Directing a messenger RNA into yeast mitochondria

by

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ABSTRACT

Mitochondria are complex eukaryotic organelles that are involved in numerous cellular tasks, most notably energy production and apoptosis. A malfunction in any of these tasks can result in a broad range of complications. Mitochondrial DNA mutations, whether inherited or acquired, cause impaired respiratory chain functioning. This, in turn, leads to decreased production of energy and formation of free radicals, which result in a series of phenotypic expressions, otherwise known as mitochondrial disorders.

There are many diseases associated with non-functional mitochondrial genes. Therefore, targeting mRNAs that mediate the synthesis of a functional copy of the protein into mitochondria can be used for therapeutic purposes. As an initial step, here we investigate the import of mRNAs into the mitochondria of budding yeast, *Saccharomyces cerevisiae*, using a *lacZ* mRNA reporter construct.

The untranslated 3' and 5' regions of mRNAs are thought to play a key role in directing and regulating the activity of a mRNA. Here, we hypothesized that a 3' untranslated region of *OXA1* mRNA may direct mRNAs into mitochondria. This region has been previously shown to direct different mRNAs into the vicinity of mitochondria. We also used a mitochondria-specific translation signal at the 5' untranslated region of *COX2* mRNA to investigate presence and functionality in mitochondria.

We successfully designed the constructs and transformed them into *S cerevisiae*. In addition, we performed further extensive experimental analysis to examine the activity of our designed mRNAs in yeast.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Ach	Acetylcholine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AD	Alzheimer's disease
Amp	Ampicillin
bp	Base Pair
cDNA	Complimentary Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DA	Dopamine
ETC	Electron Transport Chain
EtOH	Ethanol
EtBr	Ethidium bromide
FFE	Free Flow Electrophoresis
H_2O_2	Hydrogen Peroxide
kb	Kilobases or Kilobase pairs
LiOAc	Lithium acetate
LB	Luria-Bertani
M.P	Master Plate
mRNA	Messenger RNA
µm	Micrometer
MQ H_2O	Milli-Q water (Ultra pure water)
mt	Mitochondria

mtDNA	Mitochondrial DNA
mitoribosome	Mitochondrial ribosome
NA	Noradrenaline
nDNA	Nuclear DNA
OXPHOS	Oxidative Phosphorylation
PD	Parkinson's Disease
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RPM	Revolutions per Minute
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SGD	*Saccharomyces* Genome Database
5-HT	Serotonin
ssON	Single-stranded oligonucleotide
SDS-PAGE	Sodium dodecyle sulfate-Polyacrylamide Gel Electrophoresis
SN	Substantia Nigra
$^{\bullet}O_2$	Superoxide
SOD	Superoxide Dismutase
SGA	Synthetic Genetic Array
tRNA	Transfer RNA
YPD	Yeast Extract Peptone Dextrose

LIST OF FIGURES

LIST OF TABLES

1: Introduction

CHAPTER 1: INTRODUCTION

1.1 Mitochondria

Mitochondria (mt) are found in most eukaryotic cells. They are referred to as the "powerhouse" of the cell because of their primary function to convert organic matter into energy via the process of oxidative phosphorylation (OXPHOS) (Lu, 2009). They generally have their own genome and gene expression machinery, and can occupy up to 25% of the cell's cytoplasm (Figure 1.1[a]). They are usually a few micrometers (μm) long and up to one μm wide (Becker *et al.*, 2003). The mt has two membranes; an outer membrane and an inner membrane that are separated by an intermembrane space. The inner membrane is folded up to form compartments referred to as cristae. The internal semi-fluid mt space is called the matrix (Figure 1.1[b],[c]) (Fox, 2009).

The generally accepted model of mt origin is the endosymbiotic theory. This theory claims that eukaryotic cells have evolved from prokaryotic ones, and that mt are the direct descendants of bacterial endosymbionts (Margulis, 1976). These efficient, energy-converting organelles are highly conserved through the course of evolution and have a number of prokaryotic-like characteristics. Therefore, studying biological pathways of simple prokaryotes and eukaryotes may shed some light on important mt pathways in higher eukaryotes such as humans (Gray *et al.*, 1999).

Mt play a central role in many cellular functions, including bioenergetics, apoptosis, and the metabolism of amino acids, lipids, and iron (Sickmann *et al.*, 2003). In the next sections some important properties of mt will be discussed.

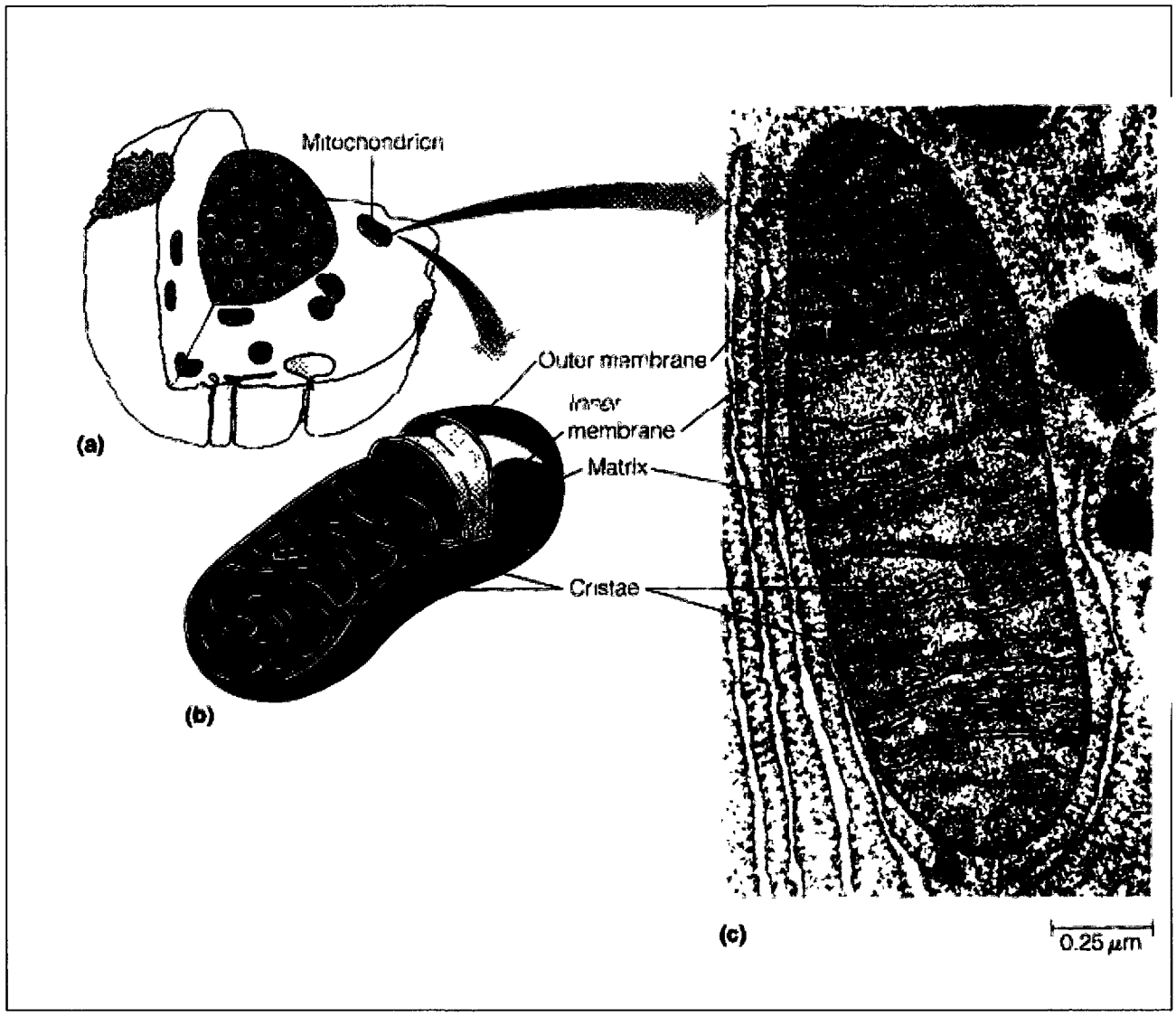

Figure 1.1: The Mitochondrion. (a) A cross-section view showing the relative numbers and size of the mt within an animal cell. (b) A schematic illustration of mt structure showing its outer and inner membranes, matrix and cristae. (c) An electron micrograph of a mt in a rat pancreas cell also showing the 4 components that make up the mt (Becker *et al.*, 2003).

1.1.1 Mitochondrial Genetics

The mt chromosome or mtDNA is a circular, double-stranded molecule that is maternally inherited. Human mtDNA is thought to contain 37 genes and is ~17kb in size (Figure 1.2). There are many mt chromosome copies per mt, often hundreds or thousands, depending on the type of cell (Pulkes and Hanna, 2001). In higher eukaryotes, there are 13 nuclear-encoded genes, which code for proteins that are directly involved in mt respiratory chain (Garcia *et al.*, 2007).

In the yeast *Saccharomyces cerevisiae*, about 1-45 mt can be found in a typical haploid cell. *S cerevisiae*'s mt DNA is ~78kb in size (Figure 1.2) (Griffiths *et al.*, 2000). There are 11 genes that produce polypeptides involved in the mt respiratory chain, eight of which are encoded by nuclear genes. The exact roles of all these genes are not well described but many are thought to be involved in enzyme assembly and stability. Three proteins (Cox1p, Cox2p and Cox3p) are encoded by mt genes and are translated inside the mt by mt ribosomes or mitoribosomes. These 3 endogenous proteins are a part of complex IV of the respiratory chain found in the inner mt membrane (Figure 1.3) (Horan *et al.*, 2005). The respiratory complexes are conserved from yeast *S cerevisiae* to human with the exception of complex I, which is replaced by three NADH dehydrogenases in *S cerevisiae* (Lemaire and Dujardin, 2008).

There are two main roles for mtDNA: to code for proteins that make up the OXPHOS system, and to code for transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and other proteins used in mt protein synthesis. Most mt proteins of eukaryotic cells are encoded by nuclear genes, synthesized by cytoplasmic ribosomes, and then transported into the mt via specialized import system (Foury and Kucej, 2001).

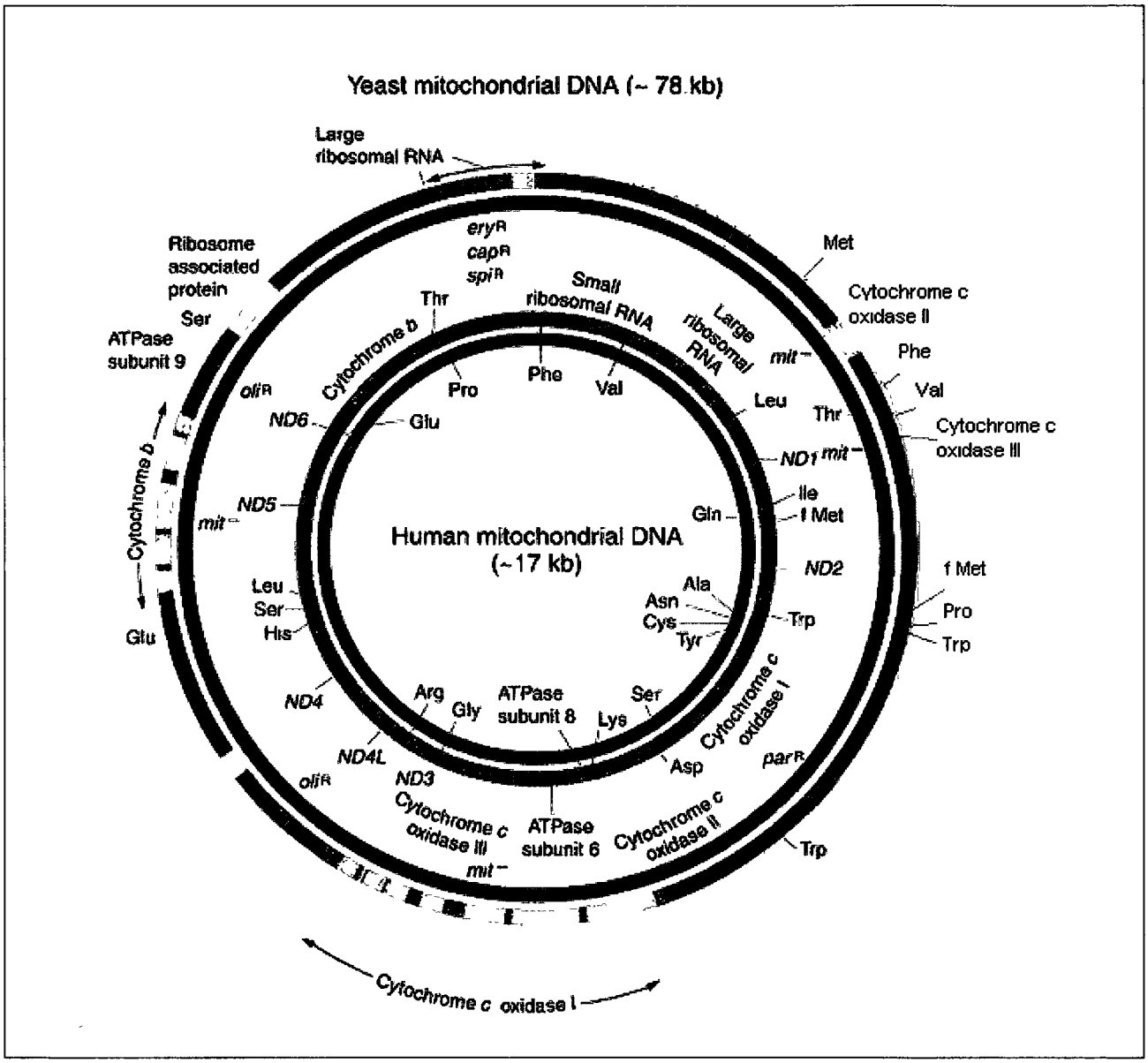

Figure 1.2: Maps of yeast and human mtDNAs showing the circular double-stranded nature of the DNA. As shown, yeast mtDNA is ~ 4 times larger than human mtDNA (modified from Griffiths *et al.*, 2000).

1.1.2 Energy Production

The survival of most aerobic cells, tissues and organisms depends on their ability to use oxygen to extract energy. This occurs through a series of metabolic reactions referred to as OXPHOS. In eukaryotes, energy is produced, in form of adenosine triphosphate (ATP), by first converting glucose to pyruvate through a process called glycolysis that occurs in the cytoplasm. Pyruvate is then transported across the mt inner membrane to be oxidized to carbon dioxide (CO_2) via a process called Krebs cycle, which also generates NADH and $FADH_2$ (Kurland and Andersson, 2000). These coenzymes store energy that is extracted by OXPHOS, the final biochemical pathway that produces ATP. Electrons are passed on from these coenzymes to oxygen (O_2), which is then reduced to water (H_2O) in a series of redox reactions termed the electron transport chain (ETC) (Figure 1.3). These reactions provide enough energy that would pump the protons (H^+) from the matrix, across the inner membrane, and into the intermembrane space. This H^+ gradient creates an electrochemical gradient that causes ATP synthase to phosphorylate adenosine diphosphate (ADP) forming ATP (Tuppen *et al.*, 2010).

The OXPHOS machinery is made up of over 80 different polypeptides, organized into 5 trans-membrane complexes. The ETC is made up of 5 complexes (Subunits I-V) and are embedded in mt inner membrane. Of the 13 nuclear-encoded mt genes (subunits) mentioned above, seven are involved in complex I, one is involved in complex III, three are involved in complex IV, and two are involved in complex V. Cytochrome *c* is found on the outer surface of mt inner membrane and is an essential component of the ETC. It transfers electrons between complex III and IV. If significantly altered or removed from the system, the ETC is blocked and energy production stalls (Lemaire and Dujardin, 2008).

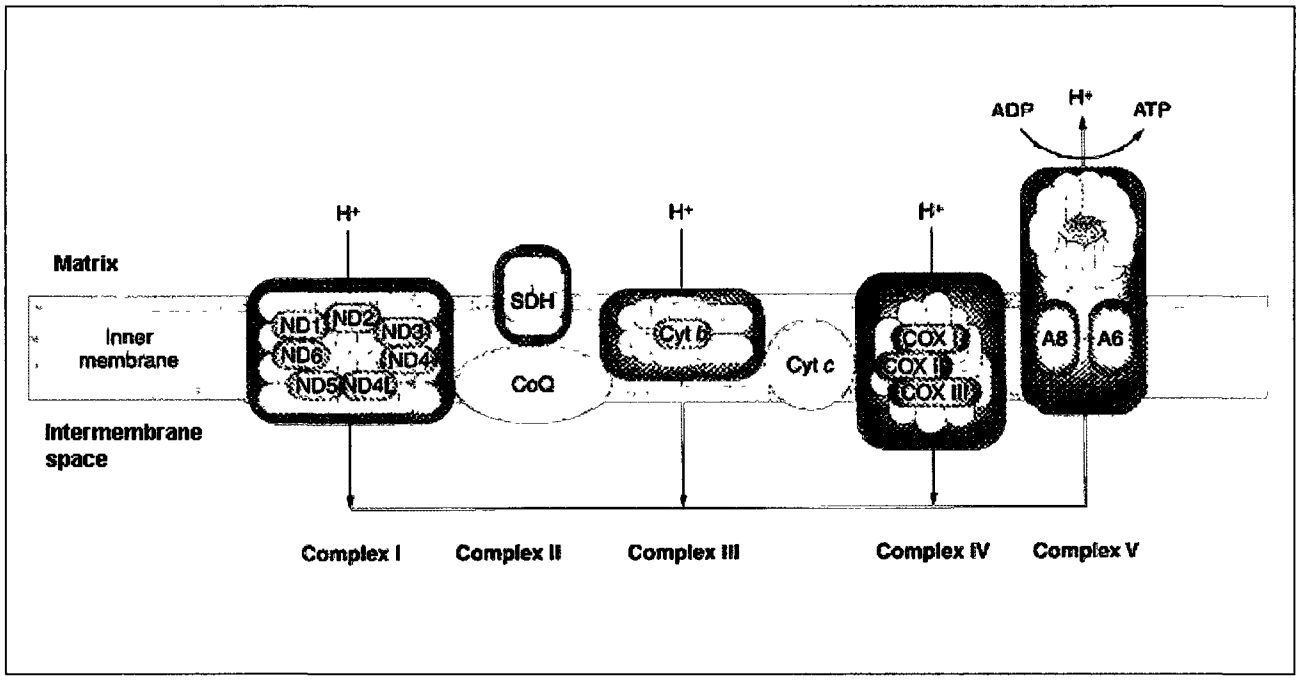

Figure 1.3: A schematic diagram of the mitochondrial electron transport chain (Mt ETC) showing its 5 complexes and cytochrome *c*. Arrows indicate the electron flow as they get passed on from the first complex to the fifth complex, where O_2 acts as a terminal electrons acceptor and reduces them to H_2O. This redox reaction generates energy that drives H^+ from the matrix, across the inner membrane, and into the intermembrane space. An electrochemical gradient is then created to produce ATP from ADP (modified from Griffiths *et al.*, 2000).

