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RNA polymerase III and RNA polymerase II promoter complexes are heterochromatin barriers in *Saccharomyces cerevisiae*

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The chromosomes of eukaryotes are organized into structurally and functionally discrete domains. Several DNA elements have been identified that act to separate these chromatin domains. We report a detailed characterization of one of these elements, identifying it as a unique tRNA gene possessing the ability to block the spread of silent chromatin in *Saccharomyces cerevisiae* efficiently. Transcriptional potential of the tRNA gene is critical for barrier activity, as mutations in the tRNA promoter elements, or in extragenic loci that inhibit RNA polymerase III complex assembly, reduce barrier activity. Also, we have reconstituted the *Drosophila gypsy* element as a heterochromatin barrier in yeast, and have identified other yeast sequences, including the CHA1 upstream activating sequence, that function as barrier elements. Extragenic mutations in the acetyltransferase genes *SAS2* and *GCN5* also reduce tRNA barrier activity, and tethering of a *GAL4/SAS2* fusion creates a robust barrier. We propose that silencing mediated by the Sir proteins competes with barrier element-associated chromatin remodeling activity.

Keywords: boundaries/*HMR*/promoters/tRNA/silencing

Introduction

All organisms regulate expression of their genome for proper development and survival. In a given mammalian cell type, ~90% of the genome is transcriptionally silent (Allis and Gasser, 1998); therefore, a key question in eukaryotic biology is how individual cells coordinate the expression of only a fraction of their genome at specific times and within specific cell types. A large degree of this specificity is due to the proper spatial and temporal expression of distinct subsets of transcription factors that bind to gene regulatory sequences (Tjian and Maniatis, 1994). Recently, long-range chromatin structure effects on transcription have been recognized as an equally critical determinant in developmental, tissue-specific and inducible gene expression (Felsenfeld, 1996).

In the interphase nucleus, chromatin is physically and functionally organized into active and inactive domains (Lamond and Earnshaw, 1998). Euchromatin is transcriptionally active and is maintained in this open state through the action of specific promoter, enhancer and locus control region sequences (Bulger and Groudine, 1999). Heterochromatin, originally defined as chromosomal loci remaining condensed during interphase (Eissenberg *et al*., 1995; Weiler and Wakimoto, 1995), is generally transcriptionally repressed, but can contain embedded active genes (Wakimoto and Hearn, 1990; Sun *et al*., 2000). The structure and function of heterochromatin are mediated by sequences that nucleate the formation of repressor protein complexes that silence the region.

It has been proposed that chromatin boundary or insulator elements function to delimit the domains of silencer and enhancer function (Gerasimova and Corces, 1996; Geyer, 1997; Bell and Felsenfeld, 1999; Sun and Elgin, 1999). Insulators are defined as DNA sequences that block enhancer activation of a promoter when located between the two elements, preventing inappropriate gene activation. Barriers are sequences that block the spread of heterochromatin, thereby separating domains of active and inactive chromatin (Sun and Elgin, 1999).

The yeast *Saccharomyces cerevisiae* contains chromosomal loci that share molecular features with metazoan heterochromatin. Haploid wild-type yeast encode non-expressed copies of the mating-type specific *MAT***a** and *MAT*α genes at the *HMR* and *HML* loci, and a variably transposed active copy of *MAT***a** or *MAT*α at the *MAT* locus (Loo and Rine, 1995; Haber, 1998; Lustig, 1998; Stone and Pillus, 1998). Although identical in sequence to genes expressed at the *MAT* locus, the genes at *HMR* and *HML* are silenced due to the formation of a repressive chromatin structure. Flanking DNA sequences designated *HMR*-E and *HMR*-I are required for silencing. These silencer elements are composed of autonomously replicating sequences (ARS) that bind the origin recognition complex (ORC), and of sites that bind the proteins Rap1p and Abf1p. These proteins nucleate the assembly of a specific complex on the silenced DNA that contains the Sir proteins (Sir1p, Sir2p, Sir3p and Sir4p) and other factors involved in maintaining repression (Loo and Rine, 1995). Gene non-specific silencing in yeast is also observed near telomeric regions of chromosomes (Gottschling *et al*., 1990), and within the rDNA repeats (Smith and Boeke, 1997).

Previously, we reported the presence of barrier sequences flanking the heterochromatic *HMR* locus in *S.cerevisiae* (Donze *et al*., 1999). Detailed analysis of the telomere-proximal barrier shows that a specific tRNA gene is necessary and sufficient for barrier activity. tRNA genes are transcribed by RNA polymerase III (Pol III), and contain promoter elements (*box* A and *box* B, also known as internal control regions, or ICRs) within the transcribed sequences that bind essential transcription factors. Mutations that compromise the activity of Pol III transcription factors TFIIIC and TFIIIB impair barrier activity of this tRNA gene. We demonstrate that this barrier is important for proper regulation of the adjacent *GIT1* gene on chromosome III, since deletion of this $tRNA^{Thr}$ leads to

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SIR4-dependent repression of *GIT1*. Finally, we show that the promoter of the *CHA1* gene, adjacent to *HML*-I, can function as a barrier to silencing. Analysis of these elements has led us to propose passive and active models of barrier function.

Results

The HMR right barrier element is a unique tRNA^{Thr} gene

The *HMR*-E silencer alone is sufficient to completely repress *a1* gene transcription when located upstream. Inserting a DNA fragment containing a putative barrier between the *HMR*-E silencer and the *a1* gene blocked the spreading of silenced chromatin and allowed expression of *a1*, which was assayed by patch mating (Donze *et al*., 1999). When transformed into a *MATα hmrΔ* strain, the *a1* gene on the plasmid is silenced by *HMR*-E, allowing the cells to retain the α mating phenotype. However, a functional barrier inserted between *HMR*-E and the *a1* gene blocked the spread of silencing, thus allowing *a1* expression leading to a non-mating phenotype.

Deletion analysis of the *HMR* downstream barrier (Figure 1) revealed that the centrally located tRNA^{Thr} gene [designated tRNA^{Thr1a} [AGT] CR1 in the Saccharomyces Genome Database (SGD), referred to here as *HMR* tRNA^{Thr}] and its flanking regions were both necessary and sufficient for heterochromatin barrier activity. Plasmids that lacked the tRNA gene (pRO367, 386 and 390) showed a complete absence of barrier function, indicated by the formation and growth of diploid cells in the patches due to silencing of the *a1* gene. Each test construct that contained the *HMR* tRNA^{Thr} gene showed little or no mating in the assay (pRO392, 366, 385, 466, 388, 391 and 387), indicating barrier activity and subsequent expression of *a1*. Interestingly, a minimal tRNA^{Thr} sequence of 119 bp (Figure 1, pRO387) showed a reduced level of barrier activity, as indicated by the formation of a limited number of colonies in the patch. This partial activity suggested that regions immediately flanking the *HMR* tRNA^{Thr} also contributed to blocking the spread of heterochromatin.

Deletion of HMR tRNA^{Thr} leads to partial repression of a downstream gene

We next wanted to ask whether deletion of the tRNA^{Thr} sequence would lead to the spread of silencing and whether this would affect the next downstream gene, *GIT1*, the promoter of which is 3.6 kb from *HMR*-I. Strains containing a precise 85 bp chromosomal deletion of the *HMR* tRNA gene were constructed, and *GIT1* expression was assayed by RNA blot analysis. Deletion of the *HMR* tRNA sequence consistently showed a reduction in *GIT1* mRNA levels that was *SIR4* dependent (Figure 2), demonstrating that in the absence of a barrier element, the *HMR* domain can partially repress *GIT1* expression.

