Dynamic association of capping enzymes with transcribing RNA polymerase II

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The C-terminal heptad repeat domain (CTD) of RNA polymerase II (pol II) is proposed to target pre-mRNA processing enzymes to nascent pol II transcripts, but this idea has not been directly tested in vivo. In vitro, the yeast mRNA capping enzymes Ceg1 and Abd1 bind specifically to the phosphorylated CTD. Here we show that yeast capping enzymes cross-link in vivo to the 5’ ends of transcribed genes and that this localization requires the CTD. Both the extent of CTD phosphorylation at Ser 5 of the heptad repeat and the binding of capping enzymes decreased as polymerase moved from the 5’ end of the heptad repeat and the binding of capping enzymes increased as polymerase moved from the 5’ end before heat shock to a mix of phosphorylated and nonphosphorylated forms at 5’ and 3’ ends after heat shock (O’Brien et al. 1994). It is not known, however, if the state of CTD phosphorylation or the protein composition (Otero et al. 1999) of the elongation complex changes as pol II travels through a gene.

The importance of the phosphorylated CTD for pre-mRNA capping is supported by genetic and biochemical evidence [Cho et al. 1997; McCracken et al. 1997a; Rodriguez et al. 2000]. The yeast-capping enzymes Ceg1 (which adds the unmethylated GpppRNA cap) and Abd1 (which methylates the cap to form m7GpppRNA) bind directly to the phosphorylated CTD in vitro (Cho et al. 1997; McCracken et al. 1997a; Yue et al. 1997). An outstanding question is whether the capping enzymes actually associate with pol II elongation complexes in vivo in a manner that requires CTD phosphorylation.

Results and Discussion

Capping enzymes localize to sites of transcription in vivo

We used in vivo formaldehyde cross-linking, chromatin immunoprecipitation (ChIP, Strahl-Bolsinger et al. 1997), and PCR analysis of precipitated DNA to ask whether capping enzymes are physically present on transcribed genes in budding yeast. Formaldehyde treatment produces a network of protein–protein and protein–DNA cross-links that, in theory, permits detection of proteins that are directly or indirectly in contact with a particular DNA sequence. In Figure 1A, cross-linked chromatin from isogenic RPB1 and rpb1-1 strains [Nonet et al. 1987] with a HA epitope tag on the Rpb3 subunit of pol II were immunoprecipitated with either monoclonal anti-HA or polyclonal antibodies against the CTD of Rpb1, Abd1, or Ceg1. The anti-CTD antibody reacted equally well with biotinylated [YSPTSPS]4 synthetic peptides that were either unphosphorylated or phosphorylated at Ser 2 or Ser 5 of each repeat [Fig. 1C, lanes 2–4]. Anti-Abd1 recognizes a single band and anti-Ceg1 recognizes a doublet in Western blots of yeast whole-cell extract [McCracken et al. 1997a; Wang and Shuman 1997; data not shown]. ChIP with anti-myc monoclonal antibody in a strain with this epitope tag on Abd1 gave similar results to the polyclonal anti-Abd1 antibody (data not shown).

The experiment in Figure 1A shows that the capping enzymes Ceg1 and Abd1, as well as the Rpb1 and Rpb3

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subunits of pol II, cross-link in vivo to the 5’ ends of the constitutively transcribed ACT1, ENO2, and TEF1 genes. In contrast, little or no cross-linking occurred at the silent mating type locus, HMR (Fig. 1A, lanes 7,11,15,19), the nontranscribed telomere VI R, or the mitochondrial gene COX3 (data not shown).

When transcription was inhibited by shifting the rpb1-1 ts mutant to 37°C for 45 min, the cross-linking of capping enzymes and pol II were significantly diminished (Fig. 1A, lanes 6,10,14,18), whereas heat shock of the isogenic RPB1 strain had little effect (Fig. 1A, lanes 8,12,16,20). The HA-Rpb3 protein is stable in rpb1-1 cells at 37°C for >60 min [data not shown], yet it does not cross-link to DNA at this temperature, providing strong support for the specificity of the in vivo cross-linking reaction. In summary, the experiment in Figure 1A shows that capping enzymes bind in vivo to the 5’ ends of transcribed genes and that this localization requires ongoing transcription.

