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Regulation of gene expression by chromatin boundary elements

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REGULATION OF GENE EXPRESSION BY CHROMATIN BOUNDARY ELEMENTS

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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ABSTRACT

Boundary elements consisting of barriers and insulators are genomic sequence elements that along with their associated DNA-binding proteins block the spread of heterochromatin into euchromatic regions or prevent the targeted activation of promoters from distal/proximal enhancers, respectively. In *Saccharomyces cerevisiae*, the deletion of RPD3, a histone deacetylase, results in an extended SIR protein-mediated silencing effect bypassing a tRNA<sup>thr</sup> barrier element adjacent to the cryptic mating locus, *HMRa*. We mutagenized *rpd3Δ* strains and identified suppressor mutants through a genetic screen that no longer displayed this enhanced silencing effect. Our results identified *BRE1* and *BRE2*, which are either directly or indirectly responsible for the tri-methylation of histone H3K4 and H3K79, as effectors of the *rpd3Δ* extended silencing effect at *HMRa*. We hypothesize that the increased silencing effect in *rpd3Δ* mutants is the result of a redistribution of SIR proteins which become concentrated at the *HMRa* region in response to a global change in the acetylation and/or methylation state of histones contingent on *RPD3*, *BRE1*, and *BRE2*. ETC, or Extra-TF<sub>III</sub>C, sites are genomic elements which bind the RNA Polymerase III transcription factor, TF<sub>III</sub>C. ETC sites contain B-box promoter sequences normally associated with RNA Polymerase III promoters, and their locations are over-represented between divergently transcribed RNA Polymerase II genes. Our results show that the transcription of *TFC6*, which codes for a DNA-binding component of TF<sub>III</sub>C, is auto-regulated by TF<sub>III</sub>C which binds to the *ETC6* site in the *TFC6* promoter region. Inhibition of TF<sub>III</sub>C binding to the *ETC6* site results in increased *TFC6* expression from its own promoter, and transcription of *TFC6* is inversely correlated with TF<sub>III</sub>C binding to the *ETC6* site. The *TFC6* promoter is also down-regulated when its own gene product is over-expressed. We present here a novel function of gene regulation where a Pol III transcription factor directly (auto) regulates a Pol II gene. Our
results also point to how this regulation might be mediated by an insulator-like function of TFIII which can implicate the functionality of Extra-TFIII sites in other eukaryotes.
CHAPTER ONE

INTRODUCTION
Regulation of gene expression in eukaryotic cells involves a myriad of different genes turned on or off and expressed in different temporal arrays. This is the hallmark in the control of gene expression which dictates cellular responses to stimuli and cellular differentiation in eukaryotic organisms. Different activating or repressive transcription factors are employed at differing times to regulate the initiation or inhibition of transcription of different regions of chromosomes in isogenic cells. Any disruption in cellular processes that result in the misregulation of gene function and altered patterns of gene expression are key features of abnormal cell proliferation or cancerous cells. Studies are consistently revealing that acquired epigenetic abnormalities participate with genetic alterations to cause this misregulation. The silencing of genes by DNA methylation and the alteration of the underlying chromatin environment by histone modifications have led to the realization that genetics and epigenetics cooperate at all stages of cell development. A primary factor in optimal cellular function is the control of transcription, which is highly regulated so that genes are expressed only where and when needed in the cell.

The transcription of DNA in eukaryotic cells occurs via the multi-subunit RNA polymerase complexes which converts the DNA base sequence into an RNA strand with the same sequence of one of the DNA strands (with uracil replacing thymine). The eukaryotic core RNA polymerases have 12-20 different protein subunits and require many other accessory proteins for proper transcription. The transcriptional initiation of a gene generally begins with the recruitment of a preinitiation complex (PIC), which is positioned at or near the transcription start site of the gene. Proteins known as general transcription factors combined with the RNA polymerase complex constitute the PIC, the assembly of which is required to initiate transcription. The RNA polymerase separates the two strands of DNA and positions a ribose nucleoside tri-phosphate (rNTP) at the transcriptional start site. Once initiated, RNA polymerase can proceed to copy the
coding strand by polymerizing nucleotides 5’ to 3’ complementary to the template strand. RNA polymerases form a “clamp” structure to bind to DNA. Since many eukaryotic genes are very long, the polymerase must hold on tightly to have the high processivity required to make it to the end of the gene. Inside the RNA polymerase complex is a “transcription bubble” of about eight nucleotides where the newly synthesized RNA is base paired with the DNA template strand near the active site. This base pairing is critical to maintaining the integrity of the DNA:RNA:polymerase complex. Specific regulatory sequences within the DNA mark the start and the ends of the regions to be transcribed and determine which strand of DNA is used as the template for RNA synthesis. RNA polymerase does not require a primer to initiate synthesis, but is directed to the proper site on DNA by nearby cis-acting sequences called promoters.

Most genes have additional transcription factor binding sites at regions from less than a hundred to several thousands of base pairs from the promoter. These sequences are called enhancers, as they stimulate the frequency of transcription initiation from a promoter. Enhancers can be located upstream of genes, within introns, and even downstream of the gene. Enhancer function is typically orientation and distance independent unlike proximal promoters. Much of our direct knowledge of how enhancers might activate transcription comes from studies of yeast Upstream Activation Sequences (UAS), the yeast counterpart of metazoan enhancers (Farrell, Simkovich et al. 1996). Current evidence suggests that UAS-bound proteins interact with components of the transcription complex to increase the probability that the PIC will find (have a stable interaction with) the promoter. Current theories suggest that DNA is looped out to accommodate enhancer-promoter interactions. Another proposed mechanism for enhancer activity has included the targeting of chromatin-modifying enzymes to the promoter region, thus making the promoter more accessible to the transcription machinery.
The regulation of transcription involves the utilization of transcriptional activators or repressors which can increase or decrease the rate of transcription of its target gene. Transcriptional activators such as Gal4, one of the first characterized transcription factors in yeast, have multiple domains that can fold and function independently. These proteins typically contain N-terminal DNA binding domains of various types along with a nuclear localization signal for import into the nucleus (Figure 1.1).

![Figure 1.1. Generic transcription factor bound to sequence element.](image)

Transcriptional activators bind to sequence elements (e.g. promoters, enhancers or UAS in yeast) located either upstream or downstream of the +1 site. They are typically polypeptide monomers that dimerize and bind to sequence palindromes in the genome, but different transcription factors can also form heterodimers. These monomers typically contain an N-terminal DNA-binding domain (BD) that also contains a nuclear localization signal, a dimerization domain (hatched domain between AD and BD), and a C-terminal activation domain (AD) rich in acidic amino acids.

Examples of DNA-binding domains include zinc modules, homeodomains, and bZIP or bHLH. There is also a domain for the dimerization of two independent monomers that are followed with activation domains which are rich in acidic amino acids near the C-terminal end. These activating regions can also consist of glutamine rich or proline rich domains. These transcription factors are brought in proximity to promoters where they function to activate or repress.
holoenzyme recruitment. Unlike prokaryotes which utilize a single RNA polymerase to transcribe their whole genome, eukaryotic cells contain three distinct RNA polymerases which partially overlap in subunit composition and are recruited by different general transcription factor complexes to transcribe different types of RNA molecules. RNA Polymerase I (Pol I), which is primarily located in the nucleolus, transcribes the larger RNA responsible for the main structural and catalytic center of the ribosome (rRNA) (Paule and White 2000).

Weaver, R.F. Molecular Biology, 3rd Ed. 2005.

Figure 1.2. Generic RNA polymerase I promoter. The RNA polymerase I gene promoter elements consists of the UPE or upstream promoter element in gold and the “core” region in blue containing the transcriptional start site or +1 site.

The RNA Pol I promoter region consists of a “core” region bound by a complex of proteins called SL1 (Figure 1.2). This complex is essential to recruit Pol I to rRNA genes. The SL1 complex contains TBP and three TAFs (110, 63, and 48). The UPE or upstream promoter region is bound by a single polypeptide, named UBF (UPE-binding factor) in human cells, or UAF (upstream activation factor) in yeast. *In vitro* transcription of rDNA genes by Pol I requires both UBF and SL1 (Paule and White 2000).

RNA Polymerase II (Pol II), found throughout the nucleoplasm, transcribes the heterogeneous nuclear RNA (hnRNA). This hnRNA consists of mostly protein coding mRNAs, along with most small nuclear spliceosomal RNA. The Pol II complex contains 12 protein subunits, and the largest subunit (RPB1) has an unusual carboxy terminal domain (CTD) with nearly perfect repeats of the amino acids YSPTSPS (27 in yeast, 52 in human), and the functions of the CTD are highly regulated by phosphorylation on these residues. The IIA form of Pol II has
an unphosphorylated CTD and is the initiating form of the complex. The IIO form of Pol II is the elongating form, which is phosphorylated on its CTD. The CTD, which is essential for viability in yeast, also binds to components of chromatin remodeling complexes, and is involved in the splicing, termination, capping and polyadenylation of mRNAs. Besides RNA polymerase II, other factors are recruited to the promoter including the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIF, a mediator complex, and the TATA-binding protein (TBP) which is associated with TFIID. Generic Pol II promoters contain an initiator region around the +1 or CAP site, a TATA box, a TFIIB recognition element, and upstream and downstream promoter elements (Figure 1.3).

Weaver, R.F. Molecular Biology, 3rd Ed. 2005.

Figure 1.3. Generic RNA polymerase II promoter. The upstream element in gold binds an activating transcription factor. BRE in purple is the TFIIB, a non-sequence specific general transcription factor, recognition element. The TATA box in red is an AT rich sequence about 25 bases upstream of the CAP site and binds the TBP or TATA-binding protein. The initiator (Inr) in green is centered around the CAP site. The DPE or downstream promoter element is located downstream from the CAP site.

The ordered assembly on Pol II genes is TFIID (which is also associated with numerous TAFIIs or TBP-associated factors), TFIIB, Pol II which requires the presence of TFIIF, then the other factors. TFIIB links TBP with the polymerase, TFIIA imparts directionality to the preinitiation complex; TFIIF functions as a helicase to unwind the strands of DNA allowing the polymerization of nucleotides complementary to the template strand and is stimulated by TFIIE.
(Hahn 2004). TF II F is hypothesized to direct the polymerase to preformed TF II D:TF II A:TF II B complexes. There are other Pol II promoter regions such as GC boxes which can bind the transcription factor SP1 and CCAAT boxes which binds CTF (CCAAT binding factor) or CEBP (CCAAT/Enhancer binding protein) (Hahn 2004).

RNA Polymerase III (Pol III) mainly transcribes the transfer RNA (tRNA) molecules that read the information encoded in the mRNA and translates it into protein sequence, but also transcribes the 5S ribosomal RNA, RNase P (RPR1), the U6 small nuclear spliceosomal RNA (SNR6), the cytoplasmic RNA of the signal recognition particle (SCRI), and Alu repeats (Paule and White 2000; Geiduschek and Kassavetis 2001; Huang and Maraia 2001). There are three types of RNA Polymerase III (Pol III) promoters which are grouped according to the organization of the binding sites for the core transcription factors TF III B and TF III C (Figure 1.4). Type I consists of the 5S ribosomal gene and contains an intragenic promoter region ICR (or Internal Control Region) which consists of a Box A and Box C sequence flanking an intergenic element (IE).

Transcription of the 5S gene requires the additional gene-specific factor TF III A. In yeast, 5S RNA genes are arranged in tandem with the 35S RNA genes where there are arrays of 100-200 copies of these genes. In contrast, the 274 tRNA genes with Type II promoters are distributed throughout the genome (Percudani, Pavesi et al. 1997; Hani and Feldmann 1998). The Type II promoter consists of intragenic A and B box elements, and includes all tRNA genes. The Type III promoter is a hybrid consisting of Box A and B elements and a TATA sequence in yeast. Human Type III promoters contain no Box A or Box B elements, but do contain TATA and downstream and upstream promoter elements.

The focus of this dissertation is principally on the Type II mechanism involved in tRNA transcription where both the presence of the Pol III complex and the transcription potential of the
tRNA gene can function as a chromatin boundary; and the Box B promoter sequence potentially functions as an insulator element.

Adapted from Schramm and Hernandez, (2002)

**Figure 1.4. Different types of RNA polymerase III promoters.** Type 1 promoter (5S) contains intragenic A and C boxes flanking an intergenic element (IE). All three elements of the Type I promoter are referred to as the Internal Control Region (ICR). Type 2 promoter (tRNA) contains an A and B box. Type 3 promoter (Hs U6) contains a Distal Sequence Element (DSE), a Proximal Sequence Element (PSE), and a TATA box all located upstream of the transcription start site (TSS) similar to RNA Polymerase II genes. The *Saccharomyces* U6 gene contains an intragenic A box, a TSS upstream TATA box, and a B box located surprisingly downstream of the transcription termination sequence (TTTT). Each promoter type above contains a plus number above the transcription termination sequence (TTTT) indicating the length of the gene in base pairs.

In tRNA gene (Type II) transcription initiation, the Pol III specific transcription factor TFIII C is recruited to the two intragenic sequences Box A and Box B downstream of the transcription start
site and remains tethered to these highly conserved regions of DNA (Figure 1.5). Next, TF\textsubscript{III}C recruits the Pol III transcription factor TF\textsubscript{III}B that contains the TATA-binding protein (TBP) subunit, and is essential for transcription initiation.

Robert Weaver, 4\textsuperscript{th} ed., (2008)

**Figure 1.5. RNA polymerase III Type II (tRNA) transcription initiation.** The transcription factor complex TF\textsubscript{III}C, through its DNA binding subunits, binds to Box A and Box B sequences within the coding region of the tRNA gene. TF\textsubscript{III}C recruits the transcription factor TF\textsubscript{III}B which contains TATA-binding protein (TBP) that participates in TF\textsubscript{III}B stability. TF\textsubscript{III}B then recruits RNA polymerase III where processive transcription can now occur. Reinitiation of transcription may or may not require the TF\textsubscript{III}C complex.

TF\textsubscript{III}C thus indirectly influences start site selection (Bartholomew, Meares et al. 1990). Bound TF\textsubscript{III}B then recruits and correctly positions RNA Polymerase III at the transcription start site
where transcription of the tRNA gene is initiated. TF\textsubscript{III}B-DNA complexes are competent to direct initiation and multiple rounds of transcription after TF\textsubscript{III}C complexes are stripped (Kassavetis, Braun et al. 1990). Mechanistically, TF\textsubscript{III}C bound at the Box A and Box B promoter elements opens the DNA duplex for the advancing Pol III (Bardeleben, Kassavetis et al. 1994).

Gene expression in eukaryotes is not simply regulated by the direct binding of repressors and activators to promoter elements along with the recruitment of a preinitiation complex, but also by the structure of the chromatin in which the given gene resides. Eukaryotic DNA is packaged and highly condensed in the nucleus through its association with histone proteins and additional non-histone chromatin associated proteins. Chromatin typically exists as two distinct states: euchromatin and heterochromatin (Figure 1.6).

**Figure 1.6. Types of chromatin.** Euchromatin (green nucleosomes), early replicating, transcriptionally active, hyperacetylated histone tails, less condensed structure; Heterochromatin (red nucleosomes), late replicating, transcriptionally repressed, hypoacetylated histone tails, more condensed structure with the binding of heterochromatin-associated proteins. Heterochromatin-associated proteins (e.g. Sir complex) are in yellow.

Euchromatin is the term that is associated with regions of the nucleoprotein complexes that are transcriptionally active, early replicating, and generally have hyperacetylated N-terminal histone
tails. Heterochromatin refers to a more condensed structure that is minimally transcribed, late replicating, and generally associated with hypoacetylated histone tails (Weiler and Wakimoto 1995).

The maintenance of the silent and/or open state has been shown to be heritably stable for ten generations or more (Pillus and Rine 1989). These two states of chromatin are not necessarily static since the chromatin environment of the underlying DNA can change between the open and closed states depending on changes in histone modifications, the binding of chromatin-associated proteins, or the activity of different signaling proteins in the cell. Heterochromatic gene silencing differs from simple repression in that larger regions of chromosomes, not just individual promoters, are repressed. Not only is the transcriptional potential of any gene dependent on the underlying chromatin environment, but all aspects of eukaryotic DNA function (transcription, replication, recombination, repair) must occur within the context of chromatin where the nucleosome, a nucleoprotein complex, is the fundamental unit.

The nucleosome consists of an octamer of four core histone proteins H3, H4, H2A, and H2B (two of each) around which approximately 147 bp of DNA are wrapped, and also contains ~20-60 bp of linker DNA which connects adjacent nucleosomes (~ 20 bp in S. cerevisiae, ~40-50 in humans). These proteins are abundant and comprise ~50% of total nuclear protein. The core histones (H2A, H2B, H3, and H4) are small, positively charged (basic) proteins (20-25% lysine and arginine) that share a conserved domain which folds into a conserved structure, the histone fold domain. This protein structure is composed of three α-helices separated by two short loops. The structure mediates the formation of head to tail dimers of specific pairs of histones (H2A + H2B, H3 + H4), which mediates the assembly of the nucleosome core particle on DNA.

Nucleosomes are extremely stable protein-DNA complexes under physiological conditions because of 14 contact points that occur between the histone core and the DNA duplex, with >120
direct atomic interactions (Luger, Mader et al. 1997). The numerous contact points are well-suited for the packing function that is required for chromatin, especially during mitosis. In higher eukaryotes, a histone H1 linker protein binds to linker DNA that adjoins two adjacent nucleosomes and participates heavily in chromatin remodeling through phosphorylation of H1 during the condensation of chromosomes during mitosis (Allan, Cowling et al. 1981).

The histone proteins are mainly globular but have N-terminal tails that extend out from the nucleosome core and are also partly responsible for the compaction of chromatin, but are subject to different covalent modifications (e.g. acetylation, methylation, and phosphorylation) at specific residues. The specific pattern of histone modifications can determine whether transcriptional activators or RNA polymerase can bind to and/or function on the underlying DNA.

There are over 60 different residues on histones where modifications have been detected either by specific antibody binding or by mass spectrometry (Figure 1.7). Extra complexity comes from the fact that methylation at lysines or arginines may be one of three forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines. These modifications have been shown to recruit other histone modification enzymes and/or chromatin remodeling factors (Luger, Mader et al. 1997; Lorch, Zhang et al. 1999; Wu and Grunstein 2000). It has been shown that the N-terminal tails of histone H4 play a vital role in establishing a heterochromatin environment, since deletion of the N-terminal tails abolished the establishment of heterochromatin (Kayne, Kim et al. 1988). The covalent modifications that occur on the N-terminal tails of histone proteins are hypothesized to represent a “histone code” indicating the expression state of the underlying chromatin (Strahl and Allis 2000; Turner 2000; Jenuwein and Allis 2001). Effector proteins translate this code by binding to specific modifications on the
histones. The binding of these effector proteins then can lead to further chromatin modifications which can ultimately dictate particular expression states of the underlying chromatin.

Figure 1.7. Summary of the most common post-translational histone modifications on the N-terminal histone tails and inside the nucleosome core. Depicted above is a schematic of the amino terminal tails of histones H3, H4, H2A, and H2B (only one each shown for clarity) extending out from the nucleosomal core. The N-terminal tails are subjected to different post-translational modifications depending on the specific amino acid residue and position. Shown above are the amino acids, K (Lysine), R (Arginine), S (Serine) with their numbered position along with the modification. Me is methylation (Red), Ac is acetylation (Light Blue), P is phosphorylation (Purple), and Ub is Ubiquitylation (Gold).

Histone modifications are also known to reinforce or inhibit one another, with one modification affecting the likelihood of other modifications. The acetylation of lysine residues, the
methylation of lysine and arginine residues, the phosphorylation of serine residues, and ubiquitylation of lysine residues on histone proteins have all been implicated in the activation of transcription; whereas certain residues of histone proteins with modifications of methylation, ubiquitylation, sumoylation, deamination, and proline isomerization have been implicated in transcriptional repression.

Histone proteins H3 and H4 that have acetylated lysine residues typically have roles in transcriptional activation and sometimes even in repression (Fisher-Adams and Grunstein 1995; Megee, Morgan et al. 1995); whereas the N-terminal tails of H3 and H4 are hypoacetylated in silenced heterochromatin (Braunstein, Rose et al. 1993; Suka, Suka et al. 2001). The acetylation state of the histone tails is believed to determine DNA-histone compaction by virtue of the interacting charges between the modified amino acid and the DNA backbone. The DNA backbone is negatively charged and wrapped around a positively charged histone core. The acetylation of lysine residues seems to partially relieve DNA-histone compaction by neutralizing the lysine residue’s positive charge.

Three distinct classes of histone deacetylases (HDAC) have been discovered: class I, II, and the class III NAD$^+$-dependent enzymes of the SIR (Silent Information Regulator) family. The histone deacetylase, Sir2p, preferentially deacetylates H4K16 in vitro and is essential in establishing in vivo silencing in S. cerevisiae (Imai, Armstrong et al. 2000; Meijsing and Ehrenhofer-Murray 2001; Tanny and Moazed 2001; Suka, Luo et al. 2002). In general, HDACs do not have preference for a particular acetyl group but some, like Sir2p, do have specificity for a particular histone such as Hda1p for H3 and H2B, and Hos2p for H3 and H4 (Vaquero, Scher et al. 2006).

Lysine methyltransferases have enormous specificity compared to acetyltransferases as they usually modify only one lysine residue on a single histone and can either be activating or
repressive to transcription (Bannister and Kouzarides 2005). The methylation of lysine 4 on H3 appears to be a euchromatic imprint in a wide range of organisms (Strahl, Ohba et al. 1999). In other higher eukaryotes, including Schizosaccharomyces pombe (S. pombe), methylation of lysine 9 on H3 (H3K9) and lysine 27 on H3 (H3K27) are characteristic methylation marks in heterochromatic regions. These heterochromatic regions include the presence of the chromodomain-containing Heterochromatin Protein 1 (HP1) and a significant lack of acetylation marks. HP1 mediates the recruitment of additional chromodomain-containing Histone Methyl Transferase (HMT) activity to establish heterochromatic states in higher eukaryotes. There are only three known histone H3 methylation sites in yeast – H3K4, H3K36, and H3K79 and each has a specific distribution pattern. Dot1p is a lysine methyltransferase in yeast specific for H3K79 which is unexpectedly located in the nucleosome core (Feng, Wang et al. 2002; van Leeuwen, Gafken et al. 2002). The methylation of lysine 79 of histone H3 is thought to prevent the binding of Sir proteins (van Leeuwen, Gafken et al. 2002). The methylation of histone H3 lysine 4 (H3K4) in S. cerevisiae is mediated by Set1p (Briggs, Bryk et al. 2001).