The HMR tRNA^{Thr} barrier is expressed when located downstream of a silencer

Since our results showed that *HMR* tRNA^{Thr} was necessary for barrier function, we wished to determine whether the tRNA positioned downstream of the *HMR*-E silencer was active when it acted as a barrier. To assess whether the

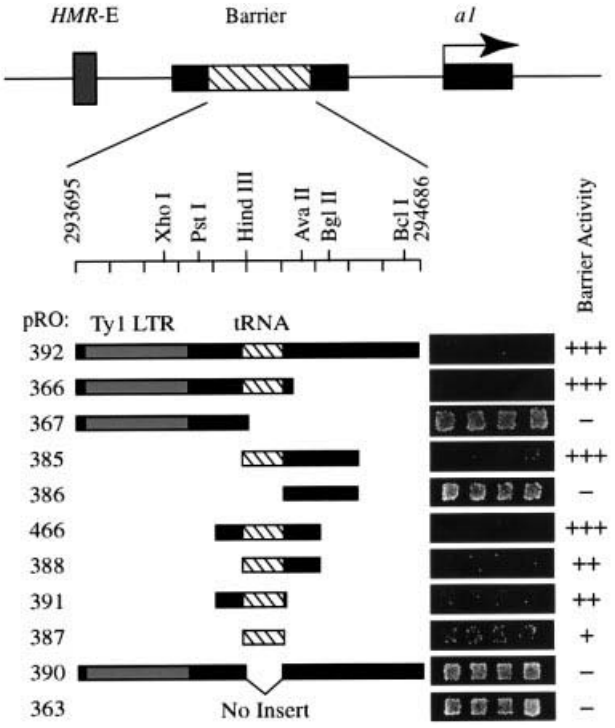

Fig. 1. Deletion analysis of the *HMR* telomere-proximal barrier element. Fragments of the 1.0 kb *HMR* barrier element were cloned between *HMR*-E and the *a1* gene of pRO363, transformed into ROY113 (*MATα hmrΔ*), and transformants tested for barrier activity in a mating assay. Barrier activity leads to expression of the *a1* gene and a non-mating phenotype; growth indicates lack of barrier function. Relative +/– barrier activity was determined from multiple experiments. SGD chromosome III coordinates of the 1.0 kb fragment are listed at the ends of the scale, and each tick mark corresponds to 100 bp. Numbers to the left correspond to pRO plasmid numbers.

HMR tRNA^{Thr} was transcribed, we inserted a 19 bp extension at the 3′ end of the gene to distinguish its transcript from those of the other seven copies of tRNA^{Thr} present in the *S.cerevisiae* genome (Cherry *et al*., 1997; Mewes *et al*., 2000). The modified tRNA displayed unaltered barrier activity as compared with the wild-type gene in the mating assay (Figure 3A, tRNA^{Thr}+19). Total RNA from the same transformants used in the barrier experiment was resolved on an acrylamide gel, electroblotted and probed with an oligonucleotide complementary to the transcribed sequence of tRNA^{Thr1a}. Figure 3B shows the presence of the wild-type tRNA^{Thr1a} transcripts in all cells, and the modified transcript in cells carrying the plasmid containing the marked tRNA^{Thr}+19 gene, demonstrating that the barrier tRNA was not silenced when located downstream of the *HMR*-E silencer. Additionally, a modified Sup53 tRNA^{Leu} gene that exhibited barrier activity (Figure 4B) efficiently suppressed a *lys2-801* amber mutation when located downstream of *HMR*-E (our unpublished data), which confirmed that a barrier tRNA gene downstream from *HMR*-E is not silenced.

RNA Pol III transcribed genes are not necessarily barrier elements

Since the *HMR* tRNA^{Thr} gene showed robust barrier activity, we asked whether other RNA Pol III promoters, those of the 5S rRNA and U6 RNA genes, displayed barrier activity. As shown in Figure 4A, neither gene was

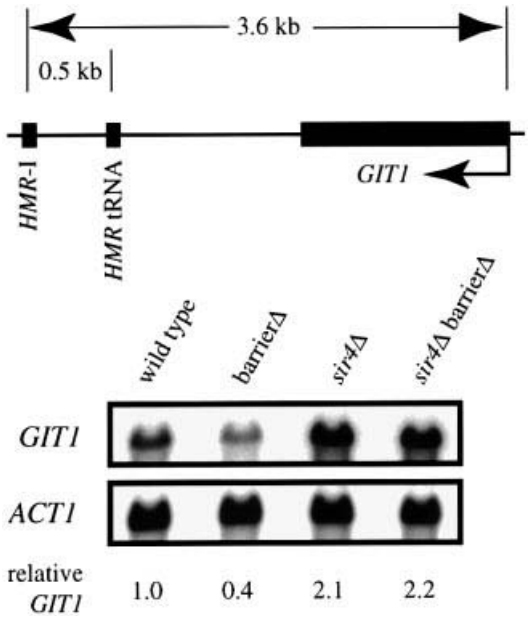

Fig. 2. Deletion of *HMR* tRNA^{Thr} from *S.cerevisiae* chromosome III results in partial repression of the downstream *GIT1* gene. Isogenic wild-type (ROY1685) and barrierΔ (ROY1681) strains in *SIR4* and *sir4Δ* backgrounds (ROY1679, *sir4Δ*; ROY1675, *sir4Δ* barrierΔ) were grown in YPD, and total RNA was prepared for northern analysis. *GIT1* expression was normalized to *ACT1*.

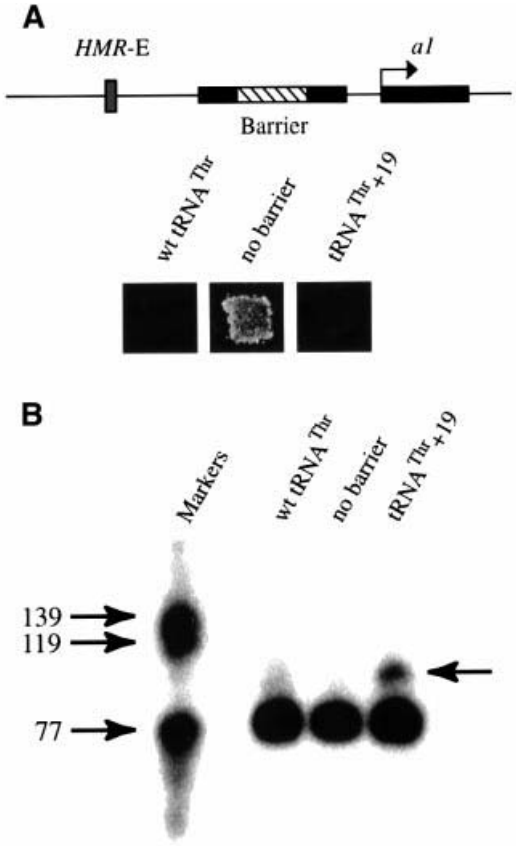

Fig. 3. The tRNA^{Thr} CR1 gene is expressed when downstream of the *HMR*-E silencer. A 19 bp extension was inserted into tRNA^{Thr1a} CR1 between the *box* B site and the transcription termination site to distinguish its transcript from the other seven tRNA^{Thr1a} genes in *S.cerevisiae*. Plasmids pRO363 (no barrier), pRO466 (*HMR* tRNA^{Thr} barrier) and pRO519 (tRNA+19 bp) were transformed into ROY1863 and URA+ transformants isolated. The same transformants tested in the barrier assay were grown in liquid media for RNA isolation. (**A**) The extended tRNA^{Thr1a} CR1+19 gene retains full barrier activity. (**B**) Expression of the tRNA^{Thr}+19 gene; the arrow indicates the extended transcript.

as effective as the *HMR* tRNA^{Thr} gene in its ability to block the spread of silencing from *HMR*-E. The U6 gene showed slight activity in one orientation (pRO461), but the barrier activity of the 5S gene was indistinguishable from the no-barrier control (pRO463 and pRO464).

