A Dual Interface Determines the Recognition of RNA Polymerase II by RNA Capping Enzyme

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RNA capping enzyme (CE) is recruited specifically to RNA polymerase II (Pol II) transcription sites to facilitate cotranscriptional 5′-capping of pre-mRNA and other Pol II transcripts. The current model to explain this specific recruitment of CE to Pol II as opposed to Pol I and Pol III rests on the interaction between CE and the phosphorylated C-terminal domain (CTD) of Pol II largest subunit Rpb1 and more specifically between the CE nucleotidytransferase domain and the phosphorylated CTD. Through biochemical and diffraction analyses, we demonstrate the existence of a distinctive stoichiometric complex between CE and the phosphorylated Pol II (Pol II). Analysis of the complex revealed an additional and unexpected polymerase-CE interface (PCI) located on the multihelical Foot domain of Rpb1. We name this interface PCI1 and the previously known nucleotidytransferase/phosphorylated CTD interface PCI2. Although PCI1 and PCI2 individually contribute to only weak interactions with CE, a dramatically stabilized and stoichiometric complex is formed when PCI1 and PCI2 are combined in cis as they occur in an intact phosphorylated Pol II molecule. Disrupting either PCI1 or PCI2 by alanine substitution or deletion diminishes CE association with Pol II and causes severe growth defects in vivo. Evidence from manipulating PCI1 indicates that the Foot domain contributes to the specificity in CE interaction with Pol II as opposed to Pol I and Pol III. Our results indicate that the dual interface based on combining PCI1 and PCI2 is required for directing CE to Pol II elongation complexes.

In eukaryotic cells, RNA polymerase II (RNA Pol II) and its associated factors carry out transcription of pre-mRNAs, snRNAs, telomerase RNA, and other noncoding RNAs. Pol II also couples transcription to nuclear processes including pre-mRNA modifications (1–4), mRNA export, and chromatin reconfiguration (5–7). Coupling RNA processing with synthesis is presumed to be critical in restricting the temporal window during which unmodified transcripts are vulnerable to degradation by endogenous ribonucleases (8–10) and directing RNA processing factors to sites of Pol II transcription at specific steps during Pol II progression through a gene. The Pol II elongation complex coordinates these transactions to help orchestrate control over gene expression (for review, see Ref. 11). Such coordination is mediated by specific and reversible interactions among Pol II and the factors involved in elongation.

The formation of RNA 5′ cap structure, m7GpppN, is the first transcription-coordinated RNA modification event, and it occurs as soon as the transcript attains ~25 nucleotides (12–15). The RNA cap is formed in three enzymatic steps (16, 17): (i) removal of the 5′ γ-phosphate catalyzed by the RNA triphosphatase; (ii) attachment of a GMP to the 5′ diphosphate by the guanylyltransferase; (iii) methylation of the 5′ guanine by the cap methyltransferase. The first two of these steps are closely linked and coupled to Pol II transcription in most organisms. Mammals combine their triphosphatase and guanylyltransferase into a bi-functional capping enzyme (CE) encoded by a single gene. Fungal CEs comprise the two enzymes in a tightly associated complex, as exemplified by the Saccharomyces cerevisiae CE, a heterotetrameric complex consisting of the guanylyltransferase Ceg1 and triphosphatase Cet1 subunits (18) (for review, see Ref. 19). Emerging evidence has also suggested non-catalytic roles of the CE in transcription regulation. For instance, stimulation of CE by HIV Tat promotes read-through of the viral genome (20, 21). CE has been implicated in the formation of an early elongation checkpoint, as has been observed in 5′-paused Pol II complexes (15, 22–25). Cotranscriptional capping is effected by the specific interaction between a CE and its cognate RNA polymerase that supports a stoichiometric CE-polymerase complex (13, 26), as exemplified in certain viral transcription/processing systems in each of which the transcription of viral genes is mediated by a single virus-encoded RNA polymerase (27).

The issue of class specificity in CE recruitment arises in eukaryotes because of the existence of three related RNA poly-

SEC, size-exclusion chromatography; OB, oligonucleotide binding; PCI, polymerase CE interface; FCC, Foot, nucleic acid cleft, and CTD; FOA, 5-fluoroorotic acid; TAP, tandem affinity purification.
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...merases, Pol I, Pol II, and Pol III, that share five subunits (Rpb5, -6, -8, -10, and -12) but synthesize different classes of RNAs (28). Capping is specifically targeted to Pol II transcription complexes (1, 3) after the phosphorylation of Pol II by TFIIH during transcription initiation (29). Importantly, mRNAs that are forced to be transcribed by Pol I, Pol III, or T7 RNA polymerase are uncapped (30–32). The mechanism for the specific recruitment has been ascribed to the interaction between CE and the phosphorylated C-terminal repeated domain (CTD) of Rpb1, a domain that is unique to the Pol II largest subunit (1, 3, 33, 34). The absence of CTD in Pol I and Pol III (35, 36) suggests that the CTD imparts the Pol II apparatus with a unique ability to regulate interactions with various factors including the CE in a phosphorylation-dependent manner. It is known that CTD phosphorylation occurs reversibly on the serine-2, -5, or -7 residues in its heptadepptide motif, 1YSPTSPS7. It is also known that CTD phosphorylation patterns correlate with discrete stages of transcription (37, 38). CE binding to Pol II is stimulated by the phosphorylation at either Ser-2 or Ser-5 positions in the CTD (39, 40), but Ser(P)-5 catalyzed by the Kin28 kinase of TFIIH predominates during early stages of transcription (41–43). The binding per se does not require the presence of transcript in Pol II, as has been observed either in the absence of RNA polymerization events (3) or with the purified polymerase (33). Interestingly, the elongation factor Spt5 of the fission yeast Schizosaccharomyces pombe seems to contribute a CE recruitment function in vivo that overlaps with the function provided by the CTD (45, 46). Although evidence exists for a functional interaction between Spt5 and CE in the budding yeast S. cerevisiae (47), it remains unclear if this interaction is critical in targeting CE to Pol II transcription because of a lack of biochemical and structural data on this issue. Because S. cerevisiae Spt5 also participates in Pol I transcription (48), Spt5 is not likely to be a decisive factor in the specific recruitment of CE to Pol II in budding yeast, although it may contribute a salubrious effect on the proper assembly of CE into the early elongation complex.

