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The *Saccharomyces cerevisiae TRT2* tRNA^{Thr} gene upstream of *STE6* is a barrier to repression in *MAT*α cells and exerts a potential tRNA position effect in *MAT*a cells

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ABSTRACT

A growing body of evidence suggests that genes transcribed by RNA polymerase III exhibit multiple functions within a chromosome. While the predominant function of these genes is the synthesis of RNA molecules, certain RNA polymerase III genes also function as genomic landmarks. Transfer RNA genes are known to exhibit extra-transcriptional activities such as directing Ty element integration, pausing of replication forks, overriding nucleosome positioning sequences, repressing neighboring genes (tRNA position effect), and acting as a barrier to the spread of repressive chromatin. This study was designed to identify other tRNA loci that may act as barriers to chromatin-mediated repression, and focused on *TRT2*, a tRNA^{Thr} adjacent to the *STE6* α2 operator. We show that *TRT2* acts as a barrier to repression, protecting the upstream *CBT1* gene from the influence of the *STE6* α2 operator in *MAT*α cells. Interestingly, deletion of *TRT2* results in an increase in *CBT1* mRNA levels in *MAT*a cells, indicating a potential tRNA position effect. The transcription of *TRT2* itself is unaffected by the presence of the α2 operator, suggesting a hierarchy that favors assembly of the RNA polymerase III complex versus assembly of adjacent α2 operator-mediated repressed chromatin structures. This proposed hierarchy could explain how tRNA genes function as barriers to the propagation of repressive chromatin.

INTRODUCTION

RNA polymerase III is predominantly responsible for the transcription of small cellular RNA molecules including tRNAs, 5S RNA, 7SL RNA and in *Saccharomyces cerevisiae*, the *SNR6* gene encoding the spliceosome U6 RNA. Transcription of tRNA genes is mediated by the stepwise assembly of the $TF_{III}C$ transcription factor complex onto the *box* A and *box* B internal control region promoter elements, followed by recruitment of the TATA binding protein (TBP) containing complex $TF_{III}B$. Once all transcription factors are in place, the RNA polymerase III enzymatic complex is recruited to initiate high level transcription of its target genes (1–3). These RNAs are extremely abundant in dividing cells, as tRNAs alone can account for as much as 15% of total RNA in log phase *S.cerevisiae* (4). This number suggests that tRNA genes are transcribed at an amazingly high rate during log phase growth (compared to RNA polymerase II genes), averaging approximately 10^4 transcription cycles/tRNA gene/generation, or roughly two cycles per second. This high rate of transcription can be explained in part by a facilitated recycling model in which an assembled RNA polymerase III complex is transferred from the termination site to the initiation site, remaining assembled on the tRNA gene through multiple rounds of transcription (5–7).

Such a persistently organized RNA polymerase III complex could also explain several observed 'extra-transcriptional' roles of tRNA genes within chromosomes. In *S.cerevisiae*, actively transcribed tRNA genes have been shown to direct Ty element integration (8–10), override nucleosome positioning signals (11), exert repressive position effects on neighboring RNA polymerase II promoters (12–15), act as replication fork pause sites (16), and act as a barrier to the propagation of heterochromatic repression, by blocking the spread of silent chromatin at the *HMR* locus (17). Of particular interest is the dichotomy that in certain cases a tRNA gene is capable of protecting a neighboring gene from repression (at *HMR*), while in other instances tRNA genes can directly repress or exert a negative influence on transcription of an adjacent RNA polymerase II gene, a process referred to as tRNA-mediated gene silencing (14) or tRNA position effect (15). While these types of effects have been observed in a limited number of cases (both natural and engineered), the genome-wide effects of the location of RNA polymerase III complex formation on neighboring chromosomal loci are largely unstudied.

We have previously described the heterochromatin barrier effect attributed to the *HMR-tRNA* (*tRNA^{Thr}[AGU]C*) on *S.cerevisiae* chromosome III. This *tRNA^{Thr}* gene prevents the spread of Sir protein-mediated gene silencing from the adjacent *HMR* locus in both reporter constructs and along the native chromosome (17). We asked if tRNAs adjacent to other repressed loci in *S.cerevisiae* could also function as

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barriers to repression of neighboring genes. *TRT2* (coding for *tRNA^{Thr}[CGU]K*) is a single copy *tRNA^{Thr}* gene that lies just upstream of the α2 operator sequence that regulates the *MAT***a** cell-specific *STE6* gene on *S.cerevisiae* chromosome XI. We specifically selected this locus for study as another example of a tRNA gene located adjacent to a repressed region of chromatin, and asked whether this tRNA gene might act as a barrier to the spread of repression. The α2 operator binds the Mcm1p/α2p complex, and initiates *MAT*α cell-specific repression of *MAT***a** specific genes such as *STE6* via multiple mechanisms, including nucleosome positioning (18,19), the recruitment of Ssn6p, Tup1p, and their associated histone deacetylases (20–23), and direct interaction with transcriptional machinery (24,25). This study asked whether *TRT2* served as a barrier to α2 operator-mediated repression in *MAT*α cells, and revealed that the same tRNA gene both protects the adjacent *CBT1* gene from α2 operator repression in *MAT*α cells, and exerts a potential negative tRNA position effect on *CBT1* in *MAT***a** cells. This is the first example of a tRNA gene that displays multiple types of extra-transcriptional functions at the same locus.

