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# 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 $GpppN_1$-. 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, $m^7GpppN_1$-. (13, 42).

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, 31) but is inactive for covalent enzyme-GMP complex formation unless also bound to Cet1 (6). The carboxyl-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* (*pce1*$^{+}$) 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* Δ*ceg1*

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Δ*cet1* 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 pct1 and pce1 was observed. pct1 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 ceg1Δ1::HIS3* [pRS316-CEG1] [11]), YSB230 (*MATa ura3-52 leu2-3,112 his3Δ200 ceg1Δ1::HIS3* [pRS315-ceg1-63] [12]), YSB533 (*MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202 cet1Δ1::TRP1* [pRS316-CET1] [48]), YSB719 (*MATα ura3-52 leu2Δ1 trp1Δ63 his3Δ200 lys2Δ202 cet1Δ1::TRP1 ceg1Δ3::LYS2* [pRS316-CEG1-CET1] [48]), FWP101 (*h⁺ ura4::fbp1-lacZ leu1-32 ade6-M210 his7-366*), FWP112 (*h⁻ ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366*), and TE696 (*h⁺ ura4-294 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 with 5-fluoroorotic acid (5-FOA), and other yeast manipulations were performed by standard methods (2, 15).

The experiment shown in Fig. 1A was carried out as follows. The *CET1*/*CEG1* double shuffling strain YSB719 (48) was transformed with pRS423-pct1⁺ (2μm, *HIS3*, expressing pct1 protein from the *CET1* promoter). His⁺ isolates were subsequently transformed with the following *LEU2*/2μm plasmids: vector (pRS425 [44]); *CEG1* (pRS425-CEG1 [48]); *MCE* (211-597) (pAD5-MCE [211-597] [48]); *pce1⁺* (pDB20L-pce1⁺, which expresses pce1 under the control of the *ADH1* promoter); and *CGT1* (pRSL-CGT1, which expresses Cgt1 from its own promoter). Leu⁺ His⁺ transformants were tested for growth in the presence of 5-FOA to shuffle out the *CEG1* and *CET1* genes carried on pRS316-CEG1-CET1 (48).

The experiment shown in Fig. 3D was performed as follows. YSB719 (Δ*ceg1* Δ*cet1*) was transformed with *LEU2* plasmids expressing various GTases: Ceg1 (pRS425-CEG1); MCE (211-597) (pAD5-MCE[211-597]); pce1 (PDB20L-pce1⁺); and Cgt1 (pRSL-CGT1). Leu⁺ isolates were subsequently transformed with *HIS3* plasmids that carry different alleles of *CET1* (48): *cet1* (265-549) (*pRS423-CET1[Pro + 265-549]*); *cet1-446* (pRS313-cet1-446 [P245A, W247A]); *cet1-401* (pRS313-cet1-401 [D422A]); or *cet1-438* (pRS313-cet1-438 [C330W]). Leu⁺ His⁺ transformants were grown in the presence of 5-FOA to shuffle out pRS316-CEG1-CET1.

**Isolation of guanylyltransferase genes from *S. pombe* and *C. albicans*.** YSB230, 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 YSB230 (*ceg1-63*) 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 used 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).

**Isolation of the *S. pombe* RNA triphosphatase gene.** A 0.9-kb fragment carrying the ORF of the *pct1⁺* gene (GenBank accession number AL355012) was amplified from an *S. pombe* cDNA library (9) using oligonucleotide primers SpCET1start and SpCET1stop. For the amplification of a 0.8-kb fragment carrying the ORF that lacks the residues 1 to 42, SpCET1 (40Met) replaced SpCET1start. The 0.9- and 0.8-kb PCR products were subcloned into pCR-Blunt II-TOPO to generate pCR-pct1 and pCR-pct1 (43-303), respectively.

**Disruption of the *pct1⁺* gene.** The *S. pombe pct1* ORF was disrupted using a PCR-based approach as described by Bahler et al. (3). Oligonucleotides KO1 and KO2 (see Table A1 in Appendix) were used to PCR amplify a *kanMX6*-containing fragment from pFA6a-3HA-kanMX6. The amplified fragment was used to transform a diploid *S. pombe* strain (derived from mating FWP101 and FWP112) to G418 resistance, thus replacing one wild-type allele of *prt1* with a *kanMX6*-marked disruption allele. Homologous recombination at the *prt1* locus was confirmed by both PCR analysis and Southern blotting. Azygotic asci were dissected on yeast extract agar (YEA) medium to determine the phenotype of the disruption in haploid progeny.

**Isolation of the RNA triphosphatase gene of *C. albicans*.** The *CaCET1* ORF was amplified from a *C. albicans* genomic DNA library (30) with oligonucleotides CaCET1-5′orf and CaCET1-3′orf derived from the published sequence (52). A 1.56-kb amplified fragment was subcloned into pCR-Blunt II-TOPO (pCR-CaCET1orf).

