Divergent Subunit Interactions among Fungal mRNA 5’-Capping Machineries

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The Saccharomyces cerevisiae mRNA capping enzyme consists of two subunits: an RNA 5’-triphosphatase (RTPase) and GTP:mRNA guanylyltransferase (GTase). The GTase subunit (Ceg1) binds to the phosphorylated carboxyl-terminal domain of the largest subunit (CTD-P) of RNA polymerase II (pol II), coupling capping with transcription. Ceg1 bound to the CTD-P is inactive unless allosterically activated by interaction with the RTPase subunit (Cet1). For purposes of comparison, we characterize here the related GTases and RTPases from the yeasts Schizosaccharomyces pombe and Candida albicans. Surprisingly, the S. pombe capping enzyme subunits do not interact with each other. Both can independently interact with CTD-P of pol II, and the GTase is not repressed by CTD-P binding. The S. pombe RTPase gene (pct1) is essential for viability. Pct1 can replace the S. cerevisiae RTPase when GTase activity is supplied by the S. pombe or mouse enzymes but not by the S. cerevisiae GTase. The C. albicans capping enzyme subunits do interact with each other. However, this interaction is not essential in vivo. Our results reveal an unexpected diversity among the fungal capping machineries.

The “cap” structure is a specific modification of the 5’ end of mRNA found in eukaryotic cells and most of their viruses. It consists of a 7-methylguanosine moiety attached to the 5’ terminus via a 5’-5’ linkage. Cellular mRNA capping enzyme is bifunctional, consisting of RNA 5’-triphosphatase (RTPase) and GTP:mRNA guanylyltransferase (GTase) activities. In the first step of capping, RTPase removes the γ-phosphate from the 5’ end of the RNA substrate to leave a diphosphate end. GTase subsequently transfers GMP from GTP to form the structure GpppN1-. The third activity, RNA (guanine-7-)methyltransferase, adds a methyl group to the N-7 position of the guanine cap to form the “cap 0” structure, m’GpppN1-.  

Capping enzyme from Saccharomyces cerevisiae is a complex of RTPase and GTase subunits (27). These polypeptides are encoded by the CET1 and CEG1 genes, respectively, and both are essential for cell viability (41, 49). Mammalian capping enzyme is a single bifunctional polypeptide composed of an amino-terminal RTPase domain and a carboxyl-terminal GTase domain. The mammalian gene complements null and conditional mutants of CEG1 and/or CET1 (25, 26, 29, 51, 54).

Ceg1 has a high degree of amino acid similarity to the GTase proteins/domains from viruses and metazoans, and all are thought to use a common reaction mechanism (16, 50). In contrast, Cet1 does not resemble viral or metazoan phosphatases. The metazoan RTPase domain is a member of the protein tyrosine phosphatase (PTP) superfamily (31, 46, 50, 51, 54).

Cellular capping enzymes are recruited to the phosphorylated carboxyl-terminal domain of the largest subunit of RNA polymerase (pol) II (CTD-P) (5, 31, 54; for review, see references 18, 40, and 42). S. cerevisiae Ceg1 binds directly to CTD-P (6, 51) but is inactive for covalent enzyme-GMP complex formation unless also bound to Cet1 (6). The carboxy-terminal region (amino acids [aa] 265 to 549) of Cet1 is sufficient for catalytic activity, while its middle part (aa 235 to 265) binds and increases the activity of Ceg1 bound to CTD-P (6, 48). The mammalian GTase domain interacts with CTD-P, whereas the RTPase domain does not (26, 54). In contrast to the S. cerevisiae GTase inhibition (6), the mouse GTase activity is stimulated by binding to CTD-P (19).

In the present study, we characterize and compare the GTases and RTPases from the fungi Schizosaccharomyces pombe and Candida albicans using both biochemical and genetic approaches. An S. pombe homolog of CEG1 (pct1) and C. albicans homologs of CEG1 (CGT1) and CET1 (CaCET1) have been isolated and function in S. cerevisiae (43, 52, 53). More recently, an S. pombe RTPase gene (pct1) was isolated and characterized (35). pct1 resembles the catalytic region of Cet1 and CaCet1 but lacks the conserved region for binding the GTase (6, 39, 48). Deletion of the pct1 gene in S. pombe is lethal. pct1 supports the cell viability of an S. cerevisiae Δceg1
Δcet1 strain when coexpressed with either pce1 or Cgt1 but not with Ceg1. Therefore, some species-specific interactions between capping enzyme subunits must exist. Unlike the S. cerevisiae and C. albicans GTases and RTPases, no tight association between pce1 and pce1 was observed. pce1 binds to CTD-P independently of pce1, and pce1 does not require allosteric activation by RTPase. C. albicans GTase and RTPase do interact, but unlike S. cerevisiae capping enzyme, the subunit interaction is not absolutely required for their functions in vivo. This study reveals an unexpected diversity among the fungal mRNA capping systems.