The influence of the histone code is expanding in relation to the methylation state of both lysine and arginine residues. Since methylation of lysine residues can also occur as a mono-, di- or tri-methylation on a single lysine residue, new evidence is beginning to show a differential but gradual distribution of di- and tri-methylation patterns between promoter regions and open reading frames (Cuthbert, Daujat et al. 2004; Carrozza, Li et al. 2005; Joshi and Struhl 2005; Keogh, Kurdistani et al. 2005). This significantly adds to the complexity of the underlying histone code in determining open or closed chromatin states. Currently, there is only one known H3K4me3 demethylase in budding yeast, JhD2p (Liang, Klose et al. 2007). Due to limiting histone lysine demethylases, the methylation of lysine residues seems to represent a more long-term epigenetic mark for maintaining chromatin states, as DNA replication and semiconservative
nucleosome distribution appears to be the sole means to “dilute” histone lysine methylation below a critical threshold level.

The phosphorylation of serine residues by specific kinases on N-terminal histone tails is most prominent during cell division. This modification is spatially and temporally correlated with mitotic and meiotic chromatin condensation (Wei, Mizzen et al. 1998). Many protein phosphorylation cascades may have a more direct effect on gene expression through the phosphorylation of chromatin at serine residues. Snf1p, a kinase in budding yeast has been shown to target H3S10.

The relatively large modification of ubiquitylation on lysine residues can be found mainly on the histone proteins H2A and H2B. The modification of H2BK123 in yeast is mediated by Rad6/Bre1, an E2 ubiquitin-conjugating/E3 ubiquitin-ligase, and is required for the subsequent trimethylation of H3K4 and H3K79 which is associated with transcriptional activation (Zhu, Zheng et al. 2005). The chromatin-modifying functions of ubiquitylation are somewhat unclear. It more than likely recruits additional chromatin factors, but may also function to physically keep chromatin open by a “wedging” process, given its large size.

The genomes of multi-cellular organisms consist of different hypothetically demarcated regions of gene expression based on whether or not the underlying DNA is in an open or closed conformation. The demarcation line between these differing regions of expression can occur on sequence elements between regions of differing chromatin states, resulting in position effect silencing of adjacent genes and/or blocked transcriptional activation of nearby gene promoters. These sequence elements along with their associated DNA-binding proteins are known as boundary elements and are functionally characterized as either barriers that block the spread of heterochromatic silencing into regions of open and transcriptionally active genes, or as insulators that block the transcriptional activation of genes by enhancers from distal (or even proximal)
genomic regions (Figure 1.8). Enhancer blocking only occurs if the insulator is situated between the enhancer and promoter, not if it is placed elsewhere.

![Figure 1.8. Two types of boundary elements: barriers and insulators.](image)

Most studies on enhancer-blocking deal primarily with protein-protein interactions that may interfere with the activity of complexes mediating normal enhancer-promoter communication. Compound elements are sequence elements that contain closely-spaced enhancers and enhancer-blockers that are typically polar in nature (West, Gaszner et al. 2002). Some blocking elements (insulators) do appear to show polarity function in an orientation-dependent manner, unlike enhancers which are typically both orientation- and distance-independent (Hark, Schoenherr et al. 2000; Bell, West et al. 2001). A wide variety of enhancer-blocking elements have been identified (mainly in *Drosophila*) and an increasing number are being found in vertebrates (Kellum and Schedl 1991).
Saccharomyces cerevisiae, commonly known as budding yeast, is unicellular, but grows as individual isogenic cell colonies descended from one original cell on plated media; and was the first eukaryotic organism to have its genome sequenced, thus making all genes and their sequences available for study in all aspects of gene regulation, including chromatin boundary elements. The yeast genome is relatively easy to manipulate, which makes it a very desirable model for analyzing the mechanisms involved in chromatin remodeling and gene regulation. Also, study of the yeast model is very desirable because many of the proteins in yeast are conserved through evolution and are homologous to proteins in higher eukaryotic organisms, which can provide insight into the function of genes across species. The established model for the study of boundary elements (barriers) in Saccharomyces is best exemplified by a tRNA gene (tDNA) on chromosome III immediately downstream of the cryptic mating-type loci, HMRa.

In Saccharomyces cerevisiae, heterochromatic silencing occurs at the cryptic-mating loci HMRa (Homothallic Right) and HMLa (Homothallic Left), ribosomal DNA, and telomeres. In most strains, HMLa contains a cryptic copy of the MATα genes and HMRa contains a cryptic copy of the MATα genes (Figure 1.9). Homothallic strains of S. cerevisiae have the ability to interconvert their mating types either from a to α, or from α to a, as frequently as once every other generation. The ability of S. cerevisiae to switch haploid mating types depends on the HO gene. The HO gene encodes a sequence-specific endonuclease that cleaves the mating type locus to create a double strand break in the DNA. The repair of the cleavage occurs by a mechanism similar to gene conversion using homologous recombination machinery, in which a silent copy of the mating-type genes at either HMR or HML is copied into the transcriptionally active euchromatic MAT locus (Haber 1998). Most laboratory strains do not contain an active copy of the HO gene, so a stable haploid state can be maintained for study using various genotype combinations after meiotic recombination and sporulation.
Within the yeast nucleus, \( HMRa \) (or \( HMR \)) exists as 12 ordered nucleosomes arranged in six pairs of closely spaced nucleosomes separated from one another by longer linkers and spans ~3.5kb of DNA (Mastrangelo, Weinstock et al. 1992; Ravindra, Weiss et al. 1999).

The sequence elements flanking both \( HM \) loci are silencing initiators termed E and I silencers (Essential and Important). The E and I silencer elements contain ARS (Autonomously Replicating Sequences) which are binding sites for the Origin Recognition Complex (ORC). The E silencer is primarily responsible for the initiation of silencing with the binding of Abf1p (Autonomously replicating sequence Binding Factor), Rap1p (Repressor Activator Protein), and ORC; whereas the I silencer only binds Abf1p and ORC (Moazed 2001; Huang 2002). ORC and Rap1p are only required for the initiation of silencing and not any other subsequent silencing event. At \( HMR \), silencing is most robust between the two silencer sequences, E and I, where the haploid-specific cryptic mating type genes \( (a1a2) \) are positioned (Rusche, Kirchmaier et al. 2003).
In *S. cerevisiae*, silenced domains consist of continuous distributions of SIR proteins along the chromosome that are targeted by hypoacetylated nucleosomes, and are thought to form an ordered, compact structure that restricts transcription (Hecht, Strahl-Bolsinger et al. 1996; Strahl-Bolsinger, Hecht et al. 1997; Lieb, Liu et al. 2001; Rusche, Kirchmaier et al. 2002). Interestingly, the inheritance of silenced chromatin domains has been shown to be remarkably stable during both mitosis and meiosis (Grewal and Klar 1996) and the tails of histones H3 and H4 are unacetylated at most positions (Braunstein, Rose et al. 1993; Braunstein, Sobel et al. 1996; Suka, Suka et al. 2001).

The propagation of silent heterochromatin through *HMR* is sequence-independent and mediated by the Sir proteins after the initiation of silencing at the E and I silencers. The Sir complex, which contains Sir2p, Sir3p, and Sir4p, is recruited to the silencers through the initiating DNA-binding proteins of ORC1p, Rap1p, and Abf1p (Figure 1.10). Sir1p binds to ORC1p and sets the stage for the binding of the other Sir proteins. Sir4p binds next and is known to interact with both Sir1p and Rap1p. The binding of Sir4p to the silencer does not require Sir2p or Sir3p but instead is responsible for the initial recruitment of Sir2p. Sir4p is required for the binding of Sir3p which also possibly binds to Abf1p. Sir2p not only functions in the structural integrity of silencing at heterochromatic regions of yeast, but is also a histone deacetylase which is dependent on **Nicotinamide Adenine Dinucleotide (NAD⁺)** as a cofactor (Rusche, Kirchmaier et al. 2003).

In the propagation of heterochromatin (silencing), the enzymatic deacetylation by Sir2p of the adjacent nucleosome coupled with the hydrolysis of NAD⁺ creates sites on histones where Sir3p and Sir4p can bind with higher affinity to the N-terminal histone tails of the adjacent nucleosomes. The Sir complex then proceeds to propagate along an array of deacetylated nucleosomes.
Downstream of the I silencer sequence at the HMR locus is a boundary element of the barrier type which consists of a $tRNA^{thr}$ gene. Deletion of this tDNA ($tRNA$ gene) can lead to a significant loss of boundary element function. Also, mutations that affect the activity of the RNA Polymerase III transcription factors TF$_{III}C$ or TF$_{III}B$ impairs the barrier function of this tDNA. Disruption of the barrier can occur from tDNA mutations in the $tRNA^{thr}$ promoter, which

Adapted from Rusche, Kirchmaier, and Rine, Mol. Biol. Cell, v13, p2207, 2003

**Figure 1.10. Initiation and propagation of heterochromatic silencing at the E silencer through the HMR locus in S. cerevisiae.** A) Initial recruitment of DNA-binding proteins Orc1p, Rap1p, and Abf1p along with the Sir proteins in an ordered assembly. B) Sir2p deacetylates the adjacent nucleosome creating a high affinity binding site for Sir3p and Sir4p. C) Propagation of silent heterochromatin begins with the deacetylation action of Sir2p and the ordered assembly of Sir proteins. D) A repetition of this process leads to silencing of the entire region.
eliminates a nucleosome-free gap by inhibiting the binding of the entire Pol III complex, or by mutations in either the GCN5 or SAS2 genes that encode histone acetyltransferases, which affect global chromatin processes (Donze, Adams et al. 1999). Another gene that could play a role in the integrity of the barrier element by affecting global processes is the histone deacetylase RPD3 (Jambunathan, Martinez et al. 2005).

RPD3 or Reduced Potassium Dependency encodes the catalytic subunit of two functionally different histone deacetylase complexes, is conserved between humans and yeast, and can either repress or lead to activation of transcription when targeted to promoters. Two Rpd3 complexes have been characterized and have been designated as Rpd3 small and large complexes, Rpd3(S) and Rpd3(L). Rpd3(S) functions within coding regions of genes to prevent erroneous transcription initiation by recognizing Set2p methylated histones (H3K36) through its Eaf3p chromodomain where it deacetylates histones within these transcribed sequences (Carrozza, Li et al. 2005). Rpd3(L) functions specifically at repressed promoters through contacts with DNA binding repressor proteins (Keogh, Kurdistani et al. 2005).

Curiously, while the deletion of RPD3 leads to higher global acetylation levels, at the same time, silencing is enhanced at the cryptic mating loci (specifically HMR) and telomeres (Vannier, Balderes et al. 1996), even overriding the tDNA barrier element adjacent to the HMR locus (Jambunathan, Martinez et al. 2005). In rpd3Δ mutants, it was found that a fraction of Sir2p molecules was delocalized from the nucleolus (rDNA) and became enriched at the regions of DNA adjacent to telomeres and at the silent HM loci (Santos-Rosa, Bannister et al. 2004; Venkatasubrahmanyam, Hwang et al. 2007; Zhou, Zhou et al. 2009). Through our own experimentation with an ADE2 marker gene inserted downstream of the tDNA boundary element, strains deleted for RPD3 showed that silencing bypasses the tDNA barrier element and represses the inserted ADE2 marker gene (Figure 1.11).
The laboratory created ADE2 inserted strains harbor an intrinsic mutation in its genomic ADE2 gene, making the parent strains auxotrophic for adenine. The silencing of this ectopic ADE2 gene results in the build-up of intermediates in the adenine biosynthesis pathway which are converted to a red pigment and reflected as a colony color phenotype.

The silencing paradox that exists in rpd3Δ strains is evident by the fact that Rpd3p is a histone deacetylase, the action of which is associated with heterochromatin. Intuitively, the deletion of RPD3 should result in the increase of global histone acetylation levels and thus a decrease or an abolishment of silencing. What is the mechanism of this rpd3Δ silencing effect? What role would Rpd3p play in halting the enhanced spreading of silencing heterochromatin? Are there any other extraneous effectors mediating this silencing effect? Does Rpd3p play a

**Figure 1.11. Deletion of RPD3 affects the spreading of silencing heterochromatin at HMRα.**

Both wild-type (WT) and rpd3Δ representations of the HMR region are depicted above with E and I silencers in blue, the mating type specific genes (a1a2) in green, the barrier tRNA\(^{thr}\) in brown, and the inserted ADE2 gene either active (white) or silenced (red). The spreading of silencing (represented by the red arrow) begins at the silencers and is halted at the barrier in WT cells where the ADE2 gene is active and produces a white colony color phenotype in S. cerevisiae cells. Silencing in rpd3Δ cells bypass the barrier element and the spreading of silencing heterochromatin proceeds through to repress transcription of the ADE2 gene, thus resulting in a red colony color phenotype.
major role in boundary integrity? Rpd3 has many roles in maintaining genome integrity and one focus of this dissertation was to study the mechanisms which lead to the enhanced silencing effect in \textit{rpd3A} strains of \textit{S. cerevisiae}, which bypass the barrier element of \textit{tRNA}^{thr} at the \textit{HMR} loci.

Emerging studies are revealing that tDNAs not only function as transcription units for transfer RNA molecules essential for translation and barrier elements to heterochromatic propagation, but also perform numerous other extra-transcriptional roles which can potentially have effects on chromatin state and genomic organization. Chromatin bound Pol III complexes mediate the targeting of \textit{Ty} element integration (Chalker and Sandmeyer 1992; Kirchner, Connolly et al. 1995; Devine and Boeke 1996), the blocking of replication fork progression (Deshpande and Newlon 1996), condensin and cohesin recruitment (Dubey and Gartenberg 2007; Haeusler, Pratt-Hyatt et al. 2008), and inhibition of transcription from nearby Pol II promoters (Kinsey and Sandmeyer 1991; Hull, Erickson et al. 1994; Bolton and Boeke 2003; Simms, Miller et al. 2004) (Figure 1.12).

Yeast TF\textsubscript{III}C is a large, multi-component protein of 570 kDa consisting of six polypeptides of 138, 131, 95, 91, 60 and 55 kDa which correspond to the genes \textit{TFC3}, \textit{TFC4}, \textit{TFC1}, \textit{TFC6}, \textit{TFC8}, and \textit{TFC7} respectively. No single component of TF\textsubscript{III}C seems to be able to bind DNA on its own; instead, TF\textsubscript{III}C tends to bind as a complete complex. Studies in both budding and fission yeast initially identified the presence of chromosomal sequences that are bound by the TF\textsubscript{III}C complex, but not the other Pol III transcription factors TF\textsubscript{III}A, TF\textsubscript{III}B, or the Pol III enzymatic complex itself (Harismendy, Gendrel et al. 2003; Roberts, Stewart et al. 2003; Moqtaderi and Struhl 2004; Noma, Cam et al. 2006), and similar sites have recently been identified in human cells (Canella, Praz et al. 2010; Moqtaderi, Wang et al. 2010; Oler, Alla et al. 2010; Raha, Wang et al. 2010).
These sequences are referred to either ETC (Extra-TFIIIc) or TFIIIc-only sites in budding yeast and humans, and COCs (Chromatin Organizing Clamps) in fission yeast (Roberts, Stewart et al.)
2003; Moqtaderi and Struhl 2004; Noma, Cam et al. 2006) and contain only the intragenic Box B sequences of the Type II RNA Polymerase III genes (tRNAs) (Figure 1.13).

![Figure 1.13. The ETC (extra-TFIIIc) site.](image)

**Figure 1.13. The ETC (extra-TFIIIc) site.** ETC sites are conserved RNA Pol III type II intragenic Box B sequences which are dispersed throughout the genome. The Pol III transcription factor TFIIIc stably binds to these ETC sites which are overrepresented between divergently transcribed RNA Pol II genes.

Interestingly, the conserved regions of ETC sites in *Saccharomyces* are over-represented at divergently transcribed Pol II genes. Some of these ETC (or COCs) sites in budding and fission yeast have been shown to function as chromatin boundary elements (Noma, Cam et al. 2006; Simms, Dugas et al. 2008), however, the genome-wide function of most of these TFIIIc-bound sites remains unknown. The B box sequences are highly conserved among tRNA genes (Dieci, Percudani et al. 2000) and the interaction of TFIIIc with the B block sequence functions to some extent in a distance- and orientation-independent manner (Burnol, Margottin et al. 1993).

ETC or TFIIIc-only loci were first identified in genome-wide distribution assays of RNA Pol III transcription components using ChIP followed by microarray hybridization (ChIP-CHIP) in *S. cerevisiae*. Eight loci were identified as having only TFIIIc occupancy and were not occupied by any other Pol III factor and were found to be highly conserved among the four yeast
species: *S. cerevisiae*, *S. mikatae*, *S. bayanus*, and *S. paradoxus* (Moqtaderi and Struhl 2004) (Figure 1.14).

![LOCUS B BLOCK ALIGNMENT](image)

**Figure 1.14. Extra-TF_{III}C (ETC) sites discovered in different Saccharomyces species.** B block alignment of *ETC1* to *ETC8* including the *ZOD1* locus. The B block consensus is derived from 274 tRNAs. The *ETC* consensus is derived from four different *Saccharomyces* species. The height of each letter is directly proportional to its degree of identity across contributing species. Adjacent Pol II genes are indicated in parentheses with a hyphen.

These sites were found to contain an eleven nucleotide consensus in *S. cerevisiae*, and there is also a 100% sequence conservation of three additional nucleotides located 6 to 8 bases downstream of the B block consensus. The final C in this sequence is significantly conserved upon alignment of the B block of the 274 tRNA genes. Perfect conservation of the three additional nucleotides is found at all ETC loci but in only 21 out of 274 tRNAs. The *ZOD1* locus
differs from the other ETC sites in that it is bound by all Pol III components (Moqtaderi and Struhl 2004).

Microscopic examination of *S. pombe* nuclei showed that distant COC sites localize to form a limited number of clusters at the nuclear periphery (Noma, Cam et al. 2006). COC loci in *S. pombe* are also often positioned near CCCTC-binding factor (CTCF) binding sites suggesting a possible role in chromatin insulation. A number of ETC loci also have binding motifs for the ETS transcription factor, but the connection between ETS and TFIII_C remains unknown (Noma and Kamakaka 2010). Under strict experimental criteria, 1,865 ETC sites were identified in humans with the implication of several thousand more. In humans, the distribution of TFIII_C-occupied loci of both ETC and non-ETC types revealed a positional bias toward the transcriptional start sites (TSSs) of Pol II genes, as 181 ETC loci with the highest levels of TFIII_C showed occupancy near the TSSs of Pol II genes, with 68% being located within 1kb of a Pol II TSS (Moqtaderi, Wang et al. 2010). Also, the strongest TFIII_C-bound loci were within 200bp of CCCTC-binding factor (CTCF) sites (Noma and Kamakaka 2010).

The purpose of this study is to further our understanding of gene regulation mediated by boundary elements in *S. cerevisiae* and the mechanisms behind these particular functions. Previous studies at *HMR* have shown that although the spread of heterochromatic silencing in *RPD3* mutants is not halted at the tDNA barrier, it does not appear to affect transcription of the tRNA gene (Donze, Adams et al. 1999; Donze and Kamakaka 2001), so *RPD3* must play a role in restricting the spread of silencing at *HMR*. This different mechanism may involve global changes in histone modifications which in turn can affect the recruitment of specific chromatin-associated proteins at that region. Studies of ETC sites in yeast have suggested that they may function as chromatin boundary elements (Noma, Cam et al. 2006; Simms, Dugas et al. 2008); and mapping of the *ETC6* site to the *TFC6* promoter suggested that the TFIII_C complex might be
involved in auto-regulation of the \textit{TFC6} gene (Moqtaderi and Struhl 2004) and can potentially function as an insulator to the targeted activation of the \textit{TFC6} promoter (Figure 1.15).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.15.png}
\caption{Model of autoregulation at the \textit{TFC6} locus. Schematic of the \textit{TFC6} locus including the promoter region that contains \textit{ETC6}. The divergently transcribed Pol II genes, \textit{TFC6} and \textit{ESC2}, are in blue and the \textit{ETC6} site is in orange. \textit{TFC6} encodes the Tfc6p subunit (green) of the TF\textsubscript{III}C transcription complex (green) that either binds to \textit{ETC6} or other RNA Pol III binding sites. The binding of TF\textsubscript{III}C to \textit{ETC6} potentially functions as an insulator to the activation of \textit{TFC6} expression by a potential \textit{UAS} (blue triangles) and an unidentified \textit{UAS}-bound transcription factor.}
\end{figure}

At the \textit{TFC6} locus, our previous studies have shown that deletion of the \textit{ETC6} (B box) site results in increased relative \textit{TFC6} gene expression, whereas deletion of \textit{ETC6} plus all sequences upstream of \textit{ETC6} through to the transcription start site of \textit{ESC2} (the divergently transcribed Pol II gene at the \textit{ETC6} locus) resulted in a surprising decrease of \textit{TFC6} transcription (Simms, Dugas et al. 2008). These results suggested that the \textit{ETC6} site directly functions in the regulation of \textit{TFC6}, and that it may be due to an insulator-like activity.

In overview of this dissertation, the second chapter examines factors affecting barrier elements by elucidating the mechanisms or effectors of the enhanced \textit{rpdr3A} silencing effect that
bypasses the natural tRNA\(^{thr}\) barrier at the HMR region. Using a genetic screen, we proposed to identify potential mechanistic effectors of the rpd3\(\Delta\) silencing effect and subsequently analyze these effectors. Using UV-mutated strains of rpd3\(\Delta\) cells, we identified and analyzed seven different genes which when individually deleted, revealed that only two of the seven genes reversed the increased silencing effect in rpd3\(\Delta\) strains.

The third chapter examines a novel autoregulatory mechanism with potential insulator activity of TF\(\text{III}\)C bound at ETC6 in the TFC6 promoter. Since TF\(\text{III}\)C binds to the B box sequence of ETC6, we propose that TF\(\text{III}\)C may have a direct effect on the regulation of the TFC6 gene through either an insulator-like mechanism, or by competing for binding with an uncharacterized transcription factor that binds immediately upstream. We also propose that this novel function is the first known example of an RNA Polymerase III core transcription factor (TF\(\text{III}\)C) directly regulating an RNA Polymerase II promoter (TFC6). Finally, chapter four will include a more thorough discussion of the results of both studies and the implications of those results. This section will also include new strategies for future experiments and/or directions for these studies of barriers and insulators as boundary elements in light of new evidence.

