Interactions between Fission Yeast Cdk9, Its Cyclin Partner Pch1, and mRNA Capping Enzyme Pct1 Suggest an Elongation Checkpoint for mRNA Quality Control

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RNA polymerase II (pol II) is subject to an early elongation delay induced by negative factors Spt5/Spt4 and NELF, which is overcome by the positive factor P-TEFb (Cdk9/cyclin T), a protein kinase that phosphorylates the pol II C-terminal domain (CTD) and the transcription elongation factor Spt5. Although the rationale for this arrest and restart is unclear, recent studies suggest a connection to mRNA capping, which is coupled to transcription elongation via physical and functional interactions between the cap-forming enzymes, the CTD-triphosphatase, (ii) the diphosphate RNA end is capped with GMP by RNA guanylyltransferase, and (iii) the GpppN cap is methylated by RNA (guanine-N7) methyltransferase (1). Targeting of cap formation to pre-mRNAs depends on interactions of the capping enzymes with the phosphorylated C-terminal domain (CTD) of the largest subunit of pol II (Ref. 2 and citations therein). Recruitment of the capping apparatus to the elongation complex requires the TFIIH-associated CTD kinase (Kin28 in yeast, Cdk7 in mammals), which phosphorylates Ser-5 of the CTD heptad repeat YSPTSPS (3, 4).

Other protein-protein contacts may also be involved in coupling capping to pol II transcription elongation. The pol II elongation factor Spt5 binds directly to the triphosphatase and guanylyltransferase components of the mammalian and Schizosaccharomyces pombe capping apparatus (5, 6). The fission yeast S. pombe employs a distinctive strategy of cap targeting whereby the triphosphatase (Pct1) and guanylyltransferase (Pce1) enzymes of the capping apparatus are not associated physically with each other (as they are in budding yeast and metazoans), but instead bind independently to the phosphorylated pol II CTD and to the unphosphorylated C-terminal domain of Spt5 (6, 7). The S. pombe Spt5 CTD consists of tandem repeats of a nonapeptide motif of consensus sequence TPAYNSGSK (6).

The HIV Tat protein binds to the triphosphatase and guanylyltransferase domains of mammalian capping enzyme Mec1 and up-regulates both activities (8). Spt5 and Tat cooperate to regulate HIV transcription elongation. Spt5 and its binding partner Spt4 comprise the transcription elongation factor DSIF (DRB sensitivity-inducing factor) (9, 10). DSIF binds to pol II and, in conjunction with NELF, represses elongation at promoter-proximal positions (Ref. 11 and citations therein). Escape from the elongation delay depends on P-TEFb (positive transcription elongation factor b), a DRB-sensitive protein kinase that phosphorylates both the pol II CTD and Spt5 (11–15). P-TEFb consists of two subunits, Cdk9 and cyclin T1, and it binds to Tat via cyclin T1 (Ref. 13 and citations therein). Expression of a dominant negative version of Spt5 in human cells results in stimulation of transcription from the HIV-LTR and other promoters, implying that wild-type human Spt5 is a negative regulator of transcription (10). Depletion of Cdk9 or cyclin T in Caenorhabditis elegans embryos results in a general shutoff of pol II transcription (16). Rescue of heat-shock transcription in Cdk9-depleted embryos by co-depletion of Spt5 and Spt4 provides evidence for an inhibitory role for Spt5/Spt4 during transcription elongation in vivo (16).

A purpose of this regulatory circuit may be to ensure timely capping of the nascent pre-mRNA before committing pol II to processive elongation (8). In the case of HIV, Spt5-induced arrest at promoter-proximal sites would maximize the oppor-

The 5′-cap is the defining structural feature of eukaryotic mRNA. Consisting of m′G linked via an inverted 5′-5′ triphosphate bridge to the initiating nucleoside of the transcript, the cap is formed by enzymatic modification of pre-mRNAs as they are being synthesized by RNA polymerase II (pol II).1 Capping entails three reactions: (i) the 5′-triphosphate end of the nascent pre-mRNA is hydrolyzed to a diphosphate by RNA triphosphatase, (ii) the diphosphate RNA end is capped with GMP by RNA guanylyltransferase, and (iii) the GpppN cap is methylated by RNA (guanine-N7) methyltransferase (1). Targeting of cap formation to pre-mRNAs depends on interactions of the capping enzymes with the phosphorylated C-terminal domain (CTD) of the largest subunit of pol II (Ref. 2 and citations therein). Recruitment of the capping apparatus to the elongation complex requires the TFIIH-associated CTD kinase (Kin28 in yeast, Cdk7 in mammals), which phosphorylates Ser-5 of the CTD heptad repeat YSPTSPS (3, 4).

