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Multiple Bromodomain Genes Are Involved in Restricting the Spread of Heterochromatic Silencing at the *Saccharomyces cerevisiae* *HMR*-tRNA Boundary

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ABSTRACT

The transfer RNA gene downstream from the *HMR* locus in *S. cerevisiae* functions as part of a boundary (barrier) element that restricts the spread of heterochromatic gene silencing into the downstream region of chromosome III. A genetic screen for identifying additional genes that, when mutated, allow inappropriate spreading of silencing from *HMR* through the tRNA gene was performed. *YTA7*, a gene containing bromodomain and ATPase homologies, was identified multiple times. Previously, others had shown that the bromodomain protein Bdf1p functions to restrict silencing at yeast euchromatin-heterochromatin boundaries; therefore we deleted nonessential bromodomain-containing genes to test their effects on heterochromatin spreading. Deletion of *RSC2*, coding for a component of the RSC chromatin-remodeling complex, resulted in a significant spread of silencing at *HMR*. Since the bromodomain of *YTA7* lacks a key tyrosine residue shown to be important for acetyllysine binding in other bromodomains, we confirmed that a GST-Yta7p bromodomain fusion was capable of binding to histones *in vitro*. Epistasis analysis suggests that *YTA7* and the *HMR*-tRNA function independently to restrict the spread of silencing, while *RSC2* may function through the tRNA element. Our results suggest that multiple bromodomain proteins are involved in restricting the propagation of heterochromatin at *HMR*.

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

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

At the *HMR* silent mating locus, a transfer RNA gene and its associated RNA polymerase III complex functions as part of a boundary element that prevents the spreading of Sir protein-mediated heterochromatin

(Donze *et al.* 1999; Donze and Kamakaka 2001). However, we found that while the *HMR*-tRNA alone is sufficient to act as a barrier to silencing in a plasmid-based assay (Donze and Kamakaka 2001), when integrated back into the chromosome, the tRNA alone shows only partial barrier function (our unpublished results), suggesting that additional proteins are required to completely block silencing. Other genes coding for chromatin-associated proteins that can lead to a partial spreading of silencing through the tRNA have been identified and include *HTZ1*, *BDF1*, and *YTA7* (Ladurner *et al.* 2003; Meneghini *et al.* 2003; Tackett *et al.* 2005), and the global level of histone acetylation regulated by *SIR2* and *SAS2* influences silencing spread (Kimura *et al.* 2002; Suka *et al.* 2002). Taken together, these results suggest that both local boundary-associated factors and global chromatin modifications affect the formation of a true euchromatin-heterochromatin boundary (Kimura and Horikoshi 2004).

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

MATERIALS AND METHODS

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

The schematic of *YTA7* and amino acid positions of AAA ATPase and bromodomains was based on query of the Conserved Domain Database (Marchler-Bauer *et al.* 2005). The alignment of *S. cerevisiae* bromodomains was created using ClustalW (Chenna *et al.* 2003) and was manually adjusted to fit a structurally based alignment of bromodomains (Zeng and Zhou 2002). The GST-Yta7p bromodomain fusion was made by PCR cloning the coding sequence of *YTA7* from amino acids 1000 to 1101 into the glutathione-*S*-transferase (GST) vector pGEX-2TK (Amersham Biosciences), and the fusion protein was expressed and purified according to the manufacturer's instructions. Purified chicken core histones were purchased from Upstate (Lake Placid, NY). Histone pull-down assays were performed as described (Ladurner *et al.* 2003), and the recovered samples were run on an 18% SDS-PAGE gel and stained with Bio-Safe Coomassie stain (Bio-Rad Laboratories, Hercules, CA). Briefly, core histones were dissolved in water at 1 mg/ml, and 500-μl binding reactions contained 1× HEMG buffer (25 mm HEPES-KOH, pH 7.6, 0.2 mm EDTA, 12.5 mm $MgCl_2$, 10% glycerol, diluted from a 5× stock), 50-μg core histones, 150 mm KCl, 15 μl glutathione-Sepharose (Amersham Biosciences) equilibrated in 1× HEMG plus 150 mm KCl, and 15 μg GST-Yta7p fusion protein or an equimolar amount of GST in the controls. Binding reactions were incubated for 4 hr at 4°, and beads were collected by centrifugation and then washed four times in 500 μl 1× HEMG, 150 mm KCl, 0.01% NP-40. A total of 125 μl 1× Laemmli loading buffer was added directly to the beads for gel analysis, and unbound fractions were precipitated with trichloroacetic acid, redissolved in 25 μl 0.1 n NaOH, and brought to 125 μl in 1× Laemmli loading buffer.

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

Mutant alleles of genes affecting the spread of silencing through the boundary were then crossed into each of these *ADE2* parent strains (DDY811, DDY814, and DDY2114). Colony color assays were performed by streaking representative strains onto minimal media containing 3 μg/ml adenine, which is 10% of the normal level in this medium. Colonies were grown for 3 days and held at 4° for 1 week prior to photography on a dissecting microscope equipped with a digital imaging system. Colony color was assessed for three independent isolates for each mutant; one representative strain is shown for each.