The current model for Pol II-linked cotranscriptional RNA 5′-capping rests on the binding of CE to the phosphorylated CTD (CTD-P) (1, 3, 33, 34). The CTD is present but disordered in the Pol II crystal structure and may extend from the globular core of Pol II into bulk solution (49, 50). Structural studies of a complex between the Cgt1 subunit (Candida albicans homolog of S. cerevisiae Ceg1) and synthetic CTD heptads bearing Ser(P)-5 have revealed interactions with about 2.5 CTD repeats that are coordinated in a surface groove within the nucleotidyltransferase (NT) domain of Cgt1 (51). Given that there are 26–27 heptad repeats in the CTD of S. cerevisiae and the CE structures are highly conserved between C. albicans and S. cerevisiae, one could expect at least eight copies of CE to bind, possibly distributively, along the CTD of yeast Pol II upon CTD phosphorylation. However, it is difficult to conceive that such spatially undefined interactions can provide the precise spatial and temporal information required to target CE activity to a narrow window within the transcription cycle. Furthermore, recent experiments with human cells found that the CTD alone was not sufficient for enhancing cotranscriptional processing in vivo, suggesting the involvement of additional Pol II components and/or associated factors in the process (52). It, therefore, appears that CTD-P alone does not fully account for the Pol II specificity in CE recruitment even though CTD phosphorylation is critical for RNA capping in vivo.

Here, we present evidence for a mechanism that complements the CTD-P-only recruitment model for CE function, thus shedding light on the Pol II specificity in cotranscriptional targeting of the budding yeast CE. We demonstrate a distinctive and stoichiometric CE-Pol II IO complex that requires two interfaces on the polymerase. One interface is based on the CTD-P as expected from the common model and the Cgt1/CTD-P cocrystal structure (51). The other interface deemed important for the recognition is formed with the Pol II multihelical Foot domain (50). Mutational impairment of either interface disrupts the stability of the complex in vitro and compromises cell growth, whereas overexpression of CE suppresses the in vivo effects of the interface mutations. Mutational evidence also indicates that the Foot domain plays an essential role in imparting specificity toward Pol II as opposed to Pol I and Pol III. Our data indicate that the simultaneous formation of the dual interface is necessary and sufficient to stabilize CE binding to the polymerase. Hence, the dual interface is the physical basis for specifying RNA CE to Pol II transcription in vivo. We also show that each of the two surfaces alone is unable to support stable interactions with CE. Therefore, the post-initiation Pol II capping complex may dissociate as soon as the state of either interface is changed, a modus operandi that seems suitable for the dynamic assembly and disassembly of multiprotein complexes involved in transcription regulation.

EXPERIMENTAL PROCEDURES

RNA polymerases were purified directly from the budding yeast S. cerevisiae by applying the TAP-tagging strategy (53) with the procedures given in the supplemental Experimental Procedures. Pol IIO was generated using the in vitro phosphorylation by MAP kinase 2 (MAPK2) under a condition known to produce both Ser(P)-2 and Ser(P)-5 sites but with preference toward Ser-5 positions (supplemental Experimental Procedures). The Pol IIO was indistinguishable from that generated with the yeast TFIIK kinase, a subcomplex of TFIIH (supplemental Fig. S1B), which is responsible for in vivo phosphorylation of the CTD and the recruitment of CE (29, 54). As such, the Pol IIO generated in vitro was suitable for binding studies with CE. The heterodimeric yeast CE and its individual subunits (Ceg1 and Cet1) were expressed in Escherichia coli and purified as described in the supplemental Experimental Procedures.

Glutathione S-transferase (GST) and maltose-binding protein (MBP) fusions were constructed using PCR primers corresponding to sequence regions that each encompassed a Pol II structural domain (50), i.e. the CTD of Rpb1, Rpb1-Foot, Rpb2-Lobe (residues 216–407), Rpb5, Rpb6, and Rpb8 subunits and Rpb6 truncations. GST-CTD-P and MBP-CTD-P were generated by the same in vitro phosphorylation procedure as used for generating Pol IIO. The GST- and MBP-mediated chromatographic procedures are described in the supplemental Experimental Procedures.
Affinity capture by calmodulin-coupled beads was used in analyzing CE recognition of RNA polymerases. The calmodulin-binding peptide on the C terminus of a polymerase subunit (Rpb7 for Pol II, Rpb8 for Pol I) was derived from the TAP tag and was coupled to calmodulin beads. Detailed conditions are given in the supplemental Experimental Procedures. Size exclusion chromatography was applied to assess stabilities of the various polymerase-CE complexes under the conditions given in the supplemental Experimental Procedures. Size exclusion chromatography was used in affinity assays and size-exclusion chromatography experiments were stained with Coomassie Blue and digitized with an optical scanner (CanoScan-4400F) that allowed the immobilization of a polymerase complex on calmodulin beads. Detailed conditions are given in the supplemental Experimental Procedures.

