Poly(A) site choice and Pol2 CTD Serine-5 status govern lncRNA control of phosphate-responsive *tgp1* gene expression in fission yeast

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ABSTRACT

Expression of fission yeast glycerophosphate transporter Tgp1 is repressed in phosphate-rich medium and induced during phosphate starvation. Repression is enforced by transcription of the *nc-tgp1* locus upstream of *tgp1* to produce a long noncoding (lnc) RNA. Here we identify two essential elements of the *nc-tgp1* promoter: a TATA box ⁻³⁰TATATATA⁻²³ and a HomolD box ⁻⁶⁴CAGTCACA⁻⁵⁷, mutations of which inactivate the *nc-tgp1* promoter and de-repress the downstream *tgp1* promoter under phosphate-replete conditions. The *nc-tgp1* lncRNA poly(A) site maps to nucleotide +1636 of the transcription unit, which coincides with the binding site for Pho7 (¹⁶³²TCGGACATTCAA¹⁶⁴³), the transcription factor that drives *tgp1* expression. Overlap between the lncRNA template and the *tgp1* promoter points to transcriptional interference as the simplest basis for lncRNA repression. We identify a shorter RNA derived from the *nc-tgp1* locus, polyadenylated at position +508, well upstream of the *tgp1* promoter. Mutating the *nc-tgp1-short* RNA polyadenylation signal abolishes de-repression of the downstream *tgp1* promoter elicited by Pol2 CTD Ser5Ala phospho-site mutation. Ser5 mutation favors utilization of the short RNA poly(A) site, thereby diminishing transcription of the lncRNA that interferes with the *tgp1* promoter. Mutating the *nc-tgp1-short* RNA polyadenylation signal attenuates induction of the *tgp1* promoter during phosphate starvation. Polyadenylation site choice governed by CTD Ser5 status adds a new level of lncRNA control of gene expression and reveals a new feature of the fission yeast CTD code.

Keywords: phosphate homeostasis; polyadenylation; CTD code

INTRODUCTION

Phosphate homeostasis in the fission yeast *Schizosaccharomyces pombe* is achieved by an intricate network of positive and negative influences on the transcription of genes encoding three proteins involved in extracellular phosphate mobilization and uptake: a cell surface acid phosphatase Pho1, an inorganic phosphate transporter Pho84, and a glycerophosphate transporter Tgp1 (Carter-O'Connell et al. 2012). These genes are repressed during growth in phosphate-rich medium and induced during phosphate starvation. Induction of the phosphate-regulated genes is dependent on transcription factor Pho7 (Henry et al. 2011; Carter-O'Connell et al. 2012). Repression of *pho1* under phosphate-replete conditions is itself an active process involving protein kinases Csk1 and Cdk9, and the phosphorylation of the carboxyl-terminal domain (CTD) of the Rpb1 subunit of RNA polymerase II

(Pol2) (Carter-O'Connell et al. 2012; Schwer et al. 2014, 2015; Chatterjee et al. 2016).

Recent studies have unveiled a role for long noncoding (lnc) RNAs in phosphate-regulated expression of the *pho1* and *tgp1* genes. The *pri* lncRNA, initiating 1147 nucleotides (nt) upstream of the *pho1* mRNA transcription start site, represses *pho1* in *cis* during phosphate-replete growth (Lee et al. 2013; Shah et al. 2014; Schwer et al. 2015; Chatterjee et al. 2016). *pho1* expression from the *pri-pho1* locus is inversely correlated with the activity of the *pri* promoter, which resides in a 110-nt DNA segment preceding the *pri* transcription start site. Within the *pri* promoter, there are two elements that dictate its strength: (i) a TATA box (⁻³⁷TATATATA⁻³⁰) that is essential for *pri* transcription; and (ii) an upstream segment from -110 to -62 that augments *pri* transcription (Chatterjee et al. 2016). The

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positive contributions of the upstream segment stem, at least in part, from the presence of a HomolD sequence ($-^{75}\text{CAGTCACG}^{-68}$). Manipulations of the *pri* promoter in the context of the *pri-pho1* locus that either delete the upstream DNA segment or mutate the TATA box cause de-repression of *pho1* in phosphate-replete cells. Unlike *pho1*, expression of *pri* is independent of Pho7 (Schwer et al. 2015). The *pho1* promoter resides within a 283-nt DNA segment upstream of the mRNA start site and its activity depends on two tandem dodecamer sequences [consensus motif 5'-TCG(G/C)(A/T)xxTTxAA] that are binding sites for the Pho7 transcription factor (Chatterjee et al. 2016; Schwer et al. 2017).

In the case of *tgpl*, the lncRNA *nc-tgpl*, initiating 1865 nt upstream of the *tgpl* AUG start codon (Fig. 1A), represses *tgpl* in *cis* during phosphate-replete growth (Ard et al. 2014). *tgpl* is de-repressed when the *nc-tgpl* locus is disrupted either by deleting the *nc-tgpl* transcription start site and upstream flanking DNA (embracing the putative *nc-tgpl* promoter) or by inserting a *ura4⁺* cassette (in reverse orientation) into the 5' end of the *nc-tgpl* transcription unit (Ard et al. 2014). The *cis* and *trans* factors that control *nc-tgpl* transcription are presently unknown. However, it is clear that *pri* and *nc-tgpl* lncRNA levels are restrained by the action of the nuclear exosome; they increase sharply when the Rrp6 nuclear exosome subunit is absent. The turnover of these

FIGURE 1. Transcripts derived from the *nc-tgpl* and *tgpl* loci and plasmid reporter for dissection of *nc-tgpl* control of *tgpl* expression. (A) The tandem *nc-tgpl-tgpl* locus is shown with the *nc-tgpl* transcription start site indicated by the blue arrow and the *tgpl* ORF depicted as a green bar with arrowhead indicating the direction of transcription. The distance between the *nc-tgpl* transcription initiation site and the ATG translation start site of the *tgpl* ORF is indicated by the bracket. The two *nc-tgpl* transcripts detected by northern analysis (in panel B) are depicted as wavy lines below the DNA. (B) Northern blotting. Total RNA from wild-type (WT), *rrp6Δ*, and *mmi1Δ* cells was resolved by agarose gel electrophoresis, and ethidium bromide-stained ribosomal RNAs (EtBr, right panel) were visualized prior to transfer of the gel contents to membrane, which was hybridized to ³²P-labeled *nc-tgpl* (left panel) and *tgpl* (middle panel) DNA probes. The positions and sizes (kb) of RNA size markers are indicated on the left panel. (C) *tgpl* mRNA. The *nc-tgpl-tgpl* locus is shown with the distance between the *nc-tgpl* and *tgpl* transcription initiation sites indicated by the bracket. The DNA sequence surrounding the *tgpl* transcription start site (mapped by primer extension and indicated by the black arrow) is shown on the top line; the *tgpl* ATG translation start codon is underlined. The cDNA sequence including and preceding the poly(A) tail of the *tgpl* mRNA is shown on the bottom line; the consensus poly(A) signal is highlighted in white on black background. (D) The plasmid-borne *nc-tgpl-tgpl-pho1* reporter cassette is shown with the mapped transcription start sites of the *nc-tgpl* lncRNA and *tgpl* mRNA indicated by arrows. The *pho1* ORF is depicted as a gold bar with arrowhead. (E) The *nc-tgpl-tgpl-pho1* plasmid was introduced into *pho1Δ* yeast cells that were either *pho7⁺* (WT) or *pho7Δ*. Cells grown for 3 h in synthetic medium containing 15.5 mM phosphate (+PO₄) or in medium lacking exogenous phosphate (−PO₄) to elicit the starvation response were assayed for acid phosphatase activity by conversion of *p*-nitrophenylphosphate to *p*-nitrophenol. The y-axis specifies the phosphatase activity (A₄₁₀) normalized to input cells (A₆₀₀). The error bars denote SEM. (F) CTD mutations de-repress regulated *tgpl* expression during phosphate-replete growth. The *nc-tgpl-tgpl-pho1* reporter plasmid was introduced into *pho1Δ* cells in which the *rpb1* chromosomal locus was replaced by alleles *rpb1-CTD-WT*, *CTD-T4A*, *CTD-S7A*, or *CTD-S5₃S5A₁₁*. Cells grown in phosphate-replete medium were assayed for acid phosphatase activity. (G) Time course of the phosphate starvation response. The indicated CTD strains bearing the *nc-tgpl-tgpl-pho1* reporter plasmid were assayed for acid phosphatase activity immediately prior to and at hourly intervals after transfer to medium lacking exogenous phosphate.