RESULTS

We used a *MAT*α boundary reporter strain (DDY277) that harbors a modified *HMR* locus deleted for the *HMR*-I silencer and that also contains the downstream

TABLE 1

Strains of *S. cerevisiae* generated for this study

Strain	Genotype
DDY277	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1*
DDY282	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMRΔI*
DDY799	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7*
DDY687	*MAT*α *ADE2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1 sas2Δ::TRP1*
DDY695	*MAT*α *ADE2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1 gcn5::TRP1*
DDY800	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:sas5*
DDY802	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:sas5*
DDY803	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7*
DDY811	*MAT***a** *ade2 his3 leu2 LYS2 trp1 ura3 HMR-trnaΔ-ADE2*
DDY814	*MAT***a** *ade2 his3 leu2 LYS2 trp1 ura3 HMR-ADE2*
DDY1174	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7*
DDY1309	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7*
DDY1313	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:sas4*
DDY1316	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7*
DDY1318	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7*
DDY1340	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7*
DDY1344	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:rpd3*
DDY1345	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:rpd3*
DDY1347	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7*
DDY1665	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 rsc2Δ::TRP1*
DDY1893	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 rsc1Δ::URA3*
DDY1997	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 yta7Δ::TRP1*
DDY2078	*MAT*α *ADE2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 Tn::LEU2:yta7 sir2Δ::TRP1*
DDY2093	*MAT*α *ade2 his3 leu2 lys2Δ trp1 ura3 HMR-ADE2 Tn:LEU2:rpd3*
DDY2106	*MAT*α *ade2 his3 leu2 lys2Δ trp1 ura3 HMR-ADE2 Tn:LEU2:sas5*
DDY2114	*MAT***a** *ade2 his3 leu2 LYS2 trp1 ura3 hmrΔ::ADE2*
DDY2128	*MAT***a** *ade2 his3 leu2 lys2Δ trp1 ura3 Tn:LEU2:rpd3 hmrΔ::ADE2*
DDY2142	*MAT***a** *ade2 his3 leu2 LYS2 trp1 ura3 Tn:LEU2:sas5 hmrΔ::ADE2*
DDY2143	*MAT***a** *ade2 his3 leu2 LYS2 trp1 ura3 Tn:LEU2:rpd3 HMR trnaΔ-ADE2*
DDY2156	*MAT***a** *ade2 his3 leu2 LYS2 trp1 ura3 Tn:LEU2:sas5 HMR trna Δ-ADE2*
DDY2198	*MAT***a** *ade2 his3 leu2, LYS2 trp1 ura3 hmrΔ::ADE2 yta7Δ::TRP1*
DDY2200	*MAT***a** *ade2 his3 leu2, LYS2 trp1 ura3 HMR trnaΔ-ADE2 yta7Δ::TRP*
DDY2205	*MAT*α *ade2 his3 leu2, lys2Δ trp1 ura3 HMR-ADE2 yta7Δ::TRP1*
DDY2450	*MAT*α *ade2 his3 leu2 LYS2 trp1 ura3 hmrΔ::ADE2 rsc2Δ::TRP1*
DDY2489	*MAT***a** *ade2 his3 leu2 LYS2 trp1 ura3 HMR trna Δ-ADE2 rsc2::TRP1*
DDY2496	*MAT*α *ade2 his3 leu2 lys2Δ trp1 ura3 HMR-ADE2 rsc2Δ::TRP1*
DDY2514	*MAT*α *ade2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 spt7Δ::URA3*
DDY2596	*MAT*α *ade2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1 bdf1Δ::HIS3*
DDY2601	*MAT*α *ade2 his3 leu2 lys2Δ trp1 ura3 HMR-E-Boundary-a1 bdf2Δ::LEU2*
DDY2659	*MAT*α *ade2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1 snf2::LEU2*
DDY2716	*MAT*α *ADE2 his3 leu2 LYS2 trp1 ura3 HMR-E-Boundary-a1 htz1Δ::KanMX*

boundary sequence cloned into the *HMRa2* gene (Figure 1, top). This strain is nonmating, as the boundary blocks the spread of silencing from *HMR*-E, allowing the *a1* gene to be expressed in the *MAT*α background (Donze *et al.* 1999). To identify genes involved in restricting the spread of silencing, we subjected this strain to a transposon mutagenesis protocol (Ross-Macdonald *et al.* 1999) and selected transposon insertion mutants that acquired an α-mating phenotype, indicating that silencing was now able to spread through the boundary into *a1*. A total of 20,000 Leu+ transformants were replica plated onto *MAT***a** tester lawns to identify mutants that lost boundary function at the modified *HMR* locus as judged by their ability to mate.