For the determination of protein stoichiometry, the gels resulting from affinity assays and size-exclusion chromatography experiments were stained with Coomassie Blue and analyzed with the same protocol as used in the supplemental Experimental Procedures. X-ray crystallography was employed to reveal structural aspects of the interaction between CE and Pol II. Detailed procedures and conditions are given in the supplemental Experimental Procedures.

RESULTS

Differential Binding Affinities Underlie the Recognition of Pol II by Cellular CE—To uncover the biochemical determinants for Pol II-specific recruitment of CE, we measured the amount of yeast CE captured by Pol I, Pol IIA (Pol II unphosphorylated on its CTD), and Pol IIO after each had been immobilized on calmodulin beads (“Experimental Procedures”). As shown in Fig. 1, we observed weak interactions between yeast CE and either Pol IIA (Fig. 1, A and B, lanes 2) or Pol I (Fig. 1B, lane 4). In contrast, we observed stoichiometric interactions between CE and Pol IIO, the phosphorylated post-initiation isoform of Pol II (Fig. 1, A, lane 4, and B, lane 3). The stoichiometric values were 1.0 for CE/Poly I and 0.15 for CE/Poly IIA (Fig. 1A, inset), as measured by using the ratio of Ceg1/Rpb2; thus, the retention of CE by Pol IIO increased 7-fold when compared with Pol IIA. This result is consistent with the notion that Pol II-specific targeting of 5’ capping is dependent on CTD phosphorylation (1, 3, 29, 33, 34). These data also demonstrate a CTD phosphorylation-dependent stoichiometric interaction between a bifunctional CE and a structurally intact cellular RNA polymerase.
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We next tested whether CTD-P alone was sufficient to support the stoichiometric interaction with CE. Previous detections for CE binding to GST-CTD-P monitored formation of the radioactive CE-*GMP intermediate (1, 3) or used Western analysis (33). Due to the nature of these assays, the stoichiometry of the respective complexes remained unknown. We confirmed the interaction between CE and CTD-P using a similar assay with radioactive labeling of the CE (supplemental Fig. S1C). However, we were unable to detect CE in the complex by staining with silver (data not shown) even when a 30-fold molar excess of CTD-P was used. We concluded that CTD-P alone is not capable of sustaining the stoichiometric Pol IIO-CE complex. Together, these results suggest that cellular CE recognizes specific features in the globular structure of the respective Pol II molecule, a mechanism reminiscent of that of the viral systems (13, 26).

To assess the stability of the respective complexes, excess CE was added to both Pol IIA and Pol IIO, and interactions were characterized by size-exclusion chromatography (SEC). While CE readily separated from Pol IIA (Fig. 1C), it coeluted with Pol IIO with a stoichiometry of 1:1 (Fig. 1D). In the presence of CE, the Pol IIO (peak fractions #42–47) migrated slightly faster than Pol IIA (peak fractions #46–50), consistent with an increase in molecular weight as a result of CE binding to Pol IIO, but in the absence of CE, the two forms of Pol II migrated indistinguishably (not shown). These data indicate the formation of a stable and stoichiometric complex between CE and Pol IIO but not Pol IIA.

Additional information can be obtained from this analysis. The relative amounts of Cet1 and Ceg1 that are bound to Pol IIO persistently approach 1:1 in repeated experiments (supplemental Table S1). That this stoichiometry is equivalent to that of CE/Pol IIO (see above) suggests that the CE-Pol IIO complex contains a 1Ceg1:1Cet1 heterodimer. However, when we consider the molecular state of the excess Ceg1 and Cet1 complex, its apparent size as indicated by the migration position is more consistent with a 2Ceg1:2Cet1 heterotetramer that has been observed in SEC and the crystal structure (18). Apparently, the heterodimer found in the complex with Pol IIO is not consistent with the previously observed heterotetramer or the 1Ceg1:2Cet1 heterotrimer CE (18) nor does it explain the indistinguishably (not shown). These data indicate the possibility that the heterotetrameric and heterotrimeric states may be dynamic and could change upon the interaction with Pol IIO.

Identifying Structural Determinants for Pol IIO-CE Recognition—As pointed out in the Introduction, the CTD-P-alone model for CE recruitment does not fully explain the precision of CE binding to Pol IIO. Although the 1Ceg1:1Cet1 heterotetramer that has been observed in SEC and the crystal structure (18) suggests that the CE-Pol IIO complex contains a 1Ceg1:1Cet1 heterodimer, we were unable to detect CE in the complex by staining with silver (data not shown) even when a 30-fold molar excess of CTD-P was used. We concluded that CTD-P alone is not capable of sustaining the stoichiometric Pol IIO-CE complex. Together, these results suggest that cellular CE recognizes specific features in the globular structure of the respective Pol II molecule, a mechanism reminiscent of that of the viral systems (13, 26).