Since barrier activity was not an intrinsic property of Pol III genes, we asked whether it was specific to tRNA genes. Since the *HMR* tRNA is a tRNA^{Thr} and there are multiple copies of tRNA with threonine anticodons in the yeast genome, we tested other tRNA^{Thr} genes for barrier activity (Figure 4B). The tRNA^{Thr} genes NL1 (chromosome XIV, pRO469) and KL (chromosome XI, pRO470) showed weak barrier activity, while tRNA^{Thr} GR1 (chromosome VII, pRO471) showed robust barrier activity comparable to the *HMR* tRNA^{Thr} barrier.

The *SUP53* tRNA^{Leu} gene is a well characterized gene in studies on tRNA regulation; therefore, we asked whether it was capable of acting as a barrier. However, as shown in Figure 4B, the *SUP53* tRNA^{Leu} gene showed no barrier activity (pRO465). Detailed inspection of its DNA sequence revealed a key difference between *HMR* tRNA^{Thr} and *SUP53* in that the suppressor tRNA contains an intron that increases the *box* A–*box* B promoter element distance to 74 bp, compared with 32 bp in *HMR* tRNA^{Thr}. To test whether this larger spacing of the promoter elements affected barrier function, we deleted this intron to create a *Sup53Δ*IVS allele, with the ICR spacing reduced to 42 bp. This tRNA gene showed a dramatic increase in barrier activity (Figure 4B, pRO467).

It is interesting that tRNA^{Thr} NL1 is a weaker barrier than the *HMR* tRNA, as it has the identical coding sequence (and therefore identical *box* A and *box* B promoter elements and spacing), but differs in the flanking regions. To demonstrate further the importance of the flanks on barrier activity, we created hybrid tRNA^{Thr} NL1 genes containing either the upstream or downstream flank of the *HMR* tRNA. Figure 4C shows that substitution of either flank increases boundary activity of NL1, consistent with the results of deleting the flanks of *HMR* tRNA^{Thr} shown in Figure 1 (pRO387).

Furthermore, the tRNA NL1 gene, which is a weak barrier on its own, is converted to a strong barrier when this gene is duplicated in tandem (Figure 4C, bottom panel), suggesting that tRNA promoter occupancy may play a role in barrier function.

Mutations that inhibit the function of RNA Pol III transcription factors compromise the activity of the barrier tRNA

Having established that all Pol III transcribed genes were not competent for barrier function, we conducted a series of experiments to determine whether Pol III-mediated transcription was necessary for barrier activity by examining mutations in the *HMR* tRNA^{Thr} gene. Mutations GCC10–12/AAA in the *box* A sequence and C56/G in the *box* B sequence are known to reduce transcription of tRNA

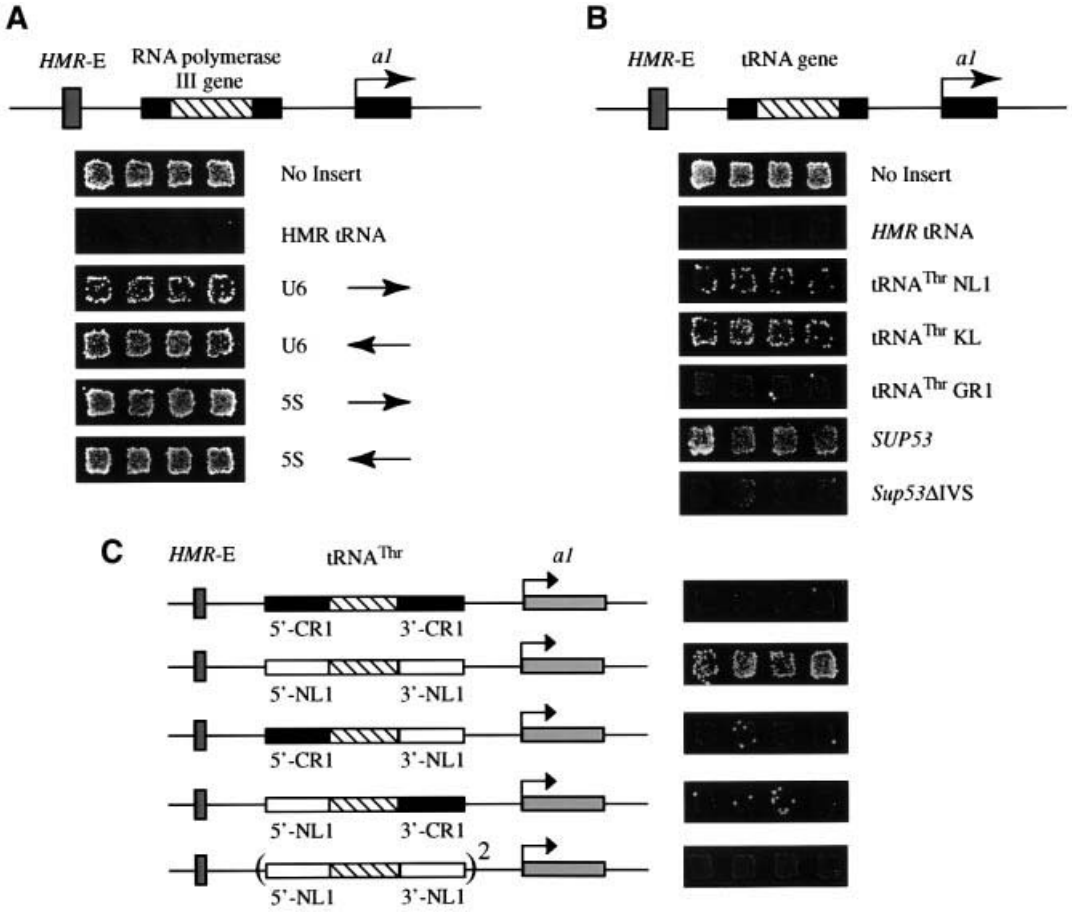

Fig. 4. (**A**) Analysis of other RNA Pol III transcribed genes as barrier elements. The *SNR6* gene (spliceosomal U6 RNA) and the *RDN5* gene (5S rRNA) were cloned into pRO363 and assayed as in Figure 1. The no insert and *HMR* tRNA plasmids are as in Figure 1, the U6 gene plasmids are pRO461 and 462, and the 5S plasmids are pRO463 and 464. (**B**) Analysis of other yeast tRNA genes for barrier activity. Plasmids containing the following *S.cerevisiae* tRNA genes as barriers were tested as in Figure 1: *HMR* $tRNA^{Thr}$(AGT) CR1 from chromosome III (pRO466); $tRNA^{Thr}$ (AGT) NL1 (chromosome XIV, pRO469); $tRNA^{Thr}$ (CGT) KL (chromosome XI, pRO470); $tRNA^{Thr}$ (TGT) GR1 (chromosome VII, pRO471); and *SUP53* $tRNA^{Leu3}$ with (pRO465) or without (pRO467) its intervening sequence (IVS). (**C**) Flanking regions of $tRNA^{Thr}$ CR1 contribute to barrier activity. The 5′ and 3′ flanking regions of $tRNA^{Thr1a}$ CR1 and $tRNA^{Thr1a}$ NL1 were swapped and tested for barrier function. The plasmid designated 5′-CR1/3′-CR1 is pRO466, 5′-NL1/3′-NL1 is pRO469, 5′-CR1/3′-NL1 is pRO479, and 5′-NL1/3′-CR1 is pRO481. A dimerized tRNA NL1 (pRO529) also functioned as a barrier to silencing (bottom panel).

genes *in vitro* (Newman *et al*., 1983) and *in vivo* (Hull *et al*., 1994), while the GGC23–25/TTT mutation, which lies between the promoter elements, has no effect on transcription. Figure 5A shows barrier assay results of the 320 bp *HMR* $tRNA^{Thr}$ fragment, which displayed full activity (pRO466), and the same fragment containing point mutations in the *box* A and *box* B promoter elements. Only the mutations known to affect transcription (GCC 10–12 AAA and C56G) resulted in a loss of barrier function, while the GGC23–25/TTT mutation had no effect. This loss of barrier activity is consistent with the severity of the transcription defects caused by these mutations *in vitro* (Newman *et al*., 1983), with C56/G showing the greatest effect.