α-Mating isolates were confirmed by backcrossing to a *MAT***a** strain containing the same reporter construct, and only tetrad isolates in which the transposon-encoded *LEU2* marker completely cosegregated with the mating phenotype (20 tetrads analyzed) were studied further. After this analysis, mutations in only four separate genes were confirmed as leading to a spread of silencing: *SAS4* and *SAS5*, encoding subunits of the *s*omething *a*bout *s*ilencing (SAS) acetyltransferase complex; *RPD3*, a histone deacetylase; and *YTA7*, a little studied gene containing a weak bromodomain homology and two domains of AAA family ATPase homology. The mating phenotypes of each independent mutant isolate are shown in Figure 1. A single mutant of the

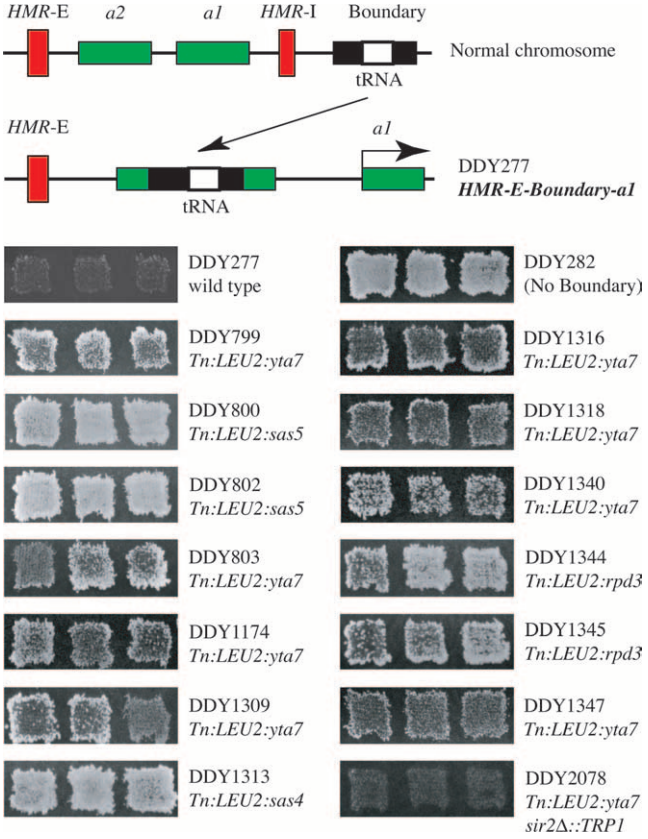

Figure 1.—Mating assay to identify extragenic mutations that lead to spreading of silencing through the *HMR* right boundary. (Top) The 1.0-kb region downstream of *HMR* containing the boundary tRNA was cloned into the *a2* gene, and the resulting construct was integrated back into chromosome III in a *MAT*α strain. The boundary element blocks the spread of silencing into *a1*, resulting in a nonmating phenotype (DDY277). The mating assay was performed essentially as described previously (Donze *et al.* 1999). DDY277 was mutagenized by transformation with a yeast genomic DNA library containing random transposon-*LEU2* insertions (Ross-Macdonald *et al.* 1999), and Leu+ recombinants were tested for mating. (Bottom) Thirteen independent isolates that showed increased silencing were confirmed, representing mutations in four different genes, *YTA7*, *SAS5*, *SAS4*, and *RPD3*. All strain genotypes are listed in Table 1.

SAS4 gene resulted in increased mating, along with two independent insertions into *SAS5* and two independent insertions into *RPD3*. Mutations in the SAS complex were previously shown to increase the silencing efficiency of a defective *hmra-e*** locus (Ehrenhofer-Murray *et al.* 1997; Xu *et al.* 1999), and *sas2* mutations were first identified as leading to increased silencing in *sir1* mutants (Reifsnyder *et al.* 1996). Since *hmra-e*** lacks the boundary element, it is likely that mutations in the SAS complex globally affect the robustness of silencing by affecting cellular levels of histone H4 lysine 16 acetylation (Meijsing and Ehrenhofer-Murray 2001; Kimura *et al.* 2002; Suka *et al.* 2002; Shia *et al.* 2005), as opposed to specifically affecting the integrity of the boundary element. Mutations in *RPD3* are also well documented as leading to a global increase of silencing at all three yeast heterochromatin loci (mating loci, rDNA, and telomeres) by an unknown mechanism (Rundlett *et al.* 1996; Vannier *et al.* 1996; Kim *et al.* 1999; Smith *et al.* 1999; Sun and Hampsey 1999).

Interestingly, eight independent isolates containing transposon insertions in *YTA7*, representing five different insertion sites, were isolated in the screen. It is not clear why *yta7* mutants would occur so frequently in the library compared to other genes. It should be noted that the screen, while calculated to be saturating, was not, as mutations in genes previously known to induce a mating phenotype in this reporter system, such as the acetyltransferase subunit of SAS, *SAS2*, and the bromodomain-acetyltransferase *GCN5*, were not recovered (Donze and Kamakaka 2001). Additionally, insertions into other genes known to be required for restriction of silencing at *HMR*, *BDF1* (Ladurner *et al.* 2003), and *HTZ1* (Meneghini *et al.* 2003) were also not recovered. However, as discussed below, direct deletion of *BDF1* or *HTZ1* does lead to a spread of silencing in this reporter assay (Figure 3). The Sir protein dependence of the observed spread of silencing is shown for a *yta7* mutant (DDY2078, Figure 1) and was also confirmed for *rpd3* and SAS mutants (our unpublished data).

