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Boundaries of transcriptionally silent chromatin in 
Saccharomyces cerevisiae

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In the budding yeast Saccharomyces cerevisiae, heterochromatic gene silencing has been found within HMR and HML silent mating type loci, the telomeres, and the rRNA-encoding DNA. There may be boundary elements that regulate the spread of silencing to protect genes adjacent to silenced domains from this epigenetic repressive effect. Many assays show that specific DNA regulatory elements separate a euchromatic locus from a neighboring heterochromatic domain and thereby function as a boundary. Alternatively, DNA-independent mechanisms such as competition between acetylated and deacetylated histones are also reported to contribute to gene insulation. However, the mechanism by which boundaries are formed is not clear. Here, the characteristics and functions of boundaries at silenced domains in S. cerevisiae are discussed.

Key words: boundary, gene silencing, S. cerevisiae, euchromatin and heterochromatin, histone modification

INTRODUCTION

Two different forms of chromatin domains exist in the nucleus: euchromatin and heterochromatin. In metazoans, euchromatin domains enriched in acetylated core histones present a DNA configuration that is accessible to replication and transcription proteins, whereas heterochromatin domains contain histone H3 methylated on lysine 9 (K9) and lysine 27 (K27) with repressor proteins that package the chromatin into an inaccessible configuration that silences gene transcription. The situation is different in the budding yeast S. cerevisiae. The N-terminal tails of histones H3 and H4 are hypoacetylated and bound by the Sir (Silent information regulator) proteins. The Sir proteins are recruited to the silenced domain, where Sir2p, an NAD+-dependent histone deacetylase, modifies the tails of histones H3 and H4 and recruits Sir3p that, in turn, promotes binding of the Sir4p/Sir2p complex to the deacetylated tails and allows heterochromatin to propagate along nucleosomes (Imai et al., 2000; Rusche et al., 2003). Several silenced regions of the S. cerevisiae genome have been detected (Fig. 1). These include the two silent mating type loci that possess silenced HMR and HML domains, the telomeres, and the rRNA-encoding DNA. Sir2p, Sir3p, and Sir4p are all required for the spread of silencing at the HMR domain, HML domain and the telomeres, although silencing of genes at rDNA domain requires Sir2p only. Next to or near these silencing domains are genes important for the growth of yeast. Thus, it is necessary for these genes to be protected from the silencing that might be propagated from the silenced domains. This suggests that regulatory elements, or “boundaries”, stop the spread of gene silencing. DNA sequence-dependent and -independent models have been proposed to explain how gene silencing is localized to particular regions of the DNA (Oki and Kamakaka, 2002; Valenzuela and Kamakaka, 2006; Kimura and Horikoshi, 2004; Donze and Kamakaka, 2002). While the mechanisms are not understood, the characteristics and functions of boundaries at all silenced loci have common features.

HMR

Characteristics of the silenced HMR region The HMR locus contains E silencer (HMR-E), open reading frames of the a2 and a1 mating type genes and I silencer (HMR-I) (Fig. 1A). Outside the HMR locus, there are tRNA gene and Ty-LTRs (long terminal repeats). Among
the four silencers (HMR-E, HMR-I, HML-E, HML-I) of the two silent mating type loci, HMR-E is perhaps the most thoroughly studied and has an ARS consensus sequence (ACS) for the binding of the origin recognition complex (ORC), as well as binding sites for two well-known transcription activators: Rap1p and Abf1p. Ty1-LTR and Ty5-LTR are present upstream of HMR-E. HMR-I has binding sites for Abf1p and ACS with Ty1-LTRs and the tRNA gene downstream of the HMR-I. The number of Ty1-LTRs on the right side of the HMR domain is not constant and varies depending upon the yeast genetic background. The GR1 gene is present near the HMR domain on Chromosome III and its relationship with silencing is not fully understood.

