (v/v) 4 x B-salts, 0.01% (v/v) trace elements and 5% (v/v) 2 x PO₄ buffer (consisting of 80 mM K₂HPO₄ and 50 mM KH₂PO₄; pH 6.9 -- KOH). Trace elements (Surzycki, 1971) contain 180 mM H₃BO₃, 77 mM ZnSO₄ · 7H₂O, 26 mM MnCl₂ · 4H₂O, 18 mM FeSO₄ · 7H₂O, 7 mM CoCl₂ · 6H₂O, 6 mM CuSO₄ · 5H₂O, 0.9 mM (NH₄)₆Mo₇O₂₄ · 4H₂O. This solution was heated to boiling and a boiling solution of sodium EDTA added to a final concentration of 0.1 M. The pH was adjusted to 6.8 using KOH. The solution was left to precipitate over a period of two weeks, filtered and the precipitate discarded. TAP was solidified by the addition of 2% (w/v) or 0.5 % (w/v) bacto agar and where necessary supplemented with zeomycin to a final concentration of 20 μg/ml.

Bacterial cells were cultured in Luria-Bertani Medium (LB; Sambrook et al., 1989) which contains 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 0.17 M NaCl. LB was solidified by the addition of 2% (w/v) nutrient agar. Where required, ampicillin was added to a final concentration 50 μg/ml. For induction experiments and blue/white colony selection, the addition of isopropylthio-β-D-galactoside (X-gal) and/or 5-bromo-4-chloro-3-indolyl-β-D-galactoside (IPTG) was necessary. Individual plates were supplemented with aliquots of X-gal (1 μg) and/or IPTG (4 μg).
Solutions and media were sterilised within at least eight hours by autoclaving at 15 psi, 121°C for 15 minutes and stored at room temperature (RT). The antibiotics and IPTG were filter sterilised by passage through a 0.22 μm pore filter and stored in aliquots at −20 °C.

2.2. *C. reinhardtii*: STRAINS AND MAINTENANCE

2.2.1. Strains

The strains used in this work are detailed in the table below. All are derived from the original isolate 137c described by G.M. Smith (Hoshaw, 1965).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Mt</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-13</td>
<td>wild type</td>
<td>+</td>
<td>J G-B</td>
<td>J G-B pers comn</td>
</tr>
<tr>
<td>cw-10</td>
<td>cell-wall deficient</td>
<td>-</td>
<td>CGC</td>
<td>Hyams and Davis, 1972</td>
</tr>
<tr>
<td>dk-97</td>
<td>complex IV deficient</td>
<td>-</td>
<td>CGC</td>
<td>Matagne et al., 1989</td>
</tr>
<tr>
<td>dum-1</td>
<td>complex III deficient</td>
<td>-</td>
<td>CGC</td>
<td>Wiseman et al., 1977</td>
</tr>
</tbody>
</table>

Table 2.1. The *C. reinhardtii* strains used in this research project.

Mt = Mating-type, J G-B = Jacqueline Girard-Bascou and CGC = *Chlamydomonas* Genetics Center (Dr E Harris, Duke University, North Carolina, USA).
2.2.2. Maintenance

*C. reinhardtii* strains were maintained on TAP agar and incubated at 25 °C under a photon flux of 45 μE/m²/s. They were streaked onto fresh TAP agar every four weeks. Cultures in use were streaked every three days. Liquid cultures were grown in TAP or HSM medium (Harris, 1989) in conical flasks (up to 1 l) or carboys (8 l). The smaller cultures were incubated at 25 °C, under a photon flux of 80 μE/m²/s and continuously shaken (120 rpm in a Gallenkamp illuminated orbital incubator). The large cultures were incubated at 25 °C, under a photon flux of 100 μE/m²/s and were stirred with a magnetic stirring bar. Air sterilised by passage through a 0.22 μm filter, was bubbled through the cultures. All liquid cultures were started by inoculating the stock culture into 25 ml of liquid medium and allowing the cells to grow to mid-log phase (~5 x 10⁶ cells/ml). An appropriate volume was subsequently transferred to the larger volume of medium.

2.2.3. Determination of cell density

Cell density was determined using a haemocytometer (Webber Scientific International Ltd). A 1 ml aliquot was removed from cells grown in liquid culture and 0.01% (v/v) tincture of iodine (19.7 mM iodine in 95% (v/v) ethanol) added. Two aliquots were removed from this and the number of cells present in each counted using the haemocytometer under a light microscope (Wild Heerbrugg microscope) at x400 magnification. From the mean of the two cell counts, the number of cells present per ml was determined by multiplying the count by 10⁴. For cultures above ~5 x 10⁶, cells were diluted ten-fold for counting.
Chapter two

2.3. BACTERIA: STRAINS AND MAINTENANCE

2.3.1. Strains

The bacterial strain used was *Escherichia coli* DH5α (F-, [φ80lacZΔM15], Δ(lacZYA-argF)U169, recA1, endA1, hsdR17(rK-, mK+), supE44, λ-, thi-1, gyrA96, relA1). This is available from Life Technologies, Inc.

2.3.2. Maintenance

DH5α cells were maintained on LB agar and streaked to single colonies. DH5α transformants carrying plasmids with the ampicillin-resistance gene, *bla*, were maintained on LB agar supplemented with ampicillin. Cells were initially grown at 37 °C for at least 16 hours and then stored at 4 °C. Fresh cultures were made every four weeks. Liquid cultures, inoculated with a single colony, were grown in sterilin tubes (Bibby Sterilin Ltd) or conical flasks. These cultures were grown overnight at 37 °C and were shaken continuously (125 rpm in a Gallenkamp illuminated orbital incubator).

2.3.3. Determination of cell density

For protein induction experiments, DH5α cells were grown until a cell density of 2 x 10^8 cells/ml was reached. This was estimated by measuring the absorbance at 600 nm of 1 ml of culture in a spectrophotometer (Unicam UV/Vis) against a blank of LB only. An absorbance of approximately 0.5 demonstrated a cell density of 2 x 10^8 cells/ml.
2.4. NUCLEIC ACID ANALYSIS

2.4.1. Restriction endonuclease digestion

Aliquots of DNA (0.5 – 5 µg) were digested using 10 units of restriction endonuclease per µg of DNA in the presence of an appropriate buffer and, where necessary, 100 µg/ml of BSA. Conditions were determined from the supplier’s instructions. Reactions were incubated at 37 °C (with the exception of Apa I, which was incubated at 25 °C) in a set volume (20 µl – 200 µl; higher concentrations of DNA were digested in a larger volume) for one hour per µg of DNA digested. For digestions with two endonucleases, conditions optimal for both were chosen.

2.4.2. Agarose gel electrophoresis of DNA

DNA fragments were separated by electrophoresis. Loading dye (40% (w/v) glycerol, 0.01% (w/v) SDS, 0.1 M sodium EDTA (pH 8.0 - NaOH) and a few grains of bromophenol blue) was diluted 1 in 5 in samples to be loaded. Samples were then loaded on to 0.6 – 1% (w/v) agarose gels cast and submerged in TAE buffer (0.04 M tris, 1 mM sodium EDTA and 17.5 mM glacial acetic acid). The fluorescent compound, ethidium bromide (Sharp et al., 1973) was also included in the gel mixture at a concentration of 0.5 µg/ml. The DNA fragments were separated from the cathode to the anode at room temperature. The conditions of electrophoresis were a constant voltage of 50 – 75 V provided by a PowerPac 300 (Biorad). Small gels (50 ml) were run in the Hoefer 10 cm cooled minigel apparatus; large gels (400 ml) were run in the Hybaid 30 cm maxigel apparatus. DNA markers of known size were run in all gels (0.5 µg of the 1 kb ladder supplied by MBI Fermentas), allowing an estimation of the size of the test DNA fragments. Separated DNA fragments were visualised at 302 nm on a UV illuminator (UVP Gel
2.4.3. Agarose gel electrophoresis of RNA

RNA (10 µg) was separated by electrophoresis in a 1.2% (w/v) agarose gel under denaturing conditions. The gel was cast in MOPS buffer (20 mM MOPS (pH 8.0 - KOH), 8 mM sodium acetate and 1.5 mM sodium EDTA) and 5% (v/v) of 40% (v/v) formaldehyde. The set gel was submerged in MOPS buffer in the Biorad 25 cm DNA sub cell™. Samples were diluted at a ratio of 8 (sample):6 (loading buffer) with a stock loading buffer (stock: 0.17 µg/ul of EtBr, 11.6% (v/v) of 40% (v/v) formaldehyde, 43% (v/v) formamide, 0.086 mM MOPS, 34 mM sodium acetate and 15 mM sodium EDTA) and incubated at 65 °C for ten minutes. The RNA was separated from cathode to anode for at least 16 hours at room temperature with a constant voltage of 50 V provided by a PowerPac 300 (Biorad). The MOPS buffer was continuously circulated using a 10200 perpex peristaltic pump (LKB, Bromma) for ten minutes before and during electrophoresis. RNA markers of known size were run in all gels (2 µg of a 0.2 – 10 kbp ladder supplied by Sigma), allowing the estimation of the size of the test RNA fragments. RNA molecules were visualised at 302 nm on the UV illuminator and photographed as before. Again, a transparent ruler was placed beside gels to be used for hybridisation experiments, allowing the distance any band of RNA has migrated to be determined from the photographic image.

Care was taken in this protocol to create an RNase free environment. Before use, water and the MOPS buffer were treated by the addition of 0.1 % (v/v) DEPC, incubated at 37 °C and then autoclaved. All other equipment was free of RNase.
2.4.4. Recovery of DNA fragments from agarose gels

DNA fragments were isolated from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN; catalogue number 28704). Manufacturer’s instructions were followed. DNA fragments were eluted in 30 μl of sterile water and stored at −20 °C until required. A 5 μl of the sample was run on an agarose gel to determine the concentration.

2.4.5. Dephosphorylation and ligation of DNA fragments

2.4.5.1. Dephosphorylation

Linearised plasmid DNA was dephosphorylated using calf intestinal alkaline phosphatase (CIAP; New England Biolabs). The reaction was performed in the appropriate buffer supplied with the phosphatase using 0.5 U of CIAP and 10 μl of buffer per μg of DNA. Conditions for dephosphorylation were 37 °C for one hour. Dephosphorylated plasmid DNA was purified from the CIAP by use of the QIAquick PCR purification kit (section 2.4.9).

2.4.5.2. Ligation

Two types of ligation were carried out: Cohesive end ligations and blunt end ligations. For cohesive end ligations, insert (50 ng) and vector DNA molecules were mixed at a 3:1 ratio. They were incubated at 16 °C for a minimum of four hours in a 10 μl solution containing T4 DNA ligase (1 U per μg of DNA; New England Biolabs) and the buffer supplied with the enzyme. For blunt end ligations, insert (100 ng) and vector DNA molecules were mixed at an equal ratio. Conditions for the ligation were the same as for cohesive end ligations except that the reactions were incubated at 37 °C for one hour instead of 16 °C for four hours.
2.4.6. Transformation

2.4.6.1. Transformation of DH5α

DH5α cells were transformed with plasmid DNA using a method derived from Cohen et al. (1972). From a fresh liquid culture of stationary DH5α cells, 100 µl was taken and used to inoculate a 10 ml aliquot of LB. This was then incubated at 37 °C for three hours (approximately $10^8$ cells/ml) and harvested at 6000 g for five minutes at 4 °C in an Eppendorf 5403 centrifuge. Pelleted cells were resuspended in 10 ml of cold (4 °C) 100 mM MgCl₂, incubated on ice for five minutes, and harvested as before. The pellet was then resuspended in 1 ml of cold (4 °C) 100 mM CaCl₂ and incubated on ice for 30 minutes. The appropriate aliquot of plasmid DNA (typically 5 µl of a ligation reaction and 1 µg of uncut parental plasmid) was then added to 100 µl of the CaCl₂ - DH5α mixture. This was incubated on ice for 30 minutes, heat shocked at 42 °C for 1 minute, and then left to cool on ice for 5 minutes. LB (900 µl) was then added and the resulting culture was incubated at 37 °C for one hour with shaking. A 100 µl aliquot of the culture was spread on to ampicillin-containing LB agar plates. For blue/white colony selection, the agar was supplemented with IPTG and X-gal. For transformations with a ligation reaction, the remaining 900 µl was pelleted at 6000 g for two minutes, resuspended in 100 µl of LB and also spread onto LB plates containing ampicillin plates. All plates were incubated at 37 °C for 16 hours and then kept at 4 °C until required.

2.4.6.2. Nuclear transformation of C. reinhardtii

C. reinhardtii cells were transformed with plasmid DNA using the glass bead transformation method derived from Kindle (1990). Cells from a fresh 25 ml culture were inoculated into 400 ml of TAP medium and grown to a density of $1 \times 10^6$ cells/ml. They were then harvested, in 200 ml aliquots, for eight minutes at 921 g, 4
°C in a Sorvall RC-5B refrigerated superspeed centrifuge (GSA rotor). The pelleted cells were then resuspended in 4 ml of TAP medium to a density of 2 x 10^8 cells/ml. A 300 µl aliquot of the cells was then added to 0.3 g of glass beads and 1 µg of plasmid DNA in a pyrex glass tube. The resulting mixture was vortexed vigorously for ten seconds. This was then diluted with 4 ml of molten TAP 0.5 % (w/v) agar (42°C) and poured onto a 2 % (w/v) TAP agar plate. All plates were wrapped in foil and incubated at 25 °C until colonies were observed.

2.4.7. Extraction of nucleic acids

2.4.7.1. Isolation of plasmid and cosmid DNA from DH5α

For small-scale isolation of plasmid DNA from transformed DH5α, two methods were used. An alkali lysis method (derived from Birnboim and Doly, 1979) was used to isolate DNA from cells transformed with ligation reactions. Ampicillin-containing LB (5ml) was inoculated with a single transformed bacterial colony and grown for 16 hours at 37 °C. An aliquot (1.5 ml) was then removed and harvested at 10000 g for 5 minutes in a Biofuge pico centrifuge (Heraeus Instruments). The pellet was resuspended in 100 µl of solution I (50 mM glucose, 25 mM tris (pH 8.0 – HCl) and 50 mM sodium EDTA) and incubated at room temperature for 5 minutes. A 200 µl aliquot of freshly prepared solution II (1 % (w/v) SDS and 0.2 M NaOH) was then added. After a 5 minute incubation at room temperature with occasional shaking, 150 µl of Solution III (2 M glacial acetic acid and 3 M potassium acetate) was added to the sample, which was then incubated on ice for five minutes. The sample was centrifuged at 10000 g for 15 minutes and the supernatant (approximately 400 µl) diluted with an equal volume of 100% (v/v) isopropanol (propan-2-ol). This was spun at 10000 g for 10 minutes and the resultant pellet was then washed in 70 % (v/v) ethanol, spun at 10000 g and then dried in a Rotovac vacuum drier. The pellet was resuspended in 50 µl sterile water.
containing RNase A (0.01 μg/μl). A 2 μl aliquot was used for subsequent digestion reactions. The QIAprep spin miniprep kit (QIAGEN; catalogue number 27106) was used to isolate plasmid DNA from *E. coli* cells only when the DNA was subsequently going to be used for sequencing, PCR and restriction enzyme mapping digestions. Plasmid DNA was extracted according to the manufacturer’s instructions. DNA was eluted in 30 μl of sterile water. A 2 μl aliquot, digested with an endonuclease known to cut the plasmid once, was used to estimate the total amount of DNA isolated (section 2.4.7.4). DNA was stored at -20 °C until required.

Large-scale preparations of both plasmid and cosmid DNA were made using the QIAGEN plasmid Maxiprep kit (QIAGEN; catalogue number 12163) according to the manufacturer’s instructions. Isolated plasmid and cosmid DNA were resuspended in 600 μl of sterile water. DNA concentration was determined as described above. DNA was stored at -20 °C until required.

### 2.4.7.2. Isolation of *C. reinhardtii* DNA

A rapid method derived from Davis *et al.* (1980) was used to isolate *C. reinhardtii* DNA. A 10 ml aliquot of cells grown to a density of 3 x 10⁶ cells/ml was harvested at 6000 g for 5 minutes in an Eppendorf 5403 centrifuge. The pelleted cells were then resuspended in 1ml of TAP and spun at 10000 g for 1 minute in a Biofuge pico centrifuge (Heraeus Instruments). The pellet was resuspended in 0.35 ml of 50 mM sodium EDTA, 20 mM tris (pH 8.0 – HCl) and 0.1 M NaCl. Proteinase K and SDS were then added to a final concentration of 0.3 mg/ml and 1.4 % (w/v) respectively and the sample was incubated at 55 °C. After two hours, 2 μl of DEPC was added to the sample, which was then incubated at 70 °C for 15 minutes. After a brief cooling on ice, potassium acetate was added to a final concentration of 0.6 M. The sample was mixed thoroughly and then incubated on ice for 30 minutes. It was then microfuged at 10000 g for 15 minutes. TE (10 mM tris (pH 8.0 – HCl) and 1 mM sodium EDTA) saturated phenol was added to the supernatant in a ratio of 1:1 and
the resultant mixture was vortexed vigorously. An equivalent volume of chloroform:IAA (24:1 (v/v) respectively) was then added. The sample was vortexed as before and then centrifuged at 10000 g for five minutes. The phenol/chloroform:IAA steps were repeated a further two times with the upper, aqueous phase formed after centrifugation. The upper aqueous phase was then diluted with 1.5 ml of 95 % (v/v) ethanol and centrifuged at 10000 g for ten minutes. The DNA pellet formed was washed with 70 % (v/v) ethanol as before (section 2.4.7.1) and resuspended in 50 μl of sterile water containing RNase A (0.01 μg/μl). A 2 μl aliquot was used to give an estimation of the total amount of DNA isolated in the protocol (section 2.4.7.4). DNA was stored at −20 °C until required.

2.4.7.3. Isolation of *C. reinhardtii* total RNA and mRNA

Total RNA was extracted using a method adapted from Goldschmidt-Clermont (1990). A 10 ml aliquot of cells grown to a density of 1 x 10⁶ cells/ml was harvested at 6000 g for five minutes in an Eppendorf 5403 centrifuge. The pelleted cells were resuspended in 0.6 ml of 0.2 M tris (pH 8.0 - HCl), 0.5 M NaCl, 0.01 M sodium EDTA and 0.2 % (w/v) SDS and freeze-thawed three times using liquid nitrogen and a 37 °C water bath. The sample was then diluted with 0.6 ml of TE saturated phenol:chloroform:IAA (25:24:1 (v/v) respectively) and centrifuged at 10000 g for 2 minutes in a Biofuge pico centrifuge (Heraeus Instruments). The upper aqueous phase was then diluted in 1.4 ml of 95 % (v/v) ethanol and centrifuged again at 10000 g for 10 minutes. The pellet was washed with 70 % ethanol (section 2.4.7.1) and resuspended in 50 μl of DEPC treated (section 2.4.3) sterile water. A 5 μl aliquot was used to determine the total amount of RNA isolated using the protocol. RNA was stored at −70 °C until required.

mRNA was extracted using the poly(A) pure mRNA purification kit (Ambion, catalogue number 1915) according to the manufacturer’s ‘basic protocol for cells’ instructions. The pelleted mRNA was resuspended in 50 μl of DEPC (section 2.4.3)
treated sterile water. A 4 µl aliquot was used for RT-PCR (section 2.4.10). RNA was stored at −70 °C until required. For both protocols, RNase contamination was reduced to a minimum.

### 2.4.7.4. Determination of extracted DNA and RNA concentration

The concentration of extracted DNA was determined by comparing its intensity under UV light (UVP Gel Documentation System) to that of a known concentration of DNA (marker) after separation by electrophoresis. A 2.5 µg aliquot of Lambda (λ) DNA cut with HindIII was used to provide a range of known concentrations DNA fragments. This forms eight bands of different sizes and intensities (Table 2.2). Bands of similar intensity were judged to contain a similar amount of DNA.

<table>
<thead>
<tr>
<th>Fragment size (bp)</th>
<th>Fragment conc. (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23130</td>
<td>1192</td>
</tr>
<tr>
<td>9416</td>
<td>484</td>
</tr>
<tr>
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</tr>
<tr>
<td>2027</td>
<td>104</td>
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<tr>
<td>564</td>
<td>29</td>
</tr>
<tr>
<td>125</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2.2. Markers used for determination of DNA concentration

The size (bp) and concentration (ng) of the fragments produced when a 2.5 µg aliquot of Lambda (λ) DNA is cut with HindIII.

The concentration of total RNA isolated was determined using a spectrophotometer (Unicam UV/Vis). A 5 µl aliquot of RNA was diluted in 995 µl of sterile water and
its absorbance measured at 260 nm against a sterile water blank. From the absorbance value, the amount of RNA (mg/ml) present in sample was determined using the following calculation: $A_{260} \times \text{dilution factor} \times 40$.

2.4.8. Hybridisation of nucleic acids

2.4.8.1. Transfer of nucleic acids from agarose gels to nylon membranes

DNA fragments separated by gel electrophoresis were transferred onto nylon membrane (Hybond-N\textsuperscript{+} - Amersham Pharmacia Biotech) using a protocol derived from the capillary transfer method (termed the Southern blot) described by Southern (1975). The photographed gel was soaked at room temperature in 1.5 M NaCl and 0.5 M NaOH (denaturing solution) for 30 minutes and then rinsed in deionised water. It was then soaked in 1 M tris (pH 7.4 – HCl) and 1.5 M NaCl (neutralising solution) for 30 minutes at room temperature. This solution was changed once (after 15 minutes) during the incubation. DNA was then transferred from the gel onto the nylon membrane using standard southern blot apparatus (fig. 2.1). After at least 16 hours, the nylon membrane was sealed in 3 MM Whatman paper and baked at 80 °C for two hours.

