Autoregulation of an RNA polymerase II promoter by the RNA polymerase III transcription factor III C ($TF_{III}C$) complex

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Extra $TF_{III}C$ (ETC) sites are chromosomal locations bound in vivo by the RNA polymerase III (Pol III) transcription factor III C ($TF_{III}C$) complex, but are not necessarily associated with Pol III transcription. Although the location of ETC sequences are conserved in budding yeast, and similar sites are found in other organisms, their functions are largely unstudied. One such site, ETC6 in Saccharomyces cerevisiae, lies upstream of TFC6, a gene encoding a subunit of the $TF_{III}C$ complex itself. Promoter analysis shows that the ETC6 B-box sequence is involved in autoregulation of the TFC6 promoter. Mutation of ETC6 increases TFC6 mRNA levels, whereas mutation immediately upstream severely weakens promoter activity. A temperature-sensitive mutation in TFC3 that weakens DNA binding of $TF_{III}C$ also results in increased TFC6 mRNA levels; however, no increase is observed in mutants of $TF_{III}B$ or Pol III subunits, demonstrating a specific role for the $TF_{III}C$ complex in TFC6 promoter regulation. Chromatin immunoprecipitation shows an inverse relationship of $TF_{III}C$ occupancy at ETC6 versus TFC6 mRNA levels. Overexpression of TFC6 increases association of $TF_{III}C$ at ETC6 (and other loci) and results in reduced expression of a TFC6 promoter-URA3 reporter gene. Both of these effects are dependent on the ETC6 B-box. These results demonstrate that the TFC6 promoter is directly regulated by the $TF_{III}C$ complex, a demonstration of an RNA polymerase II promoter being directly responsive to a core Pol III transcription factor complex. This regulation could have implications in controlling global tRNA expression levels.

The eukaryotic RNA polymerase III (Pol III) system is responsible for synthesizing transfer RNA molecules and other transcripts, which in yeast include the U6 spliceosomal RNA, 7SL RNA, 5S ribosomal RNA, *snr52* small nucleolar RNA, and the RNA component of RNaseP (1–3). Transcription by Pol III requires the activity of the multisubunit transcription factor III C ($TF_{III}C$) complex, which binds to conserved A-box and B-box Pol III promoter elements and functions to overcome chromatin repression of Pol III transcription and to recruit the $TF_{III}B$ complex (4–6). Although Pol III and its transcription factors are thought to be dedicated to transcription of these specific genes, a growing body of evidence has shown that both partial and complete chromosomally bound Pol III complexes can have effects on nearby RNA polymerase II (Pol II) promoters (7–11). Chromatin-bound Pol III complexes also mediate other extratranscriptional functions, including targeting Ty element integration (12–14), blocking replication fork progression (15), condensin and cohesin recruitment (16, 17), and direct inhibition of transcription from nearby Pol II promoters (9, 18–20).

Studies in both budding and fission yeast initially identified the presence of genome sequences that bind the $TF_{III}C$ complex, but not Pol III transcription factors $TF_{III}A$ and $TF_{III}B$ or the Pol III enzymatic complex itself (10, 21–23). Recently, similar sites have been identified in human cells (24–27). These B-box–containing sequences are referred to as either *E*xtra *T*$F_{III}C$ (*ETC*) or $TF_{III}C$-only sites in budding yeast and as chromatin organizing clamps (COCs) in fission yeast (10, 21, 23). Particular $TF_{III}C$-binding sites have been shown to function as chromatin boundary elements (8, 10), but the genome-wide function of the $TF_{III}C$-bound *ETC* sites remains unknown.

Interestingly, one *ETC* site in *Saccharomyces cerevisiae*, *ETC6*, lies within the promoter of the *TFC6* gene, which encodes a subunit of the $TF_{III}C$ complex itself. We hypothesized that the Tfc6 protein, as part of the $TF_{III}C$ complex, might autoregulate its own promoter by binding to *ETC6*. Autoregulation of gene expression is critically important in all forms of life, from its role in the lysogen/lytic growth decision of bacteriophage λ (28) to its important roles in developmental and neuronal gene expression in metazoans (29, 30). Our results identify the B-box within *ETC6* as a functional regulatory element within the *TFC6* promoter that mediates stringent autoregulation of the promoter; this regulation is sensitive to Tfc6 protein levels and binding of the $TF_{III}C$ complex. This appears to be a demonstration of a core Pol III transcription factor complex directly regulating the transcription of a Pol II-transcribed promoter, and this tight control of Tfc6p levels could be important in regulating global tRNA expression, which could have subsequent global effects on translational regulation.