MATERIALS AND METHODS

All yeast strains were derived from wild-type *S.cerevisiae* W303 (DDY2, DDY3 and DDY4, originally JRY4012, JRY4013 and JRY2334, obtained from Jasper Rine, University of California at Berkeley; genotypes of all yeast strains generated in this study are listed in Table 1). Since *TRT2* is an essential single copy tRNA gene, a 0.32 kb fragment of *TRT2* (SGD chromosome XI coordinates 46596–46919) was cloned by PCR into plasmids pRS414 and pRS415 (26) to cover deletions of the gene (plasmids pDD675 and pDD676, respectively). To construct the *trt2–cbt1*Δ::*URA3* reporter strains described in Figure 1, a 2.1 kb segment of the *TRT2* locus (coordinates 46162–48248) was amplified by PCR and cloned into pCR2.1-TOPO (Invitrogen) to make plasmid pDD689. The resulting plasmid was cut with *Spe* I and *Xho* I to remove *TRT2* and *CBT1*, and was replaced with the Spe I-Xho I *URA3* fragment from pDD588 (*URA3* cloned into Bluescript SK+) to create plasmid pDD694, *trt2–cbt1*Δ::*URA3*. The modified locus was cut out of pDD694 and transformed into the diploid strain DDY2, and URA+ recombinants were selected and screened by PCR to verify proper integration. This diploid strain was then transformed with *TRT2* plasmids pDD675 or pDD676 to cover the deletion, sporulated, and URA+ haploids were recovered. The *cbt1*Δ::*URA3* control strains were made by direct PCR knockout of *CBT1* with *URA3*, using pRS406 as template. Cells were grown on yeast minimal medium (YMD + 2% dextrose) lacking uracil to test for repression of the *URA3* marker gene. Yeast Nitrogen Base was purchased from U.S. Biologicals, and YMD + all mix contained only those nutrients required for growth of W303 strains (adenine, histidine, leucine, lysine, tryptophan, and uracil).

To make the modified chromosomal loci, pDD689 was mutagenized using the Quik-change kit (Stratagene) to delete *TRT2* (oligonucleotides DDO-96/97) from *box* A to the *box* B (chromosome XI coordinates 46747–46800). The α2 operator from coordinates 46472–46489 was deleted in the same way using oligonucleotides DDO-123/124. Plasmids containing deletions of *TRT2* and/or the α2 operator were transformed into DDY889 (*trt2–cbt1*Δ::*URA*), selected on 5-FOA, and proper integration verified by PCR. Resulting strains containing modified *STE6–CBT1* loci were backcrossed to *trt2–cbt1*Δ::*URA3* strains to obtain sibling *MAT***a** and *MAT*α versions.

For northern blot analysis, RNA was prepared as described in Iyer and Struhl (27). Northern blots contained 10 μg total RNA per lane, and were performed using Northern Max reagents (Ambion). *CBT1* northern blots were run on 1.0% agarose gels, and the *TRT2* blot in Figure 4 was run on a 1.2% agarose gel. Northern probes were generated from PCR

Table 1. Genotypes of all yeast strains generated in this study

	S.cerevisiae W303 strains	Source
DDY2	*MAT*α/*MAT***a** *ade2-1/ADE2 his3-11/his3-11 leu2-3, 112/leu2-3, 112 LYS2/lys2*Δ *trp1-1/trp1-1 ura3-1/ura3-1*	J. Rine
DDY3	*MAT***a** *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1*	J. Rine
DDY4	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1*	J. Rine
DDY889	*MAT*α *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2-cbt1*Δ*::URA3 pTRT2:LEU2*	This study
DDY890	*MAT***a** *ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2-cbt1*Δ*::URA3 ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY891	*MAT***a** *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2-cbt1*Δ*::URA3 ppr1*Δ*::HIS3 pTRT2:TRP1*	This study
DDY902	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2-cbt1*Δ*::URA3 ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY 903	*MAT*α *ade2-1 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2-cbt1*Δ*::URA3 ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY974	*MAT*α *ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 cbt1*Δ*::URA3 ppr1*Δ*::HIS3*	This study
DDY975	*MAT***a** *ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 cbt1*Δ*::URA3 ppr1*Δ*::HIS3*	This study
DDY1022	*MAT***a** *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2*Δ *ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY1024	*MAT***a** *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2*Δ *ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY1026	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2*Δ *ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY1028	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2*Δ *ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY1261	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2*Δ α*2 operator*Δ *ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY1262	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2*Δ α*2 operator*Δ *ppr1*Δ*::HIS3 pTRT2:LEU2*	This study
DDY1737	*MAT*α *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1* α*2 operator*Δ	This study
DDY1739	*MAT***a** *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1* α*2 operator*Δ	This study
DDY1740	*MAT***a** *ADE2 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1* α*2 operator*Δ	This study
DDY1742	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1* α*2 operator*Δ	This study
DDY1805	*MAT*α *ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 trt2*Δ *pTRT2:URA3 hos1::HIS3 hos2::TRP1 rpd3::LEU2*	This study
DDY1825	*MAT*α *ADE21 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2*Δ *pTRT2:URA3 hos1::HIS3 hos2::TRP1 rpd3::LEU2*	This study
DDY1956	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2*Δ *ppr1*Δ*::HIS3 pTRT2:LEU2 hda1*Δ*::KanMX*	This study
DDY2021	*MAT*α *ADE2 his3-11 leu2-3,112 lys2*Δ *trp1-1 ura3-1 trt2*Δ *ppr1*Δ*::HIS3 pTRT2:LEU2 hda1*Δ*::KanMX*	This study