RNA molecules separated by gel electrophoresis were transferred onto nylon membranes and baked in the same way as DNA fragments except gels were not incubated in denaturing and neutralising solution, but were directly set up in the blotting apparatus (fig 2.1). The transfer of RNA from agarose gels to nylon membranes is termed northern blotting.
Figure 2.1. The Southern blot

The transfer buffer (20 x SSC) is drawn from the reservoir through the gel, nylon membrane and 3 MM whatman to the paper towels by capillary action. This causes the transfer of DNA (RNA) from the gel to the membrane. The weight ensures contact between all materials during transfer. Based on a drawing from Sambrook et al. (1989).

2.4.8.2. Radiolabelling of DNA probes

DNA fragments for use as probes were radiolabelled using the Prime-It II Random Primer Labelling Kit (Stratagene, catalogue number 300383) according to the manufacturer’s instructions. Radiolabelled [α-32P]dCTP (Amersham International) was used as the labelling nucleotide. Radiolabelled double stranded products were denatured by incubating the sample in a boiling water bath for five minutes before use.
2.4.8.3. Hybridisation of DNA probes to membrane bound nucleic acids

The baked nylon membrane was placed in 5 x SSC (0.6 M NaCl and 0.06 M sodium citrate; pH 7.0 - NaOH). Once wet, it was sandwiched between two sheets of nylon mesh and placed in to a Hybaid hybridisation bottle. It was then incubated in 20 ml of Prehybridisation solution (0.1 % (w/v) Ficoll (type 400), 0.1 % (w/v) PVP, 0.1 % (w/v) BSA, 0.5 % (w/v) SDS, 5 x SSC and 100 μg/ml salmon sperm) at 65 °C in a Hybaid dual hybridisation oven. After four hours, the prehybridisation solution was replaced with 20 ml of fresh prewarmed (65 °C) hybridisation solution. This consisted of prehybridisation solution plus the denatured radiolabelled probe (section 2.4.8.2). The nylon membrane was incubated in this solution for 16 hours at 65 °C. It was then washed for 30 minutes at room temperature in 0.1 % (w/v) SDS and 2 x SSC (0.24 M NaCl and 24 mM sodium citrate; pH 7.0 - NaOH). It was then washed for a further 30 minutes in 0.1 % (w/v) SDS and 0.1 x (v/v) SSC (12 mM NaCl and 1.2 mM sodium citrate; pH 7.0 - NaOH) at 65 °C. The membrane was then sealed in Saran wrap (DOW Chemical Co) and exposed to a Biomax MS-1 X-ray film (Kodak, catalogue number z36, 304 - 9) in a cassette containing an intensifying screen (Genetic Research Instruments) at -70 °C until the image was detectable. Films were developed using the Compact X2 automatic film processor with XO developer and fixer.

2.4.9. The polymerase chain reaction (PCR)

The protocol used to amplify DNA fragments by PCR was derived from the method described by Mullis and Faloona (1987). Reaction mixtures contained 200 μM dATP, 200 μM dTTP, 200 μM dGTP, 200 μM dCTP, 1 μM of each oligonucleotide primer (forward and reverse), 2.5 U of Vent polymerase (New England Biolabs), 1 x Vent buffer (New England Biolabs), 1 μg of genomic DNA or 10 ng of plasmid DNA (template) and sterile water. Water was added to increase the total volume of the mixture to 100 μl. The DNA region of interest was then amplified by PCR in a
Genius Techne thermocycler. Reaction mixtures were incubated at 95 °C for 5 minutes. They were then cycled through 35 (genomic DNA) or 15 (plasmid DNA) rounds of amplification. Each round of amplification included a 95 °C incubation for 1 minute, a 1 minute incubation at a temperature 2 °C below the lower Tm value of the two primers and a 2 minute incubation at 72 °C. The 72 °C incubation step was extended to 6 minutes in the final cycle of amplification. A 10 µl aliquot of each completed PCR reaction was run on an agarose gel to check PCR was successful. If it was, the completed reaction was then cleaned up using the QIAquick PCR purification kit according to the manufacturer’s instructions (QIAGEN; catalogue number 28104). DNA was eluted in 30 µl of sterile water and stored at −20 °C until required. A 5 µl aliquot was run on an agarose gel to determine the concentration of the sample.

2.4.10. Reverse Transcription of RNA (RT-PCR)

Reverse transcription (RT) of mRNA isolated from *C. reinhardtii* cells was performed using the display THERMO-RT™ kit (Version 1.4: Display Systems Biotech) according to the manufacturer’s ‘reverse transcription of difficult RNA’ instructions. P2.2 (fig 4.9) was used as the primer for the cDNA synthesis. PCR of the newly synthesised cDNA strands was performed using a 5 µl aliquot of the reverse transcription reaction as the template and P1 (fig 4.9) and p2.2 as the forward and reverse primers respectively (section 2.4.9). The cDNA was cycled through 35 rounds of amplification.

2.4.11. Sequencing

For sequencing, 500 ng of plasmid DNA or 100 ng of a PCR product and 3.2 µM of primers were used. DNA was prepared for sequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems,
catalogue number 4303152) according to the manufacturer’s instructions and was sequenced using an ABI PRISM 377 DNA sequencer (Applied Biosystems).

2.5. PROTEIN ANALYSIS

2.5.1. Extraction of protein from *C. reinhardtii* cells

2.5.1.1. Extraction of crude whole cell protein

From a 25 ml culture of *C. reinhardtii* cells grown to a density of approximately 1 x 10^7 cells/ml, the absorbance at 750 nm of 1 ml of the cells was measured using a spectrophotometer (Unicam UV/Vis). The cells (15ml) were then harvested at 6000 g for five minutes at 4 °C in an Eppendorf 5403 centrifuge and the pellet resuspended in resuspension solution consisting of 0.8 M tris (pH 8.4 – HCl), 0.2 M sorbitol and 1% (v/v) β-mercaptoethanol. The volume of resuspension solution added to the pellet was relative to the previously determined absorbance reading *i.e.* for an absorbance value of 1.48, 1.48 ml of resuspension solution was added to the pellet. To 50 and 100 μl aliquots of the sample, SDS was added to a final concentration of 1% (w/v). These aliquots were heated in a boiling water bath for two minutes and loaded onto a SDS – polyacrylamide gel (section 2.5.3). Unused sample was stored at –70 °C until required. Crude whole cell protein was extracted from *Dunaliella salina* by the same method.

2.5.1.2. Extraction of mitochondrial proteins

Mitochondria were isolated from *C. reinhardtii* cells using a method derived from Nurani and Franzén (1996). A culture of 6 l was grown to a cell density of approximately 1 x 10^7 cells/ml and then harvested in a Millipore cell harvester using a 0.22 μM filter at no more than 3 psi. The concentrated cell culture was then further harvested, in 250 ml aliquots, at 10242 g for 10 minutes at 4 °C in a Sorvall
RC-5B (GSA rotor) refrigerated superspeed centrifuge (unless otherwise stated, this centrifuge was used for all subsequent spins). The cell pellet formed was then resuspended in a small volume of preparation buffer 1 (0.25 M sucrose, 50 mM MOPS (pH 7.4 – KOH), 5 mM sodium EDTA, 10 mM cysteine, 0.5 mM PMSF and 0.6% (w/v) BSA) at room temperature. It was then rapidly diluted in 75 ml of ice-cold preparation buffer 1 and loaded into a French press or a cell disrupter (Constant Systems) where the intact cells were broken at 4000 psi. The sample was then diluted in an equal volume of ice-cold preparation buffer 2 (preparation buffer 1 + 1.2% (w/v) PVP-40) and centrifuged at 480 g for 3 minutes at 4 °C. The supernatant was centrifuged at 3000 g for 4 minutes at 4 °C and then at 13300 g for 7 minutes at 4 °C to pellet crude mitochondria. The pellet was resuspended in 28ml of preparation buffer 3 (0.25 mM sucrose, 10 mM MOPS (pH 7.2 – KOH), 1 mM sodium EDTA, 0.2% (w/v) BSA and 32% (v/v) percoll) and centrifuged at 37000 g, 4 °C for 50 minutes in a Sorvall ultracentrifuge OTD65B (rotor - T865). The mitochondrial band (located towards the bottom of the tube) was isolated. This was diluted 10 times with wash solution (0.25 M sucrose, 25 mM MOPS (pH 7.2 – KOH), 1 mM sodium EDTA and 0.1 % (w/v) BSA) and centrifuged at 12000 g for 10 minutes at 4 °C. The final pellet was resuspended in 0.5 ml of wash solution. It was stored at –70 °C until required. A 1 µl aliquot was used to determine the amount of mitochondrial protein isolated in the preparation (section 2.5.2).

Mitochondrial protein extracts to be loaded on to SDS – polyacrylamide gels were prepared by diluting 50 µg of isolated mitochondria with an equal volume of 5 x Laemmli gel loading buffer (0.3 M tris (pH 6.8 – HCl), 50% (w/v) glycerol, 10% (w/v) SDS, 25 % (v/v) β-mercaptoethanol and a few grains of bromphenol blue), heating in a boiling water bath for 5 minutes and then centrifuging at 10000 g for 10 minutes in a Biofuge pico centrifuge (Heraeus instruments). The supernatant was loaded on to the gel (section 2.5.3).
Mitochondrial protein extracts to be loaded (1–5 µg for immunolabelling and 50 µg for in situ enzyme activity staining) on BN - polyacrylamide gels were prepared using a method derived from Schägger et al. (1996). A 15 µl aliquot of protein solubilising solution (1 M 6-aminocaproic acid, 50 mM bistris (pH 7.0 - HCl), 1 mM PMSF, 1 µg/ml Leupeptin and 1 µg/ml Pepstatin) was mixed with the isolated mitochondria. The sample was then solubilised with 5 µl of 10% (w/v) n-dodecyl-β-D-maltoside, incubated on ice for 15 minutes and centrifuged at 10000 g for 20 minutes at 4 °C in a Biofuge pico centrifuge (Heraeus instruments). The supernatant was then mixed with 2.5 µl of loading buffer (1 M 6-aminocaproic acid and 5% (w/v) Serva blue G) and loaded on the gel (section 2.5.4).

2.5.2. Determination of protein concentration

Protein concentration of samples was determined by the Bradford protein assay (Bradford, 1976). Aliquots (1 µl; mitochondrial membrane protein or 10 µl; purified MBP-M90 fusion protein) were diluted in 1 ml of Biorad solution (200 µl Biorad reagent (Biorad; catalogue number 5000006) + 800 µl water). The absorbance at 595 nm was measured against a blank of water plus reagent and protein concentration determined using a BSA standard (fig 2.2).

![Figure 2.2. The BSA standard curve.](image)
The standard curve was obtained for BSA as described above.
2.5.3. Denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was based on the method of Laemmli (1970). The denaturing SDS-PAGE system consisted of a resolving gel (10 or 15%) to separate proteins and a stacking gel (3.5%) to concentrate proteins prior to entering the resolving gel. The resolving gel consisted of 12.5% (v/v) of 3M tris (pH 8.8 - HCl), 25% (v/v) or 37.5% (v/v) of 40% (w/v) acrylogel 2.6 solution (B.D.H.) for a 10 or 15% acrylamide gel respectively, 1% (v/v) of 10% SDS, 0.016% (v/v) TEMED and 0.75% (v/v) of 10% (w/v) AMPS. For large gels, 32 ml of the resolving gel mixture was made. This was used to produce the gel for use in the 20 x 20 cm slab gel apparatus (Biorad). For small gels, 5 ml of the resolving gel mixture was made. A 3.55 ml aliquot of this was used to produce the gel for use in the mini-PROTEAN II electrophoresis apparatus (Biorad). Once poured, the resolving gel was overlaid with water-saturated butan-2-ol. When the gel had set (after at least 45 minutes), this layer was removed and the surface of the gel was rinsed with deionised water.

The stacking gel consisted of 25% (v/v) of 0.5M tris (pH 6.8 – HCl), 8.75% (v/v) of 40% (w/v) acrylogel 2.6 solution, 1% (v/v) of 10% (w/v) SDS, 0.1% (v/v) of TEMED and 0.5% (v/v) of 10% (w/v) AMPS in a total volume of 20 ml for large gels and 2 ml for small gels. The gel mixture was pipetted on to the set resolving gel and a comb (15 wells for large gels and 10 wells for small gels) placed in it. Once set, the comb was removed and gel was set up in the electrophoresis apparatus. Samples were then loaded into the gel and proteins were separated from cathode to anode at room temperature in running buffer (25 mM tris (pH 8.3 – HCl), 50 mM glycine and 0.1% (w/v) SDS). The conditions for electrophoresis were a constant voltage of 50 V for large gels and 200 V for small gels. The voltage was supplied by a PowerPac 300 (Biorad). Protein markers of known size were run in all gels (20
μl of 10000 to 25000 Da rainbow recombinant protein molecular weight markers; Amersham).

The gels were then either stained in a Coomassie Brilliant Blue R solution (3 mM Coomassie brilliant blue R, 50 % (v/v) of 100% (v/v) methanol and 10% (v/v) glacial acetic acid), to visualise the proteins (large gels), or used for western blotting (small gels; section 2.5.6). For Coomassie staining, gels were soaked in the coomassie solution for one hour at room temperature and continuously shaken. They were then washed in destaining solution (40% (v/v) of 100% (v/v) methanol and 10% (v/v) glacial acetic acid), under constant agitation, until only the protein bands were stained blue.

2.5.4. Blue Native polyacrylamide gel electrophoresis

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was based on the method of Schägger et al. (1995). The blue native system consisted of a resolving gradient gel (8 – 16%) to separate enzyme complexes, a stacking gel (4%) to concentrate complexes prior to separation and a 0.02% (w/v) Serva blue G cathode buffer to give protein complexes an overall negative charge during electrophoresis.

The 8% resolving gel mixture consisted of 16.7% (v/v) of 48% (w/v) acrylamide + 3 % (w/v) bis-acrylamide, 33.3% (v/v) of 1.5 M 6-aminocaproic acid + 150 mM bistris (pH 7.0 - HCl), 0.6% (v/v) of 10% (w/v) AMPS and 0.06% (v/v) TEMED. The 16% resolving gel mixture consisted of 31.7% (v/v) of 48% (w/v) acrylamide + 3 % (w/v) bis-acrylamide, 32.2% (v/v) of 1.5 M 6-aminocaproic acid + 150 mM bistris (pH 7.0 - HCl), 19.3% (v/v) glycerol, 0.3% (v/v) of 10% (w/v) AMPS and 0.03% (v/v) TEMED. The resolving gradient gel was prepared by mixing 7 ml of an 8% resolving gel mixture with 7 ml of a 16% resolving gel mixture in gradient gel pouring apparatus (Biorad). This gel mixture was used to produce the gel for use in the mini-PROTEAN II electrophoresis apparatus (Biorad). Once poured, the gel
was overlaid with water-saturated butan-2-ol. When the gel had set this layer was removed and the surface of the gel was rinsed with deionised water.

The stacking gel consisted of 8.3% (v/v) of 48% (w/v) acrylamide + 3% (w/v) bis-acrylamide, 33% (v/v) of 1.5 M 6-aminocaproic acid + 150 mM bistris (pH 7.0 – HCl), 0.8% (v/v) of 10% (w/v) AMPS and 0.08% (v/v) TEMED in a total volume of 6 ml. A 2 ml aliquot of this was pipetted on to the set resolving gradient gel and a 10 well comb placed in it. Once set, the comb was removed and the gel was set up in the electrophoresis apparatus, ready for sample loading.

Protein complexes were separated from cathode to anode at room temperature initially in 0.02% serva blue G cathode buffer (50 mM tricine, 15 mM bistris (pH 7.0 – HCl) and 0.02% serva blue G) and anode buffer (50 mM bistris (pH 7.0 – HCl)). The cathode buffer was replaced with 0.002% serva blue G buffer (as before but with 0.002% serva blue G) once the gel was half run. The conditions for electrophoresis were a constant voltage of 200 V supplied by a Powerpac 300 (Biorad). After separation, the gel was used for *in situ* enzyme staining or for western blotting.

### 2.5.5. *In situ* enzyme staining

To stain for the presence of complex IV in mitochondrial proteins separated by BN-PAGE, one lane containing the separated proteins was removed from a freshly run gel. This was destained for a few minutes in 50 mM bistris (pH 7.0 – HCl) and rinsed in deionised water. It was then incubated for one hour at 37 °C in the complex IV staining solution (0.05M sodium phosphate (pH 7.4), 0.05% (w/v) 3, 3’, 4, 4’-Tetraaminobiphenyl, 0.1% (w/v) cytochrome c and 2 μg/ml catalase). Water was added to stop the staining reaction and the gel was photographed.
2.5.6. Western blotting

Proteins separated by electrophoresis were transferred on to PVDF membranes (Immobilon-PSQ transfer membrane; MILLIPORE) using a method derived from Towbin et al. (1979). Proteins separated in denaturing gels were transferred using a Trans-Blot SD semi-dry electrophoretic transfer cell according to the manufacturer’s instructions (Biorad). Before transfer, gels and the 3 MM Whatman (six pieces per blot) were equilibrated in the transfer buffer (25 mM tris (pH 8.3 – HCl) and 193 mM glycine) for 20 minutes and the PVDF membrane was soaked in 100% (v/v) methanol for 5 minutes. The conditions for transfer were a constant voltage of 15 V supplied by a Powerpac 300 (Biorad) at room temperature. The duration of transfer was determined using the following equation: Area of gel cm² x 0.37 = minutes required for transfer. After transfer, the PVDF membrane was used immediately for immuno-detection (section 2.5.6).

Proteins separated in blue native gels were transferred using the mini trans-blot electrophoresis transfer cell equipment according to the manufacturer’s instructions (Biorad) in Towbin’s transfer buffer (25 mM tris, 192 mM glycine and 20% (v/v) methanol) at room temperature. Before transfer, gels were equilibrated in the transfer buffer for at least 10 minutes. The PVDF membrane was wet in methanol before equilibrating in transfer buffer. The conditions for transfer were a constant voltage of 100 V supplied by a Powerpac 300 (Biorad) for 1 hour. After transfer, the PVDF membrane was allowed to dry at room temperature. The ten protein lanes were then cut into individual strips, rinsed in methanol to remove the Serva blue G dye and used for immuno-detection (section 2.5.7).
2.5.7. Immuno-detection

Proteins were detected on PVDF membranes using the ECL detection kit according to the manufacturer’s instructions (Amersham LIFE SCIENCE; product RPN 2209). The primary antibodies were diluted 1:2000 (rabbit anti-M90, polyclonal), 1:2000 (mouse anti-COX III, monoclonal) and 1:5000 (mouse anti-COX I, monoclonal). The secondary antibodies (anti-rabbit Ig: horseradish peroxidase linked whole antibody (Amersham LIFE SCIENCE: product NA 934) and anti-mouse Ig: horseradish peroxidase linked whole antibody (J.-W. Taanman: royal free hospital) were both diluted 1:3000 for probing of enzyme complexes. The anti-rabbit Ig was diluted 1:10000 for probing denatured proteins. Low fat powdered milk was used as the blocking reagent.

2.5.8. Protein expression and purification

The pMAL expression system was used to express and purify the M90 protein on both a small (1 µg) and large scale (10 µg) according to the manufacturer’s instructions for the pMAL-c2 vector (New England Biolabs; catalogue number 800).

The amount of protein isolated in each eluted fraction during the large-scale preparation of the MBP-M90 fusion protein was determined using the Bradford assay (section 2.5.2). The fractions were then pooled and concentrated to give a final concentration of 1 mg/ml using a concentrating column (ym50; Amicon) in the mistral 3000i centrifuge (Sanyo).
Chapter two

2.5.9. Antibody production and optimisation

Polyclonal antibodies to the MBP-M90 fusion protein were raised in two rabbits (SK847 and SK848) by Eurogentec. Eight 200 µg samples of the MBP-M90 fusion protein were lyophilised at 10⁻¹ atmospheres in a Modulyo freeze dryer (Edwards) at -40 °C and sent to Eurogentec. Each of the rabbits was inoculated with one 200 µg aliquot of the protein (day 0) which was resuspended in 500 µl of 0.95% (w/v) NaCl and 500 µl of adjuvant. The rabbits received three subsequent booster jabs of 200 µg of resuspended protein on days 14, 28 and 56. Blood was taken from the rabbits on day 0 (preimmune - 2 ml), 38 (bleed 1 - 2 ml), 66 (bleed 2 - 20 ml + 2 ml) and 80 (final bleed - 20 ml + 2 ml) and the serum isolated and sent to our laboratory. Sodium azide was added to the 2 ml aliquots of the preimmune and final bleed serum to a final concentration of 0.01% (v/v). These serum samples were then stored at 4 °C and were used for all subsequent analysis of the M90 protein. The remaining serum was stored at -70 °C.

To check antibodies had been successfully raised to the MBP-M90 protein and to optimise the antibody concentration required for subsequent western analysis of the M90 protein, dot blotting was first performed. Aliquots of 1 µl of 20 µg/µl and 200 µg/µl MBP-M90 fusion protein were spotted on to 8 strips of PVDF membrane and allowed to dry. These strips were then probed with the serum raised against the MBP-M90 fusion protein (fig 2.3) according to the manufacturer's instructions in the ECL detection kit (section 2.5.7). Four of the strips were incubated with serum isolated from rabbit SK847 (preimmune; 1:1000 and 1:1500 and final bleed; 1:1000 and 1:1500) and the other four incubated with serum isolated from rabbit SK848 (same as SK847). The secondary antibody (anti-rabbit Ig: horseradish peroxidase linked whole antibody) was used at 1:1000 for probing.
Figure 2.3. Optimisation of antibodies for use in western analysis (1)

Aliquots of the MBP-M90 fusion protein (20 ng/μl – upper spots and 200 ng/μl – lower spots) were spotted on to PVDF membrane. These were probed with preimmune serum at a dilution 1:1000 (a) and 1:1500 (b) and final bleed serum at a dilution of 1:1000 (c) and 1:1500 (d) isolated from rabbits SK847 and SK848. The secondary antibody (anti-rabbit Ig: horseradish peroxidase linked whole antibody) was used at a dilution of 1:1000.