**LITERATURE CITED**


CHAPTER TWO

EFFECTORS OF THE RPD3 DELETE SILENCING EFFECT
INTRODUCTION

DNA in eukaryotic cells exists in the context of chromatin where the nucleosome is the fundamental unit. The nucleosome consists of 147 bp of DNA wrapped 1.7 times around histone proteins H3, H4, H2A, and H2B (2 subunits each) with N-terminal tails on each individual histone polypeptide extending out from the nucleosome core. These N-terminal tails are partly responsible for the compaction of chromatin and are subject to different covalent modifications (e.g. acetylation, methylation, and phosphorylation) at specific residues. These modifications have been shown to recruit other histone modification enzymes and/or chromatin remodeling factors (Luger, Mader et al. 1997). A combinatorial code may exist for the distribution of both type and location of histone modifications which can serve as molecular docking sites for the recruitment of specific regulatory proteins and/or transcriptional machineries.

Chromatin environments display mainly two major states of architecture, euchromatin and heterochromatin. Euchromatin is a more open state where the underlying DNA is accessible to the transcription machinery, and heterochromatin is a more closed, condensed state of compact nucleoproteins and additional protein complexes making the underlying DNA inaccessible to transcription. Regulation of regions that in either open or closed states is crucial for proper gene expression during differentiation of cell types and for overall proper genomic functioning within living cells. Numerous protein complexes interact not only with DNA but also associated chromatin proteins to modify particular histone proteins, to recruit ATP-dependent chromatin modifying enzymes, and to silence certain regions of the genome (Luger, Mader et al. 1997; Lorch, Zhang et al. 1999; Wu and Grunstein 2000).

Saccharomyces cerevisiae contains regions in its genome where the spreading of heterochromatic silencing is essential for proper functioning of the cell. These regions include the cryptic mating-type loci, HMRα and HMLα, the telomeric ends of chromosomes, and
ribosomal RNA (rRNA) genes (Rusche, Kirchmaier et al. 2003). In this study, we preferentially examined the cryptic mating locus region of *HMRa*, which is an ideal model for the initiation, propagation, and establishment of heterochromatic silencing of particular mating type genes. This silencing is initiated by the silencer sequence regions E and I that bind sequence-dependent proteins generally involved in DNA replication and transcription activation (e.g. ORC, Rap1p, Abf1p). Silencing is then propagated downstream of the silencer sequences, and at the mating loci is essential in maintaining a particular yeast haploid cell mating type, a or α.

The propagation of silent heterochromatin at *HMR* is sequence-independent and mediated by the binding of Silent Information Regulator (SIR) proteins. Sir2p is an NAD⁺-dependent histone deacetylase that is required for silencing of heterochromatic regions in yeast. The propagation of silencing proceeds downstream through the *HMR* by the deacetylation action of Sir2p on the successive nucleosomes coupled with the hydrolysis of NAD⁺ and the subsequent binding of the other Sir proteins (Sir3p, Sir4p, etc.) (Rusche, Kirchmaier et al. 2003).

Rpd3p, another histone deacetylase, is the enzymatic subunit of two characterized complexes, Rpd3(L) and Rpd3(S). Rpd3(L) represses transcription when targeted by promoter-specific transcription factors, and Rpd3(S) prevents erroneous transcription initiation within coding regions by recognizing Set2p methylated histones (at H3K36), where it then deacetylates those histones within transcribed sequences (Carrozza, Li et al. 2005; Keogh, Kurdistani et al. 2005). In yeast, *RPD3* deletion surprisingly enhances silencing at the cryptic mating loci and telomeres (Vannier, Balderes et al. 1996) even overriding the tDNA barrier element adjacent to the *HMRa* locus (Donze and Kamakaka 2001; Jambunathan, Martinez et al. 2005).

We hypothesized that the increased silencing in rpd3Δ mutants may be due to the de-repression of genes that increase silencing. This gave us the rationale to first perform a genetic
screen to identify potential effectors of the rpd3Δ silencing effect. To understand the mechanism of enhanced silencing in yeast lacking RPD3 by identifying potential effectors of this silencing effect, we used ultraviolet light to mutagenize rpd3Δ strains, which contained an inserted ADE2 marker gene downstream of the HMR locus. We identified suppressor mutants that no longer displayed an enhanced silencing phenotype. These genes were subject to individual gene replacements with the LEU2 marker gene (knockouts) and crossed back to an rpd3Δ background and assessed for a change in colony color phenotype.

At HMR in wild-type strains, the tRNA\textsuperscript{thr} gene functions as a barrier element that blocks the spread of silencing into an ectopic ADE2 marker gene. In rpd3Δ strains, silencing spreads past the barrier element and into the downstream ADE2 gene, thus silencing the expression of this gene. Silencing of the ADE2 gene results in accumulation and polymerization of P-ribosylaminoimidazole (AIR) molecules in the adenine biosynthesis pathway which results in a red/pink pigment reflected in yeast colony color. Since Sir-dependent mediated silencing also occurs at telomeres, we also took advantage of an inserted telomeric URA3 gene on chromosome VII-L to assess Sir-mediated silencing at telomeres in our genetic screen and test for mechanistic consistencies between the HMR and the telomere.

From this genetic screen, we identified seven genes which when disrupted reversed the extended heterochromatin formation at HMR in rpd3Δ backgrounds: BRE1, GDH2, BRE2, GAT3, QNS1, NPT1 and RXT3. It has been previously shown that NAD\textsuperscript{+} biosynthesis pathway genes (NPT1 and PNC1) are either directly or indirectly involved in the regulation of Sir2p activity and silencing at rDNA and telomeres (Sandmeier, Celic et al. 2002), therefore we asked if expression levels of those genes were altered by deletion of RPD3. Using Northern analysis to assess the expression of these NAD\textsuperscript{+} biosynthesis pathway genes, our studies showed no
difference in the expression levels of the genes \textit{NPT1}, \textit{QNS1}, or \textit{PNC1}, between our WT and \textit{rpd3Δ} strains. These genes were not studied further as potential effectors. There was also the potential that Rpd3p, which complexes with Ume6p to repress promoter regions, could act as a repressive transcription factor for \textit{GDH2} expression. The product of \textit{GDH2} is an NAD$^+$-dependent glutamine dehydrogenase, so the requirements of Gdh2p could potentially be affected by the availability of Sir2p and its enzymatic requirements of NAD$^+$ (Kurdistani, Robyr et al. 2002). Also, the only yeast H3K4 trimethyl demethylase, Jhd2p, contains a JmjC domain which has been shown to directly remove lysine methylation via a hydroxylation reaction that requires iron and \(\alpha\)-ketoglutarate as cofactors (Klose, Kallin et al. 2006; Tsukada, Fang et al. 2006). Because glutamate dehydrogenase (\textit{GDH2}) degrades glutamate to ammonia and \(\alpha\)-ketoglutarate, it may indirectly participate in the demethylation of histone H3K4 through Jhd2p.

Of the five genes that were knocked out and crossed back to an \textit{rpd3Δ} strain, only the \textit{bre1Δ} and \textit{bre2Δ} gene knockouts showed a loss of extended silencing at \textit{HMR}. Interestingly, there was no loss of telomeric silencing in both the double knockout strains, as growth on 5-FOA (5-flouro-orotic acid) demonstrated that the inserted \textit{URA3} gene proximal to telomeres was not expressed. \textit{BRE1} and \textit{BRE2} are both either indirectly or directly required for the trimethylation of H3K79 and H3K4, which is required for the proper regulation of silencing at \textit{HMR}, rDNA, and telomeres (Fingerman, Wu et al. 2005). Our initial experimentation through ChIP assays did not show any differences in H3K4 trimethylation along the \textit{HMR} region or regions downstream of \textit{HMR} between WT and \textit{rpd3Δ} strains.

Other studies involving histone H3K4 methylation have suggested that mutations in the methylation establishment pathway can lead to a re-distribution of Sir proteins at silenced loci (Tompa and Madhani 2007; Venkatasubrahmanyam, Hwang et al. 2007; Zhou, Zhou et al. 2009;
Ehrentraut, Weber et al. 2010). In light of this new evidence, we have modified our hypothesis of the \textit{rpd3}\textDelta silencing effect to take into account the limited availability of Sir proteins in the cell. Our adjusted hypothesis includes the supposition that \textit{rpd3}\textDelta strains result in an increase in the global acetylation of nucleosomes in euchromatic regions; this altered acetylation state can potentially cause a shift of the localization of the limited pool of Sir proteins, as their binding to some regions would be inhibited by acetylated histones, freeing them to concentrate at silenced telomeric and \textit{HMR} regions. This adjusted hypothesis and the description of future studies, which will include the utilization of Sir proteins fused with a functionally repressed \textit{E. coli} DNA methyltransferase, will be addressed in more detail in the final discussion chapter.

MATERIALS AND METHODS

All yeast strains used in this study are isogenic to W303-1a except where noted. The original parent \textit{rpd3}\textDelta strain (DDY 3133) containing \textit{ADE2} downstream of \textit{HMR} used for ultraviolet mutagenesis in the genetic screen was created previously in our lab as described (Jambunathan, Martinez et al. 2005). All oligonucleotides utilized in this study are listed in Table 2.1.

Yeast Genomic DNA Extraction (Winston Prep)

A 4 mL yeast overnight culture was grown in YPD (\textit{Yeast} extract \textit{Peptone} \textit{Dextrose}) media or YMD (\textit{Yeast Minimal} \textit{Dextrose} media if selecting for a plasmid) at 30°C with rotation for aeration. The overnight culture was spun at 2000 rpm for 5 min. The supernatant was aspirated and the pellet was resuspended in 400 μL of Winston solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris HCl pH8.0 and 1 mM EDTA). The cell suspension was then added to a microfuge tube containing ~300 μL of glass beads and 300 μL of phenol/chloroform. The samples were vortexed for 5 min in a multi-vortexer at high speed, spun for 5 min at high speed and the supernatant removed and added to another tube containing 300 μL of phenol/chloroform.
for another extraction. The samples were then vortexed for ~15 sec and spun for another 5 min. The supernatant was removed and added to a tube containing 1 mL of absolute ethanol, mixed well and incubated at room temperature for 5-10 min. The samples were then spun for 10 min at room temperature or 4°C and the ethanol was aspirated. The pellet was air-dried and resuspended in 80 μL of TE containing RNase A (60 μg/mL).

**Yeast High Efficiency Transformation**

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

**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). 120 μL of 10% SDS was added and vortexed and cells were frozen at -80°C. 1.2 mL of phenol equilibrated in NaOAc extraction buffer was prewarmed to 65°C and added to the cells and mixed well. Samples were then incubated in a water bath set
at 65°C with constant shaking for one hour and then cooled on ice for 5 min. The tubes were then spun for 10 min at high speed and the top aqueous layer was removed and re-extracted with another 1 mL of equilibrated phenol. Samples were spun again and the aqueous phase was extracted with 1 mL of phenol/chloroform 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 3 M NaOAc and 1.4 mL of absolute ethanol at -20°C for 30 min to overnight. The RNA was pelleted by centrifugation for 10 min at 4°C and washed with 300 μL of 70% ethanol (diluted in DEPC water), air dried and resuspended in 600 μL of DEPC water. Concentration (µg/µL) was determined by measuring UV absorbance at 260 nm.

Northern Blot Analysis

Yeast cultures were grown in YPD and total RNA was extracted as described above. 10 µg of total RNA for each sample was resolved on a MOPS/formaldehyde/agarose gel and blotted to Zeta–Probe (Bio-Rad) membrane by wicking in transfer buffer (0.01 N NaOH + 3 M NaCl). PCR-generated double-stranded DNA containing the T7 promoter and region of homology to mRNA target was used as the template in the generation of a radioactive RNA antisense probe. The probes were 32P-α-UTP-labeled in a T7 polymerase in vitro transcription reaction at 37°C for 30 min to 1 hr. Zeta-probe membrane was incubated in ULTRAhyb (Ambion) for a pre-hybridization step. The radioactive probe was filtered to remove unincorporated UTP and added to the membrane. Hybridization was carried out overnight at 65°C. Membranes were washed twice in 2X SSC for 5 min each in 65°C, then washed twice in 0.1X SSC for 15 min each in 65°C. Zeta-probe membrane was then placed under phosphor screen in exposure cassette for 2 hr to overnight and scanned using Typhoon scanner (LSU Genomics Facility). The PCR-generated double-stranded DNA used to generate the RNA probes are as follows: PNC1 (DDO 898/899),
\textit{NPT1} (DDO 900/901), \textit{QNS1} (DDO 906/907), \textit{NMA1} (DDO 902/903), and \textit{NMA2} (DDO 904/905).

**Genetic Screen of UV-mutated rpd3Δ Strains**

For the genetic screen, \textit{rpd3Δ} cells (DDY 3133) were diluted and plated on YPD media for single cells and subjected to 12 sec doses of 200 \(\mu\)J of ultraviolet light to initiate random mutations in the yeast genome of each plated cell. After two days of growth on YPD, white colonies were isolated and used for the library transformation to complement and identify the mutated genes. 250 ng of genomic library plasmid DNA (total conc. 0.84 \(\mu\)g/\(\mu\)L), containing a yeast \textit{LEU2} marker and a bacterial AMP-resistant (\textit{AMP') gene (Courtesy of J. Rine) was transformed by high efficiency yeast transformation (see described) to produce \(\sim\)500 colonies plated on YMD –leu, 30\% adenine. Approximately 6000 colonies were screened for each round of transformations. The transformed cells were plated on YMD –leu, 30\% adenine to produce optimal red/pink pigmented colonies if the \textit{ADE2} gene is silenced. Twenty-four high efficiency transformations of the library plasmid were performed and two transformations were plated on 100 mm plates (12 total), including one negative control plate with no plasmid DNA. The red/pink colonies were patched to –leu 30\% ADE, -leu, -ura (for \textit{URA3} marker at the telomere), and 5-FOA (5-flouroorotic acid) + all mix (growth due to silencing of \textit{URA3}) plates. Positive growth patches were inoculated in YMD –leu, 30\% ADE and yeast genomic DNA extracted (See Winston prep described). Yeast total DNA was cleaned and eluted in 50 \(\mu\)L water using PCR-clean up kit (Zymo #D4003). 3 \(\mu\)L of yeast total DNA (containing library plasmid) was electroporated (2.5 kV, 25 \(\mu\)F, 200 \(\Omega\)) into 40 \(\mu\)L of electrocompetent \textit{E. coli} (DH5α) and plated on 2xyt+ampicillin bacterial media. Library plasmid DNA was extracted from ampicillin-resistant bacteria colonies (alkaline lysis miniprep) and recovered plasmid was digested with
EcoRI to detect restriction patterns different from those of previously recovered library plasmids containing normal heterochromatin-complementing genes such as SIR2, SIR3, etc. Recovered plasmids containing potential effector open reading frames (ORFs) were re-transformed back into the original UV-mutated strain they were first transformed into and analyzed for complementation by the phenotypic change of colony color from white (mutated effector) to red/pink (complement gene). Plasmids with positive complementation were then sequenced (LSU Genomics Facility) using plasmid-specific primers (DDO 821/822) and the sequences were BLAST searched through the SGD (Saccharomyces Genome Database) (Guldener, Munsterkotter et al. 2005).

**Gene Knockouts (LEU2 Replacements)**

Gene Knockouts were generated by first PCR amplifying LEU2 from plasmid template pDD672 with primers having regions of homology to both the plasmid carrying the LEU2 gene, and the flanking regions of the genes being deleted. Eight PCR reactions containing the 2.3 kb LEU2 PCR product were verified by gel electrophoresis and purified using PCR clean-up kit (Qiagen). The linear double-stranded DNA carrying the LEU2 gene was transformed into DDY 3161 and 3160 by high efficiency transformation as described. Cells were plated on YMD-leu to select for positive LEU2 integrants. DNA was extracted from Leu+ isolates and checked for proper integration by upstream and downstream PCR. DDO oligos for PCR check for upstream and downstream integration: BRE1 (914/198) and (915/199), GDH2 (918/198) and (919/199), BRE2 (927/198) and (928/199), GAT3 (931/198) and (932/199), RXT3 (935/198) and (936/199); 198 and 199 were interchanged with 823 and 824 (Table 2.1).

Plasmid Gap Repair of wild-type and UV-mutated BRE1 and BRE2 alleles were generated by PCR-amplified upstream and downstream genomic ends: BRE1 (DDO 1007/1008) Up and
Down, BRE2 (DDO 1016/1017) Up and (DDO 1018/1019) Down in parent strain DDY3. Amplified PCR products from both up and downstream for both genes were digested with Pac I at the integrated site. The upstream fragment for both genes was also digested with Sal I. The downstream fragment for BRE1 was digested with Xba I and the downstream fragment for BRE2 was digested with Not I. All restriction sites were integrated through PCR amplification of the genomic ends. The upstream and downstream fragments for BRE1 were ligated into pCR2.1 (TOPO-Invitrogen) and subcloned into pDD637 (LEU2). The upstream and downstream fragments for BRE2 were ligated into pCR4 (TOPO-Invitrogen) and subcloned into pDD637. Both pCR2.1 and pCR4 were double-digested with Sal I/Xba I for ligation of the BRE1 fragments (into pCR2.1) and Sal I/Not I for ligation of the BRE2 fragments (into pCR4); both inserts were then subcloned into pDD637, which generated new plasmids, pDD1163 and pDD1165. Both plasmids (1163 and 1165) were digested with Pac I to linearize the DNA and transformed into DDY 3 to rescue the wild-type allele by gap repair recombination (Orr-Weaver and Szostak 1983). The newly generated BRE1 plasmid (pDD1166) was transformed into UV-mutated strain DDY 3883 to verify rescue of the UV induced mutation, and the BRE2 plasmid was transformed into DDY3971.

**Chromatin Immunoprecipitation Assays**

Chromatin Immunoprecipitation (ChIP) was done according to (Kuo and Allis 1999). Wild-type and rpd3Δ cultures were grown in YPD to an optical density of 1.5 at 600 nm. The cells were then fixed with 3.25 mL of 37% formaldehyde (Fisher #F79-1) and 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 7.4). Cells 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 the protease inhibitors, pepstatin A (1 μg/mL), leupeptin (1 μg/mL) and PMSF (100 mM), and then lysed by vortexing with glass beads for 40 min at 4°C. The lysate tube was then placed into another open tube containing 10 μL of PMSF by poking the bottom of the sample tube containing glass beads with a hot 0.5 gauge needle, spun for a few seconds and collected into the new tube. To fragment the chromatin to suitable immunoprecipitate size (200-1000 bp), the lysate was sonicated six times for 10 sec each at 25% amplitude (Branson sonicator) with 0.9 sec intermittent pulses for a total of 10 sec and keeping the samples on ice ~1 min between sonication cycles. The samples were then spun at 13,200 rpm for 10 min in 4°C to pellet insoluble material. The supernatant containing soluble chromatin was collected as whole cell extract (WCE) and used for further analysis. 100 μL of lysate was mixed with 300 μL of lysis buffer containing the protease inhibitors. Then the mixture was incubated in protein A sepharose beads (Amersham Biosciences), that were 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, and rocked for 30 min at 4°C. The beads were then spun out and 5 μL of antibody (H3K4me3) (Abcam #ab8580-100, ~1 mg/mL) was added to the supernatant. The samples were incubated overnight at 4°C with gentle rocking. The antibody reactions were spun and the supernatant was transferred to fresh tubes. 30 μL of washed and equilibrated beads was added and the samples were incubated at 4°C for 1 hour on a rotating nutator to pull down the antibody-bound H3K4me3 histone proteins cross-linked to their respective DNA locus. The tubes were spun again and the supernatant was transferred to fresh tubes as unbound fractions. The beads were washed for 5 min each with 1 mL of the following buffers: i) Lysis buffer with protease inhibitors, ii) Wash buffer I (Lysis buffer + 500 mM NaCl), iii) Wash buffer II (10 mM Tris 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% Sodium Deoxycholate, 1 mM EDTA), and iv) TE (1X). After
TE wash, the tubes were spun again to remove any residual liquid. After this step, an efficient and rapid Chelex-resin based procedure was used to isolate PCR-ready DNA (Nelson, Denisenko et al. 2006). This method utilizes 100 μL of 10% Chelex-100 (10 g/100 mL H₂O, Bio-Rad) added to the washed protein A beads and vortexed. Samples were boiled for 10 min, cooled, and 1 μL of Proteinase K (100 μg/mL) was added and the beads were incubated for 30 min at 55°C while shaking, then boiled for another 10 min. The suspension was centrifuged and the supernatant was collected. The Chelex/protein A beads fraction was vortexed with another 100 μL water, 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 475 μL of Elution buffer (1% SDS, 0.1 M NaHCO₃), and 20 μL of 5 M NaCl. The tubes were incubated at 65°C (four hours to overnight) to reverse the protein/DNA crosslinks. To each tube 20 μL of 1M Tris pH 8.0, 10 μL of 0.5 M EDTA and 1 μL of Roche Proteinase K (20 mg/mL) was added and incubated at 55°C for 30 min. The sample was extracted once with phenol/chloroform, and then precipitated in 50 μL of NaOAc, 1 μL of glycogen (Roche, 20 mg/mL stock), and 1 mL of absolute ethanol and precipitated at -20°C. The samples were then spun at 4°C for 10 min and the pellets washed with 300 μL of 70% ethanol, air dried and redissolved in 200 μL TE + RNaseA (60 μg/mL).