CTD-dependent recruitment of Ceg1 to sites of transcription

The CTD requirement for recruitment of the capping enzyme Ceg1 was tested at the HSP82 gene, which can be transcribed by the CTD-deleted form of pol II [McNeil et al. 1998]. Pol II and Ceg1 cross-linking to HSP82 was severely reduced when a rpb1-1 ts mutant carrying a control vector plasmid was shifted to 37°C [Fig. 1B, cf. lanes 7,13 with 8,14]. When the wild-type RPB1 gene was provided on a plasmid, then heat shock increased the extent of Ceg1 and Rpb3 cross-linking to HSP82 [Fig. 1B, cf. lanes 9,15 with 10,16]. In the rpb1-1 strain carrying the rpb1-ΔCTD gene on a plasmid, the only active pol II at 37°C is the form that lacks the CTD. ACTD-pol II, detected with antibody, against HA-Rpb3 was cross-linked to HSP82 at 37°C, but very little Ceg1 cross-linking was detected [Fig. 1B, lanes 12,18]. In the same cells at 25°C, however, when the rpb1-1 polymerase containing a full-length CTD was engaged [Fig. 1B, lane 11], then significant Ceg1 cross-linking to HSP82 was observed [Fig. 1B, lane 17]. Ceg1 cross-linking to HSP82, as quantified by phosphorimager analysis of the ChIP signal normalized to input, showed an 80% reduction when the Δrpb1-ΔCTD strain was shifted to 37°C [Fig. 1B lanes 17,18], whereas Rpb3 cross-linking declined by only 2% [Fig. 1B, lanes 11,12]. We conclude from the experiment in Figure 1B that the CTD is necessary for the association of Ceg1 with a transcribed gene.

Kin28-dependent recruitment of capping enzymes

The importance of CTD phosphorylation for recruitment of capping enzymes was tested in a ts mutant of Kin28 that phosphorylates Ser 5 of the heptad repeat [Hengartner et al. 1998]. After inactivation of this kinase at 37°C, a reduced but significant amount of pol II remained at ACT1 and ENO2, as detected by the anti-CTD and anti-HA-Rpb3 immunoprecipitation [Fig. 2A, lanes 8,12]. CTD phosphorylation was monitored by ChIP with a polyclonal antibody that reacts with heptads phosphorylated on Ser 5 but not with nonphosphorylated heptads or those phosphorylated at Ser 2 [see Fig. 1C, lanes 6–8]. The pol II remaining at ACT1 and ENO2 after inactivation of Kin28 had reduced reactivity with the Ser 5–PO4-specific antibody [Fig. 2A, lane 16] consistent with loss of kinase activity. Significantly, this hypophosphorylated pol II was not associated with Abd1 or Ceg1 [Fig. 2A, lanes 20,24]. We conclude that Kin28 is required for normal levels of pol II binding, CTD phosphorylation at Ser 5 or recruitment of capping enzymes to ACT1 or ENO2 as judged by ChIP assays [data not shown].

CTD dephosphorylation during elongation

Is the CTD phosphorylation and binding of capping enzymes that is established at the 5’ end of the gene main-
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cross-linking was determined by ChIP with anti-HA-Rpb3 and anti-CTD antibodies, and phosphorylated pol II was monitored with antibody specific to the Ser 5 phosphorylated CTD. Remarkably, the polymerases near the 5’ end were more highly phosphorylated than polymerases near the 3’ end, that is, the 5′:3′ ratios for phosphorylated pol II were always greater than those for total pol II (Fig. 2A, cf. lanes 5–7 and 9–11; Fig. 2B, lanes 2–4). Because the anti-phospho-CTD antibody efficiently recognizes partially phosphorylated CTD (data not shown), the ChIP assay probably underestimates the extent of dephosphorylation during elongation. We conclude that there is a net dephosphorylation of pol II at Ser 5, as it traverses the ACT1, ENO2, TEF1, GAL1, and GAL10 genes. Although pol II is dephosphorylated during elongation, phosphorylation by Kin28 still appears to be important for promoter clearance and/or early elongation (Akoulitch et al. 1995; Akhtar et al. 1996), as the pol II remaining after Kin28 inactivation was mostly confined to the 5′ ends of ACT1 and ENO2 (Fig. 2A, cf. lanes 8,12 with 6,10).