**HMR right boundary** The right boundary mediates boundary functions more prominently than the left boundary. Right boundary deletion analysis demonstrates that a small DNA fragment of the tRNA gene can block the spread of silenced chromatin and that the LTR is not sufficient, despite contributing to boundary function (Donze et al., 1999). This boundary element stops the spread of silencing at the telomere domain when it is inserted in either orientation between the telomere and a URA3 reporter gene. Another study showed that a unique tRNA Thr gene (tRNA Thr 1 [AGT] CR1) on the right side of HMR is necessary for barrier function (Donze and Kamakaka, 2001). Since the tRNA Thr gene shows robust barrier activity, the authors tested whether other yeast tRNA genes also have barrier function by inserting them between HMR-E and the reporter gene a 1. Interestingly, the tRNA Thr GR1 present on chromosome VII has nearly the same level of barrier function as the tRNA Thr. Conversely, the tRNA Thr NL1 from chromosome XIV and the tRNA Thr KL at chromosome XI have a weak barrier function. One copy of the tRNA Thr NL1 is a weak boundary, but it exhibits strong boundary function when duplicated in tandem, which indicates that tRNA promoter occupancy plays a role in boundary function (Donze and Kamakaka, 2001). Results from the same study show that Pol III transcription factor TFIIC, which binds to the box B and box A promoter elements, and TFIIB, which binds upstream of the tRNA gene, are necessary to form the barrier; although, Pol III is not required. In addition, a sas2 histone acetyltransferase (HAT) mutant showed reduced barrier function and Sas2p creates a strong barrier when present as a Gal4BD-Sas2p fusion protein (Donze and Kamakaka, 2001). A systematic genome-wide screen for proteins with barrier function was used to understand these mechanisms that establish barrier functions (Oki et al., 2004). Mutants of both the tRNA-bound transcription complex and the HAT complex (SAGA, SAS-I, NuA4 etc.), although the yeast HATs are not preferentially localized to the tRNA boundary, resulting in a loss of barrier activity, which suggests that there are two parallel pathways to mediate native barrier functions (Oki and Kamakaka, 2005).

Recent data show that tRNA-bound transcription factors contribute to recruitment of the chromatin remodeler RSC complex to tRNA to form a 700-bp region depleted of histones that centers on the tRNA gene (Dhillon et al., 2009). DNA polymerase ε and Rtt109p, the histone H3 acetyltransferase, are recruited to form and maintain this histone-depleted region and to bind transcription factors to the tRNA. The two pathways to block the spread of silenced chromatin at HMR are independent. One pathway involves the tRNA-bound Pol III transcription complex, particularly, transcription factor TFIIC. The other pathway involves a competition between histone acetyltransferase complex, SAS-I, SAGA, NuA4, and Sir2 histone deacetylases. Another study that focused on Pol III-transcription factor TFIIC shows that tRNA or binding by TFIIC were sufficient for chromatin boundary function (Simms et al., 2008). The same study shows that ETC (extra TFIIC) sites that bind TFIIC but not other elements of the Pol III complex, can function both as a barrier that blocks the spread of heterochromatin and as an insulator of gene activation. Furthermore, TFIIC binding without binding of TFIIB is sufficient for this function. Other results show that TFIIB binding is required for boundary formation; however, TFIIB binding contributes to formation of the complex at the insulator. Neither RNA pol II nor RNA pol III are required for efficient boundary formation, but specific histone modifiers and remodelers influence the insulation mediated by TFIIC as shown in Fig. 2A (Valenzuela et al., 2009).

Silencers can function to regulate silencing through the recruitment of Sir3p, and they can exhibit long-range communication between each other at the HMR domain. By mapping Rap1p at HMR using quantitative ChIP, it was also found to be present at HMR-I silencer without Rap1p binding site (Fig. 1A), suggesting that the E silencer can be near the HMR-I silencer. However, long-range communication between the two silencers is not affected by a mutation of the tRNA barrier or by deletion of nuclear pore protein Nup2p, but it is dependent on the presence of Sir3p (Valenzuela et al., 2008). Chromosome conformation capture (3C) shows that HMR and HML are preferentially co-localized (Miele et al., 2009) and that HMR and HML interacted specifically by way of the E silencer or the I silencer. A model devised shows that silencing proteins are first recruited to the HM silencers, and subsequently, HMR and HML interact with each other to form heterochromatin that may be anchored to the nuclear periphery. Direct evidence for physical associations between HML silencers and telomeres was obtained using a methyltransferase targeting assay where the bacterial DNA methyltransferase Dam was fused to the TetR binding domain and the resulting
chimera was targeted to Tet operators at the repressed locus HML. A quantitative technique that combines methylation-sensitive restriction analysis and real-time PCR reveals the methylation status of HML (and other genes), and suggests the existence of higher-order chromatin structures that consist of various silencing elements (Lebrun et al., 2003). In addition, other studies show a yKu-mediated interaction between HM loci and subtelomeric core X sequences that consists of a fold-back structure (Marvin et al., 2009).