**Preparation of recombinant protein expressed in *Escherichia coli*.** Polyhistidine-tagged full-length pct1 (his$_7$-pct1) and the residues 43 to 303 of pct1 (his$_7$-pct1 [43-303]) were expressed in *E. coli* strain BL21(DE3) transformed with pSBEThis$_7$-pct1 and pSBEThis$_7$-pct1 (43-303), respectively. Proteins were purified from soluble extracts (100,000 × *g*, supernatant fraction) through $Ni^{2+}$-nitrilotriacetic acid (NTA)-agarose (Qiagen) and CM-Sephadex C-50 (Pharmacia) resins as described previously for the purification of his$_7$-Cet1 (265-549) (37).

Plasmids pSBEThis$_7$-pce1, pSBEThis$_7$-Cgt1, and pSBEThis$_7$-CaCet1 were used to express polyhistidine-tagged pce1, Cgt1, and CaCet1, respectively, in BL21(DE3). his$_7$-pce1, his$_7$-Cgt1, and his$_7$-CaCet1 were purified from soluble extracts by $Ni^{2+}$-NTA-agarose (37). Polyhistidine-tagged GTase and RTPase subunits of *S. cerevisiae* (his$_7$-Ceg1 and his$_7$-Cet1) were expressed and purified as previously described (6, 11).

**RTPase and NTPase assay.** RTPase assays were carried out with [γ-$^{32}$P]ATP-terminated dimer RNA (pp**p**ApC; boldface denotes radioisotope) or [α-$^{32}$P] ATP-terminated trimer RNA (pp**p**ApCpC) prepared with T7 bacteriophage DNA primase as described earlier (47). Nucleotide phosphohydrolase (NTPase) activity was assayed with [γ-$^{32}$P]ATP (NEN/DuPont).

**Yeast whole-cell extract preparation and protein analysis.** Preparation of whole-cell extracts from *S. cerevisiae* and *S. pombe*, immunoprecipitation, and subsequent enzyme-GMP formation assays were carried out as described earlier (48).

The experiment shown in Fig. 1B was performed as follows. For lane 1, pAD5-CET1 (2μm, *LEU2*, of *ADH1* promoter driving HA-tagged Cet1) and pRSH-CGT1 (2μm, *HIS3*, *CGT1*) were shuffled into YSB719. For lane 2, pAD5H-pct1⁺ (2μm, *HIS3*, expressing HA-tagged Pct1 protein from the *ADH1* promoter) and pDB20L-pce1⁺ were shuffled into YSB719. For experiments with *S. pombe*, strain TE696 was transformed with pSLF273 (*ura4⁺* [10]) (lane 3), pSLF273-pct1⁺ (*ura4⁺*, *nmt1* promoter expressing triple-HA-tagged pct1 protein) (lane 4); pSGP73 (*LEU2*) (lane 5); and pSGP73-pct1⁺ (*LEU2*, *nmt1* promoter expressing triple-HA-tagged pct1 protein) (lane 6). Transformants were grown in Edinburgh minimal medium with leucine (lanes 3 and 4) or uracil (lanes 5 and 6) at 30°C, and whole-cell extracts were prepared. Immunoprecipitations were carried out with 20 μg of whole-cell extract protein and monoclonal antibody 12CA5 bound to protein A-Sepharose beads. Precipitates were incubated for 10 min at 30°C with 3 μM [α-$^{32}$P]GTP and were then analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and analyzed by immunoblotting with 12CA5 (upper panel) and PhosphorImager (lower panel).

The results shown in Fig. 3B and C were obtained as follows. YSB719 was transformed with *HIS3*/2μm plasmids expressing Ceg1 (pRSH-CEG1) or Cgt1 (pRSH-CGT1). His⁺ isolates were subsequently transformed with *LEU2*/2μm plasmids expressing wild-type or the indicated truncation mutants of *CaCET1*. CaCet1 and its derivatives were HA tagged and expressed from the *ADH1* promoter. Leu⁺ His⁺ transformants were analyzed for the ability to replace Ceg1 and Cet1 by plasmid shuffling.

Whole-cell extracts were prepared from cells expressing Cgt1 and the derivatives of CaCet1 (see Fig. 3B, lane 2, full-length CaCet1; lane 3, 203-520; lane 4, 229-520; and lane 5, 251-520). Lane 1 (vector) represents cells before plasmid shuffling, while lanes 2 to 5 show extracts from cells in which pRS316-CEG1-CET1 was shuffled out using 5-FOA. Immunoprecipitation and subsequent guanylylation assays were carried out as described for Fig. 1.

