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CHARACTERIZATION OF A NOVEL GENE INVOLVED IN MITOCHONDRIAL BIOGENESIS

BY 

YI ZHANG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

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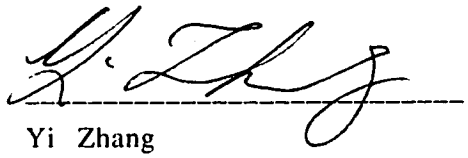

Yi Zhang
11119-84 ave.
Edmonton, AB
T6G OV9

August 17, 1993

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Characterization of a Novel Gene Involved in Mitochondrial Biogenesis submitted by Yi Zhang in partial fulfilment of the requirements for the degree of Master of Science.

Bernard Lemire

Bernard Lemire

William Bridger

Carol Cass

Frank E. Nargang
(External Examiner)

August 16, 1993

To my parents

ABSTRACT

The mitochondrial electron transport chain which is essential for yeast respiration requires about 200 nuclear gene products for its biogenesis and function. Yeast nuclear (petite) mutants have been very useful in identifying and studying these products.

This thesis describes studies on a yeast nuclear petite mutant, *tcm10*. The original *tcm10* mutant, isolated by UV-mutagenesis, can not grow on glycerol, a non-fermentable carbon source. Genetic studies have shown this phenotype is due to a single nuclear gene mutation. The *TCM10* gene has been isolated by complementation screening with a yeast genomic library, cloned, and sequenced. It consists of a 2052 bp open reading frame that encodes a 684 amino acid protein. Hydrophobicity analysis does not suggest any potential transmembrane regions. Databank searching has not identified any proteins with high sequence identity to TCM10.

The chromosomal *TCM10* locus in a haploid strain was replaced by the targeted insertion of a *TRP1* disrupted *TCM10* gene showing that the *TCM10* gene is not essential for yeast viability, but is required for respiratory function. The *TCM10* coding region was amplified by a polymerase chain reaction, and cloned into the *E. coli* expression vector, pJF118EH, behind the strong tac promoter. TCM10 expression was very poor, possibly due to the presence of low usage codons. Polyclonal antibodies were raised against the N-terminus of TCM10 overexpressed in *E. coli* as a fusion protein with trpE. The antiserum proved useful in determining the intracellular localization of TCM10. TCM10 is a mitochondrial protein associated with the inner membrane. Therefore, the TCM10 protein is encoded by a nuclear gene, synthesized in the

cytosol, and translocated into mitochondria. Its N-terminus has the characteristics of a mitochondrial targeting sequence being enriched in positively charged amino acids and devoid of negatively charged ones. However, we were unable to demonstrate *in vitro* import of the TCM10 protein into isolated mitochondria.

Due to instability of the mitochondrial genome, biochemical characterization of *tcm10* mutants was performed in strains also bearing the *pet9* allele, which product is involved in the function of ADP/ATP carrier. Several respiratory chain enzyme activities are shown to be greatly decreased compared with the wild type TCM10 controls. The cytochrome contents of *tcm10* mutants are lower than those of controls. The levels of cytochrome c_1 are most affected. Surprisingly, catalase activities in the soluble fraction from the disruption mutant cells are decreased about 50 fold as compared to a 2 fold decrease in the mutagenized mutant. This might indicate that TCM10 is only indirectly involved in mitochondrial respiration. *tcm10* mutations have pleiotropic effects on electron transport chain enzymes. Although we have been unable to firmly establish it, we believe that mutations in the TCM10 protein have their effects on mitochondrial function through their impairment of heme metabolism.

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AAC	ADP/ATP carrier
ADP	adenosine 5'-diphosphate
Amp	Ampicillin
ATP	adenosine 5'-triphosphate
bp	base pair
CAI	codon adaptation index
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CoxIII	subunit III of cytochrome *c* oxidase
COX4	gene for subunit IV of cytochrome *c* oxidase
COX5A	gene for the major form of subunit V of cytochrome *c* oxidase
CTT1	catalase T gene
CYC1	gene for iso-1-apocytochrome *c*
CYC7	gene for iso-2-apocytochrome *c*
CYT1	gene for cytochrome c_1
cyt	cytochrome
DHFR	dihydrofolate reductase
EDTA	ethylene diaminetetra acetic acid
ETC	electron transport chain
ExoIII	exonuclease III
FAD	flavin adenine dinucleotide
$FADH_2$	reduced FAD
FCCP	carbonyl cyanide p-trifluoromethyosy-phenylhydrazone
Fe/S	iron sulfur cluster (center)
FMN	flavin mononucleotide
G1P	*sn*-glycerol-1-phosphate

HEM1	gene for δ-aminolevulinate synthase
hsp	heat shock protein
IgG	immunoglobulin protein G
IM	mitochondrial inner membrane
IMS	mitochondrial intermembrane space
IPTG	isopropyl β-D-thiogalactopyranoside
ISP	import site protein
kDa	kilodalton
kb	kilobase
M	mitochondrial matrix
MAS	mitochondrial assembly
MIP	mitochondrial intermediate peptidase
MOM	mitochondrial outer membrane
MPP	matrix processing peptidase
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
NAD	nicotine adenine dinucleotide
NADH	reduced NAD
ND	not detectable
NPD	nonparental ditype
OD	optical density
OM	mitochondrial outer membrane
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PD	parental ditype

PEP	processing enhancing protein
pet	nuclear petite mutation
pI	isoelectric point
PMS	phenazine methosulphate
PMSF	phenylmethylsulfonyl fluoride
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulfate
T	tetratype
TEMED	tetramethylethylenediamine
tet	tetracycline
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
TTC	2, 3, 5-triphenyltetrazolium chloride
UV	ultraviolet
UAS	upstream activating sequence
vol	volume
wt	weight
$\Delta\psi$	membrane potential
$\Delta\mu_H$	proton motive force

A gene is represented by three letters followed by an Arabic number in italics, eg., *CYC3*; whereas, a protein will appear in plain text. A wild type gene or protein is described with capital letters; in contrast, a mutant is in small letters, eg., the wild type *TCM10* gene and the mutated *tcm10* gene.

CHAPTER I. INTRODUCTION

A. YEAST MITOCHONDRIA-THE ENERGY FACTORY

The yeast *Saccharomyces cerevisiae* is a fast-growing, unicellular eukaryote. Unlike the bacterium, *Escherichia coli*, yeast has most of its genetic material in a nucleus separated from cytoplasm; it also has most of the other organelles present in higher eukaryotic cells, such as the endoplasmic reticulum, and mitochondria. Moreover, yeast nuclear DNA replication, transcription, translation, chromosome segregation, and gene regulation are much more like a high eukaryote's (Rose and Harrison, 1969).

Yeast can grow both anaerobically and aerobically. During anaerobic growth, the energy required is produced by substrate level phosphorylation; in contrast, during aerobic growth, energy is also derived from oxidative phosphorylation within the mitochondria. Yeast cells do not normally have respiring mitochondria during anaerobic growth; the activity of the electron transport chain is significantly lower than under aerobic conditions. Dramatic increases in the activities of several electron transport enzymes can be observed after a yeast culture has been switched from anaerobic to aerobic growth conditions (Kim and Beattie, 1973). As a facultative anaerobe, *Saccharomyces cerevisiae* can utilize glucose in the presence or absence of oxygen. However, in the presence of glucose, it generates much more energy from the metabolism of glucose than from oxidative phosphorylation because glucose represses the formation of active electron transport complexes. Upon release from glucose repression, yeast will rapidly assemble active enzyme complexes into a functional electron transport chain. This feature is very

useful for biochemical and genetic studies on the expression of genes for yeast mitochondrial proteins as well as on mitochondrial assembly.

S. cerevisiae has two opposite mating types, MATa and MATα, that can mate to form stable diploids. Under conditions of carbon and nitrogen starvation, diploids will sporulate to form four ascospores, a so called tetrad, which can be separated by dissecting techniques with the help of a micromanipulator (Rose and Harrison, 1969). Studying the segregation of a marker gene amongst ascospores can discriminate between single and multiple gene mutations as well as nuclear and mitochondrial gene mutations. These characteristics make yeast a valuable system for biochemical, molecular biological, and genetic studies on mitochondrial biosynthesis.

Yeast mitochondria contain their own independently replicating mitochondrial DNA (mtDNA) genome which encodes a limited number of proteins including some subunits of the electron transport chain. However, the majority of proteins in the various mitochondrial biosynthetic pathways, including enzymes involved in energy generation and mtDNA biosynthesis is encoded by nuclear DNA, synthesized as precursor proteins in the cytoplasm, and then transported into mitochondria. There, the precursor proteins are processed and assembled into functionally active enzyme complexes (Grivell, 1989; Attardi and Schatz, 1988; Pon and Schatz, 1991).

1) Structure and Function of Mitochondria

The structure of the mitochondrion has been well studied at the subcellular level. Basically, a mitochondrion consists of a double membrane structure, that defines a four compartment entity, consisting of the outer membrane, the inner membrane, the intermembrane space, and the matrix. The inner membrane is folded to form special structures: cristae, which project towards the inside of a mitochondrion. The intramitochondrial

distribution of proteins and enzymes is not equal amongst compartments (Whittaker and Danks, 1978; Munn, 1974) (Figure 1-1). Components of the protein import apparatus are localized in both the inner and outer membranes. The electron transport chain (or respiratory chain) and the ATP synthase (or F_0F_1-ATPase) are located in the inner membrane where they carry out oxidative phosphorylation in the presence of oxygen. Most of the enzymes of the citric acid cycle (or tricarboxylic acid cycle, TCA cycle) are in the matrix where they generate reduced intermediates such as NADH that can be reoxidized through the electron transport chain. Yeast mitochondrial DNA replication, transcription, and translation occur in the matrix (Whittaker and Danks, 1978; Mathews and van Holde, 1990).

As the site of the TCA cycle and oxidative phosphorylation, the mitochondrion can well be described as the energy factory of the cell simply because the majority of ATP is made within mitochondria through respiration. ATP can also be generated by glycolysis, however, much less than by oxidative phosphorylation. Amino acid and sugar catabolism, as well as the TCA cycle, produce the reduced electron carriers, NADH and $FADH_2$ that feed into the electron transport chain. In fact, in mammalian cells as well as in yeast, 1 mole of glucose can generate six moles of reduced electron carriers (5 NADH and 1 $FADH_2$) through six dehydrogenation steps: one in the glycolytic pathway, one in the reduction of pyruvate to acetyl-CoA, and four more in the TCA cycle. The latter five steps occur in mitochondria. Reoxidation of these reduced electron carriers by the sequential action of respiratory chain enzyme complexes in the inner mitochondrial membrane produces most of the energy for ATP synthesis (Mathews and van Holde, 1990) (Figure 1-2).

It is estimated that about 70 proteins are involved in oxidative phosphorylation in most eukaryotes. They are organized into five

multisubunit enzyme complexes, namely complexes I, II, III, IV, and V (Figure I-3). The first four form the electron transport chain while complex V is the ATP synthase (Mathews and van Holde, 1990; Gennis, 1989; Hatefi, 1985). These complexes are all distributed in the mitochondrial inner membrane. Complexes I, III, and IV were first purified from bovine-heart mitochondria in the early sixties. Much more is now known about the structure and mechanism of the bovine enzymes than from other sources.

Noteworthy are several non-protein moieties that are directly involved in electron transport. They are FAD, FMN, iron-sulfur (Fe/S) clusters, hemes, and protein-attached coppers. FAD is a prosthetic group of complex II, and FMN of complex I or the NADH dehydrogenase in *S. cerevisiae*. The isoalloxazine ring portion of riboflavin participates in the oxidoreduction reactions (Figure I-4). Fe/S clusters are found in complexes I, II, and III. Fe/S clusters are formed by the covalent binding of non-heme iron atoms to the proteins via cysteine sulfurs or to other iron atoms by the sulfur bridges. There are several kinds of Fe/S clusters differing in the number of iron atoms and non-cysteine sulfur in the cluster (Figure I-5) (Mathews and van Holde, 1990). The one iron one sulfur (FeS) cluster is the simplest form; however, it is not often found in the electron transport chain. The two iron two sulfur (Fe_2S_2) cluster is found in complexes I, II, and III. The four iron four sulfur (Fe_4S_4) cluster is found in complexes I and II. The three iron four sulfur (Fe_3S_4) cluster is only found in complex II. Heme is the prosthetic group of cytochromes. The hemes in cytochrome *c* and c_1 are covalently linked to the polypeptide chain, but not those in *b*-type cytochrome. A modified form of heme, called heme A, is the prosthetic group of cytochrome aa_3 in complex IV. Two chemically identical heme A moieties are attached to the same protein, but present in different local environments (Mathews and van Holde, 1990;

Hatefi, 1985). Two copper ions are also found in complex IV. Each of the coppers is associated with a heme. Changes between the +2 and +3 oxidation states of heme iron and the +1 and +2 states of the copper ions in complex IV are responsible for the electron transfer. Therefore, the cytochromes are single electron carriers.

The NADH-ubiquinone reductase or complex I catalyzes the reduction of coenzyme Q (ubiquinone to ubiquinol) by passing electrons from NADH to ubiquinone. Complex I of bovine heart contains about 40 different polypeptide chains ranging from 10 to 70 kilodalton (kDa). Complex I can be divided into three subcomplexes: a flavin containing part (Fp), an iron-sulfur center containing part (Ip), and an extremely hydrophobic part (Hp) embedded in the inner membrane. The flavin containing subcomplex is composed of three subunits and a tightly bound FMN, whereas the iron-sulfur center containing part comprises about six subunits and a number of iron-sulfur centers. Little is known about the hydrophobic part.

Unlike mammalian mitochondria, it is now clear that yeast mitochondria do not have a typical complex I that can be inhibited by rotenone or piericidin (de Vries and Marres, 1987). In mammalian mitochondria, seven subunits of the enzyme are encoded by mitochondrial DNA. In contrast, these genes are not present on the mitochondrial DNA of *S. cerevisiae*. Two internal NADH dehydrogenases of yeast mitochondria are responsible for the oxidation of NADH produced in the matrix by the operation of the TCA cycle, but only one of them is coupled to site I phosphorylation and it is not present in cells at log phase growth. Under conditions of carbon and nitrogen limitation (during stationary phase), *S. cerevisiae* is able to synthesize the internal NADH dehydrogenase that is coupled to site I phosphorylation. However, the enzyme is insensitive to rotenone. In addition to these internal

dehydrogenases, yeast also contains an outer membrane NADH dehydrogenase that uses cytochrome *c* as the electron acceptor and is antimycin A insensitive. The internal rotenone-insensitive NADH dehydrogenase has been purified (De Vries and Grivell, 1988). It consists of a single subunit (*Mr* 53,000) with one molecule of FAD, has NADH specificity and reacts with Q_6 (the natural quinone in *S. cerevisiae*), Q_6 analogs, and ferricyanide.

Complex II or the succinate-ubiquinone reductase links the oxidation of succinate to the reduction of ubiquinone. This complex likely has four polypeptides in yeast. The yeast genes for the A and B subunits have been cloned and sequenced (Robinson and Lemire, 1992; Lombardo and Scheffler, 1989; Lombardo *et al.*, 1990). The A subunit of about 70 kDa contains a covalently bound flavin and the B subunit of about 27 kd is believed to have three iron-sulfur centers. It seems that the A and B subunits form the active site and the other two smaller subunits (C and D) act as anchors to hold the AB dimer to the membrane. A *b*-type heme is also found in one of the two small subunits. All subunits of complex II are encoded by nuclear genes, synthesized in the cytosol, and then transported into mitochondria. Succinate dehydrogenase (SDH) is the major component of this complex and thought to be composed of subunits A and B. SDH is the only membrane bound enzyme of the TCA cycle.

Complex III, also known as the ubiquinol-cytochrome *c* oxidoreductase or *bc*$_1$ complex, passes electrons from ubiquinol to cytochrome *c* (cyt *c*). In yeast, the complex consists of nine subunits. The primary sequences have been determined by cloning and sequencing the respective genes (de Vries and Marres, 1987). One subunit, cytochrome *b* (cyt *b*), is encoded by mitochondrial DNA and synthesized in mitochondria (de Zamaroczy and Bernardi, 1985). Other subunits are nuclear encoded and cytosolically

synthesized as precursors. The redox centers of complex III are cytochromes *b*562, *b*566, *c*1, and an Fe2S2 cluster. Cytochrome *c*1 (cyt *c*1) is oriented such that most of the subunit faces the intermembrane space. Cyt *b* is believed to be submerged in the inner membrane bilayer due to its inaccessibility to surface acting agents and to the extreme difficulty of extraction from the membrane (Whittaker and Danks, 1978). This asymmetric orientation of the complex is useful for the unidirectional translocation of protons. The communication between complexes I and III and complexes II and III is mediated by ubiquinone. Cyt *c* connects complex III with complex IV (Whittaker and Danks, 1978; Lenaz, 1986).

Complex IV, the cytochrome *c* oxidase, or cyt *aa*3 complex, catalyzes the last step in electron transport from cyt *c* to oxygen. There are eleven different subunits in the yeast enzyme (de Vries and Marres, 1987). The three largest subunits are encoded by the mitochondrial DNA and synthesized inside mitochondria. The six smaller subunits are encoded by nuclear DNA, synthesized in the cytosol and imported into mitochondria. The sequences of most of the subunits have been obtained by polypeptide and/or DNA sequencing. The two A-type hemes in this complex are designated *a* and *a*3 whereas the two coppers are named as Cu_a and Cu_{a3}. It has been suggested that complex IV contains two redox centers with different apparent redox potentials. The first redox center is heme *a*:Cu_a which is the recipient of electrons from cytochrome *c*, and the second is heme *a*3:Cu_{a3}. Heme *a*3 is the last electron carrier in the electron transport chain and is involved in the reduction of oxygen. Moreover, heme *a* is on intermembrane space side where it has access to cyt *c*, and heme *a*3 is on the matrix side of the inner membrane accessible to oxygen (Whittaker and Danks, 1978).

Complex V, also known as the ATP synthase or F_0F_1-ATPase, catalyzes the phosphorylation of ADP to ATP. The F_0F_1-ATPase, observed under the electron microscope as stacked particles on matrix side of the inner membrane, consists of F_0 and F_1 portions. The F_0 and F_1 portions can be easily separated by mild salt washing. The F_0 portion is integrated into the membrane bilayer and is thought to form a specific channel for the influx of protons into matrix. It also functions as an anchor for the F_1 portion. The F_1 portion contains the active site for ATP synthesis (Mathews and van Holde, 1990; Gennis, 1989). The mitochondrial F_0F_1-ATPases are composed of α, β, γ, δ, ε, OSCP (Oligomycin sensitivity-conferring protein), F_6 (coupling factor 6), IF_1 (ATPase inhibitor protein), F_B (coupling factor B), subunits as well as the mtDNA-encoded subunits 6, 8, and 9 (the DCCD-binding proteolipid). Most of genes for these subunits have been sequenced.

In addition to the complexes mentioned above, there are other less abundant enzymes that are linked to mitochondrial electron transport. Noteworthy are the mitochondrial lactate and *sn*-glycerol-3-phosphate (G3P) dehydrogenases. Mitochondrial *sn*-glycerol-3-phosphate dehydrogenase, so called Q-linked G3P dehydrogenase or glycerol-3-phosphate:Q_6 oxidoreductase, is a membrane bound enzyme catalyzing the transfer of reducing equivalents from its substrate to ubiquinone (de Vries and Marres, 1987; Cottingham and Ragan, 1980). It is normally repressed by glucose and induced by growth on glycerol.

Lactate dehydrogenases are present in several different types in yeast. These enzymes are specific for either D- or L-lactate. Two unique mitochondrial D- and L-lactate dehydrogenases (D- and L-lactate:cytochrome *c* oxidoreductases) are induced by lactate under aerobic growth conditions. Each enzyme is induced only by its specific lactate isomer. They both catalyse

the oxidation of lactate to pyruvate. The electrons are passed onto cyt *c* to enter the electron transport chain. Only the L-lactate:cytochrome *c* oxidoreductase has been well studied. Its gene has been isolated and sequenced. The open reading frame encodes a 591 amino acid precursor protein with an 80 amino acid presequence (Guiard, 1985). This enzyme has been shown to contain FMN and cytochrome b_2 (de Vries and Marres, 1987). Therefore, it is also called flavocytochrome b_2.

2) Mechanism of the Energy Generation

Three hypotheses have been put forward for the mechanism of the energy generation: the chemical coupling theory (Slater, 1953), the chemiosmotic coupling theory (Mitchell, 1969), and the conformational coupling theory (Boyer, 1964). Today, widely accepted is the chemiosmotic coupling theory, proposed by the great British biochemist, Peter Mitchell. The chemiosmotic coupling theory is able to explain the action of uncouplers and inhibitors as well as the requirement for intact mitochondria for oxidative phosphorylation. Fundamentally, this theory proposes that protons are pumped out of the matrix by the electron transport chain into the intermembrane space. As the inner membrane is a barrier for charged molecules including protons, the protons in the intermembrane space accumulate. Consequently, the proton potential or proton gradient across the inner mitochondrial membrane is built up to form the proton motive force. When protons do flow back into the matrix, they do so through the F_0F_1-ATPase. Released energy is used to drive ATP synthesis. Based on this theory, the pH of the intermembrane space should be lower than that in the matrix in an active respiring mitochondrion. In fact, evidence has shown that it is about 1.4 units lower than in the matrix. The proton gradient also generates a

membrane potential of about 0.14 volts across the membrane. As indicated in the equation:

$$\Delta\mu_H = \Delta\psi - 2.3\,RT\,\Delta pH/F$$

$\Delta\mu_H$ is the proton gradient, or the proton motive force; $\Delta\psi$ is the membrane potential in volts; ΔpH is the pH difference between the outside and the inside; F is Faraday's constant (96.5 kJ $volt^{-1}$ mol^{-1}); R is the gas constant (8.314 J/K mol), and T is the absolute temperature (K). A ΔpH of -1.4 units and a $\Delta\psi$ of 0.14 V correspond to a $\Delta\mu_H$ of +0.224 V. This value converts to 21 KJ of energy per mole of protons, suggesting that at least two protons are required for the synthesis of 1 molecule of ATP (the standard free energy change, $\Delta G^{\circ\prime}$, of ATP hydrolysis is about -32 KJ /mol).

Agents that reduce $\Delta\psi$ or ΔpH will reduce the proton motive force, and uncouple electron transport from phosphorylation (Mathews and van Holde, 1990; Gennis, 1989). For instance, valinomycin, a cyclodepsipeptide antibiotic that forms a complex with monovalent cations such as K^+, can easily cross the inner membrane. Consequently, it collapses the transmembrane potential ($\Delta\psi$) by transferring potassium ions across the inner membrane without affecting the pH difference (ΔpH). Uncouplers such as CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and FCCP (carbonyl cyanide p-trifluoromethyosy-phenylhydrazone) are weak acids. The protonated forms are electrically neutral and can easily cross the membrane. In contrast, the deprotonated forms can also cross the membrane, but at 1% the rate. Consequently, CCCP and FCCP increase the membrane permeability to protons and allow them to reach electrochemical equilibrium across the inner membrane. Thus, these uncouplers affect both $\Delta\psi$ and ΔpH (Gennis, 1989).

Respiratory chain proteins that function as proton pumps should be accessible from both the inner and outer sides of the inner membrane.

Electron carriers should also be asymmetrically oriented in the inner membrane to account for the unidirectional movement of protons. For example, cyt *c* contacts cyt *a* of cytochrome *c* oxidase only on the outer side of the inner membrane and electrons accepted are passed onto cyt *a3* which faces the matrix side. The asymmetry of respiratory chain proteins has been demonstrated by the use of antibodies, proteolytic enzymes and membrane impermeable labeling reagents that react only from the agent accessible side (Whittaker and Danks, 1978; Mathews and van Holde, 1990; Gennis, 1989).

Strong support for the chemiosmotic coupling model came from early experiments with bacteriorhodopsin, a protein able to pump protons in a light dependent reaction. One such experiment consisted of reconstituting both bacteriorhodopsin and the F_0F_1-ATPase into artificial vesicles (Figure 1-6). Upon illumination, bacteriorhodopsin begins to pump protons into the vesicles and generates a proton gradient that is able to drive ATP synthesis by the F_0F_1-ATPase. This is one of the most convincing arguments in support of phosphorylation being driven by an electrochemical potential even in the absence of an electron transport chain.

B. BIOGENESIS OF YEAST MITOCHONDRIA

Mitochondria have their own genomic DNA which encodes only a small number of products, including seven proteins involved in oxidative phosphorylation, several RNA processing enzymes, a complete set of tRNAs for interpreting mitochondrial DNA codons and some rRNAs (Attardi and Schatz, 1988; Tzagoloff and Myers, 1986). However, the majority of mitochondrial proteins, including the enzymes of mtDNA replication, transcription and translation, is encoded by nuclear genomic DNA. These

proteins are first synthesized in the cytosol and subsequently transported into mitochondria. The mitochondrial import machinery found in the outer and inner membranes mediates the import of protein across one or both membranes. There is some evidence that some tRNAs and other RNAs may be imported into mitochondria of some organisms (Doersen *et al.*, 1985; Suyama, 1986). As discussed below, some codons of mtDNA encode amino acids different from the universal codons. Therefore, the biogenesis of mitochondria is a very complicated process which requires the proper coordination of the two genetic systems.

1) Yeast Mitochondrial Genetic System

The mitochondrial DNA is distinct from the nuclear genomic DNA. First, like bacterial DNA, yeast mtDNA is circular and variable in sizes from 70 kb to 80 kb (Hollenberg *et al.*, 1970; de Zamaroczy and Bernardi, 1985). Second, the genes are loosely organized. Third, many mtDNA genes, such as cytochrome *b*, cytochrome oxidase subunit I, and large rRNA genes are interrupted by introns. Fourth, the genetic code for mtDNA is different from the universal code; UGA encodes Trp instead of a stop codon, CUN codons specify Thr instead of Leu, and AUA encodes Met instead of Ile (Attardi and Schatz, 1988). Finally, yeast mitochondrial ribosomes are more closely related to those of prokaryotes, such as in their sensitivity to antibiotics that inhibit prokaryotic ribosomal large subunit, eg. chloramphenicol. However, they are not sensitive to inhibitors of the small prokaryotic ribosomal subunit such as streptomycin and kanamycin or of the eukaryotic 80 S ribosome, such as cycloheximide (Whittaker and Danks, 1978; Mathews and van Holde, 1990).

The transcription of mtDNA is fulfilled by an RNA polymerase encoded by nuclear DNA. The same enzyme also seems to be responsible for the priming of mtDNA replication. There are about twenty transcriptional

initiation sites possessing the conserved sequence, (A/T)TATAAGTA, in the mtDNA genome (Grivell, 1989). Many genes lacking a transcriptional start site will form a part of larger transcription units and be processed by an RNA maturase. Yeast mtRNAs are unlikely capped or polyadenylated, but they usually have 5'-untranslated sequences (Groot *et al.*, 1974; Li *et al.*, 1982; Fox, 1986). The regulation of mitochondrial genes is still poorly understood and always involves nuclear-encoded proteins (Groot *et al.*, 1974; Li *et al.*, 1982; Fox, 1986).

Different physiological conditions, such as the presence of oxygen, high glucose levels, or non-fermentable carbon sources, differentially affect the expression of mtDNA (Jakovic *et al.*, 1979; Attardi and Schatz, 1988). Mitochondrial mRNA levels show no apparent decrease during anaerobic growth and only a slight decrease was found upon growth at high glucose levels. This latter effect may result from the repression by glucose on the synthesis of the nuclear-encoded mitochondrial RNA polymerase. However, under anaerobic growth or high glucose levels, both nuclear and mitochondrial encoded protein levels decrease (Woodrow and Schatz, 1979; Ibrahim *et al.*, 1973; Falcone *et al.*, 1973). The key regulatory molecule seems to be heme, not only because it is required for the assembly of the respiratory chain complexes such as the cytochrome *c* oxidase and the cytochrome *bc*$_1$ complex but also because it is actually involved in the gene regulation as discussed below. Since heme synthesis requires oxygen and is repressed by glucose, both may regulate the expression of mitochondrial proteins through the levels of heme. The regulation seems to be post-transcriptional. The overall decrease in mitochondrial protein level seems to be due to the decreased stability of unassembled subunits as well as to the decreased translation of their mRNAs. However, heme may not be the only player.

Recent studies also show that oxygen and glucose act by heme independent pathways. For example, oxygen, but not heme, induces the expression at the level of translation of the nuclear gene *PET494* (gene involved in the translation of *CoxIII* mRNA) even though no mitochondrial gene has been found under this type of regulation (Forsburg and Guarente, 1989; Zitomer and Lowry, 1992).

2) Nuclear Genomic System

The nuclear genome plays an essential role in the biogenesis of mitochondria. As mentioned above, it encodes the majority of mitochondrial proteins including those necessary for the assembly of the multimeric enzyme complexes of oxidative phosphorylation, for the expression of mtDNA, and the mitochondrial import machinery.

Ephrussi and his colleagues noted about 40 years ago that single mutations in nuclear genes could abolish respiration (Ephrussi, 1953). Several thousand of these nuclear petite mutants (so called *pet* because they form small colonies on media with limiting glucose) have been identified (Tzagoloff and Myers, 1986), which have been divided into about 200 different groups on the basis of complementation analysis. These gene products may not all be directly involved in mitochondrial respiration. For example, nuclear proteins may affect the transcription of an electron transport chain subunit or may be a component of the mitochondrial ribosome. The consequences are similar to those mutations that prevent the utilization of nonfermentable carbon sources such as ethanol or glycerol in the generation of energy through the respiratory chain. The molecular basis of most nuclear *pet* mutants reported so far is still a mystery since many of these mutants have broad effects. *Pet* mutants fall into four major groups (Attardi and Schatz, 1988). The first group is mutated in genes encoding enzymes directly involved in the electron

transport and phosphorylation systems, such as subunits of the F_1-ATPase. This type of mutation usually has a specific phenotype. For example, if a subunit of the F_1-ATPase is defective, the mitochondria will not be able to use the proton motive force for the synthesis of ATP. As a consequence, such mutants will form petite colonies on media with low levels of glucose. These mutants should, however, have a fairly normal electron transport chain but be dysfunctional in phosphorylation. In contrast, most mutants of the second group have lower levels of all mitochondrial genome encoded proteins because the nuclear gene mutation has changed one of the components necessary for mitochondrial DNA expression such as the RNA polymerase (Greenleaf *et al.*, 1986), or ribosomal proteins (Myers *et al.*, 1987). These mutations have multiple effects on mitochondrial protein function. Moreover, most of these mutants quickly lose their intact mtDNA as mitochondrial protein synthesis is necessary to maintain a wild-type mitochondrial genome in yeast cells (Tzagoloff and Myers, 1986; Attardi and Schatz, 1988). Most of these molecular lesions are still understudied. The third group contains those mutants that are affected for mitochondrial protein import or assembly. These mutations are amongst the most difficult to deal with because they always have pleiotropic effects. So far, only a few of this kind have been reported (Yaffe and Schatz, 1984). For example, the yeast *MAS1* and *MAS2* genes encode two subunits of a matrix localized protease; they have been cloned, sequenced, and shown to be essential for yeast survival. Heat shock protein 60, ISP42, and heat shock protein 70 are involved in protein import and are also essential for yeast cell viability (Cheng *et al.*, 1989; Baker *et al.*, 1990; Baker and Schatz, 1991). The fourth group contains nuclear petite mutants that affect the expression of selected mitochondrial genes. For example, the nuclear gene, *PET494*, encodes a 56 kDa protein that interacts with the 5' untranslated leader

sequence of the mRNA for subunit III of cytochrome *c* oxidase, and mediates its translation by mitochondrial ribosomes (Fox, 1986). Subsequent studies have shown that translation of *CoxIII* mRNA requires not only the PET494 protein but also the products of at least three other nuclear genes. The translation of the cyt *b* mRNA also requires multiple nuclear gene products (Attardi and Schatz, 1988).

Most nuclear genes controling mitochondrial protein biosynthesis are regulated by other nuclear genes. Many have at least one specific region on their DNA for binding of transcriptional activators or repressors that are encoded by a variety of regulatory genes (Guarente and Mason, 1983; Pfeifer *et al.*, 1987). This region is termed an upstream activation site or *UAS* in yeast. *UAS*'s are functionally similar to the enhancer sequences of higher eukaryotic cells. Those proteins that bind to *UAS*'s are themselves regulated in response to physiological signals. Another key element of most eukaryotic promoters is the short conserved sequence TATA, called the TATA box, to which the basic transcriptional factors are bound. The transcriptional initiation of genes requires the cooperation of the transcriptional activators at *UAS*'s and the transcriptional factors at the TATA box.