The detection of the MBP-M90 fusion protein when probing with final bleed serum and a very reduced signal when probing with preimmune serum from rabbits SK827 and SK848 demonstrated antibodies had been successfully raised to the protein in both rabbits. However, the high background produced after detection with the final bleed serum indicated the concentration of either or both the primary and secondary antibodies were too high. A further optimisation experiment was therefore performed using more dilute samples of antibody for probing. Serum from the final bleed of rabbit SK848 was used for this (and all other subsequent probing experiments).

Six aliquots of 5 μg of the MBP-M90 fusion protein were diluted with an equal volume of 5 x Laemmli gel loading buffer (section 2.5.1.2) and run in a denaturing gel (2.5.3). The protein samples were then transferred on to PVDF membrane by
western blotting (section 2.5.6) and the protein lanes cut in to strips. Each strip was then probed with a different combination of primary and secondary antibody. The MBP-M90 fusion protein was detected using all combinations of the antibodies. However, detection using 1:2000 primary antibody and 1:10000 secondary antibody produced the least background (fig 2.4). Antibodies were therefore used at these dilutions in subsequent experiments.

![Image of western blot with annotations](image)

**Figure 2.4. Optimisation of antibodies for use in western analysis (2)**

The figure shows the probing of an aliquot of the MBP-M90 fusion protein (5μg), which has been separated by SDS-PAGE and transferred to a PVDF membrane, with the primary antibody SK848 (final bleed) at a dilution of 1:2000 and the secondary antibody (anti-rabbit Ig: horseradish peroxidase linked whole antibody) at a dilution of 1:10000. The primary antibody binds the MBP-M90 fusion protein and the degradation product of the unstable fusion protein, MBP. Little background is present indicating the concentration of the antibodies are ideal for probing.

### 2.6. GENETIC ANALYSIS OF *C. reinhardtii*

*C. reinhardtii* cells were mated and their progeny analysed using methods derived from Levine and Ebersold (1960) and Harris (1989). Cultures for mating were restreaked three times at three-day intervals on to fresh 2% (w/v) TAP agar plates.
The cells were then restreaked on to 2% (w/v) TAP agar plates containing 1/10th the normal nitrogen concentration and grown for three days at 25 °C under a photon flux of 45 μE/m²/s. A loopful of each culture was then suspended in 2 ml of 10 mM sodium phosphate buffer (pH 7.4) and incubated at room temperature in the light. After two hours, 1 ml of the opposite mating type cultures were mixed. This culture was incubated at 25 °C under a photon flux of 45 μE/m²/s for three hours. Four 200 μl aliquots of mated cells (zygotes) were then removed from the culture and spotted on to a TAP 3% (w/v) top grade agar plate. This was incubated in the light (photon flux of 45 μE/m²/s) for 16 hours at 25 °C and then later in the dark for seven days at 25 °C to allow zygote maturation. Zygotes were then transferred on to six TAP 2% (w/v) washed agar plates and separated. The plates were held over chloroform for 30 seconds to kill unmated cells and then incubated at 25 °C under a photon flux of 45 μE/m²/s for 16 hours to allow zygote germination. The four haploid progeny of the zygotes were then separated and the plate incubated as before until colonies were visible. For spot tests, a 25 ml starter culture of the progeny cells was grown to a cell density of 1 x 10⁷ cells/ml. Aliquots of 5 μl were then spotted on to the appropriate agar plate.

2.7. GROWTH CURVES

Aliquots of 2 ml were taken from a 25 ml starter culture grown to a cell density of 1-2 x 10⁷ cells/ml and inoculated into 200 ml of either TAP or HSM medium. Cultures were then incubated at 25 °C and continuously shaken. If light was required, the cells were incubated under a photon flux of 80 μE/m²/s. Otherwise cultures were wrapped in foil. Twice daily, the number cells present in each culture was measured using a haemocytometer (section 2.2.3).
2.8. RESPIRATORY ENZYME ASSAYS OF C. REINHARDTII

2.8.1. Measurement of classical and alternative respiration

Measurement of classical and alternative respiration in C. reinhardtii was based on a method derived from Dorthu et al. (1992). A 20 ml culture of cells grown to a density of approximately $1 \times 10^7$ cells/ml was harvested at 6000 g for 5 minutes in an Eppendorf 5403 centrifuge and the pellet resuspended in 1 ml of TAP medium (section 2.7.3). A 200 μl aliquot of the concentrated cells was then diluted in 2 ml of TAP medium and its oxygen consumption measured using a Clark oxygen electrode (Rank, Bottisham) at room temperature for a duration of 15 minutes. To measure classical respiration, octyl gallate (final concentration 50 μM) and then potassium cyanide (final concentration 5 mM) were added to the cells after 5 minutes and 10 minutes respectively. To measure alternative respiration, the order of the addition of the inhibitors was reversed. The respiratory rate of the cells in the presence and absence of the inhibitors was expressed as μmol O$_2$/mg chlorophyll/h. A 10 μl aliquot of the concentrated cell sample was used to determine chlorophyll concentration (section 2.8.3).

2.8.2. Measurement of complex I, III and IV activity

Complex I, III and IV enzyme activities were assayed using whole cell homogenates in either a Sigma ZWSII dual-wavelength spectrophotometer or a Clark oxygen electrode (Rank, Bottisham). All assays were performed at room temperature.

2.8.2.1. Preparation of the cell homogenate

A 20 ml culture of cells grown to a density of $1 \times 10^7$ cells/ml was harvested at 6000 g for 5 minutes in an Eppendorf 5403 centrifuge. The pellet of cells was then resuspended in 1 ml of 3 mM phosphate (pH 7.4), 0.1 % (w/v) BSA and 0.5 mM
PMSF and sonicated for 3 x 30 seconds to lyse the intact cells. The homogenate was then stored on ice until required. A 10 μl aliquot was used to determine the amount of chlorophyll in the sample (section 2.8.3).

2.8.2.2. Complex I activity assay

Complex I activity was measured by assaying for its ability to transfer electrons from NADH to decyl ubiquinone.

\[ \text{NADH} + \text{complex I} + \text{decal ubiquinone} \rightarrow \text{NAD}^+ + \text{complex I} + \text{decal ubiquinol} \]

A 50 μl aliquot of the cell homogenate was diluted in to 2 ml of a solution containing 50 mM phosphate buffer (pH 7.4), 1 mM potassium cyanide, 50 μM octyl gallate and 100 μM NADH. NADH oxidation was initiated by the addition of decyl ubiquinone to a final concentration of 10 μM to the mix and was followed at 340 nm minus 380 nm (340 nm - NAD$^+$ and 380 nm - NADH) in the spectrophotometer. To confirm NADH oxidation was due to the activity of complex I, the assay was repeated in the presence of 20 μM of the complex I inhibitor, rotenone. Complex I activity was expressed as μmol NADH oxidised/mg chlorophyll/min.

2.8.2.3. Complex III activity assay

Complex III activity was measured by assaying for its ability to transfer electrons from decyl ubiquinol to oxidised cytochrome c.

\[ \text{decal ubiquinol} + \text{complex III} + \text{cyt c}^{\text{ox}} \rightarrow \text{decal ubiquinone} + \text{complex III} + \text{cyt c}^{\text{red}} \]

A 50 μl aliquot of the cell homogenate was diluted in to a 2 ml solution containing 50 mM phosphate buffer (pH 7.4), 50 μM octyl gallate, 1 mM potassium cyanide,
0.01% (w/v) dodecyl maltoside and 30 μM oxidised cytochrome c. The inhibitors potassium cyanide and octyl gallate were added to the assay to block complex IV and alternative oxidase activities respectively. Cytochrome c reduction was initiated by the addition of decyl ubiquinol to a final concentration of 60 μM and was followed at 550 nm minus 542 nm in the spectrophotometer. To confirm cytochrome c reduction was due to the activity the complex III, the assay was repeated in the presence of 2.5 μM of the complex III inhibitor, MOA stilbene (Glynn Laboratories of Bioenergetics). Complex III activity was expressed as μmol cytochrome c reduced/mg chlorophyll/min.

2.8.2.4. Complex IV activity assays

Complex IV activity was measured by assaying for its ability to transfer electrons from reduced cytochrome c to oxygen. This process was followed using two different methods.

\[
\text{cyt } c^{\text{red}} + \text{complex IV} + O_2 + 2\text{H} \rightarrow \text{cyt } c^{\text{ox}} + \text{complex IV} + \text{H}_2\text{O}
\]

The first method used cytochrome c oxidation as a measure of complex IV activity. For this, the same assay as that used to measure complex III activity was performed (section 2.7.2.3) except that potassium cyanide, the complex IV inhibitor, was omitted from the initial reaction mixture. The re-oxidation of cytochrome c after reduction by complex III was used to demonstrate complex IV activity.

The second method used oxygen consumption as a measure of complex IV activity. A 50 μl aliquot of the cell homogenate was diluted in to a 2 ml solution containing 0.1 M tris (pH 7.2 - HCl) and 0.002% (v/v) triton X-100 in a Clark oxygen electrode. Sodium ascorbate, TMPD and cytochrome c were then added to a final concentration of 10 mM, 50 μM and 20 μM respectively and the oxygen consumption of the cell homogenate was measured.
2.8.3. Determination of chlorophyll concentration

A 10 µl aliquot of cell sample was diluted in 1 ml of 80% (v/v) acetone, vortexed vigorously and then centrifuged at 10000 g for 2 minutes in a Biofuge pico centrifuge (Heraeus Instruments). The absorbance at 653 nm of the supernatant was then measured against a blank of 80% (v/v) acetone. Chlorophyll concentration (mg/ml) was determined using the following calculation: $A_{653} \times 27.8 \times$ dilution factor.

2.9. REDUCED MINUS OXIDISED HAEM SPECTRA

An aliquot of the mitochondrial membranes (160 µg) prepared in section 2.5.1.2. was diluted to a final volume of 500 µl with 0.25 M sucrose, 25 mM MOPS (pH 7.2 - KOH), 1 mM EDTA and 0.1% (w/v) BSA. A few grains of the oxidant potassium ferricyanide was added to the sample and its absorption spectra at 500 nm to 650 nm was then measured using an in-house built single beam spectrophotometer. A few grains of the reductant sodium dithionite was then added to the sample and its new absorption spectra was measured as before. Reduced minus oxidised haem spectra were then plotted.

2.10. MATERIALS

Restriction enzymes and their respective buffers were obtained from New England Biolabs, Stratagene and Promega. These were stored at −20 °C until required. All other reagents were obtained from Sigma Chemicals Co and BDH unless stated otherwise in the text. The equipment and kits used are referenced in the text.
CHAPTER THREE

GENETIC AND BIOCHEMICAL ANALYSIS OF
M86 AND M90

3.1. INTRODUCTION

The biogenesis of the respiratory chain requires genetic contribution from both the nuclear and mitochondrial genomes (section 1.3). Both genomes encode subunits of the respiratory enzymes. In addition, the nuclear genome codes for proteins that aid in the expression of mitochondrial genes and the assembly of the respiratory enzyme complexes.

In *C. reinhardtii*, all the subunits encoded by the mitochondrial genome have been identified and the genes sequenced (section 1.5.1). These include five subunits of complex I (ND1, 2, 4, 5 and 6), one subunit of complex III (COB) and one subunit of complex IV (COXI). Of the remaining respiratory subunits that are all encoded by the nuclear genome, only a few have been identified and their genes sequenced (section 1.5.2). These include the alternative oxidase, the Rieske iron sulphur protein of complex III, the COX III subunit of complex IV, the cytochrome c carrier protein and the α subunit and β subunit of complex V. None of the nuclear encoded proteins required for mitochondrial gene expression or respiratory enzyme assembly have been identified in the organism.

One way to identify and characterise the unknown nuclear encoded proteins required for respiratory chain biogenesis in *C. reinhardtii* is to use a mutagenic approach *i.e.* disrupt nuclear genes coding for proteins involved in this process. This would allow the genes to be cloned and the gene products to be characterised.
So far, the mutagenic approach has been used twice in *C. reinhardtii* to characterise the nuclear encoded proteins required for respiratory chain biogenesis. In 1977, a large set of nuclear mutants defective in respiratory function were generated using the mutagen MNNG (Wiseman *et al.*, 1977). From the results of enzyme assays and electron microscopic analysis (section 1.5.3.2), possible functions for the proteins absent in nine of the mutants were predicted. However, as molecular biology was still in its infancy in 1977, complete functional analysis of the mutants was not possible. The genes disrupted in the mutants were not cloned or sequenced, nor were their gene products analysed to determine a specific function. Dorthu *et al.* (1992) later generated one nuclear respiritory mutant (dn-12) using the mutagen acriflavin. However, apart from detecting a reduced complex IV activity, no further characterisation of the mutant was performed.

With the development in the past decade, of a range of new molecular techniques for the analysis of wild type and mutant *C. reinhardtii* cells, it would now be possible to characterise the Wiseman mutants further. The mutants are still available for analysis (*Chlamydomonas* Genetics Center, Duke University). However, a new ‘advanced’ mutagenic method to generate nuclear mutants has been developed that would allow simpler characterisation of the disrupted gene function. This technique, known as insertional mutagenesis, involves the transformation of a selectable marker into the nucleus of wild type *C. reinhardtii* cells (section 1.4.5.1). Mutants generated by insertional mutagenesis are: 1) random, as the marker integrates by non-homologous recombination into the nuclear genome; 2) stable, as once the marker integrates, it is maintained in the genome. Mutants are unlikely to revert to a wild type phenotype; 3) tagged. The insertional mutagen physically tags the gene it has disrupted, allowing the straightforward isolation of the mutated gene (Gumpel and Purton, 1994).

A set of respiratory mutants were generated in our laboratory using insertional mutagenesis (Turner *et al.*, 1996) in order to identify and characterise nuclear genes.
required for respiratory chain biogenesis. The *ble* construct was used as the selectable marker and was transformed into cell wall deficient *C. reinhardtii* cells (*cw10*). Respiratory mutants were isolated from the transformed cells using the following strategy:

1) Transformed cells were grown in the presence of zeomycin. Only those expressing the *ble* gene grow.

2) The zeomycin resistant cells were overlaid with TTC* and incubated in the dark. Respiratory functional cells turn purple. Respiratory defective cells remain green.

3) The phenotype of the putative respiratory mutants was confirmed by assessing for the ability of the cells to grow in the dark. Respiratory mutants are unable to grow in the dark as 1) they are unable to utilise acetate as an energy source and 2) they cannot use photosynthesis to provide the ATP required for growth.

* TTC is reduced by complex I in anaerobic conditions (P. Rich, personal communication). The compound changes from a colourless solution to a red precipitate when it is reduced, staining cells with a functional respiratory chain
purple. Cells that lack complex I, III or IV activity are unable to reduce TTC and therefore remain green. Complex I mutants are unable to reduce the compound and complex III and IV mutants are unable to provide the anaerobic conditions required for TTC reduction by complex I.

Two of the respiratory mutants, M86 and M90, generated by insertional mutagenesis were chosen for further characterisation. These were created using an intron-containing version of the ble marker in plasmid pSP115 (fig 1.11). There are three aims of the work described in this chapter: 1) Determination of the genetic location of the mutation in each mutant (nuclear, mitochondrial or chloroplastic). 2) Confirmation that the mutations have arisen due to the insertion of the ble marker. 3) Determination of the specific defect in each mutant.

3.2. RESULTS

3.2.1. Genetic analysis

3.2.1.1. Origin of the mutation in M86 and M90

Nuclear genes are inherited in a Mendelian fashion in Chlamydomonas reinhardtii cells and segregate 2:2 in tetrad progeny, whereas chloroplast and mitochondrial genes are inherited (predominantly) unparentally from the mt+ and mt− parent respectively (section 1.4.3).

To establish the origin of the mutation in the M86 and M90 mutants, they were each crossed with the wild type strain WT-13 and the resultant progeny were assessed for their ability to grow in the presence or absence of light on acetate-containing agar plates. Usually, this type of analysis requires that all four progeny from each dissected tetrad be examined. However, in the M86 and M90 crosses, the cells within the tetrad tended to undergo a rapid further division producing eight
offspring. As it was possible to distinguish between wild type and mutant progeny (respiratory defective) on the basis of growth rate, with mutant progeny producing smaller colonies, only two progeny from each tetrad dissected in the crosses were used for the analysis, a fast and a slow growing colony. For the M90 cross, 16 progeny in total were characterised and for the M86 cross, 18 were analysed.

In both the M86 (mt') x WT-13 (mt') cross and the M90 (mt') x WT-13 (mt') cross, the mutant phenotype of the slow growing progeny was demonstrated by their inability to grow in the dark (fig 3.1a and b). As the slow growing phenotype segregated 2:2 or 4:4 in all the tetrads analysed, it was concluded the mutations in M86 and M90 were inherited in a Mendelian fashion and therefore are located in the nuclear genome.

3.2.1.2. Linkage between ble and the M86 and M90 mutations

Genes that lie close to each other on a chromosome are linked i.e. they are rarely assorted independently of each other in genetic crosses. If the insertion of the ble marker has given rise to the mutations in M86 and M90, then the inheritance of ble should be linked to the inheritance of the respiratory defective phenotype.

To determine if the inheritance of ble was linked to the inheritance of the mutation in M86 and M90, the progeny analysed above were grown on acetate-containing agar in the presence of light and zeomycin. Cells synthesizing the ble protein grow in the presence of zeomycin.

Only the slow growing progeny grew under these conditions (fig 3.1c). As the slow growing progeny are respiratory defective, this result demonstrated that the ble gene and the mutations in M86 and M90 are linked. From this, it was therefore concluded that the mutations in M86 and M90 have arisen due to the insertion of the ble marker.
Figure 3.1. Genetic analysis of the M86 and M90 mutants

Progeny from crosses of M86 and M90 with WT-13 were grown in three different growth environments: a) acetate + light, b) acetate + dark and c) acetate + light + zeomycin as were the parental cells. For M86, colonies A = faster growing colonies and colonies B = slower growing colonies. For M90, the top eight progeny are the faster growing colonies and the bottom eight progeny are the slower growing colonies.
3.2.2. Growth phenotype

The nuclear genomes of M86 and M90 have been disrupted in genes that are required for respiration. The disrupted genes could potentially encode proteins that are required for a wide spectrum of functions in the cell that when absent would result in the loss of respiratory function. These include proteins involved in acetate assimilation, the citric acid cycle, the biogenesis of the classical and alternative respiratory pathways and the biogenesis of complex V.

In order to characterise M86 and M90 further and gain some insight into the type of mutation that has arisen in the mutants, the growth phenotypes of the mutants under phototrophic conditions (light + minimal medium - no carbon source), heterotrophic conditions (acetate, but no light) and mixotrophic conditions (acetate and light) were compared to the wild type strain WT-13 (fig 3.2).

The M86 and M90 growth phenotypes were similar. Under mixotrophic conditions, they grew at a reduced rate compared to the wild type cells (fig 3.2ai and aii), although they reached a similar density of cells at stationary growth of approximately $1 \times 10^7$ cells/ml. Under phototrophic conditions, the mutants grew at a similar rate to the wild type cells (fig 3.2bi and bii). Stationary growth of all the cell types was reached at cell density of approximately $3 - 5 \times 10^6$ cells/ml. Under heterotrophic conditions, the mutants showed very little growth (fig 3.2ci and cii). The wild type cells, however, grew at a similar rate to those grown under mixotrophic conditions and reached stationary growth at a cell density of approximately $1 \times 10^7$ cells/ml.

Interpretation of these results (see discussion of chapter: section 3.3.3) indicated that M86 and M90 retain some ability to synthesize ATP by respiration, although not enough to support growth in the dark. This implied that the mutants were wild type for acetate assimilation, citric acid cycle function and complex V function.
WT-13 (black), M86 (blue) and M90 (red) were cultivated in liquid medium under mixotrophic (a), phototrophic (b) and heterotrophic (c) conditions. As WT-13 has a cell wall, the cell-walled respiratory defective progeny M86-1 and M90-6, produced from the crosses of the two original cell wall deficient mutants with WT-13, were used. Growth rates were measured twice. As the results were the same, only one set of data is presented here.
It was therefore concluded that M86 and M90 are defective in factors required for the biogenesis of the classical or alternative respiratory pathways.

3.2.3. Classical and alternative respiration

As mentioned above, *C. reinhardtii* cells possess two respiratory chains: the classical respiratory pathway, which is sensitive to the inhibitor potassium cyanide and the alternative respiratory pathway, which is sensitive to the inhibitor octyl gallate (fig 3.3). To determine if the biogenesis of one or both of these pathways were defective in M86 and M90, the respiratory rates of the mutants were measured in the presence and absence of the inhibitors (table 3.1). The assay was also performed with the wild type strain, WT-13 and *cwl0*, and the mutant cells, *dum-1* (complex III defective) and *dk-97* (complex IV defective).

In the absence of the inhibitors, the M86 and M90 mutants respire at a similar rate to the wild type strain WT-13 and to the *dum-1* and *dk-97* mutants, which are defective in classical respiration. The strain *cwl0* respires at approximately double this rate. In the presence of octyl gallate only, the rate of respiration fell by at least 80% in the M86 and M90 mutants and the *dum-1* and *dk-97* mutants. The respiratory rate of the wild type cells was not significantly altered. However, the subsequent addition of potassium cyanide to these cells (WT-13 and *cwl0*), reduced their respiratory rate by approximately 80%. In the presence of potassium cyanide only, the rate of respiration was not significantly modified in any of the mutants and their rates of respiration remained comparable to the WT-13 cells. However, the subsequent addition of octyl gallate to all of the cells, reduced their respiratory rates by 80-90%.
Figure 3.3. Site of action of respiratory chain inhibitors, potassium cyanide (KCN) and octyl gallate (OG).

Table 3.1. The effect of inhibitors on the rate of respiration in *Chlamydomonas* cells

The respiratory rate of WT-13, *cw10*, *dum-1*, *dk-97*, M90 and M86 cells grown under mixotrophic conditions was determined. Cell-walled respiratory defective progeny from the genetic crosses (section 3.2.1) were used for these experiments. The respiratory rate is expressed as μmol O₂ / mg chlorophyll / h (mean of 3-5 readings ± standard error) before the addition of the inhibitors octyl gallate (OG) and potassium cyanide (KCN) and then as the % of respiratory activity left after the addition of the inhibitors.
Oxygen consumption was not completely abolished in any of the strains after the addition of one or both of the inhibitors. This probably reflects either incomplete inhibition of the respiratory pathways of other oxygen consuming processes occurring within the whole cells, such as chlororespiration.