1.1.3 Mitochondria Malfunction

Like nuclear DNA, mtDNA is also vulnerable to genetic mutations. In fact, base-substitutions rate is approximately 10 times higher than that of nuclear genes. This is thought to be as a result of mtDNA not having protective histones that are found in nuclear DNA (Carraway *et al.*, 2008). There are many factors that might be involved in mt dysfunction. Such factors include: base-substitutions, point-mutations (single base substitutions), large deletions in mtDNA, defects in the chaperoning proteins, and flaws in protein import mechanisms (Medeiros, 2008).

Many mtDNA changes are expressed as abnormal phenotypes at the cellular and organismal level. Gene mutations that affect the OXPHOS process lead to respiratory defects that severely compromise the generation of energy in cells and tissues (Smeitink *et al.*, 2001).

Another key function of mt is regulating apoptosis or programmed cell death. Apoptosis is the physiological mechanism of programmed cell death. This mechanism is vital to a healthy cell cycle. When it is time for the cell to die, a series of events are activated to ensure efficient execution of cell death (Blackstone and Green, 1999).

The mt-dependent apoptotic pathway is activated by diverse stimuli including DNA damage, heat shock, UV and gamma radiation, as well as chemotherapeutic drugs. These stimulating stressors activate some mt signaling pathways that result in permeabilization of the mt outer membrane and release of soluble proteins from the mt inter-membrane space. The release of a number of these proteins has been shown to trigger apoptosis. An example of such proteins is cytochrome *c* subunit, which relocates to the cytoplasm and couples with other proteins to help initiate apoptosis (Pradelli *et al.*, 2010).

In many instances when a malfunction occurs in mt genes associated with apoptotic regulation, the apoptotic mechanism can become unregulated and dysfunctional. In neural cells this can lead to premature cell death which is the main feature of many neurological disorders. Mt disorders can occur in association with genetic abnormalities of either nuclear or mt origin (Szewczyk and Wojtczak, 2002).

Since each mt contains many copies of mtDNA and there are a number of mt in a cell, both normal and mutant mtDNAs may co-exist in different proportions. Higher proportions of mutant alleles contribute to disease severity (Jensen *et al.*, 2000).

1.1.4 Mitochondrial Diseases

Many metabolic and neurodegenerative disorders are now known to be associated with deficient mt genes. Environment factors may also play a role in the genetically predisposed mtDNA that are related to mt disorders (Wallace, 2005). The number of mt per cell is related to cell energy demands. Generally, there are more mt in cells found in tissues and organs that require more energy to function, such as the brain (Sack Jr., 2009). When mt become nonfunctional, usually a threshold is reached before the disease symptoms appear and as the number of nonfunctional mt is increased, so does the severity of the symptoms. Examples of such diseases are Parkinson's, Alzheimer's, diabetes, etc. (Wallace and Fan, 2010).

Parkinson's disease (PD) occurs due to neuronal death in the cells of the midbrain, which produce neurotransmitters dopamine (DA), noradrenaline (NA), serotonin (5-HT), and acetylcholine (ACh). Specifically, the disease is characterized by a slow and progressive large-scale loss of DA neurons in the substantia nigra (SN) that is found in the midbrain, and the formation of protein aggregates that accumulate in the cytoplasm of the remaining DA neurons

(Kortekaas *et al.*, 2005). Clinically, patients have the symptoms of progressive rigidity, bradykinesia (slow movement), tremor and postural instability (Banerjee *et al.*, 2009).

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that affects about 10% of the population after the age of 65. The disease is characterized by 3 major pathological syndromes: senile plaques, neurofibrillary tangles, and inflammation. The most common symptom of AD is memory loss, and in advanced stages, dementia (Bouras *et al.*, 2005).

A defined cause for the development of these diseases is unknown. However, there are many factors thought to be involved in their pathogenesis most notably mt dysfunction and oxidative stress.

Mt complex I is the first enzyme of the respiratory chain and the main source of free radicals in the cell. The respiration chain consumes nearly all of molecular oxygen, leaving powerful oxidants as byproducts (Schulz and Falkenburger, 2004). In PD, the inhibition of the respiratory chain, usually due to mtDNA mutation, leads to an increase in the production of reactive oxygen species (ROS), such as superoxide ($^{\bullet}O_2$) (Yoritaka *et al.*, 1996). When an electron escapes from the mt ETC it may react with oxygen molecule to form $^{\bullet}O_2$, which is converted to hydrogen peroxide (H_2O_2) and other ROS. These reactive toxic metabolites may have a dramatic effect on mt and the cell as a whole by eliciting a cascade of events leading to mutation and apoptosis (Figure 1.4). The metabolism of DA also generates ROS, which can lead to oxidative damage that cause neurodegeneration (Stuart and Brown, 2006). In AD, an increase in ROS levels in the system is thought to cause mutations in mt genome. This leads to a decrease in cytochrome *c* oxidase activity, the terminal complex of the mt respiratory chain, resulting in neuronal degeneration (Howell *et al.*, 2005).

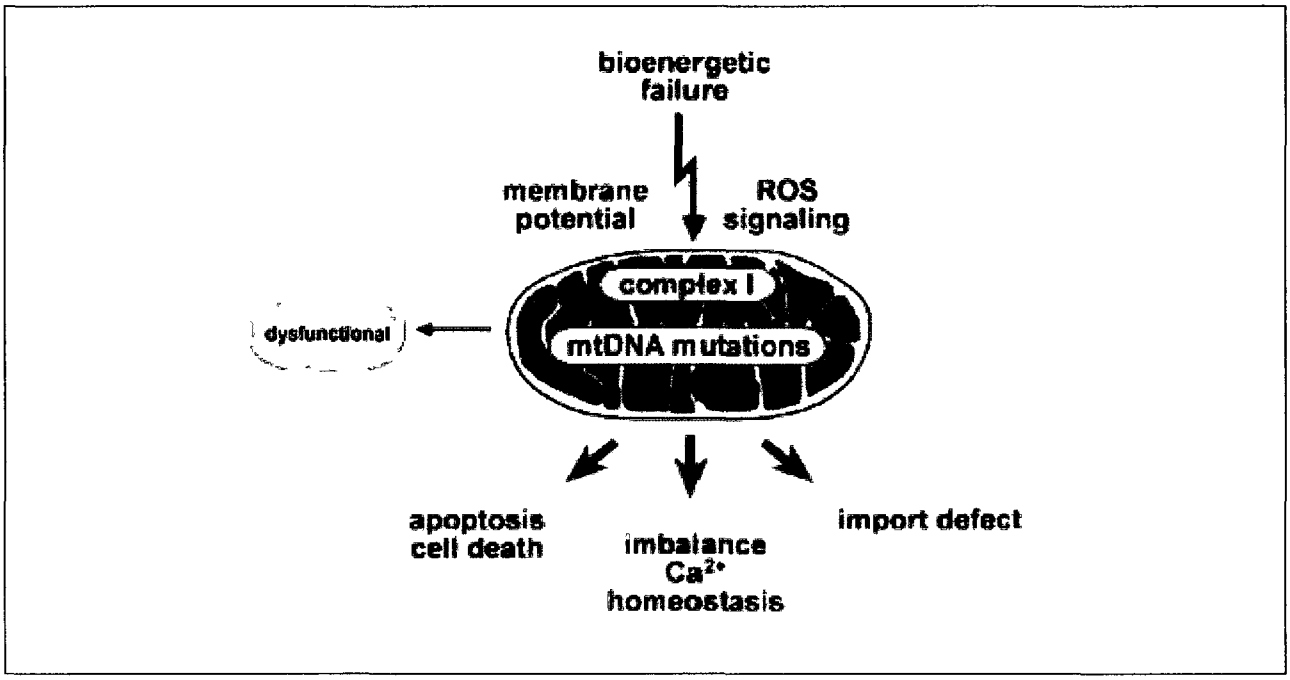

Figure 1.4: A schematic representation of mt alterations associated with mtDNA mutations. Respiratory chain malfunctions and MtDNA mutations unleash a series of effects, including an increase in the levels of ROS in the cell. These chain reactions directly affect mt key functions such as energy metabolism, apoptosis, calcium homeostasis, and cellular quality control pathways. Ultimately, neurons begin to deteriorate leading to the development of neurological diseases such as AD and PD (modified from Winklhofer and Haass, 2010).

Healthy cells maintain a certain level of intracellular ROS to keep a balanced redox. They do so by releasing and regulating metabolic enzymes or "antixodiants" such as superoxide dismutase (SOD) to break down and eliminate ROS (Pelicano *et al.*, 2003).

Epidemiological studies have shown a strong role for environmental factors in the etiology of many mt-associated diseases such as AD. Exposure to agricultural chemicals such as pesticides has been linked to increased percentage of PD and AD in humans (Schapira, 2010).

Recent evidence confirmed that there are several PD-associated genes which intersect with pathways that regulate mt function. One of the genes involved in PD development is parkin (PRKN). It is known that a mutation in this gene causes a premature termination of its translation. This loss-of-function mutation is found to contribute to the pathogenesis of PD, as well as mt damage (Gasser, 2009). Another PD-causing gene is PTEN induced putative kinase 1 (PINK1). The PINK1–PRKN pathway is found to be involved in the protection of mt integrity and function. Therefore, any mutation or disturbance in this pathway strongly impacts the mt and PD pathogenesis (Park *et al.*, 2009).

To understand the biological roles of PINK1 and PRKN, animal knockout models of PD were created by inactivating these two genes in mice. Whitworth and Pallanck describe the observations in such knockout models. The mice showed nigrostriatal physiological defects suggesting a role for PINK1 and PRKN in DA neurotransmission. They also had enlarged and swollen mt in tissues that were missing PINK1 and PRKN, suggesting that these genes influence mt integrity (Whitworth and Pallanck, 2009).

To date, there is no cure for either of these disorders. Patients depend completely on symptomatic relief treatments and drugs that slow the disease progress (Winklhofer and Haass, 2010).

Diabetes mellitus is a disease that occurs with persistent high sugar levels (hyperglycemia) in the blood. This results from defective secretion or action of the hormone insulin. Insulin is secreted by β cells in the pancreas to regulate carbohydrate metabolism. There are two forms of this disease: type 1, also called juvenile diabetes, and type 2, which usually occurs in adults. Type 1 occurs when children are born with very little or no insulin production in their system. Therefore, they need to receive insulin injections for life. This form is considered rare (Lazar, 2005). Type 2 occurs when body tissues either become resistant to insulin, or when insulin production decreases dramatically. This form is the most common metabolic disease and its side effects include blindness, renal disease, and loss of limb (Lowell and Shulman, 2005). The skeletal muscle and liver maintain normal glucose levels in the blood by signaling insulin to convert glucose to glycogen that can be stored in the muscle and liver. Defects in the skeletal muscle and liver lead to resistance towards insulin (Petersen *et al.*, 2003). Mt regulate insulin secretion from pancreatic β cells by detecting glucose levels. Thus any breakdown in this mechanism can lead to hyperglycemia and type 2 diabetes (Lowell and Shulman, 2005). Recently, evidence has linked resistance to insulin with impaired mt function, which is mainly on the basis of insufficient supply of energy to the insulin signaling pathway. Wang *et al.* were able to impair mt function by chemical treatment or genetic manipulation, which led to insulin insensitivity and decreased glucose utilization of adipose tissue in mice, rats and humans (Wang *et al.*, 2010). This strongly suggests that mt dysfunction is associated with type 2 diabetes. Furthermore, the pathological changes due to mt dysfunction can lead to disturbance of glucose homeostasis and type 2 diabetes in animals and humans. Another study that used a mouse model to explore mt dysfunction in type 2 diabetes found a decrease in the number of mt, contents of mtDNA, and respiratory enzymes. It also reported a disturbance of mt

network, which is consistent with the decline in the OXPHOS and ß-oxidation in the adipose tissues of the mice with type 2 diabetes (Choo *et al.*, 2006).

1.2 Yeast as a Model Organism

Yeast *S cerevisiae* genome is the first fully-sequenced eukaryotic genome. Its genetic system is thought to highly resemble that in humans (Hurowitz *et al.*, 2003). *S cerevisiae* is a unicellular facultative anaerobic fungus; qualifying traits of a suitable organism for biochemical and genetic studies of mt gene expression and respiratory metabolism. Yeast is easily grown, inexpensive to maintain, and is exceptionally amenable to genetic manipulations (Daum, 2000).

S cerevisiae contains approximately 6000 genes, characterized by the genome sequencing project. Approximately 1000 genes are known to have indispensible specific functions and hence are referred to as "essential" genes. The majority of the essential genes are well-characterized. The remaining 5000 genes are identified as "nonessential" genes given that their deletion does not lead to a lethal phenotype (Goffeau *et al.*, 1996). A significant number of these non-essential genes are not yet fully characterized. Numerous studies are currently focused on yeast functional genomics and proteomics to better understand gene function and the functional relationship between genes, proteins, pathways and complexes in yeast (Tong *et al.*, 2004). Recent genome-wide yeast analyses continue to provide important information about how a cell works as a system. This information can lead to our better understanding of complex diseases such as cancer and diabetes (Boone *et al.*, 2007).

Yeast genes share significant sequence identities with 30%-40% of the human disease-associated genes, making yeast an important model organism to study human diseases. These conserved genes are generally associated with a number of essential cellular mechanisms, such

as DNA replication, recombination and repair, RNA transcription, and translation (Mager and Winderickx, 2005).

Moreover, genome-wide analysis identified 466 genes whose deletion impaired mt respiration. Many of these genes were linked to human mt disorders making yeast an ideal organism to study mt-associated human diseases (Schwimmer *et al.*, 2006). An example of this is the use of yeast in studying PD disease.

Numerous studies have further substantiated the usefulness of yeast to study human mt diseases (Steinmetz *et al.*, 2002). For example, in mammalian cells, complex I is the largest complex within the respiratory chain and consists of at least 45 subunits. In bacteria, fungi and plant, complex I consist of one subunit only (Carroll *et al.*, 2006). In mt of yeast *S cerevisiae* there are 2 types of NADH dehydrogenases (Ndi1): internal and external. The internal complex (Ndi) is facing the matrix and catalyzes NADH oxidation in the matrix, and the external enzyme (Nde) is facing the inter membrane space and oxidizes NADH in the cytoplasmic space. Marella *et al* recently proposed that yeast Ndi1 can restore NADH oxidation in impaired mammalian complex I. They were able to show perfect functional integration of yeast Ndi1 protein into the mammalian respiratory chain which is highlighted by the repair of ATP production, without any side effects. As a result, they were able to reduce PD symptoms progress in patients (Marella *et al.*, 2009).

1.2.1 Translation and Mitochondria

Gene expression is the process by which information from a gene is converted into functional structures, or proteins, in the cell. This means that by controlling gene expression, cellular functions can be controlled (Berg, 2002).

Translation is part of the overall process of gene expression and is the process by which genetic information is converted from messenger RNA (mRNA) to proteins. This occurs in three steps: initiation, elongation and termination (Bauerschmitt *et al.*, 2008). In eukaryotes, a scanning model for translation initiation states that the small (40S) subunit of eukaryotic ribosome first binds to the mRNA at its capped 5'-end, and then scans the mRNA until it reaches the first start codon AUG, which triggers the initiation of translation (Kozak, 2005). Next is the elongation phase where the mRNA moves along the ribosome, reading one codon at a time. Translation ends when the ribosome reaches one of the three stop codons UAA, UAG, or UGA (Lodish *et al.*, 2000).

In prokaryotes, mRNAs are not capped at the 5' end. Instead, they show sequence conservation from positions -20 through +13 (positions are relative to the start codon at +1). The small (30S) subunit of prokaryotic ribosomes binds to the mRNA at a specific site, referred to as Shine-Dalgarno sequence. This sequence is usually 6-9 base pairs (bp) long, including a consensus sequence of 6bp (5'-AGGAGG-3') (Gualerzi and Pon, 1990). It is generally located 8-9bp upstream of the start codon. In *Escherichia coli*, the Shine-Dalgarno sequence is 7bp long (5'-AGGAGGU-3'). This sequence is complementary to the anti-Shine-Dalgarno sequence found at the 3' end of the 16S rRNA in the ribosome. After the binding of the ribosome to the mRNA, protein synthesis is initiated (Mori *et al.*, 2007). The tRNAs arrive at the 2 sites on the ribosome: the A and P sites. The A site is the entry point for the aminoacyl tRNA (except for the

first aminoacyl tRNA, fMet-tRNA$_f^{Met}$, which enters at the P site). The P site is the biding place for the peptidyl-tRNA carrying the growing polypeptide chain. Each new amino acid is added by the transfer of the growing chain to the new aminoacyl-tRNA (from P site to A site), forming a new peptide bond. The deacylated tRNA is then released from the P site and the ribosome moves one codon farther along the mRNA, transferring the new peptidyl-tRNA to the P site and leaving the A site vacant for the next incoming aminoacyl-tRNA (Buchan *et al.*, 2006). When the ribosome reaches one of the three stop codons UAA, UAG, or UGA, release factors recognize the stop codons and thus bind to the A site. The polypeptide chain is then released from the P site and the ribosomes separate into 2 subunits in a reaction driven by the hydrolysis of a GTP molecule (Sund *et al.*, 2010).

