2008

Genetic and mechanistic analysis of heterochromatin spreading in the yeast S.cerevisiae

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GENETIC AND MECHANISTIC ANALYSIS OF HETEROCHROMATIN SPREADING IN THE YEAST *S. CEREVISIAE*

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Biological Sciences

by

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August 2008
ACKNOWLEDGEMENTS

I would like to express sincere thanks to my mentor Dr. David Donze, for his invaluable guidance, and financial support. I am very grateful to my committee members Dr. Craig Hart, Dr. John C. Larkin, Dr. Patrick DiMario and Dr. Donald E. Franke, for their guidance and additionally to Dr. John C. Larkin for timely advices. I am very lucky to have wonderful colleagues who were very helpful and made the time I spent in the Donze lab an enriching and fulfilling experience. Specifically, I would like to thank Sandra Dugas and Megan Ibos for the help with media preparation and technical support. I am thankful to Nneamaka Aghochukwu for help with the mutagenesis experiments and Richard Kleinschmidt for the helpful academic discussions. I would like to thank Ms. Prissy Milligan, administrative specialist (Graduate Studies) who makes the life of graduate students so much easier.

I would like to thank my husband Ramesh Subramanian, for his unconditional love and support. Our little discussion sessions on science were very helpful throughout my research. I would also like to extend my thanks to my parents-in-law for their support and prayers.

Last but not the least I would like to thank my parents and my sister for their love and support. Their words of encouragement and faith have been a constant source of inspiration in every walk of my life.
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ABSTRACT

RNA Polymerase III transcribed tRNA genes are implicated in a wide variety of chromosome organizational functions that includes the ability to act as a boundary to heterochromatic silencing. A tRNA gene has been shown to be a major component of the barrier that prevents spreading of silencing from the HMR locus to the downstream GIT1 gene on chromosome III in S.cerevisiae. Our results suggest that additional proteins are involved in maintaining the boundary function of this tRNA gene. Mutations or deletions of the genes coding for these additional proteins have been shown to either weaken the boundary or enhance silencing. This mutational analysis identified YTA7, a novel bromodomain containing gene, as being required for full barrier activity of the HMR tRNA gene. This provided some of the first evidence demonstrating a role for bromodomain proteins in boundary function. RPD3, a histone deacetylase gene was also identified by our mutational analysis, as when deleted caused an increase in the spread of silencing downstream of the boundary tRNA gene. Our results suggest that this spreading of heterochromatic silencing occurs in spite of this tRNA gene remaining transcriptionally active. This suggests that heterochromatic silencing can bypass active regions along a chromosome to silence downstream genes.
CHAPTER ONE

INTRODUCTION
Multicellular organisms are made up of wide variety of cell types. These different cell types arise from a single fertilized egg and acquire different fates during development by a process called differentiation. Each cell type is characterized by its pattern of gene expression, that is, by the particular gene products that it produces. The principal level for controlling gene expression is at transcription as it is more energy efficient, and can be directly modulated by transcriptional regulators. These transcriptional regulators are usually DNA binding proteins that activate transcription either directly at particular promoters or through distal enhancers, and they can directly or indirectly repress transcription. The different cell types are not due to different genes but due to different patterns of transcription within cells in different regions of the embryo. This pattern initiates a cascade of events, where regulation of genes turned on (or off) at one stage, controls expression of other genes at the next stage. This regulation is very complex in multicellular eukaryotic development. The cascade of gene regulation also depends on cell signaling, which includes cell-cell interactions that define boundaries between groups of cells.

The simple unicellular eukaryote, *Saccharomyces cerevisiae*, also known as baker’s yeast can exist as one of three cell types, a haploid MATa or MATα or a diploid MATa/α. These different cell types are due to different patterns of gene expression in genetically identical cells, allowing yeast to be used as a simple model of cellular differentiation. To add to this complexity of gene regulation, eukaryotes have their DNA packaged into nucleosomes, which must be unwound during processes of replication and transcription, which creates yet another additional layer of regulation by chromatin modifying proteins.

Eukaryotic genomes do not exist as free DNA, but are part of a nucleoprotein complex called chromatin. Nucleosomes are the basic unit of chromatin and they are made of two copies each of core histones H2A, H2B, H3 and H4 (Lorch, Zhang et al. 1999; Wu and Grunstein...
2000). Approximately 146bp of DNA is wound around the histones to form nucleosomes, and additional proteins can bind to this DNA-protein complex, as part of chromatin (Kornberg 1974; Luger, Mader et al. 1997).

Chromatin is of two types: i) Heterochromatin, which replicates late in the S-phase, is hypoacetylated and transcriptionally repressed ii) Euchromatin which replicates early in the S-phase, is hyperacetylated and transcriptionally active (Weiler and Wakimoto 1995).

![Figure 1.1. Types of chromatin.](image)

**Figure 1.1. Types of chromatin.** Euchromatin (green nucleosomes), Early replicating in S phase, hyperacetylated histone tails, transcriptionally active, H3K4 methylated; Heterochromatin (red nucleosomes), Late replicating, highly condensed structure, hypoacetylated, gene poor, rich in repetitive sequences, methylation of H3K9 and binding of heterochromatin proteins (HPs).

This higher order of DNA packaging plays a crucial role in the control of gene expression. For the gene regulatory proteins and transcription factors to gain access to this tightly packed DNA, in most instances, it has to be unwound from the nucleoprotein complex. The amino terminal tails of the histone proteins are extended outwards and are enriched in basic amino acid residues (Luger, Mader et al. 1997; White, Suto et al. 2001). These residues can be subjected to a wide variety of post – translational modifications such as acetylation at lysine residues, methylation at lysine and arginine residues, phosphorylation at serine, and
ubiquitylation of lysine (Jenuwein and Allis 2001; Zhang and Reinberg 2001; Cosgrove and Wolberger 2005; Kouzarides 2007). These post-translational modifications contribute to the formation of the open or closed states of chromatin.

**HISTONE ACETYLATION**

Negatively charged DNA is highly compacted around the positively charged core histones in nucleosomes. Acetylation of histones leads to the neutralization of this positive charge on lysines (Bhaumik, Smith et al. 2007). All four core histones can be acetylated at specific lysine residues. Histone acetylation is catalyzed by a class of enzymes known as Histone acetyltransferases (HATs). The acetylation of histones is generally associated with transcriptional activation and accounts for a more open chromatin structure. Transcriptional coactivators such as Gcn5/PCAF, CBP/p300 and SRC-1, have been shown to possess intrinsic HAT activity. The Histone Deacetylases (HDACs) are class of enzymes that reverses the acetylation by the HATs. Transcriptional corepressor complexes such as mSin3a, NCoR/SMRT and NURD/ Mi-2 have subunits with HDAC activity (Shahbazian and Grunstein 2007).

**HISTONE PHOSPHORYLATION**

Histones are phosphorylated at specific residues during cell division by specific kinases. Core histone phosphorylations are usually associated with condensation of chromatin, however it is context dependent. It occurs on all four core histones and H1. H2A can be phosphorylated at all points of the cell cycle and ~15% of H2A is phosphorylated in dividing cells. H2A phosphorylation is associated with mitotic chromosome condensation. Phosphorylation of histone H2B (on Ser14 in humans and Ser10 in yeast) is associated with meiotic chromosome condensation. H2B phosphorylation is linked with apoptotic cells (Ajiro 2000) with premature chromosome condensation characterized in both yeast and mammals. Apart from chromosome
condensation phosphorylation of histones also plays a role in transcriptional activation. Histone H3S10 phosphorylation is associated with gene activation in mammalian cells. H3 phosphorylation has been shown to establish the transcription of early response genes, such as *FOS* and *JUN*. H4S1 phosphorylation has been shown to be involved in chromatin compaction during later stages of gametogenesis in mouse and *D.melanogaster* and has been shown to be important for sporulation in *S.cerevisiae* (Krishnamoorthy, Chen et al. 2006).

**HISTONE METHYLATION**

Histone methylation takes place at lysine and arginine residues. Methylated residues of H3K4, H3K36 and H3K79 are implicated in transcriptional activation, and H3K36 and H3K4 also have a direct role in transcriptional elongation (Turner 2002). The histone residues H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20 can be mono-, di- or tri- methylated by lysine methyltransferases, while arginine methylation on certain histone residues can be mono- or dimethylated in higher eukaryotes and is mediated by Protein Arginine Methyltransferases (PRMTs) (Lee, Teyssier et al. 2005; Shilatifard 2006). Both lysine and arginine methylation can cause either transcriptional activation or repression. Methylated lysines H3K9, H3K27 and H4K20 are associated with transcriptional repression. H3K9 methylation causes heterochromatinization by the recruitment of other enzymes and heterochromatin proteins containing chromodomains. H3K9 methylation is found in the fission yeast as in higher eukaryotes (Noma, Allis et al. 2001), whereas it is absent in the budding yeast (Valenzuela and Kamakaka 2006). H3K27 methylation is associated with the silencing of *HOX* gene expression in vertebrates, but appears to be absent in both budding and fission yeast (Kouzarides 2007). Trimethylated H3K4 is associated with transcriptional activation and is known to localize at the 5’ end of the gene while trimethylated H3K36 is associated with transcriptional elongation and is
known to be present at the 3’end of the gene. In yeast, H3K36me3 is recognized by the EAF3 domain of the RPD3S histone deacetylase complex and is targeted to the coding regions of genes. This association has been shown to be important for full length transcription of genes as it prevents erroneous intragenic transcription (Carrozza, Li et al. 2005; Li, Gogol et al. 2007). H3K4 is methylated by Set1 and H3K36 is methylated by Set2. Lysine methylation is reversible and many demethylases have recently been discovered. Two types of lysine demethylase domains are known, and they possess different catalytic reactions; the LSD1 domains are amine oxidases, and Jmjc domain demethylases possess hydroxylase activity (Shi, Lan et al. 2004; Bhaumik, Smith et al. 2007). Arginine methylation is reversed by the conversion of arginine to citrulline by deimination (Kouzarides 2007).

**HISTONE UBIQUITYLATION**

Ubiquitylation has been shown to occur mostly on histones H2A and H2B. Approximately 5-20% of H2A and 2-6% of H2B is ubiquitylated. H2AK119 is known to be ubiquitylated in humans while H2B is known to be monoubiquitylated at H2BK120 in humans and H2BK123 in budding yeast (Bhaumik, Smith et al. 2007; Kouzarides 2007). Monoubiquitylation of H2B by Rad6 (ubiquitin-conjugating) and Bre1 (ubiquitin-ligase) proteins in budding yeast has been shown to be associated with transcriptional activation and open chromatin (Zhu, Zheng et al. 2005). This monoubiquitylated H2B serves as a mark for Set1 and Dot1 methyltransferases, which then methylates H3K4 and H3K79, and is correlated with active transcription (Sexton, Schober et al. 2007). Deubiquitylation of H2B is catalyzed by Ubp8 and Ubp10 in *S.cerevisiae*. Ubp8 is a subunit of the SAGA chromatin remodeling complex, and its deubiquitylase activity is required for the function of SAGA, while the activity of Ubp10 is required to maintain the silenced state in budding yeast (Emre, Ingvarsdotir et al. 2005).
both ubiquitylation and deubiquitylation are necessary for transcriptional activation of certain genes depending on the context.

Figure 1.2. Post-translational modifications of core histones. Depicted above is a schematic representation of the amino terminal tails of core histones H2A, H2B, H3 and H4 extending out from a nucleosome core. These tails are highly basic and are subjected to many post-translational modifications. Shown above are some of these modifications, where P is Phosphorylation (Indigo), Me is Methylation (Dark red), Ac is Acetylation (Blue) and Ub is Ubiquitylation (green).

Hyperacetylation is generally associated with gene activation and hypoacetylation is associated with transcriptional repression. After decondensation of the genes within euchromatin after mitosis, gene regulatory proteins gain access to DNA and direct the expression of individual genes. Genes in heterochromatin regions are repressed, as the transcription complex cannot gain access to it or is prevented from elongating transcripts. The regulation of genes expression therefore depends in part on the “histone code” where the modifications described above serve as marks for other proteins and chromatin modifying complexes (Braunstein, Rose et al. 1993; Thompson, Ling et al. 1994; Strahl and Allis 2000; Turner 2000; Moazed 2001;
Thus the eukaryotic genome is organized into regions of euchromatin and heterochromatin, and these chromatin states can be stably inherited through multiple cell divisions by the maintenance of the histone code. This mode of inheritance is known as “epigenetic inheritance” (Richards and Elgin 2002). Therefore post-translational modifications of histones and resulting interaction with chromatin remodeling complexes can have profound influences on DNA replication, transcription, repair and recombination.

Transcription can be regulated at multiple levels. While proteins bound to DNA can have direct influences on transcription rates, overall chromatin structure also plays a role in transcriptional regulation. Heterochromatic gene silencing is different from gene–specific repression, in that the latter is particularly directed to a specific promoter, while silencing involves the heterochromatinization of an entire region of a chromosome (Moazed 2001). Although the initiation of silencing is sequence dependent, the spreading of silencing is sequence–independent.

![Yeast Chromosome III](image)

**Figure 1.3. Schematic representation of the HML, MAT, and HMR loci in *S. cerevisiae*.** The transcribed *MAT* locus is in the central region of chromosome III, and the cryptic *HMR* and *HML* loci are near each end of the chromosome. *HMR* and *HML* are flanked by the E and I silencers that binds the proteins Rap1p, Orc1p and Abf1p. These proteins cause the nucleation of the silencer proteins (*SIR*) which bring about the heterochromatinization of the entire locus.
Silencing was originally defined in *S. cerevisiae* at the *HMR* (Homothallic Right) and *HML* (Homothallic Left) loci, and has been studied in great detail in yeast (Figure 1.3). The *HM* loci are each flanked by E and I silencers (Essential and Important). *HMR*-E has binding sites for three factors required for proper silencing, *ORC*, Rap1p and Abf1p. The initiation of silencing at this locus is brought about by the binding of Abf1p (Autonomously replicating sequence Binding Factor), Rap1p (Repressor Activator Protein) and ORC (Origin Recognition Complex). These proteins then recruit the Sir (Silent Information Regulator) proteins – Sir1p, Sir2p, Sir3p and Sir4p (Moazed 2001; Huang 2002).

