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Research paper

Transcription factor Reb1 is required for proper transcriptional start site usage at the divergently transcribed *TFC6-ESC2* locus in *Saccharomyces cerevisiae*

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ABSTRACT

Eukaryotic promoters generally contain nucleosome depleted regions near their transcription start sites. In the model organism *Saccharomyces cerevisiae*, these regions are adjacent to binding sites for general regulatory transcription factors, and the Reb1 protein is commonly bound to promoter DNA near such regions. The yeast *TFC6* promoter is a unique RNA polymerase II promoter in that it is autoregulated by its own gene product Tfc6p, which is part of the RNA polymerase III transcription factor complex TFIIIC. We previously demonstrated that mutation of a potential Reb1 binding site adjacent to the TFIIIC binding site in the *TFC6* promoter modestly reduces transcript levels, but leads to a severe decrease in Tfc6 protein levels due to an upstream shift in the *TFC6* transcription start site. Here we confirm that Reb1p indeed binds to the *TFC6* promoter, and is important for proper transcription start site selection and protein expression. Interestingly, loss of Reb1p association at this site has a similar effect on the adjacent divergently transcribed *ESC2* promoter, resulting in a significant increase of 5′-extended *ESC2* transcripts and reduction of Esc2 protein levels. This altered divergent transcription may be the result of changes in nucleosome positioning at this locus in the absence of Reb1p binding. We speculate that an important function of general regulatory factors such as Reb1p is to establish and maintain proper transcription start sites at promoters, and that when binding of such factors is compromised, resulting effects on mRNA translation may be an underappreciated aspect of gene regulation studies.

1. Introduction

Active eukaryotic RNA polymerase II (Pol II) promoter regions within cells are characterized by nucleosome depleted regions (NDRs) in the vicinity of transcription start sites (TSS). In the budding yeast *S. cerevisiae*, this fact has been demonstrated by both *in vivo* and *in vitro* genome-wide nucleosome mapping studies (Yuan et al., 2005; Mavrich et al., 2008a; Kaplan et al., 2009; Nagarajavel et al., 2013), and further analysis confirmed a similar architecture in metazoans (Mavrich et al., 2008b). Nucleosomes adjacent to promoter NDRs, referred to as the −1 and +1 nucleosomes, appear to be relatively well positioned within populations of cells, and are thought to influence the transcriptional start site (Jiang and Pugh, 2009). Passive positioning of these promoter-proximal nucleosomes appears to be determined both by intrinsic underlying DNA sequences and nearby DNA bound proteins (Kaplan et al., 2009; Zhang et al., 2009), and maintenance of NDRs appears to involve active processes utilizing chromatin remodelers such as the RSC and ISW2 complexes (Badis et al., 2008; Hartley and Madhani, 2009; Yen et al., 2012; Ganguli et al., 2014).

In *S. cerevisiae*, several general regulatory factors (GRFs) have been directly implicated in contributing to NDR formation, and include Abf1, Cbf1, Rap1, Reb1, and Tbf1 proteins (Kent et al., 1994; Yarragudi et al., 2004; Badis et al., 2008; Hartley and Madhani, 2009; Tsankov et al., 2010; Ganapathi et al., 2011; Tsankov et al., 2011). Reb1p is a sequence-specific DNA binding protein containing two myb-like regions, and is essential for viability in *S. cerevisiae* (Morrow et al., 1989; Ju et al., 1990; Morrow et al., 1990; Morrow et al., 1993). Reb1p can bind to sites within transcriptional control regions of genes transcribed by either RNA polymerase I or RNA polymerase II (Chasman et al., 1990; Morrow et al., 1990; Wang et al., 1990), recognizing a consensus YYACCCG sequence (Liaw and Brandl, 1994). Numerous studies have revealed that Reb1p is involved in the regulation of transcription by acting as a weak activator (Brandl and Struhl, 1990; Chasman et al., 1990; Remacle and Holmberg, 1992). Furthermore, Reb1p was demonstrated to bind the terminator of the rRNA transcription unit, and was thought to be involved in the termination of transcription by RNA polymerase I (Lang and Reeder, 1993), however further studies demonstrated that this function is mediated by another myb-domain protein Nsi1p

Abbreviations: Pol, RNA polymerase; NDR, nucleosome depleted region; NFR, nucleosome free region; TSS, transcription start site.

