RNA polymerase II CTD phospho-sites Ser5 and Ser7 govern phosphate homeostasis in fission yeast

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ABSTRACT

Phosphorylation of the tandem YSPTSPS repeats of the RNA polymerase II CTD inscribes an informational code that orchestrates eukaryal mRNA synthesis. Here we interrogate the role of the CTD in phosphate homeostasis in fission yeast. Expression of Pho1 acid phosphatase, which is repressed during growth in phosphate-rich medium and induced by phosphate starvation, is governed strongly by CTD phosphorylation status, but not by CTD repeat length. Inability to place a Ser7-PO₄ mark (as in *S7A*) results in constitutive derepression of Pho1 expression in phosphate-replete medium. In contrast, indelible installation of a Ser7-PO₄ mimetic (as in *S7E*) hyper-represses Pho1 in phosphate-replete cells and inhibits Pho1 induction during starvation. Pho1 phosphatase is derepressed by ablation of the CTD Ser5-PO₄ mark, achieved either by mutating Ser5 in all consensus heptads to alanine, or replacing all Pro6 residues with alanine. We find that Ser5 status is a tunable determinant of Pho1 regulation, i.e., serial decrements in the number of consensus Ser5 heptads from seven to two elicits a progressive increase in Pho1 expression in phosphate-replete medium. Pho1 is also derepressed by hypomorphic mutations of the CTD kinase Cdk9. Inactivation of the CTD phosphatase Ssu72 attenuates Pho1 induction in wild-type cells and blocks Pho1 derepression in *S7A* cells. These experiments implicate Ser5, Pro6, and Ser7 as component letters of a CTD coding “word” that transduces a repressive transcriptional signal via serine phosphorylation.

Keywords: phosphate-responsive gene expression; acid phosphatase; phosphate transporter

INTRODUCTION

Phosphate homeostasis in the fission yeast *Schizosaccharomyces pombe* is governed by an intricate signaling network entailing positive and negative influences on the transcription of genes encoding proteins involved in extracellular phosphate mobilization and uptake, specifically a cell surface acid phosphatase Pho1 and an inorganic phosphate transporter Pho84 (Carter-O’Connell et al. 2012). Upregulation of the expression of these genes during phosphate starvation depends on the transcription factor Pho7 (Henry et al. 2011; Carter-O’Connell et al. 2012), a member of the zinc binuclear cluster family of fungal DNA-binding transcription regulators (MacPherson et al. 2006). The protein kinase Csk1 is a negative regulator of the phosphate response, as judged by the fact that *pho1* expression is constitutively turned on in *csk1Δ* cells under phosphate-replete conditions (Henry et al. 2011; Carter-O’Connell et al. 2012; Schwer et al. 2014). Csk1 is a CDK-activating kinase with several physiological targets, including the kinases Cdc2, Mcs6, and Cdk9 (Saiz and Fisher 2002; Pei et al. 2006). Mcs6 and Cdk9 figure

prominently in fission yeast transcription via their phosphorylation of the carboxy-terminal domain (CTD) of the Rpb1 subunit of RNA polymerase II (Pol2) and/or the carboxy-terminal domain (CTD) of the transcription elongation factor Spt5 (Pei et al. 2006; Viladevall et al. 2009).

The Pol2 CTD consists of tandemly repeated heptapeptides of consensus sequence Y¹S²P³T⁴S⁵P⁶S⁷. The inherently plastic CTD structure is modulated by phosphorylation of the Tyr1, Ser2, Thr4, Ser5, and Ser7 residues and by *cis-trans* isomerization of the prolines (Corden 2013; Eick and Geyer 2013). With up to 128ⁿ potential CTD primary structures (where *n* is the number of heptads), the CTD provides information about the state of the transcription machinery—a CTD code—that is “read” by diverse CTD receptor proteins that control transcription, modify chromatin structure, and catalyze or regulate mRNA capping, splicing, and polyadenylation (Corden 2013; Eick and Geyer 2013).

Informational rules for the CTD code in fission yeast have been elucidated by genetically manipulating the composition

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and structure of the Pol2 CTD, which consists of 25 YSPTSPS repeats connected to the body of the Rpb1 subunit via four degenerate repeats (the CTD “rump”) that deviate in size and/or sequence from the consensus heptad. A CTD composed of the rump plus 12 or more native heptads suffices for normal growth of *Schizosaccharomyces pombe* under all laboratory conditions tested (Schneider et al. 2010). By introducing alanines and conservative mutations in lieu of Tyr1, Ser2, Pro3, Thr4, Ser5, Pro6, and Ser7 in every consensus heptad of a fully functional Rpb1-CTD array (comprising the rump plus 14 consensus heptad repeats), we determined that (i) Tyr1, Pro3, Ser5, and Pro6 are essential for viability, by the criterion that alanine substitution is lethal, whereas Ser2, Thr4, and Ser7 are not essential; and (ii) *Y1F*, *Y1F* + *S7A*, *S2A* + *S7A*, and *T4A* + *S7A* mutants are viable, signifying that phenylalanine is functional in lieu of Tyr1 and that Ser5 is the only strictly essential phosphorylation site in fission yeast (Schwer and Shuman 2011; Schwer et al. 2012).

A connection between the Pol2 CTD and phosphate homeostasis emerged when we applied RNA-seq to gauge globally the impact of the loss of the four inessential CTD phosphoacceptors. This analysis illuminated how individual letters of the Pol2 CTD code affect the expression of limited and distinct sets of genes. To wit, CTD mutations *S2A*, *Y1F*, *S7A*, and *T4A* elicited $\geq$ twofold dysregulation of only 4.4%, 1.4%, 1.2%, and 0.14% of the annotated fission yeast protein-coding RNAs, respectively (Schwer et al. 2014). CTD letters Thr4 and Ser7 were identified as novel components of the phosphate response pathway, on which the *T4A* and *S7A* mutations had opposing effects. *T4A* reduced the expression of the genes encoding Pho1 and the Pho84 (without affecting the expression of the *pho7* or *csk1* genes that regulate the phosphate response pathway), while *S7A* increased Pho1 expression (Schwer et al. 2014).