Results

Inhibition of $TF_{III}C$ Binding to *ETC6* Results in Increased *TFC6* Transcript Levels. We used a combined transcript mapping, bioinformatics, and mutational approach to identify the potential promoter elements upstream of *TFC6*. 5′-RACE analysis was performed to map transcriptional start sites, which were identified at bases minus 46, 96, 98, 104, and 110 from the annotated *TFC6* translational start site (Fig. 1*A*, detailed in Fig. S1). Mapping of the start sites allowed us to focus on the upstream region to identify promoter elements. Comparison of the *TFC6* promoter regions from five budding yeast species revealed regions of high conservation in addition to the *ETC6* site B-box sequence. Regions containing six or more bases common to all five species over a 12- base stretch were designated as promoter boxes 1–7, as shown schematically in Fig. 1*A* (and at sequence level detail in Fig. S1). These 12-bp boxes were mutated on plasmids and reintegrated into the yeast genome, and Northern blot analysis for *TFC6* mRNA was performed for each mutant.

The results in Fig. 1*B* show that the major effects were seen clustered across promoter mutants 3, 4, and 5. Mutant 3 significantly decreased *TFC6* mRNA level, and this mutant is compromised for growth due to limiting *TFC6* expression, as complementation with a *TFC6* plasmid restores normal growth (Fig. S2). Mutants 4 and 5, which both span the *ETC6* site, show a twofold increase in *TFC6* mRNA levels, which is consistent with our previous results deleting this site (8). These results are consistent with mutant 3 affecting a transcription factor binding site and with the *ETC6* B-box being involved in negative regulation of the *TFC6* promoter.

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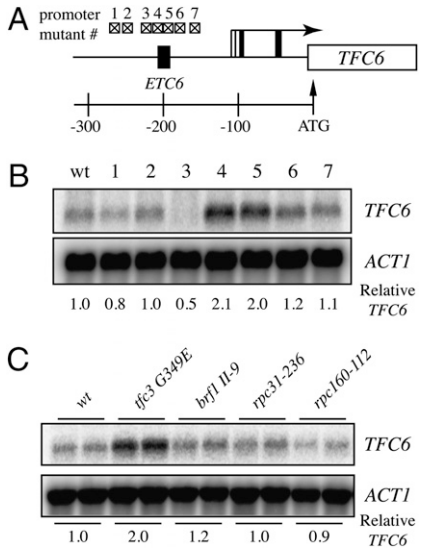

Fig. 1. Characterization of the *S. cerevisiae TFC6* promoter suggests autoregulation by the $TF_{III}C$ complex. (*A*) Transcriptional start sites upstream of *TFC6* were mapped by 5′-RACE analysis and are detailed in Fig. S1. *TFC6* promoter regions of highest homology among five budding yeast species are designated as promoter boxes 1–7 and are also detailed in Fig. S1. (*B*) Mutant promoters were reintegrated into the yeast chromosome, and relative *TFC6* mRNA levels were determined by Northern blotting. Expression was determined from three independently isolated strains for each mutation; one each is shown here. (*C*) Temperature-sensitive mutation in *TFC3*, but not in other Pol III mutations, results in increased *TFC6* transcript levels. Strains containing mutant alleles of $TF_{III}C$, $TF_{III}B$, and Pol III components were grown at permissive temperature (30 °C) and then pulsed for 1 h at the nonpermissive temperature (37 °C) before RNA extraction and Northern analysis.

