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Characterization of FMP35: A novel gene and its role in mitochondrial DNA stability

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Abstract

Mitochondria are essential organelles for all eukaryotic organisms with very few exceptions. The life-giving processes contributed by mitochondria are the end result of many proteins that are encoded within the mitochondria. Many nuclear encoded proteins give mitochondrial DNA (mtDNA) the high stability needed so that life can thrive.

Saccharomyces cerevisiae (baker's yeast) has historically been a model organism for mitochondrial function studies. These yeast are categorized as facultative anarobes; meaning that they are able to respire or ferment depending on media available. Functional mitochondria allow baker's yeast to thrive on a 3-carbon medium (ρ+), while mitochondrial dysfunction due to mtDNA defects do not allow growth on the same medium (ρ-). The ease of visualizing this phenotype and culturing these organisms has made *S. cerevisiae* an important tool for mitochondrial studies.

Nuclear encoded proteins such as Abf2p and Ilv5p have been implicated in offering a degree of stability to mtDNA. Many nuclear proteins have been localized to mtDNA, creating an essential DNA-protein complex called a nucleoid. One protein that has been defined as a mitochondrial protein is Fmp35p. This is a novel protein that remains uncharacterized.

A fmp35Δ::URA3 gene knockout yields a ρ- phenotype as illustrated by a respiration loss assay. Furthermore, a significant decrease in direct repeat recombination has been described by this study. A less significant increase in polymerase slippage within microsatellites has also been documented. It is the conclusion of this study that Fmp35p plays a role in a recombination pathway that gives rise to wild type yeast with a

full complement of functional mtDNA. When this gene is defective and the protein is not produced yeast will not thrive.

Background and Significance

Introduction

Mitochondria are essential organelles for all eukaryotic organisms with very few exceptions. Through respiratory processes mitochondria create vital molecules of adenosine triphosphate (ATP). A single molecule contains powerful bonds between phosphate atoms that release energy upon hydrolysis and fuel the organism. In addition to ATP production mitochondria produce Heme and some amino acids (Schatz, 1995). Heme is a prosthetic group of cytochromes, which aid in electron transport within the mitochondria (Gonzales, 1990). Not only have mitochondria been looked upon as a force behind life, but it has also been implicated in disease and death. *Harrison's Principles of Internal Medicine* mentions many diseases that have been linked to mitochondrial dysfunction (reviewed Kasper *et al.*, 2005) and apoptosis originates in the mitochondria (Douglas, 1998). For this reason many aspects of the mitochondria are under scrutiny. To seek answers to mitochondrial DNA (mtDNA) function would lead researchers not only to solutions as to why these diseases exist, but unveil possible therapies.

Origin of Mitochondria

To understand where we are going it is very important to note where we have been. The origin of the mitochondria can still be debated today. Two schools of thought exist: autogenous origin and endosymbiotic theory. The more unpopular idea of autogenous origin states that the mitochondrion originated from the nucleus (Scheffler, 1999). As evolution occurs this took time and many steps. Currently there is little

information to bolster this theory. There is no evidence of intermediate species with mitochondria-nuclear hybrid organelles. However, it does possibly explain where mtDNA originated from and why there is a shared responsibility of mitochondrial proteins between the nucleus and the mitochondrion.

The widely accepted idea of endosymbiotic theory was first purposed by FJR Taylor in 1974 (Sheffler, 1999). The theory was based on the idea that a protoeukaryote able to use glycolysis and fermentation took up a primitive prokaryote by endocytosis. The primitive prokaryote must have been cabable of oxidative phosphorylation giving the cell a new organelle that is able to carry out this process. In 1992 the symbiotic theory stood up to further cirtical review and now finds its way to most textbooks (Sheffler, 1999).

Evidence that supports this theory lies within a wide array of organisms that have an incredible diversity of mitochondria. The first, and most primitive do not contain mitochondria at all. Archezoa is a group of eukaryotic organisms that do not contain mtDNA. Other organisms like the *Reclinomonas americana* contain mitochondria that consist of 97 genes. These freshwater organisms' mitochondria contain 23 genes for components of the electron transport chain as compared to 13 in mammals. Many other genes are present that cannot be found in higher eukaryotes (Sheffler, 1999). This may be a representation of what a new fusion between two different organisms may have looked like. Of course, this symbiotic relationship ushers along with it redundancies in genetic code. Through the course of evolution many higher eukaryotes have reduced redundancy by delegating much of the protein requirements to the nuclear genome.

Further mitochondrial studies have been undertaken to draw a more distinct line of lineage. Through rRNA comparison most plant mitochondria have been found to be related to the alpha subdivision of purple bacteria (reviewed in Sheffler, 1999). Other animal, ciliate and fungal mitochondria have been shown to be more distantly related but this may be interpreted that more than a single prokaryote was taken up by a single protoeukaryote. This may have occurred at different times in different organisms.

Today there are still studies being done to prove that mitochondria are not just transplantable machines that will function within any cell. Previous studies have shown that a human cell with mouse mitochondria will not function and prosper. Human cells have been shown to be incompatible with mitochondria from other species. However, recently chimpanzee and gorilla mitochondria have been able to function and support human cell respiration (reviewed in Sheffler, 1999).

Life Cycle of *Saccharomyces cerevisiae*

The life cycle of *Saccharomyces cerevisiae* can be convoluted. The organism does not have to follow a traditional eukaryotic life cycle. Steps such as proliferation, mating, and meiosis do occur in the life cycle; however, not necessarily in that common order. Two specialized cell types can be found in yeast denoted as *a* and α. In either form the cell would be haploid. In addition these haploid forms can mate (or fuse) to create a diploid *a*/α cell. The remaining portion of the diploid life cycle is dependent on the MAT locus. In the haploid this locus can determine whether the yeast is *a* or α by replicative transposition (reviewed in Watson *et al.*, 1992). Sex determination can be simplified as flanking genes (HMLα and HMRa) store the sex determining genes until

HO endonuclease creates a division in the MAT locus. Gene conversion inserts a copy of the gene into the lesion allowing for sex determination (reviewed in Watson *et al.*, 1992).

The entire life cycle depends on this starting position of the cell. When an *a* haploid cell is placed near an α haploid they mate nearly 100% of the time. This occurs due to cofactor pheromones that are released by both cell types (reviewed in Herskowitz, 1988). A release of this cofactor causes neighboring opposite sexed cells to arrest in G1 phase of the cell cycle. Fusion then occurs between the two cells and the cell cycle is reactivated in synchrony (reviewed in Herskowitz, 1988). This new *a*/α diploid is unable to further mate with either haploid cell type but instead undergoes meiosis and spore formation resulting in more haploid cells (reviewed in Herskowtiz, 1988).

In general new cells are generated from budding. Yeast can double its number approximately every 100 minutes (reviewed in Herskowitz, 1988). Budding has been witnessed in both haploid and diploid species. The bud starts as a small outgrowth of the mother cell during S phase. Throughout G2 and M phases the growth continues to enlarge finally cleaving during late M phase (reviewed in Herskowitz, 1988). This proliferation can continue as long as environmental conditions allow.

Saccharomyces cerevisiae As A Research Model

S. cerevisiae was the first eukaryote to have its complete genome sequenced (Goffeau *et al.*, 1996). Biochemical pathways have also been well defined. *S. cerevisiae* has been shown to have a very compact nuclear genome. Almost 6,000 open reading frames (ORF) have been identified in yeast or 70% of the genome. A protein is encoded approximately every 2kb of code (reviewed in Goffeau *et al.*, 1996). Most research

organisms are chosen due to the amount of current knowledge known as well as ease of use. Yeast in particular *S. cerevisiae* offers both to the researcher.

The budding yeast, *S. cerevisiae*, has been widely used and is defined as a facultative anaerobe due to the organism's ability to grow in the absence of a functional mitochondrion on a fermentable carbon source. The ability of this organism to switch between respiration to fermentation (diauxic shift) may be one of the primary lures to its use as a research model (reviewed in Ohlmeier *et al.*, 2004). This shift has been exhaustively studied as well. Green fluorescent protein (GFP) labeling has been able to illustrate a morphological change within mitochondria. While in an environment rich in glucose the mitochondria are abnormal in appearance and non-functioning. Soon after a switch to glycerol medium there is a rapid induction of gene transcription within mitochondria resulting in a more normal looking, functioning organelle (reviewed in Sheffler, 1999). In addition to these well defined pathways *S. cerevisiae* are single celled organisms that are easy to grow and can reproduce asexually as well as sexually. However, it is important to point out that the relative size of the yeast mitochondrial genome versus other known mitochondrial genomic sizes. *S. cerevisiae* mitochondria do contain a sizable 85.8 kilobases (kb) while human (16.5 kb) and insect (15 to 20 kb) mitochondria contain a smaller genome (Lecrenier and Francoise, 2000; Saito, Tamura and Aotsuka, 2005). On the other hand watermelon (330 kb) and muskmelon (2,500 kb) possess weightier mtDNA (Alberts *et al.*, 2002). Although there is obvious diversity among eukaryotic organisms many parallels are still being drawn between human and yeast mitochondrial functions thus making this organism a viable research model.

***Saccharomyces cerevisiae*: Mitochondrial Genome**

The physical structure of *S. cerevisiae* mtDNA (smtDNA) is still under debate. Since 1996 scientists have been trying to differentiate between closed circular models and the more identifiable linear model. Through different in-gel techniques linear segments as well as circular DNA have been identified in the smtDNA. Most of these molecules range from 1,000 kb to 60 kb (reviewed in Lecrenier and Foury, 2000). However during these tests it is still possible to visualize a minority of circular species. This may mean that the circular examples are functional, complete genomes while the linear are units that may be under replication (reviewed in Lecrenier and Foury, 2000).

A total of 55 mitochondrial genes have been confirmed in yeast to date (SGD, 2005). The 85.8 kb code for many proteins essential to glycolysis (COX genes, ATP synthase subunits, etc.). The genome contains seven to eight replication origin (ori) elements, as well as codes for rRNA (21S and 15S subunits), and tRNA (24 varieties) (Foury *et al.*, 1998). Unlike human mtDNA (hmtDNA), yeast have been shown to contain introns and may have to take part in self splicing to complete successful translation (Lodish *et al.*, 2000). Dispersed among the genes of smtDNA are long stretches of A+T regions that are interrupted by G+C regions (Foury, 1998). These G+C clusters have been associated with the aforementioned ori sequences and may serve as structural components of the mtDNA (Zamaroczy and Bernardi, 1986).

In total there are approximately 50 copies of the mitochondrial genome in haploid yeast cells while it is double in diploid cells. The mitochondria are typically found within the margin of a cell arranged in an elongated cylindrical branched structure. Inside, the genome is further organized into a protein-DNA complex also called the nucleoid

(reviewed in MacAlpine *et al.*, 2000). This complex is under control from proteins that are encoded in the nuclear genome of the organism. Proteins such as Abf2p, Mgt1p, Ilv5p, Mgm101p and others either directly or indirectly affect the nucleoid complex (MacAlpine *et al.*, 2000, Zuo *et al.*, 2002). A nucleoid structure is important not only for proper expression of genes, but these proteins also can influence appropriate segregation mtDNA to offspring cells (See "*S. cerevisiae* mitochondrial proteome").

The expression of these genes is essential for mitochondrial function; however, it is not necessarily essential for our yeast research model. As previously discussed *S. cerevisiae* are known as facultative anaerobes. Although the mitochondria within are nonfunctional, the yeast may survive if offered media, such as glucose. While these yeast may still be able to flourish, the respiratory deficient mitochondria would be termed ρ- (rho minus). Wild type, fully functional mitochondria are referred to as ρ+ (rho plus) due to their ability to fully respire and contain a full compliment of genetic information. Other yeast and human cell lines have been created to study the effects of a complete lack of mtDNA. These cells are referred to as ρ^0 (rho null).

Fully functional mitochondria require many proteins and a concert of acute reactions to safeguard the integrity of mtDNA. Yeast mtDNA is riddled with microsatellites and other hazards that may put these essential genes in danger.

***Saccharomyces cerevisiae*: Mitochondrial Proteome**

Genomic DNA encodes a majority of proteins that control and stabilize mtDNA. Recent studies have reported more than 400 nuclear genes encode mitochondrial proteins (Karlberg *et al.*, 2000). These nuclear encoded proteins are generated a distance away from the mitochondria on which they have been localized. Recently it has been

discovered that many of these proteins contain weakly conserved mitochondrial targeting sequences (MTS)(Ohlmeier *et al.*, 2004). One report shows the mitochondrion is only behind the nucleus and cytoplasmic vesicles as a protein dense organelle (Huh *et al.*, 2003). Specifically proteins like Mgm101p, Abf2p, and Ilv5p are very important in the viability of a mitochondria. Deletions in any of these proteins can have a lethal effect on the mitochondria (Kaufman *et al.*, 2000; MacAlpine *et al.*, 2000; Zuo *et al.*, 2002; and Sia *et al.* 2003).

When specific genes are discussed a range of complications can arise from knockout models. These models can divulge different mtDNA phenotypes and lead researchers to the proteins function. Mgm101p is necessary for mitochondria to undergo respiration. A deletion in this gene results in cells that cannot grow for more than 10 divisions (Zuo *et al.*, 2002). It was further shown that a strain carrying a temperature sensitive allele of Mgm101p contained half the complement of mtDNA per cell division eventually yielding a mitochondrion that was inactive (Zuo *et al.*, 2002). Abf2 is another nuclear encoded gene that displays the importance of protein influence on mitochondria. The protein bends around DNA to most likely function as a packaging protein in the nucleoid (MacAlpine *et al.*, 2000). Deletions in this gene produce nucleoids with altered protein distribution and cells that cannot respire (MacAlpine *et al.*, 2000). This collection of proteins that all are associated with and affect mitochondria is referred to as the mitochondrial proteome. Understanding the proteome and how it interacts with mtDNA is becoming increasingly important to understanding mitochondrial function.

Mitochondrial Metabolism

When high carbon media is available for yeast (i.e., glucose) mitochondria look abnormal and do not need functioning respiratory pathways to live; however when a smaller carbon source (i.e., glycerol) is available the mitochondria seem to almost immediately react, genes are turned back on, and necessary proteins are produced. This rapid change is necessary so that the yeast can continue to consume a three-carbon source and produce ATP. Metabolism is tightly controlled on many levels. At the genetic level metabolic control is being explored by upstream activating or repressing sequences that influence protein production. For example, CYB2 encodes for lactate cytochrome C oxidoreductase. Cyb2p is localized in the intermembrane space (reviewed in Scheffler, 1999). This gene is controlled by two positively acting cis elements and one negative (reviewed in Scheffler, 1999). One of these positive elements binds Hap1 and another binds Hap 2/3/4/5. These complexes increase the production of the enzyme so that the cell can maintain homeostasis. Hap 1 has been identified as an important complex that controls many aspects of ATP metabolism because of its sensitivity to O_2 levels within the cell (reviewed in Scheffler, 1999).