The best characterized of the regulated nuclear genes, *CYC1*, encodes the mitochondrial protein iso-1-cytochrome *c*. This gene is regulated at the transcriptional level by a set of physiological signals such as oxygen, heme, and carbon source. The *CYC1* gene contains two *UAS*'s: *UAS1* (activated by HAP1) and *UAS2* (activated by HAP2/3/4); these form two independent regulatory systems (Forsburg and Guarente, 1989; Grivell, 1989). The HAP1 system (*UAS1*) controls the induction of *CYC1* by heme. The binding of HAP1 to *UAS1* is heme-dependent *in vitro*, suggesting that heme interacts directly with the HAP1 protein. An internal deletion of about 200 amino acids in the

sequence of HAP1 results in a heme-independent, constitutive phenotype for *CYC1* expression *in vivo*. These experiments suggest that this 200 amino acid internal sequence functions to prevent HAP1 from binding to *UAS1* in the absence of heme. Moreover, the internal sequence has a motif similar to other heme binding proteins. HAP1 is also involved in the regulation of other genes besides *CYC1*, such as *CYC7* (iso-2-cytochrome *c*), *CTT1* (catalase T), and *CYT1* (cytochrome c_1).

The HAP2/3/4 (*UAS2*) system, is a heteromeric regulatory complex that controls the repression of *CYC1* by glucose. HAP2 and HAP3 can bind DNA independently. They can also form a complex in solution. HAP4 has two functional domains: an acidic C-terminal region which is the major activation domain for the entire complex, and an amino terminal region required for DNA binding of the complex. HAP4 transcription is induced when cells are switched from glucose to a non-fermentable carbon source, while HAP2 and HAP3 are transcribed constitutively. It seems that HAP4 is the principal regulatory subunit of the complex. The HAP2/3/4 complex is also involved in the regulation of other genes, such as *COX4* (subunit IV of cytochrome *c* oxidase), *COX5A* (the major form of subunit V_A of cytochrome *c* oxidase), and *HEM1* (δ-aminolevulinate synthase, a heme biosynthetic enzyme).

C. MITOCHONDRIAL PROTEIN IMPORT AND ASSEMBLY

The vast majority of mitochondrial proteins is encoded by nuclear genes and synthesized as precursor proteins in the cytoplasm. Most of these precursor proteins contain amino-terminal presequences that are cleaved off after import into mitochondria. The presequences, also called signal sequences or targeting sequences, are usually enriched in positively charged

amino acid residues and devoid of negatively charged ones. Some mitochondrial proteins do not have the cleavable targeting sequences, such as MAS 70, the ADP/ATP carrier and apocytochrome *c* (Gennis, 1989; Hase *et al.*, 1983 and 1984; Hines *et al.*, 1990). In these cases, the targeting information is located with the mature protein sequence.

Import of proteins into mitochondria has been extensively studied. Import involves the following steps; first, after translation, presequences direct precursor proteins to mitochondria and are recognized by the receptor-like proteins on the outer membrane. Second, the precursor proteins are translocated into mitochondria at contact sites or sites where the two membranes are in close proximity. The membrane potential across the inner membrane is required to cross the inner membrane. ATP is often required and is thought to be involved in maintaining proteins in loosely folded or import competent conformations. Since proteins must undergo unfolding to pass through contact sites and subsequent refolding after import, a number of assembly factors known as molecular chaperones are involved. Third, processing of precursor proteins with cleavable prequences takes place after the presequences emerge from the inner membrane (Figure I-7). For proteins in the intermembrane space, a second process may also be involved as discussed below. Finally, the imported and processed proteins are sorted to their destinations and assembled into functional complexes.

1) The Features of Mitochondrial Targeting and Sorting Sequences

Cleavable mitochondrial targeting sequences are short amino-terminal segments enriched in positively charged and hydroxylated amino acids. They usually lack acidic amino acids or extended stretches of hydrophobic amino acids. A few exceptions to these common features have been reported. The

targeting sequence of yeast ADP/ATP carrier appears to be internal (Pfanner *et al.*, 1988; Smagula *et al.*, 1988; Adrian *et al.*, 1986). The targeting sequence of the 17 kDa subunit of the yeast cytochrome *bc*$_1$ complex is rich in acidic amino acids rather than basic (van Loon *et al.*, 1983). There are two general criteria used to functionally identify targeting sequences: the deletion of a targeting sequence will result in non-import of the protein; the fusion of a targeting sequence to the amino terminus of a cytosolic protein should result in the import of the hybrid protein (Hurt *et al.*, 1987; Horwich *et al.*, 1985 and 1986).

There is no consensus sequence in the targeting sequences of mitochondrial precursor proteins. However, many show a tendency to form amphiphilic α-helices. This property seems to be more important than a sequence motif. It has been shown that an artificial presequence containing only four kinds of amino acids (Arg, Leu, Ser, and Gln) is functional as long as it is amphiphilic (Allison and Schatz, 1986; Roise *et al.*, 1988). Although net positive charge is important, it is not sufficient. Analysis of presequences has shown that the positively charged amino acids tend to cluster on one side of a helical wheel representation and leave hydrophobic amino acids on the other side. Amphiphilic helices may be able to interact with negatively charged phospholipids in membranes. This interaction may help in the recognition and translocation of precursor proteins across membranes. If amphiphilic helices involved in targeting are to interact with the receptor-like proteins on the outside of mitochondria, they should be exposed on the proteins' surfaces. The cytosolic protein mouse dihydrofolate reductase (DHFR) contains an amphiphilic helix buried in its interior. This helix can function as a targeting signal only if placed at the amino terminus of the protein (Hurt and Schatz, 1987).

In addition to matrix targeting signals, sorting signals are required for imported proteins to get to other intramitochondrial locations, i.e., the inner membrane and the intermembrane space. In some cases, sorting signals are segments of uncharged, hydrophobic residues downstream of the matrix targeting signals. Removal of the sorting sequences will result in the proteins being imported into matrix. The mechanism by which sorting signals exert their effects on targeting remains controversial (Glick *et al.*, 1992; Hartl *et al.*, 1987).

2) Mitochondrial Outer Membrane Import Receptors and Import Machinery

A series of proteins function at contact sites between the outer and inner membranes to mediate the import of mitochondrial precursor proteins. Imported mitochondrial precursor proteins are recognized by protein receptors on the outer membrane and then transfered to contact sites. Several proteins have been identified in *Neurospora crassa* and *Saccharomyces cerevisiae* that function either as proteinaceous receptors or as parts of a proteinaceous channel. These are located on the outer membrane and include MOM19, MOM72 and MOM38 in *N. crassa* (Pfanner *et al.*, 1987; Söllner *et al.*, 1989), and MAS70, p32 and ISP42 in *S. cerevisiae* (Baker and Schatz, 1991; Hines *et al.*, 1990; Pain *et al.*, 1990; Murakami *et al.*, 1990; Vestweber *et al.*, 1989). Most are identified by specific antibodies against individual outer membrane proteins or by a crosslinking assay. The antibody binding should inhibit or slow down the import of precursor proteins because of competition for binding sites. Mitochondrial protein import can be blocked at low temperatures allowing the association between precursor proteins and receptors to be identified by cross-linking with specific reagents.

MOM19 and MOM72 (for mitochondrial outer membrane) have been identified as receptors in *N. crassa*. MOM19 is able to mediate the import of most mitochondrial proteins, including MOM72. In contrast, MOM72 is specific for AAC import. MOM19 and MOM72 have overlapping functions; both can mediate AAC import, and may serve as back-up receptors for each other (Söllner *et al.*, 1989; Söllner *et al.*, 1990). Both are not exclusively found in contact sites but are distributed over the whole outer membrane surface. About 50% of MOM72 and 15% of MOM19 are in contact sites as studied by electron microscopy and immunogold labeling.

The DNA sequences of the MOM19 and MOM72 genes have been determined. Both contain putative membrane-anchor sequences at their amino termini and carboxy-terminal hydrophilic domains that apparently protrude into the cytosol. A membrane bound 17 kDa proteolytic fragment of MOM19 is still capable of mediating the import of F1-ATPase ß-subunit (F1ß), but not of other precursor proteins. This suggests that different recognition sites may exist on MOM19. Mild protease treatment of MOM72 releases a 60 kDa soluble fragment and greatly slows AAC import. The 60 kDa portion may contain the binding site for AAC. About 25% of AAC can be imported by MOM19 after the removal of MOM72 (Söllner *et al.*, 1990).

The yeast counterpart of MOM72 is MAS70 (for mitochondrial assembly), a 70 kDa outer membrane protein. MAS70 import has been studied as a model for import of proteins to the outer membrane (Hines *et al.*, 1990). The N-terminal 11 amino acids contain mitochondrial targeting information followed by a hydrophobic membrane anchor found from residues 9 to 38 (Hase *et al.*, 1984). The C-terminal region of MAS70 has no effect on targeting and sorting. Treatment of MAS70 with low levels of trypsin also releases a 60 kDa hydrophilic domain. MAS70 function seems to be located in this

hydrophilic domain since the import of several precursor proteins as well as the binding of AAC are inhibited by trypsin treatment. Anti-MAS70 immunoglobulin protein G (IgG) inhibit import of the F1ß subunit, AAC, and several other precursor proteins into isolated mitochondria by about 50 to 80%. They have little effect on the import of porin. Furthermore, mitochondria isolated from a *mas*70 mutant have decreased import rates for several precursors. MAS70 is mainly found at the contact sites. It may act as an import receptor on the outer membrane. That the MAS70 gene is not essential for cell viability, has led to the prediction that there are other receptors on the outer membrane. The yeast 17 kDa outer membrane protein is thought to be the homologue of MOM19 (Pon and Schatz, 1991).

Another receptor is a 32 kDa outer membrane protein, p32. The p32 protein was identified by using anti-idiotypic antibodies raised against the primary antibodies recognizing the signal peptide of subunit IV of cytochrome *c* oxidase (pCoxIV) (Pain *et al.*, 1990). The approach was based on the belief that some of the anti-idiotypic antibodies would mimic the pCoxIV signal peptide and thus be able to bind an import receptor. The p32 protein is probably not synthesized with a cleavable presequence (Murakami *et al.*, 1990). It is enriched at contact sites and can not be digested by low levels of trypsin, yet has epitopes exposed on the outer membrane surface that can be recognized by anti-p32 antibodies. Its role as an import receptor is supported by the observations that mutations of the p32 gene, *MIR*32, and anti-p32 antibodies both block the import of several precursor proteins.

ISP42 (import site protein 42) was first identified by photo-crosslinking of a precursor protein stuck at the import site. Its gene has been sequenced and shows a single open reading frame capable of encoding a polypeptide of *Mr* 41,983. Surprisingly, the predicted amino acid sequence resembles that of

a hydrophilic protein. However, a series of experiments have shown that ISP42 is an outer membrane protein involved in the mitochondrial import of precursor proteins. ISP42 does not have a cytosolic domain. ISP42 is believed to function as a part of the transmembrane channel for the import of precursor proteins across the outer membrane. It is, however, not exclusively found at contact sites. ISP42 is essential for yeast viability and for protein import and so is thought to catalyze a central step in protein import (Vestweber *et al.*, 1989; Baker *et al.*, 1990). Its *N. crassa* counterpart, known as MOM38, has significant sequence identity (Kiebler *et al.*, 1990) that may suggest its central role in protein import (Pfanner *et al.*, 1991).

The mitochondrial import machinery is suggested to be composed of multimeric proteins that form proteinaceous channels at contact sites and allow the passage of precursor proteins. The structure and function of these channels remain to be elucidated.

3) Import and Sorting of Mitochondrial Proteins

After recognition by receptor-like proteins on the outer membrane, precursor proteins are translocated into or across the mitochondrial membranes. Proteins destined to the outer membrane, such as porin or MAS70 in yeast, are simply inserted and assembled into it without proteolytic cleavage. External ATP is required, but a membrane potential is not necessary (Freitag *et al.*, 1982; Gasser and Schatz, 1983; Pon and Schatz, 1991).

Proteins destined to the matrix pass through both membranes at contact sites in one step. The membrane potential ($\Delta\psi$) is required for the initial insertion of the presequence into the inner membrane. It may have an electrophoretic effect on the positively charged presequences or on positively charged regions in precursors lacking cleavable targeting sequences. Once inserted, a membrane potential is no longer needed for

completion of the transport. Matrix factors are believed to mediate further translocation.

ATP hydrolysis is required for the import process. ATP is used to maintain precursor proteins in import-competent (unfolded) conformations outside mitochondria. Nascent polypeptide chains are imported into mitochondria at lower levels of ATP than the corresponding completed precursors. Also, denatured, but not native polypeptides can be imported independent of ATP. The sensitivity of nascent and denatured polypeptides to protease digestion suggests that they are in loosely folded conformations (Pfaller and Neupert, 1987; Pfanner *et al.*, 1991; Verner and Schatz, 1987). Imported precursors will associate with the matrix factors, mitochondrial heat shock protein 70 (mhsp70) and heat shock protein 60 (hsp60) to complete their folding to mature proteins. ATP is required for both the initial association of imported precursors with mhsp70 and the dissociation from mhsp70 and hsp60 (Krieg *et al.*, 1991).

Presequences of imported proteins are proteolytically removed in the matrix even before the completion of import. The processing is carried out by a soluble matrix protease. This enzyme is composed of two non-identical subunits encoded by the two nuclear genes: *MAS1* and *MAS2*. The two subunits share some sequence similarity and both genes are essential for yeast viability (Yaffe and Schatz, 1984; Yaffe *et al.*, 1985).

Both MAS1 and MAS2 subunits are about 50 kDa in size. The holoenzyme is a stable 100 kDa heterodimer (Yang *et al.*, 1988; Yang *et al.*, 1991). Precursors may bind via their N-terminal matrix targeting sequences to the MAS2 subunit in the preassembled holoenzyme followed by presequence removal. However, it is not clear whether the MAS2 subunit alone can carry out the proteolysis or whether both subunits function sequentially. Matrix

processing peptidases (MPP) of *Neurospora* as well as of rat show homology to the MAS2 subunit of yeast, while the processing enhancing protein (PEP) in *Neurospora* and the mitochondrial intermediate peptidase (MIP) in rat resemble the role of the MAS1 subunit. In *Neurospora*, precursors are thought to bind PEP followed by proteolytic processing once MPP binds to the complex (Hawlitschek *et al.*, 1988; Hartl and Neupert, 1990). In contrast, MPP and MIP in rat are two independent enzymes; MIP seems to function only on intermediates from a first cleavage by MPP in a two-step proteolytic processing (Isaya, *et al.*, 1990; Isaya *et al.*, 1992). These processing proteases share some common features; they can only cleave precursors and have no activity on mature forms of mitochondrial proteins and they all need divalent cations such as Mg^{++}, Mn^{++}, or Co^{++} to function.

Yeast mhsp70 and hsp60 are two members of a small family of molecular chaperones that interact with newly synthesized polypeptide chains or unfolded proteins and direct the correct (re)folding *in vivo* (Beckmann *et al.*, 1990; Martin *et al.*, 1991; Landry and Gierasch, 1991; Kang *et al.*, 1990).

Mitochondrial mhsp70 links the import of precursor proteins with their refolding in the matrix. The isolation of a cross-linked trimeric complex of ISP42, mhsp70, and a partially imported precursor has suggested an early interaction between presequences and mhsp70 (Baker and Schatz, 1991). The preferred binding sites of the *E. coli* protein, GroEL, also a chaperone, are amphipathic helical regions similar to mitochondrial targeting sequences (Landry and Gierasch, 1991). Therefore, mhsp70 might interact with the presequences of precursors as they emerge from contact sites, and "pull" the rest of the protein into the mitochondrion. The interaction between precursors and mhsp70 is transient. Imported proteins are not completely folded after their release from mhsp70s. mhsp70 interacts with incoming

precursors to maintain them in unfolded conformations and mediates their transfer to the mitochondrial chaperonin, hsp60. ATP or GTP hydrolysis is required for the interaction of precursors and mhsp70, for the completion of the polypeptide chain translocation, and for the release of imported proteins from mhsp70 (Krieg *et al.*, 1991; Baker and Schatz, 1991; Kang *et al.*, 1990). The removal of presequences by the MAS protease occurs after or during the release of precursor proteins from mhsp70s (Krieg *et al.*, 1991).

Yeast hsp60 is a nuclear encoded mitochondrial protein with a cleavable presequence. It is a 60-kDa protein with high sequence identity with GroEL of *E. coli*. Like GroEL, hsp60 is a homo-oligomer of 14 subunits that mediates the folding of proteins. Krieg and co-workers have shown that at low temperatures (8°C), *in vitro* imported pre-hsp60 is transiently bound to mhsp70; after removal of its presequence, hsp60 is released from mhsp70 and accumulates as loosely folded intermediates of low molecular mass (Kreig *et al.*, 1991). Those intermediates resemble the unfolded GroEL monomer at low temperatures. Upon raising the temperature to 30°C, the intermediates are assembled to hsp60 14-mers. This assembly requires pre-existing hsp60 14-mer (Cheng *et al.*, 1990). Although the mechanism of refolding by hsp60 is not clear, it is speculated that like GroEL, hsp60 14-mer associates with unfolded polypeptide chains and releases them with the assistance of at least one other factor (chaperone 10) and ATP hydrolysis. Both mhsp70 and hsp60 are essential for cell viability. A temperature sensitive mutant of hsp60 has been isolated. Mitochondria of this mutant import proteins at the non-permissive temperature, but can not assemble them properly so that misfolded, insoluble aggregates are generated (Glick *et al.*, 1992; Cheng *et al.*, 1989).

The majority of inner membrane proteins are assembled in complexes associated with mitochondrial respiration. They generally contain a typical mitochondrial targeting sequence. Even though some inner membrane proteins have been shown to be sorted via the matrix, this is not the general case. Subunit 9 of the F_0-ATPase in *Neurospora* and higher eukaryotes is cytosolically synthesized with a twice-cleaved N-terminal presequence (Viebrock *et al.*, 1982). After the removal of the presequence, the mature protein sorts to the inner membrane presumably by association with other subunits. However, in yeast, subunit 9 is encoded by the mitochondrial DNA and made without a cleavable presequence (Tzagoloff and Meagher, 1972; Sierra and Tzagoloff, 1973; Macino and Tzagoloff, 1979). Another inner membrane protein, ADP/ATP carrier (AAC), has a distinct import pathway. Its targeting sequence is non-cleavable and assigned to three segments in the mature AAC polypeptide chain. AAC is inserted into contact sites after being recognized by the receptors, MOM19 and/or MOM72. It then moves laterally out of the contact site to its inner membrane location. The distinct import of AAC may result from the special arrangement of the targeting sequences.

Soluble proteins of the intermembrane space include cyt *c*, cyt b_2, and cyt *c* peroxidase (Daum *et al.*, 1982). Proteins that expose a large hydrophilic domain towards the intermembrane space with a small anchor attaching them to the inner membrane include cyt c_1, and the Fe/S protein of complex III. With the exception of cyt *c*, they are synthesized as precursors with complex amino-terminal presequences cleaved in the two steps. The presequences of cyt b_2, cyt c_1, and possibly cyt *c* peroxidase are composed of typical mitochondrial targeting signals followed by sorting sequences that contain the information for their final location in the intermembrane space (Hurt and van Loon, 1986; van Loon *et al.*, 1987). The sorting sequence

contains a stretch of uncharged, hydrophobic amino acid residues flanked by charged residues. The matrix targeting sequence is removed by matrix processing protease, and the sorting sequence is cleaved by a different protease at the outer surface of the inner membrane (Glick *et al.*, 1992; Hartl *et al.*, 1987). In contrast, the presequence of the Rieske Fe/S protein is cleaved twice in the matrix (Hartl *et al.*, 1986; Hartl and Neupert, 1989).

The import of proteins into the intermembrane space is still controversial. The focus of the argument is on whether the proteins get into the intermembrane space via the matrix. Extensive studies have been carried out on the import of cyt c_1 and cyt b_2. Two hypotheses have been put forward; the "stop-transfer model" and the "conservative sorting model" (Fig. I-8). In the stop-transfer model, the sorting sequences of cyt b_2 and cyt c_1 (van Loon and Schatz, 1987; Glick *et al.*, 1992), and cyt *c* peroxidase (Kaput *et al.*, 1982) act as stop transfer signals to prevent the mature proteins from passing through the inner membrane. Instead, they will continue to cross the outer membrane and move laterally into the intermembrane space. As the matrix-targeting sequences have already crossed the inner membrane, they will be proteolytically cleaved off. The sorting sequences are removed in the intermembrane space and the mature proteins are generated (Glick *et al.*, 1992). The ability of a hydrophobic sequence to act as a stop-transfer signal has been reported in other translocation systems (Blobel, 1980) as well as in the case of yeast outer membrane receptor MAS70 (Hase *et al.*, 1983 and 1984). Moreover, the sorting sequences of cyt c_1 and cyt b_2 are both about 20 amino acids long which is in good agreement with the thickness of the membrane bilayer (Glick *et al.*, 1992). The hydrophobic amino acid residues may interact with the fatty acyl groups while flanking charged residues may interact with the charged head groups of phospholipids in the membrane bilayer.

In contrast to the stop-transfer model, the conservative sorting model proposes that precursors are transported into the intermembrane space via the matrix. After the removal of the matrix targeting sequence by matrix protease, the intermembrane space sorting signal sequences direct the export of the intermediates across the inner membrane into the intermembrane space (Hartl *et al.*, 1987). The reexport process does not need a membrane potential and is very similar to the secretory process in prokaryotes. The conservative sorting hypothesis is consistent with the general concept of the endosymbiosis of mitochondria. According to this hypothesis, the sorting sequences of cyt c_1 and cyt b_2 act as the signals for the intermediate forms to be exported across the inner membrane after they are exposed by the removal of the targeting sequences (Hartl *et al.*, 1987; Hartl and Neupert, 1989).

The major differences between the two hypotheses are on the appearance of precursor proteins in the matrix en route to the intermembrane space, on the ATP requirement for sorting, and on the role of hsp60. In the conservative sorting model, mature proteins should transiently appear in matrix; hsp60 might be required to keep the intermediates in a transport-competent conformation; ATP hydrolysis would be required for the release of proteins from hsp60. Neither hsp60 nor ATP hydrolysis are necessary for import via the stop-transfer model. Additionally, the stop-transfer model predicts that the import machineries in the two membranes have to separate in order for a protein to move laterally into the inner membrane. The conservative sorting model predicts import through a continuous proteinaceous channel (Glick *et al.*, 1992; Hartl *et al.*, 1987; Hartl and Neupert, 1989).

Apocytochrome *c* is synthesized in the cytosol without a cleavable presequence. The import of apocytochrome *c* into the intermembrane space is distinctly unique. A membrane potential across the inner membrane is not required. Surprisingly, its import does not use contact sites, receptors in the outer membrane, nor ATP (Hennig and Neupert, 1981; Nicholson *et al.*, 1988; Dumont *et al.*, 1991). Apocytochrome *c* may cross the outer membrane by interactions either with the lipid bilayer or with non-receptor proteins. Once it reaches the intermembrane space, apocytochrome *c* is trapped by the heme lyase (or holocytochrome *c* synthase) attached on the outer surface of the inner membrane. The heme lyase catalyses the attachment of heme to apocytochrome *c* to form cytochrome *c* which is not able to transverse the outer membrane. The heme lyase is not enriched at contact sites (Dumont *et al.*, 1991).

Many imported mitochondrial proteins involved in respiration contain cofactors or coenzymes. Their maturation often involves the attachment of heme as for cytochrome *c* and cytochrome c_1, the assembly of iron-sulfur clusters as for several subunits of complexes I, II, and III, and the attachment or insertion of cofactors such as NAD, FAD or FMN. Very little is known about when, where, and how this occurs. In a few cases, attachment occurs prior to assembly of functional complexes (Werner *et al.*, 1974; Woodrow *et al.*, 1979). The attachment of heme to apocytochrome *c* is after its appearance in the intermembrane space (Dumont *et al.*, 1991). The attachment of heme to cytochrome c_1 takes place after the cleavage of the matrix targeting sequence and is tightly coupled to the maturation of cyt c_1 (Hartl and Neupert, 1989). The formation of iron-sulfur clusters in the Rieske Fe/S protein occurs in the matrix and seems to be a prerequisite for the second cleavage of the presequence (Hartl *et al.*, 1986). The attachment of cofactors

may occur at early or later stages of import depending on the individual proteins. In addition, very little is known about the assembly of the mitochondrial respiratory chain complexes (Grivell, 1989). A model for assembly of the cyt *bc1* complex proposes that subcomplexes are formed before the entire complex is. Many questions still remain to be answered such as; 1) How are these subcomplexes formed? 2) How do the subcomplexes find each other and assemble? 3) Are chaperones involved in the assembly?.

Overall, the biogenesis of mitochondria is a very complex process that involves the interaction of two genetic systems at both transcriptional and translational levels. Proteins synthesized in the cytosol as precursors have to be translocated into mitochondria through a complicated process. Proteins encoded by mitochondrial DNA are normally synthesized in mitochondria without processing. Functional multisubunit complexes are formed after the attachment of essential prosthetic groups. The regulation of these processes is far from fully understood.

D. THESIS PROBLEM

Mitochondrial respiration is essential for yeast growth on non-fermentable carbon sources like ethanol, glycerol, and lactate. Respiration requires a large number of nuclear encoded proteins that are directly or indirectly involved in electron transport chain function.

The yeast nuclear petite mutant, *tcm10*, ceases to grow on media with glycerol as the sole carbon source. The aim of this work was to identify and characterize the defect present in this mutant that prevents normal mitochondrial function. To do this, the wild-type *TCM10* gene was cloned and sequenced, the intracellular location of the protein product was determined,

and a range of biochemical analyses were performed to further define the effects of mutant alleles.

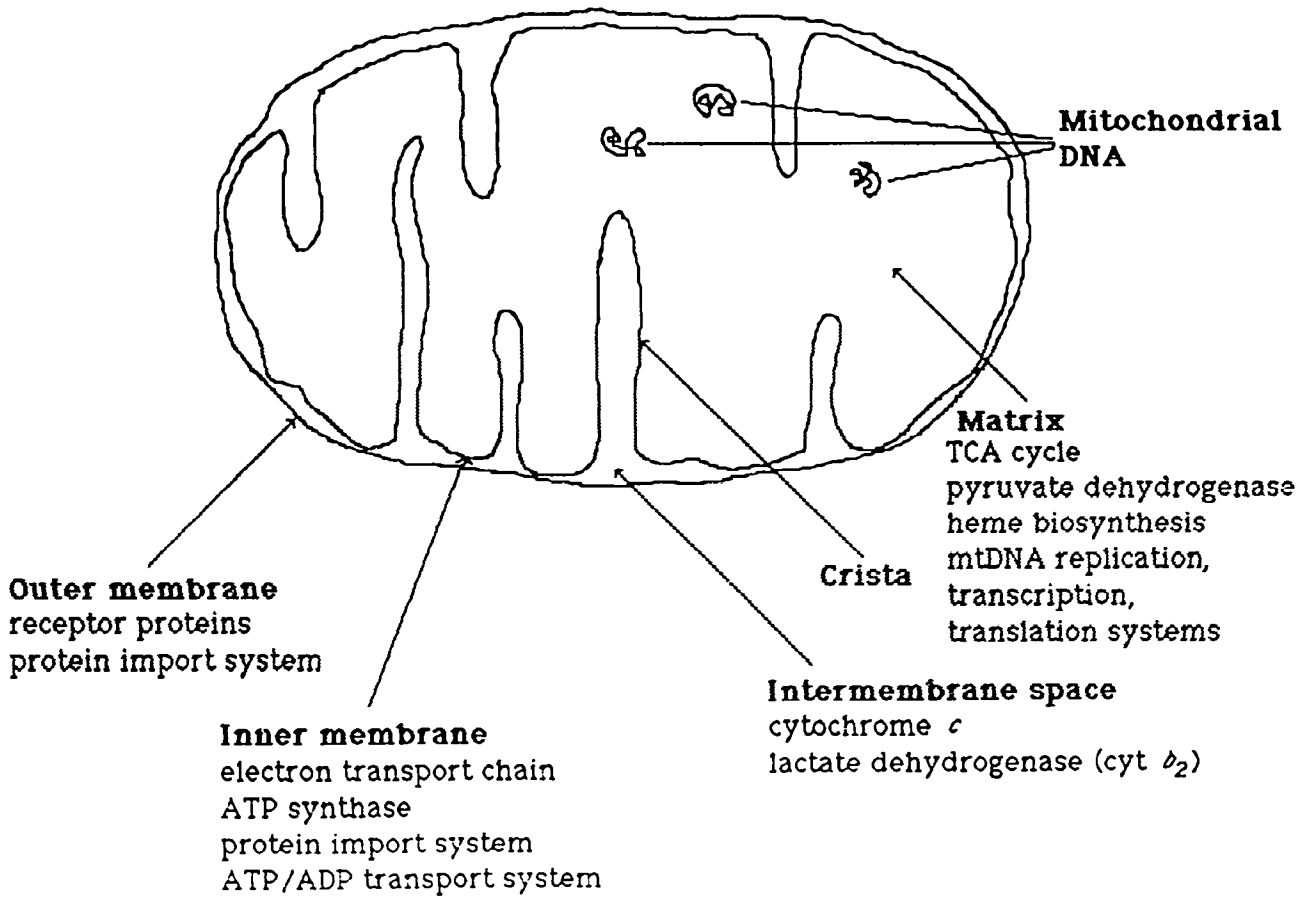


Figure I-1 A schematic diagram of the four compartments of a mitochondrion. The principal enzymes and pathways in each compartment are shown. Circular mitochondrial DNAs are also shown. (Modified from Mathews and van Holde, 1990)

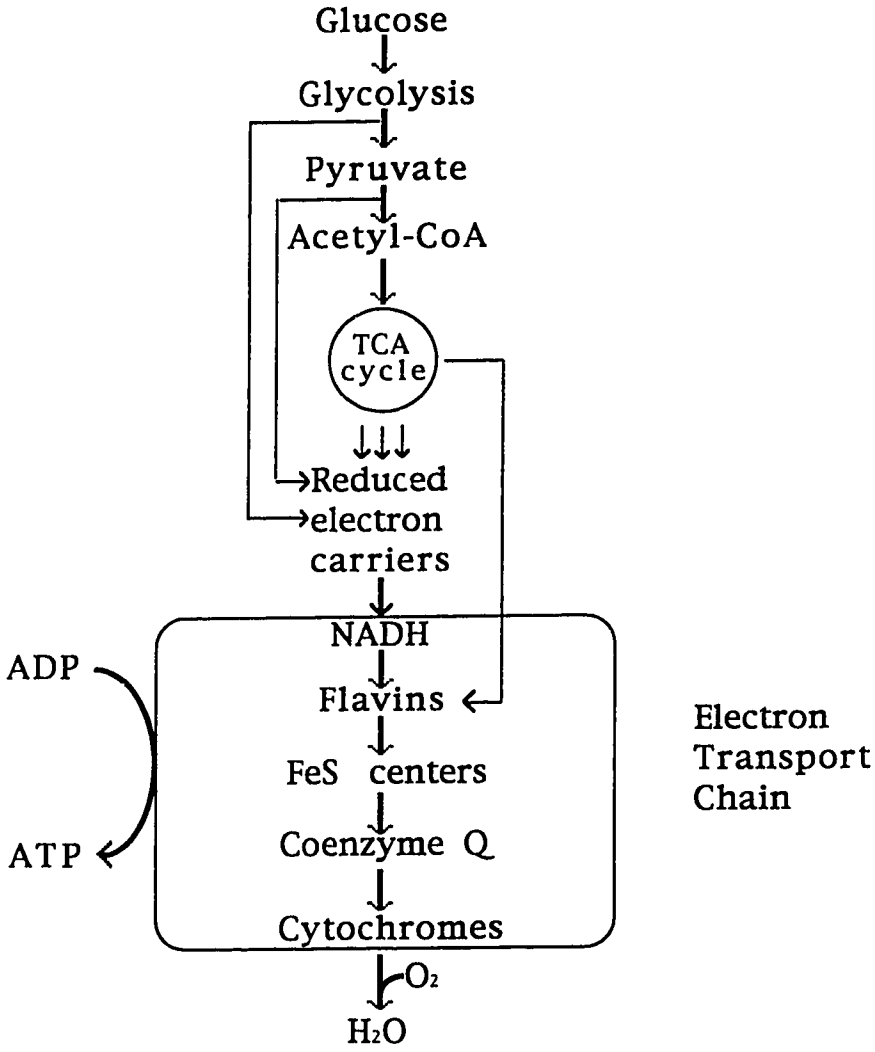


Figure I-2 An overview of respiration in eukaryotic cells. 1 $FADH_2$ and 3 NADH in the TCA cycle, 2 more NADH in the glycolysis and the pyruvate dehydrogenation are generated from 1 glucose. The narrow arrows indicate the transfer of hydrogen to NAD^+ and FAD^+. The thick arrows indicate the direction of each reaction (Modified from Mathews and van Holde, 1990).

