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TFIIIC Binding Sites Function as both Heterochromatin Barriers and Chromatin Insulators in *Saccharomyces cerevisiae*[▽]

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Chromosomal sites of RNA polymerase III (Pol III) transcription have been demonstrated to have "extra-transcriptional" functions, as the assembled Pol III complex can act as chromatin boundaries or pause sites for replication forks, can alter nucleosome positioning or affect transcription of neighboring genes, and can play a role in sister chromatid cohesion. Several studies have demonstrated that assembled Pol III complexes block the propagation of heterochromatin-mediated gene repression. Here we show that in *Saccharomyces cerevisiae* tRNA genes (tDNAs) and even partially assembled Pol III complexes containing only the transcription factor TFIIIC can exhibit chromatin boundary functions both as heterochromatin barriers and as insulators to gene activation. Both the *TRT2* tDNA and the *ETC4* site which binds only the TFIIIC complex prevented an upstream activation sequence from activating the *GAL* promoters in our assay system, effectively acting as chromatin insulators. Additionally, when placed downstream from the heterochromatic *HMR* locus, *ETC4* blocked the ectopic spread of Sir protein-mediated silencing, thus functioning as a barrier to repression. Finally, we show that *TRT2* and the *ETC6* site upstream of *TFC6* in their natural contexts display potential insulator-like functions, and *ETC6* may represent a novel case of a Pol III factor directly regulating a Pol II promoter. The results are discussed in the context of how the TFIIIC transcription factor complex may function to demarcate chromosomal domains in yeast and possibly in other eukaryotes.

Eukaryotic genomes are organized into structurally and functionally distinct domains as one layer of transcriptional regulation to allow the expression of particular sets of genes when required and to restrict their expression when necessary. Mechanisms of activation usually involve DNA-bound transcription factors that recruit RNA polymerase or general transcription factors or recruit proteins that promote the formation of chromatin structures compatible with RNA polymerase preinitiation complex formation and transcriptional elongation. Repressive chromatin domains can inhibit gene expression at either of these stages. Chromatin boundary elements function to separate chromosomal domains so that regulatory regions of one domain do not inappropriately influence adjacent domains, either by insulating promoters from activation or by acting as a barrier to propagating repressive heterochromatin (55, 58).

Evidence has accumulated over the past several years that RNA polymerase III (Pol III) promoter sequences, mainly studied using tRNA genes (tDNAs), can possess an intrinsic chromatin boundary activity. This was first demonstrated at the heterochromatic *HMR* locus in *Saccharomyces cerevisiae*, as the downstream tDNA is a critical component of the barrier that prevents the inappropriate spreading of silencing from *HMR* (16), and the characterization of this activity was the first demonstration of a natural chromatin boundary in yeast. Another yeast tDNA, *TRT2*, was shown to prevent the spread of repression from the *MAT***a** cell-specific *STE6* gene in *MAT*α cells (51).

In eukaryotes, Pol III is devoted to the transcription of small RNAs participating in basic cellular functions such as protein synthesis (tRNAs, 5S rRNA), pre-mRNA processing (U6 snRNA), and protein secretion (7SL RNA) (19, 46) and has recently been shown to effect micro-RNA expression (5). Additionally, a considerable fraction of the megacopy *Alu* repetitive elements in primates and B1 elements in mice can contain active Pol III promoters (12, 26, 54). The transcription initiation of 5S rRNAs and tDNAs is dependent on internal control regions (ICRs), which are transcription factor binding sites that lie within the transcribed DNA sequence. Within tDNAs, the ICR is formed by two nonadjacent conserved elements, *boxA* and *boxB*. The *boxB* consensus is conserved in all eukaryotes (GGTTCGANTCC; the underlined C residue is invariant and essential for efficient Pol III complex assembly and transcriptional activity). These ICR elements together form the specific binding site for the multisubunit transcription factor TFIIIC that upon binding to DNA directs the assembly of another multiprotein transcription factor, TFIIIB, to a less conserved region immediately upstream of the transcription start site, which is then followed by the recruitment of Pol III (19, 29). Mutation of the invariant cytosine residue in *boxB* inactivates both TFIIIC binding to (3) and Pol III transcription of (40) tDNAs.

Pol III-transcribed RNAs are generally very abundant. For example, tRNAs represent approximately 15% of the total RNA of exponentially growing *S. cerevisiae* cells (57), implying that there are on the order of 3×10^6 tRNA molecules per yeast cell. Thus, each of the 274 nucleus-encoded (24) and additional mitochondrially encoded tDNAs of this organism

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must be (on average) transcribed on the order of 10^4 times per generation (or approaching about twice per second, given a 90-min generation time), a value that is considerably higher than the maximal transcription initiation frequency of one initiation every 6 to 8 s estimated for RNA Pol II-transcribed genes in yeast (31). Based on these estimates, one could argue that tDNA and other Pol III promoters are constantly occupied by active transcription complexes.

In addition to active Pol III-transcribed genes, several studies have identified genomic sites that contain partial complexes containing the Pol III transcription factor TFIIIC (and in one case also TFIIIB) but are not occupied by the polymerase itself (21, 37, 42, 47). In *S. cerevisiae*, these chromosomal locations are called *ETC* (extra TFIIIC) sites, and in *Schizosaccharomyces pombe* they are referred to as COC (chromosome-organizing clamp) sites. In *S. pombe*, particular COC sites act as heterochromatin barriers, but no distinct function was demonstrated for the *ETC* sites in *S. cerevisiae*. Interestingly, these nontranscribed TFIIIC binding regions are overrepresented in the intergenic regions of divergently transcribed genes, suggesting a possible function in genome demarcation.

Since tDNAs or simply bound TFIIIC can act as a chromatin barrier element by blocking the spread of heterochromatin, we hypothesized that such chromatin-bound complexes might also function as insulators to gene activation, which would expand the role of the Pol III complex as a chromosomal boundary or landmark element. We show here that in *S. cerevisiae* both tDNAs and *ETC* elements can block the activation of genes when juxtaposed between promoters and upstream activation sequences (UAS), in essence functioning as chromatin insulators. We further demonstrate that *ETC* sites in *S. cerevisiae* can also function as heterochromatin barriers and that TFIIIC binding in the absence of TFIIIB is sufficient for both insulator and barrier activities. Finally, we identify in yeast TFIIIC binding sites that possess insulator-like functions in their natural contexts, suggesting a broader role for the Pol III complex in regulating Pol II genes and in genome organization.

