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Transcription Independent Insulation at TFIIIC-Dependent Insulators

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ABSTRACT

Chromatin insulators separate active from repressed chromatin domains. In yeast the RNA pol III transcription machinery bound to tRNA genes function with histone acetylases and chromatin remodelers to restrict the spread of heterochromatin. Our results collectively demonstrate that binding of TFIIIC is necessary for insulation but binding of TFIIIB along with TFIIIC likely improves the probability of complex formation at an insulator. Insulation by this transcription factor occurs in the absence of RNA polymerase III or polymerase II but requires specific histone acetylases and chromatin remodelers. This analysis identifies a minimal set of factors required for insulation.

THE eukaryotic genome can be divided into two chromatin states. Heterochromatin, which in most metazoans constitutes the vast majority of the genome, is condensed and contains primarily repetitive DNA sequences while euchromatin is accessible chromatin that is gene rich. Euchromatic and heterochromatic domains form a mosaic along the chromosome and often these functionally competing chromatin states reside adjacent to one another.

Gene activity in eukaryotes occurs within the context of these chromatin domains and is regulated by DNA sequence elements. Enhancers and locus control regions (LCRs) positively regulate genes while silencers repress genes. Typically these regulatory elements function within specific chromatin domains and contribute toward the formation of these domains. Enhancers and LCRs bind various combinations of transcription factors, which in turn recruit accessory proteins such as histone modifiers, chromatin remodelers and histone subtypes to open chromatin domains thereby generating a euchromatic state that is amenable to stable gene activation. Silencers on the other hand bind specific factors and recruit histone modifiers and repressor proteins that spread and encompass DNA sequences into a condensed state that is inaccessible to various enzymatic probes.

The euchromatic and heterochromatic domains are separated from one another by DNA regulatory elements called insulators (Bi and Broach 2001; Fourel *et al.* 2004; Valenzuela and Kamakaka 2006). Insulators are integral to proper gene regulation and have many of the same properties as promoters and occasionally are promoters of genes (Donze and Kamakaka 2001; Fourel *et al.* 2002; Bartkuhn *et al.* 2009). Insulators bind various transcription factors and these factors recruit chromatin-modifying activities to delineate chromatin domains.

Yeast tRNA insulators: Protein translation requires tRNAs encoded by tRNA genes. In eukaryotes, tRNA genes utilize specific multisubunit transcription factors TFIIIC and TFIIIB to mediate synthesis of tRNA by RNA pol III (Geiduschek and Kassavetis 2001). Transcription of a tRNA gene involves the binding of TFIIIC to conserved intragenic promoter elements called box A and box B. TFIIIC binding leads to recruitment of TFIIIB to a ∼50-bp AT-rich region upstream of the start site of transcription. TFIIIB recruitment to the gene results in the recruitment of RNA pol III and transcription of the gene (Schramm and Hernandez 2002). These motifs within and upstream of a tRNA gene affect its transcription efficiency and function.

In eukaryotes the tRNA genes are a special form of repetitive DNA, present as multiple copies in the genome and are either arranged as small clusters or individual dispersed copies throughout the genome (Percudani *et al.* 1997). Since tRNA genes are dispersed throughout the genome this leads to questions of whether their location influences other chromosomal processes. The presence of a tRNA gene results in replication pausing at the gene (Deshpande and Newlon 1996), nucleosome positioning immediately adjacent to the gene (Morse *et al.* 1992), and preferential retroviral integration immediately upstream of the gene (Kirchner *et al.* 1995; Devine and Boeke 1996). Furthermore, in the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, tRNA genes have been found to function as chromatin insulators restricting the spread of heterochromatin (Donze *et al.* 1999; Donze and Kamakaka 2001; Noma *et al.* 2006; Scott *et al.* 2006).

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Silenced chromatin domains in the yeast *S. cerevisiae* and *S. pombe* are distinct, use different repressor proteins, and are formed via different mechanisms (Dhillon and Kamakaka 2002; Grewal and Moazed 2003). Silencing in *S. cerevisiae* is mediated by the Sir proteins while in *S. pombe*, silencing initiation utilizes the RNAi machinery and involves the association of Swi6p repressor protein with histones. Interestingly, in both systems, tRNA genes are able to block the spread of silencing. A $tRNA^{Thr}$ gene located adjacent to the silenced *HMR* locus in *S. cerevisiae* blocks the spread of silencing, and deleting this gene leads to an increased spread of Sir-mediated silencing (Donze *et al.* 1999; Donze and Kamakaka 2001). Similarly in *S. pombe*, clusters of tRNA genes mediate barrier activity restricting the spread of Swi6p containing heterochromatin (Noma *et al.* 2006; Scott *et al.* 2006). In both organisms, data indicate that the transcription factors that bind the tRNA genes are required for insulation. In *S. cerevisiae*, mutations in TFIIIC and TFIIIB but not pol III affect tRNA-mediated insulation (Donze and Kamakaka 2001), while in *S. pombe* TFIIIC binding has been shown to be required for insulation (Noma *et al.* 2006).

Besides acting as insulators to the spread of silenced chromatin, TFIIIC bound elements also act as insulators to repressed chromatin (Simms *et al.* 2004, 2008; Valenzuela *et al.* 2006) suggesting that tRNA genes help partition the genome into functionally distinct domains.

The sequences of tRNA genes are highly conserved and the substitution rate of sequences within functional tRNA genes is less than that observed at protein encoding genes suggesting a strong selection (Withers *et al.* 2006). Pseudogenes are DNA sequences that show homology with a known functional gene but where mutations have rendered the gene product inactive. In tRNA pseudogenes, high rates of nucleotide substitutions are observed during the death and degeneration of the genes (Withers *et al.* 2006). Several tRNA relics have been identified in *S. cerevisiae* and *S. pombe*. Genomewide mapping of the transcription factors TFIIIC, TFIIIB, and RNA pol III identified sites in *S. cerevisiae* that bind either TFIIIC alone or TFIIIC and TFIIIB but not RNA pol III, suggesting ancient genes (Harismendy *et al.* 2003; Roberts *et al.* 2003; Moqtaderi and Struhl 2004). Nine such sites called *Extra TFIIIC* (*ETC*) loci have been identified in *S. cerevisiae*. Similarly in *S. pombe* TFIIIC binding sites devoid of RNA pol III have also been reported called chromosome-organizing clamp (COC) loci (Noma *et al.* 2006). Interestingly most of the COC sites are located adjacent to RNA pol II transcribed genes and may aid in regulating these genes.

To investigate the molecular mechanism by which RNA pol III transcription factors function in insulation we decided to use *ETC* loci to determine the minimal factor requirement for tRNA promoter-mediated insulation in *S. cerevisiae*. We find that merely recruiting TFIIIC to a DNA element will insulate a gene, but the presence of TFIIIB improves the ability of TFIIIC to insulate. Efficient insulation by TFIIIC does not require either RNA pol II or RNA pol III, but mutations in specific histone modifiers and remodelers affects TFIIIC-mediated insulation, thus delineating the key components required for insulation.

MATERIALS AND METHODS

Strains, plasmids and oligonucleotides used in this study are listed in Table 1, Table 2, and Table 3, respectively.

Strain preparation: Deletions and integrations were performed by homologous recombination, using PCR products amplified with the Expand High Fidelity PCR System (Roche) and standard lithium acetate high-efficiency transformation procedure (Ito *et al.* 1983). In strain LOU162 (*HMR-pUC18::URA3 sir2*Δ), the 1.2-kb wild-type barrier element located to the right of the *HMR* locus was deleted and replaced by a 1.2-kb DNA fragment derived from pRS. The *URA3* cassette was then integrated at the pRS sequences, 590 bp downstream from the *HMR-I* silencer. B box-containing elements were PCR amplified and used to replace the *URA3* cassette at pRS sequences in strain LOU162. After transformation, FOA-resistant colonies were analyzed by PCR and crossed to obtain the final phenotypes.

Chromatin Immunoprecipitation: Cells were grown in YPD to an OD_{600} of 2.0, and cross-linked with 1% HCHO for 10–15 min followed by neutralization of the HCHO with 0.125 m glycine. Cells were washed with PBS and FA-140 and lysed in FA-140 with protease inhibitors using glass beads and a bead beater.

Lysed cells were sonicated twice, first with a Diagenode Bioruptor and then with a Branson cup-horn sonicator. This sequential sonication was important as it resulted in DNA with an average length of 300 bp. The sonicated chromatin solution was centrifuged to remove insoluble cellular debris.

Immunoprecipitation reactions were performed with the desired antibodies and protein A/G beads overnight. H3, Rpc40-HAp, Tfc1-HAp, and Pol II were chromatin immunoprecipitated using Rabbit polyclonal antibodies—ab1791 against the C terminus of H3 (Abcam), the Anti-HA.11 antibody (from Babco), or the Pol II monoclonal antibody 8WG16 (from Covance), in combination with protein G plus/protein A agarose (Calbiochem). Protein Bdp1-TAP was immunoprecipitated with IgG-sepharose 6 Fast Flow (Amersham Biosciences). Beads were washed sequentially with buffer FA-140, FA-500, LiCl/Det, and finally TE.

The immunoprecipitated chromatin was eluted off the beads by the addition of 10% Chelex-100 and incubated at 100° for 10 min. Proteinase K was added to the mixture and incubated at 55° for 30 min followed by a second incubation at 100° for 10 min. The DNA bound to the Chelex beads was eluted off of the beads with water.

The input DNA and immunoprecipitated DNA were quantitated using the PicoGreen dsDNA quantitation reagent (Molecular Probes) and a Perkin Elmer Viktor[3] Fluorescence spectrophotometer. (Lambda DNA was used to construct a standard graph).

Equal amounts of immunoprecipitated DNA and input DNA (usually between 50 pg and 200 pg) were used for real-time PCR analyses.

Real-time PCR-based amplification of the DNA was performed using specific primers. All primer pairs were initially screened (on average we tested three pairs of primers for each PCR fragment) for the absence of primer dimers or cross-

