MINIREVIEW

RNA Polymerase II Carboxy-Terminal Domain Kinases: Emerging Clues to Their Function

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The cloning of the largest subunit of RNA polymerase II (pol II) from mouse and Saccharomyces cerevisiae in 1985 (3, 28) revealed a remarkable and highly conserved domain known as the pol II carboxy-terminal domain (CTD). This domain has intrigued researchers interested in the mechanisms regulating gene expression ever since its discovery, because of both its simplicity and its complexity. The CTD is simple in the sense that it consists entirely of repeats of the 7-amino-acid consensus sequence YSPTSPS. The mouse (28) and human (125) CTDs consist of 52 repeats, of which 21 exactly match the consensus while 20 differ at only a single position (Table 1). This same consensus sequence is conserved in other eukaryotes, with 27 repeats in budding yeast (18 exact and 5 with a single difference) (3, 45) and, more importantly, that phosphorylation of the CTD varies during the transcription cycle (54, 73). These insights stimulated searches for the CTD-specific kinase, but it was first suggested by the finding that the CTD is phosphorylated (13) and, more importantly, that phosphorylation of the CTD during purification (28), while effects on transcription. Promoter-specific requirements for the CTD are not restricted to yeast, as they have also been observed using promoters derived from other organisms. A CTD-less form of pol II, for example, is active in nonspecific RNA polymerase assays and for both basal and activated transcription from several promoters in crude extracts, including the adenovirus major late and Drosophila actin 5C promoters (12, 58, 132, 133). Other promoters, typified by the murine dihydrofolate reductase promoter, however, require the CTD in vitro (2, 11, 54, 117). These results demonstrate that the CTD does not perform an essential role during transcription but instead overcomes or compensates for rate-limiting steps that are inherent to specific promoters. The basis for CTD dependence is not completely understood; one study investigating the cis-acting elements that influence the CTD requirement suggests that the presence of a consensus TATA box or other proximal elements can contribute to CTD independence in vitro (10), whereas another attributed the CTD requirement to the upstream activating sequence (105). Thus, although the promoter-specific requirement for the CTD during transcription is firmly established, the basis for this selectivity is not clearly understood, and it might be conferred or reversed by different cis-acting sequences and their respective promoter-bound factors.

VARIABLE CTD REQUIREMENT DURING TRANSCRIPTION

To understand the functions of the CTD kinases, it is first necessary to summarize the present view of the roles of the CTD itself. The CTD is essential for viability in mouse, yeast, and Drosophila (4, 8, 86, 133), although mutants with deletions that remove approximately half of the repeats are still viable. CTD truncations reduce the levels of several transcripts tested in yeast (105), but not all pol II-dependent promoters are affected. This differential requirement for the CTD can be recapitulated in nuclear extracts (68) and thus is due to direct effects on transcription. Promoter-specific requirements for the CTD are not restricted to yeast, as they have also been observed using promoters derived from other organisms. A CTD-less form of pol II, for example, is active in nonspecific RNA polymerase assays and for both basal and activated transcription from several promoters in crude extracts, including the adenovirus major late and Drosophila actin 5C promoters (12, 58, 132, 133). Other promoters, typified by the murine dihydrofolate reductase promoter, however, require the CTD in vitro (2, 11, 54, 117). These results demonstrate that the CTD does not perform an essential role during transcription but instead overcomes or compensates for rate-limiting steps that are inherent to specific promoters. The basis for CTD dependence is not completely understood; one study investigating the cis-acting elements that influence the CTD requirement suggests that the presence of a consensus TATA box or other proximal elements can contribute to CTD independence in vitro (10), whereas another attributed the CTD requirement to the upstream activating sequence (105). Thus, although the promoter-specific requirement for the CTD during transcription is firmly established, the basis for this selectivity is not clearly understood, and it might be conferred or reversed by different cis-acting sequences and their respective promoter-bound factors.

REGULATION OF CTD ACTIVITY BY PHOSPHORYLATION

The initial purification of pol II revealed three forms, designated pol IIA, pol IIB, and pol IIO (107), that differed in the electrophoretic mobility of the largest subunit in sodium dodecyl sulfate-polyacrylamide gels. Form IIB results from promoter-specific requirement for the CTD during transcription is firmly established, the basis for this selectivity is not clearly understood, and it might be conferred or reversed by different cis-acting sequences and their respective promoter-bound factors.

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to 20 phosphates in the mammalian form IIO (135), and IIA is hypophosphorylated (13). Several lines of evidence indicate that forms IIA and IIO are functionally distinct and that CTD phosphorylation is likely to be important in vivo. First, in transcription reactions using equimolar amounts of forms IIA and IIO, form IIA was recruited four times more efficiently into the transcription preinitiation complex (23). Second, form IIA converted to IIO concomitant with or shortly after recruitment of pol II into preinitiation complexes (23). This preferential recruitment of form IIA over IIO is likely due to interactions between the hypophosphorylated CTD and the other general transcription factors. In support of this idea, pol IIA has been reported to associate directly with both the TATA-binding protein (TBP) (119) and the mediator complex (59, 62, 88).