The Sir proteins are recruited to the target region in a sequential manner and through protein-protein interactions (Rusche, Kirchmaier et al. 2003). Sir1p binds to Orc1p and sets a flag for the binding of other Sir proteins. Sir4p binds next and is known to interact with both Sir1p and Rap1p. Sir4p does not require Sir3p or Sir2p for its binding. Since Sir4p exists as a complex with Sir2p, binding of Sir4p results in the recruitment of Sir2p. Sir4p is also required for the binding of Sir3p. Sir3p binds to Rap1p, Sir4p and possibly Abf1p as well (Rusche, Kirchmaier et al. 2003).

Sir2p is a NAD$^+$-dependent histone deacetylase (Sauve, Celic et al. 2001; Jackson and Denu 2002). Sir2p deacetylates histones H3 and H4 of the nearby nucleosome which creates high affinity binding sites for Sir3p and Sir4p. Since Sir4p exists as a complex with Sir2p, binding of Sir4p brings in Sir2p which then deacetylates the histones H3 and H4 of the next nucleosome in an NAD$^+$ dependent manner (Landry, Sutton et al. 2000; Smith, Brachmann et al. 2000; Moazed 2001). This causes the silencing to spread along successive nucleosomes across several kilobases of DNA. The yeast mating loci have long served as an important model system for studying the mechanism of heterochromatin formation.
So, where does this self-propagating heterochromatinization stop? What prevents it from spreading into the transcriptionally active euchromatin region? Specialized DNA sequences referred to as boundary elements exist along chromosomes, and their function is to delimit chromosomal domains. Boundary elements can be of two types: barriers, and insulators.

Figure 1.4. Sequential events associated with heterochromatic spreading in *S. cerevisiae.*
A) Initial recruitment of Sir proteins by the silencer binding proteins Orc1p, Rap1p, and Abf1p,
B) Sir2p by virtue of its histone deacetylase activity deacetylates the histone tails of the neighboring nucleosome creating a high affinity binding site for other silencer proteins,
C) Sequential recruitment of additional Sir proteins to the deacetylated tails,
D) Repetition of this process from step B) leading to the heterochromatinization of the entire locus.

Barrier elements are DNA sequences that prevent heterochromatin from spreading into neighboring active euchromatic regions, while insulators are elements that prevent inappropriate
activation of a gene when placed between a promoter and an enhancer (Labrador and Corces 2002; West, Gaszner et al. 2002). These boundary elements therefore function to help organize the complex eukaryotic genome into active and inactive regions by preventing regulatory elements of one domain from influencing another domain. Insulators have been suggested to form higher order loop structures within chromosomes, and also appear to affect post-translational modifications of histones (Kimura and Horikoshi 2004).

![Diagram of chromatin boundaries]

**Figure 1.5. Chromatin boundaries in eukaryotes.** Barrier elements (green oval) prevents heterochromatin (shown by red deacetylated nucleosomes) from spreading into active euchromatin regions (depicted by green nucleosomes), which are generally hyperacetylated. The inappropriate activation of a promoter (P) by an enhancer (E) is prevented by insulators (green oval) when present between them.

Proposed mechanisms of barrier function suggest that they can either be i) a physical barrier composed of a large DNA bound protein complex, which separates the two states of chromatin by restricting the ability of the silencing proteins, especially Sir2p to access the neighboring nucleosomes and thus prevent the spreading of deacetylation of histones or ii) an active barrier that recruits chromatin modifying enzymes which counteracts the silencing mechanism by acetylation of histones and thus creating an active chromatin (Donze and
Studies in *Schizosaccharomyces pombe*, by Noma et al. 2006, show that silencing at the *MAT* locus involves the methylation of histone H3 at lysine 9, which recruits the heterochromatin protein Swi6. A reduction in Swi6 and H3K9me and an increase in H3K4me are observed at the boundary – suggestive of an active barrier.

**MATING IN YEAST AND GENE REGULATION**

The heterochromatic regions of the budding yeast *S.cerevisiae* include the mating loci, the rDNA locus and the telomeres. There are two mating types in yeast, ‘a’ and ‘α’. The genetic information on the *MAT* locus determines the mating type of the yeast cell. Accordingly, a cell can be either *MATα* or *MATa*. Only the opposite mating types can mate with each other.

*MATα* cells produce an ‘a’ specific pheromone called the a-factor, while *MATα* cells produce an α-specific pheromone called the α-factor. *MATα* cells have receptor for α-factor called Ste2p and *MATα* cells have a receptor for a-factor, called Ste3p. These are G protein-coupled receptors and are present on the cell membranes of the mating cells (Shi, Kaminskyj et al. 2007). When ‘a’ and ‘α’ cells come in close proximity, the secreted pheromones of the opposite mating types bind on the respective receptors, which is then followed by a series of responses. The two cells stop dividing, and proteins required for the mating process are produced and they mate with each other by a process called “shmooing”, in which they produce a projected structure off their surface directed towards each other, and this structure is called a “shmoo”. Once they mate to produce a diploid *MAT a/α* cell, they are unable to mate with another diploid cell or the haploid mating types. This is because the genes encoding the pheromones or the receptors are not transcribed. The *MAT a* locus encodes two proteins, a1p and a2p, while the *MATα* locus encodes α1p and α2p (Ducker and Simpson 2000). α1p is regulates the expression of α-specific genes by binding to the promoter of these genes and *MATα2* is
responsible for the repression of a-specific genes in α cells (Yuan, Stroke et al. 1993). In MATα cells, a-specific genes are expressed by default as neither the MATα1 nor MATα2 proteins are produced.

**Figure 1.6. Representation of sexual cycle in S.cerevisiae.** The a (blue) and α (yellow) cells respond to the pheromones produced by the opposite mating type and arrest in G1 phase of the cell cycle. They produce extended appendages called shmoos, resulting in the fusion of the cell membranes, followed by nuclear fusion resulting in a diploid MATα/α cell (green). This diploid cell under limiting nutrient conditions can undergo meiosis to produce four spores which are enclosed in a sac called ascus. Under favorable conditions this sac ruptures releasing the spores which can then resume vegetative growth.

**MAT α CELLS**

α2 protein in α cells acts as a repressor for a-specific genes. It binds cooperatively with Mcm1p which exists as a homodimer at the α2 operator, a region upstream of the a-specific genes. α2p also exist as a homodimer and binds on either side of Mcm1p (Tan and Richmond 1998). They interact not only with Mcm1p but also with each other. Binding of α2p then facilitates the binding of the repressor complex, Ssn6-Tup1p which when recruited causes the
repression of α-specific genes in α cells (Wahi, Komachi et al. 1998). α1p activates the expression of α-specific genes such as Ste3p. α1p again binds Mcm1p but it also binds another protein Ste12p which together in this case, activate transcription of α-specific genes (Yuan, Stroke et al. 1993).

**Figure 1.7. Regulation of α-specific gene expression in MATα cells.** a) α1p binds to the Mcm1p homodimer and Ste12p to activate α specific genes b) a homodimer of α2p binds to a homodimer of Mcm1p at a-cell specific gene (asg) promoter and results in the repression of asg expression in α cells, which is mediated by the Ssn6p- Tup1p corepressor complex.

**MATα CELLS**

In MATα cells, Ste12p along with the homodimer of Mcm1p binds to the promoter of α-specific genes and activates their transcription (Hwang-Shum, Hagen et al. 1991). Neither α1p nor α2p are involved in the expression of α-specific genes. The promoter of α-sgs contains binding sites for both Mcm1p and the activator protein Ste12p. A homodimer of Mcm1p binds
cooperatively with the Ste12p at asg promoters, and brings activates transcription of asgs in *MATa* cells (Primig, Winkler et al. 1991).

**MATa/α (DIPLOID CELLS)**

The *MATa* cell mates with an α cell to form a diploid *MATa/α* cell. The diploid *MATa/α* cells cannot mate any further. They do not express the pheromones or the pheromone receptor genes. However, they can undergo meiosis and sporulation and give rise to four haploid cells. In the diploid cells neither the asgs nor the αsgs are expressed. Only three cell-type specific genes are expressed: α2, a1 and a2. Also, the haploid–specific genes (hsgs) are repressed.

![Diagram](image)

**Figure 1.8. Regulation of α-specific gene expression in *MATa* cells.** A homodimer of Mcm1p bound at the asg-promoter interacts with Ste12 which binds to a specific DNA sequence called pheromone response element (PRE) to activate transcription of asgs in *MATa* cells. The absence of α1p and α2p in these cells prevents the expression of αsgs (α–specific genes) in *MATa* cells.

*MATa1* which does not play a role in the expression of asgs plays an important role in the diploid cells. It binds to α2p, and the resulting a1p-α2p heterodimer binds at the promoter region of α1 gene, thus preventing its transcription and thus the expression of αsgs in a/α cells. They also bind to the promoters of the hsgs and repress their transcription (Goutte and Johnson 1994). The hsgs genes are expressed only in the haploid a or α cells but not in a/α diploid cells. Binding of α2p with Mcm1p represses the expression of asgs in the diploid cell. α2p in the a1p-α2p heterodimer complex recruits the Ssn6p-Tup1p complex at the operator sequences of the hsgs, thus causing repression of these genes (Johnson 1995). Although, a2p is expressed in the diploid
cells, its function is still undetermined. Mcm1p is a transcriptional regulatory protein that is expressed in all cell types and activates the transcription of a-sgs in \( a \) cells and represses the transcription of a-sgs in \( \alpha \) cells and \( a/\alpha \) cells.

Figure 1.9. Regulation of diploid specific gene expression in \( MAT\alpha/\alpha \) diploid cells. a) The a1p-\( \alpha 2p \) complex binds to hsg promoter regions and recruits Ssn6p-Tup1p corepressor to prevent the expression of hsgs in diploid cells, b) This complex also binds to the \( \alpha 1 \) promoter region and inhibits its transcription. c) Homodimer of \( \alpha 2p \) binds to a homodimer of Mcm1p at asg promoters and causes the repression of asg expression in \( \alpha \) cells, which is brought about by the Ssn6p- Tup1p corepressor complex.
Mcm1 belongs to the family of MADS box (Mcm1 Agamous Deficiens Serum response factor) proteins and binds to DNA via its recognition helix similar to homeodomain proteins (Acton, Zhong et al. 1997). STE12 belongs to a homeodomain superfamily of proteins and binds to a specific DNA sequence called the Pheromone Response Element (PRE) (Dolan and Fields 1991). Thus even the simple unicellular eukaryote, S. cerevisiae, has a complex set of regulatory proteins which determines the pattern of expression of genes in a given cell type, which in turn determines the sex of the cell.

The HML locus carries a cryptic copy of the MATa genes while the HMR carries a cryptic copy of MAT a genes (Moazed 2001; Rusche, Kirchmaier et al. 2003). Transcription from the HML and HMR loci is silenced by Sir protein mediated heterochromatin formation as discussed above, thus preventing the genes at these loci from being transcribed in normal yeast cells. However, these strains are homothallic in that they can switch their mating types as often as once every generation (Haber 1998). This switching in mating type is catalyzed by HO endonuclease that cleaves the recipient MAT locus which accepts the sequence from either one of its two donors, the HML or the HMR. Most laboratory strains have a mutation in HO that prevents the switching of the mating types.

A barrier-type boundary element has been characterized downstream of the HMR heterochromatic locus. A tRNA gene has been shown to be a major component in preventing the spreading of silencing from the HMR to the downstream GIT1 gene on chromosome III in S. cerevisiae (Donze and Kamakaka 2001). The tRNA genes have highly conserved box A and box B internal promoter sequences, which are bound by a large transcription factor complex. TFIIIC, a large protein complex of > 500KD, is made up of six subunits. TFIIIC recognizes box B, but binds to both box A and box B, followed by the recruitment of TFIIIB. TFIIIB is made up
of three proteins; the TBP (TATA Binding Protein), TFIIIB Related Factor (Brf1) and Bdp1. Together they form the preinitiation complex, directing the binding of RNA polymerase III. This assembly accounts for an extremely large complex of \( \sim 1.5 \) MDa, consisting of 26 polypeptide subunits (Kassavetis, Blanco et al. 1992; Kassavetis, Joazeiro et al. 1992; Donze and Kamakaka 2001). Also, Pol III transcribed RNAs are very abundant, accounting for 15\% of total RNA in yeast (Warner 1999). Thus, it is hypothesized that tRNA genes and other Pol III transcribed genes are constantly occupied by transcription complexes. These complexes are bound with high affinity that increases the processivity of the enzyme complex. These two factors, i) the large size of the protein complex and the ii) constant occupancy of the transcription complexes, taken together, might account for the boundary function of the \( HMR \) tRNA gene. The large protein complex might disrupt the sequential spreading of silencing by SIR proteins, creating a nucleosome free region, or, the continuous occupancy of TFIIIC and TFIIIB might recruit histone acetyltransferases and other enzymes which then create a competition between these enzymes and the deacetylases (Donze and Kamakaka 2001; Kimura, Umehara et al. 2002).

The \( S.pombe \) inverted repeat boundary elements flanking the heterochromatic \( MAT \) locus have been shown to contain box B sequences that recruit the transcription factor TFIIIC without recruiting RNA Po IIII. Numerous sites have been identified across the fission yeast genome that are bound by TFIIIC, but not Pol III (Noma, Cam et al. 2006). Further, mutations in the Pol III transcription factors TFIIIC and TFIIIB have been shown to impair barrier activity of the \( HMR \) tRNA\(^{Thr}\) gene (Donze and Kamakaka 2001).

