A Conserved Domain of Yeast RNA Triphosphatase Flanking the Catalytic Core Regulates Self-association and Interaction with the Guanylyltransferase Component of the mRNA Capping Apparatus*

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The 549-amino acid yeast RNA triphosphatase Cet1p catalyzes the first step in mRNA cap formation. Cet1p consists of three domains as follows: (i) a 230-amino acid N-terminal segment that is dispensable for catalysis in vitro and for Cet1p function in vivo; (ii) a protease-sensitive segment from residues 230 to 275 that is dispensable for catalysis but essential for Cet1p function in vivo; and (iii) a catalytic domain from residues 275 to 539. Sedimentation analysis indicates that purified Cet1p(231–549)p is a homodimer, Cet1p(231–549)p binds in vitro to the yeast RNA guanylyltransferase Ceg1p to form a 7.1 S complex that we surmise to be a trimer consisting of two molecules of Cet1p(231–549)p and one molecule of Ceg1p. The more extensively truncated protein Cet1p(276–549)p, which cannot support cell growth, sediments as a monomer and does not interact with Ceg1p. An intermediate deletion protein Cet1p(246–549)p, which supports cell growth only when overexpressed, sediments principally as a discrete salt-stable 11.5 S homo-oligomeric complex. These data implicate the segment of Ceg1p from residues 230 to 275 in regulating self-association and in binding to Cet1p. Genetic data support the existence of a Ceg1p-binding domain flanking the catalytic domain of Cet1p, to wit: (i) the ts growth phenotype of 2μ CET1(246–549) is suppressed by overexpression of Ceg1p; (ii) a ts alanine cluster mutagenesis of Cet1p(201–549) can be bypassed when the catalytically active mouse triphosphatase (Cet1p) and guanylyltransferase (Ceg1p) proteins interact to form a heteromeric complex (1, 2, 8), whereas in mammals, autonomous triphosphatase and guanylyltransferase domains are linked in cis within a single polypeptide (Mce1p) (9–13). Genetic evidence suggests that the physical linkage of the mammalian triphosphatase and guanylyltransferase domains is essential in vivo. For example, a lethal yeast cet1 deletion can be complemented by expression of MCE1 but only if the catalytically active mouse triphosphatase domain is linked in cis to a catalytically active guanylyltransferase domain (2, 12). Our isolation of CET1 in a genetic screen for high copy suppressors of temperature-sensitive mutations of Ceg1p suggested that interaction of the endogenous yeast triphosphatase and guanylyltransferase in trans may also be essential in vivo (2). Although analyses of the spectrum of ceg1-ts mutations that could be suppressed by Cet1p overexpression showed clustering of such lesions within a C-terminal domain of Ceg1p (2, 14), there is, as yet, no physical identification of a Cet1p-binding site on Ceg1p.

A variety of qualitative methods have been employed to document Cet1p-Ceg1p interaction in vivo (by yeast two-hybrid reporter assay) or in vitro (far Western blotting or co-immunoprecipitation/Western blotting) (1, 14, 15). A drawback of these studies is that they provide limited insight into the nature of the complexes formed, and the results have not been integrated with a genetic assessment of the function of a presumptive Ceg1p-binding site on Cet1p. Our approach has been to study the interaction of purified recombinant Cet1p with purified recombinant Ceg1p by zonal velocity sedimentation (2). In this method, the purity and concentration of the input proteins, as well as the extent of complex formation (manifest as a shift in the sedimentation peaks), are apparent from the gradient profiles. Moreover, the native size of the Cet1p-Ceg1p complexes can be gauged from the sedimentation behavior. We have shown (2) that the N-terminal deletion mutant Cet1p(201–549)p forms a discrete complex with Ceg1p, in near quantitative yield, that is stable to centrifugation in a glycerol gradient.

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Thus, the catalytic domain and the Ceg1p-binding site reside within Cet1(201–549)p, which is fully functional in vivo.

Here we present further physical and genetic analysis of the yeast RNA triphosphatase that includes the following: (i) delineation of a domain structure of Cet1(201–549)p by limited proteolysis; (ii) definition by serial N- and C-terminal deletions of Cet1(241–549) as a minimum functional domain sufficient for cell growth at 25–37 °C; (iii) physical characterization of the Cet1p N-terminal deletion mutants and their complexes with Ceg1p; (iv) demonstration of suppression of the temperature-sensitive growth phenotype of the deletion mutant CET1(246–549) by overexpression of Ceg1p; (v) characterization of a lethal deletion mutant, CET1(276–549), which encodes a monomeric enzyme with full catalytic activity that is unable to interact with Ceg1p; and (vi) restoration of the in vivo function of Cet1(276–549)p by fusion in cis with the guanylyltransferase domain of the mouse capping enzyme. Our results suggest that a protease-sensitive segment of Cet1p located proximal to the core catalytic domain regulates both Cet1p–Cet1p self-association and heteromerization with Ceg1p.