Prompted by these initial findings, we aim here to further interrogate the role of the Pol2 CTD in the expression of Pho1 acid phosphatase during phosphate-replete and phosphate-starved states and in the transcriptional control of the phosphate-responsive *pho1* and *pho84* genes. We address the effects of (i) CTD length; (ii) single and combinatorial subtraction of potential phosphorylation sites, including Ser5; (iii) genetic manipulation of kinases; and (iv) inactivation of the CTD phosphatase Ssu72. Our results implicate Ser5, Pro6, and Ser7 as letters in a coding “word” that specifies repression of the phosphate regulon when phosphate is replete.

RESULTS

Effect of Pol2 CTD length on regulated Pho1 expression

To determine the impact of the CTD length on the induction of Pho1 under conditions of phosphate starvation, we exploited a set of fission yeast strains with chromosomal *rpb1* alleles encoding either the native wild-type CTD array (29 re-

peats, consisting of the rump plus 25 consensus heptads) or CTDs serially truncated at their C termini so as to comprise 26, 20, 18, 16, or 13 repeats (i.e., the rump plus 22, 16, 14, 12, or 9 consensus heptads) (Schneider et al. 2010). Cells grown in liquid culture in YES medium were washed and then incubated for 3 h either in synthetic medium containing 15.5 mM phosphate (phosphate-replete) or in medium lacking exogenous phosphate to elicit the starvation response. Acid phosphatase activity (a gauge of Pho1 enzyme level) was quantified by incubating suspensions of serial dilutions of the phosphate-replete or phosphate-starved cells for 5 min with *p*-nitrophenylphosphate and assaying colorimetrically the formation of *p*-nitrophenol. The basal phosphatase activity of wild-type *rpb1* cells with 29 repeats was increased 4.5-fold by phosphate starvation (Supplemental Fig. S1). Truncating the CTD had little effect on Pho1 activity under phosphate-replete conditions (Supplemental Fig. S1). CTD truncations also had little effect on the induction of Pho1 activity by phosphate starvation (Supplemental Fig. S1). Thus, CTD length is not a key determinant of the phosphate starvation response.

Effect of eliminating CTD phosphorylation sites, individually and pairwise

We surveyed our ensemble of viable *rpb1*-CTD mutants for effects on the Pho1 phosphate starvation response. The “wild-type” CTD control in these experiments consists of the rump plus 14 consensus heptads; the mutants have alanine or a conservative substitution in every one of the 14 heptads, either singly or in pairwise combinations (Schwer and Shuman 2011; Schwer et al. 2012). Phosphatase activity of *rpb1*-CTD-WT cells was increased fivefold by phosphate starvation (Fig. 1). Eliminating the Tyr1 or Ser2 hydroxyl groups

FIGURE 1. Effect of eliminating CTD phosphorylation sites on phosphate homeostasis. Isogenic heterothallic *S. pombe* *rpb1*-CTD strains in which 14 heptad repeats (either wild-type YSPTSPS or mutated heptads as specified) are appended to amino acid 1577 of Rpb1 (11,12) were grown in liquid culture in YES medium. Cells were then harvested, washed with water, and incubated for 3 h in PMG medium containing 15.5 mM phosphate (+ phosphate) or lacking exogenous phosphate (– phosphate). Pho1 phosphatase was assayed by conversion of *p*-nitrophenylphosphate to *p*-nitrophenol. The *y*-axis specifies the phosphatase activity (A_{410}) normalized to input cells (A_{600}).

in the *Y1F* and *S2A* strains had little effect on phosphatase activity under phosphate-replete conditions, but it did dampen the induction of Pho1 after 3 h of phosphate depletion, to 39% and 35% of the wild-type control (Fig. 1). When we tested a *Y1F*•*S2A* double mutant, we found that basal phosphatase activity was increased slightly, but the phosphatase activity after phosphate depletion remained at the same diminished level seen in the *Y1F* and *S2A* single mutants (Fig. 1). Thus, Tyr1 and Ser2 act concordantly to promote the induction of Pho1 during phosphate starvation.

The basal phosphatase activity of *T4A* cells in phosphate-rich medium was eightfold lower than wild type. *T4A* cells responded to phosphate starvation (12-fold induction), but the level of induced phosphatase activity was only 29% that of wild-type cells 3 h post-starvation. Thus, the Thr4 CTD mark exerts a positive effect on Pho1 expression, independent of exogenous phosphate status. In an *S2A*•*T4A* double mutant, basal phosphatase activity was largely restored, but there was hardly any increase in Pho1 activity at 3 h after phosphate depletion (Fig. 1).

In contrast, *S7A* cells displayed a high level of phosphatase activity in phosphate-rich medium, equivalent to the Pho1 activity of wild-type cells that were phosphate-starved for 3 h (Fig. 1), signifying that Ser7 is essential to repress Pho1 expression when phosphate is available. *S7A* cells further increased their phosphatase activity by 25% at 3 h after phosphate depletion. As noted previously (Schwer et al. 2014), the super-induction of Pho1 by *S7A* was eliminated in the *T4A*•*S7A* strain, in which the low basal and induced phosphatase activities echoed the *T4A* single mutant (Fig. 1). Apparently, the Thr4 mark is important for Pho1 expression even when a repressive signal is removed. In the *S2A*•*S7A* double mutant, we found that the derepressed pattern characteristic of *S7A* was maintained, albeit at lower magnitude, i.e., the basal phosphatase activity in *S2A*•*S7A* cells was threefold higher than wild-type, but 60% less than *S7A* (Fig. 1).

An instructive finding emerged from analysis of the *rpb1-CTD-S7E* strain, in which Ser7 in all heptads was replaced by the phosphomimetic glutamic acid. *S7E* cells had diminished basal phosphatase activity and failed to mount an inductive response after 3 h of phosphate starvation (Fig. 1). It would appear that Pho1 expression is governed strongly by CTD Ser7 status, whereby inability to place a Ser7-PO₄ mark (as in *S7A*) results in constitutive derepression of Pho1, and indelible installation of a Ser7-PO₄ mimetic (as in *S7E*) inhibits Pho1 induction.