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
JRY19a	*MAT***a** *ura3-52 leu2-3,112 trp1 his4-519*	J. Rine
ROY113	*MATα hmrΔ::bglII-bclIΔ ade2 LYS2*	
ROY1862	*MATα hmrΔ::bglII-bclIΔ tfc3G349E^{ts} ADE2 LYS2*	
ROY951	*MATα HMRa2 (no boundary) -a1-hmr-IΔ(bclI-bclI) ade2 LYS2*	
ROY4545	*MATα HMRa2::(3xETC9)-a1-hmr-IΔ(bclI-bclI) ade2 LYS2*	
ROY4546	*MATα HMRa2::(tDNA^{thr}-CR1)-a1-hmr-IΔ(bclI-bclI) ade2 LYS2*	
ROY1205	*MATα hmrΔ::bglII-bclIΔ hat1Δ::HIS3 ade2 LYS2*	
ROY1510	*MATα hmrΔ::bglII-bclIΔ sas3Δ::HIS3 ADE2 lys2*	
ROY1528	*MATα hmrΔ::bglII-bclIΔ sas2Δ::TRP1 ADE2 lys2*	
ROY4185	*MATα hmrΔ::HIS3 eafΔ::kanMX ade2 LYS2*	
ROY4184	*MATα hmrΔ::HIS3 ada2Δ::kanMX ade2 LYS2*	
ROY1154	*MATα hmrΔ::bglII-bclIΔ spt3-203Δ::TRP1 ade2 lys2*	
ROY4547	*MATα hmrΔ::HIS3 rtt109Δ::kanMX ADE2 lys2*	
ROY3512	*MATα hmrΔ::bglII-bclIΔ isw2Δ::kanMX ade2 LYS2*	
ROY4186	*MATα hmrΔ::HIS3 rsc2Δ::kanMX ade2 LYS2*	
ROY4187	*MATα hmrΔ::HIS3 htz1Δ::kanMX ade2 LYS2*	
ROY1685	*MATα HMR(S288C) ADE2 lys2*	
ROY3931	*MATα HMR(S288C) TFC1-3xHA::kanMX ade2 LYS2*	
ROY4548	*MATα HMR(S288C) BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX ADE2 lys2*	
ROY4561	*MATα HMR-tRNAΔ(S288C) BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX ADE2 lys2*	
ROY4191	*MATα HMRaΔp-barrierΔ::URA3 sir2Δ::TRP1 ade2 LYS2*	
ROY4549	*MATα HMRaΔp-barrierΔ::tDNA^{thr}-CR1 BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX ADE2 (lys2?)*	
ROY4550	*MATα HMRaΔp-barrierΔ::*2x*ETC9 BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX ADE2 (lys2?)*	
ROY4551	*MATα HMRaΔp-barrierΔ::*3xBbox *SIR2 TFC1-3xHA::kanMX ADE2 LYS2*	
ROY4552	*MATα HMRaΔp-barrierΔ::*3xBbox *sir2Δ TFC1-3xHA::kanMX ADE2 LYS2*	
ROY4553	*MATα HMRaΔp-barrierΔ::*3xBbox *SIR2 BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX ADE2 LYS2*	
ROY4554	*MATα HMRaΔp-barrierΔ::*3xBbox *sir2Δ BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX ADE2 LYS2*	
ROY4555	*MATα HMRaΔp-barrierΔ::ETC2 SIR2 TFC1-3xHA::kanMX ADE2 LYS2*	
ROY4556	*MATα HMRaΔp-barrierΔ::ETC2 sir2Δ TFC1-3xHA::kanMX ADE2 LYS2*	
ROY4557	*MATα HMRaΔp-barrierΔ::ETC2 SIR2 BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX ADE2 lys2*	
ROY4558	*MATα HMRaΔp-barrierΔ::ETC2 sir2Δ BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX ade2 LYS*	
ROY4559	*MATα HMRaΔp-barrierΔ::*1x*ETC9 RSC2 ADE2 LYS*	
ROY4560	*MATα HMRaΔp-barrierΔ::*1x*ETC9 rsc2Δ ADE2 LYS2*	
ROY4562	*MATα HMR(S288C) TFC1-3xHA::kanMX RSC2 ADE2 LYS2*	
ROY4563	*MATα HMR(S288C) TFC1-3xHA::kanMX rsc2Δ ADE2 LYS2*	
ROY4564	*MATα HMR(S288C) BDP1-TAP::HIS3MX6 RPC40-3xHA::kanMX rsc2Δ ADE2 lys2*	
GRY104	*MATα HMRaΔp-tRNA-a1 RSC2*	
GRY108	*MATα HMRaΔp-tRNAΔ-a1 RSC2*	
BUY1240	*MATα HMRaΔp-tRNA-a1 rsc2Δ*	
BUY1243	*MATα HMRaΔp-tRNAΔ-a1 rsc2Δ*	
ROY1685	*MATα HMR -tRNA RSC*	
ROY1681	*MATα HMR-tRNAΔ RSC2*	
BUY1202	*MATα HMR-tRNA rsc2Δ*	
BUYXXX	*MATα HMR-tRNAΔ rsc2Δ*	

hybridization. Furthermore, only primer pairs with similar amplification efficiencies were used.

Quantitative chromatin immunoprecipitation analysis was performed using a Corbett Life Science Rotor Gene 6000 machine. The detection dye used was SYBR Green (2× Immomix from Bioline and Platinum SYBR Green from Invitrogen). Real-time PCR was carried out as follows: 95° for 5 min (1 cycle), 95° for 15 sec, 53–58° for 20 sec, and 68–70° for 20 sec (45 cycles). The fold difference between immunoprecipitated material (IP) and total input sample for each qPCR amplified region was calculated as described in Litt *et al.* (2001), following the formula IP/Input = $(2^{\text{InputCt} - \text{IPCt}})$.

All of the graphs shown represent the mean values and standard errors of at least two independent cross-linked samples with each sample being immunoprecipitated twice with the same antibody with the exception of RNA pol II in Figures 1 and 2.

RESULTS

Mapping the transcription machinery: To study the contribution of various tRNA bound factors in insulator function we initially quantitatively mapped the distribution of various subunits of TFIIIC, TFIIIB, and RNA pol III to the native tRNA boundary at *HMR*. The tRNA gene present at the *HMR* boundary also has sequence

TABLE 2

Plasmids used in this study

Plasmid	Insert	Source
pRS425	*LEU2-2μ*	
pRO329	*SIR3-LEU2-2μ*	
pRO363	*HMR-E-a2::(Eco*NI-*Not*I-*Bam*HI-*Kpn*I-*Eco*NI*)-a1-hmr-I*Δ*(bclI-bclI)-URA3*	
pRO791	*HMR-E-a2::(Eco*NI-*Not*I-*Bam*HI-*Kpn*I*-*Eco*NI*)-a1-hmr-I*Δ*(bclI-bclI)-URA3* **Kpn*I site is unique	
pRO847	*HMR-E-a2::(*1x*ETC9)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pRO848	*HMR-E-a2::(*1x*ETC9)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMR-E*	
pRO850	*HMR-E-a2::(*2x*ETC9)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMR-E*	
pRO851	*HMR-E-a2::(*3x*ETC9)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMR-E*	
pRO852	*HMR-E-a2::(ETC2)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pRO853	*HMR-E-a2::(*pUC18-*ETC2*-pUC18*)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pRO854	*HMR-E-a2::(ETC2)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMR-E*	
pRO856	*HMR-E-a2::(*1x[pUC18-3xBbox-pUC18]*)-a1-hmr-I*Δ*(bclI-bclI)*. With the 3 consecutive consensus B boxes toward *HMR-E*	.
pRO857	*HMR-E-a2::(*3x[pUC18-3xBbox-pUC18]*)-a1-hmr-I*Δ*(bclI-bclI)*. With the 9 consensus B boxes toward *HMR-E*	
pRO858	*HMR-E-a2::(*4x[pUC18-3xBbox-pUC18]*)-a1-hmr-I*Δ*(bclI-bclI)*. With the 12 consensus B boxes toward *HMR-E*.	
pRO859	*HMR-E-a2::(*1xPPM2-ETC*)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMR-E*	
pRO861	*HMR-E-a2::(*2xPPM2-ETC*)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMR-E*	
pRO862	*HMR-E-a2::(*3xPPM2-ETC*)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pRO466	*HMR-E-a2::(tDNA*^{thr}*-CR1)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pDD441	*HMR-E-a2::(tDNA*^{thr}*-CR1)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMR-E*	D. Donze
pRO495	*HMR-E-a2::(tDNA*^{thr}*-CR1* 3′ mutated*) -a1-hmr-I*Δ*(bclI-bclI)* With B box toward *HMRa1*	
pRO470	*HMR-E-a2::(tDNA*^{thr}*-KL)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pRO452	*HMR-E-a2::(tDNA*^{thr}*-KL)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMR-E*	
pRO471	*HMR-E-a2::(tDNA*^{thr}*-GR1)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pRO469	*HMR-E-a2::(tDNA*^{thr}*-NL1)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pRO465	*HMR-E-a2::(tDNA*^{leu}*-SUP53)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	
pRO461	*HMR-E-a2::(snRNA U6)-a1-hmr-I*Δ*(bclI-bclI)*. With B box toward *HMRa1*	

homologs located elsewhere in the genome. We therefore devised oligonucleotides that would allow us to quantitatively map the distribution of transcription factors specifically across a 1-kb region centered on the *HMR*-tRNA insulator. We used a control probe for all our ChIP analysis located 500 bp from TEL6R in an intergenic region. There are no binding sites for any of the RNA pol III transcription factors in this region of the genome.

To map the distribution of various subunits of the tRNA transcription machinery across the region we used strains containing tagged proteins Tfc1, Bdp1, and Rpc40 (Roberts *et al.* 2003). Tfc1 is an essential subunit of the TFIIIC complex while Bdp1 is an essential subunit of TFIIIB and Rpc40 is an essential subunit of RNA pol III and RNA pol I. These three subunits were used as representatives of the three complexes required for tRNA transcription. The tagged strains (ROY 3931 and ROY4548) had no significant growth defects and behaved similarly to an untagged strain (ROY1685), suggesting that the tags had not severely compromised their essential functions though the Bdp1-tagged strain dominantly recruited RNA pol II (see supporting information, Figure S1).

Mapping the distribution of Tfc1 across the silenced *HMR* domain (Figure 1A) demonstrated that this protein had a unique binding site adjacent to *HMR* at the tRNA^{THR} gene. Using nested probes we found increased binding of TFIIIC in regions upstream of the tRNA and at the tRNA but not downstream of the gene (compare probes IV, IV′, and V). A similar binding profile was observed for Bdp1 with increased binding upstream of the gene (Figure 1B). The binding for Rpc40 (subunit of RNA pol III) was present both upstream as well as downstream of the gene (Figure 1C). These profiles are consistent with the observations that the transcription activators for the tRNA bind the 5′ and coding regions of the gene while pol III transcribes across the entire gene.

Having mapped the distribution of RNA pol III transcription machinery at the *HMR* tRNA insulator, we next mapped these proteins at other loci. We mapped these factors at specific tRNA genes as well as the U6 gene, which is also transcribed by RNA pol III (Figure 2). At transcribed tRNA and U6 genes, our quantitative data indicated that the levels of RNA pol III and Bdp1 were approximately equivalent at all loci compared. In contrast the levels of TFIIIC varied from locus to locus. There was an elevated level of Tfc1 at

TABLE 3

Oligonucleotides used for ChIP and qPCR

Region	Oligos	Sequence
HMR locus		
3′HMR-E (I)	LOU 191	CCCGTCCAAGTTATGAGCTTAATC
	L-95	AAAACCAGGAGTACCTGCGCTTATTCT
HMR-a1 (II)	LOU 199	TGGCGGAAAACATAAACAGAACTCT
	LOU 83	GTGTAATGTATGTTGCTCTACTTTAGTC
5′ HMR-I (III')	LOU 168	GAAGAGACTTATGATCAACATAATTTTGC
	LOU 173	CATATACGAAAATGTTGGTGACATGTAATC
3′ HMR-I (III)	L-97	TTAATACCTTTAAATGTTGAGGTAAATAGC
	L-98	GCTAAAGTGTGTGGAAAAACATTTTCTTGT
5′ HMR-tRNA^{thr} (IV)	LOU 201	CACCAATTCCGCATCTGCAGATTA
	L-96	GGTAGAATGACCTAGAATGACCCATCC
HMR-tRNA^{thr} (IV')	LOU 201	CACCAATTCCGCATCTGCAGATTA
	LOU 120	GGGTGTCACCGAATAACGTG
3′ HMR-tRNA^{thr} (V)	L-108	TACCGTTATTCGGAGATCTCTTACGG
	L-109	GTGACGCACTGAATGTCATCAAAAG
5′ Tyδ14 (VI)	L-104	CATAAGACGAGTTCTTCTATATCCGGTG
	L-107	CCTATTTTGCGTATTCCTATGTTGGTG
GIT1 (G)	Rol 120	CAACTTTGATCGACCTGTCTGACTGAA
	Rol 123	GTTGAATTTCACAACTATTTGGTATCC
HMR-tRNA^{thr} (IX)	R197	GAGACCAGGTTTATTCAACCGGTAAC
	Lou120	GGGTGTCACCGAATAACGTGAT
ETC loci and *RPL26B*		
PPM2-ETC	L-193	ATTATACTATCAGAACGCTCCGGCTTC
	L-195	CATGCGACTTGAACGATTCTAAGATGT
ETC2	L-165	AGTGCACAATGACGGATCATGTACTTT
	L-166	CCACTTCTATGGGCACCATACATATCA
5′ ETC9 (X)	L-78	TTTAGAACTATAACCTGCTGTCACTGG
	L-79	TCGGAAGCTCCAATAAATAAAGGATACA
ETC9 (XI)	L-190	CAGGAAAATCAAAAGACATGACGCAT
	L-192	AAAACCGGATAATACCAGGTCAGCTTC
RPL26B (XII)	L-87	AAAGTATTAGCCCGTTCTCAGTGCTTC
	L-88	CGGAAATTCACTCATGTTTTCTATCGT
Telomeric regions		
Tel 7.5	Rol 118	GTGGAAAGTATCGAGTTATGTGTACCT
	Rol 119	GTCATTCAAATACAGTGGGAAGTCTAC
Tel 0.5	LOU 189	GCGTAACAAAGCCATAATGCCTCC
	LOU 190	CTCGTTAGGATCACGTTCGAATCC
HMR-I region		
HMR-I::tDNA-CR1	L-82	CGTGGACTCCAACGTCAAAG
HMR-I::2xETC9	L-83	AGTGCTTTACGGCACCTCGAC
HMR-I::1xETC9 (VII)	L-163	CGTGAACCATCACCCTAATCAAG
	L-191	CTGGACATGAAAAATGACGGAATACTG
HMR-I::1xETC9 (VIII)	L-85	ATACGACTCACTATAGGGCGAATTG
	L-86	CGGCCGCTCTAGAACTAGTGGAT
HMR-I::ETC2	L-163	CGTGAACCATCACCCTAATCAAG
HMR-I::3xBbox	LOU 143	TCGCCCTATAGTGAGTCGTATTACG
Pol III transcribed genes		
tDNA^{thr} CR1 (IV')	LOU 201	CACCAATTCCGCATCTGCAGATTA
	LOU 120	GGGTGTCACCGAATAACGTG
tDNA^{thr} GR1	L-139	AGACAATCCCTTTATGTTTCATGTGCGTA
	L-140	ATGGATGGCGCGATAATTCTATACC
tDNA^{thr} NL1	L-143	CCGGTTTTCTCAAGTTCTGAGCTTCTA
	L-144	CCATCAGGCATGTTTACCGTAGAATAA
tDNA^{thr} KL	L-187	AGTATAGCGGAGCCACAAATTTAGCAG
	L-188	AAATAAAATTTCAAATGCCCTCTGTGG
SUP53	L-149	GTCCTGTACTTCCTTGTTCATGTGTG
	L-150	CTGTGTGTTCTCGTTATGTTGAGGAA
snR6	L-151	GTATTTCGTCCACTATTTTCGGCTAC
	L-153	AGGGGAACTGCTGATCATCTCTGTATT