**EXPERIMENTAL PROCEDURES**

**CET1 Deletion Mutants—**A series of N-terminal deletion mutants of CET1 was constructed by PCR amplification with mutagenic sense-strand primers that introduced an Ndel restriction site and a methionine codon in lieu of the codons for Glu 210, Asn 220, Asn 230, Lys 240, and Ile 275. The PCR products were digested with Ndel and BamHI and then inserted into yeast plasmid pET1–5′3′ (CEN TRP1) (2). The mutated genes were named according to the amino acid coordinates of their polypeptide products, i.e., CET1(211–549), CET1(221–549), CET1(231–549), CET1(241–549), and CET1(257–549). C-terminal truncation mutants CET1(201–539) and CET1(201–529) were constructed by PCR amplification using antisense primers that introduced translation stop codons in lieu of the codons for Glu 210, Asn 220, Asn 230, Lys 240, and Ile 275. The PCR product was digested with Ndel and BamHI and then inserted into yeast plasmid pET1–5′3′. Expression of the deleted alleles in these plasmids is under the control of the natural CET1 promoter.

**2 μ CET1 Expression Vectors—**Restriction fragments containing CET1(201–549), CET1(241–549), and CET1(276–549) were excised from the respective pCET1–5′3′-based plasmids and inserted into the yeast expression vector pYX232 (2 μ TRP1). In the resulting plasmids, expression of the yeast RNA triphosphatase is under the control of the yeast TRP1 promoter.

**Expression and Purification of Recombinant Cet1p Derivatives—**The Ndel/BamHI fragments encoding Cet1p(201–549), Cet1p(211–549), Cet1p(221–549), and Cet1p(257–549) were excised from their respective pCET1–5′3′ plasmids and inserted into pET16b to generate plasmids pET-CET1(201–549), pET-CET1(211–549), pET-CET1(221–549), pET-CET1(231–549), pET-CET1(241–549), pET-CET1(257–549), and pET-CET1(276–549). Induced expression of the His-tagged truncated Cet1 proteins was performed as described (2). The proteins were purified from soluble bacterial lysates by Ni2+-agarose column chromatography (2). The polypeptide compositions of the imidazole eluate fractions were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing recombinant Cet1p protein were pooled and dialyzed against buffer C (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100). Protein concentration was determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard. The enzyme preparations were stored at −80 °C.

**Expression and Purification of Recombinant Ceg1p—**The CEG1 gene was inserted into a customized T7-based expression plasmid (a derivative of pET16b) in such a way as to fuse the 459-amino acid CEG1-coding sequence in frame to an N-terminal 29-amino acid leader peptide containing 10 consecutive histidine codons sequence (MGSHHHHHH- HHHSSGGSHEGRSRSVPH). The plasmid was transformed into Escherichia coli BL21(DE3). A 500-ml culture was grown at 37 °C in LB medium containing 0.1 mg/ml ampicillin until the A600 reached 0.6. The culture was adjusted to 0.4 mM isopropyl-1-thio-β-D-galactopyranoside and 2% ethanol and then incubation was continued for 21 h at 17 °C. Cells were harvested by centrifugation and stored at −80 °C. All subsequent procedures were performed at 4 °C. Thawed bacteria were resuspended in 25 ml of buffer A (50 mM Tris-HCl, pH 7.5, 0.2 mM NaCl, 10% glycerol) at a concentration of 1×108 cells/ml; the suspension was incubated on ice for 10 min and then sonicated for 30 s. Triton X-100 was added to a final concentration of 0.1%, and sonication was repeated to reduce the viscosity of the lysate. Insoluble material was removed by centrifugation for 45 min at 18,000 rpm in a Sorvall SS34 rotor. The soluble extract was applied to a 2-ml column of Ni2+-agarose (Qiagen, Germany) that had been equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with the same buffer and then eluted stepwise with 5 ml of buffer B (50 mM Tris-HCl, pH 8.0, 0.2 mM NaCl, 10% glycerol, 0.05% Triton X-100) containing 50, 100, 200, 500, and 1000 mM imidazole. The polypeptide compositions of the column fractions were monitored by SDS-PAGE. Ceg1p was retained on the Ni2+-agarose column and recovered in the 0.2 mM imidazole eluate. The 0.2 mM imidazole fraction was dialyzed against buffer C. The Ceg1p enzyme preparation was stored at −80 °C.