Previous studies suggested that the \( HMR \)-tRNA gene was the major element required to block silencing at \( HMR \). The boundary tRNA gene when inserted between \( HMR \)-E and the \( a1 \) gene on chromosome III, at the wild type \( HMR \) locus on a plasmid-based assay, was able to stop
silencing from spreading from HMR. Though the HMR-tRNA gene alone was able to act as an efficient boundary in a plasmid-based assay, it showed only partial barrier activity when integrated back in to the chromosome. This suggested that additional proteins are involved in the complete blocking of silencing. The potential existence of additional factors led us to conduct a genetic screen to identify genes whose products are required for complete heterochromatic barrier activity at the HMR-tRNA region.

The second chapter in this thesis deals with the identification of these additional genes involved in boundary function. Using a transposon mutagenesis strategy coupled with direct deletion mutagenesis, we identified multiple different genes containing bromodomain homology that are involved in blocking the silencing at the HMR boundary. Mutations in these genes compromised boundary function, causing silencing to spread beyond the tRNA gene and into a downstream marker gene. YTA7, a novel bromodomain containing gene was identified multiple times in the screen. Specific mutations in the YTA7 bromodomain or the AAA (ATPases Associated with a variety of cellular Activities) ATPase domains lead to the spread of silencing beyond the HMR-tRNA gene. Other genes identified by this mutational analysis were SAS4 and SAS5, part of the SAS HAT complex and RPD3, part of multiple HDAC complexes.

The third chapter is a detailed analysis of the effects of RPD3 deletion on heterochromatin spreading. It is of particular interest to study RPD3 function; since RPD3 is a class I histone deacetylase, deletion of this gene would be expected to decrease silencing. On the contrary, we and others (De Rubertis, Kadosh et al. 1996; Sun and Hampsey 1999) have observed increases in the spread of silencing, in rpd3Δ mutants. Surprisingly, we found that transcription of the tRNA gene is not affected by this increased spread of silencing. Therefore, we propose that heterochromatic silencing can bypass the boundary, to silence the downstream gene.
We hypothesize two possibilities 1) The silencing in rpd3Δ strains is interrupted at the tRNA gene but somehow continues to spread to the downstream gene; 2) the tRNA gene might remain active by a looping mechanism, allowing the heterochromatic HMR region to come in direct contact with the downstream gene.

LITERATURE CITED


CHAPTER TWO

MULTIPLE BROMODOMAIN GENES ARE INVOLVED IN RESTRICTING THE SPREAD OF HETEROCHROMATIC SILENCING AT THE S. CEREVISIAE HMR-tRNA BOUNDARY*

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INTRODUCTION

Proper regulation of gene expression is of utmost importance for development and survival of all forms of life, as regulated transcription of complex genomes is necessary for cellular energy economy and the precise patterns of gene expression required for cellular differentiation. Transcriptional of genomes is regulated at multiple levels, which can involve large regions of chromosomes, individual nucleosomes, or by direct effects on the transcription machinery at individual promoters (Kimura and Horikoshi 2004). At the level of chromatin in eukaryotes, active euchromatic, and predominately inactive heterochromatic chromosomal domains alternate along chromosomes, and mechanisms must exist to prevent regulatory elements from each domain from influencing gene expression patterns of adjacent domains. Chromatin boundary elements (Donze and Kamakaka 2002; West, Gaszner et al. 2002; Donze 2004; Kimura and Horikoshi 2004) serve this purpose, as boundaries can either prevent a distal enhancer from activating gene expression inappropriately (insulators), or prevent the unrestricted spreading of heterochromatin (barriers).

The budding yeast *S. cerevisiae* contains limited heterochromatic regions at its telomeric and silent mating loci, and these chromosomal regions are important model systems for studying the establishment and propagation of silenced chromatin (Rusche, Kirchmaier et al. 2003), and also for the study of chromatin boundaries and global mechanisms that restrict heterochromatic propagation (Bi and Broach 1999; Donze, Adams et al. 1999; Fourel, Revardel et al. 1999; Kimura, Umehara et al. 2002; Suka, Luo et al. 2002; Ladurner, Inouye et al. 2003; Meneghini, Wu et al. 2003; Tackett, Dilworth et al. 2005). Silenced chromatin in yeast is maintained by the targeted regional action of the Sir proteins (Silent information regulators), where the Sir2p histone deacetylase establishes the histone acetylation state required for heterochromatin
formation as part of a proposed histone code (Jenuwein and Allis 2001), although the specificity of such a code is currently being debated (Kurdistani, Tavazoie et al. 2004; Dion, Altschuler et al. 2005). Deacetylation of histones allows higher affinity binding of Sir3p (Carmen, Milne et al. 2002), which then recruits the Sir4p/Sir2p complex, allowing heterochromatin to propagate along nucleosomes (Hoppe, Tanny et al. 2002; Rusche, Kirchmaier et al. 2002).

At the HMR silent mating locus, a transfer RNA gene and its associated RNA polymerase III complex functions as part of a boundary element that prevents spreading of Sir protein mediated heterochromatin (Donze, Adams et al. 1999; Donze and Kamakaka 2001). However, we found that while the HMR-tRNA alone is sufficient to act as a barrier to silencing in a plasmid-based assay (Donze and Kamakaka 2001), when integrated back into the chromosome, the tRNA alone shows only partial barrier function (our unpublished results), suggesting that additional proteins are required to completely block silencing. Other genes coding for chromatin associated proteins that can lead to a partial spreading of silencing through the tRNA have been identified, and include HTZ1, BDF1, and YTA7 (Ladurner, Inouye et al. 2003; Meneghini, Wu et al. 2003; Tackett, Dilworth et al. 2005), and the global level of histone acetylation regulated by SIR2 and SAS2 influences silencing spread (Kimura, Umehara et al. 2002; Suka, Luo et al. 2002).

Taken together, these results suggest that both local boundary associated factors and global chromatin modifications affect the formation of a true euchromatin-heterochromatin boundary (Kimura and Horikoshi 2004).

In order to assess what other chromatin proteins participate in boundary formation, we used transposon mediated mutagenesis and direct gene knockouts in a yeast boundary element dependent reporter strain to identify mutations that led to a spread of silencing through the HMR-tRNA boundary element, which included approximately 400 base pairs to either side of the
tRNA. In addition to identifying several factors known to be involved in regulating the spread of silencing, we identified a novel bromodomain-containing gene, YTA7, which additionally contains two separate AAA family ATPase domains. We also find that mutation of several different bromodomain genes leads to a general loss of boundary function. This suggests that bromodomains may be key effectors that recognize and maintain euchromatic histone modification patterns necessary for boundary integrity. Epistasis analysis implicates independent roles for YTA7 and the HMR-tRNA in restricting silencing, while RCS2 may be functioning through the RNA polymerase III complex.

MATERIALS AND METHODS

All yeast strains used in this study were isogenic to W303-1a. The parent boundary reporter strain used in the mating assays (DDY277) was derived from a cross of a similar strain, ROY962, previously described (Donze, Adams et al. 1999). The yeast transposon mutagenized library was obtained from Mike Snyder (Yale University), and mutagenesis was performed as described (Burns, Grimwade et al. 1994; Ross-Macdonald, Sheehan et al. 1997; Ross-Macdonald, Sheehan et al. 1999). All transposon insertion strains were created in DDY277, and identification of mutagenized genes was performed using the vectorette PCR method as described (Ross-Macdonald, Sheehan et al. 1999). Mating assay strains containing specific mutations were constructed by direct homologous recombination mediated replacement using standard methods, or by crossing previously constructed mutant alleles into the DDY277 background. Mating assays were performed as described (Donze, Adams et al. 1999).

The schematic diagram of YTA7 and amino acid positions of AAA ATPase and bromodomains was based on query of the Conserved Domain Database (Marchler-Bauer, Anderson et al. 2005). The alignment of S. cerevisiae bromodomains was created using ClustalW
(Chenna, Sugawara et al. 2003), and was manually adjusted to fit a structural based alignment of bromodomains (ZENG and ZHOU 2002). The GST-Yta7p bromodomain fusion was made by PCR cloning the coding sequence of YTA7 from amino acids 1000-1101 into the glutathione-S-transferase vector pGEX-2TK (Amersham Biosciences), and the fusion protein was expressed and purified according the manufacturers instructions. Purified chicken core histones were purchased from Upstate (catalog #13-107). Histone pull-down assays were performed as described (Ladurner, Inouye et al. 2003), and the recovered samples were run on an 18% SDS-PAGE gel, and stained with Bio-Safe Coomassie stain (Bio-Rad Laboratories, Inc.). Briefly, core histones were dissolved in water at 1 mg/ml, and 500 μl binding reactions contained 1X HEMG buffer (25 mM HEPES-KOH, pH 7.6, 0.2 mM EDTA, 12.5 mM MgCl₂, 10% glycerol, diluted from a 5X stock), 50 μg core histones, 150 mM KCl, 15 μl glutathione-Sepharose (Amersham Biosciences) equilibrated in 1X HEMG plus 150 mM KCl, and 15 μg GST-Yta7p fusion protein, or equimolar amount of GST in the controls. Binding reactions were incubated for 4 hours at 4C, beads were collected by centrifugation, then washed 4 times in 500 μl 1X HEMG, 150 mM KCl, 0.01% NP-40. 125 μl 1X Laemml loading buffer was added directly to the beads for gel analysis, and unbound fractions were precipitated with trichloroacetic acid, redissolved in 25 μl 0.1 N NaOH, and brought to 125 μl in 1X Laemmli loading buffer.

To construct the HMR-ADE2 reporter strains, a plasmid containing the 8.2 kb Sac I-Sal I HMR fragment was mutagenized to create a Bam HI site 180 base pairs downstream of the HMR-tRNA (pDD657). The 2.5 kb Bgl II fragment of ADE2 (STOTZ and LINDNER 1990) was cloned into this site such that ADE2 was transcribed away from HMR, to create plasmid pDD659. HMR-trnΔ-ADE2 was constructed by site directed mutagenesis of pDD659 to delete the tRNA coding sequence (pDD661). Plasmid pDD833, hmrΔ-ADE2 was created by subcloning the Sac I-Sal I
fragment of pDD659 into Bluescript cut with Sac I and Xho I to destroy the polylinker Xho I site creating pDD833. The resulting plasmid was digested with Sna BI and Xho I to remove HMR, ends blunted with Klenow polymerase, then religated to create pDD837. Each of these ADE2 plasmids were linearized and transformed into yeast strain DDY142, which contains an hmrΔ::URA3 allele. Transformants were selected on minimal media lacking adenine, and individual colonies were patched and replica plated onto minimal media lacking uracil and media containing 5-FOA to identify isolates that had become Ade+ and uracil auxotrophic. Proper integration was then confirmed by Southern blot analysis.

Mutant alleles of genes affecting the spread of silencing through the boundary were then crossed into each of these ADE2 parent strains (DDY811, DDY814, and DDY2114). Colony color assays were performed by streaking representative strains onto minimal media containing 3 μg/ml adenine, which is 10% of the normal level in this medium. Colonies were grown for three days, and held at 4C for one week prior to photography on a dissecting microscope equipped with a digital imaging system. Colony color was assessed for three independent isolates for each mutant, one representative strain is shown for each.

RESULTS

Identification of Mutations in Genes Coding for Potential Boundary Proteins

We used a MATα boundary reporter strain (DDY277) that harbors a modified HMR locus deleted for the HMR-I silencer, and that also contains the downstream boundary sequence cloned into the HMRA2 gene (Figure 2.1, top). This strain is non-mating, as the boundary blocks the spread of silencing from HMR-E, allowing the a1 gene to be expressed in the MATα background (Donze, Adams et al. 1999). To identify genes involved in restricting the spread of silencing, we subjected this strain to a transposon mutagenesis protocol (Ross-Macdonald, Sheehan et al. 30
selected transposon insertion mutants that acquired an α mating phenotype, indicating that silencing was now able to spread through the boundary into a1. 20,000 Leu+ transformants were replica plated onto MATα tester lawns to identify mutants that lost boundary function at the modified HMR locus as judged by their ability to mate.

α-mating isolates were confirmed by backcrossing to a MATα strain containing the same reporter construct, and only tetrad isolates where the transposon encoded LEU2 marker completely co-segregated with the mating phenotype (20 tetrads analyzed) were studied further. After this analysis, mutations in only four separate genes were confirmed as leading to a spread of silencing: SAS4 and SAS5, encoding subunits of the SAS acetyltransferase complex, RPD3, a histone deacetylase, and YTA7, a little studied gene containing a weak bromodomain homology and two domains of AAA family ATPase homology. The mating phenotypes of each independent mutant isolate are shown in Figure 2.1, bottom. A single mutant of the SAS4 gene resulted in increased mating, along with two independent insertions into SAS5 and two independent insertions into RPD3.

Mutations in the SAS complex were previously shown to increase the silencing efficiency of a defective hmra-e** locus (Ehrenhofer-Murray, Rivier et al. 1997; Xu, Kim et al. 1999), and sas2 mutations were first identified as leading to increased silencing in sir1 mutants (Reifsnyder, Lowell et al. 1996). Since hmra-e** lacks the boundary element, it is likely that mutations in the SAS complex globally affect the robustness of silencing by affecting cellular levels of histone H4 lysine 16 acetylation (Meijsing and Ehrenhofer-Murray 2001; Kimura, Umehara et al. 2002; Suka, Luo et al. 2002; Shia, Osada et al. 2005), as opposed to specifically affecting the integrity of the boundary element. Mutations in RPD3 are also well documented to lead to a global increased of silencing at all three yeast heterochromatin loci (mating loci, rDNA, and telomeres).
by an unknown mechanism (Rundlett, Carmen et al. 1996; Vannier, Balderes et al. 1996; Kim, Benguria et al. 1999; Smith, Caputo et al. 1999; Sun and Hampsey 1999).