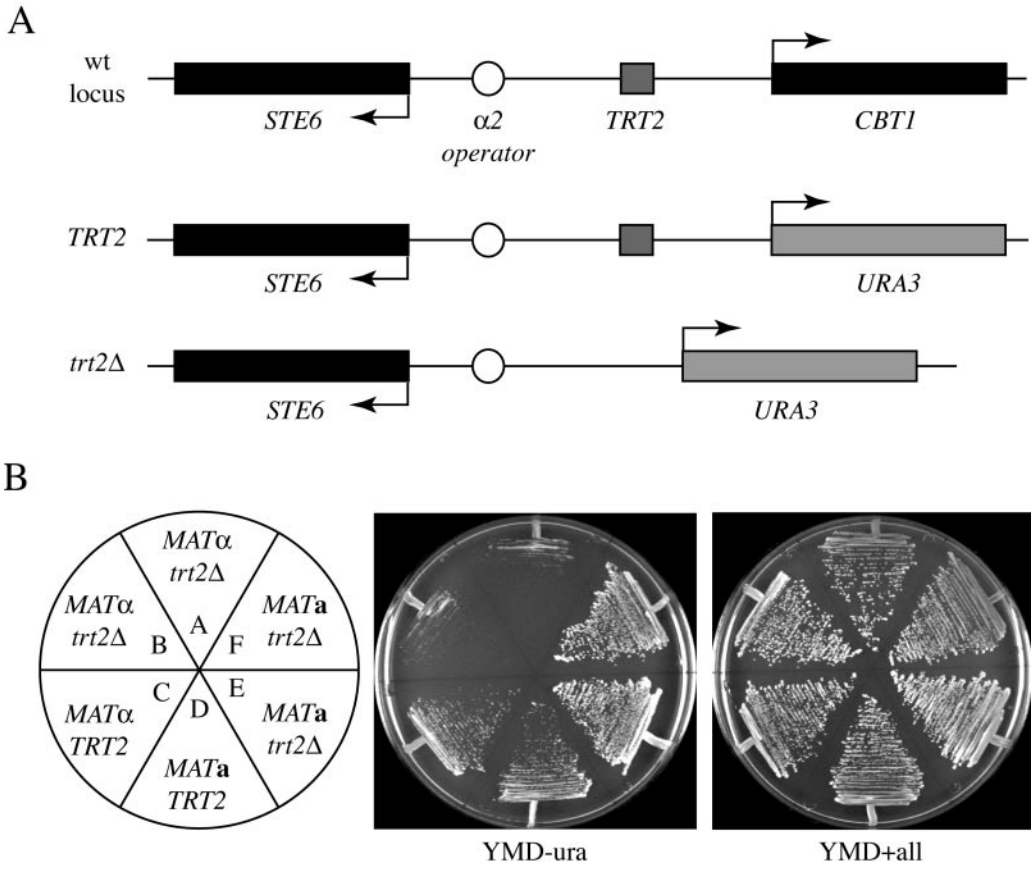

Figure 1. A *URA3* marker gene is repressed when inserted upstream of the *STE6* α2 operator site in *S.cerevisiae* chromosome XI. (**A**) The wild-type *STE6–CBT1* region of chromosome XI is depicted on top. *URA3* was inserted by homologous recombination upstream of the *STE6* α2 operator to either delete the *TRT2* $tRNA^{Thr}$ gene (DDY890, DDY891, DDY902, and DDY903), or to retain the intervening *TRT2* gene (DDY974 and DDY 975). (**B**) Each strain was streaked on YMD lacking uracil (YMD − ura) and incubated for 2 days. *MAT*α strains lacking *TRT2* showed inhibited growth on medium lacking uracil, while all strains grew equally on minimal YMD containing uracil (YMD + all).

products of the first 600 bp of each gene (except for *TRT2*, where the entire gene was amplified) that included a T7 RNA polymerase promoter attached to the downstream primer. These PCR products were used as templates to synthesize radiolabeled riboprobes using the Ambion Strip-EZ kit. All oligonucleotide sequences used for knockouts, PCR clonings, probe templates, and mutagenesis reactions are available on request.

HDA1 deletion in the *trt2*Δ strain was made by standard PCR knockout protocols using the plasmid pUG6 as a template (28). The *hos1 hos2 rpd3* strains were made by crossing *trt2*Δ strains with strain DY6445 (*MAT*α *ade2 can1 his3 leu2 trp1 ura3 hos1::HIS3 hos2::TRP1 rpd3::LEU2*), a gift from David Stillman (University of Utah).

Chromatin immunoprecipitation was performed as described in Kuo and Allis (29). Antibodies used were anti-acetyl-histone H3 and anti-acetyl-histone H4 from Upstate (catalog No. 06–599 and 06–866). An aliquot of 5 µl of a 1 : 10 dilution of DNA recovered from the immunoprecipitates was used to program PCR reactions (*Taq* polymerase purchased from Promega), and the same volume of a 1 : 40 dilution was used for the input controls. PCR conditions were 95°C for 2 min (initial denaturation), 95°C × 30 s, 55°C × 30 s, 72°C × 60 s (28 cycles).