**Binding experiment with CTD peptides.** Peptides with four repeats of the CTD heptapeptide consensus sequence (YSPTSPS) 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, [α-$^{32}$P]GTP and enzyme-GMP reaction buffer (5) were added to guanylylate 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 using anti-$His_6$ 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 protein similar to the carboxyl-terminal region of Cet1 and CaCet1 from *C. albicans* (52). This fragment has two internal stop codons. We amplified a corresponding DNA fragment from *S. pombe* cDNA library and found that those stop codons are removed as part of an intron. The resulting PCR product encodes a 35.4-kDa polypeptide with 303 residues (GenBank accession number CAB90131) containing motifs conserved in fungal and viral cap RTPases (21, 23, 24). These motifs have also been found in Ctl1/Cth1, a second RTPase from *S. cerevisiae* that is not required for capping (33, 37), and an RTPase from *Plasmodium falciparum* (20, 45). While our work was in progress, the identification of the *S. pombe* gene was independently reported and the gene was designated *pct1* (35).

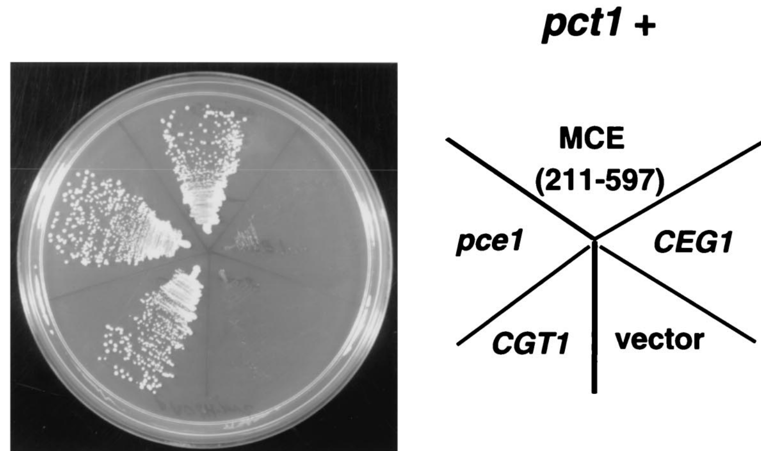
We purified recombinant polyhistidine-tagged protein ($his_7$-pct1) and carried out RTPase assays (data not shown). pct1 released [$^{32}$P]Pi from a [γ-$^{32}$P]ATP-terminated dinucleotide (**p**ppApC) and trinucleotide (**p**ppApCpC). This activity was dependent upon divalent cations, since it was seen in the presence of magnesium or manganese but was inhibited by EDTA. Other fungal RTPases are more active with magnesium than with manganese (24, 34, 37). In contrast, we found that pct1 is more active in the presence of manganese and that manganese has a lower optimal concentration than magnesium (0.2 versus 2 mM, data not shown). We found that pct1 leaves a diphosphate end, which could subsequently act as a substrate for guanylylation. Like Cet1 (24) and CaCet1 (34), pct1 hydrolyzed the β-γ phosphodiester bond of ATP in the presence of manganese but not of magnesium (data not shown). Taken together, these results demonstrate that pct1 is a metal-dependent RTPase/NTPase related to the other fungal RTPases.

**The *pct1* gene is essential for viability.** *S. cerevisiae* contains two RTPases related to pct1: the essential capping enzyme subunit Cet1 and the nonessential Ctl1/Cth1. pct1 could correspond to either of these proteins. To determine whether the *pct1$^+$* gene is essential for viability, one copy of the gene was disrupted in a diploid *S. pombe* strain. Tetrads dissected from this strain never produced more than two viable spores (data not shown). Furthermore, none of the viable spores contained the marker for the *pct1$^+$* deletion. Microscopic examination of the missing colonies showed that spores germinated and that colonies reached the 16-cell stage before ceasing growth. We conclude that pct1 is essential for viability and is therefore likeliest to be the true capping enzyme triphosphatase of *S. pombe*.

**pct1 is a capping enzyme RTPase but does not associate with GTase.** To further test if *pct1$^+$* is involved in mRNA capping, it was expressed in *S. cerevisiae* and tested for complementation of a *CET1* deletion. Unlike *CaCET1* (52), *pct1$^+$* could not support viability in a *CET1* deletion strain (35; data not shown). We speculated that pct1 might not be able to replace Cet1 because it does not interact with and allosterically activate Ceg1 (6). Previously a Δ*ceg1* Δ*cet1* strain was used to show that Cet1 lacking the region for interaction with Ceg1 (aa 235 to 265) supported cell viability in the presence of mouse capping enzyme GTase (MCE [211-597]) but not with Ceg1 (48). We used the double deletion strain to see if pct1 could function in the presence of other GTases (Fig. 1A). Overexpression of Ceg1 and pct1 could not support cell growth. However, when Ceg1 was replaced with either MCE (211-597), the *S. pombe* GTase pce1, or *C. albicans* Cgt1, cells grew as well as the wild-type strains. pct1 (43-303) supported cell growth as well as the full-length protein did (data not shown). Therefore, we conclude that *pct1$^+$* encodes a functional cap RTPase but is unable to functionally interact with *S. cerevisiae* Ceg1.