In contrast to the preferential association of pol IIA with preinitiation complexes, elongating RNA polymerase is highly phosphorylated (13), and dephosphorylation stimulates recruitment of pol II into preinitiation complexes (23). Second, form IIA is converted to IIO concomitant with or shortly after initiation (94). This temporal association of CTD phosphorylation with a key transition point in the transcription cycle implicates CTD phosphorylation in regulating the early stages of transcription. Third, the association of CTD-binding proteins such as the pol II mediator complex (59, 62, 88); Prp40 of transcription. And the in vivo function of the phosphorylation sites within the CTD, and the potential redundancy between forms IIO and IIA are likely driven by these differential associations with subsets of CTD-binding proteins. A remaining challenge will be to determine whether binding of the CTD-associated factors is mutually exclusive, occurring in a progressive and stepwise manner, or whether these factors can associate simultaneously with the CTD to a single intact mRNA-generating assembly.

### IDENTIFICATION OF THE CTD KINASES

With accumulating evidence that the CTD is regulated by phosphorylation, searches for the responsible kinases naturally followed. Standard kinase assays using either intact pol II, CTD fusion proteins, or CTD peptides as substrates were used to identify kinases capable of phosphorylating the CTD repeats in vitro. Several CTD kinases were identified using this strategy (9, 24, 29, 31, 33, 67), and independent biochemical (35, 41, 74, 77, 109) and genetic (69, 85, 99) searches for proteins with general roles in transcription identified additional factors with CTD kinase activity. Discerning whether these kinases actually target the CTD in vivo has been a more difficult task, due to the requirement of the CTD for viability, the repeated nature of the phosphorylation sites within the CTD, and the potential redundancy between the CTD kinases. Here I concentrate my discussion on a set of conserved CTD kinases with well-estab-

### TABLE 1. Conservation of the CTD repeat sequence

| Organism | No. of repeats | % Identity | GenBank accession no. |
|----------|----------------|------------|----------------------|
|          | Total | Exact<sup>a</sup> | Single difference<sup>b</sup> | Y | S | P | T | S | P | S |
| Homosapiens | 52 | 21 | 20 | 100 | 83 | 98 | 87 | 98 | 100 | 50 |
| Mus musculus | 52 | 21 | 20 | 100 | 83 | 98 | 85 | 98 | 100 | 50 |
| Drosophila melanogaster | 45 | 2 | 15 | 87 | 78 | 82 | 42 | 73 | 71 | 20 |
| Arabidopsis thaliana | 40 | 16 | 12 | 95 | 95 | 98 | 72 | 90 | 88 | 42 |
| Caenorhabditis elegans | 37 | 10 | 12 | 82 | 86 | 94 | 62 | 97 | 94 | 38 |
| Schizosaccharomyces pombe | 29 | 20 | 1 | 96 | 90 | 93 | 93 | 100 | 93 | 96 |
| Saccharomyces cerevisiae | 27 | 18 | 5 | 93 | 96 | 96 | 93 | 96 | 96 | 97 |
| Dictyostelium discoideum | 24 | 18 | 4 | 89 | 96 | 100 | 80 | 100 | 83 | 83 |

<sup>a</sup> Number of repeats that exactly match the consensus.

<sup>b</sup> Number of repeats that differ from the consensus at a single position.

### TABLE 2. CTD-binding proteins

| Protein or complex | Specificity<sup>a</sup> | Function | Comments | Reference(s) |
|--------------------|-------------------------|----------|----------|--------------|
| TBP                | U                       | TATA-binding protein | Essential for transcription initiation | 119 |
| Mediator           | U                       | Transcription coactivator | Holoenzyme subunits; CTD binding subunits unknown | 59 |
| Rsp5               | U                       | Ubiquitin ligase | Requires WW domain to bind CTD | 17, 83 |
| Spt4/Spt5          | U, P                    | Elongation | DSIF; binds both phosphorylated and unphosphorylated CTDs | 70, 123 |
| Elongator          | P                       | Transcription elongation | Six-subunit complex; CTD binding subunit unknown | 89 |
| CA150              | P                       | Transcription elongation | Requires FF domain to bind CTD | 14 |
| Ceg1               | P                       | Capping guanylytransferase | Serine 5 specific; requires Km28/Cdk7 | 102, 128 |
| Abd1               | P                       | Capping methyltransferase | Associated with pol II during transcription | 79, 106 |
| Prp40              | P                       | Splicing | Binds CTD with both FF and WW domains | 83 |
| CstF50             | P                       | Cleavage/poly(A) factor | Polyadenylation cleavage stimulatory factor subunit | 80 |
| Pcf11              | P                       | Cleavage/poly(A) factor | Component of CFI A | 7 |
| Pta1               | P                       | Cleavage/poly(A) factor | Component of CFI A (CPSF) | 102 |
| Ess1/Pin1          | P                       | Peptidyl-prolyl isomerase | Requires WW domain to bind CTD; 3′ end processing | 84 |
| Nrd1/SCAF8         | P                       | 3′ end formation | Directs poly(A)-independent 3′ end formation | 25, 92, 130 |