Interestingly, eight independent isolates containing transposon insertions in \textit{YTA7} were isolated in the screen, representing five different insertion sites. It is not clear why \textit{yta7} mutants would occur so frequently in the library compared to other genes. It should be noted that the screen, while calculated to be saturating, was not, as mutations in genes previously known to induce a mating phenotype in this reporter system were not recovered, such as the acetyltransferase subunit of SAS, \textit{SAS2}, and the bromodomain-acetyltransferase \textit{GCN5} (DONZE and KAMAKAKA 2001). Additionally, insertions into other genes known to be required for restriction of silencing at \textit{HMR}, \textit{BDF1} (Ladurner, Inouye et al. 2003) and \textit{HTZ1} (Meneghini, Wu et al. 2003) were also not recovered. However, as discussed below, direct deletion of \textit{BDF1} or \textit{HTZ1} does lead to a spread of silencing in this reporter assay (Figure 2.3). The Sir protein dependence of the observed spread of silencing is shown for a \textit{yta7} mutant (DDY2078, Figure 2.1), and was also confirmed for \textit{rpd3} and SAS mutants (our unpublished data).

\textit{YTA7} contains AAA family ATPase domains and a single bromodomain module, schematically depicted in Figure 2.2A. Since bromodomains have been demonstrated to be acetyllysine binding modules (Dhalluin, Carlson et al. 1999; Hudson, Martinez-Yamout et al. 2000; Jacobson, Ladurner et al. 2000; Owen, Ornaghi et al. 2000) and have been suggested to mediate their functions by binding to acetylated histones and other chromatin associated proteins (Zeng and Zhou 2002; Yang 2004; de la Cruz, Lois et al. 2005), it seemed likely that Yta7p may be functioning through histone recognition. The bromodomain protein Bdf1p is also known to have an anti-silencing function at \textit{HMR}, as \textit{bdf1} mutants show decreased gene expression and increased Sir3p association in regions adjacent to yeast silenced loci (Ladurner, Inouye et al.
2003). Alignment of the \textit{YTA7} bromodomain with other yeast bromodomains (Figure 2.2B), however, reveals that \textit{YTA7} lacks a critical conserved tyrosine residue required for acetyllysine recognition by P/CAF, Gcn5p and Bdf1p via a key hydrogen bond formation within the binding pocket (Dhalluin, Carlson et al. 1999; Owen, Ornaghi et al. 2000; Ladurner, Inouye et al. 2003). Yta7p does contain a serine and threonine at the same location.

Due to the absence of this key tyrosine residue, we wished to test whether the Yta7p bromodomain module was able to bind to histones. We cloned the coding sequence of the \textit{YTA7} bromodomain into a GST (glutathione-S-transferase) fusion vector, and purified the GST-Yta7p fusion protein from \textit{E. coli} cells. This fusion protein was used in a pull-down assay with purified chicken erythrocyte core histones. Figure 2.2C shows that GST-Yta7p efficiently binds to histones H3 and H4, compared to no binding of GST alone to the histones. This result shows that despite lacking the conserved tyrosine, the Yta7p bromodomain is a functional histone binding domain \textit{in vitro}. The bromodomain protein Bdf1p has also been shown to restrict the spread of silencing in yeast (Ladurner, Inouye et al. 2003), and the histone binding domain of the transcription factor CTF-1 is essential for its heterochromatin barrier activity (Ferrari, Simmen et al. 2004), suggesting a role for histone binding proteins in blocking silencing.

The \textit{S. cerevisiae} genome contains 10 genes with bromodomain homologies (aligned in Figure 2.2B). Two of these genes are essential for viability, the RSC complex genes \textit{STHI} and \textit{RSC4}. Since loss of Bdf1p and Yta7p functions lead to an apparent spreading of silencing, we asked if bromodomain proteins in general contain boundary and/or anti-silencing functions. We created a set of strains containing deletions or disruptions of each non-essential bromodomain containing gene in strain DDY277 to assess spreading of silencing across the boundary element using the mating assay described in Figure 2.1.
Figure 2.1. Mating assay to identify extragenic mutations that lead to spreading of silencing through the *HMR* right boundary. (Top) The 1.0 kb region downstream of *HMR* containing the boundary tRNA was cloned into the *a2* gene and the resulting construct was integrated back into chromosome III in a *MATα* strain. The boundary element blocks the spread of silencing into *a1*, resulting in a non-mating phenotype (DDY277). The mating assay was performed essentially as described previously (Donze, Adams et al. 1999). DDY277 was mutagenized by transformation with a yeast genomic DNA library containing random transposon-*LEU2* insertions (Ross-Macdonald, Sheehan et al. 1999), and Leu+ recombinants were tested for mating. (Bottom) Thirteen independent isolates that showed increased silencing were confirmed, representing mutations in four different genes, *YTA7*, *SAS5*, *SAS4*, and *RPD3*. All strain genotypes are listed in TABLE 1.
Figure 2.2. \textit{YTA7} encodes a histone binding bromodomain protein. (A) Schematic diagram of the predicted \textit{YTA7} open reading frame, showing the relative locations of the two AAA family ATPase domains (amino acids 449-580 and 771-957), and the single bromodomain (amino acids 1003-1091) as determined by query of the Conserved Domain Database (Marchler-Bauer, Anderson et al. 2005). (B) Alignment of yeast bromodomain sequences reveals that \textit{YTA7} lacks a key conserved tyrosine residue (arrow, shown in red) that is critical for histone binding in other bromodomains, but contains a tandem serine-threonine in the same position (green). (C) GST-Yta7p pulldown assay reveals that the bromodomain of Yta7p specifically retains histones H3 and H4. Reactions and washes were carried out in 150 mM KCl (see METHODS). Increasing KCl to 500 mM in binding and wash steps reduced the level of histone binding by approximately 50% (data not shown).
The results shown in Figure 2.3 demonstrate that loss of function of several bromodomain proteins lead to a spreading of silencing. Deletions of \textit{RSC2}, \textit{YTA7}, or \textit{BDF1} lead to the greatest loss of boundary function, while deletion of \textit{RSC1}, \textit{GCN5}, or \textit{BDF2} shows a weaker phenotype. Deletions of \textit{SPT7} or \textit{SNF2} do not show spreading of silencing as determined by this assay. As controls for spreading of silencing at \textit{HMR}, we assayed mutations in \textit{SAS2} and \textit{HTZ1}, and observed the expected spreading of silencing as indicated by increased mating compared to the control strain DDY277 (Figure 2.3). The \textit{htz1} mutants showed a consistently variegated phenotype in this assay, with patches derived from some colonies mating and others not.

We next wanted to test epistatic relationships among bromodomain factors and the \textit{HMR}-tRNA boundary element. Since deletion of the \textit{HMR}-tRNA leads to a near complete mating phenotype in the mating assay, we developed a second system for assessing the effects of tRNA deletion on heterochromatin spread at \textit{HMR}. The \textit{S. cerevisiae} \textit{ADE2} gene was integrated downstream of \textit{HMR} (referred to here as \textit{HMR-ADE2}), and isogenic strains were created that were \textit{HMR-trna\Delta-ADE2} or \textit{hmr\Delta-ADE2}. Repression of \textit{ADE2} expression leads to the accumulation of a red pigment derived from the Ade2p substrate (\textit{Fisher} 1969).

Phenotypes of these parent strains are shown in the top row of Figure 2.4B: strains containing a normal \textit{HMR} locus, or a deletion of \textit{HMR} (including both silencers) give rise to all white colonies, demonstrating expression of \textit{ADE2} at this integration site; deletion of only the \textit{HMR}-tRNA gives rise to a variegated phenotype with some sectored pink colonies arising due to epigenetic silencing of \textit{ADE2}.

These \textit{ADE2} reporter strains were crossed to strains containing mutations that resulted in spreading of silencing in the mating assay. Mutation of \textit{RPD3} resulted in completely red colonies.
with or without the tRNA present. This complete penetrance of the rpd3 phenotype prevented any epistatic analysis using this assay. Mutation of SAS5 resulted in a variegated but mostly pink-red colony phenotype, which was unchanged when the HMR-tRNA gene was deleted. Again, based on the qualitative nature of this assay, it was impossible to determine epistatic effects of sas5Δ trnaΔ mutations.

![Diagram](image)

**Figure 2.3. Mutation of multiple bromodomain containing genes results in spreading of silencing through the HMR boundary.** Specific deletions or insertions were created or crossed into DDY277 to test their effects on boundary function in the mating assay described in Figure 1. Null mutations in YTA7, BDF1, RSC2, and SAS2 led to a significant spread of silencing, while mutation of GCN5, RSC1, BDF1, and HTZ1 showed a weaker phenotype. Mutation of SPT7 or SNF2 showed no phenotype.

Deletion of YTA7 in HMR-ADE2 strains yielded a variegated pink-white phenotype, similar in magnitude to the HMR-trnaΔ-ADE2 strain, however in yta7Δ trnaΔ strains, a darker red color was observed in a greater percentage of colonies, including some completely red colonies, demonstrating a more robust spread of silencing when both mutations are present.
This additive effect suggests that \textit{YTA7} functions independently of the \textit{HMR}-tRNA in restricting the spread of silencing. \textit{RSC2} deletion shows a weakly variegated phenotype in the \textit{HMR-ADE2} background, with rare silenced colonies that were somewhat darker red. Deletion of the \textit{HMR}-tRNA in the \textit{rsc2}\textsuperscript{Δ} background did not increase the severity of the phenotype, suggesting that \textit{RSC2} and the \textit{HMR}-tRNA may function through the same pathway in restricting the spread of silencing. We attempted to determine epistatic relationships among bromodomain containing genes, but we were unable to obtain \textit{yta7-rsc2} or \textit{yta7-bdf1} double mutants both by crosses of single mutant strains or by homologous recombination, suggesting a synthetic lethality of these mutant combinations in our W303 background (our unpublished results). Importantly, when \textit{HMR} was deleted in each of these mutant backgrounds (Figure 2.4B, right column), all white colonies were observed. This confirms that phenotypes observed for each of these mutations is due to the spread of silencing from \textit{HMR}, and not due to global histone acetylation effects or spreading of silencing from the telomere of chromosome III. Deletion of \textit{SIR2} or \textit{SIR4} in these backgrounds also results in completely white colonies, confirming the Sir dependence of the phenotype (our unpublished data). Also, it should be noted that the spreading of silencing observed in all experiments described here are due to normal levels of Sir proteins, no overexpression of Sir3p was employed to obtain the observed phenotypes.

**DISCUSSION**

\textit{YTA7} Encodes a Histone Binding Chromatin Boundary Protein

The mutational analysis of boundary function described here revealed that the AAA family ATPase/bromodomain protein Yta7p plays a role in the integrity of the euchromatin/heterochromatin boundary at the \textit{S. cerevisiae HMR} locus. \textit{YTA7} contains a region
of bromodomain homology from amino acids 1003 to 1091, and two separate AAA family ATPase domains from amino acids 449 to 580 and 771 to 957 (Figure 2.2A). Comparison of the homology of the YTA7 bromodomain to other yeast bromodomains (Figure 2.2B) revealed YTA7 lacks a key tyrosine residue (conserved in most bromodomains) which makes a critical water-mediated hydrogen bond to acetyllysine in Gcn5p, and is required for the interaction of acetylated histones with Bdf1p (Dhalluin, Carlson et al. 1999; Owen, Ornaghi et al. 2000; Ladurner, Inouye et al. 2003).

However, the results shown in Figure 2.2C demonstrate that the YTA7 bromodomain is a functional histone binding module. The YTA7 sequence contains tandem serine and threonine residues at the position of the conserved tyrosine, which may contribute the necessary hydrogen bonding potential within the bromodomain binding pocket. Bioinfomatic analysis of YTA7 orthologs in related yeast species S. paradoxus, S. mikatae, and S. bayanus, and less related Debaryomyces hansenii and Yarrowia lipolytica, revealed that these YTA7 orthologs have the same serine-threonine substitutions in their respective bromodomains (Christie, Weng et al. 2004), and the same substitution is seen in both human and mouse Tif1β (ZENG and ZHOU 2002). Yta7p is also a confirmed nuclear protein, as determined in a genome-wide analysis of yeast protein localization (Huh, Falvo et al. 2003).

While this work was in progress, Tackett et al. (Tackett, Dilworth et al. 2005) published a proteomic study that also implicated Yta7p in boundary function. They found Yta7p associated with complexes containing Dpb4p, a component of the DNA polymerase ε and ISW2/CHRAC chromatin complexes, and both of these complexes are involved in regulating telomeric silencing (IIDA and ARAKI 2004). Tackett et al. also showed that deletion of YTA7 led to spreading of silencing at HMR, and most significantly, they localized Dpb4p-Yta7p complexes to sites near
HMR, HML, chromosome ends, and near the conditionally expressed and epigenetically FLO genes, suggesting a specific targeted role for Yta7p in restricting repression from multiple loci.

**Figure 2.4. YTA7 and the HMR-tRNA function through different pathways to restrict the spread of silencing at HMR.** (A) The ADE2 gene was inserted into chromosome III downstream of HMR in *S. cerevisiae* (HMR-ADE2). Isogenic strains lacking the HMR-tRNA (HMR-trnaΔ-ADE2), or lacking HMR (hmrΔ-ADE2) were also constructed. (B) Each strain was crossed into rpd3, sas5, yta7, or rsc2 mutant backgrounds, and silencing was assessed by the degree of pigmentation exhibited by the presence or absence of ADE2 expression. The combination of yta7Δ trnaΔ leads to a more silenced phenotype (more and darker red colonies), suggesting that each functions independently. No difference in phenotype is seen between strains containing rsc2Δ and rsc2Δ trnaΔ. Strains depicted in each row are: first row (wild type) DDY814, DDY811, DDY2114; second row (rpd3) DDY2093, DDY2143, DDY2128; third row (sas5) DDY2106, DDY2156, DDY2142; fourth row (yta7) DDY2205, DDY2200, DDY2198; fifth row (rsc2) DDY2496, DDY2489, DDY2450.
The results presented in this manuscript confirm the role of Yta7p in preventing the spread of silencing, and extend these results to demonstrate independent functions of Yta7p and the HMR-tRNA with an epistasis analysis (discussed below).