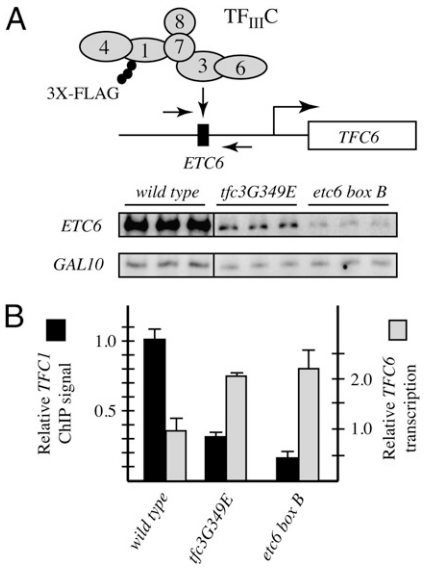

Fig. 2. $TF_{III}C$ binding to *ETC6* is inversely correlated to *TFC6* mRNA levels. (*A*) Strains containing 3×FLAG epitope-tagged *TFC1* and either *tfc3*-G349E or *etc6* B-box mutant alleles were constructed, chromatin extracts were prepared for immunoprecipitation, and relative $TF_{III}C$ association at the *TFC6* promoter was determined. (*B*) Reduction of $TF_{III}C$ binding by either mutation is correlated to increased *TFC6* mRNA levels.

To further test the hypothesis that $TF_{III}C$ binding to *ETC6* is involved in *TFC6* autoregulation, we performed *TFC6* Northern blots on strains containing conditional mutations of the RNA polymerase III machinery. The mutant *tfc3*-G349E is a temperature-sensitive allele of a $TF_{III}C$ component that reduces binding affinity (measured in vitro) of the $TF_{III}C$ complex for tDNAs (31). Mutations *brf1* II-9 and II-6 are impaired in Brf1p interaction with TBP (32), *rpc31*-236 is defective in Pol III initiation (33), and *rpc160*-112 is defective in elongation (34). The results in Fig. 1*C* demonstrate that only the *tfc3*-G349E mutant contained increased *TFC6* transcript levels. This is consistent with direct $TF_{III}C$-mediated regulation of *TFC6* transcription, and not a result of reduced Pol III activity, as the other mutations that globally impair Pol III transcription had little effect.

Inverse Correlation of $TF_{III}C$ Association at *ETC6* and *TFC6* Transcript Levels. To confirm that the mutant *etc6* and *tfc3* strains were indeed defective for in vivo binding of $TF_{III}C$ to *ETC6*, we performed chromatin immunoprecipitation (ChIP) against a carboxyl-terminal 3×FLAG-epitope tagged Tfc1p subunit (Fig. 2) in both *tfc3*-G349E and *etc6* B-box mutant strains. The B-box mutation changes a cytosine residue conserved in all $TF_{III}C$ binding sites to a guanine and is known to inhibit $TF_{III}C$ binding in vitro (35). The results in Fig. 2*A* illustrate that both mutations lead to loss of $TF_{III}C$ association with the *TFC6* promoter in vivo, and reduced binding correlates with the relative increase in *TFC6* transcript levels in the same mutants (Fig. 2*B*). The reduced ChIP signal in the *tfc3* mutant was not due to reduced levels of Tfc1p, as Western blot analysis of wild-type and *tfc3*-G349E mutant show similar Tfc1p levels (Fig. S3).

Overexpression of *TFC6* Inhibits Expression from Its Own Promoter. If Tfc6 protein levels are directly autoregulating Tfc6's own promoter, then overexpression of *TFC6* from an episomal plasmid can be predicted to reduce transcription from the endogenous chromosomal promoter. To test this hypothesis, we created diploid yeast strains that have the *URA3* ORF precisely replacing one chromosomal copy of the *TFC6* ORF (Fig. 3*A*). These strains allowed us to assess the level of *TFC6* promoter activity independently of episomal expression by assessing growth on media lacking uracil. *TFC6* was overexpressed in strain DDY4520, both from its own promoter on a high-copy plasmid containing the entire *TFC6* gene and from the *ADH1* promoter on a low-copy plasmid. Increased *TFC6* expression has no effect on growth of this strain on media lacking only histidine compared with cells transformed with the *HIS3* vector (Fig. 3*B*), showing that increased Tfc6p levels alone do not inhibit growth. However, when the same cells were plated on minimal media lacking both histidine and uracil, the average colony sizes formed by cells containing either the high-copy or *ADH1*-promoter plasmid were consistently 65–70% of controls containing empty vector. This effect is dose-dependent, as expression of *TFC6* from its own promoter on a lower-copy *ARS-CEN* plasmid reduces average colony size to only 89% of controls (Fig. S4). ChIP against *TFC1*-3×FLAG showed that overexpression of *TFC6* resulted in increased association of $TF_{III}C$ at *ETC6* (Fig. 3*C*), as the amount of *TFC6* promoter DNA immunoprecipitated was ~1.7 times the vector control. This correlated with a decrease in TATA binding protein (TBP) association at the *TFC6* promoter, as the anti-TBP ChIP signal was only ~70% compared with the vector control (Fig. 3*C*). These results show that overexpression of *TFC6* increases the degree of $TF_{III}C$ association with *ETC6* and reduces expression from its own promoter, presumably due to increased stability of $TF_{III}C$ binding to the *ETC6* site, leading to reduced TBP association.