Gene regulation and protein production are necessary for ATP synthesis. The metabolic processes within the mitochondrion are complex. Here I briefly define ATP synthesis in three pathways. Tricarboxylic acid cycle (TCA cycle), oxidation of NADH, and oxidative phosphorylation are all parts of a complex metabolic scheme. Once glycolysis has occurred the resulting NAD+ is reduced to NADH and two pyruvate molecules enter the mitochondria where they are converted to acetyl CoA (reviewed in Solomon *et al.*, 1996). At this point within the matrix acetyl CoA joins the TCA cycle.

Citrate synthesis catalyzes the condensation of Acetyl CoA with oxaloacetate to form citrate (reviewed in Scheffler, 1999). Citrate is catalyzed to its isomer isocitrate by aconitase and is eventually catalyzed by another enzyme yielding CO_2 and the first of three NADH molecules (reviewed in Scheffler, 1999). Isocitrate also undergoes dehydrogenation and decarboxylation to yield α-ketoglutarate (reviewed in Solomon *et al.*, 1996). This compound undergoes many enzymatic reactions to create the 4-carbon compound succinyl-CoA. Through substrate level phosphorylation the succinyl-CoA forms succinyl-phosphate and then ultimately succinate (reviewed in Scheffler, 1999). Succinate is further oxidized to fumarate and flavin becomes a hydrogen acceptor, taking on 2 hydrogen molecules to become $FADH_2$ (reviewed in Scheffler, 1999). Fumarate is converted to malate during hydration, and a final reaction of this malate is undergone to form oxaloacetate and NAD+ is further reduced to NADH (reviewed in Scheffler, 1999). At the end of these complicated reactions glucose has been catabolized and four molecules of ATP have been formed.

NADH and $FADH_2$ are important because they participate in the electron transport chain (ETC). Electrons made available from these molecules participate in exergonic redox reactions to drive the ETC. Finally ATP synthesis is completed by oxidative phosphorylation of ADP and P_i (reviewed in Solomon *et al.*, 1996). Four complexes make up the ETC. First, complex I is made up of NADH-ubiquinone oxidoreductase, which is responsible for a transfer of four protons from the matrix side to the intermembrane space (reviewed in Scheffler, 1999). Complex II consists of four peptides associated tightly with the inner membrane. This complex processes electrons from the oxidation of succinate to fumarate. This specific complex is most noted for

linking the ETC directly to the aforementioned TCA cycle because of its processing of succinate to fumarate (reviewed in Scheffler, 1999). Complex III contains proteins that were at the forefront of mitochondrial exploration. Namely cytochrome C was the first peptide identified from the mitochondria and plays an important role in electron transport. This complex oxidizes a substrate and transfers electrons to cytochrome-c which transfers protons across the inner mitochondrial membrane (reviewed in Scheffler, 1999). Complex IV causes cytochrome-c to be oxidized with the following electron acceptor being molecular oxygen (reviewed in Scheffler, 1999). Cytochrome-c is further reoxidized and four protons are released and enter the intermembrane space from the matrix (reviewed in Scheffler, 1999). In general the protons that are pushed along this pathway and diffuse through ATP synthase complexes causing ADP phophorylation to create ATP (reviewed in Solomon, 1996). At the end of this process three ATP are produced for every pair of electrons that enters the pathway (reviewed in Solomon, 1996). The exploited electrons create a gradient that manipulates protons causing reactions with oxygen to yield H_2O (reviewed in Solomon, 1996).

Mitochondrial DNA Recombination

Recombination is a process by which double stranded DNA (dsDNA) is cleaved and foreign DNA is translocated or inserted into this break. Assortments of nuclear encoded proteins are necessary for recombination to take place. Recombination is especially important for mtDNA because it is responsible for segregation and transfer of into daughter cells. Abf2p is a known structural protein that is essential for proper function of the mitochondria. This high mobility group (HMG) protein stabilizes the nucleoid yielding wild-type (rho+,ρ+) mtDNA (Kaufman, *et al.*, 2000). Absence of this

protein in an abf2Δ cell suppresses mtDNA recombination (Zelenaya-Troitskaya, *et al.*, 1998). Furthermore over-expression of the same gene creates cells with very high counts of both ρ+ and ρ- mitochondria suggesting that Abf2 plays a role in copy number and heredity (Zelenaya-Troitskaya, 1998). Presently a gap exists in the knowledge of the entire proteome responsible for this process.

During the normal life cycle and sexual reproduction mitochondria exhibit recombination of mtDNA (reviewed in Ling and Shibata, 2002). Over much of the cell cycle mitochondria are fairly mobile. They are closely associated with the cytoskeleton and have been recorded as moving 2 to 30μm per minute (reviewed in Scheffler, 1999). Through this movement and close association with other mitochondria there are many accounts of fission and fusion among mitochondria during the cell cycle (reviewed in Scheffler, 1999). Homologous recombination in *S. cerevisiae* is initiated first by double stranded breaks. At this point the DNA is not broken evenly but contains uneven overhanging ends. These ends then interact with the "foreign" homologous sequence creating a heteroduplex which can be referred to as a Holiday junction (reviewed in Watson *et al.*, 2004). These junctions are not stagnant; that is to say, there is mobility once the junctions are formed. The movement is unidirectional and proteins such as RecAp and Rad51p need to be involved for ATP binding and hydrolysis for branch migration (reviewed in Ling and Shibata, 2002). The most important part of recombination is how the junction is resolved. Crusciform cutting endonuclease (CCE1) also referred to as mitochondrial genome transmission (MGT1), is encoded in the nuclear genome and is responsible for junction resolution (Lockshon *et al.*, 1995). To avoid confusion I will refer to this gene as CCE1 because of a repair gene that bears the name

MGT1 as well. Experimentation with CCE1 knockouts and marker genes proved that without CCE1 present mitochondria would not transfer mtDNA to other mitochondria (Lockshon *et al.*, 1995). In this experiment the CCE1 gene was disrupted with URA3 or LEU2 marker gene. Mating between wild type rho+ haploid CCE1 species and CCE1 rho- species yielded diploid species of which, over 95% were rho- (Lockshon *et al.*, 1995). Species that were rho+ Δcce1 and rho- Δcce1 were mated and resulted in cells that were less than 1% respiratory. CCE1 gene causes a dramatic decrease in the rho- genome because of its ability to resolve a holiday junction (Lockshon *et al.*, 1995).

Implications of Microsatellite Instability

Microsatellites are regions of DNA that contain multiple bases repeated multiple times. Typically a region 1 to 8 base pairs that repeat are considered to be microsatellites. Regions of this nature tend to exhibit differences in mutation rates most likely due to DNA slippage (reviewed in Sia *et al.*, 2001). Once these mutations arise they can be passed on to future generations yielding an increased possibility of disease.

In yeast a very common tandem array region consists of CA/GT repeats at a rate 30-fold higher than what is expected for random repeat frequencies (Gendrel *et al.*, 2000). These types of arrays have been a cause for polymerase slippage in recent experiments *in vitro* (Shinde *et al.*, 2003). Simple repeats such as $(CA)_n$ and $(A)_n$ were inserted into the pUC18 vector, and two rounds of nested PCR were performed. Analysis of the products showed significant slippage. This was further analyzed to show that contractions far outnumbered expansions (Shinde *et al.*, 2003). Phenomenon like this can cause frameshift mutation (Kokoska, 1998). Experiments testing other loci showed that the frequencies of additions or deletions were different between regions; however, the

result was a framshift mutation (Kokoska, 1998). This was further illustrated by an experiment that placed a $(CA/GT)_{39}$ repeat upstream from the ARG4 promoter. The locus was impaired by the microsatellite (Gendrel, 2000). Many of these mutations can be thought of as a fidelity problem with DNA polymerase enzymes. Enzyme slippage may cause an extra addition or deletion of a repeating sequence. DNA polymerase fildelity is not the only issue surrounding frameshift and other mutations associated with microsatellites. CTG/CAG or CGG/CCG triplet repeats have also been found to take on a secondary hairpin loop structure (Moore, 1999). Hairpin loop structures have been found to create deletions if found on the template strand or additions if found on the primer strand (Moore, 1999).

Screening of yeast with increased microsatellite instability disclosed several genes that contained point mutations. Genes that were found to be mutated belonged to the yeast mismatch repair mechanism (MSH2, MSH3, MLH1, and PMS1) (Sia *et al.*, 2001). Other mutations in genes involved in replication have also been identified as increasing microsatellite instability (POL30, RFC1, and RAD27) (reviewed in Sia *et al.*, 2001). There are many different pathways in which microsatellites can damage essential information for cellular survival. Further understanding may lead to a more in-depth idea of disease pathology.

During normal mitochondrial function mtDNA is bombarded with reactive oxygen species and is replicated by a polymerase with deficient fidelity, causing genetic instability. Currently, genetic instability is recognized as an initiator of some cancers. Instability can arise from various sources including malfunction of mismatch repair mechanisms. If all systems are functional there is a low occurrence of mutation during

replication; however, if repair mechanisms are dysfunctional this mutation will not be corrected and the mutation can continue to be replicated. Any mutation that arises during replication is typically due to the resolution of the polymerase. While most polymerase activity is of very high fidelity, none is perfect. Repair mechanisms correct most mutations. When this process malfunctions mismatched bases continue to exist, causing genetic instability and possibly contribute to mitochondrial dysfunction. While there is still no direct link of mismatch repair malfunction and mtDNA instability the polymerase (POLγ) is somewhat deficient in proofreading ability (reviewed in Bianchi *et al.*, 2000). There is a dramatic increase of mismatches along microsatellites, which has been indicated in breast, colorectal, gastric, and kidney cancers (Bianchi *et al.*, 2000).

Mitochondrial Dysfunction

Diseases that affect a wide range of tissues have all implicated mtDNA mutation as a root cause. Alterations in genes coding for proteins essential for mtDNA function can cause reduction of ATP supply, generation of free radicals, and the early onset of apoptosis. Myopathies and encephalopathies are general categories of disease that are brought on by mitochondrial mutation because these tissues rely heavily on the metabolic processes of the mitochondria (i.e. oxidative phosphorylation) to thrive (Kasper *et al.*, 2005). Disease progression is typically dependent upon the heteroplasmic state of the cell. A cell denser in mutated mitochondria may show a more rapid progression of the disease (reviewed in Wallace, 2000).

The first disease attributed to mitochondrial dysfunction was Luft's disease (reviewed in Cassarino & Bennett, 1999). The exact mechanism is still unknown, but the disease causes uncoupled respiration, which manifests in a basal metabolic rate of 200%

of normal (reviewed in Cassarino & Bennett, 1999). The disease ravages a patient by increasing basal temperatures, low body weight, and is associated with spontaneous releases of mitochondrial calcium stores (reviewed in Cassarino & Bennett, 1999). This release of calcium causes sustained stimulation of respiration and loose coupling (reviewed in Cassarino & Bennett, 1999). The discovery of this disease has opened the door to finding other diseases that may be due to mitochondrial dysfunction.

Mutations that typically are responsible for human disease are large deletions, point mutations, expansions or deletions of microsatellites. Patients with these diseases typically present their illness in one of three major categories: chronic progressive external opthalmoplegia (CPEO, muscular condition associated with the eye), skeletal muscle-central nervous system syndromes, and pure myopathy (Kasper *et al.*, 2005). Mutations in the gene TK2 on chromosome 16q22 cause Mitochondrial DNA Depletion Myopathy. Specifically mutations in histidine 90 to asparagine and isoleucine 181 to asparagine have lead to mitochondrial activity losses of 14-45% (Saada *et al.*, 2001). Myclonic Epilepsy with Ragged Red Fibers (MERRF) is another disease that is brought about by a A → G transition at position 8344 (Silvestri *et al.*, 1992). This disease includes epilepsy, cerebellar ataxia, and progressive muscle weakness (Kasper *et al.*, 2005). Deletions in the γ polymerase motif B have recently been found to be responsible for CPEO (Goethem *et al.*, 2001). Other large mutations have been found to cause CPEO as well (reviewed in Goethem *et al.*, 2001).

Colorectal cancer has been studied through PCR techniques. Cells from normal and diseased tissues have been studied along with several established cancer cell lines. To characterize the findings two categories were developed, MSI-H and MSI-L. The first

is a category that was designed to illustrate a cancer that has arose due to a possible repair malfunction that may be caused by genomic mutations that encode proteins responsible for repair. MSI-L is a category that depicts cancers not caused by repair malfunction (reviewed in Bianchi *et al.*, 2000). After extensive comparisons 88 mutations were found (reviewed in Bianchi *et al.*, 2000). Many of these mutations were found in the D-loop or along several mononucleotide tracts (reviewed in Bianchi *et al.*, 2000).

Mutations have also been mapped in breast, gastric, and renal cancer. Mitochondrial instability is a common thread that links all of these cancers. Since there are many polyhaploidy mitochondria per cell, one might think that a mutation in one mitochondrion may not affect the entire system or organism. However, there is a clonal expansion among the cancerous haplotype (reviewed in Bianchi *et al.*, 2000). Two scenarios may account for the homoplasmic state of mutated mitochondria. One, mutated mtDNA may have a selective advantage and replace its wild type counterpart and further expand with replication (reviewed in Bianchi *et al.*, 2000). Two, the mutated mitochondrion is responsible for self replicating and may over replicate to compensate for its lack of function (reviewed in Bianchi *et al.*, 2000).

Neurological diseases have also indicted mitochondrial dysfunction. Brain activity requires energy supplies and functioning mitochondria. MtDNA mutations and pathological free radical reactions may decrease mitochondrial function (reviewed in Cassarino & Bennett, 1999). The electron transport chain (ETC) may be most affected, and once impaired there is a tremendous decrease in ATP production (reviewed in Cassarino & Bennett, 1999). There is also evidence that reduced ATP production may not be the most severe consequence of ETC dysfunction. While protons and electrons are

being shuffled through the ETC free radicals are being produced that are ultimately reduced by molecular oxygen to water. However, if the ETC is blocked at a middle point of the reaction the radicals never reach the end point and are released (reviewed in Cassarino & Bennett, 1999). Free radicals are linked to being responsible for oxidation of cellular constituents like proteins, lipids, and DNA (reviewed in Cassarino & Bennett, 1999). During normal aging processes ETC efficiency is decreased and reactive oxidants like peroxide and superoxide radicals increase (reviewed in Cassarino & Bennett, 1999). This may lead to further mtDNA degradation because mtDNA lacks protective components like histones (reviewed in Cassarino & Bennett, 1999). It is possible that these low levels of radicals may cause further mtDNA mutation in a person with preexisting mutations causing them to be predisposed to a neurodegenerative disease (reviewed in Cassarino & Bennett, 1999).

Many diseases with varying pathologies have been linked to mtDNA mutation. Further understanding of genes and specific mutations may lead to therapies. While mtDNA can be very difficult to study in many species, yeast offer researchers a tremendous opportunity for study.

Preliminary Studies

FMP35 Discovery

FMP35 is a novel gene with very little known about its function with mitochondria. This experiment will be a preliminary characterization as to how this gene may function. FMP35 was first sequenced from the IX chromosome in 1997 (Churcher *et al.*, 1997). The gene was first referred to as YIL157C for its placement on the chromosome. The "C" at the end of this nomenclature is of importance as it denotes the genes placement on the crick strand of DNA. The gene is known to be 594 base pairs (bp) in length that runs from nucleotide 46949 to 47542 on the crick strand. The gene is translated into a protein containing 197 amino acids. In 2004, experimentation led to localization of the gene product to *S. cerevisiae's* mitochondria. In unpublished communication to the *Saccharomyces* Genome Database the name was then changed to FMP35 to illustrate the localization of this protein to the mitochondria (Rehling *et al.*, 2004). Since its discovery FMP35 has been found in few publications. I hope that this study will start to characterize this gene as playing an important role in mitochondrial genome stability.