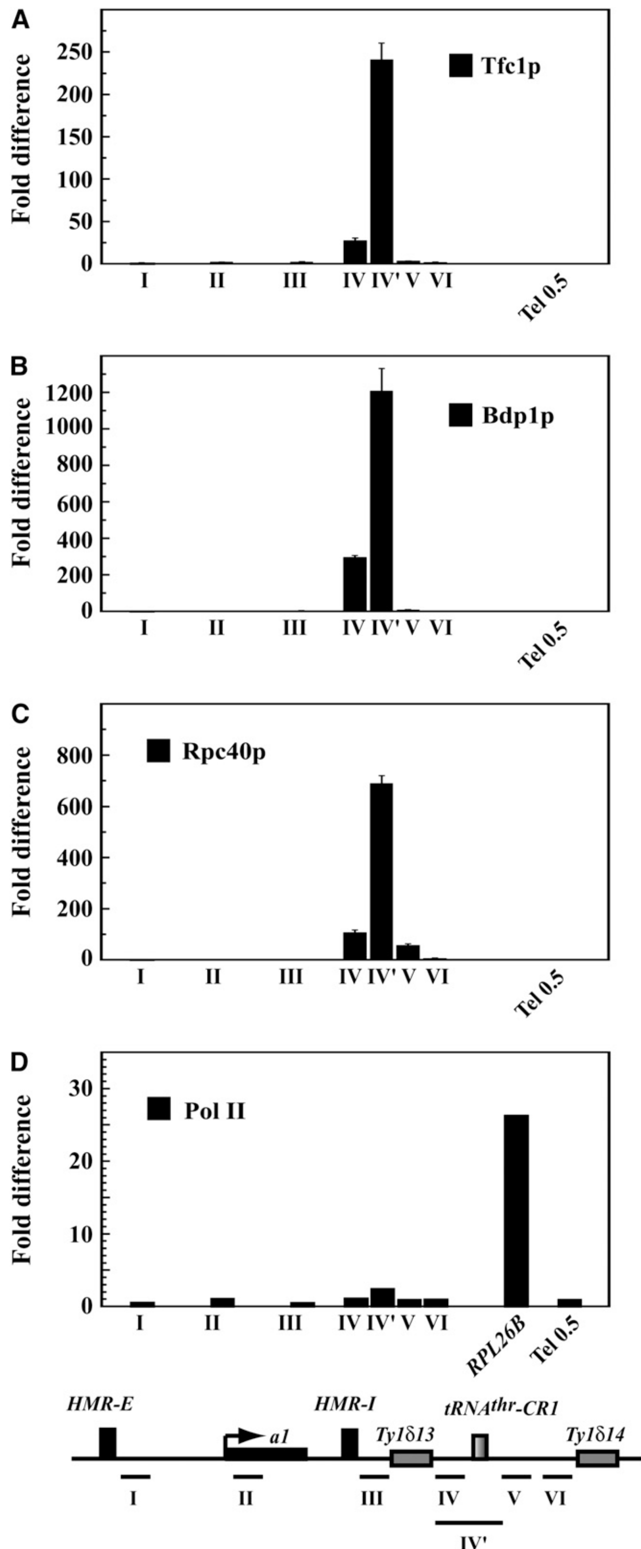


FIGURE 1.—Binding of RNA pol III and pol II factors at the silenced *HMR* domain. The distribution of Pol III and Pol II factors was analyzed by quantitative chromatin immunoprecipitation using real-time PCR. The fold difference between immunoprecipitated material (IP) and total input sample for each qPCR amplified region was calculated as described in LITT *et al.* (2001). IP/Input ratios were normalized to the telomeric region on chromosome VI (Tel 0.5). Positions of the PCR products are shown in the schematic diagram. (A) Binding of TFIIIC was analyzed using HA-tagged Tfc1 in strain ROY3931. (B and C) TFIIIB subunit Bdp1 and RNA Pol III subunit Rpc40 were mapped in strain ROY4548 using Bdp1-TAP and Rpc40-HA-tagged proteins. (D) Distribution of Pol II was analyzed in strain ROY1685 using a Pol II monoclonal antibody that recognized the C-terminal heptapeptide repeat of the large subunit of RNA Pol II. The promoter of the *RPL26B* gene was included in the analysis as a positive control for Pol II localization.

tRNA^{THR}-GR1 and tRNA^{THR}-KL while tRNA^{THR}-NL1 and *SUP53* had a twofold reduction in levels of Tfc1 and the U6 gene had a very significant reduction in the level of Tfc1 compared to the *HMR* tRNA.

All *ETC* loci bind TFIIIC and some of these loci also bind TFIIIB but none of these loci bind RNA pol III (ROBERTS *et al.* 2003; MOQTADERI and STRUHL 2004). These loci are scattered throughout the yeast genome. We next mapped the RNA pol III factors at a few *ETC* loci that bind the transcription factors TFIIIB and/or TFIIIC but do not recruit RNA pol III (Figure 2). We analyzed three different *ETC* loci: we investigated binding at a previously identified pseudo tRNA^{ARG} gene located on chromosome VII between genes *TIM21* and *RPL26B* (ROBERTS *et al.* 2003). In this article we will refer to this locus as *ETC9*. We also investigated two previously identified loci on chromosome XV—one located in the coding region of *PPM2*, which we will refer to as *PPM2-ETC* and the second located between genes *PPM2* and *ARG8* called *ETC2* (ROBERTS *et al.* 2003; MOQTADERI and STRUHL 2004). All three loci have sequences with homology to the B box present at all RNA pol III transcribed genes and in close proximity to these B-box sequences is a sequence with homology to a consensus A box.

Upon mapping RNA pol III subunit Rpc40 at these loci (Figure 2C) we found that unlike the tRNA genes, *ETC2* and *PPM2-ETC* did not bind RNA pol III to any significant level. *ETC9* bound significant amounts of Bdp1 though the levels were lower compared to the functional tRNA genes (Figure 2B). The other *ETC* loci did not bind any detectable Bdp1. *ETC2* and *ETC9* both bound Tfc1 while the *PPM2-ETC* locus did not bind any pol III factors (Figure 2A). These results confirm the previous observations that *ETC2* and *ETC9* are genuine *ETC* loci (MOQTADERI and STRUHL 2004) but *PPM2* is not a functional *ETC* locus in the W-303 strain background used in this study.

In *S. pombe*, RNA pol II is found at the IR insulators along with TFIIIC (NOMA *et al.* 2006) and we therefore mapped RNA pol II at the *HMR* tRNA using a monoclonal antibody (8WG16) specific for the unmodified large subunit of RNA pol II (Figure 1D). This analysis demonstrated the absence of the large subunit of RNA pol II across the entire *HMR* domain and we also did not observe any RNA pol II at or near the tRNA gene. We also tested the distribution of RNA pol II at other tRNA

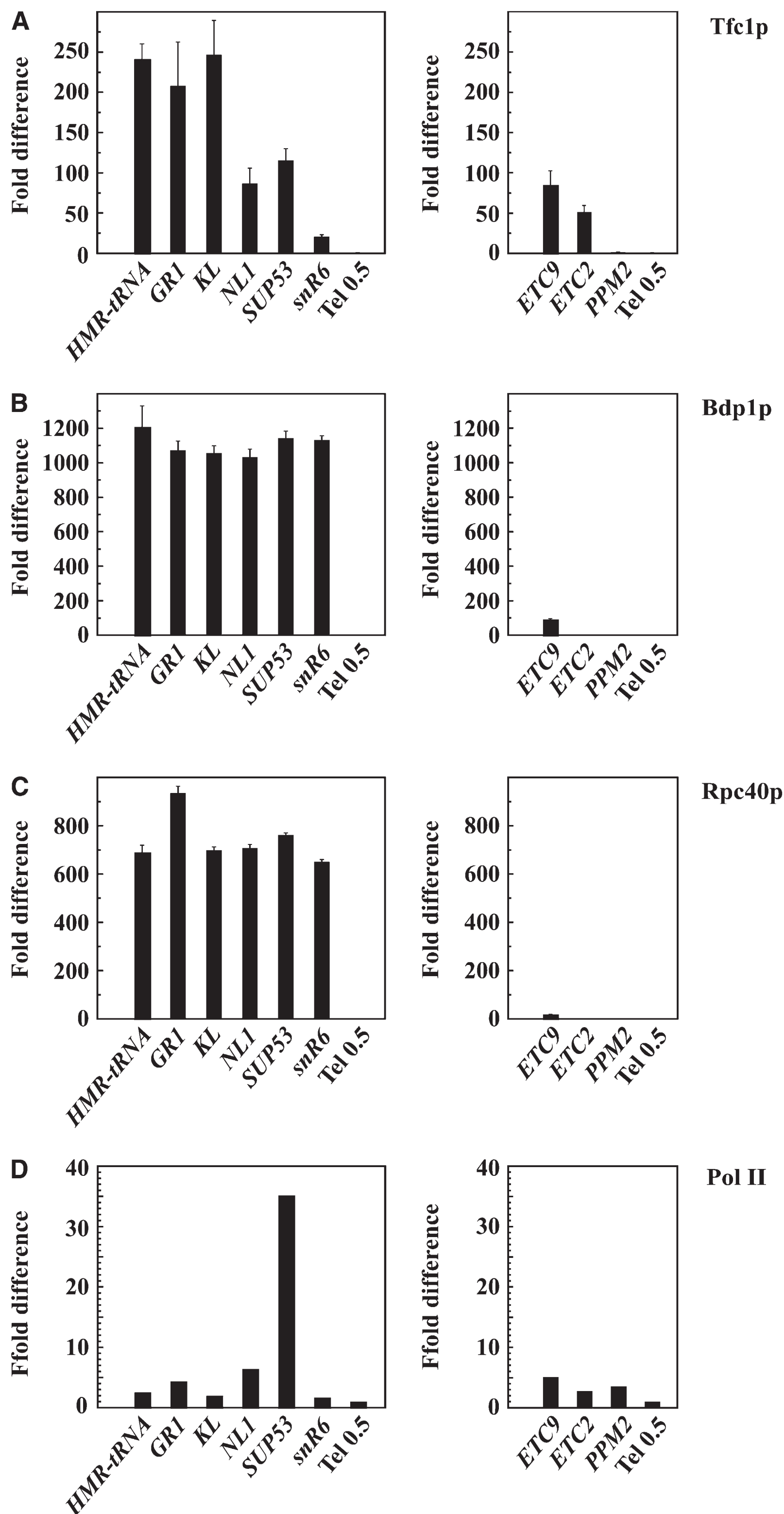
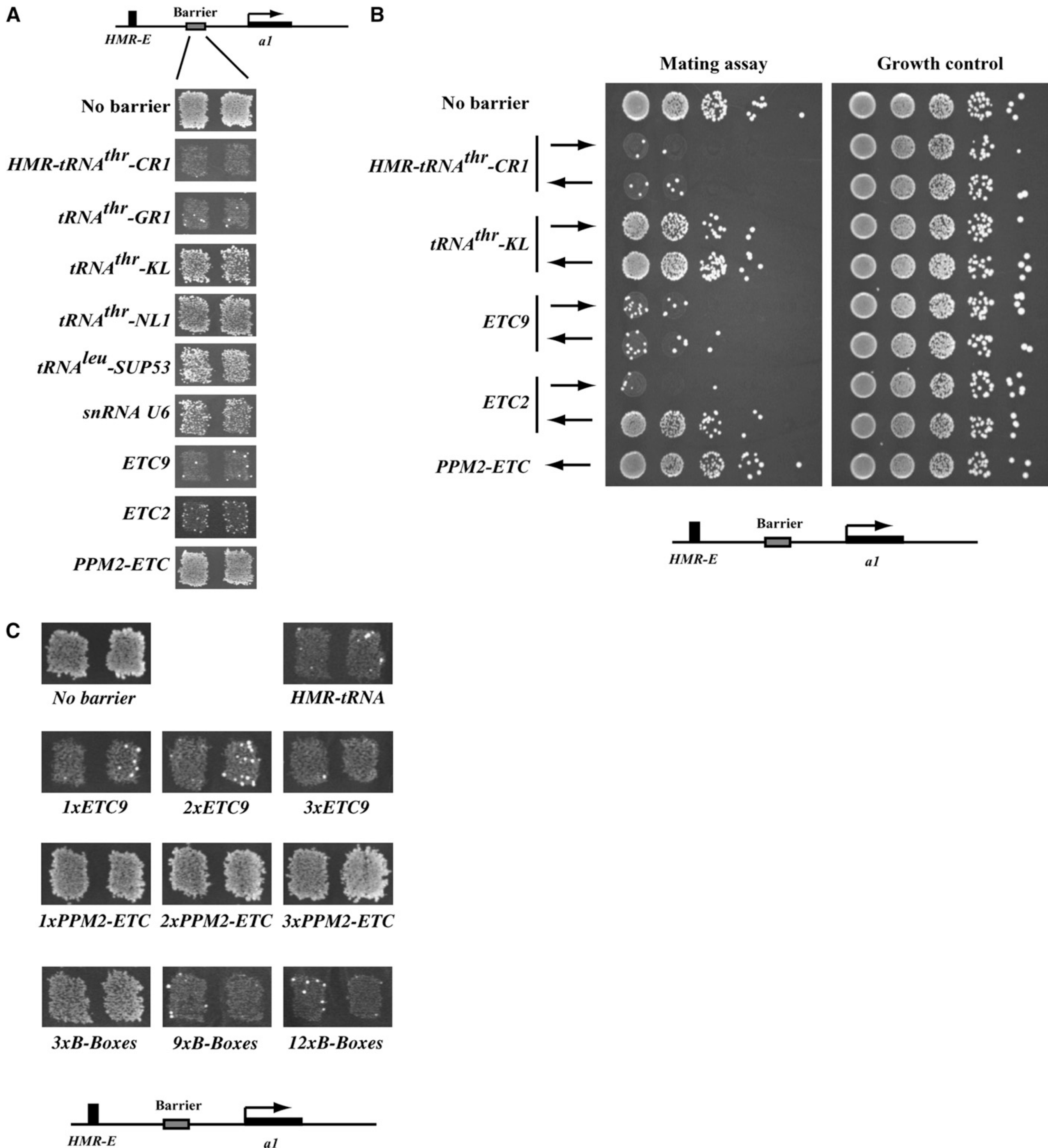