Meanwhile, a relationship between the regulation of boundaries and the cell cycle is evident (Kirchmaier and Rine, 2006; Lazarus and Holmes, 2011). Aside from the influence of nucleosome structure, the histone variant Htz1p is enriched in euchromatic regions outside the HMR domain and could interfere with the spread of silencing (Meneghini et al., 2003). Conversely, in htz1Δ cells, Sir2p and Sir3p spread into the neighboring euchromatic domains that include anti-silenced genes such as...
GIT1, which reveals that Htz1p has a role in boundary function.

**HMR left boundary** To examine the function of the HMR left boundary, the left boundary element was inserted between the HMR-E silencer and a1 in an HMR-I deleted strain and expression of a1 was monitored by mating analysis. The left boundary stops repression from the HMR-E silencer, but this repression is not as strong as that found for the right boundary (Donze et al., 1999). Other studies show that Dpb4-related chromatin complexes regulate both the left and the right boundary. These complexes contain more than 15 chromatin-associated proteins, including DNA pol ε holoenzyme (Pol2p, Dpb2p, Dpb3p, Dpb4p), chromatin remodeling proteins Itc1p, Isw2p, and Dls1p, the AAA ATPase Yta7p, the histone acetyltransferase Sas3p, and an RNA polymerase core subunit Rph2p (Tackett et al., 2005). All of these proteins are present in both the pole and chromatin remodeling complexes. The left side of HMR-E, the relationship of the Ty5-LTR and the Ty1-LTR with the formation of heterochromatin is not understood.

Silencing could be prevented from spreading into neighboring euchromatin by deletion of the boundary element. However, many factors associated with euchromatin can also prevent silencing including histone H3 methylated on lysine 36 (K36). The anti-silencing function of methylated H3K36 depends upon Set2p, a histone ortholog of histone H1, which modifies boundary function such as deacetylation and may inhibit silencing (Veron et al., 2006).

**HML**

**Characteristics of the silenced HML region** The HML locus contains the E silencer, as well as the open reading frames of the α2 and αl mating type genes, and the I silencer. HML-E has binding sites for ORC, Rap1p, and Sum1p (I2) while HML-I has binding sites for Abf1p and ORC (Irlbacher et al., 2005). The CHA1 gene is approximately 2 kb downstream from the HML-I silencer (Fig. 1B). Between the HML-E and HML-I silencers, histone hypoacetylation-mediated silencing is detected at a high level that decreases beyond the HML-I silencer (Bi et al., 1999). Like HMR silencing, the Sir protein complex is required to establish and maintain silencing.

**HML boundary** No natural chromatin boundary flanking the HML domain was found, although the UAS\textsubscript{Bfr} (upstream activation sequence of ribosome protein genes) that bind several Rap1p molecules can function as a boundary by forming a gap between nucleosomes in chromatin that stops the migration of the Sir protein complex (Bi and Broach, 1999). The high level of silencing inside HML and little silencing outside HML suggests two models are possible: a boundary model and a directional model. Mating experiments show that neither HML-I nor the sequence flanking the HML-I silencer can stop silencing results that disprove the boundary model. When its natural orientation is reversed, the HML-I silencer decreases silencing inside HML and silencing outside the HML centromere proximal to HML-I increases. This suggests that the HML-I silencer works between the active and inactive domains in one orientation (Bi et al., 1999; Bi and Broach, 2001). The CHA1 promoter that is normally repressed by the Sir complex is approximately 2 kb downstream of the HML-I silencer. However, in the presence of serine, CHA1 transcription is induced, which suggests that it plays a role in the boundary formation (Donze and Kamakaka, 2001). The LB sequence, a 7-kb domain that is not silenced, functions as a boundary upstream of the HML-E silencer and may function as a promoter (Bi, 2002) (Fig. 1B).