RESULTS

TRT2 can protect an integrated *URA3* marker gene from α2 operator repression

STE6 is a *MAT***a** cell-specific gene that is repressed in *MAT*α cells by an upstream α2 operator sequence. Several α2 operator sequences, including this particular one, have been shown to be orientation independent in plasmid-based *lacZ* reporter gene assays (30), so we wished to determine if repression was also bi-directional in a chromosomal context. Also, since the *TRT2* $tRNA^{Thr}$ gene lies between this α2 operator and *CBT1*, the next RNA polymerase II transcribed gene upstream of *STE6*, we tested whether *TRT2* acts as a barrier to repression of *CBT1*.

To test the hypothesis that repression spreads bi-directionally from a chromosomal α2 operator, and that the *TRT2* gene acts as a barrier to α2 operator-mediated repression, we constructed yeast strains that contained *URA3* integrated into chromosome XI in place of *CBT1*, upstream of the α2 operator site at the *STE6* locus. Two sets of strains were constructed (Figure 1A), one that retained *TRT2* between the α2 operator and *URA3*, and a second that replaced both *CBT1* and *TRT2* with *URA3*. Figure 1 shows the results when these strains were streaked on minimal media lacking uracil. *MAT*α

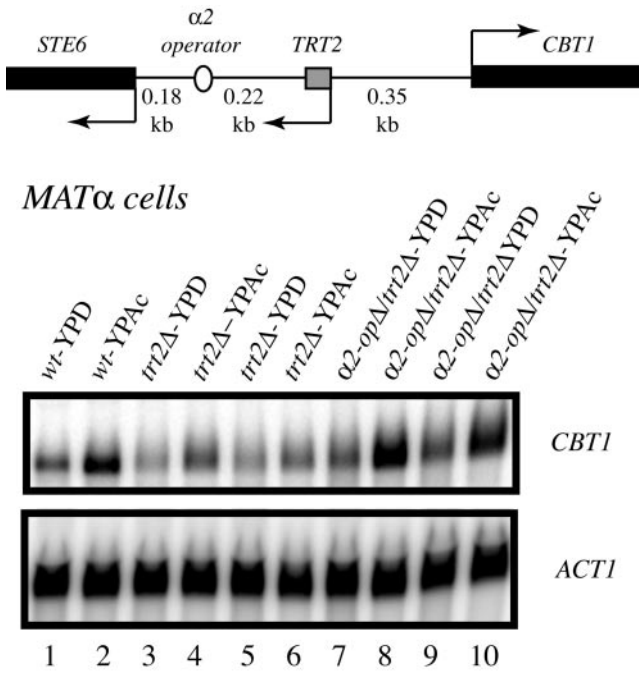

Figure 2. Deletion of *TRT2* results in the repression of *CBT1* transcription in *MAT*α cells. Total RNA was isolated from strains containing a wild-type *STE6–CBT1* locus (DDY4, lanes 1 and 2), a mutant locus deleted for *TRT2* (DDY1026, lanes 3 and 4, DDY1028, lanes 5 and 6), and a mutant locus containing deletion of both *TRT2* and the α2 operator (DDY1261, lanes 7 and 8, DDY1262, lanes 9 and 10). Odd numbered lanes contain RNA isolated from cells grown on dextrose as a carbon and energy source (YPD), and even numbered lanes from cells grown on acetate (YPAc), which induces *CBT1* transcription. *CBT1* mRNA levels were reduced approximately 3-fold in strains lacking only *TRT2*. Results from two independent isolates of each mutant strain are shown.

*trt2–cbt1*Δ*::URA3* strains (Figure 1B, DDY 902 and DDY 903, wedges A and B) are considerably compromised for growth on YMD media lacking uracil compared to isogenic *MAT*α (DDY974) or *MAT***a** (DDY975) strains containing *TRT2* between the operator and *URA3* (wedges C and D). *URA3* is not completely repressed in these strains, as extended incubation eventually leads to formation of colonies. This delay in growth suggests that repression can spread from the α2 operator in both directions along chromosome XI and inhibit *URA3* expression.

Interestingly, *MAT***a** *trt2–cbt1*Δ*::URA3* strains grow slightly better on YMD lacking uracil than *MAT***a** strains containing *TRT2*, suggesting that in the absence of α2 operator-mediated repression in *MAT***a** cells, *TRT2* may exert a repressive tRNA position effect on the *URA3* reporter (compare DDY975, wedge D with DDY890 and 891, wedges E and F). These results prompted us to further investigate the effects of deleting *TRT2* on the expression of *CBT1*, the gene naturally upstream of *STE6* on chromosome XI, in both *MAT***a** and *MAT*α cells.

Deletion of *TRT2* from chromosome XI in *MAT*α cells inhibits induction of *CBT1* when cells are grown on acetate, and inhibition is dependent on the α2 operator

Cytochrome B termination (*CBT1*) is a gene required for proper maturation of cytochrome b mRNA in *S.cerevisiae*, and is essential for respiratory growth on non-fermentable