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tunity for recruitment of Mecl to the elongation complex, by a multiplicity of Mecl interactions with Tat, Sp5, and/or pol II CTD-PQ2. Recent studies show that Tat directly stimulates the cotranscriptional capping of nascent HIV pre-mRNA (17). An inference is that Tat serves a dual role in promoting capping during the transcription arrest and in activating P-TEFb to then override the elongation block.

It is unclear whether and how the capping apparatus fits into the SpTS/P-TEFb axis during cellular gene expression. Here, by studying the macromolecular interactions of the S. pombe capping apparatus, we identify a S. pombe Cdk9 homolog as a binding partner for the S. pombe RNA triphosphatase Ptc1. A C-terminal domain of S. pombe Cdk9, separate from the kinase domain, suffices for binding to Ptc1 in vitro and in vivo. We identify Pch1 as the cyclin partner of SpCdk9 and show genetically that SpCdk9/Pch1 are the functional orthologs of the S. cerevisiae CTD kinase Bur1/Bur2, a putative fungal counterpart of P-TEFb (18). Based on these findings, we invoke an Sp5-induced elongation checkpoint, the purpose of which is to recruit the capping enzymes, in which P-TEFb-mediated release from the elongation block may be facilitated by contacts between P-TEFb and a component of the capping apparatus.

EXPERIMENTAL PROCEDURES

Yeast 2-Hybrid Screen—The screen was performed as described previously using BD-Pce1 and BD-Pct1 as the bait (7). Plasmid DNA recovered from the strains that tested positive for both HIS3 and lacZ expression was used as the template for PCR amplification of the S. pombe DNA insert with flanking primers specific for the AD-fusion plasmid. The PCR products were gel-purified and then sequenced. The AD plasmid clones were recovered after transformation into Esche-
richia coli DH5α. The BD plasmids SpCdk9 and Pch1 2-Hybrid Fusion Plasmids—Gene fragments encoding truncated versions of S. pombe Cdk9 were generated by PCR amplification using sense primers that introduced a NcoI site at the 5′-end of the truncated coding region and an antisense primer that introduced an BamHI site immediately downstream of the intended stop codon. The PCR products were digested with NcoI and BamHI and then inserted into the 2-hybrid BD fusion vector pAS2-1. Missense mutations K65A, E83A, D184N, and T212A in the kinase domain were introduced into the BD-SpCdk9-(1–385) plasmid. The full-length cDNA encoding S. pombe cyclin Pch1 was amplified by PCR from a cDNA library and inserted into the 2-hybrid AD fusion vector pGAD-GH. All of the inserts were sequenced to ensure that the genes were fused in-frame to AD or BD and that no unwanted coding changes had been introduced during amplification and cloning.

Recombinant Proteins from Bacteria and Protein Affinity Chromatography—S. pombe RNA triphosphatase Ptc1 was produced in E. coli as an N-terminal His6-tagged fusion and purified from soluble bacterial lysates by Ni-agarose chromatography as described previously (7, 19). GST-Cdk9-(386–591), GST-Cdk9-(386–525), and GST-Bur1-(411–657) were produced in bacteria by cloning the inserts were sequenced to ensure that no unwanted coding changes had been introduced during amplification and cloning.

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by adding SDS to a 1% final concentration. The products were analyzed by electrophoresis through a 12% polyacrylamide gel containing 0.1% SDS. Phosphorylated polypeptides were visualized by autoradiographic exposure of the dried gel. The phosphate acceptor proteins were as follows: recombinant GST-Spt5-(801–990) or purified untagged Spt5-(801–990) containing the C-terminal nonapeptide repeat array of S. pombe Spt5 (6); recombinant GST-Pol2 containing the complete array of 52 heptapeptide repeats of human pol II (a gift from Karen Lee and Rob Fisher); purified GST alone (6); or calf thymus histone H1 (purchased from Roche Molecular Biochemicals).

**RESULTS**

**Novel Interaction of Pct1 with a S. pombe Cdk9 Homolog**

- A 2-hybrid screen of 100,000 transformants for triphosphatase-interacting proteins using a Gal4 DNA-binding domain (BD)-Pct1 fusion as bait yielded 16 His² isolates, two of which contained plasmids encoding the Gal4 activation domain (AD) fused in-frame to C-terminal fragments of a predicted 591-amino acid S. pombe polypeptide with extensive similarity to human and Drosophila Cdk9 (Fig. 1). Thus, we named this previously uncharacterized S. pombe gene product SpCdk9.