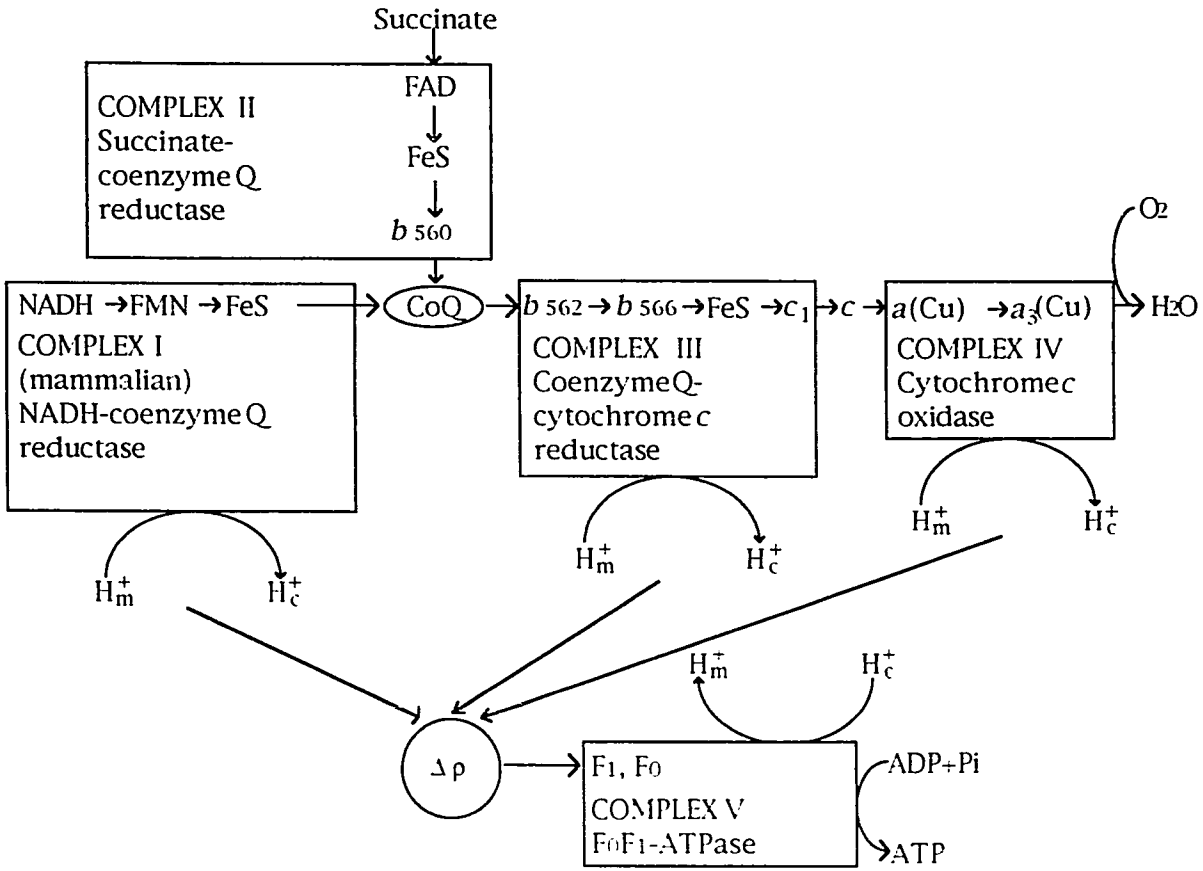
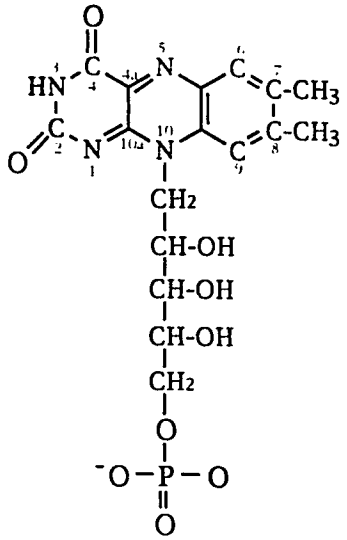
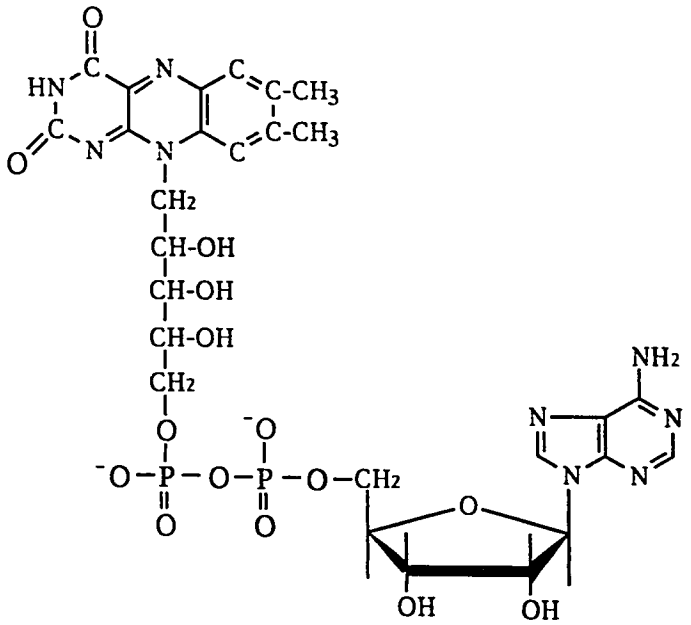


Figure I-3 Multisubunit complexes in oxidative phosphorylation with prosthetic groups. Complexes I, II, III, and IV are involved in electron transport, whereas complex V is involved in ATP synthesis using the energy derived from electron transport. FeS, iron-sulfur center; *a*, *b*, and *c*, cytochromes *a*, *b*, *c*; H_m^+ and H_c^+, protons on the matrix and cytosolic sides of the inner membrane; $\Delta\rho$, the proton motive force generated from proton translocation (Adapted from Hatefi, 1985).



Flavin mononucleotide (FMN)



Flavin adenine dinucleotide (FAD)

Figure I-4 Molecular structures of FMN and FAD. As prosthetic groups of the electron transport chain, FMN and FAD are involved the electron transfer in oxidoreduction reactions through the electron pairs between N_1 and N_5. The molecule of FMN without phosphate group is called riboflavin. The aromatic ring system in FMN and FAD is called the isoalloxazine ring system. (Adapted from Mathews and van Holde, 1991)

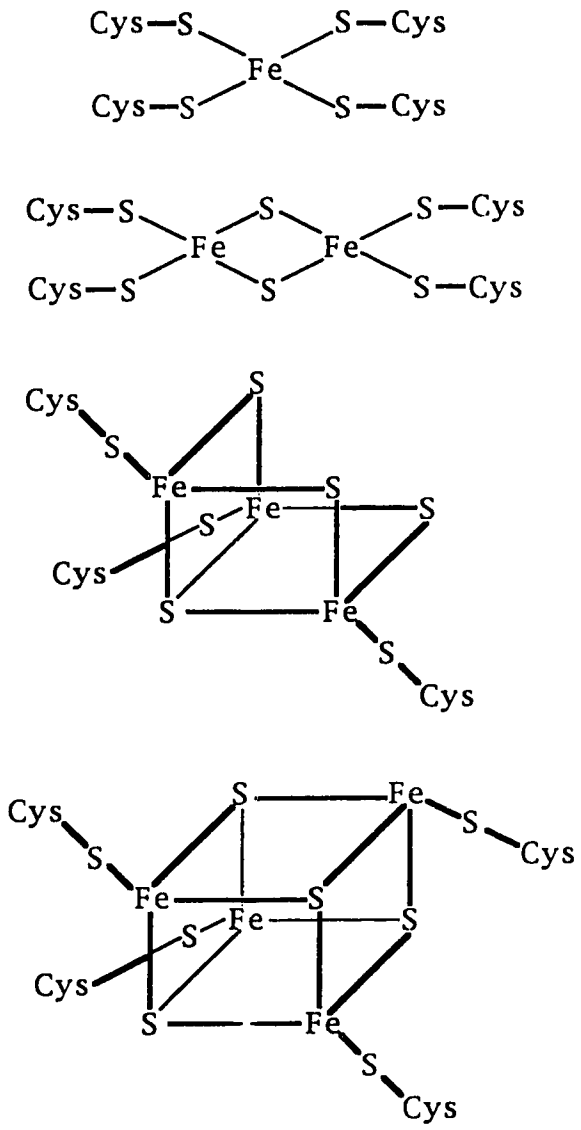


Figure I-5 Structures of four kinds of iron-sulfur clusters. The iron atoms are linked by the sulfide groups of cysteines from the same polypeptide chain.

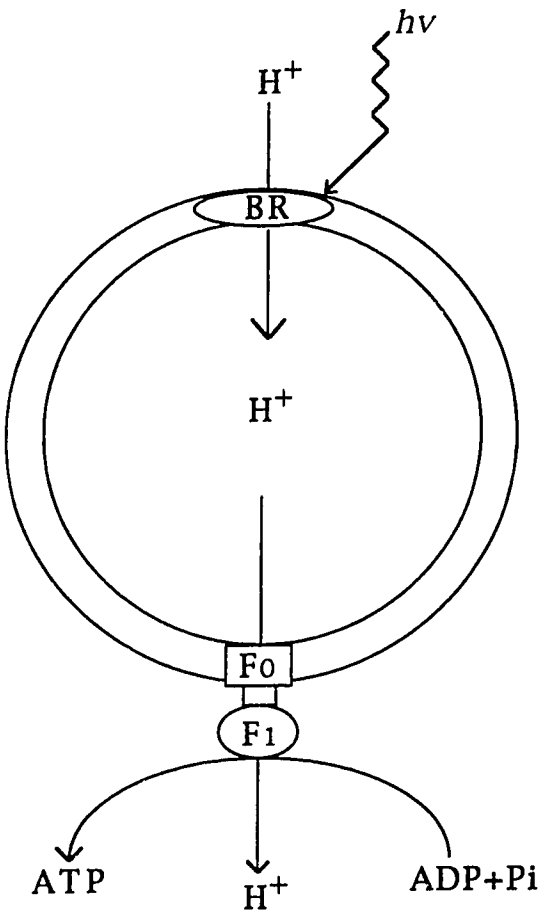


Figure I-6 Scheme for ATP synthesis in phospholipid vesicles reconstituted with *H. halobium* bacteriorhodopsin (BR) and mitochondrial F_oF_1-ATPase . Protons are pumped into the vesicle from the external medium upon illumination (*hv*); this forms a proton gradient. The energy released from the outflow of protons through the F_oF_1-ATPase drives ATP synthesis.

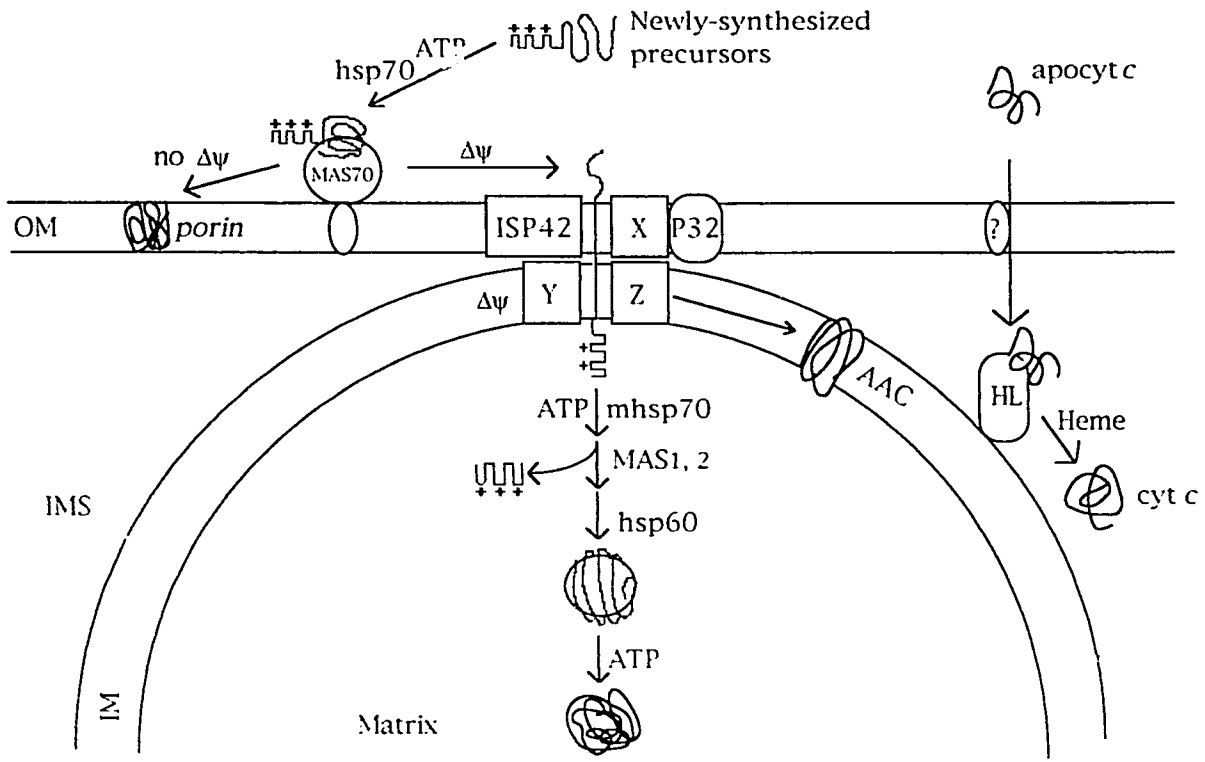


Figure 1-7 The general view of protein import into mitochondria. hsp70, the heat shock protein 70 in the cytoplasm, mhsp70, mitochondrial heat shock protein 70;hsp60, heat shock protein 60; mas1, 2 subunits of the matrix processing protease; HL, apocytochrome c heme lysae; ISP42, and p32, two known outer membrane proteins involved in protein import; X, Y, and Z, unknown parts of the import machinery; ?, the unknown component on the outer membrane for *cyt c* import; $\Delta\psi$, the membrane potential; AAC, the ADP/ATP carrier; MAS70, the outer membrane receptor protein; OM, the outer membrane; IM, the inner membrane; IMS, the intermembrane space. See text for more details (Modified from Baker and Schatz, 1991).

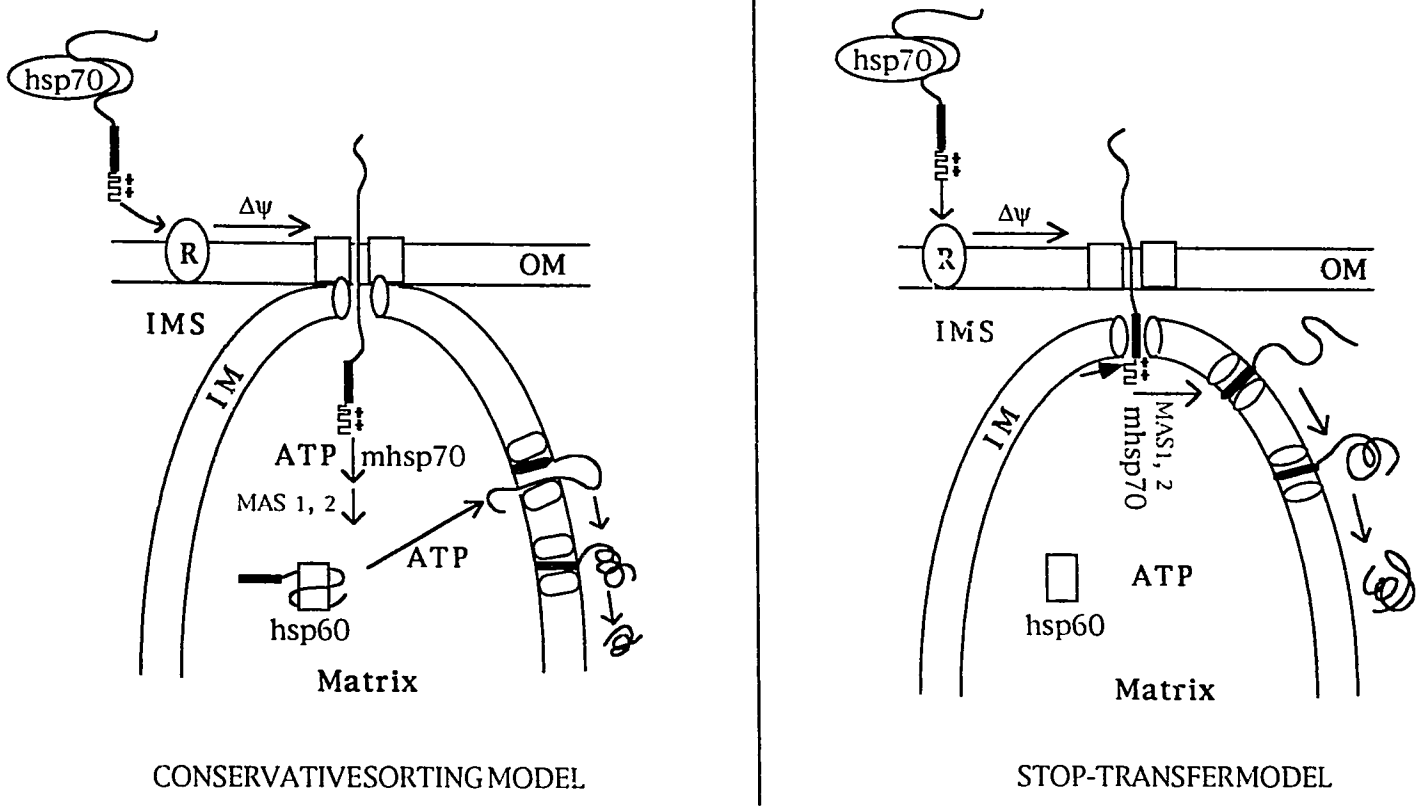


Figure I-8 The two models for import of proteins into the intermembrane space. R, receptor proteins on the outer membrane; Other abbreviations are described in the legend for **Figure I-7.** See text for details. (Modified from Glick *et al.*, 1992)

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CHAPTER II. MOLECULAR AND GENETIC STUDIES OF A NOVEL MUTATION AFFECTING THE YEAST MITOCHONDRIAL RESPIRATORY CHAIN

A. INTRODUCTION

Respiration competent mitochondria are essential for the facultative yeast, *Saccharomyces cerevisiae*, to grow aerobically on non-fermentable carbon sources. During aerobic growth, energy is mainly produced by oxidative phosphorylation through the electron transport chain inside mitochondria. In the 1960's, it was established that anaerobically grown yeast contain respiration-incompetent mitochondria called promitochondria. These promitochondria contain mtDNA, a typical mitochondrial DNA expression system, and even parts of the oxidative phosphorylation machinery (Schatz, 1965; Schatz *et al.*, 1968; Criddle and Schatz, 1969; Damsky *et al.*, 1969). Promitochondria differentiate into respiration-proficient mitochondria upon switching to aerobic growth. Differentiation is a very complicated process requiring the expression of over 200 nuclear genes (Tzagoloff and Dieckmann, 1990). Therefore, studying nuclear petites will help us to further understand the communication between nuclear and mitochondrial genomes and the control of the normal function of mitochondria.

Note: The isolation of original mutagenized tcm10 mutant was carried out by B. Lemire, and the two plasmids, pTCM10-510 and pTCM10-530, were isolated by K. Robinson.

A nuclear mutant, tcm10, was isolated and shown to be deficient in oxidative phosphorylation; it could not grow on nonfermentable carbon sources, such as glycerol. The *tcm10* mutant forms petite colonies on media with a low concentration of glucose. The *TCM10* gene has been isolated, cloned, and sequenced. No significant sequence identity to any known protein has been found by data bank searching.

B. MATERIALS AND METHODS

1) Strains

S. cerevisiae strains MH125 (MATa, leu2-3, leu2-112, ura3-52, trp1, his4, his3), MH124 (MATα, leu2-3, leu2-112, ura3-52, trp1, his4, his3), DAU1 (MATα, ade2, ura3-del), LB355-7D (MATa, met, ura), LB355-1B (MATα, met), MS10 (MATα, kar1-1, leu2-3, canR, ρ^o), a4rho0 (MATa, ade1, lys2, ρ^o) were used in this study. *E. coli* strain, UT580 [Δ(lac-pro), rk^-, mk^+, Tn10, supD, lacIq, F'traD, $pro^{+'}$, LacZΔM15] was used for manipulating and maintaining plasmids and as a host in the preparation of single-stranded DNA sequencing templates.

2) Media

Media used for yeast growth were YPD (1% yeast extract, 2% peptone, 2% D-glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol vol/vol), SD (0.67% yeast nitrogen base, 2% D-glucose), SG (0.67% yeast nitrogen base, 3% glycerol vol/vol), sporulation medium (1% potassium acetate, 0.2% yeast extract, 0.02% D-glucose). SD+CAA and SG+CAA media were made by adding 0.5% casamino acids to SD and SG media, respectively. Drop-out media SDC-ura, SDC-ade, SDC-trp, SDC-his, SDC-leu, and SDC-lys were made by adding the required individual amino acids and bases (adenine and/or uracil) to SD media and leaving out the amino acid and/or base indicated. Luria broth (LB)

and M9 media (Sambrook *et al.*, 1989) were used for *E. coli* growth. Two percent agar was used for solid media and 0.1 mg/ml of Ampicillin (Amp) was used for selection of antibiotic resistant transformants. All materials used in media were from Difco, Michigan; except, agar from GIBCO-BRL, USA; the amino acids and nucleotides from SIGMA, USA. All other chemicals were from BDH Chemical Inc., Toronto.

3) Isolation of TCA Cycle Mutants

A protocol for isolation of TCA cycle mutants was adapted from the isolation of mutants in the electron transport chain (de Kok *et al.*, 1975). Mutagenesis with ultraviolet (UV) light was used to induce mutations in yeast (Spencer *et al.*, 1989). Haploid cells of the wild type, MH125, were grown in YPD for 48 hours. Cells were diluted 10 fold, put in petri-dishes, and UV-irradiated with a 254 nm UV lamp (1.4 J/m^2 sec) for 9 minutes (about 5% survival). After the mutagenesis, cells were diluted and spread to give about 300 colonies on each YPD plate. Colonies on the master YPD plates were replicated onto YPG plates and non-growing colonies were picked onto YPD plates. Replicas of these second YPD plates were grown for 1.5 days and overlaid with 10 ml of 67 mM sodium phosphate buffer pH 6.8, 0.1% 2,3,5-triphenyltetrazolium chloride (TTC), 1.5% agar. TTC is an electron acceptor of cytochrome *c* oxidase and flavoproteins, and becomes dark red when reduced by respiration competent colonies. Only dark red colonies were chosen after overnight growth. YPG^- and TTC^+ colonies were picked onto YPD plates containing the pH indicator, bromocresol purple (0.003%). Acid producing colonies turn the indicator yellow around the colonies; these were scored after a 48 hour incubation. Fifteen of 47 YPG^- and TTC^+ colonies were acid producing. Mutants of this pool enriched in TCA cycle defects are called 'tcm' mutants for TCA cycle mutation.

4) Genetic Characterization of the *tcm10* Mutant

tcm10 was mated to the wild type strain, DAU1, and diploids were selected. These were sporulated on sporulation media for 4-6 days. Cells were treated with 1:4 dilution of ß-glucuronidase (115,000 units/ml; SIGMA, USA) at 30°C for about 10 min and tetrads were dissected by micromanipulation. tcm10-9B (Table II-1) was selected from a tetrad showing 2^-: 2^+ segregation on YPG. Furthermore, tcm10-15D and tcm10-19D (Table II-1) were selected from tetrads generated by mating tcm10-9B with MH125. The genotype of each haploid was tested for the presence of the markers: *ura3*, *ade2*, *trp1*, *lys2*, *leu2*, and *his3* and *4*. The mating type of each mutant strain was determined by mating with LB355-1B and LB355-7D and assessing complementation of their methionine auxotrophies.

5) Isolation of *TCM10* Gene from Yeast Genomic Library

tcm10-9B was used to isolate the *TCM10* gene by complementation screening of a yeast genomic library (Rose *et al.*, 1987). Transformants able to grow on YPG were regrown in YPD liquid media to an OD_{600} of 1 and harvested. Cell pellets were washed and resuspended in 200 µl SCE buffer (1 M sorbitol, 0.1 M $NaPO_4$, pH 7.0, 0.06 M EDTA). Zymolyase 20,000 (0.5 mg/g wet cell) and 0.05 volumes of ß-mercaptoethanol were added and incubated at 37°C for 1-2 hours to achieve complete spheroplasting. 400 µl of a 0.2 N NaOH, 1% SDS solution was added to break open the cells. This was followed by the addition of 300 µl of 3 M potassium acetate buffer pH 4.8 to precipitate proteins on ice for 5 min. The sample was centrifuged at 14,000 x *g* for 10 minutes and the supernatant ethanol precipitated. Finally, the DNA pellet was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Two complementing plasmids were isolated and named pTCM10-510 and pTCM10-530 (Figure II-1).

6) Construction of Plasmids YCplac33-TCM10(1#) and (2#)

The plasmid pTCM10-510 was digested with HindIII and run on a 0.8% agarose to clearly separate each fragment. Four small HindIII fragments were isolated from the gel by the glass wool method (Heery *et al.*, 1991) and ligated with likewise cut vector YCplac33. Plasmids carrying four different fragments were isolated and transformed into the mutant tcm10-15D to test their abilities to complement for growth on glycerol. Only a 2.7 kb fragment could complement tcm10-15D for growth on YPG. Plasmids with the 2.7 kb inserts in each orientation were isolated and named YCplac33-TCM10(1#) and (2#) (Figure II-2).

7) Nested Deletions of *TCM10* Gene

YCplac33-TCM10(1#) and (2#) were first digested with KpnI and then by XbaI. The nested deletion protocol was a modification of Promega's Erase-a-Base™ system. Ten µg of each double cut YCplac33-TCM10(1#) and (2#) were dissolved in 60 µl of 1 x Exonuclease III buffer (66 mM Tris-HCl pH 8.0, 0.66 mM $MgCl_2$), and incubated at 35°C for 2 minutes before the addition of 350 units of Exonuclease III. Five µl aliquots were removed at one minute intervals from 0 to 9 minutes and mixed on ice with 15 µl of S1 mix (40.5 mM potassium acetate pH 4.6, 0.34 M NaCl, 1.35 mM $ZnSO_4$, 7% glycerol, 5 units of S1 nuclease). After all the time points were taken, the S1 nuclease digestion was carried out at room temperature for 30 minutes. Digestion was stopped by the addition of 1 µl of S1 stop solution (0.3 M Tris-base, 0.05 M EDTA, pH 8.0) and heat inactivation at 70°C for 10 min. Then, 2 µl of Klenow mix (1.5 µl of 10 x nick translation buffer, 1 unit of Klenow) (Maniatis *et al.*, 1982) and 2 µl of dNTP mix (0.125 mM of each) were added to each sample and incubated at 37°C for 5 min. Finally, ligation was carried out at room temperature for 2 hours with 1 unit of T4 DNA ligase (GIBCO-BRL, USA) (Sambrook *et al.*, 1989). The

plasmids were transformed into UT580 and transformants were screened by restriction digestion of miniprep DNA (Birnboim and Doly, 1979; Morelle, 1990).

8) Preparation of DNA Sequencing Templates

Double stranded DNA sequencing templates were made by linearizing miniprep plasmid DNA with HindIII Since one HindIII site in the polylinker sites was digested away by Exonuclease III, the HindIII site left at the distal end is unique. Sequential digestions with RNase A (20 µg of RNase A per miniprep) at 37°C for one hour, and proteinase K (20 µg of proteinase K per miniprep) for another hour were performed. The samples were twice extracted with phenol/chloroform/iso-amyl alcohol (24:24:1). When necessary, single stranded DNA templates were made by subcloning into pBluescript II (SK^-) vector (Stratagene, USA). UT580, carrying these plasmids, were infected with helper phage (M13K07) and grown for four hours at 37°C. Single stranded DNA templates were isolated as described (Maniatis *et al.*, 1982). DNA sequence was determined by the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977). The entire sequence was obtained for both strands from the DNA sequencing lab of this department using either denaturing polyacrylamide gels or the Applied Biosystem 373A DNA Sequencer, USA.

9) PCR Amplification of *TCM10* Gene

Two oligonucleotide primers were used to amplify the *TCM10* gene from the amino terminal methionine of the open reading frame to the distal HindIII site in YCplac33-TCM10(2#) (Figure II-3). The NdeI linearized plasmid YCplac33-TCM10(2#) served as PCR template. Ten µl of 10 x polymerase buffer (500 mM KCl, 100 mM Tris-HCl pH 8.4, 25 mM $MgCl_2$, 200 µg/ml gelatin), 10 µl of an 8 mM dNTP solution (2 mM of each dATP, dTTP, dGTP, and dCTP), 10 µl of 3

μM primer 1 and 3 μl of 10 μM of primer 2, 1 μg of template DNA and H_2O were mixed to 100 μl. 2.5 units of Taq polymerase (GIBCO-BRL, USA) were added to each reaction. The PCR reaction mix was kept on ice before cycling was initiated. Each cycle contains a denaturation step at 94°C for 30 sec, an annealing step at 48°C for 30 sec, and a polymerization step at 72°C for 2 min. The cycle was repeated 25 times. The optimal $MgCl_2$ concentration was 4.0 mM. The amplified products were precipitated by the addition of 1/2 volume of 7.5 M NH_4OAc, 9 volumes of EtOH. The resuspended PCR product was digested with BamHI and HindIII, and inserted into the vectors pBluescript II for *in vitro* expression, pJF118EH for expression in *E. coli* (Figure II-4), and pEH71 for expression in yeast under control of the alcohol dehydrogenase 1 (ADH1) promoter (Figure II-5).

10) Construction of a *TCM10* Disruption Mutant

The *TCM10* gene amplified by PCR was cloned into pJF118EH vector (Figure II-4) as a 2.4 kb BamHI/HindIII fragment. The marker gene, *TRP1*, was inserted at the unique SstI site. This construct, pJF118EH-TCM10::TRP1 (Figure II-6) was cut with BamHI and ScaI and transformed into MH125. Integrants at the *TCM10* locus arising from homologous recombination events were selected for tryptophan prototrophy.

11) Miscellaneous Methods

Published procedures were used for yeast (Hinnen *et al.*, 1978) or *E. coli* (Maniatis *et al.*, 1982) transformation, for miniprep of plasmids from *E. coli* (Morelle, 1990) and yeast (Baker and Schatz, 1987), and for isolation of DNA fragments from agarose gel with glass wool (Heery, 1990) or NA45 DEAE membrane (Schleicher & Schuell Inc., USA). Restriction enzyme digestions and ligations were performed as described by the supplier (GIBCO-BRL, USA). Standard methods of yeast genetics (Sherman *et al.*, 1983) were used for

mating, sporulation of diploids, and dissection of tetrads by micromanipulation.

C. RESULTS AND DISCUSSION

1) tcm10 - A Mitochondrial Respiration Defective Mutant

The mutant, tcm10, was isolated by UV-mutagenesis of the wild type strain, MH125. It exhibited defective mitochondrial respiration as indicated by its inability to grow on YPG. TTC staining indicates that tcm10 is not ρ^-. It appears that the TCA cycle in tcm10 may also be defective as indicated by the accumulation of acidic metabolites on plates containing the pH indicator, bromocresol purple. tcm10-9B was isolated by microdissecting tetrads arising from diploids of tcm10 and DAU1; 11 complete tetrads were examined and gave a $2^-:2^+$ segregation on YPG. A $2^-:2^+$ segregation of the mutant phenotype is indicative of a single gene mutation (Sherman and Wakem, 1991). Two other mutants, tcm10-15D and tcm10-19D, were also isolated from tetrads of diploids produced by mating tcm10-9B and MH125. tcm10-15D and tcm10-19D (Table II-1) were backcrossed with MH124 and MH125 respectively. All complete tetrads examined from both diploids showed $2^-:2^+$ segregation for growth on YPG and for the leucine marker. We concluded that the *tcm10* mutant is caused by a single nuclear gene mutation.

2) The *TCM10* Gene Is on Chromosome XV

The chromosomal location of the *tcm10* locus has been assigned to the yeast chromosome XV by meiotic mapping. We studied the linkage between *tcm10* and marker genes by examining the segregation pattern of these two genes in four spores of each dissected ascus. There are three types of tetrads from a heterozygous diploid for two selective markers, *AB X ab*; parental

ditype (PD; *AB, AB, ab, ab*), nonparental ditype (NPD; *aB, aB, Ab, Ab*), and tetratype (T; *AB, Ab, ab, aB*) (Sherman and Wakem, 1991). The ratios of these tetrads can be used to deduce the linkage of the *tcm10* gene with a selected marker. If *tcm10* is unlinked to the marker gene giving, random assortment will lead to a ratio of PD : NPD : T 1: 1: 4; if *tcm10* is linked to the marker gene, there will be an excess of PD over NPD asci, if *tcm10* is centromere-linked, there will be a reduction in the proportion of T asci.