**Glycerol Gradient Sedimentation—**Aliquots of the Ni2+-agarose preparations of truncated Cet1p proteins (typically −30 μg) were mixed with catalase (25 μg), BSA (25 μg), and cytochrome c (25 μg) in 0.2 ml of buffer G (50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol). Where indicated, the mixture also contained recombinant Ceg1p (−30 μg). The mixtures were incubated on ice for 45 min and then layered onto a 4.8-m1 15–30% glycerol gradient containing buffer G. The gradients were centrifuged in a Beckman SW50 rotor at 50,000 rpm for 16 h at 4 °C. Fractions (0.2 ml) were collected from the bottoms of the tubes. Aliquots (20 μl) of odd-numbered fractions were analyzed by SDS-PAGE along with samples of the input protein mixtures for each gradient. Polypeptides were visualized by staining with Coomassie Blue dye.

**Yeast Strains—**Strain YBS20 (MATa trpl his3 ura3 leu2 ade2 can1 cet1:LEU2 p360-CET1) is deleted at the chromosomal locus encoding RNA triphosphatase. Growth of YBS20 depends on maintenance of plasmid p360-CET1 (CEN URA3 CET1). Strain YBS50 (MATa leu2 ade2 trpl his3 ura3 can1 cet1:hisG cet1::LEU2 p360-CET1/CEG1) is deleted at the chromosomal loci encoding RNA triphosphatase and guanylyltransferase. Growth of YBS50 is contingent on the maintenance of plasmid p360-CET1/CEG1 (CEN URA3 CET1 CEG1), which contains the CEG1 and CET1 genes under the control of their natural promoters.

**Isolation of cet1-ts Mutants—**The CET1 gene was amplified in vitro by the standard PCR. The standard PCR protocol was modified to contain either (i) a reduced concentration of dATP (0.08 mM) relative to the other three dNTPs (each at 2 mM); (ii) 0.08 mM dATP plus 0.2 mM manganese chloride; or (iii) 2 mM dATP plus 0.2 mM manganese chloride. The PCR products were digested with Ndel and BamHI. The mutagenized CET1 DNA fragments from 10 separate PCR reactions (using PCR reaction condition i, and each using conditions ii and iii) were separately inserted into CET1–5′3′, and the transformants were transformed into E. coli. After amplification in vivo, 10 pooled plasmid libraries were prepared, each from approximately 3,000 ampicillin-resistant colonies harvested directly from the agar plates. These DNA libraries were transformed into yeast strain YBS20. Trp+ transformants (150 for each transformed pool) were patched on agar medium lacking tryptophan at 30 °C and then replica-plated on 5-FOA medium at 30 °C to eliminate the wild type CET1 allele on a CEN URA3 plasmid. Six to fifteen percent of the transformants were unable to grow on 5-FOA. The surviving isolates, which had lost the CET1 URA3 plasmid, were replica-plated on YPD agar and incubated at 30 °C (permissive temperature) and 37 °C (nonpermissive temperature). Those able to grow at 30 but not 37 °C were selected. Plasmid DNA was recovered from candidate ts mutants, amplified in vitro in E. coli, and restigated for the conditional growth phenotype by plasmid shuffle. In this way, we obtained a collection of cet1-ts mutants. Fifteen of these mutant cet1 clones were mapped at the nucleotide level by DNA sequencing.

**Candida albicans CET1 Deletion Mutants—**The complete C. albicans genome was sequenced by the 520-base-pair DNA triphosphatase CaCet1p (15) was amplified by PCR from a C. albicans DNA library (16) using a sense primer that introduced an Ndel site at the translation stop codon and an antisense primer that introduced a BamHI site immediately 3′ of the translation stop codon. The PCR product was digested with Ndel and BamHI and then inserted into a customized yeast expression vector pYN132 (CEN TRP1), a derivative of pYX132 in which a unique Ndel site replaced the Ncol site of pYX132. The resulting
pling plasmid was named pCaCet1(1–520). N-terminal deletion mutants of CaCET1 were constructed by PCR amplification with mutagenic sense-strand primers that introduced an NdeI restriction site and a methionine codon in lieu of the codon for Asp178, Gln195, Tyr202, or Asn216. The PCR products were digested with NdeI and BamHI and then inserted into pYNI32 to yield plasmids pCaCet1(179–520), pCaCet1(196–520), pCaCet1(203–520), and pCaCet1(217–520). In these vectors, expression of the C. albicans polypeptide is under the control of the TPII promoter.