Additionally, the study by Tackett et al. identified roles for DNA polymerase ε, Isw2, and Sas3-Spt16 chromatin remodeling complexes in boundary function. We had previously tested mutations in SAS3 (Donze and Kamakaka 2001) and SPT16 (our unpublished results), and saw no spreading of silencing in our mating assay. Their analysis of the effect of SAS3 deletion showed a reduction in transcript levels of genes adjacent to HMR (GIT1 and YCR095C), but is unknown if this reduction is Sir protein dependent, as expression of these genes was not reported in a sirΔ or hmrΔ background.

Our genetic analysis also uncovered a role for RSC (Remodels the structure of chromatin) complex proteins in boundary function. RSC is a multi-subunit, highly abundant, and essential chromatin remodeling complex first purified from S. cerevisiae cells (Cairns, Lorch et al. 1996). Genome wide analysis of the location of the RSC complex has demonstrated that RSC is targeted to many tRNA genes in vivo (Ng, Robert et al. 2002), suggesting a potentially direct connection between the function of the HMR-tRNA and the RSC complex in heterochromatin boundary function. Our epistasis analysis demonstrating no difference in phenotype between rsc2Δ and rsc2Δ trnaΔ strains shown in Figure 2.4B is consistent with this proposed connection.

Yta7p appears to function independently of the HMR-tRNA, as deletion of both the cis-element and trans-factor leads to a more silenced phenotype (Figure 2.4B). We could not determine the epistatic relationships between the HMR-tRNA and BDF1, BDF2, RSC1, or GCN5 in this assay, as strains containing deletions of these genes grew as all white colonies in the HMR-ADE2 background, and had no apparent effect on colony color when in HMR-trnaΔ-ADE2
background (our unpublished results).Mutations in $BDF2$, $RSC1$, and $GCN5$ all exhibited a weaker phenotype in the mating assay, an effect that may be too weak to affect the $ADE2$ promoter. However, we observed a strong increase in silencing upon deletion of $BDF1$ in the mating assay, but this mutation did not give rise to pigmented colonies in the $HMR$-$ADE2$ background. It should be noted that $bdf1$ strains are very slow growing, and it is uncertain how growth rate might affect the accumulation of pigment in $ADE2$ silenced strains.

The Role of Multiple Chromatin Associated Proteins in Maintaining Euchromatin-Heterochromatin Boundaries

Since the discovery and characterization of a discrete heterochromatin boundary downstream of the $HMR$ locus (Loo and Rine 1994; Donze, Adams et al. 1999; Donze and Kamakaka 2001), many mutations have been uncovered that alter the integrity of the convergence point of heterochromatin with downstream euchromatin at this locus. As described here and in other studies, in addition to mutation of the $HMR$-tRNA or its associated RNA polymerase III complex proteins, loss of function of chromatin associated complexes containing $Yta7p$, $Rsc2p$, $Rpd3p$, $Sas2$, 4, or 5 proteins, $Htz1p$, or $Bdf1p$ lead to spreading of silencing through the $HMR$ boundary element. These effects appear to be either targeted to the boundary region in the case of the RNA polymerase III complex, $Yta7p$, and $Bdf1p$ (Donze and Kamakaka 2001; Ladurner, Inouye et al. 2003; Tackett, Dilworth et al. 2005), or may act more globally to affect histone acetylation levels, as has been suggested for the SAS complex (Kimura, Umehara et al. 2002; Suka, Luo et al. 2002).

Several recent studies have demonstrated that direct targeting of transcription factors or components of chromatin modifying complexes can act as a barrier, preventing the spread of SIR mediated heterochromatin in yeast (Donze and Kamakaka 2001; Fourel, Boscheron et al. 2001; Chiu, Yu et al. 2003; Jacobson and Pillus 2004; Oki, Valenzuela et al. 2004). The barrier effect
created by acetyltransferase targeting was not necessarily due to the enzymatic activity of acetyltransferase fusions, but did require the presence of other components of the respective complexes (Chiu, Yu et al. 2003; Jacobson and Pillus 2004; Oki, Valenzuela et al. 2004), suggesting that targeting of a specific factor results in the recruitment of multiple proteins. Global models of heterochromatin barrier function suggest that acetyltransferases and deacetylases establish “negotiable borders” or “fuzzy boundaries” by convergence of the effects of their activities, without the need for a specific barrier element or localized targeting (Fourel, Magdinier et al. 2004; Kimura and Horikoshi 2004).

However, while both targeted and negotiable models are consistent with current data, the effects of global genome alteration (such as in a sas2 background) on the normal targeting of chromatin-associated proteins is not well defined. Any natural targeting of proteins occurs within the context of normal cellular chromatin acetylation. Therefore while domains of global histone acetylation may be negotiable in mutant strains, such genome-wide effects may lead to specific alterations in acetylation state dependent localization of proteins, such as the targeting of bromodomains.

Taken together, the current evidence suggests that euchromatin-heterochromatin boundaries are determined by both site-specific interactions and global influences. Sir protein dependent silencing in yeast appears to propagate by successive Sir2p deacetylation, Sir3p binding to deacetylated nucleosomes, and recruitment of Sir4p-Sir2p complex, allowing spreading of the heterochromatic chromatin along a nucleosomal array after initial targeting of the SIR complex at silencers or telomeres (Hoppe, Tanny et al. 2002; Rusche, Kirchmaier et al. 2002). In certain cases, simply excluding nucleosomes by binding of LexA to multimerized sites can block the spread of Sir protein mediated heterochromatin propagation in S. cerevisiae (Bi,
Yu et al. 2004), perhaps by simply distancing the next nucleosome from the previous Sir2-3-4-nucleosome complex. Part of the function of the HMR-tRNA may involve creating such a nucleosomal gap, as fully assembled RNA polymerase III complexes footprint close to 150 base pairs (Chedin, Ferri et al. 1998; Kassavetis, Kumar et al. 1998), approximately the length of DNA occupied by a nucleosome. However, since the HMR-tRNA alone cannot provide full boundary function in a chromosomal context (our unpublished results), other chromatin-associated factors are clearly involved.

Additional studies will be required to identify the roles of bromodomains and other histone binding proteins like CTF-1 (Ferrari, Simmen et al. 2004) in establishing heterochromatin-euchromatin boundaries. Bdf1p has been shown to protect histone H4 from Sir2p deacetylation in vitro (Ladurner, Inouye et al. 2003), suggesting one possible mechanism of bromodomain action in restricting silencing. However, bromodomains are known to bind to acetylated proteins other than histones (Yang 2004; de la Cruz, Lois et al. 2005), therefore the effects on boundary establishment reported in this study may involve more than bromodomain-histone interactions. The results presented here suggest that the RSC complex may be acting through the HMR-tRNA, which is supported by the fact that RSC preferentially associates with tRNA genes in vivo (Ng, Robert et al. 2002). However, it is currently unknown whether RSC is targeted to assembled RNA polymerase III genes, or if prior RSC binding promotes RNA polymerase III complex assembly. Yta7p appears to be targeted to a limited set of chromosomal sites (Tackett, Dilworth et al. 2005), and while the mechanism of this targeting is unknown, it may involve the histone acetylation state near the boundary. The variability of bromodomain mutant phenotypes on boundary function may reflect a varied array of target proteins for bromodomain binding, or could be a function of varied effects on the different multi-subunit
complexes in which most bromodomain factors have been identified. It will be of interest to
determine the acetylation requirements for Yta7p binding at heterochromatin boundaries, and
how mutations that cause specific or global alterations in the acetylation states of histones, or
possibly other chromatin associated proteins, affect the targeting and function of boundary
associated bromodomain factors.

**TABLE 2.1**

Strains of *S. cerevisiae* generated for this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDY277</td>
<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1</td>
</tr>
<tr>
<td>DDY282</td>
<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMRΔI</td>
</tr>
<tr>
<td>DDY799</td>
<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1  Tn::LEU2:yta7</td>
</tr>
<tr>
<td>DDY687</td>
<td>MATα ADE2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1  sas2Δ::TRP1</td>
</tr>
<tr>
<td>DDY695</td>
<td>MATα ADE2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1  gen5::TRP1</td>
</tr>
<tr>
<td>DDY800</td>
<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1  Tn::LEU2:sas5</td>
</tr>
<tr>
<td>DDY802</td>
<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1  Tn::LEU2:sas5</td>
</tr>
<tr>
<td>DDY803</td>
<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1  Tn::LEU2:yta7</td>
</tr>
<tr>
<td>DDY811</td>
<td>MATα ade2 his3 leu2 LYS2 trp1 ura3 HMR-trnaΔ-ADE2</td>
</tr>
<tr>
<td>DDY814</td>
<td>MATα ade2 his3 leu2 LYS2 trp1 ura3 HMR-ADE2</td>
</tr>
<tr>
<td>DDY1174</td>
<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1  Tn::LEU2:yta7</td>
</tr>
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<td>DDY1309</td>
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<td>DDY1997</td>
<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1  rta7Δ::TRP1</td>
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<tr>
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<td>MATα ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1  Tn::LEU2:yta7  sir2Δ::TRP1</td>
</tr>
<tr>
<td>DDY2093</td>
<td>MATα ade2 his3 leu2 lys2Δ trp1 ura3 HMR-ADE2  Tn::LEU2:rpΔ3</td>
</tr>
<tr>
<td>DDY2106</td>
<td>MATα ade2 his3 leu2 lys2Δ trp1 ura3 HMR-ADE2  Tn::LEU2:sas5</td>
</tr>
<tr>
<td>DDY2114</td>
<td>MATα ade2 his3 leu2 LYS2 trp1 ura3  hmrΔ::ADE2</td>
</tr>
<tr>
<td>DDY2128</td>
<td>MATα ade2 his3 leu2 lys2Δ trp1 ura3  Tn::LEU2:rpΔ3  hmrΔ::ADE2</td>
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<tr>
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<td>DDY2205</td>
<td>MATα ade2 his3 leu2, lys2Δ trp1 ura3 HMR-ADE2  yta7Δ::TRP</td>
</tr>
<tr>
<td>DDY2450</td>
<td>MATα ade2 his3 leu2 LYS2 trp1 ura3  hmrΔ::ADE2  rsc2Δ::TRP1</td>
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</table>

(Table 2.1 continued)
DDY2489   MATα ade2 his3 leu2 LYS2 trp1 ura3 HMR trna Δ-ADE2 rsc2::TRP1  
DDY2496   MATα ade2 his3 leu2 lys2Δ trp1 ura3 HMR-ADE2 rsc2Δ::TRP1  
DDY2514   MATα ade2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 sp7Δ::URA3  
DDY2596   MATα ade2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1 bdf1Δ::HIS3  
DDY2601   MATα ade2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 bdf2Δ::LEU2  
DDY2659   MATα ade2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1 snf2Δ::LEU2  
DDY2716   MATα ADE2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1 htz1Δ::KanMX  

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CHAPTER THREE

ANALYSIS OF $RPD_3$ EFFECTS ON BOUNDARY FUNCTION
INTRODUCTION

The open and closed state of chromatin determines whether a gene is transcriptionally active or repressed. This open or closed state is brought about by enzymes that effect the post-translational modifications of the extended N-terminal tails of the core histones. Two classes of enzymes play an important role i) Histone acetyltransferases (HATs), that acetylate lysine residues in histones and promote transcriptional activation of underlying genes and ii) Histone deacetylases (HDACs) that remove the acetyl groups and contribute to transcriptional repression. Often, these activities bring about localized effects modifying one or a very small set of genes as they can be associated with a specific transcription factor (Goodsell 2003). Other modifications of histones include methylation, phosphorylation, and ubiquitylation. The histone code hypothesis suggests that the combinatorial distribution of these different modifications act as a direct read out for regulatory proteins and transcriptional machineries to bind and result in transcription or repression of a given gene or a group of genes.

Heterochromatic regions of the budding yeast, *S. cerevisiae* include the mating loci, the rDNA locus and the telomeres, and provide well studied examples of the effects of histone modifications on transcriptional regulation. Silenced chromatin in yeast is maintained by the Silent Information Regulator (SIR) proteins. Sir2p has histone deacetylase activity which when recruited causes the local deacetylation of histone tails of the neighboring nucleosomes, thus creating high affinity binding sites for Sir3p, and Sir4p which is bound to Sir2p. This allows the propagation of silencing along the entire locus in a sequence independent manner. Barrier elements are DNA sequences that prevent heterochromatin from spreading into the downstream euchromatic regions. It has been shown that silencing from *HMR* in yeast is restricted from spreading into the downstream region of Chromosome III by the *HMR* tRNA<sup>Thr</sup> gene that acts as
a boundary (Donze and Kamakaka 2001). In addition to genes involved in Pol III transcription, several additional genes have been shown to be involved in tRNA gene barrier function, such as \textit{YTA7}, \textit{SAS2}, \textit{SAS5} and \textit{GCN5}. These genes have been identified by a transposon mutagenesis strategy, and when mutated have been found to weaken the \textit{HMR} boundary (Jambunathan, Martinez et al. 2005). One other gene that was identified to play a role in barrier activity by mutational analysis was \textit{RPD3}. Rpd3p is a class I histone deacetylase and is known to associate with Sin3p corepressor. In both yeast and mammals, the Sin3p-Rpd3p complex mediates repression at specific promoters, as their recruitment brings about a localized heterochromatic region (Pile and Wassarman 2000). The enzymatic activity of \textit{RPD3} is important for transcriptional repression and, it deacetylates histone H3 and preferentially lysines 5 and 12 of histone H4 (Rundlett, Carmen et al. 1996; Kadosh and Struhl 1998; Deckert and Struhl 2002).