During the isolation of tcm10-15D and tcm10-19D mutants, I dissected 35 tetrads from the diploid of MH125 and tcm10-9B and obtained 32 complete tetrads. All 32 complete tetrads were tested for growth on YPG, SDC-ade, SDC-leu, and SDC-trp plates to determine the segregation of *TCM10*, *ADE2*, *TRP1*, and *LEU2* genes. Only those four genes could show segregation in the tetrads from the diploids of MH125 and tcm10-9B (Refer to Table II-1). All those tetrads have shown $2^+:2^-$ segregation on those selective media, which indicates the single nuclear gene mutation. When I compared the segregation of *TCM10* with three other markers, *LEU2*(on chromosome III), *TRP1* (on chromosome IV), and *ADE2* (on chromosome XV), I found the following interesting results: 32 tetrads examined for the segregation of *TCM10* and *LEU2* have shown 5 PD : 7 NPD : 20 T, which is close to the 1:1:4 ratio expected for unlinked genes; 28 tetrads examined for the segregation of *TCM10* and *TRP1* have shown 5 PD : 4 NPD : 19 T, also close to the 1:1:4 ratio. These results show that the *TCM10* gene is not linked to either the *LEU2* or the *TRP1* genes. Both *LEU2* and *TRP1* are centromere linked (Sherman and Wakem, 1991). However, when I examined 32 tetrads for the segregation of *TCM10* and *ADE2*, we found 9 PD : 2 NPD : 21 T. The excess of PD over NPD tetrads indicates that *TCM10* is linked to *ADE2* gene. Therefore, the *TCM10* gene is located on the same chromosome as *ADE2*, which is chromosome XV.

The linkage of *TCM10* and *ADE2* is shown at the 5% of significance level according to the statistics and the distance between *TCM10* and *ADE2* is 57.2 centimorgan (cM) (Sherman and Wakem, 1991).

3) Nucleotide Sequence of the *TCM10* Gene

Two plasmids, pTCM10-510 and pTCM10-530, were isolated by complementation screening of a yeast genomic library in the shuttle vector, YCp50 (Rose *et al.*, 1987). Retransformation of either of these two plasmids into tcm10-9B conferred growth on YPG to all transformants. These two plasmids carry overlapping inserts since both produce a 2.7 kb band after HindIII digestion. Double digestion of both plasmids with HindIII and SstI resulted in the disappearance of the 2.7 kb band and the appearance of two smaller bands of 2.1 kb and 0.6 kb (Figure II-7). The SstI site is unique in pTCM10-530. Therefore, both contain the same 2.7 kb HindIII fragment later shown to encode the *TCM10* gene.

The four smallest bands from a HindIII digestion of pTCM10-510 were recovered from a 0.8% agarose gel inserted into YCplac33, and transformed into tcm10-15D (Figure II-2) to test its ability to complement the *tcm10* mutation. Only the plasmid with the 2.7 kb fragment could restore growth on glycerol as a carbon source. Two plasmids, YCplac33-TCM10 (1#) and (2#), were isolated with the 2.7 kb insert in opposite orientations (Figure II-2). The sequence of the entire 2.7 kb fragment was obtained from both sense and antisense strands. The sequencing strategy is described in Figure II-8. The entire sequence is shown in Figure II-9.

Several lines of evidence suggest that the cloned 2.7 kb fragment contains the wild-type *TCM10* gene. First, the two complementing plasmids isolated both contain the identical fragment. Second, complementation occurs with single copy vectors carrying the *TCM10* gene. No other genes

able to complement or suppress the *tcm10* mutation were isolated during the genomic library screening. Third, the disruption mutant made using the cloned fragment displays the same phenotypes as tcm10-15D and tcm10-19D (see below and Chapter III). Fourth, the diploid arising from the mating of tcm10-19D and a *tcm*10 disruption mutant is unable to grow on glycerol. This shows that the disrupted chromosomal locus is the same as the mutated locus in tcm10. Fifth, when the disruption mutant was mated with MH124 and sporulated, the tetrads showed stable $2^-:2^+$ cosegregation of the YPG^- and TRP^+ markers. This shows that the *TCM10* gene disruption by the *TRP1* marker gene is tightly linked to the YPG^- phenotype as expected for a disruption arising from homologous recombination. Therefore, I believe that the wild type *TCM10* gene has been cloned and not a suppressor. Suppressor genes have been reported to complement a mutation when present on high copy number plasmids (Rose and Broach, 1991) or even when a extra copy is present (Davis *et al.*, 1981).

The sequence of the 2.7 kb fragment shows a single open reading frame. The 2052 bp long coding region encodes a putative protein of 684 amino acids with a mass of 79.7 kDa if translation starts at the first ATG. The TCM10 protein is a basic protein with an estimated pI of 9. There are no acidic amino acids in the first 54 residues. In contrast, this N-terminal region contains 7 basic amino acids and 10 hydroxylated amino acids. Even though it does not form a perfect amphipathic helix with positively charged residues clustered to one side of the helix (Figure II-10), it could serve as a mitochondrial targeting signal since the positively charged amino acids in the presequences of yeast mitochondrial proteins such as δ-ALA synthase, MAS1, and SDH1 do not form typical amphipathic helicies (Hartl *et al.*, 1989; Robinson and Lemire, 1992). The Kyte Doolittle hydrophobicity profile does

not show any highly hydrophobic region long enough to span the membrane bilayer (Figure II-11). However, it does not exclude the possibility that TCM10 is a membrane protein since a similar situation was noted for ISP42 (an integral mitochondrial outer membrane protein) (Baker *et al.*, 1990).

In the *TCM10* upstream region, two TATA consensus sequences are located at -153 (TATATA) and -126 (TATA). AT rich regions are also found downstream of the open reading frame and may function as polyadenylation signals. The sequence TACTAAC, thought to be essential for pre-mRNA splicing in *S. cerevisiae* (Langford *et al.*, 1983), is not found within the open reading frame. Therefore, *TCM10* likely does not contain introns. The predicted amino acid sequence of TCM10 was used in computer searches against the PIR34 and Swiss Prot-23 databanks using the IG-SUITE program package. No protein with high identity was found. The top score was 22% identity and had numerous gaps over the entire protein sequence. Moreover, no shorter stretch of high identity was found. Therefore, *TCM10* is a previously unidentified gene involved in yeast mitochondrial respiration. Interestingly, the reverse strand of the 3'-noncoding region of the *TCM10* gene has shown 99.2% DNA sequence identity with the gene 1 of the elongator methionine-accepting transfer RNA (*EMT1*) in *S. cerevisiae*. However, nothing is known about this *EMT1* gene due to the unpublished results.

4) N- and C-terminal Regions Important for TCM10 Function

During the sequencing of the gene, several truncated versions of *TCM10* were constructed. Two of these were made by cloning the regions of the open reading frame upstream and downstream of the SstI site. YCplac33 was used as a vector for most of these complementation tests while pEH71 with the ADH1

promoter was used in the others (Figure II-12). Neither of these fragments could complement the *tcm10* mutation, suggesting that the entire TCM10 protein is important for function (Figure II-12).

5) TCM10 Is Not Essential for Yeast Viability

The plasmid, pJF118-TCM10::TRP1, was constructed by inserting the *TRP1* marker gene at the SstI site of the PCR amplified *TCM10* gene. This PCR product expressed behind the ADH1 promoter (Figure II-5) could complement the *tcm10* mutation. pJF118-TCM10::TRP1 was used for the disruption of the chromosomal *TCM10* gene. After digestion with BamHI and ScaI, the products were transformed into the wild type strain MH125. As shown in Figure II-6, the fragment containing disrupted *tcm10* gene is able to recombine with the chromosomal *TCM10* gene and replace it with the *TRP1* disrupted, inactive version. If *TCM10* were essential for yeast viability, this recombination event would be lethal in a haploid strain.

Eighteen tryptophan prototrophs were isolated. Four of these 18 colonies could not grow on YPG. However, only one was shown to be ρ^+ after complementation with the ρ° strain, MS10. This single isolate could be complemented for growth on glycerol with the plasmid YCplac33-TCM10(1#) and was named tcm10dsr2c. A whole cell PCR (Sathe *et al.*, 1991) was used to confirm the insertion of *TRP1* gene at the SstI site of the chromosomal *TCM10* locus (Figures II-6, II-13). With the p1 and p2 primers, a 1.2 kb fragment is expected from the disruption mutant cells (Figure II-13, lane 7) and a 0.5 kb fragment from the wild type cells (lane 5). With the p1 and p3 primers, fragments of 1.5 kb and 0.7 kb were expected in the disruption mutant (lane 14) and the wild type cell (lane 12), respectively. Digestion of these PCR products from the disruption mutant reveals the 0.8 kb *TRP1* marker gene as well as the common fragments present in both disrupted and non-disrupted

TCM10 loci (lanes 8, 9, and 10; lanes 15, 16, and 17). This result suggests that the size difference between PCR products from *TCM10* locus disrupted and non-disrupted strains was due to the insertion of *TRP1* marker gene at SstI site in the *TCM10* locus. Further confirmation was from genetic analysis. The disruption mutant, tcm10dsr2c, was mated with the wild type strain MH124. The resultant diploids were sporulated and tetrads were dissected. The cosegregation of the inability to grow on glycerol with the TRP prototrophy was found in all 9 tetrads examined (only tetrads with 4 viable spores were counted). tcm10dsr2c was also mated with tcm10-19D; diploid cells could not grow on glycerol but were TRP^+ (Table II-2). Taken together, these data indicate that tcm10dsr2c is a null mutant of the *TCM10* gene.

The *tcm10* mutation increases the frequency of partial or total loss of the mitochondrial DNA. I have also observed that tcm10dsr2c becomes ρ^- at a high rate as the cells enter stationary phase by examining the nongrowth on YPG of diploids which were made by mating tcm10-15D (a) and tcm10dsr10 (a) with the ρ^0 strain, MS10 (α).

In conclusion, we have demonstrated that the *tcm10* mutant arises from a single nuclear gene mutation, that the *TCM10* gene is not essential for yeast viability, and that the TCM10 protein may influence the stability of the mitochondrial DNA. The *TCM10* gene sequence did not provide clues as to its function. Further studies are focused on location of the protein inside the yeast cell and the range of effects caused by this mutation.

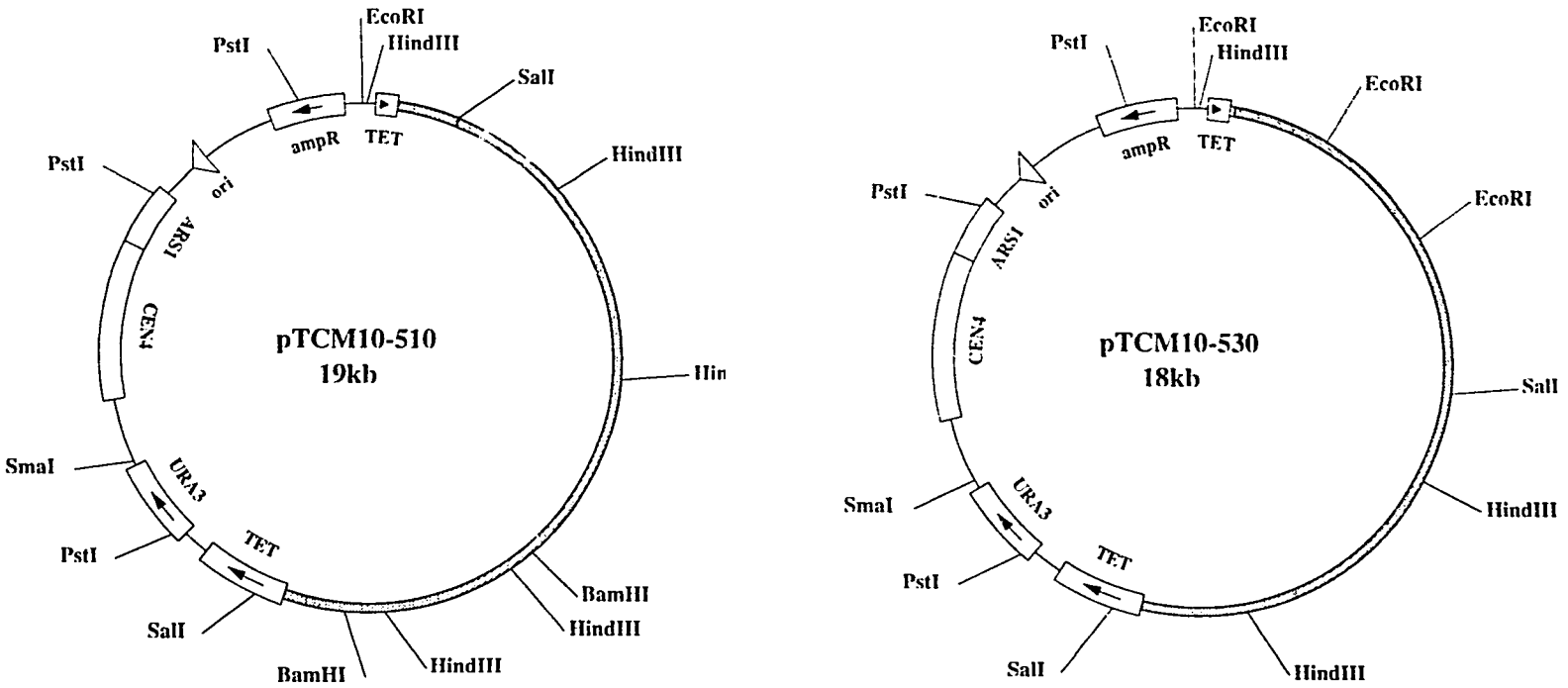


Figure II-1 Maps of the two plasmids able to complement the *tcm1C* mutation. The genomic inserts are shown as stippled fragments. The abbreviations are; ampR, ß-lactamase gene; ARS1 and CEN4, two loci for DNA replication and maintenance in yeast; TET, tetracycline resistant gene which is disrupted by the genomic inserts; URA3, the gene encoding orotidine-5'-phosphate decarboxylase, an enzyme required for the biosynthesis of uridine monophosphate; ori, replication origin required in *E. coli*. Some restriction enzyme sites are also shown.

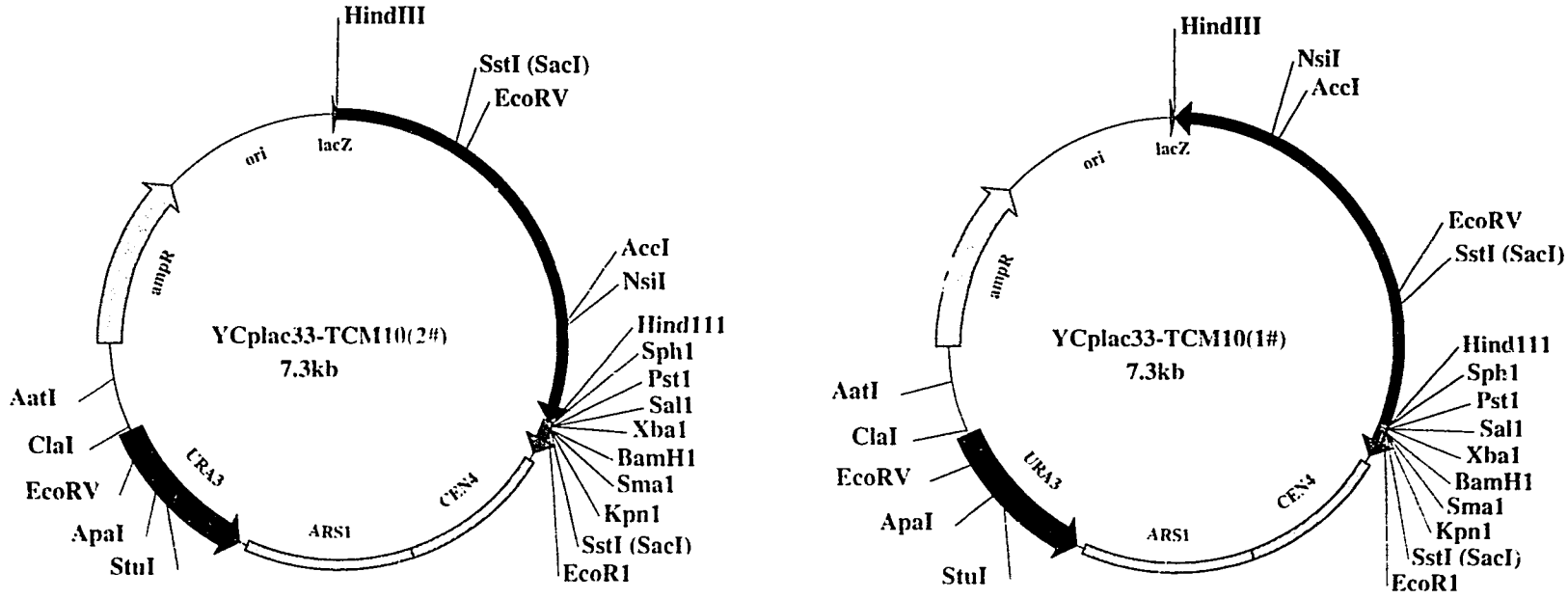


Figure II-2 The plasmids YCplac33-TCM10 (1#) and YCplac33-TCM10 (2#) carry the *TCM10* gene in two orientations. The solid arrow represents the 2.7 kb HindIII fragment originating from pTCM10-510. The arrow represents the direction of translation of the *TCM10* gene. YCplac33-TCM10 (1#) and (2#) are named based on the size of SstI fragment: (1#) contains a 0.6 kb fragment and (2#) contains a 2.1kb fragment. lacZ, ß-galactosidase. Other abbreviations are as for Figure II-1.

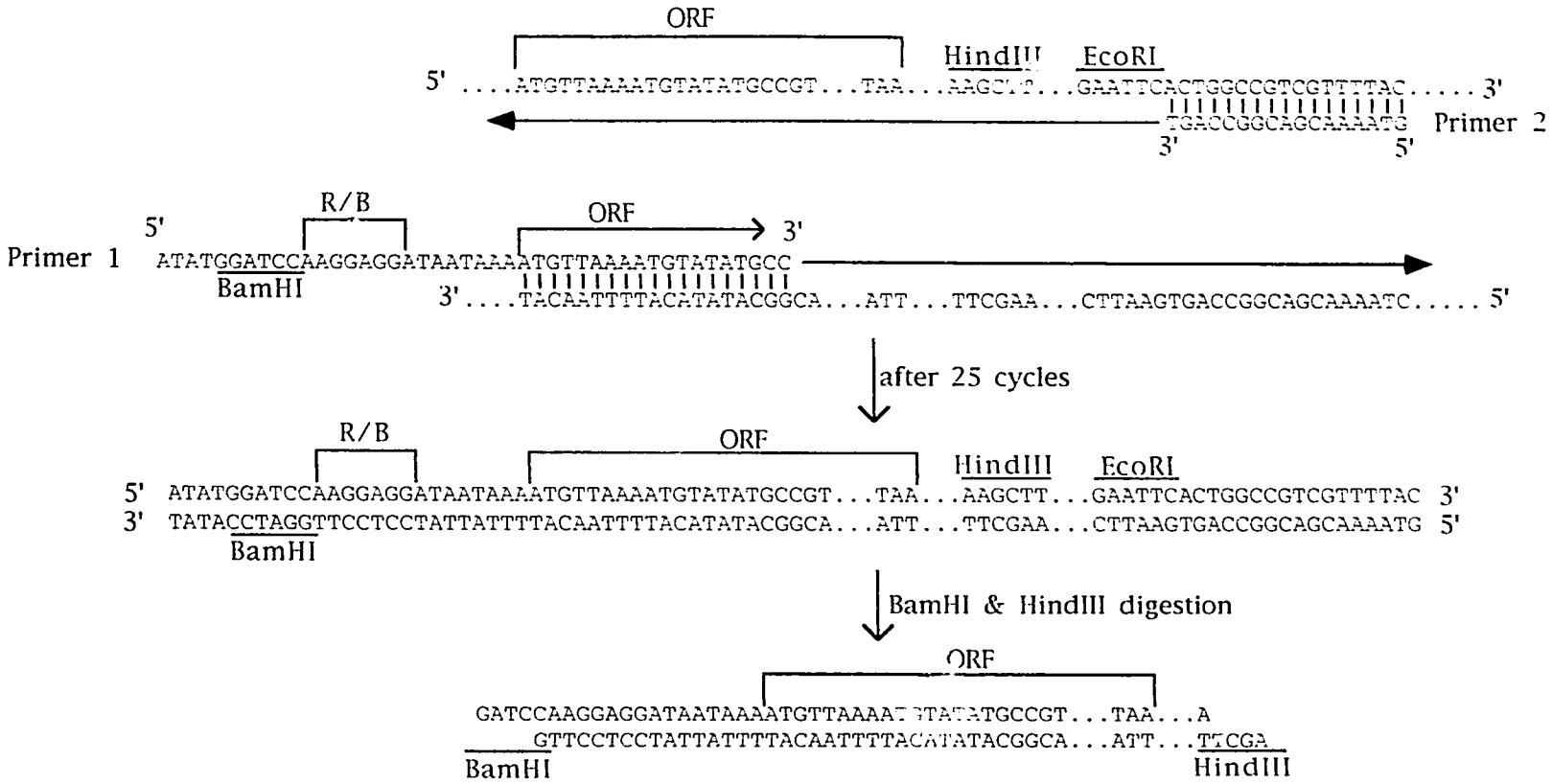


Figure II-3 Scheme for the PCR amplification of the *TCM10* gene. Two primers were used; primer 1 has a BamHI site at the 5' end followed by a ribosomal binding site, R/B (AAGGAGG), a spacer region of 8 nucleotides, and the first 19 nucleotides of the *TCM10* reading frame, primer 2 is the M13 forward universal primer. ORF, the open reading frame of the *TCM10* gene; nucleotides that are not shown are indicated with dots. Sequence between the HindIII and EcoRI sites is identical to the polylinker of pUC19. PCR template was NdeI digested YCplac33-TCM10 (2#). The solid arrowheads indicate the newly synthesized sequences extended from the two primers. After BamHI and HindIII digestion, the resultant fragment was cloned into the vector, pJF118EH.

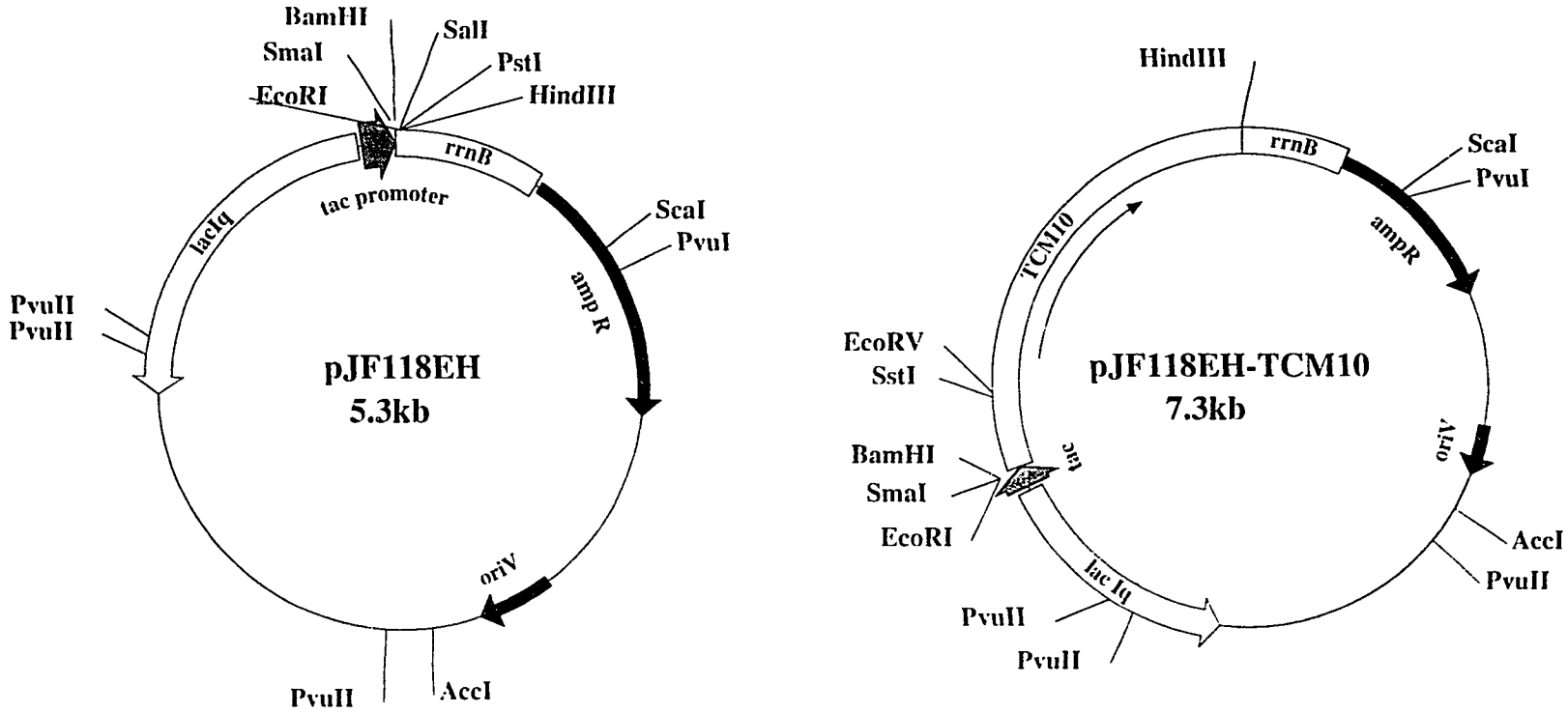


Figure II-4 The plasmid pJF118EH-TCM10 places the *TCM10* open reading frame under control of the tac promoter. PCR amplified *TCM10* fragment was inserted at the BamHI and HindIII sites of the vector pJF118EH, which contains a strong, IPTG-inducible tac promoter. lacIq, the lac repressor gene; rrnB, transcription termination signals from the rrnB operon. pJF118EH-TCM10 plasmid was used for overexpression of the TCM10 protein *in vivo*.

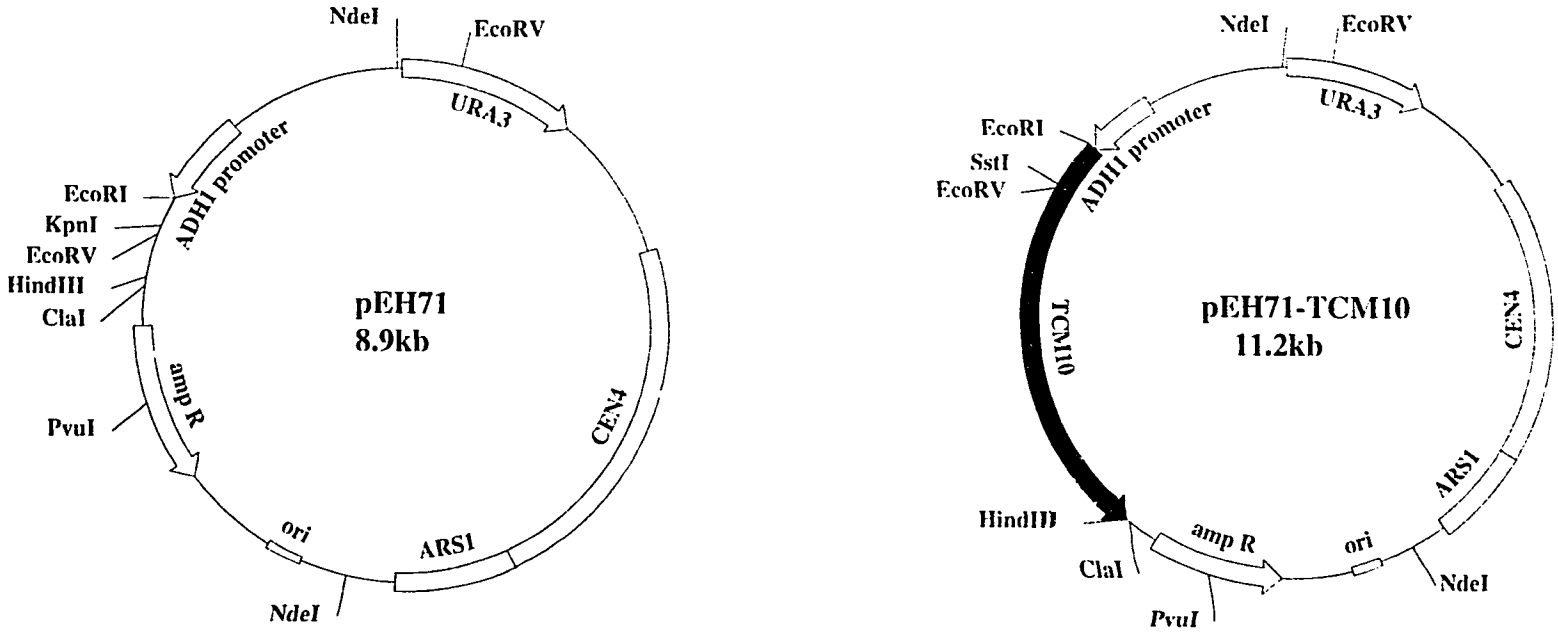


Figure II-5 pEH71-TCM10 places the *TCM10* gene under control of the *ADH1* promoter. It was used for expression in yeast. The 2.4 kb EcoRI and HindIII fragment from pJF118EH-TCM10 was inserted into likewise cut pEH71. The open reading frame of *TCM10* is represented as the thick solid arrow after the *ADH1* promoter. The direction of the arrow also indicates the direction of translation. Other abbreviations are as in Figures II-2, 3, 4.