Chimeric Yeast-Mammalian Capping Enzymes—Genes encoding fusion proteins composed of an N-terminal segment derived from yeast Cet1p and the catalytically active C-terminal guanylyltransferase domain of the mouse capping enzyme [Mce1p(211–597)] were constructed as follows. The CET1 coding sequences from residues 201–549, 275–549, and 301–549 were PCR-amplified using the respective pET-based CET1 plasmids as templates and an antisense primer that changed Val246 to His and introduced an NdeI restriction site at the C-terminal dipeptide His548–Met549. The PCR products were digested with NdeI and then inserted into the NdeI site of pXI-MCE1(211–597) (CEN TPII) to yield in-frame fusion genes CET1(201–547)-MCE1(211–597), CET1(276–547)-MCE1(211–597), and CET1(301–547)-MCE1(211–597). Expression of the chimeric genes in these plasmids is under the control of the TPII promoter.

RESULTS

Probing the Structure of Yeast RNA Triphosphatase by Limited Proteolysis—Recombinant Cet1p(201–549) containing a short N-terminal His tag was subjected to proteolysis with increasing concentrations of chymotrypsin, trypsin, and V8 proteases (Fig. 2). SDS-PAGE analysis of the undigested protein preparation revealed a 44-kDa polypeptide (Fig. 2A) corresponding to His-tagged Cet1p(201–549) (predicted size of 43 kDa). Sequencing of this species by automated Edman chemistry after transfer to a polyvinylidene difluoride membrane confirmed that the N-terminal sequence (GHHHHHH) corresponded to that of the recombinant protein beginning from the second residue of the His tag. Apparently, the triphosphatase suffered removal of the initiating methionine during expression in E. coli. Initial scission of Cet1p(201–549) at low concentrations of chymotrypsin yielded a predominant ~39-kDa species with N-terminal sequence RNVPW resulting from chymotryptic cleavage at Tyr241/Arg242 (Fig. 2A). At 2-fold higher protease concentration, this species was converted to an ~36-kDa polypeptide with N-terminal sequence QSINVK, arising via cleavage at Leu259/Gln259 (Fig. 2A). Even higher chymotrypsin concentrations yielded two major products, a ~26-kDa polypeptide with its N terminus at Gln259 and a ~12-kDa fragment with N-terminal sequence KSQSPI resulting from scission at Tyr445/Lys446 (Fig. 2A). These two species persisted at the highest levels of chymotrypsin tested (8-fold greater than the amount sufficient to cleave all of the input protein at least once), although some of the protein was digested to a mixed cluster of low molecular fragments. One of these fragments retained an N terminus at Gln259, whereas two novel fragments were generated via cleavage at Tyr280/Arg281 (RVGLST) or Tyr461/Ile462 (IHDNXT). The evolution of the digestion pattern with increasing protease concentration suggested the sequence of chymotryptic cleavages diagrammed in Fig. 1B. The key point here is that the principal chymotrypsin-accessible sites (Tyr241 and Leu259) are located in the N-terminal portion of Cet1p(201–549).

Initial scission by V8 protease occurred between Glu231 and Ile232 to yield a ~39-kDa polypeptide with N-terminal sequence ISASSK (Fig. 2C). At higher V8 concentrations, this fragment was converted transiently to a ~32-kDa species with the same N terminus (Fig. 2C). Even higher levels of V8 led to the transient appearance of a ~26-kDa fragment with N-terminal sequence LDAHHTL via scission at Glu334/Leu335 (Fig. 2C). Also arising was a ~12-kDa species with N-terminal residue Ile232, this fragment was presumably generated when the larger precursor was cleaved at Glu334. This 12-kDa species persisted at the highest V8 levels tested, whereas the ~26-kDa fragment originating at Leu232 was converted into an ~19-kDa species with the same N terminus (Fig. 2C).