- Two different AD-Cdk9 fusion clones were isolated in the 2-hybrid screen: AD-Cdk9-(313–591) and AD-Cdk9-(330–591). The His² and lacZ² phenotypes required cotransformation with BD-Pct1 and AD-Cdk9-(313–591) plasmids and neither fusion plasmid activated the HIS3 or lacZ reporter genes when co-transformed with the BD or AD vectors (Fig. 1A).

SpCdk9 was not isolated in a 2-hybrid screen for binding partners for the S. pombe guanylyltransferase Pce1. This same screen did uncover the positive interactions between Pce1, the pol II CTD, and Spt5 (6, 7). Furthermore, a directed 2-hybrid interaction assay using BD-Pce1 and AD-Cdk9-(313–591) plasmids failed to activate reporter gene expression (not shown).

We conclude that SpCdk9 interacts uniquely with the triphosphatase component of the S. pombe mRNA capping apparatus.

**An Autonomous Pct1-binding Domain of SpCdk9**

- SpCdk9 consists of an N-terminal protein kinase domain and a C-terminal Pct1-binding domain. The amino acid sequence of SpCdk9 is most closely related to the sequences of the HsCdk9 (372 amino acids) and DmCdk9 (401 amino acids) subunits of human and Drosophila P-TEFb (21) and to the essential S. cerevisiae cyclin-dependent kinase Bur1 (657 amino acids), which phosphorylates the pol II CTD and is the putative functional equivalent of Cdk9 in budding yeast (18). SpCdk9 and Bur1 are 200–250 amino acids larger than the metazoan Cdk9 proteins; this difference is attributable to the presence of C-terminal domains in the fungal Cdk9 proteins that have no counterpart in HsCdk9 and DmCdk9 (Fig. 1B). The fission yeast, budding yeast, and metazoan Cdk9 proteins display sequence similarity throughout their N-terminal kinase domains. (Residues conserved in all four Cdk proteins are indicated by dots.)
The Coomassie Blue-stained gel is shown. 

**Fig. 2.** S. pombe Cdk9 binds Pct1 in vitro. Panel A, purified Pct1 (5 μg) was mixed with GSH beads containing immobilized GST (lane 2), GST-Cdk9 (386–591) (lane 3), or GST-Bur1 (411–657) (lane 6), or with GSH beads alone (lane 5). Aliquots comprising 50% of the bead-bound eluate fractions (lanes 2, 3, 5, and 6) were analyzed by SDS-PAGE, along with 2.5 μg of the recombinant Pct1 (lane 1), GST-Cdk9-(386–591) (lane 4), and GST-Bur1-(411–597) (lane 7) protein preparations. The Coomassie Blue-stained gel is shown. Panel B, aliquots (1 μl) of the fractions analyzed in panel A, lanes 1–7, were assayed for triphosphatase activity (19). The extent of 32P release from [γ-32P]ATP is shown.

The C-terminal polypeptides Cdk9-(313–591) and Cdk9-(330–591) that were isolated in the 2-hybrid library screen contain short segments derived from the protein kinase domain. We subsequently found that an explicitly engineered shorter AD fusion, to Cdk9-(386–591), which starts downstream of the Cdk domain, was just as active in the 2-hybrid reporter assays as the constructs isolated originally (Fig. 1A). We conclude that the kinase domain does not contribute to the Pct1-binding site of SpCdk9, which resides entirely within the C-terminal domain. By testing a series of deletions of SpCdk9 in the 2-hybrid interaction assay, we delineated an 82-amino acid segment from residues 442 to 523 that sufficed for Pct1 binding (Fig. 1A).

**Pct1 binds directly to the SpCdk9 carboxy domain in vitro**—We used affinity chromatography to analyze the interaction of Pct1 with SpCdk9 in vitro. A glutathione S-transferase (GST)-Cdk9-(386–591) fusion protein (Fig. 2A, lane 4) was immobilized on GSH- Sepharose beads, which were then mixed with purified Pct1. The input Pct1 (lane 1) was analyzed by SDS-PAGE along with the material that bound to the GSH resin and was subsequently stripped off with glutathione. We found that the Pct1 protein bound to the GSH beads containing the GST-Cdk9-(386–591) fusion (lane 3), but did not bind at all to GSH beads alone (lane 5) or to GSH beads containing just GST (lane 2). The input Pct1 and glutathione eluate fractions were also assayed for triphosphatase activity. Approximately 55% of the input activity was retained on the GSH beads containing GST-Cdk9-(386–591) (Fig. 2B, lanes 1 and 3). Control assays showed that the GST-Cdk9-(386–591) fusion protein by itself had no triphosphatase activity (lane 4) and that there was negligible retention of triphosphatase activity on the GSH beads (lane 5) or the GSH beads containing GST (lane 2). Pct1 also bound specifically to the shorter fusion GST-Cdk9-(386–523) (data not shown).