Mt has its own translation machinery. However, only ~1% of mt proteins are encoded inside mt. The rest of the proteins are imported into the mt by a complex protein import pathway. For this, mt proteins are generated as precursors carrying specific targeting signals that direct them towards mt (Becker *et al.*, 2008). While mitoribosomes and general translation factors are similar to their prokaryotic counterparts, many features of mt translation appear to be different from that in either prokaryotic cells or the cytoplasm of eukaryotic cells.

In mt, translation initiation is also based on a sequence signal upstream of the start codon AUG on the mt mRNA, much like Shine-Dalgarno sequence in prokaryotes. However, this signal which is also found on the 5' untranslated region (UTR) of the mRNA, does not seem to have complementarity to the mitoribosome (Folley and Fox, 1991). In addition, translational activators encoded by nuclear genes seem to be required to stimulate the translation of specific mt mRNAs (Mittelmeier and Dieckmann, 1995).

1.3 Mitochondria-Associated Gene Therapy

Due to its importance in the development and progress of a number of diseases, mt genome has become an important candidate for gene therapy. In general, this technique aims to correct defective disease-causing genes by genetically engineering and introducing the corrected gene into human cells to produce functional proteins (Koene and Smeitink, 2009). There are several generic approaches to gene therapy, including: gene transfer, repair, and knock-out (Collombet and Coutelle, 1998).

Gene transfer involves the replacement of the mutated gene with a healthy copy of the gene. This is done by identifying and isolating the gene of interest, then sending the "normal" gene into the cell to replace the defective one via a carrier, often called a vector. These vectors are either viral or non-viral. Part of the challenge in gene therapy is choosing the most suitable vector for treating a certain disease (Suter *et al.*, 2006). Generally, when the functional gene is inserted into the cell, it produces a protein which then, in case of mt gene therapy, gets sent to the mt. This protein enters the mt via special import machinery located on the outer mt membrane (Laurence *et al.*, 2009).

In gene repair, the abnormal gene can either be swapped for a normal gene through homologous recombination or it can be repaired through selective reverse mutation, which returns the gene to its normal function. This method uses single-stranded DNA oligonucleotides (ssONs) that are complementary to the wild-type sequence. They are introduced to the cell to anneal to the mutated target sequence and produce a mismatch. They proceed to fix the mismatched sequence using DNA damage repair pathways (McLachlan *et al.*, 2009).

Gene knock-out refers to the inactivation of the mutated gene. This is possible by replacing the mutated gene with a non-active gene. In this case, endogenous homologous recombination is often used for the swapping of the genes (Dragatsis and Zeitlin, 2001).

There are many challenges with using gene therapy to treat mt-associated disorders. Some of these challenges include: regulation of introduced genes, application of introduced genes to cells which no longer enter the mitotic phase, and carcinogenic potential of such procedures (Kagawa *et al.*, 2001). Vector size restrictions, host immune response to the introduced gene, incomplete gene inactivation, and a possible interference of the introduced gene with neighboring gene expression are other complications that contribute to challenges of gene therapy (Mueller and Flotte, 2008). Direct introduction of mtDNA is possible; however, to date, it has not been successfully implemented yet. The natural import mechanisms for nucleic acids are only known for a few RNA molecules, including yeast tRNA which has been successfully introduced into *S cerevisiae* mt (DiMauro and Mancuso, 2007). These methods are primarily aimed at overcoming the barriers of the outer and inner membranes of the mt.

1.3.1 Study Plan

Background

In this study, a possible import of mRNAs into *S. cerevisiae* mt is explored using the principles of mt-vicinity localization and mt translation.

In addition to carrying protein coding sequences, mRNAs also bear other information that can affect expression, stability and localization of mRNA. For example, their UTRs hold critical information about regulatory signals used in translation. Also there is evidence that UTRs often carry signals that are needed for subcellular localization of mRNAs (Sylvestre *et al.*, 2003b).

A mRNA sorting to the vicinity of mt seems to be crucial for the function of this organelle. It has been previously shown that the 3'-UTR of yeast *OXA1* gene is required for the localization of this mRNA to the vicinity of mt. Yeast *OXA1* is a nuclear gene whose sequence is conserved from prokaryotes to eukaryotes. It codes for Oxa1p, a cytochrome *c* oxidase assembly protein (Bonnefoy *et al.*, 1994). Using a genome-wide approach, it was discovered that mRNAs containing a certain sequence in their 3'-UTRs appear to be localized near mt. However, due to technical limitations associated with such studies; it is still not clear whether the mRNA enters the mt or it simply attaches to the outside of mt membrane (Marc *et al.*, 2002).

Subcellular RNA localization is used by eukaryotic cells to achieve high local concentrations of proteins. It is also required for efficient import of proteins to the nucleus and organelles in somatic cells. Most of these proteins are targeted to their destination on the basis of signals in their peptide sequences. These localization signals are termed the "postal codes" of mRNAs as they are needed to ensure successful delivery of mRNAs to their destinations. Such "postal codes" are found in the 3'-UTR of a number of genes (Jansen, 2001).

Since more than 98% of mt proteins are encoded by the nucleus one may expect that mt sorting may be needed for some of these mRNA to ensure proper function inside mt. Generally, localizing a mRNA to its site of action is more efficient than protein localization, since one mRNA can be a template for multiple rounds of translation (Fünfschilling and Rospert, 1999).

It has recently been shown that human *OXA1* mRNA can be sorted to the vicinity of yeast mt and functionally replace yeast *OXA1*. In addition, it was shown that this sorting was mediated by yeast proteins that recognize the human 3'-UTR of *OXA1* mRNA. It was therefore concluded that the recognition mechanism of *OXA1* 3'-UTR was conserved throughout evolution and that it was needed for the activity of Oxa1p function (Saint-Georges *et al.*, 2001).

In a separate study, introduction of mutations in the 3'-UTR (158bp) of yeast *OXA1* gene showed a complete respiratory deficiency in yeast mt. This was explained by the inability of *OXA1* to be sorted near mt, providing further evidence for the activity of *OXA1* mRNA 3'-UTR in sorting this mRNA near mt (Margeot *et al.*, 2002).

Ribosomes, also referred to as polysomes when translating mRNAs, are generally found in the cytoplasm but they can also be localized to the surface of mt. It is thought that mt-bound polysomes may contribute to mt import of the proteins made by these polysomes (Margeot *et al.*, 2005). These ribosomes are expected to initiate translation like most other eukaryotic ribosomes by binding at the 5'-CAP structure of mRNAs and scanning the mRNA for the first available start codon. However, mitoribosomes are thought to resemble prokaryotic ribosomes and recognize a common feature to all yeast mt mRNA 5'-UTR, which is a highly conserved octanucleotide sequence 5'-UAUAAAUA-3' and hence initiate translation (Green-Willms *et al.*, 1998). This consensus sequence represents a conserved ribosome binding site for mt mRNAs similar to the Shine-Dalgarno sequence in prokaryotic mRNAs.

S cerevisiae mt mRNA 5'-UTRs are uncapped and contain AUG start codon sequences upstream of the initiator AUG. They also generally contain the conserved consensus sequence as well as a long Adenine/Uracil (A/U)-rich tail (Dunstan *et al.*, 1997).

For example, the 5'-UTR (54bp) of yeast mt *COX2* gene was found to be essential for the translation of this gene inside the mt. *COX2* codes for cytochrome *c* oxidase subunit II, the terminal member of mt inner membrane ETC. It is expressed by a nuclearly encoded translational activator protein, Pet111p, which interacts with *COX2* 5'-UTR to promote translation. These translation activators tend to be gene-specific (Bonnefoy *et al.*, 2001).

When *COX2* ribosome binding site was mutated, the translation activity of this mRNA in mt was eliminated. This finding provided further evidence that mitoribosomes recognize a common feature of the 5'-UTR that is needed for successful recognition of mRNA and translation. Unlike Shine-Dalgarno sequence, this unique octanucleotide sequence does not seem to occur at a fixed distance from the start codon of mRNAs. Also, there is no complement for the sequence at the 3' end of 16S rRNA (Green-Willms *et al.*, 1998).

Objective and Study Design

The objective of this thesis is to investigate our ability to direct a designed mRNA into *S cerevisiae* mt. This would be done using the principles of mt mRNA localization and translation machinery in mt. If successful our long term goal would be to follow the same principles to direct therapeutic mRNAs into human mt.

To this end mRNAs containing reporter ß-galactosidase (ß-gal) genes are designed with or without 3'- and 5'-UTR that may direct mRNAs into mt and mediate translation using mitoribosomes. These mRNAs contain the 3'-UTR of yeast *OXA1* gene to direct the reporter ß-gal mRNA into mt. A 5'-UTR derived from yeast *COX2* gene that contain mitoribosomal binding site is used to examine the ability of mitoribosomes to translate the reporter genes that are imported into mt. Several measures, such as the presence of a translation inhibitory structure, are taken to reduce the affinity of cytoplasmic translation machinery to the designed mRNA constructs.

Chapter 2: Materials and Methods

CHAPTER 2: MATERIALS AND METHODS

2.1 Media Preparation

Standard sterilization techniques were used in all experiments. All media were stored at 4°C.

2.1.1 Luria-Bertani (LB)

To make 1L of LB media: 10.0g of Bacto-Trypton (Peptone), 5.0g of Yeast Extract, and 10.0g of Sodium chloride (NaCl) were dissolved in 1000ml distilled water (dH_2O) and the pH was adjusted to ~7.5. The mixture was autoclaved, allowed to cool down, then stored at 4°C. To make 1% LB agar plates; the same ingredients were used as well as 10.0g of agar, which was autoclaved separately then mixed and poured in plates.

2.1.2 Yeast Extract Peptone Dextrose (YPD)

To make 1L of YPD media; 2 flasks were used: in flask A, 20.0g Peptone and 10.0g Yeast Extract were dissolved in 900ml dH_2O. In flask B, 20.0g of Glucose were dissolved in 100ml dH_2O. The flasks were autoclaved separately then mixed.

2.1.3 Media lacking Uracil (-URA)

To make 1L of –URA agar plates; 3 flasks were used: in flask A, 6.74g of Yeast Nitrogen Base (YNB) (containing Ammonium sulfate) and 2.0g of –URA Drop out powder were dissolved in 200ml dH_2O. In flask B, 20g of Glucose were dissolved in 100ml dH_2O. Finally, in flask C, 20.0g of agar were dissolved in 700ml dH_2O. Next, the 3 flasks were autoclaved separately, and then mixed before they were poured, and stored at 4^0C. To make –URA liquid media; the same ingredients were used without the agar component.

2.1.4 Ampicillin

Stock Amp with a concentration of 50.0mg/ml was prepared and stored at -20°C. The antibiotic was added to the media to a final concentration of 50.0µg/ml. Therefore, for every 1.00ml of tempered media, 1.00µl of Amp was added.

2.1.5 Freezer Stocks

All freezer stocks were prepared as 2:1 ratio of cells to 60% glycerol and were stored in -80°C.

2.1.6 Strains and Plasmids

Strains used throughout the study are listed in Table 2.1.

The expression vector used was p416 GALL (Figure 2.1). It was ~5.5kb in size and it contained galactokinase promoter (GALL-pro), and *lacZ* reporter gene that was cloned as an *XbaI/BamHI* cassette downstream of the promoters. The plasmid also contained Uracil (URA3) and Ampicillin resistance (Amp) genes for selective growth of yeast and *E coli*, respectively in minimal media (Mumberg *et al.*, 1994).

Table 2.1: Strains and plasmids used in this study.

Organism / Plasmid	Strain / Plasmid	Reference
Saccharomyces cerevisiae	BY4741, *MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 yku70*Δ mutants	Alamgir *et al* (2010)
Saccharomyces cerevisiae	DAN1, *MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 yku70*Δ mutants	Alamgir *et al* (2010)
Escherichia coli	DH5α, F^- *fhuA2Δ(lacZ-argF)U169 Φ 80dlacZΔM15 endA1 hsdR17 deoR nupG thi-1 supE44 gyrA96 relA1 recAI phoA* λ^-	Bryant *et al.* (2009)

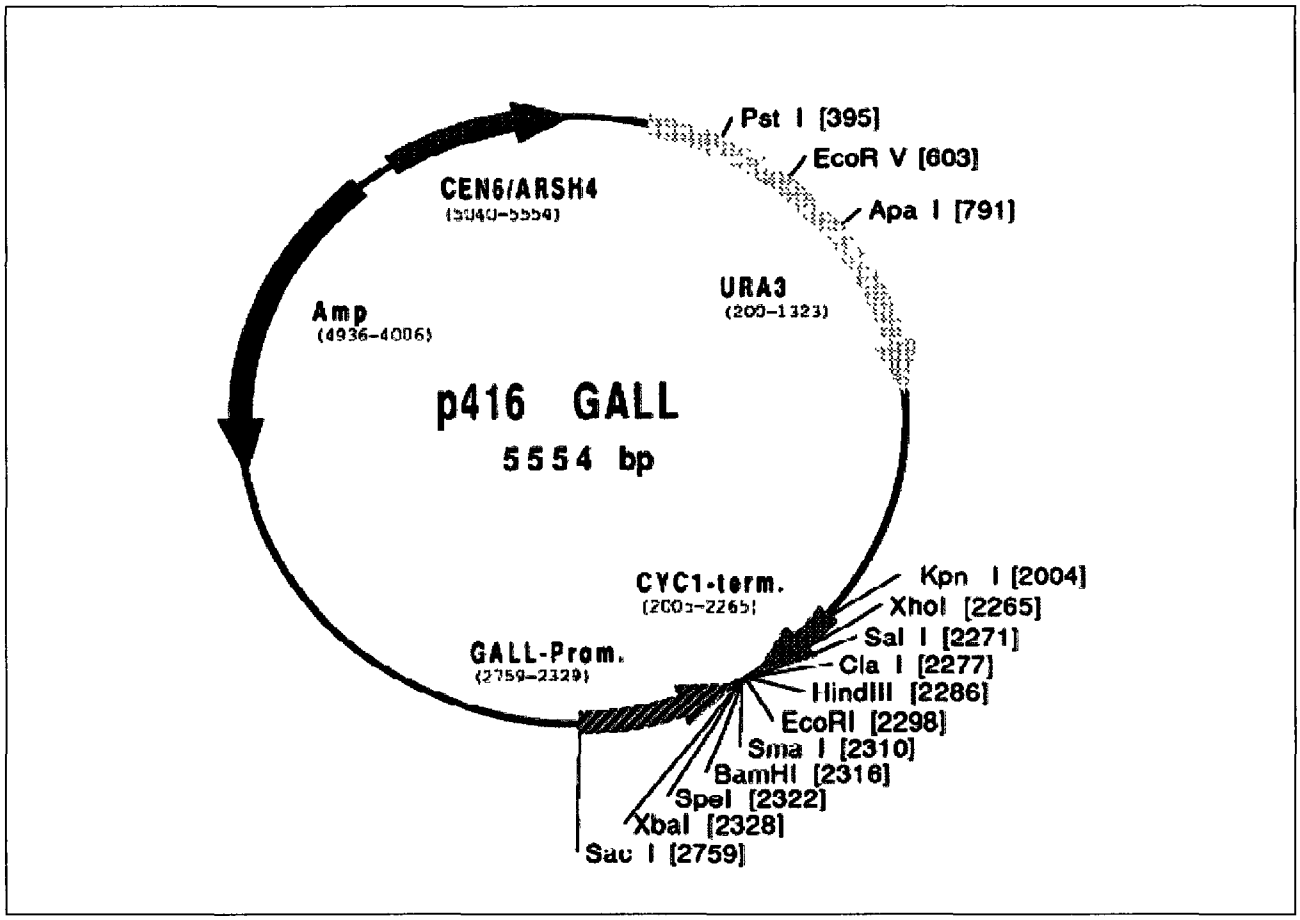

Figure 2.1: Plasmid map of p416 GALL showing its Uracil (URA3) and Ampicillin resistance (Amp) genes, which enable transformed cells to grow in a media lacking Uracil (-URA) and containing Amp, for yeast and *E. coli* selection, respectively. The map also shows the location of the GALL promoter (GALL-Prom.) and different restriction enzyme sites (modified from Mumberg *et al.*, 1994).

2.2 Molecular Techniques

2.2.1 Chromosome extraction

Standard methodology was used to extract chromosomes from yeast *S cerevisiae* BY4741 (Sambrook *et al.*, 2000). All yeast strains used were from this background (Table 2.1). The culture was grown in 5.00ml Luria-Bertani (LB) media overnight, in a 30°C / 200 RPM incubator shaker. Chromosome extraction included three basic steps: cell lysis, DNA clean up, and DNA isolation. The cells were lysed by vortexing with glass beads and STET lysis buffer. For every 1.00ml cell culture, 2.00g glass beads were added. Residues bound to DNA were removed by precipitation with Ammonium acetate ($C_2H_3O_2NH_4$). Finally, DNA was precipitated in ice cold ethanol (EtOH), since it is insoluble in alcohol.

2.2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was performed following standard methodology. The three steps of PCR include: denaturing the double stranded DNA, annealing the primers to DNA templates, and extending the newly synthesized strand to form a semi conservative double stranded DNA. Denaturation step was set up at a very high temperature to break the bonds holding the nitrogen bases in DNA. This was performed at the temperature of 95°C for 1 min. Annealing step was set up at a low temperature to allow the primers to attach themselves to single stranded DNA templates (2 minutes at 52°C). Finally, elongation step was set up at a medium temperature to allow the thermostable DNA enzyme to copy the DNA strands in the 5'→3' direction (4 minutes at 72°C). This enzyme catalyzes the polymerization of deoxyribonucleotides into a DNA strand, leaving amplified DNA products with Adenine (A) overhangs at their 3' ends. The three step reaction was repeated 30 times. Each PCR cycle doubles the amount of DNA exponentially.

In these experiments, isolated yeast DNA was used as a template and *Taq* DNA polymerase was used as a catalytic enzyme. A combination of three short DNA fragments (primers) were used (Table 2.2); a 35 base pair (bp) forward and reverse primers (3'-YOXA1UTR-F and 3'-YOXA1UTR-R, respectively), which were complementary to yeast *OXA1* 3'-UTR, and a 21bp GALL-Pro primer.