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As a first step toward this end, we mapped the interaction using the subcomplexes of CE and Pol II that are structurally stable when isolated in solution. These included the Ceg1 (guanylyltransferase) and Cet1 (triposophatase) subunits of the yeast CE, the Rpb4-Rpb7 subcomplex (consisting of Pol II subunits Rpb4 and Rpb7), and the 10-subunit Pol II lacking Rpb4-Rpb7. In experiments described in the supplemental Data, Section 2.1, we determined that the stable and stoichiometric interaction with CE requires only the 10-subunit core of Pol IIO (supplemental Fig. S2C); the Rpb4-Rpb7 subcomplex is dispensable for this interaction. Moreover, a stable complex was observed between the 12-subunit Pol IIO and Ceg1 but not Cet1 (supplemental Fig. S2, A and B), suggesting that Cet1 is not critical for the interaction with the polymerase.

To further probe the structural features of Pol IIO that might be involved in recruiting CE, we copurified the 12-subunit Pol IIO with recombinant Ceg1-Cet1 by SEC and crystallized the complex (supplemental Fig. S3A). Similar attempts using the 10-subunit Pol IIO did not yield crystals at all. SDS-PAGE analysis revealed Ceg1, Cet1, and Pol II subunits in the crystals (supplemental Fig. S3B). The crystals diffracted weakly, and native anomalous data were eventually collected to 4.7 Å at the zinc edge (supplemental Table S2). Crystalllographic phases were experimentally determined using the multi-crystal approach that proved effective for resolving the electron density of Pol II (58). The experimental map of the cocrystal showed Pol II density that matched the known atomic model (59) (Fig. 2A), validating the experimental phase values for the complex. We did not refine the Pol II model against the cocrystal data due to the low resolution. Additional protein-like density not ascribed to Pol II was observed adjacent to the multihelical Foot domain of Rpb1 (Fig. 2, A and B). As a control, diffraction data from Pol IIO crystals obtained in the absence of CE were analyzed, but no significant density was observed adjacent or near to the Rpb1 Foot domain (not shown). These data indicated that the extra density was not due to a folded portion of Pol II. The possibility that the CTD-P on Pol IIO might fold up and account for the additional density was also diminished by the fact that the CTD-P tail of Pol IIO was as susceptible to chymotrypsin digestion as the unstructured CTD of Pol IIA (supplemental Fig. S3C).

To account for the additional density, we attempted to manually dock domains within the CE structure (18) into the complex. We found that neither the NT domain of Ceg1 subunit nor the Cet1 subunit could be docked into this density, whereas several differently oriented models of the oligonucleotide binding (OB) domain of Ceg1 could be placed inside the density (supplemental Fig. S3D), indicating a local rotational disorder in the cocrystal. A difference Fourier ($2F_{o} - F_{c}$) map phased by the unrefined Pol II model revealed density also at the same location (not shown), but the density was much weaker than that based on the experimental phases. This was taken as an additional indication for the disorder. This structural disorder could reflect potential different modes of CE binding to Pol IIO in the absence of a transcription bubble and other factors. Nevertheless, the location of the additional experimental density adjacent to a surface on the Foot domain (Fig. 2B) indicated that...
this Pol II domain might interact with CE and most likely via the OB domain. The Rpb1 Foot is an independently folded domain in the Pol II structure (50). The Foot domain is conserved among Pol IIs from different species (supplemental Fig. S4A), but it is one of the two regions that vary the most when comparing sequences of the largest subunits from Pol I, Pol II, and Pol III (supplemental Fig. S4, B–D). The structural volume of this domain differs noticeably between Pol I and Pol II (60). These observations suggest a model wherein the Foot domain, in concert with the CTD-P, presents surface determinants for the specific recognition of Pol II by CE. We designed biochemical and functional experiments to investigate this model. We designated the crystallographic interface between the CE OB and Pol II Foot domain as PCI1 (polymerase CE Interface 1) (Fig. 2B) and the previously defined CE interface on CTD-P (51) as PCI2. Based on the Pol II structure, the polymerase component of PCI1 consists of the secondary structural elements H2, H4, and LP (H4-H5), the loop between H4 and H5 (Fig. 2B) of the Foot domain.

**Biochemical Characterization of the Pol IIO-CE Interfaces**—When we monitored CE binding to different subdomains of Pol II (supplemental Fig. S5A) using GST-mediated affinity chromatography, we observed weak interactions between CE and an isolated Foot domain, in agreement with the crystallographically observed PCI1. Furthermore, we confirmed the Foot as the only non-CTD site for CE recognition (supplemental Fig. S5B; details are in supplemental Data, Section 2.2). No interactions were found between an isolated Cet1 and the Pol II domains (supplemental Fig. S5C), consistent with the result described above that Ceg1, but not Cet1, was essential for binding.

