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Effects of a putative Reb1 protein binding site on IME4 sense and antisense transcription and sporulation in *Saccharomyces cerevisiae*

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Effects of a putative Reb1 protein binding site on *IME4* sense and antisense
transcription and sporulation in *Saccharomyces cerevisiae*

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biological Sciences

by

Milele Ramsay

B.S. Xavier University of Louisiana, 2004

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Abstract

Genome transcription is much more widespread than has been traditionally thought because our view of a “gene” or “transcription unit” has changed dramatically over the past 4 to 5 years with the identification of many different non-coding ribonucleic acids. In the yeast, *Saccharomyces cerevisiae*, meiosis and sporulation are an important part of the life cycle and *IME4* gene expression is required for these processes. *IME4* sense transcript levels of expression are influenced by the level of its complementary non-coding antisense strand by mechanisms that are currently unknown. The $\alpha 1$ - $\alpha 2$ heterodimer binding in the downstream 3' region of *IME4* is one component required for repression of *IME4* antisense transcription. However, this thesis shows that the general regulatory protein Reb1 is also required in this system. Reb1 involvement is most likely to create a nucleosome-free zone in the promoter region of the *IME4* antisense strand therefore contributing to transcription.

Keywords: Reb1 site mutant, *IME4*, sporulation, antisense transcription, regulation of ncRNA, $\alpha 1$ - $\alpha 2$ repression, RNA strand-specific qPCR analysis

Introduction

Cell-type circuit in Saccharomyces cerevisiae

The budding yeast *Saccharomyces cerevisiae* is a model unicellular organism that is extensively studied to better understand molecular processes in eukaryotic cells. There are three distinct cell types including **a**, alpha (α), and **a/alpha** (α) types based on the allele present at the mating-type locus. The MAT**a** type and the MAT α type are both haploid with regard to DNA content. The **a** cell and the alpha cell mate with each other, ultimately combining cytoplasm and undergoing nuclear fusion, to produce a single nucleus with a diploid complement of chromosomes, the **a/alpha** cell-type. The **a/alpha** cell can undergo meiosis and sporulation to produce four haploid cells (Herskowitz, 1988). The cell-type can be characterized by the genes that are active in the life of the cell when it is exposed to certain environments. For each *Saccharomyces cerevisiae* cell-type, there are specific genes that are active and inactive as a result of interactions between the specific genes and proteins functioning as transcription regulators (Herskowitz, Rine, and Strathern, 1992). The MAT**a** locus encodes the **a1** and **a2** proteins and the MAT α locus encodes proteins $\alpha1$ and $\alpha2$. In the α cell, the protein $\alpha1$, along with Mcm1 protein, binds to promoters of α -specific genes and promoters of haploid-specific genes resulting in transcriptional activation. The $\alpha2$ protein along with Mcm1 protein represses transcription of genes specific to the **a** cell-type in α cells. In **a** cells, **a**-specific genes are active and α -specific genes are not because $\alpha1$ is not present. In the diploid **a/alpha** cell-type, only proteins **a1**, **a2**, and $\alpha2$ are expressed. There is no $\alpha1$ so the α -specific genes are not expressed while the presence of $\alpha2$ ensures that the **a**-specific genes are not expressed. The **a1**- $\alpha2$ protein

complex inhibits expression of the $\alpha 1$ protein and the haploid-specific genes (*hsg*). A diagram of the yeast cell types and the **a1**- $\alpha 2$ regulatory pathway is shown in Figure 1.

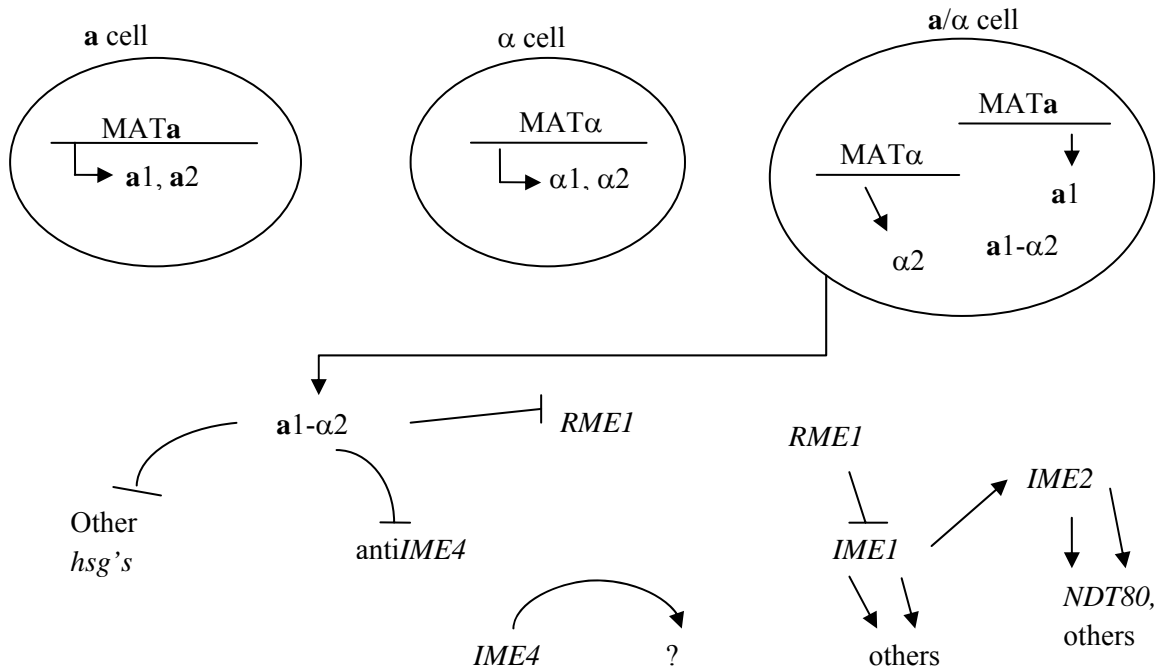


Figure 1. Diagram of yeast cell types and **a1**- $\alpha 2$ regulatory pathway. *hsg* = haploid-specific gene

Transcription and repression by a1- $\alpha 2$ protein complex

In eukaryotes like *Saccharomyces cerevisiae*, transcription is a complex process because of the way that DNA is packaged in the nucleus. The helical DNA is wrapped around a number of protein complexes called nucleosomes, and each nucleosome is comprised of histones. A pair of four different histones: H2A, H2B, H3, and H4, create an octamer, plus the helical DNA, and a single H1 histone make up a nucleosome. The DNA is wrapped one and three-fourths turns, or about 145 base pairs, on each nucleosome. This bead-like structure is then coiled around itself and coiled again to create the chromosome structure. The result of the tight packaging of DNA in a eukaryotic cell is that the DNA must be unwound and separated from the nucleosomes in

order for genes to be transcribed into RNA. The transcriptional activating proteins and repressing proteins, in addition to other proteins involved in the initiation of transcription, must have access to the DNA, specifically the promoter and operator of target genes (reviewed by Cairns, 2009)

In *Saccharomyces cerevisiae*, the **a1- α 2** protein complex functions with other proteins to accomplish repression. Studies by Harashima et al (1989) found that the α 2 protein domain required for repression is different from the α 2 protein domain required for the protein complex formation and interaction in **a1- α 2**. They identified another protein termed the *AAR1* product for **a1- α 2** repression, now known as Tup1, which is also associated with the **a1- α 2** protein complex. Tup1 is part of a general co-repressor complex that contributes to repression of many classes of genes, including glucose repressible genes and DNA damage-inducible genes, among others (reviewed by Malave and Dent, 2006). During cell type regulation, Tup1 and the **a1- α 2** protein complex work together to influence repression of haploid-specific genes, and the gene responsible for the α 1 protein. Mukai et al (1991) found that Tup1 interacts with protein complex **a1- α 2** to influence expression of the *MAT α* gene. Tup1 also was found to interact with the protein Ssn6 to act as a repressor of transcription in yeast (Keleher, 1992). Furthermore, Gavin et al (2000) found that Tup1 along with α 2 proteins may block Mcm1 protein transcriptional activity. Tup1 contains a helical N-terminal section for interaction with Ssn6, and the C-terminal section has 7 tryptophan-aspartate (WD) domain that folds into a propeller structure for interaction with other proteins like α 2 (reviewed by Malave and Dent, 2006). Studies by Huang et al (1997) showed that histones H3 and H4 are also involved in **a1- α 2** repression and when these histones are truncated and/or have mutations, the repression

mediated by **a1- α 2** is decreased. The Tup1-Ssn6 repressor complex recruits other proteins to the complex which in turn interact with proteins involved in initiating transcription, including a component of the RNA polymerase II holoenzyme, inhibiting their activity (reviewed by Smith and Johnson, 2000 and Malave and Dent, 2006). Tup1 also interacts with N terminal tails H3 and H4 and also with several histone deacetylases (HDAC's) (Watson et al, 2000). This, in turn, leads to repression.

In addition to cooperating with the Tup1-Ssn6 protein complex, the **a1- α 2** protein complex has its own specific DNA binding affinity (Jin et al 1999). The consensus **a1- α 2** site, which showed the most repression in comparison to naturally occurring **a1- α 2** binding haploid-specific gene operators in β -galactosidase assays, was 5'-TCATGTAATTAATTACATCA-3'. Li et al (1998) determined the structure of the **a1- α 2** heterodimer in complex with DNA containing a string of A's between the sequences that are in contact with the proteins. Komachi et al (1994) showed that the α 2 protein specifically binds to tryptophan-aspartate (WD) repeats in Tup1 carboxyl terminus. Repression mediated by **a1- α 2** also requires the N-termni of histones H3 and H4 (Huang et al, 1997). They showed that mutations in the N-termini of these histones derepressed **a1- α 2** induced repression and truncated versions of these histones increased the derepression even further.

More recently, studies using microarrays have been utilized to identify targets of the **a1- α 2** transcription factor, which include genes involved in mating in haploid cells, mating-type switching, recombination, and other cellular processes (Nagaraj et al (2004). Also in 2004, Galgoczy et al used chromatin immunoprecipitation (ChIP) experiments to identify genes that were bound by the **a1- α 2** protein haploid and diploid cells *in vivo*, by using antibodies against

the $\alpha 2$ protein. This study confirmed all genes previously shown to be repressed by **a1- $\alpha 2$** and also revealed, by using transcriptional profiling analysis with microarrays and phylogenetic comparison, some open reading frames whose transcription was not known to be regulated by **a1- $\alpha 2$** . Although a great deal is known about the yeast cell-type circuit (Sprague 2005), all **a1- $\alpha 2$** -DNA binding events are still not fully explained.

Meiosis, Sporulation, and IME4

As mentioned above, haploid **a** and α cells will mate (fuse) to form a diploid **a/ α** cell, which can go through meiosis and sporulation under particular nutritional conditions when nitrogen and carbon are not present. Haploid yeast cells do not go through meiosis because the *RME1* gene, a haploid-specific gene that encodes a protein that represses meiosis, is expressed; however in diploid cells this gene is repressed by **a1- $\alpha 2$** (Mitchell and Herskowitz, 1986). Covitz et al (1991) showed evidence that Rme1 protein contains zinc fingers, which are nucleic acid-binding motifs, and these regions allow transcriptional repression. As a result of this repression of *RME1* by **a1- $\alpha 2$** , *IME1* can be expressed.

RME1 represses *IME1*, the key transcriptional activator of meiosis in *Saccharomyces cerevisiae*. *IME1* product contains an activation domain which activates expression of genes involved in meiosis including the *IME2* transcript (Smith et al 1993). Guttman-Raviv et al (2002) identified a kinase activity of Ime2 protein, and this activity is required for targeting Ime1 for degradation by proteasomes. Ime2 also influences other genes involved in the meiotic pathway and sporulation. *IME2* is necessary for the expression of additional transcriptional activators such as Ndt80, which activates genes specific to the middle phase of sporulation (Pak and Segall, 2002). They found that Ime2 inactivates the repressor, Sum1, of Ndt80. Pierce et al (2003) found that Sum1 and Ndt80 have overlapping binding-site sequences and, suggested that

these proteins may compete for these sites during meiosis. The genes activated during initiation of meiosis include those necessary for DNA replication and chromosome segregation (reviewed by Honigberg and Purnapatre, 2003). In summary, meiosis is a complex process involving many different genes.

IME4, or inducer of meiosis 4, is a gene whose mRNA is highly elevated in cells undergoing sporulation and was found to be a key player in the pathway that leads to meiosis and sporulation (Shah and Clancy, 1992). Certain nutritional environmental conditions such as reduced nitrogen and carbon, in turn triggers a transcriptional response that ultimately allows an **a/α** cell to undergo meiosis. The diploid cell forms four ascospores, each being haploid in DNA content within one mother cell (reviewed by Herskowitz, 1988). Ime4 protein is likely to have RNA-directed methyltransferase activity resulting in its role in activating sporulation (Clancy et al, 2002). *IME4* gene also has a binding site for the protein complex **a1-α2** less than 200bp from the stop codon. Investigation in the Clancy laboratory is consistent with the results of Hongay et al (2006) that *IME4* transcription may be regulated by the expression of its own antisense transcript in **a/α** diploid cells.

Reb1 protein: a general regulatory factor

The Reb1 protein binding site is present in operators and promoters in rDNA genes transcribed by RNA polymerase I and genes transcribed by RNA polymerase II. Reb1 involvement in regulation is vital to many pathways in molecular processes of the budding yeast. Reb1 protein has several different and potentially antagonistic roles including activation, repression, silencing of RNA polymerase II transcribed genes, and influence in RNA polymerase I and RNA polymerase II mediated transcription. The Reb1 (rRNA enhancer binding) protein was first observed by Morrow et al (1989). They found Reb1 protein binds DNA at a specific

sequence in the region of DNA where the ribosomal RNA is transcribed by RNA polymerase I. The region where Reb1 protein binds was found between genes in a region of sequence previously found to be an enhancer element for RNA polymerase I. The location of the enhancer allows the elements it binds to influence activity upstream and downstream of the binding event. Reb1 has been previously called GRF2 or general regulatory factor 2 (Chasman et al, 1990) because of its binding in the upstream activating sequence of genes transcribed by RNA polymerase II. The Reb1 binding site in DNA is also involved in terminator activity of RNA polymerase I (Lang and Reeder, 1993). Lang and Reeder further found that Reb1 protein interacts with a T-rich element in the DNA and suggested its role was to stall transcription by RNA polymerase I and thereby influence the release of the newly transcribed RNA molecule (1995). More recently, Sanchez-Gorostiaga et al (2004) uncovered evidence suggesting that Reb1 protein may play a role in blocking the replication fork at its barriers which are located 3' to the coding region of ribosomal DNA.

Studies by Morrow et al (1990) describe a consensus binding site for the Reb1 protein which is 5'-CCGGGTAA-3'. They also found that the Reb1 protein is a single polypeptide chain that is phosphorylated and has an apparent mass of 125,000 Da by methods of sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). According to the *Saccharomyces cerevisiae* database (www.yeastgenome.org), Reb1 protein is comprised of 810 amino acids with a calculated mass of 91,874 Daltons. Kulkens et al (1992) showed that a mutation in the binding site sequence for Reb1 protein interrupts binding and results in decreased transcription from RNA polymerase I at the rDNA enhancer. Liaw and Brandl (1994) found the optimal consensus sequence for Reb1 protein binding to be 5'-

GNCCGGGGTAACNC-3'. In budding yeast, the silencing of genes is also affected by Reb1 protein binding to the silencer in *K. lactis* (Sjostrand et al, 2002).

Studies from Wang et al (1990) found Reb1 protein binding sites in promoters of genes transcribed by RNA polymerase II, actively repressing transcription. Reb1 protein also binds DNA upstream activating sequence (UAS) of the gene *CLB2*, and this action reduces expression (Van Slyke and Grayhack, 2003). In apparent conflict with a role in repression, Reb1 protein along with other proteins associated in a complex, promotes high basal transcription levels (Remacie and Holmberg, 1992). In reference to transcriptional activation, Schuller et al (1994) found that mutations in a Reb1 protein binding site reduces transcription of the fatty acid synthase gene *FAS1* and that Reb1 protein is involved in activation of the *FAS2* gene.

Reconciling these observations, it has been observed that the binding of Reb1 protein to its consensus sequence in upstream activation sequences of genes directly effects the positioning of nucleosomes (Scott and Baker, 1993). Nucleosome positioning is important because RNA polymerase II needs access to its target DNA. Fedor et al (1988) identified a protein-binding sequence in the upstream activating sequence of galactose response genes that is necessary for the arrangement of nucleosomes and therefore affects the actual structure of the tightly packed DNA in its chromatin structure. Angermayr and Woodlaw (2003) and Angermayr et al (2003) confirmed the requirement for Reb1 protein binding sites in the promoters of yeast *GCY1* gene induced by Gal4p, and the yeast profilin promoter, function to keep the region accessible to the transcription machinery, free of nucleosomes.

More recent studies have shown that Reb1 binding contributes to specific positioning of nucleosomes on chromatin including location of nucleosome free regions, where transcription start sites are located (Martinez-Campa et al, 2004; Raisner et al, 2005; Koerber et al, 2009).

There is evidence that Reb1 recruits chromatin remodelers, mainly the Rsc complex (Hartley and Madhani et al, 2009), that create nucleosome-free regions in the vicinity of transcription start sites, thereby improving accessibility of the DNA to proteins involved in transcriptional regulation like TATA-binding factor among others.

We have observed a potential Reb1 protein binding site located in the 3' region of the *IME4* gene, suggesting Reb1 could bind to the 5' region of the antisense *IME4* strand, as seen in Figure 2.

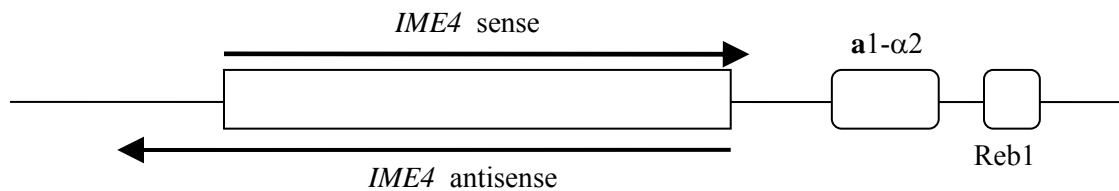


Figure 2. Schematic of *IME4* gene. Located on chromosome VII in *Saccharomyces cerevisiae*. It is drawn from 5' to 3' including the 3' region downstream of the stop codon.

This discovery in *Saccharomyces cerevisiae* led us to propose that the Reb1 binding site is also in other yeast species in the 3' region of *IME4* homologs. A search in the yeast Genome database, using the program ClustalW aligned four species of yeast where this is indeed the case (Figure 2).

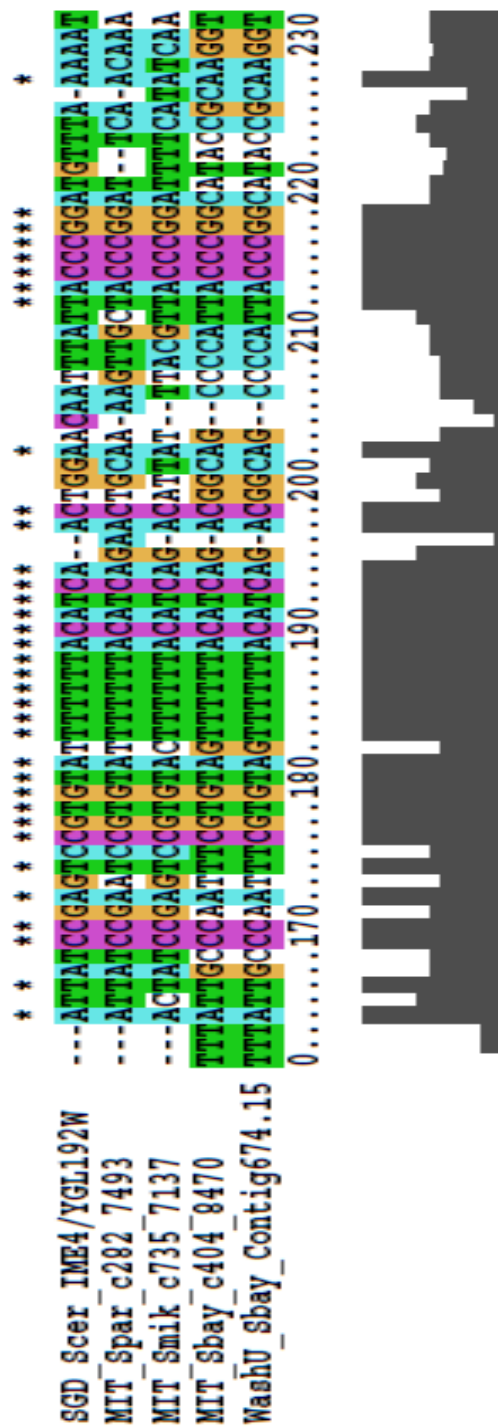


Figure 3. Multiple sequence alignment of a 3' region, downstream of the IME4 gene stop codon in *Saccharomyces cerevisiae* (top row). Conserved bases from 182 to 194 represent the $\alpha 1$ - $\alpha 2$ protein complex binding site, from base 213 to 217 represent the Reb1 protein binding site. 2nd row is *Saccharomyces paradoxus*, 3rd row is *Saccharomyces mikatae*, and the last two rows are *Saccharomyces bayanus*.

The focus of this thesis was to determine whether the Reb1 binding site plays a role in the expression or regulation of sense and antisense *IME4* RNA. One approach was to create a mutant Reb1 protein binding site in the *IME4* gene downstream sequence and to characterize the effects of this mutation in **a** and α haploid cells and **a**/ α diploid cells from the genus *Saccharomyces cerevisiae*. In addition, verification of any transcriptional activity regulated by the presence of Reb1 protein at its binding site in the antisense mRNA transcript of *IME4*, in **a**, α , and **a**/ α cells from *Saccharomyces cerevisiae* will be examined. This thesis will also study the expression patterns of the *IME4* sense and antisense strands in **a**/ α diploid *Saccharomyces cerevisiae* cells along with Reb1 protein binding effects on strand specific transcription. I hypothesize that the Reb1 general transcription factor has transcriptional activity in the antisense strand of the *IME4* gene and that this could in turn influence *IME4* sense strand production. The results show that mutations disrupting efficient binding of Reb1 protein to its target DNA sequence in the *IME4* gene context indeed affects expression of sense and antisense *IME4* mRNAs and in turn have an impact on sporulation in diploid yeast.

Materials and Methods

Growth media for E. coli and yeast

- LB: 1% Bactotryptone, 0.5% Bacto-yeast extract, 0.5% NaCl, pH 7.5 plus 100 µg per mL ampicillin when selection for plasmids was required.
- YEPD: 1% Bacto-yeast Extract, 2% Bacto-peptone, 2% glucose
- SC: 0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose, 0.2% Drop-out mix, 1% agar, plus histidine, tryptophan, leucine, and adenine (20µg/mL each)
- SC casamino acids: 0.67% Bacto-yeast nitrogen base without amino acids, 1% dextrose, 0.5% casamino acids, 20mg adenine, 1% agar
- PSP: 1% potassium acetate, 0.067% Yeast nitrogen base, 0.1% Bacto-yeast extract, pH 5.5, plus tryptophan, histidine, and leucine (20µg/mL of each)
- SPM: 1% potassium acetate, plus histidine, tryptophan, leucine, and uracil (20µg/mL of each)

Microscopy

8µL of cells were placed on a plain glass microscope slide (3"x1" Corning), covered with a microscope cover glass (Fisherbrand), and observed using the Nikon Labophot microscope at 40 X magnification.

General molecular biology techniques

Agarose gel electrophoresis for DNA analysis

A 1% agarose in 1X TBE buffer (100mM Trizma base, 88mM Boric acid, 1mM EDTA [ethylenediaminetetraacetic acid disodium salt dehydrate]) gel was placed in a gel box filled with 1X TBE buffer. Loading buffer was added to the DNA samples to be run in a 1:4 volume ratio of loading buffer to DNA sample. The DNA samples were then loaded along with Kb

ladder 250bp-12Kb (Stratagene) and the gel run-time ranged from 30 minutes to an hour, at 100 volts. The gel was then stained with diluted ethidium bromide (1 μ g/mL) for 10 minutes, rinsed with deionized water and then a picture was taken under ultraviolet light (BIORAD Geldoc2000).

Northern blot hybridization for RNA analysis

Northerns were performed on purified RNA following the protocol from Brown et al (2004).

Restriction enzyme digest

For the restriction digest, 3-5 μ L plasmid DNA with up to 1 μ L restriction enzyme (Promega), along with 2 μ L of the appropriate 10X buffer (Promega) and sterile water, for a total volume of 20 μ L, was pipetted into a 1.5mL tube. For some experiments the total volume was multiplied, so the components of the reaction were multiplied by the same amount. The tube was placed in a 37°C heating block for at least 30 minutes, followed by agarose gel electrophoresis.

Plasmid DNA purification

All plasmids used in this study were isolated and purified from the *E. coli* colonies using the StrataPrep Plasmid Miniprep Kit from Stratagene.