PCR product was purified via QIAquick PCR purification kit, using the manufacturer specifications (QIAquick Spin Handbook, 2008). With the exception that 40µl of Milli-Q water (MQ H_2O) was added to the centre of the QIAquick column to elute DNA.

Table 2.2: Sequences for 3' primers used in PCR.

Oligonucleotide name	Sequence (5'→3)
3'-YOXA1UTR-F	CGC**GGATCC**ATTAATAACAAAAAATGAATAAAGGC
3'-YOXA1UTR-R	CGC**GGATCC**TCCAAATGATTATTTCAAGCAATAAA
GALL-pro	ATTAACCCTCACTAAAGGGAA

Legend:

Underlined sequence = *BamHI* restriction sites used for cloning

2.2.3 Phenol-Chloroform Extraction of Mt RNA

All solutions were prepared with water treated with Diethyl pyrocabonate (DEPC). To do this, DEPC was added to MQH_2O at a ratio of 1:1000, and the mixture was left at room temperature overnight to allow DEPC to destroy the RNase. The next day, the solution was autoclaved. In a separate tube, 1ml of DEPC was mixed with 9ml EtOH and was added to the DEPC-treated water (final concentration 0.1%). The solution was mixed well and was left at room temperature overnight before it was autoclaved again.

Isolated mt (100µl) were mixed with 200µl RNA extraction buffer (4M Guanidine thiocyanate, 25mM Sodium citrate (pH 7.0), 0.5% Sarcosyl, and 0.1M β-mEtOH, with β-mEtOH added immediately before use), and heated at 65°C for 1 min. Next, 30µl 2M sodium acetate (NaOAc) was added and mixed. Equal volume (330µl) of Phenol:Chloroform:Isoamyl alcohol mix (25:24:1) was added to extract crude RNA. Contents of the solution were separated into 3 phases by centrifugation at 12,000g (~ 14000 RPM) for 15 min at 4°C. The aqueous phase containing total RNA, which under acidic conditions is usually the top layer, was transferred to a fresh tube. This aqueous phase was then extracted two times with one volume of chloroform (330µl). Phenol-chloroform extraction was repeated to completely remove DNA and protein contaminations from the interphase and phenol or organic phase, which are usually found in the bottom layer. RNA was precipitated by adding an equal volume (~330µl) of 100% isopropanol. The tube was inverted a few times to mix the solution and then it was incubated at -20°C overnight. Mt RNA was pelleted by centrifugation at maximum speed for 7 min at 4°C. The supernatant was discarded and the pellet was washed briefly with 1ml of cold 70% EtOH by vortexing and then centrifuging at maximum speed for 2 min at 4°C. EtOH was gently removed with a fine pipette tip. After that, the pellet was allowed to air dry by inverting the tube on a

paper towel in the incubator at 37°C for 1-5 min. Finally, mt RNA was resuspended in 50µl DEPC MQH_2O then stored at -80°C.

2.2.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR reaction consists of 2 steps: complementary DNA (cDNA) synthesis, and real time PCR. In cDNA synthesis, mt RNA samples (15µl) were mixed with the reagents from BioRad RT-PCR kit, according to the manufacturer's specification (4µl 5x iScript Reaction Mix, 1µl iScript Reverse Transcriptase, and 15µl Nuclease free water). The mixture was incubated first at 25°C for 5 min., and at 42°C for 30 min., following a third incubation at 85°C for 5 min., and finally briefly at 4°C. In real time PCR phase, 5µl of cDNA was added to a mixture of BioRad reagents (10µl IQ SYBR green supermix, 6µl MQH_2O, 2µl Forward primer 1 (MA-*LacZ* F 5'-TTGAAAATGGTCTGCTGCTG-3'), and 2µl Reverse primer 2 (MA-LacZ R 5'-TATTGGCTTCATCCACCACA-3'). The reaction mixture (25µl) was added to a flat-top PCR tube and placed in a RT-PCR cycler (ROTOR GENE RG-3000 from Corbett research). The reaction ran for 44 cycles as follows: 30 sec. hold at 95°C, then 30 sec. hold at 55°C, followed by 20 sec. hold at 72°C. Resulting data were analyzed using Rotor-Gene Real-Time Analysis Software 6.0.14, and RNA concentrations were determined using NanoDrop Nd-100 Full-spectrum UV/Vis Spectrophotometer.

2.2.5 Agarose gel electrophoresis

In brief, in a 200ml flask: 0.40g agarose was diluted in 40.0ml 1X TAE buffer solution and was heated for 1min. to melt the agarose particles. The mixture was left to cool down to ~50°C before adding 2µl of 10mg/ml Ethidium bromide (EtBr). EtBr is used to visualize DNA or RNA bands. It fluoresces under UV light when intercalated into DNA or RNA. The gel mixture was swirled and poured onto the tray immediately after. When the gel solidified at room temperature (RT), the comb was carefully removed and samples were loaded into the wells. Samples were mixed with a loading buffer before adding the mixture to the gel. Loading buffer contains Bromophenol Blue which gives the buffer its dark blue colour. The dye is easily visualized when loading samples to the wells as it co-sediments with the DNA. The gel ran for 45min. to1 hr, at a constant voltage of 80-95V and a current of 500mA. Volumes generally used in agarose gel electrophoresis are listed in Table 2.3.

Table 2.3: General volumes used in agarose gel electrophoresis.

DNA ladder		Sample	
100 bp / 1 kb DNA ladder	1 µL	Sample	5 µL
Loading buffer	1 µL	Loading buffer	1 µL
MQ H_2O	10 µL	MQ H_2O	6 µL
Total volume	**12 µL**	**Total volume**	**12 µL**

2.2.6 Western Blot

Western blot analysis was performed over a 2 day period and was composed of the following stages: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), blotting, blocking, washing, and visualizing.

2.2.6.1 SDS-PAGE

Standard procedure was used to run an 8% SDS-PAGE, which is the recommended concentration for a large protein size. SDS-PAGE is composed of 2 gels: resolving and stacking gels. The resolving gel is made first. In a 50ml Falcon Tube ingredients in Table 2.4 were mixed. Protogel and Protogel buffer are commercially made by National Diagnostics (Catalogue #EC-890 and EC-892, respectively). Protogel, its buffer, and MQ H_2O were added first and swirled to mix. Then 30% Ammonium per sulfate (APS) (0.30g APS in 1ml MQ H_2O) and commercially prepared Tetramethylethylenediamine (TEMED) (Bioshop Canada (Cat. #TEM001.25)) were added. The mixture was quickly swirled and poured into the apparatus until it reached the marked line. A 1.5mm Bio-Rad mini gel apparatus with same size combs and glass slides were used in all experiments. Together, APS and TEMED catalyze the polymerization of acrylamide in SDS-PAGE.

While waiting for the resolving gel to solidify, the stacking gel was prepared. In a 50ml Falcon Tube ingredients in Table 2.5 were mixed in the same manner listed above. Protogel stacking buffer was commercially made by National Diagnostics (Cat. #EC-893). APS and TEMED were added only when the gel was ready to be poured.

Table 2.4: Recipe for making an 8% resolving gel for SDS-PAGE with ingredients listed in order of addition.

Ingredients	8% (µl)
Protogel	2136
Protogel buffer	2080
MQ H_2O	3696
30% APS	28
TEMED	8

Table 2.5: Recipe for making a stacking gel for SDS-PAGE with ingredients listed in order of addition.

Ingredients	4% (µl)
Protogel	520
Protogel stacking buffer	960
MQ H_2O	2440
30% APS	8
TEMED	4

Once the resolving gel solidified the apparatus was tipped over to drain remaining liquids. The stacking gel was then poured on top of the resolving gel and the comb was inserted immediately. While waiting for the gel to solidify, the rest of the apparatus was assembled and samples were prepared according to the following guideline. Equal amount of loading buffer dye was mixed with protein samples. Dye mix is composed of 95% Laemmli Sample Buffer (2% SDS, 25% Glycerol, 0.01% Bromophenol Blue, and 62.5mM Tris-HCL, pH 6.8) and 5% β-Mercaptoethanol (β-mEtOH), purchased from Bioshop Canada (Cat.# MER002.100). β-mEtOH is used to denature proteins by reducing the intra and inter-molecular disulfide bonds of the proteins to allow proper protein separation by size. After protein samples were combined with the dye mix, they were heated at 95°C for 3-5min. then centrifuged at max. speed (15,000RPM) for 5sec.

By this time the stacking gel solidified and the apparatus was ready to use. The comb was slowly removed, and the gel cassette was inserted in the tank, which was then filled with 1X resolving gel buffer (10X Resolving gel buffer: 250mM Tris-Base, MQ H_2O, 2500mM Glycerol/Glycine, and 1% SDS– pH 8.3 adjusted with 10M HCl). Samples were then loaded into the gel. For 1.5mm spacers the lane capacity is 30µl-40µl. Also, 5µl protein ladder (Precision Plus Protein Kaleidoscope Standards from BioRad, Cat. # 161-0375) was loaded directly into gel, without being heated. The gel ran at RT with a voltage of 140V and a current of 137mA for 1 hr.

2.2.6.2 Blotting

After running the gel, the following items were soaked in transfer buffer (20mM Tris-HCl, 150mM Glycine, and 20% Methanol - pH 8.0): 2 transfer sponges, 2 blotting papers, and 1 nitrocellulose membrane cut to the shape and size of the gel. At all time, the nitrocellulose membrane was handled with a clean forceps from one edge only. The "blotting sandwich" was prepared by placing a previously soaked sponge on the black side of the sandwich apparatus. Then a blotting paper was placed over the sponge, followed by the gel. After that, the previously soaked nitrocellulose membrane was placed on top of the gel and another blotting paper was placed over the membrane, followed by another sponge. Air bubbles were pressed out with gloved fingers and the sandwich apparatus was clamped and placed in the correct orientation inside the transfer tank. A stirrer bar and an ice block were also inserted into the tank, which was filled with transfer buffer. Protein samples were allowed to transfer from the gel to the nitrocellulose membrane overnight in the cold room at a constant current of 180mA.

2.2.6.3 Blocking and Washings

The next day, block solution (5% w/v) was prepared (2.5g skim powder milk and 50ml TBST detergent buffer (20mM Tris-HCl, 137mM NaCl, and 0.1% Tween 20 - pH 7.6) in a 50ml Falcon tube, and kept on ice. Primary (1'AB) and secondary (2'AB) antibodies were prepared by mixing the manufacturer's recommended concentrations of antibody to block solution.

The nitrocellulose membrane, now containing the protein samples, is incubated with block solution and gentle shaking, with the protein side facing upwards, for 1 hr at RT.

Block solution was collected back to the falcon tube, after blocking, and stored in -20°C for re-use. The nitrocellulose membrane was incubated with 1'AB solution and gentle shaking for 1hr at RT. Afterwards, 1'AB solution was collected back into the tube and stored in -20°C

for re-use. The nitrocellulose membrane was washed with TBST buffer alone for 30 min. while adding new TBST buffer every 5 min. Subsequently, the nitrocellulose membrane was incubated with 2'AB solution and gentle shaking for 1hr. After that, 2'AB solution was collected back into the tube and stored in -20°C for re-use. The block solution, 1'AB, and 2'AB solutions can be re-used 3-10 times or stored in the freezer for 6 months. The nitrocellulose membrane was washed with TBST buffer as described above.

2.2.6.4 Visualization

After the washings, the nitrocellulose membrane was drained on a paper towel before it was placed on a piece of transparent sheet. Chemiluminescent substrate (Pierce Supersignal West Pico or West Femto, Cat. # PI34080 or PI095, respectively) for detecting Horseradish peroxidase (HRP) conjugated probes was mixed in a microtube (1.5ml) and pipetted over the whole nitrocellulose membrane surface, with the protein side facing upwards. The membrane was incubated for 5 min. After that, another piece of transparent sheet was placed on top to sandwich the membrane.

The membrane was then exposed to a photographic film for few seconds depending on the strength of protein signals. Protein bands should luminesce on the film. After, the film was placed in the developer (1:5) until the bands start to appear, then it was rinsed with tap water and placed in the fixer (1:10) for few seconds, and was rinsed with tap water.

A CCD camera (Alpha Innotech HD2) was also used to visualize the protein bands and capture a digital image of the western blot.

2.2.7 β-gal liquid assay

Transformed yeast cells were grown in 5ml –URA liquid media containing glucose overnight at 30°C / 200 RPM incubator shaker. The next day, they were pelleted at 4000 RPM for 5 min. and washed with 5ml dH_2O twice before they were diluted to OD_{600} ~0.25 in fresh –URA liquid media containing galactose. Cells were incubated at 30°C / 200 RPM until OD_{600} ~1.0. Next, the cells were washed and re-suspended in Z-buffer. Mt were isolated from the samples as described in the next section. The OD_{600} of suspension was recorded. Then 200µl of mt suspension were mixed with 800µl Z-buffer, 20µl of 0.1% SDS and 50µl of chloroform. The solution was vortexed for 15sec. before incubation at 30°C heat block for 15 min. After that, 200µl of ortho-Nitrophenyl-β-galactoside substrate (ONPG) (4mg/ml stock) was added and the solution was vortexed for 5sec. and incubated 30°C heat block. The mixtures were monitored for change of color. When the solution turned yellow the reaction was stopped by adding 500µl 1M Sodium carbonate (Na_2CO_3). Then the absorbance at 420nm and 550nm were measured relative to a blank of 1ml Z-buffer and 0.5ml Na_2CO_3. Finally, β-gal activity was calculated in Units as follows:

$$\text{Unit} = 1000*(OD_{420} - 1.75*OD_{550}) / (\text{Time}*\text{Volume}*OD_{488}).$$

Where,

U refers to units of β-gal activity
OD_{420} refers to the optical density of 0-nitrophenol and cell debris
OD_{550} refers to the optical density of cell debris
OD_{488} refers to mt density in the sample at the start of the assay
t refers to time of reaction in minutes
V refers to the volume of cell culture used in mille liters

2.2.8 Mitochondria Isolation

Two methods were used to extract mt from yeast *S cerevisiae*, the first was accomplished using a "Yeast Mt Isolation kit" (Sigma, MITOISO3), which was supplied with 8 reagents in concentrated form. Yeast mt was also isolated using a classical approach.

Aliquots of 20 OD_{600} of induced yeast samples were pelleted at 3500 RPM for 7 min. Cells were then washed in 5-6 volumes of water and pelleted again. Next, the pellets were resuspended in 2ml of 1x "Buffer A" and incubated or 15min. at 30°C / 200 RPM incubator shaker. After that, cells were pelleted at 3000 RPM for 7 min. and resuspended in 1ml of 1x "Buffer B". $OD_{600(1\ 10)}$ was measured and the total OD of cell suspension was calculated to be used as a reference value. Lyticase concentration was optimized by using 1.5 units of lyticase per OD of cell suspension. Cells were incubated with lyticase at 30°C / 200 RPM incubator shaker and the $OD_{600(1\ 10)}$ was measured every 5 min. to monitor for a 30-40% decrease in the absorbance from the initial reading. Once the desired absorbance was reached, the reaction was stopped by centrifuging the mixtures at 4500 RPM for 5 min. at 4°C. The resulting spheroplast pellets were kept on ice before they were resuspended in 1ml "Lysis Buffer" and incubated on ice for 5 min. Then, 2 volumes of 1x "Storage Buffer" were added to the cells before they were pelleted in a microcentrifuge at 3300 RPM for 10min. at 4°C. The supernatant liquid was then transferred into clean sterile microtubes and centrifuged in a microcentrifuge at 13,300 RPM for 10min. at 4°C. Finally, the pellets were resuspended in 250µl "Storage Buffer". This "crude mt" was stored in -20°C.

Standard methodology was used to manually isolate mt from yeast *S cerevisiae* (Meisinger *et al.*, 2006). Spheroplasts were formed by a combination of enzymatic digestion and differential centrifugation of the yeast cell wall, as described in (Meisinger *et al.*, 2006).

Spheroplasts were then further purified using free flow electrophoresis (FFE) sample preparation protocol (Zischka *et al.*, 2003). Spheroplasts were suspended in "STP Buffer" (0.6M Sorbitol, 10mM Tris-HCL pH~7.4, 1mM PMSF) to a concentration of 0.15g/ml. Cell membranes, on ice, were disrupted by vortexing the spheroplasts with acid washed / soda lime glass beads (~ same size of pellet) for 15min. instead of disrupting them with glass Teflon homogenizer. Cell debris was removed by centrifugation at 3750 RPM for 5 min. at 4°C, twice. Mt was sedimented from the supernatant at 12,200 RPM for 12min. at 4°C. Raw mt fraction was washed (1 volume pellet) with "SET Buffer" (0.25M Sucrose, 1mM EDTA, 10mM Tris-HCL pH ~7.4, 1mM PMSF). Supernatant was transferred to a new tube and mt were sedimented at 12,000 RPM for 12min. at 4°C. Finally, crude mt were resuspended in 500ml "Separation Buffer" and then stored at -20°C.

A sample of the isolated mt was purified by zone electrophoresis using BD Free Flow Electrophoresis (FFE) system, using the manufacturer's specifications (BD Diagnostics, 2007).

2.2.9 Restriction Digest

Digestion reactions were prepared and incubated at the temperature for the duration specified by the enzyme manufacturer. For most reactions, mixtures were incubated overnight at 37°C, and then stored at -20°C. An example of a restriction digest reaction is shown in Table 2.6.

Plasmids were purified before and after enzymatic digestion, using QIAquick plasmid purification kit, using the manufacturer's specifications (QIAquick Spin Handbook, 2008). With the exception that 40µl of MQ H_2O was added to elute the purified DNA. This step was repeated once more using 20µl of MQ H_2O.

Table 2.6: An example of a restriction digest using *BamHI* enzyme, listed in order of addition.