To evaluate the role of the CTD-based PCI2, we produced MBP-CTD, a construct of intact yeast CTD fused with MBP (maltose-binding protein). We phosphorylated it to produce MBP-CTD-P by the same procedure as for generating Pol IIO. SEC results showed that MBP-CTD-P did not form a stable complex with CE (Fig. 3, A and B). We reasoned that formation of the stable complex might require the presence of both PCI1 and PCI2 and, hence, produced an MBP-Foot fusion protein. This construct was combined in trans with MBP-CTD-P and incubated with CE. Each of the proteins in this mixture migrated independently through the SEC column (Fig. 3C). The GST-Foot protein was tested as well and produced similar results (not shown). Therefore, in contrast to Pol IIO, neither PCI1 nor PCI2 alone nor their cop-

![Figure 2. X-ray diffraction analysis of Pol IIO-CE complex. A. shown is an electron density map of the Pol IIO-CE complex. The map (green, contoured at 1.0σ) was experimentally phased by using anomalous scattering from the zinc ions bound in the Pol II molecule and, hence, was not biased by any existing model. The refined 12-subunit model (gray and black) of Pol II (59) was readily fitted into the density. The density (CE density) adjacent to the Rpb1 Foot domain (brown and red) is considered to be that of the OB domain of Ceg1 based on the docking experiment shown in supplemental Fig. S3D. B. a stereo-plot of the electron density (blue, 1.0σ) attached to the surface of Foot (brown and red) is shown. The contact surface is contributed by secondary structural elements H2, H4, and LP (H4-H5) of the Foot domain.](image-url)
resence as separate proteins could support formation of a stoichiometric complex with CE.

To combine PCI and PCI2 in cis, we expressed a soluble MBP fusion protein with the Rpb1 module consisting of tandem domains of the Foot, nucleic acid cleft, and CTD (FCC) in their natural order (Rpb1 861–1733) (Fig. 4A). This protein, named BFCC (B for Pol II) module herein, was partially purified using the MBP-amylose affinity chromatography and then phosphorylated in the same way as for Pol II (Fig. 4B). The impurities in the preparation proved to be breakdown products as they all bound to the amylose resin; they likely formed high molecular weight aggregates as indicated by their SEC elution positions, possibly via the Cleft domain, which is mostly buried in the Pol II tertiary folding but exposed in this construct. The phosphorylated (BFCCo) but not the unphosphorylated (BFCCa) module formed a stable and stoichiometric complex with CE (Fig. 4, C and D). It is worth noting that the profile of unbound Ceg1-Cet1 complex (Fig. 4D) does not spread into the fractions that correspond to elution positions of the bound CE. This characteristic is persistent throughout the SEC runs including that shown in Fig. 1C. Because this binding was phosphorylation-dependent and the breakdown products in the BFCC preparation were not responsive to the CTD phosphorylation (hence CTD-less) as determined by mobility shift in SDS-PAGE (Fig. 4B), the stable complex in Fig. 4C was deemed to have resulted from interactions with the BFCCo protein (top band). The breakdown products common to the BFCCa and BFCCo preparations as separate proteins could support formation of a stoichiometric complex with CE.

FIGURE 4. Linked Foot and CTD-P domains recapitulate the stable interaction between Pol II and CE. A, shown is the BFCC module (magenta) in the Pol II structure. The CTD is not crystallographically determined but is represented by the wavy line to indicate its unfolded state. B, time-course of MAPK2 phosphorylation of the BFCC module fused to MBP. Incubation durations are indicated on top. BFCCa and BFCCo denote the unphosphorylated and phosphorylated forms, respectively. Contaminating proteins (asterisks) in the partially purified samples proved not responsive to the phosphorylation. C, CE separates from the BFCCa module (MBP-BFCCa) in SEC. E and F, SEC experiments demonstrating that AFCCo and CFCCo, the phosphorylated modules with a Foot domain from Pol I and Pol III, respectively, do not form a stable complex with CE.

FIGURE 3. Separated Foot and CTD-P domains do not support stable interactions with CE. A, shown is a SEC profile of the recombinant CTD-P with the peak fraction indicated by the upward arrow. B, CE separates from the recombinant CTD-P in SEC (upward arrows). The downward arrow approximately marks a potential peak position for a stable complex. C, copresence of the CTD-P and Foot domains in trans does not support formation of a stable complex with the CE. The staggered profiles of each protein (upward arrows) indicate their separation. A complex formed among the proteins would create a comigration peak (approximated by the downward arrow) ahead of the individual peaks. Note that the CTD is unfolded and its migration is abnormally slow. A contaminating protein is marked by an asterisk. MK, an M₉₆ marker.
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A minimum CTD length of 13- to 14-heptad repeats is required for stable interaction with CE in vitro. A, the protein sequences of truncated CTDs in the BFCC modules used in this analysis are shown. The numbers of heptad repeats are indicated on top, and the binding results from SEC experiments are given below each sequence. Note that the italicized amino acid ID in the 10CTD construct corresponds to the last 10 non-consensus residues found at the C terminus of yeast CTD. B, the BFCCo with 16 CTD heptads forms a stable complex with CE in SEC. The peak profile of the BFCCo-CE complex is marked below the SDS gel. The elution fraction number increases in the direction of the arrow (top). Asterisks indicate the contaminating proteins that were not responsive to the phosphorylation as shown in supplemental Fig. S6A. A M, marker (250, 130, 95, 72, 55 and 36 kDa, from the top) was loaded in the left-most lane for B–E. C, the BFCCa with 16 CTD heptads does not form a stable complex with CE in SEC. Note the profile of the unbound CE peak (marked below the gel) did not spread into the migration positions of the complex. D, the BFCCo with 14 CTD heptads forms a stable complex with CE in SEC. Both the Ceg1 and Cet1 subunits of CE were found in the peak fractions corresponding to the complex (marked below the gel). E, the BFCCo with 12 CTD heptads does not form a stable complex with CE in SEC. No proteins of CE could be seen in the fractions corresponding to the complex.