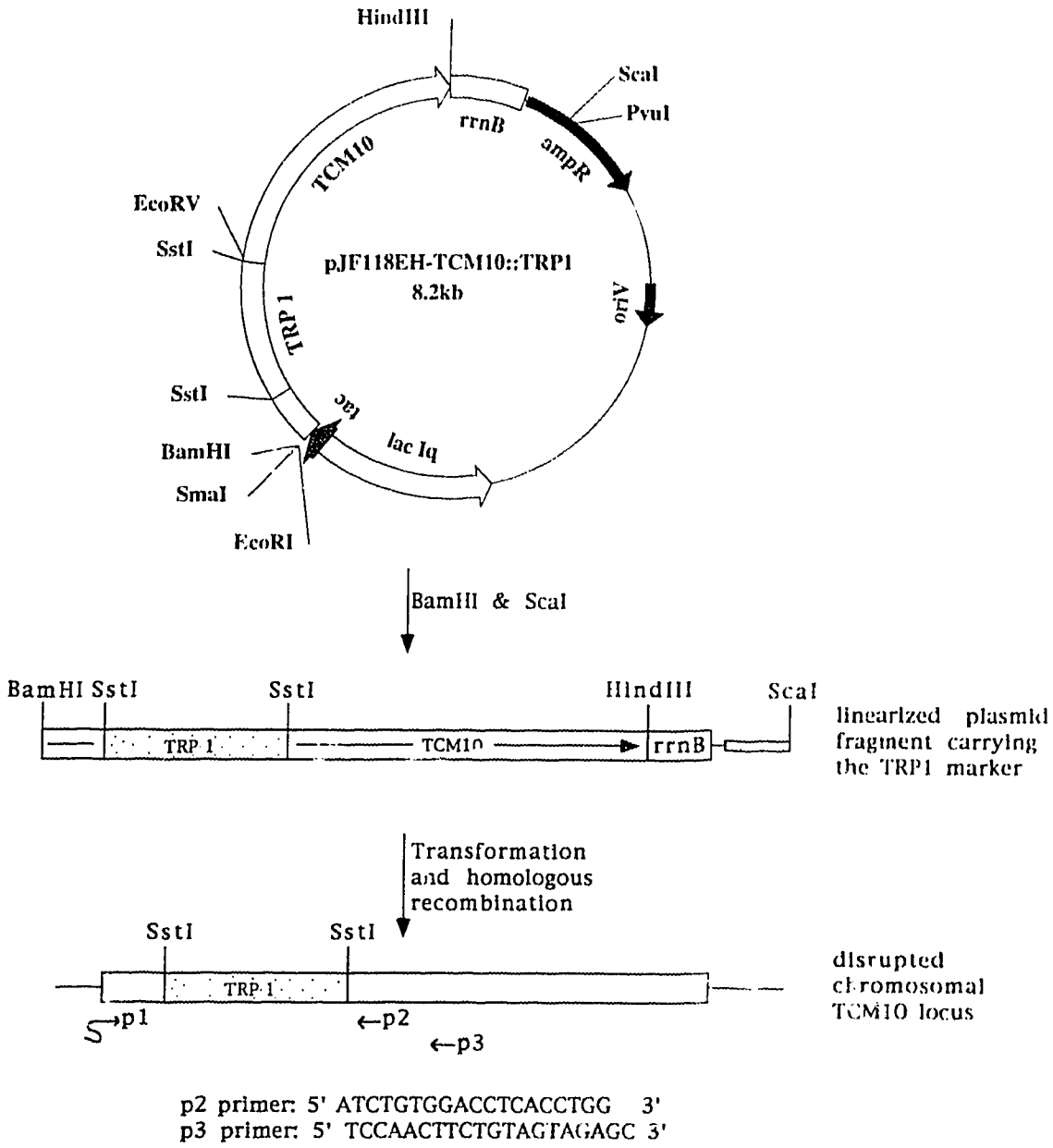
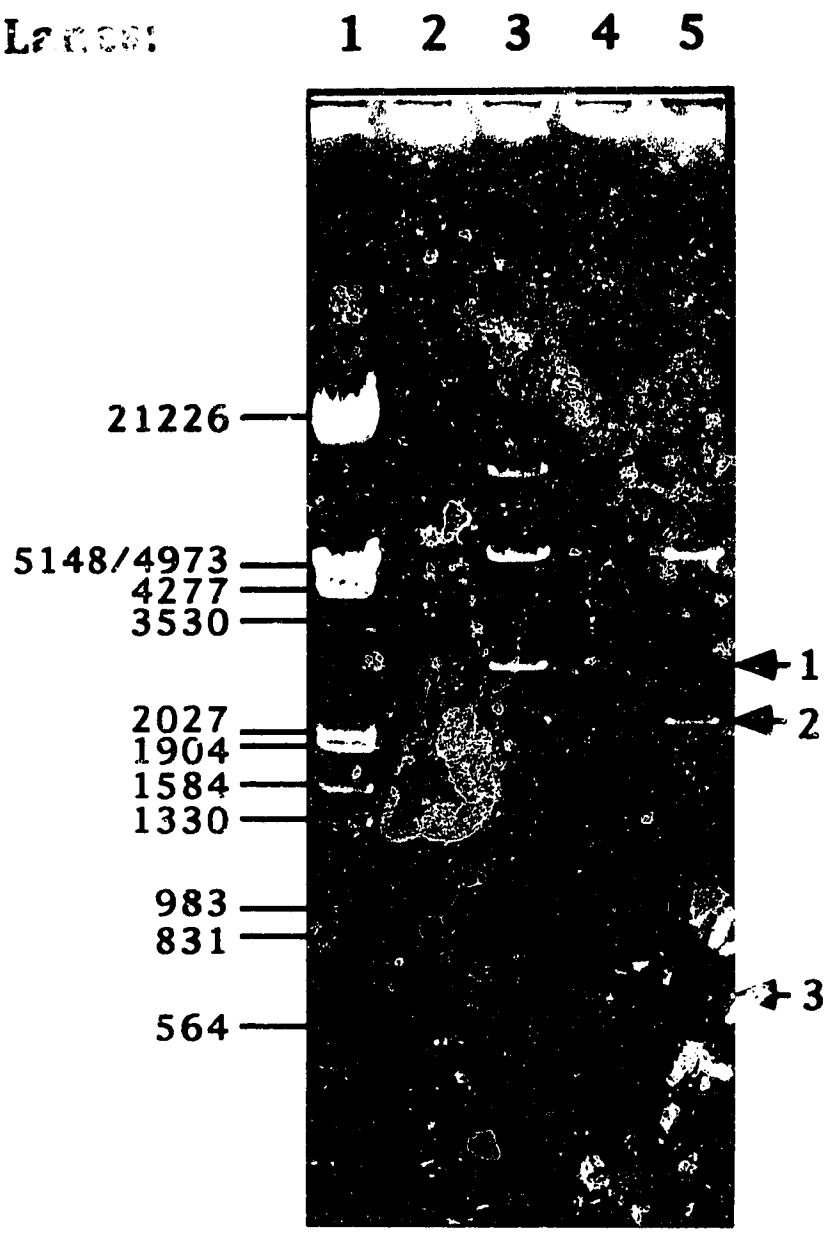


Figure II-6 Scheme for the disruption of the chromosomal *TCM10* gene. The fragment cut with BamHI and ScaI from the plasmid pJF118EH-TCM10::TRP1 is shown. It was used for targeted disruption of the chromosomal *TCM10* gene. The disrupted chromosomal *TCM10* gene is also shown with *TRP1* marker gene. The oligonucleotide primers used for confirmation of the disruption by the whole cell PCR are shown as arrows indicated as p1, p2, and p3. The p1 primer is primer 1 as described in Figure II-3. The p2 and p3 primers were used for DNA sequencing of the *TCM10* gene. The fragment of p1-p2 and p1-p3 are about 1.2 kb and 1.5 kb (*TRP1* gene is about 0.8 kb), respectively. See previous figures for abbreviations used.

Figure II-7 pTCM10-510 and pTCM10-530 contain overlapping inserts. HindIII and HindIII/SstI digestions were carried out on both plasmi Lane 1, λ-DNA cut with EcoRI and HindIII as a standard; lanes 2 and 3, HindIII digestion of pTCM10-510 and pTCM10-530, respectively; lane 4 and 5, HindIII/SstI digestion of pTCM10-510 and pTCM10-530, respectively. The sizes (in base pairs) and positions of the marker bands are indicated on the left of the picture (bands of 5148 and 4973 are not resolved). Arrows on the right are: (1) the common 2.7 kb HindIII band found in both pTCM10-510 and pTCM10-530 (lanes 2 and 3); (2) the common 2.1 kb and (3) the common 0.6 kb HindIII/SstI bands in both pTCM10-510 and pTCM10-530 (lanes 4 and 5). A 0.8% agarose gel was used.


Lanes:
1 2 3 4 5
21226
5148/4973
4277
3530
2027
1904
1584
1330
983
831
564
1
2
3

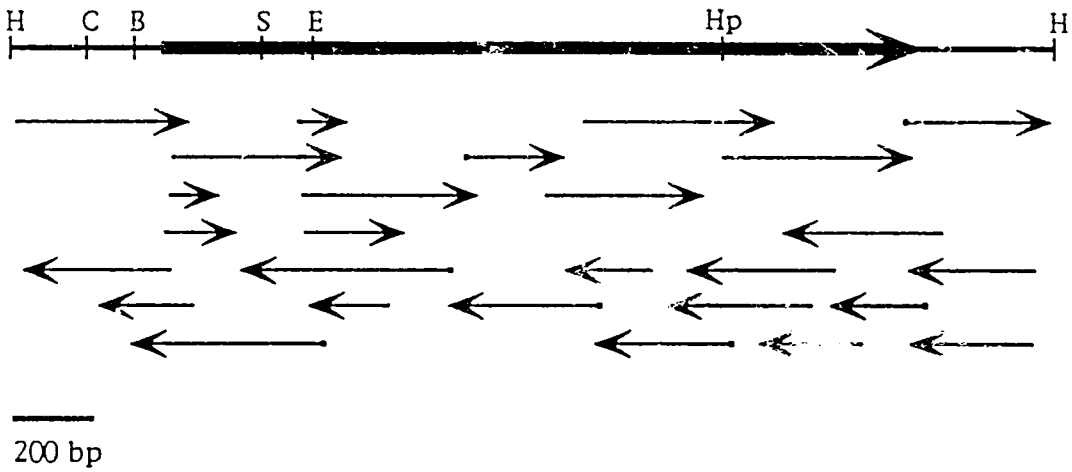


Figure II-8 Sequencing strategy for the *TCM10* gene. Some useful restriction sites are shown above the sequence. H, HindIII site; C, ClaI site; B, BglII site; S, SstI site; E, EcoRV site; and Hp, HpaII site. The open reading frame is shown as thick arrow within the sequence. The entire sequence was determined on both strands. bp, base pair.

```
                                                    AAGCTTGTAA GAACGTATGAATGAAAGCACTAATTGACAAAAAACTG    -56
AATTTTGTCAAAA AGCTCTATGTTTCAGAATGATAAAAAGGAGAGTTAAAAATA AATGGACAGTGAAT   285
ACAGCCAAATGC GATAGCTTTCGCAGAAACAGTACAATTAAAGATCAACAC TGTTATTCTAATTATAGC   214
AGGATCGATGTT ACATCTCTTTTGCTCTCTAATCGGAGCTGAAAAAAAAAAAAAAAAGCGTATATAGTGAT   143
CTACGCTGAT  AACATTATAGATTGCATAAAAGGGAAAAAGTA  TCAAGATCTATTCTCGCGGACA    72
CACTTTA     GTACTGGCAAGAATGGGCTTTCAAATCTT  TCACTTTAATGTTCTTATTGC     -1

M   L   F   C   I   C   R   A   Y   S   Q   I   L   A   Q   M   V   T     18
ATG TT  AA TGT ATA TGC CGT GCC TAT TCC CAG CCT TTG GCT CAA ATG GTC ACT    54

S   P   L   F   K   H   M   G   S   A   G   T   Y   T   I   L   P   I     36
TCA CCG CTG TTT AAA CAT ATG GGT AGC GCA GGG ACA TAC ACA ATT CTA CCT ATT   108

T   N   L   R   H   L   S   T   K   N   C   F   L   K   I   K   S   N     54
ACA AAT CTG CGC CAT TTA TCT ACG AAA AAC TGT CCA TTG AAA ATT AAA TCA AAT   162

R   S   E   P   L   Q   F   G   D   F   E   R   Q   V   P   C   S   E     72
CGT TCA GAA CCT TTG CAG TTT GGC GAT TTT GAA CGA CAG GTG CCA TGT TCT AGG   216

K   S   G   S   S   K   N   V   Q   K   R   L   Y   E   L   R   Q   L     90
AAG AGC GGA TCT TCG AAA AAT GTT CAG AAG CGT TTA TAC GAG CTC CGA CAA TTG   270
                                                    Sst I
K   T   V   L   S   E   T   F   G   V   T   E   Y   A   S   F   F   E    108
AAA ACT GTA TTA AGT GAG ACT TTT GGA GTA ACT GAA TAC GCC TCT TTT TTT GAA   324

S   L   F   N   A   L   H   I   N   N   C   I   E   L   E   K   K   K    126
TCT CTA CGA AAT GCC CTG CAT ATA AAT AAT TGC ATA GAA AAT GAA AAG AAG AAA   378

L   L   Y   D   I   I   L   H   Q   H   E   L   Y   P   E   V   A   F    144
CTT TTA TAT GAT ATC ATT TTG CAT CA  CAC GAG TTA TAC CCA GAG GTG GCA AGA   432

K   I   G   F   Y   L   P   G   E   V   H   R   W   F   W   Y   H   I    162
AAA ATT GGA TTT  AT CTA CCA GGT GAG GTC CAC AGA TGG TTC TGG TAT CAT ATT   486

F   K   S   E   S   F   N   H   Y   L   F   L   L   K   S   D   V   L    180
CCA AAA TCA GAA TCT TTT AAT CAC TAT CTT TTT CTT TTG AAA AGT GAT GTT CTT   540

L   F   T   S   N   Y   C   T   R   F   T   N   R   L   I   K   G   T    198
CTC TTT ACT TCT AAC TAC TGT ACG AGA TTT ACC AAT AGG CTG ATA AAA GGA ACT   594

E   M   E   P   Q   L   A   T   F   Q   I   F   L   H   D   E   T   N    216
GAG ATG GAG AG  CAG TTA GCC ACG TTC CAA ATA TTT TTG CAT GAT GAC ACA AAC   648

I   K   F   I   M   E   K   V   L   K   L   H   T   F   D   S   L   I    234
ATA AAA TTT ATA ATG GAA AAA GTT CTG AAG TTG CAT ACA TTT GAC AGC TTA ATC   702

A   L   V   N   G   L   V   K   A   K   N   F   R   F   I   K   V   F    252
GCT CTA GTA AAT GGC CTT GTT AAG GCG AAA AAT TTT AGG TTC ATT AAA GTA TTT   756

I   Q   A   L   L   Q   K   L   E   Q   H   C   Y   S   G   P   D   G    270
ATT CAA GCT CTA CTA CAG AAG TTG GAA CAG CAC TGT TAT TCT GGT AAA GAT GGA   810

A   .   Q   K   N   L   R   Y   V   F   F   N   N   T   L   L   Y   Y    288
GCA A   CAA AAG AAC CTG CGT TAT GTC AAG TTC AAC AAT ACC TTA CTT TAT TAC   864

L   L   K   S   G   N   V   E   L   F   I   P   T   F   Q   E   E   L    306
CTT TTG AAA AGT GGA AAT GTT GAG TTA TTC ATC AAA ACC TTT CAA GAG GAA CTG   918

K   F   I   V   S   S   G   L   L   N   H   I   D   G   N   D   H   I    324
AAA TTC ATT GTA AGC TCA GGA TTG CTT AAT CAC ATT GAT GGA AAC GAC CAC ATA   972
```

```
L   N   F   P   I   H   H   Y   L   N   L   L   R   I   S   N   R   Q     342
TTG AAT TTT CCC ATT CAC CAC TAC TTG AAT CTG CTC CGA ATA TCT AAT AGG CAG   1026

E   E   L   F   N   V   I   S   C   L   Q   S   S   P   L   M   K   Y     360
GAG GAG CTT TTC AAT GTA ATT TCC TGC TTA CAA AGC AGC CCA CTG ATG AAA TAT   1080

K   L   F   E   E   F   L   M   G   E   L   I   A   S   F   Q   A   F     378
AAA TTG TTC AAG GAG TTT TTA ATG GGT GAA TTA ATT GCA TCT TTT CAA GCC TTT   1134

R   D   P   K   L   V   C   K   Y   L   L   S   S   Y   S   S   K   A     396
CGT GAC CCG AAA TTG GTG TGT AAA TAT CTC CTC TCA TCA TAC AGC TCA AAG GCG   1188

S   A   N   I   L   N   A   L   G   I   W   G   W   L   Y   H   S   K     414
TCT GCT AAT ATT CTG AAT GCC CTA GGG ATT TGG GGG TGG CTC TAT CAT TCA AAA   1242

S   T   T   L   T   A   P   T   L   A   R   E   L   K   N   K   N   N     432
TCA ACG ACT TTG ACA GCT CCT ACG TTA GCA AGA GAG CTG AAG AAC AAA AAT AAT   1296

I   L   P   N   T   M   R   I   G   S   P   V   T   V   P   I   L   T     450
ATT CTA CCG AAC ACA ATG CGA ATA GGA TCA CCA GTG ACT GTT CCA ATT TTA ACT   1350

E   L   Y   R   S   L   L   S   S   S   S   V   S   L   E   S   G   Q     468
GAA CTG TAT AGA AGC CTT TTA TCT TCG AGT TCT GTT TCA CTG GAA AGT GGT CAA   1404

F   K   N   C   L   L   D   L   Y   Y   K   Y   K   S   F   L   S   E     486
TTC AAG AAT TGT CTT CTT GAT TTA TAT TAC AAG TAC AAG AGT TTT CTT TCC GAG   1458

E   A   H   K   Y   R   Y   W   R   N   D   T   G   I   L   N   V   F     504
GAA GCT CAT AAA TAC AGG TAT TGG AGA AAT GAT ACC GGA ATC CTC AAT GTT TTC   1512

L   N   Y   I   R   F   Q   A   R   E   P   R   L   A   Y   N   V   L     522
CTA AAT TAT ATT AGA TTC CAA GCT CGT GAA CCA AGG CTC GCC TAC AAT GTT CTT   1566

L   D   F   Y   S   Q   P   F   A   K   K   V   V   L   T   T   T   L     540
CTG GAT TTT TAT TCT CAA CCT TTC GCC AAA AAA GTG GTT TTA ACT ACC ACG CTA   1620

C   P   F   S   I   V   A   Y   K   N   H   T   L   T   Q   A   E   L     558
TGT CCC TTT TCC ATA GTA GCC TAT AAA AAT CAT ACA TTA ACG CAG GCT GAA CTA   1674

S   E   L   L   Q   V   M   H   K   N   G   V   P   L   T   F   K   F     576
TCA GAA TTG CTA CAG GTG ATG CAT AAA AAT GGG GTG CCT TTA ACG TTT AAA TTT   1728

C   S   A   M   V   M   H   Y   V   K   M   R   D   E   K   G   A   R     594
TGT TCT GCA ATG GTA ATG CAT TAT GTC AAA ATG AGA GAC GAA AAA GGA GCA CGC   1782

S   W   Y   N   K   I   L   F   G   G   F   E   I   R   H   M   A   L     612
TCC TGG TAC AAT AAG ATA CTC TTC GGG GGC TTT GAA ATC AGG CAT ATG GCT TTG   1836

I   Q   I   I   K   D   Q   G   W   P   F   P   K   N   F   D   E   T     630
ATA CAG ATA ATA AAA GAC CAA GGC TGG CCG TTT CCC AAA AAC TTT GAC GAA ACT   1890

L   L   T   E   L   V   E   N   N   N   I   K   E   P   T   D   S   T     648
TTA CTG ACG GAA TTG GTG GAA AAT AAT AAC ATT AAA GAA CCT ACT GAT AGC ACC   1944

L   F   T   D   E   D   M   F   E   E   D   G   K   P   R   F   N   D     666
CTG TTT ACT GAC GAA GAT ATG TTC GAA GAA GAT GGC AAG CCT AGA TTT AAT GAT   1998

D   D   V   N   K   C   T   N   I   I   R   E   T   L   K   S   L   N     684
GAT GAT GTT AAT AAA TGT ACA AAT ATT ATA AGA GAG ACG TTG AAA AGT CTA AAT   2052

*
TAA                                                                       2055
```

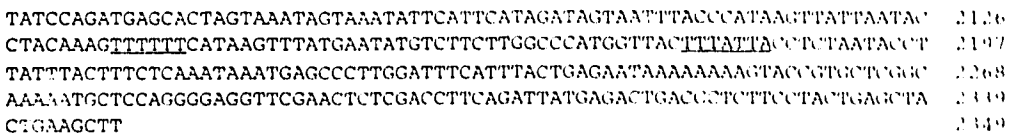


Figure II-9 Nucleotide sequence of the *TCM10* gene and flanking regions. The sequence of the entire 2.7 kb genomic insert is shown. The *TCM10* open reading frame starts with the methionine codon at position +1 and ends with a stop codon, TAA, at position +2053. Two putative TATA boxes and polyadenylation signals each are underlined in the 5' and 3' flanking regions of the *TCM10* reading frame, respectively. Only the sense strand is shown. The deduced TCM10 protein sequence is also shown above the nucleotide sequence.

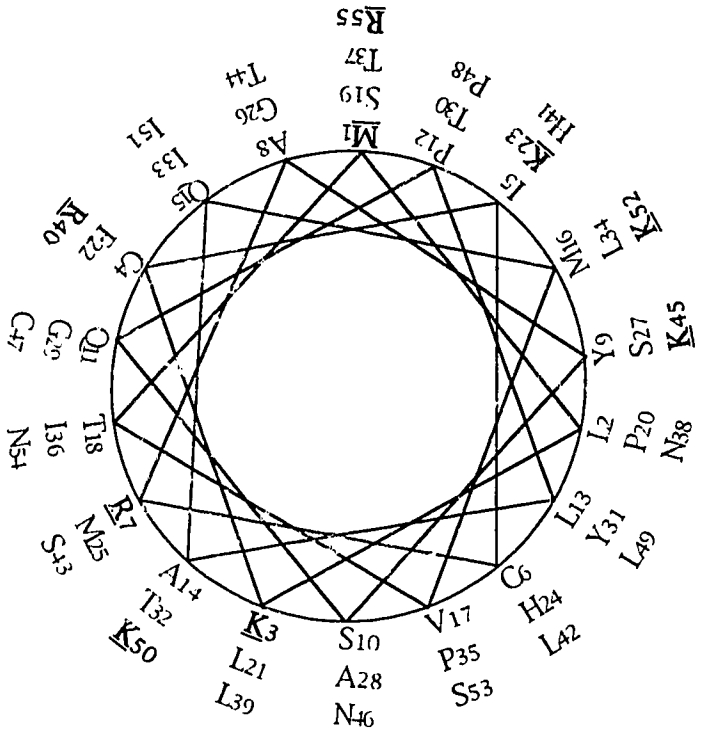


Figure II-10 Amphipathic helical wheel of the first 55 amino acid residues. The charged residues are shown in bold and underlined. The 9 positively charged amino acid residues are not clustered.

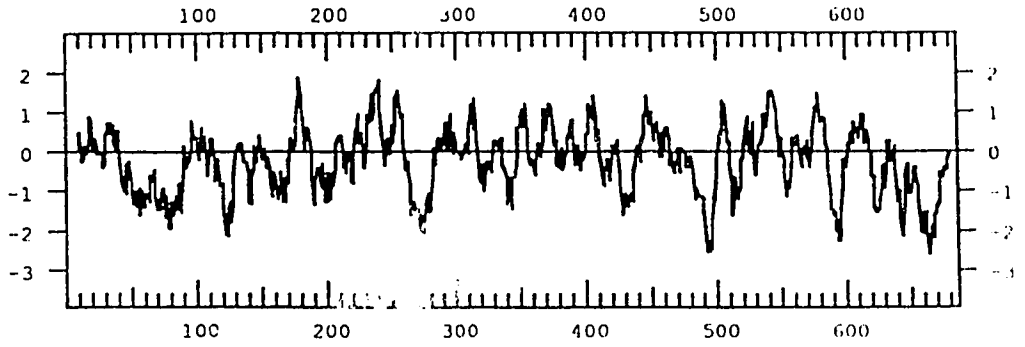


Figure II-11 Kyte Doolittle hydrophobicity profile of TCM10 protein. The window used was 11 amino acid residues. The verticle scale represents hydrophobicity values with positive values indicating the degree of hydrophobicity. The horizontal axis represents the tcm10 amino acid sequence.

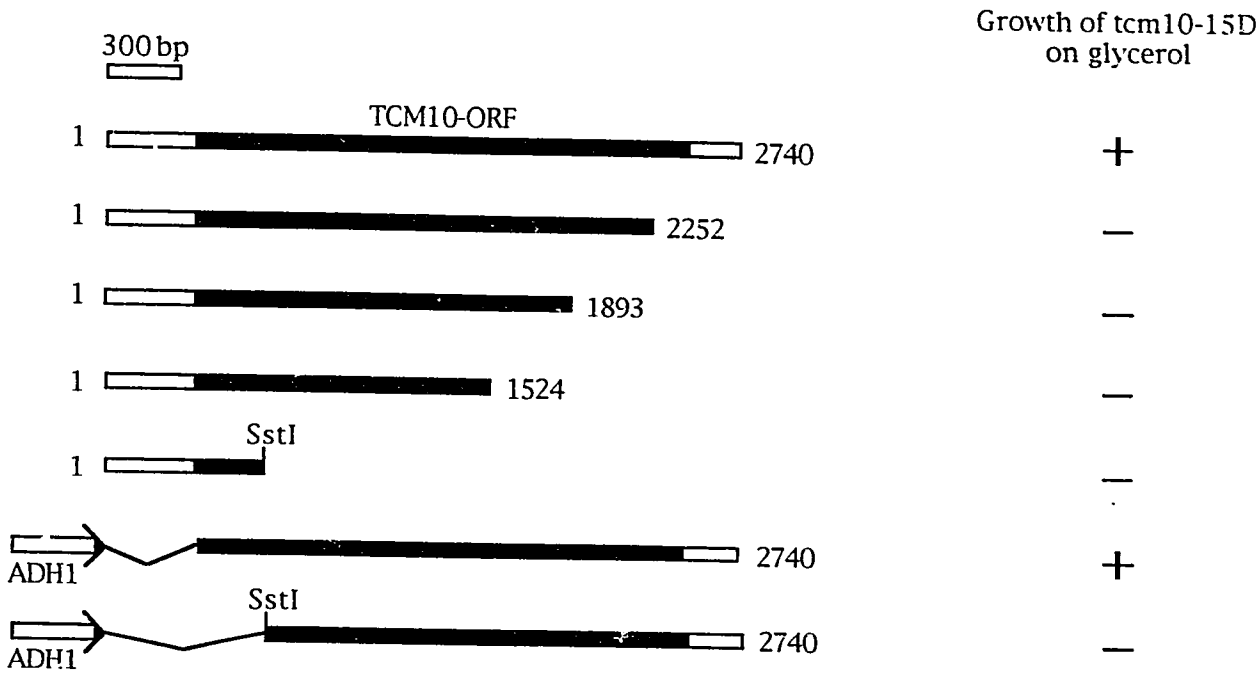
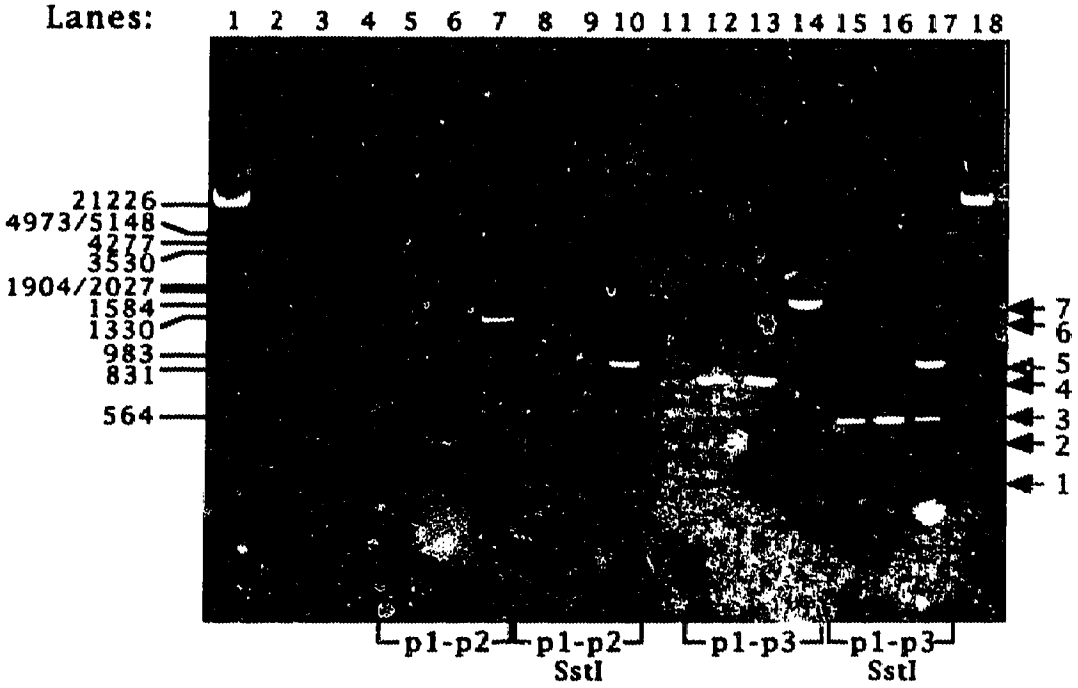


Figure II-12 Complementation by plasmids with different N- and C-terminal deletions. The DNA fragments under the control of ADH1 (Alcohol Dehydrogenase 1) promoter are PCR amplified products (see text). The TCM10 reading frame is shown as filled area. +, growth; -, no growth on glycerol.

Figure II-13 Confirmation of the *TCM10* gene disruption. Lanes 1 and 18 are the digests of λ-DNA with EcoRI and HindIII, sizes are indicated on the left of the picture; lanes 2, 3, and 4 are negative controls without the addition of cells to the PCR reactions, but contain primers p1 and p2, p1 and p3, and p1, respectively; lanes 5 and 6 are the PCR products of the wild type MH125 and the mutant tcm10-15D with p1 and p2, respectively; lane 7 is the PCR products of the disruption mutant tcm10dsr2c with p1 and p2; lanes 8, 9, and 10 are the SstI digests of the PCR products of lanes 5, 6, and 7, respectively. Lane 11 is the digests of pBR322 with HaeIII (587, 540, 504, 458, and 434 bp from the top to the bottom, respectively). Lanes 12 to 17 are in the same order as lanes 5 to 10 except with the primer combination of p1 and p3. The primers used and the SstI digestion are also indicated beneath the corresponding lanes to make comparison easier. Refer to Figure II-6 for primer information. The scheme is shown underneath the picture panel. Open bars show the PCR fragments without insertion, the inserts, *TRP1* gene, are at SstI site and shown as solid bars. The positions of each PCR fragment as well as fragments resulted from their SstI digestion are indicated by arrows on the right of the picture. B, BamHI site; S, SstI site. The p1-p2 PCR fragments are 0.5 kb (arrow 2, lanes 5 and 6) with MH125 and tcm10-15D cells, and 1.3 kb (arrow 6, lane 7) with tcm10dsr2c cells; two 0.25 kb fragments (indicated by arrow 1) and an extra 0.8 kb fragment (arrow 5, lanes 10 and 17) in tcm10dsr2c are released after SstI digestion. Meanwhile, the p1-p3 PCR fragments are 0.7 kb (arrow 4, lanes 12 and 13) with MH125 and tcm10-15D cells, and 1.5 kb (arrow 7, lane 14) with tcm10dsr2c cells; two fragments of 0.25 kb (arrow 1) and 0.5 kb (arrow 3, lanes 15, 16, and 17) and an extra 0.8 kb fragment are released after SstI digestion. The ruler is 250 bp.


Lanes:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
21226
4973/5148
4277
3530
1904/2027
1584
1330
983
831
564
7
6
5
4
3
2
1
p1-p2
p1-p2
SstI
p1-p3
p1-p3
SstI

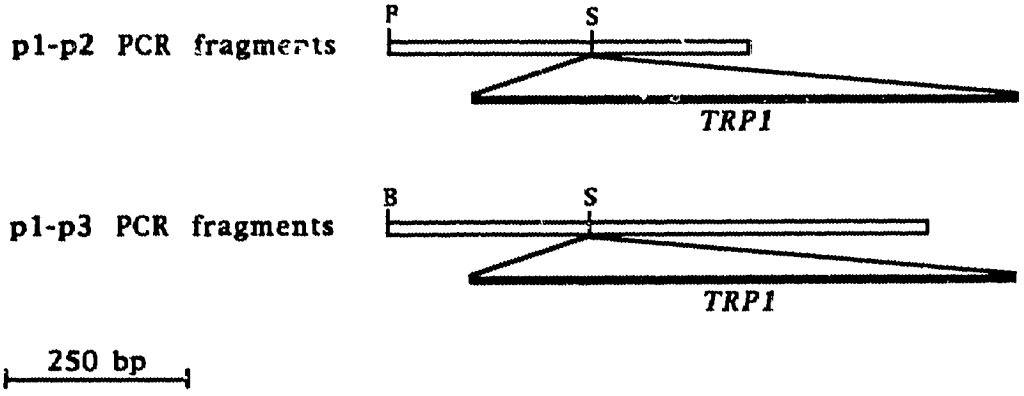

p1-p2 PCR fragments
P
S
TRP1
p1-p3 PCR fragments
B
S
TRP1
250 bp

Table II-1 Genotypes of the strains used in this study. Prototrophic markers are indicated in capital letters; in contrast, auxotrophic genes are in small letters. Both tcm10 and tcm10-9B have become ρ^-.

strains	mating type	Genotype
DAU1	α	*ade2*, *ura3*-Δ
MH124	α	*leu2*-3, -112, *ura3*-52, *trp1*, *his3*, *his4*
MH125	a	*leu2*-3, -112, *ura3*-52, *trp1*, *his3*, *his4*
tcm10	a	*leu2*-3, -112, *ura3*-52, *trp1*, *his3*, *his4*, *tcm10*, ρ^-
tcm10-9B	α	*ade2*, *LEU2*, *ura3*, *TRP1*, *his3*, *his4*, *tcm10*, ρ^-
tcm10-15D	a	*ade2*, *LEU2*, *ura3*, *trp1*, *his3*, *his4*, *tcm10*
tcm10-19D	α	*ade2*, *LEU2*, *ura3*, *trp1*, *his3*, *his4*, *tcm10*

Table II-2 Complementation analysis of the disruption mutant, tcm10dsr2c. tcm10dsr2c was mated with the wild type, MH124, and the mutant, tcm10-19D. Results are shown with + and -. +, growth; -, no growth at all. See Materials and Methods for more information about media used.

	Mating types	YPD	YPG	SDC-trp	SGC-trp
tcm10dsr2c	a	+	−	+	−
tcm10-19D	α	+	−	−	−
MH124	α	+	+	−	−
tcm10dsr2c X MH124	a/α	+	+	+	+
tcm10dsr2c X tcm10-19D	a/α	+	−	+	−

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CHAPTER III. FUNCTIONAL STUDIES ON THE YEAST TCM10 PROTEIN

A. INTRODUCTION

Nuclear petite mutants have been used to study many functions of mitochondrial proteins such as protein import mechanism, energy generation, mitochondrial DNA expression, and communication between nuclear and mitochondrial genomes. Many nuclear gene petites have multiple effects on mitochondrial protein import or respiration, making it difficult to separate primary effects from secondary ones.