lncRNAs by the exosome is aided by Mmi1 (Ard et al. 2014; Shah et al. 2014), a YTH domain-containing protein that recognizes determinants of select removal (DSR) in the target RNA (Harigaya et al. 2006; Yamashita et al. 2012; Chatterjee et al. 2016; Wang et al. 2016).

Whereas there are divergent views on the role of heterochromatin in the lncRNA-mediated repression of fission yeast phosphate-responsive genes, the simplest model is that transcription of the upstream lncRNA in *cis* is what interferes with expression of the downstream genes encoding Pho1 or Tgp1 in phosphate-replete cells, likely by displacing the Pho7 transcription factor from the *pho1* and *tgpl* promoter DNAs (Ard et al. 2014; Chatterjee et al. 2016). By extension, the simplest view of the response to phosphate starvation is that it causes cessation of lncRNA transcription across the downstream gene promoter (in a manner as yet uncharted) and alleviates the downstream interference.

We are interested in elucidating the signals that affect this dynamic during phosphate starvation and in the role of the Pol2 CTD in establishing or overriding the repressed state imposed by lncRNA synthesis. Here we focus on the repression of *tgpl* by *nc-tgpl*. We address the following questions: (i) What comprises the *nc-tgpl* promoter? (ii) Does *nc-tgpl* promoter strength correlate with *tgpl* repression? (iii) How do CTD phospho-site mutations affect *nc-tgpl* and *tgpl* expression? (iv) Does mutating DSR-like elements in the *nc-tgpl* RNA affect *tgpl* phosphate-responsiveness? (v) Is there a connection between *nc-tgpl* lncRNA 3' end formation and *tgpl* promoter activity?

In the course of answering these questions, we identified a novel short polyadenylated RNA derived from the *nc-tgpl* transcription unit and found that mutation of its polyadenylation signal abolished the de-repression of the downstream *tgpl* promoter elicited by CTD Ser5Ala phospho-site mutation. Ser5Ala promotes utilization of the short RNA poly(A) site located upstream of the *tgpl* promoter, thereby diminishing transcription of the lncRNA that overlaps and interferes with the *tgpl* promoter. We find that mutating the *nc-tgpl*-short RNA polyadenylation signal attenuates induction of the *tgpl* promoter during phosphate starvation. Polyadenylation site choice responsive to Pol2 CTD status comprises a new means of lncRNA control of gene expression.

RESULTS

Two transcripts derived from the *nc-tgpl* locus

Northern analysis of total RNA from phosphate-replete wild-type *S. pombe* cells using *nc-tgpl* and *tgpl* probes failed to detect transcripts from either locus. In contrast, in a *mmi1Δ* strain, bearing a *mei4* mutation that suppresses the lethality of *mmi1Δ* (Sugiyama and Sugioka-Sugiyama 2011), we detected two discrete transcripts that hybridized to the *nc-tgpl* probe: a ~1.9 kb RNA corresponding to the *nc-tgpl* lncRNA; and a ~4.6 kb RNA that extended through the

tgpl gene and was therefore recognized by the *tgpl*-specific probe (Fig. 1A,B). The *nc-tgpl* lncRNA and the *nc-tgpl*-*tgpl* read-through transcript were also detectable by northern analysis of RNA from *rrp6Δ* cells (Fig. 1B). A transcript corresponding to the ~2.5 kb *tgpl* mRNA (Ard et al. 2014) was not detected in phosphate-replete *mmi1Δ* or *rrp6Δ* cells, affirming that *tgpl* is tightly repressed during growth in phosphate-rich medium (more so than *pho1*, which is expressed at a basal level in phosphate-replete cells).

tgpl mRNA initiation and polyadenylation sites

Reverse transcriptase primer extension analysis located the 5' end of the *tgpl* mRNA to a single site 42 nt upstream of the start codon of the *tgpl* open reading frame (Fig. 1C; Schwer et al. 2017). Thus, the *tgpl* mRNA transcription start site is 1823 nt downstream from the *nc-tgpl* lncRNA initiation site (Fig. 1C). Here we mapped the site of polyadenylation of the *tgpl* mRNA by 3' RACE using as template total RNA isolated from phosphate-starved wild-type cells. Sequencing of 14 individual cDNA clones revealed that 43% (6/14) had the identical junction to a poly(A) tail at a site 2102 nt downstream from the *tgpl* transcription start site (Fig. 1C). The dominant *tgpl* poly(A) site is located 19 nt downstream from a fission yeast AAAUAA polyadenylation signal (Fig. 1C; Mata 2013). Seven additional poly(A) junctions, six recovered once and one recovered twice among the 14 cDNA clones, were distributed within a 277-nt segment (Supplemental Fig. S1). The most proximal poly(A) site was located 1940 nt downstream from the transcription start site, and the most distal poly(A) site was situated 2216 nt from the transcription start site. The most distal poly(A) site is preceded by two tandem fission yeast AAUAAA polyadenylation signals (Supplemental Fig. S1; Mata 2013).

We also performed 3' RACE analysis using RNA isolated from phosphate-replete *mmi1Δ* cells that express the *nc-tgpl*-*tgpl* read-through transcript. Of 13 individual cDNA clones sequenced, four had a poly(A) tail at the position corresponding to the dominant poly(A) site of the *tgpl* mRNA (Supplemental Fig. S2). Four of the cDNA clones had a poly(A) tail at the position corresponding to the most distal poly(A) site of the *tgpl* mRNA (Supplemental Fig. S2). The majority of the poly(A) sites of the *nc-tgpl*-*tgpl* read-through transcript (9/13) were downstream from fission yeast polyadenylation signals. Five other poly(A) junctions were each recovered once (Supplemental Fig. S2), two of which were identical to "singlet" poly(A) sites detected by 3' RACE of the *tgpl* mRNA.