The first step in Pol III transcription is the binding of TFIIIC to *box* B and *box* A promoter elements. This is followed by the recruitment of TFIIIB, which binds upstream of the tRNA gene. The final step in transcription is the recruitment of the polymerase subunits. Since mutations in the promoter elements affected barrier activity, we wished to determine whether mutant alleles of TFIIIB, TFIIIC or Pol III also affected barrier activity. Since each of these RNA Pol III complexes is essential for cell viability, we performed the experiments using temperature-sensitive alleles at the permissive temperature, where only subtle phenotypes were evident. However, as shown in Figure 5B, a strain mutant in a TFIIIC DNA binding component, *tfc3G349E* (Lefebvre *et al*., 1994; Arrebola *et al*., 1998), resulted in a subtle but consistent loss of barrier activity. Mutations in *brf1* (Andrau *et al*., 1999), the gene coding for the 70 kDa component of TFIIIB, showed a strong reduction in barrier function, as demonstrated by a significant increase in the level of mating (Figure 5B, lower panel). The *rpc31-236* mutation in a polymerase subunit (Thuillier *et al*., 1995) that is competent for pre-initiation complex assembly, but defective in initiation of Pol III transcription, did not show significant loss of barrier activity. While we cannot at this time conclude whether RNA Pol III transcription itself is required to block the spread of heterochromatin, our results indicated that efficient TFIIIC and TFIIIB assembly was required for barrier activity.

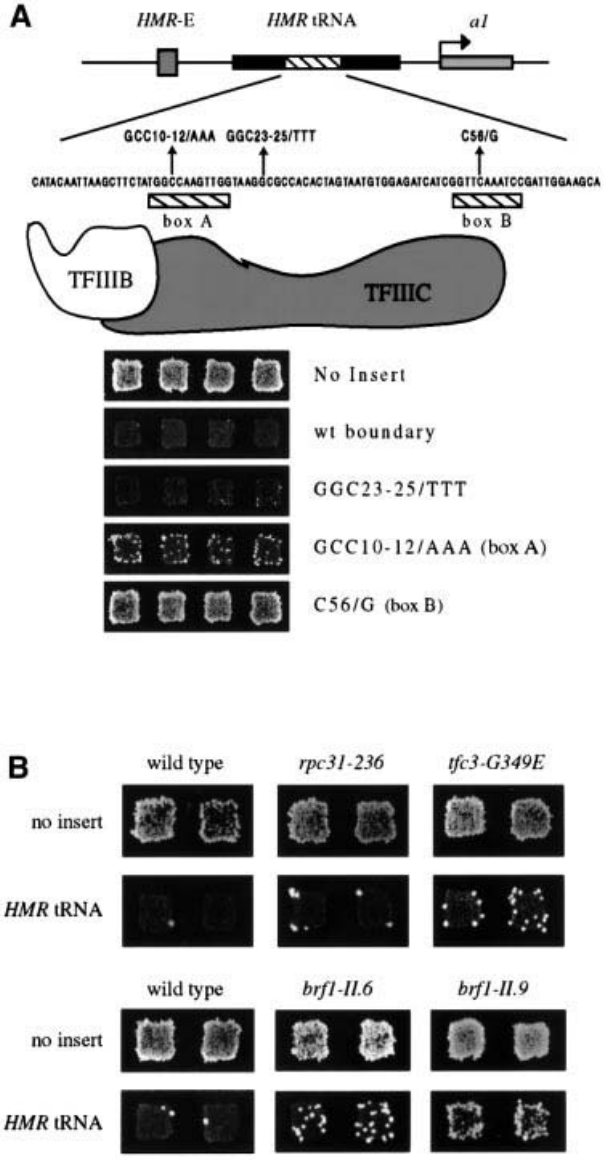

Fig. 5. (**A**) Barrier activity of mutant tRNA^{Thr1a} CR1 genes. *Box* A and *box* B promoter elements were mutagenized in pRO466 and tested for barrier activity. The mutant *HMR* tRNA barrier plasmids are pRO499 (GGC23–25/TTT), pRO498 (GCC10–12/AAA) and pRO468 (C56/G). (**B**) Analysis of barrier activity of tRNA^{Thr1a} CR1 in strains carrying conditional mutations in RNA Pol III factors. Upper panel, wild-type (ROY1863), *rpc31-236* (ROY1861) and *tfc3-G349E* (ROY1862) strains were transformed with pRO363 or pRO495 (pRO363 containing the same insert as pRO391 in Figure 1, but in the opposite orientation) and tested as in Figure 1. Lower panel, wild-type (ROY1864), *brf-II.6* (ROY1539) and *brf-II.9* (ROY1531) strains were transformed with pRO363 or pRO495 as above.

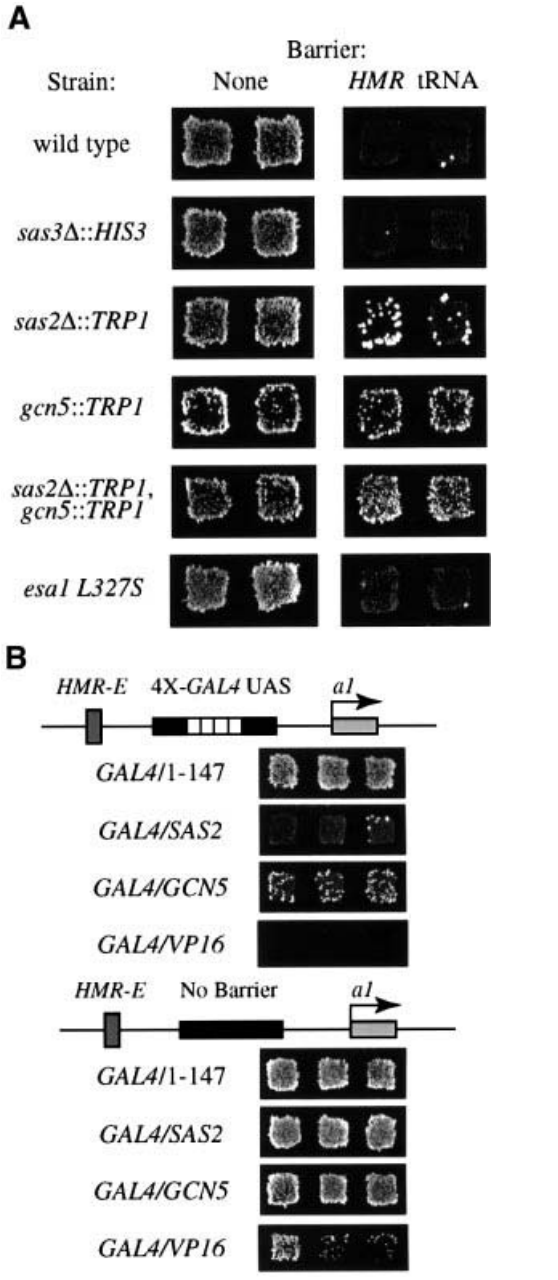

Fig. 6. (**A**) Effects of mutations in acetyltransferase genes on barrier activity. Strains isogenic to ROY1864 (wild type) containing deletions of *SAS3* (ROY1510) or *SAS2* (ROY1528), disruption of *GCN5* (ROY1544), or a conditional point mutation in *ESA1* (ROY1643) were constructed and barrier activity of the *HMR* tRNA was tested in each. (**B**) Tethering of acetyltransferases or a transcriptional activator can create a barrier to heterochromatin. Plasmids expressing the *GAL4* DNA binding domain (pLP493), *GAL4/SAS2* (pLP646), *GAL4/GCN5* (pLP871) or *GAL4/VP16* (pRO541) were co-transformed with pRO486 (4 × *GAL4* UAS between *HMR*-E and *a1*) or pRO363 (no barrier) into ROY1864, and mating assays performed as above.