**S. pombe Cdk9 interacts with S. pombe cyclin Pch1**—Metazoan P-TEFb is composed of Cdk9 and cyclin T subunits (22). In considering a candidate cyclin partner for SpCdk9, we focused on S. pombe Pch1 (23), an essential 342-amino acid polypeptide that resembles mammalian cyclin T (Fig. 3A). We found that full-length Pch1 displayed a 2-hybrid interaction with the isolated N-terminal kinase domain of SpCdk9 (Cdk9N), which did not interact with the isolated C-terminal triphosphatase-bind- ing domain of SpCdk9 (Cdk9ΔC) (Fig. 3A). The 2-hybrid interaction was stronger with the isolated kinase domain (scored as ++ based on its colony size) than with the full-length BDCdk9 fusion protein. *S. pombe* Pch1 also resembles the S. cerevisiae cyclin Bur2 (24), which together with the Cdk9-like Bur1 kinase comprises the putative S. cerevisiae P-TEFb ortholog (18). Thus, we envision that SpCdk9 and Pch1 are the constituents of *S. pombe* P-TEFb.

**S. pombe Cdk9 and Pch1 are functional orthologs of S. cerevisiae Bur1 and Bur2**—As an initial genetic test of the hypothesis that SpCdk9/Pch1 are functionally related to P-TEFb, we asked whether *S. pombe* Cdk9 and Pch1 can function

![Image](https://example.com/image.png)
Pch1 or SpCdk9 alone could not complement the bur2° temperature-sensitive phenotype, whereas coexpression of Pch1 and SpCdk9 from single-copy plasmids did rescue bur2° growth at 37 °C (Fig. 5). Although the coexpression of SpCdk9 and Pch1 did complement bur1° and bur2° strains (Fig. 4A), control experiments showed that the complementation was attributable solely to the introduction of an additional copy of the BUR1 gene (not shown). This agrees with the earlier report that BUR1 is a dosage suppressor of bur2Δ (24).

Our conclusions are that: (i) SpCdk9/Pch1 is a functional ortholog of Bur1/Bur2 and (ii) there is a species-specific interaction between the Cdk and cyclin subunits. To consolidate the point that the SpCdk9/Pch1 functional interaction is specific, we tested whether a different S. pombe cyclin, Mcs2, could substitute for Pch1 in the yeast complementation assay. Mcs2 is an essential cyclin that partners with a kinase subunit Mcs6 to form the fission yeast ortholog of the CTD kinase component of TFIIH (25). We found that a yeast CEN plasmid expressing Mcs2 was unable to complement bur1Δ or rescue the bur2Δ ts phenotype when cotransformed with a plasmid expressing SpCdk9 (not shown). Thus, SpCdk9 and Pch1 comprise a genuine Cdk/cyclin pair.

Mutations of the Kinase Active Site and T-loop Abolish SpCdk9 Function in Vivo—Initial studies of Pch1 isolated from S. pombe extracts showed that the cyclin was associated with a CTD kinase activity (23); however, the identity of the catalytic subunit was not established. Given that the Bur1/Bur2 complex is important for transcription in S. cerevisiae and that it phosphorylates the pol II CTD (18, 24), we can tentatively implicate similar activities to SpCdk9/Pch1. We addressed this issue genetically using budding yeast as a surrogate model. We constructed yeast bur2Δ strains transformed with the indicated plasmids or plasmid pairs (see Experimental Procedures) for plasmid nomenclature) and individual transformants were selected on appropriate drop-out media. Colonies were patched on drop-out medium and then streaked on agar plates containing 0.75 mg/ml 5-FOA. The plates were photographed after incubation for 3 days at 30 °C. B. mutations of the protein kinase active site abolish Cdk9 function in vivo. bur1Δ p360-BUR1 was transformed with TRP1 plasmids containing the indicated alleles of SpCdk9 plus a ADE2 PCH1 plasmid. bur1Δ complementation was tested by plasmid shuffle. Lethal mutations (scored as – ) were those that failed to support growth during selection on 5-FOA at either 23 or 30 °C. E83A cells were viable, but displayed cs and ts growth defects (see the legend to Fig. 6). The point mutations were introduced into BD-Cdk9(ΔC) and tested for 2-hydrid interaction with Pch1 as described in the legend to Fig. 3.