PCR product purification

PCR products used for cloning were purified using the Wizard PCR Preps DNA Purification System (Promega) followed by the StrataPrep PCR Purification Kit (Stratagene).

DNA sequencing

For sequencing, 300-400ng plasmid DNA and 6 pmol of a primer (Sigma-Aldrich), along with ddNTPs, buffer, and DNA polymerase were put into a PCR tube. PCR was performed in a

thermocycler programmed for 96°C for 1 minute followed by 50 cycles of 96°C for 20 seconds, 48°C for 10 seconds, 60°C for 4 minutes, then held at 10°C. The newly synthesized DNA strands were isolated by pipetting the PCR reactions on a sephadex column for size selection and removal of the ddNTPs. The sample was resuspended in formamide and injected into the sequencer for analysis (all sequencing performed by Robin Rowe). Sequences from the trace files were copied and BLASTed against the yeast genome (www.yeastgenome.org) to compare the mutagenized sequences with the yeast genome reference sequence.

Recombinant DNA plasmid construction

Site-directed mutagenesis

A putative Reb1 mutant was constructed by site-directed mutagenesis of the 3' end of the *IME4* gene contained in plasmid pRM2b using forward and reverse primers ANOTHERReb1, substituting a HindIII restriction enzyme site for the Reb1 binding site. The 50 µL mutagenesis reaction contained 50-100 ng template plasmid pRM2b, 200 ng of each primer, 5 µL 10X PfuUltra reaction buffer (Stratagene), and 0.5 µL (1.25 units) of Pfu polymerase (Stratagene) in sterile water. A thermocycler from Bio-Rad was programmed for 94°C for 4 minutes followed by 18 cycles of 94°C for 1 minute, 55°C for 1min, and 65°C for 18 minutes to complete the mutagenesis. Next, 20 µL of this reaction was transferred to a new 1.5 mL tube and digested with 10 units of restriction enzyme DpnI in a 37°C heating block for one hour. The DpnI-treated DNA from the mutagenesis reaction was then transformed into XL10-Gold Ultra Competent *E. coli* cells (Stratagene) following the Stratagene protocol. The plasmid DNA was isolated from the colonies and the presence of the mutation was confirmed by restriction digest using HindIII and EcoRI. Isolates showing the desired restriction pattern were then further analyzed by DNA

sequencing using primers 2190 Reverse, Cys-Rich Forward, Cys-Rich Reverse, MTase Forward, and MTase Reverse. A list of all primers and their sequences can be found in Table 1.

| Table 1. Primers used in this study. All primers obtained from Sigma-Aldrich. | |
|---|---|
| Primer | Sequence (5' → 3') |
| ANOTHER Reb1 Forward | AACTGGAACAATTTATTAAGCTTATGTTTAAAAATT GTTGTCG |
| ANOTHER Reb1 Reverse | GCGACAACAATTTTAAACATAAGCTTAATAAATTG TTCCAGTT |
| +2190 reverse | GCATCTAGATTCGCTATTCCCACAGTTTCC |
| -444 Forward | GCGCTCGAGCATTGGCGACACACCTAAAA |
| Cys-rich Forward | TTTTGAATTCCACCCAGGATTAATTGAGTGC |
| Cys-rich Reverse | TTTTGTGCGACTTGCAAGCATGACGGAAT |
| MTase Forward | TTTTGAATTCCATTGTATCAAAAAGGCTTTA |
| MTase Reverse | TTTTGTGCGACGCTCTTCATAAACTCCTGGTACTT |
| newActin Forward | TTTCTCCACCACTGCTGAAA |
| newActin Reverse | ACGGACATCGACATCACACTT |
| 1143 Forward | TCTTTGGGTGACGGGTAGAG |
| 1328 Reverse | TTACCCTTTAAGCCCAACAA |
| myo1 2725 Forward | GCGCAAAATCTTGAAGAAGC |
| myo1 2725 Reverse | AAAGACTGGGAGCTCTGTTCC |
| IME4 region 2 Forward | GTCAGGAAAATCACCCAGGA |
| IME4 region 2 Reverse | TTTTCACTCGCAGTTTCACG |
| ADHBgl Forward | ATCAAGATCTAGAAGCTTTGGACTTCTTCG |
| ADHBgl Reverse | TCGAAGATCTGGTAGAGGTGTGGTCAATA |
| IME4 Reb1 psx okay2 Forward | ATTATCCGAGTCCGTGTA |
| IME4 Reb1 psx okay2 Reverse | CCATGGGAAAGAACCGACCAAG |
| RPA190 3000 Forward | AGATCCGGTTATTTGCAACG |
| RPA190 3000 Reverse | CAATCAGTGCTGATGGGTTG |
| pSX Forward | AAACTCGAGATTATCCGAGTCCGTGTA |
| pSX Reverse 2 | AAACTCGAGCCATGGGAAAGAACCGACCAAG |
| TAP42 Forward 1 | GCGCAACCAAAAAATAACAAAGA |
| TAP42 Reverse 1 | TTGTTAATCGTATCAAGACTAAA |
| IME4 Region 4 Forward 1 | ACGAAATGGATGTCGAGAGGA |
| IME4 Region 4 Reverse 2 | TGGAATTGTTGTTTTTGTTC |
| | |

Construction of *ADHI* terminator plasmid

Cloning of the *ADHI* terminator sequence was done by first creating primers, *ADH* Bgl forward and *ADH* Bgl reverse, which complement a region upstream of the 3' terminator part

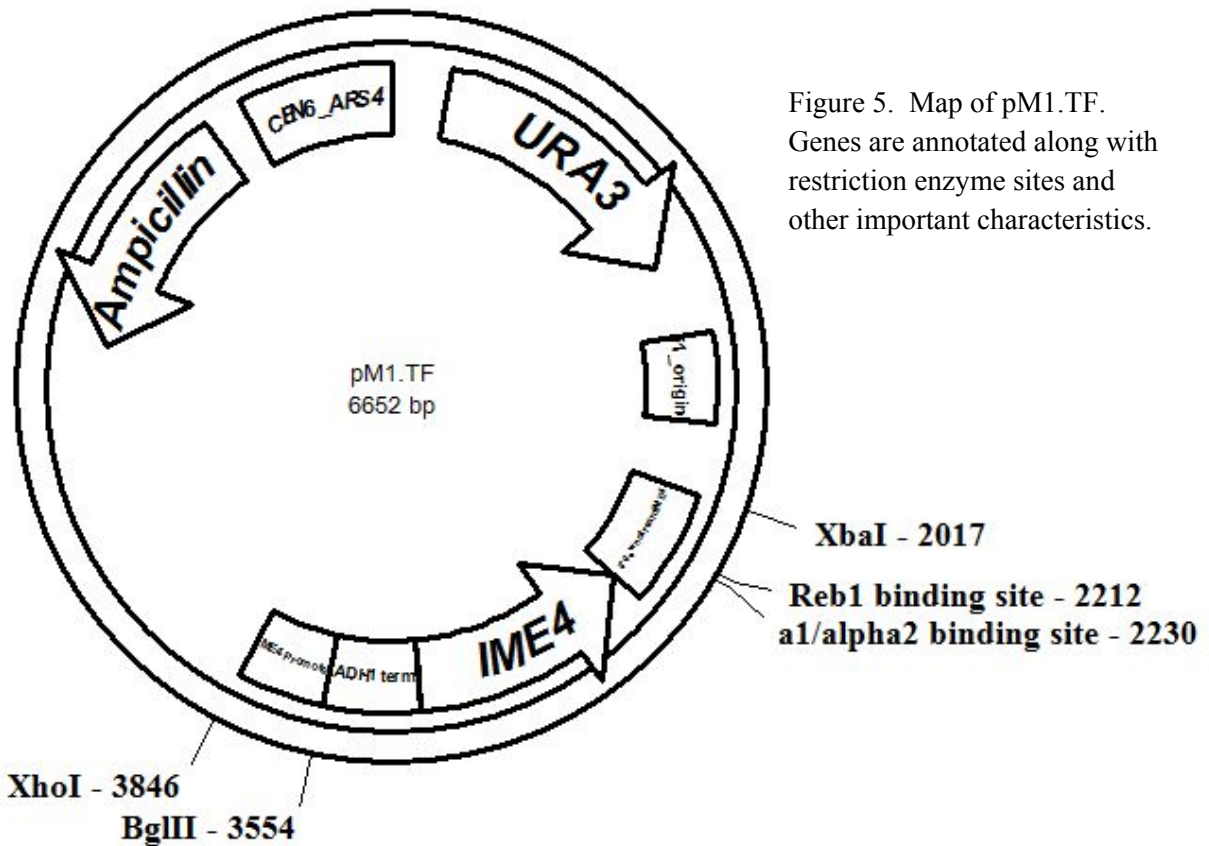
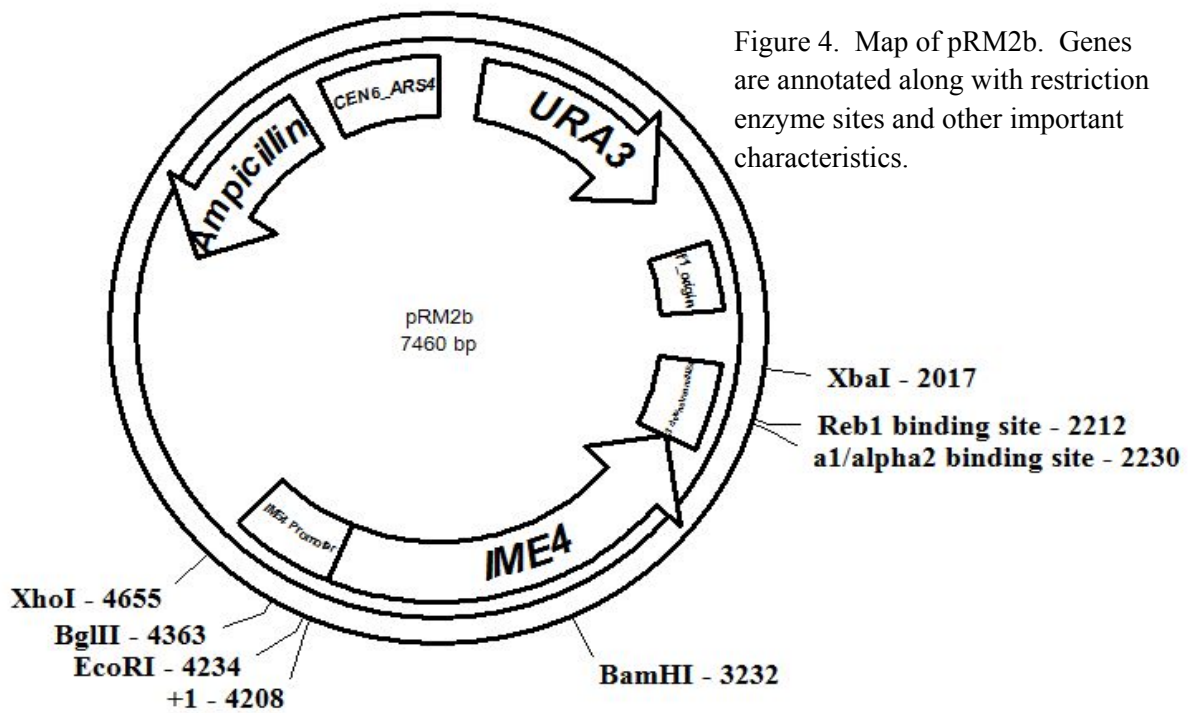
and downstream of the 5' part of the template plasmid pAD-Gal4-2.1 (Stratagene), to produce a PCR fragment about 300 base pairs long with restriction enzyme BglII sites on the ends. The PCR reaction tubes contained 5 μ L template DNA, 10 μ L 5X Green GoTaq Reaction Buffer (Promega), 1 μ L of each primer (50 pmol), 1 μ L PCR Nucleotide Mix (40nmol/ μ L, Promega), 32.5 μ L sterile water, and 0.5 μ L GoTaq polymerase (2.5 units, Promega). The PCR program was: 94°C for 4 min, then 30 cycles of 95°C for 30 seconds-55°C for 30 seconds-72°C 45 seconds, then 72°C for 3 minutes, then 4°C hold (BIORAD Thermocycler). Next, agarose gel electrophoresis was done using 10 μ L of the PCR reaction; the gel ran for 30 minutes. Next the PCR product was purified, then 10 μ L this PCR product was digested with 5 μ L (50 units) BglII in a 100 μ L total volume digest and incubated overnight. Next, 4 μ L of either vector pRM2b, pRM2bmutE, or pRMmut5 were digested with 1 μ L (10 units) BglII and 1 μ L (10 units) BamHI in a 40 μ L total volume digest and incubated for 2 hours. The restriction enzymes in the digestions were heat inactivated at 65°C for 5 minutes. The cut vectors were then phosphatase treated for 1 hour at 37°C using 10 units of calf intestinal phosphatase (CIP; New England BioLabs). 30 μ L cut DNA vector was added to a 1.5 mL microfuge tube along with 6 μ L 10X buffer (New England BioLabs), 0.25 μ L (2.5 units) CIP (New England Biolabs), and 23.75 μ L sterile water then incubated. The phosphatase and BglII enzymes were inactivated by heat at 65°C for 5 minutes. The cut vectors and the PCR product with BglII ends were then purified. Next, the clean cut vectors were ligated to the PCR product in a ligation reaction containing the following in a 1.5 mL tube: 10 μ L PCR product, 10 μ L purified digested vector, 4 μ L 10X T4 DNA Ligase buffer (New England BioLabs), 15 μ L sterile water, and 1 μ L (400 units) T4 DNA

Ligase (New England BioLabs). The ligation reaction tube was incubated overnight in 4°C in a styrofoam box half filled with tap water.

The ligation reaction was then used to transform XL1-Blue Competent *E. coli* cells (Stratagene) following the Stratagene transformation protocol, selecting for the ampicillin resistance gene on the plasmid. Next, single colonies were numbered, picked with sterile toothpicks and patched on LB+ampicillin plates and individual isolates inoculated into 1 mL LB+ampicillin to grow overnight at 37°C with shaking.

The overnight cultures were then centrifuged at maximum speed for 1 minute to pellet cells, the supernatant was discarded, and the plasmid DNA was purified. Aliquots of the plasmid DNAs were then digested with restriction enzymes to identify isolates in which the desired manipulation had been accomplished. A list of all plasmids utilized in this study is in Table 2.

| Table 2. All plasmids used in this study | | |
|--|--|---------------------------------|
| Plasmid | Description | Source |
| pRS316 | <i>CEN6 ARSH4 URA3 Amp^r fl-ori</i> | ATCC |
| pRM2b | pRS316 - <i>IME4</i> (Figure 3) | Rowan Madison |
| pRM2bmutE | pRM2b with HindIII sequence replacing nt 1995-2000 relative to <i>IME4</i> start codon | This study |
| pRMmut5 | pRM2b with KpnI sequence replacing nt 1973-1978 relative to <i>IME4</i> start codon | This study |
| pSX178 | 2 μ , <i>AMP</i> , <i>ori</i> , <i>lacZ</i> , <i>CYC1</i> (TATA region) UAS, <i>URA3</i> | Guarente L. and Mason T, (1983) |
| p31 | pSX178 – wild type <i>IME4</i> 3' region spanning 180 bp including nt1947 to 2127 relative to <i>IME4</i> start codon | This study |
| p5-2 | p31 with KpnI sequence replacing nt 1973-1978 relative to <i>IME4</i> start codon | This study |
| p53 | p31 with HindIII sequence replacing nt 1995-2000 relative to <i>IME4</i> start codon | This study |
| pAD-GAL4-2.1 | 7.7 kb phagemid vector containing <i>ADHI</i> terminator (<i>t_{ADHI}</i>) (1168-1318 bp) | Stratagene |
| pM1.TF | pRM2b with <i>ADHI</i> terminator (1160-1495 bp in pAD-GAL4-2.1) replacing a region spanning from nt -155 to +977 relative to <i>IME4</i> start codon, removing the ATG (Figure 4) | This study |
| pME.TF | pRM2bmutE with <i>ADHI</i> terminator (1160-1495 bp in pAD-GAL4-2.1) replacing a region spanning from nt -155 to +977 relative to <i>IME4</i> start codon, removing the ATG | This study |
| pM5.TF | pRMmut5 with <i>ADHI</i> terminator (1160-1495 bp in pAD-GAL4-2.1) replacing a region spanning from nt -155 to +977 relative to <i>IME4</i> start codon, removing the ATG | This study |



Yeast Transformation

Yeast transformation was generally done by the method of Gietz et al (1992).

Saccharomyces cerevisiae strains to be transformed were streaked on YEPD media plates and grown overnight at 30°C. 25 µL scraped cells were suspended in 1mL sterile water and pelleted at maximum speed in a microcentrifuge (Eppendorf Centrifuge 5415 C) for 5 seconds. The supernatant was discarded and the following added on top of the pellet in order: 240 µL polyethylene glycol (50% w/v), 36 µL 1.0M lithium acetate, 50 µL salmon sperm carrier DNA (boiled and quick-chilled on ice), 5 µL transforming plasmid DNA, and 20 µL sterile water, then incubated at room temperature for 20 minutes. The sample was vortexed at least one minute to resuspend pellet in transformation mix then incubated for 20 minutes at 42°C. The cells were pelleted at maximum speed for 10 seconds followed by removal of the supernatant, and then the cells were resuspended in 300 µL sterile water by pipetting up and down. 150 µL of the cell suspension was spread per SC-uracil plate and incubated at 30°C to select for transformed cells. Colonies grew in 2-4 days.

For some experiments, a higher efficiency transformation method was used (Linda Hoskins/Hahn Lab). First, 5 mL YEPD was inoculated with a half-filled loop of freshly grown YYF101 MATa/MATα cells and grown overnight at 30°C with shaking at 225 rpm. Then 0.5 mL culture was diluted 1:10 with 4.5 mL sterile YEPD and 1mL diluted culture's optical density was measured at 660nm; it was 0.2, so the remaining 4.5 mL overnight culture was added to 40.5 mL fresh YEPD along with the initial 5mL diluted culture into a 250 mL flask and grown up to the exponential phase, about 4 hours at 30°C with shaking at 225 rpm. The cells were pelleted at 3000 rpm for 5 minutes at room temperature, the supernatant discarded, the cells

washed with 10 mL sterile water then centrifuged again. The supernatant was discarded and the cells were resuspended in 1 mL sterile water and transferred to a 1.5 mL tube and centrifuged again. The supernatant was discarded and the cells resuspended in 1 mL sterile TE/LiOAc (made from 10X TE [0.1M Tris-HCl, 0.01M EDTA, pH 7.5] and 10X LiOAc [1M LiOAc pH 7.5, adjusted with diluted acetic acid]) and centrifuged. The supernatant was carefully discarded using a pipette and the cells were resuspended in 250 μ L TE/LiOAc. For each separate transformation, 50 μ L yeast cells, 5 μ L plasmid DNA, and 10 μ L carrier salmon sperm DNA (2mg/mL) were mixed in a sterile 1.5 ml tube. Then, 300 μ L sterile polyethylene glycol (40% polyethylene glycol 4000, 1X TE, 1X LiOAc, made from sterile 50 % polyethylene glycol 4000, 10X TE, and 10X LiOAc) was added to the tube and mixed thoroughly. The transformation reaction tubes were then incubated at 30°C for 60 minutes with occasional gentle shaking. Next 40 μ L dimethyl sulfoxide (DMSO) was added to the transformation reactions then the cells were heat-shocked in a 42°C water bath for 15 minutes. The tubes were microfuged for 10 seconds, the supernatant removed, the cells resuspended in 1 mL 1X TE then microfuged again for 10 seconds. The cells were resuspended in 1 mL 1X TE then 100 μ L cells were spread on each labeled SC casamino acids medium plate and incubated at 30°C for 2-4 days.

Reporter constructs

The pSX forward 2 and pSX reverse primers were used to PCR a region downstream of the IME4 stop codon. The PCR reaction tubes contained 5 μ L template DNA, 10 μ L 5X Green GoTaq Reaction Buffer (Promega), 1 μ L of each primer (50 pmol), 1 μ L PCR Nucleotide Mix (40 nmol/ μ L, Promega), 32.5 μ L sterile water, and 0.5 μ L GoTaq polymerase (2.5 units, Promega). The PCR program was: 94°C for 4 min, then 30 cycles of 95°C for 30 seconds-55°C

for 30 seconds-72°C 45 seconds, then 72°C for 3 minutes, then 4°C hold (BIORAD Thermocycler). Plasmid pSX178 was cut with XhoI as was the PCR product, and the enzyme was inactivated and the cut vector was phosphatase treated as stated earlier. The PCR product and the cut vector were then purified. The PCR product with XhoI ends was then ligated to the cut pSX178 in a 1.5 mL tube containing the following: 10 µL clean PCR product, 10 µL clean cut vector, 4 µL 10X T4 DNA Ligase buffer (New England BioLabs), 15 µL sterile water, and 1 µL (400 units) T4 DNA Ligase (New England BioLabs). The ligation reaction tube was incubated overnight in 4°C in a styrofoam box half filled with tap water.

The ligation reaction was then used to transform XL1-Blue Competent *E. coli* cells (Stratagene) following the Stratagene transformation protocol, selecting for the ampicillin resistance gene on the plasmid. Next, single colonies were numbered, picked with sterile toothpicks and patched on LB+ampicillin plates. Individual isolates were initially analyzed by colony PCR using the pSX forward 2 and pSX reverse primers to determine presence of the clone. Colonies that contained the desired clone were miniPreped and digested with XhoI to check for the insert. Finally, the clone was sequenced using primers to determine the orientation of the insert and that no unwanted mutations occurred during the PCR of the insert.

For colony PCR, in a 1.5 mL tube a small amount of single colonies were picked with a sterile toothpick and put into 50 µL sterile water. Next, the cells were vortexed at maximum speed, then incubated in a 95°C heating block for 10 minutes. The cells were vortexed again then centrifuged at maximum speed (Eppendorf Centrifuge 5417C, 14000 rpm), and 2.5 µL of the supernatant was used as the template in a 50 µL PCR reaction.

β -galactosidase reporter assays

Strains were grown on SC-ura plates overnight at 30°C to maintain plasmid, and then strains were inoculated in 5 mL cultures of SC, PSP, or YEPD and grown in 30°C with 225 rpm shaking overnight. Cells were harvested by centrifugation at 2000 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 250 μ L breaking buffer (100 mM Tris-Cl pH 8, 1 mM dithiothreitol, 20% glycerol) then 0.5 mm glass beads (Sigma) were added to a level right under the meniscus of liquid, which was about 2.5 grams of glass beads. Next, 12.5 μ L phenylmethylsulfonyl fluoride or PMSF (40 mM in 100% isopropanol) was added to each sample followed by vortexing at maximum speed in 15 second bursts until cells were lysed (about 9 times) with cells chilled on ice between bursts. Then, 250 μ L breaking buffer was added and mixed well followed by removal of the liquid by plunging a 1000 μ L pipette to the bottom of the sample and transferred to a new 1.5mL tube. Samples were then centrifuged at maximum speed in a microfuge for 15 minutes. Next, 100 μ L of the clarified extract was added to 900 μ L “Z” buffer (0.06M Na₂HPO₄- 7 H₂O, 0.04M NaH₂PO₄-H₂O, 0.075% KCl, 0.0246% MgSO₄, 0.27% beta-mercaptoethanol, pH 7) in a glass tube (12x75 mm, VWR) and incubated at 28°C for 5 minutes. The hydrolysis reaction was initiated by the addition of 200 μ L *o*-nitrophenyl- β -D-galactoside or ONPG (4 mg/mL in “Z” buffer) then incubated at 28°C until the samples turned yellow. At this point, the reaction is terminated by the addition of 500 μ L of 1M Na₂CO₃. The time ONPG was added and the time Na₂CO₃ was added was recorded in a table for calculating specific activity. The optical density was measured at 420 nm (Beckman DU-64 Spectrophotometer) and recorded. Next the Bradford Assay for protein concentration was performed. The Bradford (BioRad) reagent was diluted five-fold in deionized water then filtered

through Whatman 540 paper. The extract was diluted 1:10 in sterile water then 5 μ L was added to 1 mL diluted Bradford reagent followed by measuring and recording the optical density at 595 nm (Beckman DU-64 Spectrophotometer). The standard curve ranged from 0 to 150 micrograms per mL. The specific activity of the extracts was calculated according to the following equation: $(OD_{420} \times 1.7) / (0.0045 \times \text{protein (mg/mL)} \times \text{extract (mL)} \times \text{time (minutes)}) = \text{SA (nmole/minute/mg protein)}$ (Amberg et al, 2005).

Growth and Sporulation

Yeast strains were grown overnight at 30°C on SC-caa media plates, then cells were transferred to 10 mL SC-caa media and grown overnight at 30°C with 225 rpm shaking. Cells were diluted 1:10 in 10 mL PSP media in a sterile tube and incubated for 24 hours at 30°C then transferred to a 15 mL tube, and centrifuged for 3 minutes. The supernatant was discarded as much as possible, and then the cells were washed in 10 mL sporulation media. Cells were centrifuged for 3 minutes, supernatant was discarded, and the cells were resuspended in 10 mL SPM, supplemented as required by the strain, transferred to a sterile flask, and incubated at 30°C with shaking 225 rpm for a time interval. 8 μ L of the cell culture was put on a microscope slide and live pictures were taken with a Nikon Eclipse E800 at 600 X magnification.