The *tgpl* gene is annotated in Pombase (www.pombase.org) as containing a putative 46-nt intron interrupting the 3' end of a predicted open reading frame that, assuming the intron is spliced out in the mature *tgpl* mRNA, would encode a predicted polypeptide of 543 amino acids. Our 3' RACE analysis of the *tgpl* mRNA from phosphate-starved fission yeast, which used sense strand primers

corresponding to sequences 5' of the predicted intron, yielded polyadenylated cDNA clones that, in every case (14/14), contained the 46-nt segment thought to comprise an intron (Supplemental Fig. S1). We conclude that this element is not a bona fide intron and is not spliced out during phosphate starvation, the only time when *tgpl* mRNA is known to be transcribed. (The 46-nt segment is also retained in all of the 13 cDNA clones obtained by 3' RACE of the *nc-tgpl-tgpl* read-through transcript in phosphate-replete *mmi1Δ* cells [Supplemental Fig. S2].) The corrected 1584-nt open reading frame in the *tgpl* mRNA encodes a 528-aa protein (Supplemental Fig. S1) that contains a C-terminal Lys528 instead of a C-terminal 16-aa peptide that would have been encoded had the intron been spliced.

Plasmid-based reporter assay to study regulated expression of *tgpl*

Whereas the regulation of *pho1* expression is conveniently studied by measuring acid phosphatase enzyme activity on the surface of live yeast cells, analyses of *tgpl* expression have relied on steady-state *tgpl* RNA levels. Because our goal here was to dissect what drives *nc-tgpl* lncRNA expression and its influence on *tgpl* transcription, we developed a plasmid-based reporter system inspired by the recently described *pri-pho1* plasmid reporter, which recapitulated the features of regulated *pho1* expression that had been determined for the chromosomal *pri-pho1* locus (Chatterjee et al. 2016). As depicted in Figure 1D, we isolated a fragment of the *nc-tgpl-tgpl* locus, spanning 301 nt upstream of the *nc-tgpl* transcription initiation site (encompassing the putative *nc-tgpl* promoter) and the entire 1865 nt segment between the *nc-tgpl* transcription start site and the *tgpl* translation start codon (containing the putative *tgpl* promoter), and fused it to the *pho1* ORF and its native 3' flanking DNA. We cloned the *nc-tgpl-tgpl-pho1* reporter cassette into a fission yeast plasmid, which we then introduced into a *pho1Δ* strain in which the chromosomal *pho1* gene had been deleted. Plasmid-bearing *pho1Δ* cells grown to mid-log phase in liquid culture in phosphate-replete YES medium were washed and then incubated for 3 h in synthetic medium containing 15.5 mM phosphate (+PO₄) or in medium lacking exogenous phosphate (−PO₄) to elicit the starvation response. Acid phosphatase activity was quantified by incubating suspensions of serial dilutions of the cells for 5 min with *p*-nitrophenylphosphate and assaying colorimetrically the formation of *p*-nitrophenol. Product formation in the linear response range, normalized to cell density, is plotted on the *y*-axis in Figure 1E. The low basal phosphatase activity of *nc-tgpl-tgpl-pho1* cells in phosphate-replete medium was increased 10-fold after 3 h of phosphate starvation. Moreover, the basal activity and the starvation response of the *nc-tgpl-tgpl-pho1* reporter was attenuated in a *pho7Δ* strain that lacks the Pho7 transcription factor. Thus, the plasmid-borne DNA

element containing the *nc-tgpl* gene and the *tgpl* transcription start site suffices to confer phosphate-regulated expression of the acid phosphatase reporter.

Effect of CTD phospho-site mutations on regulated *tgpl* expression

Previous studies had shown that repression of *pho1* expression from the *pri-pho1* locus (in either chromosomal or plasmid contexts) in phosphate-replete cells is affected by Pol2 CTD phosphorylation status, as gauged by the impact of CTD mutations that eliminate particular phosphorylation marks (Schwer et al. 2014, 2015; Chatterjee et al. 2016). For example, inability to place a Ser7-PO₄ mark in *S7A* cells de-repressed *pho1*. Limiting the number of serine-5 CTD sites to three consecutive Ser5-containing CTD heptads also de-repressed *pho1*. In contrast, inability to place a Thr4-PO₄ mark in *T4A* cells hyper-repressed *pho1* under phosphate-replete conditions. Here, to test the impact of these CTD mutations on regulated *tgpl* expression, we introduced the *nc-tgpl-tgpl-pho1* reporter plasmid into *pho1Δ* cells in which the *rpb1* chromosomal locus was replaced by alleles *rpb1*-CTD-WT, -CTD-T4A, -CTD-S7A, or -CTD-S5₃S5A₁₁ (Schwer and Shuman 2011; Schwer et al. 2012). Whereas the *T4A* allele had no effect on *nc-tgpl-tgpl-pho1* reporter activity in phosphate-replete cells, the *S7A* and *S5₃S5A₁₁* CTD variants elicited fivefold and eightfold increases in activity compared to the wild-type CTD (Fig. 1F).

The time dependence of the phosphate starvation response of the *nc-tgpl-tgpl-pho1* reporter in the *rpb1*-CTD-WT, -CTD-T4A, and -CTD-S7A strains was monitored by scoring acid phosphatase activity immediately prior to and at hourly intervals after transfer of the cells to medium lacking exogenous phosphate (Fig. 1G). The onset of acid phosphatase accumulation in *T4A* cells occurred with a slight delay (≤1 h) compared to *rpb1*-CTD-WT, though the slope of the increase was similar in both strains. Whereas the *S7A* strain started with a higher basal level of acid phosphatase prior to phosphate starvation, the time course of induction of the *nc-tgpl-tgpl-pho1* reporter was similar to that of *T4A* cells.

nc-tgpl transcription: What comprises a *nc-tgpl* promoter?

To address this question, we constructed a plasmid reporter in which the *pho1* ORF was fused immediately downstream from a genomic DNA segment containing nucleotides −301 to +6 of the *nc-tgpl* transcription unit (Fig. 2A). Because this plasmid generated vigorous acid phosphatase activity when introduced into a *pho1Δ* strain (Fig. 2B), we surmised that the 301-nt segment embraces a *nc-tgpl* promoter and potential regulatory elements. Absence of the Pho7 transcription factor had no effect on the activity of the *nc-tgpl-pho1* reporter (Fig. 2C). To demarcate *cis*-acting elements, we serially truncated the 5' flanking DNA to positions

FIGURE 2. HomolD and TATA boxes are essential for *nc-tgp1* promoter activity and regulation of *tgp1*. (A) Plasmid reporter of *nc-tgp1* promoter activity. The *pho1* ORF (beginning at the underlined ATG translation initiation codon in blue font) was fused downstream from a fragment of genomic DNA containing the *nc-tgp1* transcription start site (indicated by the black arrow above the DNA sequence) and 301 nt of 5' flanking *nc-tgp1* DNA (presumed to include the *nc-tgp1* promoter). Serial truncations of the upstream margin of the 5' flanking *nc-tgp1* DNA were made at positions -211, -134, -98, -71, and -55 (indicated by red arrows below the DNA sequence) relative to the *nc-tgp1* transcription start site. A putative TATA element is outlined by a blue box. Three putative HomolD elements are shaded in gold (forward orientation) or orange (reverse orientation). (B) Acid phosphatase activity of *pho1Δ* cells bearing the indicated *nc-tgp1* promoter-driven *pho1* reporter plasmids. (C) Acid phosphatase activity of *pho1Δ pho7⁺* (WT) and *pho1Δ pho7Δ* cells bearing the -301 *nc-tgp1* promoter-driven *pho1* plasmid. (D) Acid phosphatase activity of *pho1Δ rpb1-CTD* strains bearing the -301 *nc-tgp1-pho1* plasmid. (E,F) The indicated mutated versions of individual HomolD sites (hd-1, hd-2, and hd-3) and the TATA box of the *nc-tgp1* promoter (shown at right in panel F) were introduced into the -301 *nc-tgp1-pho1* reporter and phosphate-replete *pho1Δ* cells bearing the indicated *nc-tgp1-pho1* reporter plasmids were assayed for acid phosphatase activity (panel E). (F) The HomolD and the TATA box mutations of the *nc-tgp1* promoter were introduced into the *nc-tgp1-tgp1-pho1* reporter plasmid (shown at the top of the panel). Phosphate-replete *pho1Δ* cells bearing the indicated reporter plasmids were assayed for acid phosphatase activity.