To assess the functional significance of PCI1, we replaced the Foot domain in BFCC with its counterparts from Pol I and Pol III to produce the AFCC and CFCC (A for Pol I; C for Pol III) modules, respectively. These domain-swapped constructs were partially purified and phosphorylated (supplemental Fig. S6A) but failed to form stable complexes with CE (Fig. 4, E and F), indicating that the Foot domains of Pol I and Pol III cannot functionally substitute for that of Pol II and that the Pol II Foot is required for stable association with CE. This biochemical result offers an explanation for the observation that a CTD-tailed mutant Pol III is not able to impart RNA processing to its transcripts in vivo (52). Introduction of the analogous domain-swapped mutations into yeast RPB1 conferred lethality (see below), confirming that the Rpb1 Foot domain is essential for Pol II function in vivo. The possibility that other Pol II subunits (e.g. Rpb5 and Rpb6) might be involved in the interaction with CE was ruled out through the additional experiments described in the supplemental Data, Section 2.2.

A Minimum CTD Length in Pol II Is Required for Stable Interaction with CE—We probed PCI2 by evaluating the minimal length of CTD that is capable of maintaining interactions with CE. Mutant BFCC proteins were produced by terminating the CTD domain at the carboxyl ends of the 16th, 14th, 12th, and 10th heptad repeats (Fig. 5A). Again, the degradation products evident in these preparations were not responsive to the phosphorylation as judged by mobility in the SDS-PAGE (supplemental Fig. S6A). In SEC performed at 20 °C, CE formed stable complexes with the construct of 16CTD in a phosphorylation-dependent manner (Fig. 5B, C and D). Characteristically, again, the profile of unbound CE did not spread into positions of the complexed CE (Fig. 5C). Complexed CE was also found with BFCCo(14CTD) in the SEC profile (Fig. 5D). However, we were not able to discern the formation of a stable complex with BFCCo(12CTD) (Fig. 5E) or the 10CTD module (not shown), as no CE was detectable by Coomassie staining. However, a slight shift (0.5–1 fraction, 0.15 ml/fraction) of the CE profile toward the peak of BFCCo(16CTD) was noticed, which might suggest a certain level of unstable interactions with the 12CTD module. Overall, these results indicate that CE requires at least 14 linear heptads on the polymerase to form a stable complex at 20 °C in vitro in this assay. It should also be noted that the last 10 non-consensus residues from the very C terminus of yeast CTD are probably not involved in CE binding, as they were present in the 10CTD construct (Fig. 5A), and no interaction were detected (data not shown). These biochemical data correlated well with phenotypes of the strains (shown below) bearing the same CTD truncations in Rpb1; namely, that the 12CTD allele could not support yeast growth at 18 °C and only allow very slow growth at 30 and 37 °C and the 10CTD allele is inviable at all the temperatures tested.

These observations indicate that a CTD containing 13–14 heptad repeats on the yeast Pol II is required and sufficient for...
the stable interaction with CE. This notion is consistent with earlier findings that the *S. pombe* CE requires 12–14 CTD heptads for interaction in a two-hybrid assay (61) and that human Pol II with 15 heptads from either the N- or C-terminal half of the CTD supports capping *in vivo* (62). When considered together with the *C. albicans* structure mentioned above in which the PC12 encompasses ~2.5 CTD heptads (51), our data suggest a possibility that the yeast CE might interact with 2–3 consecutive heptads between registers 11–14 on the native Pol II molecule; an alternative possibility is that formation of the complex might require 2–3 consecutive heptads at a certain linker distance from the body of the polymerase – a relaxed form of the former model. Such a restriction of CE interaction to a specific segment within the CTD could be a mechanism that explains why CTD di-heptads appear to be functional units of the CTD (63).

**Functional Significance of the Pol II-CE Interface**—To assess the importance of PCI1 and PCI2 for yeast growth, we used the plasmid shuffle strategy (64) to substitute a wild-type (WT) *RPB1* in the yeast with a copy of *rpb1* carrying mutations in PCI1 or PCI2. Both the WT and mutant alleles were under the control of the native promoter of *RPB1* (“Experimental Procedures”). As shown in Fig. 6A, when the Rpb1 Foot was substituted by A-Foot and C-Foot, its counterparts from Pol I and Pol III (sequences aligned in supplemental Fig. S4B), or by FtLP and FtH2, cells were not viable (Fig. 6A, Leu/FOA). These results are consistent with the observed loss of CE interactions with the corresponding AFCCo and CFCCo modules in the SEC assays (Fig. 4, E and F). The C-Foot and FtLP mutations displayed a dominant negative growth effect over WT *RPB1* allele born on the URA3 plasmid that was used for shuffling (Fig. 6A, −Leu + FOA). These results are consistent with the observed loss of CE interactions with the corresponding AFCCo and CFCCo modules in the SEC assays (Fig. 4, E and F).