YTA7 contains AAA family ATPase domains and a single bromodomain module, schematically depicted in Figure 2A. Since bromodomains have been demonstrated to be acetyllysine-binding modules (Dhalluin *et al.* 1999; Hudson *et al.* 2000; Jacobson *et al.* 2000; Owen *et al.* 2000) and have been suggested to mediate their functions by binding to acetylated histones and other chromatin-associated proteins (Zeng and Zhou 2002 ; Yang 2004; de la Cruz *et al.* 2005), it seemed likely that Yta7p may be functioning through histone recognition. The bromodomain protein Bdf1p is also known to have an antisilencing function at *HMR*, as *bdf1* mutants show decreased gene expression and increased Sir3p association in regions adjacent to yeast silenced loci (Ladurner *et al.* 2003). Alignment of the *YTA7* bromodomain with other yeast bromodomains (Figure 2B), however, reveals that *YTA7* lacks a critical conserved tyrosine residue required for acetyllysine recognition by P/CAF, Gcn5p, and Bdf1p via a key hydrogen bond formation within the binding pocket (Dhalluin *et al.* 1999; Owen *et al.* 2000; Ladurner *et al.* 2003). Yta7p does contain a serine and threonine at the same location.

Due to the absence of this key tyrosine residue, we wanted to test whether the Yta7p bromodomain module was able to bind to histones. We cloned the coding sequence of the *YTA7* bromodomain into a GST fusion vector and purified the GST-Yta7p fusion protein from *Escherichia coli* cells. This fusion protein was used in a pull-down assay with purified chicken erythrocyte core histones. Figure 2C shows that GST-Yta7p efficiently binds to histones H3 and H4, compared to no binding

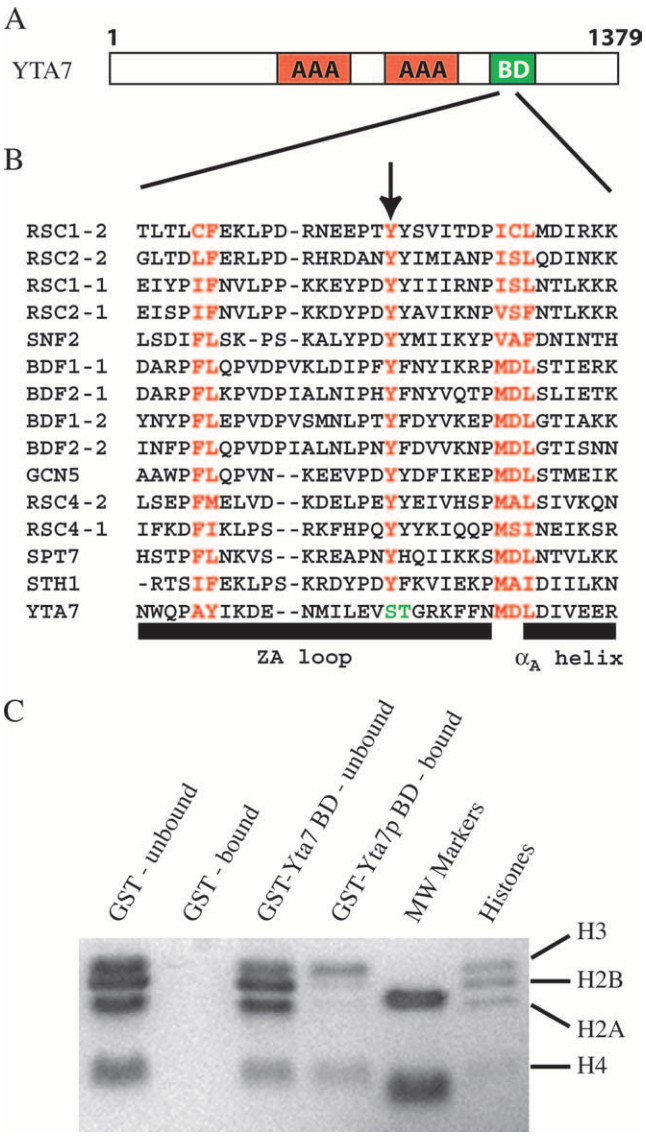

FIGURE 2.—*YTA7* encodes a histone-binding bromodomain protein. (A) Schematic of the predicted *YTA7* open reading frame, showing the relative locations of the two AAA family ATPase domains (amino acids 449–580 and 771–957) and the single bromodomain (amino acids 1003–1091) as determined by query of the Conserved Domain Database (MARCHLER-BAUER *et al.* 2005). (B) Alignment of yeast bromodomain sequences reveals that *YTA7* lacks a key conserved tyrosine residue (arrow, red) that is critical for histone binding in other bromodomains, but contains a tandem serine-threonine in the same position (green). (C) The GST-Yta7p pull-down assay reveals that the bromodomain of Yta7p specifically retains histones H3 and H4. Reactions and washes were carried out in 150 mM KCl (see MATERIALS AND METHODS). Increasing KCl to 500 mM in binding and wash steps reduced the level of histone binding by ~50% (data not shown).