–211, –134, –98, –71, and –55 upstream of the *nc-tgp1* transcription start site. The acid phosphatase activity of the plasmid-bearing *pho1Δ* cells (Fig. 2B) indicated that the 5′ flanking 134-nt segment sufficed for *nc-tgp1* promoter-driven expression. Truncation to –98 and –71 reduced acid phosphatase activity to 52% and 40% of that driven by the –134 promoter; further truncation to –55 effaced acid phosphatase activity (Fig. 2B). To query whether CTD mutations influence *nc-tgp1* promoter-driven transcription, we introduced the –301 *nc-tgp1•pho1* reporter plasmid into the *pho1Δ rpb1-CTD-WT*, *-CTD-T4A*, *-CTD-S7A*, and *-CTD-S53•S5A11* strains and measured acid phosphatase expression (Fig. 2D). *nc-tgp1* promoted *pho1* activity was identical in the WT and *S53•S5A11* strains, from which we conclude that the de-repression of the *nc-tgp1–tgp1•pho1* reporter in *S53•S5A11* cells was not caused by a decrease in transcription from the *nc-tgp1* lncRNA promoter. The *nc-tgp1•pho1* reporter activity in *T4A* and *S7A* cells was 25% and 35% higher than that in WT cells, respectively (Fig. 2D).

HomolD and TATA boxes are essential for *nc-tgp1* promoter activity

The sequence of the 301-nt DNA segment flanking the *nc-tgp1* start site is shown in Figure 2A. The promoter deletion analysis suggested the presence of an essential transcriptional element in the interval between –56 and –71 and a stimulatory element located between –72 and –134. Our inspection of these segments disclosed three sequences that are potential HomolD-box elements (shaded yellow and orange in Fig. 2A). The consensus HomolD element 5′-CAGTCAC(A/G) functions as a Pol2 promoter signal in fission yeast, especially in genes encoding ribosomal proteins (Witt et al. 1993, 1995; Gross and Käufer 1998). The HomolD sequence is a binding site for the essential fission yeast transcription factor Rrn7 (Rojas et al. 2011). The putative HomolD box closest to the *nc-tgp1* transcription start site (–⁶⁴CAGTCACA–⁵⁷; box 3 in Fig. 2A) is a perfect match to the HomolD consensus sequence. The candidate HomolD element furthest from the start site (–⁹⁰CAGTCAAA–⁸³; box 1 in Fig. 2A) deviates by one nucleobase from the consensus sequence. Putative box 2 (–⁸⁰TGTGACTC–⁷³), situated between boxes 1 and 3 in the opposite orientation, is a single-base variant GAGTCA CA of the HomolD consensus (Fig. 2A). Our previous studies of the *pri–pho1* locus implicated a HomolD box as a driver of *pri* lncRNA transcription and its ensuing repression of *pho1* during phosphate-replete growth (Chatterjee et al. 2016).

Here, to evaluate whether any of the HomolD boxes play a role in lncRNA regulated *tgp1* expression, we introduced into the *nc-tgp1–tgp1•pho1* reporter multiple nucleotide changes, at six out of eight positions in box 1, or at seven out of eight positions in boxes 2 or 3 (Fig. 2F). *pho1Δ* cells transformed with the mutated *nc-tgp1–tgp1•pho1* reporters hd-1, hd-2, and hd-3 were grown in phosphate-replete medium and tested for acid phosphatase activity in parallel with cells bearing

the WT *nc-tgp1–tgp1•pho1* plasmid. Whereas mutations hd-1 and hd-2 had no effect on the phosphate-repressive state of the *nc-tgp1–tgp1•pho1* locus, the hd-3 mutation elicited a de-repression of reporter activity, to a level 29-fold higher than the WT control (Fig. 2F). To judge whether this apparent loss of phosphate regulation was caused by a change in the activity of the *nc-tgp1* promoter, we introduced the hd-1, hd-2, and hd-3 mutations into the *nc-tgp1•pho1* reporter plasmid (Fig. 2E). The salient findings were that whereas hd-1 and hd-2 mutations had no significant effect, *nc-tgp1* promoter activity was lowered 30-fold by the hd-3 mutation (Fig. 2E). We conclude that HomolD box 3 is an essential component of the *nc-tgp1* promoter and that down-regulation of *nc-tgp1* promoter-driven lncRNA transcription by its mutation leads to increased activity of the downstream *tgp1* promoter in phosphate-replete cells.

The *nc-tgp1* promoter also contains a TATA box sequence –³⁰TATATATA–²³. To query the role of the TATA element in *nc-tgp1* function, we introduced a TATA mutant (GCGCGC GC) into the *nc-tgp1–tgp1•pho1* reporter and found that it (akin to the hd-3 mutant) resulted in a 40-fold increase in acid phosphatase activity under phosphate-replete conditions (Fig. 2F) that correlated with an eightfold decrement in the activity of the *nc-tgp1* promoter (Fig. 2E). Thus, the TATA box, too, is a key determinant of *nc-tgp1* promoter function and phosphate repression of the flanking *tgp1* promoter.

Effect of CTD mutations on *tgp1* promoter activity

To interrogate the *tgp1* promoter, we constructed a plasmid reporter in which a genomic DNA segment containing nucleotides –871 to +42 of the *tgp1* transcription unit (with +1 being the mRNA start site) was fused to the *pho1* ORF (Fig. 3). This plasmid generates vigorous acid phosphatase activity when introduced into a *pho1Δ* strain (Schwer et al. 2017). Acid phosphatase activity driven by the *tgp1* promoter reporter plasmid is reduced by 85% either: (i) in a *pho7Δ* strain background; or (ii) by mutation of the Pho7 DNA binding site 5′-TCGGACATTCAA located at nucleotides –192 to –181 upstream of the *tgp1* transcription start site (Schwer et al. 2017). The effects of CTD mutations on the activity of the *tgp1•pho1* reporter are shown in Figure 3. Activity was nearly identical in the *rpb1-CTD WT*, *T4A*, and *S53•S5A11* strains. Thus, the de-repression of the *nc-tgp1–tgp1•pho1* locus seen in *S53•S5A11* cells is not reflective of increased activity of the *tgp1* promoter *per se*. Whereas *tgp1* promoter-driven *Pho1* expression in *S7A* was 38% higher than in WT cells, this effect is much less than the fivefold increase in expression of *Pho1* from the *nc-tgp1–tgp1•pho1* reporter elicited by the *S7A* allele.