Contribution of CTD Ser5 to regulated Pho1 expression

In light of the findings above regarding the importance of Ser7 in Pho1 regula-

tion, we aimed to gauge the impact of altered Ser5 content on phosphate homeostasis. Ser5 is the only phosphoacceptor amino acid in the Pol2 CTD that is strictly essential for growth of fission yeast. Replacing all of the Ser5 residues in the consensus heptads with alanine is lethal (Schwer and Shuman 2011). Through the analysis of chimeric CTDs, composed of the rump plus a total of 14 Ser5-containing and Ala5-containing heptads, in assorted combinations, we showed that as few as two Ser5 heptads sufficed for vegetative growth (Schwer et al. 2012). Immunoblotting cellular Rpb1 with phospho-specific antibodies showed that the intensity of the Ser5-PO₄ mark was proportional to the number of Ser5 heptads in the CTD. Furthermore, the intensity of the Ser7-PO₄ mark also correlated with the number of Ser5 heptads (Schwer et al. 2012). Therefore, we asked whether reduced Ser5 content might elicit a derepression of Pho1 akin to what we observed in *S7A* cells.

At the outset, we compared Pho1 regulation in wild-type *rpb1-CTD-(S5)₁₄* cells to that of three different chimeras in which the CTD contained a mixture of seven S5 heptads and seven S5A heptads, arrayed such that (i) the S5 and S5A heptads alternated (with heptads 1, 3, 5, etc. being S5A and 2, 4, 6, etc. being S5), (ii) the proximal half were S5A and the distal half were S5, or (iii) the proximal 7 heptads were S5 and the distal 7 heptads were S5A. The *(S5A)•(S5)₇*, *(S5A)₇•(S5)₇*, and *(S5)₇•(S5A)₇* strains displayed seven-, six-, and fourfold greater phosphatase activity than wild-type *(S5)₁₄* cells under phosphate-replete conditions and they elaborated greater than wild-type phosphatase activity in response to 3 h of phosphate starvation (Fig. 2A). Thus, even a 50% reduction in the Ser5 content of the 14-heptad CTD perturbed the phosphate regulatory axis.

We then proceeded to survey a series of chimeric CTDs consisting of 6, 5, 4, 3, or 2 Ser5-containing heptads linked to 8, 9, 10, 11, or 12 S5A heptads, respectively. The salient findings were that serial decrements in the number of consensus Ser5 heptads from seven to two elicited a progressive increase in Pho1 expression in phosphate-replete medium

FIGURE 2. Contribution of CTD Ser5 to regulated Pho1 expression. Cells with Rpb1-CTDs composed of the indicated mixtures of S5 heptads and S5A heptads (A), or CTD-MCE fusions in which the 14 heptads were all wild-type, S5A, or P6A (B), were assayed for Pho1 activity after incubation for 3 h in PMG medium containing 15.5 mM phosphate (+ phosphate) or lacking exogenous phosphate (– phosphate).

(Fig. 2A). Indeed, the basal activity of $(S5)_2(S5A)_{12}$ cells was 13-fold greater than the wild-type $(S5)_{14}$ cells. The level of phosphatase activity in $(S5)_2(S5A)_{12}$ cells did not increase after 3 h of phosphate starvation (Fig. 2A).

Replacing all of the Ser5 residues in the consensus heptads with alanine is lethal because the Ser5- PO_4 mark is needed for recruitment of the fission yeast mRNA capping enzymes RNA triphosphatase and RNA guanylyltransferase to the Pol2 elongation complex (Pei et al. 2003; Schwer and Shuman 2011; Doamekpor et al. 2014). Replacing all of the Pro6 residues in the consensus heptads with alanine is lethal because Pro6 is necessary to inscribe the Ser5- PO_4 mark needed for capping enzyme recruitment (Schwer et al. 2012). The lethality of *rpb1-CTD-S5A* and *rpb1-CTD-P6A* can be rescued by covalently fusing mammalian capping enzyme (MCE, a bifunctional RNA triphosphatase-guanylyltransferase) to the mutant Rpb1-S5A and Rpb1-P6A polypeptides. We find here that Pho1 expression in the *rpb1-CTD-S5A-MCE* and *rpb1-CTD-P6A-MCE* fusion strains was derepressed in phosphate-replete medium vis-à-vis the control *rpb1-CTD-WT-MCE* fusion strain (Fig. 2B).

These experiments establish that CTD coding letters Ser5, Pro6, and Ser7 are essential for the repression of Pho1 activity under phosphate-rich conditions.

Mapping the 5' ends of mRNAs and a noncoding RNA transcribed from a clustered phosphate response regulon

The fission yeast phosphate transporter Pho84 and the acid phosphatase Pho1 are encoded by adjacent co-oriented genes on chromosome II (Fig. 3A). Previously, we used reverse transcriptase primer extension analysis to map the 5' end of the *pho1* mRNA to a single start site 51 nucleotides upstream of the AUG start codon of the *pho1* open reading frame (Schwer et al. 2014). Here, we used the same strategy to locate the 5' end of the neighboring *pho84* gene. A ^{32}P -labeled DNA primer complementary to nucleotides -87 to -108 upstream of the *pho84* open reading frame was annealed to total yeast RNA and then subjected to reverse transcription. The RT primer extension product was analyzed by denaturing PAGE in parallel with a

chain-terminated sequencing ladder generated by DNA polymerase-catalyzed extension of the same ^{32}P -labeled primer annealed to a DNA template extending from nucleotides -500 to +130 of the transporter gene. Two 5' ends were thereby located 151 and 149 nt upstream of the start codon of the *pho84* open reading frame (Fig. 3B).