MATERIALS AND METHODS
DNA cloning. All cloning was carried out using standard techniques (2). PCR was carried out with Vent DNA polymerase (New England Biolabs). Oligonucleotides used in this study are listed in the Appendix (see Table A1). Also listed in the Appendix are plasmids used for subcloning and recombinant protein expression (see Table A2) and for expression of proteins in yeast (see Table A3).

Genetic manipulations of S. cerevisiae and S. pombe. S. cerevisiae and S. pombe strains used in this study were YSB244 (MATa ura3-52 leu2-3,112 his3Δ200 ceg1Δ::HIS3 [pRS316-CEG1] [11]), YSB280 (MATa ura3-52 leu2-3,112 his3Δ200 ceg1Δ::HIS3 [pRS316-Ceg1] [12]). YSB53 (MATa ura3-52 leu2Δ1 trplΔ63 his3Δ200 lys2Δ202 cet1Δ::TRP1 [pRS316-CEG1] [48]). YSB71 (MATa ura3-52 leu2Δ1 trplΔ63 his3Δ200 lys2Δ202 cet1Δ::TRP1 [pRS316-CGT1] [48]). YSB719 (MATa ura3-52 leu2Δ1 trplΔ63 his3Δ200 lys2Δ202 cet1Δ::TRP1 [pRS316-CGT1-CEG1] [48]). YSB101 (his4-1Δ::ura3-lacZ leu1-32 ade6-M210 his7-366), FW1122 (his4-1Δ::ura3-lacZ leu1-32 ade6-M216 his7-366), and TE696 (his4-1Δ::ura3-294 leu1-32 [T. Enoch, Harvard Medical School]).

Plasmids were introduced into yeast using a modified lithium acetate transformation protocol (14). Medium preparation, the plasmid-shuffling technique (14) and restriction analysis. One clone was processed for subcloning of a 1.56-kb amplification fragment was subcloned into pCR-Blunt II-TOPO (pCR-Blunt II-TOPO) (lane 2); pDB20-pce1 (pce1−), which expresses pce1 under the control of the ADH1 promoter); and CGT1 (pRLS-CGT1, which expresses Cgt1 from its own promoter). Leu4 His+ transformants were grown in the presence of 5-FOA to shuffle out the CET1 and CEG1 genes carried on pRS316-CEG1-CEG1 (48).

The experiment shown in Fig. 3D was performed as follows. YSB19 (Δcet1Δ ceg1Δ) was transformed with pLEU2 plasmids carrying various GTases: Ceg1 (pRS425-CGE1); MCE (211-597) (pSDS-MCE[211-597]) [48]; pce1− (pDB20-pce1−), which expresses pce1 under the control of the ADH1 promoter); and CGT1 (pRLS-CGT1, which expresses Cgt1 from its own promoter). Leu4 His+ transformants were transfected for growth in the presence of 5-FOA to shuffle out the CET1 and CEG1 genes carried on pRS316-CEG1-CEG1 (48).

Isolation of guanylytransferase genes from S. pombe and C. albicans. YSB280, which carries the ceg1-63 conditional allele (12), was transformed with an S. pombe cDNA library cloned into plasmid pDB20 for S. cerevisiae expression (4, 9). Approximately 200,000 Ura+ transformants were screened for restoration of growth at the restrictive temperature. Five transformants were selected after 2 or 3 days at 37°C. As all five positive clones displayed identical restriction patterns, one representative clone was sequenced and designated pDB20-pce1−. The C. albicans CEG1 homolog was isolated similarly, screening 63,000 Ura+ transformants of YSB280 (cet1-63) for rescue at the restrictive temperature. A C. albicans genomic DNA library was used (30). Six Ura+ transformants were selected and rescreened. All six proved to be independent but overlapping genomic isolates by restriction analysis. One clone was processed for subcloning and sequencing and was designated pRS-CGT1. We note that other groups have also shown that a similar approach to clone pce1− (43) and CGT1 (53).

The open reading frame (ORF) of CGT1 was amplified from a C. albicans genomic DNA library (30) with oligonucleotide primers CGT1-5′-orf and CGT1-3′-orf. A 1.4-kb amplified fragment was subcloned into pCR-Blunt II-TOPO (Invitrogen) (pCR-CGT1orf).
Binding experiment with CTD peptides. Peptides with four repeats of the CTD heptapeptide consensus sequence (YSPSPS) were synthesized at the Biopolymers Facility in the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School. Peptides with either serine or phosphoserine at position 5 were created. Each peptide was biotinylated at the N terminus to allow binding to Streptavidin-coated magnetic beads (Dynabeads M280 Streptavidin; Dynal, Inc.). Binding to beads was performed in phosphate-buffered saline buffer plus 0.01% Triton X-100, and conjugated beads were washed several times with the same buffer to remove free peptide.