RNA Isolation and cDNA synthesis

Isolation of RNA from yeast cells was performed following the RNeasy Midi Kit (Qiagen) protocol with the DNaseI treatment. RNA was quantified following the BIORAD Experion RNA StdSens Analysis Kit or the Nanodrop3000. Next, BIORAD iScript Select cDNA Synthesis Kit was used to do a reverse transcriptase reaction to make DNA from mRNA expressed in the yeast cells. The primers used for the cDNA library include the Oligo(dT)₂₀

primer provided in the kit, and the gene specific primers, which will be discussed more in the results.

Quantitative Real-Time PCR

Real-Time PCR was performed on the cDNA products following the iQ SYBR Green Supermix and MYiQ Single Color Real-Time PCR Detection System from BIORAD. Primers used for the Quantitative RT-PCR include newACTforward and newACTreverse for detection of the control gene actin, TAP42 forward and reverse primers also for detection of the control gene *TAP42*, and a number of different sets of *IME4* primers to detect the *IME4* gene sense and anti-sense DNA strands (Table 1). The program for the RT-PCR was as follows: Cycle 1 was one step at 95°C for 3 minutes, Cycle 2, which was repeated 40 times, was step 1 at 95°C for 10 seconds then step 2 at 55°C for 30 seconds. Data collection and real-time analysis was enabled during cycle 2. Cycle 3 was one step at 95°C for 1 minute, cycle 4 was one step at 55°C for 1 minute, and cycle 5, which was repeated 81 times, was one step with temperature rising from 55°C to 95°C within 30 seconds. Melt curve data collection and analysis was enabled during cycle 5. The PCR Quantification Detailed reports were printed out and the percent of the target gene was calculated as a percent of actin and/or as a percent of TAP42 according to the following equation: % of Control Gene = $(2^{(\text{Control Gene Ct} - \text{Target Gene Ct})}) \times 100$.

Results

Identification of a haploid-specific UAS (upstream activating sequence) 3' to the IME4 coding region

Although there is evidence of expression of sense and antisense *IME4* transcripts, the factors that influence this expression are unknown. Previous strand specific *IME4* mRNA studies in the Clancy laboratory detected the presence of both sense and antisense mRNA in wild type **a/α** *Saccharomyces cerevisiae* cells after sporulation was induced. To verify these initial findings, RNA was isolated from wild type *IME4* **a**, **α**, and **a/α** haploid and diploid yeast grown in SC *caa* media. Northern blot hybridization was performed on the RNA after it was separated by size in an agarose gel (Figure 6). The RNA was transferred to a nylon membrane, which was then incubated with *IME4* sequence-specific DNA probe. The *IME4* gene probe was double-strand, thus detecting both sense and antisense transcripts. Shown in Figure 6 is RNA from haploids in lanes 1 and 2, and diploids in lanes 3 and 4 of a blot incubated with the double strand *IME4* gene probe. Transcripts from the *IME4* region were detected in both haploid and diploid cells. The probe detecting the *IME4* antisense RNA has sequence identical to the *IME4* sense strand, thus binding its complement, the antisense strand. Conversely, the probe for the *IME4* sense RNA has sequence identical to the *IME4* antisense strand, which binds and detects the complement sense strand. Thus, both sense and antisense *IME4* RNAs are detected by this probe.

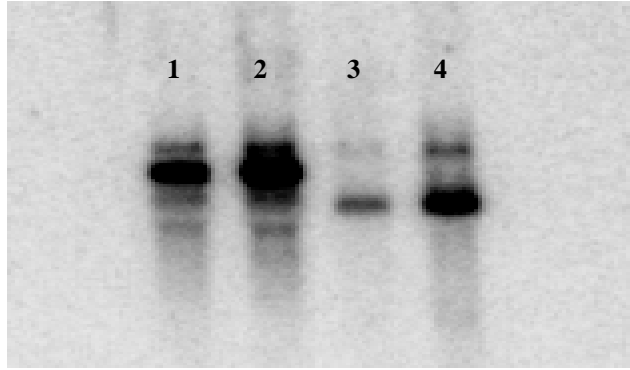


Figure 6. Northern Hybridization of wild type *Saccharomyces cerevisiae*. Lanes 1 and 2 contain RNA from haploid cells. Lanes 3 and 4 contain RNA from diploid cells. The probe was radiolabeled DNA fragment internal to the *IME4* gene

In a second experiment, diploid strain YYF101 **a**/ α was transformed with plasmids containing the wild type *IME4* gene (pRM2b) or a mutant allele (pRMmut5) lacking a functional **a**1- α 2 site after *IME4* (see Figure 2), or vector control (pRS316). Strain YYF101 was chosen because the *IME4* gene has been removed and replaced by the *TRP1* gene. In Figures 7A and 7B, the blot contains RNA from diploid **a**/ α cells that carried the wild type and from those cells that carried the **a**1- α 2 site mutant (pRMmut5). In Figure 7A, the blot incubated with a single-stranded T7-transcribed RNA probe (*IME4* antisense probe) detected *IME4* sense strand only in the wild type (Lane 3). When the blot was incubated with *IME4* sense probe (a single-stranded T7 transcript), in Figure 7B, the *IME4* antisense strand was detected only in the **a**1- α 2 site mutant. These findings are consistent with Hongay et al (2006). Thus, the *IME4* mRNA is expressed only in **a**/ α cells whereas the antisense RNA is observed in haploid cells. Investigation of this **a**1- α 2 site mutant in the downstream region of *IME4* confirmed that this mutation allows antisense transcription in diploid MAT**a**/MAT α cells and this reduces sense strand accumulation which can lead to a sporulation defect.

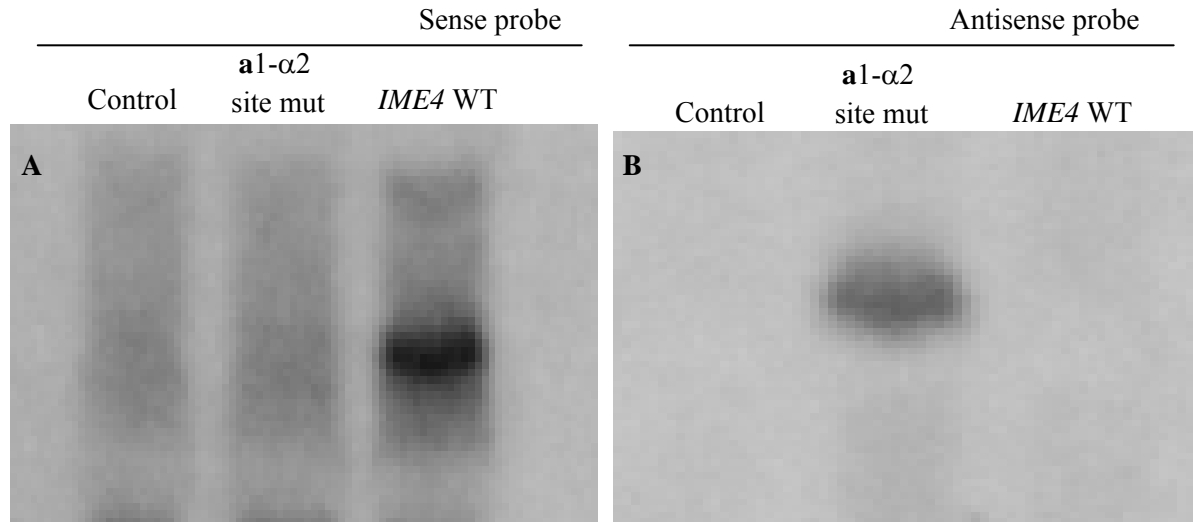


Figure 7. Northern blot of MAT α /MAT α *IME4* knockout yeast. (A) Lane 1 contains RNA from cells that carried the vector only-pRS316, lane 2 contains RNA from cells that carried the a1-α2 site mutant pRMmut5, and lane 3 contains RNA from cells that carried the wild type-pRM2b. (B) Same as in (A).

Based on the northern results that the antisense *IME4* strand is transcribed, further investigation about the region before the start site of the antisense transcript was conducted. We wanted to further define sequences responsible for expression and regulation of the two *IME4* RNAs. A model of meiosis regulation is shown in Figure 8.

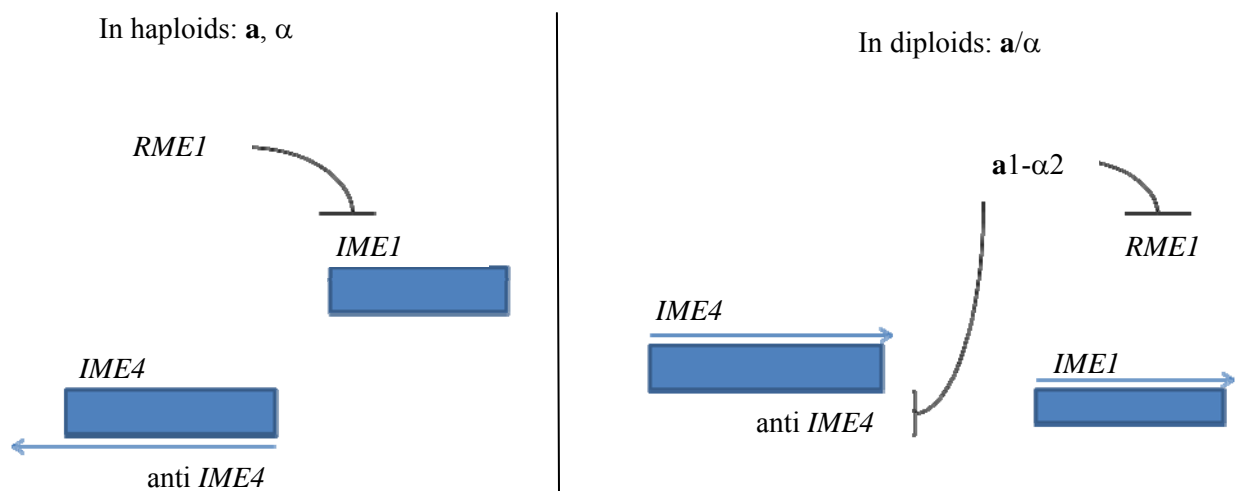


Figure 8. Diagram of meiosis regulation in *Saccharomyces cerevisiae*.

These results open the questions including what are the mechanisms by which antisense transcription occurs and how do the mechanisms decrease the sense strand.

Initially, a 392 base pair segment of DNA, starting with the stop codon of the *IME4* gene in *Saccharomyces cerevisiae*, was aligned with other yeasts using the *Saccharomyces cerevisiae* database fungal alignment tool (Figure 3). The $\alpha 1$ - $\alpha 2$ binding site is conserved among these other yeasts, whereas most of the rest of the 3' region is not highly conserved. The nucleotide sequences that comprise an apparent Reb1 binding site were present in most of the yeasts in this group, suggesting that this was an important sequence element for some aspect of gene expression. Comparison of this Reb1 binding site sequence to the Transfac database, which contains information about transcription factors and their target genes and regulatory binding sites, identified the sequence as the consensus binding site for Reb1 (www.gene-regulation.com). The Reb1 binding site sequence identified in the database from the multiple sequence alignment is consistent with genome-wide studies that also identified this as a strong potential binding site (Liaw et al, 1994 and Houseley et al 2008).

Site-directed mutagenesis to create a Reb1 mutant

A putative Reb1 mutant was constructed by site-directed mutagenesis of the 3' end of the full-length *IME4* gene contained in plasmid pRM2b. PCR with forward and reverse primers ANOTHERReb1 substituted a HindIII restriction enzyme site, which is 5'-AAGCTT-3', for the Reb1 binding site, which is 5'-ACCCGG-3'. The specific base changes are shown in Figure 9.

| | | | |
|---|--------------|------------|--|
| | $\alpha 2$ - | $\alpha 1$ | |
| Wild Type: CATC CGGGT AATAAATTGTTCCAGTT TGATGTAAAAAAATACACGG ACTCGGATAA | | | |
| Reb1 site | a1/a2 site | | |
| Δ Reb1: CA AAGCTT AATAAATTGTTCCAGTTGATGTAAAAAAATACACGGACTCGGATAA | | | |
| Δ a1/a2: CATCCGGGTAATAAATTGTTCCAGT CCATGG AAAAAAATACACGGACTCGGATAA | | | |

Figure 9. Putative Reb1 binding site mutant sequence. Mutations in the Reb1 protein binding site and also in the a1/a2 site in our mutant plasmids. The wild type sequence is shown for reference.

The ligation mix was then transformed into *E. coli* colonies that were grown overnight; then single colonies were minipreped to isolate the plasmid DNA. The plasmids were initially analyzed by restriction enzyme digest with EcoRI and HindIII restriction enzymes. These enzymes were utilized because the EcoRI site is present in the IME4 gene and the HindIII site was introduced by the mutagenesis reaction.

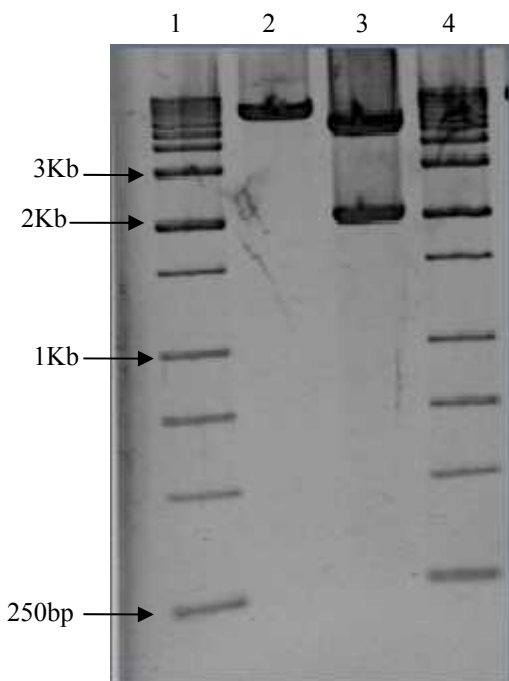


Figure 10. EcoRI and HindIII Restriction Digest pRM2bmutE. Lanes 1 and 4 contain the Kb DNA ladder 250bp to 12 Kb. Lane 2 contains pRM2b. Lane 3 contains pRM2bmutE.

Plasmids containing both the *IME4* gene along with the mutant Reb1 binding site contained a 2000 base pair fragment of DNA when digested with EcoRI and HindIII, as seen in lane 3 on the agarose gel in Figure 10. As expected, the control plasmid pRM2b (lane 2) is 5 kb with no additional fragments observed. In this experiment, there were 6 out of 20 randomly-selected colonies whose plasmids contained the 2 kb fragment. These six plasmids were further analyzed by sequencing the region of the desired mutation using the 2190 reverse primer and comparing that to the *Saccharomyces cerevisiae* genome with BLAST (Figure 11). The entire insert containing the full-length *IME4* gene was also sequenced using -444 forward primer, 2190 reverse primer, Cys-rich forward and reverse primers, MTase forward and reverse primers, and IME4 Region4 reverse and forward primers (See Table 1) and then compared with the genomic DNA of *Saccharomyces cerevisiae* (Appendix A). The resulting plasmids, which contained the potential Reb1 binding site mutation but no other changes in the *IME4* gene, were retained for further analysis.

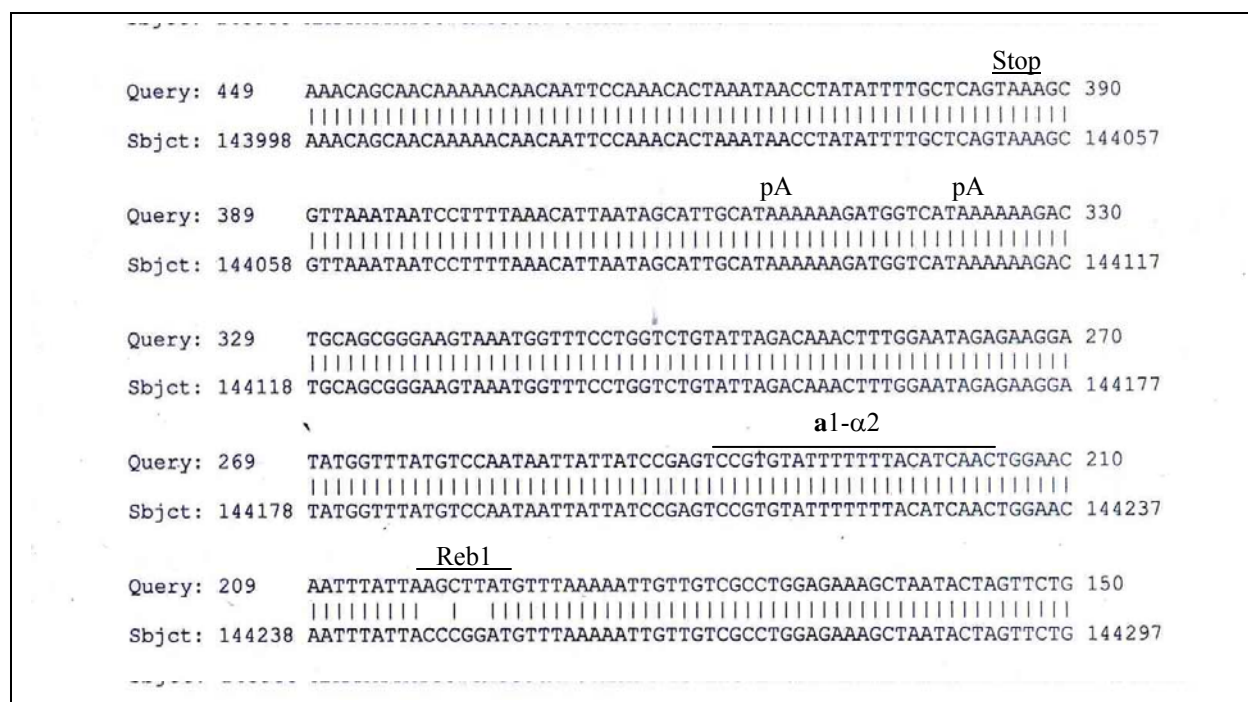


Figure 11. BLAST results comparing pRM2bmutE with the *S. cerevisiae* genome database. (www.yeastgenome.org). The Reb1 site mutant is from base 196 to 201 of the query sequence. *IME4* Stop codon starts at base 144052 of the subject

β-galactosidase assays

We tested the hypothesis that the 3' *IME4* region contains a haploid-specific transcriptional regulatory region. Transcription promoting activity of the 3' region of the *IME4* gene, where the Reb1 and a1- α 2 binding sites are located, was tested using beta-galactosidase assays. A 190 base pair region spanning nucleotides 144 to 334 downstream of the *IME4* stop codon was cloned into a yeast high copy vector with a *lacZ* reporter gene, a schematic is shown in Figure 12. Plasmid pSX178 contains an *E. coli lacZ* reporter fused downstream of a portion of the yeast *CYC1* gene. The *CYC1* region includes the TATA box and transcription and translation initiation sites but lacks the upstream activation sequence present in the full length gene. This plasmid also contains the yeast *URA3* and *E. coli Amp^R* (confers ampicillin resistance) genes for selection in yeast and *E. coli*, respectively and is maintained in high copy

by the origin of replication located on the plasmid. The plasmid pSX178 is the empty vector, p31 has the wild type *IME4* downstream region, p5-2 has the *IME4* downstream region from pRMmut5 which has the $\alpha 1$ - $\alpha 2$ binding site converted to a KpnI restriction enzyme site, and p53 has the downstream *IME4* region from pRM2bmutE with the Reb1 binding site switched to a HindIII restriction enzyme site.

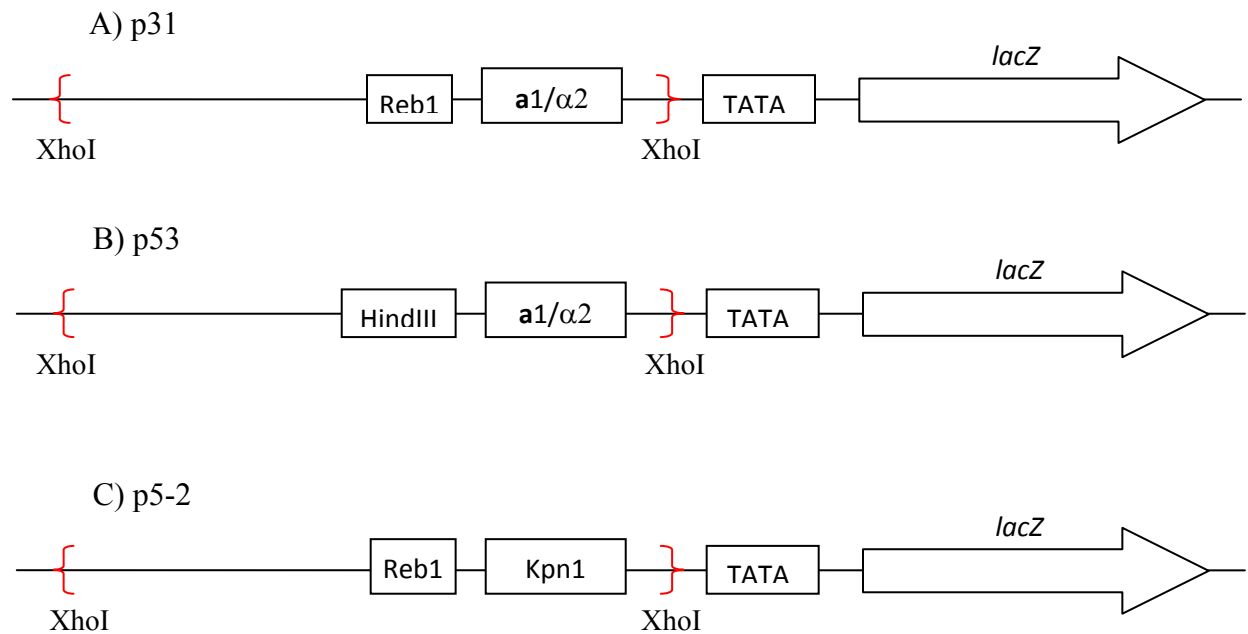


Figure 12. Beta-galactosidase Reporter constructs. A 190 bp region downstream of *IME4* stop codon, XhoI sites indicated by the red brackets, was cloned into a vector containing the *lacZ* reporter gene. Panel A shows the wild type *IME4* named p31, panel B shows the mutant Reb1 binding site from *IME4* named p53, and panel C shows the $\alpha 1/\alpha 2$ binding site mutant named p5-2.

To confirm the presence of the beta-galactosidase constructs, restriction enzyme digest followed by agarose gel electrophoresis was performed on the plasmids from the transformed *E. coli*. Figure 13 shows a picture of the agarose gel of the restriction digest of the plasmids with XhoI restriction enzyme. Restriction enzyme XhoI cuts double stranded DNA at the sequence 5'-CTCGAG -3'; this site is located on both ends of the insert. A digestion in which the correct

sized fragment was cut from the plasmid confirmed the presence of the insert and its orientation. Lane 1 contains the 12 Kb DNA ladder for reference fragment size. Lane 2 shows the empty vector plasmid. Lanes 3 through 5 contain the beta-galactosidase constructs which have two bands in each lane. It is clear that the beta-galactosidase constructs for assays were successfully cloned because of the band at 190 base pairs. The insert orientation was confirmed by sequencing (not shown). These constructs were then used to assay for any transcriptional activity.

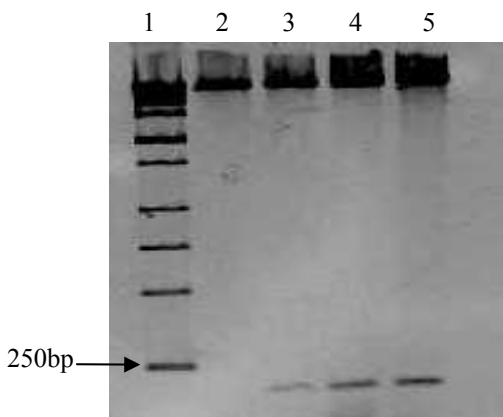


Figure 13. Restriction Digest of *lacZ* constructs with XhoI. Lane 1 contains the Kb DNA ladder 250bp to 12Kb, lane 2 contains the pSX178 vector, lane 3 contains p31, lane 4 contains pR53, and lane 5 contains p5-2. (see Figure 11)

Each of the four reporter plasmids was transformed into diploid MAT α /MAT α , haploid MAT α , and haploid MAT α *IME4*-knockout yeast strain YYF101. Transcription can be determined indirectly by determining whether *lacZ* beta-galactosidase activity is detected. If the *lacZ* gene is expressed, the enzyme beta-galactosidase is synthesized, and is detected by hydrolysis of a substrate called *o*-nitrophenyl- β -D-galactoside (ONPG). Soluble extracts from each transformed strain were assayed for beta-galactosidase activity. Absorbance of light at 420

nm for each assay measured hydrolysis of *o*-nitrophenyl- β -D-galactoside (ONPG). The absorbance was used to calculate specific activity of the beta-galactosidase enzyme. Specific activity of beta-galactosidase was determined by utilizing the equation described in Materials and Methods.