Two recent studies have identified in fission yeast *rrp6Δ* cells (which lack a catalytic subunit of the nuclear exosome) a long noncoding (lnc) RNA transcribed from the ~1.6-kb

FIGURE 3. 5'-end mapping of RNAs transcribed from the fission yeast phosphate regulon. (A) Schematic illustration of the adjacent *pho84*⁺ (SPBC8E4.01c) and *pho1*⁺ (SPBP4G3.02) genes on fission yeast chromosome II, with the open reading frames denoted by arrows in the direction of mRNA synthesis. The experimentally mapped 5' ends of the *pho84* and *pho1* mRNAs and the intervening noncoding *prt* RNA are indicated by their distance (nt) from the translations start sites of the *pho84* and *pho1* mRNAs. (B) To map the 5' ends of the *pho84* and *prt* RNAs, we analyzed the reverse transcriptase primer extension product (lanes *) in parallel with a series of DNA-directed primer extension reactions that contained mixtures of standard and chain-terminating nucleotides (the chain terminator is specified above the lanes). The primer extension products were analyzed by electrophoresis through a 42-cm denaturing 8% polyacrylamide gel and visualized by autoradiography of the dried gel. The 5' RNA ends are denoted by ◀ to the right of the gel. The coding strand DNA sequences flanking the *pho84* and *prt* transcription start sites are shown at right, with the start sites denoted by ↑. (C) ^{32}P -labeled oligonucleotide primers complementary to *pho1* and *act1* mRNAs (left panel), *pho84* mRNA RNA (top right panel), or *prt* RNA (bottom right panel) were annealed to total RNA from *pho7*⁺ (WT) or *pho7Δ* strains and extended with reverse transcriptase. The reaction products were analyzed by denaturing PAGE and visualized by autoradiography.

region between the *pho84* and *pho1* genes and extending through the *pho1* open reading frame (Lee et al. 2013; Shah et al. 2014). This lncRNA, named *prt*, has been implicated as a negative regulator of *pho1* expression (Lee et al. 2013; Shah et al. 2014). Here we report that the *prt* transcript is detectable in wild-type *rrp6*⁺ cells by primer extension analysis with a ³²P-labeled DNA primer complementary to the intergenic region. PAGE analysis of the reverse transcriptase primer extension product in parallel with a DNA-templated sequence ladder mapped the *prt* start site to an A position located 1198 nt upstream of the *pho1* start codon (Fig. 3B). The 5' end of *prt* in wild-type cells that we obtained by primer extension is at the same position reported for *prt* in *rrp6Δ* cells, as determined by PCR amplification across the 5'/3' junctions of decapped and intramolecularly ligated RNA circles, followed by cloning and sequencing of individual junctions (Shah et al. 2014).

Differential requirements for Pho7 for expression of *prt* and the flanking mRNAs

Primer extension analysis was used to gauge the role of Pho7 in the expression of the three transcription units that comprise the phosphate-responsive gene cluster. Total RNA from wild-type and *pho7Δ* cells was annealed to a mixture of two ³²P-labeled DNA primers complementary to mRNAs encoding actin and Pho1. Discrete gene-specific extension products for both mRNAs were detected in a reaction programmed by RNA from wild-type cells, but only the actin product was detected in the reaction templated by RNA from *pho7Δ* cells (Fig. 3C). Primer extension reactions with a probe specific to *pho84* revealed that transcription of the phosphate transporter gene was eliminated in *pho7Δ* cells (Fig. 3C). In contrast, the *prt* primer extension product was unaffected by *pho7Δ* (Fig. 3C). We conclude that *prt* transcription initiation is not dependent on the Pho7 transcription factor.

Effects of Pol2 CTD mutations on expression of phosphate-responsive mRNAs

The cell-based enzyme assays presented above showed that Pho1 was derepressed under phosphate-replete conditions by Pol2 CTD mutations that altered the number of Ser7 and Ser5 coding letters. To correlate these findings with transcription of the phosphate-responsive gene cluster, we analyzed RNA levels by primer extension templated by total RNA isolated from wild-type and mutant yeast strains that had been grown in liquid culture in YES medium, washed, and then incubated for 3 h either in synthetic medium containing 15.5 mM phosphate (phosphate +) or in medium lacking exogenous phosphate (phosphate −) (Fig. 4A). Actin mRNA was assayed as an internal control. We found that the *pho1* mRNA and *pho84* mRNA primer extension products were (i) increased under phosphate-replete condi-

tions by the *S7A*, (S5)₃(S5A)₁₁, and (S5)₇(S5A)₇ CTD alleles that derepressed Pho1 enzymatic activity; and (ii) decreased under phosphate-replete conditions by the *T4A*, *T4A*•*S7A*, and *S7E* CTD alleles that hyper-repressed basal acid phosphatase enzymatic activity (Fig. 4A). The *pho1* and *pho84* mRNAs were induced in wild-type, *T4A*, and *T4A*•*S7A* cells after 3 h of phosphate starvation, but the mRNA inductive response was blunted in *S7E* cells (Fig. 4A).

The radiolabeled *pho1* primer extension product was quantified and normalized to the internal actin control, these values were then expressed as ratios to the level of *pho1* mRNA in wild-type cells in phosphate-replete medium (Fig. 4B). We also performed independent measurements of *pho1* mRNA levels by RT-qPCR with *pho1*-specific primers, again normalized to an internal actin mRNA control and expressed for each strain in +/− phosphate conditions as the ratio to *pho1* mRNA in wild-type cells in phosphate-rich medium (Fig. 4C). The two methods revealed concordant mRNA expression patterns, whereby *pho1* transcription is derepressed by CTD alleles (S5)₃(S5A)₁₁, *S7A*, and (S5)₇(S5A)₇ and hyper-repressed by *T4A*, *T4A*•*S7A*, and *S7E*. The key point here is that the Ser7 and Ser5 CTD coding letters exert repressive effects on the Pho7-dependent mRNA transcription units under phosphate-replete conditions.

