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Autoregulation of an RNA polymerase II promoter by the RNA polymerase III transcription factor III C (TF\textsubscript{III}C) complex

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Edited by Jasper Rine, University of California, Berkeley, CA, and approved March 25, 2011 (received for review December 20, 2010)

Extra TF\textsubscript{III}C (ETC) sites are chromosomal locations bound in vivo by the RNA polymerase III (Pol III) transcription factor III C (TF\textsubscript{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 \textit{Saccharomyces cerevisiae}, lies upstream of TFC6, a gene encoding a subunit of the TF\textsubscript{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\textsubscript{III}C also results in increased TFC6 mRNA levels; however, no increase is observed in mutants of TF\textsubscript{III}B or Pol III subunits, demonstrating a specific role for the TF\textsubscript{III}C complex in TFC6 promoter regulation. Chromatin immunoprecipitation shows an inverse relationship of TFC6 occupancy at ETC6 versus TFC6 mRNA levels. Overexpression of TFC6 increases association of TF\textsubscript{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\textsubscript{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 splicesome RNA, 7SL RNA, 5S ribosomal RNA, \textit{srr2} small nucleolar RNA, and the RNA component of RNase P (1–3). Transcription by Pol III requires the activity of the multisubunit transcription factor III C (TF\textsubscript{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\textsubscript{III}C 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\textsubscript{III}C complex, but not Pol III transcription factors TF\textsubscript{III}A and TF\textsubscript{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 Extra TF\textsubscript{III}C (ETC) or TF\textsubscript{III}C-only sites in budding yeast and as chromatin organizing clamps (COCs) in fission yeast (10, 21, 23). Particular TF\textsubscript{III}C-binding sites have been shown to function as chromatin boundary elements (8, 10), but the genome-wide function of the TF\textsubscript{III}C-bound ETC sites remains unknown.

Interestingly, one ETC site in \textit{Saccharomyces cerevisiae}, ETC6, lies within the promoter of the TFC6 gene, which encodes a subunit of the TF\textsubscript{III}C complex itself. We hypothesized that the Tfc6 protein, as part of the TF\textsubscript{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 \(\lambda\) (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\textsubscript{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\textsubscript{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. L4, 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. L4 (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. 1B show that the major effects were seen clustered across promoter mutants 3, 4, and 5. Mutant 3 significantly decreased TFC6 mRNA levels, 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.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1019175108/DCSupplemental.
Tfc6 protein levels are directly autoregulating Tfc6 similar Tfc1p levels (Fig. S3). Western blot analysis of wild-type and the B levels in the same mutants (Fig. 2 binding correlates with the relative increase in results in Fig. 2 transcription, and rpc160 interaction with TBP (32), permissive temperature (37 °C) before RNA extraction and Northern analysis. To further test the hypothesis that TFIIIC binding to ETC6 is involved in TFII6 autoregulation, we performed TFII6 Northern blots on strains containing conditional mutations of the RNA polymerase III machinery. The mutant tfc3-G349E is a temperature-sensitive allele of a TFIIIC component that reduces binding affinity (measured in vitro) of the TFIIIC complex for tDNAs (31). Mutations brf1 I–II and II–II are impaired in Brf1p interaction with TBP (32), rpc1p-236 is defective in Pol III initiation (33), and rpc160-112 is defective in elongation (34). The results in Fig. 1C demonstrate that only the tfc3-G349E mutant contained increased TFII6 transcript levels. This is consistent with direct TFIIIC-mediated regulation of TFII6 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 Tfc6 Association at ETC6 and TFII6 Transcript Levels. To confirm that the mutant etc6 and tfc3 strains were indeed defective for in vivo binding of TFIIIC to ETC6, we performed chromatin immunoprecipitation (ChIP) against a carboxy-terminal 3xFLAG-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 TFIIIC binding sites to a guanine and is known to inhibit TFIIIC binding in vitro (35). The results in Fig. 2A illustrate that both mutations lead to loss of TFIIIC association with the TFII6 promoter in vivo, and reduced binding correlates with the relative increase in TFII6 transcript levels in the same mutants (Fig. 2B). 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 TFII6 Inhibits Expression from Its Own Promoter. If Tfc6 protein levels are directly autoregulating Tfc6’s own promoter, then overexpression of TFII6 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 TFII6 ORF (Fig. 3A). These strains allowed us to assess the level of TFII6 promoter activity independently of episomal expression by assessing growth on media lacking uracil. TFII6 was overexpressed in strain DDDY4520, both from its own promoter on a high-copy plasmid containing the entire TFII6 gene and from the ADH1 promoter on a low-copy plasmid. Increased TFII6 expression has no effect on growth of this strain on media lacking only histidine compared with cells transformed with the HIS3 vector (Fig. 3B), 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 TFII6 from its own promoter on a lower-copy ARS-CE/N plasmid reduces average colony size to only 59% of controls (Fig. S4). ChIP against TF1C-3xFLAG showed that overexpression of TFII6 resulted in increased association of TFIIIC at ETC6 (Fig. 3C), as the amount of TFII6 promoter DNA immunoprecipitated was ~1.7 times the vector control. This correlated with a decrease in TATA binding protein (TBP) association at the TFII6 promoter, as the anti-TBP ChIP signal was only ~70% compared with the vector control (Fig. 3C). These results show that overexpression of TFII6 increases the degree of TFIIIC association with ETC6 and reduces expression from its own promoter, presumably due to increased stability of TFIIIC binding to the ETC6 site, leading to reduced TBP association.