The presence of a respiratory rate in M86 and M90 in the absence of inhibitors indicated that either the classical or the alternative respiratory pathway was functional in the mutants. The insensitivity of M86 and M90 to potassium cyanide, but sensitivity to octyl gallate demonstrated that the mutants, like dum-1 and dk-97, lack classical respiration, but are able to respire by the alternative respiratory pathway.

3.2.4. Complex I activity

As M86 and M90 are capable of some ATP production by respiration, this indicates that at least one of the proton-pumping complexes (I, III and IV) is functional in the mutants. The presence of alternative respiration but absence of classical respiration (which involves complexes III and IV) in the mutants indicates that it is complex I that is functional in the mutants and is responsible for the production of ATP. To confirm this, the ability of the mutants to transfer electrons from NADH to ubiquinone in the presence and absence of the complex I inhibitor rotenone was assessed (fig 3.4 and table 3.2). As C. reinhardtii cells contain the alternative NADH dehydrogenases that catalyse the same electron transfer event as complex I (section 1.2.2), rotenone was added to determine the contribution by complex I. Complex I activity was also assayed in the wild type strain cw10 and in the respiratory defective strains dum-1 and dk-97 (table 3.2).
Figure 3.4. The assay used to measure complex I activity and site of action of the inhibitor rotenone.

![Diagram of electron transport chain](image)

Table 3.2. Complex I activity in *Chlamydomonas* cells

Total cellular NADH dehydrogenase activity (total) and rotenone NADH dehydrogenase activity (+ rotenone) was determined in cell homogenates of the *cw10*, *M90*, *M86*, *dum-1* and *dk-97* strains. The cell wall deficient forms of the mutants were used for this analysis. NADH dehydrogenase activity is expressed as μmols NADH oxidised / mg chlorophyll / min (mean of four readings ± standard error). Complex I activity was deduced from these rates (absolute; c) and is expressed as a % of the total NADH dehydrogenase activity (d).
All strains were able to transfer electrons from NADH to ubiquinone in the absence of rotenone (table 3.2 - column a). The rate of the NADH oxidation was not significantly different for any of the samples. On addition of rotenone, the rate of NADH oxidation in all the strains was reduced by approximately 40% (table 3.2 - column b). The complex I activity deduced from these data was similar for all the samples and was therefore, approximately 40% that of the total NADH dehydrogenase activity (table 3.2 - columns c and d). This value is similar to that determined by Duby and Matagne (1999) in their study of the complex I mutant dum-24.

The results showed that complex I function is present in M86 and M90 and therefore it was concluded that the ATP production attributed to respiration in the growth curves is due to the activity of complex I.

3.2.5. Assay of complex III and IV function

As the classical respiratory pathway is not functional in M86 and M90, it was predicted that either complex III and/or complex IV was defective in M86 and M90. Previous work in our lab has demonstrated that the carrier molecule cytochrome c, the third component of the classical respiratory pathway, is present in the mutants (S. Purton, unpublished data). For this, whole cell protein from the mutants and the wild type strain, WT-13, were separated on a Laemmli gel and stained with 3, 3', 5, 5'-tetramethylbenzidine (TMBZ), which binds to haem-containing proteins. In wild type C. reinhardtii, this stain binds to cytochromes f (32 kDa), b6 (20 kDa), c1 (25 kDa) and c (12 kDa). As the amount and size of cytochrome c detected in the mutants was similar to WT-13, it was concluded that the protein is probably functional in M86 and M90 and therefore, has not caused the absence of classical respiration observed in the mutants.
To determine if complex III was functional in M86 and M90, the ability of the mutants to transfer electrons from ubiquinol to oxidised cytochrome c in the presence and absence of MOA stilbene, the complex III inhibitor, was assayed (fig 3.5). Complex III activity was also assayed in the wild type strain cw10 and the mutant strain dum-1 (complex III defective). The inhibitors octyl gallate and potassium cyanide were included in each assay to prevent the removal of the ubiquinone by the alternative respiratory pathway and the reoxidation of cytochrome c by complex IV, respectively.

In the absence of MOA stilbene, the M86 and M90 mutants reduced cytochrome c at a similar rate to the wild type strain cw10 (table 3.3). Little activity (8% of cw10) was detected in the dum-1 mutant. This result was expected as dum-1 lacks complex III function (Dorthu et al., 1992).

In the presence of MOA stilbene, the rate of cytochrome c reduction fell to 11% and 8% in the M86 and M90 mutants respectively and 6% in the cw10 strain (table 3.3). This confirmed that the assay was measuring complex III function. Complex III activity was not completely abolished in any of the strains. It is possible that the inhibitor is not able to reach all the complex III molecules within the assay sample and therefore the function of the complex is not completely inhibited.

The results indicated therefore that M86 and M90 are not defective in complex III function.

To determine if complex IV was functional in M86 and M90, the ability of the mutants to transfer electrons from cytochrome c to molecular oxygen was assayed. This electron transfer event was assayed using two different approaches: following cytochrome c oxidation and the oxidation of diaminobenzidine (DAB).
Figure 3.5. The assay used to measure complex III activity (cyt c = cytochrome c) and the site of action of the inhibitor MOA stilbene.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of cytochrome c reduction</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmols cytochrome c reduced / min / mg chlorophyll</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>+ MOA stilbene</td>
</tr>
<tr>
<td>cw10</td>
<td>1.95 (±0.13)</td>
<td>0.13</td>
</tr>
<tr>
<td>M90</td>
<td>1.91 (±0.35)</td>
<td>0.16</td>
</tr>
<tr>
<td>M86</td>
<td>1.73 (±0.27)</td>
<td>0.19</td>
</tr>
<tr>
<td>dum-1</td>
<td>0.16 (±0.07)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 3.3. Complex III activity in *Chlamydomonas* cells

Total cellular complex III activity (total; mean of three readings ± standard error) and MOA stilbene complex III activity (+ MOA stilbene; mean of two readings) was determined in cell homogenates of the *cw10*, *M90*, *M86* and *dum-1* strains. The cell wall deficient forms of *M86* and *M90* were used for this analysis. Complex III activity is expressed as μmols cytochrome c reduced / min / mg chlorophyll.
3.2.5.1. Cytochrome c oxidation

Cytochrome c oxidation by complex IV was followed in M86, M90 and cwI0 by repeating the assay used to measure complex III activity, but in the absence of potassium cyanide (fig 3.6). It was predicted that reoxidation of the cytochrome c reduced by complex III would be observed if complex IV function was present in M86 and M90.

The assay showed that the wild type strain cwI0 was able to reoxidise the cytochrome c reduced by complex III (fig 3.7). As this reoxidation did not occur in the presence of the complex IV inhibitor potassium cyanide, this result confirmed that the assay measured complex IV function. The mutants M86 and M90 were unable to reoxidise cytochrome c in the presence or absence of potassium cyanide. This strongly indicated that both mutants lack complex IV function.

3.2.6.2. DAB oxidation

DAB reacts with cytochrome oxidase (fig 3.8) in gluteraldehyde fixed cells in the presence of catalase to form an electron dense polymerised oxidation product that precipitates along the surfaces of the mitochondrial cristae, thereby staining them. This staining of the cristae can be viewed under an electron microscope.
Figure 3.7. Complex IV activity in *Chlamydomonas* cells

Complex IV activity was assayed in *cw10* (black), M90 (red) and M86 (blue) cell homogenates using a spectrophotometer in the presence and absence of potassium cyanide (KCN). An increase in absorption indicates cytochrome c reduction and a decrease in absorption indicates reoxidation of the reduced cytochrome c.
Figure 3.8. DAB oxidation by cytochrome oxidase

The ability of the M86 and M90 mutants to oxidise DAB was assessed in collaboration with J. Olive (Institut J. Monod, Université Denis Diderot, Paris). The mutants were DAB stained and photographed under a transmission electron microscope (fig 3.9), using a method derived from Wiseman et al. (1977). The assay was also performed with the wild type strain WT-13.

The mitochondrial WT-13 cristae were stained with DAB (fig 3.9). The outlines of the cristae were clearly defined. DAB staining was absent in M86 and M90 (fig 3.9). This also strongly indicated that complex IV function is absent in the mutants.

On the basis of the results of the two assays to measure complex IV function, it was concluded that M86 and M90 are respiratory defective due to the absence of complex IV function.

3.2.6. Assembly of complex IV

The results presented in section 3.2.5. demonstrate that M86 and M90 lack complex IV function. To determine if this complex is assembled in the mutants, mitochondrial extracts of the mutants were analysed for the presence of a diagnostic signal arising from the $a$ and $a_3$ cytochromes in complex IV. Cytochromes $a$ and $a_3$ absorb light in the visible region, giving a characteristic peak at 603nm in yeast and mammals and at 609nm in C. reinhardtii.
Figure 3.9. DAB staining of *Chlamydomonas* cells

Guanidinium fixed WT-13, M86 and M90 cells were stained with DAB and then viewed under a transmission electron microscope. Magnification was at 39000x. Cell-walled progeny from the M86 and M90 genetic crosses were used for this assay.
The absence of these peaks indicates a lack of (or defective) assembly of complex IV. The cytochrome content of the wild type strain cw10 was also assayed.

Two sets of data are presented in figure 3.10 that show the results of this analysis. In figure 3.10a, potassium ferricyanide was not added to fully oxidise the mitochondrial extracts before measuring the oxidised absorption spectra of the sample and plotting the reduced minus oxidised spectra. In figure 3.10b, potassium ferricyanide was added. As mitochondria are usually oxidised when isolated from whole cells, the oxidation of the extracts with potassium ferricyanide was not expected to significantly affect the reduced minus oxidised haem spectra for the samples.

The absorption peaks for cytochrome $a$ and $a_3$ were absent in M86 and present at a very reduced amount in M90 compared to cw10 (fig 3.10a and b). This indicated that complex IV was not assembled correctly in M86 and assembled at only a very reduced level in M90 compared to wild type.

The mutants also possessed the characteristic absorption peaks for the complex III cytochromes (fig 3.10a and b), although these were hidden in the M86 sample in the second assay due to contamination of the sample with cytochrome $b_{559}$ from the chloroplast, which absorbs light in the same region as the cytochromes from complex III (fig 3.10b). Complex III cytochromes include the $b_L$ and $b_H$ cytochromes, which absorb light at 562nm and the $c_1$ cytochrome, which absorbs light at 553nm. The $c_1$ peak was only clearly defined when the mitochondrial extracts were fully oxidised with potassium ferricyanide before measuring the reduced minus oxidised haem spectra (fig 3.10b). This result indicated that complex III is assembled in M86 and M90. As complex III activity was detected in the mutants (section 3.2.5), this result was expected.
Figure 3.10. Haem spectra of *Chlamydomonas* cells

The absorption spectra of oxidised (potassium ferricyanide) and reduced (sodium dithionite) cw10, M86 and M90 mitochondrial extracts was measured and the reduced minus oxidised spectra plotted (cw10 = black, M86 = blue and M90 = red). In figure 3.10a, the spectra of the cells before the addition of sodium dithionite was used as the oxidised spectra. Potassium ferricyanide was not added.
Chapter three

The absorption peaks for $b_L$, $b_H$ and $c_1$ in the mutants were approximately half the height of those in the $cwl0$ sample (fig 3.10a and b). This suggested that $cwl0$ contains double the number of complex III enzymes than the mutants, which was unexpected as 1) the mutants reduced cytochrome $c$ at a similar rate to $cwl0$ in the complex III activity assay (table 3.3) and 2) the reduced minus oxidised absorption spectra was performed using the same concentration of mitochondrial proteins for each strain. The phenotype may have arisen due to a knock-on effect caused by the absence of complex IV activity in the mutants *i.e.* as complex IV was not active, the number of complex III enzymes present in the cell was reduced as the function of complex III was no longer required. Why the mutants therefore reduced cytochrome $c$ in the complex III activity assay at a similar rate to $cwl0$ is unclear, although as the measurements were made using cell homogenates, it is likely that they do not reflect the true activity of the complex III enzymes in the samples.

3.3. DISCUSSION

3.3.1. Nuclear origin of the M86 and M90 mutations

The results of the genetic analysis showed that the mutations in M86 and M90 are of a nuclear origin. The respiratory defective phenotype is inherited at a ratio of 2:2 in the progeny. It was possible to tell this by eye once the progeny from the dissected zygotes had grown into visible colonies, as progeny of two different phenotypes (wild type and respiratory defective) were present at a 2:2 ratio. The wild type progeny grew faster and formed dark green colonies whereas the respiratory defective progeny grew slower, forming smaller, light green colonies, a phenotype that has been observed before (Dorthu *et al.*, 1992).
3.3.2. The M86 and M90 mutations were caused by the insertion of ble

The results of the linkage experiments demonstrated that the genes disrupted in the M86 and M90 mutants are linked to the ble gene. They also indicated, as zeomycin resistance was not found in any of the wild type progeny (a phenotype expected if multiple copies of the marker had integrated into the genome), that only one copy of the ble marker had integrated into the nuclear genome. When cloning the genes disrupted in the mutants, it is important that only one copy of the marker, the one that is linked to the mutation, has integrated into the genome. The reason why will become clear in chapter four.

3.3.3. Growth phenotype of M86 and M90

The growth phenotypes of M86 and M90 allowed several conclusions to be drawn about the type of mutation that has occurred in the mutants.

The similar rate of growth of M86 and M90 under phototrophic conditions (light without acetate) compared to WT-13 (fig 3.2b) demonstrated the mutants are wild type for photosynthetic function as under these conditions, wild type *C. reinhardtii* cells are only able to produce the ATP required for cell division by photosynthesis. The similar rate of growth under these conditions also indicated that the mutants are likely to contain a fully functional citric acid cycle, the major source of respiratory substrates. This cycle plays a central role in intermediary metabolism and if the activity of the cycle is reduced in M86 and M90, the mutants would have been expected to show reduced growth under phototrophic conditions compared to WT-13.

Under mixotrophic conditions (light + acetate), wild type *C. reinhardtii* cells are able to both respire and photosynthesise to produce the ATP required for cell
division. M86, M90 and WT-13 grow at a faster rate under mixotrophic conditions than under phototrophic conditions (fig 3.2a and b). This demonstrates that all cell types are utilising acetate as an energy source. The rate of growth is slightly slower in the mutants compared to the wild type strain however (fig 3.2a). This demonstrates that the mutants are not utilising the acetate as effectively as WT-13.

The ability of M86 and M90 to utilise acetate as an energy source allowed a number of conclusions to be drawn about the phenotype of the mutants. 1) It demonstrated that the mutants are able to assimilate acetate and contain a functional citric acid cycle. 2) It also indicated that the mutants are able to produce ATP via the respiratory chain. This demonstrated firstly that the mutants possess a functional complex V and secondly, as *Chlorella reinhardtii* possesses two respiratory pathways and the mutants showed a reduced rate of growth compared to wild type under the mixotrophic conditions, that the function of one of these proton pumping pathways is defective in the mutants. Such mutants have been isolated before (dum-18 and 19) and shown to grow at enhanced rates in the presence of acetate (Colin *et al.*, 1995). These lack the function of the classical respiratory pathway.

The very reduced growth of M86 and M90 under heterotrophic conditions (dark + acetate) compared to WT-13 (fig 3.2c), demonstrates that the mutants are defective in respiration as under these conditions, wild type *Chlorella reinhardtii* cells are only able to produce the ATP required for cell division by respiration. The small amount of growth observed in the mutants could be due to either 1) a leak of light into some of the cultures allowing the mutants to photosynthesise and grow or 2) the presence of enough ATP in the cells to allow one more cell division once they are transferred from mixotrophic to heterotrophic conditions.

It is surprising that M86 and M90 do not divide at a faster rate under heterotrophic conditions as the mutants grow at an enhanced rate in the presence of acetate under mixotrophic conditions compared to that in the absence of acetate under
phototrophic conditions. The absence of cell division under heterotrophic conditions may indicate that ATP levels have to reach a certain threshold before they support active cell division. If this is the case, then under mixotrophic conditions, due to ATP production by photosynthesis, this threshold has already been overcome, so ATP production via the protonmotive force generating alternative pathway is able to enhance growth.

3.3.4. M86 and M90 lack classical respiratory function

The results of the growth analysis indicated that M86 and M90 lack the function of either the protonmotive force generating classical or alternative respiratory pathway. The assay measuring respiratory function in M86 and M90 in the presence and absence of the inhibitors potassium cyanide and octyl gallate demonstrated that the function of the classical pathway was absent in the mutants.

It was only possible to identify the defective respiratory pathway in the mutants by observing the effect that the pathway inhibitors had on respiration. This was because, under the conditions of the oxygen electrode, if one pathway is non-functional, the other pathway will increase its respiratory rate to a level similar to that observed for cells that are able to respire by both respiratory pathways. This phenotype arises as oxygen is a limiting factor for respiration in the oxygen electrode. The similar rates of respiration of the wild type strain, WT-13, and the two mutants, dum-1 and dk-97, which lack classical respiratory function, support this theory (table 3.1). In situ studies in higher plants show, when oxygen is not limiting and in the absence of stress, that respiration occurs predominantly by the classical respiratory pathway (Vanlerberghe and McIntosh, 1997).

The increased respiratory rate measured in the cell wall deficient strain, cw10, compared to walled wild type strain, WT-13, may have arisen due to the increased ability of cw10 to absorb oxygen from the surrounding environment due to the
absence of a fully formed cell wall. As oxygen is limiting, this would explain the increase in the rate of respiration observed in cw10 and the decreased respiratory rate observed in the walled wild type and mutant strains.

The absence of classical respiration but presence of alternative respiration in M86 and M90 indicate that the increased growth observed for the mutants under mixotrophic conditions compared to growth under phototrophic conditions is very likely to arise due the function of the protonmotive force generating alternative respiratory pathway (fig 3.11). The demonstration that complex I, the only protonmotive force generating complex of the alternative pathway, is functional in the mutants supports this as does recent work by Duby and Matagne (1999) with the dum-24 mutant. This mutant lacks both complex I and III function and does not show enhanced growth under mixotrophic conditions.

![Diagram of electron flow through alternative respiratory pathway](image)

**Figure 3.11. The flow of electrons through the alternative respiratory pathway**

The transfer of electrons through complex I to the alternative oxidase results in the transfer of protons \( n = 4 \) or 5) from the mitochondrial matrix to the intermembrane space. This contributes to the protonmotive force, which results in the production of ATP through complex V. Complex II and the alternative NADH dehydrogenases can also contribute electrons to the alternative pathway. However, transfer of electrons through these complexes does not contribute to the protonmotive force.
3.3.5. M86 and M90 lack complex IV function

The ability of M86 and M90 to transfer electrons from ubiquinol to cytochrome c, but their inability to accept electrons from cytochrome c demonstrate that the absence of classical respiration in the mutants has arisen due to the loss of complex IV function. The results of the haem spectra analysis demonstrate that in M86, the complex is not assembled and in M90, the complex is assembled but only in very reduced amounts compared to wild type.

3.4. CONCLUSION

The results of this chapter demonstrate that M86 and M90 lack classical respiration due to the loss of complex IV function. In M86, complex IV is not assembled and in M90, the complex is assembled but only in very reduced amounts compared to a wild type strain. The loss of complex IV function in the mutants has arisen due to the insertion of the ble marker into a nuclear gene required for the biogenesis of the complex.

From the analysis of mutants lacking specifically complex IV activity in the yeast S. cerevisiae, the absence of a wide spectrum of genes could result in the phenotypes observed in the two mutants. These include genes that are required for the expression of the mitochondrial cox1 gene, the assembly of complex components into the inner mitochondrial membrane, the import of copper into the mitochondria and the synthesis of the haems groups or genes that encode subunits of the complex. To determine the function of the genes disrupted in M86 and M90, all these avenues need to be investigated. This analysis is the subject of the next two chapters.
CHAPTER FOUR
CLONING AND SEQUENCING OF THE GENE DISRUPTED IN THE M90 MUTANT

4.1. INTRODUCTION

Insertional mutagenesis, the mutation of genes through the insertion of a known piece of DNA (insertional mutagen) into the genome, has become an extremely useful research tool for generating mutants with a variety of phenotypes (section 1.4.5.1). The method not only creates novel mutants, due to the random integration of the insertional mutagen, but also tags the genes that have been disrupted thereby facilitating their isolation.

The technique has been used to generate large collections of nuclear mutants in model organisms such as *Drosophila, Caenorhabditis elegans, maize, Antirrhinum majus* and *Arabidopsis thaliana*. Transposable elements are commonly used as the insertional mutagens in these organisms (Martienssen, 1998) although the T-DNA of *Agrobacterium tumefaciens* (Azpiroz-Leehan and Feldmann, 1997) is the predominant insertional mutagen used to generate mutants in *A. thaliana*.

In *C. reinhardtii*, transforming DNA serves as the insertional mutagen. These mutagens are simple plasmids, which contain marker genes such as the *ARG7, NIT1, CRY1* and *ble* genes that confer a selectable phenotype to cells once the marker has integrated into the nuclear genome (section 1.4.5.1). The *ARG7* (Debuchy et al., 1989) and *NIT1* (Fernandez et al., 1989) markers allow cells lacking the corresponding gene to grow in the absence of arginine or on nitrate as the sole nitrogen source, respectively. The *CRY1* (Nelson et al., 1994) and *ble* (Stevens et al., 1996) markers allow cells to grow in the presence of cryptopleurine/emetine and zeomycin/phleomycin respectively.

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Cloning of genes mutated by insertional mutagenesis is achieved in two steps. 1) Isolation of the genomic DNA flanking the mutagen. In most cases, this flanking region contains part of the gene of interest that has been disrupted by the mutagen. 2) Isolation of the wild type copy of the gene of interest. A wild type genomic library of the organism under study is screened with the flanking sequence to isolate a clone containing the gene. In *C. reinhardtii*, integration of the mutagen is often accompanied by large deletions (tens of kilobases) of genomic DNA. Therefore, the gene of interest may be some distance from the flanking sequence. In these cases, it is necessary to perform a chromosome walk using overlapping clones isolated from the wild type genomic library to isolate the gene of interest.