MATERIALS AND METHODS

Strains containing ectopically inserted tDNAs or *ETC* sites were made by a standard two-step replacement strategy. For modification of the *GAL1-10* locus, the *URA3* gene was amplified with primers containing homology to the *GAL1* and *GAL10* coding regions and integrated by homologous recombination to create the *gal1-10 intergenic*Δ::*URA3* strain DDY 2606. Integrants were verified by PCR analysis at both ends of the inserted *URA3*. Plasmid pDD866, containing 2.4 kb of the *GAL1-10* locus (KpnI-SpeI fragment cloned into Bluescript SK+, SGD chromosome II coordinates 277624 to 280057) was constructed, and a BamHI site was introduced at coordinate 278542 between UAS_G and *GAL10* by site-directed mutagenesis to create pDD901. Wild-type and *boxB* mutant tDNAs and *ETC* sites were cloned into this BamHI site, resulting plasmids were linearized and transformed into DDY 2606, and 5-fluoroorotic acid-resistant colonies were isolated. Mutant *boxB* sequences were created using site-directed mutagenesis to change the invariant cytosine residue to guanine (*boxB* consensus, GGT TCGANTCC [invariant C underlined]). This mutation inactivates Pol III genes by inhibiting TFIIIC binding. The resulting isolates were confirmed by both PCR and DNA sequencing to verify proper integration of the TFIIIC binding sites at *GAL1-10*. Insertion of *TRT2* between UAS_G and *GAL1* was performed in a similar manner, by inserting the BamHI site at coordinate 278710 (pDD872). The *TRT2* fragment contained sequences between chromosome XI coordinates 46730 and 46826, and the *ETC4* fragment spanned chromosome VII coordinates 1010900 to 1010990.

Strains containing the *ETC4* site adjacent to the silenced *HMR* locus were constructed in a similar fashion. DDY 811 and DDY 814 were described previously (32). Plasmid pDD662 contains a 2.5-kb *ADE2* fragment (chromosome XV coordinates 566829 to 564325) inserted downstream of *HMR* (SacI-SalI *HMR* fragment) at SGD chromosome III coordinate 295736, with *HMR*-tDNA deleted and replaced with an SphI site. Wild-type and *boxB* mutant *ETC* sites were cloned into the SphI-cut plasmid, and the resulting plasmids were linearized and integrated into an *hmr*Δ::*URA3 sir4*Δ strain (DDY 631). Ade-positive isolates were screened for a nonmating phenotype, 5-fluoroorotic acid resistance, and uracil auxotrophy to indicate the integration of the *HMR* fragment and were confirmed for proper integration by PCR analysis. Positive integrants were then transformed with a *SIR4*-expressing plasmid and crossed to DDY 511, and resulting diploids were sporulated to obtain SIR-positive isolates containing the *HMR-ADE2* constructs with *ETC4* inserted in place of *HMR*-tDNA. Strains containing modifications at the *STE6-CBT1* locus were made as described previously (51). All chromosomal coordinates were derived from the *Saccharomyces* Genome Database (www.yeastgenome.org).

Chromatin immunoprecipitation was performed as described previously (48) using Chelex-100 modification (39) to deproteinize the final DNA. Primer sequences used for PCR analysis are available on request. Anti-FLAG epitope antibody was purchased form Sigma Chemical Co. (Anti-FLAG-M2, catalog number F3165). Northern blots were performed as described by Simms et al. (51), and each was analyzed on a Molecular Dynamics PhosphorImager. Quantitation of mRNA levels of each mutant was performed three times using at least two independent isolates, and quantitative results represent all replicates performed, with representative blot images shown. Growth on galactose was on yeast nitrogen base minimal medium (catalog number Y2025; U.S. Biologicals) containing 2% galactose as the sole carbon source and supplemented to cover all other auxotrophies. Epigenetic silencing of *ADE2* was assayed as described previously (32).

RESULTS

Insertion of a tDNA into an ectopic site creates an insulator to gene activation. In order to directly test the hypothesis that tDNAs can function as insulators, we inserted wild-type and mutant versions of the *TRT2* tDNA into the *GAL1-10* locus to ask whether this tDNA could block the activation of *GAL* gene expression by the well-characterized UAS_G. We have previously characterized a barrier-like activity for this tDNA both ectopically (16) and in its native location (51). *TRT2* fragments were cloned between UAS_G and *GAL10* or *GAL1* (Fig. 1A) and then integrated into the *gal1-10 intergenic* Δ::*URA3* strain (DDY 2606) (see Materials and Methods). Resulting strains were grown in raffinose, and *GAL* transcription was induced by the addition of galactose to a final concentration of 2%. Total RNA was isolated and analyzed by Northern blot analysis using *GAL10* or *GAL1* probes, and growth on galactose plates was assessed.

When *TRT2* was inserted in either orientation between UAS_G and *GAL10*, the ability to grow on galactose as a sole carbon source was completely abolished, suggesting that *TRT2* insulated the *GAL10* promoter from the UAS (Fig. 1B, compare wedges 1 and 2 to wedges 3, 4, 11, and 12). The insertion of an inactive *trt2* gene with a *boxB* point mutation abolished this insulator effect, as indicated by normal growth on galactose (wedges 5, 6, 11, and 12), while maintaining the same UAS-*GAL10* promoter spacing as in the insulated strains. The insertion of *TRT2* on the *GAL1* side of the UAS also prevented growth on galactose, presumably by insulating *GAL1* from the UAS (wedges 7, 8, and 15), while the mutant *trt2* did not prevent growth (wedges 9, 10, and 16).