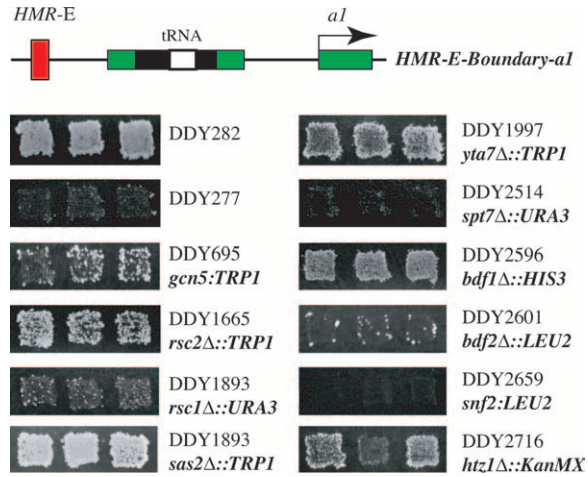

FIGURE 3.—Mutation of multiple bromodomain-containing genes results in spreading of silencing through the *HMR* boundary. Specific deletions or insertions were created or crossed into DDY277 to test their effects on boundary function in the mating assay described in Figure 1. Null mutations in *YTA7*, *BDF1*, *RSC2*, and *SAS2* led to a significant spread of silencing, while mutation of *GCN5*, *RSC1*, *BDF1*, and *HTZ1* showed a weaker phenotype. Mutation of *SPT7* or *SNF2* showed no phenotype.

of GST alone to the histones. This result shows that, despite lacking the conserved tyrosine, the Yta7p bromodomain is a functional histone-binding domain *in vitro*.

The bromodomain protein Bdf1p has also been shown to restrict the spread of silencing in yeast (LADURNER *et al.* 2003), and the histone-binding domain of the transcription factor CTF-1 is essential for its heterochromatin barrier activity (FERRARI *et al.* 2004), suggesting a role for histone-binding proteins in blocking silencing. The *S. cerevisiae* genome contains 10 genes with bromodomain homologies (aligned in Figure 2B). Two of these genes, the RSC complex genes *STH1* and *RSC4*, are essential for viability. Since loss of Bdf1p and Yta7p functions lead to an apparent spreading of silencing, we asked if bromodomain proteins in general contain boundary and/or antisilencing functions. We created a set of strains containing deletions or disruptions of each nonessential bromodomain-containing gene in strain DDY277 to assess the spreading of silencing across the boundary element using the mating assay described in Figure 1. The results shown in Figure 3 demonstrate that loss of function of several bromodomain proteins leads to a spreading of silencing. Deletion of *RSC2*, *YTA7*, or *BDF1* leads to the greatest loss of boundary function, while deletion of *RSC1*, *GCN5*, or *BDF2* shows a weaker phenotype. Deletion of *SPT7* or *SNF2* do not show spreading of silencing as determined by this assay. As controls for spreading of silencing at *HMR*, we assayed mutations in *SAS2* and *HTZ1* and observed the expected spreading of silencing as indicated by increased mating compared to the control strain DDY277 (Figure 3). The *htz1* mutants showed a consistently variegated phenotype in this assay, with patches derived from some colonies mating and others not.

We next wanted to test epistatic relationships among bromodomain factors and the *HMR*-tRNA boundary element. Since deletion of the *HMR*-tRNA leads to a