Unlike most eukaryotes, yeast can survive the loss of part or all of its mitochondrial DNA; these states are designed ρ^- and ρ°, respectively (Dujon, 1981). All ρ^- and ρ° cells are deficient in mitochondrial protein synthesis. Moreover, mitochondria tend to lose their DNA partially or entirely when mitochondrial protein synthesis is impaired (Attardi and Schatz, 1988). Mutations in some nuclear genes can also result in the loss of mitochondrial DNA, especially, those affecting mitochondrial gene expression such as the mitochondrial RNA polymerase (Lustig *et al.*, 1982), maturases for intron splicing of mitochondrial mRNA (McGraw and Tzagoloff, 1983), mitochondrial aminoacyl-tRNA synthetases, and proteins of the mitochondrial ribosome (Myers *et al.*, 1985). The loss of mitochondrial protein synthesis results in a defective respiratory chain that will certainly affect the membrane potential which is required for import of most mitochondrial proteins (Hartl *et al.*, 1989; Pfanner *et al.*, 1988). This secondary effect may be the reason why some respiratory-deficient mutants have a higher frequency of ρ^- or ρ° mutations.

The *tcm10* mutants, tcm10-15D and tcm10dsr2c, lose their mtDNA at high frequencies making functional studies very difficult. Grown on YPD 0.6% media, about 80% of mutant cells are ρ^- by stationary phase. To overcome this problem, the *pet9* mutation was introduced into tcm10-15D and tcm10dsr2c to create TCM10-pet9-1A, tcm10-pet9-1C, tcm10-pet9-9B, and TCM10-pet9-9C mutants. *pet9* mutants are defective in the gene for the major mitochondrial ADP/ATP carrier and this mutation is lethal to pet9 cells without a complete mitochondrial DNA (Mattoon *et al.*, 1979). However, the function of the PET9 protein is not clear (Mattoon *et al.*, 1979). The activities of respiratory chain enzymes in the *tcm10/pet9* double mutants were measured and compared with those of control single *pet9* mutants. Cytochrome contents were also measured. In studying the localization of TCM10, I used polyclonal antibodies against the N-terminal 87 amino acid residues of the TCM10 protein. The TCM10 protein is mitochondrial and membrane associated. In this chapter, I will present a biochemical characterization of the *tcm10* mutation.

B. MATERIALS AND METHODS

1) Strains and Media

Strains and media are as described in Chapter II. Additional strains used include D273-10B A1 (MATα, met6), D360-2D (MATα, pet9, his7, ade1, ade2) and D360-7D (MATa, pet9, his7, ade1, ade2). Additional media include YPD 0.6% (1% yeast extract, 1% peptone, 0.6% glucose, 0.12% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, pH 6.2, wt/vol), lactate media (0.3% yeast extract, 0.1% glucose, 0.05% $CaCl_2$, 0.05% NaCl, 0.06% $MgCl_2$, 0.1% KH_2PO_4, 0.1% NH_4Cl, 2.0% lactic acid, vol/vol, pH to 5.5). Additional *E. coli* strains, BNN103 [(lacIOP2YA)U169, proH$^+$, lon, araD135, strA, thi, hflA15D, cbr::Tn10], and DH5α [F$^-$, φ80dlacZΔM15Δ(lacZYA-argF)U169,

recA1, endA1, hsdR17(rK , mK+), supE44, λ-, thi, gyrA, relA1] were used in this study. UT580 was used as the host for expressing the fusion protein and general gene cloning.

2) Overexpression of TCM10 in *E. coli*

pJF118EH-TCM10 transformed *E. coli* cells were grown in LB+Amp broth to an OD_{600} = 0.5 at 37°C; isopropylthio-β-D-galactoside (IPTG; GIBCO-BRL, USA) was added to a final concentration of 1 mM. Cells were collected periodically for up to 10 hours. pJF118EH transformed cells were harvested 3 hours after IPTG was added. At each time point, 1 ml of culture was pelleted, resuspended in 0.1 ml TEN buffer (50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 0.3 M NaCl), and treated with lysozyme (10 µg/ml cells; Sigma, USA) on ice for 15 minutes. Two µl of 10% Nonidet P-40 (octyl-phenoxypolyethoxyethanol; SIGMA, USA) and 1 µl of 1 M phenylmethylsulfonyl fluoride (PMSF; SIGMA, USA) were added to the digestion solution. The mixture was then sonicated with Braun Sonic-1510 (B. Braun Instruments, USA) at 100 W for 3 x 3 sec at 4°C to shear DNA. The samples were centrifuged at 14,000 x *g* for 10 min, the pellets were washed twice in TEN buffer and dissolved in 50 µl of urea-SDS cracking buffer (0.01 M $NaPO_4$ pH 7.2, 1% ß-mercaptoethanol, 1% SDS, 6 M urea) at 30°C for 30 min. 25 µl of 3 x Laemmli sample buffer (Laemmli, 1970) was added to each pellet fraction and 50 µl was added to each supernatant fraction. Samples were incubated at 95°C for 4 min followed by centrifugation at 14,000 x *g* for 1 min before being loaded on 10% SDS polyacrylamide gels.

3) Construction and Expression of the Plasmid pATH11-tcm10BS

Since the 0.6 kb BamHI/SstI fragment of pJF118EH-TCM10 could not be inserted directly in pATH11 in frame with the *trpE* gene (see Chapter II), it was first inserted into the likewise cut vector pMTL22 to make the plasmid, pMTL22-tcm10BS (Figure III-1). Thereafter, the 0.6 kb BamHI/XhoI fragment

of pMTL22-tcm10BS was inserted in the BamHI and SalI sites of the vector pATH11 (Koerner *et al.*, 1991) to create pATH11-tcm10BS (Figure III-2) which was used to express a trpE-tcm10 fusion protein. Extraction of the expressed fusion protein was done both in small and large scales (Koerner *et al.*, 1991).

4) Electroelution of trpE-tcm10BS Fusion Protein and Immunization

Extracted fusion protein from a large scale expression of pATH11-tcm10BS transformed cells was run on a 10 cm, 1.5 mm thick, 10% SDS polyacrylamide gel. The gel was run at 200 V for 45 min, stained with Coomassie blue for 20 min, and destained for 2-3 hrs. The fusion protein band was excised, cut into 1 mm cubes, and soaked in elution buffer (0.1% SDS, 50 mM NH_4HCO_3) for 5 min before electroelution (Hunkapiller *et al.*, 1983). Polyclonal antibodies were raised in rabbits using electroeluted fusion protein as antigen (Sambrook *et al.*, 1989; Sunbar and Schwoebel, 1990).

5) Western Blot Analysis

Proteins separated on 10% SDS-polyacrylamide gel were transferred to nitrocellulose membranes (0.45 micron; Bio-Rad, USA) using the electrophoretic transfer system ET20 as described by the supplier (Tyler Research Instruments, Canada). The transfer was at 250 mA for 45 min at room temperature.

The detection of transferred proteins was carried out with the ECL detection reagents as described by the supplier (Amersham International, UK). Peroxidase-conjugated affinipure F(ab')2 fragment goat anti-rabbit immunoglobulins (Jackson Immunoresearch Laboratories Inc., USA) was used as a second antibody.

6) Isolation of the *tcm10/pet9* Double Mutants

D360-2D (MATα) was mated with tcm10-15D (MATa) and tcm10dsr2c (MATa), respectively. The diploids were sporulated on sporulation medium for 4 days and tetrads were dissected. The four spores from each tetrad were then tested for growth on YPG before and after mating with tcm10-19D, tcm10-15D, D360-2D, or D360-7D (Table III-1). There are four possible genotypes; *TCM10/PET9*, *tcm10/pet9*, *TCM10/pet9*, and *tcm10/PET9*. Among the four tetrads, only *TCM10/PET9* spores can grow on YPG. *TCM10/pet9* spores are able to grow on YPG only after mating with a *tcm10* mutant (tcm10-15D or tcm10-19D) but unable to grow after mating with *pet9* mutant (D360-2D or D360-7D). *tcm10/PET9* spores are able to grow on YPG after mating with a *pet9* mutant but unable to grow after mating with a *tcm10* mutant. *tcm10/pet9* spores can not grow on YPG even after mating. A *tcm10/pet9* double mutant and a *TCM10* wild type control, *TCM10/pet9*, were isolated by this complementation approach.

7) Miscellaneous Methods

Published procedures were used for preparation of submitochondrial particles (Robinson *et al.*, 1991), for the isolation of intact mitochondria (Gasser *et al.*, 1982), for the isolation of subcellular fractions (van Loon *et al.*, 1986), for carbonate extraction of the TCM10 protein (Fijiki *et al.*, 1982), for the sonication of isolated mitochondria (Glick *et al.*, 1992), for TCA precipitation (Maniatis *et al.*, 1982), for measuring the accessibility to externally added proteinase K of TCM10 protein in isolated mitochondria (Glick *et al.*, 1992), for *in vitro* transcription and translation of *TCM10*, and for protein import into isolated mitochondria (Robinson and Lemire, 1992). Enzyme assays were as published (Robinson *et al.*, 1991). Fumarase activity assays were measured the conversion of fumarate to malate as described (Racker, 1950); catalase activities measured the disappearance of H_2O_2 as

described (Aebi, 1974). Cytochrome contents were determined with reduced minus oxidized spectra as described (Williams, 1960). SDS polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970). Tetrad dissection was performed with a de Fonbrune micromanipulator (Technical Products International Inc., USA). Freshly made hemin chloride (Calbiochem, USA) in 0.1 N NaOH was used at 15 mg per liter of YPG media to test the growth of *tcm10* mutants (Labbe-Bois, 1990). 40 µg/ml δ-aminolevulinic acid (δ-ALA) was used for the growth of *hem-1* mutant on YPD and YPG plates (Guarente and Mason, 1983)

C. RESULTS AND DISCUSSION

1) Overexpression of the TCM10 protein in *E. coli*

The overexpression of many prokaryotic and eukaryotic proteins in *E. coli* has been made possible through the use of recombinant DNA technology. Efficient expression can produce protein levels of up to 50% of total *E. coli* cell protein (Schoner *et al.*, 1985). Many overexpressed proteins form inclusion bodies or granules of aggregated protein. These inclusion bodies are coprecipitated with cell debris during low speed centrifugation. Polypeptides in inclusion bodies are often resistant to proteolytic degradation and require high concentrations of urea to solubilize them (Schoner *et al.*, 1985).

Our attempt to overexpress the TCM10 protein in *E. coli* was aimed at generating antigen for raising polyclonal antibodies. The plasmid, pJF118EH-TCM10, places the *TCM10* gene under control of the inducible *tac* promoter (Figure II-4). Expression of TCM10 in UT580 was examined periodically up to 10 hrs after IPTG induction and compared with those in pJF118EH transformed cells. However, no overexpressed TCM10 protein was observed (Figure III-4).

In order to explore the possibilities of protease degradation and strain dependence, I also examined the differences in the protein contents of IPTG induced and uninduced cells at each time point in the *E. coli* strains, BNN103 and DH5α. Similar results were observed (Figure III-5, Figure III-6). The failure to overexpress the TCM10 protein might be due to several reasons. A 75 kDa band was observed in induced but not in uninduced Coomassie Blue stained sample of the 30 min and 1 hr time points in DH5α and BNN103 (panels B in Figures III-5 and 6), respectively. However, this band was not consistently observed and we were not able to confirm its identity as TCM10 protein. Protein stability may have been a factor.

There have been reports that low usage codons limit the rates of protein translation *in vivo* (Robinson *et al.*, 1984; Bonekamp *et al.*, 1985). The introduction of a few low usage codons into a highly expressed gene *in vivo* can decrease its expression dramatically; this effect was only observed for expression off a high copy plasmid (Robinson *et al.*, 1984). Eight low usage codons have been identified in *E. coli*; these are AGG (Arg), AGA (Arg), CGA (Arg), CGG (Arg), AUA (Ile), CUA (Leu), CCC (Pro), and UCG (Ser). There are 8 AGG, 10 AGA, 5 CGA, 2 UCG, 3 CCC, 13 CUA, and 15 AUA codons (56 in total) in the *TCM10* coding sequence. Twenty-three of the 31 Arg residues are encoded by low usage codons. Therefore, it may not be surprising that the TCM10 protein could not be overexpressed *in vivo* under a strong promoter.

The Codon Adaptation Index (CAI) for *TCM10* is 0.108 in *S. cerevisiae* and 0.183 in *E. coli* (Sharp and Li, 1987). These values approach the lowest values in both species as the range of CAI values for 165 genes in *E. coli* is from 0.2 to 0.9, and that of 106 genes in yeast is from 0.1 to 0.95 (Sharp and Li, 1987). The CAI value measures the synonymous codon usage bias due to natural selection and mutation. Low usage codons should disappear in highly expressed genes

such as ribosomal protein genes. The CAI uses a reference set of highly expressed genes from a species to assign the relative values to each codon, and a score for a gene is calculated from the frequency of use of all codons in that gene. This value can be used to compare the usage of each codon within the same or amongst different species.

2) High Level Expression of the trpE-tcm10 Fusion Protein

As attempts to overexpress the full length TCM10 protein in *E. coli* were not successful, expression as a fusion protein was attempted. The N-terminal 87 amino acid residues of TCM10 were fused in frame with the *E. coli* trpE protein, which encodes anthranilate synthase (Yanofsky *et al.*, 1989), by a short linker of 5 amino acid residues; Lys Glu Asp Asn Lys. This fusion protein has a predicted mass of 48 kDa. Such fusion proteins are expected to be relatively stable due to the presence of host polypeptide sequence, and to be produced at relatively high levels.

The pATH vectors, which allow for the creation of C-terminal fusions to trpE, are high copy number vectors that use the strong inducible *trp* promoter to produce insoluble fusion proteins. The expression plasmid, pATH11-tcm10BS, was expressed in tryptophan-free medium to which was added the tryptophan analog indoleacrylic acid (IAA) to relieve repression and attenuation at the trpE promoter. Cells were harvested 2 hrs after the addition of IAA, treated with lysozyme, and lysed with detergent. The insoluble fractions were isolated, analyzed by SDS-PAGE, and stained with Coomassie blue (Figure III-7). The fusion protein was expressed at a high level as an inclusion body that could be sedimented by centrifugation at 3300 x *g* for 10 min.

The fusion protein was eluted by electrophoresis and used as antigen to raise a rabbit polyclonal antibody.

3) Anti-TCM10 Antiserum Recognizes the TCM10 Protein in Yeast

There are several pieces of evidence to demonstrate that the anti-TCM10 antiserum recognizes the TCM10 protein in yeast. First, as shown in Figure III-8, a 70 kDa protein band is detected with this serum in MH125, and mutants transformed with the complementing plasmids. In contrast, this band is absent in both mutagenized and disruption mutants. In addition, there is no 70 kDa band detected with the preimmune serum (Figure III-8). We believe that the 70 kDa protein is the TCM10 protein because its appearance is correlated with the presence of the complementing plasmid carrying the *TCM10* gene. Second, the *in vitro* translated TCM10 protein can be aligned with the 70 kDa protein after they are run on the same gel (Figure III-9). The antiserum raised against the trpE-tcm10 fusion protein recognizes the TCM10 protein but also recognizes a 49 kDa protein in both membrane and soluble fractions of all cells (Figure III-8). We speculated that this anti-TCM10 antiserum cross-reacts with other yeast proteins rather than the 49 kDa protein being a degradation product because the 49 kDa is also present in the disruption mutant.

4) TCM10 Is a Peripheral Membrane Protein

Cellular proteins can be subdivided into soluble or membrane bound proteins. Soluble proteins function in an aqueous environment such as the cytosol or the mitochondrial matrix, and usually require movement in the fluid environment. Examples include transcription initiation factors or regulatory factors that associate or dissociate with DNA or mRNA as required to control expression. Membrane bound proteins are restricted to the membrane and usually function locally. Membrane bound proteins can be divided into two categories; peripheral and integral proteins. Peripheral proteins are associated with the membrane by interactions with other

proteins or with phospholipid (Gennis, 1991). These interactions are usually weak and can be disrupted with high pH or high salt concentrations. In contrast, integral proteins are embedded in the membrane bilayer. They are usually very difficult to extract without detergents. Fujiki and colleagues have reported that peripheral proteins can be solubilized or washed off of isolated intracellular membranes using Na_2CO_3 (pH 11) solutions (Fujiki *et al.*, 1982).

Membrane and soluble fractions were isolated from yeast cells grown to stationary phase on YPD 0.6%. As shown in Figure III-8, the anti-TCM10 serum detects the TCM10 protein as a 70 kDa protein in membranes from MH125, and from tcm10-15D and tcm10dsr2c carrying the plasmid YCplac33-TCM10(1#). It is not detected in tcm10-15D, or the disruption mutant, tcm10dsr2c. The majority of the TCM10 protein is associated with the membrane fractions; only a small amount is present in soluble fractions (approximately 80-90% in membrane fractions).

In order to explore whether TCM10 is a peripheral or an integral membrane protein, membrane fractions were treated with 100 mM Na_2CO_3, pH 11, and the released proteins were separated by centrifugation. All samples were subjected to TCA precipitation and acetone washing. Then equal amounts of protein were electrophoresed and transferred for Western blot analysis. The TCM10 protein is exclusively found in the supernatant fraction (Figure III-9). Reprobing the membrane with anti-SDHB (subunit B of yeast succinate dehydrogenase, a known peripheral membrane protein) showed that all SDHB had also been extracted. I did not show that the Na_2CO_3 treatment does not extract integral membrane proteins due to the lack of a suitable antibody, but it has been reported that about 21% of total mitochondrial proteins remain

with the membranes after Na_2CO_3 treatment (Fujiki *et al.*, 1982). Therefore, TCM10 appears to be a peripheral membrane protein.

As indicated in Figure III-9, additional proteins detected by the anti-tcm10 serum appear after the TCA precipitation and acetone washing treatment. SDHB could be detected only after these treatments. We speculated that acetone washing could remove lipids that surround these proteins during the preparation of the membrane fractions, and consequently, improve their transfer to nitrocellulose membranes and/or expose epitopes for recognition by anti-TCM10 and anti-SDHB sera.

5) TCM10 Is a Mitochondrial Protein

MH125 was grown in lactate medium to stationary phase and subjected to subfractionation. Mitochondrial, cytosolic, and microsomal fractions were collected and subjected to Western blot analysis. TCM10 protein is only found in the mitochondrial fraction (Figure III-10).

6) TCM10 Is Located on the Matrix Side of the Inner Membrane

There are four possible locations for a peripheral membrane protein such as TCM10; the cytosolic or intermembrane space surfaces of the outer mitochondrial membrane, or the outer or matrix surfaces of the inner membrane. The outer and inner membranes are impermeable to proteases. The outer membrane can be disrupted by either mild detergent or osmotic shock treatments while leaving the inner membrane intact (Glick *et al.*, 1992; Hartl *et al.*, 1987). In this experiment, the osmotic shock procedure was used to disrupt the outer membrane without damaging the inner membrane (Figure III-11, lanes 6-10). First, if TCM10 is on the cytosolic surface of the outer membrane, it should be degraded by protease treatment of intact mitochondria and we should have seen the disappearance of TCM10 band in the intact mitochondria (Figure III-11, lane 2). Second, if TCM10 is associated

with the outer or inner membranes but protruding towards the intermembrane space, it should be protected from protease digestion in the intact mitochondria but degraded after disrupting the outer membrane. As result, we should see the TCM10 band present in the intact mitochondria treatment (panel A, lane 2) and should have seen the disappearing of the TCM10 bands with the decreasing concentration of sorbitol (panel A, lanes 6-10). Third, if TCM10 is inside the inner membrane, it should be protected from protease digestion unless both membranes are permeabilized by detergents. Thus, we should see that TCM10 bands do not change with the decreasing concentration of sorbitol (panel A, lanes 6-10) but disappear after both membranes are disrupted by Triton X-100 (panel A, lane 5). Cyt b_2, an intermembrane space protein, and SDHB, a peripheral inner membrane protein protruding to the matrix, are used as the controls to monitor the disruption of the outer membrane and integrity of the inner membrane.

In such a protease experiment, the TCM10 protein is only digested after disruption of the inner membrane (panel A, lane 5). The TCM10 protein is still present when the outer membrane is osmotically disrupted (panel A, lanes 6-10); in contrast, the control protein, $cytb_2$, is progressively digested with decreasing sorbitol concentrations that disrupt the outer membrane (panel B, lanes 6-10). SDHB is protease resistant after the disruption of the outer membrane, but digested in the presence of the detergent Triton X-100 (panel B, lanes 6-10 and lane 5). In addition, the TCM10 protein is not released as a soluble protein from the outer membrane disrupted mitochondrial preparations without proteinase treatment, and in contrast to cyt b_2 is only found in the pellet fraction along with SDHB (panels A and B, lanes 3 and 4). Therefore, TCM10 is localized on the matrix side of the inner membrane.

The panel A was overexposed after developed in the ECL developing reagent (Amersham, USA). The overexposure causes the appearance of those extra bands as well as a high background (Figure III-11, panel A). We also observed differences in TCM10 migration after SDS-PAGE between the isolated MH125 membrane fraction from the French pressure cell lysis and isolated mitochondrial preparations (data not shown). The mitochondrial preparations contain 1 mg/ml bovine serum albumin that comigrates and shifts the TCM10 band to higher mobility. Therefore, mitochondria were pelleted at 14,000 x *g* to remove BSA. Mitochondrial pellets were then resuspended in import buffer without BSA (Figure III-11, lanes 1 to 10). Each sample was precipitated with 5% TCA and washed with ice-cold acetone before being loaded and separated by SDS-PAGE. Sonication treatments of the mitochondria, which may indicate the strength of TCM10 associated with the inner membrane, were performed without the initial centrifugation to remove excess BSA (Figure III-11, lanes 11 and 12). During sonication, TCM10 is released to the soluble fraction along with the majority of *cyt* b_2. The small amount of *cyt* b_2 found in the pellet fraction could be due to entrapment in mitochondrial vesicles. A small amount of SDHB is also present in the soluble fraction being dissociated from the inner membrane by sonication. Therefore, TCM10 is not tightly associated with the inner membrane.

7) *in vitro* Import of pre-TCM10 into Isolated Mitochondria

TCM10 was transcribed with a T7 expression system (Figure III-3) and the mRNA translated in RNase treated rabbit reticulocyte lysate (Promega, USA). The *in vitro* translated TCM10 protein could not be imported into isolated mitochondria (data not shown). However, we noticed two bands migrating close together where the TCM10 protein is expected (Figure III-9, lane X). The major band is below the top faint band. We think the top band is the full

length TCM10 protein started at the first ATG in the coding region as no bands are observed in the negative control without addition of any mRNA (data not shown). We speculated that the TCM10 protein was being translated using a second ATG codon corresponding to Met16 in Figure II-9 instead of from the first ATG. As a consequence, the majority of the *in vitro* synthesized TCM10 protein would be truncated at the N-terminus with the loss of first 16 amino acid residues. The full length TCM10 may have been synthesized at too low an amount to be detected after an import reaction. We further speculated that this N-terminal truncated TCM10 protein could not be imported because of the lack of a mitochondrial targeting sequence (Attardi and Schatz, 1988; Hartl *et al.*, 1989).

8) Construction of *pet9/tcm10* Double Mutants

The *tcm10* mutants, tcm10-15D and tcm10dsr2c, become ρ^- at high frequencies. After growing to stationary phase in YPD 0.6%, fewer than 20% of cells are ρ^+. *pet9/tcm10* double mutants were constructed to overcome this problem. The *pet9* mutation affects the ADP/ATP carrier and prevents growth on nonfermentable carbon sources such as glycerol or ethanol. *pet9* strains respire and express all cytochromes, although cytochrome *a* concentrations are relatively low compared to isogenic control cells (Mattoon, 1979). One of the most useful characteristics of yeast cells with the *pet9* mutation is their inability to tolerate the ρ^- mutation.

Two *pet9* strains, D360-2D and D360-7D, were used. D360-2D was mated with both tcm10-15D and tcm10dsr2c. After sporulation, tetrads from each diploid were dissected. Only tetrads with four viable spores were counted and studied further; 8 from the D360-2D/tcm10dsr2c diploids and 11 from D360-2D/tcm10-15D diploids. The segregation of the *pet9* and *tcm10* alleles was monitored by growth on YPG after complementation with *pet9* or *tcm10*

mutants (Table III-2). 25 complete tetrads were examined for the segregation of the pet9 and tcm10 alleles. The results were; 5 PD, 6 NPD, and 14 T. The ratio of PD:NPD:T is close to 1:1:2.8. The 1:1 ratio of PD:NPD asci indicates that *pet9* and *tcm10* are not linked. There is a slight reduction in the proportion of T asci but above two third of the total asci examined, which indicates that none of them are centromere-linked.

pet9 single mutants show typical yeast colonies with a round shape and smooth edges; in contrast, *pet9/tcm10* double mutants show rough edged colonies. Cells of the double mutants tend to cluster when grown in liquid media (data not shown). Double mutants grown in YPD 0.6% for 40 hr are 100% ρ^+. Furthermore, Western blot analysis shows that the *pet9/tcm10* double mutant does not have the TCM10 protein whereas *pet9* single mutants do (Figure III-12).

9) Enzyme Assays

Several respiratory chain enzyme activities were measured using submitochondrial particles prepared from the wild type strain, MH125, single *pet9* mutants (1A and 9C), and double *tcm10/pet9* mutants (1C and 9B) (Table III-3). 1A and 1C are from the same tetrad as are 9B and 9C. Thus, 1A serves as a positive control for the *tcm10* disruption mutant, 1C, and 9C for the mutagenized mutant, 9B. The strains are not isogenic, but differences in the respiratory chain enzyme activities between 1A and 1C, and 9B and 9C should reflect the effects of the *tcm10* mutation. As shown in Table III-3, the activities of all the respiratory chain enzymes assayed were greatly affected. Succinate ubiquinone reductase activities in both disruption and mutagenized mutants are decreased to about 15% of the wild type controls, indicating that complex II is largely affected by the *tcm10* mutation. The succinate cyt *c* reductase activities involving the complexes II and III are decreased 98% in

both mutants. This result suggests that the complex III might also be affected. Succinate DCPIP reductase activities, a direct measurement of the active domains (subunits A and B) of complex II, are also decreased about 85%. NADH ubiquinone reductase activities are decreased about 75% in both mutants, whereas, the antimycin A sensitive NADH cyt *c* reductase activities are decreased about 95%. Similar results are also obtained with the glycerol-1-phosphate (G1P) cyt *c* reductase and the G1P ubiquinone reductase activities although there is a small difference between the disruption and the mutagenized mutants in the residual G1P ubiquinone reductase activities. cyt *c* oxidase activities are decreased at least 80%. No oxygen consumption was detectable in submitochondrial membranes from both the mutants with either exogenous succinate or NADH as electron donors. Overall, all the respiratory chain enzymes measured, including NADH dehydrogenase, complexes II, III, and IV, are affected with little difference between the disruption and the mutagenized mutants. Therefore, the *tcm10* mutation has pleiotropic effects on the electron transport chain.

In order to explore the possiblity that non-respiratory chain enzymes are also affected, I measured the fumarase activities in the soluble fractions. These fumarase activities reflect contributions from both cytosolic and mitochondrial fumarase activities. No obvious decreases in the mutants versus the wild type controls were observed. However, the fumarase activities in mitochondria decreased about 2 to 3 fold, but not as dramatically as those respiratory chain enzymes did. This decrease could be due to a lower rate of protein import due to a lower membrane potential normally maintained by the electron transport chain. However, we do not know whether the import of different proteins is differently affected by the decreased membrane potential.

From these results, we speculated that the *tcm10* mutation may affect one of the common prosthetic groups in the electron transport chain, such as heme or Fe/S centers, or affect mitochondrial protein import. Heme is present in complexes II, III, and IV; all show greatly decreased activities. The decreases in the activities of complexes II and IV in both mutants are basically the same but complex IV does not contain Fe/S centers. Therefore, we speculated that the cellular heme biosynthesis could be affected and be the cause of multiple effects on the electron transport chain. Direct measurement of cellular cytochromes should shed light on the role of TCM10 in yeast.

10) Cytochrome Contents of *tcm10* Mutants

The cytochromes *c*, c_1, *b*, and *a* have reduced minus oxidized spectra that are distinguishable by their absorption maxima at 550 nm, 554 nm, 563 nm, and 605 nm, respectively. The levels of each cytochrome can be quantified by measuring the absorbance differences at the following pairs of wavelengths, 550-535, 554-540, 563-577, and 605-630 nm for cytochromes *c*, c_1, *b*, and *a* (Williams, 1964).

The cytochrome spectra of submitochondrial particles of 1A, 1C, 9B, and 9C were recorded on Hitachi U-3200 spectrophotometer (Hitachi Ltd, Japan), and the amounts of each cytochrome calculated (Table III-4). cyt c_1 levels are most affected. The levels of cyt *a*, cyt *b*, cyt c_1, and cyt *c* in 1C are 88%, 68%, 6%, and 33%, respectively of the control, 1A. The situation between 9B and 9C is similar. The total cytochrome content of 1C is about 40% of that in 1A; that of 9B is about 20% of that in 9C. This result might indicate that the TCM10 protein is not involved in heme biosynthesis because there is only a two foid difference between the disruption mutant (1C) and the wild type control (1A). In addition, all of the mitochondrial enzymes involved in heme biosynthesis have been identified, cloned, and sequenced (Labbe-Rois and Labbe, 1990).

Computer searching with the TCM10 protein sequence against data banks (EMBL-23 and PIR-33) did not identify any sequence with high identity (Chapter II). In order to explore the possibility that other non-respiratory hemoproteins like catalases might be affected by the *tcm10* mutation, I examined the catalase activities in the soluble fractions. The catalase activities that measure the sum of catalases A and T might also indicate a difference in heme metabolism between the mutants, 1C and 9B, and the wild type controls, 1A and 9C. 9B, the mutagenized *tcm10* mutant contains about 52% of the catalase activity of 9C, whereas, 1C, the disrupted *tcm10* mutant has only 2% of that in 1A (Table III-4). This is the only big difference observed between the disruption and the mutagenized mutants. This result may indicate that the TCM10 protein is involved in heme transport out of mitochondria where it is synthesized. The higher catalase activities also suggest a leaky phenotype in the mutagenized strains.

In addition, as the involvement of the TCM10 protein in the heme biosynthesis is suspected, I have tested the possibility of heme complementation of the *tcm10* mutation. tcm10dsr2c and tcm10-15D cells (ρ^+) along with *hem-1* (δ-aminolevulinate synthase gene) mutant, were streaked out on YPG plates supplemented with either δ-aminolevulinic acid or hemin chloride. *hem-1* mutant cells could form normal colonies after 4 days of growth on YPG+heme, and 8 days of growth in the presence of δ-ALA. However, both tcm10dsr2c and tcm10-15D formed tiny colonies on YPG+heme plates in 4 days and ceased to grow after then. tcm10dsr2c and tcm10-15D barely grow on YPG+δ-ALA. This result suggests that the TCM10 protein is unlikely an enzyme in the heme biosynthesis, for any mutation of the enzymes involved in the heme biosynthesis should be complementable by externally added heme (Labbe-Bois and Labbe, 1990). Therefore, TCM10

protein is possibly involved in the steps after heme has been synthesized, such as the transport of heme out of mitochondria.

In conclusion, all of the respiratory chain enzyme activities measured are greatly decreased in the mutants. These results suggest that the TCM10 protein may be a common factor in the biogenesis of these enzymes. The effects on catalase activities strongly implicates TCM10 in heme metabolism, transport, or assembly. The inability of external heme to complement the *tcm10* mutation indicates that the TCM10 protein is probably not an enzyme involved in heme biosynthetic processes. Many questions remain to be answered, such as why the loss of mitochondrial DNA occurs at high frequencies and whether the broad effects on the electron transport chain are primary or secondary effects of the *tcm10* mutation.