Figure 2.—Quantification of Pol III transcription factors at tRNA genes and *ETC* loci TFIIIC, TFIIIB, and Pol III were mapped at different tRNA genes and *ETC* loci by ChIP followed by qPCR. Data were analyzed as described in Figure 1. Oligonucleotides used for the qPCR flanked the tRNA genes and the *ETC* loci. (A) Tfc1-HA in strain ROY3931 was used to map TFIIIC. (B and C) Bdp1-TAP and Rpc40-HA in strain ROY4548 were used to map TFIIIB and RNA pol III. For ease of comparison data obtained for the *HMR*-tRNA (region IV′) from Figure 1 are included. (D) RNA pol II large subunit was mapped in ROY1685 using a monoclonal antibody as described in Figure 1.

genes as well as at the three *ETC* loci (Figure 2D). All tRNA genes except *SUP53* had extremely low levels of RNA pol II protein in their immediate vicinity. There were significant levels of RNA pol II near *SUP53* presumably because this gene is located in a very short intergenic region immediately adjacent to a RNA pol II regulatory region. None of the *ETC* loci bound significant amounts of RNA pol II, suggesting that pol II recruitment to *ETC* elements does not occur in *S. cerevisiae*. This obviously does not rule out the possibility that *ETC* loci facilitate or regulate pol II-mediated transcription of neighboring genes.

Our results on the distribution of tagged pol III factors are consistent with previously published results on the



distribution of pol III factors (Harismendy *et al.* 2003; Roberts *et al.* 2003; Moqtaderi and Struhl 2004), demonstrating that the presence of the tags does not affect the distribution of these proteins. However, interestingly, when we mapped RNA pol II in the Bdp1/Rpc40 tagged strain we surprisingly observed robust levels of RNA pol II immediately upstream of the tRNA genes tested (Figure S1, A and data not shown). This binding was present at all the tRNA genes tested and was lost when a internal promoter of a tRNA gene was deleted, demonstrating that pol II was being recruited by these tagged proteins (Figure S1, A). In contrast to the tagged Bdp1 strain (ROY4548), in the tagged Tfc1 strain (ROY3931) we did not observe any significant recruitment of RNA pol II (Figure S1, B). In future, care will need to be taken in the use of tagged proteins.

***ETC* loci function as insulators:** Specific tRNA genes function to block the spread of heterochromatin and previous experiments had demonstrated that mutations in TFIIIC and TFIIIB weakened insulation but muta-

tions in RNA pol III had no effect, suggesting that transcription was not necessary for insulation (Donze and Kamakaka 2001). We decided to take advantage of the differential binding of pol III factors at *ETC* loci to determine the minimal DNA binding transcription factors necessary for insulation. A modified *HMR* locus was created by the insertion of a 300-bp fragment containing a tRNA gene or *ETC* loci between the *HMR-E* silencer and the *MAT***a***1* gene. In strains where a functional insulator was present, silencing emanating from the silencer would be blocked and the reporter gene would be insulated and be active. If the insulator were not able to function, then the gene would be repressed. These strains were assayed for barrier activity using a mating assay. If the DNA fragment had barrier activity, then the strain would be a nonmater and conversely, if barrier activity was absent, then the silent domain would spread to repress the *MAT***a***1* reporter gene and the strain would mate. This analysis (Figure 3A) indicated that $tRNA^{THR}$-KL, $tRNA^{THR}$-NL1, *SUP53*, and the U6 gene were unable to block the spread of silencing while only $tRNA^{THR}$-GR1 and the *HMR*-$tRNA^{THR}$ were able to block the spread of silencing. These data also indicated that the steady state levels of Tfc1 at specific sequences did not correlate with the ability of those sequences to block the spread of silencing since the $tRNA^{THR}$-KL gene on chromosome XI, which had the same levels of Tfc1 bound (see Figure 2) as $tRNA^{THR}$-GR1 on chromosome VII and the *HMR* $tRNA^{THR}$ on chromosome III, was not as efficient in blocking the spread of silencing when inserted between the *HMR-E* silencer and the a1 reporter gene.

We next analyzed the ability of the *ETC* loci to function as insulators (Figure 3A). *ETC2* and *ETC9* were both able to block the spread of silencing while *PPM2-ETC* was not able to block the spread of silencing. Both *ETC2* and *ETC9* contained lower levels of Tfc1 than $tRNA^{THR}$-KL, $tRNA^{THR}$- NL1 or *SUP53*, indicating that the absolute levels of TFIIIC binding at their native sites in the genome are not a good indicator of whether an element will function as an insulator. These results collectively also suggest that Tfc1-bound DNA elements might be necessary for insulation but were not sufficient for insulation since $tRNA^{THR}$-KL and $tRNA^{THR}$ -NL1, and *SUP53* bound Tfc1 but were not able to block the spread of silencing.

The *HMR* $tRNA^{THR}$ gene that functions as an insulator does so in either orientation (Donze and Kamakaka 2001). We therefore decided to test whether the *ETC* loci could also function in both orientations or whether they were orientation dependent (Figure 3B). Using the same mating assay (coupled with serial dilutions) we found that *ETC9* functioned as a barrier in both orientations but interestingly *ETC2* only functioned in one orientation while *PPM2-ETC* was still not able to function as an insulator in either orientation. The reason for the orientation dependence of *ETC2* is currently not known.

***ETC* multimerization improves barrier function:** The *ETC* fragments tested were not fully functional as insulators. We therefore asked if multimerization of the *ETC9* fragments would improve their ability to function as insulators. We generated constructs with one, two, or three tandem copies of *ETC9* loci inserted between the silencer and the reporter gene. Mating assays indicated that three copies of *ETC9* inserted between the silencer and the reporter gene resulted in slightly better insulation (Figure 3C). We also multimerized *PPM2-ETC*, which was unable to block silencing when present as a single copy. Inserting two or three copies between the silencer and the reporter also did not result in barrier activity, further demonstrating that *ETC-PPM2* was not a bona fide *ETC* locus.

Since *ETC-PPM2* was unable to block silencing and since *ETC2* functioned only in one orientation, we decided to generate a construct with a tandem array of consensus TFIIIC binding sites. In *S. pombe*, these sites recruit TFIIIC but not RNA pol III and the three TFIIIC binding sites (B boxes) present in a tandem array have been shown to function as insulators and restrict the spread of Swi6p-repressed heterochromatin in this organism (Noma *et al.* 2006). We therefore decided to ask whether TFIIIC binding alone could block the spread of Sir proteins in *S. cerevisiae*. We inserted three consensus B-box binding sites in a tandem array between the silencer and the reporter gene and moni-

Figure 3.—*ETC* loci function as barriers to Sir-mediated silencing. (A) Various sequences were cloned between the *HMR-E* silencer and the *MAT***a***1* gene in pRO363, and analyzed for their ability to block silencing in strain ROY113 (MATα *hmr*Δ). Sequences that block silencing, result in transcription of the *MAT***a***1* gene and an inability of the strain to mate and form diploids on selective plates. Presence of diploid colonies on selective plates indicates an absence of barrier function. Plasmids used were pRO363 (no barrier), pRO466 (*HMR*-$tRNA^{THR}$), pRO471 ($tRNA^{THR}$-GR1), pRO470 ($tRNA^{THR}$-KL), pRO469 ($tRNA^{THR}$-NL1), pRO465 (tRNA-SUP53), pRO461 (snRNA-U6), pRO850 (*ETC9*), pRO853 (*ETC2*), and pRO861 (*ETC-PPM2*). (B) DNA fragments were cloned in both orientations between the *HMR-E* silencer and the *MAT***a***1* gene at *HMR* in pRO363 and tested for their ability to block silencing as described in Figure 3A. For the mating-assay, 10-fold serial dilutions were spotted on selective plates top spread with a mating lawn. Plasmids used were pRO363 (no barrier), pRO466 and pDD441 (*HMR*-$tRNA^{THR}$), pRO470 and pRO452 ($tRNA^{THR}$-KL), pRO847 and pRO848 (*ETC9*), pRO852 and pRO854 (*ETC2*), and pRO859 (*ETC-PPM2*). (C) Varying numbers of *ETC9* loci, *ETC-PPM2*, and B-box sequences were inserted between the *HMR-E* silencer and the *MAT***a***1* gene at *HMR* in pRO363 and analyzed for their ability to block silencing. Barrier activity was tested as in Figure 3A. Plasmids used were pRO363 (no barrier), pRO466 (*HMR*-$tRNA^{THR}$), pRO848 (1x*ETC9*), pRO850 (2x*ETC9*), pRO851 (3x*ETC9*), pRO859 (1x*PPM2-ETC*), pRO861 (2x*PPM2-ETC*), pRO862 (3x*PPM2-ETC*), pRO856 (3x B boxes), pRO857 (9x B boxes), and pRO858 (12x B boxes).

tored expression of the reporter gene (Figure 3C). Our mating data showed that unlike *S. pombe*, these three TFIIIC B-box sites were not sufficient to significantly block the spread of silencing. We then asked whether increasing the number of TFIIIC binding sites would lead to insulation. We inserted either 9 or 12 B-box sites and monitored insulation. Upon inserting either 9 or 12 sites, we now observed insulation, suggesting that increased TFIIIC binding was able to restrict the spread of silencing.