Even though the Sin3-Rpd3 complex causes transcriptional repression upon recruitment to a specific promoter, absence of Sin3 or Rpd3 proteins causes an increase in silencing at certain repressed loci (Sun and Hampsey 1999) and mutations in \textit{RPD3}, have been shown to increase silencing at all three heterochromatic loci in yeast (mating loci, rDNA, and telomeres) by an unknown mechanism (Rundlett, Carmen et al. 1996; Vannier, Balderes et al. 1996; Kim, Benguria et al. 1999; Smith, Caputo et al. 1999; Sun and Hampsey 1999). These observations are contrary to what is expected upon deleting a HDAC.

Despite spreading of silencing through the boundary in \textit{rpd3Δ} strains, our chromatin immunoprecipitation studies show the presence of bound Brf1p, a TFIIB transcription factor, at the tRNA gene suggesting a functional tRNA. Northern blot analysis confirms that the expression of the tRNA gene remains unaltered in \textit{rpd3Δ} strains. Our ChIP experiments further demonstrate that the binding of Sir3p to downstream \textit{ADE2} marker gene is increased in \textit{rpd3Δ}
strains relative to WT. These suggest that silencing somehow bypasses the active tRNA gene yet propagates into the downstream chromosomal region in *rpd3Δ* mutants. We speculate on how silencing can bypass an active gene and propagate down a chromosome.

**MATERIALS AND METHODS**

**Yeast Genomic DNA Extraction (Winston Prep)**

A 4 ml of yeast overnight culture was grown in YPD (Yeast extract Peptone Dextrose) media or YMD (minimal media if selecting for a plasmid) at 30°C. The overnight culture was spun at 2000rpm for 5 minutes. The supernatant was aspirated and the pellet was resuspended in 400 μl of Winston solution (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris HCl pH8.0 and 1mM EDTA). This was then added to a microfuge tube containing ~ 300 μl of glass beads and 300 μl of phenol: chloroform. It was vortexed for 5 minutes in a multivortexer at high speed, spun for 5' and the supernatant removed and added to another tube containing 300μl of phenol: chloroform. The tubes were vortexed and spun for another 5 minutes. The supernatant was removed and added to a tube containing 1 ml of absolute ethanol, mixed well and incubated at room temperature for 5 minutes. The tubes were spun for 10 minutes at room temperature and the ethanol was aspirated. The pellet was air dried and resuspended in 80μl of TE containing RNase A (60μg/ml).

**Yeast RNA Extraction**

A culture of 35 ml was grown to an optical density of 1.0. The cells were pelleted and washed with 1 ml of DEPC water and resuspended in 1 ml of extraction buffer (50 mM NaOAc, 10 mM EDTA, HOAC pH 5.0). 120μl of 10% SDS was added and vortexed. 1.2 ml of phenol equilibrated in NaOAc extraction buffer was prewarmed to 65°C and added to the cells and mixed well. They were then incubated in a water bath set at 65°C with constant shaking for one
hour and then cooled on ice for 5 minutes. The tubes were spun for 10 minutes at high speed and the top layer was removed and re-extracted with 1 ml of phenol. The tubes were spun again and the aqueous phase was extracted with 1 ml of phenol/chloroform/isoamyl alcohol equilibrated in ANE buffer (10 mM NaOAc, 100 mM NaCl, 1 mM EDTA pH 6.0). RNA was then precipitated with 1/10 volume of 3M NaOAc and 1.4 ml of absolute ethanol at -20°C for 30’ to overnight. The RNA was pelleted by centrifugation for 10’ at 4°C and washed with 300 µl of 70% ethanol, air dried and resuspended in 600 µl of DEPC water. The concentration was determined by measuring UV absorbance at 260 nm.

**Chromatin Immunoprecipitation Assays**

Chromatin Immunoprecipitation was done according to Kuo and Allis. Wild type and *rpd3Δ* cultures were grown in YPD to an optical density at 600 nm of 1.5. The cells were fixed with 3.25 ml of 37% formaldehyde and were incubated at room temperature for 20 min with gentle swirling. Cells were pelleted and washed once with 10 ml of 1X Phosphate buffered saline PBS (pH 7.3). They were resuspended in lysis buffer( 50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1% Triton X-100 and 0.1% Sodium deoxycholate) containing protease inhibitors, pepstatin A (1 µg/ml), leupeptin (1 µg/ml) and PMSF (100 mM) and cells were lysed by vortexing with glass beads for 40 minutes at 4°C. The lysate was piggy backed into another tube by poking the bottom of the tube containing glass beads with a hot 0.5 gauge needle, spun for a few seconds and collected in to a new tube.

To fragment chromatin, the lysate was sonicated six times for 10 secs each at 25% amplitude (Branson sonicator) by keeping the samples on ice intermittently. The samples were spun at 4°C to pellet insoluble material. The supernatant containing soluble chromatin was collected and used for further analysis. 100 µl of lysate was mixed with 300 µl of lysis buffer
containing the protease inhibitors. The lysate was precleared using protein A sepharose beads (Amersham Biosciences) that was washed thrice with lysis buffer containing protease inhibitors and resuspended in lysis buffer containing 200 μg/ml of salmon sperm DNA and 500 μg/ml BSA for 30 minutes at 4°C. The beads were spun out and 5 μl of monoclonal Anti- FLAG M2 Ab (SIGMA, ~1mg/ml) or Anti- MYC Ab (Upstate) was added to the supe. It was incubated overnight at 4°C with gentle rocking. The antibody reactions were spun and the supes were transferred to new tubes. Around 30 μl of equilibrated beads was added and the tubes were incubated at 4°C for 1 hour on the nutator to pull down the FLAG- tagged BRF1 or MYC tagged SIR3 respectively. The tubes were spun and the supes were transferred to new tubes as unbound fractions. The beads were washed for 5 minutes each with 1 ml of the following buffers i) Lysis buffer with protease inhibitors ii) Wash buffer I (Lysis buffer + 500mM NaCl) iii) Wash buffer II (10 mM Tris 8.0, 250 mM LiCl, 0.5% NP-40,0.5% Sodium Deoxycholate, 1 mM EDTA iv) TE (1X)

After TE wash, the tubes were spun again to remove any residual liquid. After this step a fast chromatin immunoprecipitation assay by Nelson et al was used (Nelson, Denisenko et al. 2006). 100μl of 10% chelex 100 (10g/100 ml H2O, Bio-Rad) was added to the washed protein A beads and vortexed. Samples were boiled for 10 minutes, cooled, and 1μl of Proteinase K (100μg/ml) was added and the beads were incubated for 30 minutes at 55°C while shaking, and boiled for another 10 minutes. The suspension was centrifuged and the supernatant was collected. The chelex/protein A beads fraction was vortexed with another 100 μl H2O, centrifuged again, and the supernatant was combined with the previous supernatant.

The eluate was used directly in the PCR reaction. For input controls 10 μl of the whole cell extract was mixed with 475μl of Elution buffer (1% SDS, 0.1M NaHCO3), and 20 μl of 5M
NaCl. The tubes were incubated at 65°C overnight to reverse the crosslinks. To each tube 20 μl of 1M Tris pH8.0, 10 μl of 0.5M EDTA and 1 μl of Roche Proteinase K (20 mg/ml) was added and incubated at 55°C for 30 minutes. The sample was extracted once with phenol/chloroform, and to the top layer 50 μl of NaOAc, 1μl of glycogen (Roche, 20 mg/ml stock), and 1 ml of absolute ethanol was added and precipitated at -20°C. The samples were spun at 4°C for 10 minutes and the pellets washed with 300 μl of 70% ethanol, air dried and redissolved in 200 μl TE + RNase A (60 μg/ml).

**Colony Color Assay**

The strains used in this assay contained the *ADE2* gene integrated downstream of the *HMR* domain containing the modified tRNA+19. Both WT and *rpd3Δ* strains were grown in YPD to an optical density of 1.0 at 600 nm. This corresponds to approximately 10⁷ cells/ml. Cells were diluted in YPD medium, and 50, 100 and 150 cells were plated on to YMD plates that contained 30% of normal levels of adenine (final concentration 9 μg/ml). The plates were incubated at 30°C for three days and were shifted to 4°C for a week before photographing on microscope equipped with a digital imaging system.

**Strains and Plasmids**

To construct the *HMR-tRNA+19–ADE2* strains, plasmid pDD 571 containing the modified tRNA+19 gene was cut with *Sac II/ Bgl II*, and the tRNA gene sequence was gel purified. Plasmid pDD 1065, having the *HMR-tRNAΔ-ADE2* and marked with *URA3* was cut with *Sph I*. To clone the tRNA+19 into *HMR* tRNAΔ- *ADE2* by gap repair, DDY 150 was co-transformed with the *Sph I* digested pDD 1065 and the gel purified tRNA+19, and colonies that were able to grow on both uracil and adenine dropout media were selected. Isolates were screened by PCR to check for the presence of the tRNA+19, and were verified by DNA
sequenceing. One plasmid isolate was linearized with Sac I+Sal I, transformed into DDY 631 and Ade+ recombinants were selected.

To create $rpd3\Delta::URA3$ strains, the $URA3$ gene was PCR amplified with oligonucleotides DDO 590/591, which contain 20 bases of homology to $URA3$, and 50 bases to $RPD3$, and the product was transformed into DDY 1391, and Ura+ colonies selected. DDY 1391 contains $BRF1$ tagged with FLAG epitope. This created a strain that is both $rpd3\Delta$ and has its $BRF1$ tagged. The strains obtained were designated DDY 3589 and DDY 3590. Strains 3579 was crossed to 3590 to isolate strains DDY 3681 and 3683 that have the modified tRNA with the $ADE2$ downstream of it and are $rpd3\Delta$ and also have their $BRF1$ tagged with the FLAG epitope. DDY 1990 was crossed to DDY 3686 to get DDY 3833 which has its $BRF1$ tagged with FLAG and $SIR3$ with MYC epitopes. It is also $rpd3\Delta::URA3$ and contains the modified tRNA gene. DDY 3835 has both $BRF1$: FLAG and $SIR3$: MYC tags. It has the modified tRNA gene but is wild type for $RPD3$.

**Northern Blot Analysis**

Yeast cultures were grown on YPD and total RNA was extracted as described above. 3.5μg of total RNA was resolved on a 1% agarose gel and electroblotted to zeta–probe (Bio-Rad) membrane. The probes were end labeled with $^{32}$P using polynucleotide kinase, and hybridization was carried out overnight at 50°C. The blots were probed with either the 19bp extension specific probe 5’- TCCGCAAGATTACTGCGGCTGCTTC or the tRNA$^{Thr}$ probe.

**Yeast High Efficiency Transformation**

Yeast cultures were grown in YPD at 30C with constant shaking to an optical density at 600nm of 0.7, and the pelleted cells were resuspended in 1X TEL (1ml of 1X TEL per 10 ml culture) and were left to rock overnight at room temperature. The next day cells were pelleted,
resuspended in 100μl of 1X TEL per 10ml culture incubated at room temperature for 30minutes. 100μl of competent cells, 5μl of Salmon sperm DNA and 1μg of the plasmid DNA was mixed in an eppendorf tube and incubated again for 30minutes. 700μl of 40% PEG/TEL was added to each tube and incubated at room temperature for 60minutes without shaking. 88μl of DMSO was added to each tube, mixed and the cells were subjected to heat shock at 42C for 10minutes. The cells were spun gently at 8000rpm for 30seconds, pellets washed with 300μl of water and resuspended in 400μl of water. 200μl was plated on to each YM selection plates.

**Stocks**

10X TEL: 1M LiAc, 100mM Tris HCl pH 7.5, 10mM EDTA; PEG/TEL: 10ml of 10X TEL, 40g PEG (sigma P-3640); SSDNA: 10mg/ml water sonicate 1min (three times), boil 10 min and use.