For each binding reaction, 250 µg of peptide-linked Dynabeads was incubated with 6 or 7 pmol each of polyhistidine-tagged GTase and/or RTPase protein for 1 h at room temperature in binding buffer (20 mM HEPES-KOH at pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 10% [vol/vol] glycerol, 100 mM potassium acetate, 0.1% [vol/vol] Triton X-100, 0.02% [vol/vol] NP-40, and 0.1% bovine serum albumin). After several washes with the same buffer, [γ-32P]GTP and enzyme-GMP reaction buffer (5) were added to guanylate GTase. After incubation for 50 min at room temperature, the reaction was stopped by addition of sample loading buffer. Bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting for 50 min at room temperature, the reaction was stopped by addition of sample loading buffer. Bound proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using anti-His, monoclonal antibody (Clontech) and autoradiography to detect radiolabeled GTase-GMP intermediate (E-GMP).

### RESULTS

**S. pombe pct1** is an essential gene that encodes a capping enzyme RNA triphosphatase. We searched the S. pombe genome database (Sanger Center, Cambridge, United Kingdom) for proteins with significant similarity to the yeast capping enzyme RNA triphosphatase (49). A BLAST search found a gene fragment on chromosome I that encodes a hypothetical enzyme RNA triphosphatase. We searched the database for proteins with significant similarity to the yeast capping enzyme RNA triphosphatase (49). A BLAST search found a gene fragment on chromosome I that encodes a hypothetical enzyme RNA triphosphatase (49). A BLAST search found a gene fragment on chromosome I that encodes a hypothetical enzyme RNA triphosphatase.

We tested for an interaction between Pce1 and Pct1, the two S. pombe capping enzyme subunits. First, we coexpressed his-7-pct1 and untagged pce1 in E. coli and purified them from the soluble fraction with Ni²⁺-NTA-agarose. This histidine-tagged pct1 bound to the agarose, but pce1 was found only in the flowthrough fraction (data not shown). Therefore, we conclude that pct1 encodes a functional cap RTPase but is unable to functionally interact with S. cerevisiae Ceg1.

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review, see references 18, 40, and 42). Unlike the mammalian GTase domain (19), Ceg1 bound to CTD-P is inhibited for covalent enzyme-GMP complex formation unless Cet1 is also present (6). pce1 has been shown to bind CTD-P, but its activity was not tested in that context (31). As there was no observable interaction between pce1 and pct1 (Fig. 1B), two questions were raised. First, how is pct1 recruited to the pol II transcription complex? Second, does pce1 resemble Ceg1 in being inhibited by binding to the CTD?

The CTD is composed of a tandemly repeated heptad with
the consensus sequence YSPTSPS (7). The heptapeptide consensus repeat is phosphorylated at several positions in vivo, predominantly at serines 2 and 5 (32). Genetic and in vivo cross-linking experiments showed that phosphorylation of serine 5 is critical for the recruitment of *S. cerevisiae* capping enzyme to the pol II complex (28, 36, 38). Accordingly, synthetic CTD peptides with four tandem repeats of heptapeptide were prepared, one unphosphorylated and one in which all serine 5 positions are phosphorylated. These were conjugated to beads and used for in vitro binding experiments with the fungal GTase and RTPases (Fig. 2). Bound proteins were assayed both by immunoblotting and by enzyme-GMP intermediate formation.

First, we tested Ceg1 and/or Cet1 (Fig. 2, lanes 1 to 6). As previously demonstrated using glutathione transferase-CTD fusions (6), Ceg1 preferentially interacts with the CTD-P peptide, either alone or complexed with Cet1 (Fig. 2, lanes 4 and 6). Cet1 associated with CTD-P only via its interaction with Ceg1 (Fig. 2, compare lanes 2 and 6). Ceg1 on the CTD-P could only be labeled with [γ-32P]GTP in the presence of Cet1 (Fig. 2, lower panel, lanes 4 and 6). These results confirmed our earlier finding that Cet1 positively regulates the GTase activity of Ceg1 bound to CTD-P (6).