Initial cleavage by trypsin yielded a large fragment retaining the N-terminal His tag (Fig. 2B). At the highest level of trypsin tested, the protein was converted to a stable ~27-kDa doublet arising via closely spaced cleavages at Lys256/Ala257 (leaving N-terminal sequence ALQSIIN) and Lys264/Asp265 (sequence DLKIDP) (Fig. 2B).

The experimentally determined protease cleavage sites within Cet1p(201–549) are annotated in the primary structure in Fig. 1A. The instructive point of this analysis is that the principal or prominent sites of accessibility to all three proteases are clustered within a short polypeptide segment from Glu231 to Lys259. We infer that this segment is either disordered or surface-exposed. In contrast, the distal portion of the protein is relatively protease-insensitive and likely to comprise a folded domain.

Deletion Analysis of Yeast RNA Triphosphatase Defines a Minimal Functional Domain in Vivo—We reported previously that expression of the truncated derivative Cet1p(201–549) on a single copy plasmid under the control of the natural CET1 promoter fully complemented the growth of a Delta1 deletion strain of S. cerevisiae, whereas expression of the more extensively truncated derivatives Cet1p(246–549) and Cet1p(301–549) did not complement (2). In light of the proteolysis results
suggesting a domain boundary downstream of residue 201, we
constructed a series of finer N-terminal deletion alleles as
follows: CET1(211–549), CET1(221–549), CET1(231–549), and
CET1(241–549). The margins of the latter two alleles were
placed at Glu231 and Tyr241, the residues that were cleaved by
trypsin (2). This result delineates a short peptide segment between
residues 241 and 245 that is required for Cet1p function in vivo
when the triphosphatase is expressed from its own promoter.

Previously, we showed that deletion of 30 or 60 amino acids
from the C terminus of Cet1p resulted in loss of function in vivo
(2). Here, we tested by plasmid shuffle a finer series of C-terminal
truncation mutants and found that CET1(201–539) complemented
growth of YBS20 on 5-FOA, whereas CET1(201–529) did not (data not shown). A mutant allele, CET1(241–549), in
which both termini were truncated to their respective func-
tional boundaries, also complemented Δcet1 (data not shown).

**Effects of N-terminal Deletions on Triphosphatase Activity**—
The N-terminal deletion mutants of Cet1p were expressed in
bacteria as His-tagged fusions and purified from soluble lysates
by Ni2+-agarose column chromatography (Fig. 4A). Triphos-
phatase activity was determined by assaying manganese-
dependent ATP hydrolysis (3) as a function of input enzyme. The specific activities of CET1(211–549)p, CET1(221–549)p,
CET1(231–549)p, and CET1(241–549)p were comparable to
that of CET1(201–549)p (Fig. 4B). This was in keeping with the
expectation that any Cet1p derivatives that were active in vivo
should retain catalytic activity in vitro. Note that CET1(246–
549)p, which did not complement Δcet1 growth, was also active
in vitro (Fig. 4B). The instructive finding was that CET1(276–
549)p also retained full triphosphatase activity in vitro (Fig.
4B). This result delineates an N-terminal margin of the cata-
lytic domain at a point only 29 residues upstream of catalytic
motif A.

**Overexpression of CET1**—We tested whether activity of the
CET1(246–549) or CET1(276–549) deletion alleles in vivo
could be augmented by increased gene dosage and/or expres-
sion of the genes under the control of a strong yeast promotor.
To do this, we cloned CET1(246–549) and CET1(276–549), as
well as full-length CET1 and CET1(201–549) (the positive con-

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**Fig. 2. Limited proteolysis of Cet1(201–549)p.** Reaction mixtures (10 μl) containing 50 mM Tris-Cl, pH 8.0, and 5 μg of purified His-
Cet1(201–549)p were incubated with increasing amounts of either chymotrypsin (A), trypsin (B), or V8 protease (C) for 15 min at 22 °C. The
amounts of protease added to each reaction, proceeding from left to right
within each titration series, were as follows: chymotrypsin (20, 50, 100,
200, 300 ng); trypsin (0.5, 2.0, 5.0, 10, 25 ng); V8 (25, 50, 100, 250, 500
ng). Control mixtures were incubated without protease (−). The reac-
tions were quenched by the addition of SDS sample buffer, and the
digests were analyzed by electrophoresis through a 15% polyacrylamide
gel containing 0.1% SDS. Photographs of the Coomassie Blue-stained
gels are shown. The positions and sizes (kDa) of marker proteins are
denoted in single-letter code.