Northern blot analysis of *GAL* gene expression of these strains confirmed an insulator-like effect. When wild-type *TRT2* was inserted between *GAL1* and UAS_G, galactose-induced *GAL1* transcripts were practically undetectable, while this insertion had no effect on *GAL10* (compare lanes 1 and 2 to lanes 7 and 8 in Fig. 1C for both *GAL10* and *GAL1* probes).

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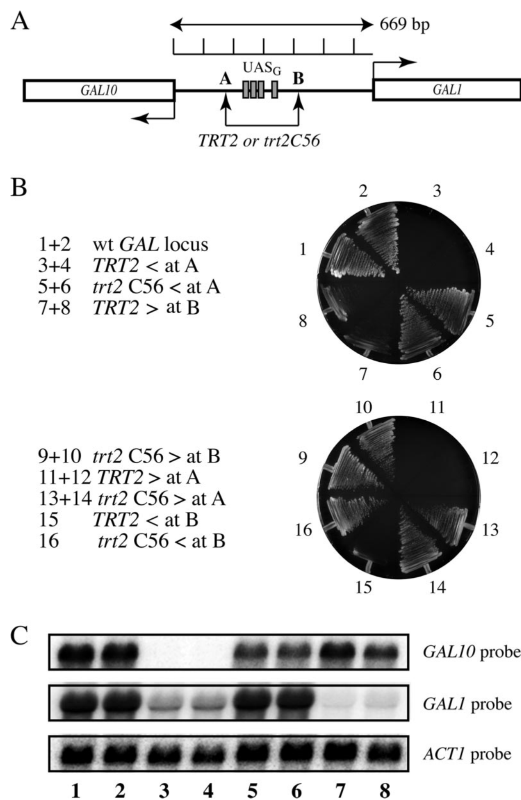

FIG. 1. Insulator activity of a tDNA inserted at the *GAL1-10* locus. Insertion of a functional tDNA between UAS$_G$ and *GAL10* (at A) or *GAL1* (at B) promoters can insulate the promoter from activation. (A) Schematic diagram of modified *GAL1-10* loci. Functional and mutant tDNA sequences were cloned into the *GAL1-10* intergenic region and integrated into chromosome II. Tick marks on the scale bar are in 100-bp increments. (B) Resulting strains were streaked onto minimal media with galactose as the sole carbon source. Arrows refer to the orientation of the *boxB* sequence relative to the *GAL* locus depicted in panel A. (C) Cells were grown in raffinose to mid-log phase and induced with galactose, and total RNA was isolated and analyzed by Northern blotting with *GAL10* or *GAL1* probes. Wedges in panel B and lanes in panel C correspond to the following strains: 1 and 2, DDY 2861 and DDY 2862 (wild-type [wt] *GAL* locus); 3 and 4, DDY 3256 and DDY 3257, *TRT2* inserted between UAS$_G$ and the *GAL10* gene at site A; 5 and 6, DDY 3266 and DDY 3267, *trt2 boxB* mutant inserted between UAS$_G$ and *GAL10* at site A; 7 and 8, DDY 3268 and DDY 3269, *TRT2* inserted between UAS$_G$ and *GAL1* (site B). Strains in wedges 9 to 16 in panel B are described in Table 1.

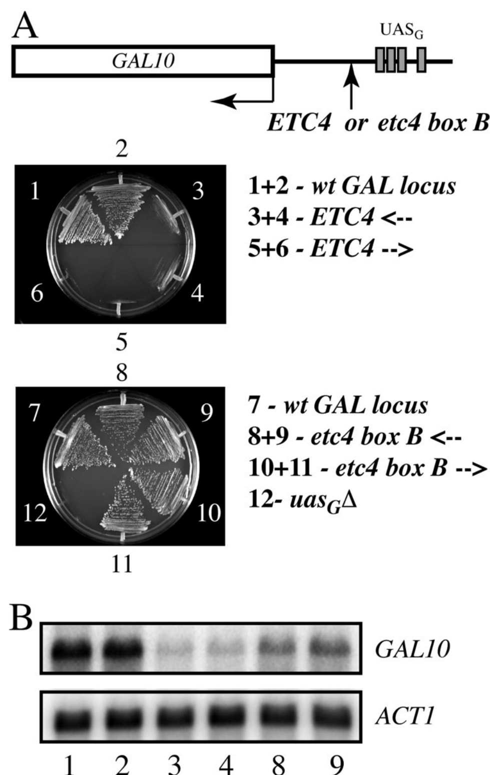

FIG. 2. A single *ETC* site can insulate the *GAL10* promoter from UAS$_G$. (A) Wild-type and mutant *ETC4* sequences were inserted between *GAL10* and UAS$_G$ as described for Fig. 1. Wedges: 1 and 2, DDY 3 and DDY 2864, wild-type (*wt*) *GAL* locus; 3 and 4, DDY 3770 and 3771, *ETC4* inserted between UAS$_G$ and *GAL10*, *boxB* oriented toward *GAL10*; 5 and 6, DDY 3995 and DDY 3773, *ETC4* orientation opposite from that for wedges 3 and 4; 7, DDY 2861, wild-type *GAL* locus; 8 and 9, DDY 3757 and DDY 3758, *etc4 boxB* mutant inserted, same orientation as for wedges 3 and 4; 10 and 11, DDY 3754 and DDY 3760, *etc4 boxB* mutant, opposite orientation; 12, DDY 2674, *uas$_g$*Δ. (B) Northern blot analysis of GAL10 expression of representative strains was performed as described for Fig. 1.

This is a defining characteristic of an insulator, in that it blocks the activation of a gene only when placed between the transcription factor binding site and the promoter. A slightly different result was seen when *TRT2* was placed between *GAL10* and the UAS, as both genes were inhibited. *GAL10* transcripts were undetectable (Fig. 1C, lanes 3 and 4), but *GAL1* was also reduced. The insertion of an inactive *trt2 boxB* mutant sequence had a minimal effect on the level of *GAL10* transcripts (Fig. 1C, lanes 5 and 6) and did not affect growth on galactose (Fig. 1B). These results demonstrate that a functional tDNA positioned between a UAS and promoter in yeast has the potential to behave as a typical eukaryotic insulator, blocking enhancer/UAS promoter communication when placed between the two elements. Since the insertion of *TRT2* on the *GAL1* side had no effect on *GAL10* expression, the assembled Pol III complex did not appear to be sterically interfering with the binding of Gal4p to the UAS in this case, or transcript levels of both genes would have been reduced.