<sup>a</sup> Specificity for binding to unphosphorylated (U) or phosphorylated (P) CTD.
lished connections to transcription or RNA processing, first summarizing their initial characterization as CTD kinases and then addressing the mechanistic basis for their distinct roles.

**Cdk7/Kin28.** The discovery that the CTD is phosphorylated after preinitiation complex formation in a reconstituted transcription system suggested that one of the general transcription factors possessed CTD kinase activity. This activity was found to copurify with and reside in TFIH (35, 74, 109). The TFIH CTD kinase activity consists of a cyclin-dependent kinase (Cdk) and its associated cyclin partner, designated Cdk7-cyclin H in mammalian cells and Kin28-Cell1 in *S. cerevisiae*. A major difference between the yeast and mammalian factors is that in mammalian cells Cdk7 also possesses Cdk-activating kinase activity, whereas Cdk-activating kinase activity in *S. cerevisiae* is encoded by a separate gene, *CAK1* (52). The in vivo function of the TFIH kinase has been explored thoroughly in *S. cerevisiae*, and as expected of a general transcription factor, *KIN28* is essential for viability (110). Whole-genome analysis of mRNA levels in a *kin28* temperature-sensitive mutant demonstrated that synthesis of nearly all Pol-II-dependent transcripts ceases rapidly at the nonpermissive temperature (48). Consistent with its characterization as a CTD kinase in vitro, phosphorylation of the Pol II CTD decreased dramatically in a *kin28* mutant strain at the nonpermissive temperature (120). Combined, these results present a compelling case that Kin28/Cdk7 acts as a CTD kinase, stimulating transcription in vivo.

**Cdk8/Srb10.** Although the CTD is essential for viability, strains containing *rpb1* truncations that remove approximately half of the heptad repeats are still viable (4, 86). In yeast these CTD truncations cause cold-sensitive and heat-sensitive growth and defects in transcription of some, but not all, promoters in vivo (105). Selection for genomic suppressor mutations that reverse the CTD truncation cold-sensitive phenotype identified several *SRB* genes (87, 116) that encode proteins associated with Pol II to form the Pol II holoenzyme (62). Relevant to this discussion is that two of the genes identified in this selection, *SRB10* and *SRB11* (designated Cdk8 and cyclin C in metazoans), encode a Cdk-cyclin pair (62, 114) that copurifies with Pol II in some holoenzyme preparations from mammals and yeast (18, 38, 76, 113). The association of Srb10-Srb11 in Pol II-containing complexes and the identification of *srb10* and *srb11* mutations as suppressors of CTD truncations suggested that they might target the CTD. Indeed, Srb10 can phosphorylate the CTD in vitro, and holoenzymes purified from *srb10* mutant strains are defective for CTD kinase activity (69). In sharp contrast with these results, both an *srb10A* null allele and *srb11* mutations that abolish repression have no detectable effect on CTD phosphorylation in vivo (27, 102). Thus, either the activity of other CTD kinases masks the effect of *srb10A*, the effects of *srb10A* on CTD phosphorylation are more subtle and will require better detection methods, or Srb10 is not a CTD kinase in vivo and its transcriptional effects are due to phosphorylation of other substrates. In support of this last model, several other Srb10 substrates have been identified. Srb10 phosphorylates at least three site-specific DNA-binding proteins in yeast, affecting their activity by different mechanisms; Srb10-dependent phosphorylation stimulates Gal4 activation (45), inhibits the Msn2 activator by blocking its nuclear localization, and targets Gcn4 for proteosome-mediated degradation (19). In addition, human Cdk8 inactivates TFIH through phosphorylation of its cyclin H subunit, although this regulatory system is not conserved in yeast (1). Thus, although Srb10/Cdk8 possesses CTD kinase activity in vitro, it is presently unclear how many of its transcriptional effects are mediated through the CTD and how many are mediated through alternative substrates in vivo.