FMP35 Interactions

Interactions among proteins have been well identified. More recently these interactions have been looked upon with increasing importance. For example, Abf2p is known to play a significant role in nucleoid stability. Other proteins like Kgd2p have been found to be closely associated with Abf2p. When Kgd2p is knocked out nucleoid stability is markedly decreased (Kaufman *et al.*, 2000). This is just one example of how a protein-protein interaction may play an important role in DNA stability. These

interactions have been utilized in two hybrid assays. Fmp35p has been associated with two reported assays. First, Fmp35p was pulled out of a two hybrid assay when Atp14 was used as bait (Ito *et al.*, 2001). ATP synthase is responsible for the synthesis of ATP from ADP and P_i (reviewed in Arselin *et al.*, 1996). Atp14 is a gene that encodes for the h subunit of ATP synthase (Arselin, 1996). It is difficult to draw conclusions from such studies; however, they are invaluable in identifying possible pathways in which a particular protein might participate.

Through a yeast two-hybrid assay Fmp35p has also been found to interact with Ilv5p (Mirando, 2004). The ramifications of this interaction are of particular interest to this study because of Ilv5p's purposed function. Ilv5p has been shown to be required in the process that controls mtDNA divisions into nucleoids (MacAlpine, *et al.*, 2000). Ilv5p encodes for acetohydroxy acid reductoisomerase (Petersen, *et al.*, 1983) and in concert with the general amino acid control pathway regulates the number of nucleoids present in a cell as well as distribution within the cell (MacAlpine, *et al.*, 2000). This may indicate the importance of Fmp35p in this or closely associated functions.

Sumoylation of Fmp35p

Sumoylation is a post-translational modification that a protein undergoes much like phosphorylation, acetylation, methylation, etc. Other molecules known as ubiquitin-like polypeptides have been shown to regulate aspects of cell cycle progression, protein degradation, cell polarity, and morphogenesis (reviewed in Wykoff & O'Shea, 2005). Small ubiquitin-related modifier (SUMO), as the name implies, is much like ubiquitin. The molecule functions by covalently binding to lysine residues in the target protein (Johnson, 2004). While the SUMO-protein interaction pathway has been defined the

outcome of this interaction can vary greatly depending on the substrate (Johnson, 2004). Although the effects of this interaction can vary it should be highlighted that within these different types of interactions proteins modified with SUMO can have implications on further protein-protein interactions. In *S. cerevisiae* SMT3 encodes for SUMO. The isopeptidase Ulp1 processes SUMO while the conjugation to a protein requires an E1 heterodimer (reviewed in Wykoff & O'Shea, 2005). Proteins associated with the septin complex were originally characterized as being modified by SUMO (reviewed in Wykoff & O'Shea, 2005). Experiments that have mutated lysine residues in the septin complex have shown that cells continue to divide (reviewed in Wykoff & O'Shea, 2005); however, this is not always the case. The polymerase sliding clamp is essential for processivity that is associated with polymerase. When Pol30 is mutated and replaces a lysine with an arginine it has implications on the site essential for SUMO but also inhibits ubiquitilation. These cues control DNA replication versus error-free DNA repair (Hoege, *et al.*, 2002). In total 82 proteins were found to be associated with SUMO in Wykoff and O'Shea's (2005) experiment. Fmp35p was among these proteins. This implies that Fmp35p undergoes a level of control that is not found with all proteins.

FMP35 Bioinformatics

The advent of bioinformatics has given researchers unprecedented information and speed in which information is obtained. An *in silico* approach is invaluable to obtaining preliminary information about a gene of interest. Basic Local Alignment Search Tool (BLAST) and multiple sequence alignment were used to discover orthologs that may exist. This may yield an understanding of what Fmp35p's role is in yeast.

FMP35 published sequence was obtained from the *Saccharomyces Genome Database* (Appendix II). This sequence has been used in recent BLAST searches using the National Center for Biotechnology Information (NCBI) databases. Searches were performed on both the nucleotide sequence (blastn) and the translated nucleotide sequence (tblastx). The "nr" (non-redundant) database was chosen for each search. The nucleotide searches yielded very little homology with other genes in the database. However, the translated search proved that the protein sequence shared a degree of homology with other organisms (Appendix II).

Homologene is another part of the NCBI website that allows for quick reference to related proteins found in other organisms. A query of an accession number or protein name will initiate the program to output other proteins in its database that may be considered to be homologous (Appendix II). As of October 2005 the program contained over 160,000 gene groups. If other related proteins are identified and characterized more fully it may guide further study of this protein. Currently few organisms (other yeast species and fungus) with a protein homologous to Fmp35p have been identified using this program (Appendix II).

Collection of other information through use of many other programs has also been completed in an effort to further understand what this gene and protein product look like, its composition, and how it may be related to other known genes/proteins. DNAStar is a program suite that is composed of sequence analyzing programs. Selected material that describe the gene's base composition and amino acid composition and be found in Appendix II. Programs like this aid in many aspects of a study, but restriction enzyme

site identification was of particular importance during the planning of the cloning strategy (described in Materials and Methods).

Construction of Respiration Loss Rate Test

The respiration loss rate assay is used to first identify strains of *S. cerevisiae* that have lost mitochondrial function. Identification of these mutants is important so that a frequency of this phenotype can be established and compared to the wild type frequency obtained under the same conditions. The assay utilizes a mixture of two different media to obtain growth of colonies under different conditions. YPD media contains a six-carbon source (dextrose) so that yeast are able to ferment; breaking dextrose down to CO_2 and ethanol. YPG media consists only of glycerol, three-carbon source. Yeast are only able to respire on media containing this hydrocarbon. In a respiration loss assay YPG media is combined with 0.1% dextrose. This mixture allows all (mutated and non-mutated) yeast to grow until the dextrose is exhausted. At this point the strains able to respire continue to grow while the strains that lack wild type mitochondrial function remain a distinguishably smaller colony (Koprowski *et al.*, 2002, reviewed in Phandis and Sia, 2004). All large wild type colonies are counted, as are the petite colonies that lack mitochondrial function. A change in the respiration phenotype is quantified as a frequency. In repeated trials a median from each trial is averaged and compared to the wild type frequency.

Construction of Direct Repeat Recombination Assay (EAS reporter strain)

This study focuses on the phenomenon of recombination. Large sections of mitochondrial DNA can be deleted through a direct-repeat mediated deletion (reviewed in Phadnis, 2005). These deletions have been linked to various diseases and tend to occur

in an age dependent manner in humans (reviewed in Phadnis, 2005). A reporter gene was constructed using $arg8^m$ and the COX2 gene. Arg8m is a derivative of ARG8 nuclear encoded gene; however, this gene can be expressed in the mitochondria (reviewed in Sia, 2000). In this reporter $arg8^m$ is inserted after the first 99bp of the COX2 gene (Phadnis, 2005). This construct was placed into cells with ARG8 nuclear deletion so that the yeast are able to grow on Arg- medium due to $arg8^m$ expression; however, respiration is not possible due to the COX2 mutation. In the instance of a direct repeat mutation the arg8m gene is deleted and the COX2 gene is again complete giving the cell a Arg^-, respiration $^+$ phenotype.

Construction of Microsatellite Assay (CAB reporter strain)

Microsatellite stability has also been looked upon as a possible pathway causing changes in phenotype. The microsatellite assay uses a frameshift reporter to measure the rate of alteration within the mitochondrial microsatellites. Microsatellites have a lower degree of stability due to an increased frequency of expansions or deletions (reviewed in Sia, 2000). For this assay the reporter gene was constructed by inserting out of frame poly(AT) or poly(GT) tracts within the *cox*3 gene. This *cox*3 gene is also fused with $arg8^m$ (Sia, 2000). This gene is a derivative of the *ARG8* gene that can be expressed within the mitochondria (reviewed in Sia, 2000). This results in $arg8^m$ being out of frame (it is held in the 2+ position). Strains with the genomic *ARG8* deletion and the gene construct are phenotypically Arg^- therefore they are unable to grow in media lacking arginine. However, if there is a change within the microsatellite region the $arg8^m$ gene will be read back in frame and the yeast will now be Arg^+. The mutation rate can then be visualized by phenotype and calculated by fluctuation analysis.

Materials and Methods

Construction of fmp35Δ::URA3 Cassette

URA3 was utilized as a selective gene so that future colonies that grew in the absence of uracil in the media could be identified as more likely containing the gene knockout. To construct the knockout, primer sequences coding for FMP35 were utilized in conjunction with URA3 coding region. The upstream primer encoded for a portion of FMP35 at the 5' end and concluded with URA3 code (5'-GGA TAT TTA CAG ACA AAT AGC ACC ATA CTT GCT ATT AAA TTG GTG TCT TGG | CAG ATT GTA CTG AGA GTG CAC C-3') (Integrated DNA Technologies). The downstream primer also included a portion of FMP35 and URA3 sequence (5'-CTC TCC GTA CCA GTC CAC GAC ATA AAA GTA AAA GAT AAA ACA GAC TAT AC|C TGT GCG GTA TTT CAC ACC GC-3') (Integrated DNA Technologies). These primers were important as they were utilized in a reaction with pRS406. This plasmid contains URA3 and the above primers contain homology to this gene for annealing during PCR.

The total PCR reaction was completed with a master mix for 10 reactions. A single reaction is described by the following components. Platinum Pfx polymerase and accompanying reagents (Invitrogen) were utilized in developing the construct. 5μl of 10x Pfx PCR buffer was added to a tube that contained 40μl of dH_20. 1.5μl of dNTP (10mM) was then added to the reaction. 1μl of $MgSO_4$ (50mM) was then added. 0.5μl of the Sense and Antisense primer (20μM) were both added to the reaction. 1μl of the template pRS406 was then added to the mixture. Lastly 0.5μl of Pfx Platinum Polymerase was added and the reaction was mixed by vortex and aliquoted into 10 different tubes for thermocycling. The thermocycler was run on a gradient program to distinguish the best

temperature for future reactions. The first cycle of the reaction was 94 °C for 30 seconds; a denaturing step. Second an annealing temperature was reached. Due to the gradient program one reaction was at each of the following temperatures (°C): 50.5, 51.3, 52.7, 54.4, 56.4, 58.3, 60.3, 62.6, 64.0, and 64.7. Finally an extension phase of 72 °C for 2 minutes activated the polymerase. These cycles were repeated 30 times.

Transformation Into Yeast Strains

The fmp35Δ::URA3 cassette was transformed into three different strains of baker's yeast. DFS188 is a wild type strain that was utilized in screening assays for rho phenotype. EAS748 and CAB193 were utilized in characterization assays. 15µl of carrier DNA (salmon sperm) was added to 100µl of yeast. 20µl of PCR product was then added and the contents were mixed. The mixture was incubated at 37°C on a shaker at ~200rpm for 30 minutes. 1ml of 40% PEG/0.1M LiOAC/TE was added and mixed to the tube. The mixture was incubated at 30°C on a shaker at ~200rpm for 30 minutes. The mixture was then incubated at 45°C for 15 minutes. The contents were then plated onto appropriate media. The end result of this process was yeast that contained the disrupted FMP35 gene within the plasmid. Due to the homology of approximately 50 bp the plasmid and genomic DNA would undergo recombination and integrate disrupted FMP35 into the genome.

Whole Cell PCR

To evaluate integration of the fmp35Δ::URA3 into the three strains whole cell PCR was utilized. An upstream primer FMP35 F-88 (5'-GTG GAG AGA AAG AAA GCG C-3') (Integrated DNA Technologies) was employed along with a downstream primer FMP35 R+227 (5'-CAG CTA TTC GCT TTC TCC TC-3') (Integrated DNA

Technologies) to evaluate the insertion of the knockout within the yeast species. A resulting band of ~315bp was interpreted as yeast having an intact FMP35 gene. Yeast containing the knockout of this gene did not produce a band. This was due to incompatibility to the downstream primer. The PCR started with 25µl of BioMix Red and 1.5µl of each primer (20µM each). The template was added by transferring a small amount of a colony to the reaction tube. The reaction was placed into a thermocycler which was set for 94°C for 2 minutes. This denaturing cycle was then followed by 10 rounds of 94°C for 15 seconds, 55°C for 15 seconds, and finally an extension cycle at 72°C for 1 minute.

A 1% agarose gel was used to visualize DNA. 0.50g of agarose was weighed and poured into a sterile bottle. 50ml of 1x TBE was measured and added to the same bottle. The mixture was heated in a microwave on a medium power setting for 1 minute. The bottle was stirred and careful attention was paid that it had not boiled out of the bottle. The bottle was placed back into the microwave and heated until a the agarose was in solution (approximately 20 seconds). The bottle was allowed to cool at room temperature for approximately 10 minutes. 5µl of ethidium bromide was added to the solution and the bottle was gently stirred. The mixture was poured into a gel mold and cooled at 6°C for at least 10 minutes. While the gel was cooling a specimen was generated for agarose gel visualization. The mixture contained 3µl of 6x loading dye, 5µl of PCR product, and 10µl of H_2O.

Once the gel had solidified it was placed into a chamber filled with 1x TBE. 5µl of 1kb ladder was added to the first lane of the gel and any sample created above was added to the remaining lanes. The chamber was closed and the power supply was

attached. The gel was run between 85 and 95 volts until satisfactory separation was seen with a ultraviolet light source.

Respiration Loss Assay

The respiration loss assay was designed to illustrate the phenotypic change from a wild type rho+ cell to rho-. The assay uses YPG media plus 0.1% dextrose. This allows colonies to be displayed independent of rho+ or rho- to phenotype. However, after the small amount of the six-carbon source is consumed only the rho+ colonies continue to grow. The rho- colonies remain small (petites) and can be distinguished from the wild type yeast due to size difference.

Yeast was then streaked on a YPD plate so that single colonies could be distinguished. This YPD plate was incubated at 30°C for three days. Twenty colonies were sterilely chosen at random and individually placed into separate aliquots of 100µl dH_2O. The colony was well vortexed and 5µl was transferred to 500µl of dH_2O. This mixture was again well vortexed and 5µl of this dilution was diluted yet again into 500µl of dH_2O. Finally 100µl of this mixture was plated onto YPG +0.1% dextrose media and incubated for at least three days at 30°C. A total of 20 plates were used; one plate representing each colony chosen.

Once the incubation period was completed the colonies were counted. Wild type and petite colonies were tallied separately. Finally a percentage was calculated and reported. This assay was completed twice in the same manner.