ETC sites can function both as an insulator to gene expression and as a barrier to repression. Although *ETC* sites have been identified in several studies as sites that bind TFIIIC but not the rest of the Pol III complex (21, 37, 47), little has been done to identify potential functions for the *ETC*, or extra TFIIIC, sites. Interestingly, most of the *ETC* sites identified lie between divergently transcribed genes. We hypothesized that bound TFIIIC alone might be able to function as an insulator between regulatory elements of divergently transcribed promoters.

In order to test the hypothesis that *ETC* sites can function as insulators, we cloned a 90-bp fragment of the *ETC4* site between the *GAL10* gene and UAS$_G$ as described for Fig. 1 above. Strains were constructed containing both wild-type *ETC4* and *etc4 boxB* mutant sequences in both orientations and integrated into the *GAL* locus. Independent colony isolates were streaked onto minimal medium containing galactose as the sole carbon source. The results in Fig. 2 indicate that

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when the wild-type *ETC4* site is inserted between the UAS and the promoter, growth on galactose is impaired (Fig. 2A, wedges 3 to 6, compared to wild-type growth in wedges 1 and 2), indicating that the *GAL10* gene is again insulated from the UAS, presumably due to the binding of the TFIIIC complex to the *ETC4* sequence. However, when the *boxB* mutant *etc4* site (presumably unable to bind TFIIIC) was inserted at the same location (Fig. 2A, wedges 8 to 11), the cells retain their ability to grow on galactose. Deletion of just the UAS_G also prevents growth on galactose, as expected (Fig. 2A, wedge 12). Northern blot analysis of *GAL10* transcripts shows a slightly different result than with *TRT2*, as the wild-type *ETC4* sequence does not completely inhibit GAL10 induction (Fig. 2B). This is most likely due to the fact that *TRT2* recruits the entire Pol III complex, while *ETC* sites recruit only TFIIIC (see Fig. 4 below and Discussion).

Since tDNAs can function both as barriers to repression and as insulators to activation, we next asked whether *ETC4* could also act as a barrier to Sir protein-mediated silencing. We have previously constructed a reporter system that contains the *ADE2* gene cloned downstream from the *HMR* silent mating locus (32), which grows as pure white Ade-positive colonies on minimal medium containing limiting adenine (Fig. 3A). Deletion of *HMR*-tDNA from this region results in an epigenetic spread of silencing, yielding a mixture of white (unsilenced), red-to-pink (partially silenced), and white-red sectored colonies (Fig. 3B). Removal of the tDNA therefore partially weakens the *HMR* barrier so that silencing ectopically spreads in a variegated fashion. Replacement of the tDNA with *ETC4* in either orientation restores the white-colony phenotype (Fig. 3C and D), suggesting that bound TFIIIC alone is sufficient to stabilize the barrier. Replacement with a mutant *etc4* containing the *boxB*-inactivating mutation yields a mixed-colony-color phenotype similar to that of *tdna*Δ strains (Fig. 3E and F). The silencing of *ADE2* in the *etc4 boxB* strains is Sir dependent, as subsequent deletion of *SIR4* results in white colonies (Fig. 3G and H). We have previously confirmed that the silencing of *ADE2* in this system is due to heterochromatin spreading from *HMR*, as strains deleted for the *HMR* silencers also yield pure white colonies (32).

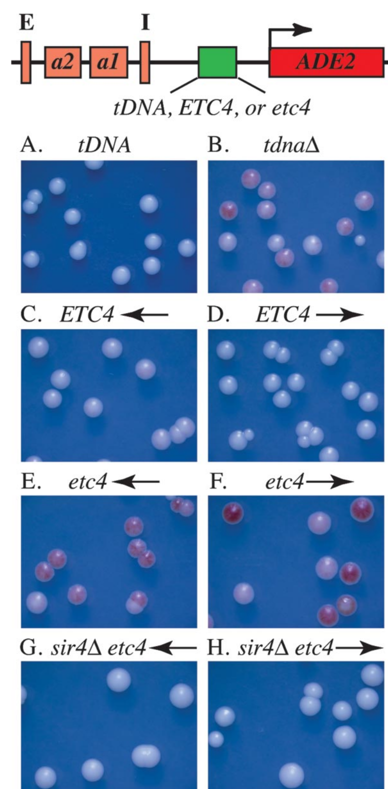

FIG. 3. The *ETC4* sequence can function as a barrier to heterochromatin spreading. Strains containing the *ADE2* gene recombined downstream of *HMR* were used to assess ectopic spreading of silencing, as indicated by the formation of pink-to-red colonies on minimal medium containing limiting adenine. (A) DDY 814; *ADE2* inserted downstream of *HMR* is protected from heterochromatin position effects by the natural tDNA barrier element, as indicated by the formation of all-white colonies. (B) DDY 811; deletion of *HMR*-tDNA weakens the barrier, resulting in variegated *ADE2* expression. (C and D) DDY 3724 and DDY 3743; replacement of the tDNA with *ETC4* in either orientation restores barrier function. (E and F) DDY 3815 and DDY 3812; replacement of the tDNA with *boxB etc4* mutant does not restore barrier function. (G and H) DDY 3817 and DDY 3811; silencing in panels E and F is Sir protein dependent.