Table 2.1. Oligos used in this study:

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDO 59</td>
<td>5’-GAATTCGTTAACCGGATCCACTAGGGACGTTAAGGTTTG-3’ – tRNA Up</td>
</tr>
<tr>
<td>DDO 60</td>
<td>5’-GAATTCGTTAACCGGATCCCTTTTTGCCATGGCATG-3’ – tRNA Down</td>
</tr>
<tr>
<td>DDO 184</td>
<td>5’-GTTAAGCTCACTAGCTATAGGGC-3’ – T7 Primer</td>
</tr>
<tr>
<td>DDO 198</td>
<td>5’-GCACCTCTCAGTACAAATCTGC-3’ – pRS universal RC, upstream</td>
</tr>
<tr>
<td>DDO 199</td>
<td>5’-CCGCACAGATGCAGAAGGAG-3’ – pRS universal RC, downstream</td>
</tr>
<tr>
<td>DDO 478</td>
<td>5’-TGACTAAGTAGAGGAACAGAACTAGT-3’ – HMR B-1</td>
</tr>
<tr>
<td>DDO 479</td>
<td>5’-TCTCATACGTTAATTTATGAACCTAC-3’ – HMR B-2</td>
</tr>
<tr>
<td>DDO 480</td>
<td>5’-TCAATTGATTTAATGACTAGTCTGG-3’ – HMR C-1</td>
</tr>
<tr>
<td>DDO 481</td>
<td>5’-CAATGCAATTTGATAAAACCATAG-3’ – HMR C-2</td>
</tr>
<tr>
<td>DDO 482</td>
<td>5’-GCCGATATAATTTATCATGTTTGG-3’ – HMR D-1</td>
</tr>
<tr>
<td>DDO 483</td>
<td>5’-TCCTAAATTCGTGACAAATTTTC-3’ – HMR D-2</td>
</tr>
<tr>
<td>DDO 484</td>
<td>5’-CCAATCCCGATCTGCAGATTACCT-3’ – HMR-dDNA-E1</td>
</tr>
</tbody>
</table>

(Table 2.1 continued)
**RESULTS**

**Genetic Screen of Genomic Library Transformed Into Ultraviolet-Mutated rpd3Δ Strains.**

*RPD3* deleted strains exhibit a red pigment phenotype due to the ectopic silencing of a downstream *ADE2* marker gene. This marker gives an indication of whether or not silencing has occurred.
spread from \textit{HMR} through the \textit{tRNA} barrier element to repress \textit{ADE2} expression and can be optimally assessed by growth on minimal media with 15-30\% adenine supplement (Figure 2.1).

\textbf{Wild-Type colonies} \hspace{1cm} \textbf{\textit{rp}d3\textit{A} colonies}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Visual and schematic representations comparing the wild-type and \textit{rp}d3\textit{A} colony color phenotype. Wild-type colonies of \textit{Saccharomyces} typically show a white to cream color demonstrating that the spread of silencing heterochromatin from the silencer sequences at \textit{HMR} is halted at the \textit{tRNA}\textsuperscript{thr} barrier allowing the inserted downstream \textit{ADE2} gene to be expressed. \textit{rp}d3\textit{A} colonies repress expression of the inserted downstream \textit{ADE2} gene and can display a pink to a much darker red color depending on the degree of silencing of the \textit{ADE2} gene in each cell of the colony and the free adenine available in the growth media. The strains shown are DDY 3136 (WT) and DDY 3133 (\textit{rp}d3\textit{A}).}
\end{figure}
We also assessed effects of silencing at the telomeres, as the *HMR-ADE2* strain also contained an inserted *URA3* marker on chromosome VII proximal to the telomere ends. *URA3* expression was assessed by growth on minimal media containing 5-FOA (Boeke, LaCroute et al. 1984). Subjecting *rpd3Δ* strains with doses of UV-radiation typically induces random mutations in the yeast genome such as point mutations and pyrimidine dimers. Phenotypical analysis of any such mutations that would cause a loss-of-function in a potential effector of the increased silencing effect should show a reversion of colony color from the red/pink of *rpd3Δ* to the wild-type white/cream.

For establishing these random mutations across the yeast genome, *rpd3Δ* cells (DDY 3133) were spread on YPD plates for the growth of isogenic yeast colonies derived from a single colony. The plates were then exposed to 200 µJ of ultraviolet light for ~20 sec. Nine YPD plates with *rpd3Δ* cells were treated with ultraviolet light and three were untreated control plates. After UV exposure and growth at 30°C for two days, colonies containing mutations resulting in reversion from the red colony color to the white colony color phenotype were isolated. UV-mutagenesis produced 27 isolates (white colonies) from *rpd3Δ* strains after screening over 20,000 colonies. The colony isolates change to a white color was verified by second streaks on YPD media and YMD+all with 30% adenine.

Individual mutated isolates were then transformed with a genomic plasmid library by high efficiency transformation (see Methods) and plated on YMD -leu to select for cells containing the library plasmids and grown at 30°C for two to three days. Colony growth analysis was to strictly observe any growing colonies that had reverted back to the original red colony color phenotype, suggesting either complementation or suppression of the UV induced mutation. The genomic library plasmids contained average insert sizes of 8-12 kb. Because of the compact
nature of the yeast genome, each plasmid contained multiple ORFs, thus verification of potential ORFs would require analysis of individual genes contained on each plasmid. 18 out of the 27 white colony revertant UV-mutated strains exhibited red colony isolates after transformation of the genomic plasmid library after screening >12,000 colonies per strain transformed (Table 2.2).

**Table 2.2. UV-mutated rpd3Δ strains transformed with potential effector genes from genomic library plasmids.**

<table>
<thead>
<tr>
<th>Strain</th>
<th># of minipreps (isolates)</th>
<th>Genes In Plasmid Unique EcoRI patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3352</td>
<td>7 then 14(2 sets)</td>
</tr>
<tr>
<td>2</td>
<td>3368</td>
<td>SIR3</td>
</tr>
<tr>
<td>3</td>
<td>3408</td>
<td>#2(BRE2, GAT3) and #4(SIR4)</td>
</tr>
<tr>
<td>4</td>
<td>3351</td>
<td>#2 and #4</td>
</tr>
<tr>
<td>5</td>
<td>3369</td>
<td>#6(BRE2),7(SIR3),10(GDH2),12</td>
</tr>
<tr>
<td>6</td>
<td>3353</td>
<td>#1A(GNS1),3A(GNS1),3B</td>
</tr>
<tr>
<td>7</td>
<td>3354</td>
<td>All transformants were dark pink</td>
</tr>
<tr>
<td>8</td>
<td>3406</td>
<td>#1A(RXT3),3A(BRE1),5A,5C</td>
</tr>
<tr>
<td>9</td>
<td>3305</td>
<td>No pink colonies</td>
</tr>
<tr>
<td>10</td>
<td>3306</td>
<td>A1, A1</td>
</tr>
<tr>
<td>11</td>
<td>3407</td>
<td>#2A, 4A, 5A</td>
</tr>
<tr>
<td>12</td>
<td>3438</td>
<td>#4A, 5B, 5C, 6A, 14A(NPT1)</td>
</tr>
<tr>
<td>13</td>
<td>3440</td>
<td>#1B</td>
</tr>
<tr>
<td>14</td>
<td>3439</td>
<td>#3A, 11A</td>
</tr>
<tr>
<td>15</td>
<td>3441</td>
<td>#1A</td>
</tr>
<tr>
<td>16</td>
<td>3442</td>
<td>#8C</td>
</tr>
<tr>
<td>17</td>
<td>3443</td>
<td>#10C</td>
</tr>
<tr>
<td>18</td>
<td>3444</td>
<td>#6C, 7A, 10C</td>
</tr>
</tbody>
</table>

Listed are the 18 *HMR-ADE2* UV-mutated *rpd3Δ* strains (white) that, upon genomic library transformation, gave rise to isolates that had reverted to the original *rpd3Δ* colony color phenotype (pink). After plasmid rescue, the plasmid inserts were sequenced and analyzed for any genes that were potentially involved in the silencing effect. The genotype of each strain is isogenic and listed in Table 2.4.

The newly transformed colonies exhibiting red colony color were isolated and the transformed plasmid containing the potential effector was recovered through yeast DNA extraction (Winston prep - see Methods). To recover the library plasmid, the Winston prep DNA was electroporated into *E. coli* for plasmid amplification and subsequent analysis. The genomic
library plasmids contained an ampicillin resistant gene which was used to select for *E. coli* cells that had retained the library plasmid on 2xyt+ampicillin bacterial media. After DNA extraction from the *E. coli* cells, the candidate library plasmid was subjected to restriction analysis using *EcoRI* and known sites in the plasmid vector to analyze any unique restriction patterns that differed from what was seen from recovered plasmids that contained any normal heterochromatin-complementing genes such as *SIR2, SIR3*, etc. The library plasmids were then sequenced using library plasmid-specific primers (DDO 821-Up and DDO 822-Down) to determine if any ORFs within the plasmid insert could be directly or indirectly responsible for participating in the silencing effect or the establishment of heterochromatin. The recovered plasmid insert sequences were BLAST (Basic Local Alignment Search Tool) searched using the *Saccharomyces Genome Database* (SGD) (Guldener, Munsterkotter et al. 2005).

We identified seven genes outside the normal heterochromatin-forming complement that were highly ranked as potential effectors of the silencing effect. These genes were *BRE1, BRE2, GDH2, GAT3, RXT3, QNS1*, and *NPT1*. Table 2.2 lists the 18 white colony strains with the number of revertants (minipreps) for each set of transformations. Any isolate containing a potential effector listed in red was used for complementation analysis. As expected in this particular genetic screen, we identified a number of recovered plasmids containing *SIR* genes, mainly *SIR3* and *SIR4*, a few of which are also listed in Table 2.2. Each of the seven genes identified in the genetic screen are functionally described as follows: *QNS1* - glutamine-dependent NAD⁺ synthetase; *NPT1* - nicotinate phosphoribosyltransferase, acts in salvage pathway of NAD⁺ biosynthesis; *BRE1* - E3 ubiquitin ligase for Rad6p, required for the recruitment of Rad6p to promoter chromatin and ubiquitylation of histone H2B on K123; H2BK123 ubiquitylation is required for subsequent methylation of histone H3 (K4 and K79);
GDH2 – NAD\(^+\)-dependent glutamate dehydrogenase, which converts glutamate to \(\alpha\)-ketoglutarate; BRE2 - Subunit of the COMPASS (Set1c) complex, which methylates histone H3K4; GAT3 - Protein containing GATA family zinc finger motifs (transcription factor activity); RXT3 - Subunit of Rpd3(L) complex; contributes to histone deacetylase activity and transcriptional repression.

QNS1, glutamine-dependent NAD\(^+\) synthetase, was recovered in three separate isolates after library plasmid transformation and NPT1, nicotinate phosphoribosyltransferase, was found in one. Also found in the genetic screen were two isolates of BRE1, E3 ubiquitin ligase for Rad6p, four isolates of BRE2, subunit of the COMPASS (Set1c) complex, two isolates of GDH2, NAD\(^+\)-dependent glutamate dehydrogenase, three isolates of GAT3, GATA family zinc finger motifs, and two isolates of RXT3, subunit of Rpd3(L) complex. BRE2 and GAT3 are two genes which are in close approximation to each other (~2 kb) in the *S.cerevisiae* genome and two isolates recovered contained both ORFs. BRE2 and GAT3 were individually identified as effectors since we recovered two independent plasmid isolates containing only the BRE2 ORF and one independent plasmid isolate containing only the GAT3 ORF. For complementation analysis, all library plasmids that were recovered and identified as having potential effectors were re-transformed back into their original UV-mutated *rpd3A* strain (white colonies). All strains, after plasmid complementation, showed a colony color phenotype change from white to red, thus further verifying that the recovered library plasmids contained genes that were potential effectors.

**Northern Analysis of NAD\(^+\) Biosynthesis Genes in Wild-Type vs. rpd3A Cells**

Since we identified multiple genes that potentially affect NAD\(^+\) levels in cells, we pursued this class of genes first. The deacetylation reaction of H4K16 by Sir2p, and thus the propagation
of silencing, relies on the cofactor nicotinamide adenine dinucleotide (NAD$^+$) to catalyze the deacetylation reaction (Imai, Armstrong et al. 2000; Landry, Slama et al. 2000; Smith, Brachmann et al. 2000). Two of the genes identified in the genetic screen, \textit{QNS1} and \textit{NPT1}, are components of the NAD$^+$ biosynthesis pathway. Since the NAD$^+$-coupled Sir2p deacetylase reaction is the foundation of heterochromatic silencing at \textit{HMR}, we hypothesized that \textit{RPD3} could either be directly or indirectly involved in the regulation of these pathway genes and thus the expression of these two genes may affect Sir2p activity. The rationale is that Rpd3p may function as a repressor of \textit{QNS1} or \textit{NPT1} expression at their own promoters and thus the deletion of \textit{RPD3} may enhance the expression of these genes which can result in increased availability of NAD$^+$ as a cofactor for the enzymatic action of Sir2p at the \textit{HMR} region.

We performed a Northern analysis on the two genes identified in the screen, \textit{QNS1} and \textit{NPT1}, and included the gene \textit{PNC1} which is responsible for the conversion of nicotinamide to nicotinic acid and thus the clearance of nicotinamide (a product of the Sir2p deacetylase reaction) which can inhibit the enzymatic action of Sir2p.

![Northern analysis](image)

\textbf{Figure 2.2. Northern analysis of NAD$^+$ biosynthesis pathway genes in wild-type vs. \textit{rpd3A} cells.} NAD$^+$ pathway genes identified in the genetic screen were analyzed for differential gene expression based on Sir2p dependence on NAD$^+$ for its proper function. Probes generated for mRNAs of \textit{QNS1}, \textit{PNC1}, and \textit{NPT1} showed no reproducible qualitative difference in gene expression in wild-type vs. \textit{rpd3A} cells. Actin (\textit{ACT1}) was used as an input control.

As shown in the Northern blot analysis (Figure 2.2), the mRNA expression of the genes \textit{QNS1}, \textit{PNC1}, and \textit{NPT1} showed no reproducible difference between wild-type (DDY 3 and 4)
and rpd3Δ (DDY 3018 and 3021) strains even after three replications of the Northern experiment. The Northern assay was also repeated in three replicates using different rpd3Δ laboratory strains, DDY 1671 and 1677, and no difference in expression was noted between WT and rpd3Δ. These consistent null results were also seen in Northern analyses of the genes NMA1, NMA2, QPT1 (involved in the NAD\textsuperscript{+} de novo pathway), and TNA1 (involved in the NAD\textsuperscript{+} salvage pathway – nicotinic acid plasma membrane permease) (data not shown). The expression of ACT1 (Actin) was used as an input (loading) control for all Northern analyses. The five remaining genes identified in the genetic screen were knocked out and subjected to genetic, phenotypic, and complementation analysis.

**Genomic Knockouts of BRE1, BRE2, GDH2, GAT3, and RXT3 and rpd3Δ Cross Analysis**

The original UV-mutated strains (white colonies) which contained random mutations in potential effector genes may or may not have had a complete loss-of-function. Such mutations can result in partially truncated proteins with a range of lower-end activity and high turnover rates, but not necessarily a complete loss-of-function. To verify that the five remaining genes were bona-fide effectors, the complete ORFs of each gene were individually knocked out and replaced with a LEU2 gene marker. This complete knockout of the coding sequence would insure that there was a complete loss-of-function of the potential effector genes. The analysis was to assess if each individual gene knockout, when crossed to an rpd3Δ strain, would derepress expression of the ADE2 gene and result in a colony color phenotype from red (rpd3Δ) to white (rpd3ΔmutX).

As exemplified in Figure 2.3, the question remains if the potential effector is directly involved in contributing to the integrity of the tRNA barrier element or if the effects are indirect and results in a gradual loss of silencing before reaching the ADE2 gene. The five separate gene
knockouts were generated through transformation of linear double-stranded DNA containing a functional \textit{LEU2} gene and regions of homology to the target loci on both the 5’ and 3’ ends of the DNA duplex into strain DDY 3161.

![Diagram](image)

**Figure 2.3. Schematic representation of the \textit{HMR} region, tRNA barrier element and inserted \textit{ADE2} gene.** \textit{rp}d\textit{3}A strains enhance silencing from \textit{HMR} downstream to the \textit{ADE2} gene. Our hypothesis states that mutating (or deleting) a potential effector gene in the \textit{rp}d\textit{3}A background will abolish the spread of silencing in the double mutant and the \textit{ADE2} gene will be expressed. Whether or not the effector contributes to the integrity of the barrier element or influences the robustness of silencing remains to be answered.

The oligonucleotides used for amplifying the \textit{LEU2} gene for each knockout were as follows: \textit{BRE1} (912/913), \textit{GDH2} (916/917), \textit{BRE2} (925/926), \textit{GAT3} (929/930), and \textit{RXT3} (933/934). The PCR-amplified linear DNA was transformed using high efficiency transformation (see Methods). Gene integrations of \textit{LEU2} for the \textit{BRE1} locus and the \textit{RXT3} locus were verified by upstream and downstream PCR. \textit{BRE2}, \textit{GAT3}, and \textit{GDH2} were verified by Southern blotting. Each newly created strain carrying one knock out each of the five genes assayed (\textit{BRE1}, \textit{BRE2}, \textit{GAT3}, \textit{RXT3}, \textit{ADE2}).
and GDH2) were subsequently crossed to an rpd3Δ (DDY 3155) strain and analyzed for colony color phenotype change in the double mutants.

Figure 2.4. BRE1 and BRE2 are effectors of the rpd3Δ silencing effect. Knockouts of either BRE1 or BRE2 in an rpd3Δ background halted the spread of silencing past the tRNA barrier to the downstream inserted ADE2 gene. The colony color phenotype was reverted from a dark red colony color in the rpd3Δ strain to a white colony color in both the bre1Δrpd3Δ and bre2Δrpd3Δ strains.

Of the five strains assayed with individual genes deleted, only strains containing mutated BRE1 or BRE2, when crossed with an rpd3Δ strain, showed a significant change in colony color phenotype from red to white, thus abolishing the enhanced silencing effect of rpd3Δ at HMR (Figure 2.4). The individual gene knockouts of GDH2, GAT3, and RXT3 all exhibited no phenotype change (red to white) when crossed to the rpd3Δ background indicating that UV mutagenesis did not produce a complete loss-of-function in these three genes. The bre1Δrpd3Δ
and \( \text{bre2Arpd3A} \) phenotype change was verified in complementation assays by transforming the plasmids containing the \( \text{BRE1} \) and \( \text{BRE2} \) coding sequences back into the double knockout strains \( \text{bre1Arpd3A} \) and \( \text{bre2Arpd3A} \), respectively. The plasmids used for complementation were the original plasmids obtained from the genomic library that contained the \( \text{BRE1} \) and \( \text{BRE2} \) ORFs in the \( \sim10 \) kb insert. Since the original library plasmids contained more than one potential ORF (e.g. some library inserts contained both \( \text{BRE2} \) and \( \text{GAT3} \)), we had to verify that \( \text{BRE1} \) and \( \text{BRE2} \) were the only genes (coding sequences) responsible for complementation of the \( \text{rpd3A} \) silencing effect. We separately transformed laboratory-generated cloned plasmids containing the coding sequence of wild-type \( \text{BRE1} \) and \( \text{BRE2} \) which were generated by gap repair techniques (see Methods – Gene Knockouts) from wild-type strains. The individual \( \text{bre1A} \) or \( \text{bre2A} \) knockout strains (no \( \text{rpd3A} \) background) created did not have any effect on the colony color phenotype compared to WT (all white) which establishes \( \text{bre1A} \) and \( \text{bre2A} \) epistatic to \( \text{rpd3A} \) in a regulatory pathway.

When analyzing enhanced silencing at the telomeres with WT, \( \text{rpd3A} \), and our generated knockout strains, including the double mutants, we took advantage of an inserted \( \text{URA3} \) at the telomeres for a growth marker and assayed for degrees of growth or non-growth on both YMD minus uracil and 5-FOA+all mix. Silencing at the telomere on chromosome VII-L showed nearly the same consistency of silencing as seen at the \( \text{HMR} \) region between WT and \( \text{rpd3A} \) strains. In WT cells, there was growth on minus uracil but no growth on 5-FOA. In \( \text{rpd3A} \) strains, there was no growth on minus uracil but growth on 5-FOA due to silencing of the \( \text{URA3} \) gene at the telomere (Table 2.3). The \( \text{bre1A} \) mutant grew slightly more on 5-FOA+all mix compared to WT and showed less growth on minus uracil compared to WT indicating that \( \text{BRE1} \) is potentially contributing to silencing at the telomeres. The \( \text{rpd3A} \) strain showed an expected extended
silencing at the telomeres with no growth on minus uracil and full growth on 5-FOA+all mix. The *bre1Δrpd3Δ* double mutant rescued partial growth on minus uracil, while there was full growth on 5-FOA+ all mix, suggesting partial de-repression of telomeric silencing.

**Table 2.3. Phenotypic analysis of silencing at telomere region (URA3) on chromosome VII-L.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>15% adenine</th>
<th>YMD -ura</th>
<th>5FOA+all</th>
</tr>
</thead>
<tbody>
<tr>
<td>3136 – WT</td>
<td>white</td>
<td>+++++</td>
<td>-</td>
</tr>
<tr>
<td>3133 – rpd3Δ</td>
<td>pink</td>
<td>-</td>
<td>+++++</td>
</tr>
<tr>
<td>3761 – bre1Δ</td>
<td>white</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>3790 – bre1Δrpd3Δ</td>
<td>white</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>3878 – bre2Δ</td>
<td>white</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>3133 – bre2Δrpd3Δ</td>
<td>white</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>3764 – gdh2Δ</td>
<td>white</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>3846 – gat3Δ</td>
<td>white</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>3767 – rxt3Δ</td>
<td>white</td>
<td>+++++</td>
<td>+++</td>
</tr>
</tbody>
</table>

*HMR* and telomeric silencing was assessed through phenotypical analysis of colony color in three separate isolates grown on YMD 15% adenine (*HMR*), YMD -ura (telomere), and growth on 5-FOA+all (telomere). Colony color on 15% adenine – white (*ADE2* expressed) or pink (*ADE2* repressed), - no growth, +very little growth, ++spotty growth, +++more growth, ++++full growth.

The *bre2Δrpd3Δ* double mutant had very little growth on minus uracil similar to *rpd3Δ* and full growth on 5-FOA+all, suggesting a very weak de-repression of telomeric silencing. The *bre2Δ* mutant showed very little growth on minus uracil and the growth on 5-FOA+all was similar to its double mutant of *bre2Δrpd3Δ*. These results indicate that the mutants, *bre1Δ* and *bre2Δ* in the *rpd3Δ* background, do not halt the spread of silencing at telomeres to the extent the same
mutations do at HMR. Interestingly, based on these initial results, BRE1 may play a role in silencing at the telomeres, while BRE2 seems to have no significant effect.