Figure 14 shows the specific activity of the wild type *IME4* 3' downstream region compared to the *IME4* 3' downstream **a1**- α 2 site mutant. Yeast strains containing either plasmid were grown in the presence of either glucose (Figure 14A) or acetate (Figure 14B). As expected from the expression pattern of the antisense RNA observed in the Northern analysis, beta-galactosidase activity was observed in both **a** and α haploids carrying the wild type and **a1**- α 2 site mutated sequences. The specific activity of the reporter was constant in the haploid cells containing the wild type and **a1**- α 2 site mutant. In the diploid cells by contrast, the activity of the reporter was repressed from the wild type plasmid--activity was essentially undetectable in these cells. This repression is significant in diploids and is relieved 9-fold in cells carrying the **a1**- α 2 site mutant versus the cells carrying the wild type. In glucose p-value was 0.0001 and in acetate p-value was 0.0000007. Surprisingly, the repression from the wild type appears even lower than in the vector only cells because of the strength of the **a1**- α 2 repression. In the **a1**- α 2 site mutant, however, activity was comparable to that of the haploids' specific activity of 17 from wild type versus 15 from the **a1**- α 2 site mutant in **a** cells ($p = 0.246$) and specific activity of 30 from wild type versus 20 from the **a1**- α 2 site mutant in α cells ($p = 0.398$) for strains in glucose. For haploids in acetate, p-value was 0.15 for **a** cells and .007 for α cells. Specific activity is typically low in diploids because the cells are larger.

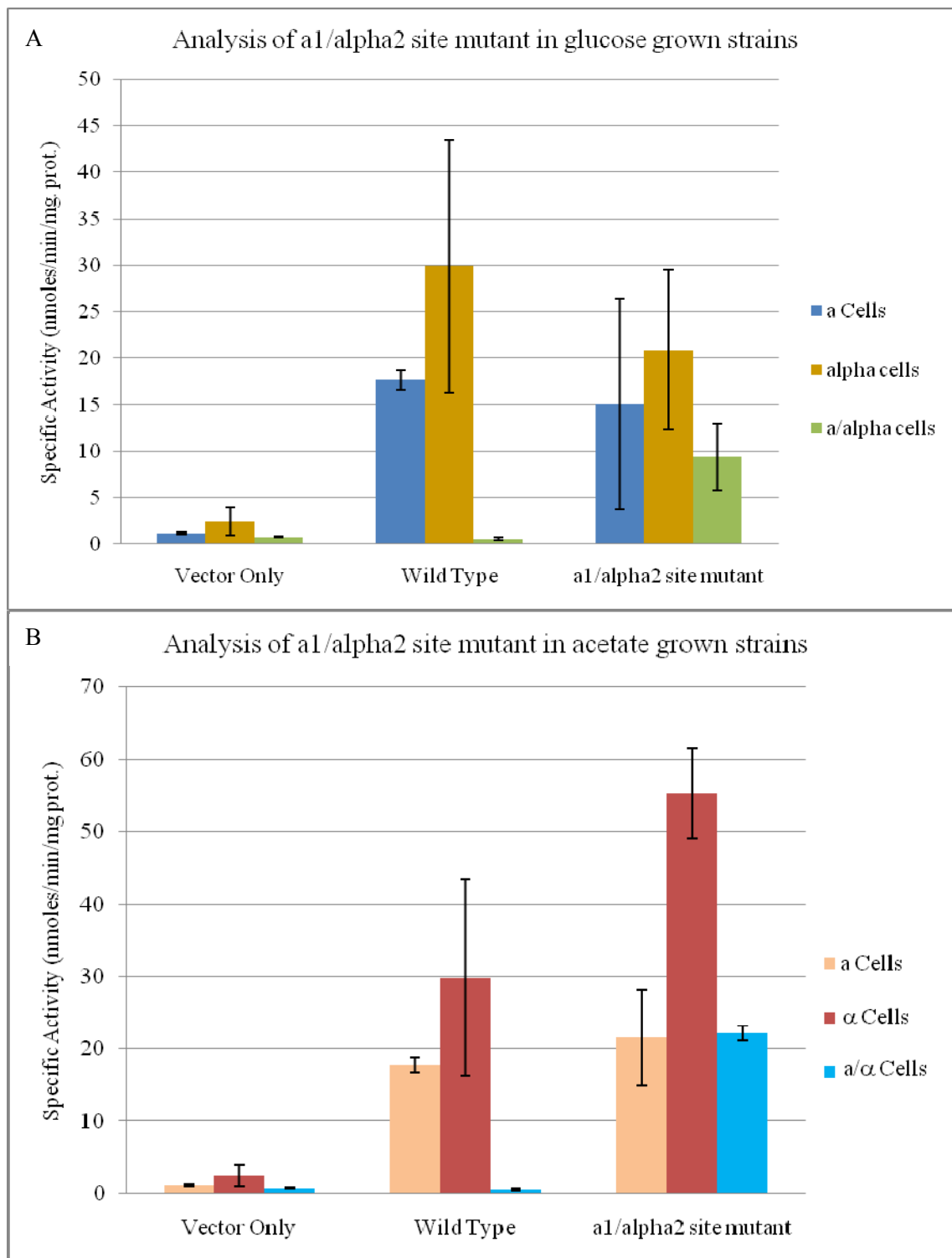


Figure 14. Specific activity of *IME4* 3' region with the $\alpha 1$ - $\alpha 2$ site mutant in *lacZ* reporter strains. (A) Yeast cultures were grown in glucose (B) Yeast cultures were grown in acetate

We conclude that this 190 bp region 3' to *IME4* contains haploid-specific UAS activity and that the **a**1- α 2 site is functional in this context, repressing UAS activity more than 10 fold.

Reb1 binding sites have most frequently been associated with activation of RNA polymerase II promoters; however they also contribute to repression of some genes. To examine their effects on UAS activity, we examined transformants carrying the wild type (pRM2b) and mutant (pRM2bmutE) forms of the sequence (Figure 11, pg. 31). Figure 15, shows the specific activity from the *lacZ* reporter of the wild type 3' downstream region of *IME4* in comparison to the Reb1 binding site mutant. Again, yeast strains were grown in the presence of either glucose (Figure 15A) or acetate (Figure 15B). The results show (Figure 15) that the specific activity of the beta-galactosidase enzyme was clearly reduced in the haploid **a** and α cells carrying the Reb1 site mutant, in comparison to the wild type. For strains in glucose, the p-values were 0.00006 and 0.0005 for **a** cells and α cells respectively. For strains in acetate, the p-values were 0.0000001 and 0.002 for **a** cells and α cells respectively. No activity was detected in the diploid **a**/ α cells, p-values were 0.298 and 0.307 for strains in glucose and acetate respectively, because of repression in *cis* by the **a**1- α 2 site on the plasmid. The specific activity that is lower in comparison to the wild type means the beta-galactosidase enzyme is not being synthesized from the mutant plasmid as much as it is from the wild type. Transcription of the *lacZ* gene is consistently less from the Reb1 binding site mutant plasmid in the context of this reporter. As shown in the figure, the Reb1 site mutant diminished promoter activity to 20% of the wild type level. This magnitude decrease is consistent with earlier work with *GCY1* (Angermayr and Bandlow, 2003) where the mutant Reb1 site diminished activity to a third of the activity of wild type cells.

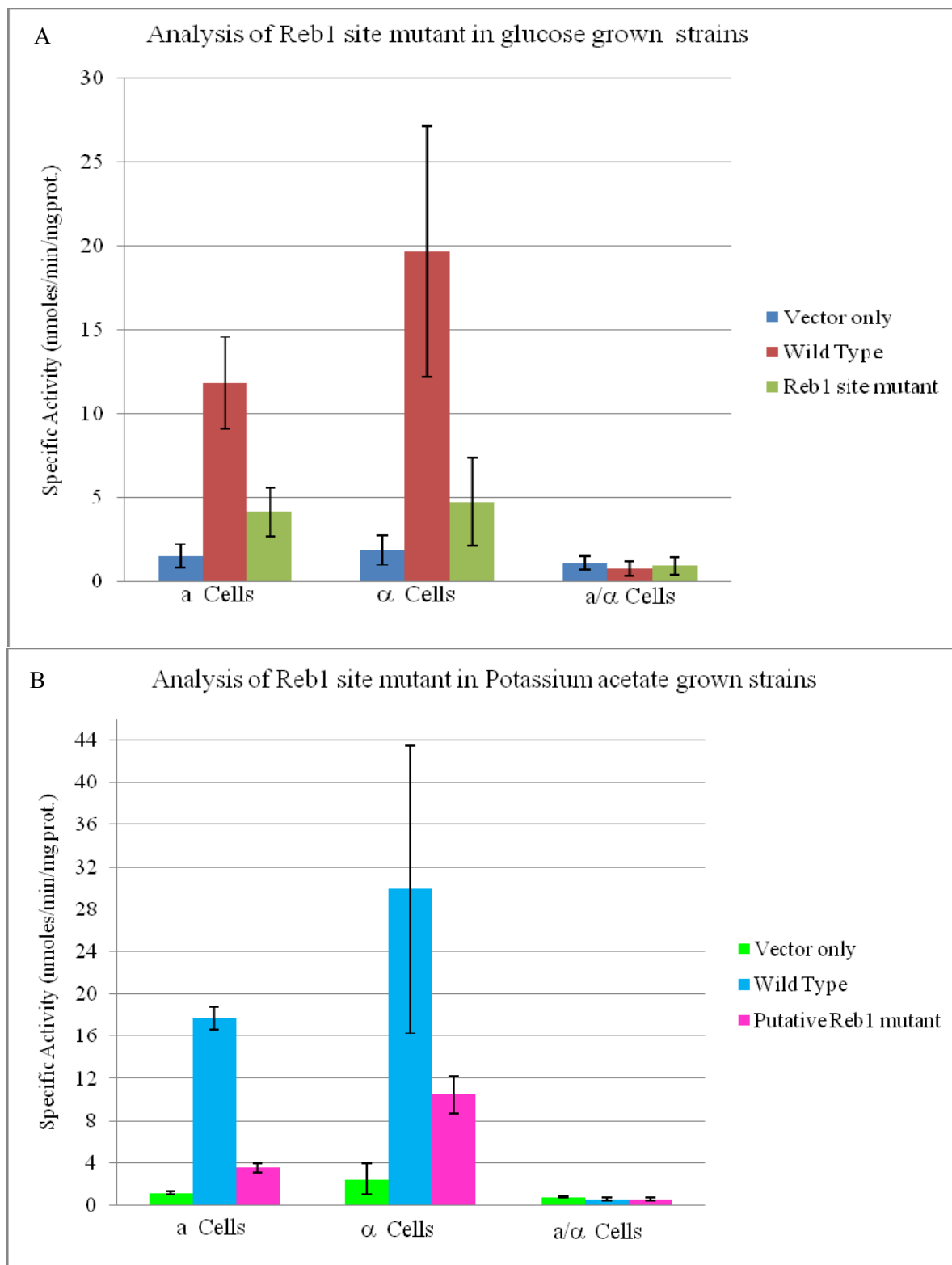


Figure 15. Specific activity of *IME4* 3' region containing the Reb1 binding site mutant in *lacZ* reporter strains. (A) Yeast cultures were grown in glucose (B) Yeast cultures were grown in acetate.

Diploid phenotype in sporulation media: Complementation assay

We wanted to observe the effects of the site-directed mutation of the Reb1 site in the context of the full-length *IME4* gene. Centromeric yeast plasmids containing either the wild type *IME4* gene (pRM2b) or the full-length gene with the Reb1 binding site mutant (pRM2bmutE) were transformed into diploid YYF101 **a**/ α cells. These cells are unable to sporulate because *IME4* is deleted from the strain. A normal *Saccharomyces cerevisiae* **a**/ α diploid cell will undergo meiosis and sporulation when environmental conditions are favorable. Typically when the diploid cell finds itself starved for nitrogen and carbon, transcription of the repressor of meiosis gene-*RME1* is repressed, and the cell can begin the meiotic cycle. The results of meiosis are the formation of four ascospores in the cell, a structure referred to as a tetrad. DIC microscopy was utilized to observe the cells to see if they contained asci after being incubated in sporulation media lacking nitrogen and carbon sources. Figure 16 shows the appearance of wild type diploid cells and the *IME4* 3' downstream region containing the Reb1 binding site mutant diploid cells at 400X magnification. The wild type cells contain tetrads in more than 80% of the field of view, while the cells with the Reb1 binding site mutant have 1 or 2 tetrads but closer to none. Table 4 shows the percentage of sporulated diploid cells observed after counting cells with tetrads. The percentages are consistent with what is seen in the microscope image.

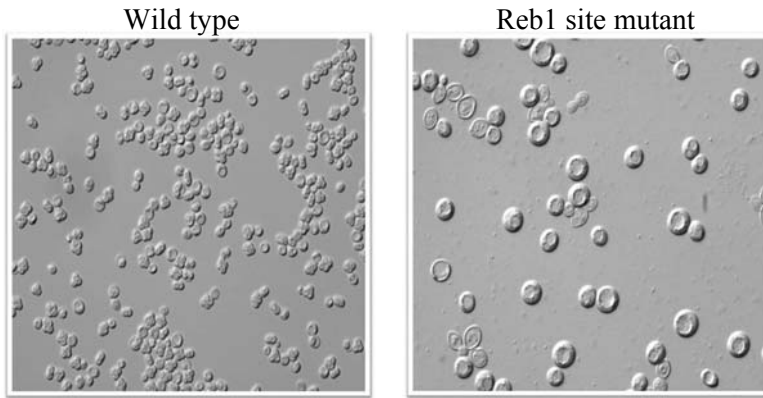


Figure 16. Phenotype of *Saccharomyces cerevisiae* after 48 hours in sporulation media. The wild type diploid cells are on the left and those containing the 3' *IME4* Reb1 binding site mutant are on the right.

| Table 3. Sporulation in MAT α /MAT α YYF101 <i>S. cerevisiae</i> | | | |
|--|------------------------------------|---------------------------------|-------------------------|
| Plasmid | Number of cells containing tetrads | Number of cells without tetrads | % of cells with tetrads |
| Empty vector | 0 | 2,852 | 0.0 % |
| Wild type <i>IME4</i> | 948 | 146 | 86.7 % |
| Reb1 site mutant #1 | 2 | 1,015 | 0.2 % |
| Reb1 site mutant #2 | 2 | 1,014 | 0.2 % |
| Reb1 site mutant #3 | 0 | 1,047 | 0.0 % |

Table 3. Sporulation Phenotype of diploid yeast. The percentage of sporulation is in the third column.

These results are surprising given the reporter assays above; Reb1 site mutant reduced transcription of the anti sense *IME4* in that assay, which in turn was expected to either enhance sporulation in α/α diploid cells or to have no effect on sporulation. Instead, the Reb1 site mutant behaved like the $\alpha 1$ - $\alpha 2$ site mutant, which allows antisense *IME4* transcription and prevents

sporulation in **a**/ α cells. These results suggested that the Reb1 site plays a different role in the context of the full-length gene than it does in the artificial reporter plasmid.

Development of a strand-specific and quantitative assay for RNA

The lack of complementation by the site-directed mutant plasmids suggested that the putative Reb1 binding site is important for some positive aspect of *IME4* expression. Thus, in order to analyze expression of sense versus antisense strand of genes, an assay had to be developed. Previous protocols were too cumbersome for this study as these used ^{32}P labeled T7-synthesized single-stranded riboprobes. The objectives of the assay were that it had to be able to clearly distinguish between the antisense and the sense strand and be quantitative at the same time. The assay also had to be reasonable for a large number of samples to be analyzed and have a very low background or no background at all. Taking into account these objectives, an assay utilizing cDNA synthesis by reverse transcriptase followed by quantitative PCR and data analysis was established for this strand-specific analysis.

To first test this strand-specific assay, **a**, α , and **a**/ α *Saccharomyces cerevisiae* were grown in glucose overnight, and the RNA was isolated from all three cell types. Primers were developed that complement each strand of the gene for actin, and used separately for the gene-specific cDNA synthesis reactions. Figure 17 shows an overview of the strand specific qPCR analysis.

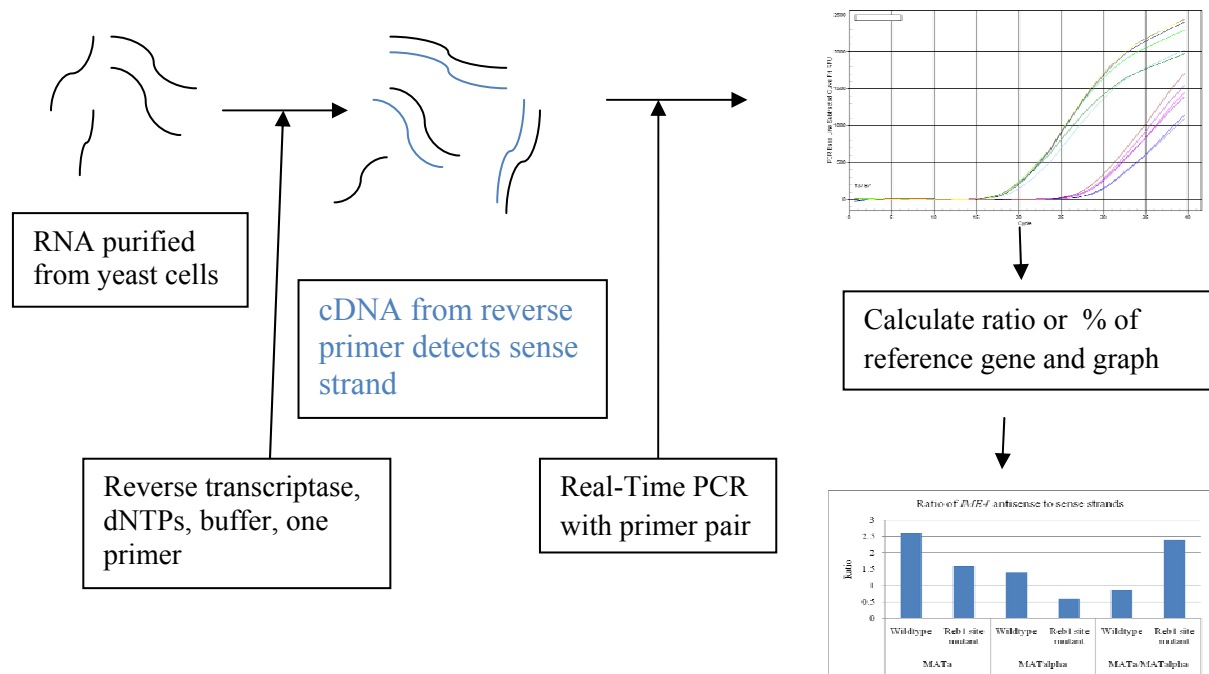


Figure 17. Flow-chart diagram of a strand-specific analysis for RNA.

The oligo dT primer was used for cDNA synthesis of RNA with poly-A tails. The newly synthesized cDNA was amplified with the Actin gene-specific primers in a qPCR analysis to determine if a specific strand is favorably expressed and the quantity of each actin strand in the different cell types. Amplification charts of the qPCR show the difference in sense strand and antisense strand quantity detected based on the threshold cycle (C_T), the number of cycles of repeated synthesis it would take to cross the threshold value where exponential growth of the sequenced fragment is at its peak. The lower the cycle number means the more abundant that target DNA sequence. The higher the cycle number means a low quantity of the target DNA sequence. Figure 18 shows the curve generated by the BIORad MyiQ Single-Color detection system for the Actin RNA analysis. The samples that crossed the threshold first include the Actin sense cDNA strand primed with the Actin reverse primer and the oligo-dT primer. The

next groups of samples that come up 10 cycles later include the Actin antisense cDNA primed with the Actin forward primer and the sample that had no primers, which shows that there is a very small amount of product made from the RNA itself as a primer. The data are shown in the form of bar graphs (Figure 19).

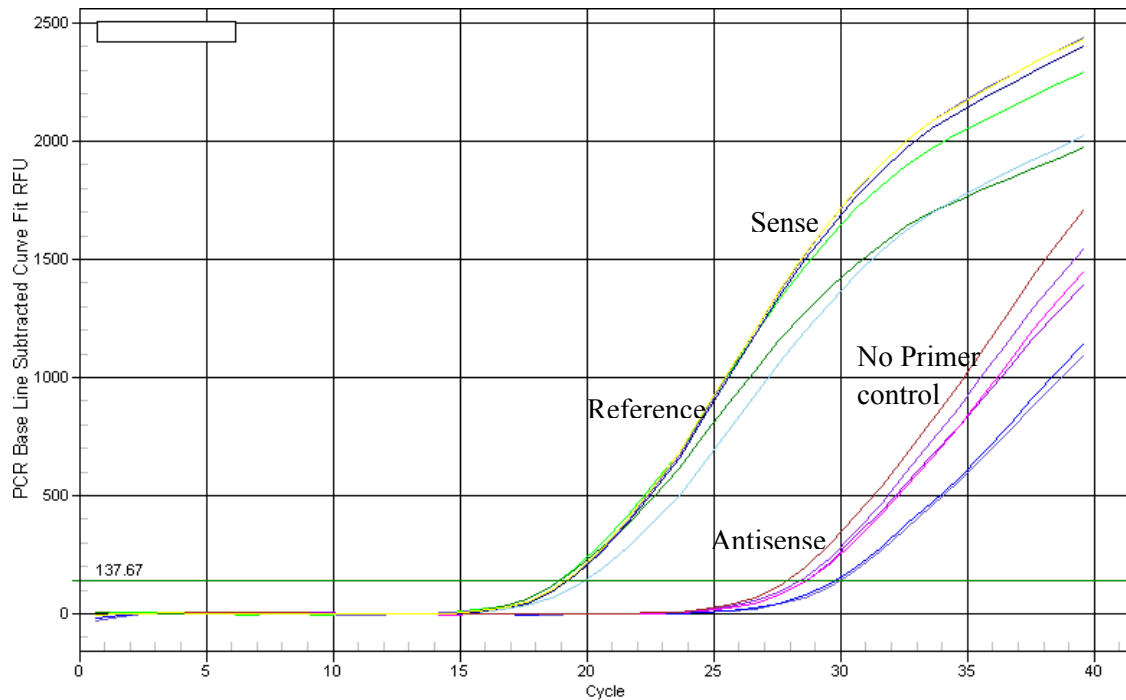


Figure 18. Amplification Chart of Actin qPCR. Actin sense DNA strand is 10 cycles quicker in appearance than the antisense strand.

Figure 18 shows the results of this analysis with the quantity of each strand expressed as a percent of the quantity of actin detected in the oligo-dT primed cDNA reaction. There is no evidence that the actin antisense RNA is transcribed so I hypothesized that only the actin sense strand will be detected in this analysis. The graph clearly shows that no antisense actin RNA was found in neither the haploid **a** and alpha cells, nor in the **a**/ α diploid cells. These results satisfy the goal of strand specificity distinguishing between the two strands. The result of this first test of the assay was for a housekeeping gene, actin, which is always expressed in cells.

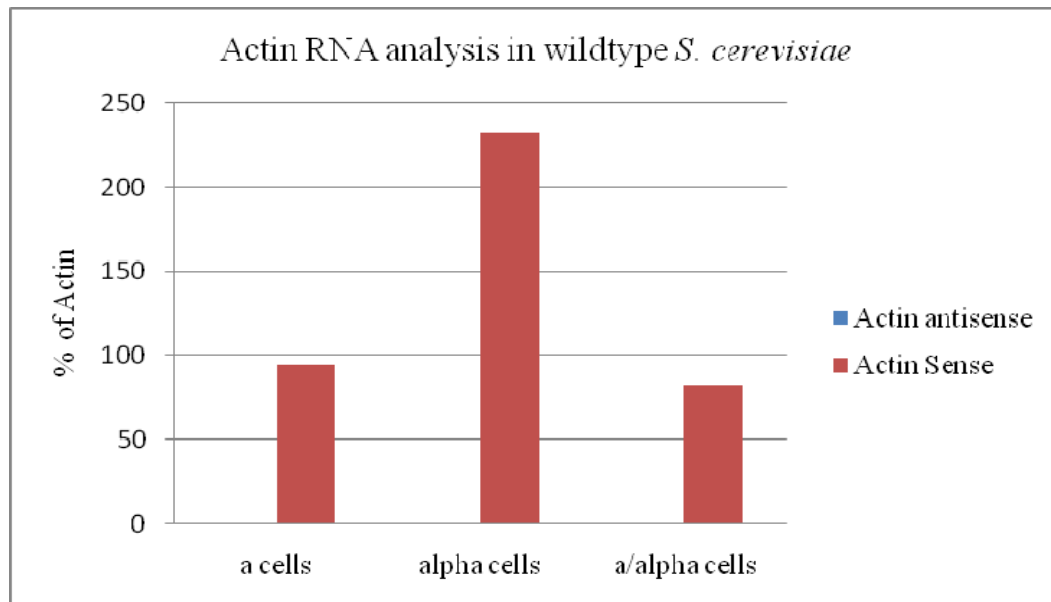


Figure 19. Strand-specific analysis of Actin. qPCR of Actin after single-primer cDNA synthesis reactions with actin forward (detects antisense) primer or reverse (detects sense) primer.