TFIIIC function was necessary for *ETC9* and B-box mediated insulation: Our ChIP data had indicated that Tfc1 bound both *ETC9* and *ETC2* at their native euchromatic loci on chromosomes VII and XV, respectively (Figure 2). Furthermore the tandem array of B boxes would be expected to bind TFIIIC as well. If these elements functioned in insulation via the RNA pol III transcription factors then conditional mutations that weaken the function of these factors should reduce the ability of the tRNA/*ETC* loci to act as insulators. Temperature-sensitive mutations in Tfc3 have been shown to affect *HMR* tRNA^{THR}-mediated insulation (Donze and Kamakaka 2001). We tested whether this mutation also led to a reduction in *ETC*-mediated insulation (Figure 4). At the semi-permissive temperature (30°), *ETC9*-mediated insulation was reduced in a *tfc3* mutant but surprisingly we did not observe any reduction in insulation mediated by *ETC2*, suggesting that *ETC2*-mediated insulation was probably due to other factors and not TFIIIC or a combination of both. We also investigated the nine B-box synthetic insulators and found that mutations in Tfc3 resulted in near complete loss of insulation from the nine B-box insulators. Due to these results we focused our further analyses on *ETC9* and the tandem B-box arrays.

Rpc40 is absent from *ETC* loci at *HMR*: Since *ETC9* was able to function as an insulator at *HMR*, we investigated whether RNA pol III was necessary for insulation. *ETC9* at its native site on chromosome VII does not bind RNA pol III and is not transcribed (Roberts *et al.* 2003; Guffanti *et al.* 2006). We inquired whether the transposed *ETC9* locus, adjacent to *HMR* on chromosome III, recruited RNA pol III. To allow a direct comparison between *ETC9* and the *HMR*-tRNA^{THR} in a sequence neutral environment, two strains were constructed in which a 1-kb fragment encompassing the *HMR*-tRNA^{THR} and the two TY LTRs were replaced with a fragment of the same length from a pRS vector. We then inserted either 2x*ETC9* or the *HMR*-tRNA^{THR} into the pRS sequence at approximately the same distance from *HMR-I* as the native *HMR*-tRNA^{THR}. We compared binding of the RNA pol III subunit Rpc40 at these loci and found nearly background levels of RNA pol III at the transposed *ETC9* locus relative to the *HMR*-tRNA insulator, suggesting that *ETC9* was most likely not transcribed even though it could function as an insulator (Figure 5A). As a control we also compared the binding of the RNA pol III transcription factor Bdp1 in these two strains (Figure 5B). While Bdp1 levels were approximately twofold reduced at *ETC9* compared to the *HMR*-tRNA^{THR} (Figure 5B) the levels were significantly above background. These results collectively suggest that RNA pol III-dependent transcription is unlikely to be required for insulation.

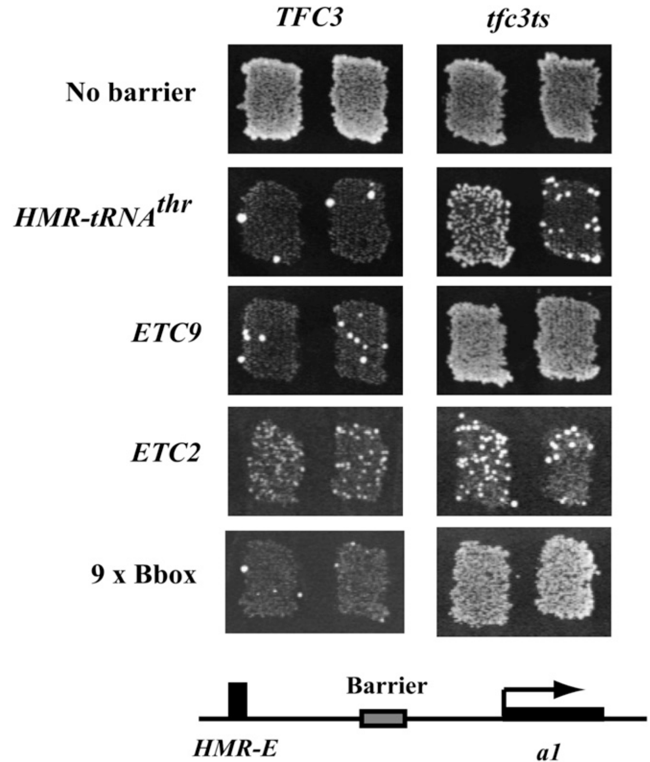


Figure 4.—Analysis of barrier activity in strains carrying conditional mutations in TFIIIC wild-type (ROY113) and *tfc3G349E^{ts}* (ROY1862) strains were transformed with vectors that contain the following sequences inserted between the silencer and the reporter gene in pRO363: no barrier (pRO363), *HMR*-tRNA^{THR} (pRO495), *ETC9* (pRO850), *ETC2* (pRO853) or 9x B box (pRO857). Barrier activity was tested as in Figure 3, at the semi-permissive temperature (30°).

We next investigated binding of the RNA pol III factors at the three B boxes and *ETC2* when these loci were integrated in place of the tRNA insulator at *HMR* on chromosome III. In *sir2*Δ strains there were significant levels of binding of Tfc1 at both *ETC2* and the 3xB-box elements but very negligible binding of Bdp1 (Figure 6, A and B). However in strains containing Sir2 and silencing, binding of Tfc1 was dramatically reduced at both elements. These results suggest that the presence of the Sir proteins blocks recruitment of TFIIIC to these elements and these data help explain why these elements were not able to function as insulators in a TFIIIC-dependent manner (see Figure 4). Furthermore the results obtained with the three B boxes (Figure 3C and Figure 6A) suggested that insulator function was most likely a result of direct competition between Sir protein spreading and TFIIIC binding at the insulator, thus suggesting that a key step in the formation of an insulator is the stable binding of TFIIIC.

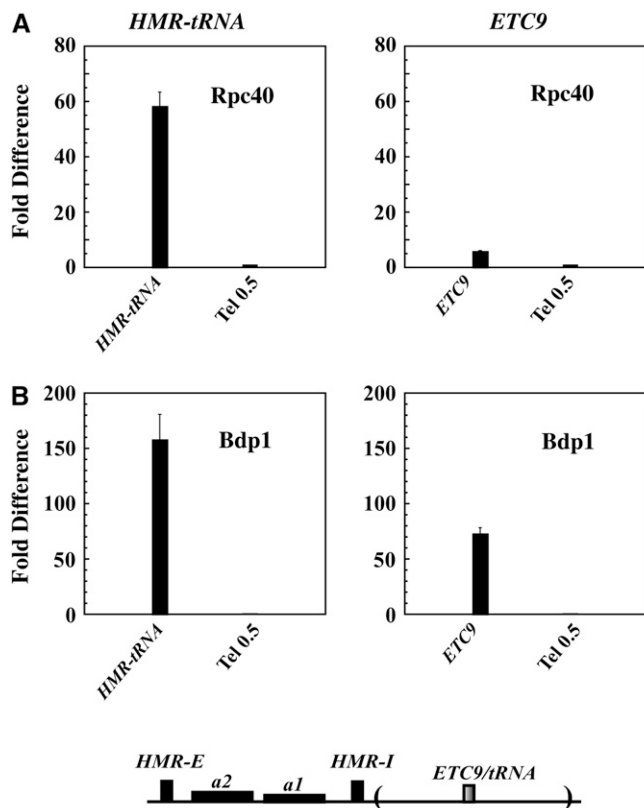


FIGURE 5.—*ETC* loci do not recruit significant levels of RNA Pol III. (A) Relative amounts of Rpc40p recruited to the tRNA barrier and *ETC9* locus at *HMR* were quantified by ChIP and qPCR, in strain ROY4549 and ROY4550, as described in Figure 1. The PCR products amplified by qPCR are shown in the schematic diagram. In strains ROY4549 and ROY4550, the 1.2-kb wild-type barrier element located to the right of the *HMR-I* silencer was deleted and replaced with 1.2-kb pRS DNA. A fragment containing the *HMR*-tRNA^{THR} barrier element (337 bp), or a fragment containing two *ETC9* loci (350 bp), was integrated within the pRS sequence. (B) Relative amounts of Bdp1p recruited to the tRNA barrier and *ETC9* locus at *HMR* were quantified in strains ROY4549 and ROY4550 by ChIP and qPCR, as described in Figure 1. The location of the PCR product amplified by qPCR is shown in the schematic diagram.

TFIIIC-mediated insulation can be overcome by increased Sir proteins: Since *ETC9* appeared to insulate genes in the absence of transcription but required TFIIIC, we hypothesized that insulation was a consequence of competition between TFIIIC binding and Sir protein spreading. This would predict that overexpressing Sir proteins should overcome the insulator.

To test this hypothesis we generated three reporter strains. In each strain the *HMR-E* silencer repressed the *MAT***a***1* gene at *HMR* on chromosome III. One strain had the *HMR* tRNA^{THR} insulator placed between the silencer and the reporter gene, the second strain had 3x*ETC9* loci inserted between the silencer and the reporter while the third strain had no insulator. In the absence of the insulator, the reporter gene was silenced, but in the presence of either the tRNA or *ETC9* loci, silencing was blocked and the gene was active (Figure 6C). We then assayed expression of the reporter in these strains following overexpression of Sir3. Sir3 overexpression significantly overcame both the tRNA and *ETC9* insulators, resulting in repression of the reporter. These results strongly support our model that insulation is a consequence of a competition between TFIIIC binding and Sir protein spreading.

Specific acetylases and remodelers are required for *ETC*-mediated insulation: At the native *HMR* tRNA^{THR} barrier, the silenced domain is restricted from spreading by the tRNA gene in conjunction with the action of specific histone acetylases and chromatin remodelers. Sas2, Eaf3, Gcn5, Isw2, and Rsc (Donze and Kamakaka 2001; Jambunathan *et al.* 2005; Oki and Kamakaka 2005) are required for efficient restriction of the silenced domain while Sas3, Hat1, and Swr1 are not. We wished to know whether the same acetylases and remodelers were utilized by the *ETC* loci to restrict the spread of the silenced domain or whether these loci utilized different enzyme complexes. We used various mutant strains lacking specific histone acetylases and chromatin remodelers and asked whether in the absence of these enzymes insulation mediated by the *ETC* locus was affected (Figure 7). We tested mutations in Hat1, Sas2, and Eaf3 as representatives of histone acetylase subunits and we tested mutations in Isw2 and Rsc2 as representatives of chromatin remodelers. Most of the mutants affected insulation mediated by *ETC9* and the 9x B-box insulators to some degree. Loss of Sas2, Eaf3, Isw2, or Rsc2 led to reduction in insulation while mutations in Hat1 and Sas3 (data not shown) had no effect. Isw2 and Eaf3 mutants had a weak effect on the tRNA-mediated insulation but had a larger effect on *ETC9* and 9x B-box-mediated insulation. This may reflect the fact that the *ETC* insulators are weaker than the tRNA and loss of these enzymes weakens these insulators further.

RSC is required for transcription factor loading at the tRNA: While histone acetylases such as Sas2 and Eaf3 are not specifically recruited to tRNA genes and function in insulation independently of the tRNA (Oki and Kamakaka 2005), the Rsc chromatin remodeler localizes to tRNA genes (Damelin *et al.* 2002; Ng *et al.* 2002) where it evicts histones (Parnell *et al.* 2008) and mutants in RSC affect tRNA-mediated insulation (Jambunathan *et al.* 2005). We therefore decided to investigate the role of RSC in insulation.