**Ceg1 is released early but Abd1 travels with pol II elongation complexes**

Ceg1 and Abd1 bind only to the phosphorylated CTD in vitro (McCracken et al. 1997a). Therefore, we reasoned that dephosphorylation during elongation might be linked to release of capping enzymes. This prediction was supported by ChIP, which showed that cross-linking of capping enzymes to 5′ ends was less than cross-linking to 5′ ends on all five genes we examined (Fig. 2A, lanes 17–19, 21–23; Fig. 2B, lanes 6,7). Unexpectedly, Ceg1 and Abd1 were not distributed in the same way along these genes however; the 5′:3′ gradients of Ceg1 cross-linking were significantly steeper than those for Abd1. This point is illustrated in the **Kin28** wild-type strain at 37°C, where the 5′:3′ ratios for Abd1 were 2.2, 3.9, and 1.7 on the **ACT1**, **ENO2**, and **TEF1** genes, respectively, compared to 8.6, 12, and 9.0 for Ceg1 (Fig. 2A, lanes 18,22). Similarly, the ChIP 5′:3′ ratios for Abd1 on the induced **GAL1** and **GAL10** genes were only 1.5 and 2.0, respectively, compared to ratios of 7.6 and 5.6 for Ceg1 on the same genes (Fig. 2B, lanes 6,7). In fact, the 5′:3′ ratios for Ceg1 on the **GAL1** genes were similar to those for the Gal4 transcription factor (Fig. 2A, cf. lanes 8,12 with 6,10). Assuming that Gal4 is localized exclusively at the promoters, this result confirmed that Ceg1 was mostly restricted to the 5′ ends of **GAL1** and **GAL10**, whereas Abd1 was more evenly distributed between the 5′ and 3′ ends.

Changes in the composition of the pol II elongation complex as it traversed the 1.3-kb **ENO2** gene were mapped in more detail using five primer pairs spaced as pol II travels to the 3′ end! To address this question, we compared the cross-linking of pol II and capping enzymes to the 5′ and 3′ ends of the constitutively expressed **ENO2**, **ACT1**, and **TEF1** genes and the inducible **GAL1** and **GAL10** genes (Fig. 2). Total pol II

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**Figure 2.** 5′–3′ distribution of pol II, and capping enzymes and the effect of Kin28 inactivation. (A) ChIP analysis of kin28-ts3 and isogenic wild type strains [Valay et al. 1993] at 25°C and 37°C. Ratios of 5′:3′ ChIP signals that are normalized to input are given below selected lanes. The normalization controls for any differences in PCR amplification efficiency between 5′ and 3′ gene segments. (B) Distribution of pol II and capping enzymes along **GAL1** and **GAL10** genes. ChIP analysis 30 min after addition of 2% galactose to a wild-type strain at 37°C. (C) Distribution of total pol II (CTD), Ser 5 phosphorylated pol II [CTD-P], Abd1, and Ceg1 along the **ENO2** gene. Results are average ChIP signals relative to input for four experiments. Standard errors were <10%. The four wild-type strains used were the same as those in Fig. 1B: **DBY154** (pFL38-Rpb1), Fig. 2A: GF262 [Valay et al. 1993]; Fig. 3: YBS2[pYGCE358-Ceg1] [Schwer et al. 1998], and the YMK16 FCP1 strain isogenic to the fcp1-1 mutant [Kobor et al. 1999]. The 5′-most PCR product was set to 100% for each antibody. PCR primers were generated to cover ~200-bp regions spaced throughout the **ENO2** gene. Distances are from the ATG to the middle of each PCR product. The **ENO2** primer pairs used in A correspond to the points at 100 and 1050 bp from the ATG.
throughout the ORF. The average values for four experiments in four different strains are plotted in Figure 2C. Of the total pol II recruited to the 5′ end of ENO2, 50% remained at the 3′ end as determined by either anti-CTD (Fig. 2C) or anti-HA-Rpb3 ChIP (Fig. 2A, lane 6; data not shown). This observation suggests that some premature termination of transcription occurs on the ENO2 gene. In contrast to total pol II, only 15% of the initial 5′ level of Ser 5 phosphorylated pol II remained at the 3′ end (Fig. 2C), with most of the loss in phosphorylation occurring within ~600 bases of the start site. Of the initial 5′ amount of cross-linked Abd1, 21% remained at the 3′ end, compared to only 6% of Ceg1. Ceg1 cross-linking fell precipitously within the first 600 bases of the transcription unit. These data demonstrate that Ceg1 is released from pol II early, while Abd1 is released later in elongation. Furthermore, the results indicate that a significant fraction of the elongating pol II carries Abd1 to the 3′ end of the ENO2 gene.