A number of strategies have been developed to clone the DNA flanking the site of plasmid integration.

a) Plasmid rescue (fig 4.1). This is the simplest of the cloning strategies and takes advantage of the presence, in the plasmid, of vector sequence containing an antibiotic resistance gene and an *E. coli* origin of replication.

This strategy has been used also in the cloning of T-DNA tagged genes whereby the T-DNA has been modified to contain *E. coli* vector sequence (Azpiroz-Leehan and Feldmann, 1997). In *C. reinhardtii*, use of plasmid rescue to clone the flanking sequence is rarely successful (Tam and Lefebvre, 1993). Although the *C. reinhardtii* insertional mutagens contain the vector sequence required for replication in *E. coli*, it is quite common, on integration into the nuclear genome, for regions of this vector sequence to be lost since, unlike the marker gene itself, there is no selection for the maintenance of the vector sequences.
1. Genomic DNA of mutant is digested with enzyme that cuts vector DNA once.

2. Fragment containing the vector DNA required for replication and selection in *E. coli* and some of the flanking sequence is religated.

3. Religated DNA is transformed into *E. coli*. Flanking sequence is isolated from transformants.

**Figure 4.1. The strategy used to clone the flanking sequence by plasmid rescue.**

RE, Ab and ori stand for restriction enzyme, Antibiotic and origin of replication respectively.

b) Inverse PCR. In this technique, recircularised fragments generated in the same way as those in plasmid rescue, are subjected to PCR using outward facing primers that anneal to a region of the mutagen. Only fragments containing the mutagen sequence and the flanking sequence will be amplified, hence allowing isolation of the flanking sequence.

c) Generation of a mutant genomic library. This is the most complicated method used to isolate the flanking sequence, but it has a high success rate. The library is constructed from fragments of the mutant genomic DNA that have been digested with an enzyme that cuts the mutagen only once. To isolate the flanking sequence, the library is screened using probes specific to the mutagen sequence. The clones that are isolated contain, in addition to the mutagen sequence, flanking genomic DNA.
The M90 respiratory mutant was created by integration into the nuclear genome of the insertional mutagen pSP115 (fig 4.2), a plasmid that contains an intron-containing version of the ble marker (Lumbreras et al., 1998).

![Diagram of ble marker pSP115]

**Figure 4.2. The ble marker pSP115**

pSP115 consists of the ble gene (filled boxes: exon 1 = 168 bp and exon 2 = 213 bp) separated by intron 1 from C. reinhardtii RBCS2 (clear box: 145 bp), the promoter region of the 5' UTR of RBCS2 (180 bp), the 3' UTR of RBCS2 (231 bp) and pBluescribe M13+ (wavy line). pBluescribe is 3204 bp. The unique restriction enzyme sites and their position in the construct are shown. The ble gene and the 5' and 3' UTR are termed the 'ble cassette'.

The results from chapter three demonstrate that M90 lacks complex IV activity. The results also showed this phenotype has arisen due to the insertion of one copy of the ble marker into the nuclear genome of the mutant. It was concluded this region contained a gene required for complex IV activity and that the insertion of the ble marker had resulted in the loss of function of this gene. The loss of gene function can be attributed to either a simple insertion event in which the gene is disrupted or the deletion of the gene caused by an insertion event accompanied by deletion of genomic DNA. In C. reinhardtii, it is quite common for genomic DNA to be deleted at the site of marker integration (Tam and Lefebvre, 1993). If the insertion of the ble marker has resulted in the deletion of genomic DNA in M90, it is possible that several more genes, situated close to one another, have also been deleted.
The aim of this chapter was to clone and sequence the genes required for complex IV activity that are disrupted or deleted in the M90 mutant. This work was started by Victoria Lumbreras in our laboratory. She isolated the genomic DNA flanking the ble marker in M90 and used this DNA to isolate two clones (cosmid 1 and cosmid 2) from a wild type *C. reinhardtii* genomic cosmid library that contained this flanking DNA. She also demonstrated these clones contained the gene or genes disrupted or deleted in the M90 mutant.

The cosmids were isolated using the following strategy. To isolate the sequence flanking the ble marker, a ‘book shelf’ genomic library of the mutant was constructed. This is a partial genomic library that contains size-selected restriction fragments. The library was constructed using 4 kb *HindIII* fragments of M90 genomic DNA that hybridised to the ble gene probe. A colony containing the ble gene was obtained from the library and by restriction mapping, a 0.4 kb fragment of sequence flanking the ble marker was isolated (fig 4.3).

![Diagram of restriction enzyme map](image)

**Figure 4.3. Isolation of the M90 flanking sequence**

The restriction enzyme map constructed of the ble containing 4 kb *HindIII* fragment isolated from the book shelf library of M90 genomic DNA. The green filled box represents the 0.4 kb flanking sequence.

An attempt was made initially to clone the flanking sequence by plasmid rescue. However, this was unsuccessful, which indicated that some or all of the vector
sequence required for replication in *E. coli* was lost on integration of the marker into the nuclear genome of M90.

To isolate the two cosmid clones, a wild type cosmid genomic library (Purton and Rochaix, 1995) of *C. reinhardtii* was screened with the 0.4 kb flanking sequence probe. The clones were demonstrated to contain the gene(s) disrupted or deleted in the M90 mutant by their ability to rescue M90 to respiratory competency (complementation) *i.e.* when the cosmids were transformed into $2 \times 10^8$ M90 cells, between 20 and 40 putative transformant colonies were obtained that were able to grow in the dark.

The aim of this chapter was to isolate and sequence, from one of the two clones isolated from the cosmid genomic library, the gene(s) required for complex IV activity that are affected in the M90 mutant.

**4.2. RESULTS**

**4.2.1. Southern analysis of the M90 putative transformants that arise after transformation with cosmid 1**

Before attempting to isolate the gene(s) that complement M90 from cosmid 1, it was important to demonstrate that the colonies that arose after transformation with cosmid 1 had arisen due to the integration of cosmid 1 into the genome and were not a result of a reversion of the mutant to a wild type phenotype.

Genomic DNA from M90, the wild type strain, *cw10* and four putative transformants from a cosmid 1 transformation was digested with *Pvu*II and probed with the 0.4 kb flanking sequence (fs) probe (fig 4.3). Previous experiments demonstrated that a 4.2 kb *Pvu*II restriction fragment hybridises to the probe in *cw10* and a 5.0 kb fragment hybridises to the probe in M90.
Figure 4.4. Southern analysis of the M90 transformants (1)

A Southern blot showing genomic DNA of M90, the cw10 (WT) strain and four M90 transformants (1 - 4) digested with Pvull and probed with the 0.4kb flanking sequence. The probe hybridised to a 4.2 kb fragment in the wild type strain (highlighted by white box) and to a 5.0 kb fragment in M90 (highlighted by black box) and the four M90 transformants.
Both fragments were predicted to be present in the transformants if they are ‘true’ transformants, the 5.0 kb band demonstrating the transformants originated from the M90 mutant and the 4.2 kb band demonstrating cosmid 1 has successfully integrated into the nuclear genome of the mutants to rescue them.

As shown in figure 4.4, fragments of 5.0 kb and 4.2 kb hybridised to the fs probe in the M90 (5.0 kb) and *cw10* (4.2 kb) genomic digests, as expected. A fragment of 5.0 kb hybridised to the fs probe in all four of the M90 transformants, as expected. However, no other fragments hybridised to the fs probe in the transformants. The 4.2 kb fragment was not present. This might be taken as evidence that the four clones were not true transformants but had arisen as the result of a spontaneous reversion to a wild type phenotype.

However, two observations suggest this is not the case. Firstly, colonies are never obtained in control transformations of M90 in which no DNA is included in the transformation mix. Secondly, cosmid DNA is commonly lost on integration into the nuclear genome. It is possible that the sequence the fs probe hybridises to is some distance from the complementing region *i.e.* the region containing the gene(s) required to rescue M90 to respiratory competency. If this is the case, the fs sequence may be lost on integration of cosmid 1 into the genomes of the transformants, whilst the complementing region is successfully integrated. If this has occurred, then the clones are true transformants. In the light of these observations, it was decided to continue with the attempt to clone the gene(s) affected in M90 from cosmid 1.

### 4.2.2. Isolation of the complementing region from cosmid 1

Cosmid 1 contains between 30 to 40 kb of genomic DNA (Purton and Rochaix, 1995). In order to obtain the sequence of the gene(s) affected in the M90 mutant,
the first step was to isolate a subclone of the cosmid that was still able to rescue M90 to respiratory competency, but small enough to sequence. The sequence of the respiratory gene(s) can then be determined from this fragment.

A restriction enzyme map of cosmid 1 and 2 was constructed using the enzymes HindIII, EcoRI and Xbal (fig 4.5). As the first step to isolate a subclone of the cosmid that complemented M90, the ability of cosmid 1 to complement M90 when pre-digested with HindIII, EcoRI and Xbal was assessed. If a restriction site is located within the gene(s), then digestion should abolish complementation. Conversely, digestion at sites that are outside the gene should not affect complementation. The aim of this was to locate the complementing region of the cosmid to a specific area within the genomic DNA.

Cosmid 1 digested with Xbal and HindIII was able to complement M90, producing on average 39 (Xbal) and 66 (HindIII) putative transformants per transformation (table 4.1). Cosmid I digested with EcoRI was unable to complement M90 producing on average one putative transformant per transformation (table 4.1). The one colony probably represents uncut cosmid DNA. These results suggested that Xbal and HindIII cut outside the complementing region of cosmid 1, whereas EcoRI cuts within this region and abolishes complementation. As there are two EcoRI sites in the cosmid 1 genomic sequence, the complementing region of cosmid 1 was therefore predicted to be situated in one of two possible locations. Either within the 16 kb HindIII – Xbal fragment or within the 3.6 kb Xbal – HindIII fragment (fig 4.6).
Site of \textit{ble} insertion in the M90 genome

\begin{figure}
\centering
\includegraphics[width=\textwidth]{cosmid1_and_2}
\caption{Restriction mapping of cosmids 1 and 2}
\end{figure}

A physical map of cosmid 1 and 2 showing the positions of the \textit{EcoRI}, \textit{HindIII}, \textit{AvrII} and \textit{XbaI} restriction enzyme sites, the location and orientation of the flanking sequence probe (green filled box) and pARG7.8 cosmid vector sequence (blue filled box). The double headed arrow indicates the region of genomic sequence that is the same in each cosmid. \( \text{\textbackslash /} \text{\textbackslash /} = 2 \text{ kb} \)
Figure 4.6. Locating the complementary region within cosmid 1

Physical map of cosmid 1 and the three subclones derived from it (names shown in blue). // = approximately 2 kb of genomic sequence.

Table 4.1. Locating the complementing region within cosmid 1

Table showing the complementation of M90 after transformation. The exogenous DNA transformed into the mutant is shown in row 1 (cosmid 1 = cos1). The bracketed enzymes indicate the DNA was digested with this enzyme before transformation. Row 2 demonstrates whether the exogenous DNA complements (C) the mutant (✓ = yes and ✗ = no). Row 3 shows the number of transformants (T) produced for each transformation (mean of 4 to 12 readings ± standard error). The No DNA sample consisted of a mock transformation of M90 in the absence of DNA.
To determine which of these fragments contained the complementing region, two subclones were constructed (fig 4.6): the pX7.6 subclone (the 7.6 kb XbaI fragment cloned into pBluescript) and the cosmid1ΔX subclone (cosmid 1 minus the 7.6 kb XbaI fragment). The ability of these subclones to complement M90 was then assessed. pX7.6 was unable to complement the mutant, whereas cosmid1ΔX was, producing on average 21 putative transformants per transformation (table 4.1). This result indicated that the 16kb HindIII – XbaI fragment of cosmid 1 contained the M90 complementing region. The absence of complementation when the cosmid1ΔX subclone was digested with EcoRI (producing on average 2 transformants per transformation – table 4.1) supported this conclusion.

The M90 complementing region of cosmid 1 was successfully located to a region within the 16 kb HindIII – XbaI fragment. However, this fragment is too large to sequence. Therefore, the next step in the isolation of a subclone containing the M90 complementing region was to locate this region to a more specific area. The pHX16 subclone was therefore constructed, containing the HindIII – XbaI fragment cloned into pBluescript (fig 4.6). The demonstration that the construct rescued M90 to respiratory competency, producing on average 87 putative transformants per transformation (table 4.1), confirmed it contained the M90 complementing sequence.

A restriction map of the subclone was constructed using the restriction enzymes SalI, SacI, XhoI and AvrII (fig 4.7). The ability of the subclone to rescue M90 to respiratory competency when digested with these enzymes was assessed. Putative transformants arose in all the digest sample transformations, producing on average 35, 26, 26 and 28 colonies respectively (table 4.2). These results indicated that all the enzymes cut outside the complementing region of the subclone. From this, it was predicted that the M90 complementing region of the subclone was located within the 1.9 kb SacI – AvrII fragment. This contains the EcoRI site that was demonstrated to be located within the M90 complementing region.
Figure 4.7. Locating the M90 complementing region in the HindIII-XbaI subclone, pHX16

Physical map of the 16 kb HindIII - XbaI fragment and the three subclones derived from it.

Table 4.2. Locating the M90 complementing region in the HindIII-XbaI subclone

Table showing the complementation of M90 after transformation. The exogenous DNA transformed in to the mutant is shown in row 1. The bracketed enzymes indicate the DNA was digested with this enzyme before transformation. Row 2 demonstrates whether the exogenous DNA complements (C) the mutant (√ = yes and × = no). Row 3 shows the number of transformants (T) produced for each transformation (mean of 4 - 12 readings ± standard error). The No DNA sample consisted of a mock transformation of M90 in the absence of DNA.
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At 1.9 kb, the *SacI* – *AvrII* fragment is small enough to sequence with ease. The fragment was therefore isolated and cloned in pBluescript to create the plasmid pAS1.9 (fig 4.7). The subclone was able to rescue M90 to respiratory competency, producing on average 79 putative transformants per transformation (table 4.2), confirming it contained the M90 complementary region.

Two conclusions can be drawn from the results of the analysis performed in this section. 1) The insertion of the *ble* marker into the genome of M90 resulted in the deletion of the gene(s) required for complex IV activity in M90. This deletion spans from the site of *ble* insertion to at least somewhere within the *SacI* – *AvrII* fragment. The location of the complementary sequence of cosmid 1 to a region situated 3.9 kb from the site of *ble* integration into the M90 nuclear genome demonstrates this. 2) All the putative transformants are true transformants. This is demonstrated by the repeated absence of transformants in the no DNA controls and the isolation of transformant colonies for only certain subclones.

4.2.3. Determination of the sequence of the cosmid 1 complementing region and the identification of an open reading frame within this.

Once the *SacI* – *AvrII* 1.9 fragment was isolated, the next step in the isolation of the gene(s) affected in M90 was to sequence the fragment and identify possible open reading frames within it.

To determine the sequence of the fragment, two subclones of the fragment were sequenced (fig 4.7): the pSE1.0 and the pEA0.9 subclones (the fragments were cloned into pBluescript). The sequence of the subclones was determined using the strategy shown in figure 4.8.
Figure 4.8. Strategy used to sequence the M90 gene

Primers T3 and T7 (hybridise to pBluescript) and ae 1 to 4 (hybridise to the fragment) were used to determine the sequence of the EcoRI-AvrII fragment (a). Primers T3 and T7, and es 1 to 4 (hybridise to fragment) were used to determine the sequence of the SacI-EcoRI fragment (b). The thick black lines represents the genomic DNA (— = 100 bp), the red arrows represent the position of primer hybridisation, the thin black lines represents the amount of sequence determined using the primer and the boxes show the sequence of these primers.
Both DNA strands of the fragments were sequenced and compared to confirm the sequence determined for each strand was correct and once completely sequenced, the fragments were joined at the EcoRI site to provide the sequence for the whole SA1.9 fragment. Sequencing across the EcoRI site using the HX16 subclone with the ae seq1 primer confirmed the two fragments shared the same EcoRI site.

To identify possible gene sequences in the SA1.9 fragment, two approaches were used. 1) The sequence was entered into a GeneMark programme (http://genemark.biology.gatech.edu/GeneMark/predictions.html), which searches for intron/exon boundaries in *C. reinhardtii* nuclear genes. 2) Consensus sequences predicted to surround the translation start and stop site in nuclear genes of *C. reinhardtii* were searched for on both strands, as were consensus sequences predicted to indicate 5' and 3' splice sites (exon/intron and intron/exon boundaries) and polyadenylation signals in *C. reinhardtii* nuclear genes (table 4.3: Silflow, 1998).

<table>
<thead>
<tr>
<th>Site on gene</th>
<th>Consensus sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation start</td>
<td>(a/c)a(a/c)(a/c)atg(g/c)c(c/g)</td>
</tr>
<tr>
<td>Translation stop</td>
<td>(c/g)taa(g/a)</td>
</tr>
<tr>
<td>5' exon/intron boundary</td>
<td>(c/a)(a/c)g/gtg(a/c)g</td>
</tr>
<tr>
<td>3' intron/exon boundary</td>
<td>(g/a)cag/(g/a)</td>
</tr>
<tr>
<td>Polyadenylation signal</td>
<td>tgtaa</td>
</tr>
</tbody>
</table>

Table 4.3. Consensus sequences predicted to indicate the sites of importance in nuclear genes of *C. reinhardtii*.

The sequence of SA1.9 is shown in figure 4.9. The results of the GeneMark and consensus sequence searches identified only one possible open reading frame (translation start to translation stop), coded by the DNA strand going in the *SacI* to *AvrII* direction (fig 4.9).
Figure 4.9. Sequence of the SacI-AvrII fragment, SA1.9.

The protein coding region of the open reading frame (ORF) with in this sequence is represented by the black upper case letters. The light blue upper case letters are the single letter abbreviations of the amino acids coded for by this sequence. donates a stop codon. The intron sequence and the genomic sequence flanking the protein coding sequence is indicated by black lower case letters. The red upper and lower case letters indicate the consensus sequences shown in table 4.3. The dark blue letters indicate restriction enzyme sites. Sequences for primers P1, P2.1 and P 2.2 are underlined.
The open reading frame is 743 bp in size, is GC rich (62.58% G + C) and possesses a bias for a G or C base in the third position of each codon (76% G or C). It also contains an intron of 424 bp (bordered by the 5’ and 3’ splice sites) and codes for a protein of 105 amino acids (fig 4.9). The consensus sequence search also identified a polyadenylation signal that is 406 bp downstream from the stop codon (fig 4.9).

This result indicates that only one gene has been partially or fully deleted in M90 and that it has been successfully cloned and sequenced.

4.2.4. Isolation of the cDNA for the predicted open reading frame

To confirm that the open reading frame predicted from the analysis of SA 1.9 was expressed and to confirm that the sequence contained an intron, the cDNA of the gene was isolated by RT-PCR as shown in figure 4.10. Primer P2.2 was designed to hybridise just upstream of the predicted polyadenylation signal and primer P2.1 was designed to hybridise just upstream of translation start (fig 4.9). Amplification of the genomic sequence using these primers was expected to produce a PCR product of 1169 bp, whereas amplification of the cDNA product was predicted to produce a product 424 bp smaller as a result of the removal of the intron sequence.

Products of the expected sizes were obtained (fig 4.10). This confirmed the open reading frame predicted in SA1.9 is expressed and that it contains an intron of the predicted size. The cDNA product was sequenced, using primers P1, P2.2 and an internal primer, P2.1, which binds downstream of the translation stop, to confirm the intron/exon boundaries (fig 4.11a). Alignment of the sequence to the genomic sequence demonstrated the removal of the intron exactly as predicted (fig 4.11b). In all other respects the two sequences were identical.
mRNA was extracted from wild type cw10 cells

Using a primer (P2.2) designed to bind the region upstream of the predicted M90 polyadenylation signal, a DNA copy (cDNA) of the M90 mRNA was generated.

The cDNA was amplified by PCR using the primers P1 and P2.2. P1 was designed to bind the region upstream of the predicted M90 translation start site.

After 35 rounds of amplification, an aliquot of the PCR reaction was run on an agarose gel.

**Figure 4.10. Strategy used to isolate the M90 cDNA**

Steps 1 and 2 describe the strategy used to generate and isolate the cDNA for M90 from wild type cw10 cells. Thin black lines represent sequence and red arrows represent primers. The sequence of these are shown in the box. The picture of the ethidium bromide stained agarose gel shows M90 cDNA was successfully isolated using this strategy. The cDNA is approximately 750 bp. The gel also shows the PCR product (gene) obtained when the *SacI-AvrII* subclone is amplified using primers P1 and P2.2. This product is approximately 1.2 kb. M = markers.
Figure 4.11. Sequencing the cDNA for M90

Figure 4.11a shows the primers P1, P2.1 and P2.2 used to determine the sequence of the M90 cDNA (a). The thick black lines represent cDNA sequence, thin black lines represent the amount of sequence determined by each primer and the red arrows represent the position of primer hybridisation to the sequence. The sequence of primer P2.1 is 5' age gaa tga gag cgc tec 3'. Figure 4.11b shows the homology between the M90 cDNA (cDNA) and the predicted ORF for the M90 gene (genomic) at the intron/exon boundary regions. Blue letters represent exon sequence and black letters represent intron sequence. \ \ = 341 bp (intron sequence). \rightarrow = splice site.
4.2.5. Northern analysis of \textit{cw10} and M90

Northern analysis was performed to determine if the RNA transcript coded by the open reading frame predicted in SA1.9 was present in the M90 mutant. Total RNA was extracted from M90 and probed with the cDNA sequence obtained in section 4.2.4. The analysis was performed with the wild type strain \textit{cw10} as a control and also to determine the approximate size of the RNA transcript.

A transcript of 1.1 kb hybridised to the cDNA in the \textit{cw10} sample (fig 4.12b). At approximately 250 bp larger than the cDNA, this extra sequence probably represents the 5' untranslated region and polyA tail of the transcript. As the transcript is easily detected, this indicates that the gene is actively expressed.