Effects of acetyltransferase mutations on barrier activity

It has been shown that yeast silent chromatin contains hypoacetylated histones (Braunstein *et al*., 1993), a feature common to heterochromatin in other eukaryotes (Jeppesen and Turner, 1993; O'Neill and Turner, 1995). Silencing at *HMR* is *SIR2* dependent, and it has recently been shown that Sir2p possesses NAD-dependent histone deacetylase activity that is required for silencing (Imai *et al*., 2000; Landry *et al*., 2000; Smith *et al*., 2000). One possible mechanism of barrier activity could be the recruitment of acetyltransferase activity to counteract the effects of Sir2p-mediated deacetylation. We tested the activity of the *HMR* tRNA^{Thr} barrier in a series of acetyltransferase mutants and the results are shown in Figure 6A. Barrier activity is markedly reduced in strains carrying a deletion of the *SAS2* gene or a disruption of *GCN5*, as observed by the increased mating due to repression of *a1*. A strain mutant for both *SAS2* and *GCN5* shows an even greater

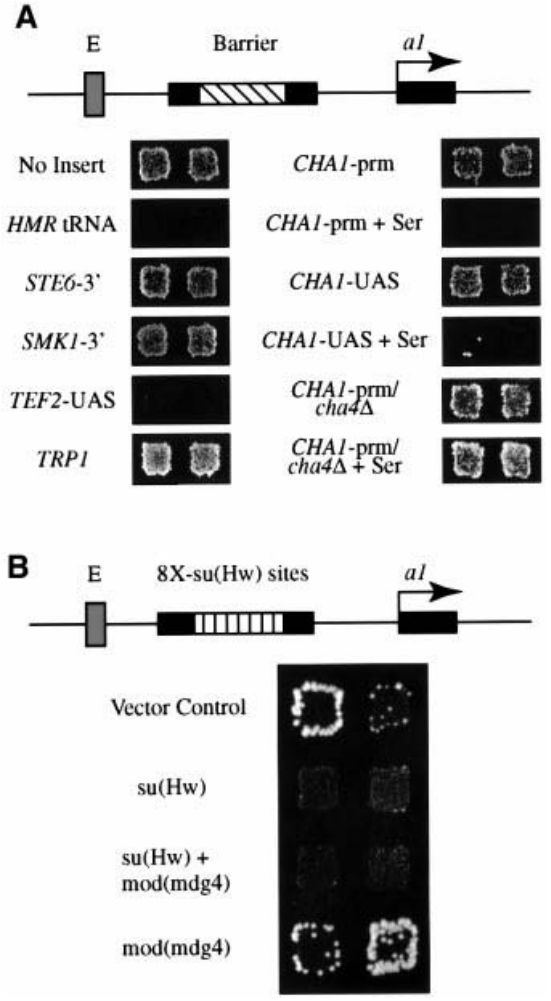

Fig. 7. (**A**) Other heterochromatin barrier sequences in *S.cerevisiae*. Plasmids containing the following *S.cerevisiae* sequences as barriers were tested as in Figure 1: *STE6*-3′ (pRO506); *SMK1*-3′ (pRO508); *TEF2*-UAS (pRO504); *CHA1* promoter (pRO510); *CHA1*-UAS (pRO512); and the *TRP1* gene (pRO262). Strain ROY1775 has a PCR-generated *cha4Δ*::*TRP1* chromosomal deletion. *CHA1* + serine plates were supplemented with 400 μM serine. (**B**) The *Drosophila gypsy* element can be reconstituted as a heterochromatin boundary in yeast. ROY1863 was transformed with pRO493, which contains eight *su(Hw)* binding sites between *HMR*-E and the *a1* gene, and plasmids expressing *su(Hw)*, mod(mdg4), or their respective vector controls.

relative loss of activity. Deletion of *SAS3* had no apparent effect on barrier activity, nor did a temperature-sensitive mutation in the essential acetyltransferase *ESA1* (Clarke *et al*., 1999).

To determine whether acetyltransferase activities could directly create a barrier effect, we cloned four Gal4p binding sites between *HMR*-E and the *a1* gene, and tested the Gal4p sites for barrier activity in the presence of various Gal4 fusion proteins. We tested strains expressing either the Gal4p DNA binding domain alone or strains expressing Gal4–Sas2 or Gal4–Gcn5 fusion proteins. Expressing the Gal4 DNA binding domain alone did not act as a barrier, suggesting that the mere presence of a protein bound to these sites is not sufficient to stop the spread of silencing. Expression of Gal4–Sas2 created a strong barrier to the spread of silencing, which was dependent on the presence of Gal4 binding sites, while Gal4–Gcn5 created a relatively weak barrier. Similarly, tethering of the potent transcriptional activator Gal4–VP16 also created a barrier to silencing (Figure 6B). Western blot analysis showed that these fusion proteins were present at equivalent levels (our unpublished data).

Other boundaries in yeast

In addition to the silent mating loci, telomeres and the rDNA locus, there are other genes in yeast that are conditionally repressed. We were therefore interested in determining whether regions flanking these genes contained barrier elements that could stop the spread of silencing from *HMR*-E. To address this question, we looked at several genes in *S.cerevisiae* where transcriptional repression is known to spread from a *cis*-acting element, and used sequences flanking these repressed regions in our barrier assay. *STE6* is a cell-type-specific gene that is repressed in α cells by mechanisms requiring Ssn6p and Tup1p (Herskowitz *et al*., 1992), and it has recently been shown that Tup1p spreads along the *STE6* gene (Ducker and Simpson, 2000). A $tRNA^{Thr}$ (KL) gene resides upstream of the *STE6* gene, but this tRNA gene is a weak barrier to *HMR*-E-mediated silencing (Figure 4B). We therefore tested a 0.6 kb fragment at the 3′ flank of the *STE6* gene for barrier activity, and as demonstrated in Figure 7A, this sequence was unable to block the spread of silencing from *HMR*-E.

SMK1 is a meiosis-specific gene that is repressed in vegetative cells by a pathway requiring *SUM1* and *HST1* (Xie *et al*., 1999). *HST1* encodes a gene homologous to *SIR2*, and probably mediates repression by similar mechanisms, since overexpression of *HST1* can partially suppress mating-type silencing defects in a *sir2* strain (Brachmann *et al*., 1995). We were therefore interested in determining whether the region flanking the downstream end of *SMK1* contained elements capable of blocking the spread of silencing. However, like the *STE6* 3′ region, this fragment also showed no barrier activity in our assay (Figure 7A).