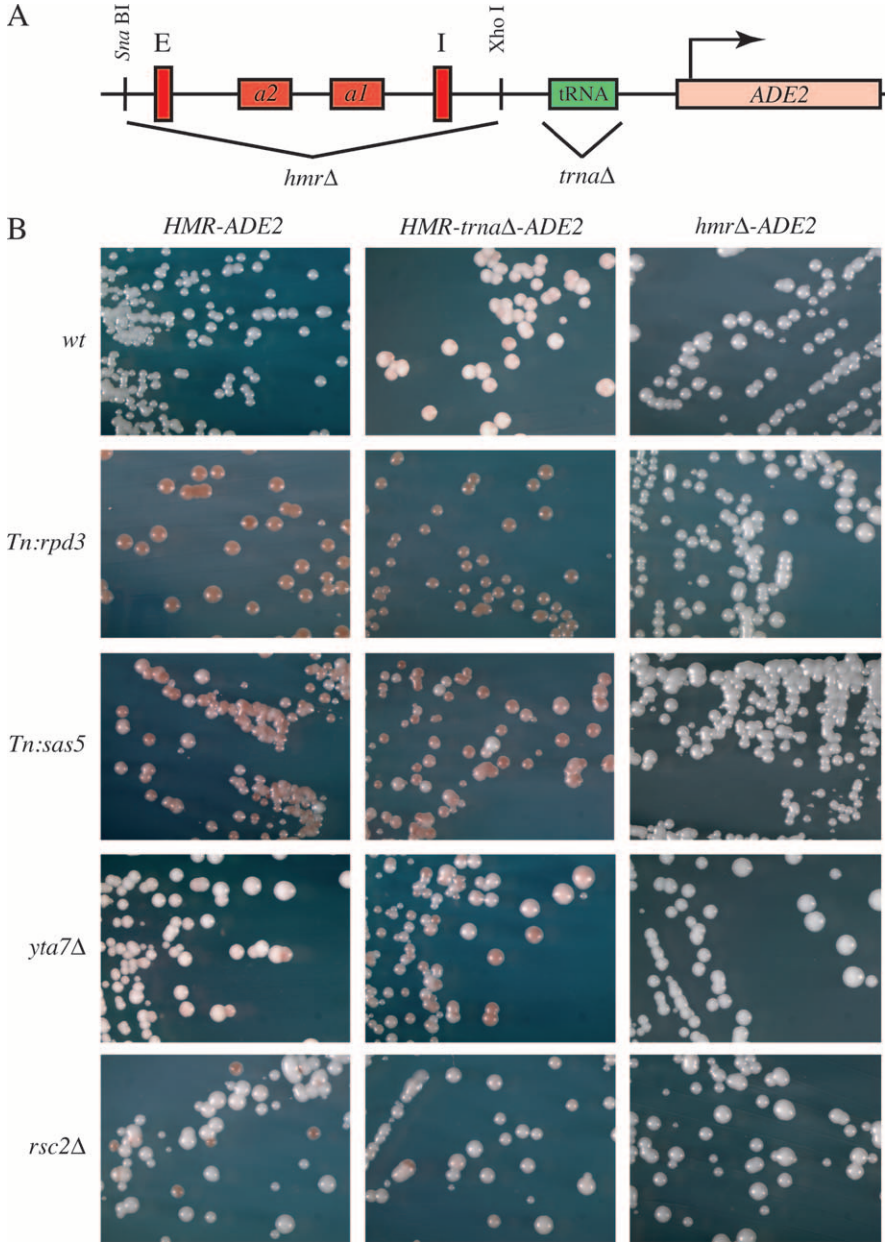

FIGURE 4.—*YTA7* and the *HMR*-tRNA function through different pathways to restrict the spread of silencing at *HMR*. (A) The *ADE2* gene was inserted into chromosome III downstream of *HMR* in *S. cerevisiae* (*HMR-ADE2*). Isogenic strains lacking the *HMR*-tRNA (*HMR-trna*Δ*-ADE2*) or lacking *HMR* (*hmr*Δ*-ADE2*) were also constructed. (B) Each strain was crossed into *rpd3*, *sas5*, *yta7*, or *rsc2* mutant backgrounds, and silencing was assessed by the degree of pigmentation exhibited by the presence or absence of *ADE2* expression. The combination of *yta7*Δ *trna*Δ leads to a more silenced phenotype (more and darker red colonies), suggesting that each functions independently. No difference in phenotype is seen between strains containing *rsc2*Δ and *rsc2*Δ *trna*Δ. Strains depicted are: first row [wild type (wt)], DDY814, DDY811, and DDY2114; second row (*rpd3*), DDY2093, DDY2143, and DDY2128; third row (*sas5*), DDY2106, DDY2156, and DDY2142; fourth row (*yta7*), DDY2205, DDY2200, and DDY2198; and fifth row (*rsc2*), DDY2496, DDY2489, and DDY2450.

near-complete mating phenotype in the mating assay, we developed a second system for assessing the effects of tRNA deletion on heterochromatin spread at *HMR*. The *S. cerevisiae ADE2* gene was integrated downstream of *HMR* (referred to here as *HMR-ADE2*), and isogenic strains that were *HMR-trna*Δ*-ADE2* or *hmr*Δ*-ADE2* were created. Repression of *ADE2* expression leads to the accumulation of a red pigment derived from the Ade2p substrate (FISHER 1969). Phenotypes of these parent strains are shown in the top row of Figure 4B: strains containing a normal *HMR* locus or a deletion of *HMR* (including both silencers) give rise to all-white colonies, demonstrating expression of *ADE2* at this integration site; deletion of only the *HMR*-tRNA gives rise to a variegated phenotype with some sectored pink colonies arising due to epigenetic silencing of *ADE2*.

These *ADE2* reporter strains were crossed to strains containing mutations that resulted in spreading of silencing in the mating assay. Mutation of *RPD3* resulted in completely red colonies with or without the tRNA present. This complete penetrance of the *rpd3* phenotype prevented any epistatic analysis using this assay. Mutation of *SAS5* resulted in a variegated but mostly pink-red colony phenotype, which was unchanged when the *HMR*-tRNA gene was deleted. Again, on the basis of the qualitative nature of this assay, it was impossible to determine epistatic effects of *sas5*Δ *trna*Δ mutations.