Effect of mutating DSR sequences in the *nc-tgp1* lncRNA

Ard et al. (2014) noted that the *nc-tgp1* RNA contains a cluster of three putative DSR sequences in the region from

FIGURE 3. Effect of *rpb1*-CTD mutations on *tgp1* promoter activity. A schematic of the -871 *tgp1* promoter-driven *pho1* reporter (Schwer et al. 2017) is shown at the top, with the Pho7 binding site in the *tgp1* promoter highlighted. Acid phosphatase activity of *pho1Δ rpb1-CTD*-WT, *rpb1-CTD*-T4A, *rpb1-CTD*-S7A, and *rpb1-CTD*-S5₃S5A₁₁ cells bearing the *tgp1•pho1* reporter plasmid is shown.

nucleotides +821 to +850 (highlighted in gold in Fig. 4A). Tandem DSRs promote RNA degradation via their recognition by the YTH-domain protein Mmi1. The canonical DSR hexanucleotide sequence is 5'-U(U/C)AAAC (Yamashita et al. 2012). The first and third hexanucleotides in the putative DSR cluster of the *nc-tgp1* lncRNA conform to the UCAAAC consensus; the middle hexanucleotide is a single-base variant UUAAAA (Fig. 4A). It was shown previously that certain variant DSR hexanucleotide motifs can augment the function of the consensus DSR element to promote RNA elimination (Yamashita et al. 2012). However, 5'-UUAAAA was not among the variant motifs tested. We have shown that the Mmi1 YTH domain binds equally well to RNA containing either a consensus UUAAAC hexamer or a UUAAAU variant (Chatterjee et al. 2016). Inspection of the *nc-tgp1* RNA sequence revealed three UUAAAU sequences and two UUAAAC sequences (highlighted in green in Fig. 4A) in addition to the DSR cluster.

To evaluate the role of the +821 to +850 DSR cluster in *tgp1* regulation, we introduced compound mutations (at five out of six positions) into each of the three hexanucleotide motifs of the *nc-tgp1-tgp1•pho1* reporter (Fig. 4B). We gauged the effect of the DSR cluster mutation on the responsiveness to phosphate starvation, as reflected in the induction of acid phosphatase activity as a function of time after transfer to medium lacking phosphate. DSR mutation delayed the onset of acid phosphatase production by 1 h, but did not affect the rate of acid phosphatase production thereafter (Fig. 4B).

Northern analysis of RNA isolated from phosphate-replete *pho1Δ* cells bearing the wild-type *nc-tgp1-tgp1•pho1* reporter plasmid showed that the ~1.9 kb *nc-tgp1* lncRNA was detect-

able, but the downstream *pho1* mRNA was not (Fig. 4C). We also detected a ~0.7-kb transcript that was recognized by the *nc-tgp1* probe, which we refer to henceforth as *nc-tgp1-short*. (The ability to detect by northern blot the lncRNA, and the short RNA, expressed from the plasmid, but not from the chromosomal *nc-tgp1* locus [Fig. 1B], likely reflects the high copy number of the fission yeast plasmid.) The salient finding was that the DSR cluster mutation elicited an increase in the abundance of the *nc-tgp1* lncRNA as well as the accumulation of a ~4 kb *nc-tgp1-pho1* read-through transcript that annealed to the *nc-tgp1* and *pho1* probes. These findings implicate the DSR cluster as a determinant of the stability of the *nc-tgp1* lncRNA, and its propensity to terminate without read-through into the downstream gene. Note that the level of the *nc-tgp1-short* RNA was not increased by the DSR cluster mutation, presumably because the short transcript terminates upstream of the DSR cluster.

Defining the 3' end of the polyadenylated *nc-tgp1* lncRNA

We mapped the site of polyadenylation of the *nc-tgp1* lncRNA by 3' RACE using as template total RNA isolated from two cellular sources that have 1.9 kb lncRNAs detectable by northern analysis: (i) *mmi1Δ* cells expressing *nc-tgp1* from the native chromosomal locus (Fig. 1B, 4D); and (ii) *pho1Δ* cells expressing *nc-tgp1* from the *nc-tgp1*(DSR-WT)-*tgp1•pho1* reporter and *nc-tgp1*(DSR-mut)-*tgp1•pho1* reporter plasmids (Fig. 4C,E). Sequencing of three, two, and four independent cDNA clones from these RNA sources, respectively, revealed (in all nine cases) identical junctions to a poly(A) tail at nucleotide 1636 of the lncRNA transcript, which is situated 17 nt downstream from a fission yeast AAUAAA polyadenylation signal (Fig. 4F; Mata 2013). Thus, the same polyadenylated lncRNA is produced from the native *nc-tgp1-tgp1* locus (Fig. 4D) and the *nc-tgp1-tgp1•pho1* reporter (Fig. 4E). The cleavage/poly(A) site of the *nc-tgp1* lncRNA (5'-UCGGA↓) is located 187 nt upstream of the *tgp1* transcription start site, within the sequence that corresponds to the Pho7 DNA binding site of the *tgp1* promoter (5'-TCGGA↓CATTC AA). Thus, transcription across the poly(A) site of the *nc-tgp1* lncRNA excludes (or displaces) Pho7 binding at the *tgp1* promoter.

Defining the 3' end of the polyadenylated *nc-tgp1-short* RNA

We mapped the site of polyadenylation of the *nc-tgp1-short* RNA by 3' RACE using as template total RNA isolated from yeast cells (not containing a plasmid) expressing *nc-tgp1* from its natural context in the chromosome (10 independent cDNA clones sequenced), from *pho1Δ* cells expressing *nc-tgp1* from the wild-type *nc-tgp1-tgp1•pho1* reporter plasmid (15 independent cDNA clones sequenced), and from *pho1Δ* cells expressing *nc-tgp1* from the *nc-tgp1*(DSR-

Our detection of a novel short poly(A)⁺ RNA derived from the *nc-tgp1* gene adds a new wrinkle to the *nc-tgp1* story. Most pertinent is the fact that Pol2 elongation complexes synthesizing the *nc-tgp1-short* RNA will undergo nascent strand 3' cleavage, and (in all likelihood) ensuing

transcription termination (Loya and Reines 2016) well upstream of the Pho7 binding site of the *tgf1* promoter [which coincides with the lncRNA poly(A) site] (Fig. 4D). This scenario raises the prospect that poly(A) site choice might modulate lncRNA activity. For example, a change in poly(A) site choice skewed toward *nc-tgf1-short* might contribute to the observed de-repression of the downstream *tgf1* promoter in phosphate-replete cells with $S5_3 \cdot S5A_{11}$ phospho-site mutations in the Pol2 CTD (Fig. 1F). If this is the case, then we would expect that the de-repressive effects of this CTD allele on the *tgf1* promoter in the *nc-tgf1-tgf1-pho1* reporter should be effaced by mutation of the *nc-tgf1-short* RNA polyadenylation signal (PAS). Indeed, this was precisely what occurred when we changed the *nc-tgf1-short* PAS in the DNA template from TTAATA (wild-type) to CCGGCG (mutant) (Fig. 5A) and gauged *tgf1* promoter-driven acid phosphatase expression in phosphate-replete $S5_3 \cdot S5A_{11}$ cells (Fig. 5B). These results indicate that the reduction in CTD Ser5 phospho-sites exerts its de-repressive effect via the *nc-tgf1-short* PAS.