We next looked at the *CHA1* gene, the promoter of which is ~2 kb downstream of the *HML*-I silencer. Moreira and Holmberg (1998) have shown that Sir4p is required for full repression of *CHA1* transcription when uninduced, suggesting that silencing from *HMR*-I may spread toward the centromere at least as far as the *CHA1* promoter. When serine is present in the medium, *CHA1* transcription is robustly induced, suggesting that the activated promoter can overcome the effects of *SIR4*-mediated repression. Figure 7A shows that the *CHA1* promoter plus UAS (*CHA1*-prm) or the UAS alone exhibited no barrier activity in media lacking serine. However, when the experiment was performed in the presence of serine, robust barrier activity was observed, comparable to the *HMR* tRNA. This barrier activity was dependent on the presence of the *CHA4* gene (Figure 7A, CHA1-prm/*cha4Δ*), which encodes a C_6 zinc cluster transcription factor that binds to the *CHA1* UAS and is required for serine induction of *CHA1* (Holmberg and Schjerling, 1996). As additional controls, we tested barrier activity of the *TEF2* UAS region identified as a barrier element in another study (Bi and Broach, 1999), and the *TRP1* gene as another RNA Pol II promoter. The *TEF* UAS was an efficient barrier in our assay (Figure 7A), while the *TRP1* gene including its

promoter was completely inactive as a barrier, consistent with previous results describing the silencing of *TRP1* at *HMR* (Sussel and Shore, 1991; Donze *et al*., 1999).

We also tested whether a known metazoan boundary could block the spread of *HMR*-E-initiated silencing in yeast. The *Drosophila gypsy* insulator element contains multiple binding sites for the suppressor of hairy wing [*su(Hw)*] protein, which is required for both enhancer blocking and heterochromatin barrier functions of the element (Holdridge and Dorsett, 1991; Geyer and Corces, 1992; Roseman *et al*., 1993). Some of the functions of *su(Hw)* also require expression of the *modifier of mdg4* [*mod(mdg4)*] protein (Georgiev and Gerasimova, 1989; Gerasimova *et al*., 1995). Kim *et al*. (1993) have demonstrated that the *gypsy* insulator does not display enhancer blocking activity in yeast, but heterochromatin barrier activity was not tested in this study. We cloned a cassette containing eight *su(Hw)* binding sites into our barrier test vector and performed the mating assay experiment with and without ectopic expression of *su(Hw)* or *mod(mdg4)* proteins. The results in Figure 7B show that full barrier activity was seen in strains expressing *su(Hw)* [with or without concomitant *mod(mdg4)* expression], but not in strains where only *mod(mdg4)* or neither protein was expressed. This result demonstrated that heterochromatin barrier activity of the *gypsy* insulator could be reconstituted in yeast when *su(Hw)* was ectopically expressed.

Discussion

The HMR right boundary is a unique and functional tRNA gene

Our functional identification of the *HMR* $tRNA^{Thr}$ as the telomere-proximal boundary of the *HMR* domain is in perfect correlation with restriction enzyme accessibility studies of the region (Loo and Rine, 1994). Those experiments mapped the downstream end of the inaccessible *HMR* chromatin to a region between the *Pst*I and *Ava*II sites on either side of the *HMR* $tRNA^{Thr}$ (SGD chromosome III coordinates 294054 for *Pst*I and 294360 for *Ava*II; see Figure 1). We had previously shown that the chromosomal deletion of a 1.0 kb region containing this tRNA and a Ty1 long terminal repeat led to *SIR*-dependent repression of *URA3* inserted downstream of the deleted sequence (Donze *et al*., 1999). In this report we have demonstrated that the *HMR* $tRNA^{Thr}$ gene and its immediate flanks were both necessary and sufficient to prevent the spread of silencing from the *HMR* locus.

tRNA genes contain regulatory sequences within the transcribed regions, called ICRs, which are the highly conserved *box* A and *box* B promoter sequences (Geiduschek and Kassavetis, 1992; Chedin *et al*., 1998; Kumar *et al*., 1998). Transcription of tRNA genes is initiated by the stepwise assembly of a large macromolecular complex, composed of the multisubunit transcription factors TFIIIC and TFIIIB, and the multisubunit RNA Pol III. TFIIIC binds directly to the *box* A and *box* B sequences, followed by recruitment of TFIIIB, which binds upstream of tRNA genes in a non-sequence-specific manner. TFIIIB binding is followed by recruitment of the catalytic RNA Pol III subunits. This assembly creates an extremely large complex of ~1.5 MDa, consisting of 26 polypeptides, and protects ~150 bp of DNA in DNase I footprint assays (Geiduschek and Kassavetis, 1992; Chedin *et al*., 1998). We have shown that mutations in the *HMR* $tRNA^{Thr}$ promoter boxes, and mutations in TFIIIB and TFIIIC subunits, resulted in a loss of barrier function, suggesting that the integrity of the complex was required for efficient barrier activity. One possible mechanism of barrier activity is that a stably bound RNA Pol III pre-initiation complex acts as a large physical block to the propagation of silencing.

The assembly of a Pol III–tRNA gene complex is known to have a number of effects on both the structure of chromatin and several DNA processes. The *SUP53* $tRNA^{Leu}$ gene can repress the level of transcription of a nearby *HIS3* gene (Hull *et al*., 1994), and Ty element retrotransposition is directed to chromosomal locations in close proximity to genes transcribed by RNA Pol III (Chalker and Sandmeyer, 1992; Kirchner *et al*., 1995; Devine and Boeke, 1996). Assembly of a Pol III complex on a tRNA gene can dominantly override nucleosome phasing normally induced by a nucleosome positioning element (Morse *et al*., 1992), and tRNA genes have also been shown to act as DNA replication fork pause sites in *S.cerevisiae* (Deshpande and Newlon, 1996). Each of these tRNA effects requires a fully assembled and functional RNA Pol III complex. Mutations that reduce Pol III factor binding or polymerase activity also abolish or reduce these secondary effects, suggesting that the entire complex is required for the observed phenomena. We see similar requirements for a fully functional Pol III–tRNA gene complex for barrier activity. Our demonstration that the *HMR* $tRNA^{Thr}$ gene transcript is produced when cloned downstream from the *HMR*-E silencer confirms that a functional RNA Pol III complex is present at the *HMR* $tRNA^{Thr}$. The expression and mutational data presented here demonstrate that this barrier tRNA is a fully functional $tRNA^{Thr}$ gene.

It is interesting that only a subset of Pol III transcribed genes function as boundaries, suggesting that there are unique features of this tRNA gene at the *HMR* locus. The requirement of the flanking sequences for full barrier function is not surprising, as several studies have shown that tRNA flanks contribute to transcriptional potential, even though the promoter elements are within the genes (Sprague *et al*., 1980; Dingermann *et al*., 1982; Raymond and Johnson, 1983; Shaw and Olson, 1984; Young *et al*., 1991; Ong *et al*., 1997). This could reflect preferential interaction of TFIIIB with flanking sequences, since TFIIIB creates an extensive footprint on the 5′ flank of tRNA genes (Huibregtse and Engelke, 1989; Kassavetis *et al*., 1989). Other studies have demonstrated that different tRNA genes with identical coding sequences are expressed at different levels due to specific upstream elements (Raymond *et al*., 1985; Ong *et al*., 1997). One study specifically demonstrated that the efficiency of TFIIIB loading varies with the 5′ flanking sequence (Joazeiro *et al*., 1996). Raymond *et al*. (1985) identified a pentadecanucleotide sequence (TTTCAACAAATAAGT) contiguous with the 5′ end of several copies of $tRNA^{Leu3}$ that is 60–80% conserved in tRNA genes that are abundantly transcribed. The *HMR* $tRNA^{Thr}$ 5′ region has 67% homology to this sequence, which may partially explain the function of the 5′ flank in barrier activity. Such