Next, cDNA synthesis specific for the sense versus antisense *IME4* gene strands was followed by qPCR to quantify the two strands from RNA of wild type strain of *Saccharomyces cerevisiae* **a**, α , and **a/α** cells. Total RNA was isolated as before, and cDNA was synthesized using primers for sense or antisense *IME4* RNAs. Then amplification was conducted using both *IME4* primers in the qPCR reactions. The results (Figure 20) show that the antisense RNA is detected primarily in haploid cells, with very little seen in **a/α** diploids. Conversely, the *IME4* sense RNA predominates in **a/α** diploid cells, as expected from previous Northern blot analysis. These experiments were repeated using primers from three different regions of the *IME4* gene and similar results were observed (data not shown).

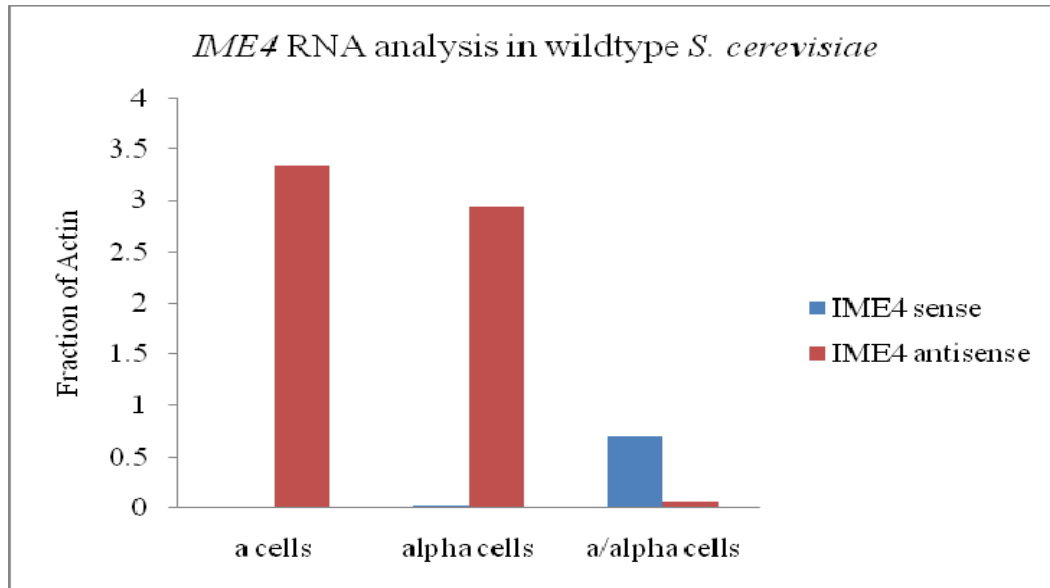


Figure 20. Strand-specific analysis of *IME4*. qPCR results of *IME4* sense and antisense single strand cDNA synthesis reactions with *IME4* Region 2 forward or reverse primer.

We conclude that this approach can be used to distinguish relative sense versus antisense RNA levels in the mutant strains.

Strand-specific qPCR analysis of Reb1 binding site mutant

After development of the strand-specific RNA analysis utilizing qPCR, *Saccharomyces cerevisiae* yeast strain YYF101 **a** and α haploid, and **a**/ α diploid strains were transformed with plasmids pRM2b and pRM2bmutE. Transformed cells were grown overnight in SC caa, glucose-rich medium then RNA was isolated and tested for *IME4* sense and antisense RNA expression by the strand-specific assay. RNA was incubated with a single primer for cDNA synthesis. Primers included the *IME4* antisense probe with sequence identical to the sense strand, *IME4* sense primer with sequence identical to the antisense, and the oligo-dT primer. The newly synthesized cDNA was then amplified following a qPCR protocol and quantified. In Figure 20, the amplification chart of from the analysis shows in the sample with the lowest threshold cycle is the oligo-dT primed cDNA amplified with actin primers because actin is the

reference gene. The next two curves that come up on the chart were the antisense and sense *IME4* cDNA samples, and vice versa depending on the cell. The last curve to come up was the control with no reverse transcriptase in the cDNA reaction enforcing the validity of the strands detected in lower cycle numbers. The quantity of each of the *IME4* strands were calculated as a percent of actin and the ratio of *IME4* antisense strand to sense strand was graphed in Figure 22. In the *IME4* wild type diploid α/α cells, there is about a five cycle difference in the sense strand versus the antisense strand (Figure 21). This is partial evidence that there is more *IME4* sense strand than *IME4* antisense strand expressed in these cells in the presence of glucose, based on the lower cycle number for the sense strand to be detected.

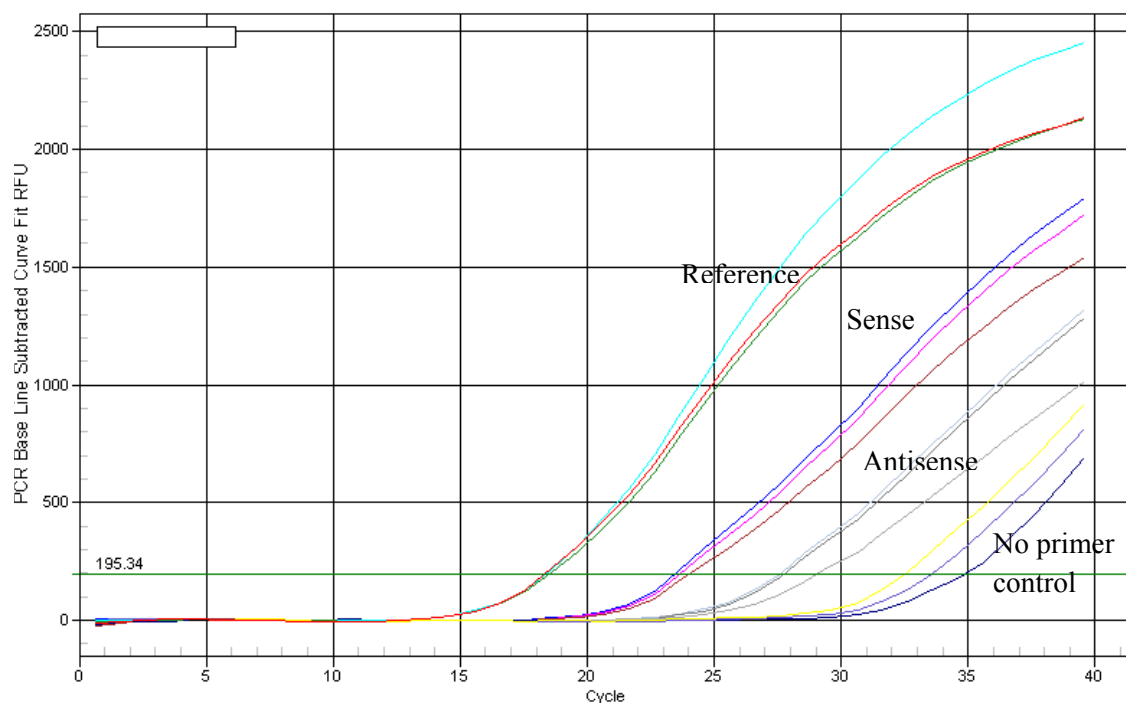


Figure 21. Amplification chart of a Strand-specific qPCR for *IME4*. cDNA from α/α cells carrying the wild type full-length *IME4* gene and 3' downstream region.

As seen in the bar graph in Figure 21, in the haploid MAT α and MAT α cells, the *IME4* antisense strand was expressed about twice as much in the wild type than in the Reb1 site mutant.

However, diploid MAT α /MAT α cells showed an opposite pattern compared to what is observed in the haploids; the *IME4* antisense strand was expressed about three times less in the wild type than in the Reb1 site mutant.

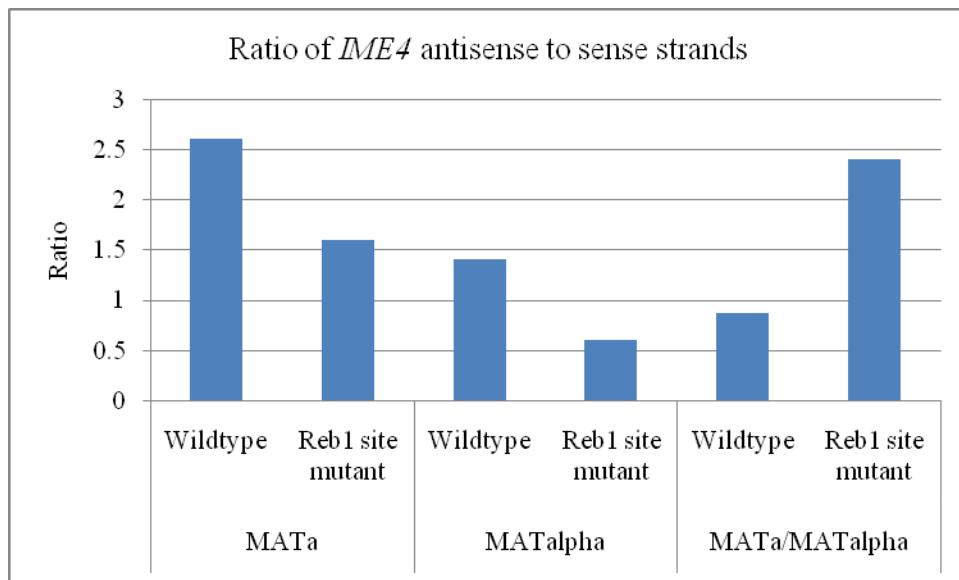


Figure 22. Ratio of *IME4* sense to antisense strands in *IME4* knockouts. RNA was isolated from cells grown in glucose and strand-specific assay detected the separate strands.

Further RNA analysis of diploid *Saccharomyces cerevisiae* transformed with either the empty vector plasmid pRS316, the full length *IME4* on plasmid pRM2b, the full length *IME4* with Reb1 binding site mutant on plasmid pRM2bmutE, or the full length *IME4* with the $\alpha 1$ - $\alpha 2$ site mutant on plasmid pRMmut5 was performed to examine how nutrition affects the *IME4* antisense expression. These transformants were grown in liquid SC caa overnight to reach the exponential growth phase followed by 1:10 dilution of the cells in PSP media for 24 hours to grow. Next the transformants were incubated in liquid sporulation media for 3, 4, or 7 hours, then their RNA was isolated and quantified. Then strand-specific cDNA synthesis of the *IME4*

sense and antisense strands was carried out followed by qPCR for quantification. Actin and *TAP42* housekeeping genes were used as a reference in calculating the quantity of each strand. Efficiency of the *TAP42* primers in qPCR analysis was also done on a serial dilution of a plasmid containing *TAP42* to verify the primers function correctly. Figure 23 shows the standard curve generated by the qPCR reaction, showing clearly that the lower the concentration of the target DNA, the higher the threshold cycle for product or newly synthesized DNA in the PCR reaction is detected. The r^2 value of 0.99 suggests that human error is at a minimum in technique and set-up of the assay.

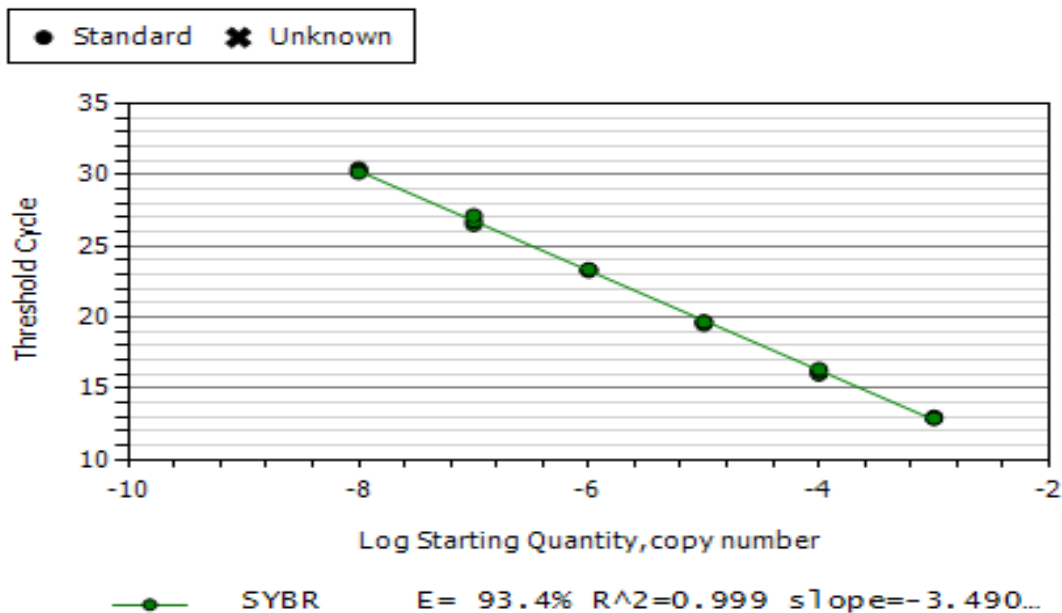


Figure 23. Standard curve for *TAP42* primers. Efficiency of *TAP42* primers amplification of its target DNA in a serial dilution of a plasmid carrying the *TAP42* gene.

The graph in Figure 24 shows the result of the qPCR analysis with the quantity of each strand calculated as a percent of actin for the cells grown in sporulation media for 3 hours. RNA from the diploid cells carrying the empty vector pRS316 had no *IME4* strands detected, which was expected because pRS316 has no *IME4* sequence incorporated into it. In the cells that were

carrying the wild type *IME4* gene on plasmid pRM2b, sense and antisense *IME4* strands were detected and quantified in reference to actin. There was about seven times as much sense *IME4* detected than antisense, which had little to no detection, in the wild type. In cells carrying *IME4* plus a mutant Reb1 protein binding site (pRM2bmutE), *IME4* sense strand was detected six times lower than it was in the wild type, and there is detection of *IME4* antisense strands also at 2-3 times more than in the wild type. In the cells carrying *IME4* along with the $\alpha 1$ - $\alpha 2$ site mutant (pRMmut5) there is no detection of the *IME4* sense strand; however, the antisense strand is detected, consistent with the Northern analysis above. The results suggest the Reb1 site and the $\alpha 1$ - $\alpha 2$ site in the 3' downstream region of *IME4* are required for full expression of the sense *IME4* strand.

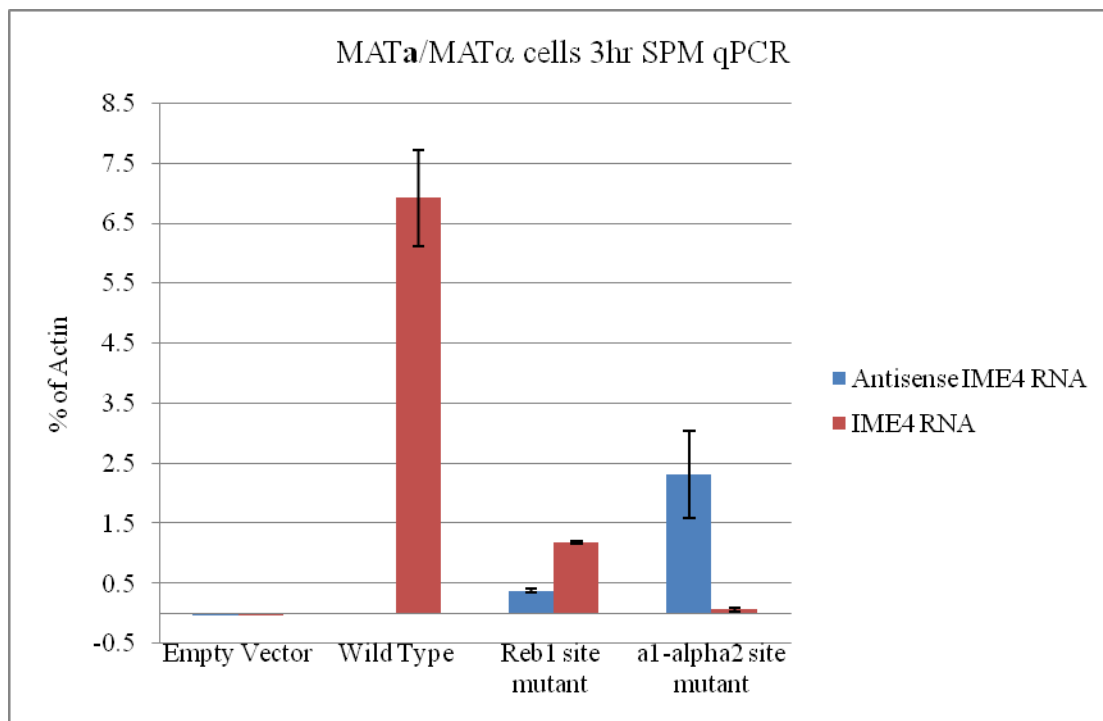


Figure 24. qPCR strand-specific analysis of diploids 3hrs in SPM. Cells were incubated in sporulation media for 3 hours before RNA was isolated from them for analysis.

The graph in Figure 25A shows the result of the qPCR analysis with the quantity of each strand calculated as a percent of actin for the cells grown in sporulation media for 4 hours. As expected, RNA from the diploid cells carrying the empty vector pRS316 had no *IME4* strands detected and was not graphed. In the cells that were carrying the wild type *IME4* gene on plasmid pRM2b, sense and antisense *IME4* strands were detected and once again quantified in reference to actin. There was about six times as much sense *IME4* detected than antisense, which had very low detection, in the wild type. In cells carrying *IME4* plus a mutant Reb1 protein binding site on plasmid pRM2bmutE, *IME4* sense strand was detected eight times lower than it was in the wild type, and detection of *IME4* antisense strands were twice as much in this mutant than in the wild type. Among the RNA isolated from the cells carrying *IME4* along with the $\alpha 1$ - $\alpha 2$ site mutant the *IME4* antisense strand was detected 9 times more than the sense strand. The results with the $\alpha 1$ - $\alpha 2$ mutant are consistent with Hongay et al (2006).

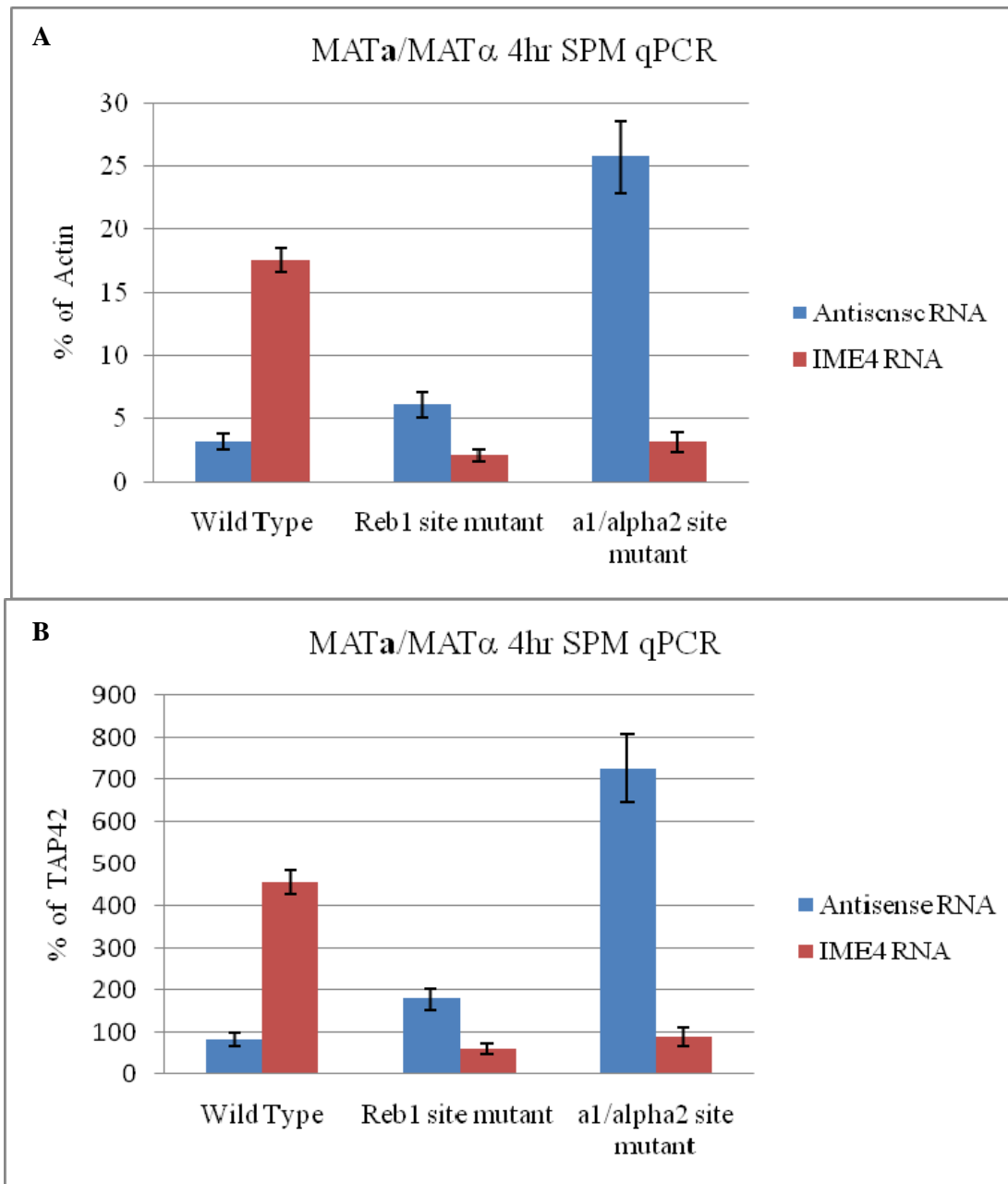


Figure 25. qPCR strand-specific analysis of diploids 4hrs in SPM..(A) *IME4* sense and antisense strands quantified as a percent of actin after 4 hours of nitrogen and carbon starvation. (B) *IME4* sense and antisense strands quantified as a percent of *TAP42* after 4 hours of nitrogen and carbon starvation.

The same samples of RNA isolated for the 4 hour analysis were assayed again except this time with oligo-dT primed cDNA amplified with primers for *TAP42* in the qPCR step. Figure 25B shows the result of the strand-specific analysis with the quantity of each strand calculated as

a percent of *TAP42*. *IME4* sense strand was detected about four times as much as the antisense strand in cells carrying the wild type plasmid. In cells carrying the Reb1 binding site mutant there was about three times as much *IME4* antisense strand detected than sense strand. In the RNA samples from the cells carrying the $\alpha 1$ - $\alpha 2$ site mutant plasmid, *IME4* antisense strand was detected 8 times more than *IME4* sense strand. These results with *TAP42* as a reference are consistent with the results obtained using actin as a reference.

This same strand-specific assay was performed on diploid MAT α /MAT α YYF101 transformants exposed to sporulation media for 7 hours to observe if the expression patterns were similar to that of the 3 and 4 hour samples. Actin was used as the reference gene for the calculations so quantity of each strand was calculated as a percent of actin. The results, in Figure 26, showed an expression pattern similar to that of the 4 hour samples, however, on a much smaller scale. In the diploid cells carrying the wild type 3' *IME4* downstream region (pRM2b), there was 4 times more sense *IME4* RNA detected than antisense *IME4* RNA. In the cells carrying the Reb1 site mutation (pRM2bmutE), *IME4* antisense strand was detected about 3 times more than the sense strand. Furthermore, in cells carrying the $\alpha 1$ - $\alpha 2$ site mutant (pRMmut5) *IME4* antisense was detected 10 times more than the sense strand, which was barely detected at all. The mutant plasmids generated a mixed pattern of *IME4* sense versus antisense RNA accumulation, and more antisense with less sense *IME4* versus in the wild type. These results together show that Reb1 protein does indeed play a role in the expression of the *IME4* gene in *Saccharomyces cerevisiae*. Reb1 is necessary for the proper levels of *IME4* sense and antisense transcripts, which are in turn required for sporulation to occur.