Autoregulation of *TFC6* Is *ETC6* Site B-Box–Dependent and Tfc6p-Specific. If increased binding of $TF_{III}C$ to *ETC6* is indeed responsible for reduced growth on media lacking uracil, then a strain with *URA3* driven by a *TFC6* promoter containing the defective B-box within *ETC6* would be insensitive to overexpressed *TFC6* when grown on media lacking uracil. Strain DDY4521 is identical to DDY4520 except for the presence of the C-to-G mutation in the *ETC6* B-box upstream of the *URA3* marker. The results in Fig. 4*A* confirm that the inhibition is

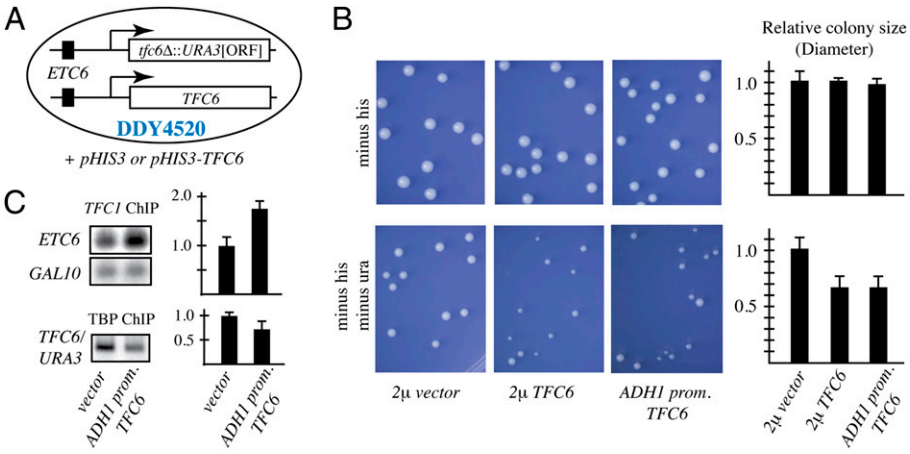

Fig. 3. Overexpression of Tfc6p down-regulates gene expression driven by the *TFC6* promoter and increases the association of the $TF_{III}C$ complex to *ETC6*. (*A*) Diploid strain DDY4520 was constructed to contain the *URA3* ORF integrated in place of the *TFC6* ORF on one copy of chromosome IV to test the effects of episomal *TFC6* overexpression. (*B*) Vector controls, 2μ *HIS3* TFC6, or ARS-CEN *HIS3 ADH1*-promoter-*TFC6* plasmid transformants were plated on media lacking histidine or both histidine and uracil, and colony sizes were measured after 3 d (minus histidine) or 5 d (minus histidine and minus uracil) at 25 °C. (*C*) ChIP of *TFC1*-3×FLAG strains transformed with vector or *ADH1* promoter-*TFC6* plasmid show increased Tfc1p association and decreased TBP at *ETC6* when *TFC6* is overexpressed. Quantitative results were averaged from three separate determinations.

mediated through the *ETC* site, as when *TFC6* is overexpressed in the strain containing the mutant B-box; no reduction of colony size is observed on media lacking uracil.