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 $Ni^{2+}$-NTA-agarose. This histidine-tagged pct1 bound to the agarose, but pce1 was found only in the flowthrough fraction (data not shown). Therefore, the recombinant proteins do not interact. This could be because another protein in yeast is required to mediate an interaction. To test this, we next expressed a hemagglutinin (HA) epitope-tagged pct1 in *S. cerevisiae* and *S. pombe*. We carried out immunoprecipitations of whole-cell extracts using the monoclonal antibody 12CA5 (8, 10), and these were assayed with [α-$^{32}$P]GTP to detect any GTase-GMP complex (Fig. 1B, lower panel). Lane 1 shows a positive control in which HA-tagged Cet1 and Cgt1 (the *C. albicans* GTase) are coexpressed in the *S. cerevisiae* Δ*ceg1* Δ*cet1* strain. Cgt1 can bind *S. cerevisiae* Cet1 in vivo, as previously suggested by yeast two-hybrid assay interactions (52). Under the same conditions, HA-tagged pct1 could not coprecipitate pce1 when coexpressed in *S. cerevisiae* (Fig. 1B, lane 2). To make sure there was not a species-specific mediator of interactions, HA-tagged pct1 was expressed in *S. pombe* (Fig. 1B, lanes 4 and 6). Although HA-tagged pct1 was efficiently precipitated (Fig. 1B, upper panel), no associated GTase could be detected. Based on these results, we come to the surprising conclusion that the *S. pombe* GTases and RTPases are not tightly associated, as they are in *S. cerevisiae* and *C. albicans* (27, 52).

**Independent interactions of pce1 and pct1 with CTD-P.** It is thought that RTPases are guided to the polymerase via the interaction between the GTase and CTD-P (5, 31, 54; for



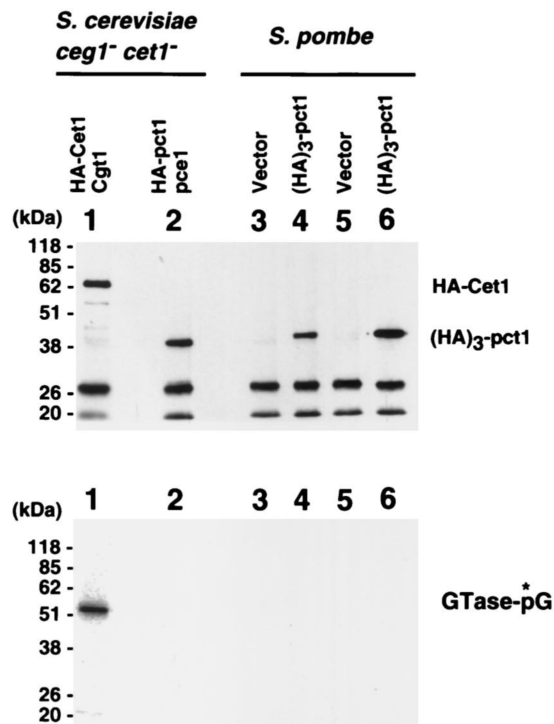




FIG. 1. pct1 is an mRNA-capping RTPase but does not associate with pce1. (A) Complementation by plasmid shuffling. pct1 plus the indicated GTases were tested for the ability to replace Ceg1 and Cet1 as described in Materials and Methods. (B) Immunoprecipitation and GTase-GMP formation assay. Whole-cell extracts were prepared from strains derived from *S. cerevisiae* YSB719 (lanes 1 and 2) or *S. pombe* TE696 strain (lanes 3 to 6) expressing the indicated proteins. RTPases were immunoprecipitated (immunoblot shown in top panel) and pellets were tested for the presence of GTase (bottom panel), as described in Materials and Methods. The asterisk denotes radioactive phosphate.