**TABLE 3.1**

Oligos used in the study

| DDO 1031 | 5'- AGT TTT TAG TAC GAT TGC ATT GTC-3’  | *HML* UPSTREAM OF I SILENCER |
| DDO 1032 | 5'- CCA GCT TTT ATG TCA TAG TAT TTT C -3’ | *HML* DOWNSTREAM OF I SILENCER |
| DDO 1027 | 5'- CAT AAC ACT GAC ATC TTT AAC AAC-3’ | *ADE2* CHIP WITH 1028 |
| DDO 1028 | 5'- CTA ATA TAC CAA CTG TTC TAG AAT-3’ | *ADE2* CHIP WITH 1027 |
| DDO 767 | 5'- TCC GCA AGA TTA CTG CGG CTG CTT-3’ | PLUS 19 SPECIFIC PROBE |
| DDO 597 | 5'- ACA AAA GAG GAT ACT AAG GTT-3’ | *RPD3 KANMX* CHECK UPSTREAM |
| DDO 593 | 5'- AAG TCA TTT ACC CAG GGG TG-3’ | *RPD3* DELETE CHECK DOWNSTREAM |
| DDO 591 | 5'- GAG TGG GAA GTA TAT ACG ATA ATA GTG AAA CAA AAG AAG AAA AGT GCT CCT TAC GCA TCT GTG CGG-3’ | *RPD3* DELETE PRS UNIVERSAL BOTTOM |
| DDO 590 | 5'- CAA TTG CGC CAT ACA AAA CAT TCG TGG CTA CAA CTC GAT ATC CGT GCA GGC AGA TTG TAC TGA GAG TGC-3’ | *RPD3* DELETE PRS UNIVERSAL TOP |
| DDO 198 | 5'- GCA CTC TCA GTA CAA TCT GC-3’ | PRS UNIVERSAL RC, UPSTREAM |
| DDO 199 | 5'- CAG CAT TCA GTA AGG AGG AGG GC-3’ | PRS UNIVERSAL RC, DOWNSTREAM |
| DDO 484 | 5'- CCA ATT CCG CAT CGT CAG ATT ACT T-3’ |
| DDO 485 | 5'- TTC ATT ATT TTT CAG ATG ACG-3’ |
| DDO 59 | 5’ GAA TTC GGG CAT GGA TCC CAT ACT CGA AGG GTA GTT GG-3’ |
| DDO 60 | 5’ GAA TTC GTT AAC GGA TCC GAT TTT TCC ATT CGC CAT GC-3’ |
RESULTS

When the RPD3 histone deacetylase was deleted, we observed an increase in spreading of silencing from the HMR to the ADE2 gene downstream of the boundary tRNA. In order, to further study the mechanism of this observation, strains were constructed that have the ADE2 gene inserted downstream of the HMR tRNA gene in chromosome III. The HMR tRNA gene in these strains was also modified to carry an additional 19 bp at 3’ end of the tRNA to distinguish this particular tRNA\textsubscript{Thr} transcript from those of the seven other copies of the same tRNA gene in the S.cerevisiae genome (Cherry, Ball et al. 1997; Mewes, Frishman et al. 2000; Donze and Kamakaka 2001). This unique 19bp sequence was used to design a specific probe for this particular tRNA. The modified strain described above (Figure 3.1) was used throughout our study. The boundary tRNA gene normally prevents silencing from spreading to the downstream ADE2 gene. Earlier studies in our laboratory (Jambunathan, Martinez et al. 2005), have shown that mutations in RPD3 compromise boundary function by allowing the silencing to spread to the downstream ADE2 gene, giving rise to red colonies.

Figure 3.1. Schematic representation of designer strain constructs. Yeast strains were created that contain the modified HMR locus having the extended tRNA gene, and ADE2 located downstream of HMR.

Construction of Designer Strains to Assay Spreading of Silencing, Brf1p Occupancy, and HMR tRNA Gene Expression Simultaneously

A colony color assay was used to assess the extent of the spread of silencing in rpd3\Delta strains. The ADE2 reporter strain was crossed to strains that were either rpd3\Delta or rpd3\Delta sir4\Delta. Mutation of ADE2 or heterochromatic repression of ADE2 transcription leads to the
accumulation of a red pigment in yeast cells due to a block in the adenine biosynthetic pathway, resulting in a red colony color phenotype on medium containing limiting adenine. Expression of sufficient Ade2p gives rise to white colonies on medium containing limiting amounts of adenine. Mutation of RPD3 caused HMR silencing to spread to the ADE2 gene resulting in red colonies (Figure 3.2, panel A). Mutation of SIR4 in the reporter strain with a wild type RPD3 resulted in white colonies (Figure 3.2, panel B), and white colonies were observed for the rpd3Δsir4Δ double mutant strains (Figure 3.2, panel C). This demonstrates that deletion of RPD3 results in Sir protein mediated silencing to spread from HMR to the ADE2 gene downstream of the boundary tRNA gene.

Figure 3.2. Silencing spreads to the gene downstream of HMR tRNA boundary in rpd3Δ strains. The ADE2 gene was inserted into chromosome III downstream of HMR tRNA. Both rpd3Δ and rpd3 Δ sir4 Δ strains were constructed. In rpd3Δ strains silencing at HMR spread to the downstream ADE2 gene. Red colonies were obtained in strains where only the RPD3 was deleted (PANEL A), while white colonies were obtained when SIR4 was deleted (PANEL B) or both RPD3 and SIR4 were deleted (PANEL C).
In Spite of Silencing of the Downstream ADE2 Gene in rpd3Δ Strains, the Pol III Transcription Factor Brf1p Is Still Bound to the HMR-tRNA Gene

The RNA Pol III transcription apparatus is interesting in that it binds tightly and stably at the Pol III gene and directs multiple rounds of transcription. Pol III transcribed tRNAs are very abundant, accounting for 15% of total RNA in yeast (Warner 1999). The high levels of tRNA transcripts observed suggest that tRNA genes and other Pol III transcribed genes are likely to be constantly occupied by transcription complexes. This hyperprocessivity together with the magnitude of the complex pose a tightly bound physical barrier for the Sir proteins to spread to the downstream gene (Chedin, Ferri et al. 1998; Donze and Kamakaka 2001). Since deletion of RPD3 led to the spread of silencing, our next question was about the functional state of the boundary tRNA gene. We anticipated that the tRNA gene would also be silenced in rpd3Δ strains, so we asked whether the tRNA gene is bound by components of the Pol III transcription complex. To address this question Chromatin Immunoprecipitation (ChIP) was performed. An HMR-tRNA+19-ADE2 strain that has BRF1 tagged with a FLAG epitope was crossed to an rpd3Δ strain. Brf1p is a component of TFIIIB transcription factor complex. Anti FLAG antibody was used to immunoprecipitate formaldehyde-cross linked sonicated chromatin from wild-type and rpd3Δ strains. ChIP was performed as described in Materials and Methods. The immunoprecipitated DNA was analyzed by PCR. Due to the fact that silencing appears to spread completely through the boundary in rpd3Δ strains, we expected to see absence or a reduction in the amount of immunoprecipitated DNA at the HMR tRNA gene. Surprisingly, the binding of TFIIIB was completely unaffected in rpd3Δ strains.

Deletion of RPD3 Does Not Result in Inhibition of Transcription of the HMR-tRNA Gene

After observing that Brf1p occupancy at the HMR tRNA was not affected in rpd3Δ strains, we next wanted to determine whether the tRNA gene was indeed transcriptionally active
in these strains. Northern blot analysis was performed to analyze the tRNA gene expression. As described earlier the 19bp addition before the 3’ termination site of the tRNA served as a distinguishing mark between this particular tRNA\textsuperscript{Thr} gene and seven other copies of the same gene in the \textit{S.cerevisiae} genome.

![Diagram of HMR locus and tRNA gene expression analysis](image)

**Figure 3.3. Pol III transcription factor Brf1p remains bound at the HMR tRNA gene in rpd3Δ strains.** A) Schematic representation of the HMR locus with the E and I silencers, the HMR tRNA gene and the \textit{ADE2} gene downstream of it. The location of regions spaned by the detection PCR promers are regions marked A, B, C and D. Primer A corresponds to a tRNA gene used as control present upstream on chromosome III. Primer B spans \textit{HMR} near the I silencer, primer C at the boundary tRNA and primer D downstream of the boundary tRNA, near the promoter region of \textit{ADE2}. B) Chromatin Immunoprecipitation was carried out in both \textit{WT} and \textit{rpd3Δ} strains in which \textit{Brf1p}, a component of transcription factor TFIIIB, was tagged with a \textit{FLAG} epitope. An \textit{rpd3Δ} strain which did not have its \textit{BRF1} tagged with the \textit{FLAG} epitope was used as negative control. The samples were analyzed by PCR with primers depicted above. Identical signals were obtained at the tRNA in both wild type and \textit{rpd3Δ} strains, suggesting the presence of bound \textit{Brf1p} and thus the presence of bound transcription complex. The right side of the panel 3.3 B corresponds to the Input controls for the respective strains.
Total RNA from WT, RPD3, sir4Δ and rpd3Δsir4Δ strains was isolated for northern analysis, electroblotted and probed with the 19bp specific probe, then the blot was stripped and reprobed with an oligonucleotide complementary to total tRNA^Thr as a loading control. Despite the spreading of silencing into ADE2, the expression of the boundary tRNA gene again appeared to be unaffected by RPD3 deletion. This result demonstrates that silencing spreads along chromosome III downstream of the HMR tRNA gene in rpd3Δ strains, without affecting the expression of the tRNA.

Figure 3.4. The expression of the boundary tRNA gene persists in rpd3Δ strains. DDY 3580 was crossed to rpd3Δ, sir4Δ and rpd3Δsir4Δ strains. Northern blots were probed with a probe specific for the 19 base pair extension added to the HMR tRNA gene. The tRNA gene is expressed in rpd3Δ (lanes 3, 4 & 5), rpd3Δsir4Δ (lanes 6&7) and sir4Δ strains (lanes 8&9). The blots were re-probed with an oligonucleotide complementary to the transcribed sequence of tRNA^Thr. A WT strain with an unmarked HMR tRNA gene served as a negative control (lane 1).
**Sir3p Spreading Downstream of HMR Is Increased in *rpd3Δ* Strains**

Since the silencing was observed to spread to the downstream *ADE2* gene as indicated by colony color, in spite of the tRNA gene still being expressed, our next step was to confirm that Sir proteins were actually spreading along the chromosome in *rpd3Δ* strains compared to the wild-type strains. As a marker for Sir protein binding we used a strain containing epitope tagged *SIR3*. We compared the extent of Sir3p spreading in *rpd3Δ* strains versus *WT* strains by chromatin immunoprecipitation. The *HMR*-tRNA+19-*ADE2* strain with its *BRF1*, tagged with a *FLAG* epitope was crossed to strains that had their *SIR3* tagged with *MYC* epitope. The *BRF1* occupancy at the tRNA gene was analyzed simultaneously with the spreading of the Sir3 protein across the *HMR*, tRNA gene and the downstream *ADE2* gene. Our results demonstrate that spreading of Sir3p across the *HMR* and to the *ADE2* gene was increased in the *rpd3Δ* strains relative to *WT* strains. Brf1p remained bound at the tRNA gene as in Figure 3.3. This demonstrates the apparent bypassing of the Pol III complex by the silencer proteins.

**Figure 3.5. Sir3p spreading is increased in *rpd3* mutants relative to wild type yeast.** The top panel shows the location of primer A (*HMR* region), primer B (boundary tRNA), and primer C (promoter region of *ADE2*). PCR analysis of the immunoprecipitated *FLAG* tagged Brf1p indicated that Brf1p remained bound at the tRNA gene in both *rpd3Δ* and WT strains (bottom panel row labeled FLAG). PCR analysis of immunoprecipitated *MYC* tagged *SIR3* showed increased amount of immunoprecipitated DNA downstream of HMR in *rpd3Δ* compared to the WT (bottom panel row labeled MYC, lanes B & C). Row labeled IC represents input controls.
Figure 3.6. Quantitative analysis of Sir3p spreading in rpd3Δ and WT strains

Autoradiogram to quantitate the increased spreading of Sir3p to the downstream ADE2 gene in rpd3Δ and WT strains. Lanes 1&2 correspond to rpd3Δ mutants and 3&4 are WT strains. Lanes 1&3 received primer sets A&B; while lanes 2&4 received primer sets A&C. As seen in the figure the intensity of the lower bands, which represents Sir3 spreading in both WT and rpd3Δ mutants is almost the same at the tRNA region (lanes 1&3), while the intensity of the lower band is higher in rpd3Δ mutants (lane 2) than the WT strain (lane 4) at the promoter region of the ADE2 gene suggesting an increased spreading of Sir3 in rpd3Δ mutants. Quantitation of the radiogram using imagequant software, shown by the graph on the right suggests that the amount of Sir3p spreading at the tRNA region was 1.46 times more in the rpd3Δ strain than in the WT strain, while the amount of Sir3p spreading was 1.09 times more at the ADE2 promoter region in rpd3Δ strain than in the WT strain, thus suggesting an overall increase in the spreading of Sir3p in rpd3Δ mutants than the WT.

In order to confirm the qualitative observation of Sir3 spreading in rpd3Δ strains compared to the WT, a quantitative PCR analysis was performed. Each reaction mix received two sets of primers. The primer set that corresponds to a region in HML (primer A) was common to all reactions. Two bands were expected for all the samples, a larger molecular weight band corresponding to the HML region near the I silencer (~600bp), and a lower sized band (~300bp) was obtained depending upon the primer set the samples receive; either B or C. Primer set B corresponds to the tRNA region, while primer set C corresponds to the promoter region of ADE2.
gene. As observed in our ChIP PCR reactions in Figure 3.5, we expected a more intense lower band for the \textit{rpd3}\textDelta{} mutants at the \textit{ADE2} region, if there was an increased spreading of Sir3p compared to the \textit{WT} (primer C). Lower bands of similar intensity were expected at the tRNA region (primer B) for both the \textit{WT} and \textit{rpd3}\textDelta{} strains. The \textit{rpd3}\textDelta{} strains exhibited increased enrichment of \textit{ADE2} promoter DNA sequences when compared to wild type (Figure 3.6, lane 2). Therefore the Sir proteins actually spread to the downstream \textit{ADE2} gene in \textit{rpd3}\textDelta{} mutants even in the presence of a functional tRNA gene, while this spreading is restricted in \textit{WT} strains.