We initially determined the phenotype of RSC mutants on the native *HMR*-tRNA boundary. We generated two isogenic MATα strains that differed at the *HMR* boundary. In both strains, the native promoter of the *MAT***a***1* gene at *HMR* was deleted (*HMRa* Δ *p*) and a functionally active *MAT***a***1* gene under its own promoter was inserted in the intergenic region between the insulator and the *GIT1* gene. In the wild-type strain the tRNA insulator was intact while in the barrier-deleted strain, the tRNA gene was deleted. The *MAT*α strains

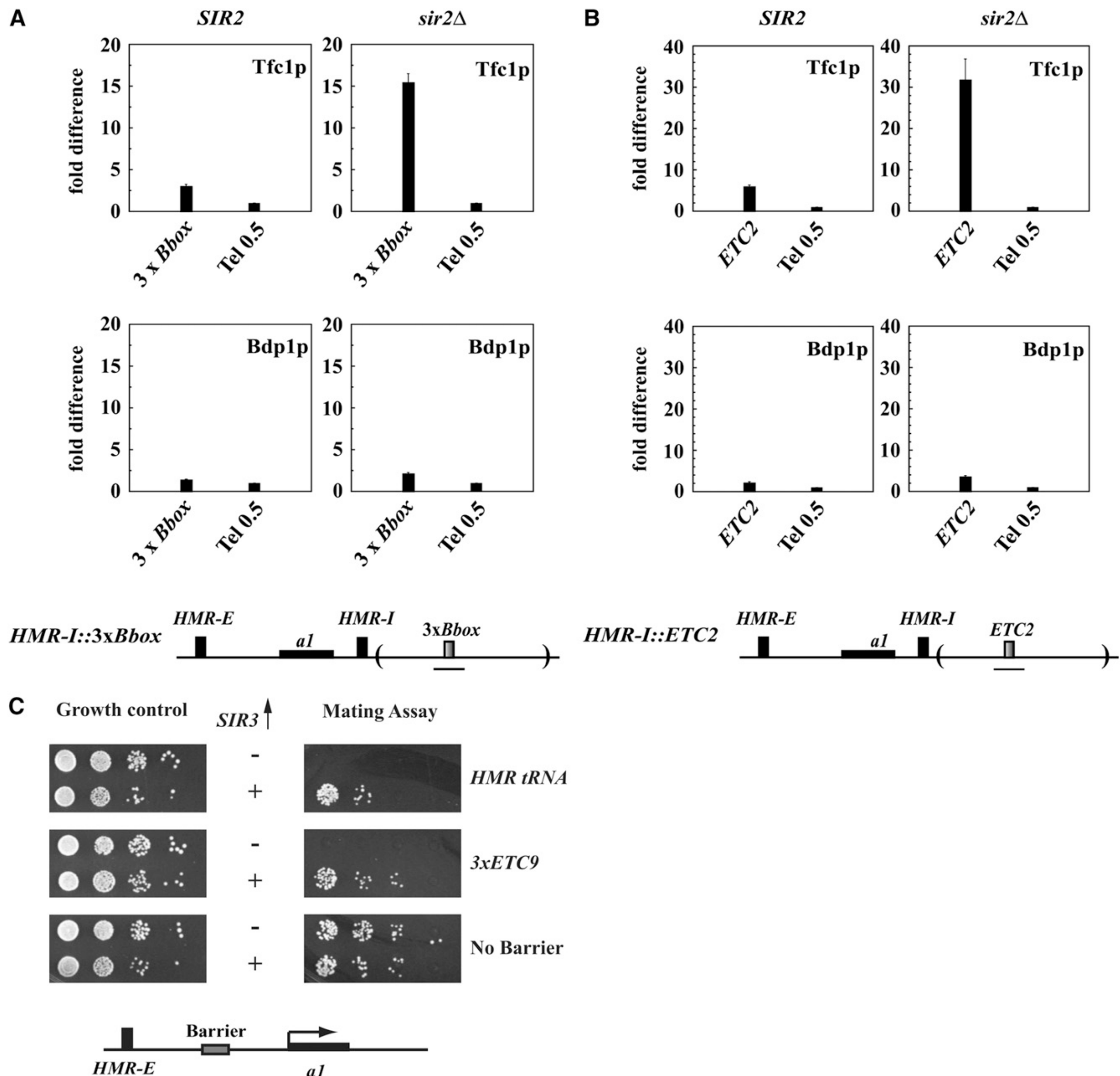


FIGURE 6.—Tfc1p but not Bdp1p are recruited to *ETC2* and 3x B boxes. (A) The relative amounts of Tfc1p recruited adjacent to the *HMR* locus by 3x B-box sequences in the presence and absence of the Sir proteins were measured in strains ROY4551 (*HMR-3xBbox TFC1-HA SIR2*) and ROY4552 (*HMR-3xBbox TFC1-HA sir2*Δ). ChIP and qPCR data were analyzed as described in Figure 1. The PCR products amplified by qPCR are shown in the schematic figure. The relative amounts of Bdp1 recruited adjacent to the *HMR* locus by 3x B-box sequences in the presence and absence of the Sir proteins were measured in strains ROY4553 (*HMR-3xBbox BDP1-HA SIR2*) and ROY4554 (*HMR-3xBbox BDP1-HA sir2*Δ). ChIP and qPCR data were analyzed as described in Figure 1. The PCR products amplified by qPCR are shown in the schematic figure. In these strains, the 1.2-kb wild-type barrier element located to the right of the *HMR-I* silencer was deleted and replaced with 1.2 kb pRS DNA. A 33-bp fragment containing the 3x B-box element was integrated within the pRS sequence. (B) The relative amounts of Tfc1p recruited to the *HMR* locus by *ETC2* sequences in the presence and absence of silencing were measured in strains ROY4555 (*HMR-ETC2 TFC1-HA SIR2*) and ROY4556 (*HMR-ETC2 TFC1-HA sir2*Δ). ChIP and qPCR data were analyzed as described in Figure 1. The oligos used for qPCR analysis flank the *ETC2* element, as shown in the schematic diagram. The relative amounts of Bdp1p recruited to the *HMR* locus by *ETC2* sequences in the presence and absence of silencing were measured in strains ROY4557 (*HMR-ETC2 BDP1-HA SIR2*) and ROY4558 (*HMR-ETC2 BDP1-HA sir2*Δ). ChIP and qPCR data were analyzed as described in Figure 1. The oligos used for qPCR analysis flank the *ETC2* element, as shown in the schematic diagram. In these strains, the 1.2-kb wild-type barrier element located to the right of the *HMR-I* silencer was deleted and replaced with 1.2 kb pRS DNA. A 200-bp fragment containing the *ETC2* barrier element was integrated within the pRS sequence. (C) Competition between Sir proteins and barrier elements. Different barrier elements integrated at the *HMR* locus were tested for their ability to block silencing generated by endogenous amounts of Sir proteins (strains transformed with pRO425) or under conditions of Sir3 overexpression (strains transformed with pRO329-*SIR3*). In strains ROY4546 (*HMR::tRNA*) and ROY4545 (*HMR::3xETC9*) the barrier element was integrated between the *HMR-E* silencer and the *MAT***a***1* gene, and strain ROY951 (*HMR*-no barrier) was used as a control. Barrier activity was tested in mating assays as described in Figure 3.

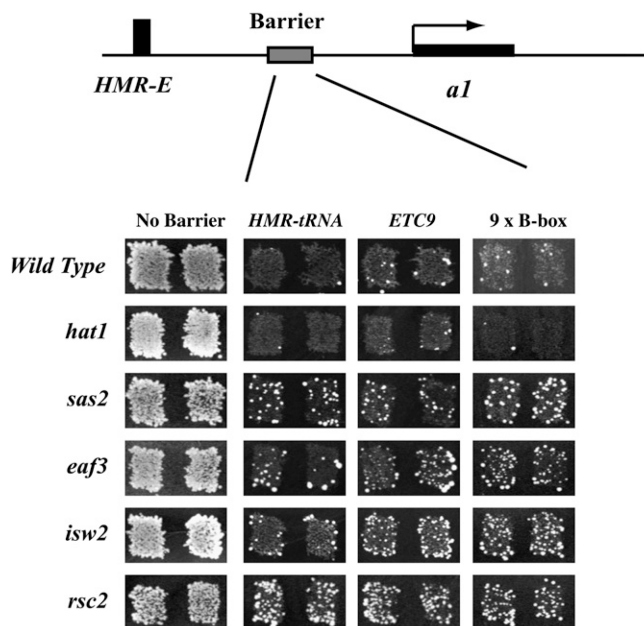


Figure 7.—B box-mediated barrier activity depends on specific histone-modifying and chromatin-remodeling proteins. Barrier function of *ETC9* and B box-containing elements was assayed in wild-type and mutant strains with impaired histone -modifying or chromatin-remodeling activities. Strains ROY113 (MATα *hmr*Δ), ROY1205 (MATα *hmr*Δ *hat1*Δ), ROY1528 (MATα *hmr*Δ *sas2*Δ), ROY4185 (MATα *hmr*Δ *eaf3*Δ), ROY3512 (MATα *hmr*Δ *isw2*Δ) and ROY4186 (MATα *hmr*Δ *rsc2*Δ) were transformed with plasmids pRO363 (no barrier), pRO466 (*HMR*-tRNA^THR^), pRO850 (*ETC9*), pRO857 (9x B box), and transformants were tested for barrier activity as in Figure 3.

were monitored for expression of the *MAT***a***1* reporter gene by mating assays. When the reporter is repressed, the strains should mate with a tester strain, form diploids, and grow on selective plates but when the reporter is active the strain should be unable to form diploids and should not grow on selective plates. Our analyses indicated that as predicted, in the wild-type strain the *MAT***a***1* gene residing outside the *HMR* boundary was fully active, while in the tRNA delete strain there was no apparent insulation and the reporter gene was almost fully repressed (Figure 8A). In contrast, in a *rsc2*Δ strain we observed significant loss of tRNA-mediated insulation as manifested by repression of the reporter gene thus confirming the previous results (shown in Figure 7).

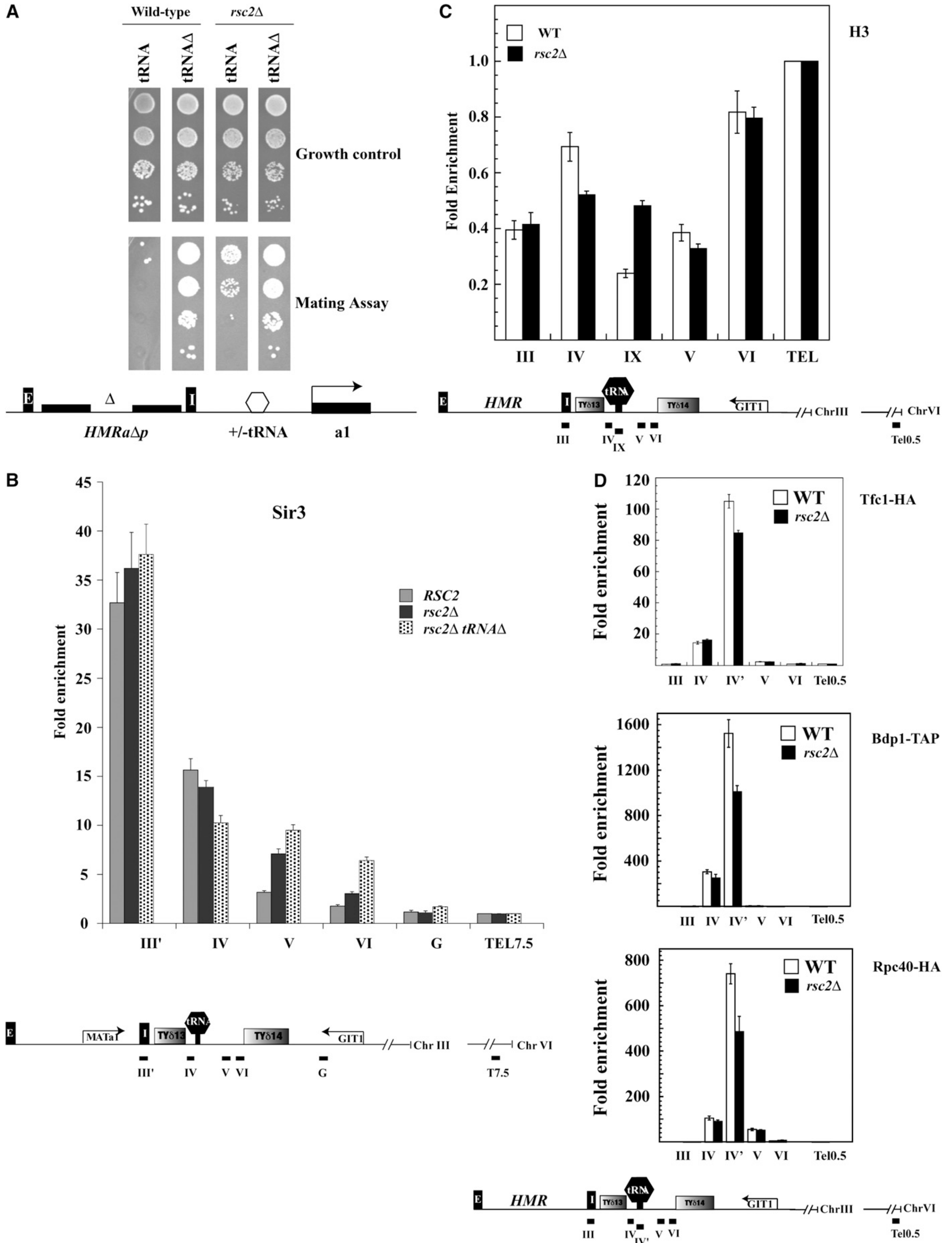
Mutations in RSC phenotypically weaken barrier activity mediated by the tRNA at *HMR*. We therefore asked whether the distribution of Sir3p was altered in cells deficient for these activities. We mapped the distribution of Sir3p in a wild-type strain and a strain lacking Rsc2 (Figure 8B). Quantitative ChIP experiments indicated that the lack of Rsc2 led to a reproducible twofold increase in the levels of Sir3p outside of the HMR tRNA^THR^ barrier. These results suggested that RSC directly or indirectly functioned to regulate Sir protein spreading at *HMR*. These results were also consistent with the observation that loss of RSC led to partial loss of tRNA-mediated insulation (see Figure 8A).