in S. cerevisiae in lieu of the putative P-TEFb subunits Bur1 and Bur2. We constructed yeast bur1Δ and bur2Δ strains suitable for plasmid shuffle. The bur1Δ mutation was lethal, while the bur2Δ strain was viable but slow-growing at 30 °C and unable to grow at 37 °C. The instructive findings were that, whereas expression of SpCdk9 or Pch1 alone could not complement bur1Δ, coexpression of SpCdk9 and Pch1 did complement growth of the bur1Δ strain (Fig. 4A). In this experiment, the S. pombe genes were on single-copy CEN plasmids marked with either TRP1 or ADE2. Complementation of bur1Δ was effective whether a TRP1 SpCdk9 plasmid was cotransformed with an ADE2 PCH1 plasmid or a TRP1 PCH1 vector was cotransformed with ADE2 SpCdk9 (Fig. 4A). SpCdk9 could not support growth of bur1Δ cells when cotransformed with an extra copy of BUR2 on a CEN plasmid driven by the native BUR2 promoter (Fig. 4A) or by the TPI1 promoter (not shown).

Additional experiments showed that expression of either Pch1 or SpCdk9 alone could not complement the bur2Δ temperature-sensitive phenotype, whereas coexpression of Pch1 and SpCdk9 from CEN plasmids did rescue bur2Δ growth at 37 °C (Fig. 5). Although the coexpression of the S. cerevisiae kinase Bur1 with the S. pombe cyclin Pch1 also rescued the bur2Δ ts phenotype (Fig. 5), control experiments showed that the complementation was attributable solely to the introduction of an additional copy of the BUR1 gene (not shown). This agrees with the earlier report that BUR1 is a dosage suppressor of bur2Δ (24).

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SpCdk9 that are predicted, based on crystal structure of the activated Cdk2-cyclinA-substrate complex (26), to be constituents of the kinase active site. (The residues that were mutated are shaded in Fig. 1B). We found that the K65A and D184N mutations abolished SpCdk9’s ability to complement bur1Δ when coexpressed with Pch1 (Fig. 4B). The inactivating mutations were then introduced individually into the 2-hybrid fusion protein BD-SpCdk9ΔC and tested for interaction in vivo with AD-Pch1. Neither K65A nor D184N affected the 2-hybrid interaction, as gauged by histidine prototrophy (Fig. 4B). Thus, the lethality of the kinase active site mutations is likely not attributable to effects on interaction of SpCdk9 with its cyclin partner. Rather, the results suggest that SpCdk9 protein kinase activity is essential for its in vivo function.

We also tested the effects of alanine substitution for Thr-212, a conserved residue (Fig. 1B) corresponding to the regulatory phosphorylation site in the T-loop of Cdk2. The T212A mutation abolished SpCdk9 complementation of bur1Δ and did not affect the interaction of the kinase domain with Pch1 in the 2-hybrid assay (Fig. 4B). These results suggest that SpCdk9 function may be regulated by phosphorylation of the T-loop, either by autophosphorylation or phosphorylation by a separate activating kinase.

Mutation of Glu-83 of SpCdk9 Elicits a Severe Conditional Growth Defect—Glu-83 of SpCdk9 corresponds to the conserved glutamate of the so-called PSTAIRE helix characteristic of Cdks. In the crystal structure of the activated Cdk2-cyclin A-substrate complex, the glutamate side chain forms an ion pair with the essential lysine (equivalent to Lys-65 of SpCdk9) that coordinates the α-phosphate of ATP (26). We replaced Glu-83 of SpCdk9 with alanine and tested the mutant enzyme for bur1Δ complementation when coexpressed with Pch1. Although E83A supported the growth of bur1Δ cells under FOA selection at 30 °C, the SpCDK-E83A mutants displayed a severe temperature-sensitive and cold-sensitive growth phenotype on rich medium, i.e. they were unable to form colonies at either 23 or 37 °C, although they were viable at 30 °C (Fig. 6). E83A cells were also unable to form colonies on YPD agar at 18 °C (not shown). We found that an E83A mutant of the 2-hybrid fusion protein BD-SpCdk9ΔC retained its 2-hybrid interaction in vivo with AD-Pch1. The strength of the E83A-Pch1 interaction was ++ at both 30 °C and 23 °C. Thus, we infer that the conditional growth phenotype of the E83A cells was likely not caused by a defect in the interaction of SpCdk9 with Pch1, but rather a conditional defect in kinase activity resulting from loss of the ion pair to the essential lysine that binds ATP.