Ingredient	Volume (µl)
MQ H_2O	12.2
10X Nebuffer *BamHI*	2.00
100X BSA	0.20
BamHI	0.60
DNA	5.00
Total volume	**20.00**

2.2.10 Plasmid Extraction

Plasmids were extracted from transformed cells via QIAprep Miniprep kit, using the manufacturer specifications (QIAprep Miniprep Handbook, 2006). In sterile microtubes, 1.00ml of cells were added and centrifuged at max. speed (15,000 RPM) for 1min. The supernatant was discarded, and the process was repeated twice to quantify plasmids extracted. The last step (elution from column) was repeated twice, using 50µl of MQ H_2O. Isolated plasmids were stored in -20°C.

2.2.11 Ligation

Ligation trials were set up at the temperature and duration specified by the enzyme manufacturer. For most trials, mixtures were kept in the cold room at 16°C water bath overnight, and then stored at -20°C. An example of a ligation set up is shown in Table 2.7.

Table 2.7: An example of a ligation trial with ingredients listed in order of addition.

Ingredient	Experimental 1 (µl)	Experimental 2 (µl)	Control 1 (µl)	Control 2 (µl)
MQ H_2O	7.70	0.20	15.7	16.9
Ligase buffer	2.10	1.90	2.10	2.10
BamHI-digested PCR product	8.00	15.8	0.00	0.00
BamHI-digested p416	1.00	1.00	1.00	1.00
Ligase enzyme	1.20	1.10	1.20	0.00
Total volume	**20.00**	**20.00**	**20.00**	**20.00**

2.2.12 Making competent cells

E coli culture was grown in 5.00ml LB media overnight, in a 37°C / 200 RPM shaker incubator. The next day, a fresh subculture was prepared by adding 1.20ml of overnight cells into 50.0ml LB media. The fresh subculture was left in the 30°C shaker incubator for few hours (usually ~2hrs), until the optical density (OD_{600}) of 0.4-0.6 was reached. Then, 10.0ml of the fresh subculture were transferred into a falcon tube, and was centrifuged at 4°C for 7min., at 3000rpm. To the pellet, 1.00ml of ice cold 50mM Calcium chloride ($CaCl_2$) was added. The cells were mixed well before adding more of the ice cold $CaCl_2$ (6.00ml). The mixture was incubated on ice for 1hr before it was centrifuged at 4°C for 7min., at 3000rpm. To the pellet, 1.00ml of the ice cold $CaCl_2$ was added, and the cells were incubated overnight at 4°C.

2.2.13 *E. coli* Transformation

Overnight competent cells (150µl) were mixed with ligation mixture (10.0µl of each ligation tube), or target DNA (usually 10-50 ng), by gently swirling the tube. For positive control, 150µl competent cells were mixed with 1.00µl of an intact purified plasmid (10 ng). The tubes were incubated on ice for 1hr, and were heat-shocked in a water bath at 42°C, for 90sec. To each tube, 1.00ml of LB media was added, before they were incubated at 37°C for 1-1.5hrs. The cells were then centrifuged at max. speed for 1 min. To each pellet, 50.0µl of LB media was added, and the cells were mixed by gently swirling the tube. The contents were plated on LB agar plate containing 50µg/ml Amp. All plates were incubated at 37°C overnight. Transformed cells were screened for by enzymatic digestion followed by fractionation using agarose gel electrophoresis.

2.2.14 Yeast transformation

Yeast were grown in 5.00ml YPD media overnight, in a 30°C / 200 RPM shaker incubator. The overnight culture was diluted to OD_{600} ~0.2-0.4 and was incubated until it reached OD_{600} ~1.0. The following was carried out in RT. Cells were pelleted at 3000 RPM for 2 min. and washed with 10ml MQH_2O. They were pelleted again and resuspended in 1ml 0.1M Lithium acetate (LiOAc). Cells were pelleted a third time and resuspended in 200-250µl LiOAc. For each transformation, 50µl of cell suspension were aliquoted to a sterile eppendorf tube (microtube) and the following components were added: 2-5µl of Rec. p416, 10µl salmon-sperm DNA (ssDNA), which was boiled for 3min. and incubated on ice for 5 min. before use, 240µl of 50% Polyethylene glycol (PEG), and 36µl of 1M LiOAc. The tubes were then incubated at 30°C for 1hr. Following incubation, 40µl of Dimethyl sulfoxide (DMSO) was added to each tube, before they were incubated again at 42°C for 15min. Cells were pelleted at 8000 RPM for 2 min., and resuspended in 110µl MQH_2O. Finally, cells were plated on –URA plates and incubated in 30°C for 2 days.

2.3 Construction of plasmids containing target mRNAs

DNA was isolated from yeast *S cerevisiae* and was used as a template in PCR to be amplified by the forward and reverse primers 3'-YOXA1UTR-F and 3'-YOXA1UTR-R, which contain sequences complementary to the target region in the template; the 3'-UTR of yeast *OXA*. The resulting PCR product was purified via QIAquick PCR purification kit. The Purified PCR product was then digested with *BamHI*, to create sticky ends, for 4hrs at 37°C, and then stored at -20°C.

Plasmid p416 was purified via QIAquick plasmid purification kit. The purified plasmid was digested with *BamHI* for 4hrs at 37°C, and then stored at -20°C. The completion of restriction digestion reaction with purified PCR product and plasmid was confirmed on an agarose gel.

The digested PCR product and the plasmid were purified for a second round using QIA purification kits as above. Purified PCR product and plasmid were then ligated overnight in the cold room at 16°C water bath, and then stored at -20°C.

Ligated plasmids were transformed into *E. coli* DH5α. The obtained *E coli* colonies were screened for recombinant plasmids. For this, plasmids were extracted from individual colonies and first digested with *BamHI* for 4hrs at 37°C. The digested plasmids were visualized on a 1% agarose gel. Those that appeared to have an insert were subjected to a second screening test by digesting with *ClaI* overnight at 37°C. Agarose gel (1%) electrophoresis was performed after the digestion to screen for a difference in band sizes. The recombinant plasmids carrying the inserted 3'-UTR sequence were stored at -20°C to be used in subsequent experiments.

The 5'-UTR primers were designed based on Kozak's hair pin theory (1989a). The structure was 111bp and consisted of the following; a complement of the XbaI overhang, a hairpin loop, an ATG start codon and the Kozak sequence, a stop codon, and the mt translation initiator gene; COX2 (Table 2.8).

Table 2.8: Sequence for single-stranded 5' primer.

Oligonucleotide	Sequence (5' → 3')
5'TI COX2-a	**CTAGCCAGCTTACGCCCGCCAAACAGGCGGGCGTAAG CTGACC GCGTAATAGTATTAACATATTATAAATAGA CAAAAGAGTCTAAAGGTTAAGATTTATTAAAATGC**
5'TI COX2-b	CTAGGCATTTTAATAAATCTTAACCTTTAGACTCTTTTGT CTATTTATAATATGTTAATACTATTACGCCATGGTCAGCT TACGCCCGCCTGTTTGGCGGGCGTAAGCTGG

Colour legend:

Purple = XbaI overhang

Blue = hairpin loop

Green highlights = Kozak sequence

Pink = stop codon

Yellow highlights = mt translation initiator

Red underline = translation initiation signal

Red = COX2 gene sequence

The 5'TI COX2-a primer was diluted with 150 µL of MQ H_2O, and 5'TI COX2-b was diluted with 162 µL of MQ H_2O to make a stock solution concentration of 1 µg/µL. Then, 40 µL of both primers where combined with 25 µL of T4 DNA ligation buffer. This mix was placed in a heat bath at 98°C for 3 minutes. Samples were then left to cool down slowly in a styrofoam box for 3 hours, while uncovering the lid every 30 min. to expedite the cooling process. After they cooled to ~30°C, they were heated again to 55°C and then allowed to cool down before they were stored at -20°C. This procedure was repeated to make a mutated version of this primer; 5'-cox2-109.

Recombinant p416 carrying the cloned 3'-UTR of yeast *OXA1* gene was then digested with *XbaI* for 5 hours at 37°C. Next, the digest was visualized with 1% agarose gel. Digested plasmids were purified via QIAquick plasmid purification kit. The purified plasmids were ligated with the double-stranded 5' primer overnight at 16°C water bath. After that, candidates were digested again with *XbaI* for 2hrs at 37°C in order to eliminate background plasmids (those without the insert). Plasmids form the obtained clones were subjected to a two separate restriction digestion reactions with *StyI* and *BglI* to further confirm the presence of inserts. Digested DNA fragments were then visualized on 1% agarose gel.

Finally, two purified clones were sent for sequencing at the Ottawa General Hospital. Consequently, Basic Local Alignment Search Tool (BLAST) was used to search for sequences of the cloned 3' and 5' inserts. This confirmed that our plasmid construction was a success.

To make a control construct carrying 5' -UTR only, the recombinant p416 above was digested with *BamHI* to cut the 3'-UTR insert out of the plasmid, and relegated to itself leaving the plasmid with 5' insert only.

Chapter 3: Results

CHAPTER 3: RESULTS

3.1 Study design

mRNA sorting to the vicinity of mt seems to be critical for the activity of this organelle. It has been previously shown that the 3'-UTR of yeast *OXA1* mRNA is necessary for the localization of this mRNA to the vicinity of mt. However, due to the limitation of the localization techniques used in such studies, it is not clear whether the mRNA enters the mt or simply attaches to the outside of the mt (Corral-Debrinski *et al.*, 2000). Translation in mt seems to be mediated by a conserved sequence. It has been reported that mt ribosomes recognize a common feature to all yeast mt mRNA 5'-UTR, which is a highly conserved octanucleotide sequence 5'-UAUAAAUA-3'. The 5'-UTR of yeast *COX2* mt mRNA was found to be essential for the translation of this gene inside the mt. When *COX2* was mutated, the translation activity in mt was eliminated (Mulero and Fox, 1993).

Here, we tested the ability of yeast *OXA1* 3'-UTR to target a mRNA to yeast mt. To do so, we designed a chimeric mRNA, *in vitro*, using *lacZ* gene. First, we placed yeast *COX2* 5'-UTR in front, as well as yeast *OXA1* 3'-UTR at the 3' end of a *lacZ* mRNA on an expression plasmid. Then, we transformed yeast cells with the designed construct and observed its expression.

3.1.1 Designing and Cloning 3'-UTR

Mt translation machinery theory was used to investigate the import of the customized mRNA into yeast mt. The first phase of this project was completed by cloning yeast *OXA1* 3'-UTR at the 3'-end of *lacZ* gene in p416 (Figure 3.1).

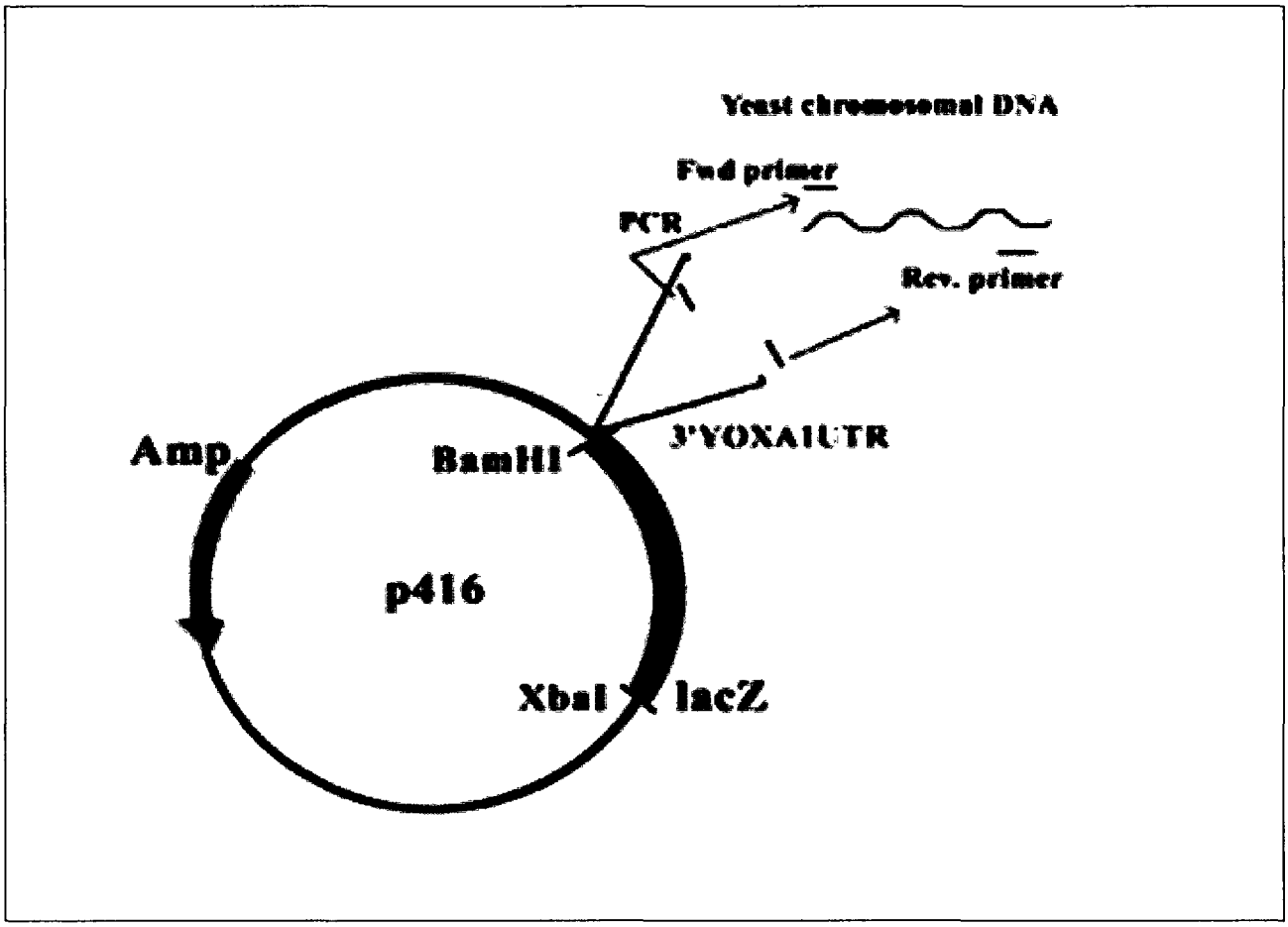

Figure 3.1: Constructed vector p416 containing an Ampicillin resistant gene, a *lacZ* reporter gene, and the inserted PCR product yeast *OXA1* 3'-UTR. The plasmid was ~5.5kb in size and also included a galactokinase promoter upstream of *lacZ* gene.

Ribosomes are non-membranous organelles that are either found suspended freely in the cytosol, or bound to the rough endoplasmic reticulum in eukaryotes. Polysomes, also referred to as ribopolysomes, are clusters of ribosomes bound to the surface of mt (Alberts *et al.*, 2002).

Previous studies showed that some mRNAs are localized to the vicinity of mt, and that ribopolysomes may contribute to the import of proteins into mt (Margeot *et al.*, 2005). It is believed that the 3'-UTR of certain mRNAs plays a role in directing the gene towards the mt. Yeast *OXA1* mRNA gene codes for cytochrome *c* oxidase assembly protein, and is found in prokaryotes and eukaryotes. A study that induced a mutation in the 3'-UTR (158bp) (Table 3.1) of yeast *OXA1* showed a complete respiratory deficiency in mt. It was suggested that *OXA1* did not get translated nearby the mt because the sequence that was thought to lead it there was altered (Sylvestre *et al.*, 2003a) and hence unable to localize the mRNA.

We hypothesized the 3'-UTR of *OXA1* may in fact lead the mRNAs inside mt. Therefore, we cloned the 3'-UTR of this gene into our plasmid p416. We amplified the target sequence using PCR and yeast total genomic DNA as a template. Figure 3.1 shows the result of the amplification of the target region. We introduced *BamH1* restriction site into the amplifying primers and used this sequence to digest the PCR product. We digested p416 using *BamH1* which cuts the plasmid at a unique site at the 3' end of *LacZ* gene. We then re-ligated the plasmid in the presence of 1000 fold excess digested insert. After transformation of *E coli* with the ligation mixture, we screened more than 25 clones for the presence of insert into p416 plasmid.

Table 3.1: Sequence of yeast *OXA1* 3'-UTR that was inserted into the plasmid.

	Sequence (5'→3)
OXA1	AAAAAATGAATAAAGGCTCTATATCTCTCTGTAAATATAAAAATAT AAAACTCAAACCCTCGATAGGCGGGACCAAATTTTTTCTCTCTCAG CAGTGGATTGTATACATTTACCACGAAAATTGTTTATTGCTTGAAAT AATCATTTGGATTCTTAAT

Figure 3.2 shows an image from the agarose gel of the digested constructs by *BamH1*. It clearly indicates that the PCR product was cloned into the expression vector (L3 and L5). The observed 10kb band corresponds to the size of the intact p416. In L4 no band was released suggesting that the plasmid did not contain an insert (background plasmid used as a control). The released lighter band that appears 158nt in size corresponds to an insert (red arrows in Figure 3.2).

To further confirm the presence of an insert into these vectors, potential recombinant plasmids were digested with *ClaI*. This enzyme has a restriction site on the vector p416 shortly downstream from the *BamHI* site, and on the *lacZ* itself. The presence of an insert would make one of the bands, released by *ClaI*, heavier than the one in the background (p416) (Figure 3.3: L2, L4). This was indeed observed in the gel (Figure 3.3: L3, L5). In Figure 3.3, L3 and L5 indicate the presence on an insert (see red arrows) into the vector (L2 and L4).

To confirm the presence of the insert and the successful cloning of the target DNA we sequenced the candidate plasmids. The sequencing results confirmed the presence of the insert in the correct orientation.