Next, we carried out similar experiments with the GTases and RTPases from *C. albicans* and *S. pombe*. Like Ceg1, GTases from these two fungi (Cgt1 and pce1) specifically bind to CTD-P, whether or not the RTPase is present (Fig. 2, middle panel, lanes 9 to 12 and 15 to 18). In surprising contrast to Cet1 (lane 2), both RTPases (CaCet1 and pct1) can also bind directly and specifically to CTD-P, independently of the GTase subunits (Fig. 2, upper panel, lanes 8 and 14). Other species-specific differences were noted in the assay for enzyme-GMP formation (Fig. 2, lower panel). The *C. albicans* capping enzyme subunits behaved like those of *S. cerevisiae* in that Cgt1 required the presence of CaCet1 to remain active when bound to CTD-P (Fig. 2, lower panel, lanes 10 and 12). In contrast, *S. pombe* pct1 was efficiently guanylated even in the absence of pct1 (compare lanes 16 and 18 with lanes 4, 6, 10, and 12).

The interaction between capping enzyme subunits in *S. cerevisiae* is thought to provide two functions: delivery of the RTPase to the polymerase and preservation of GTase activity on the CTD-P. We find that neither of these activities is required in the *S. pombe* system, supporting our proposal that these two capping activities can function in vivo without any detectable interaction (Fig. 1). The *Candida* system appears to be intermediate between the other yeast systems. The RTPase can independently interact with the CTD-P, but interaction between subunits stimulates GTase activity.

The interaction between Cgt1 and CaCet1 may not be absolutely required in vivo. Both two-hybrid assays (52) and immunoprecipitation experiments (Fig. 1B, lane 1) demonstrate interactions between the RTPases and GTases from *S. cerevisiae* and *C. albicans*. The GTase interaction region of Cet1 has been localized to residues 235 to 265 (22, 48). CaCet1 closely resembles Cet1 in this region (Fig. 3A), including four residues (P245, W247, W251, and P253) known to be important for Cet1-Ceg1 association (22, 48). This region is essential for CaCet1 to support cell viability in a Δcet1 strain, i.e., when GTase activity is supplied by Ceg1 (39). Considering this and the results from Fig. 2, it would be predicted that the *C. albicans* capping enzyme would require the subunit interaction to support viability, just as the *S. cerevisiae* enzyme does. However, we found that the *S. pombe* RTPase, which does not have the conserved domain for GTase interaction, rescued a Δceg1 Δcet1 strain when combined with the *C. albicans* GTase Cgt1 (Fig. 1A). As it is extremely unlikely that pct1 interacts with Cgt1, this result suggests that Cgt1 activity may not be dependent on an interaction with an RTPase in vivo.

To address whether the Cgt1-CaCet1 interaction is essential in vivo, we tested four N-terminal deletion mutants of CaCet1 for the ability to support viability of a Δceg1 Δcet1 strain when combined with either Ceg1 or Cgt1 (Fig. 3B). CaCet1 (203-520) carries the GTase interaction region and rescued cells with both GTases. CaCet1 (229-520) and CaCet1 (251-520) lack the interaction region and could not support viability in the presence of Ceg1. However, both supported cell growth
FIG. 3. The interaction between Cgt1 and CaCet1 is not absolutely required for their function in vivo. (A) Sequence alignment between CaCet1 (GenBank accession number O93813, residues 193 to 292) and Cet1 (O13297, residues 232 to 310). Protein sequence similarity searching was carried out on the National Center for Biotechnology Information Web server using the BLAST algorithm (1), and sequence alignments were made using SEQVU. Letters represent the single-letter amino acid code, and numbers represent the positions of the amino acid residues. Boxed residues denote identities, and shaded residues indicate similar amino acids. Asterisks indicate the residues that are important for the Cet1-Ceg1 interaction (48). The residues used for the deletion of Cet1 and CaCet1 are shown. (B) Deletion analysis of CaCet1. The indicated deletions of CaCet1 were tested for the ability to replace Cet1 (see Materials and Methods) in the presence of Ceg1 or Cgt1. Plates are shown after 3 days at 30°C. (C) Immunoprecipitation and GTase-GMP formation assay for CaCet1 derivatives and Cgt1, respectively (see Materials and Methods). Upper panel, immunoblotting with 12CA5; and lower panel, autoradiography. (D) Coexpression of Cet1 mutants and various GTases in S. cerevisiae. Plasmid shuffling with the indicated genes was carried out as described in Materials and Methods. After 2 days, 5-FOA-resistant cells were spotted on new plates and were further incubated either at 30 or 37°C. + indicates that the cells form colonies after 3 days. − indicates that colonies were not observed after 7 days. Note that the mouse capping enzyme results are from Takase et al. (48).
with Cgt1. CaCet1 (269-520) creates a deletion that impinges upon the catalytic domain: this deletion was not viable with either GTase and did not produce a stable protein (data not shown). Therefore, C. albicans Cgt1 can function without RTPase sequences that are essential for interaction with the S. cerevisiae Ceg1.