Pct1 Binds to the C-terminal Domain of Bur1—The Pct1-binding domain of SpCdk9 is related structurally to the segment of S. cerevisiae Bur1 downstream of the kinase domain, to an extent of 62 positions of amino acid side chain identity or similarity within a 173-amino acid segment of SpCdk9 (Fig. 1B). The 82-amino acid Pct1-binding module encompasses 28 positions of identity or similarity. This raises the prospect that the interactions of Cdk9/Bur1 with RNA triphosphatase are conserved among fungi. Indeed, we found that Pct1 bound specifically in vitro to a fusion protein composed of GST triphosphatase to an affinity-purification with Ni-agarose to achieve an affinity-purification of the bound material. We also found that S. cerevisiae RNA triphosphatase Cet1 (which is structurally similar to Pct1) binds to the C-terminal domain of SpCdk9 (data not shown).

The C-terminal Domain Is Important for SpCdk9 Function in Vivo—Our identification of SpCdk9 as a binding partner for RNA triphosphatase prompts the question: is the interaction between the two proteins relevant in vivo? Having localized a triphosphatase-binding element within the C-terminal domain of SpCdk9, distinct from the kinase catalytic domain, we asked whether deletion of the C-terminal domain impacts on SpCdk9 complementation of bur1Δ. We found that expression of the Cdk9ΔC protein (containing amino acids 1–385) supported growth of bur1Δ cells under FOA selection at 30 °C. However, the ΔC mutant displayed a severe cold-sensitive phenotype when grown on rich medium at 23 °C (Fig. 6) and 18 °C (not shown) and a partial temperature-sensitive phenotype at 37 °C (Fig. 6). Even at permissive temperature (30 °C), the ΔC mutant formed smaller colonies than did “wild-type” SpCDK9 cells (Fig. 6). We surmise that the C-terminal domain containing the Pct1-binding site is important for SpCdk9 function in vivo. Consistent with this idea, we found that the deletion variant SpCdk9 (1–523), which contains the Pct1-binding site, complemented the bur1Δ mutation without eliciting cold-sensitive or temperature-sensitive growth defects (not shown).

Protein Kinase Activity of Recombinant SpCdk9/Pch1—Recombinant baculoviruses were engineered to express native SpCdk9 or His-tagged Pch1 under the control of the viral polyhedrin promoter. The tagged Pch1 allele was constructed as so as to place a TEV protease cleavage site at the junction between the short N-terminal His tag and the start of the Pch1 polypeptide sequence. Soluble lysates prepared from insect cells coinfected with the SpCdk9- and His-Pch1-expressing viruses were adsorbed to Ni-agarose to achieve an affinity-purification of the His-tagged Pch1 and any associated polypeptides (Fig. 7A). A ~43-kDa polypeptide corresponding to His-Pch1 was the predominant species recovered by elution of the bound material.
with 150 mM imidazole. The initial identification of this species as His-Pch1 was predicated on the following evidence: (i) treatment of the peak imidazole eluate with TEV protease resulted in conversion of the 43-kDa polypeptide to a 41-kDa polypeptide, whereas the electrophoretic mobility of the other polypeptides in the mixture was unaffected; and (ii) Edman sequencing of the 41-kDa digestion product after transfer from the SDS gel to a polyvinylidene difluoride membrane yielded the N-terminal sequence Gly-Ala-Met-Gly-Glu-Val-Ile-Lys, which corresponds precisely to the predicted sequence of the recombinant Pch1 protein immediately flanking the TEV protease cleavage site. The imidazole eluate also contained a prominent 68-kDa polypeptide, consistent with the predicted size for SpCdk9.

The peak imidazole eluate fraction was subjected to zonal velocity sedimentation in a glycerol gradient. The sedimentation profile of the component polypeptides was determined by SDS-PAGE (Fig. 7B). The 43-kDa Pch1 polypeptide peaked in fractions 13–15, with a prominent shoulder on the heavy side of the peak. The 68-kDa polypeptide also peaked in fractions 13–15, with a slight shoulder on the heavy side. Attempts to sequence the 68- and 43-kDa polypeptides present in the peak glycerol gradient fraction by Edman chemistry were unsuccessful, suggesting that their N-terminal amino groups were blocked by covalent modification. This problem was circumvented by tryptic digestion of the peptides in situ in an excised gel slice, followed by MALDI-TOF mass spectroscopy of the digestion products. The peptide fingerprinting analysis unambiguously identified the 68-kDa species as *S. pombe* Cdk9 and confirmed the identification of the 43-kDa species as Pch1 (data not shown). The finding that untagged SpCdk9 coeluted with
Histagged Pch1 during the Ni-agarose step indicates that SpCdk9 and Pch1 form a heteromeric complex in the absence of any other yeast proteins.