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(Reiter et al., 2012). Recently, Reb1p was uncovered to have a roadblock function that terminates progressing Pol II transcription, which in turn restricts pervasive cryptic transcription and readthrough transcription in the yeast genome (Colin et al., 2014). Also, Reb1p was reported to interact with the RSC (remodels the structure of chromatin) complex, and is involved in the formation of nucleosome free regions (NFR) (Raisner et al., 2005; Wippo et al., 2011).

Previous results from our lab demonstrated that the *TFC6* promoter is autoregulated by its own gene product Tfc6p, which is one component of the six-polypeptide RNA polymerase III (Pol III) transcription factor complex TFIIIC (Kleinschmidt et al., 2011). Forced overexpression of Tfc6p increased *in vivo* binding of the entire TFIIIC complex to the *TFC6* promoter (at its binding site referred to as *ETC6*, extra TFIIIC-6), and overexpression also resulted in decreased expression of a marker gene driven by the *TFC6* promoter. This Tfc6p mediated Pol II regulation was unexpected, as TFIIIC predominately regulates Pol III promoters, and this was a unique finding of a Pol III transcription factor directly regulating a Pol II promoter. The same study demonstrated that mutation of the *TFC6* promoter just upstream of *ETC6* (encompassing a sequence identical to the Reb1p consensus binding site) results in a slow growth phenotype that is rescued by episomal expression of Tfc6 protein, and results in ~50% reduction in *TFC6* mRNA levels (Kleinschmidt et al., 2011). More detailed analysis of yeast containing this Reb1p binding site mutation demonstrated that a 5′-extended *TFC6* mRNA was produced, which led to an approximately 15-fold reduction in Tfc6 protein levels (Wang et al., 2014).

Based on our previous work indicating that ectopic overexpression of Tfc6p leads to increased TFIIIC complex binding to *ETC6* and downregulation of *TFC6* promoter activity, and given the fact that an apparent Reb1p consensus binding site is located just upstream of the *ETC6* site, we wanted to verify whether Reb1p actually binds to the *TFC6* promoter, and how both Reb1p and TFIIIC complex binding mediate regulation of *TFC6*. Both *in vivo* and *in vitro* experiments described here demonstrate that Reb1p indeed binds to and is required for normal *TFC6* expression, and while it does not appear to be involved in autoregulation, Reb1p activity affects normal transcription start site selection at *TFC6*. Metabolic depletion of Reb1p phenocopies the *TFC6* promoter mutant in that the depleted cells contain increased levels of *TFC6* mRNA with an extended 5′-end, and also show reduced Tfc6 protein levels under Reb1p depleted conditions. Interestingly, loss of Reb1p binding to this promoter site also results in a 5′-extension and reduced translation of the divergently transcribed *ESC2* gene. Finally we show that nucleosome occupancy within this intergenic region is altered when Reb1p binding is compromised, which may contribute to the observed alteration in transcription start site usage.

2. Materials and methods

2.1. Yeast strains and growth media

All yeast strains were generated from the W303-1a background. The construction of Reb1 consensus binding site mutant strain DDY4300, containing a 12-bp mutation in the *TFC6* promoter, was described previously (Kleinschmidt et al., 2011). 3X-FLAG epitope tagged strains used for Western blot analysis were constructed by amplifying plasmid P3-FLAG-KanMX (Gelbart et al., 2001) with ~65 bps flanking sequence on each side of the stop codon of the target gene. Specific yeast strains for Reb1p depletion were constructed by integrating the *GAL1* promoter upstream of the *REB1* open reading frame. Plasmid pFA6a-KanMX6-p*GAL1* (Longtine et al., 1998) was amplified with primers DDO1793/1794 containing 65 bps of homology upstream of the *REB1* start codon and 63 bps homology downstream of the start codon, attached with 20 bp homology to either end of the KanMX-*GAL1* promoter cassette. The PCR product was purified and transformed into a diploid yeast strain DDY5240 that was previously engineered to contain a 9X-Myc-*TRP1* epitope tag at the end of both copies of the Reb1 coding sequence using plasmid pYM6 (Knop et al., 1999) and oligos DDO-1591/1592. Transformed colonies were selected on YPGal plates containing 200 μg/ml geneticin. Resistant colonies were confirmed by PCR with primer sets DDO-466/1797 and DDO-1797/RR00013. Positive PCR products were further confirmed by sequencing, and the corresponding strains were sporulated to obtain haploids. G418 resistant haploids were re-confirmed by PCR with the same primer sets. Analogous nourseothricin resistant strains were constructed by transforming the KanMX strains with a PCR product amplified from the NatMX6-pGAL cassette in plasmid pFA6a–NatMX6-p*GAL1* (Hentges et al., 2005; Van Driessche et al., 2005) using oligos SWAP F and SWAP R to interchange the marker genes.