**TABLE 3.2**

Strains of \textit{S. cerevisiae} generated for this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
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<tr>
<td>DDY 3686</td>
<td>\textit{MAT@ ade2 his3 leu2 lys2 trp1 ura3 HMR-tRNA+19- ADE2 BRF1:3X FLAG: KanMX rpd3\textDelta{}::URA3}</td>
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<td>DDY 3688</td>
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<tr>
<td>DDY 3682</td>
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<tr>
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<tr>
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<td>DDY 3712</td>
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</tr>
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</tr>
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<td>DDY 3835</td>
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</tr>
<tr>
<td>DDY 3683</td>
<td>\textit{MATa ade2 his3 leu2 lys2 trp1 ura3 sir2\textDelta{}:: LEU2 HMR-tRNA+19- ADE2}</td>
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</table>
LITERATURE CITED


CHAPTER FOUR

DISCUSSION
The eukaryotic genome is organized into regions of open and closed chromatin states. Euchromatin refers to the open form of chromatin while the heterochromatin is a more condensed state of chromatin. These different states of chromatin are kept separated by boundary elements (Valenzuela and Kamakaka 2006). Promoters, enhancers and boundary elements, and the proteins that bind to them ensure that only specific genes are expressed at certain times. Boundary elements are of two types, insulators and barriers (Gerasimova and Corces 2001; West, Gaszner et al. 2002; Capelson and Corces 2004). Promoters can be activated by enhancer elements that reside several kilobases away from the gene. The inappropriate activation of a gene by enhancers is prevented by insulators, which when placed between an enhancer and a promoter prevents the activation of the promoter by the enhancer. Silencer proteins depend on certain DNA sequences for their nucleation whereas their spreading is sequence-independent and the spreading of the silencer proteins along a chromosome is prevented by barrier elements. Therefore, insulator and barrier elements are DNA sequences that regulate the expression of genes and help in the organization of the genome. At the heterochromatic HMR locus in yeast, a tRNA gene and its immediate flanking regions are known to act as a boundary in preventing the spread of silencing from the HMR to the downstream genes. Experiments by Donze and Kamakaka (2001) suggests that not all tRNA genes have the ability to act as boundary elements and that the immediate flanking regions of the HMR-boundary tRNA gene are required for full barrier activity (Donze and Kamakaka 2001). NLI, a different tRNA^{Thr} gene, in spite of having identical coding sequence and internal control elements as the HMR tRNA gene, was shown to exhibit a weak barrier activity as its flanking regions are different from the HMR tRNA gene. Though the HMR-tRNA alone was able to act as an efficient boundary in a plasmid based assay (Donze and Kamakaka 2001), when integrated back into the chromosome at its endogenous
location, the tRNA alone showed only partial barrier function, suggesting that additional proteins that
interact with the more distal flanking regions might be involved in completely blocking silencing.

Our experiments in chapter 2 suggest possible reasons why these flanking regions might be important. The flanking region of the HMR-tRNA gene may function to recruit various proteins and chromatin remodeling factors, which enhance or stabilize the barrier activity of the tRNA gene. YTA7, a gene that encodes a protein containing a bromodomain and two separate AAA family ATPase domains was identified by mutational analysis as an accessory factor in boundary function (Figure 2.1).

Another study by Tackett et al. also identified YTA7 as a barrier component using a proteomic approach (Tackett, Dilworth et al. 2005). Their findings demonstrate that YTA7 is associated with Dpb4p, a component of the DNA polymerase ε complex. They also showed a direct association of the Dpb4p-Yta7p complexes at the HMR, HML, chromosome ends, and at conditionally expressed FLO genes. They further found a preferential enrichment of this complex at the HMR boundary tRNA and demonstrated that deletion of YTA7 leads to increased spread of silencing. Our epistatic analysis also suggests that YTA7 acts independently of the tRNA gene in restricting the silencing from spreading to the downstream genes (Figure 2.4).

Alignment of the YTA7 bromodomain with other yeast bromodomains revealed the absence of a highly conserved tyrosine residue in YTA7; this tyrosine has been demonstrated to be required for acetyllysine binding in other bromodomains. However, Yta7p has a serine and threonine at the same location. Barrier assay experiments done in our lab (unpublished data) suggest that point mutation of serine and or the threonine in the bromodomain of YTA7 to alanine had minimal effect on the boundary function while complete deletion of the
bromodomain led to a loss of boundary function. This suggests that Yta7p binds to histones differently than other characterized bromodomains.

However, when a highly conserved lysine residue in the AAA Walker A motif of the ATPase domain was mutated to alanine or arginine, we observed an increased spread of silencing suggesting the weakening of the boundary (Figure 4.1). LEX-1, a bromodomain and ATPase domain containing gene found in C. elegans, shares sequence similarity to yeast YTA7 and has been shown to modulate chromatin to enhance transcription from repetitive DNA sequences (Tseng, Armstrong et al. 2007). This might suggest that AAA ATPase domain of YTA7 may be involved in chromatin remodeling which in turn is important for maintaining the boundary function at the tRNA gene.

![Site-directed mutagenesis of YTA7 bromodomain and AAA ATPase domains](image)

**Figure 4.1. Site-directed mutagenesis of YTA7 bromodomain and AAA ATPase domains.** Site-directed mutagenesis was performed to mutate the bromodomain of YTA7 at the serine and/or threonine site to alanine. A conserved lysine residue within the ATPase walker A motif was mutated to alanine or arginine. These mutations were integrated back into MATα strain where the 1Kb region downstream of HMR containing the boundary tRNA gene is cloned into the a2 gene. Single amino acid mutations of the bromodomain had a minimal effect on the boundary function as the cells remained essentially non-mating, while complete deletion of the bromodomain led to loss of YTA7 function. Mutation of the ATPase domain also led to a complete loss of boundary activity, as cells exhibited a mating phenotype.

Other bromodomain containing genes that weakened the boundary when mutated include BDF1, RSC2, GCN5, RSC1 and BDF2. Ladurner et al (2003) have shown that the bromodomain
of BDF1 binds acetylated histones H3 and H4 and functions in restricting the spread of SIR proteins, thus contributing to barrier function to demarcate the euchromatin–heterochromatin junction. In order to further understand the importance of bromodomain containing genes in maintaining the boundary at HMR, mating assay experiments were performed that involved deletion of BDF1, YTA7, and RSC2. Deletion of these genes resulted in the weakening of the boundary tRNA and thus led to the spread of silencing while mutation of GCN5, RSC1 and BDF2 had minimal effect on the boundary (Figure 2.3).

Epistatic analysis suggests that RSC might act in the same pathway as the boundary tRNA gene in restricting the spread of silencing, as rsc2ΔtrnaΔ double mutants shows similar phenotype as each single mutant (Figure 2.4). Genome-wide location studies of the RSC complex by Huck Hui Ng et al (2002) demonstrate that it is preferentially targeted to tRNA gene promoters, and RSC might preferentially associate with a majority of Pol III promoters (Ng, Robert et al. 2002). They further suggest that RSC might be recruited to tRNA genes by the components of the Pol III transcription machinery and thus RSC might associate with the Pol III promoters in which the Pol III machinery is stably bound. RSC mutants have been shown to exhibit attenuation of Pol III transcription (Soutourina, Bordas-Le Floch et al. 2006). Studies by Roberts et al (2006) show that at least part of the Pol III machinery resides at tRNA genes even during conditions of repressed transcription, and studies by Parnell et al (2008) further show that RSC remains bound at PolIII loci during repressive conditions (Roberts, Wilson et al. 2006; Parnell, Huff et al. 2008). This binding of both RSC and the Pol III machinery at the tRNA genes helps in maintaining these genes free from nucleosomes during both favorable and repressive conditions and their persistence helps in the rapid reactivation of these genes when the conditions improve to normal. Consistent with this hypothesis, loss of RSC has been shown to increase the
nucleosome density at these tRNA genes (Parnell, Huff et al. 2008). These observations lead to a hypothesis that both RSC and tRNA genes may cooperate to create a nucleosome free region to restrict silencing.

Ladurner et al. (2003), have suggested that Bdf1 acts as a barrier to the spread of heterochromatin at telomeres and a mating locus in yeast by competing with Sir2 for binding to acetylated histone H4 tails. They mixed purified GST-Bdf1p bromodomain protein with purified histones and Sir2p in vitro, and showed that the bromodomain protein protected histone H4 from deacetylation. They also observed spreading of Sir3 to euchromatic regions when the acetyl-lysine binding ability of Bdf1 was lost due to the mutation of the conserved tyrosine to phenylalanine. This suggests that an important function of bromodomains may be to protect acetylated histones from HDACs.

Braglia et al. (2006), have demonstrated that HMG box proteins Nhp6a and b have a role in tRNA gene transcription and are required by the HMR-tRNA gene in maintaining the heterochromatin barrier function (Braglia, Dugas et al. 2007). They suggest that the binding of the TFIIIB complex may be enhanced by bending of upstream DNA by Nhp6 proteins. Studies conducted by us and others demonstrate the barrier function at HMR is very complex, as many factors are involved in maintaining this chromatin boundary. YTA7, BDF1, and other bromodomain containing genes studied so far might account for maintaining global histone modifications such as increased acetylation levels at the boundary region. Additional factors like the presence of bound RSC at the tRNA region, and the requirement of a non-histone HMG protein Nhp6 for tRNA gene transcription and boundary function adds to this complexity. The large size of the Pol III initiation complex along with the above mentioned proteins might pose a physical barrier to the SIR proteins or the increased acetylation levels at the boundary might
counteract the deacetylation by Sir2p and thus prevent the spreading of other Sir proteins. The regular interval or the density of the nucleosomes may be disrupted at the boundary region thus creating a gap between the histone tails available for deacetylation by Sir2p. Based upon the results obtained by us and others we can say that none of the above processes are mutually exclusive from each other.

Several studies, including our results in Chapter 3 show that deletion of $RPD3$, a class I histone deacetylase, causes an increase in the spread of silencing, which is contrary of what is expected. The Sin3p-Rpd3p complex, upon recruitment to a promoter, cause deacetylation of the histones over the localized region and lead to repression of the genes. Therefore $RPD3$ is generally associated with transcriptional repression (Kadosh and Struhl 1998). Mutations in $RPD3$ have been shown to increase silencing at all three heterochromatic loci in $S.\textit{cerevisiae}$ (telomeres, mating loci and rDNA) by an unknown mechanism (Rundlett, Carmen et al. 1996; Vannier, Balderes et al. 1996; Kim, Benguria et al. 1999; Smith, Caputo et al. 1999; Sun and Hampsey 1999). Our results demonstrate that silencing in $rpd3\Delta$ mutants spreads downstream of the $HMR$-boundary tRNA, yet the tRNA gene remains active. Our ChIP analysis confirms the presence of bound Brf1p, a TFIIIB component at this tRNA gene, both in the wild type and $rpd3\Delta$ mutants (Figure 3.3). Our northern blot analysis further demonstrates that the expression of the boundary tRNA gene remains unaltered both in the wild type and $RPD3$ mutants. These experiments suggest that heterochromatic silencing can apparently bypass the active regions (in this case the tRNA) along a chromosome to silence the downstream genes. The spreading of Sir3p is seen to be increased in $rpd3\Delta$ mutants than the WT (Figure 3.5). Hence we propose that heterochromatic spreading can bypass the boundary to silence the downstream gene. We hypothesize two possibilities 1) The silencing in $rpd3\Delta$ strains is interrupted at the tRNA gene
but somehow continues to spread to the downstream gene; 2), the tRNA gene might remain active by a looping mechanism, allowing the heterochromatic \textit{HMR} region to come in direct contact with downstream sequences.

\textbf{Figure 4.2. Heterochromatic gene silencing can bypass boundary elements.} (Top), heterochromatic silencing (red nucleosomes) from \textit{HMR} is prevented from spreading into the euchromatic region (green nucleosomes) by the tDNA that acts as a component of a complex barrier on chromosome III in \textit{S.cerevisiae}. (Bottom) In \textit{rpd3} mutants, this heterochromatic silencing spreads to the region downstream of the tRNA gene, inspite of this tRNA remaining transcriptionally active, suggesting a possible looping of the tRNA gene.

RNA Pol III genes have been implicated in wide variety of functions within a chromosome. As discussed earlier, the \textit{HMR} tRNA gene functions as a barrier to silencing in \textit{S.cerevisiae} on chromosome III (Donze and Kamakaka 2001). Studies in Donze lab conducted by Simms et al. indicate that \textit{TRT2}, a tRNA\textsuperscript{Thr} gene exhibits position effect. This tRNA protects the upstream \textit{CBT1} gene from the repressive effect of the \textit{α2} operator in \textit{MATα} cells, while reducing the expression of the same \textit{CBT1} gene in \textit{MATa} cells, thus exhibiting opposite position effects in different cell types (Simms, Miller et al. 2004). Other extra- transcriptional activities of tRNA genes include replication fork pausing, Ty element integration and overriding nucleosome
position sequences. Studies of the *Schizosaccharomyces pombe* mating locus by Noma et al. (2006) have shown that TFIIIC binds to B-box sequences of the boundary elements at the ends of this heterochromatic region (Noma, Cam et al. 2006). They further demonstrate the presence of DNA bound TFIIIC without TFIIIB or PolIII scattered across the entire *pombe* genome, which they called the COC (chromosome–organizing clamp) loci. They also demonstrate clustering of TFIIIC at the nuclear periphery into 5-10 bodies and further suggest that the box B sequences to which TFIIIC binds help in the attachment of these sites to the nuclear periphery which might lead to the formation of “higher-order” chromatin loop structures.

The ETC loci (Extra TFIIIC loci) in *S.cerevisiae* are also known to be present across the budding yeast genome and also show the association of TFIIIC without Pol III binding. This suggests the possibility that they might be involved in the formation of higher-order loop structures as well. These findings support our hypothesis of the tRNA gene remaining active by looping out.

It is unknown why silencing spreads through the boundary in the rpd3Δ mutants. A possible explanation of why this happens in rpd3Δ mutants and not in WT strains might be due to changes in global chromatin conformation. Studies conducted in our lab (Richard Kleinschimdt, unpublished data) suggest that histone methylation may be altered in rpd3Δ mutants, which may have global effects.

One subject for the future study would be to test the hypothesis regarding the possible looping of tRNA gene in rpd3Δ strains. This can be done by a chromatin conformation capture (3C) technique, where two regions in a chromosome that are separated but are looped into a close proximity are physically cross-linked by formaldehyde treatment. If the HMR region and the region downstream of the tRNA gene are close to each other due to the looping of the tRNA
gene in rpd3 mutants, it can be verified by the 3C method, which could provide an explanation for the spreading of silencing from the HMR downstream of the tRNA gene in rpd3 mutants.

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