To directly assess poly(A) site choice, we performed RNase probe protection analysis of RNA isolated from *rpb1-CTD-WT* and $-S5_3 \cdot S5A_{11}$ cells bearing the *nc-tgf1-tgf1-pho1* reporter with a wild-type or mutant *nc-tgf1-short* PAS. The 170-nt wild-type 32 P-UMP-labeled RNA probe (Fig. 5C,

lane1) is complementary to the wild-type *nc-tgf1* lncRNA throughout a 133-nt segment that spans the 3' cleavage/polyadenylation site of the *nc-tgf1-short* RNA. RNase T1 cleavage of any probe annealed to lncRNA yields a 138-nt protected fragment; cleavage of probe annealed to the polyadenylated *nc-tgf1-short* RNA yields a shorter 86-nt protected fragment (Fig. 5C, lanes 3–6). Protection of these two fragments from T1 digestion depended on prior annealing to RNA from cells bearing the PAS-WT reporter (Fig. 5C, lane 2). The presence in all RNase digests of a 43-nt T1-resistant fragment corresponding to the longest interval between G nucleotides of the labeled probe (lanes 3–6), affirmed that the probe was present in excess over the complementary cellular *nc-tgf1* RNAs. The probe protection analysis revealed that utilization of the short PAS site increased in the *rpb1-CTD-S5_3 \cdot S5A_{11} strain (lanes 5,6) vis à vis the wild-type (lanes 3,4). The ratio of the *nc-tgf1-short* to lncRNA T1-protected fragments is a measure of *nc-tgf1-short* PAS choice. The short RNA/lncRNA ratio values (mean $\pm$ SEM of RNA preparations from three independent yeast cultures, with two technical replicates of the RNase protection for each RNA sample) were 0.955 ± 0.03 for the *rpb1-CTD-WT* strain and 1.92 ± 0.03 for the *rpb1-CTD-S5_3 \cdot S5A_{11} strain. Control experiments using a probe complementary to the *nc-tgf1* transcript with the mutant *nc-tgf1-short* PAS (lane 7) affirmed that the PAS**

FIGURE 5. Mutating the poly(A) signal of the *nc-tgf1-short* RNA abolishes de-repression of the *tgf1* promoter by CTD S5A mutation. (A) The short RNA polyadenylation signal (PAS) in the *nc-tgf1-tgf1-pho1* reporter cassette (highlighted in white font on black background) was replaced by the mutant sequence shown in red font below the wild-type PAS. (B) Acid phosphatase activity of *pho1Δ rpb1-CTD-WT* or *rpb1-CTD-S5_3S5A_{11}* cells bearing *nc-tgf1-tgf1-pho1* reporter plasmids with wild-type (WT) or mutant (mut) short RNA PAS elements. (C) RNase protection analysis of *nc-tgf1-short* PAS utilization in *rpb1-CTD-WT* cells (CTD WT; lanes 3,4,9) or *rpb1-CTD-S5_3S5A_{11}* cells (CTD Mut; lanes 5,6,10) bearing *nc-tgf1-tgf1-pho1* reporter plasmids with wild-type (PAS-WT; lanes 3–6) or mutant (PAS-Mut; lanes 9,10) short RNA PAS elements was performed as described in Materials and Methods. The RNase T1-digested samples (T1+) and untreated controls (T1–; lanes 1,7) were analyzed by denaturing PAGE. The positions and lengths (nt) of radiolabeled DNA size markers (lane 11) are indicated on the right.

mutation effaced the shorter protected species and yielded exclusively a signal for the *nc-tgp1* lncRNA (Fig. 5C, lanes 9,10).

Mutating the poly(A) signal of the *nc-tgp1-short* RNA attenuates the *tgp1* phosphate starvation response

We gauged the effect of the inactivating TTAATA (wild-type) to CCGGCG (mutant) change in the *nc-tgp1-short* RNA PAS on the kinetics of *tgp1* promoter-driven acid phosphatase expression from the *nc-tgp1-tgp1-pho1* reporter during phosphate starvation. The PAS mutation elicited a delay in the onset of Pho1 accumulation and a decrement in the rate of Pho1 production during the 6 h interval after transfer of the cells to phosphate-free medium (Fig. 6), e.g., such that the acid phosphatase activity of PAS-Mut cells at 5 h post-starvation was equivalent to the activity of PAS-WT cells after 3 h of starvation. These results imply that transcription from the *nc-tgp1* promoter persists during phosphate starvation (at least during the time frame analyzed) to an extent that a *nc-tgp1-short* PAS mutation, which biases toward *nc-tgp1* lncRNA synthesis, significantly blunts *tgp1* promoter activity. The fact that a PAS mutation does not completely prevent *tgp1* promoter induction during phosphate starvation suggests that there is also a component of *nc-tgp1* promoter down-regulation in phosphate homeostasis.

FIGURE 6. Mutating the poly(A) signal of the *nc-tgp1-short* RNA attenuates the induction of the *tgp1* promoter during phosphate starvation. Cells bearing the *nc-tgp1-tgp1-pho1* reporter in which the *nc-tgp1-short* RNA polyadenylation signal (PAS) was either wild-type or mutant were assayed for acid phosphatase activity immediately prior to and at hourly intervals after transfer to medium lacking exogenous phosphate.

DISCUSSION

lncRNA repression of phosphate-starvation genes in fission yeast

Fission yeast phosphate homeostasis entails repressing the *tgp1* and *pho1* genes during phosphate-replete growth and inducing/de-repressing them under conditions of phosphate starvation. Here we contribute to an emerging appreciation of the role of lncRNAs transcribed co-directionally from upstream *nc-tgp1* and *pri* genomic loci flanking *tgp1* and *pho1* in enforcing the repressive status, in particular by delineating shared features that govern transcription of the *nc-tgp1* and *pri* lncRNAs. Within the *nc-tgp1* promoter, there are two essential elements: a HomolD box ($^{-64}$ CAGTCACA $^{-57}$) and a TATA box ($^{-30}$ TATATATA $^{-23}$). We find that mutations in either the HomolD or TATA sequences in the context of the *nc-tgp1-tgp1* reporter result in de-repression of the *tgp1* promoter in phosphate-replete cells. HomolD and TATA elements are also present and positioned similarly in the *pri* lncRNA promoter, where they are important for *pri*-dependent repression of *pho1* expression (Chatterjee et al. 2016), and in the promoter for the *S. pombe* U3 snoRNA (Nabavi and Nazar 2008).

Our results focus attention on the HomolD element as a likely target of phosphate responsiveness, whereby HomolD functions positively in *nc-tgp1* lncRNA transcription when phosphate is available, presumably via binding of an activating transcription factor to the HomolD sequence. Because loss of *nc-tgp1* and *pri* transcription suffices to de-repress *tgp1* and *pho1*, the simplest model for phosphate starvation-induced *tgp1* and *pho1* expression is that starvation elicits a signaling pathway that eventually shuts off the *nc-tgp1* and *pri* promoters (Ard et al. 2014; Chatterjee et al. 2016), conceivably by an inactivating modification of the transcription factor that recognizes HomolD in the lncRNA promoters. Maldonado and colleagues have shown that the HomolD sequence is recognized by the essential fission yeast transcription factor Rrn7 (Rojas et al. 2011) and that phosphorylation of Rrn7 by casein kinase 2 (CK2) inhibits its ability to bind to HomolD DNA in vitro (Moreira-Ramos et al. 2015). By mutating three predicted CK2 phosphorylation sites in Rrn7 to alanine, they showed that (i) replacing Thr67 with alanine uniquely eliminated its CK2 phosphorylation in vitro; and (ii) binding of Rrn7-T67A to HomolD DNA was unaffected by CK2 (Moreira-Ramos et al. 2015). We reasoned that if Rrn7 Thr67 phosphorylation during phosphate starvation is the basis for shut-off of HomolD-driven lncRNA transcription, then prevention of such phosphorylation by T67A mutation might block the starvation induction of phosphate-responsive genes. Therefore, we disrupted the native *S. pombe* *rrn7⁺* gene in a diploid strain by insertion of a *ura4⁺* marker and introduced a mutant *rrn7-T67A* allele at the *leu1* locus. After sporulation to obtain a haploid *rrn7::ura4⁺ leu1::rrn7-T67A* strain, we verified that the *rrn7-T67A* strain grew as

well as *rrn7*⁺ on YES medium. Testing for induction of Pho1 phosphatase activity upon shift to phosphate-free medium showed no effect of *rrn7-T67A* on the extent of Pho1 accumulation (not shown), leading us to surmise that Rrn7 Thr67 phosphorylation is not a decisive event in the phosphate starvation response.