To be sure that the Candida capping enzyme subunits were not interacting via sequences outside of the known interaction domain, we tested for interactions in yeast lysates. The CaCet1 derivatives were HA epitope tagged, so we tested for coimmunoprecipitation of Cgt1 using the 12CA5 monoclonal antibody. Precipitates were tested for Cgt1 guanylylation by adding \([\alpha-32P]GTP\) to the pellets (Fig. 3C). Levels of CaCet1 and its derivatives were comparable in immunoprecipitates (Fig. 3C, upper panel). In contrast, Cgt1 was coprecipitated only with full-length CaCet1 and CaCet1 (203-520). No Cgt1 was detected in association with CaCet1 (229-520) and CaCet1 (251-520) (Fig. 3C, lower panel). These results suggest that residues 203 to 229 of CaCet1, equivalent to the Ceg1 interaction region of Cet1 (aa 235 to 265), are essential for its association with Cgt1. However, unlike the situation for the S. cerevisiae enzyme, the subunit interaction of the C. albicans enzyme is not absolutely required for cell viability.

Previously, we demonstrated that the Cet1-Ceg1 interaction becomes dispensable if Ceg1 is replaced with MCE (211-597). A complete deletion or a double point mutation in the interaction region of Cet1 (Cet1 [265-549] or cet1-446) is lethal in combination with Ceg1 but supports growth when coexpressed with MCE (211-597) (48). In contrast, two other temperature-sensitive alleles mutated in the catalytic region of Cet1 (cet1-401 [D422A] and cet1-438 [C330W]) become lethal if Ceg1 is replaced with MCE (211-597) supplied GTase activity. This is presumably because MCE (211-597) does not bind Cet1 and therefore cannot stabilize these mutants (48). We tested whether Cgt1 and pce1 behave like MCE (211-597) or Ceg1 (Fig. 3D). Both fungal GTases could support growth of cells with Cet1 (265-549) or cet1-446 at both 30 and 37°C. Somewhat surprisingly, Cgt1 could not support viability when combined with either cet1-401 (D442A) or cet1-438 (C330W), suggesting that it might not interact with Cet1 to the same extent as Ceg1. The same results were obtained with pce1, consistent with the lack of interaction between S. pombe capping enzyme subunits. The fact that Cgt1 behaves in vivo like MCE (211-597) and pce1 suggests that Cgt1 functions independently of the interaction with RTPase in vivo.

**DISCUSSION**

In the present study, we demonstrate that different yeasts use divergent strategies for linking transcription and the multiple reactions that make up mRNA capping. The best characterized fungal system is from the budding yeast S. cerevisiae (summarized in Table 1). In this yeast, the interaction between the Ceg1 and Cet1 subunits is essential for viability. Ceg1 carries out guanylylation of the mRNA but also interacts with Cet1 and the phosphorylated CTD, thereby targeting the capping enzyme to the transcription complex. Cet1 plays two crucial roles in capping. Its carboxyl-terminal region (aa 265 to 549) catalyzes the RTPase reaction (37, 49). A second region (aa 235 to 265) binds to Ceg1 (6, 22, 29, 48) to allosterically activate Ceg1 when it is bound to CTD-P (6). The Cet1/Ceg1 interaction can also serve to stabilize Ceg1 activity in vitro in the absence of CTD binding (17), although we have not observed significant lability of Ceg1 under our conditions (E.-J. Cho and S. Buratowski, unpublished data). Exchange of other GTases for Ceg1 show that activation of Ceg1, not delivery of Cet1 to the transcription complex, is the essential function for the Ceg1-Cet1 interaction (48).

In surprising contrast to the S. cerevisiae system, the S. pombe RTPase and GTase function independently of each other. No interaction could be detected either in vitro or in vivo (Fig. 1). To our knowledge, pct1 is the first capping RTPase that is not tightly associated with a GTase, either because it binds directly to the phosphorylated CTD of polymerase (Fig. 2). Also, the GTase pce1 does not require allosteric activation because it is fully active when bound to CTD-P (Fig. 2).

We and others (35) have found that pct1<sup>+</sup> does not complement an S. cerevisiae Δcet1 strain when Ceg1 is the GTase (Fig. 1A). Since pct1 lacks the region for interaction with GTase, one interpretation is that pct1<sup>+</sup> cannot complement the Δcet1 deletion because Ceg1 cannot guide pct1 to the pol II complex (35). Our results instead suggest that pct1 does not complement because it cannot bind and activate Ceg1. pct1 functions in Δcge1 Δcet1 cells when it is fused to MCE (211-597) (35). We find that linkage between these two proteins is unnecessary (Fig. 1A), indicating that pct1 does not require any GTase chaperone to the pol II complex.