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**Fig. 3. Effect of N-terminal deletions on CET1 function in vivo.** Yeast strain YBS20 was transformed with CEN TRP1 plasmids con-
taining the indicated N-terminal deletion alleles of CET1. A control
transformation was performed using the TRP1 vector. Trp+ isolates
were streaked on agar plates containing 0.75 mg/ml 5-FOA. The plates
were photographed after incubation for 3 days at 30 °C.
The positions and sizes (in kDa) of marker proteins are indicated on the left. B, triphosphatase activity. Reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 2 mM MnCl₂, 1 mM γ-[^32]P/ATP, and Cet1p as specified (dialyzed Ni²⁺-agarose fraction) were incubated for 15 min at 30 °C. The reactions were quenched by adding 2.5 μl of 5 M formic acid. The release of[^32]P, from γ-[^32]P/ATP was quantitated by scanning the TLC plate with a FUJIX BAS2000 Bio-Imaging Analyzer.

FIG. 4. Triphosphatase activity of N-terminal deletion mutants of Cet1p. A, polypeptide composition. Aliquots (4 μl) of the dialyzed Ni²⁺-agarose preparations of recombinant Cet1p(201–549) (Δ200), Cet1p(211–549) (Δ210), Cet1p(221–549) (Δ220), Cet1p(231–549) (Δ230), Cet1p(241–549) (Δ240), Cet1p(246–549) (Δ245), and Cet1p(276–549) (Δ275) were electrophoresed through a 12.5% polyacrylamide gel containing 0.1% SDS. Polypeptides were visualized by staining with Coomassie Blue dye. The positions and sizes (in kDa) of marker proteins are indicated on the left. B, triphosphatase activity. Reaction mixtures (10 μl) containing 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 2 mM MnCl₂, 1 mM γ-[^32]P/ATP, and Cet1p as specified (dialyzed Ni²⁺-agarose fraction) were incubated for 15 min at 30 °C. The reactions were quenched by adding 2.5 μl of 5 M formic acid. An aliquot (2.5 μl) of the mixture was applied to a polyethyleneimine-cellulose TLC plate, which was developed with 0.5 M LiCl, 1 mM formic acid. The release of[^32]P, from γ-[^32]P/ATP was quantitated by scanning the TLC plate with a FUJIX BAS2000 Bio-Imaging Analyzer.

FIG. 5. The temperature-sensitive phenotype of 2μ CET1(246–549) cells is suppressed by 2μ CEG1. A. YBS20 was transformed with 2μ TRP1 plasmids containing CET1 or the indicated N-terminal deletion alleles of CET1 under the control of the TRP1 promoter. Trp⁺ FOA-resistant cells were selected and streaked on YPD agar; the plates were incubated at 30 °C. B. YBS20 was transformed with 2μ URA3 plasmids containing CET1 or 2μ URA3 plasmids. Individual Ura⁺ transformants were streaked on SC agar medium lacking uracil. The plates were incubated for 4 days at each 25 or 37 °C as indicated.

The salient finding was that Δcet1 cells transformed with 2μ TIPI-CET1(246–549) did give rise to FOA-resistant colonies at 25 and 30 °C but not at 37 °C (data not shown). Thus, Cet1p(276–549)p, which is catalytically active in vitro, cannot sustain cell growth even when overexpressed in vivo. The salient finding was that Δcet1 cells transformed with 2μ TIPI-CET1(246–549) did give rise to FOA-resistant colonies at 25 and 30 °C but not at 37 °C (data not shown).

To characterize better the growth of the 2μ TIPI-CET1(246–549) strain, FOA-resistant cells selected at 25 °C were streaked on YPD plates at 25, 30, and 37 °C in parallel with CET1 and CET1(201–549) controls. The 2μ TIPI-CET1(246–549) strain displayed a clear-cut ts phenotype at the restrictive temperatures of 34 or 37 °C (Fig. 5A, Δ245). Additional experiments revealed that growth of Δcet1 on 5-FOA at 25 °C could also be complemented by a CEN plasmid containing CET1(246–549) driven by the TIPI promoter. The CEN TIPI-CET1(246–549) strain was unable to grow on YPD medium at 34 or 37 °C and grew more slowly than the 2μ TIPI-CET1(246–549) strain at 30 °C (not shown). Thus, whereas increased promoter strength and gene dosage both serve to enhance the in vitro activity of Cet1p(246–549)p at 25–30 °C, these maneuvers are insufficient to sustain cell growth at higher temperatures.