TFIIIC binding but not TFIIIB binding is required for boundary activity of *ETC4*. To this point, we have made two assumptions regarding the *ETC4* site in both our insulator and barrier assays. The first is that when moved to an ectopic location, *ETC4* behaves as in its native location, in that it binds TFIIIC but not TFIIIB. Second, we have assumed that the mutation of the conserved cytosine in *boxB* inhibits TFIIIC binding as completely as it does in tDNAs (3). In order to confirm these assumptions and the role of TFIIIC in creating boundaries, we crossed our boundary reporter strains with strains engineered to have the FLAG epitope attached to Tfc1p, the Tau95 subunit of TFIIIC, and to FLAG-tagged Brf1p strains, marking the 70-kDa subunit of TFIIIB. The resulting strains were analyzed by chromatin immunoprecipitation using anti-FLAG antibody. Figure 4A shows the expected enrichment over background (no antibody controls) for both Tfc1p and Brf1p at the *TRT2-GAL* locus. When *TRT2* was replaced with *ETC4*, Tfc1p enrichment was observed, but Brf1p enrichment was not, confirming that the *ETC4* site binds only TFIIIC but not TFIIIB when moved to an ectopic location. Replacing with the *boxB* mutant *etc4* site, which lacks insulating activity, showed no enrichment for either tagged protein compared to the no-antibody control, confirming that the point mutation inhibits TFIIIC binding as predicted and correlating TFIIIC binding with insulator activity. A distal control tDNA showed equal enrichment in each strain. Similar results were obtained for the comparable *HMR-ADE2 ETC4* barrier strains, shown in Fig. 4B. These results demonstrate that TFIIIC binding alone, in the absence of TFIIIB, is associated with both barrier and insulator activities in these strains.

tDNAs and *ETC* sites can exhibit insulator-like properties in their natural context. We next asked whether Pol III binding sites in their natural contexts actually exhibit insulator-like properties. To address this possibility, we revisited the *STE6-CBT1* locus. Our previous work demonstrated that *TRT2*, a tDNA that lies between divergently transcribed *STE6* and *CBT1*, exerts an apparent inhibitory position effect on *CBT1* in *MAT***a** cells, where *STE6* is active (51). Complete deletion or *boxB* point mutation of *TRT2* resulted in an increase in *CBT1*

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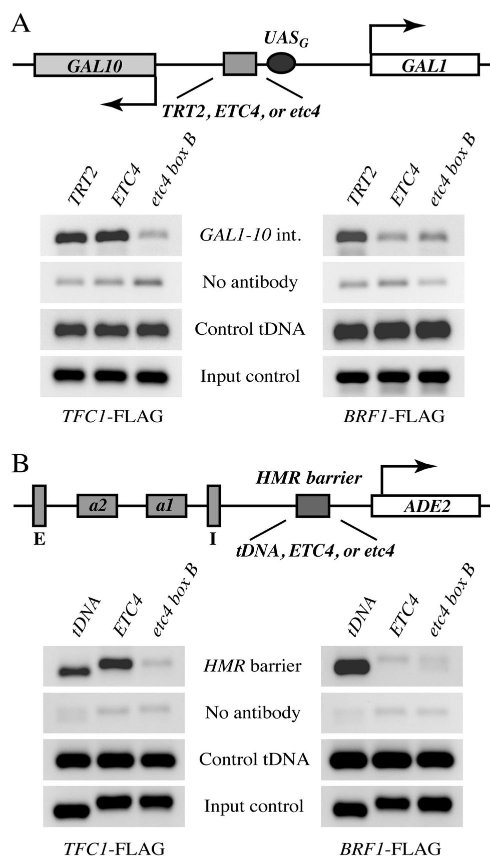

FIG. 4. Chromatin immunoprecipitation analysis of Pol III factors at tDNA and *ETC4* boundaries. Strains containing *TRT2* or *ETC4* at the *GAL* locus (A) or the native tDNA or *ETC4* at *HMR* (B) and expressing the FLAG epitope attached to either the TFIIIC subunit Tfc1p or to the TFIIIB subunit Brf1p. Only tDNA-proximal sequences were immunoprecipitated in Brf1p-FLAG strains, while both tDNA- and *ETC4*-associated regions were enriched in Tfc1p-FLAG strains. No enrichment over background (no-antibody control) was seen at *etc4 boxB* mutant loci, confirming that the mutation inhibits TFIIIC binding. The control tDNA, *tN*(*GUU*)*C*, located near the centromere of chromosome III, was equally enriched in each tagged background.

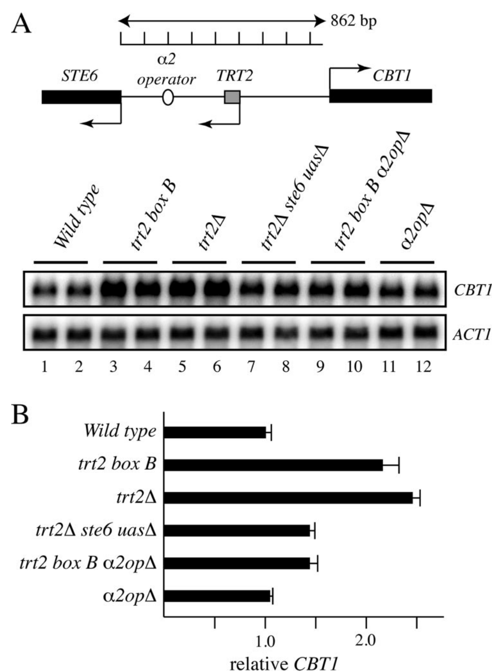

FIG. 5. *TRT2* functions as an insulator in its natural context. (A) Northern blot analysis of *CBT1* expression in *MAT***a** strains containing mutations in the *TRT2* tDNA and *STE6* regulatory elements. Mutation of *TRT2* results in an ~2.4-fold increase in *CBT1* expression (compare lanes 1 and 2 to lanes 3 to 6), which is indicative of a tDNA position effect. Further deletion of the *STE6* UAS (from the α2 operator to the *TRT2* gene; lanes 7 and 8) or just the α2 operator (only the Mcm1p binding site; lanes 9 and 10) reduces this increase, demonstrating that part of the observed increase in *CBT1* expression is due to inappropriate activation of *CBT1* by the *STE6* regulatory sequences, which is suggestive of an insulator-like activity of *TRT2*. Deletion of the α2 operator alone has no effect on *CBT1* transcription (lanes 11 and 12). (B) Relative expression of *CBT1* was normalized to *ACT1* levels for each construct. The results are averaged from three separate determinations. All modifications were chromosomally integrated, and genotypes are given in Table 1.

expression in *MAT***a** cells. Current interpretations of tDNA position effects on Pol III transcription have suggested that the presence of the Pol III complex has a negative influence on neighboring Pol II promoters, hypothesized to be due to factors such as nucleolar localization or nucleosome-positioning effects (4, 33, 56). We reasoned that an alternative hypothesis, an insulator-like activity of assembled Pol III complexes, could also explain the increased *CBT1* expression observed for *MAT***a** *trt2*Δ strains. In *MAT***a** cells, the transcription factor Mcm1p binds to a sequence within the α2 operator to activate the transcription of *STE6* (18). We hypothesized that in the absence of a functioning tDNA and assembled Pol III complex, the increase in *CBT1* expression upon *TRT2* mutation could be due to inappropriate activation of *CBT1* by α2 operator-bound Mcm1p in *MAT***a** cells.