The peak glycerol gradient fraction containing SpCdk9 and Pch1 was tested for protein kinase activity with a variety of potential phosphate acceptor protein substrates. Activity was gauged by transfer of $^{32}$P from [$\gamma$-$^{32}$P]ATP to the acceptor protein to form a phosphoprotein adduct detectable by SDS-PAGE and autoradiography (Fig. 7D). We readily detected phosphoryltransfer to the C-terminal domain of S. pombe Sp5, which spans amino acids 801–990 and consists of 18 tandem repeats of a nonapeptide motif (consensus sequence TPANWNSGSK) (6). Recombinant SpCdk9/Pch1 phosphorylated the nonamer array of Sp5 in the context of a GST Sp5 fusion protein or as tag-free Sp5 (801–990) (Fig. 7D). No phosphoryltransfer was detected to GST alone. The GST Sp5 fusion protein was a better acceptor than free Sp5 (801–990), perhaps because the GST-fused version is a dimer, whereas the isolated Sp5 CTD is monomeric (6). SpCdk9/Pch1 also phosphorylated the pol II CTD derived from mammalian pol II, consisting of 52 tandem repeats of the CTD heptapeptide motif (Fig. 7D). In contrast, histone H1 was not a substrate for the kinase. No labeled polypeptides were detected in the absence of an exogenous acceptor.

The kinase activity profile across the glycerol gradient was gauged using GST Sp5 as a substrate (Fig. 7C). We detected a single discrete peak of kinase activity centered at fractions 13–15, coincident with the peak of the SpCdk9 and Pch1 polypeptides. An apparent sedimentation coefficient of 6.0 S was calculated by comparison to marker proteins (catalase, 11.2 S, 248 kDa; bovine serum albumin, 4.4 S, 66 kDa; and cytochrome c, 1.9 S, 13.4 kDa) that were centrifuged in a parallel gradient. We surmise that the active kinase is a heterodimer of the 68-kDa SpCdk9 and 43-kDa Pch1 polypeptides.

**DISCUSSION**

mRNA capping is coupled to transcription elongation via physical interactions between the cap-forming enzymes, the phosphorylated pol II CTD, and the elongation factor Sp5 (5, 6, 17, 27). Here we report that fission yeast RNA triphosphatase, the enzyme that initiates cap formation, interacts with SpCdk9, an ortholog of the Cdk9 subunit of metazoan P-TEFb. These findings provide a rationale for the arrest and subsequent reactivation of pol II elongation at promoter proximal sites (Fig. 8).

Mammalian Sp5 in the context of DSIF interacts with pol II and arrests elongation. Sp5 also negatively regulates heat shock transcription in vivo in C. elegans (16). We presume that Sp5 in S. pombe has a similar function and we propose that the elongation arrest provides a kinetic window during which the capping enzymes Pct1 and Pce1 can be recruited to the elongation complex, via contacts with the Sp5 CTD nonamer array and also with the pol II CTD-PO$_4$$_4$. We envision that cap formation occurs on the nascent chains within the arrested pol II complexes and that the presence of Pct1 on the pol II complex facilitates the recruitment of S. pombe P-TEFb, composed of SpCdk9 and its cyclin partner Pch1, via direct contacts between Pct1 and the C-terminal Pct1-binding domain of SpCdk9. By analogy with the mammalian system (13, 28), the proper positioning and/or activation of P-TEFb would trigger its phosphorylation of the pol II CTD and/or Sp5, thereby releasing the elongation complex from the arrest and committing pol II to productive elongation.