Overexpression of Guanylyltransferase Suppresses the ts Phenotype of 2μ TIPI-CET1(246–549)—We showed previously that overexpression of Cet1p from a 2μ plasmid suppressed certain ts mutations in the yeast guanylyltransferase Ceg1p. We hypothesized that the ts guanylyltransferase has diminished affinity for Cet1p at restrictive temperature (37 °C) and that increasing the level of wild type Cet1p drives Ceg1p-Cet1p heteromerization by simple mass action (2). We have since found that the ceg1–25 mutant can be suppressed by 2μ TIPI-CET1(201–549) but not by 2μ TIPI-CET1(246–549) (data not shown). These results suggest that deletion of the Cet1p segment from residues 201 to 245 may compromise its interaction with Ceg1p at 37 °C, a scenario that may explain the ts phenotype of the 2μ TIPI-CET1(246–549) strain.

Is the genetic interaction between these cap-forming enzymes reciprocal, i.e. can overexpression of the guanylyltransferase suppress the growth defect of a conditional mutant of the triphosphatase? To address this question, we transformed the 2μ TIPI-CET1(246–549) strain with a 2μ URA3 CEG1 plasmid and tested the growth of Ura⁺ isolates at permissive and restrictive temperatures. Control cells transformed with a 2μ
URA3 CET1 plasmid grew at 25 and 37 °C, whereas cells transformed with the 2μ URA3 vector grew at 25 °C only (Fig. 5B). Remarkably, the introduction of CEG1 on a 2μ plasmid rescued growth at 37 °C and did so as effectively as wild type CET1, as judged by colony size (Fig. 5B). 2μ CEG1 also suppressed the ts growth phenotype of the CEN TP1-CET1(246–549) strain (not shown). These experiments suggest that Cet1(246–549)p is defective for interaction with Ceg1p at 37 °C in vivo.

Suppression of cet1-ts Mutations by 2μ CEG1 Is Allele-specific—In order to examine the allele specificity of the genetic interaction between the yeast triphosphatase and guanylyltransferase, we isolated a collection of temperature-sensitive cet1-ts alleles from separate pools of mutagenized CET1 clones. To do this, we performed PCR amplification of the CET1 gene under reaction conditions designed to promote nucleotide misincorporation by Taq polymerase. The PCR products from separate amplification reactions were restricted and cloned into an '-specific—vector. The coding changes affecting 28 different residues (Fig. 6). None of the mutations were located within the essential triphosphatase motif A, B, and C. Many of the changes were tightly clustered within several discrete segments of the carboxyl domain of the enzyme, e.g., the S518P, L519P, and F523L cluster (Fig. 6). All of the cet1-ts alleles were recessive to wild type CET1, i.e., growth at 37 °C could be restored to the cet1-ts strains by transforming them with a CEN URA3 plasmid bearing the wild type CET1 gene (not shown). Remarkably, none of the cet1-ts mutants could be rescued to growth at the restrictive temperature by transformation with a 2μ URA3 plasmid bearing the CEG1 gene (not shown). Therefore, suppression of the conditional growth phenotype of yeast triphosphatase mutations by overexpression of guanylyltransferase was highly specific for the 2μ CET1(246–549) allele.

Physical Characterization of N-terminal Deletion Mutants of Cet1p and Their Interaction with Ceg1p—The native sizes of several of the N-terminal deletion mutants of Cet1p were investigated by sedimentation of the recombinant enzymes through 15–30% glycerol gradients containing 100 mM NaCl. At the same time, we tested the interaction of the Cet1p deletion mutants with recombinant Ceg1p by mixing the proteins in vitro and sedimenting the mixture through a glycerol gradient. Marker proteins (catalase, BSA, and cytochrome c) were included as internal standards in every glycerol gradient. After centrifugation, the polypeptide compositions of the gradient fractions were analyzed by SDS-PAGE.