**Cdk9/P-TEFb.** A search for factors in *Drosophila* extracts that stimulate transcript elongation identified an activity designated positive transcription elongation factor b (P-TEFb) (78). Sensitivity of P-TEFb to the protein kinase inhibitor DRB (5,6-dichloro-1-b-D-ribofuranosylbenzimidazole) suggested that it possessed protein kinase activity, and upon purification P-TEFb was indeed capable of phosphorylating the Pol II CTD (77). Cloning of the P-TEFb subunits revealed that it is a Cdk consisting of the Cdk9 catalytic subunit and cyclin T (37, 96, 97, 137). This was particularly informative, because an independent study also connected Cdk9 to elongation; Cdk9 was previously identified as a human immunodeficiency virus type 1 (HIV-1) Tat-associated protein kinase, known as PITALRE or Tak, that stimulates elongation from the HIV-1 promoter (37, 41). HIV-1 transcription is regulated partly during elongation; binding of the virally encoded Tat protein to the TAR sequence of the nascent HIV-1 RNA stimulates progression of a paused RNA polymerase through recruitment of P-TEFb (115). This might reflect an analogous and generally applicable mechanism for stimulating transcription in vivo, since most cellular transcription is sensitive to DRB (108) and an early elongation block at the *Drosophila* HSP70 promoter is similarly overcome by recruitment of P-TEFb upon heat shock induction (71).

The role of P-TEFb during transcription has been studied extensively in vitro. P-TEFb acts on early elongation complexes, stimulating the transition from abortive to productive elongation (78), and this stimulation requires the CTD (77). P-TEFb had no detectable effect on purified Pol II, however, and was not required in a fractionated system, suggesting that its stimulatory effect occurs by overcoming factors present in the crude extract that inhibit elongation (95). Two factors that confer a requirement for P-TEFb have been purified: DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) (122, 126). DSIF consists of the human homologs of Spt4 and Spt5, which were independently implicated in elongation control in yeast (39). In extracts immunodepleted of either DSIF or NELF, P-TEFb is no longer required for productive elongation (123, 126), arguing that the only function of P-TEFb is overcoming these inhibitors. Two models can readily explain the role of P-TEFb and its relationship to DSIF and NELF; one proposes that the combination of DSIF and NELF inhibits elongation and that P-TEFb phosphorylates and inactivates either DSIF or NELF, thereby releasing the polymerase. Alternatively, phosphorylation of the CTD by P-TEFb counteracts or blocks the inhibitory activity of DSIF. There is presently evidence to support both of these models; P-TEFb phosphorylates both the CTD (77) and the Spt5 subunit of DSIF in vitro (50, 57).

**CTDK-I.** The first CTD kinase was purified from *S. cerevisiae* by assaying for an activity that altered the electrophoretic mobility of a CTD-glutathione S-transferase fusion protein (67). This activity, CTDK-1, consists of a three-subunit Cdk encoded by *CTK1, CTK2*, and *CTK3* (65, 112). Phosphoryla-
tion of the CTD is altered but not abolished in a ctk1Δ strain, providing evidence that CTDK-1 contributes to CTD phosphorylation in vivo (65, 102). Several results suggest that CTDK-1 performs a role during transcription elongation: purified CTDK-1 stimulates elongation in vitro (66); ctk1Δ strains are sensitive to 6-azauracil (85), a trait commonly exhibited by elongation factor mutants; and ctk1Δ mutants display synthetic phenotypes in combination with mutations in the elongation factor genes SPT4, SPT5, and PRP2 and the ELP genes (51, 70). Like SRB10, CTK1 is not essential for viability at 30°C (65), and whole-genome analysis reveals that only a subset of yeast transcripts are affected (D. Skaar and A. Greenleaf, personal communication).

Svj1/Bur1. A genetic selection designed to identify general transcriptional regulators identified another yeast CTD kinase. Recessive mutations in BUR1 and BUR2 increase transcription from a suc2 promoter mutant in which the upstream activating sequence has been deleted, suggesting that BUR1 and BUR2 act as repressors of this inactivated promoter (99). BUR1 is identical to SGV1, a gene identified previously in a screen for proteins involved in recovery from the mating factor-induced signal transduction pathway (49). The finding of BUR1/SGV1 in a transcription selection suggests that its role during mating factor recovery is indirect, mediated through effects on gene expression. Like the other CTD kinases described above, Burl is a Cdk, requiring the Burl cyclin for activity (85, 127). An unbiased search for Burl substrates using a joint immunoprecipitation–kinase assay revealed two major specific substrates: Rpb1 and Burl itself (85; G. Prelich, unpublished results). Burl coimmunoprecipitates a subpopulation of Rpb1 and can phosphorylate a CTD–β-galactosidase fusion protein. Double-mutant analysis provided additional evidence that Burl is functionally related to the CTD; bur1 and bur2 mutants display synthetic phenotypes in combination with rpb1 CTD truncations, ctk1Δ, and with a mutation in the fcp1 CTD phosphatase.