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

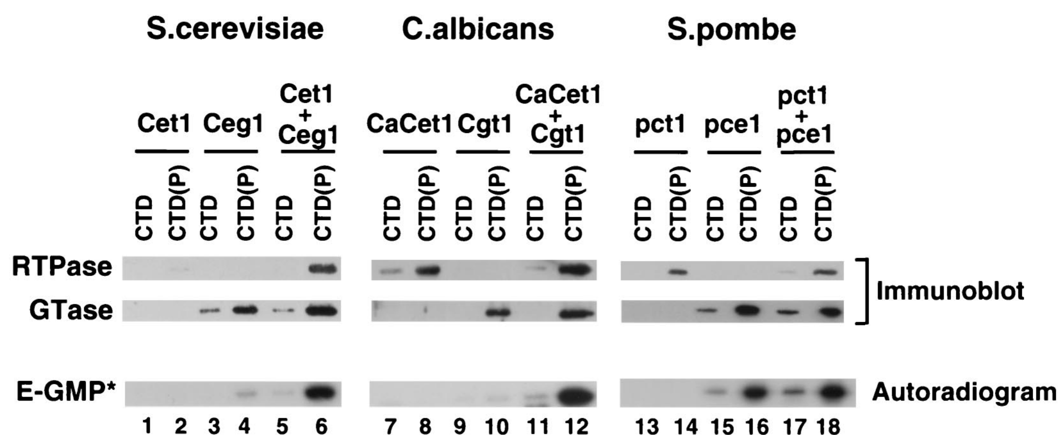



FIG. 2. Different interaction patterns between the CTD and fungal capping enzyme subunits. CTD peptides consisting of four heptapeptide repeats were conjugated to beads. The peptides were either unphosphorylated (CTD) or phosphorylated at all four serine 5 positions (CTD-P). Beads were incubated with recombinant fungal GTases and RTPases as described in Materials and Methods. Proteins bound to peptides were incubated with [$\alpha$-$^{32}$P]GTP to assay guanylyltransferase activity. Bound proteins were analyzed by SDS-PAGE followed by transfer to nitrocellulose membranes. Blots were analyzed by immunoblotting with anti-His$_6$ monoclonal antibody (upper panels) and autoradiography (bottom panel). The asterisk denotes the position of the radioactive phosphate.

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 [$\alpha$-$^{32}$P]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* pce1 was efficiently guanylylated even in the absence of pct1 (compare lanes 16 and 18 with lanes 4, 6, 10, and 12).

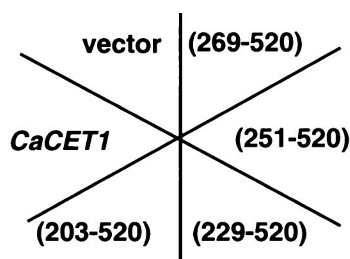
The interaction between capping enzyme subunits in *S. cerevisiae* is thought to provide two functions: delivery of the RTPase to the polymerase and preservation of GTase activity on the CTD-P. We find that neither of these activities is required in the *S. pombe* system, supporting our proposal that 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 Δ*cet1* 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 Δ*ceg1* Δ*cet1* 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.

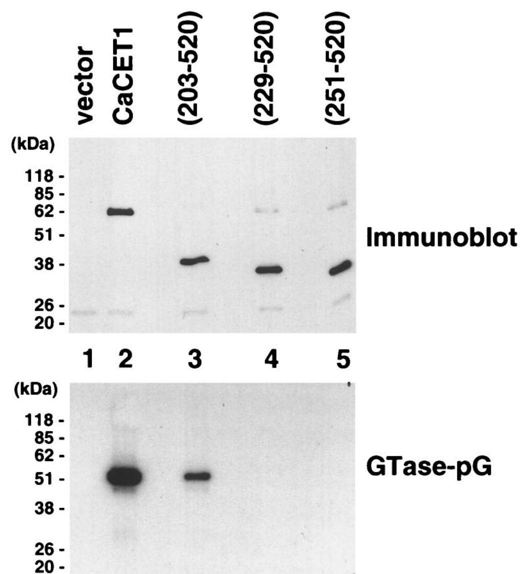
To address whether the Cgt1-CaCet1 interaction is essential in vivo, we tested four N-terminal deletion mutants of CaCet1 for the ability to support viability of a Δ*ceg1* Δ*cet1* strain when combined with either Ceg1 or Cgt1 (Fig. 3B). CaCet1 (203-520) carries the GTase interaction region and rescued 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

A

B



C




D

| | | *CEG1* | | *MCE(211-597)* | | *CGT1* | | *pce1⁺* | |
|---|---|---|---|---|---|---|---|---|---|
| | | 30 °C | 37 °C | 30 °C | 37 °C | 30 °C | 37 °C | 30 °C | 37 °C |
| *cet1(265-549)* | | - | NT | + | + | + | + | + | + |
| *cet1-446* | (P245A W247A) | - | NT | + | + | + | + | + | + |
| *cet1-401* | (D422A) | + | - | - | NT | - | NT | - | NT |
| *cet1-438* | (C330W) | + | - | - | NT | - | NT | - | NT |

NT = not tested.

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 [$\alpha$-$^{32}$P]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 CaCet1and 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 when 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).