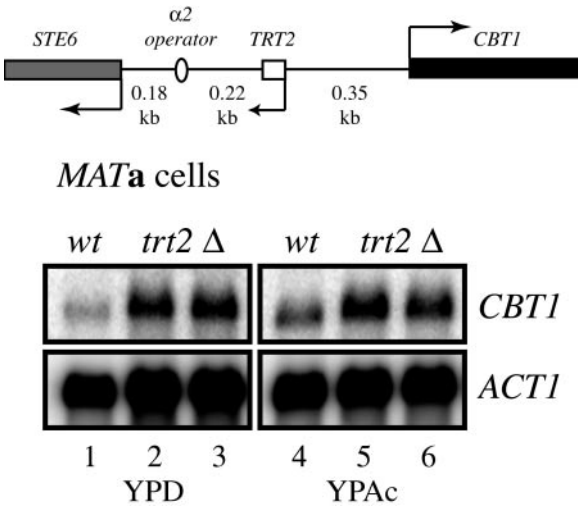

Figure 3. Deletion of *TRT2* results in an increase in expression of *CBT1* in *MAT***a** cells. Wild-type *MAT***a** *S.cerevisiae* (DDY3, lanes 1 and 4), and *MAT***a** *trt2*Δ (two independent isolates, DDY1022 lanes 2 and 5, and DDY1024 lanes 3 and 6), were grown on YPD (lanes 1–3) or on YPAc (lanes 4–6) and total RNA isolated. Northern blots were probed for *CBT1* mRNA as in Figure 2.

carbon sources such as acetate and ethanol (31). *CBT1* is located 862 bp upstream of *STE6*, placing it approximately 680 bp from the α2 operator. We observed that growth of wild-type *MAT*α *S.cerevisiae* in media containing acetate as a sole carbon source (YPAc) resulted in a 3-fold induction of *CBT1* mRNA compared to cells grown in dextrose (YPD, Figure 2, compare lanes 1 versus 2). We then asked whether *CBT1* expression is affected by deletion of *TRT2*. Since *TRT2* is an essential single copy tRNA gene, it was first deleted in a diploid strain, the deletion was covered with an episomal copy of *TRT2* (pDD676, pRS415:*TRT2:LEU2*), and the resulting diploid strain was sporulated to obtain *MAT*α *trt2*Δ:p*TRT2:LEU2* cells. Deletion of *TRT2* from chromosome XI in *MAT*α cells reduced both the basal and induced levels of *CBT1* expression to approximately 40% of normal levels as analyzed by northern blot analysis (Figure 2, lanes 3 and 4, 5 and 6 compared to lanes 1 and 2). This repression was dependent on the α2 operator, as deletion of both *TRT2* and α2 operator sequences restored the normal levels of *CBT1* mRNA induction (Figure 2, lanes 7–10). This result demonstrates that repression spreads along chromosome XI upstream of the α2 operator in the absence of *TRT2*, suggesting that *TRT2* functions as a barrier to α2 operator-mediated repression of *CBT1* in *MAT*α cells.

Deletion of *TRT2* from chromosome XI in *MAT***a** cells results in an increase in expression of *CBT1*

When *CBT1* expression from a *trt2*Δ chromosome was analyzed in *MAT***a** cells, the opposite effect was observed. Figure 3 shows the results of northern blot analysis of wild-type and *MAT***a** *trt2*Δ strains probed for *CBT1* message. Deletion of *TRT2* in *MAT***a** cells leads to increased levels of *CBT1* mRNA in either YPD or YPAc media, suggesting that in its native context in *MAT***a** cells, *CBT1* may be subject to a tRNA position effect (Figure 3, compare lane 1 to lanes 2 and 3, lane 4 to lanes 5 and 6). The increased level of transcription of *CBT1* in *trt2*Δ strains is consistent with observation of the

strains analyzed in Figure 1, as *MAT***a** *trt2–cbt1*Δ::*URA3* strains grew slightly better than *MAT***a** *cbt1*Δ::*URA3* strains on YMD–uracil media.

Transcription of *TRT2* is unaffected by α2 operator-mediated repression

Since *TRT2* is a single copy tRNA gene, its expression level can be assayed directly by northern blotting. We next asked if the α2 operator affects expression of *TRT2* itself. Figure 4 shows *TRT2* expression levels in wild-type and α2 operator deleted *MAT***a** and *MAT*α strains. After normalization to the *ACT1* signal, no significant difference in the level of *TRT2* RNA was seen in *MAT*α versus *MAT***a** cells, therefore *TRT2* is apparently unaffected by the presence of an adjacent active α2 operator (Figure 4, lanes 1 and 2). To further confirm that the *TRT2* gene is refractory to α2 operator repression, the operator site was deleted in both *MAT*α (Figure 4, lanes 3 and 4) and *MAT***a** (lanes 5 and 6) strains, and again no difference in *TRT2* levels was seen. These results demonstrate that RNA polymerase III transcription of *TRT2* is completely impervious to α2 operator-mediated repression.

Altered histone acetylation does not appear to be responsible for the spread of repression along a *trt2*Δ chromosome

The recent literature has described multiple yeast histone deacetylases as interacting with the Ssn6p/Tup1p complex to repress transcription. Increased histone H4 acetylation at the *STE6* promoter is observed in class I histone deacetylase (HDAC) *hos1 hos2 rpd3* triple mutant strains (23), however, loss of Rpd3p function affects both repression and activation of *STE6* (32). Derepression of Ssn6–Tup1 regulated genes *SUC2* and *MFA2* is observed in triple *hos1 hos2 rpd3* strains (23). Other Ssn6–Tup1 regulated genes, such as *ENA1* appear to require the class II HDAC *HDA1* for repression, and it has been reported that *STE6* is partially derepressed in either *hda1* or *rpd3* strains (21). The Ssn6–Tup1 protein complex has been shown to physically interact with all of these HDACs *in vitro* (21–23).