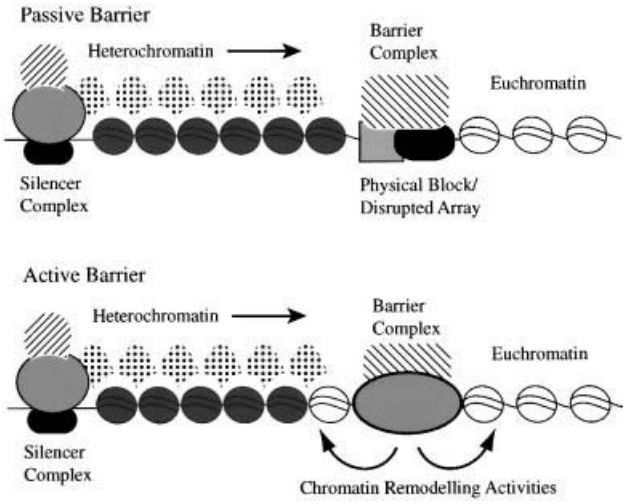

Fig. 8. Models of heterochromatin barrier activity. Silencer bound complexes nucleate the spread of histone deacetylation and the binding of heterochromatin-associated proteins along the silenced region. This structure is propagated until a barrier is reached. In the passive barrier model, a large multiprotein complex stably bound to DNA physically interferes with the propagation of the heterochromatic structure. In the active model, chromatin-modifying activities (e.g. acetyltransferases or nucleosome remodeling complexes) are recruited to the barrier to modify histones and perhaps other chromatin-associated factors. The modified nucleosomes would be less efficient substrates for incorporation into heterochromatin.

differences in tRNA genes may result in more stable interaction of certain gene flanks with TFIIIB and the Pol III complex, increasing the probability that Pol III complex formation efficiently competes with heterochromatin formation. The loss of barrier activity seen in the *brf*1 mutant backgrounds is consistent with this hypothesis. The barrier activity of a multimerized tRNA NL1 may also be due to increasing the probability of Pol III complex formation and occupancy. An alternative possibility is that the *HMR* $tRNA^{Thr}$ flanks interact with additional factors involved in barrier activity. The increase in barrier activity resulting from the deletion of the *SUP53* intron suggests that promoter element spacing is also important in tRNA barrier function. This result is in accordance with studies of the transcriptional promoter strength of tRNA genes. Natural tRNA sequences have *box* A–*box* B separations of ~30–90 bp, but optimal expression is seen when the boxes are only 30–60 bp apart (Baker *et al*., 1987; Fabrizio *et al*., 1987).

A general theme of RNA Pol III genes as chromosomal domain boundaries is emerging. As described above, a number of effects on chromatin processes are caused by the presence of nearby tRNA genes. Recently, two other types of boundary-like activities have been reported for RNA Pol III genes. Alu elements are repetitive sequences in primate genomes, most of which contain functional Pol III promoters. (Deininger and Batzer, 1999). Willoughby *et al*. (2000) have demonstrated that a human Alu element flanking the keratin-18 gene can confer position-independent and copy-number-dependent transgene expression in mice. Mutation of the *box* B site of this Alu element abolishes this protection against position effects. In *Schizosaccharomyces pombe*, mapping of centromeric proteins Swi6 and Mis6 by chromatin immunoprecipitation has defined discrete domains of interaction of these two proteins within centromeric chromatin, and a pair of tRNA genes demarcate the transition zone (Partridge *et al*., 2000). There may be special features of chromatin assembled RNA Pol III complexes, in addition to their large size, that may prevent the propagation of a given chromatin structure.

Potential mechanisms of barrier activity

How do barrier sequences prevent the spread of heterochromatin? The RNA Pol III complex has a cumulative mass of 1.5 MDa and footprints ~150 bp on a tRNA gene (Chedin *et al*., 1998; Kassavetis *et al*., 1998). Such a large, stably bound complex could be a physical impediment to heterochromatin spreading by disrupting the binding of *SIR* complex proteins. Nucleosome mapping of silenced *HMR* and *HML* domains reveals a distinctive *SIR*-dependent positioned dinucleosome array, which may be important for propagation and stability of the heterochromatic state (Weiss and Simpson, 1998; Ravindra *et al*., 1999). By creating a large gap in this array, a tightly bound RNA Pol III complex may passively disrupt this SIR protein-mediated nucleosomal organization and stop the propagation of heterochromatic structure (Figure 8).

Acetylation of histones and other chromatin substrates plays a major role in the regulation of higher order chromatin structure and transcriptional competence of chromosomal loci (Sterner and Berger, 2000), and several histone acetyltransferases (HATs) have been identified. Hyperacetylation of histones is associated with increased general DNase I sensitivity and transcriptional potential of chromosomal domains (Hebbes *et al*., 1994), while hypoacetylation is a characteristic of inactive heterochromatin (Lin *et al*., 1989). Yeast heterochromatin is known to be hypoacetylated (Braunstein *et al*., 1993), and deacetylation of Lys16 of histone H4 appears to be particularly important for the maintenance of SIR protein-mediated silencing (Johnson *et al*., 1990). Results from several laboratories (Imai *et al*., 2000; Landry *et al*., 2000; Smith *et al*., 2000) suggest that Sir2p-catalyzed deacetylation of histones, and possibly other chromatin substrates, is a key activity in the maintenance of yeast heterochromatin. Our observation of an apparent loss of barrier activity in strains with mutations in the HAT genes *GCN5* and *SAS2* suggests another possible mechanism of barrier function: that of competing chromatin-modifying activities (Figure 8). In this model, Sir2p-mediated deacetylation is propagated from the silencer until it meets a barrier element that stably recruits one or more acetyltransferases that actively compete with the effects of silencer-recruited Sir2p. Although no acetyltransferase has yet been demonstrated to be associated with the RNA Pol III complex in yeast, HAT activity associated with human TFIIIC has been identified (Kundu *et al*., 1999). While this HAT has no yeast homolog, one or more of the other *S.cerevisiae* HATs may have redundant functions in yeast Pol III activity.

One caveat to the active model is that a null mutation of *GCN5* has been shown to increase silencing at telomeres (Sun and Hampsey, 1999), and strains lacking Sas2p show a restoration of silencing at a weakened *HMR* locus (Reifsnyder *et al*., 1996; Ehrenhofer-Murray *et al*., 1997). Thus, the strengthening of silencing by these mutations

may simply override the barrier activity in our assays. Even if this is the case, these results suggest that barrier activity may arise from an underlying competition between the chromatin remodeling and silencing activities at the interface of euchromatin and heterochromatin. However, the ability of tethered *GAL4/SAS2* to block silencing (Figure 7) suggests that the recruitment of an acetytransferase complex can be sufficient for formation of a heterochromatin barrier in yeast. *GAL4/VP16* expression can also create a strong barrier in our assay system, possibly due to the recruitment of chromatin-modifying factors such as Gcn5p.

The ability of the *TEF2* UAS regions to function as boundaries would also fit either the physical block or the competing activity models, which are not mutually exclusive. Bi and Broach (1999) have proposed that chromatin-bound Rap1p at the *TEF2* UAS may act as a barrier by creating a nucleosomal 'hole' that inhibits the migration of silencer nucleated Sir complex. However, since Rap1p can act as either a repressor or an activator of promoters (Shore, 1994; Morse, 2000), one can not rule out the possibility that Rap1p barrier activity may be due to recruitment of chromatin remodeling factors associated with gene activation. It is interesting that Rap1p shares this characteristic of being an activator or a repressor with CTCF (Klenova *et al*., 1993), a vertebrate protein recently identified as a boundary binding factor (Bell *et al*., 1999; Bell and Felsenfeld, 2000; Hark *et al*., 2000). The observed barrier effect of the *su(Hw)* protein in yeast may also fall into this category, as it has been reported to have properties consistent with activation (Parkhurst and Corces, 1986) and repression (Gerasimova *et al*., 1995; Gerasimova and Corces, 1996) of transcription.