Aside from the presence of boundary sequences, anti-silencing negatively regulates silencing by way of several chromatin factors that compete with one another to form active or inactive chromatin. By forming a hyperacetylated zone, histone acetyltransferases Esa1p or Gcn5p can counteract transcriptional silencing that requires deacetylated histones (Chiu et al., 2003). A genetic screen revealed genes that encode proteins with a role in boundary formation such as HHO1 that encodes the yeast ortholog of histone H1, which modifies boundary function and may inhibit silencing (Veron et al., 2006).

**TELOMERES**

**Characteristics of the silenced telomere domain**

Telomeres are regions of repeated DNA at the end of chromosomes that contain elements known as X element core sequences (core X), subtelomeric repeats (STR), Y' elements, subtelomeric anti-silencing regions (STAR), and telomeric repeats (TG\textsubscript{13} repeats) (Fig. 1C). There are two types of telomere: one with only X elements and the other with both X and Y' elements. The X elements contain an ACS sequence and, in most instances, an Abf1p binding site while Y' elements contain a helicase-encoding sequence, an ACS sequence, and STAR. TG\textsubscript{13} repeats or/and STR are present between core X and Y' elements. In addition, Rap1p binds to TG\textsubscript{13} repeats at the terminal telomeres to form a loop structure (Pryde and Louis, 1999) (Fig. 2B).

At telomeres, Rap1p recruits Sir4p, and Sir2p binds the DNA upon interaction with Sir4p, which causes deacetylation of the N-terminal tails of histones H3 and H4. Sir3p/Sir4p bind to the deacetylated histone N-terminal tail and recruit new Sir2p/Sir4p complexes. This cycle of Sir complex binding followed by histone modification spreads gene silencing from the telomeres toward centromeric regions of the chromosomes. Consistent with
ChIP analyses, models show that the telomere might fold, allowing it to approach the subtelomeric domain to stabilize interactions between Rap1p, Sir proteins, and histones (Strahl-Bolsinger et al., 1997).

**Telomere boundaries** Subtelomeric anti-silencing regions (STARs) including the STR sequences of the X region and the 3’-terminal Y’-STAR of Y’ element have anti-silencing properties (Fourel et al., 1999). Normally, the URA3 gene is repressed by telomeric silencing, but when the STARs are inserted between URA3 and the telomere domain, the URA3 gene is protected from silencing. Other experiments show that anti-silencing activity requires Tbf1p and Rebp1 binding. By contrast, others report a sequence-independent boundary (Suka et al., 2002; Kimura et al., 2002) (Fig. 2B). Sas2p belongs to a family of histone acetyltransferases and is required for the acetylation on lysine 16 of histone H4 (H4K16). Without Sas2p, silencing spreads from 3 kb to 15 kb to overcome the near sub-telomeric chromatin. The propagation of silencing is limited to 3 kb when H4K16 at adjacent euchromatin is acetylated by Sas2p because this acts like a boundary by competing with Sir2p-mediated hypoacetylation and restricts the spread of Sir3p.

Increased H4K16 acetylation in old cells with a simultaneous decrease of Sir2p, and a longer lifespan in sas2Δ cells, suggest chromatin changes that occur through H4K16 acetylation regulated by Sir2p and Sas2p are also related to aging (Dang et al., 2009).