Direct Repeat Recombination Assay

The EAS748 strain of yeast was used for this assay. Yeast were initially plated onto SD-ARG plates to ensure that all colonies chosen for the assay were ARG+

colonies. The plate was incubated overnight at 30°C. Colonies were chosen and streaked onto a SD-URA plate so that single colonies could be isolated for the assay. This plate was incubated at 30°C for three days. 20 single colonies were chosen from this plate and placed into separate aliquots of 100µl dH_2O. The colony was well mixed by vortex. 5µl of this specimen was then transferred to another aliquot of 500µl dH_2O. This tube was again vortexed well to mix. 5µl of this sample was transferred again to another aliquot of 500µl dH_2O. This tube was again vortexed to mix. 50µl of the original tube was plated onto a YPG plate for each colony chosen. 100µl of the final dilution was plated onto YPD media for each colony chosen. All plates were incubated for three days at 30°C.

Growth was counted after the allotted incubation. All colonies from all plates were tallied. This assay was completed three times in the same manner.

Microsatellite Assay

For this assay the CAB193 strain of knockout was used. The yeast was originally plated onto SD-URA to ensure that the fmp35Δ::URA3 knockout was present. This plate is incubated overnight at 30°C. Yeast was chosen from this plate and streaked onto another SD-URA plate and incubated at 30°C for 3 days so that single colonies can be chosen. 20 single colonies were chosen from this plate and placed into separate aliquots of 100µl dH_2O. A bench vortex was used for all mixing steps. 5µl of this specimen was then transferred to another aliquot of 500µl dH_2O and mixed. 5µl of this sample was transferred again to another aliquot of 500µl dH_2O and mixed. 95µl of the original tube was plated onto a SD-ARG plate for each colony chosen. 50µl of the final dilution was plated onto YPD media for each colony chosen. All plates were incubated for three days at 30°C.

After the allotted incubation colony growth was counted. All colonies from all plates were tallied. This assay was completed once.

Mutation Rate

To interpret the above fluctuation assays it is imperative to quantitatively assign a mutation rate to the knockout strain. These calculations are based on work carried out by Lea and Coulson (1949). The mutation rate (mean number of mutations) is determined by the median number of mutations observed per selective plate.

Sample Data Set (Recombination Assay 04/08/2005)

Plate #	Control (Colony Count)	Experiment (Colony Count)
1	221	26
2	124	28
3	261	35
4	189	37
5	262	43
6	226	47
7	189	48
8	189	50
9	147	54
10	164	58
11	260	67
12	345	78
13	261	79
14	272	104
15	153	116
16	154	118
17	228	119
18	382	126
19	261	173
20	281	716

1st average: 228.5 ½ average: 114.2 2x average: 456.9

Colonies that fall below ½ average and above 2x average counts are omitted. A second average is calculated without counting the omitted plates. In this case no plates

need to be omitted therefore the average remains 228.5. The median is 62.5 this number is used to calculate r_o.

R_o=median/the dilution factor

In this case the dilution factor is ½ because 50µl of the undiluted specimen was plated onto YPG media. $R_o = 125$.

Next r_o/m is calculated. First Lea and Coulson supplied a table in their paper that allows for m (estimated average number of mutational events). To do the table supplies a r_o value of 117 and a r_o/m value of 4.5. 117 is subtracted from the calculated r_o value above, 125. 8 is then divided by 15 and multiplied by 0.1.

$8/15 = 0.53 \quad 0.53*0.1 = 0.053$

This number is then added to 4.5 from the table yielding 4.55. To calculate m r_o is divided by r_o/m.

$m = 125/4.55 = 27.47$

The second average is then divided the dilution factor of the control. The average number of mutational events (m) is then divided by this number. The result is the number of mutations per cell division.

$228.5/(1/10000) = 2.29 \times 10^6$

$27.47/2.29 \times 10^6 = 1.20 \times 10^{-5}$

In this sample experiment 1.20×10^{-5} mutations per cell division were calculated. This can then be compared to wild type experiments to determine if there is a change in mutational events when these conditions are present.

PCR of wild-type FMP35

To obtain copies of wild type FMP35 wild-type DFS strain of yeast was used. Wild-type DNA template was obtained by plasmid isolation. 5.0ml of a yeast culture in LB was grown overnight. The culture was centrifuged at maximum speed for two minutes and the supernatant was decanted. The pellet was resuspended in 500µl of H_2O and transferred to a 1.5ml tube. The tube was spun and the supernatant was decanted. 200µl of Southern Lysis Buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM TrisCl, 1mM EDTA) was added and the tube was mixed by inversion. 200µl of phenol:chloroform:isoamyl alcohol (25:24:1) was then added. Approximately 0.3g of acid washed beads was also added to the mixture. The tube was vortexed for three minutes. 200µl of TE buffer was then added. The tube was centrifuged for five minutes at maximum speed (approximately 13,000 rpm). The aqueous layer was transferred to a fresh tube without disturbing the white interface. 1ml of 100% ethanol was then added to the tube and mixed by inverting the tube 6 to 8 times. The mixture was allowed to incubate at –20°C for one hour. The tube was then centrifuged for two minutes at maximum speed. The supernatant was discarded and the pellet was resuspended in 400µl of TE plus 0.5µl of Rnase (63mg/ml). The mixture was incubated for 30 minutes at 37°C. 500µl of phenol was added and the tube was vortexed. The mixture was centrifuged for five minutes at maximum speed. The aqueous phase was transferred to a fresh tube. 10µl of 4M ammonium acetate was added along with 1ml of 100% ethanol. The tube was inverted 6 to 8 times to mix and incubatyed at –20°C for one hour. The tube was centrifuged for 2 minutes and the supernatant was discarded. The resulting pellet air dried and was resuspended in 30µl TE.

To amplify and add cloning restriction sites primers encoding both FMP35 and the appropriate restriction site were used (5'-GGA ACC GGG AAT TCC CAC GAC ATA AAA GTA AAA G-3' and 5'-CGG AAC CGA AGC TTC AGA CAA ATA GCA CCA TAC-3'). 5µl of 10x Accu buffer was added to 34µl of H_2O in a fresh tube. 1µl of MgCl2 (50mM), 5µl of dNTP (10mM), 1.5µl of each primer (20µM), and 1µl of a 1:50 dilution of template were all added to the tube. The tube was vortexed briefly and 1µl of polymerase was added. The first cycle of the reaction was 94 °C for 30 seconds; a denaturing step. Second an annealing temperature was reached 55°C. Finally an extension phase of 72 °C for two minutes activated the polymerase. These cycles were repeated 30 times.

Digestion of Vector and Insert

To further prepare a gene that could be introduced into yeast the newly generated FMP35 gene had to be ligated into an appropriate vector. Both the insert and the vector, pRK2 were digested with HindIII and EcoRI. To digest the plasmid 10µl of the plasmid was placed into a fresh tube along with 15µl of H_2O. 3µl of compatible 10x buffer and 1µl of each enzyme was then added and the tube was gently mixed. The tube was incubated at 37°C for two hours. 20µl of the PCR product was digested by adding 4µl of H_2O, 3µl of the 10x buffer, and 1.5µl of each enzyme. This mixture was incubated at 37°C for two hours.

Ethanol precipitation was used to isolate the digested DNA. 200µl of H_2O was added to each tube. 20µl of 3M NaAC was also added along with 600µl of 95% ethanol. Each tube was mixed by inversion and then vortexed for three minutes. The tubes were placed into –80°C for 30 minutes. After incubation the samples were centrifuged at

maximum speed for 10 minutes. The supernatant was carefully removed and a wash of 70% ethanol was added to the tube. The samples were again centrifuged for five minutes. After the centrifugation the supernatant was removed and the tubes were dried by vacuum. Finally the samples were resuspended in 20μl of H_2O.

Ligation of Vector and Insert

Ultimately it is important that the insert and vector be joined into one molecule for transformation. To do this a ligation protocol is used. 10μl of PCR product and 10μl of vector are added into one tube. 2μl of 10x ligase buffer was then added. Finally 1μ of DNA ligase was added and the tube is gently mixed. The tube was incubated at 14°C overnight.

Bacterial Transformation

To amplify the copies of the newly created wild-type gene joined with pRK2 the plasmid is put into *Escherichia coli*. While in *E. coli* the plasmid will be replicated many times as the culture grows. Two different methods were used to transform the bacteria. The *E. coli* used in these protocols are not ampicillin resistant. If the transformation is successful the plasmid carries a resistant ampicillin gene and will allow the bacteria to grow in the presence of the antibiotic. This affords one the opportunity to choose colonies that are likely to contain the plasmid.

The first method of bacterial transformation is electroporation. A brief electrical pulse is used to temporarily open the membrane of electrically competent cells. 40μl of competent cells were mixed with 1μl of the ligation reaction. The mixture was transferred to a cuvette compatible with electroporation. The electroporator was set to 1450V and the cuvette was placed into the apparatus. The electroporation was initiated

and the duration of electrocution was recorded as four milliseconds. 1ml of LB was immediately added to the cuvette and the mixed. The content of the cuvette was transferred to a fresh tube and the *E. coli* were incubated at 37°C for one hour. The cells were transferred to an LB+AMP plate and incubated at 37°C overnight. Electroporation is a very high yield method of bacterial transformation. At times the efficiency can be so high that single colonies cannot be distinguished on the plate.

A lower yield bacterial transformation method can result in fewer colonies growing which allows an easier selection of single colonies. 200µl of chemically competent cells were aliquotted into a tube (1 x10^8 to 5x10^8 cells). 10µl of the ligation reaction was added to the tube, mixed, and incubated for 30 minutes on ice. The tube was then transferred to a heat block and heated to 42°C for exactly two minutes. The cells were returned to ice for one to two minutes. 1ml of LB was added to the mixture and it was incubated at 37°C for 15 minutes. The tubes were centrifuged to collect the bacteria and the supernatant was removed leaving approximately 200µl remaining in the tube. The pellet was resuspended and plated onto an LB+AMP plate. The plate was incubated overnight at 37°C.

Plasmid Isolation and Digestion

To ensure that the ligation worked, it was necessary to isolate the plasmid from the bacteria in which it was transformed and digest the resulting plasmid. ClaI and BamHI were used as diagnostic restriction enzymes. When the proposed plasmid with insert is digested with these enzymes the result should be two fragments that can be visualized on a 1% agarose gel. One fragment should be approximately 5 kb while the other should be approximately 1.5 kb.

1.5ml of a yeast culture in LB was grown overnight. The culture was centrifuged at maximum speed for two minutes and the supernatant was decanted. The pellet was resuspended in 100µl of GTE. 200µl of 0.2M NaOH/SDS was added and incubated at room temperature for five minutes. 400µl of phenol:chloroform:isoamyalcohol (ratio 25:24:1) was added and the mixture was centrifuged at maximum speed for five minutes. Approximately 800µl was of supernatant was transferred to a fresh tube and 1ml of 100% ethanol was added. The tube was vortexed and incubated at –20°C for ten minutes. The tube was then centrifuged at maximum speed for five minutes at room temperature. The supernatant was discarded and the remaining pellet was washed with 200µl of 70% ethanol. After centrifugation the ethanol was removed and the pellet was further dried under vacuum. Finally the pellet was resuspended in 50µl of RNase in TE (100µg/ml) and incubated at 37°C for 15 minutes.

Since ClaI and BamHI are enzymes that react in very different buffers a two step digest had to be completed. ClaI was used as the first enzyme. 14µl of H_2O, 3µl of the plasmid, 2µl of the ClaI 10x buffer, and1µl of ClaI were all mixed in a tube and incubated for one hour at 37°C. After incubation 6µl of H_2O, 3µl of BamHI 10x buffer, and 1µl of BamHI was all added to the tube. The tube was mixed and incubated for 1.5 hours at 37°C.

Once a clone was complete the mutated DFS strain would have been transformed with the clone. A series of respiration loss assays would have been completed to calculate a respiration loss frequency. I would expect that the frequency would return to the wild type rate of 0.3%. This preliminary study does not include the completed cloning strategy.

Results
FMP35 Knockout

The intent of the following steps was to first introduce a marker gene (URA3) into the position where wild-type FMP35 exists. This was possible because of homologous recombination. Different models have been used to illustrate this phenomenon; however, there are common traits to all recombination rationales. First base pairs of homology (in this case the 50 base pairs of FMP35, see figure 7) allow for strand invasion. The newly introduced strand associated with the plasmid is introduced into genomic DNA after it has been nicked. Once the homologous regions have base paired a junction forms. This junction is mobile and can introduce further upstream or downstream DNA into the wild-type genome. Finally the junction is resolved by enzymatic activity. The final product of this process is possibly a genome that contains the intended marker gene. Any future attempts by the cell to transcribe the wild-type gene will only cause the marker gene to be expressed. Therefore in this case FMP35 will no longer be expressed in these cells.

To determine the effects of a genome that no longer contained functioning FMP35 it was first necessary to ensure that the gene was not expressed. Once the construct was created and transformed into the yeast; analysis was run via whole cell PCR. Primers used in this reaction allow for amplification of FMP35; therefore, yeast that did not contain an

FMP35 knockout in DFS188

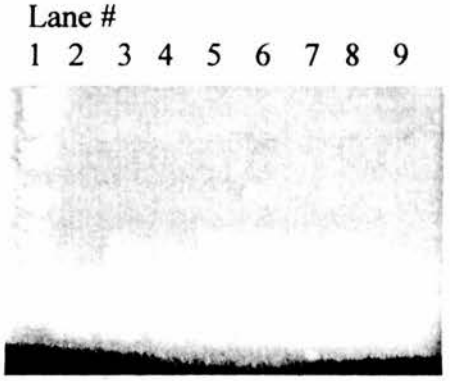


Figure 8: PCR of DFS strains shown on a 1% agarose gel. Specimens without a band at ~350 bp are knockout strains cataloged for assay testing. Lane 1contains a 1kb ladder. Strain #9-16 shown in sequential order starting in lane #2.

expected band of approximately 350 base pairs (bp) will not produce Fmp35p. In the DFS188 strain eight specimens were analyzed, three of which contained the knockout (specimens: 9, 11, and 16-See figure 8). Ten CAB193 transformants were examined. Six of specimens proved to contain the knockout (specimens: 3, 6, 7, 8, 9, and 10-See figure 9). A total of ten EAS748 transformants were also looked at using this same procedure. Four specimens were obtained that were incapable of producing a band (specimens: 1, 2, 5, and 6-See figure 9).

FMP35 knockout in EAS748 and CAB193

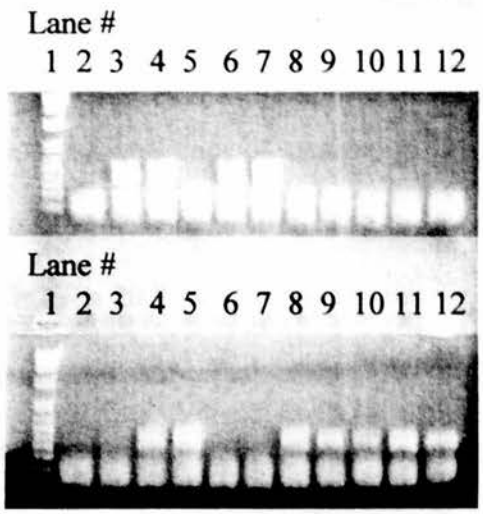


Figure 9: PCR of CAB and EAS strains shown on a 1% agarose gel. Specimens without a band at ~350 bp are knockout strains cataloged for assay testing. Lane 1contains a 1kb ladder. Top portion contains CAB strain #1-10 shown in sequential order starting in lane #3. Bottom portion contains EAS strain #1-10 starting in lane #2. Lane #2 on top portion is a DFS positive control. Lane #12 on the bottom portion is a test whole cell PCR specimen.