The mechanism of *CHA1* UAS barrier activity appears to suggest that disruption of the nucleosomal array alone may not be sufficient to form a barrier. In the uninduced state, the *CHA1* TATA box is packaged within an array of positioned nucleosomes. This positioning in the repressed state is dependent on both Sir4p and the nucleosome remodeling complex RSC (Moreira and Holmberg, 1998, 1999). However, upstream of the *CHA1* TATA box is a 250 bp region constitutively free of nucleosomes. This region encompasses the *CHA1* UAS sites, which bind the Cha4p activator in both the induced and uninduced states. Upon addition of serine, *CHA1* transcripts are quickly induced in a *CHA4*-dependent manner, and nucleosome positioning at *CHA1* is disrupted. This situation of a repressed but constitutively bound activator is similar to that seen when *HMR*-E is positioned upstream of the *HSP82* promoter (Lee and Gross, 1993; Sekinger and Gross, 1999). Also, as shown in Figure 6, expression of the Gal4p DNA binding domain is not sufficient to create a barrier at *GAL4* UAS sites. Therefore, in these situations, simply creating a nucleosomal gap is not sufficient for barrier activity, so we propose that upon induction these DNA-bound factors recruit additional proteins or mediate chromatin remodeling, which leads to formation of a barrier to silencing.

In summary, we have identified the *HMR* downstream barrier as the $tRNA^{Thr}$ CR1 gene, and demonstrated that a fully assembled RNA Pol III complex is required to resist the spread of heterochromatin. This and other recent studies suggest a general role for RNA Pol III genes in demarcating the genome. The observed barrier activity of *HMR* $tRNA^{Thr}$, *CHA1* UAS and the *TEF2* UAS suggest active mechanisms by which these elements can disrupt silenced chromatin. Elucidation of the mechanisms by which these sequences resist Sir-mediated silencing will tell us much about the interface between euchromatin and heterochromatin, and how these regions are restricted to their respective domains.

Materials and methods

Yeast strains

Saccharomyces cerevisiae W-303 background strains generated in our laboratory for this study are as follows:
ROY 113, *MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 hmrΔ*::bgl-bcl (Donze *et al*., 1999);
ROY1863, *MATα his3-11 leu2-3,112,112 trp1-1-1 ura3-1-1 hmrΔ*::bgl-bcl;
ROY1864, *MATα his3-11 leu2-3,112–3,112 lys2Δ trp1-1 ura3-1 hmrΔ*::bgl-bcl;
ROY1861, *MATα his3-11 leu2-3,112 trp1-1 ura3-1 rpc31–236 hmrΔ*::bgl-bcl;
ROY1862, *MATα his3-11 leu2-3,112 trp1-1 ura3-1 tfc3-G349E hmrΔ*::bgl-bcl;
ROY1531, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ*::bgl-bcl *brf1Δ::HIS3* [*brf1* II.9 *ARS CEN LEU2*];
ROY1539, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ*::bgl-bcl *brf1Δ::HIS3* [*brf1* II.6 *ARS CEN LEU2*];
ROY1510, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ*::bgl-bcl *sas3Δ::HIS3*;
ROY1528, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ*::bgl-bcl *sas2Δ::TRP1*;
ROY1544, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ*::bgl-bcl *gcn5::TRP1*;
ROY1639, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ*::bgl-bcl *sas2Δ::TRP1 gcn5::TRP1*;
ROY1643, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 hmrΔ*::bgl-bcl es*a1*-L327S;
ROY1675, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 sir4Δ::LEU2* $tRNA^{Thr}$1a CR1Δ;
ROY1679, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 sir4Δ::LEU2*;
ROY1681, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1* $tRNA^{Thr}$1a CR1Δ;
ROY1685, *MATα his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*;
ROY1775, *MATα his3-11 leu2-3,112 trp1-1 ura3-1 hmrΔ*::bgl-bcl *cha4Δ::TRP1*.

Plasmids

Barrier test plasmids were constructed in pRO363, which contains the *Sac*I–*Sal*I *HMRΔI* fragment from pRO22 (Donze *et al*., 1999) cloned into pRS406 (Sikorski and Hieter, 1989) and containing a synthetic polylinker with a unique *Bam*HI site cloned into the *Eco*NI site of the *a2* gene. All fragments tested were cloned by PCR amplification with *Bam*HI sites in the primers. Mutant tRNAs were created using the Quik-Change Mutagenesis Kit (Stratagene), and mutations were in the same *box* A and *box* B positions as described (Newman *et al*., 1983) for pRO499, pRO498 and pRO468, or by inserting 19 bp between *box* B and the transcription termination site for pRO519. The *SUP53* gene was the *Bam*HI fragment as described (Hull *et al*., 1994), and the intron was deleted using site-directed mutagenesis as described above. Hybrid $tRNA^{Thr1a}$ genes used in the flanking region swap experiment were made by PCR cloning each region and joining the swapped fragments at the *Hin*dIII site at the start of the *HMR* tRNA sequence. Final test clones of each barrier sequence were verified by either manual or automated dideoxy DNA sequencing. SGD (Cherry *et al*., 1997) coordinates of each fragment tested are available on request. Designations of tRNA genes are those listed in the Munich Information Centre for Protein Sequences (MIPS) Database (Mewes *et al*., 2000).

Expression plasmids for *su(Hw)* and *mod(mdg4)* were constructed by first cloning the *ADH1* promoter from pCF113 (*ARS CEN URA3 ADH1* promoter in pRS416; gift from Catherine Fox) into pRS414 to create an *ARS CEN TRP1 ADH1* promoter expression plasmid (pRO489). To construct pRO494 [*ADH1-su(Hw) ARS CEN URA3*], the *su(Hw)* cDNA was cloned into pCF113. *ADH1*-mod(mdg4) *ARS CEN TRP1* (pRO497)

contained the *mod(mdg4)* cDNA in pRO489 (cDNAs were gifts from Victor Corces and Dolan Ghosh). Eight *su(Hw)* binding sites were cloned into pRO490 (a *LEU2* version of pRO363) to create pRO493. Plasmids pLP493, pLP646 and pLP871 were a generous gift from Lorraine Pillus.

Mating assays

Mating assays to determine barrier activity were performed as described (Donze *et al*., 1999) with selection on yeast minimal media plates supplemented with dextrose (YMD) plus adenine, leucine, lysine and tryptophan. In experiments using *lys2Δ* strains, the selection medium was YMD plus adenine, histidine, leucine and tryptophan, and in the *gypsy* element experiment, the selection medium was YMD plus adenine and lysine.

RNA analysis

Expression of the marked tRNA+19 gene was determined as described (Krieg *et al*., 1991). The blot was probed using a ^{32}P-end-labeled 76mer oligonucleotide complementary to the tRNA^{Thr1a} coding sequence using the oligonucleotide hybridization and washing protocol supplied with the membrane (Bio-Rad Zeta Probe). Northern blot analysis was performed using the NorthernMax kit (Ambion), and blots were probed with RNA complementary to the first 600 nucleotides of either *GIT1* or *ACT1*. Riboprobes were synthesized using Maxiscript *in vitro* transcription reagents (Ambion). Quantitative analysis was performed on a Molecular Dynamics Storm 820 PhosphorImager.

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