No transcripts hybridised to the probe in the M90 sample (fig 4.12b). As equal amounts of \textit{cw10} and M90 total RNA were probed with the cDNA (fig 4.12a), this result shows that the M90 mutant does not express the mRNA coded by the gene located within SA1.9. This indicates that the open reading frame represents the gene required for complex IV activity that has been deleted in the M90 mutant. The gene from now on will be called \textit{COX90}.

4.2.6. Southern analysis of the genomic sequence of M90 at the site of \textit{ble} insertion

The results of the complementation analysis with cosmid 1 demonstrate the insertion of the \textit{ble} marker into the nuclear genome of M90 has resulted in a deletion of least 3.6 kb of genomic DNA, from the site of \textit{ble} insertion to the \textit{AvrII} site. Southern analysis of M90 was performed to confirm this deletion had occurred and also to investigate the extent of the deletion in the genomic DNA of the mutant. The analysis was also performed with the wild type strain \textit{cw10} as a control.
Figure 4.12. Northern analysis of the M90 mutant

The agarose gel (a) shows size fractionated RNA extracted from cw10 and M90 cells. The northern blot (b) shows a transcript of approximately 1.1 kb hybridises to the cDNA of the open reading frame located in SA1.9 in the cw10 sample.
Genomic DNA was extracted from M90 and *cwilo* and digested with *Hind*III and *Avr*II. To confirm the deletion of genomic DNA in M90 at the site of *ble* insertion, the mutant was probed with the 6.7 kb *Avr*II – *Hind*III fragment, which was isolated from cosmid 1 (fig 4.13c). This probe contains the 3.6 kb of sequence that is predicted to be absent in the M90 mutant. Based on the restriction map of cosmid 1 and the M90 mutant at the site of *ble* insertion, a fragment of 6.7 kb was expected to hybridise to the probe in the *cwilo* digest and a fragment of 3.8 kb was expected to hybridise to the probe in the M90 digest (fig 4.13c).

Fragments of the expected sizes hybridised to the probe in both the *cwilo* and M90 digests (fig 4.13a). The absence of a second fragment hybridising to the probe in the M90 sample, which would have been expected if the insertion of *ble* had only disrupted genomic sequence, confirms that the 3.6 kb of genomic sequence is absent in the mutant.

To investigate the extent of the deletion in M90 mutant, the Southern blot was stripped and re-probed with the 5.2 kb *Hind*III – *Hind*III fragment, which was isolated from cosmid 1. A fragment of 5.2 kb was expected to hybridise to the probe in the wild type *cwilo* sample.

A fragment of the expected size hybridised to the probe in the *cwilo* sample (fig 4.13b). No fragment hybridised to the probe in the M90 sample (fig 4.13b). This result indicated that this sequence has been lost in the mutant and suggests that the genomic DNA deletion in the M90 mutant extends from the site of *ble* insertion to past the left-hand *Hind*III site (fig 4.13c). This represents a region of at least 23 kb of genomic sequence. The result implies that the complete sequence of *COX90*, which lies within this genomic sequence, is absent in M90.
Figure 4.13. Southern analysis of M90

The Southern blots show genomic digests of cw10 (wild type) and M90 probed with the 6.7 kb AvrII - HindIII fragment (a) and the 5.2 kb HindIII - HindIII fragment (b) from cosmid 1. The physical map of the wild type (cw10) and the M90 genomic DNA at the site of ble integration (c) indicates the size of the fragments the probes will hybridise to in the cw10 and M90 digests. The red box represents the M90 gene and the dotted lines indicate the extent of the genomic deletion in M90. ▶️ = direction of expression
4.2.7. Analysis of cosmid 1 transformants

To gain further insight into the mechanism of complementation, six randomly selected M90 transformants, rescued to respiratory competency by transformation with cosmid 1, were analysed to investigate the number of integrated copies of the cosmid and the pattern of integration of the cosmid sequence.

Genomic DNA was extracted from the six transformants and digested with HindIII and AvrII. This was probed with the 6.7 kb AvrII – HindIII probe and then with the 5.2 kb HindIII – HindIII probe. The fragments that hybridised to these probes are shown in figure 4.14a and b.

The results of the first probing show that the mutant 3.8 kb fragment (fig 4.13a) is present in all of the transformants (fig 4.14a). This indicates that cosmid 1 has not integrated by homologous recombination to rescue the mutants. The mutant band would be lost if this had occurred.

The additional bands present in this blot have arisen due to the integration of cosmid sequence into the genome of the transformants. In transformants 2 and 3, only one copy of the cosmid has integrated (one additional band) whereas in transformants 1, 4, 5 and 6, two to three copies of the cosmid had integrated (two or three additional bands). Integration of multiple copies of exogenous sequence is commonly seen when using the glass bead transformation system (Gumpel and Purton, 1994). The multiple copies of the cosmid sequence within the genome may have arisen due to the integration of cosmid 1 at different sites within the genome or due to the recombination of the cosmid sequence prior to integration, thereby generating multiple copies of the cosmid sequence at the same locus (Gumpel et al., 1995).
Figure 4.14. Southern analysis of the M90 transformants

The blot shows genomic DNA from six randomly selected M90 transformants rescued to respiratory competency by cosmid 1 digested with *Hind*III and *Avr*II and probed with the 6.7 kb *Avr*II - *Hind*III fragment (a) and the 5.2 kb *Hind*III - *Hind*III fragment (b) isolated from cosmid 1.
The presence of bands of the predicted size (6.7 kb) in transformants 2, 3, 4 and 5 and of other sizes in transformants 1, 4, 5 and 6, which demonstrate the loss of a restriction enzyme site, indicate that sometimes cosmid sequence is lost on integration.

The result of the second probing (fig 4.14b) confirms that cosmid sequence is lost on integration in two ways. 1) Only two out of the six transformants contain this region. 2) A fragment of the expected size (5.2 kb) and another size is present in transformants 5 and 1, respectively.

4.3. DISCUSSION

4.3.1. Complex IV function is absent in M90 due to the deletion of one gene

The results of this chapter show that the loss of complex IV activity in the M90 mutant has arisen due to the deletion of one gene, the COX90 gene, which encodes a protein of 105 amino acid residues.

The identification of only one possible open reading frame in the smallest complementing region of cosmid 1, the 1.9 kb SalI – AvrII fragment, pAS1.9 (sections 4.2.2 and 4.2.3) initially indicated that the function of only one gene was required to restore complex IV activity to M90. Confirmation of this was provided by three results. 1) The isolation of the cDNA for this open reading frame (section 4.2.4). 2) The demonstration of the absence of the RNA transcript of this gene in the M90 mutant (section 4.2.5). 3) The demonstration that complementation of M90 by SA1.9 was abolished by digestion with EcoRI and other enzymes (ApaI and StuI) that cut within the predicted gene sequence (data not presented). The first two results showed that the predicted open reading frame is actively expressed and also that its product is absent in M90. The third result shows that disrupting the gene results in the loss of complementation in M90.
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The assignment of COX90 to a region located at a distance of 3.6 kb from the site of ble insertion in M90 (section 4.2.2) initially indicated that the gene was deleted, or partially deleted, in the mutant. Support for the deletion of the whole gene was provided when it was shown that sequences located immediately downstream and 23 kb upstream of COX90 were also absent in the mutant (section 4.2.6). This result strongly suggested that the whole of the genomic sequence bordered by these sequences, including COX90, is absent in the mutant. The demonstration that part of the gene sequence, contained within the EcoRI – AvrII fragment of SA1.9, was absent in the mutant (data not presented) confirmed that the gene was deleted in the mutant. This latter result also indicated that only one copy of COX90 is present in the nuclear genome of C. reinhardtii.

4.3.2. Insertional mutagenesis as a tool for gene isolation

The cloning of the M90 gene demonstrates that insertional mutagenesis can be used successfully to isolate novel genes associated with phenotypes of interest. Although a number of C. reinhardtii nuclear genes have been cloned using this method (Kindle, 1998), the work presented in this thesis demonstrates the first cloning of a gene using the ble marker as the insertional mutagen. The work also represents the first cloning of a nuclear gene associated with a respiratory defect in C. reinhardtii by use of an insertional mutagen.

It is very simple to generate a large collection of stable, null mutants using insertional mutagenesis. However, use of the technique has a number of potential pitfalls and these had to be taken into account when cloning COX90.

1) The mutations that result in the defective phenotype in the mutant may not have arisen due to mutagen insertion and therefore are not tagged. In Arabidopsis, only 35 – 40 % of mutants generated by transformation with the T-DNA insertional mutagen arise due to T-DNA insertion (Azpiroz–Leehan and Feldmann, 1997).
C. reinhardtii, this number is greater at 75%. Since random spontaneous mutations occur at a very low rate, most untagged mutations have been suggested to arise due to deletions caused by abortive integration events of the marker (Kindle, 1998). It is therefore necessary to check that the affected gene is tagged with the insertional mutagen by genetic analysis before attempting to clone it. A detailed genetic analysis was therefore carried out for M90 prior to cloning the gene (chapter three). This clearly demonstrated that it was tagged.

2) More than one copy of the insertional mutagen may have integrated into the genome of the mutant. In C. reinhardtii, it is quite common for multiple copies of the mutagen to integrate into the genome when using glass bead transformation as a system to introduce the DNA into the cell (Kindle, 1990). Multiple integration could occur at distinct sites or at one locus. As mutagen sequence is used as a molecular tag to clone the affected gene, multiple mutagen insertions would complicate this procedure. This problem did not arise with M90 as the results of genetic (section 3.2.1) and molecular analysis (Lumbreras and Purton – unpublished data) demonstrated that only a single copy of the mutagen had integrated into the genome.

3) Large genes, containing many introns, are more likely to be knocked out by the insertional mutagen than smaller genes as they represent a bigger target. In the case of M90, in respect to molecular characterisation, it was fortunate that the affected gene was relatively small.

4) The insertion of the marker may have resulted in the deletion of a large amount of genomic sequence. This occurs frequently in C. reinhardtii insertional mutants (Tam and Lefebvre, 1995), where deletions of up to 23 kb have been shown to arise. Large deletions can cause complications for the following reasons.
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a. Genes located close to essential nuclear genes may not be tagged, since deletion would frequently result in a lethal mutation. Some other strategy must be used to isolate these genes. In M90, the demonstration that a deletion of at least 23 kb has occurred at the site of ble marker insertion (section 4.2.6) indicates that no genes essential for cell viability are absent or disrupted in the mutant. As the deletion is so large and C. reinhardtii nuclear genes are typically separated by only a few kilobases, it is likely though that some genes, in addition to the gene of interest, have been deleted in the mutant. The function of these genes can not be critical for cell growth and survival as M90, apart from lacking respiratory function, appears to be wild type in all other respects including photosynthetic function, motility and mating.

b. The sequence flanking the mutagen can be situated some distance away from the affected gene. Consequently, genomic clones obtained using the flanking sequence probe may not contain the affected gene. Chromosome walking would have to be carried out to obtain the gene, in which each overlapping clone is tested for its ability to complement the mutant (Ferris, 1995). To overcome this problem, a genomic library that contains large genomic inserts should be screened. This increases the likelihood of isolating a clone containing both the flanking sequence and the gene of interest. A cosmid genomic library, which contains genomic inserts of 40 kb, was therefore screened to isolate the affected gene in M90. The isolation of two clones that rescued the mutant to respiratory competency demonstrated the affected gene had been successfully isolated.

4.3.3. Characterisation of the gene required for complex IV activity in M90

The determination of the sequence of the gene affected in the M90 mutant once the subclone of the complementing cosmid had been isolated and sequenced (section 4.2.2 and 4.2.3) was not straightforward. It was not possible to identify the open reading frame of the gene within the sequence by simply searching for a stretch of
sequence bordered by start and stop codon. When this was attempted, as the nuclear genome of *C. reinhardtii* is GC rich and stop codons (TAA) occur infrequently within it, multiple overlapping open reading frames, mostly fictional, were identified within the sequence of the subclone. This problem does not arise in organisms with an AT rich nuclear genome, such as that of *Dictyostelium*. As stop codons occur so frequently in the genome of this organism, not many open reading frames arise and when they do, they are likely to be correct.

Instead, to identify the open reading frame of the gene affected in M90, it was necessary to search the sequence of the subclone for open reading frames that also contain consensus sequences predicted to surround translation start and stop sequences and intron boundaries. This was done manually and with the aid of the GeneMark programme (section 4.2.3).

The gene affected in M90 contains features in common with other nuclear genes of *C. reinhardtii* that have been characterised (section 4.2.3) as these typically contain at least one intron, are GC rich and are biased for the presence of a G or C in the third position of each codon. It has been suggested that the codon bias aids the expression of gene in the nucleus (Silflow, 1998). No 5' regulatory regions, such as the TATA and CAAT boxes, were identified in the flanking region of the gene. However, a possible polyadenylation signal was identified within the 3' flanking region. The isolation of the cDNA for the gene using a primer located close to this signal region (section 4.2.4) demonstrated that this signal was likely to function as the polyadenylation signal for the gene.

The cloning and sequencing of the gene required for complex IV activity in M90 was performed in order to allow further investigation into its specific role in complex IV function. The use of this gene to determine its function is the subject of the next chapter.
CHAPTER FIVE
THE ROLE OF THE AFFECTED GENES IN RESPIRATORY MUTANTS M86 AND M90

5.1. INTRODUCTION

The biogenesis of the respiratory chain has been studied predominantly in *S. cerevisiae* using mutational analysis. It has been demonstrated that the absence of a wide spectrum of nuclear-encoded proteins results specifically in the loss of complex IV assembly and function in this organism (table 5.1). These proteins include those involved in a) the expression of mitochondrial genes that encode subunits of the complex (COXI, II and III), b) the import, synthesis and attachment of the cofactor components and c) the assembly of the complex (Grivell, 1995; Grivell et al., 1999). The proteins also include the nuclear-encoded subunits of the complex (Grossman and Lomax, 1997; Poyton and McEwen, 1996).

The results of chapter three show that complex IV function was absent in the *C. reinhardtii* nuclear respiratory mutants M86 and M90. The results also indicate that this was due to a reduced or partial assembly of the complex in M90 and a lack of assembly of the complex in M86. The aim of this chapter was to determine the specific function of the genes required for complex IV function that are affected in M86 and M90. If the biogenesis of complex IV in *C. reinhardtii* is similar to that of *S. cerevisiae*, these genes could possess one of many possible functions (table 5.1).
<table>
<thead>
<tr>
<th>Role</th>
<th>Protein name</th>
<th>Specific function</th>
</tr>
</thead>
<tbody>
<tr>
<td>COXI expression</td>
<td>nam1p, nam2p, mrs2p</td>
<td>- Processes coxl precursor RNA</td>
</tr>
<tr>
<td></td>
<td>pet309p</td>
<td>- Stabilises coxl mRNA and aids translation</td>
</tr>
<tr>
<td></td>
<td>mss51p</td>
<td>- Aids translation coxl and insertion into the inner membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>COXII expression</td>
<td>pet309p, mrs2p</td>
<td>- Processes and stabilises cox2 mRNA</td>
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<tr>
<td></td>
<td>pet111p</td>
<td>- Aids translation initiation of cox2</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COXIII expression</td>
<td>pet309p, mrs2p</td>
<td>- Processes and stabilises cox3 mRNA</td>
</tr>
<tr>
<td></td>
<td>pet54p, pet122p, pet494p</td>
<td>- Aids translation cox3 and insertion into the inner membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cofactor import and synthesis</td>
<td>cox17p, sco1p, cox10p</td>
<td>- Aids copper import into the mitochondria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Aids insertion of copper into the inner membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Involved in haem A biosynthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assembly factor</td>
<td>cox14p, cox15p, pet100p, pet191p, pet117p, cox11p</td>
<td>- Aids in the assembly of complex IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex subunit</td>
<td>COX IV, Va, Vb, VI, VII, VIIa, VIIb, COX VIII, VIA</td>
<td>- Required for assembly and function of complex IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Required for regulation of complex IV function?</td>
</tr>
</tbody>
</table>

**Table 5.1. Proteins required for complex IV assembly and function in *S. cerevisiae***

The table shows some of the proteins identified by analysis of complex IV mutants in *S. cerevisiae* that are involved in complex IV assembly and function. COXI, II and III are the subunits of complex IV encoded by the mitochondrial genome. The other complex subunits (COXIV, Va, Vb, VI, VIa, VIIb, VII, VIIa and VIII) are encoded by the nuclear genome. Note that the COXVIII and VIA protein subunits are not essential for complex IV assembly and function.
5.2. RESULTS 1 – ANALYSIS OF THE COX90 PROTEIN

The absence of complex IV activity in M90 has arisen due to the deletion of one gene (COX90), which encodes a protein of 105 amino acids (chapter four). In order to determine a role for this protein in complex IV activity, the aims of this chapter were to 1) analyse the COX90 protein sequence to investigate whether the primary sequence would provide clues as to the protein function and 2) investigate the size, abundance and cellular location of the COX90 protein using antibodies raised to the recombinant protein.

5.2.1. Analysis of the COX90 protein sequence

The analysis of the COX90 protein sequence included the analysis of its composition, a homolog search, a search for functional motifs within the sequence and analysis of its secondary structure for the presence of a mitochondrial targeting sequence and transmembrane spanning domains.

The compositional analysis of the COX90 protein sequence was performed using the Lasergene protein analysis programme. The results of this analysis are shown in tables 5.2 a and b. COX90 is a relatively small, neutral protein. It has a molecular weight of 11682.40 Da, an isoelectric point of 7.10 and contains at least one of all the 20 amino acids.

Homologs to COX90 were searched for by comparing its protein sequence to all the protein sequences maintained within the NCBI and MITOP databases and also to conceptional translations of all the EST sequences maintained within the TIGR database (table 5.3). The BLASTP (NCBI), tBLASTn (TIGR) and FASTA (MITOP) analysis programmes were used to make these comparisons. No significant matches were found in any of the searches.
Chapter five

a)  

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Whole protein</th>
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<tr>
<td>Molecular Weight</td>
<td>11682.40 kDa</td>
</tr>
<tr>
<td>Length</td>
<td>105aa</td>
</tr>
<tr>
<td>1 microgram =</td>
<td>85.599 pMoles</td>
</tr>
<tr>
<td>Molar Extinction coefficient</td>
<td>16620 ± 5%</td>
</tr>
<tr>
<td>( \text{A(280)} ) =</td>
<td>0.70 mg/ml</td>
</tr>
<tr>
<td>Isoelectric point</td>
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<tr>
<td>Charge at pH 7</td>
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</table>

b)  

### Whole protein composition analysis

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<tr>
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<th>% by weight</th>
<th>% by frequency</th>
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<tr>
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<tr>
<td>Acidic (DE)</td>
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<td>Polar (NCQSTY)</td>
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<td>Hydrophobic (AILFWV)</td>
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<tr>
<td>A Ala</td>
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<tr>
<td>C Cys</td>
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<tr>
<td>D Asp</td>
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<td>0.95</td>
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<tr>
<td>E Glu</td>
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<td>F Phe</td>
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<tr>
<td>H His</td>
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<td>K Lys</td>
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<td>6.78</td>
<td>6.67</td>
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<tr>
<td>M Met</td>
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<td>2.86</td>
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<td>3.46</td>
<td>3.81</td>
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<td>5.94</td>
<td>6.67</td>
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<td>W Trp</td>
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<tr>
<td>Y Tyr</td>
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<td>5.59</td>
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<tr>
<td>B Asx</td>
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<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Z Glx</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
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<td>0</td>
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</tr>
<tr>
<td>. Ter</td>
<td>0</td>
<td>0.00</td>
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</table>

Table 5.2. Analysis of the COX90 protein sequence

The COX90 protein sequence was analysed using a protein analysis programme. Characteristics of the whole protein are shown in table a and the results of the analysis of the amino acid composition of the protein are shown in table b.
The search for conserved functional motifs within the COX90 protein sequence and analysis of its secondary structure was performed using the PROSITE, Pfam, SignalP, MitoProt, DAS and TMpred secondary databases (table 5.3). The PROSITE and Pfam analysis and SignalP and MitoProt analysis found no functional motifs or mitochondrial targeting signals within the protein sequence. However, the DAS and TMpred analysis indicated that the protein contains a possible 20 amino acid transmembrane spanning domain located towards the N-terminal region of the protein. The transmembrane spanning domain predicted by the DAS programme (amino acid 25 to 44) is shown in figure 5.1. The TMpred programme predicted the domain to be in a similar location (amino acid 29 to 48).

The analysis of the COX90 protein sequence did not suggest a possible role for the protein in complex IV function. However, the results showed a possible transmembrane domain was present in the sequence, suggesting that the protein may be located in the inner or outer mitochondrial membrane.