**ChIP (Chromatin Immunoprecipitation) of H3K4me3 at HMR in WT vs. rpd3Δ**

BRE1 encodes the E3 ubiquitin ligase for Rad6p (E2), which in an E2-E3 complex together is recruited to promoter regions and ubiquitylates histone H2B on K123. This ubiquitylation is required for the subsequent trimethylation of histone H3K4 by Set1p and H3K79 by Dot1p. Set1p is the catalytic subunit of the COMPASS complex which mono-, di-, or tri-methylates H3K4. Trimethylation of H3K4 is normally associated with active transcription, particularly in the coding regions of genes (Bernstein, Humphrey et al. 2002; Santos-Rosa, Schneider et al. 2002). BRE2 encodes a functional subunit of the COMPASS (Set1p) complex. Since both BRE1 and BRE2 are either directly or indirectly involved in the trimethylation of H3K4 and H3K79, we hypothesized that there might exist a qualitative difference in the trimethylation status of H3K4 at the HMR region and downstream loci through to the ADE2 gene in strains of WT, rpd3Δ, and the double mutants, bre1Δrpd3Δ and bre2Δrpd3Δ.

We have already shown that the silencing effect of rpd3Δ at HMR is abolished in both the double mutants of bre1Δrpd3Δ and bre2Δrpd3Δ. If BRE1 or BRE2 have a direct effect on the methylation status of the underlying chromatin as effectors, we hypothesize that there would be reduced enrichment of trimethylated H3K4 (H3K4me3) in the silenced regions downstream of the tRNA barrier element in rpd3Δ strains and higher enrichment of H3K4me3 at these same regions in both the WT and double mutant strains of bre1Δrpd3Δ and bre2Δrpd3Δ. We performed chromatin immunoprecipitation (ChIP) using an antibody specific for H3K4me3 to immunoprecipitate regions of HMR and downstream loci enriched in H3K4me3 (Figure 2.5). The PCR primer sets used to amplify enriched regions include four regions in and between the
two silencer sequences, E and I (A-D), the tRNA barrier element (E), and three regions in the inserted downstream ADE2 gene (F-H).

![Diagram of HMR and ADE2 loci with oligo primer sets labeled A through H.](image)

**Figure 2.5.** ChIP (chromatin immunoprecipitation) of targeted H3K4me3 along the HMR and downstream loci in wild-type vs. rpd3Δ cells. Schematic representation of the HMR region with oligo primer sets labeled A through H at the regions where immunoprecipitated H3K4me3 cross-linked to DNA was PCR amplified. Shown below the schematic are the qualitative enrichment levels of PCR-amplified regions A through H in WT and rpd3Δ strains tested. Also included is a no antibody control (No Ab) which shows levels of non-specific background enrichment and an input control for loaded DNA quantities.

As shown in Figure 2.5, there was no significant (or reproducible) difference in the enrichment of H3K4me3 across the HMR and downstream region in WT vs. rpd3Δ cells. In fact, qualitative enrichment levels appear to be almost identical in all regions assayed between the two strains, with regions B, D, and E (Intra-HMR, I silencer, and tRNA gene) possibly showing slightly less enrichment than the other sites in both strains (although Input Control for B and D also shows less enrichment). We also followed up these ChIP experiments by assaying the enrichment of
H3K4me3 in the double mutants of bre1Δrdp3Δ (DDY 3790) and bre2Δrdp3Δ (DDY 3918) using the same PCR amplified regions comparing rpd3Δ and WT (data not shown). There was also no reproducible difference in the trimethylation status of the entire HMR region using the same primer sets A through H in both double mutants. Consistent with these null ChIP results was the lack of consistent levels of immunoprecipitated H3K4me3 protein from Western blot experiments in WT, rpd3Δ, and both double mutant strains tested.

COS7 is a protein of undefined function but is a member of the DUP380 subfamily of conserved, often subtelomerically-encoded proteins; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies (Spode, Maiwald et al. 2002). The COS7 gene was previously used by Kirmizis et al. (2007) as a positive control in ChIP assays for 5’ enrichment of H3K4me3 levels (Kirmizis, Santos-Rosa et al. 2007). We employed the same strategy and unlike this published report, our results showed equivalent levels in enrichment of H3K4me3 at both the 5’ and 3’ ends of COS7 (whereas trimethylation enrichment is reported as progressively decreasing from the 5’ to the 3’ end of the ORF). These results, along with the acquisition of newer evidence on the mechanisms of silencing at telomeres involving the redistribution of Sir proteins, (Santos-Rosa, Bannister et al. 2004; Venkatasubrahmanyam, Hwang et al. 2007; Ehrentraut, Weber et al. 2010), have prompted us to refine both our model and hypothesis of the mechanism by which enhanced silencing occurs at HMR in rpd3Δ cells. Our adjusted hypothesis also reflects the role that BRE1 and BRE2 play in the establishment of extended silencing (methylation).

DISCUSSION

Previous results in our laboratory showed an enhanced silencing effect at HMR in rpd3Δ strains, which bypassed the downstream tRNA barrier element. A novel bromodomain
containing gene, YTA7, was also shown to be required for restricting the propagation of silencing independent of the tRNA barrier element (Jambunathan, Martinez et al. 2005). These results implicate other factors can be involved in abolishing the spreading of heterochromatin such as other bromodomain-containing proteins, along with factors involved in the proper functioning of barriers. Alternatively, there are potential factors besides the ubiquitous Sir proteins involved in the establishment, propagation, and maintenance of the increased silencing effect at HM, telomeres, and ribosomal DNA loci in the rpd3Δ background. Using a genetic screen involving UV-mutated rpd3Δ yeast strains, our results identified two effectors of the rpd3Δ silencing effect that are essential for the extended spreading of heterochromatin which bypasses or spreads through the tRNA barrier element at HMRa. These two effectors were identified as BRE1 and BRE2. BRE1 encodes an E3 ubiquitin ligase for Rad6p and exists as a complex between the two, where the heterocomplex is recruited to promoter regions and is responsible for the ubiquitylation of H2BK123. This ubiquitylation of H2BK123 is required for the subsequent trimethylation of H3K4 by the COMPASS complex and trimethylation of H3K79 by Dot1p. Because of the direct methylation function of both these genes, it was of interest to analyze this histone modification status of the HMR region.

Although we took advantage of the inserted URA3 gene on chromosome VII-L near the telomere to assess silencing at telomeric ends, our main focus was to identify potential effectors of heterochromatic silencing at HMR, since the goal and the focus of our studies was to elucidate the mechanisms involved in the enhanced spread of silencing past the tRNA barrier element. Based on the limited amount of preliminary data generated from growth assays that depend on a marker gene at a telomere, there appears to be effects on silencing occurring at telomeres mediated by BRE1.
Strains deleted for RPD3 not only exhibit enhanced silencing at HM loci, telomeres, and rDNA, but they also have a sporulation defect in homozygous diploid strains (rpd3Δ/rpd3Δ). To determine if BRE1 or BRE2 contributed to this defect, we attempted to sporulate the homozygous diploids rpd3Δbre1Δ/rpd3Δbre1Δ and rpd3Δbre2Δ/rpd3Δbre2Δ on carbon source-limiting nitrogen-rich nutrient media and observe if these double mutant strains would correct the sporulation defect that occurs in the homozygous rpd3Δ/rpd3Δ strains. Both diploid double mutants showed no change of function in the sporulation/meiosis defect in rpd3Δ strains. After 3-4 days on nutrient limiting media at 30C and using light microscopy, we saw no formation of sporulated tetrads from the diploids on YPD media. Another phenotype of an rpd3Δ strain is its sensitivity to ethidium bromide during cell growth and mitosis. Neither of the double mutants showed a decrease in growth sensitivity to ethidium bromide (growth on ethidium bromide-supplemented YPD was not enhanced in the double mutants) compared to WT.

The results we obtained in our ChIP assays were surprising since previous studies of the COS7 gene have shown that there are progressive differences in H3K4me3 enrichment between promoter and coding regions with a gradual decline of H3K4me3 levels from the 5’ to the 3’ end (Kirmizis, Santos-Rosa et al. 2007). Another gene, PPH3, encodes the catalytic subunit of an evolutionarily conserved protein phosphatase complex containing Psy2p and the regulatory subunit Psy4p. PPH3 is required for cisplatin resistance and is involved in the activation of Gln3p (Hoffmann, Jung et al. 1994). PPH3 is a constitutively active gene positively regulated by the trimethylation of H3K4 by Set1p and has been employed as a positive control in previous ChIP studies using antibodies specific for H3K4me3 (Santos-Rosa, Schneider et al. 2002). Our ChIP assays at the genomic locus of PPH3 also resulted in equivalent enrichments of H3K4me3 contrary to previously reported results.
These inconsistent and/or irreproducible ChIP results throughout the entire study shed doubt on the quality of the polyclonal H3K4me3 antibody (AbCam #ab8580). The antibody used was reportedly tested by the manufacturer with a blocking assay where the antibody detects a 17 kDa band on a Western blot which is completely blocked by the addition of histone H3K4me3 peptide. Partial blocking was also observed with addition of histone H3K4me2 peptide (http://www.abcam.com/Histone-H3-tri-methyl-K4-antibody-ChIP-Grade-ab8580.html). The specificity of the antibody used in our assays was not tested by the manufacturer in vivo, and our results may reflect differences in binding of the antibody by short peptides versus the native histone H3 protein.

The elusive mechanism of enhanced silencing in rpd3Δ strains at HM, telomeres, and rDNA have perplexed researchers for over fifteen years. With the growing complexity of the ‘histone code’ hypothesis, many studies have tried to elucidate the mechanism of silencing by the pattern of histone modifications using genome-wide high-throughput ChIP-ChIP based assays. Research on chromatin has also delved into the degree of chromatin-associated proteins or other factors that directly bind to chromatin along with the specific modifications on particular amino acid residues on histone proteins. More recent studies have looked to how indirect effects can possibly play a role in the establishment or disruption of silencing heterochromatin. Our adjusted hypothesis includes the supposition of indirect effects on how BRE1 and BRE2 contribute to establishing the extended silencing in rpd3Δ strains. Chapter four of this dissertation will further expand on our modified hypothesis and concomitant strategy in future experimental design and execution, while at the same time taking into consideration the results of new studies, which report intriguing evidence of indirect mechanisms to these silencing effects.
Table 2.4. Strains of *S. cerevisiae* used in this study:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDY</td>
<td>MATa ade2 his3 leu2 lys2 trp1 ura3 VII-URA3-TEL ppr1A::TRP1 HMR-ADE2 rpd3A::KanMX</td>
</tr>
<tr>
<td>DDY 2</td>
<td>MATa/Ma ade2/ADE2 his3/his3 leu2/leu2 LYS2/lys2A trp1/trp1 ura3/ura3</td>
</tr>
<tr>
<td>DDY 3</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2A trp1-1 ura3-1 *GAL can1-100</td>
</tr>
<tr>
<td>DDY 4</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2A trp1-1 ura3-1 *GAL can1-100</td>
</tr>
<tr>
<td>DDY 19</td>
<td>MATa his4</td>
</tr>
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<td>MATa his4</td>
</tr>
<tr>
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</tr>
<tr>
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<td>MATa ADE2 his3 leu2 lys2 ADE2 trp1 ura3 rpd3A::LEU2</td>
</tr>
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<td>MATa ADE2 his3 leu2 lys2 trp1 ura3 rpd3A::KanMX HMR wild type</td>
</tr>
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</tr>
<tr>
<td>DDY 3133</td>
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</tr>
<tr>
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</tr>
<tr>
<td>DDY 3881-3884</td>
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<tr>
<td>DDY 3970-3973</td>
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<tr>
<td>DDY 3984-3986</td>
<td>MATa ade2 his3 leu2 lys2 trp1 ura3 VII-L-URA3-TEL HMR-ADE2 ppr1A::TRP1 bre1A</td>
</tr>
<tr>
<td>DDY 3987-3989</td>
<td>MATa ade2 his3 leu2 lys2 trp1 ura3 VII-L-URA3-TEL HMR-ADE2 ppr1A::TRP1 bre2A</td>
</tr>
</tbody>
</table>

**LITERATURE CITED**


CHAPTER THREE

AUTOREGULATION OF AN RNA POLYMERASE II PROMOTER BY THE RNA POLYMERASE III TRANSCRIPTION FACTOR III C (TFIIC) COMPLEX *

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INTRODUCTION

The eukaryotic RNA polymerase III (Pol III) system is responsible for synthesizing transfer RNA molecules and other transcripts, which in yeast include the U6 spliceosomal RNA, the 7SL RNA, the 5S ribosomal RNA, the snr52 snoRNA, and the RNA component of RNaseP (Paule and White 2000; Geiduschek and Kassavetis 2001; Huang and Maraia 2001). Transcription by Pol III requires the activity of the multi-subunit transcription factor complex TF\textsubscript{III}C, which binds to conserved A-box and B-box Pol III promoter elements, and functions to overcome chromatin repression of Pol III transcription and to recruit the TF\textsubscript{III}B complex (Burnol, Margottin et al. 1993; Kundu, Wang et al. 1999; Geiduschek and Kassavetis 2001). While Pol III and its transcription factors are thought to be dedicated to transcription of these specific genes, a growing body of evidence has shown that both partial and complete chromosomally bound Pol III complexes can have effects on RNA Polymerase II (Pol II) promoters (Donze and Kamakaka 2001; Simms, Miller et al. 2004; Noma, Cam et al. 2006; Scott, White et al. 2007; Simms, Dugas et al. 2008). Chromatin bound Pol III complexes also mediate other extra-transcriptional functions including targeting Ty element integration (Chalker and Sandmeyer 1992; Kirchner, Connolly et al. 1995; Devine and Boeke 1996), blocking of replication fork progression (Deshpande and Newlon 1996), condensin and cohesin recruitment (Dubey and Gartenberg 2007; Haeusler, Pratt-Hyatt et al. 2008), and direct inhibition of transcription from nearby Pol II promoters (Kinsey and Sandmeyer 1991; Hull, Erickson et al. 1994; Bolton and Boeke 2003; Simms, Miller et al. 2004).

Studies in both budding and fission yeast initially identified the presence of genome sequences that bind the TF\textsubscript{III}C complex, but not the other Pol III transcription factors TF\textsubscript{III}A, TF\textsubscript{III}B, or the Pol III enzymatic complex itself (Harismendy, Gendrel et al. 2003; Roberts,
Stewart et al. 2003; Moqtaderi and Struhl 2004; Noma, Cam et al. 2006). Recently, similar sites have been identified in human cells (Canella, Praz et al. 2010; Moqtaderi, Wang et al. 2010; Oler, Alla et al. 2010; Raha, Wang et al. 2010). These B-box containing sequences are referred to either as ETC (Extra TFIII C) or TFIII C-only sites in budding yeast, and COCs (Chromatin Organizing Clamps) in fission yeast (Roberts, Stewart et al. 2003; Moqtaderi and Struhl 2004; Noma, Cam et al. 2006). Particular TFIII C binding sites have been shown to function as chromatin boundary elements (Noma, Cam et al. 2006; Simms, Dugas et al. 2008), but the genome-wide function of the TFIII C bound ETC sites remains unknown.

Interestingly, one ETC site in Saccharomyces cerevisiae, ETC6, lies within the promoter of the TFC6 gene, which encodes a subunit of the TFIII C complex itself. We hypothesized that the Tfc6 protein, as part of the TFIII C complex, might autoregulate its own promoter by binding to ETC6 and function as an insulator. Autoregulation of gene expression is critically important in all forms of life, from its role in the lysogen/lytic growth decision of bacteriophage λ (Ptashne 2005), to having important roles in developmental and neuronal gene expression in metazoans (Crews and Pearson 2009; Hobert 2011). Our results identify the B-box within ETC6 as a functional regulatory element within the TFC6 promoter that mediates stringent autoregulation of the promoter; this regulation is sensitive to Tfc6 protein levels and binding of the TFIII C complex. This appears to be the first demonstration of a core Pol III transcription factor complex directly regulating the transcription of a Pol II promoter, and this tight regulation of Tfc6p levels could be important in regulating global tRNA expression, which could have subsequent global effects on translational regulation. Our results also implicate a potential UAS immediately upstream of ETC6 that potentially binds an activating transcription factor which gives us clues to the mechanism of TFC6 gene activation/regulation and TFIII C’s role as an insulator.
MATERIALS AND METHODS

5’RACE analysis was performed using the First Choice RLM-RACE kit (Ambion-Applied Biosystems #AM1700). Construction of the promoter mutants is described in the legend to Figure 3.1. Each mutant intergenic region was re-integrated into chromosome IV by transformation into strain DDY3453 (etc6Δ::URA3) and selection on 5-FOA media, and were verified by PCR of genomic DNA, and digestion of the PCR products with Drd I to verify the presence of the mutation. DDY3453 was created by standard yeast knockout techniques using oligonucleotides DDO-792 and 793 to amplify URA3 from plasmid pRS406 (Sikorski and Hieter 1989). All oligonucleotide sequences are listed in Table 3.2. Northern blot analyses were performed as described in Chapter Two (Methods).

Plasmids expressing TFIII subunits Tfc1p, Tfc3p, and Tfc6p were constructed by PCR amplification of each gene plus approximately 500 base pairs upstream and downstream from yeast genomic DNA using the high fidelity Phusion DNA polymerase (New England Biolabs, F-530S). Functional expression was verified by complementation of mutant strains. TFC4 was subcloned from a previously characterized plasmid PCF1 (kindly provided by Ian Willis). Each gene was cloned into the HIS3 marked pRS series of ARS-CEN and 2μ vectors (Sikorski and Hieter 1989; Kurdistani and Grunstein 2003).

Chromatin immunoprecipitation (ChIP) was performed as described in Chapter Two (Methods), using the TFC1-3X-FLAG allele crossed into the appropriate strains. Anti-FLAG monoclonal M2 was from Sigma (F1804), and anti-yeast TBP from Santa Cruz Biotechnology (sc-33736). Quantitation of ChIP signals was determined by radioactive PCR according to (Kurdistan and Grunstein 2003), except that samples were resolved on 1.2% agarose gels. ChIP signals were normalized to the background PCR signal generated using primers homologous to
the non-TFIII C binding *GAL1-10* intergenic region (oligos DDO1023 and 1024) to control for background and sample variation. All quantitative ChIP results were averaged from three independent determinations.

*TFC6* promoter-*URA3* ORF reporter strains were constructed by standard yeast recombination methods, using oligonucleotides DDO-1201 and DDO-1202 homologous to the ends of the *URA3* open reading frame plus 50 bases immediately upstream and downstream of the *TF6* open reading frame to amplify the coding sequence of *URA3*. Ura+ recombinants expressing *URA3* from the *TF6* promoter were slow growing on media lacking uracil (required 4-5 days to appear) and were slightly temperature sensitive; therefore all colony growth experiments were performed at 25°C. To compare colony sizes, *tfc6Δ::URA3* cells were transformed with empty pRS vector (Sikorski and Hieter 1989; Christianson, Sikorski et al. 1992) or *HIS3* marked TFIII C subunit expressing plasmids and plated on minimal media lacking histidine. His+ isolates were grown in liquid media lacking histidine and plated at ~50 colonies/plate on media lacking histidine, and media lacking both histidine and uracil. Plates were incubated at 25°C for three days (minus histidine) or five to six days (minus histidine and uracil) before photographing. Relative colony sizes from 30-50 colonies were measured and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/).

**Table 3.1. Plasmids used and/or generated in this study:**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS406 <em>URA3</em> vector</td>
<td>(Sikorski and Hieter 1989)</td>
</tr>
<tr>
<td>pRS413 ARS-CEN <em>HIS3</em> vector</td>
<td>(Sikorski and Hieter 1989)</td>
</tr>
<tr>
<td>pRS423 2μ <em>HIS3</em> vector</td>
<td>(Christianson, Sikorski et al. 1992)</td>
</tr>
<tr>
<td>pDD1098 <em>TF6</em>-ESC2 intergenic region (~640 bp) in Bluescript SK+</td>
<td>(Simms, Dugas et al. 2008)</td>
</tr>
<tr>
<td>pDD1179 <em>TF6</em> in pRS413, ARS-CEN <em>HIS3</em></td>
<td>This study</td>
</tr>
<tr>
<td>pDD1184 <em>TF6</em> promoter mutant #1</td>
<td>This study</td>
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<td>pDD1185 <em>TF6</em> promoter mutant #2</td>
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<td>pDD1188 <em>TF6</em> promoter mutant #5</td>
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</tr>
<tr>
<td>pDD1189 <em>TF6</em> promoter mutant #6</td>
<td>This study</td>
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</tbody>
</table>

(Table 3.1 Continued)
We used a combined transcript mapping, bioinformatic and mutational approach to identify the potential promoter elements upstream of TFC6. 5' - RACE analysis was performed to map transcriptional start sites, which were identified at bases minus 46, 96, 98, 104, and 110 from the annotated TFC6 translational start site (Figure 3.1).
Figure 3.1. Comparison of the TFC6 promoter region among budding yeast species. Sequences were aligned using Clustal software available through the Saccharomyces Genome Database website. Shaded boxes covering regions of highest homology were designated as sites 1-7, and were mutated as described below; mutagenic oligonucleotides are listed in Table 3.2. Transcription start sites marked in red were determined by 5’ RACE analysis. The numbers above each arrow refer to the location of the start site relative to the start codon (Met), and the numbers in parentheses are the number of independent 5’ RACE clones obtained for that start site. The region of the ETC6 B-box is marked by the green shading.

Promoter mutants were constructed by site-directed mutagenesis of plasmid pDD1098 which contains the TFC6-ESC2 intergenic region (from Saccharomyces Genome Database chromosome IV coordinates 1198718 to 1199357, www.yeastgenome.org, as of 20 October 2010) cloned into Bluescript SK+. Each mutant had a 12 base pair region replaced with a Drd I restriction enzyme site (Quick-Change kit, Stratagene), which allowed scrambling of 10-12 base pairs within each region, and initial restriction digest screening of mutants, which were verified by DNA sequencing.
Mapping of the start sites allowed us to focus on the upstream region to identify promoter elements. Comparison of the TFC6 promoter regions from five budding yeast species revealed regions of high conservation in addition to the ETC6 site B-box sequence. Regions containing six or more bases common to all five species over a twelve base stretch were designated as promoter boxes 1-7, as shown schematically in Figure 3.2A and at sequence level detail in Figure 3.1. These 12-base pair boxes were mutated on plasmids, re-integrated into the yeast genome, and Northern blot analysis for TFC6 mRNA was performed for each mutant.