To ask if this position effect was due to the insulator function of *TRT2*, we created mutant yeast strains with *TRT2* either deleted or containing the *boxB* mutation to inactivate Pol III complex assembly and *TRT2* transcription. We then constructed yeast strains that additionally contained a deletion within the α2 operator, specifically the Mcm1p binding site within the *STE6* UAS. An analysis of *CBT1* expression in these strains is shown in Fig. 5.

Deletion or mutation of *TRT2* resulted in an increase in *CBT1* expression (compare Fig. 5A, lanes 3 to 6 with lanes 1 and 2; quantitation in Fig. 5B) characteristic of a tDNA position effect. However, when either the entire *STE6* UAS or just the α2 operator site (the Mcm1p binding site) was also deleted, the increase in transcription of *CBT1* was partially mitigated. These results indicated that at least part of the increased expression due to tDNA deletion was due to the inappropriate activation of *CBT1* by transcription factors (Mcm1p) bound to the *STE6* UAS and not solely due to factors such as subnuclear localization or nucleosome positioning exerting a direct negative effect on the neighboring Pol II promoter.

We conducted a similar analysis on the *ETC6* site, which lies between *TFC6* and *ESC2* on chromosome IV. This was an interesting locus to study; as *TFC6* encodes a component of the TFIIIC complex, it has been suggested that *ETC6* may mediate the autoregulation of *TFC6* (37). As shown in Fig. 6, chromo-

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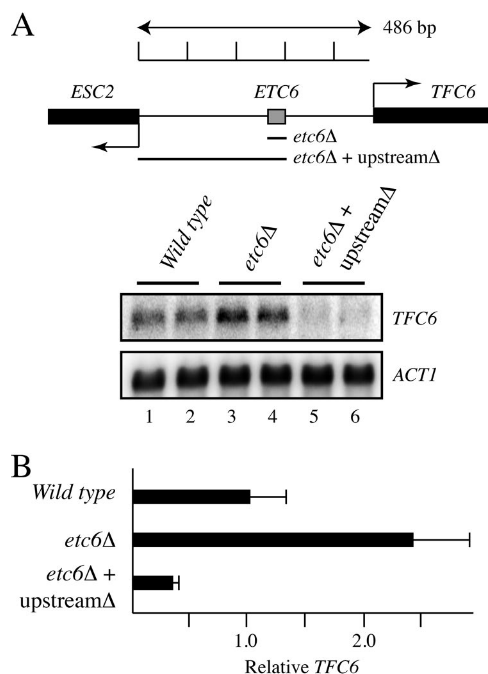

FIG. 6. *ETC6* displays an insulator-like activity in its natural context. (A) Strains were created that contained chromosomal deletions of either the *ETC6* site *boxB* sequence or both *ETC6* and the upstream region. Deletion of *ETC6* resulted in an ~2.5-fold increase in *TFC6* expression, which was reduced upon deletion of potential upstream activating sequences. Strains used were wild-type DDY 363D and 3637 (lanes 1 and 2), DDY 4115 and 4117 containing the *etc6*Δ mutation (lanes 3 and 4), and DDY 4114 and 4120 containing the *etc6*Δ+upstreamΔ mutation (lanes 5 and 6). (B) Quantitation was performed as described for Fig. 5, and six independently isolated *etc6*Δ strains were analyzed.

somal deletion of *ETC6* results in a subtle yet reproducible 2.4-fold increase in *TFC6* transcripts (confirmed with six independent isolates). To test if this deletion allowed an uncharacterized upstream UAS to activate *TFC6*, we made strains deleted for both *ETC6* and the entire upstream region back to the *ESC2* start codon. These strains showed a marked decrease in *TFC6* transcripts (Fig. 6), which resulted in a slow-growth phenotype that yielded colonies of variegated size (D. Donze, unpublished observations), presumably due to limiting *TFC6* synthesis. The increase in *TFC6* transcripts upon deletion of *ETC6* coupled with a subsequent decrease upon the deletion of upstream sequences is consistent with an insulator-like function of *ETC6*.

DISCUSSION

A growing body of evidence suggests that genes transcribed by RNA Pol III, mainly the tDNAs, can be involved in various extratranscriptional functions throughout eukaryotic genomes (10, 51). Mostly studied with the yeast *Saccharomyces cerevisiae*, these additional functions include directing the periodic integration of Ty elements (1, 9, 14, 35) and the integration of a *Dictyostelium* retrotransposon (52), creating pause sites for replication fork progression (13), the dominant overriding of nucleosome positioning sequences (38), and creating the apparent repression of transcription from neighboring RNA Pol II promoters (4, 30, 34, 51) and, conversely, the protection of neighboring Pol II genes from transcriptional repression due to propagating heterochromatin structures or the effects of other transcriptional repressors (16, 42, 45, 49, 51). Most recently, a role for the Pol III complex in sister chromatid cohesion has been demonstrated (17), as well as a role in recruiting the condensin complex to chromosomes (11, 23). Some of these genomic effects were believed to require a fully functional RNA Pol III complex bound to a tDNA, but recent data indicate that partial Pol III complexes bound to DNA are in some cases sufficient to impart certain extratranscriptional activities (37, 42). Given that *S. cerevisiae* contains 274 tDNAs scattered throughout its genome (22), and vertebrates contain in addition to tDNAs many repetitive elements capable of recruiting the Pol III apparatus (54), such extratranscriptional effects may exert a substantial effect on genome-wide chromosomal organization in eukaryotes. This study expands the role of tDNAs and *ETC*/COC sites as potential chromatin-organizing elements, demonstrating an additional insulator activity of DNA-bound Pol III complexes.