Abd1 is recruited to sites of pol II transcription independent of Ceg1

Although Abd1 cross-linking is resistant to the RNase treatment in the ChIP protocol [see Materials and Methods], it is possible that Abd1 remains associated with elongation complexes by a mechanism that requires recognition of its substrate: the 5′ cap guanylate added by Ceg1. We tested this possibility using two ceg1 ts mutants that are defective for capping at restrictive temperature (Schwer et al. 1998). Ceg1 cross-linking to the ACT1, HSP82, and ENO2 genes was abolished in both ts strains at 37°C [Fig. 3, lanes 16,18], but not in the wild type [Fig. 3, lane 14]. In contrast to the complete loss of Ceg1 cross-linking in the ts strains at 37°C, significant amounts of Abd1 remained associated with transcription complexes on all three genes examined [Fig. 3, lanes 10,12]. For reasons we do not understand, Ceg1-13 had no significant effect on Abd1 recruitment at the nonpermissive temperature [Fig. 3, lane 12], whereas ceg1-3 partially reduced Abd1 recruitment [Fig. 3, lane 10]. The ratio of Abd1 at the 5′ and 3′ ends of the ENO2 gene was unaffected by either ceg1 ts allele, indicating that Abd1’s ability to travel with pol II is independent of Ceg1 [data not shown]. Ceg1 inactivation also had no effect on pol II recruitment or CTD phosphorylation on Ser 5 [data not shown] at the ACT1, ENO2, and HSP82 genes. We conclude that protein–protein interaction rather than Abd1–substrate interaction is responsible for localizing Abd1 to pol II elongation complexes.

Fcp1-dependent release of capping enzymes from elongation complexes

The experiment in Figure 2C suggests that dephosphorylation of the CTD could actually be the signal that releases capping enzymes from pol II elongation complexes. This hypothesis was tested using a ts mutant of the Fcp1 CTD phosphatase. We reasoned that if Fcp1 dephosphorylates elongation complexes and if dephosphorylation signals release of capping enzymes, then inactivation of Fcp1 should reduce the 5′:3′ gradient of capping enzymes. Fcp1 is required for re-cycling of pol II and for expression of most genes in yeast (Cho et al. 1999; Kobor et al. 1999). At the permissive temperature of 25°C, the fcp1-1 ts mutant [Kobor et al. 1999] grows slowly and has reduced pol II recruitment to ENO2, as expected for a partial recycling defect [data not shown]. ChIP analysis at 25°C and 37°C shows that Fcp1 inactivation had no effect on the 5′:3′ distribution of total pol II [Fig. 4A, lanes 3,4] but greatly reduced the 5′:3′ gradient of Ser 5 phosphorylated pol II [lanes 5,6]. Note that the
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5’-3’ ratio for Ser 5 phosphorylated pol II was only 1.4-fold higher than for total pol II [3.9 vs. 2.8; Fig. 4A, lanes 4,6] after Fcp1 was inactivated. These results show that Fcp1 inactivation reduced dephosphorylation during elongation, as expected. Fcp1 inactivation also reduced the cross-linking of phosphorylated CTD at the 5’ end, suggesting an initiation defect. Most important, Fcp1 inactivation significantly reduced the 5’-3’ ratios of Abd1 and Ceg1 cross-linking on ENO2 [Fig. 4A, cf. lanes 7,8 and 9,10].

To examine the effect of Fcp1 inactivation on elongation complexes along the ENO2 gene in more detail, we analyzed immunoprecipitates with the panel of five primer pairs used in Figure 2C. In the fcp1-1 strain at 25°C, total pol II, phosphorylated pol II, Ceg1, and Abd1 were distributed in a similar way to wild-type strains [Fig. 2C, Fig. 4B, open symbols]. When Fcp1 was inactivated at 37°C, however, cross-linking of phosphorylated pol II, Abd1, and Ceg1 were all significantly increased at the 3’ end relative to the 5’ end [Fig. 4B, filled symbols]. In the isogenic FCP1 wild-type strain, a shift to 37°C had no effect on the distribution of total pol II, phosphorylated pol II, or capping enzymes [data not shown; see also Fig. 2A, WT strain]. This experiment shows that reduced CTD dephosphorylation during elongation in the fcp1-1 mutant at 37°C prevented the dissociation of capping enzymes and, therefore, implies that CTD dephosphorylation is at least part of the signal for release of capping enzymes.