Autoregulation of TFII6 Is ETC6 Site B-Box-Dependent and Tfc6p-Specific. If increased binding of TFIIIC to ETC6 is indeed responsible for reduced growth on media lacking uracil, then a strain with URA3 driven by a TFII6 promoter containing the defective B-box within ETC6 would be insensitive to overexpressed TFII6 when grown on media lacking uracil. Strain DDDY4521 is identical to DDDY4520 except for the presence of the C-to-G mutation in the ETC6 B-box upstream of the URA3 marker. The results in Fig. 4A confirm that the inhibition is
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 TFIIIC 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 TFIIIC 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 TFIIIC complex binding to ETC6 and reduce TFC6 promoter activity, whereas overexpression of TFIIIC should have no effect because it is already in excess. Plasmids (2μ) containing TFC1, TFC3, TFC4, and TFC6 were separately transformed into strain DDY4403 (TFC3 promoter-URA3, similar to DDY4520, but in the S288C background) and plated on media lacking both histidine and uracil. Colonies sizes were determined at 6 days of growth, and the results are shown in Fig. 4B. 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 TFIIIC Association at Multiple Loci.** Because TFIIIC binding at ETC6 was increased upon overexpression of Tfc6p, we tested other B-box–containing loci by ChIP for enrichment of the TFIIIC 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 TFIIIC 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 TFIIIC 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 TFIIIC 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 TFIIIC-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 TFIIIC binding site ETC6 in S. cerevisiae, which lies in the promoter of the TFC6 gene encoding a subunit of the TFIIIC complex itself. The location of this site was noted by Moghtaderi and Struhl in their study characterizing ETC sites (23), and they suggested the possibility that TFIIIC 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 TFIIIC binding to ETC6 results in increased TFC6 transcript levels, whereas overexpression of Tfc6p increases association of the TFIIIC 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 TFIIIC 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 TFIIIC 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
expression of TFC6 (Brf1p) elevate 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 TFC6 complex. This fact raises the question as to why yeast need to maintain such stringent control of Tfc6p expression and therefore of TFC6 activity. Because we observe that overexpression of Tfc6p differentially increases the Tfc1p ChIP signal at several loci (Fig. 5), we speculate that altered Tfc6p levels might differentially regulate TFC6 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 TFC6 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. 5. Each mutant intergenic region was reintegrated into chromosome IV by transformation into strain DDY4353 (etc6Δ::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. DDY4353 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 TFC6 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-5305). Functional expression was verified by complementation of mutant strains. TFC4 was subcloned from a previously characterized plasmid.

![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 TFIIIC subunits in DDD4403. 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.](image-url)

![Fig. 5. Overexpression of TFC6 increases TFC6 association at multiple genomic loci. Strain DDDY4381 (TFC1-3xFLAG) was transformed with either empty vector or pDD1234 (ADH1 promoter-TFC6) to overexpress Tfc6p. Binding of Tfc6p 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.](image-url)
PCR signal generated using primers homologous to the non-TFIIIC binding site according to Kurdistani and Grunstein (53), except that samples were re-analyzed. Quantitation of ChIP signals was determined by radioactive PCR method using [3H]dCTP from Amersham (Uppsala, Sweden). Anti-yeast TBP was from Santa Cruz Biotechnology (sc-12,733). Yeast recombination methods, using oligonucleotides DDO-1201 and DDO-1202 homologous to the regions upstream of genes transcribed by RNA polymerase III, were used to select for 


Ghielmini et al. EMBO J 10:3350-3357.


ACKNOWLEDGMENTS. We sincerely thank Ian Willis, Robyn Moir, and Bob White for helpful discussions on this work and Giorgio Dieci for critical reading of the manuscript. This work was funded by Grant MCB-0817823 (to D.D.) from the National Science Foundation.