Bur1 and Ctk1 each possess characteristics expected of a yeast P-TEFb homolog: both are Cdns that can phosphorylate the CTD, and mutations in both genes cause 6-azauracil-sensitive growth and exhibit genetic interactions with known elongation factors, suggestive of a role during elongation (70, 85). Their amino acid sequences are nearly equally similar to that of Cdk9, although one evolutionary comparison suggests that Burl is the yeast P-TEFb homolog (72). The answer to whether Burl, Ctk1, or both are yeast P-TEFb will rely upon more detailed biochemical investigations into their proposed role during elongation or tests for their ability to be exchanged either in vivo or in vitro.

DISTINCT ROLES OF THE CTD KINASES AND THEIR MECHANISMS

An important concept emerging from recent studies is that the designation CTD kinase is a useful but simplistic categorization, since the CTD kinases clearly have distinct functions in vivo. The distinctions between the CTD kinases are readily detected at the genetic level in yeast. First, KIN28 and BUR1 are essential for viability, whereas srb10Δ and ctk1Δ strains are viable and display only slight growth defects at 30°C. Second, mutations in the individual CTD kinase genes display largely nonoverlapping phenotypes; bur1 and kin28 mutations do not cause an Srb− phenotype, and srb10 and kin28 mutations do not cause a Bur− phenotype. Furthermore, in the combinations tested, overexpression of one kinase does not suppress mutations in the others (64; G. Prelich, unpublished results). Third, different effects on global gene expression patterns have been detected by microarray analysis in CTD kinase mutants. A genome-wide decrease in transcription occurs in a kin28 temperature-sensitive strain, whereas only 3% of the transcripts are affected in an srb10 deletion strain, and most of those transcripts increase (48). Limited effects have also been observed in both ctk1Δ and bur1 mutant strains (D. Skaar, A. Greenleaf, and G. Prelich, unpublished results). Despite these differences, there is some functional overlap between the yeast CTD kinases. A bur1 mutation is lethal with ctk1Δ and shows allele-specific effects with kin28 mutations, but no combinatorial effects are observed with srb10Δ (70, 85). Combined with the finding that bur1 and ctk1Δ mutations, but not kin28 or srb10Δ mutations, are sensitive to 6-azauracil (85), this suggests that BUR1 and CTK1 might have partially overlapping roles during elongation and that they are more closely related to each other than to KIN28 or SRB10. Distinctions between the CTD kinases have also been observed at the biochemical level; where the kinases have defined activities in complex assays, they are not biochemically interchangeable. For example, the TFIIH kinase cannot substitute for P-TEFb in a transcription elongation assay (77, 121), and CTDK-I cannot substitute for TFIIH activity in a fractionated transcription system (104).

What specific biochemical mechanisms can account for the different phenotypic effects of the CTD kinases? Four simple models can be envisioned (i) different phosphorylation site specificities within the CTD, (ii) differential temporal activation during the transcription cycle, (iii) phosphorylation of other substrates, and (iv) differential requirement at subsets of promoters. Although the characterization of the CTD kinases is far from complete, as discussed below, support for all of these possibilities has emerged.

Target specificity within the CTD. Because the CTD consensus heptad repeat YSPTSPS contains five potential phosphate acceptor residues, an important initial characterization for any CTD kinase is to define its target specificity within the CTD consensus repeat. Phosphoaminoacid analysis of mammalian form IIO revealed that the CTD contains more phosphoserine than phosphothreonine, and the amount of phosphothreonine was consistent with phosphorylation of threonine within nonconsensus repeats (13, 134). Although phosphotyro- sine was not detected initially, subsequent inclusion of tyrosine phosphatase inhibitors during extract preparation revealed equivalent amounts of phosphotyrosine and phosphothreonine (9). The specificity of the individual CTD kinases has been characterized both by assaying for phosphorylation of wild-type and mutant CTD peptides and by measuring reactivity with the phospho-CTD-specific monoclonal antibodies H5 (phosphoserine 2 specific) and H14 (phosphoserine 5 specific) (91). Experiments that rely solely upon reactivity with the H5 and H14 antibodies should be interpreted with some caution, as cross-reactivity occurs at high antigen concentrations (20). Mammalian Cdk7 and Cdk8 (100, 101, 103, 118) and their yeast counterparts Kin28 and Srb10 (40, 120) each phosphorylate serine 5, although one study found that the Cdk8-con-
taining NAT complex phosphorylates both serine 2 and serine 5 (113). The specificity of Cdk9 is less clear; one group found that Cdk9 phosphorylates serine 5 in vitro (100), another study found that Cdk9-cyclin T phosphorylates serine 2 and that its specificity is expanded to include serine 5 in the presence of the HIV-1 Tat protein (136), and RNAi-mediated depletion of Cdk9 in Caenorhabditis elegans results in specific loss of serine 2 phosphorylation in vivo (K. Blackwell, unpublished results). The specificity of Ctk1 has not been examined directly, but ctk1Δ strains are defective for the increase in serine 2 phosphorylation observed during the diauxic shift (90) and for the association of serine 2-phosphorylated pol II within coding regions (20). Phosphorylation of the CTD by Bur1 results in reactivity with the H14 monoclonal antibody, suggesting that serine 5 is its primary target within the CTD (85).