The results in Figure 3.2B show that the major effects were seen clustered across promoter mutants 3, 4, and 5. Mutant 3 results in a significant decrease in TFC6 mRNA, and this mutant is compromised for growth due to limiting TFC6 expression, as complementation with a TFC6 plasmid restores normal growth (Figure 3.3). Mutants 4 and 5, which both span the ETC6 site, show a 2-fold increase in TFC6 mRNA levels, which is consistent with our previous results mutating this site (Simms, Dugas et al. 2008). These results are also consistent with mutant 3 affecting a transcription factor binding site, and with the ETC6 site B-box being involved in negative regulation of the TFC6 promoter.

To further test the hypothesis that TFIIIc binding to ETC6 is involved in TFC6 auto-regulation, we performed TFC6 Northern blots on strains containing conditional mutations of the RNA polymerase III machinery. The mutant tfc3-G349E is a temperature sensitive allele of a TFIIIc component that reduces binding affinity (measured in vitro) of the TFIIIc complex for tDNAs (Lefebvre, Ruth et al. 1994). Mutations brf1 II-9 and II-6 are impaired in Brf1p interaction with TBP (Andrau, Sentenac et al. 1999); rpc3l-236 is defective in Pol III initiation (Thuillier, Stettler et al. 1995); and rpc160-112 is defective in elongation (Dieci, Hermann-Le Denmat et al. 1995).
Figure 3.2. Characterization of the *S. cerevisiae* *TFC6* promoter suggests autoregulation by the *TFIIIC* complex. A) Transcriptional start sites upstream of *TFC6* were mapped by 5'- RACE analysis, and are detailed in Figure 3.1. *TFC6* promoter regions of highest homology among five budding yeast species are designated promoter boxes 1-7, also detailed in Figure 3.1. B) Mutant promoters were re-integrated into the yeast chromosome, and relative *TFC6* mRNA levels determined by Northern blotting. Expression was determined from three independently isolated strains for each mutation; one each is shown here. C) Temperature sensitive mutation in *TFC3*, but not other Pol III mutations result in increased *TFC6* transcript levels. Strains containing mutant alleles of *TFIIIC*, *TFIIIB*, and Pol III components were grown at permissive temperature (30°C), then pulsed for one hour at the non-permissive temperature (37°C) before RNA extraction and Northern analysis.
The results in Figure 3.2C demonstrate that only the tfc3-G349E mutant contained increased $TFC6$ transcript levels. This is consistent with direct TF_{III}C mediated regulation of $TFC6$.

**Figure 3.3.** $TFC6$ promoter mutant #3 exhibits a slow growth phenotype due to insufficient $TFC6$ expression. Wild type (DDY3630) and promoter mutant #3 (DDY4301) strains were transformed with either HIS3 vector (pRS413) or the same vector containing the $TFC6$ gene (pDD1179), and streaked onto minimal media lacking histidine. The slow growth phenotype of strain DDY4301 is complemented by the $TFC6$ expressing plasmid. Identical complementation results were obtained using two independent promoter mutant #3 isolates, and also in strain DDY4114 (described in Simms et al, 2008), which has the $ETC6$ site and the upstream region deleted.

The results in Figure 3.2C demonstrate that only the tfc3-G349E mutant contained increased $TFC6$ transcript levels. This is consistent with direct TF_{III}C mediated regulation of $TFC6$.
transcription, and not a result of reduced Pol III activity, as the other mutations that globally impair Pol III transcription had little effect on TFC6 mRNA levels.

**Inverse Correlation of TFIII C Association at ETC6 and TFC6 Transcript Levels.**

To confirm that the mutant etc6 and tfc3 strains were indeed defective for *in vivo* binding of TFIII C to ETC6, we performed chromatin immunoprecipitation (ChIP) against a carboxy-terminal 3XFLAG-epitope tagged Tfc1p subunit (Figure 3.4) in both *tfc3*-G349E and *etc6* B-box mutant strains. The B-box mutation changes a cytosine residue conserved in all TFIII C binding sites to a guanine, and is known to inhibit TFIII C binding *in vitro* (Newman, Ogden et al. 1983). The results shown in Figure 3.4A illustrate that both mutations lead to a loss of TFIII C association with the TFC6 promoter *in vivo*, and reduced binding correlates with the relative increase in TFC6 transcript levels in the same mutants (Figure 3.4B).

**Over-Expression of TFC6 Inhibits Expression From Its Own Promoter.**

If Tfc6 protein levels are directly autoregulating its own promoter, then overexpression of TFC6 from an episomal plasmid was predicted to reduce transcription from the endogenous chromosomal promoter. In order to test this hypothesis, we created diploid yeast strains that have the *URA3* open reading frame precisely replacing one chromosomal copy of the TFC6 open reading frame (Figure 3.5A). These strains allowed us to assess the level of TFC6 promoter activity independent of episomal expression by assessing growth on media lacking uracil. TFC6 was overexpressed in strain DDY4520, both from its own promoter on a high-copy plasmid containing the entire TFC6 gene, or from the *ADH1* promoter on a low copy plasmid. Increased TFC6 expression has no effect on growth of this strain on media lacking only histidine compared to cells transformed with the *HIS3* vector alone (Figure 3.5B), showing that increased Tfc6p levels alone do not inhibit growth.
Figure 3.4. TFIIIC binding to ETC6 is inversely correlated to TFC6 mRNA levels. A) Strains containing 3X-FLAG-epitope tagged TFC1 and either tfc3-G349E or etc6 B-box mutant alleles were constructed, chromatin extracts prepared for immunoprecipitation and relative TFIIIC association at the TFC6 promoter determined. B) Reduction of TFIIIC binding by either mutation is correlated to increased TFC6 mRNA levels.
However, when the same cells were plated on minimal media lacking both histidine and uracil, the average colony sizes formed by cells containing either the high-copy or ADH1-promoter plasmid were consistently 65-70% of controls containing the empty vector. This effect is dose dependent, as expression of TFC6 from its own promoter on a lower copy ARS-CEN plasmid reduces average colony size to only 89% of controls (Figure 3.6 A & B). ChIP against TFC1-3XFLAG showed that overexpression of TFC6 resulted in increased association of TF_{III}C at ETC6 (Figure 3.5C), as the amount of TFC6 promoter DNA immunoprecipitated was ~1.7 times the vector control. This correlated with a decrease in TATA binding protein (TBP) association at

Figure 3.5. Overexpression of Tfc6p down-regulates gene expression driven by the TFC6 promoter, and increases the association of the TF_{III}C complex to ETC6. A) Diploid strain DDY4520 was constructed to contain the URA3 open reading frame (ORF) integrated in place of the TFC6 ORF on one copy of chromosome IV to test the effects of episomal TFC6 overexpression. B) Vector controls, 2μ HIS3 TFC6, or ARS-CEN HIS3 ADH1-promoter-TFC6 plasmid transformants were plated on media lacking histidine or both histidine and uracil, and colony sizes were measured after three days (minus histidine) or five days (minus histidine minus uracil) at 25C. C) ChIP of TFC1-3X-FLAG strains transformed with vector or ADH1 promoter-TFC6 plasmid show increased Tfc1p association, and decreased TBP at ETC6 when TFC6 is overexpressed. Quantitative results were averaged from three separate determinations.
the TFC6 promoter, as the anti-TBP ChIP signal was only ~70% compared to the vector control (Figure 3.5C). These results show that overexpression of TFC6 increases the degree of TFIII C association with ETC6, and reduces expression from its own promoter, presumably due to increased stability of TFIII C binding to the ETC6 site, leading to reduced TBP association.

Figure 3.6. Tfc6p inhibition of Its own promoter is dose dependent. A) Strain DDY4403 containing the TFC6 promoter driving URA3 was transformed with empty HIS3 vector, low copy ARS-CEN-TFC6 plasmid, or high copy 2μ-TFC6 plasmid. Platings and colony size determinations were as in Fig. 3.5 and as described in the methods. B) Bar graph represents the measurement of at least 30 colonies from three separate images. C) Western blot analysis to estimate the relative level of overexpression of TFC6. The same plasmids used in A and B were modified to contain the identical triple FLAG epitope that is integrated as a single copy in the haploid strain DDY4107 (pDD1244 = 2μ TFC6- 3XFLAG, pDD1245 = ARS-CEN TFC6-3XFLAG). DDY4107 was then transformed with either empty HIS3 vector (pRS413) to measure normal Tfc6p levels, or each of the TFC6-FLAG plasmids. Total protein extracts from each strain were prepared and equal protein amounts were immunoprecipitated with anti-FLAG M2 monoclonal antibody (Sigma), and the concentrated immunoprecipitates were analyzed by Western blotting using the same antibody. Relative amounts were assessed by scanning the blot on a Pharmacia Typhoon 8600 Phosphorimager on the chemiluminescent setting, and using ImageQuant software to measure the relative signals.

Auto-Regulation of TFC6 is ETC6 Site B-Box Dependent and Tfc6p Specific.

If increased binding of TFIII C to ETC6 is indeed responsible for reduced growth on media lacking uracil, we predicted that a strain with URA3 driven by a TFC6 promoter containing the defective B-box within ETC6 would be insensitive to overexpressed TFC6 when grown on media lacking uracil.
Figure 3.7. Downregulation of the TFC6 promoter by Tfc6p requires the ETC6 B-box and is specific to TFC6 overexpression. A) Strain DDY4521 was constructed to contain a mutant B-box linked in cis- to the TFC6 promoter driving URA3. TFC6 was overexpressed as in Fig. 3.6., and was unable to downregulate the TFC6 promoter containing the mutant B-box, as indicated by no change in colony sizes. B) Overexpression of other TFIIIC subunits in DDY4403. High-copy 2µ plasmids encoding each gene driven by its native promoter were transformed into the URA3 reporter strain and plated on minimal media lacking histidine and uracil, and colony sizes relative to the vector control were determined as in Fig. 3.6.
Strain DDY4521 is identical to DDY4520 except for the presence of the cytosine to guanine mutation in the ETC6 B-box upstream of the URA3 marker. The results in Figure 3.7A confirm that the inhibition is mediated through the ETC site, as when TFC6 is overexpressed in the strain containing the mutant B-box, no reduction of colony size is observed on media lacking uracil.

We next asked if overexpression of other TF_{III}C subunits would affect URA3 expression from the TFC6 promoter. Large-scale proteomic studies of yeast protein expression have estimated the number of protein molecules per yeast cell (Ghaemmaghami, Huh et al. 2003), and the results suggest that Tfc3p, Tfc4p, and Tfc6p are the most limiting components of the TF_{III}C complex. Tfc1p appears to be present in large excess, and Tfc7p and Tfc8p are at intermediate levels. Indeed, this apparent excess of at least Tfc1p and Tfc7p was determined to exist as a chromatographically separable sub-complex in yeast extracts (Manaud, Arrebola et al. 1998). We confirmed that Tfc6p is limiting relative to Tfc1p, as Western blots of protein extracts from strains containing the identical triple FLAG epitope on each gene show a large relative excess of Tfc1p expression compared to Tfc6p (Figure 3.8.). We also determined by Western blotting that Tfc1p levels are not significantly affected by the tfc3-G349E temperature sensitive mutation used in this study (Figure 3.9.). These endogenous ratios suggest that overexpression of other limiting subunits might also increase the level of TF_{III}C complex binding to ETC6 and reduce TFC6 promoter activity, while overexpression of TFC1 should have no effect since it is already largely in excess. 2μ plasmids containing TFC1, TFC3, TFC4, and TFC6 were separately transformed into strain DDY4403 (TFC6 promoter-URA3, similar to DDY4520, but in the S288C background) and plated on media lacking both histidine and uracil. Colony sizes were determined at six days of growth, and the results are shown in Figure 3.7B. As expected, overexpression of TFC1 had no effect on cell growth, nor did overexpression of TFC4.
Figure 3.8. Western blot analysis of yeast strains containing identical carboxy-terminal triple-FLAG epitope tags on TFC1 (DDY4381), TFC6 (DDY4107), or BRF1 (DDY844). Total protein extracts from each strain were prepared and equal protein amounts were immunoprecipitated with anti-FLAG M2 monoclonal antibody (Sigma), and the concentrated immunoprecipitates were analyzed by Western blotting using the same antibody. Tfc6 protein levels are limiting compared to Tfc1p and Brf1p levels.

Figure 3.9. Tfc1p levels are not significantly affected by the tfc3-G349E temperature sensitive mutation. Whole cell extracts from TFC1-3XFLAG strains were prepared and equivalent protein amounts were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were subjected to Western blot analysis with anti-FLAG primary antibody. No significant difference was seen in the wild type versus tfc3-G349E mutant.
Expression of *TFC3* appeared to inhibit growth slightly, but not as much as *TFC6*. These results demonstrate that the *TFC6* promoter is preferentially sensitive to increased levels of its cognate gene product.

**Overexpression of *TFC6* Results in Elevated TFIII C Association at Multiple Loci.**

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<tr>
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**Figure 3.10.** Overexpression of *TFC6* increases TFIII C association at multiple genomic loci. Strain DDY4381 (*TFC1-3XFLAG*) was transformed with either empty vector or pDD1234 (*ADH1* promoter-*TFC6*) to overexpress Tfc6p. Binding of TFIII C was assessed at several B-box sites by ChIP using anti-FLAG antibody, each of which showed increased enrichment when Tfc6p was overexpressed. As in Figure 3.5, determinations were performed in triplicate, normalized to the *GAL* locus signal, and one pair of lanes is shown for each locus.

Since TFIII C binding at *ETC6* was increased upon overexpression of Tfc6p, we tested other B-box containing loci by ChIP for enrichment of the TFIII C complex. The results in Figure 3.10 demonstrate that all loci tested, which included three tDNAs, the *ZOD1/UFO1* locus, and *ETC4*
and *ETC5*, an increase in TF\(_{III}\)C association was observed upon episomal expression of *TFC6*. The magnitude of this increase varied from 1.2-fold to over 2-fold. Despite this seemingly general increase in TF\(_{III}\)C binding, we have not yet identified any tDNAs or other loci that show altered levels of Pol III transcription (see discussion).

**DISCUSSION**

Although Pol III is dedicated to the transcription of tDNAs and a handful of other RNAs, genome wide ChIP studies in yeast have demonstrated the presence of the transcription factor complex TF\(_{III}\)C at chromosomal locations not associated with the Pol III complex (Harismendy, Gendrel et al. 2003; Roberts, Stewart et al. 2003; Moqtaderi and Struhl 2004; Noma, Cam et al. 2006). Recently, similar studies using human cells and high-throughput sequencing detection (ChIP-Seq) have also demonstrated the presence of such sites beyond yeast (Canella, Praz et al. 2010; Moqtaderi, Wang et al. 2010; Oler, Alla et al. 2010; Raha, Wang et al. 2010). These loci have been referred to as *ETC* (extra TF\(_{III}\)C) sites, COC (chromatin organizing clamps), or TF\(_{III}\)C-only sites, and have been shown to affect expression of neighboring Pol II genes by acting as chromatin boundary elements (Noma, Cam et al. 2006; Simms, Dugas et al. 2008). This study set out to further characterize the role of the TF\(_{III}\)C binding site *ETC6* in *S. cerevisiae*, which lies in the promoter of the *TFC6* gene encoding a subunit of the TF\(_{III}\)C complex itself. The location of this site was noted by Moqtaderi and Struhl in their study characterizing *ETC* sites (Moqtaderi and Struhl 2004), and they suggested the possibility that TF\(_{III}\)C might regulate this promoter.

Our results confirm their speculation, as we show that *ETC6* is a functional promoter element of the *TFC6* gene that mediates autoregulation of *TFC6* expression in response to Tfc6 protein levels. We show that inhibition of TF\(_{III}\)C binding to *ETC6* results in increased *TFC6* transcript levels, while overexpression of Tfc6p increases association of the TF\(_{III}\)C complex at
ETC6 and inhibits expression from the TFC6 promoter. These results suggest that Pol II transcription of TFC6 is sensitive to the level of its own protein product, a product that is part of what was previously thought to be a dedicated core Pol III transcription factor. While such crosstalk between Pol II transcription factors and Pol III promoters has been described for the Octamer binding proteins and the SNAPc complex in mammalian systems (Schramm and Hernandez 2002), they appear to be general Pol II transcription factors that act on a limited subset of Pol III promoters. Therefore, this does appear to be the first demonstration of a core Pol III factor regulating Pol II transcription.

The results presented here also begs the question of how does the TFIII C complex inhibit expression from its own promoter. Data in Figure 3.5 show reduction in TBP association at the TFC6 promoter when TFC6 is episomally overexpressed; while this may be due to direct inhibition of TBP binding, this reduction might also be a consequence of other mechanisms. We previously suggested (Simms, Dugas et al. 2008) that inhibition may occur via an insulator-like mechanism, with bound TFIII C inhibiting upstream transcription factors from recruiting a productive pre-initiation complex at the transcription start site. However, since the key TFC6 promoter element (mutant site #3) is immediately upstream of the ETC6 B-box, we also consider that TFIII C and the putative transcription factor may be in competition for binding to the same region of DNA.

While much work has been done on the global control of Pol III transcription by the Maf1 mediated pathway (Willis and Moir 2007; Ciesla and Boguta 2008; Goodfellow, Graham et al. 2008), few studies have looked at the role of the regulation of expression of the Pol III transcription factors themselves. In yeast, over-expression of TFIII B70 (Brf1p) elevates expression of promoter mutant tDNAs (Sethy-Coraci, Moir et al. 1998). In mammalian cells, overexpression
of Brf1 stimulates Pol III transcription, while shRNA inhibition of Brf1 expression reduces oncogenic transformation and tumor formation in a mouse model (Johnson, Dubeau et al. 2008; Marshall, Kenneth et al. 2008). These results indicate that levels of the Pol III transcription factors can have critical roles in regulating Pol III transcription and cell proliferation.

Autoregulatory circuits have been identified as key components controlling gene expression, and have evolved in organisms from bacteriophage to humans (Crews and Pearson 2009). Bacteriophage λ uses its CI repressor protein to both positively regulate its own expression, and then negatively regulate itself when the cellular concentration of the protein reaches proper levels, a key circuit in maintaining the inducible lysogenic state (Ptashne 2005). Neuronal terminal differentiation genes in C. elegans are controlled by autoregulated terminal selector transcription factors, and disruption of this process can lead to defective neuron function (Hobert 2011); many other instances of autoregulation could be cited (Crews and Pearson 2009). Given that, the results presented here suggest that in yeast there exists a tight regulation of TFC6 expression that maintains its protein product as a limiting component of the TFIII complex. This fact raises the question as to why do yeast need to maintain such a stringent control of Tfc6p expression, and therefore TFIII activity. Since we observe that overexpression of Tfc6p differentially increases the Tfc1p ChIP signal at several loci (Figure 3.10), we speculate that altered Tfc6p levels might differentially regulate TFIII occupancy genome-wide, and possibly differentially affect expression levels of tRNAs and other Pol III transcripts. Recent studies have shown that slowly translated rare/sub-optimum codons play a role in fine-tuning translational regulation and protein stability and activity (Crombie, Boyle et al. 1994; Zalucki and Jennings 2007; Zhang, Hubalewska et al. 2009; Tuller, Carmi et al. 2010; Zhang, Saha et al. 2010), therefore altered Tfc6p levels might differentially affect the production of tRNAs decoding these
regulatory codons, potentially having global effects on translational regulation. Although we have not yet detected any differences in Pol III transcription upon overexpression of Tfc6p (from a limited set of Pol III transcribed genes tested), a genome-wide analysis may reveal particular tDNAs whose expression is altered. Since many tDNAs are present in multiple copies in the yeast genome, such differences may only be revealed by tagging of individual loci to distinguish altered expression levels.

The work presented here is significant in that it appears to be the first demonstration of a core Pol III transcription factor that can directly regulate transcription from a Pol II promoter, that this stringent regulation could potentially be important in global gene expression, and adds another potential avenue of crosstalk among the different RNA polymerase systems (Conesa, Ruotolo et al. 2005). Additionally, since ETC-like sites have now been confirmed in human cells, the role of the TFIII C complex in genome organization and global control of gene expression may be more prevalent than previously realized.

Table 3.3. Strains of S. cerevisiae used in this study:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Created</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDY3</td>
<td>MATa ADE2 his3-11 leu2-3,112 lys2Δ trpl-1 ura3-1</td>
<td>Donze Lab</td>
</tr>
<tr>
<td>DDY232</td>
<td>MATa ADE2 his-3-11 leu2-3,112 LYS2 trpl-1 ura3-1 1 rcp31-236 hmrΔ</td>
<td>Donze Lab</td>
</tr>
<tr>
<td>DDY237</td>
<td>MATa ADE2 his-3-11 leu2-3,112 LYS2 trpl-1 ura3-1 1 tfc3-G349E hmrΔ</td>
<td>Donze Lab</td>
</tr>
<tr>
<td>DDY246</td>
<td>MATa ade2-1 his-3-11 leu2-3,112 LYS2 trpl-1 ura3-1 1 rpc160-A1::HIS3 p-rpc160-112</td>
<td>Donze Lab</td>
</tr>
<tr>
<td>DDY247</td>
<td>MATa ade2-1 his-3-11 leu2-3,112 LYS2 trpl-1 ura3-1 1 rpc160-A1::HIS3 p-rpc160-112</td>
<td>Donze Lab</td>
</tr>
<tr>
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<td>Donze Lab</td>
</tr>
<tr>
<td>DDY269</td>
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<td>Donze Lab</td>
</tr>
<tr>
<td>DDY416</td>
<td>MATa ADE2 his-3-11 leu2-3,112 lys2Δ trpl-1 ura3-1 1 brf1Δ::HIS3 p-brf1 II.9 hmrΔ</td>
<td>Donze Lab</td>
</tr>
<tr>
<td>DDY420</td>
<td>MATa ADE2 his-3-11 leu2-3,112 lys2Δ trpl-1 ura3-1 1 brf1Δ::HIS3 p-brf1 II.6 hmrΔ</td>
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<tr>
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<td>This Study</td>
</tr>
<tr>
<td>DDY4312</td>
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</tr>
<tr>
<td>DDY4077</td>
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<td>This Study</td>
</tr>
<tr>
<td>DDY4376</td>
<td>MATa ADE2 his3-11 leu2-3,112 LYS2 trpl-1 ura3-1 1 tfc3-G349E TFC1-3XFLAG::KanMX</td>
<td>This Study</td>
</tr>
<tr>
<td>DDY4381</td>
<td>MATa ADE2 his3-11 leu2-3,112 LYS2 trpl-1 ura3-1 1 TFC1-3XFLAG::KanMX</td>
<td>This Study</td>
</tr>
</tbody>
</table>

(Table 3.3 Continued)
DDY4403 MATa/MATα ADE2/ADE2 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/lys2Δ0 met15Δ0/MET15 TRP1/TRP1 This Study
ura3Δ0/ura3Δ0 TFC6/αf6Δ::URA3

DDY4520 MATα/MATa ade2-1/ADE2 his3Δ1-11/11 leu2-3,112/leu2-3, 112 lys2Δ/lys2Δ trp1-1/trp1-1
ura3-1/ura3-1 TFC6/αf6Δ::URA3
This Study

DDY4521 MATa/MATα ade2-1/ADE2 his3Δ1-11/11 leu2-3,112/leu2-3, 112 lys2Δ/lys2Δ trp1-1/trp1-1
ura3-1/ura3-1 TFC6/αf6Δ::URA3
ura3-1/ura3-1 TFC6/αf6Δ::URA3
This Study

All strains are isogenic to S. cerevisiae W-303 except for DDY4403, which is in the S288C background (BY4743 parent). Most experiments were confirmed with at least three independent isolates, only those isolates depicted in the figures are listed. Strains 4403, 4520, and 4521 contain the URA3 open reading frame expressed from the TFC6 promoter as described in the text and methods.