Hyper-repression of Pho1 by CTD mutations correlates with elevated basal *prt* RNA level

The *prt* RNA is implicated in repression of *pho1* under phosphate-replete conditions, i.e., a deletion of the *prt* promoter element causes derepression of *pho1* mRNA and phosphatase activity (Lee et al. 2013; Shah et al. 2014). We found here that the hyper-repressing *rpb1*-CTD-*S7E* allele increased the level of the *prt* RNA in phosphate-replete cells, as gauged by primer extension analysis (Fig. 4A), or by RT-qPCR, which indicated that the basal *prt* RNA level was 5.3-fold higher in *S7E* cells than in wild-type (Supplemental Fig. S2). The *T4A* and *T4A*•*S7A* alleles that reduced *pho1* expression in phosphate-rich medium also elicited 2.3-fold and 3.3-fold increases in *prt* RNA (Supplemental Fig. S2). The RT-qPCR data revealed lowered *prt* RNA levels 3 h after phosphate starvation of wild-type, *T4A*, and *T4A*•*S7A* cells (Supplemental Fig. S2), concomitant with the induction in *pho1* mRNA levels at 3 h post-starvation (Fig. 4). Whereas the *S7E* mutant did evince a decrement in *prt* RNA at 3 h after phosphate starvation, the post-starvation level of *prt* was still 2.6-fold greater than that of wild-type cells in phosphate-replete medium (Supplemental Fig. S2), which might explain the attenuated induction of acid phosphatase activity (Fig. 1) and *pho1* mRNA (Fig. 4) in phosphate-starved *S7E* cells. The derepressing *S7A* allele had little effect on *prt* RNA level in phosphate-replete cells (Supplemental Fig. S2), suggesting that the *S7A* effect is not solely via *prt*.

Previous microarray analysis of the transcriptional response of fission yeast as a function of time after transfer to

FIGURE 4. Effects of CTD mutations on expression of phosphate-responsive mRNAs. (A) ³²P-labeled primers complementary to *pho1*, *pho84*, and *act1* mRNAs (top panel) or *prt* RNA (bottom panel) were annealed to total RNA isolated from the indicated fission yeast strains that had been incubated for 3 h in PMG medium containing 15.5 mM phosphate (+ phosphate) or lacking exogenous phosphate (– phosphate). After primer extension with reverse transcriptase, the reaction products were analyzed by denaturing PAGE and visualized by autoradiography. (B) The *pho1* primer extension product in panel A was quantified and normalized to that of *act1* measured for the same RNA sample. The bar graph shows the fold-change in *pho1* relative to the wild-type + phosphate control (defined as 1.0). (C) RT-qPCR analysis was performed as described in Materials and Methods. The level of *pho1* transcript was normalized to that of *act1* measured for the same RNA sample. The bar graph shows the fold-change in *pho1* RNA relative to the wild-type + phosphate control (defined as 1.0). Each datum in the bar graph is the average of values from RT-qPCR analyses of RNAs from three independent yeast cultures. The error bars denote SEM.

phosphate-free medium (Carter-O’Connell et al. 2012) showed no increase in *pho1* mRNAs after 30 min, a twofold increase after 1 h, and a fourfold induction after 2 h. There is a lag between mRNA induction and the elaboration of active acid phosphatase on the surface of wild-type cells. Acid phosphatase was evident at 3 h after transfer to phosphate-free medium (but not at 2 h) and then increased steadily up to at least 6 h (Supplemental Fig. S3), consistent with ongoing translation of the induced *pho1* mRNA. These findings informed our choice of 3 h as a standard time to assess the effects of CTD mutations on pre- and post-starvation Pho1 expression. In the case of the *T4A*, *T4A•S7A*, and *S7E* mutants that reduced Pho1 activity at 3 h post-starvation, tracking the starvation response over a longer time frame (6 h) revealed that the induction of acid phosphatase activity was delayed, but not abolished (Supplemental Fig. S3). The *T4A* strain started to accrue acid phosphatase activity at 4 h, which increased steadily thereafter, with a similar slope (albeit shifted to the right) as the wild-type strain (Supplemental Fig. S3). The *T4A-S7A* strain evinced a similar delay, with a slightly higher slope during recovery (conceivably because of the loss of the repressive Ser7 residue) (Supplemental Fig. S3). Because the *T4A*-mutated and wild-type cells had similar *pho1* mRNA levels at 3 h post-starvation, we surmise

that the phosphatase activity lag reflects delayed onset of *pho1* transcription induction by Pol2 lacking the Thr4 coding letter. The salient finding was that *S7E* cells displayed a longer lag (5 h) and a more shallow increase in acid phosphatase activity (Supplemental Fig. S3), consistent with the lower *pho1* mRNA level seen at 3 h after starvation in *S7E* cells.

rrp6Δ delays the induction of both *pho1* and *pho84*

Deletion of the gene encoding the Rrp6 subunit of the fission yeast nuclear exosome elicits the accumulation of *prt* lncRNA, as gauged by Northern blot analysis (Lee et al. 2013; Shah et al. 2014). Increased *prt* in turn dampens the induction of *pho1* in response to phosphate starvation (Lee et al. 2013; Shah et al. 2014). In light of the present results showing the concordant effect of certain CTD mutations on *pho1* and *pho84* mRNA levels (Fig. 4A), we tracked by RT-qPCR and enzyme activity assays the phosphate starvation response in wild-type *rrp6⁺* and *rrp6Δ* cells (Fig. 5). The onset of Pho1 phosphatase accumulation in *rrp6Δ* cells occurred between 5 and 6 h after transfer to phosphate-free medium, i.e., with a ~3-h delay compared to the inflection point of Pho1 accumulation between 2 and 3 h post-transfer in the wild-type strain (Fig. 5A). Whereas *pho1* and *pho84*

FIGURE 5. *rrp6Δ* delays the induction of both *pho1* and *pho84*. (A) Fission yeast *rpb1⁺ rrp6⁺* (WT) and *rpb1⁺ rrp6Δ* strains were grown in YES medium to A_{600} of 0.5 to 0.7. The cells were harvested, washed in water, and after withdrawing an aliquot to measure Pho1 activity (time 0), the cells were transferred to PMG medium lacking exogenous phosphate. Pho1 activity was assayed after incubation for the times specified. (B,C) Total RNA isolated from WT and *rrp6Δ* cells at time 0 and at 3 and 6 h post-transfer to phosphate-free medium was used to assay by RT-qPCR the levels of *pho1* and *pho84* mRNAs (B) and *prt* RNA (C). The transcript levels were normalized to those of *act1* measured for the same RNA samples. The bar graph shows the fold-change in RNA relative to the WT time 0 value (defined as 1.0). Each datum in the bar graphs is the average of values from RT-qPCR analyses of RNAs from three independent yeast cultures. The error bars denote SEM.

mRNA levels were induced in wild-type cells by 3 h post-starvation, there was no detectable increase in either transcript in *rrp6Δ* cells after 3 h of phosphate starvation (Fig. 5B). However, *pho1* and *pho84* mRNA levels did increase after 6 h post-starvation of *rrp6Δ* cells (Fig. 5B), signifying a concordantly delayed induction of both phosphate-responsive genes.