**FIGURE 6. Phenotypes of the PCI1 and PCI2 mutants.** A, growth effects due to Foot swaps and deletion (A-Foot, C-Foot, and Δ-Foot) as tested with 20-fold serially diluted cultures are shown. FOA is toxic to URA3-containing cells and, thus, allows the LEU2-linked alleles to be assayed in the absence of the WT gene. The LEU2-linked RPB1 (WT) served as the positive control, and the empty LEU2 shuffle plasmid (vector) served as the negative control. B, frequencies of the LEU2 plasmids carrying a Foot-swap, Foot deletion, or PCI2 (CTD shortening) mutant rpb1 to transform the shuffle strain that contains RPB1 on the URA3 plasmid are shown. Constant amounts of competent cells and plasmid DNA (key below) were used. C, locations of the mutated residues in Rpb1 Foot domain are shown. Mutated PCI1 residues in the FtH4, FtH2, and FtLP elements are colored red, magenta, and yellow, respectively. D, the PCI1 and PCI2 mutations impair yeast growth. The shuffle assays were performed at 37 °C (not shown), 30 °C (upper left), and 18 °C (lower left). The former two conditions yielded similar phenotypes. The slow-growth defects of PCI1 worsened at 18 °C, indicating cold sensitivity. The PCI1 mutations also slowed yeast growth (right panels). Shortening the CTD to 12 heptads severely impaired growth at 30 °C and prevented growth at 18 °C; the 10CTD allele could not support growth at all temperatures. E, the PCI2 mutations dominantly impair yeast growth at lower temperature (18 °C). The LEU2-marked plasmids carrying rpb1 with the PCI1 and PCI2 mutations were each transformed into a WT strain derived from W303. The selection and growth of LEU+ strains were carried out on SC−Leu plates, and growth rates were assessed using 20-fold serial dilution.

The PCI2 mutations impair yeast growth at lower temperature (18 °C). The LEU2-marked plasmids carrying rpb1 with the PCI1 and PCI2 mutations were each transformed into a WT strain derived from W303. The selection and growth of LEU+ strains were carried out on SC−Leu plates, and growth rates were assessed using 20-fold serial dilution.
To evaluate the importance of Pol II surface residues that are located within PCI1, we made three groups of alanine substitutions in the secondary elements H2, H4, and LP (H4–H5) of the Foot domain (Fig. 2B) and named them Fth2 (K924A/L928A/E931A/K938A), Fth4 (K984A/L988A/K991A/D992A/E995A), and Ftlp (K1003A/E1005A/R1012A) (Fig. 6C). These rpbl alleles supported viability but resulted in slow growth at 30 °C, and the defects became more pronounced at 18 °C (Fig. 6D, left panels).

The PCI2 mutant with a CTD identical to that in the 10CTD module used in the SEC experiment was not able to support growth at all temperatures tested (Fig. 6D, right panels), the 12CTD mutant grew extremely slowly at 30 °C and was inviable at 18 °C, the 14CTD and 16CTD alleles were apparently wild type at 30 °C, but the 14CTD mutant exhibited cold sensitivity. These observations are similar to those made previously by Young and co-workers (65) and Corden and co-workers (66) and support the conclusion that a minimum of 11–12 CTD repeats is required for budding yeast viability. This minimum CTD length required for viability is nearly identical to the aforementioned threshold length, 13–14 repeats, for forming a stable complex with CE in vitro. Combined with the biochemical results, our data pinpoint the impairment of PCI2 and loss of CE interactions as another detrimental consequence of CTD shortening beyond a minimum length in addition to the disruption of Mediator interactions, 3′-processing, and termination (2, 11, 38).

To test for dominant effects of the non-lethal mutations over an endogenous RPB1, we transformed the LEU2-marked PCI1 and PCI2 alleles (under the native RPB1 promoter) into a WT strain (a derivative of W303). As shown in Fig. 6E, the PCI1 surface mutants (Fth4, Fth2, and Ftlp) did not affect growth in the WT background. The 14CTD and 12CTD alleles displayed dominant negative effects at the lower temperature (18 °C), which reflected the low frequency and small colony size in the initial transformation (Fig. 6B). Similar dominant effects had been noticed for S5A substitutions in the CTD and for the combination of Mediator interactions, 3′-processing, and termination (2, 11, 38).

DISCUSSION

The Dual-interface CE Recruitment Model—The biochemical, structural, and functional data presented here allow us to propose a dual-interface model that explains the physical basis for the specific targeting of RNA capping to Pol II transcripts (Fig. 8). In this model CE forms a stoichiometric complex with Pol IIO, the phosphorylated, post-initiation isoform of Pol II; the specificity of this interaction is achieved by combining the two determinants, PCI1 and PCI2, in cis as they occur in a native Pol IIO molecule. PCI1 is unique to Pol II and, thus, contributes to the isozyme specificity during recruitment of CE. PCI2 is also unique to Pol II and allows further differentiation between the phosphorylated and unphosphorylated forms of Pol II. Our functional data suggest that the dual interface may be metastable in nature, as a mutated interface of either PCI1 or PCI2 is prone to inactivation by cold (Fig. 6, D and E), and impairing either PCI1 or PCI2 is detrimental to the recognition by CE in vitro (Figs. 4 and 5) and yeast survival in vivo (Fig. 6A). In summary, CE targeting depends upon the combined PCI1 and PCI2, which is a characteristic of elongating Pol II molecules.