The *HMR* tRNA^THR^ gene has been shown to reside in a histone-depleted region (Oki and Kamakaka 2005) and RSC is involved in evicting nucleosomes from chromatin (Saha *et al.* 2006). It was therefore possible that RSC was required to evict nucleosomes at the *HMR* tRNA insulator and loss of RSC would then be predicted to result in the reformation of a nucleosome at the tRNA insulator. We quantitatively mapped the distribution of histone H3 in a wild-type strain (ROY4562) and in a strain lacking Rsc2 (ROY4563) (Figure 8C). Consistent with previous data (Oki and Kamakaka 2005), histones were depleted at the *HMR* tRNA in the wild-type strain. In a *rsc2*Δ strain, histone H3 levels increased at the tRNA insulator, suggesting that RSC played a direct or indirect role in nucleosome eviction at the tRNA. *HMR-I* is normally nucleosome free due to binding of silencer proteins and in a Rsc2 mutant the levels of H3 at *HMR-I* were unchanged, demonstrating that this RSC-dependent H3 eviction effect was restricted to the tRNA insulator.

We next mapped the distribution of RNA pol III transcription factors Tfc1, Bdp1, and Rpc40 in wild-type strains (ROY4562 and ROY 4548) and in strains lacking Rsc2 (ROY4563 and ROY4564). Our data demonstrated that occupancy of all three factors was reduced in a Rsc2 mutant compared to the wild-type strain. While the reduction was small, we observed the same effects with all three RNA pol III transcription complexes and these effects were observed in different strains, suggesting that these effects are significant. The fact that we did not observe large changes in Tfc1, Bdp1, or H3 occupancy at the tRNA in a *rsc2*Δ strain was consistent with our phenotypic data, demonstrating that loss of Rsc2 resulted in only a partial loss of insulator activity.

RSC was also required to evict nucleosomes at *ETC9-HMR*: To investigate the mechanism of RSC-mediated insulation at the *ETC* loci we first mapped histone occupancy at the *ETC9* locus on chromosome VII in the presence and absence of Rsc2. We used probes at the promoters of *TIM21* and *RPL26B* genes that flank *ETC9* as well as a probe at the *ETC9* locus itself. Using qPCR we found that in wild-type strains histone H3 occupancy was reduced at all three probes on chromosome VII compared to control probes near the telomeres of chromosome VIR (Figure 9A). In the absence of Rsc2, we did not observe any significant change in histone occupancy at these sites, indicating that histone eviction/sliding at this site was either not dependent on the RSC complex or was redundant with other chromatin remodelers/modifiers.

We then mapped histone occupancy at the *ETC9* locus when this locus was transposed and inserted adjacent to *HMR* on chromosome III. If the mechanism of insulation were conserved between the tRNA and *ETC9* then we would expect nucleosome loss at the


A
Wild-type
rsc2Δ
tRNA
tRNAΔ
tRNA
tRNAΔ
Growth control
Mating Assay
E
Δ
I
HMRaΔp
+/-tRNA
a1
B
Sir3
RSC2
rsc2Δ
rsc2Δ tRNAΔ
Fold enrichment
III'
IV
V
VI
G
TEL7.5
E
MATa1
I
TYδ13
tRNA
TYδ14
GIT1
Chr III
Chr VI
T7.5
C
WT
rsc2Δ
H3
Fold Enrichment
III
IV
IX
V
VI
TEL
HMR
ChrIII
ChrVI
Tel0.5
D
Tfc1-HA
Bdp1-TAP
Rpc40-HA
IV'

HMR-ETC9 insulator at this site. We mapped histone H3 distribution with several probes at the *HMR-I* silencer as well as probes adjacent to *HMR-ETC9* in the presence and absence of Rsc2 (Figure 9B). Histone H3 was depleted at the *HMR-I* silencer as was expected due to the binding of ORC and Abf1 to the silencer. Furthermore there was reduced H3 binding at the *HMR-ETC9* insulator. However the H3 levels at *HMR-ETC9* were not identical to that observed for *ETC9* at its native site on chromosome VII. Interestingly upon loss of Rsc2, histone levels increased almost twofold at the *HMR-ETC9* locus but not at the control telomeric loci, suggesting that RSC was directly or indirectly involved in evicting nucleosomes from the insulator. *HMR-I* is normally nucleosome free due to binding of silencer proteins and in a Rsc2 mutant the levels of H3 at *HMR-I* were unchanged, demonstrating that this RSC dependent H3 eviction effect was restricted to the TFIIIC-dependent *ETC9* insulator.

DISCUSSION

Heterochromatin domains are formed by the recruitment of silencing complexes to silencers followed by the propagation of these complexes along the nucleosomal filament (Rusche *et al.* 2003). The extent of heterochromatin spreading is dependent upon the amount of the silencing complexes but DNA elements can actively block the spread of silencing complexes by creating local regions of chromatin that are refractory to the binding and spreading of repressor proteins (Oki and Kamakaka 2005). These DNA elements are often referred to as barriers. At the native *HMR* domain a tRNA gene promoter functions to block the spread of silencing (Donze *et al.* 1999; Donze and Kamakaka 2001). tRNA genes are also barriers to heterochromatin in *S. pombe* even though heterochromatin in *S. pombe* is different from silenced chromatin in *S. cerevisiae* (Noma *et al.* 2006; Scott *et al.* 2006). Given that this property of tRNA genes is conserved we set out to identify a minimal set of factors that are required for tRNA gene-mediated barrier function in *S. cerevisiae.*

TFIIIC is necessary for insulation: Our study showed that 9x B boxes alone were able to function as an insulator and TFIIIC was able to block the spread of silencing in the absence of TFIIIB or RNA pol III. However, this insulation was not robust and multiple binding sites for TFIIIC were required for efficient insulation. This analysis does demonstrate that TFIIIC binding to a DNA element, in the absence of TFIIIB or RNA pol III, can insulate genes from repression. A very recent report also demonstrates that a single ETC locus that only recruited TFIIIC (*ETC4*) is also able to function as an insulator (Simms *et al.* 2008). While this report did not quantitatively measure binding of the various factors it is consistent with our conclusions that TFIIIC binding to a DNA sequence can result in insulation even in the absence of TFIIIB or RNA pol III.

Our result also suggested that occupancy of the TFIIIC binding sites was dynamic and in direct competition with the spreading Sir repressors. In the presence of three B boxes, Sir proteins displaced or prevented TFIIIC from binding but nine B boxes were able to insulate presumably because the probability of binding of TFIIIC to chromatin at any particular time was greatly increased. A similar situation was previously observed with tRNA genes. A tRNA gene (tRNA^{THR}-NL1) that was not effective as an insulator in one copy, became an effective insulator when two copies were inserted (Donze and Kamakaka 2001). Furthermore, overexpressing the Sir proteins partially overcame an effective barrier (tRNA and *ETC9*) and these data collectively suggest that dynamic competition between TFIIIC binding and Sir repressor protein spreading determines the extent to which silenced domains spread.

While the ability to recruit TFIIIC is necessary for a DNA element to function as an insulator it is not sufficient. We showed that in the absence of the Sir proteins, TFIIIC could bind to three B boxes, but in the presence of the Sir proteins this binding was significantly reduced and the 3x B boxes were unable to insulate a reporter gene. Similarly we showed that some tRNA^{THR} genes efficiently recruited TFIIIC and TFIIIB at their original sites in euchromatin, but when these genes were moved to the silenced *HMR* domain, they were unable to block the spread of silencing emanating from the *HMR-E* silencer. DNA sequences upstream and downstream of various tRNA genes have been shown to affect the stability

Figure 8.—Chromatin structure analyses at the *HMR*-tRNA insulator. (A) RSC affected the *HMR* tRNA boundary. The *HMR* tRNA boundary mating assay shown with wild-type and *rsc2*Δ cells carrying either the wild-type boundary or a 70-bp tRNA deletion of the boundary. Tenfold serial dilutions of overnight cultures with a starting A_{600} of 1.0 were spotted on fully supplemented minimal medium (growth control) or minimal medium with the mating tester lawn (mating assay). Strains tested were GRY104 (HMRaΔp-a1 RSC2), GRY108 (HMRaΔp -tRNAΔ-a1 RSC2), BUY1240 (HMRaΔp -a1 rsc2Δ), and BUY1243 (HMRaΔp -tRNAΔ-a1 rsc2Δ). (B) Mapping the distribution of Sir3p in wild-type and Rsc2 mutants. The distribution of Sir3p was determined in a wild-type (ROY1685) strain, a tRNAΔ strain (ROY1681), a *rsc2*Δ strain (BUY1202), and a tRNAΔ *rsc2*Δ strain (ROY). ROY1685 data are from Dhillon *et al.* (2009) and are shown here for ease of comparison. (C) Histone H3 at the *HMR*-tRNA. The histogram represents quantitative ChIP data to study the distribution of histone H3 from a wild-type or an *rsc2*Δ strain. Strains used were ROY4562 (*Tfc1-HA RSC2*) and ROY4563 (*Tfc1-HA rsc2*Δ). (D) Binding of RNA pol III transcription factors at the silenced *HMR* domain in the presence and absence of Rsc2. Binding of TFIIIC was analyzed using HA-tagged Tfc1 in strains ROY4562 (*Tfc1-HA RSC2*) and ROY4563 (*Tfc1-HA rsc2*Δ) TFIIIB subunit Bdp1 and RNA Pol III subunit Rpc40 were mapped in strains ROY4548 (*RSC2*) and ROY4564 (*rsc2*Δ) using Bdp1-TAP and Rpc40-HA-tagged proteins. ROY4548 (BDP1-TAP RPC40-HA RSC2) ROY4564 (BDP1-TAP RPC40-HA rsc2Δ) PCR products amplified by qPCR are shown in the schematic diagrams.