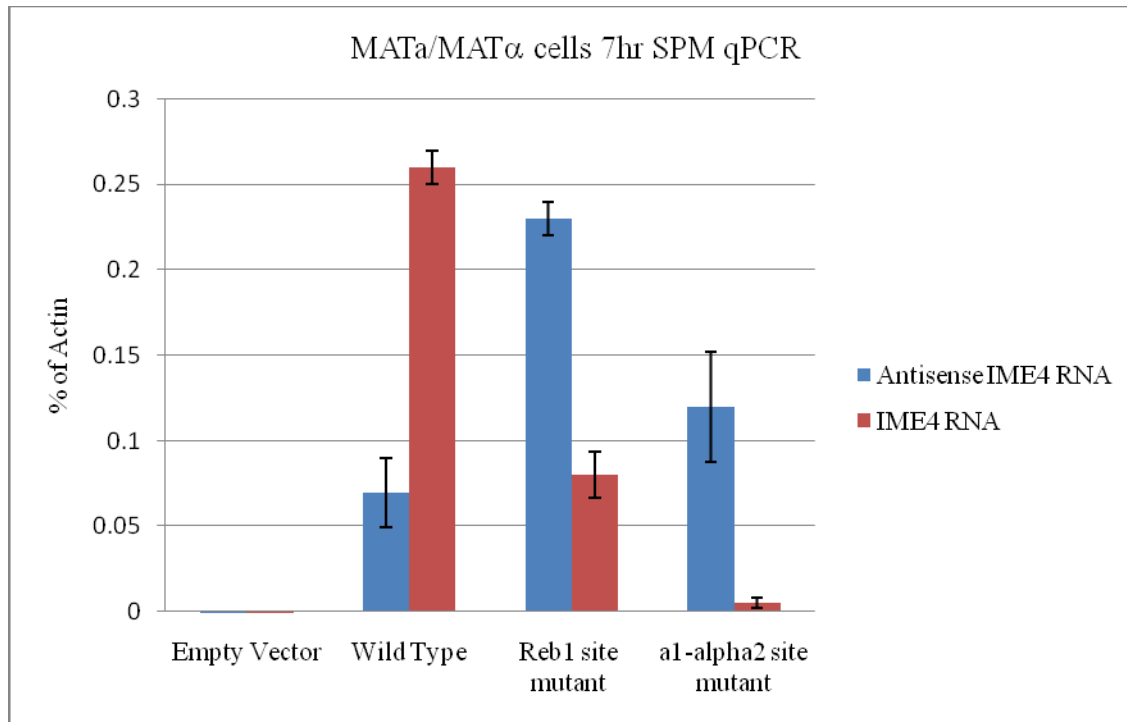


Figure 26. qPCR strand-specific analysis of diploids in 7 hrs SPM. Cells were incubated in sporulation media for 7 hours before RNA was isolated from them for analysis. The quantity of each strand was calculated as a percent of actin

In the wild type, Reb1 and $\alpha 1$ - $\alpha 2$ binding at their sites in the 3' region of *IME4*, keep *IME4* sense RNA at a level necessary for sporulation, and *IME4* antisense at a manageable level so as to have no effect on sporulation. In contrast to the wild type, when the $\alpha 1$ - $\alpha 2$ site is mutated and the Reb1 binding site is still functional, transcription of sense *IME4* is somehow disrupted and the antisense *IME4* transcription is detected. Moreover, in the Reb1 site mutant, with a functioning $\alpha 1$ - $\alpha 2$ site, there is not enough sense *IME4* detected to allow sporulation, and there is more antisense *IME4* detected than sense *IME4* RNA. In order to analyze if transcription of sense *IME4* RNA is truly affecting antisense *IME4* RNA, transcription of the sense strand must be demolished without altering the 3' downstream region of the gene. This will help better understand if $\alpha 1$ - $\alpha 2$ and Reb1 have separate roles in strand-specific expression of the *IME4* gene.

Construction of an ADH1 terminator plasmid

Two broadly different models were considered to explain the discrepancy between the results with the *lacZ* reporter gene and the full-length *IME4* gene. The differences are expected to reflect the underlying mechanisms by which their sites are active. One difference between the two is that transcription from the *IME4* promoter of the full-length gene could enter the 3' region, leading to chromatin reorganization in response to RNA polymerase II passage or termination. In this model repression is disrupted by transcription and needs to be re-established, thereby imposing a requirement for Reb1. In the second type of model, sequence context of the 3' end is the critical determinant of the requirement for Reb1. Nucleosome positions are determined by a combination of DNA sequence and the response of the remodeling complex to bound activators. If the first model is correct, then disrupting transcription from the *IME4* promoter will relieve the requirement for Reb1 in $\alpha 1$ - $\alpha 2$ repression. We expect that in the absence of sense-strand transcription, Reb1 will not be required, as for the *CYC1-lacZ* reporter construct. Alternatively, if the sequence context or nature of the promoter is more important, then disrupting sense-strand transcription will have no effect: the requirement for Reb1 for $\alpha 1$ - $\alpha 2$ repression will remain.

The results of the qPCR led to the question of whether transcription through the *IME4* sense RNA would have an effect on $\alpha 1$ - $\alpha 2$ repression from the 3' downstream region. Moreover, would the Reb1 site still be functional in antisense *IME4* expression, and how will the site-directed mutant affect the expression detected in strand-specific qPCR assays above. To examine if full transcription of the sense *IME4* strand would have an effect on or regulate the role of Reb1 in expression of the antisense RNA, a terminator sequence from enzyme alcohol dehydrogenase 1 or *ADH1*_{term} was inserted into the middle of the *IME4* gene so the sense *IME4*

promoter and 5' half of the coding region is missing. Restriction digest with XbaI and XhoI initially confirmed that the *ADHI* terminator sequence was successfully cloned into the plasmids pRM2b, pRM2bmutE and pRMmut5. Figure 27 shows a picture of the restriction digest product after agarose gel electrophoresis. The 12 kb DNA ladder is in lanes 1 and 18, and in lanes 3 - 17 are the potential clones after digesting with XbaI and XhoI. A potentially successful clone is expected to have a band pattern similar to the positive control in lane 2 which has two distinctive bands. There is one band at the 1800 base pair mark containing the *ADHI* terminator sequence insert, and one band between the 4kb and the 5 kb marks, the rest of the plasmid. A diagram of the 1800 base pair insert is shown in Figure 28.

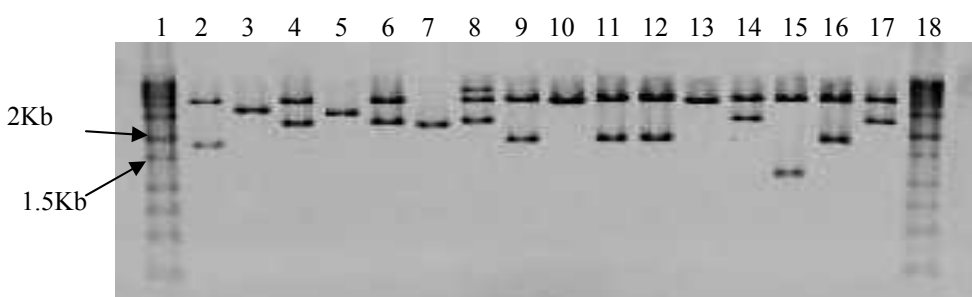


Figure 27. Restriction Digest with XbaI and XhoI to characterize possible *ADHI* terminator sequence clones. Lanes 1 and 18 contain the Kb DNA ladder. Lane 2 contains the positive control. Lanes 3 – 17 contain potential successful clones.



Figure 28. Diagram of *ADHIterm* clone insert from pM1.TF. The insert is approximately 1800 base pairs.

Plasmids with the desired restriction digest pattern were then sequenced with six primers: ADH Bgl forward, ADH Bgl reverse, IME4 Region4 Forward, IME4 Region 4 reverse, IME4 pSX okay2 reverse, and -444 forward, to verify the inserted PCR product and its orientation, and that no other mutations were introduced (Figure 29).

Identities = 270/271 (99%), Gaps = 1/271 (0%), Frame = +1 / +1

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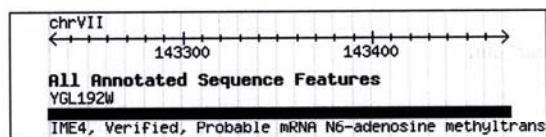
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            |||
Sbjct: 141886 ACGTTTAACGATGCCCAAAGAACATCGAGAATCCTCTATATAGAGTATAAACAGGGCAT 141945

Query: 121     TCTACATTGGTATAGAGGTGCCTTAGGTATATAGTGGTACCGGTCACATAAATTGACGGG 180
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Sbjct: 141946 TCTACATTGGTATAGAGGTGCCTTAGGTATATAGTGGTACCGGTCACATAAATTGACGGG 142005

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Sbjct: 142006 AATATGCGTCTTTAGGCGGCTTTTGGCGTACAGTATTGTTCTTTCTTCGTGCTTGTGAA 142065

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Score = 158.8 bits (1018), Expect = 2.4e-97, P = 2.4e-97 [[Retrieve Sequence / ORF Map / Genome Browser](#)]