Defining the specificity of the CTD kinases within the heptad repeat has become more important with the realization that phosphorylation of serine 2 and serine 5 of the CTD consensus repeat has different functional consequences. Both serine 2 and serine 5 are essential in yeast and therefore do not perform completely redundant functions (124). Furthermore, mutations that suppress a CTD containing alanines at position 2 do not suppress a mutant CTD containing alanines at position 5 (129). An important distinction between serine 2 and serine 5 phosphorylation emerged from a recent chromatin immunoprecipitation study (63). The phosphoserine 5-specific antibody H14 recognized pol II that cross-linked to the promoter-proximal region, whereas pol II that cross-linked to promoter-distal regions was recognized by the phosphoserine 2-specific monoclonal antibody H5. These results indicate that CTD phosphorylation is a dynamic process, switching from a serine 5-phosphorylated form to a serine 2-phosphorylated form during the transcription cycle. Consistent with the idea that the site of phosphorylation and not just overall CTD phosphorylation is important, the mammalian capping guanylyltransferase is stimulated by CTD phosphorylated on serine 5, not on serine 2 (46), and a yeast capping enzyme mutant is synthetically lethal with an *pb1* allele containing serine 5-to-alanine mutations in the CTD but not with an allele containing serine 2-to-alanine mutations (102). These observations should trigger additional investigations into whether other phospho-CTD-binding proteins are differentially affected by phosphorylation of serine 2 or serine 5.

As discussed above, CTD kinase specificity can be defined by the ability to phosphorylate amino acids at specific positions within the consensus CTD heptad repeat. A related type of substrate specificity is the ability to phosphorylate nonconsensus CTD repeats. Although more than half of the CTD repeats conform to the consensus (Table 1), nonconsensus repeats are more common in the C-terminal half of the CTD. Is phosphorylation of each repeat equivalent, or is its location within the CTD important? Do the nonconsensus repeats have a role in CTD regulation? These issues have been examined in several studies (8, 36, 124), with the results suggesting that the repeats are not all equivalent. It will be interesting to determine how much of this difference is due to their phosphorylation state. Towards this end, Cdk8 and Cdk7 differed in their ability to phosphorylate N-terminal repeats versus the more divergent C-terminal repeats (101), although the functional consequences of this preference remain unknown.

**Differential timing or activation during the transcription cycle.** Biochemical analysis suggests that CTD phosphorylation has at least three distinct roles during transcription: inhibiting preinitiation complex (PIC) formation, stimulating promoter escape, and stimulating productive elongation. These sequential functions of CTD phosphorylation during the transcription cycle could in principle result from two distinct temporal mechanisms: the kinases could be present constitutively in RNA polymerase complexes but be sequentially regulated, or they could be recruited in an active form to the CTD in a sequential manner. Evidence in support of both of these models has now surfaced. A biochemical comparison of purified Kin28 and Srb10 revealed identical substrate specificities despite their opposing roles in stimulating and inhibiting transcription. While investigating this paradox, Hengartner et al. (40) found that in holoenzyme preparations that contained both Srb10 and Kin28, Srb10 was capable of phosphorylating the CTD only prior to PIC formation, consistent with its role as an inhibitor, whereas Kin28 was capable of phosphorylating the CTD only after initiation. This temporal activation model is consistent with the opposing effects of Srb10 and Kin28 on transcription despite the apparently equivalent biochemical activities of the purified enzymes. Implicit in this model, however, is the requirement for both a Kin28-specific inhibitor prior to initiation and an Srb10-specific inhibitor subsequent to initiation, but direct evidence for these inhibitory activities has not been provided.

Other studies have provided evidence for the alternative model, which posits sequential association of the CTD kinases with pol II during the transcription cycle. TFIIH is a component of PICs but is released shortly after promoter escape, when the elongating chain is approximately 20 to 50 nucleotides long (98, 131, 136). The differential association of CTD kinases has also been detected in vivo; in a ChIP assay Kin28 is specifically associated with promoter-proximal DNA fragments (63), while Ctk1 associates with both promoter and coding regions (20).