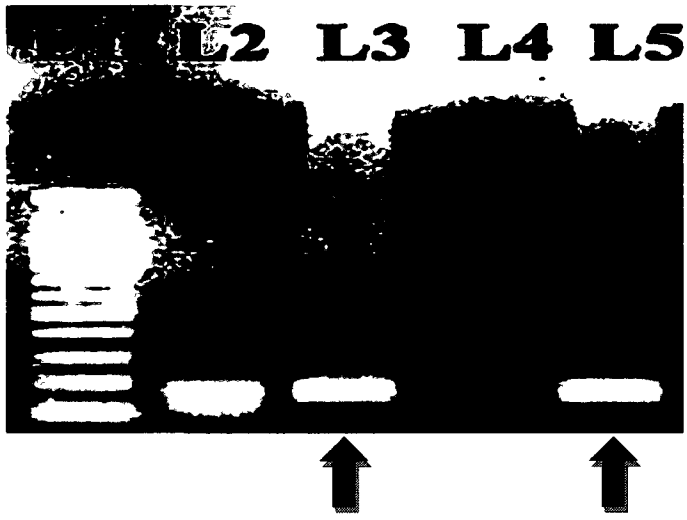

Figure 3.2: Agarose gel of purified recombinant p416 digested with *BamHI*, compared to a 100bp DNA ladder (L1), PCR product (L2), and purified intact p416 digested with *BamHI* (L4). The lighter bands represent the cloned inserts (L3, L5) (red arrows). There was no sample in (L4).

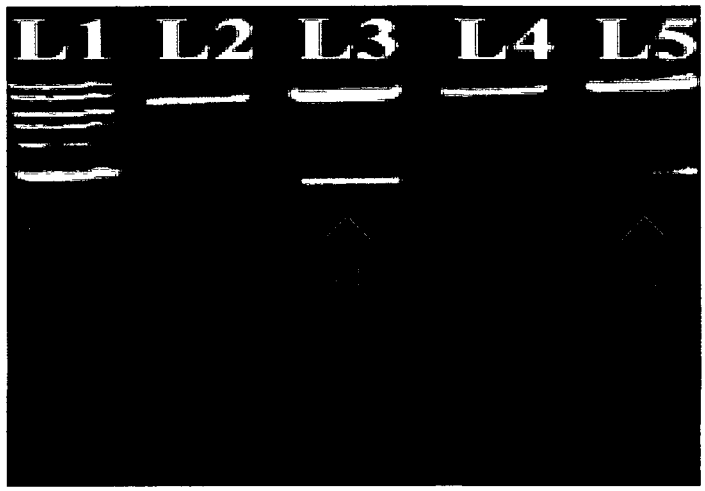

Figure 3.3: Agarose gel of purified recombinant p416 digested with *ClaI*, compared to a 1Kb DNA ladder (L1), and purified intact p416 digested with *ClaI* (L2, L4). The heavier released bands indicate the presence of an insert (L3, L5 – red arrows).

3.1.2 Designing and Cloning 5'-UTR

Three different constructs were designed as in Table 3.2 and Figure 3.4. In these constructs, a strong inhibitory hairpin structure is designed to prevent translation of the mRNA by cytoplasmic ribosome (Kozak, 1989a).

It has been shown that a strong stem-loop structure, located on the mRNA between its 5'-end and the first AUG, prevents the ribosome from scanning the mRNA. Instead, the ribosome ends up stalling at the 5'-end of the structure (Kozak, 1989a). Therefore, if our designed mRNA, which is targeted towards yeast mt, binds to cytoplasmic ribosomes, it should not be translated there. We believe that these ribosomes will not be able to scan the hairpin on the mRNA. The sequence of this hairpin (35bp) was chosen from a study by Vega Laso *et al.* (1993), who demonstrated the effectiveness of this hairpin at preventing the advancement of the 40S ribosomal subunit along the mRNA strand (Vega Laso *et al.*, 1993).

Table 3.2: Sequences used in the 3 designed constructs.

	Sequence (5'→3)
Hairpin	CAGCTTACGCCCGCCAAACAGGCGGGCGTAAGCTG
COX2 gene	AGTATTAACATATTATAAATAGACAAAAGAGTCTAAAGGTTAA GATTTATTAAA
cox2-107	AGTATTAA**agatc**T**ta**AAATAGACAAAAGAGTCTAAAGGTTAAGA TTTATTAAA
XbaI	TCTAGA

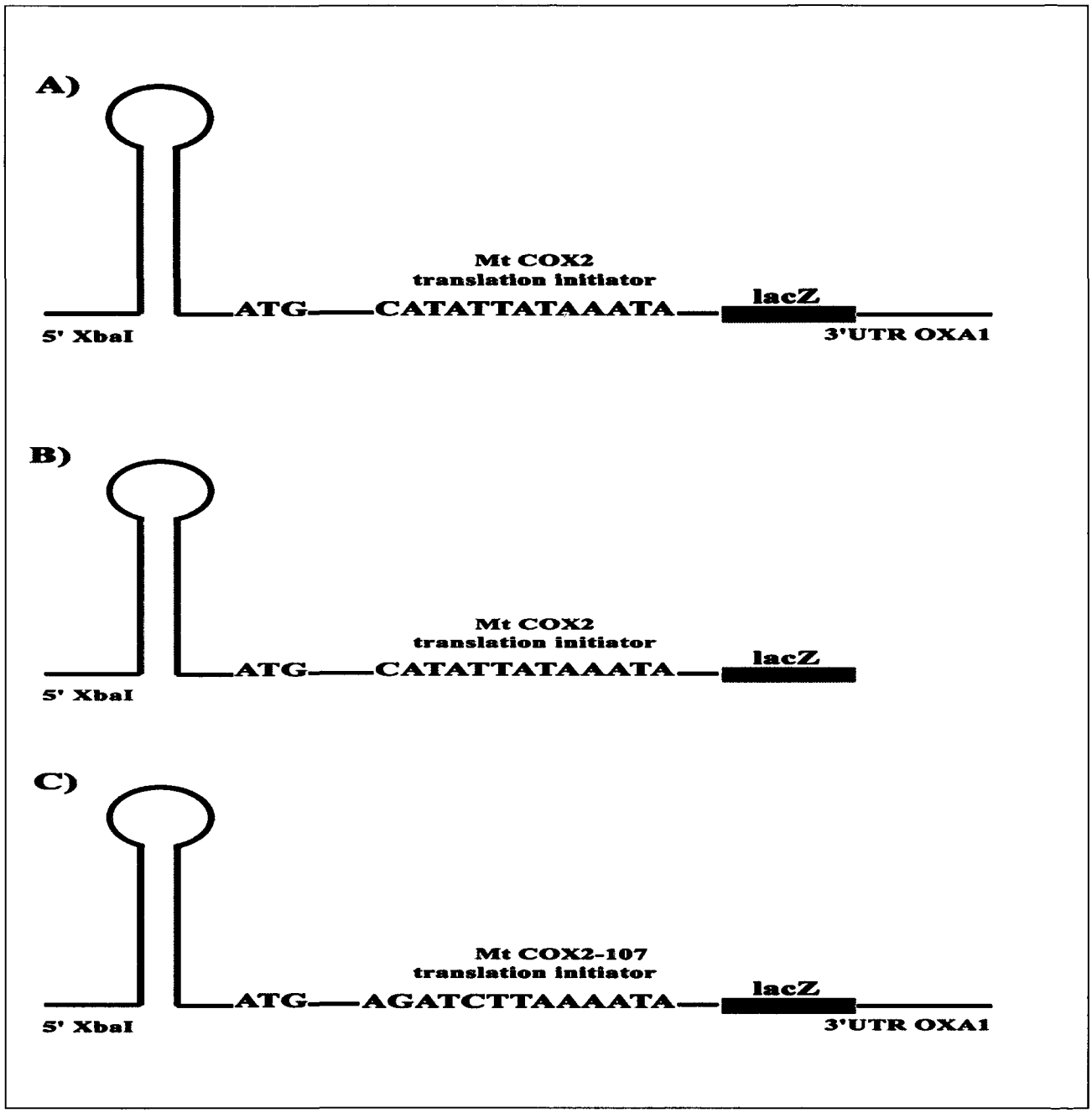

Figure 3.4: The designed constructs. (A) construct showing yeast 5'-UTR *COX2* attached to *lacZ* gene, with a hairpin loop, and 3'-UTR *OXA1* (B) construct including yeast 5'-UTR *COX2* attached to *lacZ* gene, with a hairpin loop (C) construct containing yeast non-functional mutant 5'-UTR *cox2-107* attached to *lacZ* gene, with a hairpin loop, and 3'-UTR *OXA1*. (not to scale)

If a ribosome manages to pass through the inhibitory hairpin structure above, then in theory it can produce ß-gal within the cytoplasm of the cell and hence complicate our results. To prevent this from happening, a secondary measure was taken that includes a premature out-of-frame initiation codon with a corresponding stop codon. It has been previously shown that a consensus sequence ACCAUGG, referred to as Kozak sequence, plays a major role in translation initiation in eukaryotes as it mediates the selection of the start codon AUG. (Kozak, 1984). Therefore, we placed an out-of-frame start codon, ATG, which is a part of the Kozak sequence, next to the hairpin structure (Figure 3.4). It is believed that this ATG has an inhibitory effect for the cytoplasmic translation of ß-gal since it blocks the initiation of the gene from a downstream site. According to the scanning model, if an upstream AUG codon is placed in the wrong reading frame, then the translation of the downstream gene should be suppressed (Kozak, 1989b), in this case the *LacZ* gene. We also placed an out-of-frame stop codon, TAA, a few nucleotides after the Kozak sequence as another secondary measure to ensure that the mRNA does not get translated in the cytoplasm. As a result, we have created optimal conditions, which include a consensus sequence, to ensure that the escaped cytoplasmic ribosomes are recruited to the out-of-frame start codon ATG rather than the actual *COX2* translation initiation site, which was inserted after TAA.

The third feature of this construct is the inclusion of a sequence that can mediate mt translation. As mentioned above, the 5'-UTR of yeast mt mRNA seems to be important for the translation of the gene. Evidence showed that there is a specific sequence found in the 5'-UTR of all yeast mt mRNAs. This consensus sequence (5'-UAUAAAUA-3') (see underlined sequence in table 3.2) allows the ribosomes located inside the mt to recognize the mRNA and initiate translation (Green-Willms *et al.*, 1998). There are 8 major mt mRNAs with different

characteristics such as: sequence, complexity and length. We chose the mt mRNA, *COX2*, because it has the shortest 5'-UTR sequence (54bp) (Table 3.2). *COX2* codes for cytochrome *c* oxidase subunit II, the terminal member of mt inner membrane electron transport chain (Bonnefoy *et al.*, 2001). A study compared the effects of *COX2* and several mutant forms of the gene on mt translation initiation. They found that a mutation in the 5'-UTR of the gene causes significant decrease in mRNA levels in the mt. We chose the mutant *cox2-107*, which had 6 altered nucleotides in its interior section (see bold small-cap letters in table 3.2), as a control (Dunstan *et al.*, 1997). Hence three different constructs were designed.

A fourth feature of the designed constructs was the inclusion of a modified *XbaI* overhang (Table 3.2) in order to ligate the 5'-UTR with plasmid p416, which has a restriction site for this enzyme at the 5'-end of *lacZ* (Figure 3.4). This overhang was designed in a way that would eliminate *XbaI* site after insertion into the plasmid. This allowed for the inclusion of an additional selection phase in the experiment simplifying the cloning step for making these constructs. If 5'-UTR was successfully inserted into the plasmid, then a second round of *XbaI* digestion would eliminate the plasmids that were not recombinant and did not contain the insert.

In Figure 3.4A, the first construct also contained the 3'-UTR of *OXA1* gene, which is believed to lead the mRNA towards mt, and the 5'-UTR *COX2* gene, which is believed to contain the mt translation initiator. If this construct successfully entered the mt, then a translation product should be detectable. The second construct (Figure 3.4B), contained the 5'-UTR, but lacked the 3'-UTR. Thus, we do not expect to detect translation product out of this construct, since it has no sequence to lead the construct towards the mt. The third construct (Figure 3.4C), contained the 5'-UTR of *cox2-107*, which is mutational translation initiator, and

the 3'-UTR. Therefore, even if the 3'-UTR leads the mRNA towards mt, we should not detect any translation product because the 5'-UTR translation initiator was altered.

Restriction enzyme analysis of the designed constructs indicated that they contain a *BglI* restriction enzyme site and therefore restriction digestion would allow verification of these constructs. Therefore, to screen for recombinant plasmids, candidate constructs were subjected to restriction digestion independently with *XbaI* and *BglI*. Images from the agarose gels indicate the identification of recombinant plasmids. Figure 3.5 shows *XbaI* digestion of samples. The recombinant candidate (L1 – red arrow) represents a pattern consistent with intact plasmids indicating the absence of a restriction site. The control digested plasmid p416 (L2) on the other hand had a pattern consistent with a single cut in plasmid. Similar differences between digestion patterns were observed in Figure 3.6, where samples were subjected to *BglI* digestion. The control digested plasmid (L1) was cut into 4 bands (red arrow) whereas the recombinant one (L2) was cut into 5 bands. Most notably, the control plasmid contained a thick band ~2.2kb in size (L1), while that of the recombinant plasmid was split into two bands ~2.1kb and ~1.9kb in size (L2) indicating presence of the target insert. The rest of the bands were comparable between the two samples.

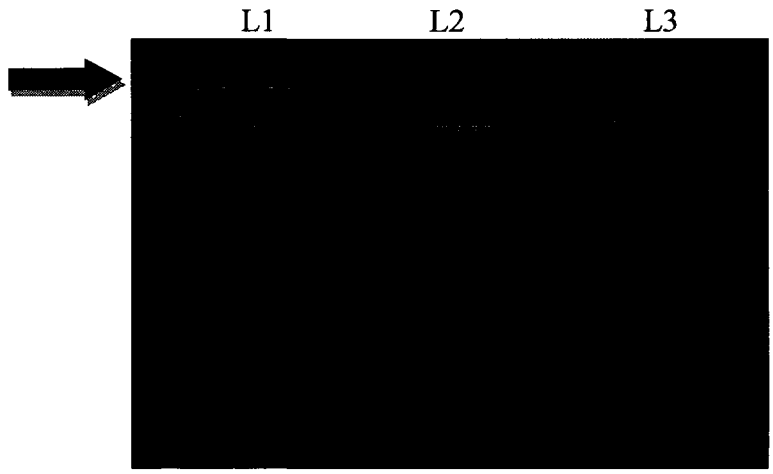

Figure 3.5: Agarose gel electrophoresis of purified samples digested with *XbaI* showing recombinant p416 candidate (L1), and digested p416 (L2), compared to 1kb DNA ladder (L3). The indigestibility of plasmid in L1 (red arrow) indicates an insertion where *Xba1* site is destroyed (L1).

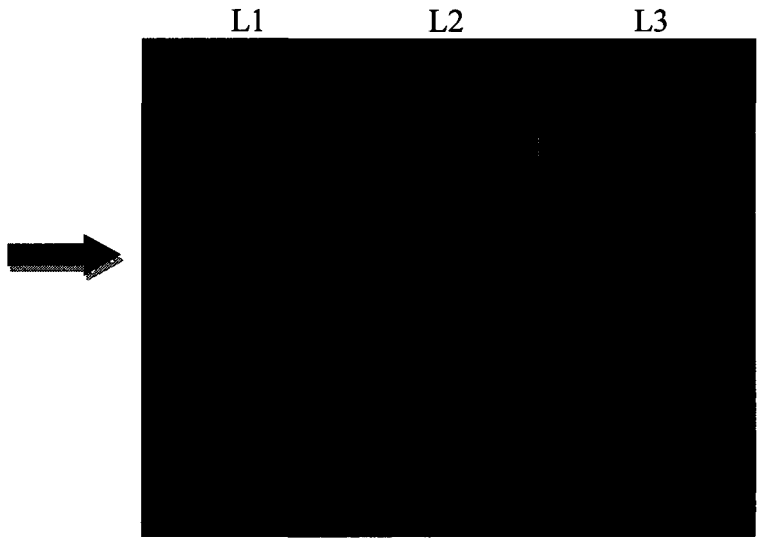

Figure 3.6: Agarose gel electrophoresis of purified samples digested with *BglI* showing digested p416 (L1), and recombinant p416 candidate (L2), compared to 1kb DNA ladder (L3). There is an expected difference in the third band cleavage in L1 and L2 (red arrow), suggesting the presence of an additional *BglI* site, where the enzyme cleaved the candidate sample (L2).

To confirm the presence of the insert and the successful cloning of the target DNA we sequenced the candidate plasmids. The sequencing results confirmed the presence of the insert in the correct orientation.

3.2 β-galactosidase assay

To test the import of the designed mRNA into mt, β-gal assay was performed in yeast cells transformed with different constructs. Yeast cells transformed with intact plasmid p416 were used as a positive control.

In theory, we may expect that the first construct, which contained 3'-UTR only, should be directed into the mt. But it may or may not be able to produce any active β-gal detected by a change of colour. The second construct, containing both 3'- and 5'-UTR should be able to enter the mt and the outcome of the translation should be detected by a change of colour. In the event that our mRNA was imported into mt and was translated there, if the translation occurred with a low efficiency, then detecting the outcome might be difficult. The third construct contained the 5'-UTR only, thus, we should not detect any outcome of the translation, since there was no sequence that would lead the construct towards the mt. Contrary to the theory, our results showed a slight change of colour in all three constructs. This prompted us to isolate the mt from yeast and perform β-gal assay directly on them, to see if we obtain more accurate results.

Indeed, the results were more promising (Figure 3.7). Analyzing the measured optical densities of three constructs showed the 3' and 5' construct had the highest β-gal activity amongst the rest (600%). These observations are in agreement with the hypothesis that 3'-UTR is mediating the entry of mRNA into mt. Interestingly, the β-gal activity of the 5' construct was slightly higher than that of the 3' construct (100% and 70%, respectively), (Figure 3.7). This finding might indicate that some cytoplasmic ß-gal activity is still present in our samples.