The capping machinery from C. albicans appears to be intermediate between that of S. cerevisiae and S. pombe. CaCet1 binds to Cgt1 via an interaction domain that is conserved in S. cerevisiae (Fig. 3A). Nonetheless, deletion analysis of CaCet1 clearly demonstrated that the Cgt1-CaCet1 interaction is non-essential in vivo, at least when these proteins are expressed from high-copy-number plasmids in S. cerevisiae (Fig. 3B and C). Based on similar deletions of CaCet1, it was proposed that CaCet1 contains a second, low-affinity site distal to aa 230 for interaction with Cgt1 (39). We could detect no association of CaCet1 (229-520) or CaCet1 (251-520) with Cgt1 (Fig. 3C). Also, CaCet1 (203-520), CaCet1 (229-520), and CaCet1 (251-520) support viability of a Δcet1 Δcge1 strain when Cgt1 is replaced with pce1 or the mouse GTase domain (data not shown), indicating that those CaCet1 mutants can function in vivo without binding to a GTase.

*Candida* capping enzyme does not seem to require either of

| Organism     | Subunits | CTD-P interaction | Stimulation of GTase by RTPase in vitro | Subunit interaction in vivo |
|--------------|----------|-------------------|----------------------------------------|-----------------------------|
| S. cerevisiae | Ceg1 (GTase) Cet1 (RTPase) | + | + | Yes, essential |
| C. albicans  | Cgt1 (GTase) CaCet1 (RTPase) | + | + | Yes, nonessential |
| S. pombe     | pce1 (GTase) pct1 (RTPase) | + | - | No |

**TABLE 1. Summary of capping enzyme subunit behavior**
the two functions assigned to the RTPase-GTase interaction. Delivery of CaCet1 to the transcription complex is not an issue because CaCet1 can bind specifically to the CTD-P (Fig. 2). In vitro, the GTases from both C. albicans and S. cerevisiae are inhibited upon binding CTD-P, but this inhibition is prevented when the RTPase is also present (6; Fig. 2). In S. cerevisiae, the Cet1-Ceg1 interaction is essential primarily to activate Ceg1 on CTD-P. In higher eukaryotes, similar pressure may have resulted in selection for the subunit interaction seen in the other yeasts. This invites speculation about how capping enzyme arrangements exist in other organisms.

In conclusion, our experiments reveal an unexpected diversity among fungal capping enzymes. Although the basic catalytic domains are highly conserved, the interactions between RTPase and GTase subunits are not. There is no interaction between the S. pombe RTPase and GTase, whereas the interaction between S. cerevisiae subunits is essential for viability. The Candida enzyme has characteristics intermediate to the other two yeasts. This invites speculation about how capping enzymes evolved. A eukaryotic progenitor system may have had two independent enzymes, similar to S. pombe. An evolutionary advantage of coupling the RTPase and GTase may have resulted in selection for the subunit interaction seen in the other yeasts. In higher eukaryotes, similar pressure may have selected for the fusion of a PTP-like phosphatase domain to the GTase domain. It will be interesting to see if any other capping enzyme arrangements exist in other organisms.

### APPENDIX

Table A1 contains a list of oligonucleotides used in this study. Table A2 lists plasmids used for cloning and expression of recombinant proteins in bacteria. Plasmids used for experiments in yeast are listed in Table A3.

#### TABLE A1. Oligonucleotides used in this study

| Name | Oligonucleotide sequence |
|------|--------------------------|
| CET1 (5’ Sal/Nco) | 5’TGCGAC ATGGTACCATCGAACACCTCTCCCTAAACA-3’ (SalI site in small capitals, NcoI site underlined) |
| CET1 (3’ SacI) | 5’TACGAAGGCTTTCTGGATATTT-3’ (SacI site in small capitals) |
| SpCET 1stop | 5’TGCGAC AAGGCTTTCTGGATATTT-3’ (SalI site in small capitals) |
| SpCET 1 (40Met) | 5’TGCGAC AAGGCTTTCTGGATATTT-3’ (SalI site in small capitals, BamHI site boldfaced, NcoI site underlined) |
| CET1-5orf | 5’TGCGAC ATGGTACCATCGAACACCTCTCCCTAAACA-3’ (SalI site in small capitals, BamHI site boldfaced) |
| CET1-3orf | 5’TGCGAC ATGGTACCATCGAACACCTCTCCCTAAACA-3’ (SalI site in small capitals, BamHI site boldfaced) |
| CET1orf | 5’TGCGAC ATGGTACCATCGAACACCTCTCCCTAAACA-3’ (SalI site in small capitals, BamHI site boldfaced) |