Identities = 230/245 (93%), Gaps = 12/245 (4%), Frame = +1 / +1

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Query: 658      TGCAGATCCTGCATGGAATATCCATATGAACCTACCATACGGTACTTGCAACGATATTGA 717
            |||
Sbjct: 143289 TGCAGATCCTGCATGGAATATCCATATGAACCTACCATACGGTACTTGCAACGATATTGA 143348

```

Figure 29. BLAST comparison of the ADH1 terminator sequence insert plasmids.

(Figure 29 continued)



The successfully cloned plasmids were named pM1.TF for the wild type IME4 + ADH1 terminator sequence in the forward orientation, pME.TF for the IME4 with mutant Reb1 binding site + ADH1 terminator sequence in the forward orientation, and pM5.TF for the IME4 with mutant $\alpha 1/\alpha 2$ binding site + *ADH1* terminator sequence in the forward orientation.

Saccharomyces cerevisiae strain YYF101 MATa/MAT α was transformed with pM1.TF, pME.TF, and pM5.TF separately following the Yeast transformation high efficiency protocol from Linda Hoskins/Hahn Lab, with a few modifications.

qPCR Strand-specific analysis of ADH1 terminator sequence plasmids

A terminator sequence from the alcohol dehydrogenase enzyme gene *ADH1* was inserted into the middle of the *IME4* gene, strategically removing the promoter and 5' region of the *IME4* sense strand, including the transcriptional start codon. The terminator sequence tells the RNA polymerase to stop transcribing; the terminator also prevents transcription read-through from any

upstream plasmid sequences. These plasmids containing the disrupted gene should help to understand if the expression of the sense strand will have an impact on the expression of the antisense strand.

YYF101 MAT α /MAT α transformants were incubated in sporulation media for 4 hours; the RNA was isolated and assayed for *IME4* strand-specific expression patterns. cDNA was synthesized using one of the following primers: oligo-dT, *IME4* region 4 forward, or *IME4* region 4 reverse. The oligo-dT primed cDNA was amplified with *TAP42* or actin primers while the *IME4* primed cDNA was amplified with *IME4* region 4 primer pair. As shown in Figure 28, the wild type has little, or no, *IME4* sense nor antisense strand produced when *IME4* is disrupted. However, when *IME4* is disrupted in either the Reb1 binding site mutant or the $\alpha 1$ - $\alpha 2$ binding site mutant, *IME4* sense strand expression is reduced while the *IME4* antisense strand expression continues as it does in the plasmids that *IME4* was not disrupted in Figure 30. The quantity of each strand was calculated as a percent of actin in Figure 30B, and as a percent of *TAP42* in Figure 30A.

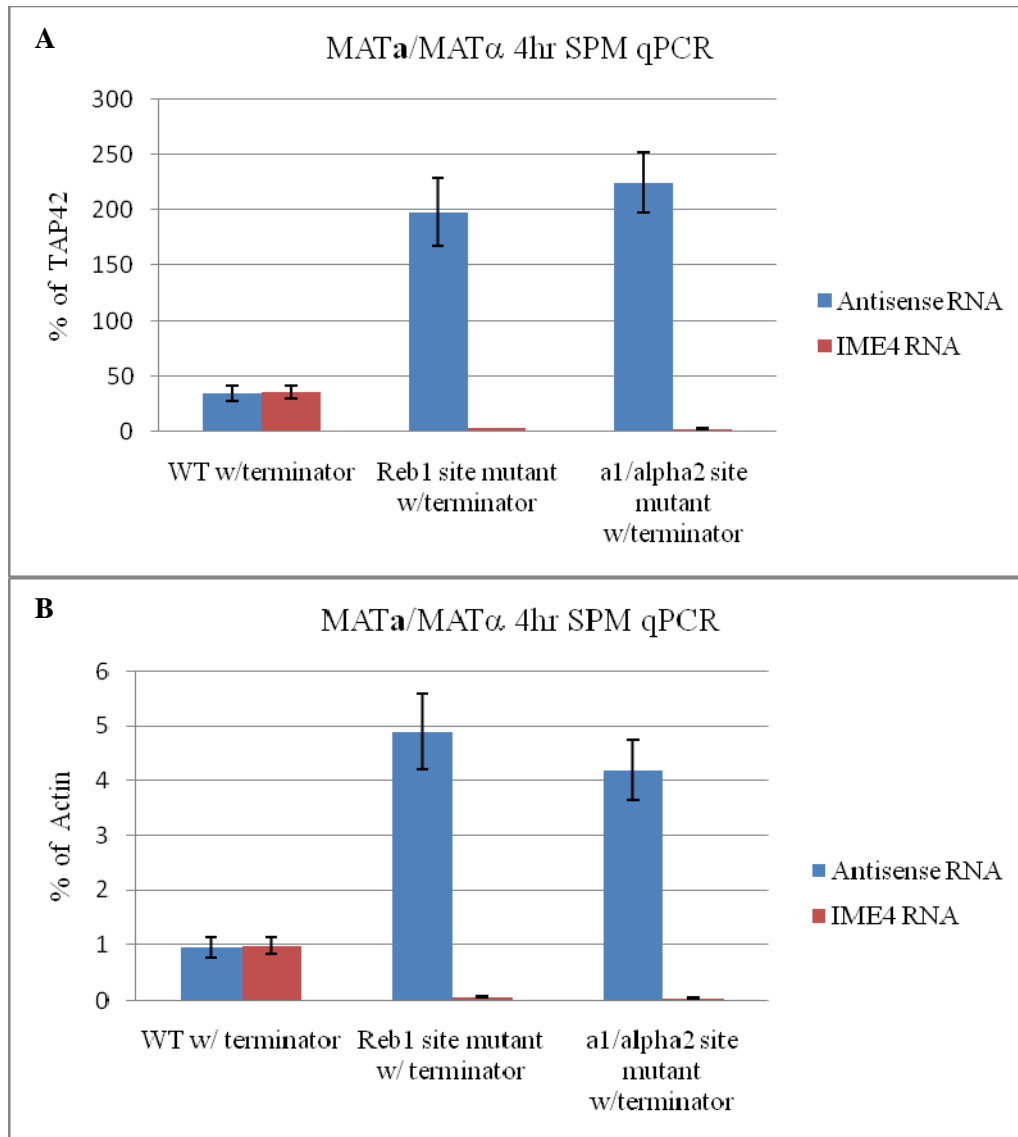


Figure 30. *ADHI* terminator insert qPCR strand-specific analysis. Strand-specific assay of RNA from diploid yeast containing the *ADHI* terminator sequence disrupting the *IME4* gene and either the wildtype *IME4* gene 3' downstream region, the 3' downstream *IME4* Reb1 binding site mutant, or the 3' downstream region of *IME4* $\alpha 1$ - $\alpha 2$ site mutant. (A) Strand quantity calculated as a percent of the *TAP42* gene. (B) Strand quantity calculated as a percent of actin.

These results show evidence that the Reb1 site is necessary for $\alpha 1$ - $\alpha 2$ repression in this sequence context, thus supporting our second model, that some aspects of this promoter sequence dictate the requirement in this context. We conclude that there may be other elements that may account for these results including the sequence and possible sequence-dependent nucleosome

positioning, and the TATA-less nature of this promoter. A model of the *IME4* antisense promoter region is shown in Figure 31.

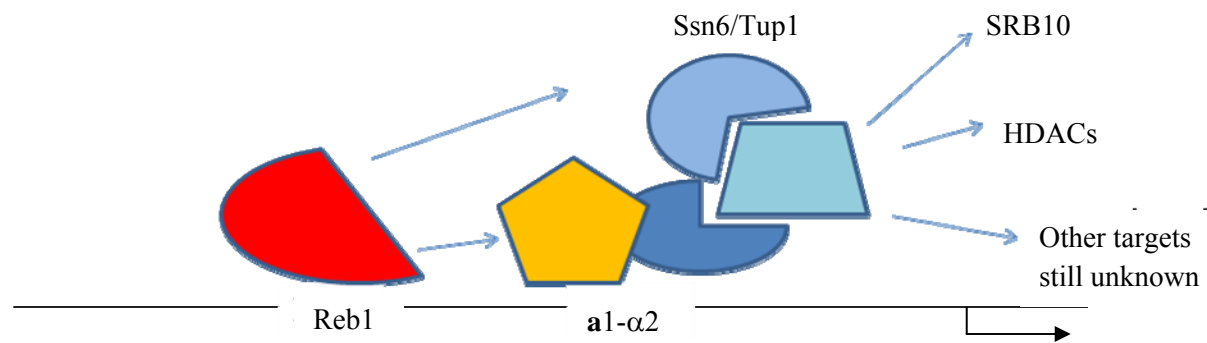


Figure 31. Model of proposed *IME4* antisense promoter. DNA is orientated from 5' to 3'.

Discussion

Summary

The focus of this thesis was to explore the factors that contribute to *IME4* transcription in *Saccharomyces cerevisiae*. *IME4* is important for sporulation in diploid cells and without it functioning properly, diploid **a**/alpha cells are unable to go through meiosis, nor form ascospores. *IME4* is important because it most likely acts on other transcripts necessary for sporulation, although this has not yet been tested directly (Clancy et al, 2002). *IME4* homologs in humans and *Arabidopsis* suggest that its role is very important (Zhong, S. et al. 2008, and Bokar et al 1997); the gene is essential in both organisms, leading to embryonic lethality in knockdown strains.

After initial studies utilizing northern blot hybridization confirmed that *IME4* is expressed in both haploids and diploids, further analysis of transcription of this gene was necessary to understand its regulation. The Northern studies conducted in the Clancy lab were a beginning to what is now seen as a very complex regulatory system involving many components. The information that is already known regarding **a**1-alpha2 functioning as a repressor presented a starting point because of the genome wide studies of transcriptional regulators that identify known and previously unknown binding sites for these transcription factors (Galgoczy et al, 2004). Strand specific analysis in various environmental conditions and cell types gave more insight and understanding of how the *IME4* gene is regulated.

The discovery of Reb1, the general regulatory factor protein, binding site within 200 base pairs of the stop codon for *IME4* was exciting because of its known regulatory functions. This putative binding site led to the hypothesis that it may be controlling transcription of the antisense

IME4 strand that is transcribed in haploid cells, and diploid cells. The purpose of this antisense strand seems to be regulation of the sense strand; however the exact mechanism is unknown (Hongay et al, 2006). The presence of the **a1**-alpha2 binding site and the Reb1 binding site both in the 3' downstream region of *IME4* sense and the 5' upstream region of its antisense led to questions regarding each factor's role in the complex regulation of this gene. This thesis attempts to answer some of these questions through examination at the level of transcription, or the synthesis of RNA from DNA, with quantification of the expressed RNA. Analysis of the RNA allows identification of which gene, or in this context, which strand of a gene is being expressed, in what quantity, and to observe the consequences. Moreover, the work addresses whether sporulation and meiosis is taking place in cells where the Reb1 protein is unable to bind to its site in the 3' downstream region of *IME4*? This thesis will also help to determine if Reb1 functioning properly in regulation of *IME4* requires **a1**-alpha 2 protein complex functioning at its binding site in the 3' downstream *IME4* region. Overall, understanding the transcription pattern of the antisense *IME4* will allow a better understanding of the complex process of meiosis and sporulation in *Saccharomyces cerevisiae*, and the roles of antisense transcription in eukaryotic gene regulation.

Verification of a haploid-specific UAS in the 3' downstream region of IME4

The observation of antisense *IME4* in the **a1**-alpha2 site mutant is evidence that the 3' region of *IME4* regulates transcriptional activity. Results from the beta-galactosidase assays were informative in this regard. Wild type haploid yeast cells showed activity from this 3' downstream *IME4* region that the diploid cells did not show. In contrast, both diploid and haploid yeast showed activity from the reporter when the **a1**-alpha2 site mutant is present in the 3' downstream *IME4* region. This observation led to the conclusion that this 3' downstream

region of *IME4* does indeed have a haploid-specific upstream activation sequence activity. These results were surprising because activity is usually observed from promoters, which are found in the 5' upstream region of a transcribed gene. Another interesting observation was that there was activity from the haploids and not the diploids, yet *IME4* has no known function in haploid cells. These results show that transcription is induced by this region and *IME4* antisense is what's being transcribed.

The Reb1 binding site present in the 3' downstream region of *IME4* was of interest because of the known regulatory functions of this abundant protein. The role of the Reb1 binding site with regard to *IME4* has not been explained. The mutated Reb1 binding site was created to examine the consequences of Reb1 not binding in the context of *IME4*. In contrast to the wild type, haploid cells carrying the site-directed mutant showed consistently decreased activity from the reporter, suggesting a positive role for Reb1 in promoter activity. However, in diploid cells, both the wild type and the Reb1 binding site mutant showed very little activity from the reporter. This repression that is seen in the diploids is due to the $\alpha 1$ - $\alpha 2$ mediated repression as observed from the beta-galactosidase assays with the $\alpha 1$ - $\alpha 2$ site mutant in which repression was relieved. Reb1 binding site does seem to have an effect on the expression of the antisense *IME4* strand as evidenced by the reduced activity of the reporter observed in the mutants in comparison to the wild type (Figure 15). From the reporter construct studies, Reb1 was observed to only influence activity in the haploid cells.

Diploid phenotype of a site-directed mutant Reb1 in the 3' downstream region of IME4

In the *lacZ* reporter assay, the Reb1 site-directed mutant had no observed altered function in the diploid cells; however the complementation assay showed another story. Diploid α/α cells transformed with plasmids carrying the full-length *IME4* gene plus the 3' downstream

region containing the mutant Reb1 site, had little or no ascopore formation taking place in nitrogen and carbon starvation. These results disagree with the beta-galactosidase reporter assay results because the activity observed was the same in wild type and Reb1 site mutants in the diploid cells being repressed in both cases. This result suggested that the Reb1 site mutant in the full-length gene caused an increase in the *IME4* antisense strand, inhibiting sporulation. This discord between the two types of experiments has been observed before. For example, Tabtiang and Herskowitz (1998) examination of Nut1 and Nut2 binding effects on *HO* gene transcription regulation in *Saccharomyces cerevisiae*, previously found the sequences in *lacZ* to be interfering with the activity of the URS2 region under examination as compared to what is seen in the northern hybridization analysis of the same region. In another example, in studies with the *PHO5* promoter, the *lacZ* –fusion reporter had transcriptional requirements that were different from the natural promoter (Martinez-Campa et al, 2004). The conclusion from these experiments is that Reb1 also must play a different role in the natural conformation with full-length *IME4* than it does with the artificial *lacZ* fusion construct. This observation was intriguing and led to the question of how much *IME4* sense is being transcribed, if any, because meiosis and sporulation is not happening.

Quantitative strand-specific analysis of IME4 RNA

The contrasting results of the beta-galactosidase assays and the complementation assay required another protocol for the purpose of quantifying the two separate RNA molecules: *IME4* sense and antisense. The site-directed mutant Reb1 did not allow sporulation; therefore the question of whether or not *IME4* was transcribed was addressed. cDNA was synthesized from RNA isolated from the different cell types, carrying either the wild type or the altered plasmid, grown in sporulation media. The appropriate negative controls for the reverse transcription

reaction were performed also to ensure the results were as accurate as possible (Haddad et al, 2007), including the no primer control and the no reverse transcriptase in the cDNA synthesis reaction. Specificity and efficiency of the primers utilized were also performed as another control or check of the protocol. Studies by Perrochi et al (2007) recently found that there can be antisense artifacts in reverse transcription reactions that may interfere with authentic signals in the analysis of RNA using micro arrays. They use actinomycin D to lower these unwanted occurrences. Analysis of the housekeeping gene actin also verified that the assay truly serves its purpose. The equation used to calculate the strand quantity has also been previously described (Pfaffl, 2001). The strand-specific analysis utilized in the work for this thesis is reliable nevertheless.

In wild type *Saccharomyces cerevisiae*, the primers designed for detection of each *IME4* strand were specific for each strand. As expected, the primer to detect the sense *IME4* did so only in the diploid **a**/alpha cells while the antisense *IME4* was detected in the haploids **a** and alpha cells. These same primers were used for analysis in the *ime4*:TRP1 knockout strains carrying the plasmid with *IME4*. The transformants that were grown in the presence of glucose showed a pattern of expression that was consistent with the analysis of the wild type yeast and the beta-galactosidase assays. Once again, in haploid cells, the antisense strand is more abundant than the sense strand, and the site-directed mutant Reb1 represses this transcription. In diploids, the pattern is reversed, with very little antisense detected in wild type with the mutated Reb1 site causing an increase in the antisense. Thus, the Reb1 site mutant caused a diploid cell to behave like the haploid cell in this respect.

The strand specific analysis in nitrogen and carbon starved cells was examined to characterize the expression pattern of the *IME4* sense and antisense strands. The results were

not surprising based on what was previously observed in this thesis. In the wild type diploid **a**/alpha cells, where *IME4* sense is necessary for meiosis and sporulation, the sense strand was detected and the antisense strand level detected was very low. In these wild type cells, **a1**-alpha2 and Reb1 function in such a way that sporulation is able to progress. The presence of **a1**-alpha2 is indispensable for *IME4* mRNA accumulation, and it most likely interacts with its co-repressors to arrange the chromatin in a way that is inaccessible for RNA polymerase II to access the antisense strand. As a result, sense transcript can be produced. Reb1 seems to be necessary for the **a1**-alpha2 function because when either the Reb1 or **a1**-alpha2 site is nonfunctioning, expression of the sense strand is severely reduced and sporulation is nonexistent when favorable conditions exist. To further explain the role of Reb1 in regards to *IME4* transcription, further strand-specific analysis was performed on RNA from yeast where *IME4* sense transcription was incomplete.

The *ADHI* terminator was utilized because the sequence is conveniently available on the Gal4 activation domain plasmid from Stratagene. Diploid yeast in sporulation media were analyzed because we wanted to obtain more information about the way in which Reb1 and **a1**-alpha2 regulate *IME4*. The results in the wild type demolished detection of the sense strand to the level of the antisense strand which is close to none. The putative Reb1 site mutant and the **a1**-alpha2 site mutants showed the same pattern of expression; low to no sense strand detected and antisense strand expression at a level that seems to be unaffected by the inserted *ADHI* sequence. RNA polymerase II cannot transcribe the sense strand because the TATA box and other promoter elements are missing from the sequence. Complete transcription of the sense *IME4* strand through the 3' region of *IME4* was abolished so that the transcription machinery will have no effect on the promoter region of the antisense *IME4* strand. These results suggest

that Reb1 is indeed required for $\alpha 1$ - $\alpha 2$ repression of *IME4*. Reb1 most likely contributes by sequence dependent mediated nucleosome positioning due to the TATA-less nature of the promoter of the *IME4* antisense strand.

Reb1 is a very important regulator in the *Saccharomyces cerevisiae* genome, as evidenced by its many binding sites. Some regulatory factors that have more than one binding site have different functions depending on its arrangement in the sequence, binding of regulatory factors, and environmental conditions (Harbison et al, 2004). Reb1 binding in the 3' downstream region of *IME4* may influence the location of a nucleosome which can in turn influence the processes that can occur at genes in the affected region. The question now is if there are other instances where $\alpha 1$ - $\alpha 2$ repression requires extra factors such as Reb1 and if so what are they. The mechanism for the repression may be at the level of $\alpha 1$ - $\alpha 2$ maintaining in the region, or at the level of the Tup1 co-repression or one of its targets. This proposed nucleosome positioning has been studied for other repressors such as the $\alpha 2$ -mcm1 repressor of α -specific genes (Morohashi et al, 2006). Disrupting the sequence by insertion led to a loss of nucleosomes at the region under examination, and this loss relieved but did not demolish the repression there. Morohashi et al (2007) further showed that a nucleosome can block binding of a transcriptional activator, thereby influencing repression. In addition, Martinez-Campa et al (2004) examined how the sequence dictates whether histone H4 tails and bromodomain factor-Bdf1 are required for transcription activation and chromatin remodeling at the *PHO5* promoter. They found that in the context of *PHO5*, when the TATA box is poorly accessible or absent, interaction between histone H4 tails acetylated lysines and Bdf1 is required for TFIID to be maintained on the promoter and activation of transcription. By analogy, Reb1 in the antisense *IME4* promoter region perhaps may be facilitating TFIID.

Reb1 binding sites more recently have been linked to the formation of the nucleosome free region or NFR, where the transcription start is located. Raisner et al (2005) showed that the Reb1 site is in a region free of nucleosomes but flanked by two H2A.Z nucleosomes, in the context of the *SNT1* promoter. Mutation in the Reb1 binding site effectively increases nucleosome localization in the region. Reb1 in the antisense strand of *IME4* may be contributing to a nucleosome free region there, to allow transcription of this strand. The important aspect of these studies on nucleosome localization in turn led to examination of the actual pathway that leads to the chromatin arrangement at promoters (Hartley and Madhani, 2009) and Reb1 is required. Moreover, Koerber et al (2009) found that Reb1 is required for positioning of the -1 nucleosome, which is about 230 base pairs upstream of the transcriptional start sites, in *Saccharomyces cerevisiae*. The -1 nucleosome is at the nucleosome free region-proximal border suggesting again that Reb1 is required for the strategic positioning. An ‘atlas’ of nucleosome positions has been compiled from genome-wide studies (<http://atlas.bx.psu.edu/yeast-maps/yeast-index.html>).

The Reb1 binding site is associated with the expression of a non-coding RNA, the antisense *IME4*. Non-coding RNA is not translated to protein but some have been shown to have specific functions in gene regulation (reviewed by Harrison et al, 2009). These non-coding RNA's are distinct from transfer RNAs, ribosomal RNAs, small nuclear RNAs, and small nucleolar RNAs and are exclusively transcribed by RNA polymerase II. A recent study by Drinnenburg et al (2009) revealed that RNA interference, the gene-silencing pathway that is induced by double-stranded RNA is present in the budding yeast *Saccharomyces castellii* and has been lost in *Saccharomyces cerevisiae*. Drinnenburg et al also found proteins Dcr1 and Ago1 that contain RNaseIII domains suggesting they are involved with Dicer-like activity. This

distinct RNA interference pathway was reconstituted in *Saccharomyces cerevisiae* by overexpressing the Ago1 and Dcr1 proteins. This pathway for non-coding RNA can be used to further study transcription regulation in *Saccharomyces cerevisiae*. Additional analysis of the antisense *IME4* transcript perhaps may determine if it functions by utilizing this RNA interference pathway in other budding yeast.

The function of the non-coding RNA is now being studied more extensively because so many exist in the genome of *Saccharomyces cerevisiae* as well as complex eukaryotes (e.g. human). In the context of *IME4*, the antisense transcript has a regulatory role (Hongay et al, 2006), which we verified. The expression of the antisense however also depends on certain factors, and we have shown that Reb1 is one of them. Reb1 is required for the antisense *IME4* transcription as it is in the context of the yeast *GAL* gene cluster (Houseley et al, (2009). In this system, a Reb1 site was shown to be necessary for the production of a non-coding RNA antisense to the *GALI-10* gene. They showed that *GALI0*-noncoding RNA transcription recruits a methyltransferase and promotes histone deacetylation *in cis* which leads to chromatin modifications and glucose repression at *GALI-10* under certain physiological conditions. Furthermore, Bumgarner et al (2009) most recently identified two non-coding RNAs that function *in cis* to control variegated gene expression at *FLO11* in yeast. Taken together, non-coding RNAs can be important for gene expression, so the regulation of non-coding RNA expression is important as well.

Overall, there is still much to learn about regulation of the non-coding RNA and the specific functions of them. High-throughput technology to analyze the transcriptome has been used to identify many non-coding RNAs (reviewed by Beretta and Morillon, 2009). Many features of non-coding RNA suggest they are ideal regulatory molecules including their mobility

and their ability to be synthesized and degraded rather quickly in response to the environment changes, however further investigation is necessary to determine exact function of each molecule (reviewed by Beretta and Morillon, 2009). In conclusion, Reb1, found all over the genome of *Saccharomyces cerevisiae*, is found in *IME4* antisense, where it most likely aides in transcription regulation. Future experiments with chromatin immunoprecipitation will help understand the mechanism by which Reb1 regulates expression of the antisense transcript. We propose a model in which Reb1 binding in this region cleans the area, therefore, maintaining a nucleosome-free region that is necessary for transcription machinery such as transcription factors to initiate transcription of the antisense *IME4*.

References

- Amberg et al. (2005). Assay of β -galactosidase in Yeast. *Methods in Yeast Genetics*. 2005 Edition. CSHL Press. 135-138.
- Angermayr, M., and Wolfhard B (2003). Permanent nucleosome exclusion from the Gal4p-inducible yeast GCY1 promoter. *The J. Biol. Chem.* 278.13: 11026-1031.
- Angermayr, M., Ulrich O., and Wolfhard B (2003). Reb1p-dependent DNA bending effects nucleosome positioning and constitutive transcription at the yeast profilin promoter. *J. Biol. Chem.* 278.20: 17918-7926.
- Berretta J. and Morillon A. (2009). Pervasive transcription constitutes a new level of eukaryotic genome regulation. *EMBO Rep.* Sep;10(9):973-82. Epub 2009 Aug 14.
- Bokar J. A., et al. (1997). Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N6-adenosine)-methyltransferase. *RNA.* Nov;3(11):1233-47.
- Brown, T. et al. (2004). Analysis of RNA by Northern and Slot blot hybridization. *Curr. Protcl. in Mol. Biol.* 68:4.9.1-4.9.8.
- Bumgarner S.L., et al. (2009). Toggle involving cis-interfering noncoding RNAs controls variegated gene expression in yeast. *Proc Natl Acad Sci U S A.* Oct 27;106(43):18321-6. Epub 2009 Sep 30.
- Cairns BR. (2009). The logic of chromatin architecture and remodelling at promoters. *Nature.* 2009 Sep 10;461(7261):193-8. Review
- Chasman, D.L., et al (1990). A yeast protein that influences the chromatin structure of UASG and functions as a powerful auxiliary gene activator. *Genes Dev.* Apr;4(4):503-14.
- Clancy, MJ et al (2002). Induction of sporulation in *Saccharomyces cerevisiae* leads to the formation of N6-methyladenosine in mRNA: a potential mechanism for the activity of the IME4 gene. *Nucleic Acids Res* 30(20): 4509-518.
- Covitz PA, Herskowitz I, Mitchell AP (1991). The yeast RME1 gene encodes a putative zinc finger protein that is directly repressed by a1-alpha 2. *Genes Dev.* Nov;5(11):1982-9.
- Drinnenberg IA, et al.(2009). RNAi in budding yeast. *Science.* Oct 23;326(5952):544-50. Epub 2009 Sep 10.
- Fedor MJ, Lue NF, Kornberg RD (1988). Statistical positioning of nucleosomes by specific protein-binding to an upstream activating sequence in yeast. *J Mol Biol.* Nov 5;204(1):109-27.
- Galgoczy, DJ et al (2004). Genomic dissection of the cell-type-specification circuit in *Saccharomyces cerevisiae*. *PNAS* 101(52): 18069-8074.
- Gavin IM, Kladde MP, Simpson RT (2000). Tup1p represses Mcm1p transcriptional activation and chromatin remodeling of an a-cell-specific gene. *The EMBO J.* Nov 1;19(21):5875-83.
- Gietz D, et al (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* Mar 25;20(6):1425
- Guarente L, Mason T. (1983) Heme regulates transcription of the CYC1 gene of *S. cerevisiae* via an upstream activation site. *Cell.* Apr;32(4):1279-86
- Guttmann-Raviv N, Martin S, Kassir Y. (2002) Ime2, a meiosis-specific kinase in yeast, is required for destabilization of its transcriptional activator, Ime1. *Mol Cell Biol.* Apr;22(7):2047-56.

- Haddad, F et al. (2007) Potential pitfalls in the accuracy of analysis of natural sense-antisense RNA pairs by reverse transcription-PCR. BMC Biotechnology. BioMed Central. Web. <www.biomedcentral.com/1472-6750/7/21>.
- Harbison, CT, et al. (2004) Transcriptional regulatory code of a eukaryotic genome. Nature 431: 99-104.
- Harrison, B.R., Yazgan, O., and Krebs, J. E. (2009). Life without RNAi: noncoding RNAs and their functions in *Saccharomyces cerevisiae*. Biochem. Cell Biol. 87(5): 767–779.
- Harashima S. et al. (1989) Mating-type control in *Saccharomyces cerevisiae*: isolation and characterization of mutants defective in repression by a1-alpha 2. Mol. Cell. Biol. (10):4523-30.
- Hartley PD, Madhani HD. (2009) Mechanisms that specify promoter nucleosome location and identity. Cell. May 1;137(3):445-58
- Herskowitz, I., Rine J., and Strathern J. (1992) Mating-type Determination and Mating-type Interconversion in *Saccharomyces cerevisiae*. The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Gene Expression. Volume 2, 583-657
- Herskowitz, I. (1998) Life cycle of the budding yeast *Saccharomyces cerevisiae*. Microbiological Review. Dec;52(4):536-53.
- Hongay, CF., et al. (2006) Antisense transcription controls cell fate in *Saccharomyces cerevisiae*. Cell 127: 735-45.
- Hoskins, L. (2000) Yeast Transformation (high efficiency). http://labs.fhcrc.org/hahn/Methods/genetic_meth/dmso_yeast_transform.html
- Houseley, J, et al. (2008) A ncRNA Modulates Histone Modification and mRNA Induction in the Yeast GAL Gene Cluster. Mol Cell 32 : 685-95.
- Honigberg SM, Purnapatre K. (2003) Signal pathway integration in the switch from the mitotic cell cycle to meiosis in yeast. J Cell Sci. Jun 1;116(Pt 11):2137-47. Review
- Huang L, Zhang W, Roth SY. (1997) Amino termini of histones H3 and H4 are required for a1-alpha2 repression in yeast. Mol Cell Biol. Nov;17(11):6555-62.
- Jin Y, Zhong H, Vershon AK. (1999) The yeast a1 and alpha2 homeodomain proteins do not contribute equally to heterodimeric DNA binding. Mol Cell Biol. Jan;19(1):585-93.
- Keleher CA, et al. (1992) Ssn6-Tup1 is a general repressor of transcription in yeast. Cell Feb 21;68(4):709-19.
- Koerber RT, et al. (2009) Interaction of transcriptional regulators with specific nucleosomes across the *Saccharomyces* genome. Mol Cell. Sep 24;35(6):889-902.
- Komachi K, Redd MJ, Johnson AD. (1994) The WD repeats of Tup1 interact with the homeo domain protein alpha 2. Genes Dev. Dec 1;8(23):2857-67.
- Kulkens T, et al. (1992) A system to study transcription by yeast RNA polymerase I within the chromosomal context: functional analysis of the ribosomal DNA enhancer and the RBP1/REB1 binding sites. EMBO J. Dec;11(12):4665-74.
- Lang WH, Reeder RH. (1995) Transcription termination of RNA polymerase I due to a T-rich element interacting with Reb1p. Proc Natl Acad Sci U S A. Oct 10;92(21):9781-5.
- Lang WH, Reeder RH. (1993) The REB1 site is an essential component of a terminator for RNA polymerase I in *Saccharomyces cerevisiae*. Mol Cell Biol. Jan;13(1):649-58.
- Li T, Jin Y, Vershon AK, Wolberger C. (1998) Crystal structure of the MATa1/MATalpha2 homeodomain heterodimer in complex with DNA containing an A-tract. Nucleic Acids Res. Dec 15;26(24):5707-18

- Liaw, PC, and Brandl CJ. (1994) Defining the sequence specificity of the *Saccharomyces cerevisiae* DNA binding protein REB1p by selecting binding sites from random-sequence oligonucleotides." *Yeast* 10(6): 771-87.
- Malave T and Dent S. (2006) Transcriptional repression by Tup1-Ssn6. *Biochem Cell Biol.* Aug;84(4):437-43. Review.
- Martinez-Campa C, et al. (2004) Precise nucleosome positioning and the TATA box dictate requirements for the histone H4 tail and the bromodomain factor Bdf1. *Mol Cell.* Jul 2;15(1):69-81.
- Mitchell AP, Herskowitz I. (1986) Activation of meiosis and sporulation by repression of the RME1 product in yeast. *Nature.* Feb 27-Mar 5;319(6056):738-42.
- Morohashi N, et al. (2007). A nucleosome positioned by alpha2/Mcm1 prevents Hap1 activator binding in vivo. *Biochem Biophys Res Commun.* Dec 21;364(3):583-8. Epub 2007 Oct 16.
- Morohashi N, et al. (2006). Effect of sequence-directed nucleosome disruption on cell-type-specific repression by alpha2/Mcm1 in the yeast genome. *Eukaryot Cell.* Nov; 5(11):1925-33. Epub 2006 Sep 15
- Morrow BE, Ju Q, Warner JR. (1990) Purification and characterization of the yeast rDNA binding protein REB1. *J Biol Chem.* Dec 5;265(34):20778-83.
- Morrow BE, Johnson SP, Warner JR. (1989) Proteins that bind to the yeast rDNA enhancer *J Biol Chem.* May 25;264(15):9061-8.
- Mukai Y et al.(1991) AAR1/TUP1 protein, with a structure similar to that of the beta subunit of G proteins, is required for α 1- α 2 and α 2 repression in cell type control of *Saccharomyces cerevisiae*. *Mol Cell Biol.* 11(7):3773-9.
- Nagaraj VH et al. (2004) Combined analysis of expression data and transcription factor binding sites in the yeast genome. *BMC Genomics.* Aug 26;5(1):59.
- Pak J and Segall J. (2002) Regulation of the premiddle and middle phases of expression of the NDT80 gene during sporulation of *Saccharomyces cerevisiae*. *Mol Cell Biol.* Sep;22(18):6417-29
- Pierce M, et al. (2003) Sum1 and Ndt80 proteins compete for binding to middle sporulation element sequences that control meiotic gene expression. *Mol Cell Biol.* Jul;23(14):4814-25.
- Perocchi, F, et al. (2007). Antisense artifacts in transcriptome microarray experiments are resolved by actinomycin D. *Nucleic Acids Res.* 35.19 (2007).
- Pfaffl, Michael W. (2001) A new mathematical model for relative quantification in real-time RT-PCR." *Nucleic Acids Res.* 29(9): 2002-007.
- Raisner, RM, et al. (2005) Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. *Cell* 123: 233-48.
- Remacle, JE, Holmberg S. (1992) A REB1-binding site is required for GCN4-independent ILV1 basal level transcription and can be functionally replaced by an ABF1-binding site. *Mol Cell Biol.* Dec;12(12):5516-26.
- Rine J, Sprague GF Jr, Herskowitz I. (1981) rme1 Mutation of *Saccharomyces cerevisiae*: map position and bypass of mating type locus control of sporulation *Mol Cell Biol.* Oct;1(10):958-60.

- Sánchez-Gorostiaga A, et al. (2004) Transcription termination factor Reb1p causes two replication fork barriers at its cognate sites in fission yeast ribosomal DNA in vivo. *Mol Cell Biol.* Jan;24(1):398-406.
- Schüller HJ, et al. (1994) Importance of general regulatory factors Rap1p, Abf1p and Reb1p for the activation of yeast fatty acid synthase genes FAS1 and FAS2. *Eur J Biochem.* Oct 1;225(1):213-22.
- Scott EW, Baker HV. (1993) Concerted action of the transcriptional activators REB1, RAP1, and GCR1 in the high-level expression of the glycolytic gene TPI. *Mol Cell Biol.* Jan;13(1):543-50.
- Shah, J.C. and Clancy, M.J. (1992) IME4, a gene that mediates MAT and nutritional control of meiosis in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 12, 1078-1086.
- Sikorski, Robert S., and Philip Hieter. (1989) A System of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122: 19-27.
- Sjöstrand JO, Kegel A, Aström SU. (2002) Functional diversity of silencers in budding yeasts. *Eukaryot Cell.* Aug;1(4):548-57.
- Smith HE, et al. (1993) Genetic evidence for transcriptional activation by the yeast IME1 gene product. *Genetics.* Apr;133(4):775-84.
- Smith RL, Johnson AD. (2000) Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem Sci.* Jul;25(7):325-30.
- Sprague, Jr., G.F. (2005). Three-pronged genomic analysis reveals yeast cell-type regulation circuitry. *PNAS* 102, 959-60.
- Tabtiang RK, Herskowitz I. (1998). Nuclear proteins Nut1p and Nut2p cooperate to negatively regulate a Swi4p-dependent lacZ reporter gene in *Saccharomyces cerevisiae*. *Mol Cell Biol.* Aug;18(8):4707-18.
- Van Slyke, C., and Grayhack, E.J. (2003). The essential transcription factor Reb1p interacts with the CLB2 UAS outside of the G2/M control region. *Nucleic Acids Res.* 31.15: 4597-607.
- Wang H., Nicholson P.R., Stillman D.J. (1990). Identification of a *Saccharomyces cerevisiae* DNA-binding protein involved in transcriptional regulation. *Mol Cell Biol.* Apr;10(4):1743-53.
- Watson A.D., et al. (2000). Ssn6-Tup1 interacts with class I histone deacetylases required for repression. *Genes Dev.* Nov 1;14(21):2737-44.
- Zhong, S., et al (2008). MTA is an Arabidopsis Messenger RNA Adenosine Methylase and Interacts with a Homolog of a Sex-Specific Splicing Factor. *The Plant Cell.* www.aspb.org: 1-11.

Appendices

Appendix A: Sequences

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Query: 1      ACTGAATTAGAATCGTTTCA-GATGCTTTCAGTTC AACATTTAGTAACATAGTAGGAATA 59
||||| |||||||
Sbjct: 141825 ACTGA-TTAGAATCGTTTCAAGATGCTTTCAGTTC AACATTTAGTAACATAGTAGGAATA 141883

Query: 60      TCACGTTTAAACGATGCCCCAAAGAACATCGAGAATCCTCTATATAGAGTATAAACAGGGC 119
||||| |||||||
Sbjct: 141884 TCACGTTTAAACGATGCCCCAAAGAACATCGAGAATCCTCTATATAGAGTATAAACAGGGC 141943

Query: 120     ATTCTACATTGGTATAGAGGTGCCTTAGGTATATAGTGGTACCGGTCACATAAAATTGACG 179
||||| |||||||
Sbjct: 141944 ATTCTACATTGGTATAGAGGTGCCTTAGGTATATAGTGGTACCGGTCACATAAAATTGACG 142003

Query: 180     GGAATATGCGTCTTTAGGCGGCTTTTGGCGTACAGTATTGTTCTTTCTTCGTGCTTG TG 239
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Sbjct: 142004 GGAATATGCGTCTTTAGGCGGCTTTTGGCGTACAGTATTGTTCTTTCTTCGTGCTTG TG 142063

Query: 240     AAACCTTGTTATAAAACAAAGAAAGAGAAAGATCTCCCCGGCATTCTGGAAGATCGGTAACC 299
||||| |||||||
Sbjct: 142064 AAACCTTGTTATAAAACAAAGAAAGAGAAAGATCTCCCCGGCATTCTGGAAGATCGGTAACC 142123

Query: 300     GCTGGAGCTATAGGAAAGGGAGATGGATAACAGTAAAGAAAACGGACCATATAAGTTAGC 359
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Sbjct: 142124 GCTGGAGCTATAGGAAAGGGAGATGGATAACAGTAAAGAAAACGGACCATATAAGTTAGC 142183

Query: 360     ATTGGGGGTTTTCGAAAAAATAAGAATTGGCATTGAGACTTGAATTCATAAAAGTTGTA 419
||||| |||||||
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Query: 420     AGCAGGCTATGATTAACGATAAACTAGTACATTTTCTGATCCAGAATTATGATGACATCC 479
||||| |||||||
Sbjct: 142244 AGCAGGCTATGATTAACGATAAACTAGTACATTTTCTGATCCAGAATTATGATGACATCC 142303

Query: 480     TAAGAGCACCGCTTTCTGGACAGCTCAAAGATGTGTATTCACTGTACATCAGTGGCGGAT 539
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Sbjct: 142304 TAAGAGCACCGCTTTCTGGACAGCTCAAAGATGTGTATTCACTGTACATCAGTGGCGGAT 142363

```

Sequence 1. Reb1 site mutant BLAST results. BLAST results comparing pRM2bmutE, the Reb1 site mutant, with the *Saccharomyces cerevisiae* genome, using the *S. cerevisiae* database. (www.yeastgenome.org).