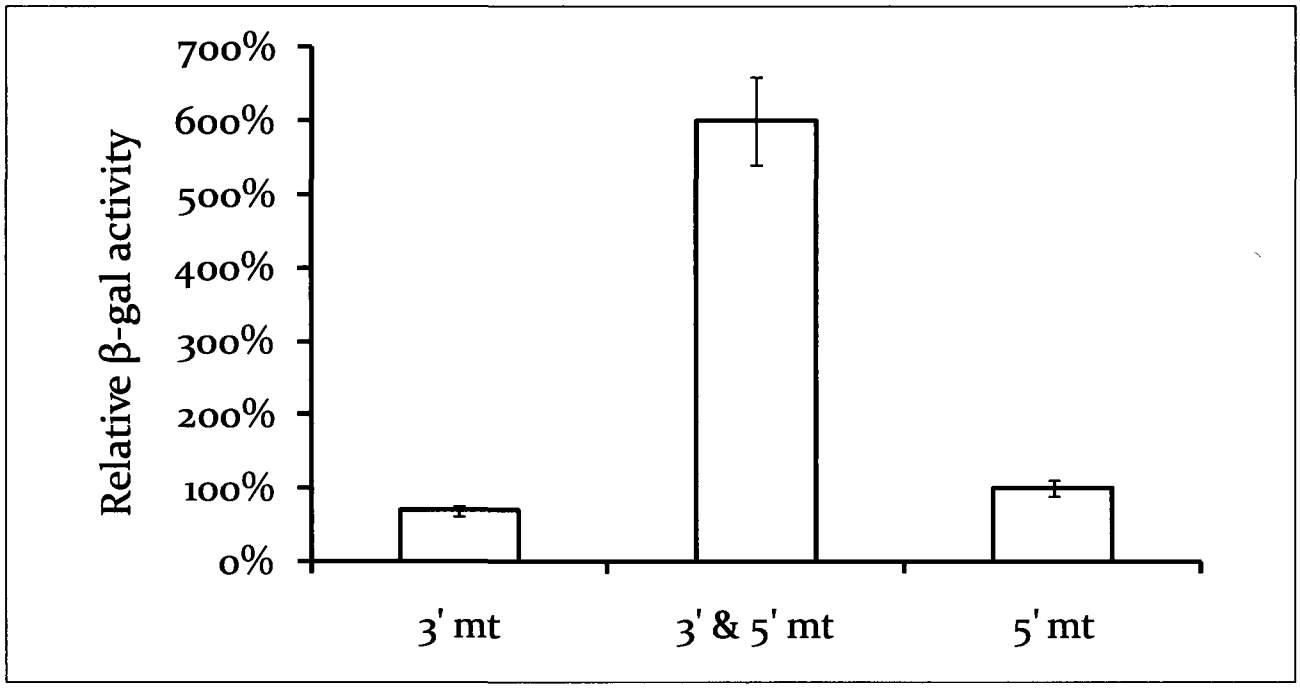

Figure 3.7: Average β-galactosidase (β-gal) activity for yeast mt isolated from the three constructs. Each experiment was repeated at least three times. The 3' and 5' construct (3' & 5'mt) had the highest β-gal activity amongst the rest (600%). Whereas, the 5' construct (5'mt) showed slightly higher β-gal activity than that of the 3' construct (3'mt) (100% and 70%, respectively).

In order to investigate further the findings obtained from the β-gal assays, we performed western blots on the isolated mt as a means for detecting any β-gal signals that might be emitted from the mt.

Mt were isolated from each yeast cell containing a different construct. Figure 3.8 shows the Protein Standard Marker (L1), as well as a clear band (~130kDa) for two samples, containing 3'and 5' construct (L2), and 5' only construct (L3). This band is the same size as β-gal protein (red arrow), which means that some β-gal is present in the isolated yeast mt samples.
The presence of a β-gal protein in L3 indicates that some cytoplasmic β-gal translation is present in our samples despite different measures that we have taken. This may stem from cytoplasmic β-gal proteins that are co-purified with mt. This is also in agreement with the observed β-gal activity for 5' construct (Figure 3.7).

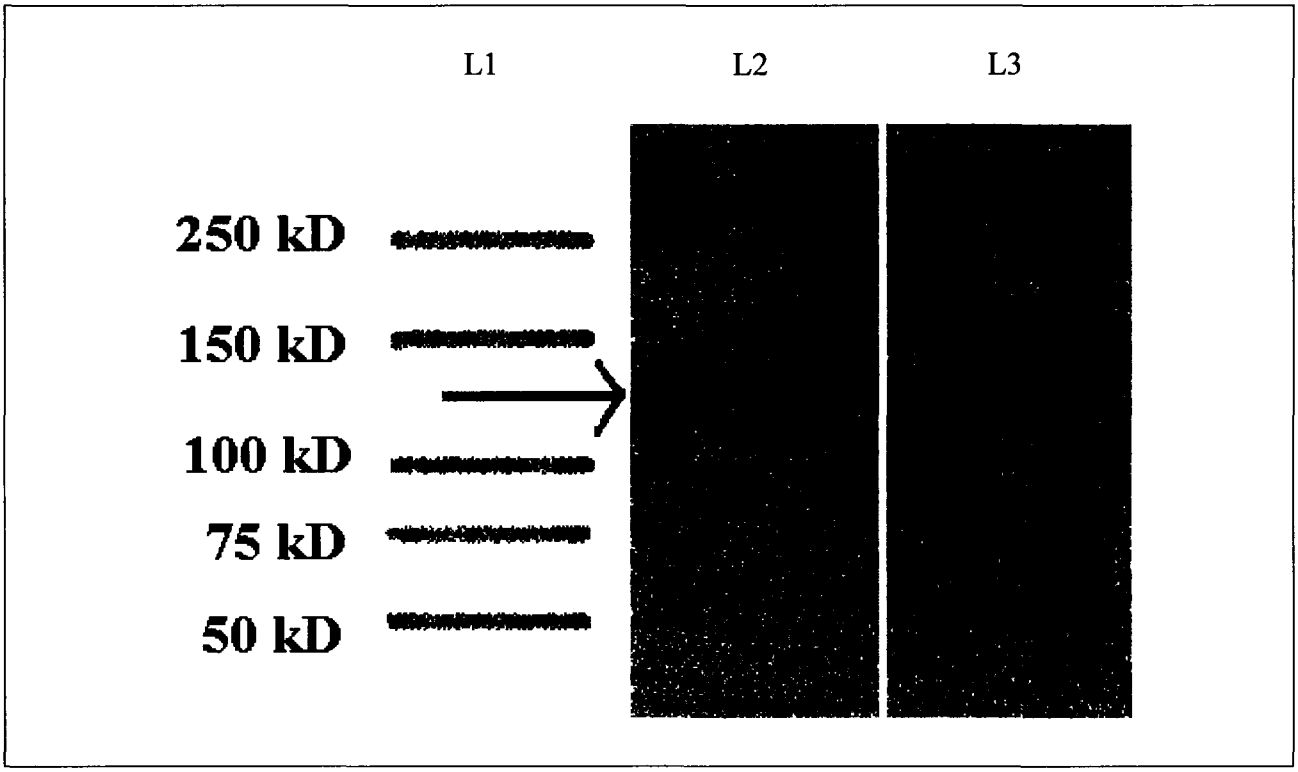

Figure 3.8: Western blot analysis of 2 samples of isolated yeast mitochondria (mt); showing the Precision Plus Protein Kaleidoscope Standard Marker from Bio-Rad (L1), 3' and 5' construct (L2) and 5' only construct (L3). The protein bands match the size of β-gal protein band (red arrow in L3) (~130 kD) suggesting the presence of β-gal protein in the samples. The band in L2 is thicker than that in L3. Equal amounts of total proteins isolated from mt were loaded on different samples.

If our target mRNAs are in fact directed into mt, we expect to detect them in purified mt. To detect β-gal mRNA signals in isolated mt, RT-PCR was performed. Detection of a β-gal activity signal may indicate that our designed mRNA was incorporated into mt and provides further support that the β-gal observed was likely produced there. First, total mt RNA was isolated using phenol-chloroform extraction method from purified mt. Isolated mRNA was used to create cDNA copies to be used in real-time PCR. Quantification of RNA was achieved by comparing the threshold cycle (C_t) values of cDNA samples from isolated mt. A wild-type (WT) standard RNA (YPL009C gene) was used as a control as it shows equal levels of expression across a range of different conditions. The average C_t value for mt carrying the 3' and 5' construct was 20.45 (Figure 3.9a). This is in comparison to the C_t value of 23.82 for mt carrying the 5' alone construct. The relative abundance of β-gal mRNA was approximately 10 fold higher for 3' and 5' construct (Figure 3.9b). RT-PCR was only repeated twice, therefore, these results are not statistically significant. The experiments need to be repeated before taking the results into consideration. However, in both trials, the trend was the same in that the gene expression for the 3' and 5' construct was higher than that of 5' only construct. This possibly will further support the notion that the 3'-UTR of *OXA1* may mediate the import of mRNAs into mt.

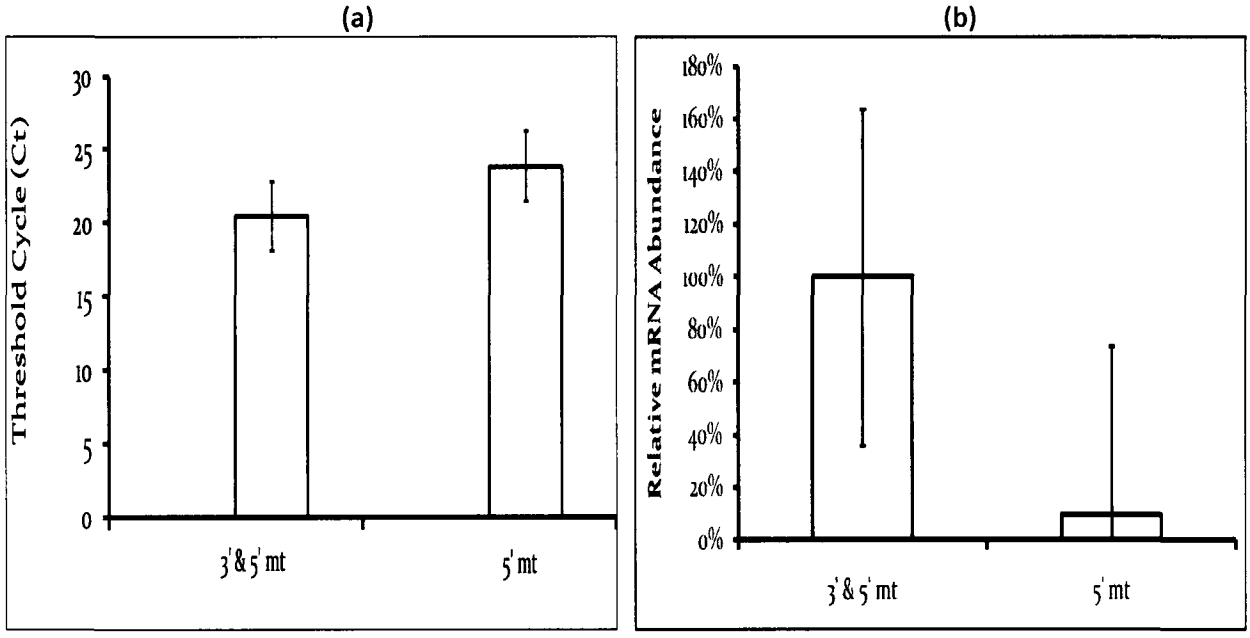

Figure 3.9: Quantitative RT-PCR analysis of RNA isolated from mitochondria (mt) samples. (a) The threshold cycle (C_t) values for the samples were calculated from the threshold cycles. (b) The relative β-gal mRNA abundance of mt containing 3' and 5' construct was set to 100% for ease of comparison. β-gal mRNA abundance of mt containing 5' construct was 10X lower than that of the 3' and 5' construct.

To prepare more mt with greater purity and with less potential contamination from cytosolic proteins, we used free flow electrophoresis (FFE) to fractionate purified mt. FFE fractionation was followed by absorbance measurements using a microplate reader. Figure 3.10 shows the profile of isolated mt after zone electrophoresis fractionation using FFE system, performed by Zischka *et al.* (2003). This is the theoretical graph, which suggests that the outcome of the FFE analysis should result in one high peak around fraction #41 that indicates the location of the highest purified mt fraction.

Figure 3.11 shows the graph of the 96 fractions collected from the purification of our mt containing 3' and 5' construct. Mt were separated by charge to mass ratio of the analytes in the sample, hence, the process is referred to as zone electrophoresis. Of the 96 samples, the most enriched fraction is that of fraction #45, (it has the highest peak with OD_{420} 0.32). This peak is comparable with that of the theoretical one that indicates high mt purity (Figure 3.10).

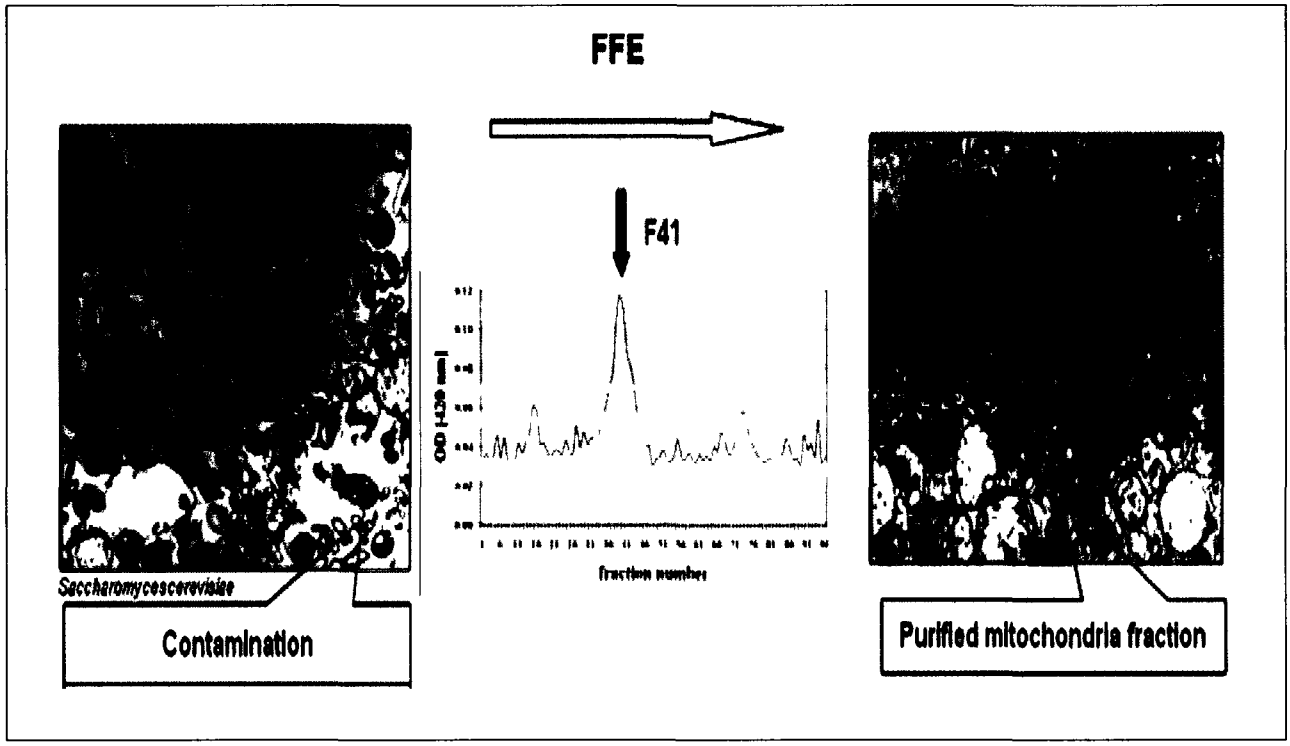

Figure 3.10: Profile of isolated mitochondria (mt) after zone electrophoresis fractionation using BD Free Flow Electrophoresis (FFE) system, performed by Zischka *et al.* (2003). The graph showed a clear high peak at fraction#41, indicating presence of the most purified mt in that sample. Zischka *et al.* (2003) also show images of yeast mt to demonstrate the accuracy of FFE system in purifying mt. The image on the left shows mt surrounded by contaminants, and after passing the sample through FFE system, mt were shown stacked side by side with minimum contaminants (image on the right) (Zischka *et al.*, 2003).

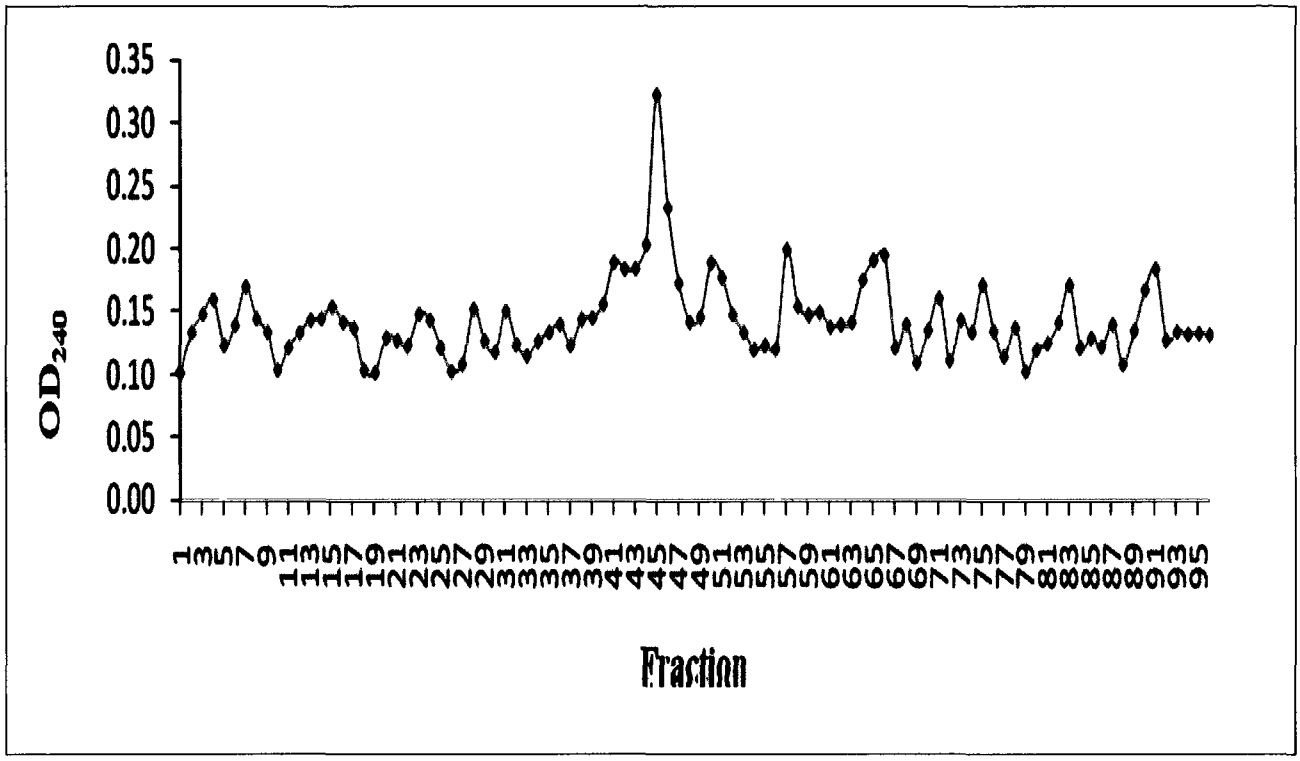

Figure 3.11: Purification of isolated mitochondria (mt), containing our 3' and 5' construct, after zone electrophoresis fractionation using BD Free Flow Electrophoresis (FFE) system. The profile showed a similar pattern of mt purification as in the previous graph. The highest peak showing at fraction #45 suggests mt sample of the highest purity (OD_{420} = 0.32).