Respiration Loss Assay

Initially it is important to monitor a respiratory phenotype. If a phenotype exists then it validates further fluctuation assays to determine the impact of the knockout in yeast. Fmp35p was found to interact with Ilv5p; a protein known to be essential in the mitochondria. It is then possible to deduce that FMP35 may also be essential for functioning mitochondria. If there is a change in respiration phenotype then FMP35 may play an important role in mitochondrial genome stability. The DFS188 fmp35Δ::URA3 specimens were monitored for mitochondrial function using a respiration loss assay. This assay measured the capability of the yeast to thrive on medium that contains a

fermentable carbon source of only 0.1%, but contains 2% glycerol for respiratory proficient yeast. Cells that contain functional mitochondria will be able to grow and form colonies that are large in size. Cells that contain dysfunctional mitochondria will only be able to consume the dextrose within the media. Once this six-carbon source is exhausted the growth of the yeast colonies will cease leaving a smaller colony. These two types of colonies (wild type and petite) can be tallied. The result is a frequency of respiration loss. A significant increase in respiratory loss was found with the knockout. The fmp35Δ::URA3 yielded a respiration loss of 6.2%. This is a 20.7 fold increase to the known wild type figure of 0.3% (Appendix II). This increase shows that among FMP35 knockout strains it is more likely to find a colony that is respiratory deficient.

To further characterize how this phenotype may arise other reporter strains with the same knockout were examined. Direct repeat recombination and microsatellite stability was a focus of this description of the FMP35 knockout.

Direct Repeat Recombination

Recombination of wild type mitochondrial DNA (mtDNA) is imperative for daughter cell survival. Mitochondria that receive DNA that contain large additions or deletions may yield a ρ- phenotype. This phenotype denotes a mitochondria that is unable to respire. A large deletion may splice out many essential genes for respiration. It is also possible that any addition present may shift essential genes out of frame or separate the gene from other essential sequences like promoters. The construct utilizes the COX2 gene, which is separated in two parts with an $ARG8^{m}$ gene. Specimens that do not recombine remain respiratory deficient and are able to grow in the absence of arginine due to the $ARG8^{m}$ gene that separates the COX2 gene. While specimens that do

recombine excise the $ARG8^m$ gene and the COX2 gene can be translated correctly. The cells now become respiratory proficient and cannot grow in the absence of arginine. Selective media is utilized to visualize these changes. To ensure that the cells utilized in this assay are ARG+ they are grown on media lacking arginine prior to starting the assay. YPD and YPG plates are utilized to select for respiratory phenotypes in the assay.

This assay effectively characterized the FMP35 knockout condition. Through fluctuation analysis wild type strain displays 8.15×10^{-5} deletion events per cell division. The EAS178 containing the FMP35 disruption displayed 1.24×10^{-5}. This is a significant 6.6 fold decrease in recombination (Table 1).

Microsatellite Stability

This assay measures the "stability of polymerase during replication" along a GT rich tract within the COX3 gene. It is essential that polymerase enzymes copy all regions with high fidelity. Enzymes that lack this ability will cause frameshifts in new strands of DNA. Genes that are essential for normal mitochondrial function may be displaced by a polymerase that cannot replicate a region with high GT content. This region also holds the $ARG8^m$ out of frame by 2 base pairs. In this scenario yeast containing this mtDNA are unable to live on media lacking arginine. However, if polymerase slippage does occur within this poly GT tract it is possible that bases will be deleted ultimately bringing $ARG8^m$ back into the correct reading frame. Once this occurs the cells will be able to flourish on media lacking arginine.

Fluctuation analysis on the wild type reporter strain yielded polymerase slippage that led to the frameshift 2.25×10^{-6} times per cell division. The FMP35 mutation led to an

increase in polymerase slippage to 3.36×10^{-6} per cell division. This is a smaller and unrepeated 1.5 fold increase in the knockout (Table 1).

Discussion

Currently, it is increasingly important to analyze mitochondrial DNA (mtDNA) stability. Dysfunction of this organelle is known to cause disease, some of which are terminal. There are 750 different proteins found within the mitochondrial proteome. It is important to further characterize how each of these proteins contribute to mtDNA stability. I propose that the gene FMP35 encodes a protein that is essential for mtDNA stability, viability, and respiration. This study outlines the general and specific mitochondrial phenotypes of *Sacharomyeces cerevisiae* that lack FMP35.

Many similarities among the mitochondrial proteome exist between *S. cerevisiae* and humans. Just in replication many orthologues are found. In yeast Mip1p is the catalytic subunit of the polymerase responsible for mtDNA replication. In humans h-Pol γ is the gene responsible for the same subunit (Lecrenier & Foury, 2000). The relevance of FMP35 to higher eukaryotes is not immediately clear. This is a novel gene found through yeast two-hybrid assay using Ilv5p as bait. ILV5 has been described as playing a vital role in mtDNA stability; however, FMP35 has yet to be fully understood. In BLAST searches proteins with high a degree of homology have only been identified in lower organisms (Appendix II). This could simply be because an ortholog has yet to be discovered in higher eukaryotes. It is also possible that a gene with little homology in amino acid sequence may still serve in a similar pathway as Fmp35p.

PCR was used to obtain a fmp35 knockout construct that utilized URA3 as a marker gene. The constructs contained a portion (approximately 50bp) of FMP35 on each end of the URA3 gene. This served as a site for homologous recombination so that the knockout could be introduced into each nuclear genome at the native FMP35 site.

Using this PCR generated knockout cassette, three strains of yeast were transformed (DFS188, EAS748, and CAB193). DFS188 was used in the respiration loss assay, which illustrated the respiratory deficient phenotype. The EAS748 strain was used in the direct repeat recombination assay; allowing us to see a decrease in recombination. The CAB193 strain was used in the microsatellite assay, further describing a possible role that the knockout may have in polymerase slippage events. PCR was utilized to ensure that the knockout cassette was in place of the wild type gene. Once these specimens were isolated the knockout strains were further analyzed.

Wild type yeast gave rise to dysfunctional mitochondria or petite colony formations at a frequency of 0.3%. There was a significant increase to 6.2% when the FMP35 gene was not present. Cells were more frequently unable to complete respiratory processes and resulted in a ρ- phenotype. The respiration loss assay measures this frequency. The unaffected yeast colonies grew to a large, wild type size by continuing to metabolize glycerol through normal processes. However, in colonies that consisted of ρ- yeast the mitochondria could not utilize glycerol and were only able to grow to a petite colony until the dextrose was exhausted in the media (see Figure 2). The increase in frequency proves that FMP35 plays a role in mitochondrial function and possibly mitochondrial genome stability or mitochondrial morphology. Further experiments were carried out to determine an exact molecular method of mutation.

Two other assays were undertaken to further look at the effects of a genome that lacked the experimental gene. Microsatellite instability and direct repeat recombination have both been described as mechanisms that can severely impact mtDNA and mitochondrial function. Polymerase slippage during replication is more prevalent along

mircrosatellite tracts of GT or AT rich regions that are found within mtDNA. If slippage occurs it may result in a frameshift mutation that causes genes within the genome to be nonfunctioning. Regions of the mitochondrial genome have homologous regions. During recombination events large portions of the genome can be excised causing the loss of potentially essential genes. Separate assays were used to measure each mechanism.

The microsatellite instability assay showed a slight 1.5 fold increase in instability. This result could implicate Fmp35p as an essential part of the mechanisms in place to control mtDNA stability during polymerase activity. To be statistically certain of this possibility the assay will need to be retested. During stretches of repeating nucleotides DNA polymerase may replicate the compliment strand with an additional base pairing or deletion. In this example a GT tract was used however CT, CA, or a number of other tracts could yield the same result. The resulting change could cause genes to fall out of frame. AGT encodes for a methionine, a signal at the beginning of most proteins. For example the following sequence shows an adenine in bold, the start of the reading frame (ie. +1) ...aatccagc**A**GT.... If the preceding sequence changes the base number that once was occupied by an adenine could be changed to a guanine simply because of the shift in our case ...aatccagca**G**T... or ...aatccag**C**AGT.... In both of these cases the AGT is now found at –1 or +2 respectively and the corresponding gene will not be transcribed into a functional mRNA.

The result of the direct repeat recombination assay seemed to merit some additional investigation. After three separate trials of this assay the average recombination event occurred 1.24×10^{-5} per cell division, a 6.6 fold decrease in

recombination compared to wild type. This indicates that Fmp35p may play an active role in recombination. Without this gene present the appropriate Fmp35p protein is not transcribed and translated. When this occurs the mitochondrial genome does not recombine appropriately. This may have drastic effects on the amount of genomic material that gets segregated to each daughter mitochondria. Other genes like CCE1 and ABF2 have already been implicated in creating problems during recombination and segregation. After replication and during some fusion events mitochondria are required to recombine at points of homology and the resulting junction must be resolved appropriately so that entire genomes can now become separated and segregate into daughter mitochondria. After replication the D-loop is typically where recombination takes place. Ultimately it is imperative that daughter mitochondria contain the appropriate compliment of mtDNA, if this does not occur the organelle is unable to function. The mitochondrion is an essential organelle for eukaryotes that utilize oxidative phosphorylation and other biochemical reactions contained within this organelle to sustain life. Without the appropriate genes present in mitochondria, respiratory enzymes and ribosomes necessary for chemical reactions will not be created. Inevitably the cell will die, because these essential proteins are not present.

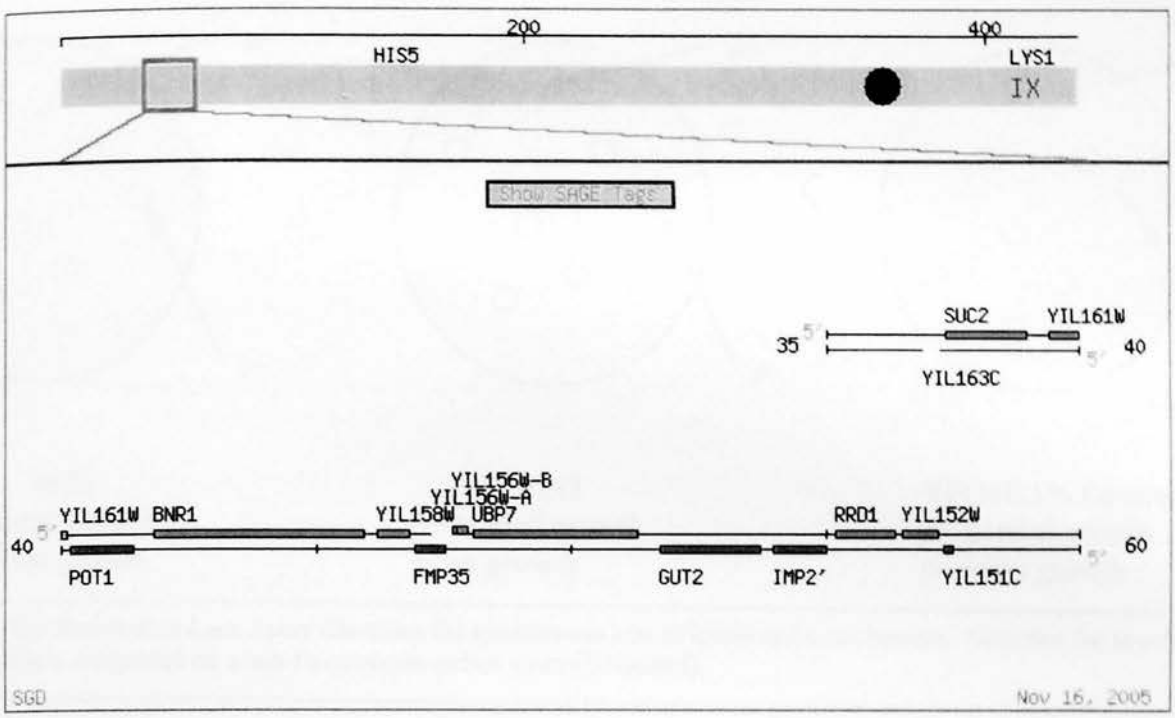
FMP35 overlaps another gene, YIL156W-A. When the knockout cassette was generated it disrupts both genes. I have begun utilizing a strategy that would further prove that FMP35 is the sole cause for the respiratory phenotype and assay results presented. The cloning strategy will need to be evaluated due to the dubious open reading frame (ORF) that is present on the Watson strand in this region (see Figure 1). During homologous recombination FMP35 gene is replaced with fmp35Δ::URA3,

essentially the URA3 marker gene. While this task has been completed the process has also displaced YIL156W-A. This is listed as a dubious ORF on *Saccharomyces* Genome Database (SGD). A dubious ORF is unlikely to be a reading frame that is used to transcribe mRNA for translation into a protein; however, a start codon (ATG) does exist and there is a substantial stretch of base pairs without a stop codon (TAA, TAG, TGA) being present until base pair 402. The region has been analyzed by BLAST and other bioinformatics techniques. The gene does not display homology with any known nucleotide or protein sequence. Though this region does not appear to be a concern it should be considered when FMP35 is under scrutiny. This cloning strategy would amplify wild type FMP35. This gene would be digested with restriction enzymes that would allow the gene to be cloned into a plasmid with compatible digested ends. After many copies of this plasmid containing the wild type gene are generated in *E. coli* the plasmids would be procured and transformed into the knockout strains. The end result of a successful cloning attempt would be yeast strain that contains wild type FMP35 but not a complete YIL156W-A. Ultimately the yeast could now create Fmp35p, but not any possible protein that YIL156W-A may create. I would predict that assay results using these "wild type" strains would return to the wild type numbers reported in this thesis.

FMP35 is most likely the gene responsible for the reported results through out these studies. The gene is essential for stable yeast containing functional mitochondria. Through this first characterization of FMP35 I have been able to report a respiration loss phenotype, changes in microsatellite stability as well a significant change in direct repeat recombination. It is most likely that this gene will be an important part of a pathway responsible for appropriate recombination and segregation of replicating mitochondria.