<table>
<thead>
<tr>
<th>Database</th>
<th>Role</th>
<th>Website</th>
</tr>
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<tbody>
<tr>
<td>MITOP</td>
<td>Stores mitochondrial DNA and protein sequences</td>
<td><a href="http://www.mips.biochem.mpg.de/proj/medgen/mitop">http://www.mips.biochem.mpg.de/proj/medgen/mitop</a></td>
</tr>
<tr>
<td>TIGR</td>
<td>Stores subset of DNA, EST and protein sequences</td>
<td><a href="http://www.tigr.org/">http://www.tigr.org/</a></td>
</tr>
<tr>
<td>Pfam</td>
<td>Screens for conserved functional motifs</td>
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</tr>
<tr>
<td>Signal P</td>
<td>Screens for target sequence</td>
<td><a href="http://www.cbs.dtu.dk/services/SignalP">http://www.cbs.dtu.dk/services/SignalP</a></td>
</tr>
<tr>
<td>MitoProt</td>
<td>Screens for target sequence</td>
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</tr>
<tr>
<td>DAS</td>
<td>Screens for transmembrane spanning domains</td>
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</tr>
<tr>
<td>TMpred</td>
<td>Screens for transmembrane spanning domains</td>
<td><a href="http://ulrec3.unil.ch/software/TMPRED_form.html">http://ulrec3.unil.ch/software/TMPRED_form.html</a></td>
</tr>
</tbody>
</table>

Table 5.3. The websites for the databases used to analyse the COX90 protein sequence
Figure 5.1. The COX90 protein transmembrane spanning domain

The hydrophobicity plot of COX90 determined using the DAS analysis programme. The dotted line cuts off a hydrophobic cluster of amino acids that may form a transmembrane spanning domain. The amino acids found in this hydrophobic cluster are highlighted in red in the sequence of the COX90 protein shown below the graph.
5.2.2. Raising antibodies to COX90

The strategy shown in figure 5.2 was used to raise antibodies to COX90. The COX90 cDNA was cloned into the multiple cloning site of the pMAL-c2 expression vector as an EcoRI - BamHI fragment. These sites were added to the ends of the cDNA by amplification with primers M90-M1 (5’gGAATTCatggccag ggcgcagat3’) and M90-M2 (5’cgGGATCCttactcgggctttgcgtcct3’), which placed an EcoRI site immediately upstream of the translation start and a BamHI site immediately downstream of the translation stop. Sequencing across the EcoRI restriction enzyme site, using the esseql primer (fig 4.8), which initiates sequencing 120 bp from the translation start in the COX90 gene sequence, confirmed that the cDNA sequence was cloned in-frame and downstream of the malE gene (fig 5.2).

The expression and isolation of the fusion protein was performed initially on a small scale to confirm that the strategy could be used to over-express and isolate COX90. To follow the induction process, protein fractions were removed from the induced E. coli cells before and sequentially after induction by IPTG, and analysed by SDS-PAGE (fig 5.3a). An increase in the presence of a protein of approximately 50 kDa was observed after IPTG induction (see arrow 1), with maximum protein present after three hours (lane 4). This band was assigned to be the MBP:COX90 fusion protein, which had been predicted to be 54.4 kDa (MBP is 42.7 kDa and COX90 is 11.7 kDa). The increased production of this protein on IPTG induction demonstrated that the COX90 protein was successfully over-expressed.

A second protein band of 42 kDa (arrow 2) was also seen to sequentially increase after induction. This was assigned to be the MBP, which is known to be 42.7 kDa. The increased production of this latter protein indicated that either 1) the MBP:COX90 fusion protein is unstable and/or partially degraded or 2) the COX90 region of the malE:COX90 gene fusion is sometimes not expressed, due to premature termination of translation.
1. The cDNA for the COX90 gene was cloned into the multiple cloning site of the pMAL-c2 expression vector downstream and in the same translational reading frame as the malE gene.

2. The plasmid was then transformed into E. coli. IPTG was added to induce the expression of the gene fusion and resulting synthesis of the MBP:COX90 fusion protein within the bacteria.

3. The fusion protein was isolated from the other bacterial proteins in one step using the MBP's affinity to amylose and maltose. It was then injected into rabbits to raise antibodies.

Figure 5.2. Strategy used to raise antibodies to the COX90 protein

Steps 1, 2 and 3 describe the strategy used to express and isolate the COX90 protein for antibody production. The sites used to clone the COX90 cDNA into the pMAL-c2 expression vector and the relative size of the COX90 protein to the MBP are illustrated. The sequence of the construct at the site of cloning, determined using the esseq1 primer (fig 4.8), is also shown.
Figure 5.3. Small scale expression and isolation of the MBP:COX90 fusion protein

The COX90-pMAL-c2 construct and pMAL-c2 vector (control) were transformed into *E. coli* and grown to log phase. SDS-PAGE gels (a) and (b) show the increasing production of the 54 kDa MBP:COX90 fusion protein (arrow 1), the 42 kDa MBP (arrow 2) and the 50.8 kDa MBP:LACZ protein (arrow 3) 0, 1, 2 and 3 hours after the addition of IPTG to the cultures. On the gel, the 54 and 50.8 kDa proteins appear smaller at approximately 50 and 48 kDa, respectively. The SDS-PAGE gel (c) shows the specific isolation of these induced proteins from the induced bacterial cell crude extracts by amylose resin purification.
A control IPTG induction experiment was performed in parallel using the pMAL-c2 vector only (fig 5.3b). In this case, a protein of approximately 48 kDa increased significantly following induction. This was assigned to be the 50.8 kDa MBP:LACZ fusion protein, which is expressed in the absence of cloning into the multiple cloning site (see arrow 3). More of this protein was produced than the MBP:COX90 fusion protein. This probably is a reflection of the relative stabilities of the MBP:LACZ and the MBP:COX90 fusion proteins.

To determine if the fusion protein could be successfully isolated from all other bacterial cell proteins by its ability to bind amylose resin, a crude extract of cells induced by IPTG for three hours (optimal conditions in induction experiment) was incubated with amylose resin. The protein that remained bound to this after washing was analysed by SDS-PAGE (fig 5.3c, lanes 1-3). Only the 54 kDa MBP:COX90 fusion protein (arrow 1) and 42 kDa MBP (arrow 2) bands were present on the gel. This demonstrated the MBP:COX90 fusion protein could be purified from all other bacterial proteins by its amylose binding ability. The purification of the 42 kDa MBP in addition to the fusion protein was not unexpected due to 1) the co-induction of both proteins on IPTG addition (fig 5.3a) and 2) the intrinsic amylose binding properties of the MBP. Only a 48 kDa band was present in the control sample (lane C), indicating the presence of purified MBP:LACZ.

On the basis of the positive results of the small-scale experiment, the MBP:COX90 was then induced and purified on a larger scale. Figure 5.4 shows the successfully purified fusion protein. This was used to raise antibodies to the COX90 protein. As the 42 kDa MBP was purified in equal quantities to the fusion protein, double the recommended amount of the purified protein sample was used to raise the antibodies. This was done commercially by the company Eurogentec.
Figure 5.4. Large scale isolation of the MBP:COX90 fusion protein.

A SDS-PAGE gel showing the protein purified (5, 10 and 25 μg) from a crude extract of IPTG induced *E. coli* cells. The protein was isolated by running the crude extract through an amylose column and then eluting in maltose. The 50 kDa protein is the MBP:COX90 fusion protein and the 42 kDa protein is the MBP.

Once antibodies were raised to the MBP:COX90 fusion protein, they were optimised for use in western analysis (section 2.5.9). The optimisation experiments demonstrated that the antibodies recognise both the MBP:COX90 fusion protein and the MBP.

5.2.3. Antibody specificity to the COX90 protein

To determine if the antibodies raised to the MBP:COX90 fusion protein recognise the COX90 moiety of the fusion protein, western analysis was performed. Purified protein sample isolated in the large-scale preparation was digested with factor Xa for 24 hours and probed with the antibodies raised to the MBP:COX90 fusion protein under optimal conditions. Factor Xa cuts between the MBP and the COX90 sequence at the recognition site: IEGR↓ (fig 5.2). Digestion of the MBP:COX90 fusion protein with the protease should separate the two proteins and therefore allow for the detection of the COX90 protein only in the western analysis. A control of uncut sample was also probed with the antibodies.

A protein of approximately 12 kDa binds to the antibodies in both the factor Xa cut and the uncut samples (fig 5.5). As the intensity of the protein binding band in the
cut sample was much greater than in the uncut sample, it was concluded firstly this protein was the 11.7 kDa COX90 and secondly, the antibodies raised to the MBP:COX90 do recognise the COX90 portion of the fusion protein.

![Western analysis of the MBP:COX90 fusion protein](image)

**Figure 5.5. Western analysis of the MBP:COX90 fusion protein**

Aliquots of 5 µg of factor Xa cut and uncut MBP:COX90 fusion protein from the large scale preparation were separated by SDS-PAGE and probed with the antibodies raised to the protein. The gel shows that a protein of approximately 12 kDa binds the probe in both the factor Xa cut and uncut sample. This is COX90. More of the protein is present in the cut sample. The large smear seen below the band could represent degraded MBP and COX90 protein that also binds to the antibodies.

### 5.2.4. Western analysis of *C. reinhardtii* and *Dunaliella salina*

The western analysis of *C. reinhardtii* was performed to: 1) show that the antibodies raised against the MBP:COX90 fusion protein were able to detect COX90 in the organism and 2) characterise the protein further and maybe from this, deduce a specific role for the protein in complex IV function.

To show the antibodies raised against the MBP:COX90 fusion protein were able to detect COX90 in *C. reinhardtii*, crude whole cell protein extracts isolated from *cw10* and M90 were probed with the antibodies under optimal conditions. The antibodies bound to a protein the size of COX90 in the *cw10* sample (fig 5.6).
Figure 5.6. Western analysis of *C. reinhardtii* and *D. salina*

Aliquots (100 µl) of crude whole cell protein extracts isolated from *D. salina* and *cw10, M90, M86, dum-1, dk-97* were separated on a denaturing gel, transferred to a PVDF membrane and probed with the antibodies raised against the MBP:COX90 fusion protein. A protein of 12 kDa was detected by the antibodies in *cw10, dum-1* and *dk-97*. Proteins of 14 and 35 kDa were detected by the antibodies in *D. salina*. The 75 kDa protein detected in the *C. reinhardtii* samples and the low intensity bands probably represent non specific binding of the primary or secondary antibody.
This demonstrates that the antibodies are able to detect the protein within C. reinhardtii. The protein was not detected in the M90 sample (fig 5.6). As the M90 mutant lacks a copy of the COX90 gene, this phenotype was expected. The nonspecific binding of the antibody to another C. reinhardtii protein (75 kDa band) indicated that equal amounts of crude protein extract were analysed in both samples.

To characterise the COX90 protein further, its synthesis in the C. reinhardtii respiratory mutants M86, dk-97 and dum-1 was assessed. M86 and dk-97 lack complex IV function and the dum-1 mutant lacks complex III function. The presence of the protein in the closely related green alga, D. salina, was also assessed. Crude whole cell protein extracts were isolated from D. salina, M86, dk-97, and dum-1 and then probed with the antibodies raised to the MBP:COX90 fusion protein.

Proteins of 14 and 35 kDa bound to the antibodies in the D. salina sample (fig 5.6). The detection of a protein (14 kDa) a similar size to COX90 indicates the protein may also be present in D. salina.

A protein the size of COX90 (11.7 kDa) bound to the antibodies in the cw10, dum-1 and dk-97 sample (fig 5.6). This demonstrates that the two mutants synthesise the protein. However, as the intensity of the protein-binding band was much greater in cw10, it was concluded that either the mutants synthesise the protein at a reduced rate compared to cw10 or the turnover rate of the protein is increased in the mutants. COX90 was not detected in M90 and M86 (fig 5.6). This result indicated that either the protein is not synthesised in the M86 mutant or the protein is degraded at a rate equal to its synthesis.

The absence of COX90 in M86 could have arisen for one of two reasons. 1) The expression of the COX90 gene is affected in M86 as a result of either a disruption of COX90 i.e. the M86 mutation is allelic to the M90 mutation, or a second gene
required for the expression of COX90. 2) A failure in the biogenesis of cytochrome oxidase and the consequent degradation of the COX90 protein. In photosynthetic mutants of *C. reinhardtii* and respiratory mutants of *S. cerevisiae*, it has been demonstrated that the absence of assembly of photosynthetic and respiratory complexes may result in the accelerated degradation or reduced synthesis of other subunits of the complex (Choquet *et al.*, 1998 and Calder and McEwen, 1991). COX90 therefore may be a subunit of complex IV that is degraded or not synthesised in M86. These two reasons can also be applied to explain the reduced abundance of COX90 in *dk-97* (fig 5.6).

Support for the role of COX90 as a subunit of complex IV is provided by the reduced abundance of the protein in the *dum-1* mutant. In *S. cerevisiae*, the loss of function of one complex has been observed to sometimes result in increased turnover of other functional, but redundant (due to the mutation) complexes. If this is occurring in the *dum-1* mutant in respect to complex III and IV, a reduction in the level of the COX90 protein, if it is a subunit of the complex, would be expected.

5.2.5. Northern analysis of the M86 mutant

To determine if the absence of COX90 in M86 had arisen due to the aberrant expression of the *COX90* gene, northern analysis of the mutant was performed. Total RNA was extracted from M86 and probed with the COX90 cDNA obtained in section 2.4.5. The analysis was also performed with *cw10* and M90 as a positive and negative control respectively.

A transcript of 1.1 kb hybridised to the COX90 cDNA in both the *cw10* and M86 samples (fig 5.7). This demonstrates that the *COX90* gene is present and expressed in M86. The transcript was absent in M90 as expected (fig 5.7).
Figure 5.7. Northern analysis of the M86 mutant

Total RNA was extracted from cw10, M86 and M90 and probed with the COX90 cDNA. A transcript of 1.1 kb hybridised to the probe in both the cw10 and M86 sample. Equal amounts of the RNA were loaded for each sample (data not shown).

It was concluded from these results that the absence of COX90 in M86 has arisen either due to the lack of translation of the COX90 transcript in the mutant or the increased turnover of the COX90 protein, as opposed to a disruption of the COX90 gene or a factor required for its expression.

5.2.6. Locating COX90 within the cell

In order to determine if COX90 functions as a subunit of complex IV, the location of the protein within the C. reinhardtii cell was established by further western analysis. It was predicted that COX90 would be located in the mitochondria as a component of complex IV if it was a subunit of the enzyme.

In order to determine if COX90 was located in the mitochondria, mitochondria were isolated from cw10, M86 and M90 and probed with the antibodies raised to the MBP:COX90 fusion protein after separation on a denaturing gel. A protein of approximately 12 kDa bound to the antibodies in the cw10 sample (fig 5.8a). As this protein represents the COX90 protein, this result indicates that the protein is located in the mitochondria in C. reinhardtii. COX90 was absent in M86 and M90 confirming again that they both lack the protein (fig 5.8a).
Figure 5.8. Locating COX90 within the *C. reinhardtii* cell

In figure (a), 50 μg aliquots of mitochondrial proteins extracted from *cw*10, M90 and M86 cells were separated on a denaturing gel, transferred to a PVDF membrane and probed with the antibodies raised against the MBP:COX90 fusion. A protein of 12 kDa was detected in the *cw*10 sample. The 30 kDa protein detected in all samples probably represents non specific binding of the primary or secondary antibody. In figure (b), the respiratory complexes of mitochondria extracted from *cw*10 (5 μg per lane) and human cells (4 μg per lane) were separated on a blue native gel, transferred to a PVDF membrane and probed with antibodies raised against the MBP:COX90 fusion protein, the yeast COXIII subunit and the human COXI subunit. The antibodies detected one complex in each sample.
To determine if COX90 is a component of complex IV, the respiratory complexes of the mitochondrial fraction isolated for cw10 in the above experiment were separated by BN-PAGE and probed with antibodies raised to the MBP:COX90 fusion protein. BN-PAGE separates active water-soluble and membrane complexes in the range from 100 to 1000 kDa. As complex IV has not been detected before in *C. reinhardtii*, two control experiments were performed in parallel to this analysis firstly to confirm any complex detected by the MBP:COX90 antibodies is complex IV and secondly, to allow the determination of the size of the complex in *C. reinhardtii*. In the first control, the respiratory complexes of the cw10 mitochondria were separated as before and then probed with anti-yeast COXIII monoclonal antibodies (DA5 – a gift from J-W Taanman, Royal Free Hospital). In the second control, respiratory complexes of human mitochondria (prepared by S. Williams, Royal Free Hospital) were separated by BN-PAGE and probed with anti-human COXI monoclonal antibodies (106EIA8 – a gift from J-W Taanman, Royal Free Hospital). Both antibodies detect complex IV.

A single protein complex was detected in all three samples (fig 5.8b). This complex is the same size in both of the cw10 samples (fig 5.8b). As the COXIII antibody binds to complex IV, this result indicated that the complex detected using the MBP:COX90 antibodies is complex IV. It was therefore concluded that COX90 is a component of complex IV.

The complex detected in the human sample is slightly smaller than the complex detected in the two cw10 samples (fig 5.8b). The *C. reinhardtii* complex IV was therefore concluded to be slightly larger than 200 kDa, the size of the human complex IV.

To demonstrate that the complex detected by the COXIII and MBP:COX90 fusion protein antibodies is complex IV, the ability of this complex to transfer electrons from cytochrome c (cyt c) to oxygen was assessed by measuring activity following
BN-PAGE. The respiratory complexes of three aliquots of the mitochondria extracted from cw10 were separated by BN-PAGE. One of the samples was incubated in a solution that stains complexes displaying complex IV function in a polyacrylamide gel brown to identify the position of complex within the gel. The reaction assayed in this process is shown in figure 5.9. The reduction of diaminobenzidine (DAB) by cyt c causes the brown staining of the complex. The other two samples were probed with the antibodies raised against the MBP:COX90 fusion protein and COXIII protein. This allowed a direct comparison to be made between the position of the complex binding the antibodies and the position of the complex displaying complex IV activity. The complex detected in the activity assay was expected to be in the same position as the complex detected by the COXIII and MBP:COX90 fusion protein antibodies if the complex detected by the antibodies is complex IV.

![Diagram of DAB and cyt c reactions](image)

**Figure 5.9. Assay used for the in-situ staining of complex IV**

The complex detected in the activity assay was in the same position as the complex detected in the antibody probing (fig 5.10). This confirms that the complex detected by the COXIII and MBP:COX90 fusion protein antibodies is complex IV.

The results from this section show that COX90 is located in the mitochondria and is a component of complex IV. From this, it was concluded that the protein is a subunit of complex IV.
Figure 5.10. *In-situ complex IV activity staining*

Two 2.5 μg aliquots of mitochondria extracted from *cw10* were run on a blue native gel to separate the respiratory complexes and then transferred to a PVDF membrane. Lanes (a) and (b) show the probing of these samples with the antibodies raised against the MBP:COX90 fusion protein (a) and the yeast COXIII protein (b). In the same experiment, a 50 μg aliquot of the *cw10* mitochondria was also run on a blue native gel. The resulting gel was incubated in the complex IV activity stain. Lane (c) shows the complexes stained during this assay. Bands of a similar size were observed in all three results.
5.2.7. Expression of *C. reinhardtii* complex IV genes

To determine the effect changes in environmental conditions have on the expression of COX subunits of *C. reinhardtii*, the COXI, COXIII and COX90 mRNA steady state levels were assessed in wild type cells (cw10) grown in various conditions. In a photosynthetic organism, growth in the absence of light or a carbon source may affect the expression of these genes, as may growth in gametogenesis-inducing conditions.

Total RNA was extracted from wild type cw10 cells that were grown in 1) the presence of a carbon source and light (mixotrophic), 2) in the absence of light, but presence of a carbon source (heterotrophic), 3) in absence of carbon, but presence of light (phototrophic) and 4) in gametogenesis-inducing conditions (mixotrophic but with 1/10th the nitrogen). This RNA was then probed sequentially with the COXI *C. reinhardtii* gene, the COXIII *C. reinhardtii* EST and the COX90 cDNA. The *C. reinhardtii* COXI and COXIII genes are located within the mitochondrial and nuclear genomes respectively. The total RNA was finally probed with *C. reinhardtii* rDNA, which hybridises to ribosomal RNA, to show equal loading. The probe details are shown in table 5.4.

<table>
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<th>Probe</th>
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<td>COXI gene</td>
<td>1.67 kb</td>
<td>PCR product generated using the primers: 745 (5' ggaattcgaactaaagaagacattcta 3') and 746 (5' cgggatcctatgacacgcgtaacagctc 3').</td>
<td>Colin et al., 1995</td>
</tr>
<tr>
<td>COXIII EST</td>
<td>1.5 kb</td>
<td>cDNA clone excised from pBluescript</td>
<td>Asamizu et al., 2000</td>
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<td>COX90 cDNA</td>
<td>0.75 kb</td>
<td>RT PCR product (fig 4.10)</td>
<td>This thesis</td>
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<tr>
<td>rDNA</td>
<td>3.6 kb</td>
<td>Genomic sequence excised from pUC8</td>
<td>Nikaido et al., 1994</td>
</tr>
</tbody>
</table>

Table 5.4. Details of the COXI, COXIII, COX90 and rDNA probes
From previous analysis, the COXI, COXIII, COX90 and rDNA probes were predicted to hybridise to a 1.7 kb (Vahrenholz et al., 1985), 1.7 kb (Perez-Martinez et al., 2000), 1.1 kb (fig 4.12, this thesis) and 3.27 kb (Michaelis et al., 1990) transcript, respectively.

The COXI, COXIII, COX90 and rRNA transcripts were successfully detected in all the cw10 samples (fig 5.11). As the equal abundance of the rRNA transcripts (slightly reduced in the phototrophically grown sample) demonstrated similar amounts of the cw10 RNA samples were probed, this result suggests that the changes in growth conditions do not alter the expression of the COX genes.

The pattern of expression of all three transcripts in the different growth conditions is the same. The transcripts detected in cw10 cells grown under heterotrophic conditions are present at a similar level to the corresponding transcripts detected under mixotrophic conditions (fig 5.11 – lanes a and b). This suggests that light has no affect on the expression of each transcript.

The transcripts detected in cw10 cells grown under phototrophic conditions are present at a slightly reduced level compared to corresponding transcripts detected under mixotrophic conditions (fig 5.11 – lanes a and c). This suggests that the absence of a carbon source down-regulates the expression of each transcript. However, as slightly less of the phototrophically grown cw10 RNA sample was loaded onto the gel (fig 5.11- rDNA), the reduced abundance of the transcripts in this sample is more likely to reflect unequal loading.

The transcripts detected in cw10 cells grown under gametogenesis-inducing conditions are present at a slightly increased level compared to the corresponding transcripts detected under mixotrophic conditions (fig 5.11 – lanes a and d). This suggests that gametogenesis upregulates the expression of each transcript.
Figure 5.11. Northern analysis of *cw10*

Total RNA was extracted from *cw10* cells grown in mixotrophic (a), heterotrophic (b), phototrophic (c) and gametogenesis-inducing (d) conditions. The blots show the sequential probing of this RNA with the *C. reinhardtii* COXI gene, COXIII EST, COX90 cDNA and rDNA genes. The sizes of the transcripts detected are indicated.
5.3. RESULTS 2 – THE BASIS OF THE DEFECT IN M86

In order to determine the specific function of the gene(s) affected in the M86 mutant, further analysis of the mutant phenotype was performed.