In addition to DNA-independent models of competition between Sir2p and Sas2p, methylation on lysine 79 of histone H3 (H3K79) by Dot1p also contributes to anti-silencing by competing with hypomethylated histone H3 at the silenced domain, and overexpression of Dot1p decreases Sir binding and disrupts silencing (Van Leeuwen et al., 2002). The histone chaperone Chz1p plays an important anti-silencing function through interactions with the histone variant Htz1p, which helps to form and maintain the boundary. When CHZ1 is deleted, decreased ubiquitination of H2B at subtelomeres caused low levels of H3K79 di-methylation and, consequently, high levels of Sir2p and Sir4p in the neighboring euchromatin (Wan et al., 2010). In addition, Rpd3p, a class I HDAC (histone deacetylase), is reported to have anti-silencing function. When the RPD3 gene is deleted, the amount of Sir2p at telomeres, HML loci, and their adjacent termini increases the extent of silencing, which indicates an important anti-silencing function of Rpd3p during the formation of heterochromatin boundaries (Zhou et al., 2009). Furthermore, rpd3Δ sas2Δ double mutants are synthetically lethal and suppressed by sir2-4 mutations. The current model is that Rpd3p histone deacetylation removes the substrate for Sir2p, and thereby blocks the propagation of silencing (Ehrentraut et al., 2010).

### rDNA

**Characteristics of the silent rDNA locus** On yeast chromosome XII, the rDNA locus RDN1 has 150 copies of a repeated 9.1-kb sequence encoding rRNA genes (Fig. 1D) (See for a review Kobayashi, 2006). The genes transcribed by RNA polymerase II are silenced although genes transcribed by RNA polymerase I or III are expressed. Ribosomal DNA silencing is different from that of the mating-type loci (HMR and HML) and telomeres, because only Sir2p silences promoters within RDN1 and silencing spreads unidirectionally, which coincides with the direction of Pol I transcription. The essential gene closest to the RDN1 locus is ACS2, which encodes an acetyl-CoA synthetase isofrom. A regulatory mechanism that can stop the silencing from encroaching upon this important neighboring gene must exist because silencing of ACS2 would negatively influence histone acetylation and global regulation of the genome.

**rDNA boundary** The long gene-free region functions as a buffer to protect essential genes from being silenced (Buck et al., 2002). Upstream of the RDN1 locus, there may be a candidate for a silencing barrier, CARL2 (Laloraya et al., 2000). At normal levels of Sir2p, the physical rDNA boundary can stop the spread of silencing within its natural limit. When Sir2p is overexpressed, a 2.4-kb tRNA gene fragment flanking upstream of RDN1 blocks silencing. TFIIIC and the histone acetyltransferases, Sas2p and Gen5p, are required for tRNA gene barrier activity. Like the HMR right boundary, the barrier activity of Rdn1p-tRNA requires Smc1p, which suggests a general requirement for cohesion in barrier function at the HMR and rDNA in S. cerevisiae (Biswas et al., 2009).

### A SYSTEMATIC SCREEN FOR BOUNDARIES

To understand the mechanism of barrier function in S. cerevisiae, Oki et al. (2004) isolated boundary proteins by genome-wide screening. They first prepared a MATα strain carrying a plasmid with a Gal4p binding domain (GDB) inserted between HMR-E and a 1 that mates with a MATα strain. If diploid colonies do not form on selective media plates, the isolated protein participates in boundary function. Fifty-five genes with boundary functions were isolated in the yeast genome such as subunits of Swi/Snf, mediator, and TFIIID, as well as subunits of the SAS-I, SAGA, Nua3, Nua4, Spt10p, Rad6p, and Dot1p complexes.

Ishii et al. (2002) constructed a boundary-trap strain at the HML domain. One UASg that interacts with the GBD-binding domain was inserted between the HML-E silencer and ADE2, and the other UASg was inserted between ADE2 and URA3. They isolated nuclear-cytoplasmic traffic proteins such as exportin, Cse1p,
Mex67p, and Los1p. These data suggest that physical tethering of genomic loci to the NPC (Nuclear pore complex) is also important for boundary formation.

To identify negative regulators of Sir2p activity, a genome-wide screen used a strain in which the URA3 reporter gene was inserted downstream of the tRNA boundary flanking HMR (Raisner and Madhani, 2008). This revealed 40 loci, 20 not described previously, that regulate the Sir complex. One of the proteins Rtt109p, originally characterized as an acetyltransferase on lysine 56 of histone H3 (H3K56), is an anti-silencing factor that unexpectedly plays a role both H3K56 and H4K16 acetylation. Studies of the rtt109Δ mutant were performed using ChIP to determine levels of Sir3p, H3K56 acetylation, and H4K16 acetylation. Sir3p was enriched within HMR, but there was little protein outside the domain. However, Sir3p levels did not increase within the reporter gene region in rtt109Δ and sas2Δ mutants, which suggests that Rtt109p and Sas2p act downstream of Sir binding to antagonize silencing, and decreased acetylation at H4K16 was observed at the reporter gene region in the rtt109Δ mutant.