Appendix I: FMP35 Gene Maps and Assay Figures

Partial Map of Chromosome IX



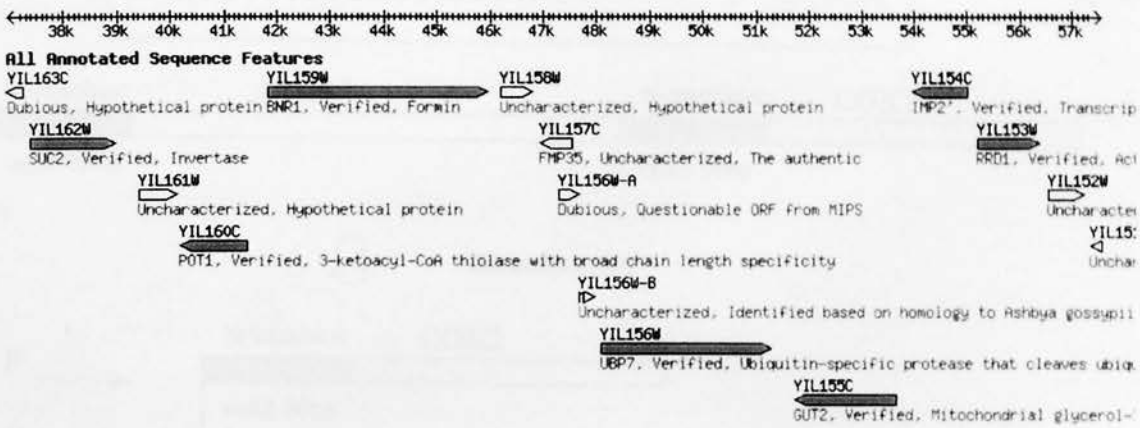


Figure1: These maps display the position of FMP35 (YIL157C) as found on chromosome IX. FMP35 is found on the Crick strand from nucleotide 47542 to nucleotide 46949. YIL156W-A can be found overlapping FMP35 on the Watson strand. (www.yeastgenome.org)

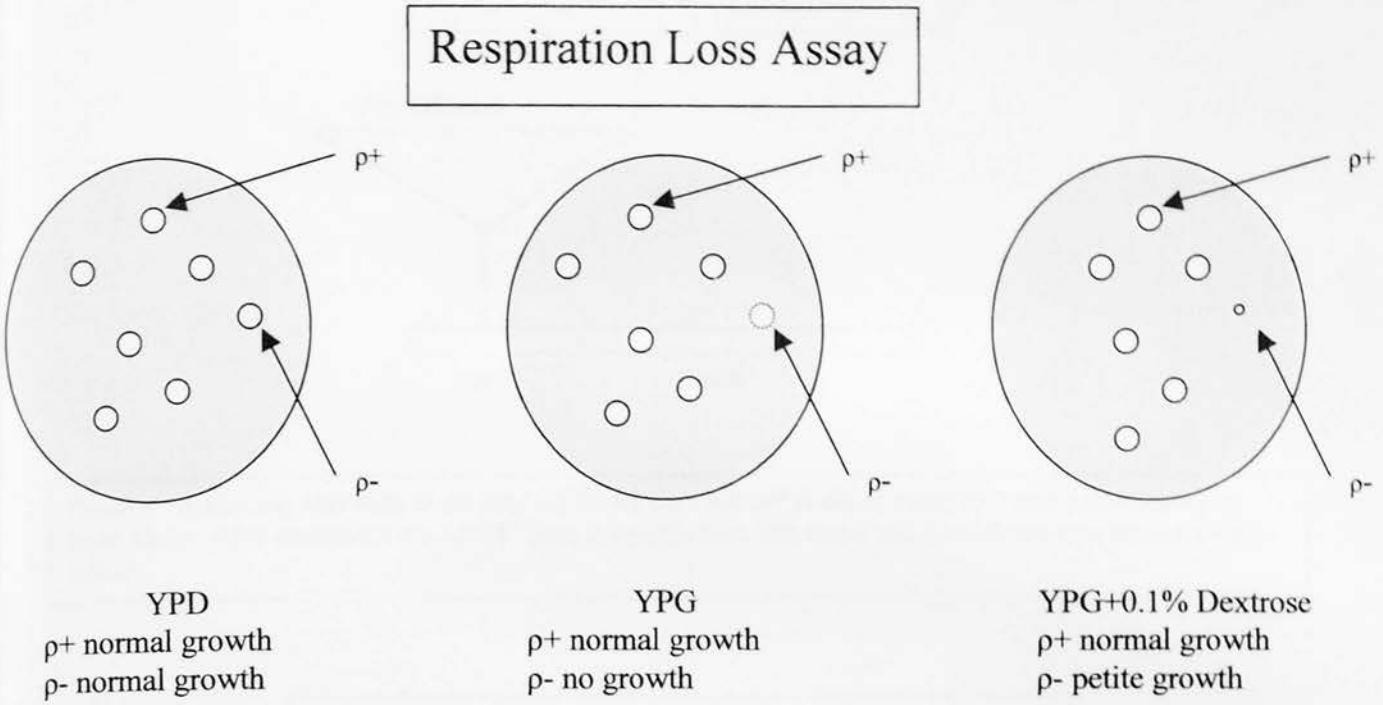


Figure 2: The Respiration Loss Assay illustrates the spontaneous loss of functional mitochondria. Selection for non-functioning mitochondria is completed on a non-fermentable carbon source (Glycerol).

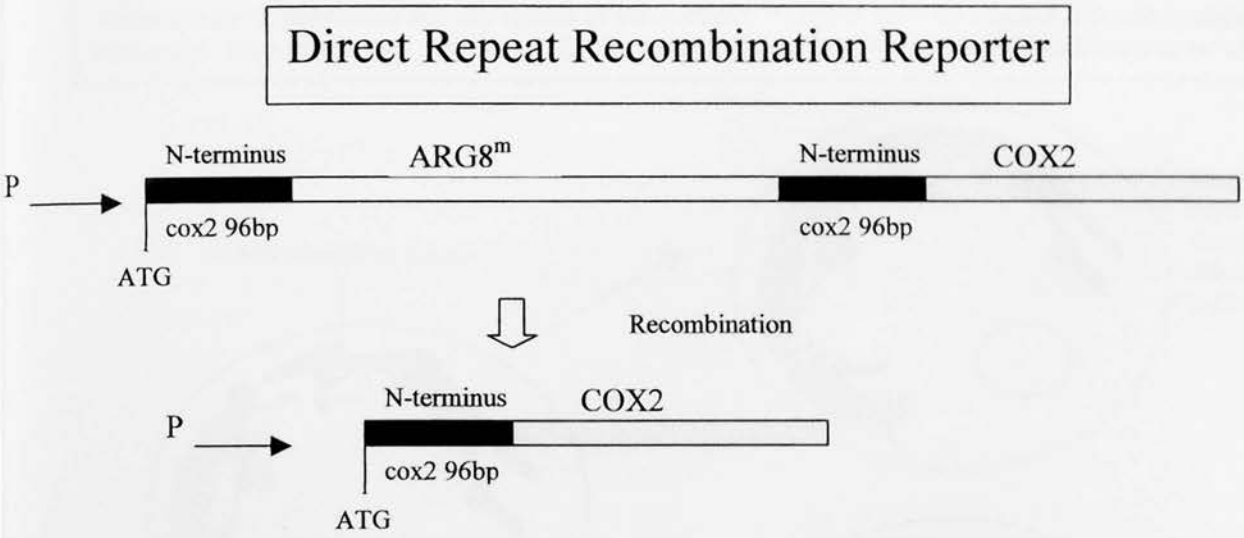


Figure 3: The Direct Repeat Recombination Assay is used to illustrate an increase or decrease in homologous recombination. With the $ARG8^{m}$ gene in place the yeast is ARG+ but unable to respire. In this condition the yeast will not grow on YPG. However, after recombination the $ARG8^{m}$ gene is removed and the yeast is now ARG- and is now able to respire with the complete COX2 gene. Growth is present on YPG.

Microsatellite Reporter

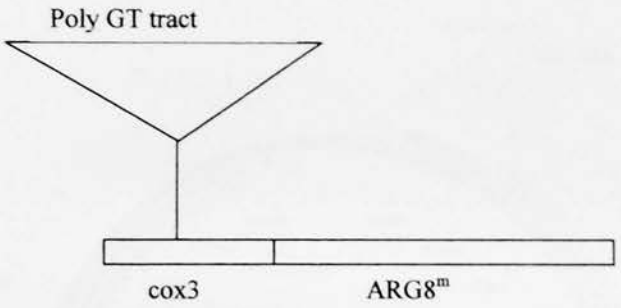


Figure 4: Before any alteration to the poly GT or AT tract $ARG8^{m}$ is out of frame by 2 base pairs making the yeast ARG-. After a mutation the $ARG8^{m}$ gene is brought back into frame and the cells are now phenotypically ARG+.

Mutated Mitochondrial Morphology

Figure 5: The figure below represents a wild type yeast cell and what daughter cells may look like after inappropriate recombination and segregation of the nucleoid. This will leave the mutated cell with a unique phenotype. Such phenotypes include inability to respire and poor distribution of mitochondria along the cells

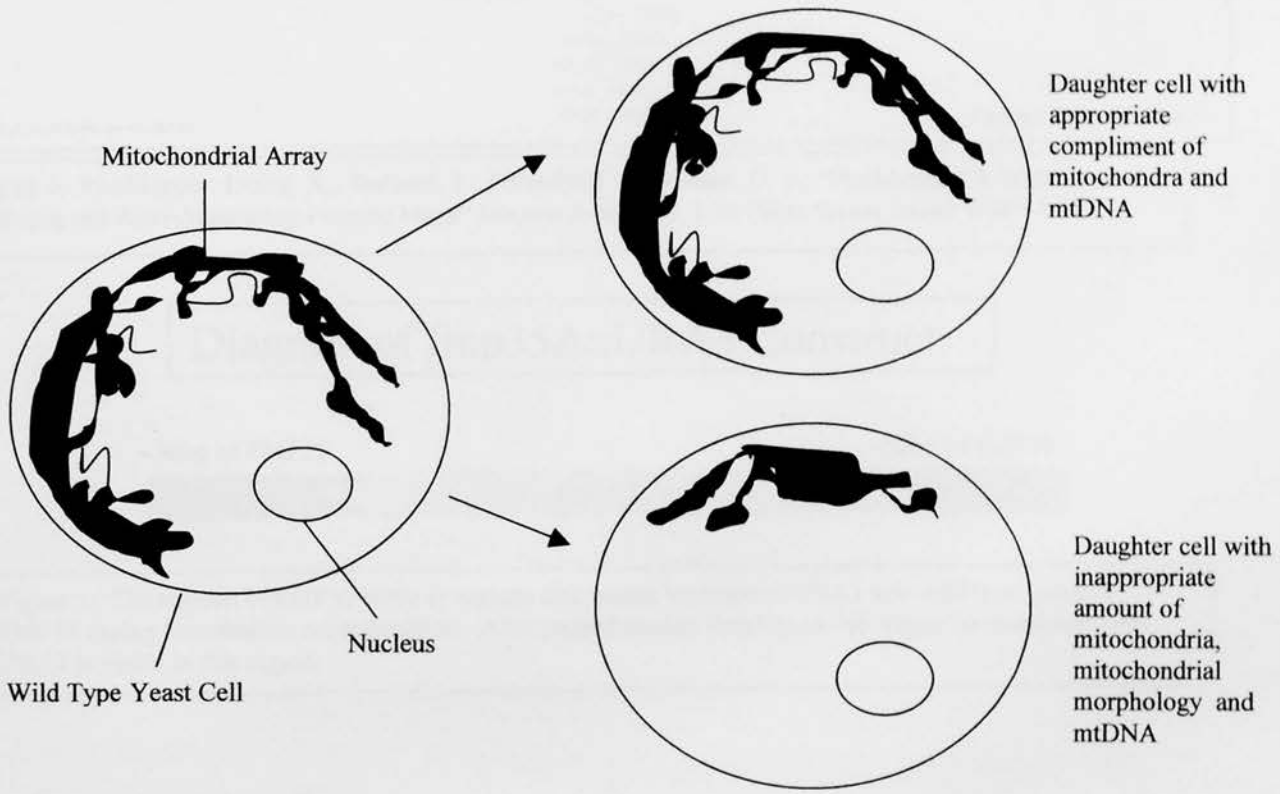


Figure of Plasmid pRS406

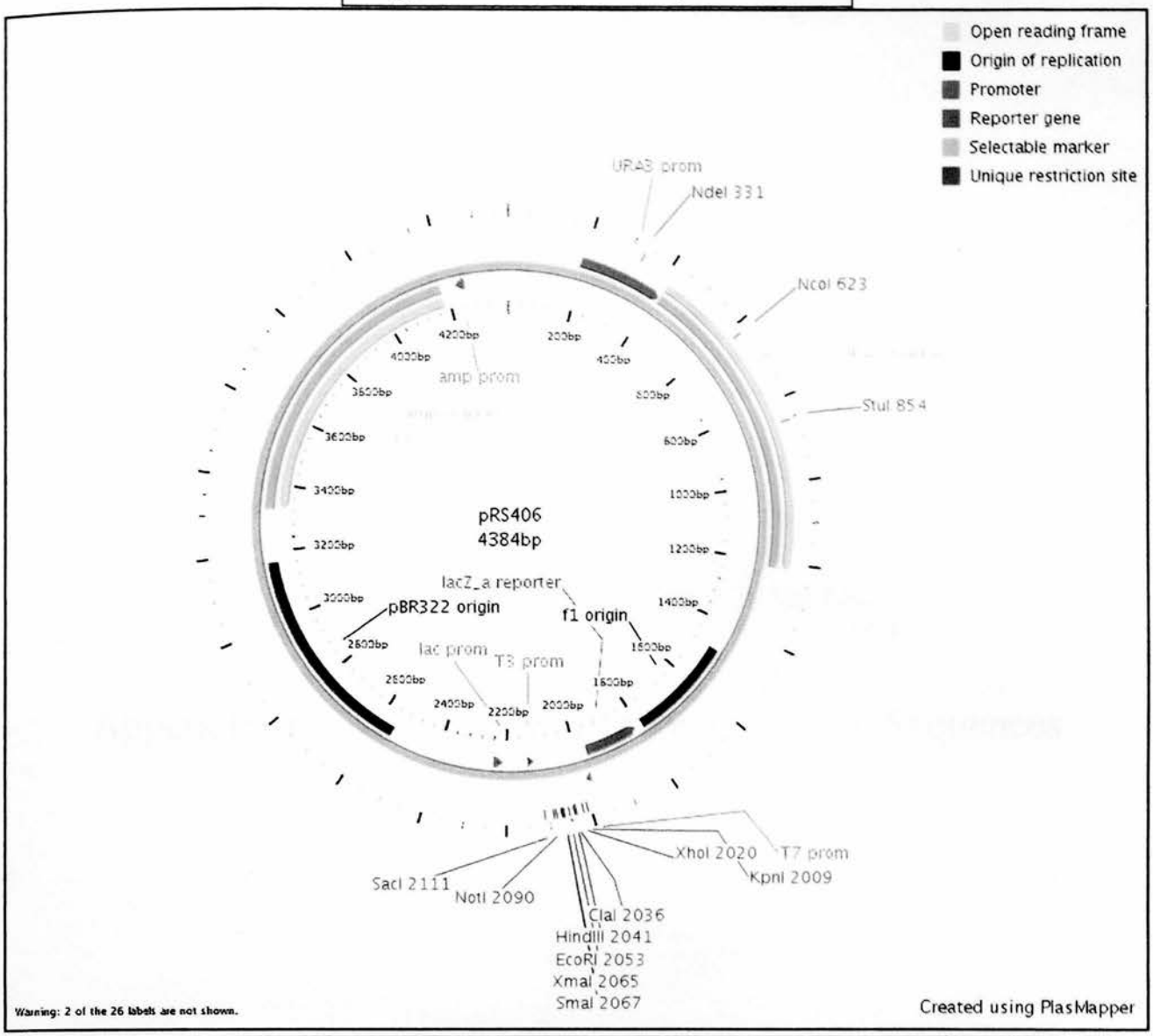


Figure 6: PlasMapper: Doing, X.; Stothard, P.; Forsythe, I. J.; Wishart, D. S.; "PlasMapper: A Web Server for Drawing and Auto-Annotating Plasmid Maps" Nucleic Acids Res. 1:32 (Web Server Issue) W660-4 2004

Diagram of fmp35Δ::URA3 Construct

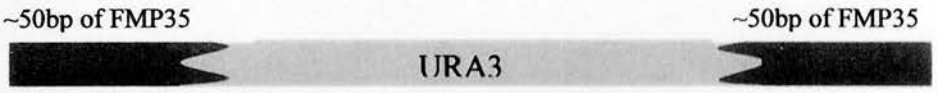


Figure 7: The regions of FMP35 serve as regions that permit insertion of URA3 into wild type genomic FMP35 during homologous recombination. After recombination Fmp35p can no longer be translated; only URA3 is viable in this region.