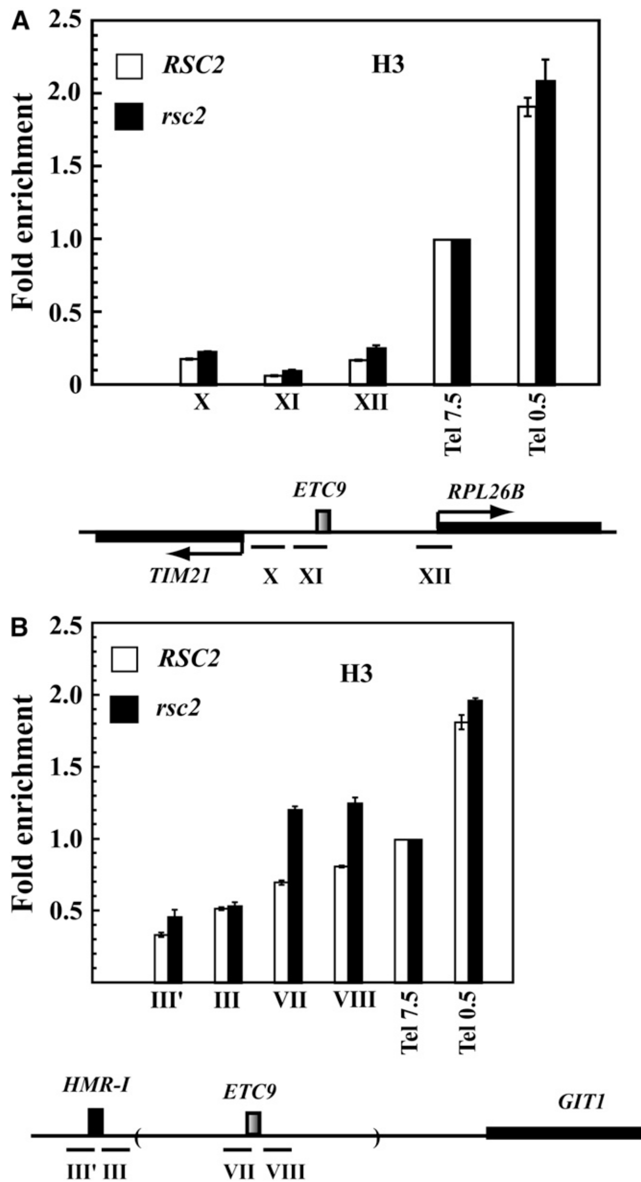


FIGURE 9.—Low nucleosome occupancy at the *ETC9* boundary depends on a functional RSC complex. Strains ROY4559 (*HMR-ETC9 RSC2*) and ROY4560 (*HMR-ETC9 rsc2*Δ) were used to analyze levels of histone H3 at the *ETC9* locus on chromosome VII (A) and at *ETC9* when this locus was integrated adjacent to the silenced *HMR* locus (B). In these strains, the 1.2-kb wild-type barrier element located to the right of the *HMR-I* silencer was deleted and replaced with 1.2 kb pRS DNA. A 174-bp fragment containing a *ETC9* element was integrated within the pRS sequences. ChIP and qPCR data were analyzed as described in Figure 1. PCR products amplified by qPCR are shown in the schematic diagrams. All data were normalized to the subtelomeric region (Tel 7.5), which was given the value of one.

of binding of TFIIIC and TFIIIB (SPRAGUE *et al.* 1980; DINGERMANN *et al.* 1982; RAYMOND and JOHNSON 1983; SHAW and OLSON 1984; RAYMOND *et al.* 1985; JOAZEIRO *et al.* 1996; ONG *et al.* 1997; DONZE and KAMAKAKA 2001; GIULIODORI *et al.* 2003). It is likely and probable that flanking sequences adjacent to TFIIIC binding sites and the internal promoter sequences that are recognized by TFIIIC play a role in determining which TFIIIC-bound promoter elements can function as insulators.

TFIIIC-mediated transcription is not necessary for insulation: We showed that *ETC9* binds TFIIIC and TFIIIB but not RNA pol III and still functioned as an insulator. The amount of RNA pol III present at *ETC9* when this locus was transposed to *HMR* was minimal and close to background. The *ETC9* data demonstrate that robust transcription and/or generation of a tRNA was unlikely to play a role in insulation. Consistent with this conclusion is the observation that there is no RNA pol III at the native *ETC9* locus (ROBERTS *et al.* 2003) and this *ETC9* locus is not transcribed (GUFFANTI *et al.* 2006). Our observation that 9x B boxes also function as insulators further demonstrates that transcription is not necessary for insulation since the B boxes alone do not recruit any RNA pol III (NOMA *et al.* 2006). Finally this conclusion is also consistent with our previous report (DONZE and KAMAKAKA 2001) that mutations in TFIIIC affect tRNA-mediated insulation but mutations in RNA pol III had no effect.

Results in *S. pombe* have shown that TFIIIC binding sites at the IR insulator do not recruit RNA pol III but are necessary for RNA pol II recruitment (NOMA *et al.* 2006). It has also been shown that these RNA pol II molecules generate noncoding transcripts at the *S. pombe* IR insulator. While at the IR elements TFIIIC recruits pol II, TFIIIC binding alone is sufficient for insulation. We do not observe any RNA pol II recruitment to *ETC* loci or tRNA genes in *S. cerevisiae*, suggesting that TFIIIC-mediated pol II recruitment is also not necessary for insulation in *S. cerevisiae*.

TFIIIC-mediated barrier function utilizes chromatin remodelers: In addition to the DNA bound transcription factors we also found that mutations in specific histone acetylases and chromatin remodelers led to a loss of insulation. We tested several mutants in histone acetylases and chromatin remodelers. Mutants in Sas2, Eaf3, Isw2, and Rsc2 all directly or indirectly affected insulation mediated by *ETC9* and the 9x B boxes while mutations in Hat1 or Sas3 (data not shown) had no effect. These same mutants also affected tRNA-mediated insulation, suggesting a commonality in the factors that affect the distribution of Sir proteins at *HMR*.

The tRNA insulator is present in a histone-depleted region of chromatin (OKI and KAMAKAKA 2005). Our current data suggest that RSC is directly or indirectly required to evict these histones at the tRNA insulator. In a Rsc2 mutant, histone occupancy at the tRNA was increased, resulting in reduced binding of the RNA pol III factors. Our current results also suggest that similar mechanisms operate at *ETC9*-mediated insulation at *HMR*. Additionally a nucleosome-free region can by itself block the spread of silencing (BI *et al.* 2004), suggesting that the creation of a nucleosome-free region may be an important step in insulation.

Genomewide mapping data demonstrate that histones are depleted at tRNA genes (Pokholok *et al.* 2005; Lee *et al.* 2007; Whitehouse *et al.* 2007; Mavrich *et al.* 2008; Parnell *et al.* 2008) and recent observations demonstrate that histone eviction around most tRNA genes is reduced in an RSC mutant (Parnell *et al.* 2008). Furthermore, a large number of RSC binding sites are located at RNA pol III transcribed genes (Damelin *et al.* 2002; Ng *et al.* 2002) and studies have shown that RSC interacts with subunits of the RNA polymerase machinery (Soutourina *et al.* 2006), suggesting a mechanism by which TFIIIC insulators may function. In this scenario, nucleosome eviction, dependent upon the RSC complex, would be required for TFIIIC binding and insulation.

An important point that needs to be borne in mind is that while RSC affected both insulation and nucleosome occupancy at the tRNA, our data are unable to unequivocally indicate whether RSC-mediated insulation is due solely to nucleosome eviction by RSC. It is entirely possible that part of the effect of RSC on insulation is due to alterations in the distribution and spreading of Sir proteins in the nucleus.

Our results collectively demonstrate that binding of TFIIIC is necessary for insulation but binding of TFIIIB along with TFIIIC likely improves the probability of complex formation at an insulator. Insulation critically depends on the ability of factors to bind stably to DNA in competition with the spreading Sir proteins. Furthermore, histone eviction mediated by RSC and possibly other enzymes may play a role in restricting the spread of silenced chromatin.

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Supporting Information

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Transcription Independent Insulation at TFIIIC-Dependent Insulators

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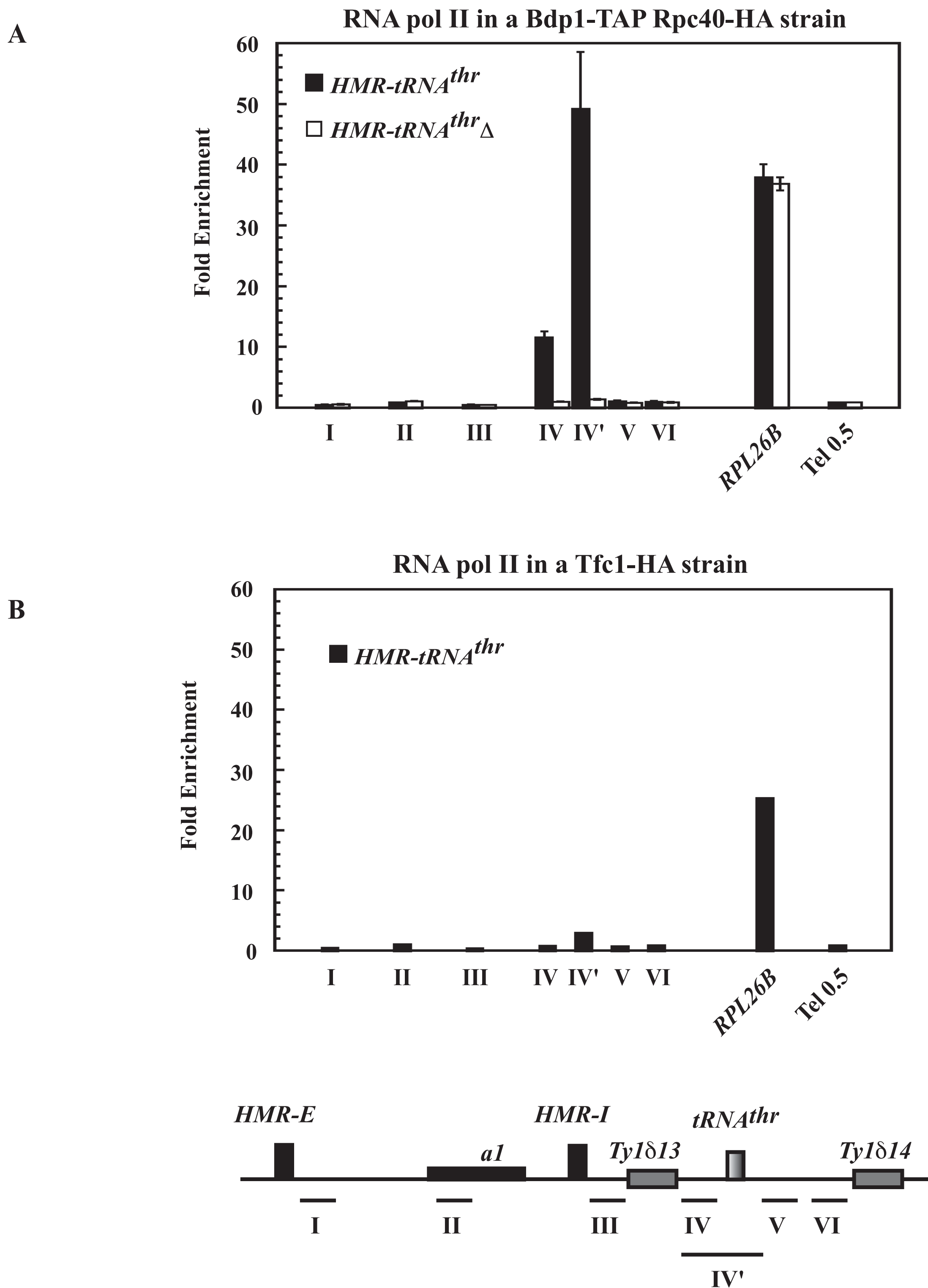

FIGURE S1.—Pol II presence at the *HMR*-tRNA boundary in strains that express different tagged proteins. (A) Binding of Pol II at the *HMR* region was analyzed in strains ROY4548 (*HMR*-WT) and ROY4561 (*HMR-tRNA*D) by ChIP and qPCR. Both strains express the tagged proteins Bdp1-TAP and Rpc40-HA. (B) Presence of Pol II at the *HMR* locus in a strain that bears the tagged protein Tfc1-HA (ROY3931) was analyzed by ChIP and qPCR. Data were expressed as fold difference with respect to the silenced telomeric region VI (Tel 0.5), as described in Figure 1. PCR products amplified by qPCR are shown in the schematic diagrams.