To assess whether HDAC recruitment by Ssn6–Tup1 at the α2 operator is responsible for *CBT1* repression in the absence of *TRT2*, we performed northern blots in *trt2*Δ strains mutated for either *hda1* or *hos1 hos2 rpd3*. Figure 5A shows that deletion of *hda1* does not relieve repression of *CBT1* in a *trt2*Δ background. The triple deletion of the class I HDACs results in even lower levels of *CBT1*, suggesting that, as for *STE6* and other genes, *RPD3* function is also required for normal activated expression (32). These results suggest that altered histone acetylation levels are not the major determinant in spreading of repression from the operator in the absence of *TRT2*.

In order to directly assess the histone acetylation state at *CBT1* in wild-type and *trt2*Δ strains, we performed chromatin immunoprecipitation using antibodies against acetylated histone H3 or histone H4. DNA immunoprecipitated from wild-type or *trt2*Δ strains was probed by PCR with multiple primer

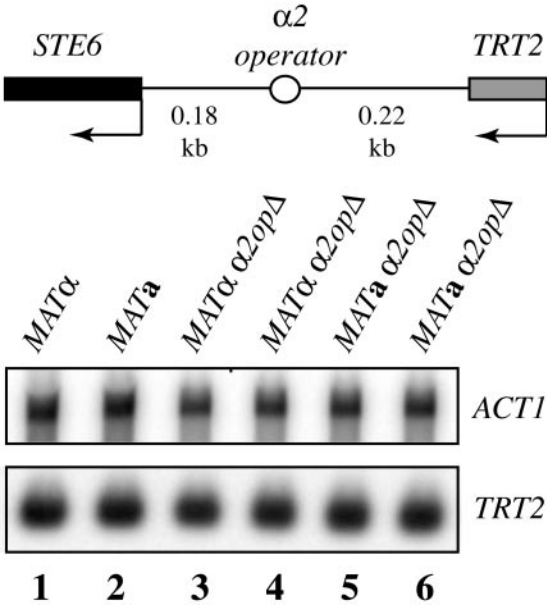

Figure 4. *TRT2* expression is unaffected by the presence of an active α2 operator site. Northern blot analysis of *TRT2* mRNA from wild-type *MAT*α and *MAT***a** strains (DDY4 and DDY3, lanes 1 and 2), α2 operator deleted *MAT*α strains (DDY1737 and DDY1742, lanes 3 and 4), and α2 operator deleted *MAT***a** strains (DDY1739 and DDY1740, lanes 5 and 6). After normalization to the *ACT1* signal, *TRT2* mRNA levels were identical in all strains.

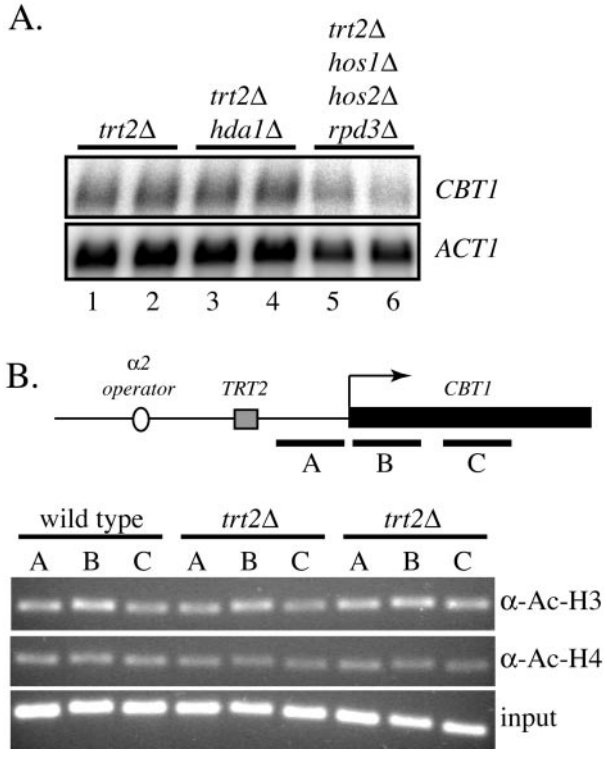

Figure 5. (**A**) Repression of *CBT1* in *trt2*Δ strains is not relieved by mutation of histone deacetylases. Northern blot analysis of *CBT1* mRNA from *MAT*α *trt2*Δ cells containing HDAC mutations. Lanes 1 and 2, *trt2*Δ (DDY1026 and 1028); lanes 3 and 4, *trt2*Δ *hda1*Δ (DDY1956 and DDY2021); lanes 5 and 6, *trt2*Δ *hos1*Δ *hos2*Δ *rpd3*Δ (DDY1805 and DDY1825). (**B**) Chromatin immunoprecipitation of wild-type and *trt2*Δ strains using anti-acetylated histone H3 and H4 antibodies. *MAT*α strains DDY4 (wild-type) and *trt2*Δ (DDY1026 and DDY1028) were grown and processed for chromatin immunoprecipitation. Primers sets for PCR analysis spanned the indicated regions (approximately 200 bp each PCR product) of the *CBT1* gene. No significant difference in the level of *CBT1* chromatin was seen in immunoprecipitates from wild-type versus *trt2*Δ strains.

sets spanning from −170 to +500 bp from the *CBT1* start codon. The data in Figure 5B showed no significant difference in the amount of immunoprecipitated chromatin between wild-type and *trt2*Δ strains. These results also suggest that gross changes in histone acetylation are not the major determinant in repression of *CBT1* in the *trt2*Δ background, and that other mechanisms of Ssn6–Tup1 repression, either nucleosome positioning or direct interaction with the transcriptional machinery, are responsible (see Discussion).