OTHER ORGANISMS

In higher eukaryotic organisms, there are similar mechanisms to regulate the genes between active and silenced chromatin domains. Known as insulators, these are divided into two types including barrier insulators that stop the propagation of silencing and separate euchromatin from the neighboring heterochromatin, and enhancer-blocking (EB) insulators that, when placed between them, interfere with enhancer-promoter interactions.

In Drosophila, the gypsy insulator is derived from the gypsy retrotransposon, a member of a family of transposable elements. Three proteins including Su (Hw), Mod (mdg4), and CP190 are members of the gypsy insulator complex. Su (Hw) and CP190 bind DNA in a sequence-specific manner whereas Mod (mdg4) is recruited to the gypsy insulator through interactions with Su (Hw) (Ghosh et al., 2001). The gypsy insulator complex can form a loop structure (Wallace and Felsenfeld, 2007). In the Abd-B locus, there are three enhancer-blocking boundaries known as MCP (Misdagastral Pigmentation), Fab-7 (Frontabdominal-7), and Fab-8. Fab-7 and Fab-8 boundary elements have binding sites for GAGA factor and CTCF, respectively. CTCF is an 82-kDa DNA-binding protein with 11 zinc fingers, and the CTCF-binding sites were identified as enhancer-blocking insulators in vertebrates (Chung et al., 1993). Recent studies using 3C methods demonstrate that CTCF mediates long-range contacts at mouse β globin (Splinter et al., 2006) and at the mouse Igf2/H19 loci (Kurukuti et al., 2006). These long-range interactions permit insulators to interact with each other, as well as with other regulatory elements. The CTCF does not bind methylated DNA and, in that case, the enhancer is not blocked (Bell and Felsenfeld, 2000; Hark et al., 2000; Kanduri et al., 2000). It is important to note that S. cerevisiae does not have a DNA methylation system.

In erythroid cells, mapping studies revealed the chicken globin domain that encompasses a cluster of globin genes (Hebbes et al., 1994). The folate receptor gene and its regulatory elements (HSA) are at the 5' end of the domain and the olfactory receptor genes are downstream. Flanking the chicken globin domain, there are constitutive hypersensitive sites that are proposed to be candidate boundaries (Chung et al., 1993). In addition, when 5' HS4 was placed between the human HS2 enhancer and its target globin gene, reduced amounts of RNA polymerase II were detected at the gene promoter. This proves that the insulator stops the spread of the polymerase from the enhancer to the promoter (Zhao and Dean, 2004).

In the fission yeast, Schizosaccharomyces pombe, the IR (inverted repeats) elements serve as heterochromatin boundaries at the mating-type heterochromatin domain by recruiting transcription factor TFIIIC complex without RNA polymerase III, and the IRC (inverted repeats surrounding centromeres) elements serve as barriers (Noma et al., 2006). Dispersed Pol III genes are near centromeres, which suggest there are functional links between the centromeric localization of Pol III genes, their transcription, and the assembly of condensed mitotic chromosomes (Iwasaki et al., 2010). An S. pombe homolog for the human lysine-specific histone demethylase, lsd1+, is significant, which creates and maintains boundaries between euchromatin and heterochromatin (Lan et al., 2007). The Lsd 1/2 complex isolated from S. pombe prevents heterochromatin spreading into euchromatin by removing mono- or di-methyl group of methylated H3K9 at boundary elements; however, when lsd1+ is deleted, the boundary element loses its function and the H3K9 methylation spread into the neighboring euchromatic region (Lan et al., 2007).
locus and the telomeres, which suggests a relationship between transposons and silencing. Functional assays show that an interaction between the integrase of Ty5 and Sir4p influences their insertion into the HM loci or the telomeres, and Ty1 is specifically targeted into the regions upstream of RNA polymerase III-dependent promoters, such as tDNAs (Zou et al., 1996; Xie et al., 2001; Bachman et al., 2005). The insertion of Ty1 is probably dependent on Sir2p and Sir3p, because Ty1 cDNA recombination increases in Sir4p mutant strains and decreases in the either Sir2p/Sir4p or Sir3p/Sir4p double mutants at high temperatures (Radford et al., 2004). When Ty1 is moved from either side of HMR to the inside locus in the Sir4p mutant strains, silencing was lost (Nishida and Ono, 2007). From the results above, the following model illustrates Ty1 transition regulated by Sir proteins because Ty1 characteristically moves upstream of tDNAs. Ty1 might tend to transfer into the HMR domain; however, this transition into the silencing domain is protected by the Sir proteins in wild-type cells. Therefore, Ty1 must be transposed to both sides of the HMR domain where little of the Sir proteins are present. Ty5 tends to be transposed to positions that are located at a distance from silencers where silencing is weakened, but not where a large amount of the Sir proteins are present. From these results, it is possible that the silencing domain protects the genome from the damage caused by the transposition of Ty transposons.