One important question regarding Pol III boundary activity centers on which components of the Pol III complex are required for boundary function. Compared to previous studies, the results presented here suggest that the requirement is highly context dependent. Earlier studies on the *HMR*-tDNA barrier suggested that both TFIIIC and TFIIIB binding, and possibly transcription by Pol III itself, were required to block silencing (16), and a tDNA heterochromatin barrier in *S. pombe* requires a fully assembled Pol III complex (50). However, the discovery of the heterochromatin barrier function of COC sites in *S. pombe* (42) challenges this requirement, as only the TFIIIC complex is bound to these sites. We show here that a single *ETC* site, confirmed to bind only TFIIIC and not TFIIIB (and therefore presumably not Pol III), can prevent the ectopic spread of silencing from the *HMR* locus. One key difference between these conflicting results is that in the previous study (16), the tDNA barrier was moved in between the *HMR*-E silencer and the *a1* gene and then the *a1* gene was used as the reporter gene. It has long been known that the *HMR*-E silencer is more robust and independent than the *HMR*-I silencer (7). In this study, we placed putative barrier elements in the natural location downstream from *HMR*-I. In this downstream location, TFIIIC binding is sufficient to stabilize a barrier that prevents the spread of Sir protein-mediated silencing. We have cloned the *ETC4* site between *HMR*-E and *a1* and have found that it functions only as a weak barrier to heterochromatin spreading when close to *HMR*-E (D. Donze, unpublished data), further demonstrating the context dependence of barrier complexes.

We also present data that TFIIIC binding sites can have a newly identified insulator function, as either a tDNA or an *ETC* sequence can block the interaction of Gal4p with the *GAL10* promoter. Chromatin immunoprecipitation analysis again confirmed that at the ectopic *ETC* site, only TFIIIC and not TFIIIB are bound in this assay system. We also demonstrated that the *TRT2* tDNA in its natural context serves as an insulator between the *STE6* and *CBT1* genes, preventing the *STE6* regulatory elements from affecting *CBT1* transcription levels. A similar insulating effect, or to use another electrical circuit analogy, a "resistor-like" effect, is conferred by the

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TABLE 1. *S. cerevisiae* strains used and generated in this study[a]

Strain(s)	Genotype[b]
DDY 3	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*
DDY 511	*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 hmrΔ::URA3*
DDY 631	*MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 sir4Δ::LEU2 hmrΔ::URA3*
DDY 811	*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-tdnaΔ-ADE2*
DDY 814	*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-ADE2*
DDY 2317, 2318	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*
DDY 2322, 2323	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2 boxB pTRT2-LEU2*
DDY 2325, 2326	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ pTRT2-LEU2*
DDY 2329, 2330	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2Δ ste6-uasΔ pTRT2-LEU2*
DDY 2333, 2335	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 trt2 boxB α2 operatorΔ pTRT2-LEU2*
DDY 2341, 2342	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 α2 operatorΔ*
DDY 2606	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal1-10 intergenicΔ::URA3*
DDY 2674	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal1-10 uasΔ*
DDY 2861, 2862	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*
DDY 2864	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*
DDY 3256, 3257	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-TRT2(←)*
DDY 3259, 3260	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-trt2 boxB(→)*
DDY 3262, 3263	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-TRT2(→)*
DDY 3266, 3267	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-trt2 boxB(←)*
DDY 3268, 3269	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal1 intergenic-TRT2(→)*
DDY 3271	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal1 intergenic-trt2 boxB(←)*
DDY 3274, 3275	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal1 intergenic-trt2 boxB(→)*
DDY 3277	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal1 intergenic-TRT2(←)*
DDY 3630, 3637	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1*
DDY 4115, 4117	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 etc6Δ*
DDY 4114, 4120	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 etc6Δ*+upstreamΔ
DDY 3724	*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-ETC4(←)-ADE2*
DDY 3743	*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-ETC4(→)-ADE2*
DDY 3754, 3760	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-etc4 boxB(←)*
DDY 3757, 3758	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-etc4 boxB(→)*
DDY 3770, 3771	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-ETC4(←)*
DDY 3773, 3995	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-ETC4(→)*
DDY 3811	*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-etc4 boxB(→)-ADE2 sir4Δ::LEU2*
DDY 3812	*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-etc4 boxB(→)-ADE2*
DDY 3815	*MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-etc4 boxB(←)-ADE2*
DDY 3817	*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-etc4 boxB(←)-ADE2 sir4Δ::LEU2*
DDY 3920	*MATα ade2-1 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 HMR-ADE2 BRF1::3XFLAG::KanMX*
DDY 3925	*MATα ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-etc4 boxB-ADE2 BRF1::3XFLAG::KanMX*
DDY 3927	*MATα ade2-1 can1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-ADE2 TFC1::3XFLAG::KanMX*
DDY 3929	*MATα ade2-1 can1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-ETC4-ADE2 TFC1::3XFLAG::KanMX*
DDY 3934	*MATα ade2-1 can1 his3-11 leu2-3,112 trp1-1 ura3-1 HMR-etc4-ADE2 TFC1::3XFLAG::KanMX*
DDY 3935	*MATα ade2-1 can1 his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 HMR-ETC4-ADE2 BRF1::3XFLAG::KanMX*
DDY 3942	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-TRT2 BRF1::3XFLAG::KanMX*
DDY 3948	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-etc4 boxB BRF1::3XFLAG::KanMX*
DDY 3951	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-ETC4 BRF1::3XFLAG::KanMX*
DDY 3958	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-TRT2 TFC1::3XFLAG::KanMX*
DDY 3966	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-etc4 boxB TFC1::3XFLAG::KanMX*
DDY 3969	*MAT***a** *his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 gal10 intergenic-ETC4 TFC1::3XFLAG::KanMX*

[a] All strains are isogenic to W303-1a. Paired strains are independent isolates of the same genotype.
[b] Arrows indicate the orientation of the *boxB* sequence within the inserted tDNA or ETC sites relative to the depiction of the individual loci in the figures.