**LITERATURE CITED**


CHAPTER FOUR
DISCUSSION
Knowledge of the regulation of eukaryotic gene expression is expanding exponentially with
the advent of genomic sequencing, particularly in *Saccharomyces cerevisiae*, the first eukaryotic
organism in which the entire genomic sequence has been determined (Goffeau, Barrell et al.
1996). Now a vast amount of genomic data is available for the approximately 6000 open reading
frames in this simple budding yeast (Oliver 1996). A large body of research has focused on the
three different eukaryotic RNA polymerases, the different types of promoters they utilize, and
the additional regulatory genomic elements involved in transcriptional regulation. These studies
include the effects on transcription by chromatin domains, along with their associated cis-
elements and trans-factors.

The cellular processes of transcription, replication, recombination, and repair all have to
occur within the environment of chromatin and the particular hetero- or euchromatic state of the
underlying domain. Regions of these different states of chromatin domains not only influence the
topological characteristics of chromatin, but also the numerous mechanisms of activation and
repression of certain genes at particular points in the cell cycle and developmental stages during
metazoan development. A simple example of a well-studied mechanism of cellular
differentiation (or cell fate) is the haploid mating type stability in *S. cerevisiae* where the cell
mating type, a or α, is dependent on the silencing of the cryptic mating loci, HMR and HML.
Loss of silencing at these HM loci can result in the cell taking on the characteristics of a non-
mating diploid.

Eukaryotic genomes are typically organized into domains containing individual genes or
gene clusters that have distinct patterns of expression. Boundary elements, consisting of barriers
and insulators, are specific sequences in the genome along with their associated DNA-binding
proteins that inhibit the spread of heterochromatin into transcriptionally active euchromatic
regions and block the ectopic activation of genes from distal enhancers, respectively. Both elements are critical for the proper transcriptomal expression of the eukaryotic genome. Studying these elements in yeast enables us to acquire information on how the genome is organized and how the cell takes on a certain characteristic that differentiates it from another cell. This dissertation has focused on the study of the functional mechanisms of boundary elements, barriers and insulators, in their natural context.

Two main models have been proposed to explain how silenced domains are restricted from spreading by barrier elements. The nuclear organization model posits that barrier elements tether chromatin to nuclear superstructures to form topologically distinct domains, and this tethering prevents the spread of silenced chromatin. The chromatin modifying model suggests that barrier elements utilize chromatin modifying activities, which modify the underlying chromatin structure, making it less amenable for the spread of silenced chromatin. There are two variations of the chromatin modifying model. One variation suggests that silenced chromatin is restricted from spreading simply as a result of the competition between chromatin-opening and condensing factors at the boundary. Silencing is restricted from spreading by this mechanism at some native yeast telomeres (Kimura and Horikoshi 2004). The other variation suggests that the binding of sequence-specific factors (e.g. TFIII C at the A and B box; TATA-like sequences for Pol III machinery components) to the barrier element creates a nucleosome-free region, and this nucleosome-free gap at the boundary is sufficient to block the spread of heterochromatic proteins (Bi, Yu et al. 2004).

The transcriptional potential of the tDNA barrier at HMR and the assembly of the RNA polymerase III transcription complex is shown to be critical for barrier function, as mutations in the tDNA promoter reduce barrier activity (Donze, Adams et al. 1999; Donze and Kamakaka
Mutations in the genes for the RNA polymerase III transcription factors Brf1p or Tfc3p, or the HMR-tRNA gene itself prevents binding of these factors and thus weakens the boundary function (Donze and Kamakaka 2001). Nhp6a and Nhp6b, which have a demonstrated role in Pol III transcription of the SNR6 (U6 RNA) gene and a subset of tDNAs, are also implicated in the barrier function of the HMR-tRNA as deletion of both genes substantially weakens the ability of the HMR-tRNA gene to block the propagation of Sir protein mediated silencing (Braglia, Dugas et al. 2007).

The deacetylase action of Sir2p has a preference for lysines 9 and 14 of H3 and K16 of H4 in vitro (Imai, Armstrong et al. 2000; Tanny and Moazed 2001), whereas in vivo all acetylatable lysines of histones H3 and H4 are fully deacetylated at silenced loci (Suka, Suka et al. 2001). Enhanced silencing in an rpd3Δ background cannot be accounted for by increased expression of SIR genes since the only Sir protein required at all three silent loci in yeast, Sir2p, weakens silencing at the HM loci when SIR2 is overexpressed (Fritze, Verschueren et al. 1997; Smith, Brachmann et al. 1998). Overexpressing SIR4 also has the same effect of weakening silencing at all three silent loci (Sussel and Shore 1991; Renauld, Aparicio et al. 1993; Smith, Brachmann et al. 1998). The same situation would presumably apply to Sir1p. Unlike the initiation of silencing at the HM loci, SIR1 is not required for silencing at telomeres (Rine and Herskowitz 1987; Aparicio, Billington et al. 1991) and all but two telomeres, III-R and IV-L, are associated with silenced chromatin (Pryde and Louis 1999; Lieb, Liu et al. 2001).

The enhanced silencing effect at HMR in an rpd3Δ strain has mechanistically eluded researchers for nearly fifteen years, but recent research has implicated a redistribution of Sir proteins across the yeast genome based on global histone N-terminal modifications (Santos-Rosa, Bannister et al. 2004; Venkatasubrahmanyam, Hwang et al. 2007; Zhou, Zhou et al. 2009).
In the early days of rpd3Δ silencing research, three separate hypotheses were proposed to explain the rpd3Δ effect on enhanced silencing (De Rubertis, Kadosh et al. 1996; Vannier, Balderes et al. 1996; Rundlett, Carmen et al. 1998). The first was based on an indirect effect of increased expression of a critical, dosage-dependent silencing factor. This was highly unlikely because as mentioned earlier, it has been shown that increased SIR2 and/or SIR4 expression does not increase silencing at the HM loci but actually reduces it (Sussel and Shore 1991; Renauld, Aparicio et al. 1993; Fritze, Verschueren et al. 1997; Cockell, Gotta et al. 1998; Smith, Brachmann et al. 1998). The second hypothesis stated that the loss of Rpd3p function causes an increase in the acetylation of the H4 N-terminal lysine residue 12 which correlates with increased silencing, and because it was determined by ChIP to be specifically acetylated in the chromatin of the silent HM loci compared to the expressed MAT locus (Braunstein, Sobel et al. 1996). Recent evidence using a more specific antibody for the lysine 12 modification contradicts this notion of a hyperacetylation state correlating with silencing (Suka, Suka et al. 2001). The third hypothesis states that the activity of an unidentified silencing factor is modulated by acetylation on internal lysine residues. Loss of deacetylase activity would then hyperactivate the silencing factor leading to stronger silencing.

In its role as a histone deacetylase, Rpd3p does not necessarily participate in ‘global deacetylation’ since the specificity is for histone proteins mainly at targeted gene promoters. The Rpd3 complex may bind directly and in a sequence-independent manner to histones or histone-binding proteins for the subsequent ‘global deacetylation’ of histones. Rpd3p is recruited to specific sites in the genome via interactions with multiple transcription factors, including Ume6p (Kadosh and Struhl 1997; Rundlett, Carmen et al. 1998) and the heterodimeric transcription factor Swi4/Swi6 (Robert, Pokholok et al. 2004). Initial studies on Rpd3p focused on its role in
the regulation of sporulation-specific genes through Ume6p, and then further studies revealed that Rpd3p regulates additional cellular functions. A set of genes occupied by Rpd3p encodes important cell cycle regulators including cyclins and cyclin-dependent kinases. This may explain, in part, the importance of Rpd3p in meiosis (Vannier, Balderes et al. 1996). Rpd3p and Sin3p occupy a total of approximately 100 genes (p<0.005) and, given that each is a component of the larger Rpd3(L) complex, are essentially associated with the same genes (Kurdistani, Robyr et al. 2002).

Through our genetic screen of UV-mutated rpd3Δ strains, we identified two genes, BRE1 and BRE2, which are involved in the enhanced silencing effect of rpd3Δ at HMR and have a tremendous influence on global histone methylation. Our results also give the indication that regulation of both euchromatic and heterochromatic environments are more complex than previously thought. We are given clues as to the occurrence of possible indirect effects of both chromatin-binding and chromatin-influencing proteins on genomic environments along with the fact that the covalent modifications on the N-terminal tails of histone proteins are in a constant state of flux. The assayed cells only reflect a particular state at a particular moment in time of the underlying chromatin, but this constant state of flux can still allow certain histone modifications to serve as epigenetic marks for chromatin states.

The histone H3/H4 tetramer would be the likely candidate to serve as a location for epigenetic marks, since the passage of the replication fork causes the two H2A-H2B dimers to disassociate from both the H3-H4 tetramer and the DNA. The H3-H4 tetramers remain associated with DNA (Kimura and Cook 2001) and are randomly distributed to the sister molecules during replication (Jackson and Chalkley 1985). In the inheritance of the heterochromatic state (silenced regions), the H3-H4 tetramers disassociated from H2A-H2B
would be hypoacetylated and Sir proteins may remain associated with the H3 and H4 histone tails after the passage of the replication fork. Newly synthesized acetylated histones could be adjacent to old H3-and H4-bound Sir2p which would influence the deacetylation of the adjacent nucleosomes creating high affinity binding sites for Sir3p and Sir4p and thus the re-establishment of silencing. The use of certain histone modifications as epigenetic marks is critical in *S. cerevisiae*, since there is no utilization of DNA modifications (i.e. CpG methylation). A new paradigm is beginning to emerge that the deacetylation of histones H3/H4 is not the only potential epigenetic mark to maintain silencing. This stands to be true as new evidence brings a higher complexity to histone modification states. Although core histones are among the most conserved proteins known in all organisms, they are also among the most diverse regarding posttranslational modifications.

Transcript levels of the two NAD⁺ biosynthesis genes detected in our genetic screen, *QNS1* and *NPT1*, were not significantly modulated between our WT and *rpd3Δ* strains. Studies have shown that only telomeric and rDNA silencing in *S. cerevisiae* are dependent on the nuclear NAD⁺ salvage pathway (Llorente and Dujon 2000) and mutations in the *SIR2* gene itself does not significantly affect overall intracellular NAD⁺ concentration (Sandmeier, Celic et al. 2002). Another Sir2 family member of HDACs (class III NAD⁺-dependent), Hst2p, is actually responsible for most of the NAD⁺-dependent deacetylase activity from whole cell extracts (Smith, Brachmann et al. 2000). It is interesting to note that the NAD⁺-dependent glutamate dehydrogenase, *GDH2*, was identified in our genetic screen. Since *GDH2* requires the cofactor NAD⁺ for its enzymatic activity of converting glutamate to α-ketoglutarate, it seems implausible that *GDH2* would be an effector, since the requirements of Gdh2p would sequester the NAD⁺ cofactor, thus inhibiting the availability of NAD⁺ to Sir2p and its effects on extended silencing at
Although GDH2 was not verified as a potential effector upon gene knockout, this scenario is still highly unlikely due to the numerous pathways for NAD\(^+\) to replenish in the cell. It may take changes in some or all of the NAD\(^+\) pathway genes to observe a significant change in NAD\(^+\) concentration that would negatively affect Sir protein-mediated silencing at HMR. Topologically, it would be very efficient for the cell to replenish the nicotinamide by-product of the Sir2p deacetylase reaction back into NAD\(^+\) near the sites of silencing. This would ensure a constant supply of NAD\(^+\) for Sir2p in the nucleus.

Rad6p is involved in the ubiquitylation of histones H2A, H2B, and H3 in vitro (Sung, Prakash et al. 1988; Haas, Reback et al. 1990) which suggests a role for Rad6p as a modifier of localized chromatin structure (Picologlou, Brown et al. 1990; Kang, Yadao et al. 1992; Liebman and Newnam 1993). Early studies implicated Rad6-mediated ubiquitylation as a regulator of silencing in both S. cerevisiae and S. pombe (Bryk, Banerjee et al. 1997; Huang, Kahana et al. 1997; Singh, Goel et al. 1998). Histone H2B is ubiquitylated by Rad6p at lysine residue 123 through its association with the E3 ubiquitin ligase, Bre1p (Robzyk, Recht et al. 2000) which was an identified effector in our genetic screen. It has been previously shown that mutation of RAD6 or histone H2BK123 completely abolishes H3K4 methylation by Set1p, which illustrates the trans-histone dependence of this chromatin modification (Sun and Allis 2002). Rad6p is also involved in many cellular processes including DNA repair, UV-induced mutagenesis, N-end rule protein degradation, sporulation, and Ty1 integration specificity (Picologlou, Brown et al. 1990; Kang, Yadao et al. 1992; Liebman and Newnam 1993; Prakash, Sung et al. 1993). From our results, and because Rad6p is associated with BRE1, there could be a mechanistic association with rpd3Δ extended silencing. Could the mechanism be that silencing is dependent upon the ubiquitylation of a silencing regulator? One posed explanation is that Rpd3p regulates expression
of \textit{RAD6} which in turn is required for silencing, but it has been shown that Rad6p protein levels are unchanged between WT and \textit{rpd3A} strains (Sun and Allis 2002).

The Bur1/Bur2 cyclin dependent protein kinase is required for histone H2B monoubiquitylation by Rad6/Bre1. Negative effects on histone monoubiquitylation and methylation can be the result of defective Bur1/Bur2-mediated phosphorylation of Rad6p on its serine residue 120 resulting in inhibited recruitment of the Paf1 complex, a Pol II elongation factor, to chromatin. Serine 120 of Rad6p has been shown to be required for silencing of telomere-associated genes, and the overall regulation of gene expression \textit{in vivo} (Wood, Schneider et al. 2005). Mutation of Rad6p serine 120 to alanine leads to a loss of telomeric silencing comparable to that seen in mutants deleted for \textit{RAD6}, \textit{BRE1}, or \textit{RTF1}, all of which are required for histone H2B monoubiquitylation and histone H3 methylation by COMPASS and Dot1p (Wood, Krogan et al. 2003).

COMPASS is a histone H3 lysine 4 (H3K4) methylase consisting of Set1p (\textit{KMT2}) and seven other polypeptides, including Swd2p, the only essential subunit. H3K4 is methylated by the Set1p methyltransferase during transcriptional elongation, through association with the Ser5-phosphorylated CTD of RNA polymerase II (Ng, Robert et al. 2003). The mechanism by which the Set1p methyltransferase of COMPASS differentially methylates H3 (mono-, di-, and tri-) is still not understood. Also, the molecular mechanisms for the histone crosstalk between histone H2B monoubiquitylation by Rad6/Bre1 and the H3K4 trimethylation by COMPASS are poorly understood. In the absence of H2B monoubiquitylation, H3K4 monomethylation is present, however, H3K4 di- and trimethylation are not detectable (Schneider, Wood et al. 2005; Shahbazian, Zhang et al. 2005). COMPASS purified from strains lacking H2B monoubiquitylation is incapable of di- and trimethylating histone H3K4 and has reduced levels
of the COMPASS subunit Swd2p. Some findings offer insight into the molecular role of Swd2p in translating the H2B monoubiquitination signal into H3 methylation (Figure 4.1). The Swd2p recruitment to chromatin via H2B monoubiquitination can bring this subunit in close proximity to COMPASS (interacting with Pol II through the Paf1 complex), resulting in their physical interactions, and therefore, H3K4 di- and trimethylation.


**Figure 4.1. Swd2p is required for translating histone crosstalk between H2B monoubiquitylation and H3 methylation by COMPASS.** COMPASS interacts with the elongating form of Pol II via its interaction with Paf1 complex. A) In the absence of H2BK123 monoubiquitylation by Rad6/Bre1, COMPASS can still interact with Pol II via the Paf1 complex and monomethylate H3K4. B) Through H2BK123 ubiquitylation, Swd2p is recruited to chromatin either directly or indirectly in a COMPASS independent manner. According to this model, the association of Swd2p with COMPASS can facilitate H3K4me3 through its association with another unspecified interaction between Swd2p and the monoubiquitylation of H2BK123. Through this mechanism dependent on ubiquitylation of H2BK123, Swd2p would also associate with Dot1p for the tri-methylation of H3K79.
In a *paf1Δ* mutant, both H3K4me2 and H3K4me3 are eliminated, but H3K4me1 is not affected. Set1p can catalyze H3K4me1 independent of *PAF1*, thus independent of Pol II elongation, but conversion to H3K4me2 or -me3 requires *PAF1* and association with Pol II. The association of Swd2p in a monoubiquitination-dependent manner can result in some posttranslational modification(s) of Swd2p which could facilitate the interaction of Swd2p with COMPASS (Lee, Shukla et al. 2007).

The proposed mechanism above is attractive since it explains how both *BRE1* and *BRE2* are participating in the methylation status of both H3K4 and H3K79. Particularly, it shows how *BRE1* is required for the trimethylation of H3K4 and H3K79, but it also indicates how *BRE2* is directly involved in the monomethylation of H3K4 and how there is a potentially allosteric involvement (through its association with COMPASS) in the enzymatic requirements of the trimethylation of H3K4; plus how this potential allosteric involvement through the association with Swd2p indirectly effects methylation of H3K79. Since both *BRE1* and *BRE2* are effectors of the *rpd3Δ* silencing effect identified in our results, it stands to reason that only the trimethylation status of H3K4 exerts its effects on Sir protein-mediated silencing, but the influence of H3K79 methylation may be far more reaching than previously thought. The question that remains is how the trimethylation of H3K4 and/or H3K79 is involved in the extended silencing effect of *rpd3Δ* at *HMR*.

An interesting and plausible hypothesis for *BRE1*’s influence on *rpd3Δ* enhanced silencing comes through the interaction of *NAT1*-acetylated Sir3p with methylated H3K79. It has been shown that the binding of Sir3p to histone peptides *in vitro* is negatively affected by the methylation and acetylation of the tails of histone H3 and H4 (Carmen, Milne et al. 2002; Santos-Rosa, Bannister et al. 2004; Liou, Tanny et al. 2005). Binding of Sir3p to histone tails
throughout the genome is mediated by the C-terminus of Sir3p (Gasser and Cockell 2001). The C-terminus of Sir3p contains a BAH domain that binds to histone H3K79. Importantly, acetylation of the BAH domain is required for the binding specificity of Sir3p for nucleosomes unmethylated at H3K79 (van Welsem, Frederiks et al. 2008) (Figure 4.2).


**Figure 4.2. Model for the interaction between Dot1p and the acetylated N-terminus of Sir3p in silencing.** Sir1 recruits the Sir 2/3/4 complex in *cis* to the E silencer at *HMRa*. Dot1p acts by methylation (me) of histone H3K79 in euchromatic regions which prevents promiscuous binding of Sir proteins, thus concentrating the Sir proteins at *HMRa* in *trans*. The N-terminus of the BAH domain of Sir3p interacts with the core domain of the nucleosome encompassing H3K79, whereby the acetylated Sir3p is able to discriminate between methylated and unmethylated H3K79 and preferably binds to unmethylated H3K79. In the absence of acetylated Sir3p, Sir3p loses its specificity for unmethylated H3K79 and binds to euchromatic regions thus diluting Sir protein concentration at *HMRa*.

With the deletion of Dot1p, binding of Sir2p and Sir3p at silent chromatin is reduced, and Sir3p becomes redistributed (San-Segundo and Roeder 2000; Ng, Feng et al. 2002; van Leeuwen, Gafken et al. 2002; Ng, Robert et al. 2003) which can reduce silencing at *HMR*. This same scenario can apply to the deletion of *BRE1* since Dot1p trimethylation at H3K79 is
dependent on the ubiquitylation of H2BK123 mediated by Rad6/Bre1 (Figure 4.1). Some genetic evidence is consistent with this model in which the N-terminus of the BAH domain of Sir3p binds histone H3K79 on the nucleosome core. Specific point mutations in the Sir3p N-terminus, the deletion of NATI (N-terminal acetyltransferase), and the deletion of DOTI each had very similar silencing phenotypes that showed reduced telomeric silencing (van Welsem, Frederiks et al. 2008). It would be interesting to see if a decrease in extended silencing occurs at HMR upon deletion of NATI in the rpd3Δ background.

Based on results by van Welsem et al. (2008), the Sir3p N-terminus binds the nucleosome core on a surface that includes histone H3K79 and acetylation of the Sir3 N-terminal alanine is required for the specificity of Sir3p for unmethylated histone H3K79. In the absence of this specificity (mutant or unacetylated Sir3p or no histone H3K79me in euchromatin), Sir3p becomes a promiscuous chromatin-binding factor, which leads to reduced Sir3p binding in silent regions, since Sir3p is in limited supply (van Welsem, Frederiks et al. 2008) (Figure 4.2). Biochemical studies (Onishi, Liou et al. 2007) demonstrate the binding of the Sir3p BAH domain to yeast nucleosomes is negatively affected by H3K79me, although it is not clear whether these interactions also occur with nucleosomes in vivo (van Welsem, Frederiks et al. 2008).