Deletion of *YTA7* in *HMR-ADE2* strains yielded a variegated pink-white phenotype, similar in magnitude to the *HMR-trna*Δ*-ADE2* strain; however, in *yta7*Δ *trna*Δ strains, a darker red color was observed in a greater percentage of colonies, including some completely red

colonies, demonstrating a more robust spread of silencing when both mutations are present (Figure 4B, fourth row, center). This additive effect suggests that *YTA7* functions independently of the *HMR*-tRNA in restricting the spread of silencing. *RSC2* deletion shows a weakly variegated phenotype in the *HMR-ADE2* background, with rare silenced colonies that were somewhat darker red. Deletion of the *HMR*-tRNA in the *rsc2*Δ background did not increase the severity of the phenotype, suggesting that *RSC2* and the *HMR*-tRNA may function through the same pathway in restricting the spread of silencing. We attempted to determine epistatic relationships among bromodomain-containing genes, but we were unable to obtain *yta7-rsc2* or *yta7-bdf1* double mutants by crosses of single-mutant strains or by homologous recombination, suggesting a synthetic lethality of these mutant combinations in our W303 background (our unpublished results).

Importantly, when *HMR* was deleted in each of these mutant backgrounds (Figure 4B, right column), all-white colonies were observed. This confirms that phenotypes observed for each of these mutations are due to the spread of silencing from *HMR* and not due to global histone acetylation effects or spreading of silencing from the telomere of chromosome III. Deletion of *SIR2* or *SIR4* in these backgrounds also results in completely white colonies, confirming the Sir dependence of the phenotype (our unpublished data). Also, it should be noted that the spreading of silencing observed in all experiments described here is due to normal levels of Sir proteins and that no overexpression of Sir3p was employed to obtain the observed phenotypes.

DISCUSSION

***YTA7* encodes a histone-binding chromatin boundary protein:** The mutational analysis of boundary function described here revealed that the AAA family ATPase/bromodomain protein Yta7p plays a role in the integrity of the euchromatin/heterochromatin boundary at the *S. cerevisiae HMR* locus. *YTA7* contains a region of bromodomain homology from amino acids 1003 to 1091 and two separate AAA family ATPase domains from amino acids 449 to 580 and 771 to 957 (Figure 2A). Comparison of the homology of the *YTA7* bromodomain to other yeast bromodomains (Figure 2B) revealed that *YTA7* lacks a key tyrosine residue (conserved in most bromodomains), which makes a critical water-mediated hydrogen bond to acetyllysine in Gcn5p and is required for the interaction of acetylated histones with Bdf1p (Dhalluin *et al.* 1999; Owen *et al.* 2000; Ladurner *et al.* 2003). However, the results shown in Figure 2C demonstrate that the *YTA7* bromodomain is a functional histone-binding module. The *YTA7* sequence contains tandem serine and threonine residues at the position of the conserved tyrosine, which may contribute the necessary hydrogen-bonding potential within the bromodomain-binding pocket. Bioinfomatic analysis of *YTA7* orthologs in the related yeast species *S. paradoxus*, *S. mikatae*, and *S. bayanus* and the less-related *Debaryomyces hansenii* and *Yarrowia lipolytica* revealed that these *YTA7* orthologs have the same serine-threonine substitutions in their respective bromodomains (Christie *et al.* 2004), and the same substitution is seen in both human and mouse Tif1β (Zeng and Zhou 2002). Yta7p is also a confirmed nuclear protein, as determined by a genome-wide analysis of yeast protein localization (Huh *et al.* 2003).

While this work was in progress, Tackett *et al.* (2005) published a proteomic study that also implicated Yta7p in boundary function. They found that Yta7p associated with complexes containing Dpb4p, a component of the DNA polymerase ϵ and ISW2/CHRAC chromatin complexes, and that both of these complexes are involved in regulating telomeric silencing (Iida and Araki 2004). Tackett *et al.* (2005) also showed that deletion of *YTA7* led to spreading of silencing at *HMR*, and most significantly, they localized Dpb4p-Yta7p complexes to sites near *HMR*, *HML*, chromosome ends, and the conditionally expressed and epigenetically regulated *FLO* genes, suggesting a specific targeted role for Yta7p in restricting repression from multiple loci. The results presented in this article confirm the role of Yta7p in preventing the spread of silencing and extend these results to demonstrate independent functions of Yta7p and the *HMR*-tRNA with an epistasis analysis (discussed below).

Additionally, the study by Tackett *et al.* (2005) identified roles for DNA polymerase ϵ, Isw2, and Sas3-Spt16 chromatin-remodeling complexes in boundary function. We previously tested mutations in *SAS3* (Donze and Kamakaka 2001) and *SPT16* (our unpublished results) and saw no spreading of silencing in our mating assay. Tackett *et al.*'s (2005) analysis of the effect of *SAS3* deletion showed a reduction in transcript levels of genes adjacent to *HMR* (*GIT1* and *YCR095C*), but it is unknown if this reduction is Sir protein dependent, as expression of these genes was not reported in a *sir*Δ or *hmr*Δ background.