The level of the *prt* transcript was 50-fold higher in phosphate-replete *rrp6Δ* cells versus wild-type, and declined by twofold and fivefold, respectively, after 3 h and 6 h of phosphate-starvation (Fig. 5C). The finding that *rrp6Δ* cells managed to induce the *pho1* and *pho84* mRNAs at 6 h post-starvation, at which the *prt* RNA level was still 10-fold higher than in phosphate-replete wild-type cells, suggests that the mere presence of *prt* RNA transcribed from the *prt* locus in *cis* is not sufficient per se to keep the flanking *pho1* and *pho84* genes durably turned off when phosphate is limiting.

Effects of manipulating protein kinases on Pho1 regulation

Pho1 expression is strictly dependent on the yeast transcription factor Pho7 (Henry et al. 2011; Carter-O'Connell et al. 2012), such that *pho7Δ* cells have virtually no acid phosphatase activity, even when starved for phosphate (Fig. 6). In contrast, *csk1Δ* cells constitutively express acid phosphatase in phosphate-replete conditions (Henry et al. 2011; Carter-O'Connell et al. 2012), at a level eightfold greater than wild type (Fig. 6). As noted previously (Henry et al. 2011; Carter-O'Connell et al. 2012), we find that all Pho1 expression in *csk1Δ* cells is abolished by *pho7Δ* (Fig. 6), implying that the gain of Pho1 expression caused by failure of Csk1 to phosphorylate one or more of its physiological targets is effected through the normal Pho7-dependent transcription program, not via activation of a bypass pathway. Similarly, the derepression of Pho1 in *rpb1-CTD-S7A* and *(S5A•S5)₇* cells was abolished by *pho7Δ* (not shown).

The cyclin-dependent kinase Cdk9 is a physiological target for activation by Csk1 (Pei et al. 2006). Cdk9, in a complex with its cyclin partner Pch1, catalyzes serine phosphorylation of the Pol2 CTD (Pei and Shuman 2003; Pei et al. 2003). Csk1 stimulates the CTD kinase activity of Cdk9•Pch1 by phosphorylating Cdk9 on residue Thr212 of the activating “T-loop” segment. The activated Cdk9•Pch1 enzyme phosphorylates the Pol2 CTD at positions Ser2 and Ser5 of the CTD heptad (Pei et al. 2006). The Cdk9-T212A mutant is refractory to activation by Csk1. Indeed, fission yeast *csk1Δ* and *cdk9-T212A* strains phenocopy each other with respect to their poor growth on minimal media and cold-sensitivity on rich medium (Pei et al. 2006). The key question here is: Do the *csk1Δ* and *cdk9-T212A* mutations elicit similar effects on phosphate homeostasis?

Pho1 was constitutively expressed in *cdk9-T212A* cells (sevenfold greater than wild-type) (Fig. 6). Moreover, the derepression of Pho1 by *cdk9-T212A* was effaced by *pho7Δ*

FIGURE 6. Effects of manipulating Csk1 and Cdk9 kinases and Su72 phosphatase on Pho1 regulation. *rpb1⁺* cells, either wild-type or bearing additional mutations as specified, were assayed for Pho1 activity after incubation for 3 h in PMG medium containing 15.5 mM phosphate (+ phosphate) or lacking exogenous phosphate (– phosphate).

(Fig. 6). These results suggest that Csk1 acts through Cdk9 to repress Pho1 expression under phosphate-replete conditions.

The phosphomimetic Cdk9 mutation T212E enhances the kinase activity of recombinant Cdk9•Pch1 about threefold compared with the wild-type enzyme that had not been activated by Csk1 and to the T212A mutant enzyme. Nonetheless, the kinase activity of Cdk9-T212E•Pch1 is threefold lower than that of wild-type Cdk9•Pch1 that had been activated by Csk1 (Pei et al. 2006). Thus Cdk9-T212E appears to be a hypomorphic mutation (Pei et al. 2003). In that vein, it is noteworthy that Pho1 was expressed constitutively in *cdk9-T212E* cells (Fig. 6).

Phosphatase Ssu72 is important for Pho1 expression

The CTD phosphatase Ssu72 is an agent of Ser5 and Ser7 dephosphorylation in the budding yeast *Saccharomyces cerevisiae* (Krishnamurthy et al. 2004; Hausmann et al. 2005; Bataille et al. 2012; Zhang et al. 2012). Ssu72 catalyzes phosphoryl transfer via a covalent enzyme-cysteinyl-S-phosphate intermediate; mutation of the active site cysteine to serine or alanine abolishes phosphatase activity. The Ssu72 protein and its phosphatase activity are essential for the viability of *S. cerevisiae* (Krishnamurthy et al. 2004). In contrast, *S. pombe* Ssu72 (SPAC3G9.04) is dispensable for growth of fission yeast (Schwer et al. 2012). To gauge the potential contribution of Ssu72 to fission yeast phosphate homeostasis, we replaced the wild-type *ssu72*⁺ gene with *ssu72-C13S*, in which the cysteine nucleophile was mutated to serine. We found that *ssu72-C13S* cells had diminished basal Pho1 activity and a feeble inductive response to phosphate starvation compared with wild-type cells (Fig. 6; Supplemental Fig. S3). RNA analysis by primer extension revealed that the *ssu72-C13S* mutation elicited hyper-repression of the *pho1* and *pho84* mRNAs under phosphate-replete conditions and attenuated the transcriptional response to phosphate depletion, such that the levels of *pho1* and *pho84* mRNAs achieved in *C13S* cells at 3 h post-starvation were about the same as those in nonstarved wild-type cells (Supplemental Fig. S4).