This report provides evidence that mRNA capping enzymes bind to pol II in vivo via independent interactions of Ceg1 and Abd1 with the phosphorylated CTD as RNA is being synthesized. The association of capping enzymes with pol II is dynamic; they bind at the 5’ end and are released further downstream. This cycle of binding and release is regulated by Kin28-dependent phosphorylation of the CTD heptad repeats on Ser 5 at the 5’ end of the gene and by Fcp1-dependent dephosphorylation of these residues within the transcription unit. Ceg1 is normally released early, while Abd1 is released later. Some Abd1 accompanies pol II at the 3’ end of the gene, suggesting that these two enzymes travel long distances together. The persistent binding of Abd1 to the elongation complex does not require interaction with the RNA cap [Fig. 3]. Why Abd1 should remain in contact with pol II at the 3’ end of a gene is unknown, and our findings raise the possibility that sites of Abd1 action are not limited to the 5’ end of the gene. Interestingly, the vaccinia virus-capping enzyme travels with the elongation complex of vaccinia RNA polymerase and serves an essential role in viral mRNA 3’-end formation as a transcription termination factor [Hagler et al. 1994].

Materials and methods
Yeasts strains and media
Yeast strains were generous gifts of R. Young, J. Greenblatt, M. Kobor, A. Greenleaf, and G. Faye. DBY154 [MATa his3–11,01 leu2–3,112 trp1–901 ade2–1 his3–11,86 trpl–1 ura3–52 MET15::HIS4–912 RPB3-1HA::LEU2 rpb1–1 trpl–1 HIS4] was made by crossing rpb1–1 into the HA-tagged Rpb3 strain Z277 [Kolodziej et al. 1990]. Yeast were grown to OD600 of 1.0 in YEPD or SC-uracil medium. Before formaldehyde cross linking and chromatin immunoprecipitation, is strains were shifted from 25°C to 37°C for 45 min.

Antibodies
Monoclonal 70A (Kenny et al. 1990) against mammalian RPA was used as a negative control. Monoclonal 12C6S detected HA-tagged Rpb3. Rabbit anti-Ceg1 [Schwer and Shuman 1996] and anti-Abd1 [Wang and Shuman 1997] have been described. Rabbit anti-Gal4 was raised against residues 1–147 and affinity purified. Rabbit anti-CTD was raised against GST-mouse CTD [1–52] and affinity purified. On Western blots, this antibody reacted with equal affinity to both phosphorylated and non-phosphorylated forms of yeast RNA pol II (data not shown). Rabbit polyclonal anti-CTD-P was raised against GST-mouse CTD [1–52] phosphorylated on glutathione sepharose beads in Hela extract followed by extensive washing in 1 M NaCl buffer as described [McCracken et al. 1997a]. It was depleted of reactivity to unphosphorylated CTD. Specificity of anti-CTD and anti-CTD-P for different phospho forms of CTD was determined by binding in FA lysis buffer [Strahl-Bolsinger et al. 1997] plus phosphatase inhibitors to biotinylated peptides (YSPTSPS), (1 nmole) immobilized on streptavidin magnetic beads. Peptides were either nonphosphorylated or phosphorylated on Ser 2 or Ser 5 [Ho and Shuman 1999]. After extensive washing in FA-lysis buffer, bound IgG was eluted with SDS sample buffer and analyzed by immunoblotting with antirabbit IgG HRP as shown in Figure 1C.

Formaldehyde cross-linking and chromatin immunoprecipitation
Chromatin was prepared as described [Strahl-Bolsinger et al. 1997] with minor modifications. After lysis of the cross-linked cells, the crude extract was sonicated to average size of 500 bp, clarified, and adjusted to 0.1% SDS and 1.0% sarkosyl in FA-lysis buffer. SDS and sarkosyl were added as above to FA-lysis buffer and FA-lysis buffer/500 mM NaCl washes. The TE wash, immunoprecipitates were digested with 10 μg of RNase A for 30 min at 37°C. Cross-linked protein/DNA complexes were eluted from protein A beads at room temperature in a buffer containing 1% SDS and 0.1 M NaHCO3. Cross-links were reversed by incubation at 65°C over night.

Quantitative PCR
PCR reactions performed under conditions that gave linear template: signal (94°C for 40 sec, 53°C for 40 sec, 72°C for 1 min; 20 cycles, reaction volume 20 μL) were carried out with 1% of the immune precipitate and 0.01% of total input DNA. 32P-dCTP (0.1 µCi) was added for the last PCR cycle. The PCR products (150–290 bp) were analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 M urea in TBE buffer. The radiolabeled products were quantified by Phosphorimager, and the signal intensities of the ChIP PCR reaction products were normalized to those of the DNAs amplified from the total input DNA template. The 70A irrelevant antibody and the HMR negative control PCR primers were used in all experiments but are only shown in selected figures.

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