Our genetic analysis also uncovered a role for *r*emodels the *s*tructure of *c*hromatin (RSC) complex proteins in boundary function. RSC is a multisubunit, highly abundant, and essential chromatin-remodeling complex first purified from *S. cerevisiae* cells (Cairns *et al.* 1996). Genome-wide analysis of the location of the RSC complex has demonstrated that RSC is targeted to many tRNA genes *in vivo* (Ng *et al.* 2002), suggesting a potentially direct connection between the function of the *HMR*-tRNA and the RSC complex in heterochromatin boundary function. Our epistasis analysis demonstrating no difference in phenotype between *rsc2*Δ and *rsc2*Δ *trna*Δ strains shown in Figure 4 is consistent with this proposed connection.

Yta7p appears to function independently of the *HMR*-tRNA, as deletion of both the *cis*-element and the *trans*-factor leads to a more silenced phenotype (Figure 4). We could not determine the epistatic relationships between the *HMR*-tRNA and *BDF1*, *BDF2*, *RSC1*, or *GCN5* in this assay, as strains containing deletions of these genes grew as all-white colonies in the *HMR-ADE2* background and had no apparent effect on colony color when in the *HMR-trna*Δ*-ADE2* background (our unpublished results). Mutations in *BDF2*, *RSC1*, and *GCN5* all exhibited a weaker phenotype in the mating assay, an effect that may be too weak to affect the *ADE2* promoter. However, we observed a strong increase in silencing upon deletion of *BDF1* in the mating assay, but this mutation did not give rise to pigmented colonies in the *HMR-ADE2* background. It should be noted that *bdf1* strains are very slow growing, and it is uncertain how growth rate might affect the accumulation of pigment in *ADE2* silenced strains.

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

Several recent studies have demonstrated that direct targeting of transcription factors or components of chromatin-modifying complexes can act as a barrier, preventing the spread of SIR-mediated heterochromatin in yeast (Donze and Kamakaka 2001; Fourel *et al.* 2001; Chiu *et al.* 2003; Jacobson and Pillus 2004; Oki *et al.* 2004). The barrier effect created by acetyltransferase targeting was not necessarily due to the enzymatic activity of acetyltransferase fusions, but did require the presence of other components of the respective complexes (Chiu *et al.* 2003; Jacobson and Pillus 2004; Oki *et al.* 2004), suggesting that targeting of a specific factor results in the recruitment of multiple proteins. Global models of heterochromatin barrier function suggest that acetyltransferases and deacetylases establish "negotiable borders" or "fuzzy boundaries" by convergence of the effects of their activities, without the need for a specific barrier element or localized targeting (Fourel *et al.* 2004; Kimura and Horikoshi 2004). However, while both targeted and negotiable models are consistent with current data, the effects of global genome alteration (such as in a *sas2* background) on the normal targeting of chromatin-associated proteins is not well defined. Any natural targeting of proteins occurs within the context of normal cellular chromatin acetylation. Therefore, while domains of global histone acetylation may be negotiable in mutant strains, such genome-wide effects may lead to specific alterations in acetylation-state-dependent localization of proteins, such as the targeting of bromodomains.

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

Additional studies will be required to identify the roles of bromodomains and other histone-binding proteins like CTF-1 (Ferrari *et al.* 2004) in establishing heterochromatin-euchromatin boundaries. Bdf1p has been shown to protect histone H4 from Sir2p deacetylation *in vitro* (Ladurner *et al.* 2003), suggesting one possible mechanism of bromodomain action in restricting silencing. However, bromodomains are known to bind to acetylated proteins other than histones (Yang 2004; de la Cruz *et al.* 2005); therefore the effects on boundary establishment reported in this study may involve more than bromodomain-histone interactions. The results presented here suggest that the RSC complex may be acting through the *HMR*-tRNA, which is supported by the fact that RSC preferentially associates with tRNA genes *in vivo* (Ng *et al.* 2002). However, it is currently unknown whether RSC is targeted to assembled RNA polymerase III genes or if prior RSC binding promotes RNA polymerase III complex assembly. Yta7p appears to be targeted to a limited set of chromosomal

sites (Tackett *et al.* 2005), and while the mechanism of this targeting is unknown, it may involve the histone acetylation state near the boundary. The variability of bromodomain mutant phenotypes on boundary function may reflect a varied array of target proteins for bromodomain binding or could be a function of varied effects on the different multisubunit complexes in which most bromodomain factors have been identified. It will be of interest to determine the acetylation requirements for Yta7p binding at heterochromatin boundaries and how mutations that cause specific or global alterations in the acetylation states of histones, or possibly other chromatin-associated proteins, affect the targeting and function of boundary-associated bromodomain factors.

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