**Other targets besides the CTD.** The different biological effects of the CTD kinases can also be mediated by phosphorylation of additional substrates besides the CTD. Determining whether the CTD is the only relevant substrate for any of the CTD kinases has been a major stumbling block, since the standard approach of asking whether mutation of the target site causes the same phenotype as mutation of the kinase is untenable with a repetitive substrate such as the CTD. This is not merely a theoretical consideration, as additional substrates have been identified for some of the CTD kinases. P-TEFB function, for example, is intimately associated with both the CTD and DSIF (the Spt5/Spt4 complex). Even though P-TEFB was demonstrated to have CTD kinase activity, it also phosphorylates the Spt5 subunit of P-TEFB (50, 57), and phosphorylation of either the CTD or Spt5 can readily explain P-TEFB’s role during elongation. In another striking example, mutations in *SRB10* suggest that it functions primarily as a transcriptional repressor of selected genes. Phosphorylation of free pol II (40) is consistent with that role, but as described above, Srb10 phosphorylates three site-specific activators in yeast (19, 45), and human Cdk8 inhibits Cdk7 activity (1). Determining how many phenotypes of the CTD kinases are actually mediated...
through the CTD ultimately will require using in vitro systems that are dependent upon the individual kinases.

**Differential requirement at subsets of promoters.** Of the four yeast CTD kinases, only Kin28 is clearly required for transcription by most yeast genes, while microarray analysis reveals more restricted defects in *srb10Δ, ctk1Δ*, and *bur1* mutant strains (D. Skaar, A. Greenleaf, and G. Prelich, unpublished observations). One simple mechanism to account for this difference is that some of the CTD kinases might be recruited to specific promoters. Unfortunately, little is known about the localization or recruitment of the yeast kinases, but this question has been addressed best for P-TEFb. For HIV-1 transcription P-TEFb is recruited to the TAR site via direct and species-specific interactions between cyclin T and an RNA-binding protein, HIV-1 Tat (115). P-TEFb also can be directly recruited to promoters by site-specific DNA-binding activators such as NF-κB and CIITA (6, 53). Recruitment is apparently restricted to subsets of activators, as Sp1 was incapable of recruiting P-TEFb. P-TEFb is also recruited to the *Drosophila* hsp70 promoter upon heat shock activation, and although heat shock factor is required for recruitment, it is not sufficient, suggesting a more complex recruitment mechanism at this promoter (71).

**THE CTD AS AN ORGANIZER**

Initial studies on the CTD centered on its role during transcription, but a solid body of evidence now points towards a broader role for the CTD in recruiting RNA processing factors to the nascent transcript. Although RNA capping, splicing, and 3' end formation can all be accomplished in vitro in the absence of transcription, these processes are all coupled and somewhat interdependent in vivo (42). It now appears that the CTD plays an active role in these processes, both by recruiting the RNA-processing factors to the nascent transcript and by directly activating them. In mammalian cells, for example, the CTD is required for efficient capping, splicing, and 3' end processing (80) and for the relocalization of splicing factors to sites of active transcription (81). The CTD requirement during splicing and 3' end processing is also observed in vitro (43, 44), even in the absence of active transcription or mRNA (56). This, combined with the detection of CTD-binding activities for several capping (22, 128), splicing (56, 83, 130), and 3' end processing factors (7), suggests that the CTD serves as an assembly platform for processing factors. The CTD might have additional roles beyond assembly, as both the yeast and mammalian capping guanylyltransferases (21, 46) are allosterically regulated by the CTD.

The role that CTD phosphorylation plays in RNA processing is just beginning to be explored, but there are already indications that phosphorylation will indeed be important. The Prp40 splicing factor and the 3' processing and polyadenylation factors CPSF, CstF, Pcf11, and Pta1 all bind preferentially to the phosphorylated CTD (7, 79, 83, 102), and the capping guanylyltransferase is specifically stimulated by serine 5 phosphorylation (47) (Table 2). For most of these proteins it is not yet known if specific kinases are responsible for regulating their interactions with the CTD. Interestingly, however, although CTD phosphorylation by Kin28, Srb10, or Ctk1 can all stimulate binding of Ceg1 in vitro, only *kin28* mutations show genetic interactions with *ceg1* mutations in vivo, suggesting that there is indeed specificity (102).