Moreover, *ssu72-C13S* eliminated the high levels of Pho1 seen in *cdk9* mutants *T212A* and *T212E* (Fig. 6). We infer that Ssu72 phosphatase activity is important for Pho1 expression, even when a repressive arm of the regulatory circuit is removed.

Genetic interaction of Ssu72 with the Pol2 CTD

In an effort to connect the Ssu72 effect on Pho1 expression with CTD phosphorylation status, we performed Western blotting of total protein from *ssu72*⁺ and *ssu72-C13S* cells with CTD phospho-specific antibodies. However, this analysis revealed no changes in the bulk phosphorylation signals for the Ser5-PO₄ or Ser7-PO₄ marks (not shown). To query a genetic interaction between Ssu72 and the Pol2 CTD, we tested for mutational synergy between *ssu72-C13S* and our collection of *rpb1* alleles with serially truncated CTDs. We found that shortening the CTD to 20, 18 and 16 repeats in the *ssu72-C13S* strain elicited progressively worsening cold-sensitive and temperature-sensitive growth defects (Fig. 7A). Indeed, a reduction to 16 repeats (rump plus 12 consensus heptads) rendered the *ssu72-C13S* strain inviable at 18°C, 20°C, and 37°C and barely viable at 30°C and 34°C. The salient finding was that the *cs* and *ts* defects of *ssu72-C13S* in the context of CTD shortened to 18 repeats (rump plus 14 consensus heptads) were suppressed by mutating the 14 Ser7 positions to alanine, i.e., *S7A* restored wild-type growth at 30°C, 34°C, and 37°C to *ssu72-C13S* cells with 18 repeats (as gauged by colony size) and enhanced growth at 18°C, 20°C, and 25°C (Fig. 7A). An identical synthetic growth defect (*cs* and *ts*) was observed when the CTD was shortened to 18 repeats in the *ssu72Δ* null strain, which was suppressed in the identical fashion by *S7A* (not shown). These results establish that the phosphatase activity of Ssu72 becomes critical for fission yeast growth when CTD length is shortened. Relaxation of this requirement by *S7A* (which precludes deposition of the Ser7-PO₄ mark) raises the prospect that Ssu72 might be an agent of Ser7 dephosphorylation in vivo in fission yeast.

FIGURE 7. Genetic and functional interaction of Ssu72 with the Pol2 CTD. (A) Exponentially growing cultures of *S. pombe* strains with the indicated chromosomal *rpb1*-CTD and *ssu72* alleles were adjusted to A₆₀₀ of 0.1, and aliquots of serial fivefold dilutions were spotted to YES agar and incubated at the indicated temperatures. (B) Fission yeast cells with genotypes as specified were assayed for Pho1 activity after incubation for 3 h in PMG medium containing 15.5 mM phosphate (+ phosphate) or lacking exogenous phosphate (– phosphate).

When we tested the *ssu72-C13S rpb1-CTD-S7A* strain for Pho1 expression, we found that inactivation of the Ssu72 enzyme effaced the constitutive expression of Pho1 that was characteristic of the *S7A* strain (Fig. 7B). *ssu72-C13S rpb1-CTD-S7A* cells resembled the *ssu72-C13S* strain with respect to decreased basal Pho1 activity and feeble induction by phosphate starvation (Fig. 7B). This result signifies that the requirement for Ssu72 for Pho1 expression cannot simply be for dephosphorylation of Ser7 (i.e., if that were the case, then *ssu72-C13S rpb1-CTD-S7A* ought to have phenocopied *S7A*).

DISCUSSION

The present study sheds new light on the fission yeast CTD code in general and its particular role in phosphate homeostasis. The information content of the code is multilayered. The most basic components are the 12 individual “letters” of the consensus heptad: Tyr1/Tyr1-PO₄, Ser2/Ser2-PO₄, Pro3, Thr4/Thr4-PO₄, Ser5/Ser5-PO₄, Pro6, and Ser7/Ser7-PO₄. Systematic CTD mutagenesis by alanine scanning and conservative substitutions has established that seven of the letters, including the Tyr1-PO₄, Ser2-PO₄, Thr4-PO₄, and Ser7-PO₄ marks, are inessential for fission yeast vegetative growth (Schwer and Shuman 2011; Schwer et al. 2012). Interrogating several of the *rpb1-CTD* mutants by RNA-seq revealed how individual coding letters govern distinct gene expression programs (Schwer et al. 2014). Insights to the vocabulary of the code emerged when mutations in two coding letters coordinately affected the expression of a common set of genes, in which case the two letters might comprise a coding “word” read by proteins that control expression of the shared target gene set. This was the case for the *Y1F* and *S2A* mutations. The most striking expression signature of the *Y1F* and *S2A* alleles was the derepression of the multi-gene iron homeostasis regulon (Schwer et al. 2014), which is normally silenced under iron-replete conditions by the DNA-binding transcription factor Fep1 (Labbé et al. 2013). We proposed that the two-letter CTD Tyr1-Ser2 coding word is necessary to transduce a repressive signal from Fep1 to the transcription apparatus.

Here we expanded on earlier findings that implicated the Pol2 CTD in the expression of phosphate-regulated genes. The salient new data implicate Ser5, Pro6, and Ser7 as component letters of a CTD word needed to dampen Pho1 expression under phosphate-replete conditions. The opposite effects of the *S7A* mutation that erases the Ser7-PO₄ mark (derepression of Pho1 in the presence of phosphate) versus the *S7E* mutation that mimics the Ser7-PO₄ mark (hyper-repression of Pho1 when phosphate is available and feeble induction of Pho1 during phosphate starvation) suggest that Ser7-PO₄ elicits Pho1 silencing.