5.3.1. Expression of the mitochondrial COXI gene.

In *S. cerevisiae*, a large number of nuclear encoded factors have been identified that are required specifically for the expression of complex IV subunits encoded by the mitochondrial genome (Pel *et al.*, 1992). To determine if the gene(s) affected in M86 encode such a factor, the expression of the *COXI* gene, the only complex IV subunit encoded by the mitochondrial genome in *C. reinhardtii*, was investigated.

Total RNA was isolated from M86 and probed with the COXI PCR product (section 5.2.7). The analysis was also performed with the wild type strain *cw*10 and the two mutant strains, M90 and M31. M31 is another strain from the collection of respiratory mutants generated by insertional mutagenesis.

The 1.7 kb *COXI* transcript was detected in the *cw*10, M90 and M31 sample (fig 5.12a). However, it was absent in the M86 sample. This indicates that the mutant is unable to express the *COXI* gene. To confirm this, the blot was stripped and reprobed with the chloroplast gene, *psbC* (details shown in table 5.5). This detects a transcript of 2 kb. Transcripts of equal intensity and the expected size hybridised to the probe in all three samples (fig 5.12a). As the expression of this gene should be wild type in all the mutants, it was concluded that equal amounts of RNA were loaded for each sample and therefore, the phenotype seen for M86 is real.
Figure 5.12. Expression of M86 mitochondrial genes

Figure (a) shows total RNA extracted from cw10, M31, M86 and M90 cells probed with the *psbC*, *COXI* and *CYTB* genes. Figure (b) shows total RNA extracted from cw10, M86 and M90 cells probed with the *psbC* and *ND2* genes. The sizes of the transcripts in each probing are indicated.
Chapter five

<table>
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<th>Probe</th>
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<th>Source</th>
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<tr>
<td>psbC gene</td>
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<td>CYTB gene</td>
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<td>PCR product generated using the primers: NAD 2.1 (5' c agctgtaacattcttctgg 3') and NAD 2.2 (5' acageccacctaat aacg 3')</td>
</tr>
</tbody>
</table>

Table 5.5. Details of the psbC, CYTB and ND2 probes

The absence of the RNA transcript in M86 indicates that the gene(s) affected in the mutant is required for the expression of the COXI transcript. To determine if the affected gene(s) was required for the expression of other genes contained in the mitochondrial genome, the expression of the CYTB and ND2 genes were examined. The ND2 and COXI genes are transcribed from the mitochondrial genome together as part of the rightward precursor transcriptional unit (fig 5.13). The CYTB gene is transcribed separately to these genes as part of the leftward precursor transcriptional unit (fig 5.13).

In order to examine the expression of the CYTB gene in M86, the RNA blot from the COXI and psbC probing was stripped and reprobed with the gene detailed in table 5.5. From previous analysis, the CYTB gene was expected to hybridise to a transcript of 1.25 kb (Michaelis et al., 1990). A transcript of the expected size was detected in all samples (fig 5.12a). This indicates that all samples are able to express the CYTB gene. However, the abundance of the transcript in M86 is reduced compared to the others (fig 5.12a). As the previous probing with psbC had demonstrated equal amounts of RNA were loaded for each sample, it was concluded that M86 is defective in CYTB gene expression.
Figure 5.13. The mitochondrial genome of *C. reinhardtii*

The two large arrows indicate the position of transcription start between the ND5 and COXI gene and also the direction of transcription. The genes amplified by PCR and used as probes.

In order to examine the expression of the ND2 gene in M86, total RNA was extracted from M86, M90 and cw10 and probed firstly with the ND2 gene (table 5.5) and then with the *psbC* gene. Transcripts of 1.15 kb and 2 kb were expected to hybridise to the ND2 (Vahrenholz *et al.*, 1985) and *psbC* probes respectively.

The ND2 transcript is detected in all three samples (fig 5.12b). The abundance of the transcript in both M86 and M90 appears reduced compared to cw10. In the case of M86, the transcript is only just detectable. As the *psbC* probing shows equal amounts of RNA were loaded (fig 5.12b), this result suggests that the M86 mutant is also defective in ND2 gene expression.
5.4. DISCUSSION

5.4.1. The role of the COX90 protein

The results of this chapter show that COX90 is a subunit of complex IV.

The absence of COX90 in M86 (fig 5.6) initially indicated that the protein might act as a subunit of complex IV. As M86 fails to assemble complex IV and the accelerated degradation or reduced synthesis of complex subunits is commonly observed in mutants of S. cerevisiae and C. reinhardtii unable to assemble respiratory or photosynthetic complexes respectively, it was concluded that maybe COX90 is a subunit of complex IV that is degraded or not synthesised in M86 as a consequence of the incorrect assembly of the complex in the mutant.

Support for this theory was provided by two results. 1) The detection of the COX90 transcript in M86 (fig 5.7). This demonstrates that the loss of the protein in M86 occurs at a post-transcriptional stage, and is not due to either an allelic mutation in M86 or mutation in a secondary gene required for the expression of the COX90 gene. 2) The reduced abundance of the protein in the complex III defective C. reinhardtii mutant, dum-1 (fig 5.6). As the increased turnover of redundant complexes (and therefore complex subunits) is sometimes observed in S. cerevisiae mutants lacking a particular complex function, this result suggested that the COX90 protein might be a subunit of complex IV.

The ability of the antibodies raised against the MBP:COX90 protein to bind to a C. reinhardtii complex (fig 5.8b) that displayed complex IV activity (fig 5.9) confirms that COX90 is a component of complex IV and therefore is a subunit of the complex. Complex IV has been purified from C. reinhardtii and its subunit composition analysed (Bennoun et al, 1995). A number of small subunits (ranging
from 10 to 14 kDa) are present in the complex. It is possible that COX90 represents one of these proteins.

Two interesting points arise from the analysis of the COX90 protein. Firstly, no homologous proteins were identified in the primary database searches. COX90 is a small, nuclear encoded protein that probably represents one of the supernumerary subunits of complex IV found in other eukaryotic species (section 1.2.5). It contains the same overall features as most of these supernumerary subunits i.e. a hydrophilic N-terminus, a hydrophobic transmembrane domain and a hydrophilic C-terminus (section 5.2.1 and Capaldi, 1990). As conservation of these supernumerary subunits between the lower and higher eukaryotic species has been demonstrated, it was expected that a homolog to the COX90 protein would be identified in the database searches.

The inability to identify a homolog to COX90 in the primary database searches could have arisen for two reasons. 1) Poor conservation between COX90 and its homolog in other species. The supernumerary subunits of different organisms show only poor conservation at the primary sequence level (Capaldi, 1990). It is possible that functional homologs to COX90 are present in the primary databases, but were not detected in the searches due to too much diversity of sequence between COX90 and its homolog. 2) COX90 represents a novel subunit of complex IV.

Attempts were made to identify a homolog to COX90 by forced alignments between the sequences of the supernumerary COX subunits from the human and yeast (S. cerevisiae and N. crassa) complex IV and the COX90 sequence (data not presented). However, as these proved unsuccessful, the true reason for the absence of a homolog to COX90 was not established.

The second interesting point to arise from the analysis of COX90 is its lack of an N-terminal mitochondrial targeting sequence. In plants, yeast and mammals, most
mitochondrial proteins encoded by the nuclear genome are initially synthesised in
the cytosol as precursor proteins that contain a cleavable N-terminal extension
sequence that targets the protein to the mitochondria (Whelan and Glaser, 1997).
This sequence is removed by a mitochondrial processing peptidase to produce the
mature protein once the protein reaches the mitochondrial matrix or its specific
location within the mitochondria.

The absence of a targeting sequence in COX90 was initially indicated by the
SignalP and MitoProt database analysis of the protein (section 5.2.1). These
databases search for features generally found in all targeting sequences, such as a
potential to form an amphiphilic structure, high content of basic and hydroxylated
amino acids and the presence of a cleavage site for the mitochondrial processing
peptidase. Further support for the targeting sequence absence was provided by the
demonstration that the protein, once expressed and integrated into complex IV, was
the same size as that predicted from its gene sequence (fig 5.6). If the protein had a
cleavable N-terminal targeting sequence, a decrease in size of the protein would
have been expected.

Examples of nuclear encoded mitochondrial proteins that do not contain an N-
terminal targeting sequence have been found in some plants species (Whelan and
Glaser, 1997) and in C. reinhardtii (the cytochrome c protein – Atteia and Franzén,
1996). These proteins are thought to be targeted to the mitochondria by an intrinsic
targeting sequence that is part of the mature protein sequence. It is possible that
COX90 is targeted to the C. reinhardtii mitochondria by a similar mechanism.

5.4.2. Expression of the respiratory genes COXI, COXIII and COX90.

Study of the external factors that regulate respiratory gene expression is an
important step towards understanding the interplay that occurs between the
chloroplast, mitochondria and nucleus to meet the constantly changing demands of
the cell. So far, the only analysis of respiratory gene expression in C. reinhardtii has been performed with cytochrome c (Felitti et al., 2000) and the alternative oxidase (Vanlerberghe and McIntosh, 1997). In the cytochrome c expression studies, by analysing the steady state-mRNA levels, it was demonstrated that cytochrome c expression is induced on illumination and on acetate addition. This induction was concluded to be mediated probably by the metabolites (possibly carbohydrates) derived from photosynthesis and acetate incorporation.

The results of the mRNA steady-state expression studies performed with the C. reinhardtii respiratory genes COXI, COXIII and COX90 (fig 5.11) are different to those obtained for cytochrome c. The expression of the COX genes are not affected by the presence or absence of acetate or light (fig 5.11 – lanes a -c). This pattern of expression could have arisen for one of three reasons. 1) The genes are constitutively expressed and not affected by changes in environmental conditions. 2) The genes are constitutively transcribed, but regulated at a translational or post-translational level. 3) The genes are induced by similar environmental changes observed for cytochrome c, but due to different experimental conditions, these changes cannot be observed. If either of the later two suggestions apply, then it is possible that the expression of cytochrome c and the COX genes are under the control of the same environmental stimuli. Further analysis needs to be performed to determine this.

The COXI, COXIII and COX90 gene expression analysis also indicates that gametogenesis induces the expression of the genes (fig 5.11 – lane d). As gametogenesis is energy-requiring and photosynthesis ceases during the process, the slight increase in the expression of respiratory genes can be explained as a response to this energy demand, although the signalling pathways that mediate this are unknown (Beck and Haring, 1996).
5.4.3. The role of the affected gene in M86

The results of this chapter show the that gene affected in M86 is required for the expression of the mitochondrial \textit{COXI} gene. This was demonstrated by the inability to detect the \textit{COXI} transcript in total RNA extracted from the mutant (fig 5.12).

As the genes contained within the mitochondrial genome are expressed initially as two large precursor transcripts (fig 1.12), the affected gene in M86 could possess one of three possible functions. 1) The gene codes for a protein that is required for the transcription of the precursor transcript containing the \textit{COXI} mRNA. However, as M86 appears to have wild type complex I function (table 3.2), which demonstrates that the complex I subunits transcribed on the same precursor transcript as \textit{COXI} are expressed, this function is unlikely. 2) The gene codes for a protein that is required for the processing of the \textit{COXI} mRNA from the precursor transcript. 3) The gene codes for a protein that is required for the stabilisation of the \textit{COXI} mRNA once processed. Either of the later two possibilities is feasible.

The reduced expression of the other two mitochondrial genes \textit{ND2} and \textit{CYTB} in M86 (fig 5.12) has probably arisen due to a secondary effect caused by either the incorrect processing of the \textit{COXI} mRNA from the precursor transcript or the lack of complex IV due to the absence of the COXI subunit. This reduction in transcript level does not appear to be significant, as wild type levels of complex I and III activity are present in the mutant (table 3.2 and 3.3).

To conclude then, the results of this chapter show that the gene affected in the M90 protein is a subunit of complex IV and the gene affected in M86 is a factor required for the expression of the mitochondrial encoded COXI protein, which is also a subunit of complex IV.
CHAPTER SIX - DISCUSSION

6.1. SUMMARY

The results of this thesis show that the respiratory defective phenotype in M86 and M90 has arisen due to the specific loss of complex IV function caused as a direct result of the integration of the \textit{ble} marker into the nuclear genome (chapter three). M86 is unable to assemble the complex whereas M90 is able to assemble the complex, but only at a very reduced level compared to wild type. All other enzyme functions required for respiration appear to be present in the mutants.

The characterisation of M90 at a molecular level demonstrates that the loss of complex IV function in the mutant has arisen due to the deletion of one gene, which codes for a protein of 105 amino acids (chapter four). The gene contains all the features of a typical nuclear gene of \textit{C. reinhardtii} e.g. it is GC rich, contains an intron and has a bias for a G or C in the third position of each codon. The ease with which the RNA and protein gene products are detected indicates that the gene is relatively highly expressed. The analysis of the gene product, the COX90 protein, show that the protein is approximately 12 kDa, possesses a putative transmembrane spanning domain and is a probable membrane spanning subunit of complex IV (chapter five). The protein does not contain an N-terminal mitochondrial targeting sequence.

The analysis of mitochondrial gene expression in M86 demonstrates that the loss of complex IV function in the mutant has arisen due to a defect in the expression of \textit{COXI}, a gene that codes for one of the core subunits of complex IV (chapter five). As the \textit{COXI} transcript is absent in M86, this indicates that the affected gene in the mutant codes for a trans-acting factor, the COX86 protein, that is required either for the transcription of the \textit{COXI} gene or the processing and stabilisation of the \textit{COXI} transcript. The expression of two other mitochondrial genes, \textit{CYTB} and \textit{ND2}, is also
reduced in the M86. This could be a secondary effect caused by the absence of COXI expression.

The biogenesis of complex IV in the yeast *S. cerevisiae* requires communication between both the nucleus and mitochondria. Both genomes encode structural subunits of complex IV. In addition, the nuclear genome encodes trans-acting factors that aid in the expression of the mitochondrial subunits, co-factor import and biosynthesis, and complex assembly. The division of the genes encoding the complex IV subunits between the nuclear and the mitochondrial genome and the determination of the role of the affected gene in M86 as a trans-acting factor required for the expression of the mitochondrial COXI gene provide support for a similar system in *C. reinhardtii*. A model demonstrating the possible nuclear-mitochondrial interactions that may occur in complex IV biogenesis in *C. reinhardtii*, which includes the roles of the affected genes in M86 and M90, is shown in figure 6.1. Further study of complex IV biogenesis in the alga is required to support or disprove this model.

6.2. FURTHER WORK

6.2.1. M86

The gene affected in M86 is a trans-acting factor required for the expression of the COXI gene. The factor either aids in the transcription of COXI, the processing of COXI mRNA from the precursor transcripts or the stabilisation of processed COXI mRNA. A number of obvious experiments could be performed that would allow the determination of the specific role of the factor in COXI expression.
Figure 6.1. A model for the action of COX86 and COX90

COX86 (a) is required for the expression of the mitochondrial COXI gene and COX90 (b) is a subunit of complex IV. Other factors involved in complex IV biogenesis are shown on the right hand side with dotted arrows. The green boxes represent genes for subunits; the red boxes are genes for factors required for the expression of the mitochondrial COXI gene; the blue box are genes for factors required for the cofactor import and biogenesis and the pink box are genes for factors required for complex assembly.
A run-on transcription assay, whereby all genes transcribed are radiolabelled, would demonstrate if the affected gene in M86 is required for the transcription of the \textit{COXI} gene. The absence of any hybridisation between the radiolabelled transcripts and \textit{COXI} DNA fixed on a solid support would indicate that the affected gene is required for the transcription of \textit{COXI}. However, the presence of the \textit{ND2} transcript in M86 (fig 5.12), which is transcribed along with the \textit{COXI} gene, and the lack of identification in \textit{S. cerevisiae} of trans-acting factors required specifically for the transcription of individual mitochondrial genes suggest that the affected gene in M86 is unlikely to code for a factor involved in \textit{COXI} transcription.

In \textit{S. cerevisiae}, mutants defective in RNA processing tend to accumulate precursor transcripts containing the affected gene (Kloeckener-Gruissem et al., 1987). The absence of accumulated precursor transcripts in M86 when probing with the \textit{COXI} gene (fig 5.12) suggests that the affected gene in the mutant is unlikely to be required for the correct processing of the \textit{COXI} mRNA from its precursor transcript. To test this, the ability of recombinant COX86 protein to cleave the \textit{COXI} precursor transcript \textit{in vitro} could be assessed.

An RNA degradation assay would demonstrate if the affected gene in M86 encodes a factor required for the stabilisation of the \textit{COXI} transcript. In this assay, radiolabelled \textit{COXI} transcript would be incubated in mitochondrial extracts of wild type and M86 cells and the rate of degradation of the transcript in the two different environments compared. A faster rate of degradation of the transcript in M86 indicates that the gene affected in the mutant is a \textit{COXI} mRNA stability factor.

The cloning of the affected gene in M86 would allow the characterisation of its gene product, the COX86 protein. This would aid in the determination of the specific function of the protein in \textit{COXI} gene expression in a number of ways. Firstly, the location of the protein within the cell could be determined using antibodies raised against the recombinant protein. If the protein is situated in the mitochondria, the
site of COXI gene expression, this would provide strong support for a direct role of COX86 in COXI gene expression. Secondly, searches of primary and secondary databases could be performed with the protein sequence. This may result in the identification of homologs to the protein or conserved motifs (such as an RNA binding motif – Draper, 1995) within the protein sequence that may indicate the specific role of the protein in COXI expression. Thirdly, RNA binding studies with the recombinant protein could be performed to determine if the protein interacts with the COXI mRNA. For this, radiolabelled COXI is incubated with COX86 and then run through a polyacrylamide gel. If COX86 binds to the COXI mRNA, the transcript will travel through the gel at a reduced rate compared to COXI mRNA only. If the protein does bind the mRNA, assessing the ability of the protein to bind deleted forms of the transcript using the same experimental technique, would identify the protein binding site within the transcript.

6.2.2. M90

The gene affected in M90 codes for a subunit of complex IV. The specific role of the protein (COX90) in complex IV function is not known. It could be required for the correct assembly of the complex, the stabilisation or dimerisation of the complex or the regulation of complex function. There is no obvious experimental approach to use to determine which of these functions applies to COX90.

However, a number of simple experiments can be performed that would further characterise the protein and maybe provide insight into its function. These include: 1) structure/function studies, whereby altered forms of the COX90 gene are transformed into the COX90 null mutant (M90) to see if the mutant is still rescued to respiratory competency. Possible alterations include site-directed changes of amino acids to alter the structure of the protein and progressive deletions to the C- or N-terminal regions of the protein. 2) Isolation of suppressor mutants of the M90
mutant phenotype and the genetic and molecular characterisation of the suppressing genes.

Other directions for future work with M90 include the analysis of the factors required for the successful expression of the affected gene in the mutant i.e. is the 5' untranslated region (UTR) of COX90 and the intron essential for its expression? It has already been demonstrated that the 3' UTR is not essential for the expression of the gene (D. Brown and S. Purton, unpublished). As the DNA sequence of the COX90 gene plus the 5' UTR and intron sequence was cloned in this thesis, the establishment of the importance of these regions could be determined simply by creating deletion constructs of the pAS1.9 plasmid (chapter four) that lack parts of the 5' UTR and intron sequence and then assessing the ability of these constructs to rescue M90 to respiratory competency. If a deletion construct is unable to rescue M90, this would indicate that the deleted region is important for gene expression. These results would provide valuable insight into *C. reinhardtii* nuclear gene expression.

6.2.3. Development of *C. reinhardtii* as a model for understanding respiration

The ability of *C. reinhardtii* to dispense with respiration and the development of a range of tools for the genetic manipulation of the alga makes it an ideal model to use to study respiration. It is possible to generate a large number of respiratory defective mutants and characterise them, as the results of this thesis demonstrate. Possible future work for this project would be to develop the organism further as a model for respiratory studies.

Simple screening methods for the identification of respiratory mutants of a particular phenotype is one way in which the organism could be developed. In the yeast, *S. cerevisiae*, two simple screening methods are used to determine the phenotype of respiratory mutants that could be developed for use in *C. reinhardtii*. 203
1) Respiratory mutants defective in complex IV function are rapidly identified using a simple colour detection test with N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD). When overlaid with the compound, mutant colonies that lack complex IV function remain the same colour, whereas wild type colonies with complex IV function turn blue (McEwen et al., 1985). Preliminary experiments with complex IV mutants in *C. reinhardtii* indicate that the screening method will work with the alga (data not presented).

2) Some respiratory mutants defective in copper import are identified by their ability to be rescued to respiratory competency by the addition of high concentrations of exogenous copper to the growth medium (Glerum et al., 1996). Initial screening of all the respiratory mutants of *C. reinhardtii* generated by insertional mutagenesis did not demonstrate if this assay could be used to identify copper import mutants in the alga as none were rescued to respiratory competency (data not presented). Development of the technique requires a copper import defective mutant that is rescued by high concentrations of exogenous copper.

Taking advantage of the development of *C. reinhardtii* genomics is another way in which the alga can be developed as a model for respiration. Many ESTs coding for proteins required for the biogenesis of the respiratory chain complexes have been identified (http://www.Kazusa.or.jp/en/plant/chlamy/EST). The use of these ESTs to perform molecular and biochemical analysis in *C. reinhardtii* would provide valuable information on the factors required for respiratory chain biogenesis in the alga and also support or disprove the model predicted for the process in figure 6.1.

A final way that would considerably improve the use of *C. reinhardtii* for respiratory studies would be the development of a simple system to isolate the complexes of the chain for subsequent biochemical analysis. One approach could be to his-tag a subunit of the complex and use its new ability to ligand nickel (due to
the presence of the histidines in the tag) to isolate the complex it is bound to. Attempts to isolate *C. reinhardtii* complex IV using this technique have already begun whereby the COX90 protein has been his-tagged at the C-terminus (D. Brown and S. Purton, unpublished).
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