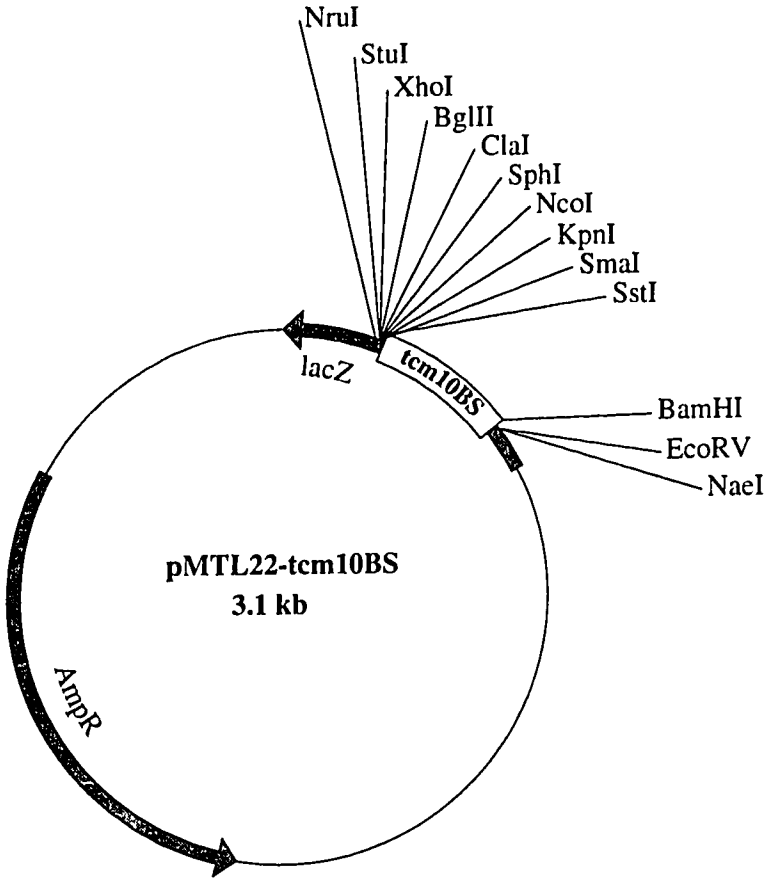


Figure III-1 Restriction map of pMTL22-tcm10BS. The BamHI/SstI *TCM10* fragment is labeled tcm10BS. The multicloning sites are also shown on both sides of the tcm10BS fragment.

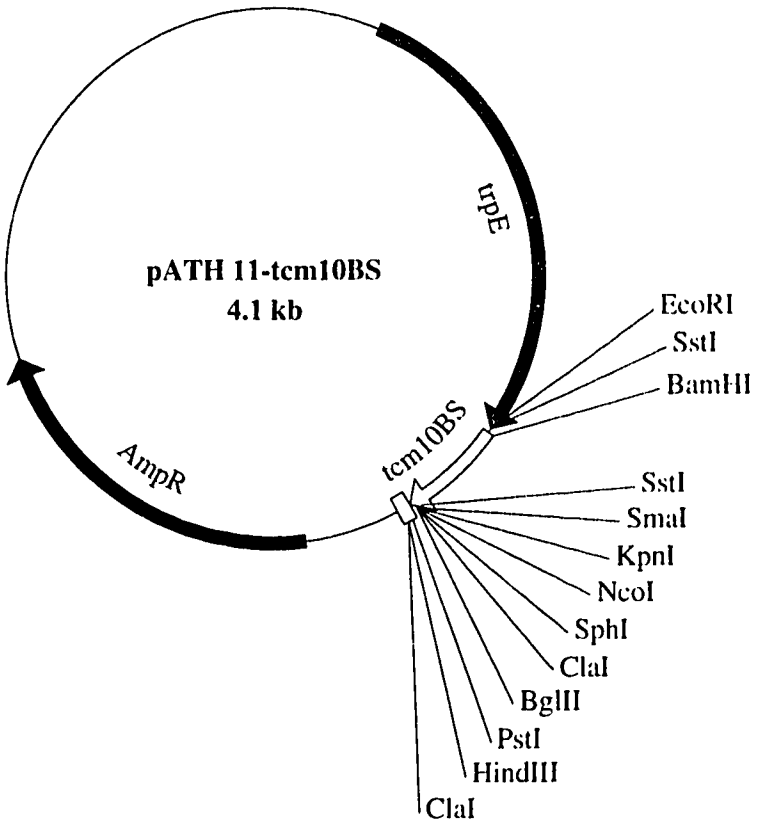


Figure III-2 Restriction map of the pATH11-tcm10BS plasmid used for expression of the trpE-tcm10BS fusion protein. The N-terminal segment of TCM10 between the BamHI and the SstI sites is linked to the C-terminus of trpE with a 5 amino acid linker (Lys Glu Asp Asn Lys). The trpE coding region is indicated as the dotted arrow and the tcm10BS coding region as the open arrow.

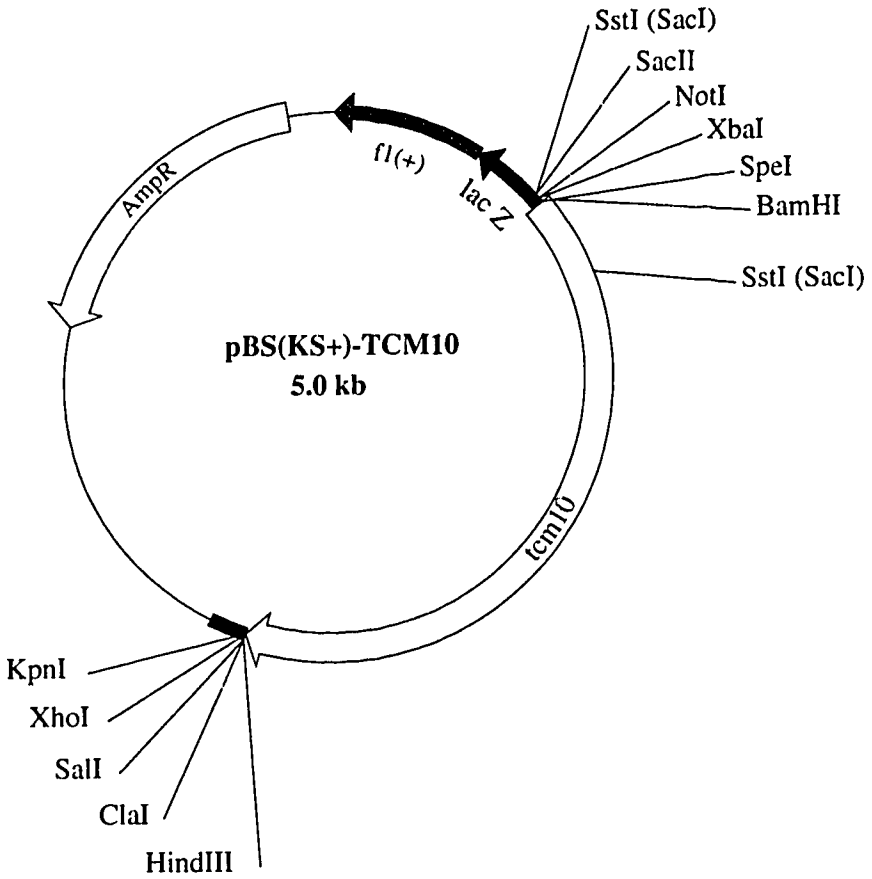


Figure III-3 Restriction map of the plasmid pBS-TCM10. This plasmid was linearized with KpnI and used for the *in vitro* transcription and translation of TCM10. The vector is pBluescript II (KS+). The *TCM10* gene is under control of the T7 promoter.

Figure III-4 Expression of TCM10 in *E. coli* UT580. The expression of TCM10 from pJF118EH-TCM10 was examined by analyzing pellet and supernatant fractions of cells at different times after IPTG induction. About one fifth of the pellet and one sixth of the supernatant of each time point from 1 ml culture were loaded on the gel. Panels A and B are profiles of the supernatants and pellets, respectively. Timing was started upon the addition of IPTG. 10% SDS polyacrylamide gels were used. The positions of size markers in kDa are indicated on the left. The predicted size of TCM10 is 79.7 kDa.

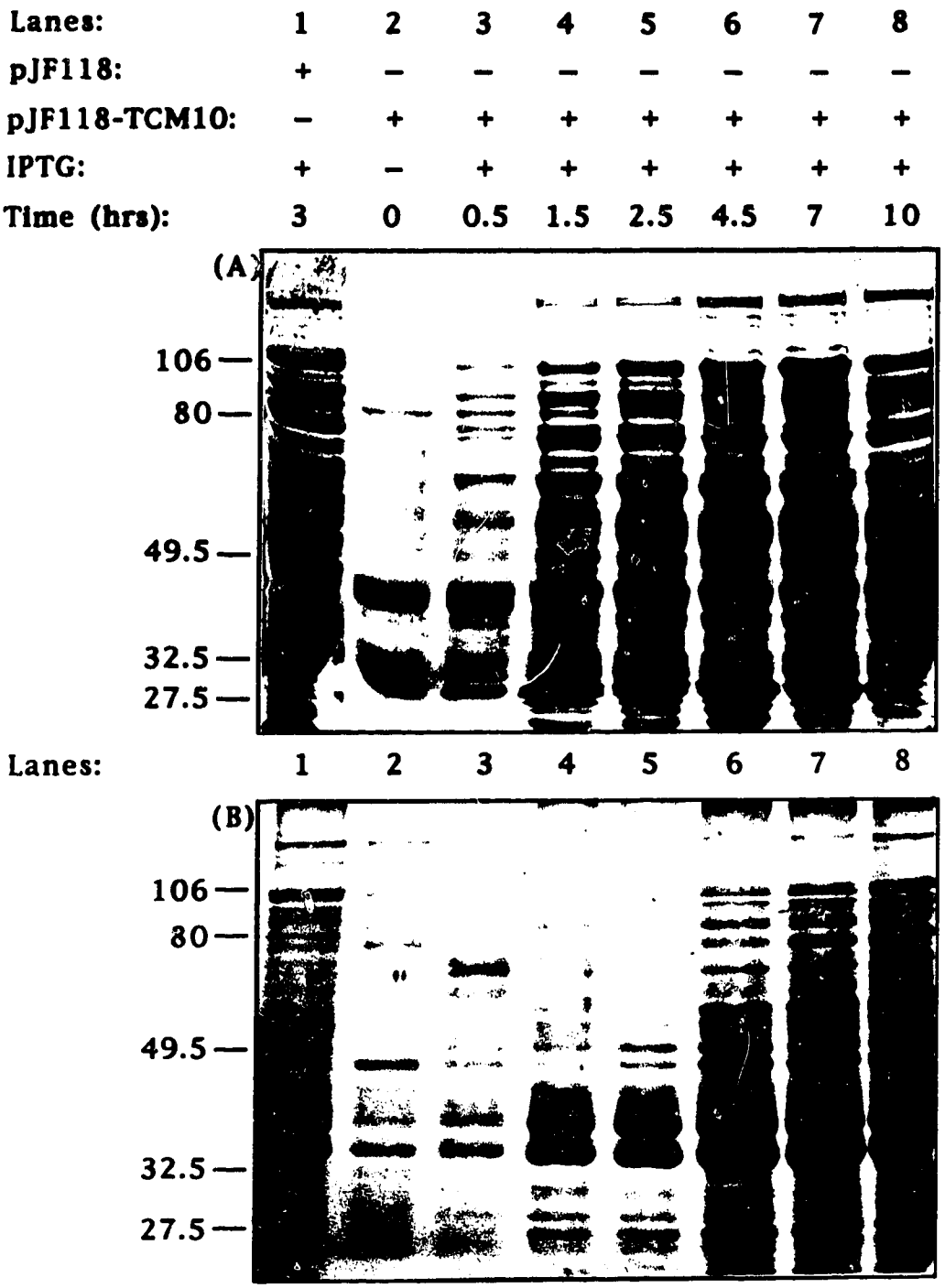
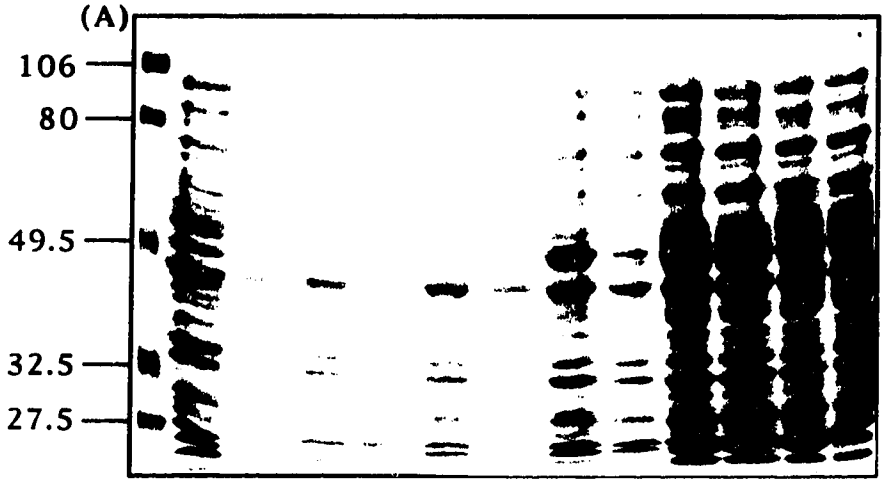

Lanes: 1 2 3 4 5 6 7 8
pJF118: + − − − − − − −
pJF118-TCM10: − + + + + + + +
IPTG: + − + + + + + +
Time (hrs): 3 0 0.5 1.5 2.5 4.5 7 10
(A)
106
80
49.5
32.5
27.5
Lanes: 1 2 3 4 5 6 7 8
(B)
106
80
49.5
32.5
27.5

Figure III-5 Expression of TCM10 in *E. coli* DH5α. Cells with the plasmid pJF118EH-TCM10 were harvested at different times as indicated. The protein patterns in supernatants (A) and pellets (B) from IPTG induced cells were compared with those from non-induced cells correspondingly at each time point. Other information is the same as Figure III-4.

Lanes:	1	2	3	4	5	6	7	8	9	10	11	12
pJF118:	+	−	−	−	−	−	−	−	−	−	−	−
pJF118-TCM10:	−	+	+	+	+	+	+	+	+	+	+	+
IPTG:	+	+	−	+	−	+	−	+	−	+	−	+
Time (hrs):	3	0	0.5	0.5	1	1	2	2	5	5	7	7



Lanes: 1 2 3 4 5 6 7 8 9 10 11 12

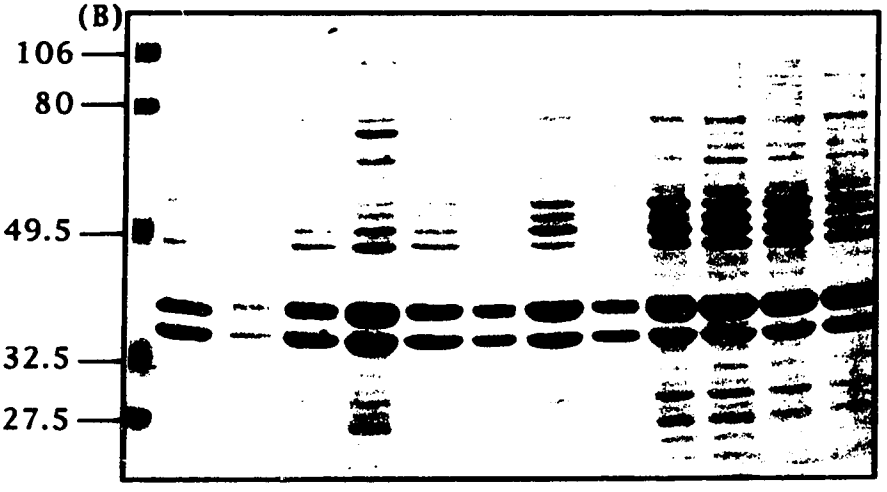
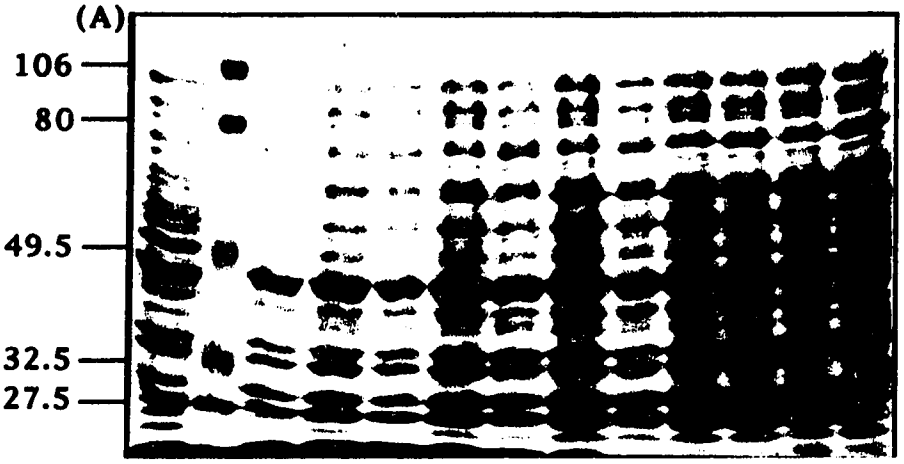


Figure III-6 Expression of TCM10 in *E. coli* BNN103. Other descriptions are the same as Figure III-5.

Lanes:	1	2	3	4	5	6	7	8	9	10	11	12
pJF118:	+	–	–	–	–	–	–	–	–	–	–	–
pJF118-TCM10:	–	+	+	+	+	+	+	+	+	+	+	+
IPTG:	+	+	–	+	–	+	–	+	–	+	–	+
Time (hrs):	3	0	0.5	0.5	1	1	2	2	5	5	7	7



Lanes: 1 2 3 4 5 6 7 8 9 10 11 12

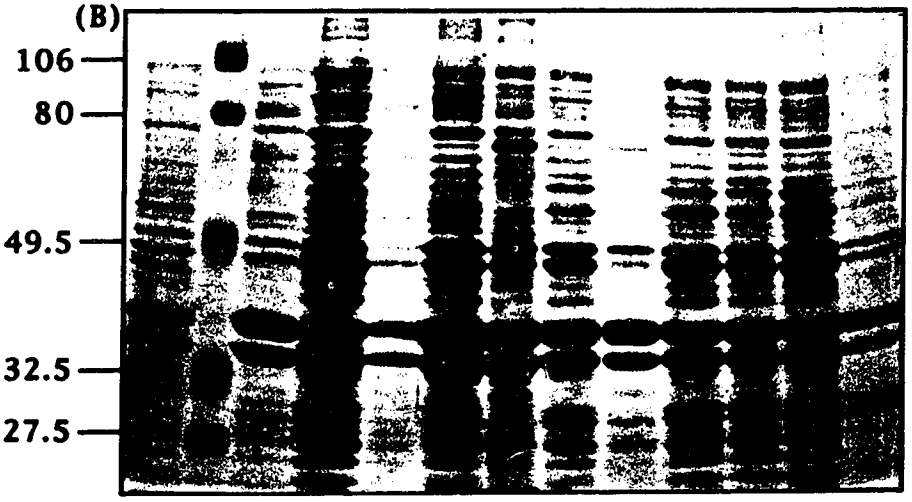


Figure III-7 Overexpression of the fusion protein, trpE-tem10BS, in *E. coli* UT580. The positions of the molecular weight marker proteins are indicated on the left. Lanes 1 and 2 are pellet fractions of UT580 with the vector pATH11, isolated in small scale and large scale preparations respectively. Lane 3 is the pellet fraction of cells with the expression plasmid isolated with the large scale procedure. The fusion protein at 48 kDa is indicated with an arrow on the right. The electroeluted fusion protein is run in lane 4. The gel was stained with Coomassie blue R-250 and destained with 10% glacial acetic acid and 25% ethanol.

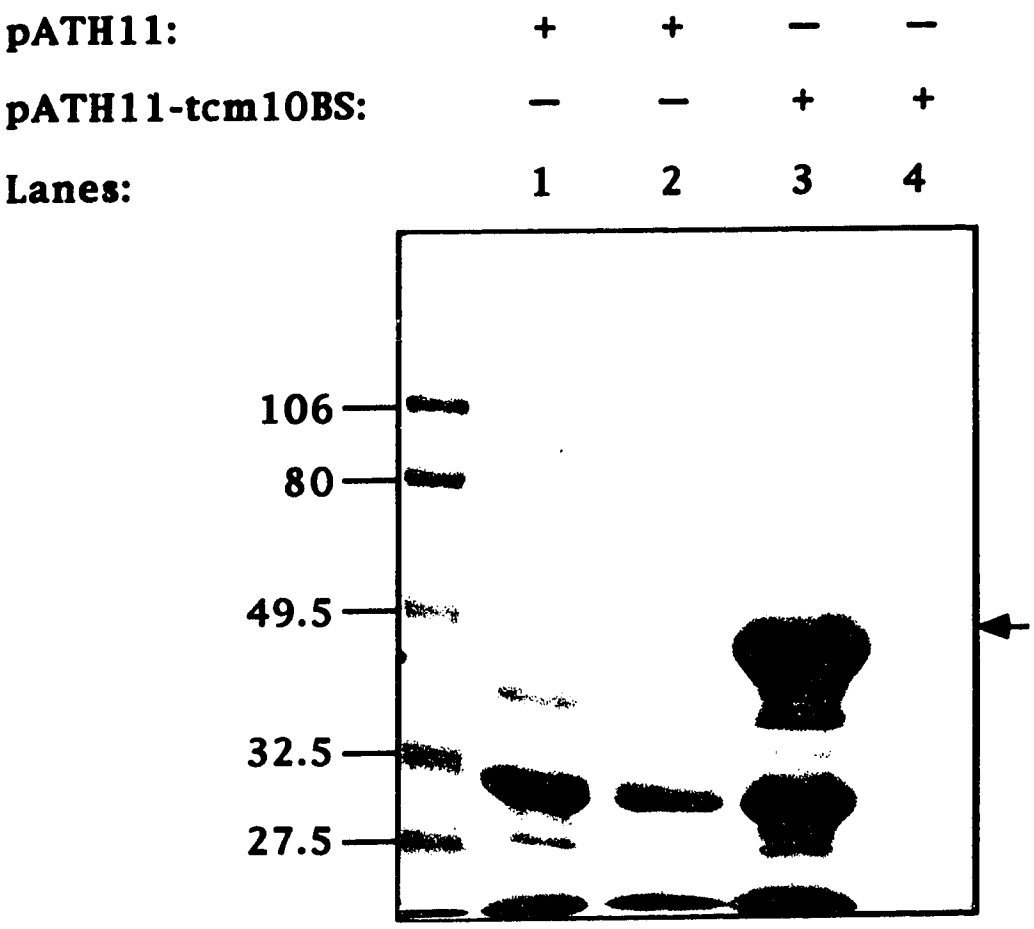

pATH11: + + − −
pATH11-tcm10BS: − − + +
Lanes: 1 2 3 4
106
80
49.5
32.5
27.5

Figure III-8 The TCM10 protein is membrane-associated. The membrane and supernatant fractions isolated by French pressure cell lysis of cultures grown in YPD 0.6% for 40 hours were run on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with the antitcm10 antiserum except lanes 1 and 2 which were probed with the preimmune serum. M, membrane fractions; S, supernatant fractions. (1#) represents the strain transformed with the plasmid YCplac33-TCM10(1#). Each lane contains 50 μg of protein except 25 μg in lanes 5 and 6. The arrow indicates the position of the TCM10 protein. The preimmune serum was used at dilution of 1:100; the anti-tcm10 serum was used at 1:200 dilution; and the second antibody, peroxidase-conjugated goat anti-rabbit antibody was used at 1:5000 dilution.

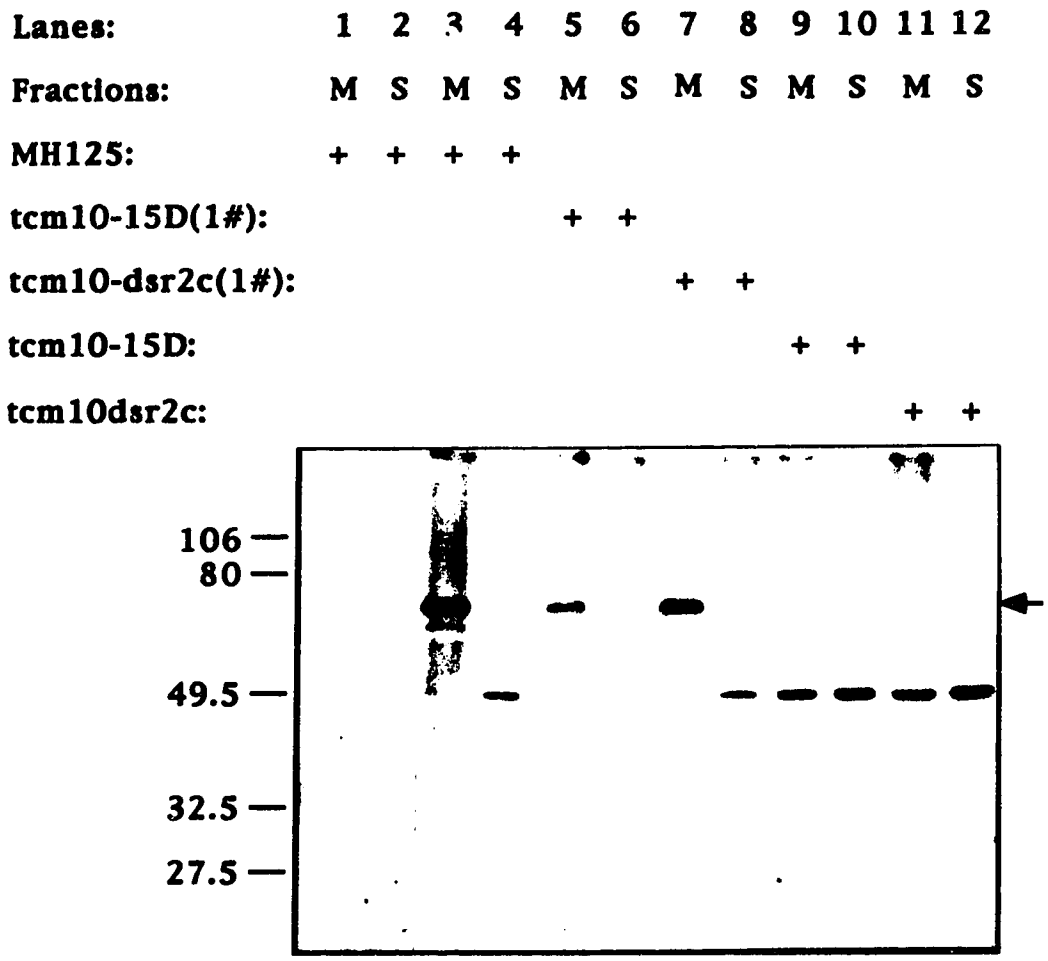

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12
Fractions: M S M S M S M S M S M S
MH125: + + + +
tcm10-15D(1#): + +
tcm10-dsr2c(1#): + +
tcm10-15D: + +
tcm10dsr2c: + +
106
80
49.5
32.5
27.5

Figure III-9 The TCM10 protein is not an integral membrane protein. Membrane fractions from MH125, tcm10-15D(1#), and tcm10dsr2c(1#), were treated with 0.1M Na_2CO_3, TCA precipitated, and examined by Western blot analysis. T, membranes not subjected to any treatments; C, control sample that contains unseparated pellet and supernatant fractions after Na_2CO_3 treatment and TCA precipitation; P and S, pellet and supernatant fractions, respectively, after Na_2CO_3 treatment and TCA precipitation. The nitrocellulose membrane was first probed with anti-tcm10 antiserum (panel A), stripped, and then reprobed with anti-SDHB antiserum (panel B). Lane X contains *in vitro* translated tcm10 protein; this lane was cut away after transfer to the nitrocellulose membrane, exposed to X-ray film (Eastman Kodak Co., USA) for 7 days, and aligned with the Western blot. 25 μg of protein was in lanes T, C, and P+S, of MH125, tcm10dsr2c(1#), and half that amount in the case of tcm10-15D(1#). The arrow in panel A indicates the TCM10 protein, whereas, in panel B, it indicates the SDHB protein. For other descriptions, refer to Figures III-4, and 8.

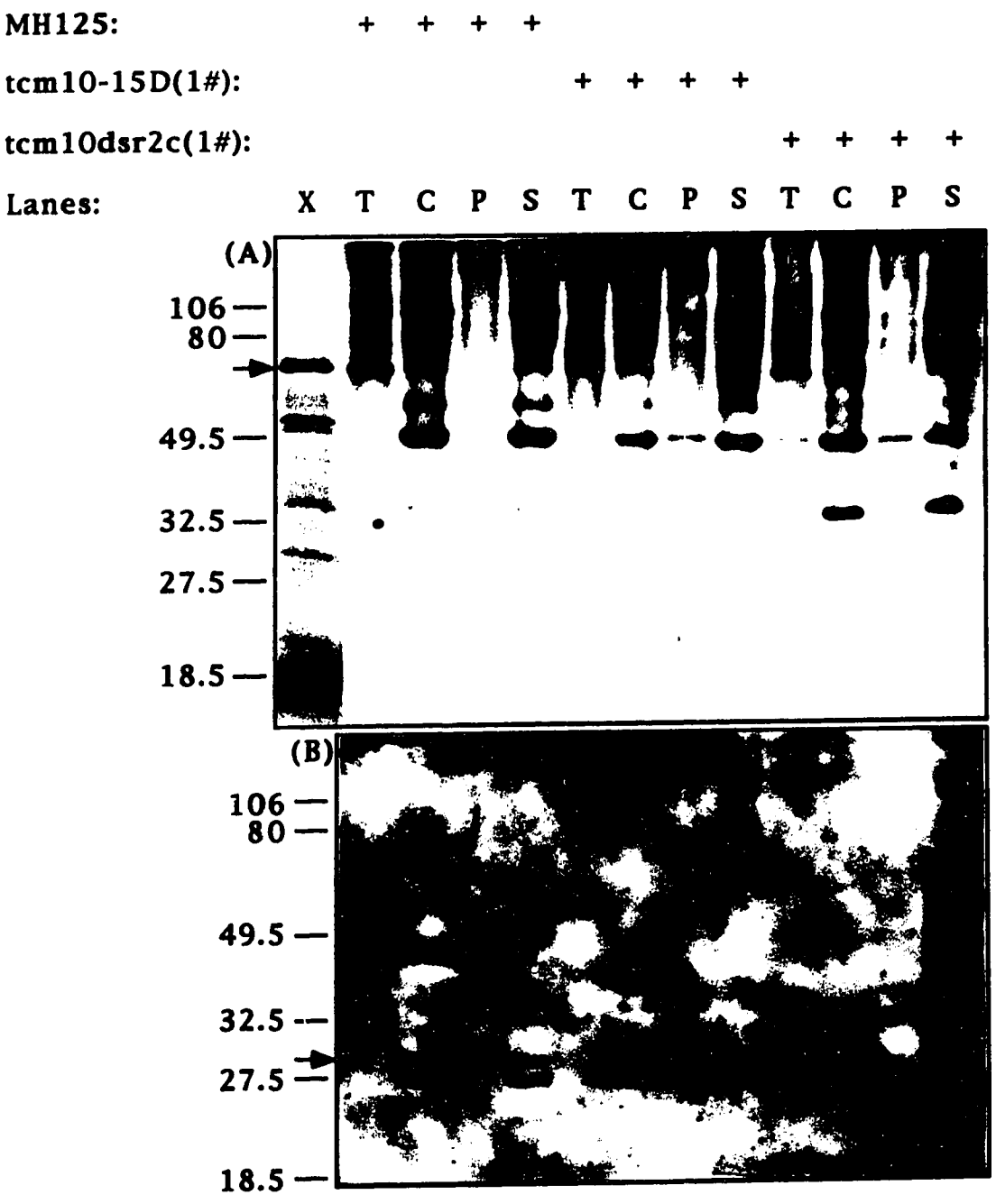

MH125: + + + +
tcm10-15D(1#): + + + +
tcm10dsr2c(1#): + + + +
Lanes: X T C P S T C P S T C P S
(A)
106
80
49.5
32.5
27.5
18.5
(B)
106
80
49.5
32.5
27.5
18.5

Figure III-10 TCM10 is a mitochondrial protein. The French pressure cell lysis membrane fractions from MH125, tcm10dsr2c, and tcm10dsr2c(1#), were run on an 8% SDS gel. Also, run were 25 µg of a mitochondrial fraction, 25 µg of a cytosolic fraction, and 12.5 µg of a microsomal fraction. The proteins were transferred to a nitrocellulose membrane, and probed with anti-tcm10 antiserum. Lanes 1, 2, and 3, membrane fractions of MH125, tcm10dsr2c, and tcm10dsr2c(1#), respectively; lanes 4, 5, and 6, mitochondrial, cytosolic, and microsomal fractions respectively from MH125 grown in YPD 0.6% at 30°C for 40 hrs. The TCM10 protein is indicated with an arrow.