Assigning a gene expression signature to the Ser5 and Ser5-PO₄ letters is complicated by the fact that replacing all Ser5 positions with alanine is lethal, because Ser5-PO₄ is essential

to recruit the mRNA capping apparatus to the Pol2 elongation complex. Here we exploited truncated CTDs and chimeric S5•S5A CTDs with progressively reduced Ser5 content to provide the first clues to the contributions of Ser5 to a specific regulatory circuit. Whereas truncating the CTD to contain a rump plus nine consensus Ser5-containing heptads had no impact on regulated Pho1 expression (Supplemental Fig. S1, the *n* = 13 strain), a chimeric (S5)_{*n*}(S5A)_{14-*n*} CTD containing seven or fewer Ser5 heptads resulted in derepression of Pho1 in phosphate-rich medium and hyper-induction during starvation. The degree of derepression increased steadily as “*n*” was decreased from seven to two Ser5 heptads.

As we show here, reduced Ser5 content mimicked *S7A* with respect to dysregulation of Pho1 expression. We speculate that Ser5 content and phosphorylation status might exert its effect on phosphate homeostasis, in some part, via Ser7 and its phosphorylation, insofar as (i) mutating all Ser5 to alanine effaces the Ser7-PO₄ mark; (ii) mutating all Ser7 to alanine has no discernible effect on the Ser5-PO₄ mark; and (iii) the signal intensity of the Ser5-PO₄ and Ser7-PO₄ marks both increase in proportion to Ser5 heptad content in the chimeric (S5)_{*n*}(S5A)_{14-*n*} strains (Schwer et al. 2012). Similarly, we infer that *P6A* dysregulates Pho1 expression in the same fashion as *S5A* and *S7A* because *P6A* precludes inscription of the Ser5-PO₄ mark (Schwer et al. 2012).

The prominent role of CTD phosphorylation in silencing Pho1 under replete conditions resonates with the findings that mutations of Csk1 and Cdk9, which together comprise a two-kinase cascade that phosphorylates the CTD, also de-repress Pho1 expression in replete medium. In contrast, inactivation of the CTD phosphatase Ssu72 blocks Pho1 expression, and does so even in genetic backgrounds that elicit constitutive Pho1 induction (e.g., *rpb1-CTD-S7A* and *cdk9-T212E*). Because the biochemical activity and substrate specificity of fission yeast Ssu72 are uncharted (and because our efforts to produce recombinant *S. pombe* Ssu72 in fission yeast in *E. coli* have yielded intractably insoluble protein), we cannot speculate presently as to the identity of the phosphoprotein substrate(s) on which Ssu72 must act to promote Pho1 expression.

Finally, our results here confirm and add to recent studies of Lee et al. (2013) and/or Shah et al. (2014) regarding the negative influence of *pri* lncRNA on the phosphate starvation response. We find that (i) *pri* is detectable by primer extension and RT-qPCR assays in wild-type fission yeast under phosphate-replete conditions; (ii) *pri* level is 50-fold higher in *rrp6Δ* cells; (iii) the 5′ ends of *pri* are the same in *rrp6*⁺ and *rrp6Δ* cells; (iv) the high *pri* level that accumulates in *rrp6Δ* cells causes a kinetic delay in the *pho1* inductive response (by ~3 h) but does not durably prevent the induction; and (v) the hyper-repression of Pho1 by CTD mutations *S7E*, *T4A*, and *T4A•S7A* in the presence of phosphate correlates with increased levels of *pri* RNA. Here we note that the *rrp6Δ* mutation elicits similar effects on the upstream *pho84* gene of the phosphate-responsive locus,

manifest by hyper-repression of *pho84* in phosphate-replete *rrp6Δ* cells and delayed induction of *pho84* mRNA after phosphate starvation. This result raises the prospect that the *pri* lncRNA acts as a repressor of both upstream and downstream protein-coding genes. (Alternatively, there could be another regulatory RNA, distinct from *pri* but also increased in *rrp6Δ* cells, that controls *pho84* expression.) Although the mechanism of *pri* action is not fully understood, current models posit that *cis*-acting elements in the nascent *pri* transcript recruit the exosome and RNAi machinery, which in turn establish a repressive state at the *pho1* gene (Lee et al. 2013; Shah et al. 2014). Our results implicate Pol2 itself (via the CTD) as a key player in controlling the phosphate starvation response.

MATERIALS AND METHODS

Assay of induction of acid phosphatase activity after phosphate starvation

Aliquots of exponentially growing *S. pombe* cultures in YES (yeast extract with supplements) medium were harvested, the cells were washed in water and adjusted to A_{600} of ~0.3 in PMG (Pombe glutamate) medium, either with 15.5 mM phosphate (+) or without phosphate (–). After incubation for 3 h at 30°C, cells were harvested, washed and suspended in water to attain A_{600} of 1.25. To quantify acid phosphatase activity, reaction mixtures (200 μ L) containing 100 mM sodium acetate (pH 4.2), 10 mM *p*-nitrophenylphosphate, and serial twofold dilutions of cells (ranging from 0.00625 to 0.1 A_{600} 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 A_{410} (*p*-nitrophenol production) to A_{600} (cells). Each datum in the bar graphs is the average of three assays using cells from three independent cultures $\pm$ SEM.

RNA analyses

Total RNA was extracted via the hot phenol method from 15 A_{600} units of yeast cells that had been incubated for 3 h at 30°C in PMG medium with 15.5 mM phosphate (+) or without phosphate (–). For RT-qPCR, 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 and adjusted to a concentration 600 ng/ μ L. Reverse transcription (RT) and gene-specific quantitative PCR (qPCR) were performed as previously described (Schwer et al. 2014). Primer extension assays (Schwer et al. 2014) were programmed by 10 μ g (for analysis of *pho1*, *pho84*, and *act1*) or 20 μ g (for *pri*) of total yeast RNA as templates for M-MuLV reverse transcriptase-catalyzed elongation of 5' 32 P-labeled oligodeoxynucleotide primers complementary to the transcripts of interest (see Supplemental Table S1 for primer sequences).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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RNA polymerase II CTD phospho-sites Ser5 and Ser7 govern phosphate homeostasis in fission yeast

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