Yeast cultures were grown in nutrient rich YPD media (1% yeast extract, 2% peptone, and 2% dextrose). *GAL* promoter conditional strains were grown in YPGal, and shifted to YPD for depletion. Colony growth assays in Fig. 1 were performed on YMD agar plates (yeast nitrogen base, U.S. Biologicals #Y2025) with the indicated dropout supplements. All yeast cultures were grown at 30 °C.

2.2. In vivo reporter assays of Reb1p function at the TFC6 promoter

HIS3 marked plasmids expressing either 3X-FLAG-*REB1* or 3X-FLAG-*NSI1* driven by the *ADH1*-promoter or empty *ADH1*-promoter vector were transformed into yeast strain DDY4521 (Kleinschmidt et al., 2011). This diploid strain has one copy of the *TFC6* open reading frame (ORF) replaced with the *URA3* ORF as a reporter gene, and cell growth on the minimal medium lacking uracil was used as an indicator of *TFC6* promoter activity. After transforming with each plasmid and selecting on media lacking histidine, cultures were grown in liquid media and plated at approximately 100 colony forming units per plate on medium lacking either histidine or lacking both histidine and uracil. Relative colony size was measured using ImageJ software (http://imagej.nih.gov/ij/).

2.3. Recombinant Reb1p DNA binding domain expression and purification

The *REB1* DNA binding domain coding sequence was cloned (essentially as described in Morrow et al., 1993) into pET30A(+) (Novagen) to include an N-terminal His-tag for overexpression and purification in *E. coli*. The targeted *REB1* sequence was amplified from full length *REB1* plasmid pDD1252 with primers DDO1510/1511 including *Nco*I and *Bam*HI restriction sites, and cloned into pET30A(+) cut with the same enzymes. BL21 *E. coli* cells containing the resulting plasmid were grown in 1 l 2XYT media in a shaker at 37 °C to mid-log phase (A600 = ~1.0). Protein induction was performed by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) to 1 mM and incubated for another 6 h before collecting cells by centrifugation and freezing at −20 °C. Cell pellets were thawed and resuspended in 25 ml lysis buffer (20 mM HEPES pH 8, 500 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF) and sonicated on ice 5 × 15 s using a Branson Model 250 sonicator equipped with a microtip and amplitude set to 70%. Triton X-100 was added to 1% and the cell suspension was incubated with gentle rocking for 30 min at 4 °C. The cell lysate was clarified by centrifugation at 10,000 × *g* for 10 min at 4 °C. Cobalt beads (Clontech TALON resin, #635502) were washed with lysis buffer before adding to the cell lysate, and the mixture was gently rocked at 4 °C for 30 min. Then, beads were gently pelleted (~4500 × *g*) and washed twice with wash buffer (20 mM HEPES pH 8, 500 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM PMSF, 5 mM imidazole). Reb1 protein was eluted three times at 4 °C with 1 ml elution buffer (250 mM imidazole, 100 mM Tris pH 8.0, 120 mM NaCl) and all eluates were pooled. Purified Reb1 protein was dialyzed in 1 l HEMG buffer (25 mM HEPES pH 7.6, 0.2 M EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1 mM PMSF) overnight at 4 °C. Aliquots were stored at −80 °C.