ETC6 site in the *TFC6* promoter. This result is particularly interesting, as it suggests that the binding of TFIIIC to *ETC6* may directly modulate *TFC6* expression, providing a potential feedback inhibition by a component of the TFIIIC complex. A detailed analysis of the *TFC6* promoter and regulatory elements will be required to verify this hypothesis. These are the first demonstrations of natural insulator-like activities in budding yeast, and the binding of TFIIIC to *ETC6* may represent the first example of a Pol III transcription factor directly regulating a Pol II promoter.

With the discovery of the *ETC* and COC loci in yeast, a key question is whether these TFIIIC binding sites are bona fide regulatory elements. The fact that TFIIIC-only binding sites exist in multiple organisms, coupled with the fact that the *ETC* loci are conserved among the budding yeast (25, 37, 47), suggests a conserved evolutionary function. It is interesting to note that in the study by Noma et al. (42), most of the COC sites in *S. pombe* lie between divergently transcribed genes, and inspection of the *ETC*-like sites reported in the three independent studies with *S. cerevisiae* reveals that six out of eight of these TFIIIC-bound *boxB* sequences also lie between divergently transcribed promoters. Additionally, one site in *S. cerevisiae* (*iYGR033C*) has been identified that appears to bind TFIIIC and TFIIIB but not Pol III (21), and this site also lies between the divergent promoters of *TIM21* and *RPL26B*. Given this propensity, we propose the hypothesis that these TFIIIC binding sites function as insulators between such divergent promoters in the compact yeast genome, and we are

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systematically mutating each of these *boxB* sites to test for effects on the transcription levels of the neighboring Pol II genes. While the number of *ETC* sites in *S. cerevisiae* are certainly limited, our data presented here suggest that other tDNAs also may serve as insulators between divergently transcribed genes in yeast, and such effects may be more widespread in *S. pombe* and other eukaryotes.

Another unresolved question is how DNA-bound TFIIIC functions as a boundary element. Our results here show that recruitment of the entire Pol III complex is not always necessary for this activity. Numerous potential mechanisms exist, some of which are subnuclear localization into a Pol II-depleted nuclear region (56), nucleosome displacement and stable occupancy by TFIIIC (16, 43), and also recruitment of chromatin remodeling complexes. With regard to barrier function, purified human TFIIIC complex has been shown to possess intrinsic acetyltransferase activity (27), but this activity has not been demonstrated in yeast. However, the chromatin remodeling complex ISW2 is recruited to tDNA loci via an interaction with the TFIIIB subunit Bdp1p (1, 20), suggesting a possible role for nucleosome sliding or displacement in tDNA boundary function. However, since TFIIIB is not recruited to *ETC* sites, it is unlikely that ISW2 is required for the *ETC* site boundary activity reported in this study.

Our results clearly demonstrate that TFIIIC binding in the absence of the rest of the Pol III machinery is capable of establishing a chromatin boundary in certain contexts, but interaction with additional chromatin modifiers that interact with TFIIIB and Pol III may assist or stabilize the formation of boundaries. Interestingly, the loss of RSC (*r*emodels the *s*tructure of *c*hromatin) complex function results in changes in both nucleosome positioning and density near tDNAs (44). Our previous genetic analysis of the *HMR*-tDNA boundary (32) demonstrated a variegated loss of barrier activity upon mutation of the *RSC2* gene, suggesting that chromatin remodeling by RSC also contributes to barrier formation. The RSC chromatin remodeling complex has been shown to be directly recruited to loci transcribed by Pol III (41), and this interaction appears to involve a direct interaction with the Rpb5p subunit conserved among all three eukaryotic RNA polymerases (53). Further complicating any interpretation of the role of the RSC complex are the observations that RSC mutations compromise the recruitment of the cohesin complex to chromosomal arms (2, 28) and that cohesin mutants are defective in barrier activity at *HMR* (15).

Recent studies have further implicated the Pol III machinery in the recruitment of the condensin class of chromosome binding and organizing proteins. Multiple studies have shown by chromatin immunoprecipitation that the condensin subunits associate with Pol III genes and TFIIIC-only binding sites and that a direct interaction occurs between condensin subunits and TFIIIB or TFIIIC (11, 23).

As for insulator function in the compact yeast genome, the mechanisms involved will likely be different from those proposed for metazoan systems, which can involve long-range chromosomal looping (36, 55). In yeast, it is likely that the Pol III complex or TFIIIC alone may simply physically block the assembly of complexes connecting the UAS to the promoter. For example, simply tethering the *Escherichia coli* LexA protein to the *GAL* locus can block Gal4p-mediated activation of a *GAL1-lac*Z fusion (8), suggesting that a simple physical presence may be sufficient to disrupt coactivator recruitment or perhaps block the propagation of histone acetylation, which has been demonstrated to occur in yeast from sites of targeted acetyltransferase binding (59).

However, chromatin boundary formation by Pol III transcription factor binding sites is turning out to be an extremely complex process, which appears to involve several active complexes such as RSC, and possibly ISW2 and direct histone acetyltransferase recruitment. Boundary function also involves direct structural components such as TFIIIC binding and the involvement of other chromatin architectural components such as bromodomain proteins (32), Nhp6 proteins (6), and, as described above, condensins and cohesions. Future work will need to focus on dissecting how these various DNA and chromatin interacting factors cooperate to create a stable chromatin boundary and to investigate the likely possibility that like promoters and enhancers, different boundaries will utilize different complements of DNA-bound and chromatin-associated factors. Also, continued genome-wide analysis of Pol III factor-mediated boundary activity will be required to determine the overall scope of these effects along eukaryotic genomes. Finally, the extent of Pol III-mediated chromosomal position effects in other eukaryotes could be far reaching, as in human chromosomes a large number of potential TFIIIC binding *boxB* sequences exist within the repetitive megacopy *Alu* elements.

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