Appendix II: FMP35 and Other Related Gene Sequences

FMP35 Sequence i

5' – 3' Crick Strand

ATGATGTTAAGACTAGTAACTAAAGGGTTGCCGAAAGTGACACCTTCTGCAGCTAAGGCTGTG
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AAGATGAGGCCAAGATCGTTTTGAAAGACAAGAACAGGCCATTGAGAATTGACAGAGAATTG
CCTGACCCAACCACGGAGAGGAGAAAGCGAATAGCTGGATTTTTGTTGTTTTCTGTGGCCATT
GGGTCCGCACTATCTTTGATCTTCAACTATGAAAAGACGGAGTCCCCCATTATCTCCAACACG
CTGTATTATATAAGACGGTCTCCAGCAACTAAAAACATACTGGGTGAGAGCATAGAATTTGAT
GGTATCATCCCTTGGGTCTACGGTGAACTGAACTCTGTGAAGGGTAGGATTAACATTACGTTT
TATATCAAGGGGGACAAAAACGTTACTGGAACCGTTAGACTGGTTGCCGATAGGAACACTCA
CGACGAAGAGTTTTTGATTCATGAATGGAGCGTCACTGCCGCTGGCCAAAAGATCGATTTGCT
AGCTGAAAATACCAAGACACCAATTTAA

YIL157W-A

5' – 3' Watson Strand

ATGGCCACAGAAAACAACAAAAATCCAGCTATTCGCTTTCTCCTCTCCGTGGTTGGGTCAGGC
AATTCTCTGTCAATTCTCAATGGCCTGTTCTTGTCTTTCAAAACGATCTTGGCCTCATCTTCAGC
GACACTATTATTGAATCTAGCGCTGGTAGAGAATGAGTGCAGCAAGGAACCACGCACTAGCA
CAGCCTTAGCTGCAGAAGGTGTCACTTTCGGCAACCCTTTAGTTACTAGTCTTAACATCATGTA
TAGTCTGTTTTATCTTTTACTTTTATGTCGTGGACTGGTACGGAGAGAAAGATCGAACTGTTTT
AAAACTGGGATTAAAATGACAAGAAGGCGCTTTCTTTCTCTCCACAATGATCAAAACAAGAA
CAAGCAAAACGCAAAGAGATGA

FMP35 + 1,000 Bases Upstream and Downstream Sequence

5' – 3' Watson Strand

ATAGTGAAGACTTAGTATTTTATGTAGTTGTGGAGCTTATATAAAGAAATGGACAATATTCGC
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CCTGCCAAGAAAAAACCGCCAAGGCAGGGTACACACCAGCATACAGAAGGCAATTGAACGCT
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ACGATAAAAATAAAATAATAATAAAGGATATTTACAGACAAATAGCACCATACTTGCTATTA
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GATCAAAGATAGTGCGGACCCAATGGCCACAGAAAACAACAAAAATCCAGCTATTCGCTTTC
TCCTCTCCGTGGTTGGGTCAGGCAATTCTCTGTCAATTCTCAATGGCCTGTTCTTGTCTTTCAA
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CAGCAAGGAACCACGCACTAGCACAGCCTTAGCTGCAGAAGGTGTCACTTTCGGCAACCCTTT
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TCCACAATGATCAAAACAAGAACAAGCAAAACGCAAAGAGATGACATTAGTAAGTACCCAAA
TGAGCTACTAACAACGCATCCGGTAATACTAACAAGAGAAATTGGTTAGGTAGGGAAACTAG

TACACATATCCATAGATTTGGTCTTGGTATCAACGTGCCTCGCGGGGATTAAACGCAATACAG
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CTATACAGATCTAAAAAAAGATGTATGAGAATGAAATGAACATGACCAATGTTGTGCTTATGGT
GAAGAAAGAAATCGGTGTGGTCCTAGATGAATCGGTTAAACACGGGGCCGGTATTGAGCACA
GAATCACCAAGAA

FMP35 gene reverse compliment
FMP35/YIL157W-A overlap
YIL157W-A gene

Fmp35p Amino Acid Sequence

MMLRLVTKGLPKVTPSAAKAVLVRGSLLHSFSTSARFNNSVAEDEAKIVLKDKNRPLRIDRELPDP
TTERRKRIAGFLLFSVAIGSALSLIFNYEKTESPIISNTLYYIRRSPATKNILGESIEFDGIIPWVYGELN
SVKGRINITFYIKGDKNVTGTVRLVADRNTHDEEFLIHEWSVTAAGQKIDLLAENTKTPI (stop)

Modified MegaBLAST Hit Table from NCBI

BLASTN 2.2.12 [Aug-07-2005]
Query: FMP35
Database: nr
Fields: query id, subject ids
1 hits found: 1_29531 gi|557761|emb|Z38059.1|SC4357
% identity: 100.00
Alignment Length: 594
Mismatches: 0
Gap opens: 0
Q. start: 1
Q. end: 594
S. start: 8571
S. end: 7978
E-value: 0.0
Bit score: 1142

TBLASTX Results from NCBI

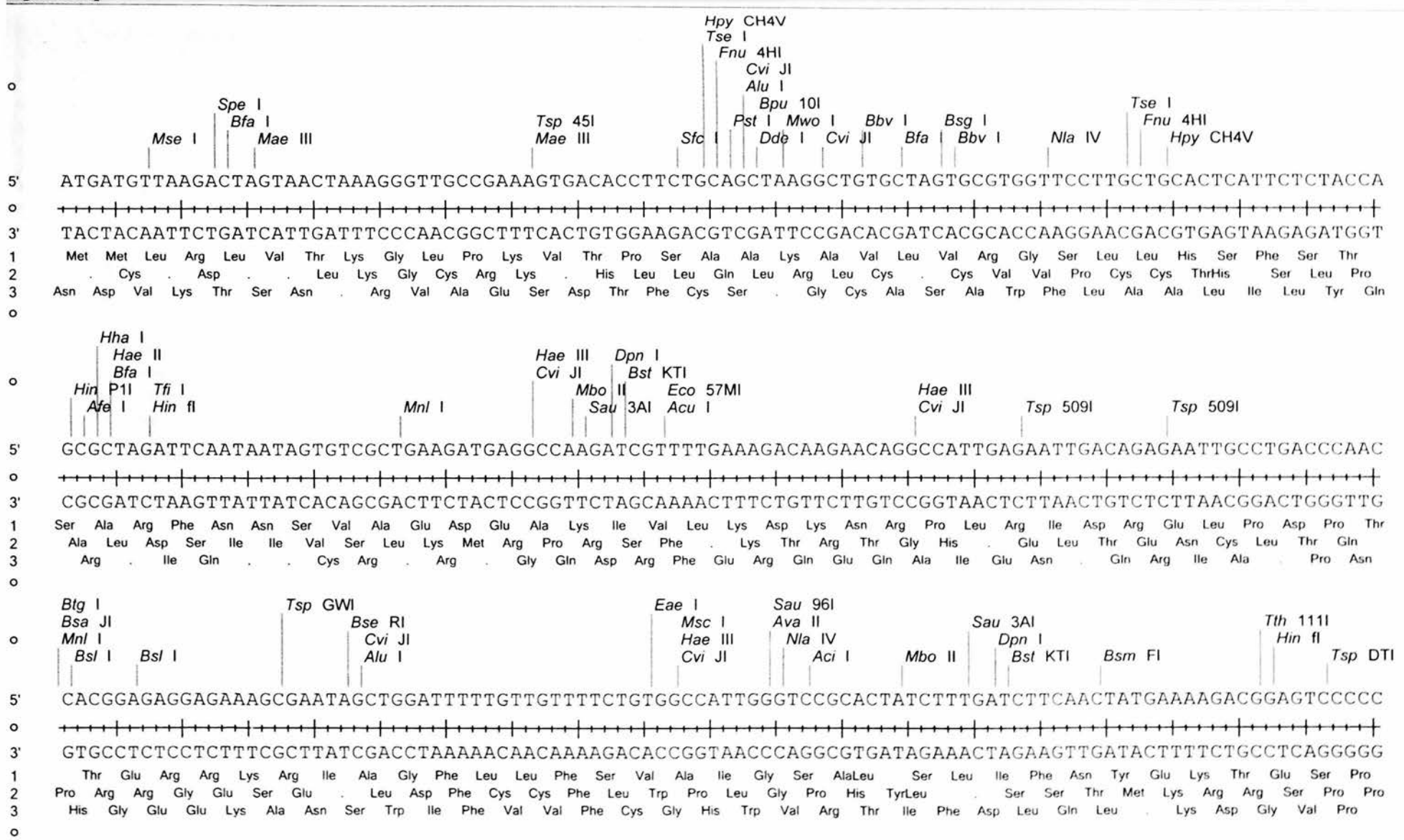
gi|557761|emb|Z38059.1|SC4357 S.cerevisiae chromosome IX lambda
gi|50289316|ref|XM_447089.1| Candida glabrata CBS138, CAGL0H0674
gi|49526114|emb|CR380954.1| Candida glabrata strain CBS138 chrom
gi|49644219|emb|CR382126.1| Kluyveromyces lactis strain NRRL ...
gi|50311110|ref|XM_455579.1| Kluyveromyces lactis NRRL Y-1140, K
gi|51895502|gb|AE016819.2| Ashbya gossypii (= Eremothecium go...
gi|47074816|ref|NM_211201.1| Eremothecium gossypii AFR299Wp (AFR
gi|68477574|ref|XM_712007.1| Candida albicans SC5314 putative...
gi|68477735|ref|XM_711927.1| Candida albicans SC5314 putative...
gi|70994895|ref|XM_747132.1| Aspergillus fumigatus Af293 hypo...
gi|50421816|ref|XM_459466.1| Debaryomyces hansenii CBS767, DEHA0
gi|49654999|emb|CR382137.1| Debaryomyces hansenii chromosome ...
gi|67528201|ref|XM_656819.1| Aspergillus nidulans FGSC A4 hyp...

gi|39968838|ref|XM_365810.1| Magnaporthe grisea 70-15 chromosome
gi|32413107|ref|XM_327033.1| Neurospora crassa strain OR74A
gi|50555647|ref|XM_505232.1| Yarrowia lipolytica CLIB99, YALI0F1
gi|49650673|emb|CR382132.1| Yarrowia lipolytica chromosome F ...
gi|30349756|emb|AL732383.1|CNS08C8W Leptosphaeria maculans : ...
gi|46109319|ref|XM_381718.1| Gibberella zeae PH-1 strain PH-1;
gi|28881344|emb|BX284751.1|NCB24N11 Neurospora crassa DNA linkag
gi|77020686|gb|AC152826.2| Mus musculus BAC clone RP23-111C21...
gi|75677735|gb|AC116569.6| Mus musculus BAC clone RP23-59O19 ...
gi|63025448|gb|AC159265.2| Mus musculus BAC clone RP23-462H20...
gi|21436704|emb|AL611984.15| Mouse DNA sequence from clone RP...
gi|62526245|gb|AC102448.15| Mus musculus chromosome 3, clone RP2
gi|50759549|ref|XM_425758.1| PREDICTED: Gallus gallus similar...

HomoloGene Results from NCBI

S. cerevisiae	FMP35	The authentic, non-tagged protein
K. lactis	mRNA gene	mRNA gene KLLA0F1095g
E. gossypii	AFR299Wp	AFR299Wp
M. grisea	hypothetical	hypothetical protein
N. crassa	hypothetical	hypothetical protein

Sequences were taken from Saccharomyces Genome Database (www.yeastgenome.com) and verified by BLAST (www.ncbi.nlm.nih.gov)