We next asked if overexpression of other $TF_{III}C$ subunits would affect *URA3* expression from the *TFC6* promoter. Large-scale proteomic studies of yeast protein expression have estimated the number of protein molecules per yeast cell (36), and the results suggest that Tfc3p, Tfc4p, and Tfc6p are the most limiting components of the $TF_{III}C$ complex. Tfc1p appears to be present in large excess, whereas Tfc7p and Tfc8p are at intermediate levels. Indeed, this apparent excess of at least Tfc1p and Tfc7p was determined to exist as a chromatographically separable subcomplex in yeast extracts (37). We confirmed that Tfc6p is limiting relative to Tfc1p, as Western blots of protein extracts from strains containing the identical triple FLAG epitope on each gene show a large relative excess of Tfc1p compared with Tfc6p (Fig. S5). These endogenous ratios suggest that overexpression of other limiting subunits might also increase the level of $TF_{III}C$ complex binding to *ETC6* and reduce *TFC6* promoter activity, whereas overexpression of *TFC1* should have no effect because it is already in excess. Plasmids (2μ) containing *TFC1*, *TFC3*, *TFC4*, and *TFC6* were separately transformed into strain DDY4403 (*TFC6* promoter-*URA3*, similar to DDY4520, but in the S288C background) and plated on media lacking both histidine and uracil. Colony sizes were determined at 6 days of growth, and the results are shown in Fig. 4*B*. As expected, overexpression of *TFC1* had no effect on cell growth, nor did overexpression of *TFC4*. Expression of *TFC3* appeared to inhibit growth slightly, but not as much as *TFC6*. These results demonstrate that the *TFC6* promoter is preferentially sensitive to increased levels of its cognate gene product.

Overexpression of *TFC6* Results in Elevated $TF_{III}C$ Association at Multiple Loci. Because $TF_{III}C$ binding at *ETC6* was increased upon overexpression of Tfc6p, we tested other B-box–containing loci by ChIP for enrichment of the $TF_{III}C$ complex. The results in Fig. 5 demonstrate that, at all loci tested, which included three tDNAs, the *ZOD1*/*UFO1* locus, and *ETC4* and *ETC5*, an increase in $TF_{III}C$ association was observed upon episomal expression of *TFC6*. The magnitude of this increase varied from 1.2-fold to over 2-fold. Despite this seemingly general increase in $TF_{III}C$ binding, we have not yet identified any tDNAs or other loci that show altered levels of Pol III transcription (*Discussion*).

Discussion

Although Pol III is dedicated to transcription of tDNAs and a handful of other RNAs, genome-wide ChIP studies in yeast have demonstrated the presence of the transcription factor complex $TF_{III}C$ at chromosomal locations not associated with the Pol III complex (10, 21–23). Recently, similar studies using human cells and high-throughput sequencing detection (ChIP-Seq) have demonstrated the presence of such sites beyond yeast (24–27). These loci—*ETC* sites, COCs, or $TF_{III}C$-only sites—have been shown to affect expression of neighboring Pol II genes by acting as chromatin boundary elements (8, 10). This study set out to further characterize the role of the $TF_{III}C$ binding site *ETC6* in *S. cerevisiae*, which lies in the promoter of the *TFC6* gene encoding a subunit of the $TF_{III}C$ complex itself. The location of this site was noted by Moqtaderi and Struhl in their study characterizing *ETC* sites (23), and they suggested the possibility that $TF_{III}C$ might regulate this promoter. Our results confirm their speculation, as we show that *ETC6* is a functional promoter element of the *TFC6* gene that mediates autoregulation of *TFC6* expression in response to Tfc6 protein levels. We show that inhibition of $TF_{III}C$ binding to *ETC6* results in increased *TFC6* transcript levels, whereas overexpression of Tfc6p increases association of the $TF_{III}C$ complex at *ETC6* and inhibits expression from the *TFC6* promoter. These results suggest that Pol II transcription of *TFC6* is sensitive to the level of its own protein product, a product that is part of what was previously thought to be a dedicated core Pol III transcription factor. Although such crosstalk between Pol II transcription factors and Pol III promoters has been described for the octamer binding proteins and the SNAPc complex in mammalian systems (38), they appear to be general Pol II transcription factors that act on a limited subset of Pol III promoters. Therefore, this does appear to be a demonstration of a core Pol III factor regulating Pol II transcription.

The results presented here also beg the question of how does the $TF_{III}C$ complex inhibit expression from its own promoter? Data in Fig. 3 show reduction in TBP association at the *TFC6* promoter when *TFC6* is episomally overexpressed; although this may be due to direct inhibition of TBP binding, this reduction might also be a consequence of other mechanisms. We previously suggested (8) that inhibition may occur via an insulator-like mechanism, with bound $TF_{III}C$ inhibiting upstream transcription factors from recruiting a productive preinitiation complex at the transcription start site. However, because the key *TFC6* promoter element (mutant site 3) is immediately upstream of the *ETC6* B-box, we