**TABLE A2. Plasmids for E. coli used in this study**

| Plasmid | Construction | Reference |
|---------|--------------|-----------|
| pBS-CET1orf (version 2) | 1.7-kb fragment was amplified from pBS-CET1 orf (6) using CET1 (5’ Sal/Nco) and CET1 (3’ SacI). This product was ligated into the Sal site of pCR-Script SK(+) (Stratagene) | This study |
| pCR-CaCET1orf | See Materials and Methods | This study |
| pBS-CaCET1 | 1.56-kb BamHI fragment from pCR-CaCET1orf was subcloned into the BamHI site of pGEM-3zf(+) (Promega) | This study |
| pBS-CaCET1 | 1.56-kb fragment was amplified from pGEM-CaCET1 using oligonucleotides CaCET1-5’ SalI and CaCET1-3’ SacI. | This study |
| pBS-CaCET1 (203-520) | 0.96-kb fragment was amplified from pGEM-CaCET1 using oligonucleotides CaCET1 (203) and CaCET1-3’ SacI. This product was ligated into the Sal site of pCR-Script SK(+) | This study |
| pBS-CaCET1 (229-520) | 0.86-kb fragment was amplified from pGEM-CaCET1 using oligonucleotides CaCET1 (229) and CaCET1-3’ SacI. This product was ligated into the Sal site of pCR-Script SK(+) | This study |
| pBS-CaCET1 (251-520) | 0.81-kb fragment was amplified from pGEM-CaCET1 using oligonucleotides CaCET1 (251) and CaCET1-3’ SacI. This product was ligated into the Sal site of pCR-Script SK(+) | This study |
| pBS-CaCET1 (269-520) | 0.76-kb fragment was amplified from pGEM-CaCET1 using oligonucleotides CaCET1 (269) and CaCET1-3’ SacI. This product was ligated into the Sal site of pCR-Script SK(+) | This study |
| pCR-pctl1 | See Materials and Methods | This study |
| pCR-pctl1 (43-303) | See Materials and Methods | This study |
| pSBEThis-pctl1 | 0.94-kb Ncol-BamHI fragment of pCR-pctl1 was subcloned into Ncol-BamHI sites of pSBEThis, (37) | This study |
| pSBEThis-pctl1 (43-303) | 0.84-kb Ncol-BamHI fragment of pCR-pctl1 was subcloned into Ncol-BamHI sites of pSBEThis, | This study |
| pCR-CGT1orf | See Materials and Methods | This study |
| pSBEThis-CGT1 | 1.4-kb Ncol-BamHI fragment from pCR-CGT1orf was subcloned into the Ncol-BamHI sites of pSBEThis, | This study |
| pSBEThis-pctl1 | 1.4-kb Ncol-Nhel (blunted) fragment from pET-PCE1 (43) was subcloned into the Ncol-Nhel sites of pSBEThis, | This study |
| pBS-MCE (211-597) | 1.2-kb fragment was amplified from p-MCE (54) using MCE-GT/NotI and MCE-B. This fragment was ligated into the Sal site of pCR-Script SK(+) | This study |
TABLE A3. Plasmids for S. cerevisiae and S. pombe used in this study