Hpy CH4V
Tse I
Fnu 4HI
Cvi JI
Alu I
Bpu 10I
Spe I
Bfa I
Tsp 45I
Pst I
Mwo I
Bbv I
Bsg I
Tse I
Fnu 4HI
Mse I
Mae III
Mae III
Sfc I
Dde I
Cvi JI
Bfa I
Bbv I
Nla IV
Hpy CH4V
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3' TACTACAATTCTGATCATTGATTTCCCAACGGCTTTCACTGTGGAAGACGTCGATTCCGACACGATCACGCACCAAGGAACGACGTGAGTAAGAGATGGT
1 Met Met Leu Arg Leu Val Thr Lys Gly Leu Pro Lys Val Thr Pro Ser Ala Ala Lys Ala Val Leu Val Arg Gly Ser Leu Leu His Ser Phe Ser Thr
2 . Cys . Asp . . Leu Lys Gly Cys Arg Lys . His Leu Leu Gln Leu Arg Leu Cys . Cys Val Val Pro Cys Cys ThrHis Ser Leu Pro
3 Asn Asp Val Lys Thr Ser Asn . Arg Val Ala Glu Ser Asp Thr Phe Cys Ser . Gly Cys Ala Ser Ala Trp Phe Leu Ala Ala Leu Ile Leu Tyr Gln
Hha I
Hae II
Bfa I
Hae III
Dpn I
Cvi JI
Bst KTI
Hin P1I
Tfi I
Mbo II
Eco 57MI
Hae III
Afe I
Hin fI
Mnl I
Sau 3AI
Acu I
Cvi JI
Tsp 509I
Tsp 509I
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3 Arg . Ile Gln . . Cys Arg . Arg . Gly Gln Asp Arg Phe Glu Arg Gln Glu Gln Ala Ile Glu Asn . Gln Arg Ile Ala . Pro Asn
Btg I
Bsa JI
Mnl I
Bsl I
Bsl I
Tsp GWI
Bse RI
Cvi JI
Alu I
Eae I
Msc I
Hae III
Cvi JI
Sau 96I
Ava II
Nla IV
Aci I
Mbo II
Sau 3AI
Dpn I
Bst KTI
Bsm FI
Tth 111I
Hin fI
Tsp DTI
5' CACGGAGAGGAGAAAGCGAATAGCTGGATTTTTGTTGTTTTCTGTGGCCATTGGGTCCGCACTATCTTTGATCTTCAACTATGAAAAGACGGAGTCCCCC
300
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2 Pro Arg Arg Gly Glu Ser Glu . Leu Asp Phe Cys Cys Phe Leu Trp Pro Leu Gly Pro His TyrLeu . Ser Ser Thr Met Lys Arg Arg Ser Pro Pro
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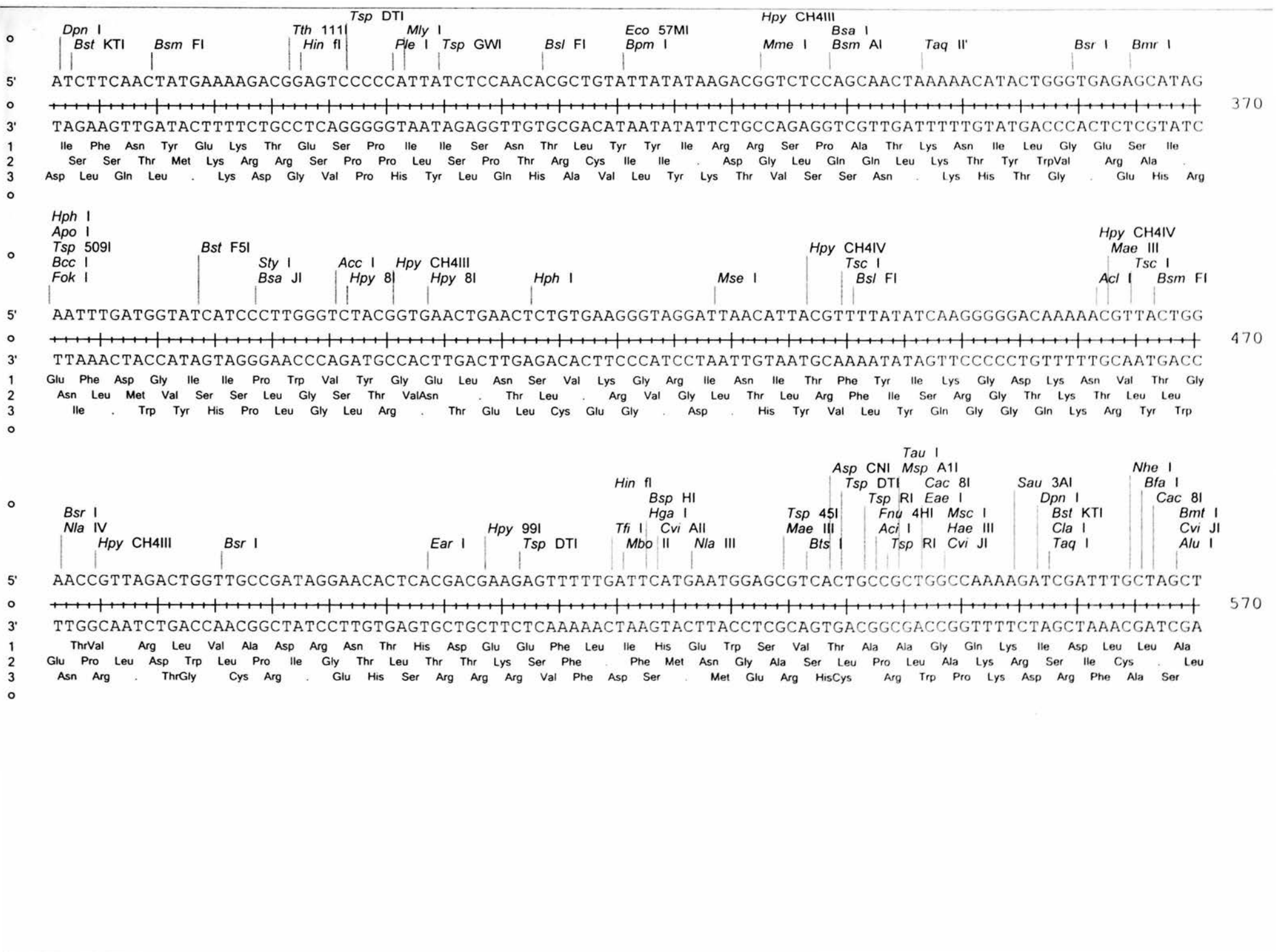
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Ple I
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Hpy CH4III
Mme I
Bsa I
Bsm AI
Taq II'
Bsr I
Bmr I
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2 Ser Ser Thr Met Lys Arg Arg Ser Pro Pro Leu Ser Pro Thr Arg Cys Ile Ile . Asp Gly Leu Gln Gln Leu Lys Thr Tyr TrpVal Arg Ala .
3 Asp Leu Gln Leu . Lys Asp Gly Val Pro His Tyr Leu Gln His Ala Val Leu Tyr Lys Thr Val Ser Ser Asn . Lys His Thr Gly . Glu His Arg
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Apo I
Tsp 509I
Bcc I
Fok I
Bst F5I
Sty I
Bsa JI
Acc I
Hpy 8I
Hpy CH4III
Hpy 8I
Hph I
Mse I
Hpy CH4IV
Tsc I
Bsl FI
Hpy CH4IV
Mae III
Tsc I
AcI I
Bsm FI
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2 Asn Leu Met Val Ser Ser Leu Gly Ser Thr ValAsn . Thr Leu . Arg Val Gly Leu Thr Leu Arg Phe Ile Ser Arg Gly Thr Lys Thr Leu Leu
3 Ile . Trp Tyr His Pro Leu Gly Leu Arg . Thr Glu Leu Cys Glu Gly . Asp . His Tyr Val Leu Tyr Gln Gly Gly Gln Lys Arg Tyr Trp
Tau I
Asp CNI
Msp A1I
Hin fI
Tsp DTI
Cac 8I
Sau 3AI
Nhe I
Bfa I
Bsp HI
Tsp RI
Eae I
Dpn I
Cac 8I
Bsr I
Nla IV
Hga I
Tsp 45I
Fnu 4HI
Msc I
Bst KTI
Bmt I
Hpy CH4III
Bsr I
Ear I
Hpy 99I
Tsp DTI
Tfi I
Cvi AII
Mae III
Aci I
Hae III
Cvi JI
Mbo II
Nla III
Bts I
Tsp RI
Cla I
Taq I
Alu I
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570
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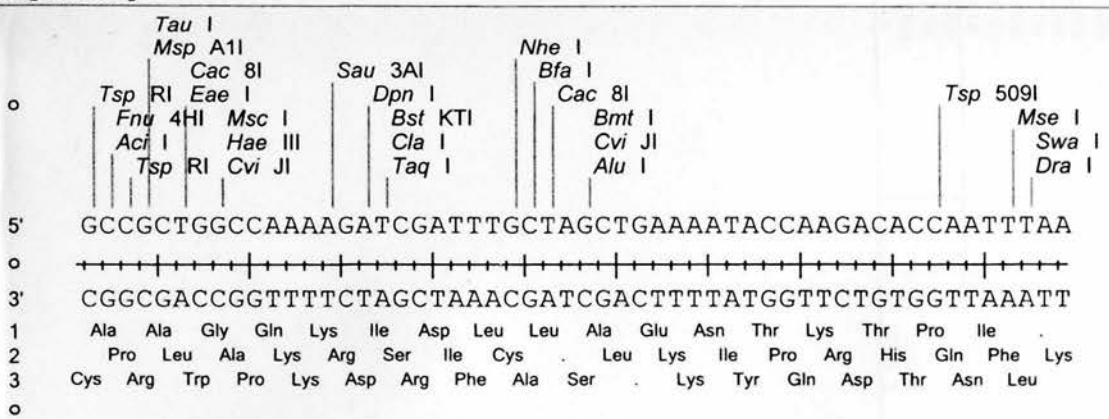
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Bst KTI
Bmt I
Mse I
Aci I
Hae III
Cla I
Cvi JI
Swa I
Tsp RI
Cvi JI
Taq I
Alu I
Dra I
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3' CGGCGACCGGTTTTCTAGCTAAACGATCGACTTTTATGGTTCTGTGGTTAAATT
1 Ala Ala Gly Gln Lys Ile Asp Leu Leu Ala Glu Asn Thr Lys Thr Pro Ile .
2 Pro Leu Ala Lys Arg Ser Ile Cys . Leu Lys Ile Pro Arg His Gln Phe Lys
3 Cys Arg Trp Pro Lys Asp Arg Phe Ala Ser . Lys Tyr Gln Asp Thr Asn Leu

Predicted Structural Class of the Whole Protein: Alpha
Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	22044.42 m.w.
Length	197
1 microgram =	45.363 pMoles
Molar Extinction coefficient	17780±5%
1 A(280) =	1.24 mg/ml
Isoelectric Point	9.58
Charge at pH 7	6.43

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	58	35.94	29.44
Acidic (DE)	22	12.31	11.17
Basic (KR)	28	18.06	14.21
Polar (NCQSTY)	47	22.78	23.86
Hydrophobic (AILFWV)	75	36.90	38.07
A Ala	14	4.51	7.11
C Cys	0	0.00	0.00
D Asp	9	4.70	4.57
E Glu	13	7.61	6.60
F Phe	8	5.34	4.06
G Gly	11	2.85	5.58
H His	3	1.87	1.52
I Ile	18	9.24	9.14
K Lys	14	8.14	7.11
L Leu	20	10.27	10.15
M Met	2	1.19	1.02
N Asn	11	5.69	5.58
P Pro	9	3.96	4.57
Q Gln	1	0.58	0.51
R Arg	14	9.92	7.11
S Ser	15	5.93	7.61
T Thr	15	6.88	7.61
V Val	13	5.85	6.60
W Trp	2	1.69	1.02
Y Tyr	5	3.70	2.54
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	1	0.00	0.51

Appendix III: Tables of Results

Table 1

Assay	Wild Type Average	fmp35Δ::URA3			fmp35Δ::URA3 Average	fmp35Δ::URA3 Standard Deviation	Fold Change
		1	2	3			
Respiration Loss	3.90E-04	7.48E-03	2.84E-03	1.55E-03	3.96E-03	3.12E-03	9.8 Increase
Respiration Loss Frequency	0.3%	8.0%	9.0%	1.6%	6.2%	4.0%	20.7 Increase
Direct Repeat Recombination Rate	8.15E-05	1.20E-05	1.58E-05	9.33E-06	1.24E-05	3.25E-06	6.6 Decrease
Microsatellite Reporter Rate	2.25E-06	3.36E-06					1.5 Increase

Table 2

Rspiration Loss Assay Trial 1			Respiration Loss Assay Trial 2			Respiration Loss Assay Trial 3		
Control	Experimental	Frequency %	Control	Experimental	Frequency %	Control	Experimental	Frequency %
368	32	8.0	397	9	2.2	518	10	1.9
444	25	5.3	406	9	2.2	840	18	2.1
454	39	7.9	422	21	4.7	1824	23	1.2
401	22	5.2	282	9	3.1	2418	18	0.7
27	24	47.1	438	19	4.2	912	18	1.9
396	31	7.3	205	8	3.8	2068	11	0.5
269	17	5.9	255	12	4.5	511	16	3.0
322	31	8.8	284	8	2.7	489	23	4.5
247	80	24.5	268	13	4.6	864	10	1.1
269	26	8.8	203	17	7.7	1172	18	1.5
247	36	12.7	1172	56	4.6	650	8	1.2
138	22	13.8	248	17	6.4	810	21	2.5
244	9	3.6	335	7	2.0	452	12	2.6
143	12	7.7	260	41	13.6	810	14	1.7
454	28	5.8	219	8	3.5	1626	15	0.9
254	23	8.3	391	9	2.3	1368	12	0.9
152	12	7.3	279	9	3.1	527	7	1.3
235	16	6.4	289	12	4.0	472	0	0.0
270	51	15.9	359	11	3.0	382	8	2.1
249	26	9.5	304	5	1.6	387	7	1.8
r_o=	260,000		r_o=	90,000		r_o=	155,000	
m=	23041.47		m=	8729.39		m=	14338.58	
mut/cell div=	7.48E-03		mut/cell div=	2.84E-03		mut/cell div=	1.55E-03	
median freq=	8.0		median freq=	9.0		median freq=	1.6	

Values in red are >2x average or <1/2 average
85% of plates were used in average calculation
Average median frequency of all trials= 6.2%

Table 3

Direct Repeat Recombination Assay T:1		Direct Repeat Recombination Assay T:2		Direct Repeat Recombiantion Assay T:3	
Control	Experimental	Control	Experimental	Control	Experimental
221	26	43	110	192	64
124	28	80	139	234	91
261	35	234	51	216	214
189	37	218	177	220	88
262	43	236	174	207	97
226	47	197	116	317	160
189	48	175	323	289	76
189	50	114	197	166	116
147	54	79	95	112	102
164	58	157	120	186	116
260	67	189	164	243	138
345	78	138	23	258	146
261	79	137	142	241	131
272	104	480	115	247	109
153	116	217	134	167	90
154	118	187	158	300	129
228	119	217	137	201	80
382	126	125	361	327	93
261	173	192	224	136	105
281	716	152	42	207	80
r_o=	125	r_o=	300	r_o=	207
m=	27.47	m=	56.82	m=	41.65
mut/cell div=	1.20E-05	mut/cell div=	1.58E-05	mut/cell div=	9.33E-06

Values in red are >2x average or <1/2 average

93% of plates were used in average calculation

Table 4

Microsatellite AssayTrial 1

Control	Experimental
26	3
161	2
137	44
98	26
44	4
67	9
98	34
87	0
68	4
103	174
38	9
131	25
94	0
112	1
148	8
130	14
73	18
148	*
60	*
20	53

r_o= 11.5

m= 7.23

mut/cell div= 3.36E-06

Values in red are >2x average or <1/2 average

* denote the plate contained an uncountable lawn

Appendix IV: Media Recipes

YPD Liquid Media
1L dH_2O
10g Yeast Extract
20g Bacteriological Peptone
20g Dextrose
Autoclave mixture to sterilize

YPD Agar Plates
1L dH_2O
10g Yeast Extract
20g Bacteriological Peptone
20g Dextrose
30g Agar
Autoclave mixture to sterilize

YPG Agar Plates
900ml dH_2O
10g Yeast Extract
20g Bacteriological Peptone
25g Agar
Autoclave mixture to sterilize
100ml 20% Glycerol (filter sterilized)

2% Glycerol/0.1% Dextrose Agar Plates
900ml dH_2O
10g Yeast Extract
20g Bacteriological Peptone
25g Agar
Autoclave mixture to sterilize
100ml 2% Glycerol (filter sterilized)
1g Dextrose

Amino Acid Mixture for Selective Plates

1g	L-adenine
1g	L-uracil
2g	L-tryptophan
1g	L-histidine
1g	L-arginine
1g	L-methionine
3g	L-tyrosine
4g	L-leucine
4g	L-isoleucine
3g	L-lysine
2.5g	L-phenylalanine
5g	L-glutamic acid

5g L-aspartic acid
7.5g L-valine
10g L-threonine
20g L-serine

To create selective plates simply omit the appropriate amino acid(s). 1.4g of the mixture was added per liter of media.

SD Complete Plates
1L dH_2O
1.7g Yeast Nigrogen Base
5.0g Ammonium sulfate
20g Dextrose
25g Agar
Autoclave mixture to sterilize
Cool to approximately 65°C
1.4g of amino acid mix

LB +Ampicillin Liquid Media
1L dH_2O
10g Tryptone
5g Yeast Extract
10g Sodium Chloride
Autoclave mixture to sterilize
Cool to approximately 65°C
1ml 1000x ampicillin

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