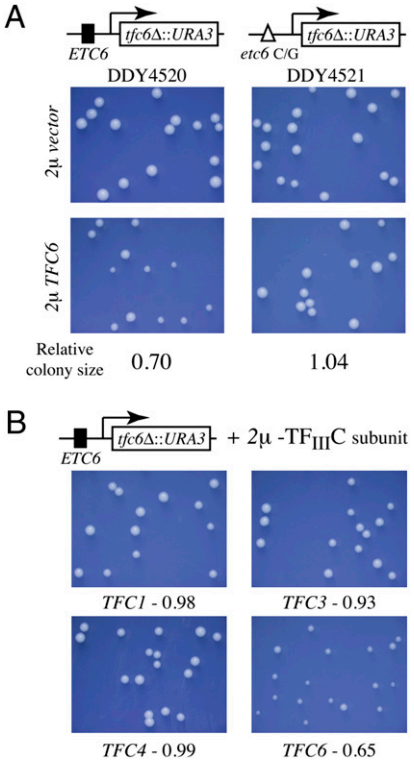

Fig. 4. Down-regulation of the *TFC6* promoter by Tfc6p requires the *ETC6* B-box and is specific to *TFC6* overexpression. (*A*) Strain DDY4521 was constructed to contain a mutant B-box linked in *cis* to the *TFC6* promoter driving *URA3*. *TFC6* was overexpressed as in Fig. 3 and was unable to down-regulate the *TFC6* promoter containing the mutant B-box, as indicated by no change in colony sizes. (*B*) Overexpression of other $TF_{III}C$ subunits in DDY4403. High-copy 2μ plasmids encoding each gene driven by its native promoter were transformed into the *URA3* reporter strain and plated on minimal media lacking histidine and uracil, and colony sizes relative to the vector control were determined as in Fig. 3.

also consider that $TF_{III}C$ and the putative transcription factor may be in competition for binding to the same region of DNA.

Although much work has been done on the global control of Pol III transcription by the Maf1-mediated pathway (39–41), few studies have looked at the role of the regulation of expression of the Pol III transcription factors themselves. In yeast, over-

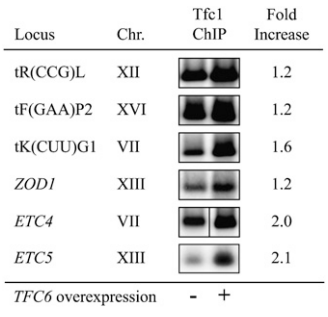

Fig. 5. Overexpression of *TFC6* increases $TF_{III}C$ association at multiple genomic loci. Strain DDY4381 (*TFC1*-3×FLAG) was transformed with either empty vector or pDD1234 (*ADH1* promoter-*TFC6*) to overexpress Tfc6p. Binding of $TF_{III}C$ was assessed at several B-box sites by ChIP using anti-FLAG antibody; each B-box site showed increased enrichment when Tfc6p was overexpressed. As in Fig. 3, determinations were performed in triplicate and normalized to the *GAL* locus signal; one pair of lanes is shown for each locus.

expression of $TF_{III}B_{70}$ (Brf1p) elevates expression of promoter mutant tDNAs (42). In mammalian cells, overexpression of Brf1 stimulates Pol III transcription, and shRNA inhibition of Brf1 expression reduces oncogenic transformation and tumor formation in a mouse model (43, 44). These results indicate that levels of the Pol III transcription factors can have critical roles in regulating Pol III transcription and cell proliferation.

Autoregulatory circuits have been identified as key components controlling gene expression and have evolved in organisms from bacteriophage to humans (30). Bacteriophage λ uses its CI repressor protein to both positively regulate its own expression and then negatively regulate itself when the cellular concentration of the protein reaches proper levels, a key circuit in maintaining the inducible lysogenic state (28). Neuronal terminal differentiation genes in *Caenorhabditis elegans* are controlled by autoregulated terminal selector transcription factors, and disruption of this process can lead to defective neuron function (29); many other instances of autoregulation could be cited (30). Given these considerations, the results presented here suggest that in yeast there exists a tight autoregulation of *TFC6* expression that maintains its protein product as a limiting component of the $TF_{III}C$ complex. This fact raises the question as to why yeast need to maintain such stringent control of Tfc6p expression and therefore of $TF_{III}C$ activity. Because we observe that overexpression of Tfc6p differentially increases the Tfc1p ChIP signal at several loci (Fig. 5), we speculate that altered Tfc6 levels might differentially regulate $TF_{III}C$ occupancy genome-wide and possibly differentially affect expression levels of tRNAs and other Pol III transcripts. Recent studies have shown that slowly translated rare/suboptimum codons play a role in fine-tuning translational regulation and protein stability and activity (45–49); therefore, altered Tfc6p levels might differentially affect the production of tRNAs decoding these regulatory codons, potentially having global effects on translational regulation. Although we have not yet detected any differences in Pol III transcription upon overexpression of Tfc6p (from a limited set of Pol III-transcribed genes tested), a genome-wide analysis may reveal particular tDNAs whose expression is altered. Because many tDNAs are present in multiple copies, such differences may be revealed only by tagging of individual loci to distinguish altered expression levels.