Chapter 4: Discussion

CHAPTER 4: DISCUSSION

Mt are essential organelles found in most eukaryotic cells. They are thought to be direct descendants of prokaryotes. Hence, mt have retained their own genetic machinery; a characteristic that makes them exceptional (Solomon *et al.*, 1999).

Mt has been the focus of research and media lately because of their link to major health issues such as neurodegenerative diseases and diabetes (Du and Yan, 2010).

OXPHOS is a metabolic pathway in mt that uses energy released by the oxidation of nutrients to produce a useful form of energy; ATP. During OXPHOS, glucose from nutrients is broken down and electrons released are passed around in a series of redox reactions to a final acceptor molecule; oxygen (Mazat *et al.*, 2010). These redox reactions release energy, which is used to form ATP. In eukaryotes, redox reactions are carried out by a series of linked protein complexes within mt, which make up the ETC. However, in prokaryotes, these protein complexes are located in the cytosol (Parikh, 2010). Any disruption in one of the steps of this complex chain reaction will affect its outcome, which is the release of energy. Such disruption is due to genetic mutations and has been linked to a number of pathologies observed such as cellular respiration defects. These defects can also result in severe and often fatal metabolic disorders that affect tissues with high energy demands like the brain, heart, or muscle (DiMauro *et al.*, 1998).

Similarly defects in mt are known to affect apoptosis. Apoptosis is the natural programmed cell death that takes place at the end of a life cycle of a cell. A malfunction in its mechanism results in an uncontrolled cell proliferation, which causes a series of serious health issues (Karbowski, 2010). This malfunction is caused by genetic mutations in mt genes that are responsible for apoptosis. Mutations in mt genes in humans have been linked to

neurodegenerative diseases. Recent studies have also implicated abnormal mt function in the pathogenesis of Parkinson's and Alzheimer's diseases. These studies establish mt dynamics as a new paradigm for neurodegenerative disease research (Navarro and Boveris, 2010).

There is no effective treatment for mt disorders. However, gene therapy is currently being explored as a mean to effectively correct mt defects. Studies showed promising developments in such treatment by selective inhibition of mutant mtDNA, and recombinant mtDNA substitution (Du and Yan, 2010).

In selective inhibition of mutant mtDNA, the idea is to genetically block the replication of mutant mtDNA to reduce the expression of defective proteins. This may be possible by either using DNA, RNA or chemical analogues to bind to cDNA or RNA targets, or restriction enzyme to cut out the mutant part of mtDNA (Bacman *et al.*, 2007).

Another approach is to replace defective mtDNA with healthy ones. A possibility would be to target corrected proteins into mt or alternatively to direct corrected mRNA into mt for translation within this organelle. However, this method is limited due to the lack of availability of human mtDNA vectors, poor mt import of large constructs, the competition of recombinant mtDNA with functional resident mtDNA, as well as lack of proven methods for effectively directing proteins and mRNA molecule to mt (Chen *et al.*, 2003).

Mt are known to contain their own protein synthesis or translation machinery. Translation is the conversion of genetic information from mRNAs to protein. In general, the overall process is conserved between eukaryotes and prokaryotes, although the eukaryotic process is more complex (Valente and Kinzy, 2003). In eukaryotes, the small (40S) subunit of ribosome binds to the mRNA at its capped 5'-end, and then scans it for the first start codon AUG, where translation is initiated (Nakamoto, 2010). In prokaryotes, the small (30S) subunit

of ribosome binds to the mRNA at the Shine-Dalgarno site, from which translation is initiated (Nakamoto, 2009).

In mt, translation initiation seems to be based on a sequence signal upstream of the start codon AUG on the mt mRNA resembling prokaryotic translation. However, this signal found on the 5'-UTR of the mRNA is not complementary to the mitoribosome. It is interesting to note that this sequence does not seem to occur at a fixed distance from the start codon of mRNAs. Translational activators encoded by nuclear genes are also required to stimulate the translation of specific mt mRNAs (Chang and Wang, 2004).

Even though mt has its own translation machinery, only a small number of mt proteins are encoded inside mt. The rest of the proteins are imported into the mt by complex protein import pathways. Therefore, mt proteins are generated as precursors, which carry specific targeting signals that direct them towards mt. The biogenesis and function of mt, thus, require coordination and communication between the mt and nuclear genomes (Wiedemann *et al.*, 2004).

It appears that mRNAs encoding mt proteins are found in the vicinity of mt. Most mt proteins are encoded in the nucleus; therefore, they need to be imported into mt. Hence, mRNAs found near mt are thought to be involved in facilitating protein transport into mt (Neupert, 1997). Localizing mRNAs is necessary to deliver newly synthesized proteins to their site of action. These mRNAs have a certain sequence in their 3'-UTRs that allows them to be localized around mt. In Chabanon *et al* (2004) researchers suggest that this sequence in 3'-UTR of mRNAs serve as a binding site for specific RNA-binding proteins that interact with motor proteins that transport mRNA to their destinations within the cytoplasm (Chabanon *et al.*, 2004). An example of such mRNA is yeast *OXA1* gene. A complete respiratory deficiency in yeast mt was observed

when mutating the 3'-UTR of *OXA1*. This mutation was observed to prevent the mRNA from being sorted near mt (Loya *et al.*, 2008), a condition which was reversed by the human 3'-UTR of *OXA1* gene suggesting that the activity of this 3'-UTR is evolutionary conserved between human and yeast.

Furthermore, mitoribosomes seem to recognize a highly conserved octa-nucleotide sequence (5'-UAUAAAUA-3') in the 5'-UTR of mt mRNA. An example of such mt mRNA is yeast mt *COX2* gene, which contains the conserved mitoribosome binding site in its 5'-UTR. Translation activity was absent for *COX2* which had a mutated ribosome binding site (Green-Willms *et al.*, 2001).

Combining the two major aspects of mt localization and translation, we designed a gene composed of *lacZ*, used as a reporter gene to produces ß-gal, the 3'-UTR (158bp) of yeast *OXA1*, and the 5'-UTR of yeast *COX2* (54bp). To prevent translation of the designed mRNA in the cytoplasm, we added an inhibitory "hairpin" loop structure between the 5'-end and the first ATG of the gene (Vega Laso *et al.*, 1993) and kept the first ATG out-of-frame with the *lacZ* (see below). If cytoplasmic ribosome binds to the mRNA, then the loop would cause the ribosome to stall at the 5'-end of the gene (Kozak, 1989a). As a secondary measure, in case cytoplasmic ribosomes fail to stall at the loop, an out-of-frame Kozak sequence, which contains ATG, and a stop codon TAA were placed next to the hairpin (Kozak, 1984). This ATG should have an inhibitory effect that would block cytoplasmic translation initiation from a downstream site. According to the scanning model, if an upstream ATG codon is placed in the wrong reading frame, then the translation should be suppressed (Kozak, 1989b).

The designed mRNA was cloned into p416 vector and resulting recombinant vector was then introduced into *S cerevisiae*. We also cloned 3 other constructs; one that carries the 3'-UTR but not the 5'-UTR, one that carries the 5'-UTR but not the 3'-UTR, and one that has both UTRs as well as a mutation in the consensus sequence of the 5'-UTR (Figure 3.4).

To check if our designed mRNA was imported into mt, we performed *in vivo* β-gal assays on the different cloned yeast candidates, each carrying a different construct, with the exception of the mutational construct, with the exception of the mutational construct for which we did not have time to complete the experiments. Unexpected β-gal activity was observed for all three constructs. This suggests that even though we have taken several measures to eliminate the production of cytoplasmic β-gal, some cytoplasmic translation of mRNAs is still taking place. Such cytoplasmic translation could complicate the interpretation of our results as it would compete with mt translation known to be significantly less efficient and abundant.

Therefore, we proceeded with examining translation activity using isolated mt (Figure 3.7). The results showed the 3' and 5' construct had the highest β-gal activity amongst the rest (600%). This finding is in agreement with the hypothesis that the 3'-UTR of *OXA1* could direct mRNA into mt and that the 5'-UTR of *COX2* can mediate translation inside mt. Interestingly the β-gal activity was also found for both the 5' construct alone and the 3'construct alone (100% and 70%, respectively) (Figure 3.7). This activity might be as a result of background contamination. It remains possible that during the purification of mt, many contaminating proteins get co-purified along with mt. These co-purified proteins represent cytoplasmic translations and compete with measurement of β-gal activity derived from mt.

There are two possible explanations for cytoplasmic translation derived from mRNA with inhibitory structure. First it is possible that ribosomes can bypass all the measures that we have

taken to prevent translation in cytoplasm. A second possibility is that mRNAs might get partially degraded and it is these degraded mRNAs that get translated. The presence of a site of mRNA cleavage after the inhibitory structure can well explain these observations. Therefore it appears that we may never be able to completely eliminate cytoplasmic translation and consequently our purified mt might always contain some of these proteins.

To better differentiate between cytoplasmic and mt translation, one approach might be to maximize the level of mRNA translation inside mt. In this context there are several issues associated with our approach. For example, the hairpin structure used to reduce cytoplasmic translation may have also interfered with mt translation machinery. This is highly unlikely because mt are believed to have a prokaryotic origin, thus their translation system should differ from that of the eukaryotic one (Chaciñska and Boguta, 2000). Mt have their own translation mechanism with unique features. Their mRNAs are uncapped, and their 5'-UTR contains AUG sequences upstream of the initiator AUG (Sylvestre *et al.*, 2003a). This means that the scanning model for translation will not work for mt. Therefore, as long as mt ribosomes recognize the consensus sequence, then an inhibitory stem-loop structure should not interfere with the ribosomes' ability to translate the mRNA.

Another problem is that *lacZ* gene might not be a good candidate for this kind of experiments; that is, it may not be a good template for optimal mt translation . The reason why we used this gene is because it is a commonly used reporter gene that codes for β-gal, whose activity is easily detected (Mumberg *et al.*, 1994). In addition, *lacZ* is a prokaryotic gene (isolated from *E. coli*) and this may make it a good candidate to study mt-based translation which resembles that of prokaryotics. *LacZ* is not present in the yeast system eliminating possible interference that might hinder the detection of its product (Matsumoto *et al.*, 1988). A major

issue with *lacZ* is that its sequence is relatively lengthy (more than 3000 nucleotides) which may interfere with the entry of its mRNA into mt. Similarly its amino acid content may not be optimized for translation inside mt.

In addition the 3'-UTR used in our study might be specific to certain genes only. In other words, the 3'-UTR might efficiently work for just yeast *OXA1* and *OXA1*-like genes. A possible explanation is that there may be other sequences within *OXA1* gene that enhance the activity of this 3'-UTR in mediating mRNA import.

Our attempt to further purify mt using a FFE system was cut short due to time constrains. This system is very promising in isolating organelles to near purify (Zischka *et al.*, 2003).

To complement our observations we then performed western blots using an anti- β-gal antibody on the isolated mt in an attempt to detect β-gal signal. In agreement with our β-gal activity assay we detected a stronger band for the 3' and 5' mt sample than the one containing 5' only mt (Figure 3.7). The bands matched the size of theoretical β-gal protein band (~130 kD). To confirm that β-gal was in fact produced in mt and was not imported into this organelle, we investigated the presence of *lacZ* mRNA in purified mt using RT-PCR. In agreement with above observations, we detected significantly more (10 fold) mRNA in the sample containing 3' and 5' mt than the one containing the 5' only mt (graph not shown).

While our work is the first of its kind to combine the features of 3'and 5'-UTRs in one gene and examine its possible translation activity inside mt, others have explored the possibility of mRNA localization to the vicinity of mt which is mediated by cytoplasmic translation.

Most mt genes require several translation regulators. These regulators facilitate the co-translational import of newly synthesized proteins (Bonnefoy and Fox, 2000). Proteins encoded by nuclear genes are first translated on cytoplasmic ribosomes and then are transported into mt.

However, strong experimental evidence suggested that cytoplasmic ribosomes bound to mt may control a co-translational import process. This process has been studied with microarrays to identify all nuclear-encoded mRNAs co-purified with mt. Results showed about half of mRNAs encoding mt proteins were found to be translated in the vicinity of the mt (Marc *et al.*, 2002).

PUF proteins are a family of translational regulatory proteins that repress the translation of their target mRNAs by binding to elements located in the 3'UTR in eukaryotic species. Puf3p is a member of this family and is localized to the outer mt surface. Puf3p binds to a consensus motif in the 3'UTR of many mRNAs encoding mt proteins (Wickens *et al* , 2002).

Saint-Georges *et al* (2008) explained that asymmetric localization of mRNA plays an important role in coordinating post-transcriptional events in eukaryotic cells. They investigated mt localization of nuclear-encoded mRNAs in various conditions in which the mRNA binding protein context and the translation efficiency were altered. From the 786 nuclear genes coding for mt proteins Saint-Georges and colleagues characterized two classes of mRNAs translated in the vicinity of mt: Class I (256 genes) and class II (224 genes). While, Class III (306 nuclear genes) included mRNAs that are translated on free cytoplasmic polysomes. Class I mRNAs were translated close to the mt and were shown to have a Puf3p binding motif in their 3'UTR. Many of them showed impaired mt localization when PUF3 was deleted. In addition, mutations in the Puf3p binding motif altered mt localization of *BCS1* mRNA. Class II mRNAs were found to have no Puf3p binding site and, hence, their asymmetric localization was not affected by the absence of PUF3. Therefore, the researchers were able to show evidence of a connection between Puf3p and the asymmetric localization of Class I mRNAs, and that their translated proteins were subsequently imported into mt (Saint-Georges *et al* , 2008).

Recently, Eliyahu *et al* (2010) tested the possible mechanism of mRNA localization to the vicinity of yeast *S cerevisiae* mt by studying mt translocase. Mt translocase is a complex of proteins located in the mt outer membrane (TOM complex) and it controls the import of a large number of nuclear-encoded proteins. Tom20 is a peripheral component of the TOM complex. To do so, they isolated mt fractions from yeast cells lacking the major import receptor, Tom20, and compared their collected mRNA to that of wild-type cells by DNA microarrays. They were able to show that yeast cells possess an mRNA mt association mode, whereby Tom20 interacts with the translated mt targeting signal (MTS), and that this interaction might be assisted by Puf3p, an RNA binding protein, presumably through interaction with specific sequences on the 3'-UTR, which leads to mRNA localization (Eliyahu *et al.*, 2010).

In another study, Garcia *et al.* (2010) studied mt localization using an improved statistical fluorescent *in situ* hybridization (FISH) technique. They fused *ATP2*, a gene that controls mRNA asymmetric localization, to *lacZ*, a reporter gene. They applied five fluorescent probes complementary to *lacZ*. To visualize the hybrid, they used a three-dimensional (3D) imaging analysis on more than 50 yeast *S cerevisiae* cells. Researchers were also interested in examining the role of the leading peptide in mRNA localization. They deleted or replaced the coding sequence (MTS) of *ATP2-lacZ* hybrid. They found that the asymmetric localization of *ATP2* mRNA to mt was considerably reduced and that the MTS is necessary for mRNA localization and translation (Garcia *et al.*, 2010).

In conclusion, to date, no mRNA has ever been shown to be imported into mt. Here we made an attempt to study the possibility of such an import using a mRNA 3'-UTR signal that is thought to be responsible for localization of mRNA to mt, as well as the principles of translation machinery within mt. Our results are promising and suggest that an import of mRNA into mt may exist. However the problem of contaminating proteins that are co-purified with mt reduces our confidence in our data. For example, it remains possible that ß-gal produced from one of the constructs have a higher affinity for binding to mt and hence is selectively co-purified with mt.

Future directions

In the future we would like to follow up by further purifying mt using a FFE approach. This may help eliminate the presence of cytoplasmic translated ß-gal in our purified samples, which can simplify the interpretation of our data. Similarly, use of mild protease that can eliminate cytoplasmic proteins leaving mt intact may increase the purity of our final isolated mt.

The same approach can be applied to *lacZ* analysis. Addition of mild RNase before the final step of mt isolation can reduce the amount of contaminating mRNAs in purified mt sample.

An interesting follow up analysis would be to examine the 3' and 5' construct which carries a non-functional translation initiation sequence. We have made this construct but due to time limitation we have not managed to investigate its activity in mt. We expect to see reduced levels of translation mediated by this construct in comparison with the 3' and 5' construct. This can give us further evidence for the presence of mt translation for our construct.

The experiment could be repeated using a different reporter gene, such as green fluorescent protein (*GFP*) gene, or chloramphenicol acetyltransferase (*CAT*) gene, which are smaller in length and therefore might be imported into mt with better efficiency. This can also help us determine the universal applicability of 3'-UTR of *OXA1* in directing mRNAs into mt.

The long term goal is to direct mRNAs into human mt as part of a gene therapeutic treatment. If this type of experiment works, then it may open the door to finding a cure for numerous disorders caused by mt diseases. If it is successful, gene therapy provides a way to fix a problem at its source. Although it is a promising treatment option for a number of diseases, including Parkinson's and Alzheimer's, the technique remains risky and requires extensive research and studies to make sure that it will be safe and effective. Gene therapy is currently only being tested for the treatment of diseases that have no other cures.

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