The HMR locus, especially near the tRNA genes and LTR sequences, was reported to be a hotspot for unselected Ty1 transpositions (Ji et al., 1993). This suggests that silencing might be regulated or controlled by a mechanism that includes Ty transposons. The Ty-LTR also influences boundary formation, and the tRNA sequence is critical for boundary formation; however, boundary function is weakened when the Ty-LTR near the tRNA is deleted (Donze and Kamakaka, 2001; Dhillon et al., 2009). Ty1 may cooperate with tDNA to form a strong boundary to protect itself from being silenced. Upstream of the HMR locus, there are also LTR sequences and a decrease of Sir3p was observed outside these LTR sequences (Meneghini et al., 2003). If the HMR domain has a loop structure, it is possible that the interaction of Ty-LTR and tDNA helps to establish boundary function. Other studies will help explain why the Ty transposons exist near the silencing domains.

It is important to determine how a boundary works in vivo. The cell cycle and boundaries, or aging and boundaries, provide good explanation. First, boundary formation is probably regulated during the cell cycle. During cell division, the silent chromatin domain that represses transcription must also be assembled and maintained (Kirchmaier and Rine, 2006). However, the cell-cycle dependence was lost when tDNA boundary sequence or cohesion that influence the silencing process are deleted (Lazarus and Holmes, 2011). In fact, cohesion was conveyed to the silencing domain (Dube and Gartenberg, 2007) and loss of SMC1 or SMC3, which belong to a protein family of chromosome condensation and cohesion, decreased the boundary function (Donze et al., 1999). Moreover, the establishment of transcriptional silencing at the telomeres depends on cell cycle progression and is influenced by histone variant Htz1p or Sas2p, a histone acetyltransferase (Martins-Taylor et al., 2011). Thus, cell cycle progression, establishment of silencing and boundary formation are all related closely. Similarly, aging also has an influence on regulating the movement of boundaries. Sir2p decreases in aging cells, especially at the telomeres where an increase of H4K16Ac causes the boundary to move in the direction of terminal (Dang et al., 2009).

Additionally, the positions of boundaries differ in individual cells, and the behavior of wild-type genes is affected by their locations, which is termed position effect variegation (PEV) (Gottschling et al., 1990). PEV is independent of DNA sequence, providing silent and expressed states that can be switched reversibly and maintained over subsequent generations (Iida and Araki, 2004). This phenomenon can be important for yeast survival. A gene that relates to a specific metabolic pathway in the sub-telomere was relocated to the telomere domain as a cluster (Wong and Wolfe, 2005). It is necessary to determine why yeast uses boundaries to regulate genes, and to determine the survival strategies yeast choose in response to environmental changes.

Additional functional assays are needed to explain how the movement of a boundary is influenced in vivo and whether the boundary is related with life phenomena such as cell differentiation and stress response. The molecular mechanism of boundary formation is important in the native life, as well as in the cell cycle and aging, and it is influenced by histone modifications, histone variants, histone chaperones, cohesion-like chromatin higher structure and intranuclear positioning.

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