The work presented here is significant in that it appears to be a demonstration of a core Pol III transcription factor that can directly regulate transcription from a Pol II promoter, in that this stringent regulation could potentially be important in global gene expression, and in that it adds another potential avenue of crosstalk between the different RNA polymerase systems (50). In addition, because *ETC*-like sites have now been confirmed in human cells, the role of the $TF_{III}C$ complex in genome organization and global control of gene expression may be more prevalent than previously realized.

Materials and Methods

5′-RACE analysis was performed using the FirstChoice RLM-RACE kit (Ambion-Applied Biosystems; #AM1700). Construction of the promoter mutants is described in the legend to Fig. S1. Each mutant intergenic region was reintegrated into chromosome IV by transformation into strain DDY3453 (*etc6*Δ::*URA3*) and selection on 5-FOA media and was verified by PCR of genomic DNA and digestion of the PCR products with DrdI to verify the presence of the mutation. DDY3453 was created by standard yeast knockout techniques using oligonucleotides DDO-792 and -793 to amplify *URA3* from plasmid pRS406 (51). The genotypes of all yeast strains used in this study are listed in Table S1, and all plasmids used are described in Table S2. All oligonucleotide sequences are listed in Table S3. Northern blot analyses were performed as described (9).

Plasmids expressing $TF_{III}C$ subunits Tfc1p, Tfc3p, and Tfc6p were constructed by PCR amplification of each gene in addition to ~500 bp upstream and downstream from yeast genomic DNA (primers and details available on request) using the high-fidelity Phusion DNA polymerase (New England Biolabs; F-530S). Functional expression was verified by complementation of mutant strains. *TFC4* was subcloned from a previously characterized plasmid

PCF1 (kindly provided by Ian Willis, Albert Einstein College of Medicine, New York). Each gene was cloned into the *HIS3*-marked pRS series of ARS-CEN and 2μ vectors (51, 52).

ChIP was performed as described (8), using the same *TFC1*-3×FLAG allele crossed into the appropriate strains. Anti-FLAG monoclonal M2 was from Sigma (F1804), and anti-yeast TBP was from Santa Cruz Biotechnology (sc-33736). Quantitation of ChIP signals was determined by radioactive PCR according to Kurdistani and Grunstein (53), except that samples were resolved on 1.2% agarose gels. ChIP signals were normalized to the background PCR signal generated using primers homologous to the non-$TF_{III}C$ binding *GAL1-10* intergenic region (oligos DDO-1023 and -1024) to control for background and sample variation. All quantitative ChIP results were averaged from three independent determinations.

TFC6 promoter-*URA3* ORF reporter strains were constructed by standard yeast recombination methods, using oligonucleotides DDO-1201 and DDO-1202 homologous to the ends of the *URA3* ORF in addition to 50 bases immediately upstream and downstream of the *TFC6* ORF to amplify the coding sequence of *URA3*. Ura+ recombinants expressing *URA3* from the *TFC6* promoter were slow growing on media lacking uracil (requiring 4–5 d to appear) and were slightly temperature sensitive; therefore, all colony growth experiments were performed at 25 °C. To compare colony sizes, *tfc6*Δ::*URA3* cells were transformed with empty pRS vector (51, 52) or *HIS3*-marked $TF_{III}C$ subunit expressing plasmids and plated on minimal media lacking histidine. His+ isolates were grown in liquid media lacking histidine and plated at ∼50 colonies/plate on media lacking histidine and on media lacking both histidine and uracil. Plates were incubated at 25 °C for 3 d (minus histidine) or for 5–6 d (minus histidine and uracil) before photographing. Relative colony sizes from 30 to 50 colonies were measured for each sample using ImageJ software (http://rsbweb.nih.gov/ij/).

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