DISCUSSION

α2 operator-mediated repression is bi-directional at the *STE6* locus

α2 operator sites mediate repression of transcription of *MAT***a**-cell-specific genes in *MAT*α cells (20), and also regulate recombination enhancer activity in mating type switching (33,34). Transcriptional repression is mediated by binding of the α2/Mcm1p complex to the operator sites, which then recruit co-repressors such as the Ssn6p/Tup1p complex. Transcriptional repression by α2 operator sequences is mediated by the further recruitment of various histone deacetylases by Ssn6p/Tup1p (21,23), by the precise stable positioning of nucleosomes at the promoter region of the regulated gene (18,19,35), and possibly by direct interaction with transcriptional machinery (24,25). Despite a degree of asymmetry of natural α2 operator sites in Mcm1p/α2p regulated genes, cloned α2 operators in either orientation are able to repress transcription of plasmid-based reporter genes (30), suggesting that repression can spread bi-directionally from an α2 operator. This observation led us to analyze whether repression from the α2 operator upstream of the *STE6* gene spreads bi-directionally on the native chromosome, and whether the *TRT2* $tRNA^{Thr}$ gene upstream acts as a barrier to such repression.

The results shown in Figures 1 and 2 show that the *STE6* α2 operator can partially repress upstream genes specifically in *MAT*α cells in a *URA3* modified, or native chromosome XI lacking *TRT2*. The results from the northern blot analysis of *CBT1* mRNA in *trt2*Δ strains shows a 3-fold repression compared to wild-type cells. This repression is clearly due to the operator sequence, as its deletion restores both the basal and induced levels of *CBT1* transcription (Figure 2). One reason for the relatively mild repression (as compared to the complete repression of *STE6* in *MAT*α cells) could be due to the relative distance between the operator and the gene. The *STE6* gene starts 182 bp from the operator, while the *CBT1* gene is 650 bp away (598 bp in the *trt2*Δ strain). This increased distance may lead to weaker repression compared to that of *STE6*. The range of repression at this locus is limited to the *CBT1* promoter, as deletion of *TRT2* had no effect on expression of *YKL207W*, the next gene centromere proximal to *CBT1* (D. Donze, unpublished data). Another possible reason for the relatively mild repression is the asymmetric nature of the *STE6* α2 operator site, which could lead to differences in repression in each direction. A plasmid-based *lacZ* reporter gene was differentially repressed by opposite orientations of this operator, with the native orientation showing 1.5-fold higher repression then the reverse orientation (30). This asymmetry may lie in an asymmetry of direction of Hda1p activity from the operator, which has been proposed for the *ENA1* promoter (21). Most likely, both distance and orientation are affecting the level of repression of *CBT1* compared to *STE6*.

TRT2 acts as a barrier to repression

Since *MAT*α cell-specific repression of *CBT1* is observed only when the *TRT2* gene is deleted (or contains only a *box* B point mutation; D. Donze, unpublished data), *TRT2* is acting as a barrier to the spread of α2 operator-mediated repression. We have previously shown that the *HMR*-tRNA (*$tRNA^{Thr}$[AGU] CR1*) acts as a barrier to the spread of silencing at the *HMR* locus, as it blocks repression of a *MATa1* reporter gene when juxtaposed between the gene and the silencer, and its deletion from the natural chromosome leads to a 60% reduction of expression of the downstream *GIT1* gene (17). When tested alongside the *HMR*-tRNA in the *MATa1* reporter gene assay, *TRT2* showed a partial barrier activity to Sir protein-mediated silencing (17), while it appears to completely prevent the spread of α2 operator repression in this study. Therefore, different tRNA genes may vary in their ability to block repression, or may have evolved specificities for different types of repression.

The upstream spread of repression from the α2 operator into *CBT1* does not appear to be mediated by major changes in histone acetylation, as suggested by the data in Figure 5. Deletion of HDACs known to be involved in Ssn6p–Tup1p-mediated repression do not result in derepression of *CBT1* in *trt2*Δ strains, and chromatin immunoprecipitation with antibodies against acetylated histone H3 or H4 show no difference in the amount of *CBT1* DNA immunoprecipitated in wild-type versus *trt2*Δ *MAT*α strains. However, it may be that specific histone deacetylation events may be responsible, which would require a detailed analysis with antibodies specific for individual acetylated residues. Tup1p has been shown to utilize multiple mechanisms to repress transcription including recruitment of HDACs (21–23), inducing the stable positioning of nucleosomes (18,19,36), and also by direct interaction with the transcriptional machinery (24,25,37). The results presented here suggest that the latter two mechanisms of Tup1 transcriptional inhibition are most likely at work in the repression of *CBT1* observed in the absence of *TRT2*. Since active tRNA genes have been demonstrated to directly override nucleosome positioning signals (11), we suggest that the barrier activity of *TRT2* is at least in part due to an ability to block the spread of phased nucleosomes emanating from the α2 operator.

