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 chromatic 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 MATa cell-specific STE6 gene in MATα 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 (GGTTGGANTCC; 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 $3 \times 10^6$ tRNA molecules per yeast cell. Thus, each of the 274 nucleus-encoded (24) and additional mitochondrially encoded tDNAs of this organism
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 DNA 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 TFIIC (and in one case also TFIIB) but are not occupied by the polymerase itself (21, 37, 42, 47). In *S. cerevisiae*, these chromosomal locations are called ETC (extra TFIIC) 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 TFIIC binding regions are overrepresented in the intergenic regions of divergently transcribed genes, suggesting a possible function in genome demarcation.

Since tDNAs or simply bound TFIIC 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 TFIIC binding in the absence of TFIIB is sufficient for both insulator and barrier activities. Finally, we identify in yeast TFIIC 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 on 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 *gali-10 intergenic::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 *UASG* 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 *SIR4* and *URA3* auxotrophs were isolated. Mutant boxB sequences were created using site-directed mutagenesis to change the invariant cytosine residue to guanine (boxB consensus, GGT TCGGANTCC [invariant C underlined]). This mutation inactivates Pol III genes by inhibiting TFIIC binding. The resulting isolates were confirmed by both PCR and DNA sequencing to verify proper integration of the TFIIC binding sites at *GAL1-10*. Insertion of TRT2 between *UASG* and *GAL10* was performed in a similar manner, by inserting the BamHI site at coordinate 278710 (pDD782). 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 566325) inserted downstream of *HMR* (SacI-Sall *HMR* fragment) at SGD chromosome III coordinate 295736, with *HMR-tDNA* deleted and replaced with an SphI site. Wild-type and boxB mutant TET sites were cloned into the SphI-cut plasmid, and the resulting plasmids were linearized and integrated into an *hmr::URA3 sir4A* strain (DDY 631). Aide-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.<sub>G</sub>. 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<sub>G</sub> and *GAL10* or *GAL1* (Fig. 1A) and then integrated into the *gali-10 intergenic::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<sub>G</sub> 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<sub>G</sub>, 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).
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 sequence between the GAL10 gene and UASG 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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**FIG. 1.** Insulator activity of a tDNA inserted at the GAL1-10 locus. Insertion of a functional tDNA between UASG 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 2864, wild-type (wt) GAL locus; 3 and 4, DDY 3770 and 3771, ETC4 inserted between UASG 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, uasGΔ. (B) Northern blot analysis of GAL10 expression of representative strains was performed as described for Fig. 1.
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 UASs 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 tDNAΔ 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).

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 MATa cells, where STE6 is active (51). Complete deletion or boxB point mutation of TRT2 resulted in an increase in CBT1

**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.
expression in \( \text{MAT} \) 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 \( \text{CBT1} \) expression observed for \( \text{MAT} \) \text{trt2}/H9004 strains. In \( \text{MAT} \) cells, the transcription factor Mcm1p binds to a sequence within the H9251 operator to activate the transcription of \( \text{STE6} \) (18). We hypothesized that in the absence of a functioning tDNA and assembled Pol III complex, the increase in \( \text{CBT1} \) expression upon TRT2 mutation could be due to inappropriate activation of \( \text{CBT1} \) by the H9251 operator-bound Mcm1p in \( \text{MAT} \) 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 \( \alpha 2 \) operator, specifically the Mcm1p binding site within the \( \text{STE6} \) UAS. An analysis of \( \text{CBT1} \) expression in these strains is shown in Fig. 5.

Deletion or mutation of TRT2 resulted in an increase in \( \text{CBT1} \) expression (compare lanes 1 and 2 to lanes 3 to 6), which is indicative of a tDNA position effect. Further deletion of the \( \text{STE6} \) UAS (from the \( \alpha 2 \) operator to the TRT2 gene; lanes 7 and 8) or just the \( \alpha 2 \) operator (only the Mcm1p binding site; lanes 9 and 10) reduces this increase, demonstrating that part of the observed increase in \( \text{CBT1} \) expression is due to inappropriate activation of \( \text{CBT1} \) by the \( \text{STE6} \) regulatory sequences, which is suggestive of an insulator-like activity of TRT2. Deletion of the \( \alpha 2 \) operator alone has no effect on \( \text{CBT1} \) transcription (lanes 11 and 12). (B) Relative expression of \( \text{CBT1} \) was normalized to \( \text{ACT1} \) levels for each construct. The results are averaged from three separate determinations. All modifications were chromosomally integrated, and genotypes are given in Table 1.

![Fig. 4. Chromatin immunoprecipitation analysis of Pol III factors at tDNA and ETC4 boundaries.](Image)

![Fig. 5. TRT2 functions as an insulator in its natural context.](Image)
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 etc6Δ mutation (lanes 3 and 4), and DDY 4114 and 4120 containing the etc6Δ + upstreamΔ mutation (lanes 5 and 6). (B) Quantitation was performed as described for Fig. 5, and six independently isolated etc6Δ 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 characterized 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 discoideum 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
ETC6 site in the TFC6 promoter. This result is particularly interesting, as it suggests that the binding of TFIIC to ETC6 may directly modulate TFC6 expression, providing a potential feedback inhibition by a component of the TFIIC 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 TFIIC 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 TFIIC-binding sites are bona fide regulatory elements. The fact that TFIIC-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. cerevisiae* reveal that six out of eight of these TFIIC-bound boxB sequences also lie between divergently transcribed promoters. Additionally, one site in *S. cerevisiae* (YGR035C) has been identified that appears to bind TFIIC and TFIIB 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 TFIIC binding sites function as insulators between such divergent promoters in the compact yeast genome, and we are

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**Table 1.** *S. cerevisiae* strains used and generated in this study

| Strain(s) | Genotype |
|-----------|----------|
| DDY 3 | MATa his3-11 leu2-3,112 lys2Δ trp1-1 ura3-1 |
| DDY 511 | MATa ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 |
| D351 | MATa his3-11 leu2-3,112 lys2A trp1-1 ura3-1 trg1Δ::LUE2 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 |
| DDY 198E | MATa his3-11 leu2-3,112 lys2A trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 |
| H9001 | MATa his3-11 leu2-3,112 lys2A trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 |
| MATa his3-11 leu2-3,112 lys2A trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 |
| MATa his3-11 leu2-3,112 lys2A trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 |
| MATa his3-11 leu2-3,112 lys2A trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 |
| MATa his3-11 leu2-3,112 lys2A trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 his3-11 ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 |

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*All strains are isogenic to W303-1a. Paired strains are independent isolates of the same genotype.*

*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.*
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 (remodels the structure of chromatin) 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-lacZ 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 euchromatic 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 megacytopl Alu elements.

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