Sir2p has been shown to be mainly localized to two distinct sub-nuclear domains, telomeres and the nucleolus (Gotta, Strahl-Bolsinger et al. 1997). The nucleolus has been proposed to serve as a reservoir for Sir2p storage, competing with subtelomeric regions and HM loci for a limiting supply of Sir2p (Maillet, Boscheron et al. 1996; Gotta, Strahl-Bolsinger et al. 1997; Smith, Brachmann et al. 1998). A global redistribution of a limited pool of Sir2–4 to euchromatin would be expected to result in weak silencing at euchromatic loci, thus the depletion of Sir proteins from heterochromatic regions resulting in dramatically reduced silencing. The fact that Sir3p is
undetectable at euchromatic loci by ChIP suggests that, compared with Sir protein association at sites near and within the silenced loci, ectopic Sir binding is much weaker and/or transient and occurs at a given gene only in a fraction of all cells (Venkatasubrahmanyam, Hwang et al. 2007).

In accordance with the view that Sir proteins can be redistributed in the nucleus, immunolocalization studies of Sir2p showed that in H4K5Q rpd3Δ cells, most of the Sir2p signal was congregated in the nucleolus instead of diffused throughout the nucleus as was observed in rpd3Δ cells with wild-type histone proteins. These observations indicate that deacetylation of H4K5 by Rpd3p is likely required for restricting the spread of Sir2p into euchromatic regions and is important for limiting heterochromatic silencing. Considering that H4K5 is one of the targets of Rpd3p, it has been postulated that boundary formation in the subtelomeric and HMR regions requires H4K5 deacetylation (Zhou, Zhou et al. 2009); again however, it is counterintuitive to rationalize how an increase in H4 acetylation would be important for the propagation of heterochromatin. When Rpd3p is absent, could an increase of H4K5 acetylation facilitate Sir2p binding? The mechanism as to how the increase of H4 acetylation caused by Rpd3p inactivation facilitates the establishment of silent chromatin remains mysterious, and requires further investigation.

The deacetylation reaction of Sir2p is distinct from that of non NAD⁺-dependent HDACs in that it not only produces nicotinamide as a by-product, but also an unusual compound, O-acetyl-ADP ribose (OAADPR) (Tanner, Landry et al. 2000), which has been proposed to influence SIR complex stability (Liou, Tanny et al. 2005). Another proposed mechanism based on recent evidence suggests histone deacetylation by Rpd3p removes the substrate for Sir2p (H4K16), so that Sir2p no longer can produce O-acetyl-ADP ribose (OAADPR) by consumption of NAD⁺ in the deacetylation reaction. In this model, OAADPR therefore is unavailable for binding to Sir3p,
preventing SIR complex propagation (Ehrentraut, Weber et al. 2010). Intriguingly, the Sir3 protein carries a domain that resembles the ATP binding pocket of AAA+ ATPases but lacks certain catalytic residues (Neuwald, Aravind et al. 1999). It therefore has been hypothesized that this domain constitutes an OAADPR binding site (Gasser and Cockell 2001). Therefore, heterochromatin spreading is stopped by the inability of Sir2p to perform histone deacetylation, to produce OAADPR, and thus to support heterochromatin spreading.

In light of recent evidence, the loss of silencing at HMR that was observed in our double mutants, bre1Δrpd3Δ and bre2Δrpd3Δ, could be the result of the loss of H3K4me3 and H3K79me3 genome-wide, which would promote the promiscuous binding of Sir proteins into euchromatic regions, thus diluting them from areas of typical heterochromatic dominance.

![Figure 4.3](image.png)

**Figure 4.3. Model of the re-distribution of Sir proteins in wild-type vs. rpd3Δ cells.** Normal silencing at HMR occurs due to adequate concentration of Sir proteins at this region with limited promiscuous binding of Sir proteins in euchromatic regions in wild-type cells. The loss of the deacetylase Rpd3p results in a loss of global deacetylation and fewer deacetylated histone targets in euchromatic regions. The limited pool of Sir proteins would concentrate higher at the normal silenced region of HMR resulting in an increase of extended silencing enough to bypass the tRNA barrier and silence the downstream ADE2 gene.
How does this relate to the global loss of deacetylation in an \( rpd3\Delta \) strain? Since our study mainly focuses on elucidating the mechanism of extended silencing at \( HMR \), we posit a model where in wild-type cells, the limiting pool of Sir proteins are concentrated enough at \( HMR \) to repress the transcription of the a-specific mating type genes, but not concentrated enough to bypass the tRNA barrier element and silence the downstream \( ADE2 \) gene (Figure 4.3).

In this model, a fraction of the silencing proteins are distributed to other chromosomal regions near euchromatic regions where deacetylated nucleosomes are always potential targets of transient or limited binding of Sir proteins. In \( rpd3\Delta \) strains, the global loss of deacetylation causes a subsequent increase in the global acetylation of nucleosomes, which would inhibit the binding of Sir proteins to euchromatic regions. The distribution of this freed pool of Sir proteins would shift to heterochromatic regions such as \( HMR \), which could override the tRNA barrier element and silence the \( ADE2 \) marker gene. So, is the increasing silencing effect of \( rpd3\Delta \) due to a global re-distribution of Sir proteins based on the acetylation status of euchromatic regions?

DamID (DNA adenine methyltransferase) methodology, first established in \( Drosophila melanogaster \), is a method used to profile chromatin-associated proteins (van Steensel and Henikoff 2000). Studies using the fusion of Sir3p and Sir4p to \( Escherichia coli \) Dam in wild-type and \( set1\Delta htz1\Delta \) strains resulted in Dam-mediated DNA methylation at sites where Sir3p or Sir4p was bound to chromatin. With no endogenous DNA methylation in \( S. cerevisiae \), the percentage of cells methylated at a particular locus provided a read-out of the chromatin association of Sir3p or Sir4p (Venkatasubrahmanyam, Hwang et al. 2007). Sir-dam fusion proteins result in methylated GATC sites at Sir protein bound sites in the genome, and can be digested by the restriction endonuclease \( DpnI \). Oligo primer sets targeted at specific genomic locations can reveal the presence or absence of site-specific methylation depending on whether that region has
been cut by \textit{DpnI} (no amplification) or not (amplification). Thus, regions in the genome that have been occupied by either Sir3p or Sir4p can be assessed by reduced amplification using PCR.

![Figure 4.4. Strategy for the genome-wide assessment of Sir proteins using Sir3- or Sir4-dam methylase fusion proteins. By utilizing Sir3p or Sir4p fused to the \textit{E. coli} dam methylase, which contains functionally reduced methylase activity due to conditional mutations in the DNA binding domain of the enzyme, we can assess the degree of transient Sir protein occupancy in euchromatic regions and in higher affinity binding heterochromatic regions by the degree of DNA methylated at GATC sequences. Methylation-specific restriction endonucleases along with oligo primer sets probing targeted genomic loci can assess limited Sir protein binding. Currently, our experimentation involves creating both Sir3p and Sir4p functional proteins fused to an \textit{E. coli} dam methylase (Figure 4.4). Dam methylase of \textit{E. coli} catalyzes the transfer of a methyl group from S-adenosyl-methionine (AdoMet) to the N6 amino group of the adenine in the sequence GATC (Hattman, Brooks et al. 1978; Geier and Modrich 1979). Our initial restriction assays using unfused wild-type dam methylase (Sir4pr-dam) showed high global DNA methylation across the entire genome. This high background made it difficult to distinguish targeted from untargeted Dam methylation. To attempt to refine the assay, we reasoned that creating catalytic mutants in the Dam methylase might improve the signal-to-noise ratio at Sir targeted sites. Based on previous studies of Dam methylase activity, we created three mutations,
P134S which contains a specific methylase activity of 66% of wild type Dam, G136A (42%), and R137L (6%) (Guyot, Grassi et al. 1993). Future studies will use unfused dam methylase mutants driven by the Sir4 promoter region for normalization of this assay. The inherent extended silencing effect of rpd3Δ at HMR shows that barrier elements such as the tRNA\textsuperscript{thr} gene can be overcome by Sir protein-mediated silencing when various chromatin processes are perturbed. As each new piece of evidence contributes to the puzzle, we are progressively getting a better understanding of the true complexity of barriers in the context of an extremely dynamic genome.

Like previous studies in our lab, our latest study (autoregulation of TFC6 by TF\textsubscript{III}C in the TFC6 promoter) is solidifying the role of TF\textsubscript{III}C binding sites as genomic elements with extra-transcriptional functions. Our results implicate the B box sequence element of type II RNA polymerase III (tRNA) genes and ETC sites in insulator function. The question remains if other ETC sites in budding yeast to humans carry the same regulatory capacity in blocking targeted activation between genomic domains. It is interesting to speculate how these B box elements might have been evolutionarily selected to function in an insulator capacity.

The yeast genome contains 274 unclustered tRNA genes which are dispersed throughout its genome of 16 chromosomes (32 in diploid strains) (Percudani, Pavesi et al. 1997; Hani and Feldmann 1998). tDNAs are represented as gene sets which redundantly code for 42 tRNA species with different codon specificities. Individual tRNA species range in copy number from 1 to 16 and correlates with both the frequency of codon occurrence on mRNAs and the intracellular amount of individual tRNAs (Percudani, Pavesi et al. 1997). Because of tDNA redundancy, and the intragenic location of the A and B blocks, genes coding for the same tRNA species in eukaryotes generally share identical TF\textsubscript{III}C-binding promoter elements, but are flanked
by divergent TF_{III}B interacting sequences (Hani and Feldmann 1998). Can varying the nuclear concentration of TF_{III}C differentially control the transcriptional outputs of the various tRNA gene families? Our results have indicated that overexpression of the TF_{III}C component *TFC6* increases relative TF_{III}C binding at representative tDNA and ETC loci (Figure 3.10). With excess TF_{III}C present, transcriptional output would be proportional to the tRNA gene copy number, but with limiting TF_{III}C the output would be determined by TF_{III}C binding affinity to these loci. It is intriguing to speculate that the cell might exploit varying TF_{III}C binding constants based on Tfc6p levels in the cell to differentially regulate the transcriptional outputs of its various tRNA gene families.

The origin of isolated B box sequences dispersed throughout the yeast genome, which are overrepresented between divergently transcribed Pol II genes, remains an interesting evolutionary notion about the multi-functionality of tRNA genes that are progressively being discovered. Are ETC loci remnants of early tRNA genes, but have lost A box sequences and functional TATA-like sequences just upstream of the RNA initiation site since these upstream sequences heavily influence Pol III transcription *in vivo* and *in vitro* by facilitating the association of TF_{III}B? However, many Pol III promoters lack canonical TATA elements and the transcriptional effects of the TATA-like sequences are quantitatively modest. Do the ETC loci have DNA sequences that result in positioned nucleosomes that do not interfere with TF_{III}C binding, but essentially block the association of TF_{III}B and Pol III? Are DNA-binding proteins bound at critical positions at the ETC loci blocking the association or recruitment of TF_{III}B and Pol III while not affecting association of TF_{III}C? Is TF_{III}C bound at the ETC loci in a conformation that precludes its association with TF_{III}B?
From an evolutionary perspective, the ETC loci may be false Pol III promoters that were derived from vestigial Pol III genes or could be just fortuitous occurrences of TF\textsubscript{III}C recognition sequences. The highly conserved promoter sequences between species surely make the former highly probable. Expanding on this idea, especially in light of the sequence conservation among related yeast species is that some (and perhaps all) ETC loci are bona-fide Pol III promoters at which the complete Pol III apparatus assembles only under specific conditions. Perhaps a modification of TF\textsubscript{III}C or TF\textsubscript{III}B in response to a specific environmental or genetic condition might permit the recruitment of the intact Pol III machinery to the ETC loci. Alternatively, a given condition might result in the dissociation of an inhibitory factor that blocks TF\textsubscript{III}B and Pol III entry at these loci; this could possibly have implications for other mechanisms of regulation of the neighboring genes. It can be difficult to distinguish between true functional elements and evolutionary remnants, though the potential to identify new classes of functional elements always exists. For example, TF\textsubscript{III}C binding sites have the capacity to function as an insulator, as either a tDNA or an ETC sequence can block the interaction of Gal4p with the GAL10 promoter. TRT2 tDNA in its natural context serves as an insulator between the STE6 and CBTL genes, preventing the STE6 regulatory elements from affecting CBTL transcription levels (Simms, Miller et al. 2004).

RNA170 (ETC5) was found as a new, non-essential 170 nucleotide non-coding RNA transcript (Olivas, Muhlrad et al. 1997; Roberts, Stewart et al. 2003). Following genome-wide ChIP-CHIP of subunits of Pol III, TF\textsubscript{III}B, and TF\textsubscript{III}C, the low levels of Pol III and TF\textsubscript{III}B that were present at this locus suggested that RNA170 may be transcribed at very low levels (Roberts, Stewart et al. 2003). ZOD1 (Zone Of Disparity), which possibly produces short transcripts, is so named because occupancy by Pol III was initially found to be disproportionately low when
compared with TFIII C occupancy (Moqtaderi and Struhl 2004). Nucleosome depletion dramatically enhances expression of both ZOD1 and ETC5 without affecting their occupancy by the Pol III machinery (Guffanti, Percudani et al. 2006). The ZOD1 locus is also bound by all components of the Pol III machinery and was initially implicated as a promoter that is bound by a transcriptionally incompetent form of the complete Pol III machinery (Stunkel, Kober et al. 1997; Moqtaderi and Struhl 2004). The ZOD1 region is riddled with poly(dT) stretches, which can act as termination signals for Pol III, so the resulting short RNAs are likely to be quickly degraded and hence difficult to detect. Although this model might imply that ZOD1 is a meaningless pseudogene or other genomic relic, the high conservation in yeast of the ZOD1 A and B blocks, as well as the conserved binding of Pol III factors to this locus suggest that any localized Pol III transcription may have a physiological function. Comparative genomic analysis revealed that the ZOD1 promoter is the only surviving portion of a tDNAffe ancestor, whose transcription capacity has been preserved throughout evolution independently from the encoded RNA product. Another TFIII C/TFIII B-associated element in the S. cerevisiae genome, iYGR033c, was also identified as a conserved tDNAArg remnant (Guffanti, Percudani et al. 2006).

In S. pombe, inverted repeat (IR) boundary elements flanking the mating-type heterochromatin domain also contain B-box sequences, which prevent heterochromatin from spreading into neighboring euchromatic regions by recruiting the TFIII C complex without Pol III. The IR elements actually contain multiple B box sequences that are also required for efficient transcription of IRs by RNA Pol II. This finding resulted in a subsequent genome-wide profiling analysis which unexpectedly identified a number of COC sites dispersed across the fission yeast genome. These TFIII C-bound sequences are tethered to the nuclear periphery in a B box dependent manner (Noma, Cam et al. 2006). Similar to the IR boundaries, multiple B boxes are
located at these TF_{III}C bound regions. Interestingly, most of these loci were found in intergenic regions between divergent Pol II genes and one of the divergent Pol II promoter regions occupied by TF_{III}C is the gene SFC3 (TFC6 ortholog in Saccharomyces), suggesting the potential for transcriptional autoregulation of SFC3 by TF_{III}C in fission yeast (Noma, Cam et al. 2006). ETC loci in budding yeast might have a role in genome organization via the tethering of specific chromosomal regions to subnuclear structures, akin to the clustering of tRNA genes near the nucleolus (Thompson, Haeusler et al. 2003).

Recent ChIP, followed by massive parallel sequencing (ChIP-Seq), data of Pol III components puts the human ETC site list at 5,474 loci, in contrast with 1,520 for the RNA Pol III subunit Rpc155, even though fold enrichments, and hence assay sensitivity, is higher for Rpc155. In these studies, an ETC locus was defined as having a TF_{III}C/Rpc155 ratio higher than 2.04, 3 s.d. above this median ratio. ETCs by this definition do not need to be completely lacking in Pol III occupancy. Under these strict experimental criteria (only 3 tRNAs passed), 1,865 ETCs were identified and the distribution of TF_{III}C-occupied loci of both ETC and some non-ETC types revealed a positional bias toward the transcription start sites (TSSs) of Pol II genes. 181 ETC loci with the highest levels of TF_{III}C occupancy were strikingly well correlated with the TSSs of Pol II genes, with 68% being located within 1 kilobase of a Pol II TSS (Moqtaderi, Wang et al. 2010). This is reminiscent of the S. cerevisiae ETCs, which are ~200–300 bp upstream of a neighboring Pol II gene. Interestingly, these studies also revealed differences in TF_{III}C occupancy at ETC loci between HeLa S3 and K562 cells suggesting that TF_{III}C binding and/or TF_{III}B recruitment at ETC loci is possibly influenced by cell type specific factors (Moqtaderi, Wang et al. 2010). While the genomic role of ETC sites has not been studied in depth, our results suggest that TF_{III}C binding sites may have general functions as boundary
elements, and in the specific example of the *TFC6* promoter, may function as an autoregulated insulator (Figure 4.5).

![Figure 4.5. TFIII potentially functions as an insulator to the targeted activation of the TFC6 promoter by a putative DNA-binding transcription factor.](image)

**Figure 4.5. TFIII potentially functions as an insulator to the targeted activation of the TFC6 promoter by a putative DNA-binding transcription factor.** The binding of the TFIII complex to the ETC6 site is autoregulated by its own protein product and inhibits the targeted activation and the assembly of a preinitiation complex either through steric hindrance or the blockage of a tracking mechanism at the TFC6 promoter resulting in lower TFC6 expression. Directional TF targeting near the TSS of TFC6 has been omitted for clarity.

Relative levels of the Tfc6 protein are lower as compared to other essential TFIII subunits (Ghaemmaghami, Huh et al. 2003; Kleinschmidt, Leblanc et al. 2011), so the auto-regulatory mechanism at the *TFC6* promoter gives credence to Tfc6p as a limited component to TFIII assembly/binding, thus having a potentially tremendous impact on maintaining cellular TFIII levels. Does this affect the overall regulation of tRNA genes? Since our results indicate that the regulation of *TFC6* is dose-dependent on the levels of its own protein product, we also investigated the global effect of *TFC6* overexpression on other Pol III promoter elements such as tRNA genes and other ETC loci in the yeast genome. Currently, there are no other studies showing that overexpression of any TFIII component causes an increase in either TFIII binding to tRNA genes or ETC sites. Future overexpression studies of *TFC6* will mainly focus on the effects at tRNA genes and the possible effects of regulatory output. ChIP-Seq analysis should
give us whole-genome data determining which TFIII-C-occupied loci shows higher enrichment upon overexpression of TFC6. Also, it is technically difficult to measure the transcriptional activity of individual tRNA genes because most are repeated. Based on ChIP-Seq data, we can assay any Pol III transcribed gene of interest by tagging the tRNA genes showing higher TFIII-C binding upon TFC6 overexpression.

The lower levels of TFC6 expression in our promoter mutant #3 also points to a potential DNA-binding protein that seems to function as a transcriptional activator for TFC6, which could participate in the assembly of an RNA Pol II initiation complex near the TSS of TFC6. Sequence analysis of this promoter region shows sequence similarity to the DNA-binding site of Reb1p. The role of Reb1p, as the putative DNA-binding transcription factor, is currently being assessed by analyzing the effects of REB1 overexpression on TFC6 promoter activity. If Reb1p is the putative transcription factor bound to promoter region #3, then overexpressing REB1 should ultimately show increased rate of growth on media lacking uracil in our TFC6 promoter-URA3 strains described in Chapter 3.

Reb1p is among the abundant so-called “general regulatory factors.” It is a multifunctional transcription factor encoded by an essential gene. The 125-kDa protein binds as a monomer to its site on DNA with the consensus YNNYYACCCG, and its DNA-binding domain is homologous to the vertebrate proto-oncogene myb. Reb1p has an architectural role, and its DNA binding is necessary and sufficient to keep nucleosomes off the DNA region spanning the core promoter (Ju, Morrow et al. 1990; Morrow, Ju et al. 1993).

An alternative hypothesis to the role of TFIII-C as an insulator can include another proposed mechanism of regulation at the TFC6 promoter region. There is the possibility of competitive binding between the unknown DNA-binding protein and TFIII-C at the short stretch of DNA
between promoter region #3 and the \textit{ETC6} site (Figure 4.6). Since the \textit{TFC6} promoter element (promoter region #3) is immediately upstream of the \textit{ETC6} B-box (~12bp), it stands to reason that \textit{TF}_{\text{III}C} when bound might occlude the binding of the transcription factor resulting in decreased \textit{TFC6} expression (Figure 4.6 B). The same situation could occur with the transcription factor bound at promoter region #3 where the assembly of the entire \textit{TF}_{\text{III}C} complex is inhibited resulting in increased \textit{TFC6} expression (Figure 4.6 A).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{competition.png}
\caption{Competition between \textit{TF}_{\text{III}C} and transcription factor (TF) for binding the limited region of putative \textit{UAS} and \textit{ETC6}. A) In the competition model of \textit{TFC6} transcription activation, the binding of the putative transcription factor to promoter region #3 occludes the binding of \textit{TF}_{\text{III}C} to the \textit{ETC6} site and activates \textit{TFC6} transcription. B) Increased levels of \textit{TF}_{\text{III}C} will bind to the \textit{ETC6} site and occlude the putative transcription factor from binding at the promoter region #3 thus lowering \textit{TFC6} expression.}
\end{figure}

It is clearly evident that chromatin boundary elements play an expanding role in the natural context of eukaryotic genomes. The extra-transcriptional effects of tDNAs (tRNA genes) and the influence they have on gene regulation and chromatin organization show that eukaryotic
genomes are not only dependent on the sequence of nucleotides on chromosomes, but are also epigenetically controlled at all stages of cellular differentiation in the life of all eukaryotic organisms.

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APPENDIX: AUTHORIZATION FOR USE OF PUBLISHED MATERIAL

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Richard Alton Kleinschmidt is the son of Mr. Robert Sere Kleinschmidt and Mrs. Bonnie Brown Oglesby. He was born in Lake Charles, Louisiana, in 1970 and graduated from A.M. Barbe High School in Lake Charles, Louisiana, in 1988. Richard received his Bachelor of Science in biology from the University of Southern Mississippi in 1995 and went on to receive his Master of Science degree in human physiology from the University of Southern Mississippi in 1996. Richard began his research career as a Polysomnography Technician at Emory University School of Medicine in Atlanta, Georgia, from 2002-2003. He continued as a Research Assistant II in molecular pathology at Children’s Hospital Institute for Research in New Orleans, Louisiana, from 2003-2006 under the guidance of Dr. Deborah Fox. He began his doctoral research in the fall of 2006 in the Department of Biological Sciences at Louisiana State University under the guidance of Dr. David Donze. Mr. Kleinschmidt will graduate with the degree of Doctor of Philosophy in biological sciences in August 2011.