In the absence of repression, deletion of *TRT2* results in elevated *CBT1* mRNA levels

Transfer RNA genes in *S.cerevisiae* have been shown to exert a phenomenon referred to as either tRNA-mediated gene silencing or tRNA position effect. In the limited number of cases studied so far, a tRNA gene can exert a repressive effect on transcription from a nearby RNA polymerase II promoter, and this repression requires a transcriptionally active tRNA gene, or at least one competent to bind $TF_{III}C$ (13–15). The genome-wide extent of tRNA position effects is unknown, as it has previously only been observed at a single native chromosomal locus, *PTR3*. However, bioinformatic analysis suggests that tRNA position effects may exert a modest but general effect on nearby RNA polymerase II promoters at many loci, and has

been suggested that position effects may regulate expression of genes that are derepressed when tRNA expression is down-regulated (15). The results shown in Figure 3 demonstrate that deletion of *TRT2* increases *CBT1* expression in *MAT***a** cells, where α2 operator-mediated repression is absent. This provides a second example of a potential tRNA position effect on a native gene, supporting the bioinformatic predictions.

It should be noted that the mechanism of tRNA position effects has not been studied in detail on native chromosomal genes. Active tRNA genes have been shown to be localized to nucleoli in *S.cerevisiae* (38,39), and a mutation in the putative psuedouridine synthetase gene *CBF5* disrupts both nucleolar localization of tRNA synthesis and suppresses tRNA-mediated gene silencing of a plasmid-based reporter gene (14). These studies have suggested that nucleolar localization may be responsible for both tRNA barrier function and tRNA position effects, however, other possibilities exist. One could speculate that inactivation of a tRNA gene could allow upstream activating sequences (UASs) from neighboring genes to inappropriately influence transcription of tRNA proximal genes, suggesting that a tRNA (or an engaged RNA polymerase III complex) might function somewhat as a classic metazoan insulator element, blocking the positive signal from the UAS.

TRT2 transcription is completely resistant to the presence of the α2 operator

Since the *box* B promoter element of *TRT2* lies only 240 bp from the *STE6* α2 operator, we wanted to ask if transcription of *TRT2* itself was affected by its proximity to the repressive element. The results in Figure 4 show that *TRT2* is unaffected by the presence of an active (*MAT*α) or inactive (*MAT***a**) α2 operator, or by deletion of the operator in *MAT*α cells. Therefore even in the presence of a nearby active operator site, a fully functional RNA polymerase III complex can form on the *TRT2* gene and carry out normal levels of transcription. This suggests a hierarchy in the assembly of the RNA polymerase III complex onto a chromosome versus the assembly and propagation of repressive structures, and such a hierarchy may shed some light onto one aspect of the mechanism of the barrier activity of tRNA genes.

Working models of RNA polymerase III transcription depict the stepwise assembly of the $TF_{III}C$ transcription factor complex onto the *box* A and *box* B sites, followed by the recruitment of $TF_{III}B$ proteins Brf1p, Bdp1p, and TBP. Once assembled, this transcription factor platform is able to recruit the RNA polymerase III enzyme complex and initiate transcription (3), in a process that no longer requires $TF_{III}C$. This sequence of events was determined largely from *in vitro* reconstitution experiments, but recent *in vivo* studies suggest a slightly different mechanism.

Chromatin immunoprecipitation studies of human cells progressing through mitosis show that as RNA polymerase III transcription decreases during mitosis, Bdp1p and polymerase subunits are mostly released from chromatin, but Brf1p and TBP remain associated with both tRNA and 5S genes (40). Studies in yeast cells during stationary phase or nutrient limited growth, conditions where RNA polymerase III transcription is markedly reduced, show that polymerase occupancy at a tRNA promoter is severely reduced, while $TF_{III}B$ subunit occupancy is only partially reduced (41,42). Interestingly, these studies show that the association of $TF_{III}C$ appears unchanged or even increased under conditions of reduced tRNA transcription. These results suggest a persistent association of at least part of the RNA polymerase III machinery with its target loci independent the transcriptional state of the gene. This partial association of RNA polymerase III transcription factors is also seen at *ETC* loci (extra $TF_{III}C$), which appear to have $TF_{III}C$ constitutively bound in the absence of $TF_{III}B$ and polymerase (43). The persistent association of RNA polymerase III factors may in one sense serve as an 'epigenetic mark' of these loci for polymerase reassembly when changing conditions require the resumption of RNA polymerase III transcription. Such persistent 'marking' of RNA polymerase III promoters may also relate to their barrier function, as it might allow a preferential reassembly of the RNA polymerase III transcription complex after replication, even if the promoter lies adjacent to silencers or other repressive operator elements.

Another feature of RNA polymerase III that may contribute to barrier function is a process called facilitated recycling. Stably bound RNA polymerase III complexes are known to direct multiple rounds of transcription *in vitro* (44,45), and an individual enzyme complex appears to be able to recycle multiple times on an individual template without the need to reform a preinitiation complex (5–7). Although observed *in vitro*, this hyper-processive and persistent occupation of the RNA polymerase III complex is likely to occur *in vivo* to account for the transcription rate required to produce the large number of tRNA molecules per yeast cell. Such a persistent occupation of tRNA genes during all phases of the cell cycle could contribute to the barrier function of tRNA genes by again physically, and perhaps enzymatically (46) preventing the spread of repressive chromatin. With regard to the data in Figure 4, the level of *TRT2* transcription from its single locus is identical with or without an active α2 operator, indicating that *TRT2* is transcribed at normal levels by the RNA polymerase III machinery even when adjacent to repressive chromatin. This suggests that RNA polymerase III complex assembly, function and persistence at *TRT2* are dominant over the encroachment of repressive chromatin structures.

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