**SUMMARY AND PERSPECTIVES FOR FUTURE STUDIES**

Our present view of CTD phosphorylation (Fig. 1) suggests a cycle in which the successive activation or recruitment of the CTD kinases regulates interactions between the CTD and CTD-binding proteins. The association of those CTD-binding proteins, through direct effects on either transcription initiation, elongation, or RNA processing, results in production of full-length processed mRNA. Imposed upon this broad picture, many details remain to be clarified, including the exact number of CTD kinases, their target specificity within the CTD repeats, the basis for their promoter specificity, the ratio of phosphorylated serine 2 to serine 5, and the relationship between phosphorylation and other modifications of the CTD (55, 75, 82). In particular, the question of how many kinases target the CTD in vivo is not yet clear; several other potential CTD kinases have been identified in mammalian cells, including Cdc2 (24), c-Abl (9), DNA-PK (33), and mitogen-activated protein kinases (31). The relevance of CTD phosphorylation to their in vivo roles needs to be evaluated further.

With increasing progress in identifying and broadly characterizing the roles of the CTD kinases, emphasis will now shift towards investigating the mechanisms that regulate their activity. All of the CTD kinases discussed in detail above are Cdkks. One might therefore predict that they utilize regulatory mechanisms similar to those used by the Cdks that are involved in cell cycle transitions (93). Although some regulatory mechanisms are shared, the relative contributions of those regulatory inputs are likely to differ. The cell cycle Cdks, for example, are regulated primarily by the abundance of the cyclin subunit, and although the CTD kinases discussed above require their cyclin subunits, the levels of the cyclins involved in CTD phosphorylation apparently do not vary. One regulatory mechanism that is likely shared between the cell cycle Cdks and the CTD kinases is activation by a Cdk-activating kinase. Phosphorylation of Cdks on a threonine residue within the regulatory T loop is necessary for Cdk activity. This threonine is conserved in Cdk7/Kin28, P-TEFb/Cdk9, Ctk1, and Bur1, but not in Srb10 or Cdk8, and Cdk-activating kinase stimulates the CTD kinase activity of Kin28 (34, 60). It is not yet known whether phosphorylation of the CTD kinases by Cdk-activating kinase varies during the transcription cycle. Regulation of the CTD kinases by other Cdk-like mechanisms, such as association with inhibitory subunits or inhibitory phosphorylation events, has not been reported to date. Another similarity between the Cdks involved in transcription and those involved in the cell cycle is that the CTD kinases might participate in feedback loops such as have been described for the cell cycle Cdks. The inhibition of TFIH kinase activity by Cdk8 (1) is one example of CTD kinase-CTD kinase interregulation. Expansion of these regulatory interactions might reveal a feedback regulatory pathway driving transcription akin to the one driving the cell cycle. Lastly, CTD phosphorylation is affected by stress (26), heat shock (31, 32), growth state (31, 91), and UV irradiation (75), but the signaling mechanisms that impinge on the CTD kinases are not yet clear and need to be explored further.
Any discussion of CTD kinases would be incomplete without briefly considering the role of CTD phosphatases in counteracting or reversing CTD kinase activity. A single conserved CTD phosphatase called Fcp1 has been purified and cloned from human and yeast cells (15, 16, 23, 61). *FCP1* is essential for viability in yeast, and the broad reduction of RNA levels observed in viable *fcp1* strains ties Fcp1 to transcription in vivo (5, 61). In vitro, Fcp1 can dephosphorylate pol II, stimulating its recruitment into initiation-competent complexes (23, 61). Interestingly, *fcp1* mutants display increased association of the serine 2-phosphorylated pol II with coding regions (20), suggesting that it recycles pol II in vivo by targeting serine 2. Clearly, it will be important to clarify the role of Fcp1 during the transcription cycle and to determine how its activity is balanced relative to the CTD kinases. Finally, if Fcp1 is indeed serine 2 specific, it suggests that a serine 5-specific CTD phosphatase awaits discovery.

The central role played by the CTD and its kinases during gene expression has been evident for many years. A story that began with a simple repetitive sequence has developed now into a complex regulatory network centered upon this domain. We can look forward to additional challenges and surprises that will be revealed by future studies into this fundamental general regulatory component.

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**FIG. 1.** A working model for sequential activities of the CTD kinases. CTD phosphorylation varies during the transcription cycle, driven by multiple CTD kinases. A working model for their sequential activities is depicted here, based on results summarized in the text. Unphosphorylated pol II is preferentially recruited to the promoter, driven by interactions with initiation factors such as TBP and the mediator complex. Shortly after initiation, the CTD is phosphorylated on serine 5 by Kin28/Cdk7, stimulating promoter escape and binding of the Ceg1 subunit of capping enzyme. Productive elongation is stimulated by P-TEFb, Ctk1, and Bur1, resulting in a shift to a form containing phosphorylated serine 2. CTD phosphorylation stimulates binding of several splicing, elongation, and 3′ end processing factors, but the responsible kinases and their order of recruitment are not clear. Subsequent dephosphorylation by the Fcp1 phosphatase allows recycling of pol II into a reinitiation-competent form. Phosphorylation of free pol II by Srb10/Cdk8 on serine 5 is proposed to block initiation.
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