TABLE 1. Summary of capping enzyme subunit behavior

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

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 through a covalent linkage or as part of a complex. There is no need for such an interaction to deliver pct1 to the transcript 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*$^+$ does not complement an *S. cerevisiae* Δ*cet1* strain when Ceg1 is the GTase (Fig. 1A). Since pct1 lacks the region for interaction with GTase, one interpretation is that *pct1*$^+$ cannot complement the Δ*cet1* 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 Δ*ceg1* Δ*cet1* 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 nonessential 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 Δ*cet1* Δ*ceg1* 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

TABLE A1. Oligonucleotides used in this study

| Name | Oligonucleotide sequence |
|---|---|
| CET1 (5′*SalNco*) | 5′-GTCGACTGCCATGGGTTACACTGACAACCCTCCTCAAACA-3′ (*Sal*I site in small capitals, *Nco*I site underlined) |
| CET1 (3′ *Sac*l) | 5′-GAGCTCAAGGGCATTTGCTTAT-3′ (*Sac*I site in small capitals) |
| SpCET 1start | 5′-GCGTCGACA**GGAT**CCATGGACCTTAAAGGTTTATTA-3′ (*Sal*I site in small capitals, *Bam*HI site boldfaced, *Nco*I site underlined) |
| SpCET 1stop | 5′-GCGGATCCGAGCTCTTAAGAATGCTCTCGTCTCAA-3′ (*Sac*I site in small capitals, *Bam*HI site underlined) |
| SpCET 1 (40Met) | 5′-GCGTCGACA**GGATCC**ATGGCAAAGATAGAAATGAACTT-3′ (*Sal*I site in small capitals, *Bam*HI site boldfaced, *Nco*I site underlined) |
| CGT1-5′orf | 5′-GC**GGATCC**ATGGTTCAATTAGAAGAAAGAGAAATT-3′ (*Bam*HI site boldfaced, *Nco*I site underlined) |
| CGT1-3′orf | 5′-GC**GGATCC**TTATTCGTCGTCACTATCTTCATAAGT-3′ (*Bam*HI site boldfaced) |
| CaCET1-5′orf | 5′-GC**GGATCC**ATGGATGTTGGATCTATTTTAAATGAC-3′ (*Bam*HI site boldfaced, *Nco*I site underlined) |
| CaCET1-3′orf | 5′-GC**GGATCC**TAGCAAATCTTGTTCAATCTATTATT-3′ (*Bam*HI site boldfaced) |
| CaCET1-5′*Sal*I | 5′-ACGCGTCGACCATGGATGTTGGATCTATTTTAAATGA-3′ (*Sal*I site in small capitals, *Nco*I site underlined) |
| CaCET1 (203) | 5′-ACGCGTCGACCATGGAAACACCTCCAATTTGGGCCCAGA-3′ (*Sal*I site in small capitals, *Nco*I site underlined) |
| CaCET1 (229) | 5′-ACGCGTCGACCATGGAAGCCATAACTAGACTTTCTGAA-3′ (*Sal*I site in small capitals, *Nco*I site underlined) |
| CaCET1 (251) | 5′-ACGCGTCGACCATGGAGTGTAGTATTACTGGTATGATA-3′ (*Sal*I site in small capitals, *Nco*I site underlined) |
| CaCET1 (269) | 5′-ACGCGTCGACCATGGAATGGGTGTATGCCAATTTTTCCA-3′ (*Sal*I site in small capitals, *Nco*I site underlined) |
| CaCET1-3′ *Sac*I | 5′GC**GGATCC**GAGCTCTAGCAAATCTTGTTCAATCTATT-3′ (*Bam*HI site boldfaced, *Sac*I site in small capitals) |
| MCE-GT*Nde*I | 5′-**GGATCC**ATATGGAACCAGGGTCAAGTGCAT-3′ (*Bam*HI site boldfaced, *Nde*I site underlined) |
| MCE-B | 5′-**GGATCC**TAGGTTGGCCGATGCAGTCTTTTGG-3′ (*Bam*HI site boldfaced) |
| KO1 | 5′ GTACGAATTCATCACGAATGGACCTTAAAGGTTTATTACATGAAGAAAACGAACTGCCTTCTATTGCGGATCCCCGGGTTAATTAA 3′ |
| KO2 | 5′ ACATTCTGATGCAAAGCAAGGAACAATTATTAAGAAATGACTTAAGAATGTCAAATAAGTAGCGAATTCGAGCTCGTTTAAAC 3′ |

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 (48). Although the *Candida* capping enzyme shows similar behavior in vitro, it appears that Cgt1 inhibition in the absence of CaCet1 may not be significant in vivo.

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 subcloning and expression of recombinant proteins in bacteria. Plasmids used for experiments in yeast are listed in Table A3.