A key sensor in the S. pombe elongation checkpoint model illustrated in Fig. 5 is the presence of the capping enzyme Pct1 on the elongation complex, rather than presence of the cap structure. (Although we do not exclude a potential role for the cap, or a cap-associated protein, in elongation control, there is no evidence as yet to invoke such a model.) Pct1 provides a physical connection between Sp5 and SpCdk9. Studies in the mammalian system show that purified recombinant hSpt5 does not interact directly in vitro with recombinant Cdk9-cyclinT1 (P-TEFb) (14), implying the existence of a bridging component. S. pombe Pct1 is the first instance in which an RNA processing enzyme is physically linked to P-TEFb. The identification of the fungal P-TEFb homologs has been elusive, in part because of the existence of multiple Cdk/cyclin pairs in S. cerevisiae that are capable of phosphorylating the pol II CTD (29). The studies of Prelich and coworkers (18, 24) are persuasive in assigning the essential Bur1/Bur2 kinase as the P-TEFb equivalent in budding yeast. The fission yeast Cdk9 protein identified by us is structurally similar to Bur1 and the similarity extends to the C-terminal Pct1-binding domain that is lacking in the metazoan Cdk9 proteins. Complementation of the S. cerevisiae bur1A and bur2A mutants by coexpression of SpCdk9 and Pch1 provides strong evidence that the fission yeast proteins are genuine orthologs of Bur1/Bur2, while highlighting the species-specificity of the Cdk-cyclin interactions. Analysis of the recombinant SpCdk9/Pch1 complex produced in baculovirus-infected insect cells shows that the S. pombe proteins comprise a bona fide protein kinase, with a putative heterodimeric quaternary structure. The capacity of SpCdk9/Pch1 to phosphorylate the CTD arrays of both pol II and Sp5 in vitro echoes the substrate specificity of metazoan P-TEFb (14, 15). The fact that the in vivo function of SpCdk9 is abolished by mutations in Lys-65, which is predicted to contact ATP, and Asp-184, which is predicted to coordinate magnesium in the Mg-ATP complex, argues that protein phosphorylation is an essential facet of SpCdk9’s biological activity. The strong conditional phenotypes elicited by the E83A mutation are in keeping with the predicted indirect involvement of this residue in catalysis via its positioning of the catalytic lysine. The finding that Thr-212 is essential for SpCdk9 function suggests that SpCdk9 is subject to allosteric activation via phosphorylation of the T-loop threonine. Yao and Prelich (30) recently reported that Bur1 is phosphorylated on its T-loop threonine by Cak1 and that T-loop phosphorylation stimulates the CTD kinase activity of the Bur1/Bur2 complex. Their surprising finding that an alanine mutation of the T-loop threonine of Bur1 does not affect cell viability (30) contrasts with our result that the
T-loop mutation of SpCdk9 is lethal in yeast. It is conceivable that the SpCdk9/Pch1 complex is more acutely dependent than Bur1/Bur2 on a regulatory phosphorylation event to attain the threshold level of kinase activity required for growth of S. cerevisiae.

The C-terminal domain of SpCdk9 that includes the Pct1-binding site is important for SpCdk9 function in vivo in bur1Δ complementation. Deletion of the C-terminal domain elicits a severe conditional phenotype with both ts and cs growth defects. The triphosphatase-binding domain of SpCdk9 is conserved in S. cerevisiae Bur1. An equivalent of the C-terminal triphosphatase-binding domain of fungal Cdk9 is lacking in metazoans, but this makes perfect sense, insofar as the RNA triphosphatase component of the metazoan capping apparatus is completely different in its tertiary structure and catalytic mechanism from the RNA triphosphatas of fungi (31, 32). Further analysis will be required to test whether the growth defects of the SpCdk9ΔC mutation are attributable, solely or partly, to the disruption of the interaction of Cdk9/Pch1 with the capping apparatus.

Commitment of pol II to processive elongation without prior acquisition of a cap is potentially wasteful, given the role of the cap in facilitating splicing of the first intron and protecting the 5'-end from premature decay. Capping may also impact on the translation machinery (Ref. 33 and citations therein). Recent studies in Drosophila suggest that ribosomes and translation factors associate with nascent pre-mRNAs within the nucleus prior to the removal of all introns and the acquisition of a poly(A) tail and that protein synthesis may occur at transcriptionally active nuclear regions (34). Cap-dependent recruitment of the translation apparatus to the nascent RNA is presumably a key event in ribosome-dependent nuclear mRNA surveillance.

We speculate that wasteful or defective rounds of transcription are avoided by the imposition of an elongation checkpoint, whereby Spt5/Spt4 plus other negative factors arrest the elongation complex shortly after promoter clearance and reversal of the arrest by is signaled by the presence of the capping apparatus on the elongation complex (Fig. 8). This step is especially relevant to the control of HIV gene expression, wherein the HIV Tat protein activates cotranscriptional mRNA capping (17), and is likely to apply to cellular gene expression, as evinced by the dual interactions of S. pombe capping enzymes with Spt5 and the P-TEFb ortholog Cdk9/Pch1. Flushing out the checkpoint model in the S. pombe system will require many steps that are beyond the scope of the present study, including: (i) genetic dissection in S. pombe of each of the putative checkpoint participants, (ii) purification and biochemical characterization of the S. pombe Spt5/Spt4 complex, and (iii) the development of in vitro transcription systems that display early elongation arrest and restart.

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