Our experiments illuminate key properties of the *nc-tgp1* lncRNA. For example, whereas it had been noted earlier that the lncRNA contains a cluster of three DSR-like hexanucleotide elements that are recognized by Mmi1 (Ard et al. 2014; Kilchert et al. 2015), the contribution of these DSRs to regulated *tgp1*-driven transcription was not addressed. Here we showed that mutating the DSR cluster resulted in a higher steady-state level of the *nc-tgp1* lncRNA and the accumulation of a longer read-through transcript spanning the lncRNA and the downstream *tgp1* promoter-driven *pho1* reporter. (These effects of *nc-tgp1* DSR mutations on the plasmid reporter echoed those of *mmi1Δ* on the chromosomally transcribed *nc-tgp1* lncRNA and *nc-tgp1-tgp1* read-through RNA.) The lack of effect of the DSR mutations on the activity of the downstream *tgp1* promoter in phosphate-replete cells simply reflects the fact that the *tgp1* promoter is already maximally repressed by transcription of the wild-type *nc-tgp1* lncRNA. However, the DSR mutations do elicit a 1 h delay in the induction of *tgp1* promoter-driven expression after phosphate starvation. In contrast, DSR cluster mutations in the *pri* lncRNA cause a significant decrement in the basal level of Pho1 expression in phosphate-replete cells and they cause a longer 2-h delay in the induction of Pho1 after phosphate starvation (Chatterjee et al. 2016).

The poly(A) site of the *nc-tgp1* lncRNA is located 187 nt upstream of the *tgp1* transcription start site and directly overlaps the binding site for transcription factor Pho7 in the *tgp1* promoter (Fig. 4D). The salient point here is that Pol2 elongation complexes synthesizing the *nc-tgp1* lncRNA will fully traverse the *tgp1* promoter. Thus, the simplest model for repression posits that *nc-tgp1* lncRNA synthesis interferes with *tgp1* promoter function by displacing Pho7 from the promoter DNA, via collision with Pol2 traveling on the segment of the *nc-tgp1* template that includes the Pho7 recognition site. An alternative view is that lncRNA transcription causes changes in chromatin status over the *tgp1* promoter (albeit not via heterochromatin) that interfere with Pho7 binding (Ard and Allshire 2016). These views are not mutually exclusive, e.g., displacement of Pho7 by Pol2 transcribing *nc-tgp1* could incite changes in nucleosome density over the *tgp1* promoter.

Comparison to regulated *SER3* expression in budding yeast

The repression of the phosphate-responsive *tgp1* gene in fission yeast by transcription of an upstream lncRNA echoes themes established for regulated *SER3* expression in *S. cerevisiae*, whereby *SER3* is tightly repressed during growth in

rich medium via transcription of the upstream *SRG1* lncRNA so as to interfere with the *SER3* promoter (Martens et al. 2004). As seen here for *nc-tgp1*: (i) The size of the polyadenylated *SRG1* lncRNA is such that its 3' end overlaps the *SER3* promoter; and (ii) mutation of the TATA box in the *SRG1* promoter de-represses *SER3* (Martens et al. 2004). *SRG1* transcription is regulated by serine availability, i.e., *SRG1* is turned on under serine-rich conditions and shut off in response to acute serine starvation, with *SER3* expression anti-correlating to that of *SRG1* (Martens et al. 2004). Whereas fission yeast *nc-tgp1* lncRNA expression is driven by a HomolD element in its promoter, the cognate transcription factor(s) of which remain unclear absent genetic evidence for a role of Rrn7, budding yeast *SRG1* expression is activated by a DNA element that contains a binding site for transcription factor Cha4 (Martens et al. 2005). Deletion of Cha4 suppresses *SRG1* expression and consequently de-represses *SER3* in serine-rich medium (Martens et al. 2005). It is noteworthy that budding yeast Cha4 is a member of the Zn₂Cys₆ family of fungal transcription regulators (Holmberg and Schjerling 1996), as is *S. pombe* Pho7 (Schwer et al. 2017). Thus, whereas budding yeast deploys a Zn₂Cys₆ factor to transcribe the lncRNA and thereby enforce the repressive state of the flanking gene, fission yeast uses a Zn₂Cys₆ factor to transcribe the flanking target gene once the repressive effect of the lncRNA is alleviated.

Influence of *nc-tgp1-short* RNA and CTD status

A short poly(A)⁺ RNA derived from the *nc-tgp1* gene adds another layer of control to the system. The poly(A) site of the *nc-tgp1-short* RNA is located 504–508 nt from the transcription start site, well upstream of the DSR cluster present in the lncRNA. Pol2 elongation complexes that synthesize the *nc-tgp1-short* RNA are expected to terminate transcription well upstream of the Pho7 site of the *tgp1* promoter. Thus, transcription of the *nc-tgp1-short* RNA would not elicit repression of the downstream *tgp1* gene, which raised a hypothesis that poly(A) site choice might figure in the regulation of lncRNA function. Indeed, we show here that poly(A) site choice biased toward *nc-tgp1-short* contributes to the de-repression of the downstream *tgp1* promoter in phosphate-replete cells bearing S5A mutations in the Pol2 CTD, and that de-repression of the *tgp1* promoter by the *rpb1-CTD-S53•S5A₁₁* allele is eliminated by mutation of the *nc-tgp1 short* RNA poly(A) signal. In the same vein, the de-repression of the *tgp1* promoter by *rpb1-CTD-S7A* (Fig. 1F) is also eliminated by mutation of the *nc-tgp1 short* RNA poly(A) signal. We find that mutation of the *nc-tgp1-short* RNA poly(A) signal attenuates induction of the downstream *tgp1* promoter in phosphate-starved cells, both with respect to the lag time to see an increase in gene expression (gauged by acid phosphatase reporter activity) and the rate of acid phosphatase accumulation.

Finally, our results reveal a new aspect of the fission yeast CTD code, in which the Ser5 phospho-site (and presumably its phosphorylation) disfavors a proximal poly(A) site in the *nc-tgp1* transcription unit, whereas reducing available Ser5 phospho-sites by S5A mutation enhances proximal poly(A) site choice. Recent studies by the Bachand laboratory have implicated the fission yeast protein Seb1 in the regulation of cotranscriptional PAS selection in coding and noncoding genes (Lemay et al. 2016; Larochelle et al. 2017). Seb1 is an RNA binding protein, essential for vegetative growth, that recognizes a 5'-(A/U)GUA(A/G) motif situated 50- to 100-nt downstream from sites of cleavage/polyadenylation (Lemay et al. 2016). Seb1 also interacts with the Pol2 CTD, though available evidence weighs against a role for the Ser5-PO₄ mark in Seb1 recruitment and function (Lemay et al. 2016). Of note, whereas there are three distinct Seb1 motifs within the 100-nt segment downstream from the cleavage/polyadenylation site of the *nc-tgp1* lncRNA, there are, in contrast, no Seb1 motifs flanking the cleavage/polyadenylation site of the *nc-tgp1-short* RNA (i.e., the nearest GUA sequence is 466 nt downstream from the short RNA cleavage/polyadenylation site). These considerations militate against Seb1 as an agent of Ser5-regulated usage of the *nc-tgp1-short* poly(A) site.