| Plasmid            | Relevant features                      | Construction                                                                 | Source or reference |
|--------------------|----------------------------------------|-----------------------------------------------------------------------------|---------------------|
| pAD5-CET1          | 2μm, LEU2, ADH1 promoter-driven        | 1.7-kb Sau3-Sal1 fragment from pBS-CET1orf version 2 was subcloned into the Sau3 and Sal1 sites of pAD5-pct1R | This study          |
| pRS131-cet1-401    | CEN/ARS, HIS3, cet1-401 (DM224)         | See reference                                                               | 48                  |
| pRS131-cet1-438    | CEN/ARS, HIS3, cet1-438 (C350W)         | See reference                                                               | 48                  |
| pRS423-CET1        | CEN/ARS, HIS3, CET1 (265-549)           | See reference                                                               | 48                  |
| pAD5-CaCET1 (203-520) | 2μm, LEU2, HA-tagged CaCET1, ADH1 promoter-driven | 1.6-kb Sal1-Sal1 fragment from pBS-CaCET1 was subcloned into the Sal1 and Sac1 sites of pAD5-CET1 | This study          |
| pAD5-CaCET1 (229-520) | 2μm, LEU2, HA-tagged CaCET1 (229-520), ADH1 promoter-driven | 1.6-kb Ncol-Sac1 fragment from pBS-CaCET1 (203-520) was subcloned into the Nco1 and Sac1 sites of pAD5-CET1 (1-265)CT1 | This study          |
| pAD5-CaCET1 (251-520) | 2μm, LEU2, HA-tagged CaCET1 (251-520), ADH1 promoter-driven | 0.9-kb Ncol-Sac1 fragment from pBS-CaCET1 (229-520) was subcloned into the Nco1 and Sac1 sites of pAD5-CET1 (1-265)CT1 | This study          |
| pAD5-CaCET1 (269-520) | 2μm, LEU2, HA-tagged CaCET1 (269-520), ADH1 promoter-driven | 0.8-kb Ncol-Sac1 fragment from pBS-CaCET1 (251-520) was subcloned into the Nco1 and Sac1 sites of pAD5-CET1 (1-265)CT1 | This study          |
| pAD5-pct1R         | 2μm, LEU2, HA-tagged pct1R*, ADH1 promoter-driven | 0.9-kb Sal1-Sal1 fragment from pCR-pct1R was subcloned into the Sal1 and Sac1 sites of pAD5 (48) | This study          |
| pAD5HF-pct1        | 2μm, leu2::HIS3::KanR*, HA-tagged pct1R*, ADH1 promoter-driven | LEU2 marker of pAD5-pct1R was swapped with HpaI-XhoI fragment from pLH7 (6a) carrying leu2::HIS3::KanR | This study          |
| pRS423-pct1        | 2μm, HIS3, pct1R*, CET1 promoter-driven | 0.9-kb Ncol-Sac1 fragment from pRS423-CET1 (Pro + 265-549) was replaced with 0.9-kb Ncol-Sac1 fragment from pSBETHis,Per1 (43-303) | This study          |
| pRS423-pct1R       | 2μm, HIS3, pct1R* (43-303), CET1 promoter-driven | 0.9-kb Ncol-Sac1 fragment from pRS423-CET1 (Pro + 265-549) was replaced with 0.8-kb Ncol-Sac1 fragment from pSBETHis,Per1 (43-303) | This study          |
| pSLF273-pct1       | ars1*, ura4*, (HA)-tagged pct1*, weak nmt1 promoter-driven | 0.9-kb BamHI fragment from pCR-pct1* was subcloned into the BamHI site of pSLF273 (10) | This study          |
| pSGP73-pct1        | ars1*, LEU2, (HA)-tagged pct1*, nmt1 promoter-driven | 0.9-kb BamHI fragment from pCR-pct1* was subcloned into the BamHI site of pSGP73 (kindly supplied by S. Forsburg) | This study          |
| pRS425-CEG1        | 2μm, LEU2, CEG1                        | See reference                                                               | 48                  |
| pRS425-CEG1        | 2μm, leu3::HIS3::KanR*, CEG1           | URA3 marker of pRS426-CEG1 (48) was swapped with Smal fragment from pUH7 (6a) carrying leu3::HIS3::KanR | This study          |
| pRS-CGT1           | 2μm, URA3, CGT1                        | See Materials and Methods                                                   | This study          |
| pRS-CGT1           | 2μm, his3::HIS3::KanR*, CEG1           | URA3 marker of pRS-CGT1 was swapped with Smal fragment from pUH7 (6a) carrying his3::HIS3::KanR | This study          |
| pRS-CGT1           | 2μm, leu::URA2::KanR*, CGT1            | URA3 marker of pRS-CGT1 was swapped with Smal fragment from pUH9 (6a) carrying ura3::URA2::KanR | This study          |
| pDB200-pce1        | 2μm, URA3, pce1*, ADH1 promoter-driven  | See Materials and Methods                                                   | This study          |
| pDB200-pce1R       | 2μm, LEU2, pce1* (43-303), ADH1 promoter-driven | 3.4-kb BamHI fragment of pDB200LDA1 (26a) was replaced with 3.74-kb BamHI fragment of pDB20-pce1R | This study          |
| pDB200-pce1        | 2μm, leu3::HIS3::KanR*, pce1*, ADH1 promoter-driven | URA3 marker of pDB20-pce1* was swapped with Smal fragment from pUH7 carrying ura3::HIS3::KanR | This study          |
| pDB200-MCE (211-597) | 2μm, URA3, MCE (211-597), ADH1 promoter-driven | 1.2-kb EcoRV-NolI (blunted) fragment from pBS-MCE (211-597) was subcloned into the NolI (blunted) site of pDB20 (4) | This study          |
| pDB200-MCE (211-597) | 2μm, HIS3, MCE (211-597), ADH1 promoter-driven | 1.2-kb EcoRV-NolI (blunted) fragment from pBS-MCE (211-597) was subcloned into the NolI (blunted) site of pDB20 (4) | This study          |
| pAD5-MCE (211-597) | 2μm, LEU2, HA-tagged MCE (211-597), ADH1 promoter-driven | Smal fragment from pUH7 carrying ura3::HIS3::KanR | See reference       |

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