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′ *SalNco*) and CET1 (3′ *Sac*). This product was ligated into the *Srf*I site of pCR-Script SK(+) (Stratagene) | This study |
| pCR-CaCET1orf | See Materials and Methods | This study |
| pGEM-CaCET1 | 1.56-kb *Bam*HI fragment from pCR-CaCET1orf was subcloned into the *Bam*HI site of pGEM-3zf(+) (Promega) | This study |
| pBS-CaCET1 | 1.56-kb fragment was amplified from pGEM-CaCET1 using oligonucleotides CaCET1-5′ *Sal*I and CaCET1-3′ *Sac*I. This product was ligated into the *Srf*I site of pCR-Script SK(+) | This study |
| pBS-CaCET1 (203-520) | 0.96-kb fragment was amplified from pGEM-CaCET1 using oligonucleotides CaCET1 (203) and CaCET1-3′ *Sac*I. This product was ligated into the *Srf*I site of pCR-Script SK(+) | This study |
| pBS-CaCET1 (229-520) | 0.88-kb fragment was amplified from pGEM-CaCET1 using oligonucleotides CaCET1 (229) and CaCET1-3′ *Sac*I. This product was ligated into the *Srf*I 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′ *Sac*I. This product was ligated into the *Srf*I 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′ *Sac*I. This product was ligated into the *Srf*I site of pCR-Script SK(+) | This study |
| pCR-pct1$^+$ | See Materials and Methods | This study |
| pCR-pct1$^+$ (43-303) | See Materials and Methods | This study |
| pSBEThis$_7$-pct1$^+$ | 0.9-kb *Nco*I-*Bam*HI fragment of pCR-prt1$^+$ was subcloned into *Nco*I-*Bam*HI sites of pSBEThis$_7$ (37) | This study |
| pSBEThis$_7$-pct1$^+$ (43-303) | 0.8-kb *Nco*I-*Bam*HI fragment of pCR-prt1$^+$ was subcloned into *Nco*I-*Bam*HI sites of pSBEThis$_7$ | This study |
| pCR-CGT1orf | See Materials and Methods | This study |
| pSBEThis$_7$CGT1 | 1.4-kb *Nco*I-*Bam*HI fragment from pCR-CGT1orf was subcloned into the *Nco*I-*Bam*HI sites of pSBEThis$_7$ | This study |
| pSBEThis$_7$pce1$^+$ | 1.4-kb *Nco*I-*Nhe*I (blunted) fragment from pET-PCE1 (43) was subcloned into the *Nco*I-*Eco*RV sites of pSBEThis$_7$ | This study |
| pBS-MCE (211-597) | 1.2-kb fragment was amplified from pG-MCE (54) using MCE-GT*Nde*I and MCE-B. This fragment was ligated into the *Srf*I 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 HA-tagged *CET1* | 1.7-kb *Sal*I-*Sac*I fragment from pBS-CET1orf version 2 was subcloned into the *Sal*I and *Sac*I sites of pAD5-pct1$^+$ | This study |
| pRS313-cet1-401 | *CEN/ARS*, *HIS3*, *cet1-401* (*D422A*) | See reference | 48 |
| pRS313-cet1-438 | *CEN/ARS*, *HIS3*, *cet1-438* (*C330W*) | See reference | 48 |
| pRS313-cet1-446 | *CEN/ARS*, *HIS3*, *cet1-446* (*P245A*, *W247A*) | See reference | 48 |
| pRS423-CET1 (Pro + 265-549) | 2μm, *HIS3*, *CET1* (265-549) | See reference | 48 |
| pAD5-CaCET1 | 2μm, *LEU2*, HA-tagged *CaCET1*, *ADH1* promoter-driven | 1.6-kb *Sal*I-*Sac*I fragment from pBS-CaCET1 was subcloned into the *Sal*I and *Sac*I sites of pAD5-CET1 | This study |
| pAD5-CaCET1 (203-520) | 2μm, *LEU2*, HA-tagged *CaCET1* (*203-520*), *ADH1* promoter-driven | 1.0-kb *Nco*I-*Sac*I fragment from pBS-CaCET1 (203-520) was subcloned into the *Nco*I and *Sac*I sites of pAD5-CET1 (1-265)-CTL1 (T. Takagi, unpublished data) | This study |
| pAD5-CaCET1 (229-520) | 2μm, *LEU2*, HA-tagged *CaCET1* (*229-520*), *ADH1* promoter-driven | 0.9-kb *Nco*I-*Sac*I fragment from pBS-CaCET1 (229-520) was subcloned into the *Nco*I and *Sac*I sites of pAD5-CET1 (1-265)-CTL1 | This study |