MATERIALS AND METHODS

Reporter plasmids

We constructed three sets of *pho1*-based reporter plasmids, marked with a kanamycin-resistance gene (*kanMX*), in which the expression of *Pho1* acid phosphatase was driven by either: (i) a tandem *nc-tgp1* (promoter+lncRNA)-*tgp1*(promoter) element; (ii) a *nc-tgp1*(promoter) element; (iii) or a *tgp1*(promoter) element. These were derivatives of pKAN-(*pho1*) described previously (Chatterjee et al. 2016) generated by insertion of genomic sequences flanking *pho1*, as follows. Plasmid pKAN-(*nc-tgp1-tgp1_{prom}-pho1*) includes a fragment of the *nc-tgp1-tgp1* locus, spanning 301 nt upstream of the *nc-tgp1* transcription initiation site (encompassing the putative *nc-tgp1* promoter) and the entire 1865-nt segment between the *nc-tgp1* transcription start site and the *tgp1* translation start codon (containing the putative *tgp1* promoter) fused to the *pho1* ORF and its native 3' flanking DNA. Plasmid pKAN-(*nc-tgp1_{prom}-pho1*) includes the 301 nt upstream of the *nc-tgp1* transcription initiation site fused to the *pho1* ORF and its native 3' flanking DNA. Plasmid pKAN-(*tgp1_{prom}-pho1*) includes the 913-nt segment upstream of the *tgp1* translation start codon fused to the *pho1* ORF and its native 3' flanking DNA. Serial truncations from the 5' end of the *nc-tgp1* promoter were generated by PCR amplification and cloned into the pKAN-(*nc-tgp1_{prom}-pho1*) reporter in lieu of the original promoter segment. Nucleotide substitutions were introduced into the reporter constructs by two-stage overlap extension PCR amplification with mutagenic primers, followed by insertion in lieu of the wild-type DNA segments. The inserts of all plasmids were sequenced to exclude unwanted mutations.

Reporter assays

Reporter plasmids were transfected into *pho1Δ* cells (Chatterjee et al. 2016). *kanMX* transformants were selected on YES (yeast ex-

tract with supplements) agar medium containing 150 μg/mL G418. Single colonies of transformants (≥20) were pooled and grown at 30°C in plasmid-selective liquid medium. Aliquots of exponentially growing cultures were harvested, washed, and suspended in water to attain A₆₀₀ of 1.25 or 2.5. To quantify acid phosphatase activity, reaction mixtures (200 μL) containing 100 mM sodium acetate (pH 4.2), 10 mM *p*-nitrophenylphosphate, and cells (ranging from 0.01 to 1 A₆₀₀ units) were incubated for 5 min at 30°C. The reactions were quenched by adding 1 mL of 1 M sodium carbonate; the cells were removed by centrifugation, and the absorbance of the supernatant at 410 nm was measured. Acid phosphatase activity is expressed as the ratio of A₄₁₀ (*p*-nitrophenol production) to A₆₀₀ (cells). Each datum in the graphs is the average (±SEM) of phosphatase assays using cells from at least three independent cultures. To test the responsiveness of *Pho1* expression to phosphate starvation, cells transformed with pKAN-(*nc-tgp1-tgp1_{prom}-pho1*) were grown in YES+G418 liquid medium, aliquots of exponentially growing cultures were harvested, washed in water, and adjusted to A₆₀₀ of ~0.3 in PMG (phthalate medium glutamate) +G418 liquid medium without phosphate. After incubation for various times at 30°C, cells were harvested, washed, and assayed for acid phosphatase activity as described above.

RNA analyses

Total RNA was extracted via the hot phenol method from 20 A₆₀₀ units of yeast cells that had been grown at 30°C in YES, YES + G418 (for selection of *kanMX* plasmids), or PMG without phosphate. The RNAs were treated with DNase I, extracted serially with phenol:chloroform and chloroform, and then precipitated with ethanol. The RNAs were resuspended in 10 mM Tris-HCl (pH 6.8), 1 mM EDTA. For northern blotting, equivalent amounts (10 μg) of total RNA were resolved by electrophoresis through a 1.2% agarose/formaldehyde gel. After photography under UV illumination to visualize ethidium-bromide-stained 18S and 28S rRNAs, the gel contents were transferred to a Hybond-XL membrane (GE Healthcare). Gene-specific probes were prepared by radiolabeling DNA fragments of the *tgp1* (nucleotides 151–1064), *nc-tgp1* (nucleotides 1–1141), or *pho1* (nucleotides 590–1293) genes. Hybridization was performed as previously described (Herrick et al. 1990) and the hybridized probes were visualized by autoradiography.

For amplification of cDNA 3' ends, we used the 3' RACE Kit (Invitrogen). In brief, 2 μg of total RNA was used as template for first strand cDNA synthesis by SuperScript II RT and an oligo(dT) adapter primer (AP). The RNA template was removed by digestion with RNase H, and the cDNA was then diluted 10-fold for amplification by PCR using gene-specific forward primers and an abridged universal reverse primer (AUAP). The PCR products were gel-purified, cloned into a pCRII TOPO vector by using the TOPO TA Cloning Kit (Invitrogen). Plasmid DNAs isolated from individual bacterial transformant colonies were sequenced. cDNAs were deemed to be “independent” when they contained different lengths of poly(dA) at the cloning junction.

Assay of *nc-tgp1* poly(A) site choice by RNase protection

To generate RNA probes for hybridization, a 134-bp DNA fragment (from nucleotides +424 to +558 of the *nc-tgp1* transcription

unit) spanning the poly(A) site of wild-type *nc-tgp1-short* RNA was amplified by PCR using primers that introduced restriction sites for cloning into the pGEM1 vector. An equivalent fragment containing the mutated polyadenylation signal was also cloned into pGEM1. The plasmids were linearized and used as templates for the in vitro synthesis of ^{32}P -UMP labeled anti-sense transcripts by T7 RNA polymerase. The RNAs were gel-purified and aliquots ($3\text{--}4 \times 10^4$ cpm) were coprecipitated with 10 μg of total RNA from *pKAN-(nc-tgp1-tgp1_{prom}•pho1)*-bearing *pho1 Δ rpb1-CTD-WT* or *rpb1-CTD-S5₃S5A₁₁* cells. After ethanol precipitation, the RNA pellets were dissolved in 20 μL hybridization buffer (80% formamide, 40 mM PIPES pH 6.4, 1 mM EDTA, 400 mM NaCl), heated at 80°C for 10 min, and then incubated at 42°C for 12–15 h. Following hybridization, 200 μL of RNase T1 (0.5 U in 10 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA) was added and the mixture was incubated at room temperature for 30 min. Digestion was quenched by adding 10 μL of 10% SDS. The samples were extracted with phenol:chloroform, ethanol precipitated, and analyzed by denaturing PAGE.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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