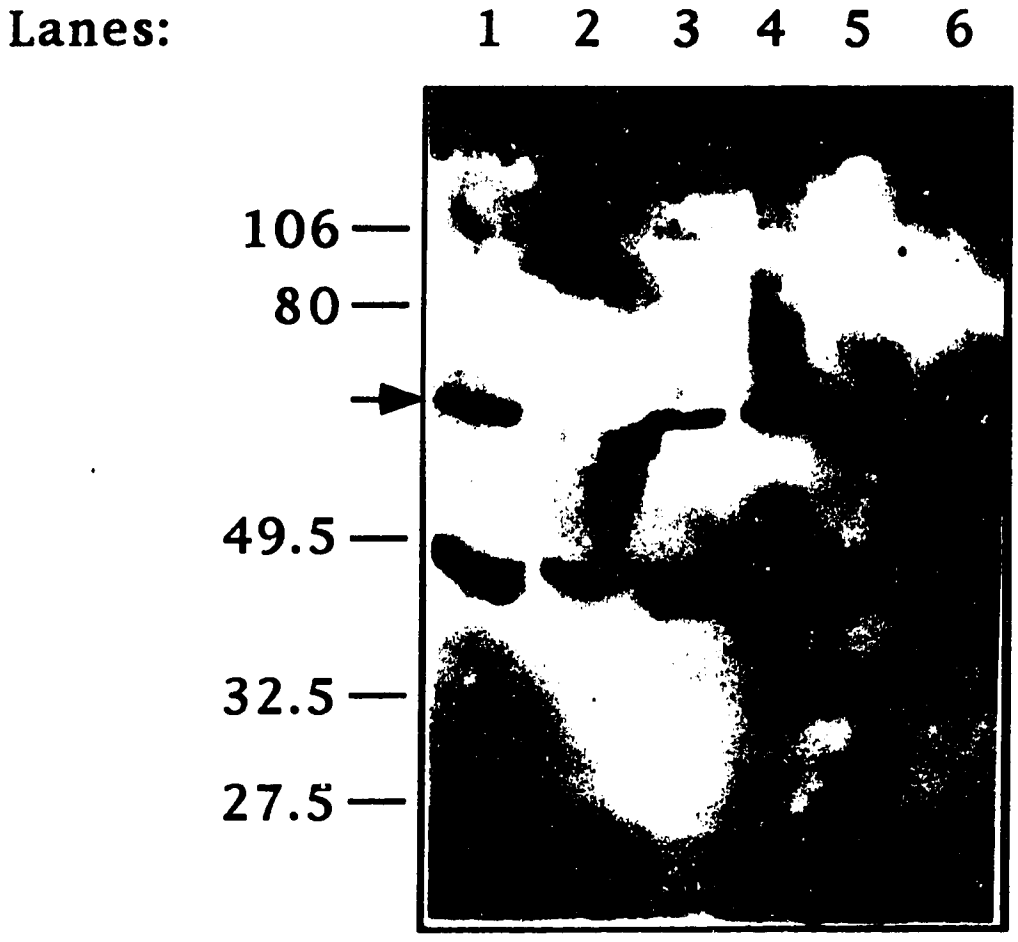

Lanes:
1 2 3 4 5 6
106
80
49.5
32.5
27.5

Figure III-11 The TCM10 protein is facing the mitochondrial matrix. Mitochondria isolated from MH125 were treated with TCA and acetone after a series of protease treatments as indicated. Each lane contains 30 µg of protein that was separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-tcm10 antiserum (panel A). The blot was stripped and reprobed simutaneously with anti-SDHB (1:200 dilution) and anti-*cyt* b_2 antisera (1:200 dilution) (panel B). Mitochondria in lanes 1 to 10 were pelleted at 14,000 x *g* to remove BSA in the mitochondrial preparations, and resuspended in the import buffer without BSA (20 mM K^+-HEPES pH 7.5, 0.3 M sorbitol) before the treatments. Samples in lanes 11 and 12 were done in the presence of BSA (1 mg/ml) without initial spinning. Lanes 3 and 4, pellet and supernatant fractions of osmotically shocked mitochondria, respectively; lanes 6, 7, 8, 9, and 10, osmotic shock of mitochondria in the presence of 0.3 M, 0.2 M, 0.1 M, 0.06 M, or 0 M sorbitol, respectively; lanes 11 and 12, pellet and supernatant fractions after sonication respectively. Protease K treatment was with 0.1 mg/ml proteinase K for 15 min at 0°C. Triton X-100 was used at 1% final concentration. P, pellet fraction; S, soluble fraction. In panel A, the arrow indicates the position of TCM10, in panel B, the solid arrow indicates cyt b_2 while dotted arrows indicate SDHB.

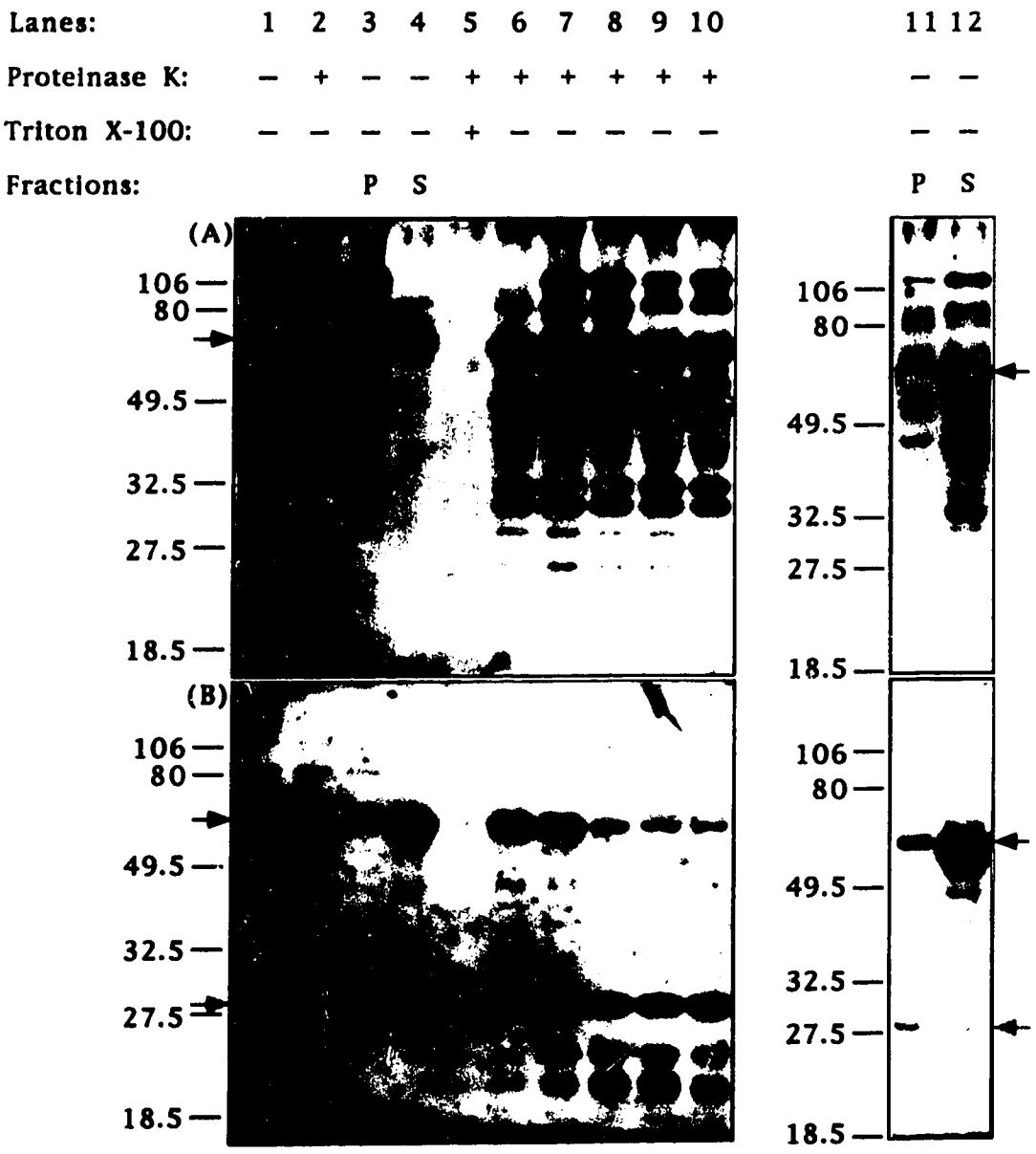

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12
Proteinase K: – + – – + + + + + + – –
Triton X-100: – – – – + – – – – – – –
Fractions: P S P S
(A)
106
80
49.5
32.5
27.5
18.5
(B)
106
80
49.5
32.5
27.5
18.5

Figure III-12 Western blot analysis of membrane proteins from single *pet9* and double *pet9/tcm10* mutants. Anti-tcm10 antiserum was used. Lanes 1 and 2, 3 and 4, 5 and 6, and 7 and 8; soluble (S) and membrane (M) fractions of 9B (*pet9/tcm10*), 9C (*pet9*), 1C (*pet9/tcm10*), 1A (*pet9*), respectively. Lanes 9 and 10; membrane and soluble fractions of MH125, respectively. The membrane and soluble fractions were isolated from 40 hour growth cultures in YPD 0.6%. Each contains 25 µg protein. The arrow points out the position of the TCM10 protein bands. Other descriptions are the same as Figure III-8.

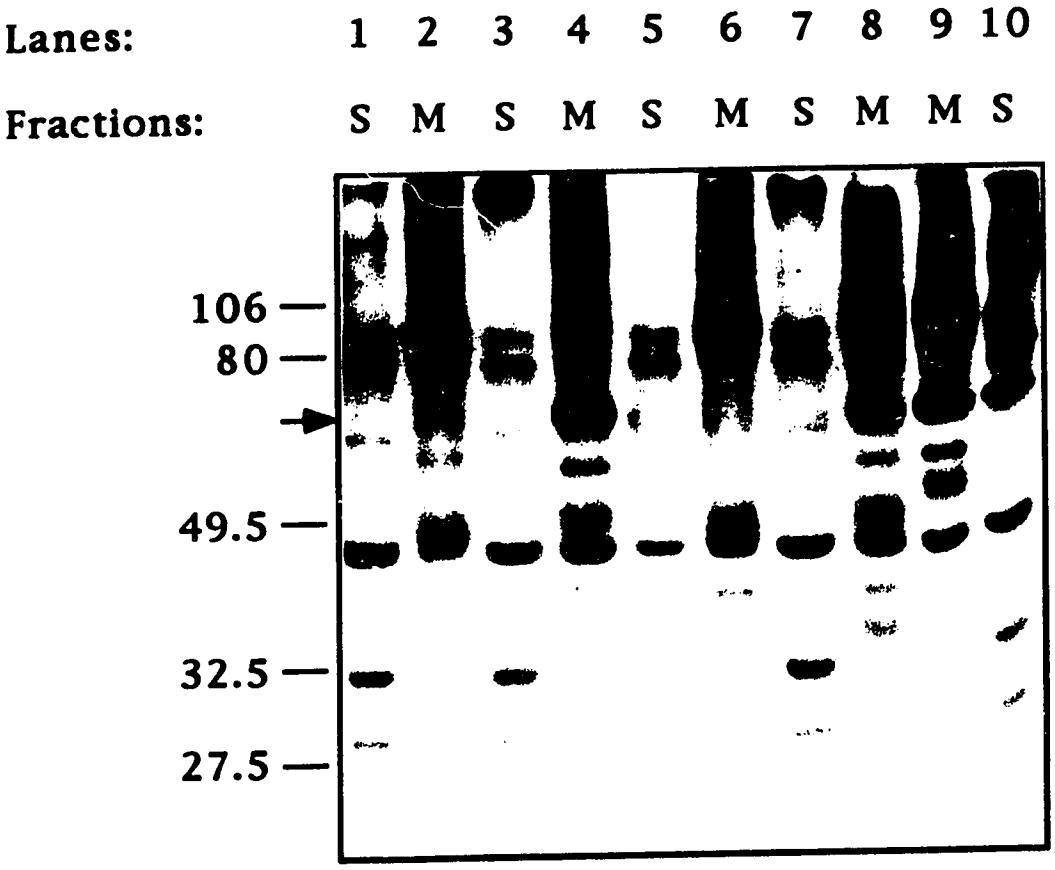

Lanes: 1 2 3 4 5 6 7 8 9 10
Fractions: S M S M S M S M M S
106
80
49.5
32.5
27.5

Table III-1 Scheme for isolation of *TCM10/pet9* and *tcm10/pet9* mutants. A, B, C, and D are the four dissected spores with different genotypes. *TCM10*, wild type *TCM10* gene; *tcm10*, mutated *TCM10* gene; *PET9* and *pet9*, wild type and mutated *PET9* genes, respectively. +, growth; -, non-growth; a and α, mating types. C can only grow on YPG after it mated with either tcm10-15D (a) or tcm10-19D (α) depending on its mating type, and the same thing to D.

				Growth on YPG after mated with the following strains			
	Mating type	YPD	YPG	D360-2D (α) (*TCM10/pet9*)	D360-7D (a) (*TCM10/pet9*)	tcm10-19D (α) (*tcm10/PET9*)	tcm10-15D (a) (*tcm10/PET9*)
A(*TCM10/PET9*)	α or a	+	+	+	+	+	+
B (*tcm10/pet9*)	α or a	+	−	−	−	−	−
C(*TCM10/pet9*)	α	+	−	−	−	−	+
	a					+	−
D(*tcm10/PET9*)	α	+	−	−	+	−	−
	a			+	−		

Table III-2 Genotypes of the *pet9/TCM10* and *pet9/tcm10* mutants. The tcm10-pet9-1A (*TCM10/pet9*) and 1C (*tcm10/pet9*) originate from the same tetrad that are sporulated from the diploid, tcm10dsr2c/D360-2D. tcm10-pet9-9B (*tcm10/pet9*) and 9C (*TCM10/pet9*) are from the same tetrad that was generated from the diploid, tcm10-15D/D360-2D.

	Genotypes
TCM10-pet9-1A	*MATa, ade1 or 2, his3 or 4 or 7, TRP1, ura3, pet9, TCM10*
tcm10-pet9-1C	*MATa, ade1 or 2, his3 or 4 or 7, ura3, pet9, tcm10::TRP1*
tcm10-pet9-9B	*MATα, ade1 or 2, his3 or 4 or 7, trp1, ura3, pet9, tcm10*
TCM10-pet9-9C	*MATα, ade1 or 2, his3 or 4 or 7, TRP1, ura3, pet9, TCM10*

Table III-3 Enzymatic activities in submitochondrial particles. Submitochondrial particles were isolated from MH125, TCM10-pet9-1A (1A), tcm10-pet9-1C (1C), tcm10-pet9-9B (9B), and TCM10-pet9-9C (9C) that were grown to stationary phase in YPD 0.6%. -, not determined. ND, not detectable; *a*, activities expressed as nanomoles of *cyt c* reduced min^{-1} mg^{-1}; *b*, nanomoles of ubiquinone-1 reduced min^{-1} mg^{-1}; *c*, nanomoles of cyt c oxidized min^{-1} mg^{-1}; *d*, nanomoles of DCPIP reduced min^{-1} mg^{-1}; e, nanomoles of fumarate converted to malate min^{-1} mg^{-1}; f, nanoatoms of oxygen consumed min^{-1} mg^{-1}. The MH125, 1A, 1C, 9B, and 9C cultures were 97, 100, 100, 95, and 100% ρ+ respectively. *cyt c*, cytochrome c. Fumarase, includes cytosolic and mitochondrial fumarase; Fumarase (mitochondria), the mitochondrial fumarase only. The protein concentrations of the submitochondrial particles and the soluble fractions of MH125, 1A, 1C, 9B, and 9C are shown in Table III-4. The protein concentrations of mitochondria of 1A, 1C, 9B, and 9C are 7.8, 1.8, 4.1, and 7.9 mg/ml, respectively.

submitochondrial particles / enzymatic activities	MH125	1A	1C	9B	9C
Succinate *cyt c* reductase[a]	72	57 (100%)	1 (2%)	1.5 (2%)	74 (100%)
Succinate ubiquinone[b] reductase	34	14 (100%)	1.8 (13%)	2.3 (14%)	17 (100%)
NADH *cyt c* reductase[a]	68	61 (100%)	1.3 (2%)	5 (6%)	84 (100%)
NADH ubiquinone[b] reductase ($\times 10^2$)	19	21 (100%)	5 (24%)	6 (25%)	24 (100%)
Glycerol-1-phosphate[a] *cyt c* reductase	45	26 (100%)	1.5 (6%)	2.7 (7%)	40 (100%)
Glycerol-1-phosphate[b] ubiquinone reductase	63	14 (100%)	4.4 (31%)	3.1 (11%)	30 (100%)
cyt c oxidase[c]	135	29 (100%)	2.7 (9%)	2 (17%)	12 (100%)
Succinate DCPIP[d] reductase	83	39 (100%)	6.5 (17%)	5 (13%)	38 (100%)
Fumarase[e]	800	280 (100%)	320 (113%)	260 (84%)	310 (100%)
Fumarase (mitochondria)[e]	—	520 (100%)	230 (44%)	170 (29%)	580 (100%)
Succinate oxidase[f]	240	210 (100%)	ND	ND	135 (100%)
NADH oxidase[f]	483	433 (100%)	ND	ND	199 (100%)

Table III-4 Hemoprotein levels in submitochondrial particles. *a*, the protein concentration measured in mg/ml; *b*, nanomoles of cytochrome per mg of protein; *c*, the activity of catalases as nanomoles of H_2O_2 reduced /mg·min. The catalase activities were measured using the soluble fraction during preparation of submitochondrial particles. conc., concentration. Other descriptions are the same as Table III-3.

	MH125	1A	1C	9B	9C
membrane fraction protein conc.[a]	18.3	25	21.5	14.3	33
cytochrome *a* [b]	35	8.2 (100%)	7.2 (88%)	5.6(110%)	5.1 (100%)
cytochrome *b* [b]	14	9.0 (100%)	6.1 (68%)	9.0 (36%)	25 (100%)
cytochrome *c1* [b]	19	14.6(100%)	1.0 (7%)	ND	7.5 (100%)
cytochrome *c* [b]	18	42.4(100%)	14 (33%)	ND	35 (100%)
total cytochromes[b]	86	74.2(100%)	28.3 (38%)	14.6 (20%)	72.6(100%)
catalases[c]	250	44 (100%)	1 (2%)	12 (52%)	23 (100%)
soluble fraction[a] protein conc.	19.6	12.2	6.5	7.5	8.4

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CHAPTER IV CONCLUSION AND HYPOTHESES

In this final chapter, I will summarize the data presented in this thesis, put forward hypotheses for the function of the TCM10 protein, and propose future experiments to test these hypotheses.

The respiration deficient mutant, *tcm10*, was complemented for growth on glycerol and the sequence of the complementing gene determined. The predicted TCM10 protein shows little sequence similarity with any proteins in current databanks. The *TCM10* gene contains a number of low usage codons possible indicating low expression in yeast. The *TCM10* protein is a mitochondrial protein associated with the matrix side of the inner membrane. Disruption of the *TCM10* gene is not lethal. Both disruption and mutagenized mutants display increased instability of the mitochondrial DNA. The electron transport chain activities are greatly decreased in both mutants. Both have about 15% residual succinate:ubiquinone reductase, succinate dehydrogenase, and cytochrome *c* oxidase activities. The NADH dehydrogenase activities in both mutants are about 25% of the wild type controls. Therefore, the *tcm10* mutation causes pleiotropic effects on the respiratory chain. The total cytochrome content of the disruption mutant is about twice that of the mutagenized strain, but is decreased 2.5 fold compared to wild type. Cytochrome c_1 levels are decreased more than 10 fold in both mutants. Surprisingly, catalase activity in the disruption mutant is decreased about 50 fold in contrast to the 2 fold decrease in the originally mutagenized mutant. This is the only significant difference observed between the two mutants. As *tcm10* has multiple effects on mitochondrial respiration, it is unlikely that the TCM10 protein is a subunit of any respiratory complex. The pleiotropism

may indicate that it is involved in the expression of mitochondrial DNA, in the biosynthesis of a cofactor required by all these enzymes, in mitochondrial protein import or in assembly.

If TCM10 is involved in the expression of the mitochondrial genome, it may do so in one of several ways: the transcription of mtDNA, mitochondrial pre-mRNA splicing, or the translation of mitochondrial mRNA. The yeast mitochondrial RNA polymerase has been isolated, and contains a 150 kDa catalytic subunit as well as a factor necessary for accurate transcriptional initiation (Winkley *et al.*, 1985; Schinkel *et al.*, 1987; Schinkel *et al.*, 1988; Ticho and Getz, 1988). Many as yet unidentified factors may exist and function to allow differential recognition of promoters (Grivell, 1989). 13 introns present in the 75 kb circular mtDNA are spliced by mitochondrial intron-encoded RNA maturases and at least 5 nuclear encoded proteins (Grivell, 1989; Séraphin *et al.*, 1989; Herbert *et al.*, 1988; Akins and Lambowitz, 1987). Some of these also function as aminoacyl tRNA synthetases. Surprisingly, some appear to be specific to individual introns (Grivell, 1989). The translation of mRNA in mitochondria requires ribosomal protein complexes, aminoacyl tRNA synthetases, and initiation factors. All these proteins are encoded by nuclear genes. Several nuclear gene products have been reported to be required for the translation of selected individual mRNAs in yeast mitochondria. PET111 and PET112 are specifically required for the translation of subunit II, PET494, PET54, and PET122 for subunit III of cytochrome *c* oxidase; CBP6, CBS1, and CBS2 are required for apocytochrome *b* translation (Grivell, 1989; Attardi and Schatz, 1989).

As the mtDNA encodes apo-cyt *b*, subunits I, II, and III of cytochrome *c* oxidase, subunits 6, 8, and 9 of the F_0F_1-ATPase, nuclear mutations that affect its expression will certainly result in a respiratory deficient phenotype.

Commonly, these mutations give rise to the loss of mtDNA. The *tcm10* mutation results in a respiratory deficient phenotype and instability of the mitochondrial genome. However, some observations argue against a function for TCM10 in mtDNA expression. Both NADH and succinate ubiquinone reductase activities are greatly decreased in *tcm10* mutants. Neither of these two enzymes contains subunits encoded by the mtDNA. Similarly, catalase levels are greatly decreased in the *tcm10* disruption mutant, suggesting that a non-mitochondrial enzyme is affected. In addition, cytochrome *b* and cyt *aa3* levels are only slightly reduced, even in the disruption mutant. For these reasons, I do not believe that the TCM10 protein is involved in mtDNA expression.

All the respiration chain enzymes measured, except the NADH ubiquinone reductase with a 4 to 5 fold decrease, contain heme as a prosthetic group (Hatefi, 1985) and have about 7-9 fold decreases in the *tcm10* mutants. Therefore, it is possible that TCM10 is involved in heme biosynthesis, in heme attachment to cytochromes, in the transport of heme out of the mitochondrial matrix, or in Fe transport and/or reduction (Urban-Grimal and Labbe-Bois, 1981). The biosynthesis of heme starts with the condensation of succinyl-CoA and glycine into 5-aminolevulinic acid (δ-ALA), a reaction catalyzed by ALA synthase, and ends in the attachment of Fe^{++} to protoporphyrin by the enzyme, ferrochelatase. In yeast, only three (the first and the last two) reactions take place in mitochondria, other steps occur in the cytosol (Labbe-Bois and Labbe, 1991). The mitochondrial enzymes have been identified and their sequences are available (Labbe-Bois and Labbe, 1991). Moreover, mutants with defects in heme biosynthesis require the addition of δ-ALA to the medium for growth (it is the precursor for the synthesis of vitamin B_{12} and siroheme which is required for cysteine and methionine synthesis).

Heme mutants with defects in the later step also accumulate fluorescent intermediates (Labbe-Bois and Labbe, 1991; Keng and Guarente, 1987). The *tcm10* mutants do not show these phenotypes. Therefore, it is unlikely that TCM10 is an enzyme of heme biosynthesis. It is also unlikely that TCM10 is involved in the attachment of heme to cytochromes for the following reasons. Firstly, the enzymes encoded by the *CYC3* gene and the CYT2 gene are responsible for the attachment of heme *c* to apocytochrome *c* (apo-cyt *c*) in the intermembrane space and the attachment of heme *c* to the intermediate form of cytochrome c_1 (Zollner *et al.*, 1992), respectively. The cytochrome *c* heme lyase, CYC3, is also involved in the import of apo-cyt *c* (Dumont *et al.*, 1987; Dumont *et al.*,1991). Secondly, if TCM10 were involved in the attachment of heme *b* to apocytochrome *b*, cytochrome *c* oxidase levels should not be affected in the mutants. Thirdly, it seems unlikely that one enzyme is responsible for both heme *b* and heme *c* attachment since these cytochromes are synthesized in different compartments (Attardi and Schatz, 1988); Finally, it is not clear why a mitochondrial protein would be responsible for the attachment of heme to catalase as its activities are also greatly decreased in the disruption mutant.

However, if TCM10 is involved in the transport of heme out of mitochondria, fully synthesized heme would remain trapped inside mitochondria in the *tcm10* mutants. This would explain the decrease in catalase activity that was observed. However, mitochondrial cytochrome levels are decreased despite the accumulation of heme in this organelle predicted by this hypothesis. Heme is also required for the function of the transcriptional activators HAP1 and HAP2/3/4 which bind to the upstream activation regions of genes encoding hemoproteins (Figure IV-1) such as *CYC1* (apo-cyt *c*), *CTT1* (catalase T), *CYT1* (apo-cyt c_1), *COX4* (subunit 4 of

cytochrome *c* oxidase), *COX5A* (subunit 5 of cytochrome *c* oxidase), *HEM1* (δ-ALA synthase) (Forsburg and Guarente, 1989) as well as one non-hemoprotein gene, *COR1* (core protein 1 in the cyt *bc1* complex) (Trumpower, 1990). In heme deficient mutants, apo-cyt *c* does not accumulate because heme is required for its transcription (Guarente and Mason, 1983). The small amount of heme synthesized in anaerobically grown cells is preferentially incorporated into other hemoproteins rather than those of the respiratory chain (Labbe-Bois and Labbe, 1991). It is thought that cells can sense the need to distribute heme to other hemoproteins rather than to cytochromes since respiration is unnecessary under anaerobic conditions. Furthermore, the apoproteins of several subunits of cytochrome *c* oxidase, catalases A and T (Woloszczuk *et al.*, 1980), and iso-1-cytochrome *c* (Matner and Sherman, 1982) are also present in greatly reduced amounts in the heme-deficient cells. Interestingly, *CYC7* (iso-2-cytochrome *c*) is not so tightly regulated, but its expression is also decreased in heme deficient mutants. Therefore, we speculate that TCM10 which is associated with the inner membrane may be part of the heme transport machinery. In *tcm10* mutants, heme synthesized in mitochondria can not be transported out into cytosol and participate in the activation of hemoprotein genes listed above (Labbe-Bois and Labbe, 1991). Decreases in mitochondrial cytochrome content may be due to impaired assembly or stability of cytochromes as a result of the reduced synthesis of nuclear subunits. The mutagenized mutant may be leaky as suggested by the higher residual catalase activities. As a secondary effect, decreased protein import may result from a decreased membrane potential caused by reduced electron transport chain activity. This may explain effects on non-heme dependent activities such as fumarase and on the instability of mitochondrial DNA in the *tcm10* mutants. Little is known about heme translocation to the

cytosol because mutations affecting intracellular heme transport are expected to be highly pleiotropic.

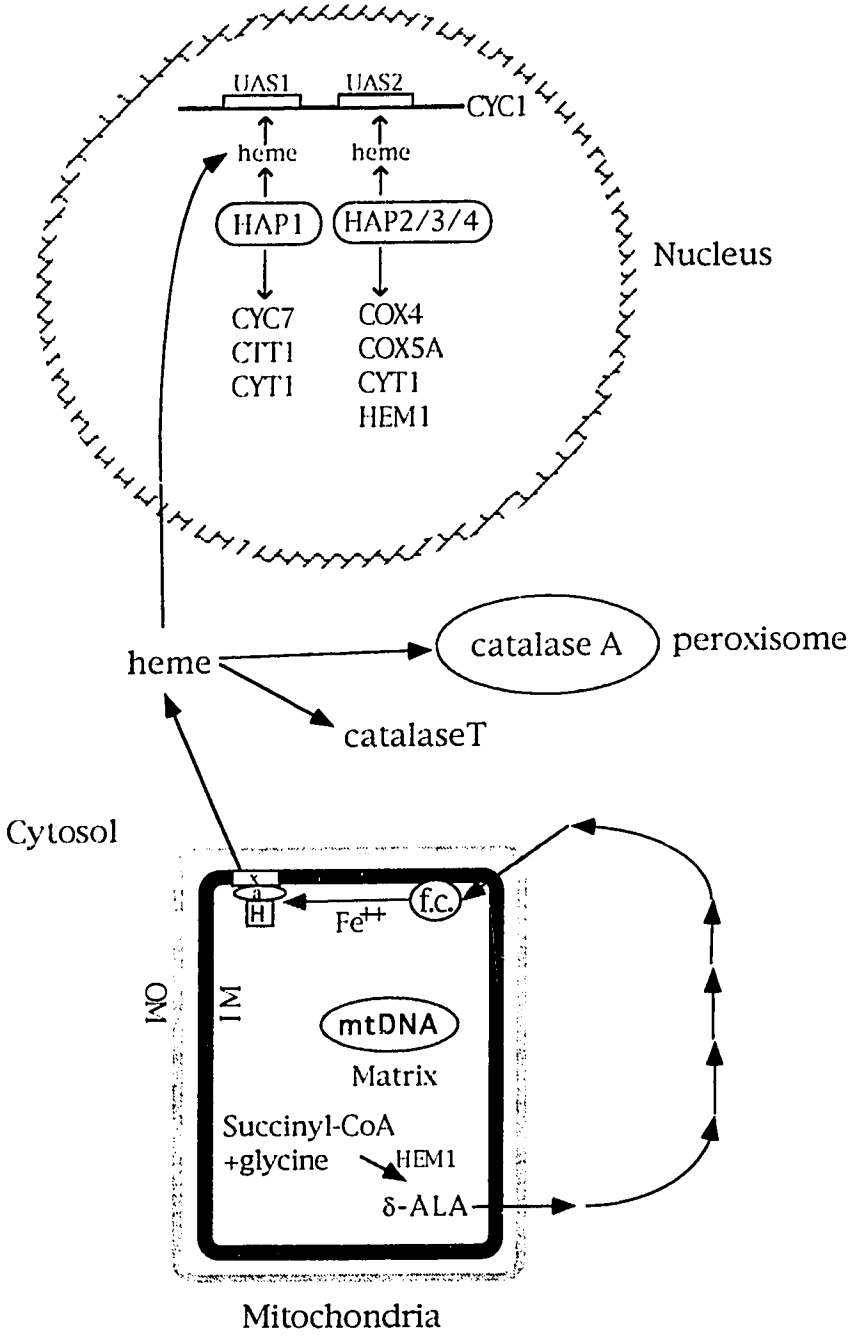
Another hypothesis is that TCM10 is involved in Fe^{++} storage inside mitochondria. Yeast cells adapting to growth on a non-fermentable carbon source will need to synthesize heme quickly. In addition, Fe is also a component of Fe/S clusters that are essential for mitochondrial respiration. Therefore, mutations affecting iron storage would be expected to be highly pleiotropic. They might also be expected to reduce cytochrome levels more than was observed. However, little is known about intracellular iron transport and its use in mitochondria and so it is difficult to rule out this possibility.

Mitochondrial protein import and assembly have been extensively studied (Attardi and Schatz, 1988; Hartl *et al.*, 1989). The localization of TCM10 to the matrix surface of the inner membrane indicates that it is unlikely to be involved in import. It is also unlikely that TCM10 is a chaperone-like protein involved in the assembly of newly imported proteins since most chaperones share high sequence identities, and the involvement of hsp70 and hsp60 has been well studied (Hartl *et al.*, 1989; Attardi and Schatz, 1988; Pfanner *et al.*, 1988; Hartl and Neupert, 1990).

At this time, I believe that TCM10 is involved in the transport of heme out of mitochondria after its synthesis on the matrix side of the inner membrane, but further experiments are necessary to test this hypothesis. One key experiment to test this hypothesis is to include the radioactively labeled (eg., ^{14}C) heme precursor, δ-ALA, in the growth medium of a *tcm10/hem1* deficient double mutant along with the control cells of *hem1* mutant and MH125. It is expected that the radioactivity would accumulate as heme inside mitochondria in the double mutant. In addition, the double *tcm10/hem1*

mutant should be isogenic to the control *hem1* single mutant in order to eliminate the possible heterogenic difference. The isogenic double *tcm10/hem1* mutant can be generated by the targetted disruption of the *TCM10* chromosomal locus in the *hem1* mutant. This experiment will provide a strong evidence to support the hypothesis if the radioactivity is accumulated inside mitochondria in the *tcm10* mutant.

Figure IV-1 A model for TCM10 function in yeast mitochondria. UAS1 and UAS2 of the *CYC1* gene are shown as open boxes in the nucleus. a, tcm10 protein; f.c., ferrochelatase; H, heme; x, the unidentified protein channel for heme translocation. OM and IM are the mitochondrial outer and inner membranes, respectively.


UAS1
UAS2
CYC1
heme
heme
HAP1
HAP2/3/4
Nucleus
CYC7
CIT1
CYT1
COX4
COX5A
CYT1
HEM1
heme
catalase A
peroxisome
catalaseT
Cytosol
x
a
H
Fe++
f.c.
OM
IM
mtDNA
Matrix
Succinyl-CoA
+glycine
HEM1
δ-ALA
Mitochondria

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