(Sequence 1 continued)

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Query: 751      TGTGTATTCACTGTACATCAGTGGCGGATACGATGATGAGATGCAGAAGTTGAGAAACGA 692
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Query: 691      TAAAGACGAGGTCTTACAGTTTGAACAGTTTGGGAATGATCTTCAGGATATTATATTGTC 632
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Sbjct: 142395   TAAAGACGAGGTCTTACAGTTTGAACAGTTTGGGAATGATCTTCAGGATATTATATTGTC 142454

Query: 631      AACGCCGCAGTCCATTCAATTTGACCAGAATCTATTAGTGGCAGATAGGCCAGAAAAAAT 572
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Sbjct: 142455   AACGCCGCAGTCCATTCAATTTGACCAGAATCTATTAGTGGCAGATAGGCCAGAAAAAAT 142514

Query: 571      TGTTTACCTGGACGTTTTTTCCTTGAAAATTCTCTATAACAAGTCCATGCCTTCTACTA 512
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Sbjct: 142515   TGTTTACCTGGACGTTTTTTCCTTGAAAATTCTCTATAACAAGTCCATGCCTTCTACTA 142574

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Sbjct: 142575   TACATTAAAAATCGTCCAGTCTTCATGTGAAGAAAAAGTGTCGAGTTTAAACAACAAAACC 142634

Query: 451      GGAAGCTGATTTCGGAAAAAGACCAACTGCTGGGTAGACTGCTTGGAGTCTGAACTGGGA 392
|||||
Sbjct: 142635   GGAAGCTGATTTCGGAAAAAGACCAACTGCTGGGTAGACTGCTTGGAGTCTGAACTGGGA 142694

Query: 391      CGTTAACGTCAGCAACCAGGGGTACCACGTGAACAACAGCAATCGCTTACAAAATTT 332
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Sbjct: 142695   CGTTAACGTCAGCAACCAGGGGTACCACGTGAACAACAGCAATCGCTTACAAAATTT 142754

Query: 331      ATTGAGGGAAAAACCATCATCTTTTCAGCTTGCTAAAGAAAGAGCCAAATACACGACCGA 272
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Sbjct: 142755   ATTGAGGGAAAAACCATCATCTTTTCAGCTTGCTAAAGAAAGAGCCAAATACACGACCGA 142814

Query: 271      GGTCATTGAATACATCCCGATATGTAGTGACTATTTCGCATGCATCTCTATTGTCCACCTC 212
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Query: 151      GGAAAATCACCAGGATTAATTGAGTGCATTCAATCAAAAATTCACTTTATCCCTAATAT 92
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(Sequence 1 continued)

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Query: 121 AATATATTCGTCATGCTTGCAAGAGCGAGCTGATCGTGAAACTGCGAGTGAAAATAAAA 180
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 |||||
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 |||||
Sbjct: 143204 TCAAAAAGGCTTTACCTGCACAGTGGATCCGTTGCGATGTGCGGAAATTTGATTTTAGGG 143263

Query: 301 TTCTTGAAAGTTTTTCGGTAGTTATTGCAGATCCTGCATGGAATATCCATATGAACCTAC 360
 |||||
Sbjct: 143264 TTCTTGAAAGTTTTTCGGTAGTTATTGCAGATCCTGCATGGAATATCCATATGAACCTAC 143323

Query: 528 GAATATCCATATGAACCTACCATACGGTACTTGCAACGATATTGAACTATTAGGTTTGCC 469
 |||||
Sbjct: 143304 GAATATCCATATGAACCTACCATACGGTACTTGCAACGATATTGAACTATTAGGTTTGCC 143363

Query: 468 TTTGCACGAACACAAGATGAAGGTATTATTTTCTTTGGGTGACGGGTAGAGCTATAGA 409
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Sbjct: 143364 TTTGCACGAACACAAGATGAAGGTATTATTTTCTTTGGGTGACGGGTAGAGCTATAGA 143423

Query: 408 ATTGGGTAAAGAGTCTTTGAATAACTGGGGTTACAACGTGATAAATGAAGTTTCATGGAT 349
 |||||
Sbjct: 143424 ATTGGGTAAAGAGTCTTTGAATAACTGGGGTTACAACGTGATAAATGAAGTTTCATGGAT 143483

Query: 348 AAAGACAAACCAACTGGGTAGAACAATTGTTACAGGTCGTACGGGTCATTGGTTAAATCA 289
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Sbjct: 143484 AAAGACAAACCAACTGGGTAGAACAATTGTTACAGGTCGTACGGGTCATTGGTTAAATCA 143543

Query: 288 TTCTAAGGAGCATTTATTGGTGGGCTTAAAGGGTAATCCAAAGTGGATCAACAAACATAT 229
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Sbjct: 143544 TTCTAAGGAGCATTTATTGGTGGGCTTAAAGGGTAATCCAAAGTGGATCAACAAACATAT 143603

Query: 228 TGACGTCGATTTGATCGTATCAATGACCAGAGAGACTAGTAGAAAGCCTGATGAACTGTA 169
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(Sequence 1 continued)

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(Sequence 1 continued)

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|||||
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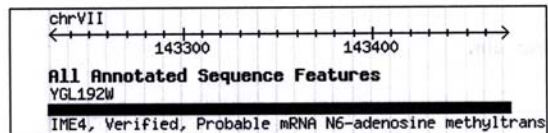
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Score = 158.8 bits (1018), Expect = 2.4e-97, P = 2.4e-97 [[Retrieve Sequence / ORF Map / Genome Browser](#)]

Identities = 230/245 (93%), Gaps = 12/245 (4%), Frame = +1 / +1

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Sequence 2. *ADHI*_{term} insert plasmids BLAST results. BLAST results comparing plasmid pM1 with the *Saccharomyces cerevisiae* genome using the *S. cerevisiae* database (www.yeastgenome.org).

(Sequence 2 continued)

```
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               |||| |   |||  ||   |||  || | ||||| ||||| ||||| ||||| |||||
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Query: 503      TTTTAAACCTAAGAGTCACTTTAAATTTGTATACACTTANNNNNNNNATAACTTATTT 444
               ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
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               ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
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(Sequence 2 continued)

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            |||
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(Sequence 2 continued)

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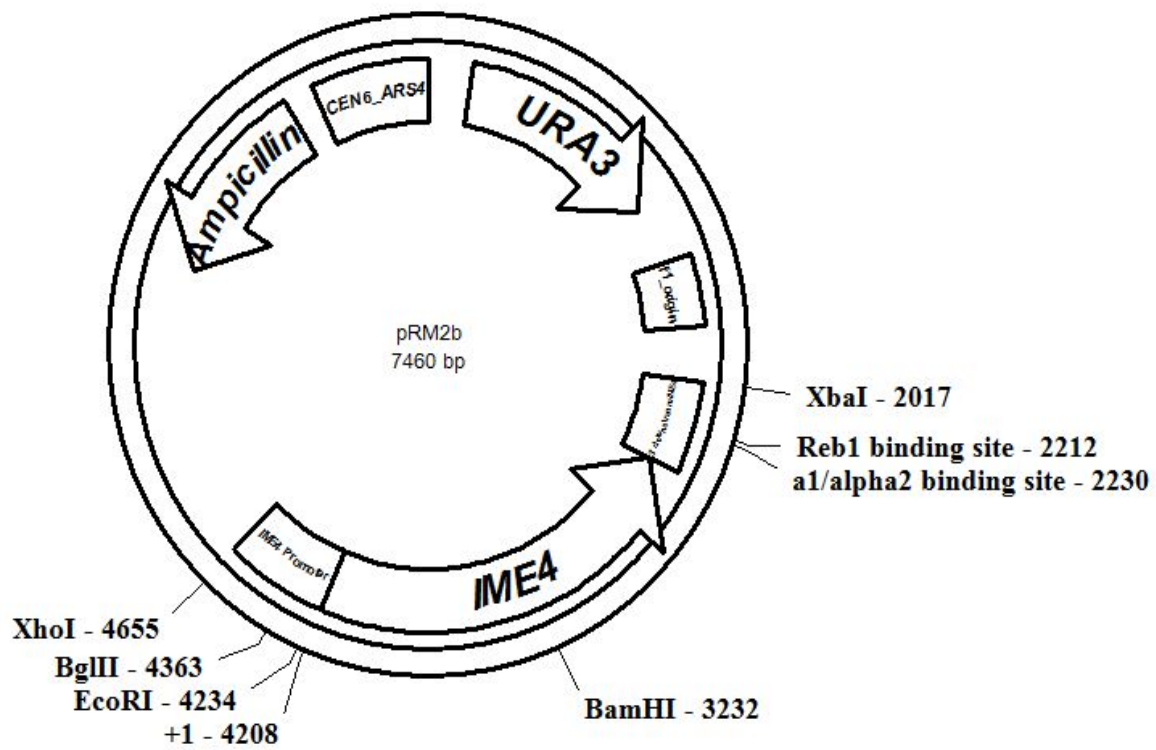
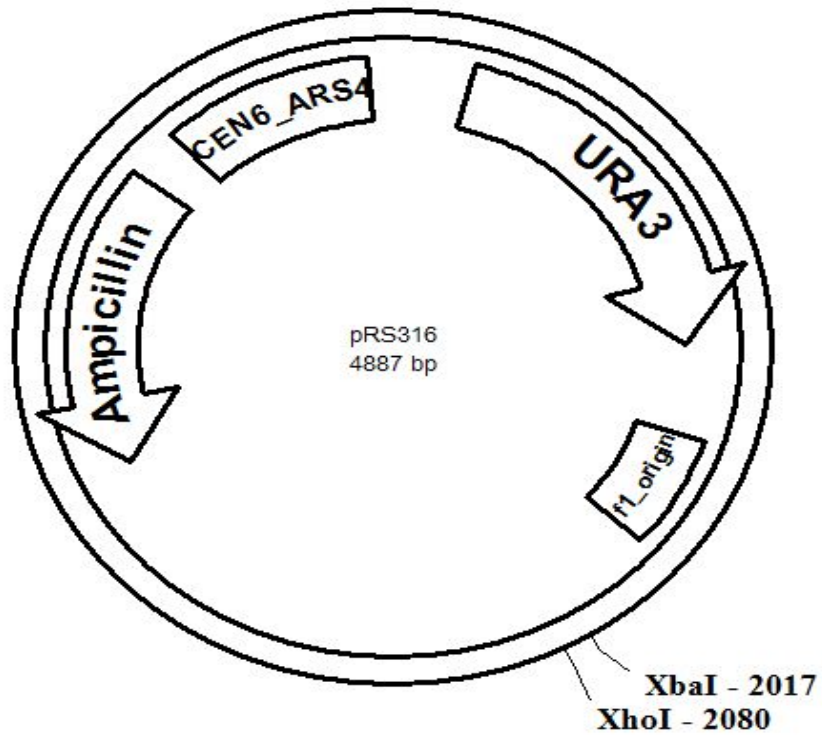
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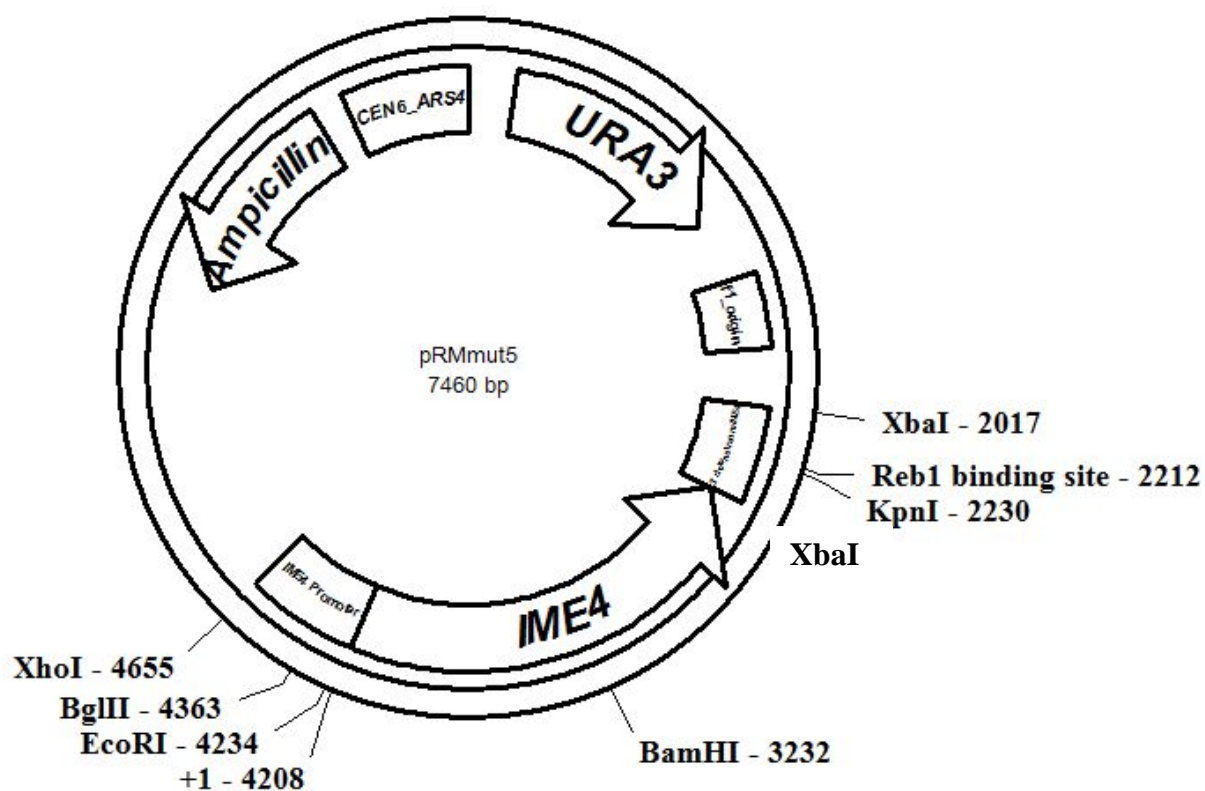
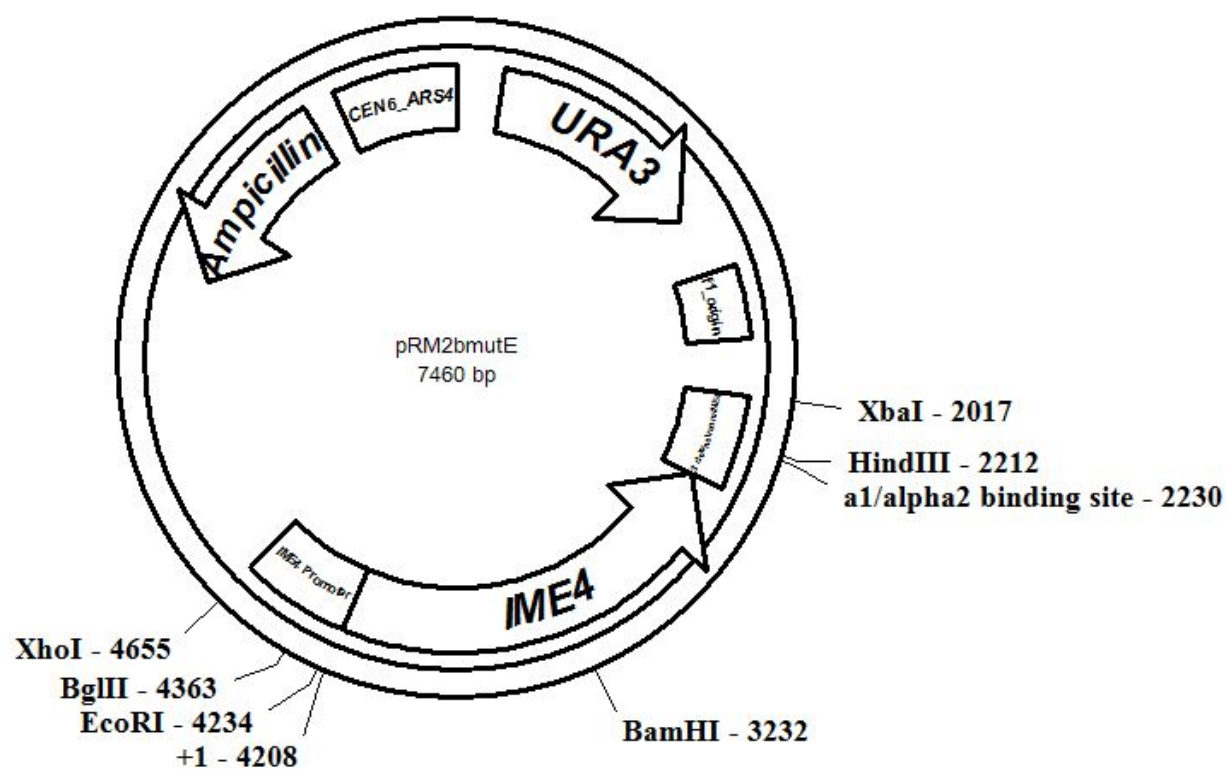
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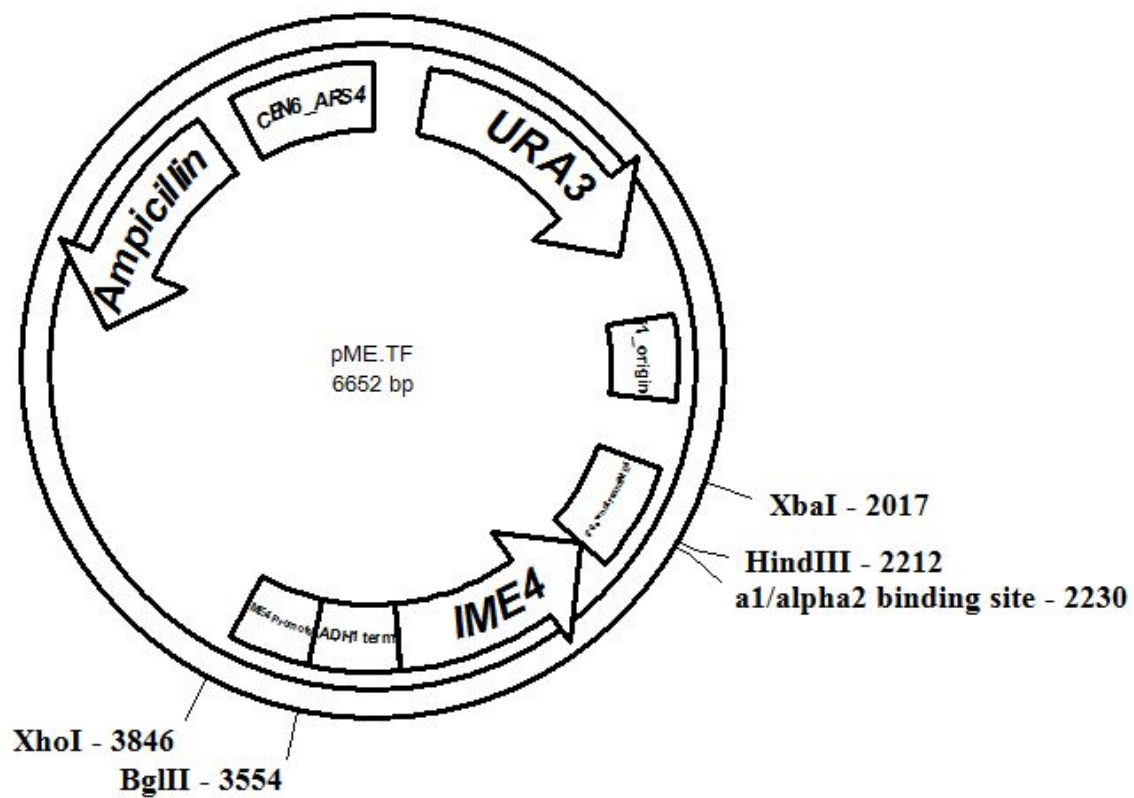
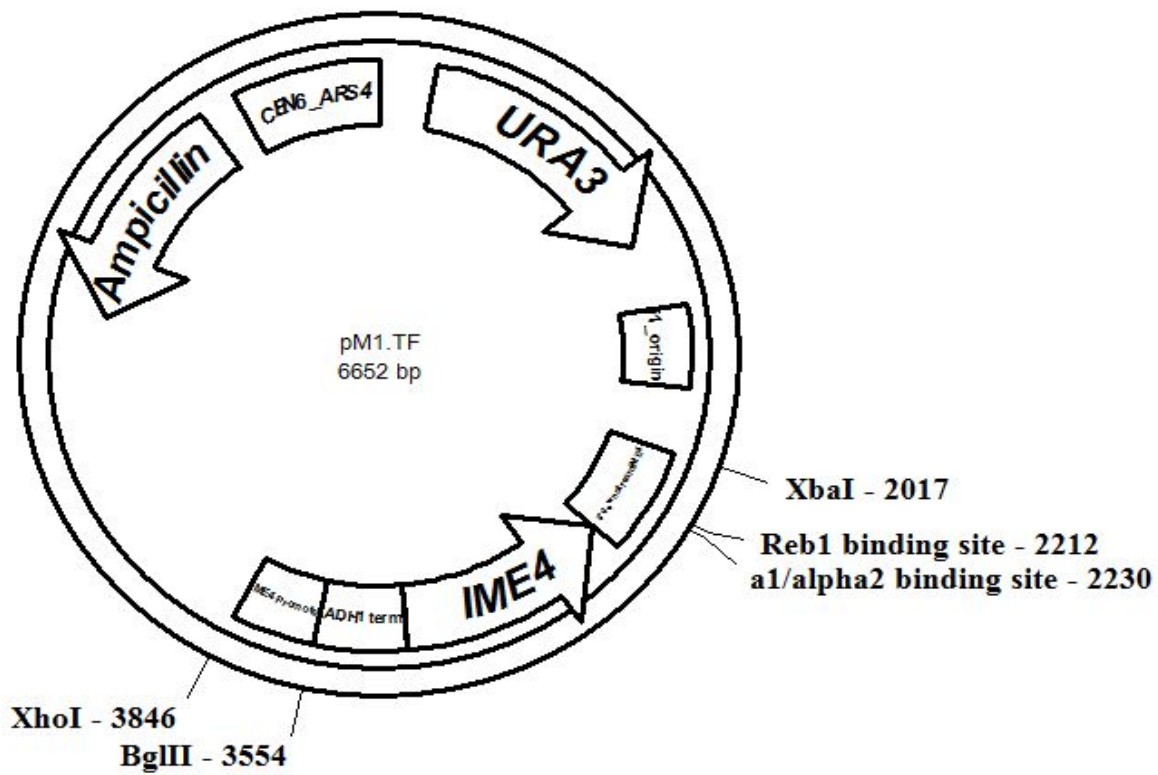
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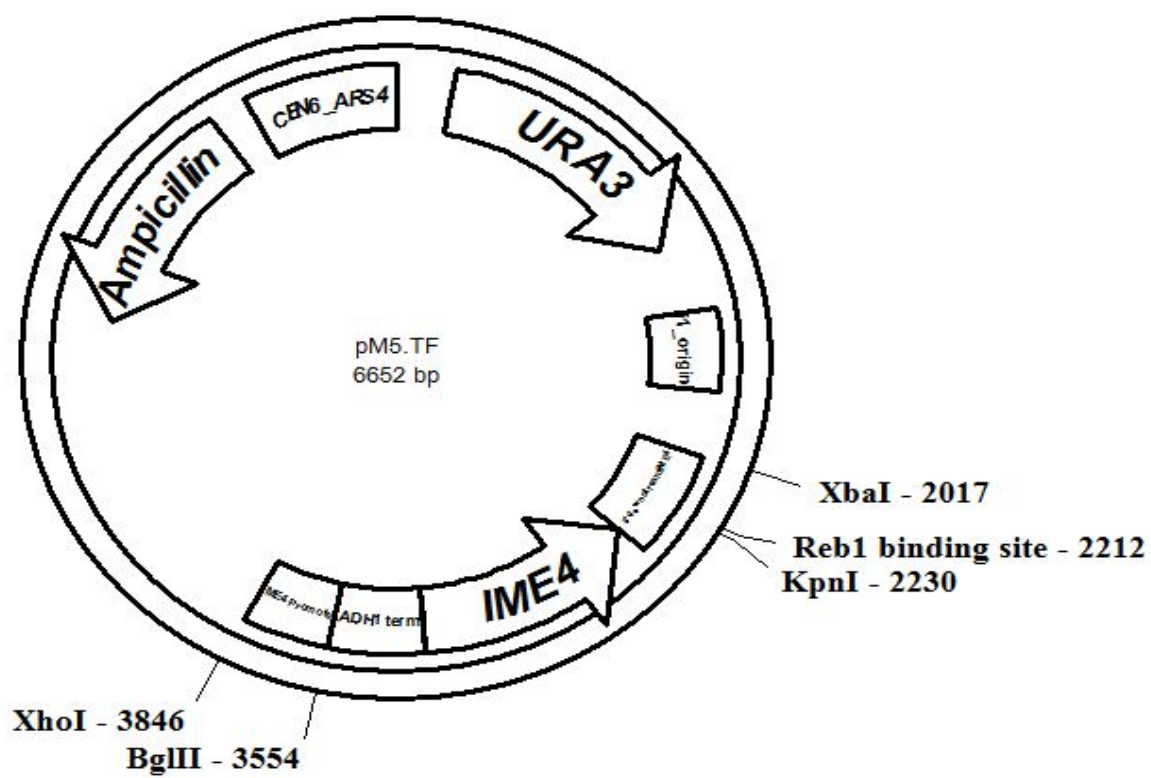
Query: 85 GCCTGGAGAAAGCTAATACTAGTTCTGGCCATATTTCAACCATTAAACACTACTAATGT 26
|||||
Sbjct: 144271 GCCTGGAGAAAGCTAATACTAGTTCTGGCCATATTTCAACCATTAAACACTACTAATGT 144330
```


Appendix: Plasmid Maps









Vita

Milele Makunda Ramsay was born in San Diego, CA in 1982. In the year 2000, she graduated from Lincoln High School, a science magnet public school. She began her undergraduate study majoring in biology at Xavier University of Louisiana in the fall of 2000, and received her Bachelor of Science degree in Biology from there in May of 2004. After working as a research associate at Louisiana State University Health Sciences Center in the Department of Nutrition located in New Orleans, Louisiana, she enrolled in the Master of Science program in Biological Sciences at the University of New Orleans in the fall of 2005. She performed her thesis research under the direction and guidance of Dr. Mary Clancy. She received a Master of Science in Biological Sciences from the University of New Orleans on December 20, 2009.