| pAD5-CaCET1 (251-520) | 2μm, *LEU2*, HA-tagged *CaCET1* (*251-520*), *ADH1* promoter-driven | 0.8-kb *Nco*I-*Sac*I fragment from pBS-CaCET1 (251-520) was subcloned into the *Nco*I and *Sac*I sites of pAD5-CET1 (1-265)-CTL1 | This study |
| pAD5-CaCET1 (269-520) | 2μm, *LEU2*, HA-tagged *CaCET1* (*269-520*), *ADH1* promoter-driven | 0.8-kb *Nco*I-*Sac*I fragment from pBS-CaCET1 (251-520) was subcloned into the *Nco*I and *Sac*I sites of pAD5-CET1 (1-265)-CTL1 | This study |
| pAD5-pct1$^+$ | 2μm, *LEU2*, HA-tagged *pct1$^+$*, *ADH1* promoter-driven | 0.9-kb *Sal*I-*Sac*I fragment from pCR-pct1$^+$ was subcloned into the *Sal*I and *Sac*I sites of pAD5 (48) | This study |
| pAD5H-pct1$^+$ | 2μm, *leu2*::*HIS3*/*Kan*$^R$, HA-tagged *pct1$^+$*, *ADH1* promoter-driven | *LEU2* marker of pAD5-pct1$^+$ was swapped with *Hpa*I-*Xho*I fragment from pLH7 (6a) carrying *leu2*::*HIS3*/*Kan*$^R$ | This study |
| pRS423-pct1$^+$ | 2μm, *HIS3*, *pct1$^+$*, *CET1* promoter-driven | 0.9-kb *Nco*I-*Sac*I fragment from pRS423-CET1 (Pro + 265-549) was replaced with 0.9-kb *Nco*I-*Sac*I fragment from pSBE-This$_I$-Pct1 (43-303) | This study |
| pRS423-pct1$^+$ (43-303) | 2μm, *HIS3*, *pct1$^+$* (*43-303*), *CET1* promoter-driven | 0.9-kb *Nco*I-*Sac*I fragment from pRS423-CET1 (Pro + 265-549) was replaced with 0.8-kb *Nco*I-*Sac*I fragment from pSBE-This$_7$-Pct1 (43-303) | This study |
| pSLF273-pct1$^+$ | *ars1$^+$*, *ura4$^+$*, (HA)$_3$-tagged *pct1$^+$*, weak *nmt1* promoter-driven | 0.9-kb *Bam*HI fragment from pCR-pct1$^+$ was subcloned into the *Bam*HI site of pSLF273 (10) | This study |
| pSGP73-pct1$^+$ | *ars1$^+$*, *LEU2*, (HA)$_3$-tagged *pct1$^+$*, *nmt1* promoter-driven | 0.9-kb *Bam*HI fragment from pCR-pct1$^+$ was subcloned into the *Bam*HI site of pSGP73 (kindly supplied by S. Forsburg) | This study |
| pRS425-CEG1 | 2μm, *LEU2*, *CEG1* | See reference | 48 |
| pRSH-CEG1 | 2μm, *ura3*::*HIS3*/*Kan*$^R$, *CEG1* | *URA3* marker of pRS426-CEG1 (48) was swapped with *Sma*I fragment from pUH7 (6a) carrying *ura3*::*HIS3*/*Kan*$^R$ | This study |
| pRS-CGT1 | 2μm, *URA3*, *CGT1* | See Materials and Methods | This study |
| pRSH-CGT1 | 2μm, *ura3*::*HIS3*/*Kan*$^R$, *CEG1* | *URA3* marker of pRS-CGT1 was swapped with *Sma*I fragment from pUH7 (6a) carrying *ura3*::*HIS3*/*Kan*$^R$ | This study |
| pRSL-CGT1 | 2μm, *ura3*::*LEU2*/*Kan*$^R$, *CGT1* | *URA3* marker of pRS-CGT1 was swapped with *Sma*I fragment from pUH9 (6a) carrying *ura3*::*LEU2*/*Kan*$^R$ | This study |
| pDB20-pce1$^+$ | 2μm, *URA3*, *pce1$^+$*, *ADH1* promoter-driven | See Materials and Methods | This study |
| pDB20L-pce1$^+$ | 2μm, *LEU2*, *pce1$^+$*, *ADH1* promoter-driven | 3.6-kb *Bam*HI fragment of pDB20LADA1 (26a) was replaced with 3.7-kb *Bam*HI fragment of pDB20-pce1$^+$ | This study |
| pDB20H-pce1$^+$ | 2μm, *ura3*::*HIS3*/*Kan*$^R$, *pce1$^+$*, *ADH1* promoter-driven | *URA3* marker of pDB20-pce1$^+$ was swapped with *Sma*I fragment from pUH7 carrying *ura3*::*HIS3*/*Kan*$^R$ | This study |
| pDB20-MCE (211-597) | 2μm, *URA3*, MCE (211-597), *ADH1* promoter-driven | 1.2-kb *Eco*RV-*Not*I (blunted) fragment from pBS-MCE (211-597) was subcloned into the *Not*I (blunted) site of pDB20 (4) | This study |
| pDB20H-MCE (211-597) | 2μm, *HIS3*, MCE (211-597), *ADH1* promoter-driven | *URA3* fragment of pDB20-MCE (211-597) was swapped with *Sma*I fragment from pUH7 carrying *ura3*::*HIS3*/*Kan*$^R$ | This study |
| pAD5-MCE (211-597) | 2μm, *LEU2*, HA-tagged MCE (211-597), *ADH1* promoter-driven | See reference | 48 |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