A Specific CTD Segment Involved in the Assembly of Pol II Cotranscriptional Capping Complex—Our data suggest a possibility that CE might interact with a particular segment of 2–3 heptads within the CTD-P when forming a specific complex with Pol IIO. This proposal is consistent with the binding of ~2.5 heptads by C. albicans CE in the cocrystal structure (51); it could also offer a physical explanation for the observation that CTD functions are confined within di-heptads (63). Factors involved in CTD-linked RNA processing and other nuclear events could use similar modes to interact with the phosphor-
Determined by CTD phosphorylation

Pol II co-IP (Rpb3-TAP tag)

w.t. FTH4 FTH2 FtLP (RBP1 allele)

α-Ceg1

α-Rpb4

Whole-Cell Extract

(kDa) w.t. FTH4 FTH2 FtLP

95 72 55 36 28

α-Ceg1

α-Rpb4

FIGURE 7. PCI1 contributes to the specific recognition of Pol II by CE in vivo. A, mutation of PCI1 reduces the association of CE with Pol II in the cell. Whole-cell extracts prepared from FOA-resistant strains bearing the PCI1 mutations were assayed by coimmunoprecipitation (co-IP) via the polymerases (using Rpb3-TAP). The left panels show a typical Western blot that reveals the levels of Ceg1 bound to Pol II in the strains, with the latter detected for its Rpb4 subunit. The right histogram shows the levels of Rpb4-normalized Ceg1 in the mutants relative to that in the WT (allele/wt). The values are the averages from four independent experiments. B, the levels of Ceg1 and Pol II remain unchanged in extracts of the various strains. A typical blotting experiment is shown on the left, and the quantification of Ceg1 and Rpb4 based on four experiments is shown on the right. C, overexpression of CE suppresses the slow-growth phenotypes of the PCI1 mutants. FOA-resistant strains bearing the PCI1 mutations were transformed with either the empty pRS423 (CEG1/α1) or CEG1-carrying vector (CEG1+). pRS423 is a multicopied yeast 2μ/H9262 plasmid bearing the HIS3 marker (69). The cultures were brought to the same cell density, serially diluted, and spotted onto the SC-His medium and incubated at 18°C.

Determinants of Cotranscriptional RNA Capping

Determination of Dynamic Regulatory Complexes by Combining Weak Interactions—The relatively weak interactions observed for isolated PCI1 and PCI2 may reflect a dynamic property of the system that may be important for cotranscriptional capping. Conceivably, this process is highly specific and temporally sensitive to ensure that CE actions are minimized toward other classes of RNAs abundant in the nucleus. In this light, a stable complex formed based on combining the individually weak PCI1 and PCI2 seems well suited for this task, as dissolution of this complex would occur rapidly upon perturbation of either interface. For instance, PCI1 and/or PCI2 may separate as a result of the CE conformational change that accompanies the capping reaction; likely, the action by the Ser-5-specific CTD phosphatase Ssu72 may weaken the PCI2 interactions at a post-capping step (70), although the mechanism that determines the order of these events is unknown. It also appears that this principle, namely combining weak interactions alongside post-translational modifications such as phosphorylation, may universally govern the dynamic assembly and disassembly of multiprotein regulatory complexes that function in transcription and possibly other pathways.

Relationship of the Dual Interface to Other Aspects of CE Recruitment—Our model is consistent with the majority of published data that implicate CTD phosphorylation in CE recruitment. In addition, the interaction based on PCI1 offers an alternative explanation for the residual coupling activities for capping (a 4-fold enhancement) by the CTD-less Pol IIA (71) and for the inability of a CTD-tailed human Pol III to instigate processing to its transcripts (52). However, our data do not address the reported inhibition of Pol II due to direct interactions with the triphosphatase Cet1 (56).
Our model highlights the binding of CE via the OB domain of Ceg1 with certain disorder, which would leave the NT domain of Ceg1 free to interact with CTD-P to form PC12. We believe this and additional disorders may have prevented the visualization of electron density for the NT domain and the entire Cet1 subunit, both present in the crystal (supplemental Fig. S3B). The absence of the NT domain in our structure is consistent with the known flexibility between NT and OB domains in guanylyltransferases (19). Also, the absence of Cet1 is consistent with the observation that Cet1 is flexibly attached to Ceg1 OB domain via a linker peptide (Cet1 241–268) (18).

Because our Pol IIO-CE complex does not contain a template and DNA/RNA hybrid, it represents at best a core structure of a complete elongation complex that may ordinarily include Spt4-Spt5. Given the functional involvement of Spt5 in early elongation and the salubrious contributions of Spt5 C-terminal region to CE recruitment as seen in S. pombe (46), the Spt5 C-terminal region might interact with parts of Cet1 and/or the Ceg1 NT domain to help orient and position the entire CE in a productive assembly on the polymerase.

Implications of the Dual Interface for RNA Early Elongation and Processing—The dominant negative effects on cell growth observed for some of the PC11 and PC12 mutants suggest that an impaired PCI1 or PC12 surface might confer defects at Pol II post-initiation steps, pointing to a role of CE in releasing the early elongation complex from promoter regions (22) or its participation in the elongation checkpoint (23, 47) either through RNA capping or by providing additional protein interactions in these complexes.

Our disclosure of the importance of the non CTD-based PCI1 for CE function has mechanistic implications for other Pol II-linked nuclear processes including splicing, 3′-processing, and mRNA export. The recruitment of factors that catalyze these activities may not be precise in the chromatin matrix if the mechanism is solely based on the CTD, because the CTD does not assume a regularly folded structure and may spatially extend over 900 Å (50). Thus, it may be useful to suggest that the targeting of these events to discrete steps in the transcription cycle may also require interactions with the globular core of Pol II so as to restrict the activities to sites of active Pol II elongation. Whether the Foot domain would again be involved in any of these processes remains to be determined.

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