The sedimentation profile for Cet1(201–549)p (Δ200) is shown in Fig. 7A. The 2μ triphosphatase (a 45-kDa polypeptide) sedimented as a discrete peak (fraction 15) just slightly heavier than the BSA peak (68-kDa). A plot of the S value of the three standards versus fraction number yielded a straight line (not shown). An S value of 5.0 was determined for Cet1(201–549)p from the internal standard curve. These results suggest that Cet1(155–549)p is a homodimer. (Note that our earlier estimate of an S value for recombinant Cet1(155–549)p (2) was based on a comparison to external markers sedimented in a different glycerol gradient.) The sedimentation profile for the mixture of Cet1(201–549)p and Ceg1p is shown in Fig. 7B. Here, the yeast proteins form a discrete complex (peak fraction 11) that sediments at a position between catalase and BSA. The sedimentation coefficient of the Cet1(201–549)p-Ceg1p complex was 7.3. This value suggested a heterotrimERIC subunit structure which, given that Cet1(201–549)p sediments as a dimer, whereas Ceg1p alone is monomeric (see below), likely consists of two molecules of Cet1(201–549)p and one molecule of Ceg1p.

The sedimentation profile for Cet1(276–549)p (Δ275) is shown in Fig. 8A. The Δ275 protein (a 34-kDa polypeptide) sedimented as a single discrete component at a position be-
between BSA and cytochrome c. The observed sedimentation coefficient of 3.6 suggests that Cet1(276–549)p is a monomer. Additional sedimentation experiments confirmed that the triphosphatase activity cosedimented with the Cet1(276–549)p polypeptide (data not shown). The sedimentation behavior of Cet1(276–549)p was unaltered in the presence of Ceg1p (Fig. 8B). Ceg1p sedimented a discrete monomeric peak intermediate between BSA and Δ275 (Fig. 8B). Ceg1p displayed the same profile when centrifuged in a gradient containing just Ceg1p and markers (data not shown). These results show that Cet1(276–549)p, although catalytically active, lacks the capacity to bind to the guanylyltransferase. We hypothesize that the lack of Ceg1p binding accounts for the failure of Cet1(276–549)p to support yeast cell growth.

Further insights into the self-association and Ceg1p-binding properties of yeast RNA triphosphatase emerged from sedimentation analyses of intermediate deletion mutants. For example, Cet1(231–549)p by itself sedimented as a discrete 4.9 S component (a presumptive homodimer) just slightly ahead of BSA (Fig. 9A). Mixture with Ceg1p resulted in the near quantitative formation of a Cet1(231–549)p-Ceg1p complex sedimenting at 7.1 S (Fig. 9B). By the same reasoning applied to the Δ200 mutant, we presume this complex to be a heterotrimer. This experiment shows that the segment from residues 201 to 230 of Ceg1p is not required for Ceg1p binding in vitro.

A dramatic change in the sedimentation behavior of the triphosphatase occurred when the protein was truncated from residue 231 to 245. The Δ245 enzyme by itself was resolved into two discrete peaks as follows: a heavy component (11.5 S) that sedimented slightly faster than catalase and a lighter component of 4.8 S (Fig. 10A). Although trace amounts of a heavy component can be discerned during gradient centrifugation of the Δ200 and Δ230 proteins (Figs. 7A and 9A), in the case of Δ245, the heavy component comprises the majority of species. The position of the heavy component of Δ245 relative to catalase (a 248-kDa tetramer of a 62-kDa subunit) suggests that the species is at least a hexamer, if not an octamer, of the 37-kDa Cet1(246–549)p polypeptide. The 4.8 S light component is a presumptive homodimer. Note that additional sedimentation studies showed that the triphosphatase activity profile mirrored the biphasic distribution of the Δ245 polypeptide, with the majority of the activity associated with the heavy component (data not shown). The stability of the oligomeric form of Cet1(246–549)p was investigated by increasing the ionic strength of the protein sample (to 0.25, 0.5, or 1.0 M NaCl) and then centrifuging the Δ245 and internal standards through glycerol gradients containing 0.25, 0.5, or 1.0 M NaCl. These experiments showed that the sedimentation coefficient of the heavy and light components and the distribution of the Δ245 polypeptide between the two components was unaffected by salt up to 1 M NaCl (data not shown).

Mixing Δ245 with Ceg1p resulted in a shift of the light
component to form a Cet1(246–549)p-Ceg1p complex sedimenting at 7.1 S (Fig. 10B, fractions 11–13). A fraction of the Ceg1p also became associated with the heavy component (Fig. 10B, fraction 3). The ratio of the Ceg1p to Δ245 proteins was much lower in the heavy component complex than in the 7.1 S complex (a putative heterotrimer).

Finally, the sedimentation behavior of Cet1(241–549)p (Δ240) constituted a forme fruste of the aberrant behavior of Δ245, meaning that Δ240 by itself was resolved into two discrete peaks of 12 S (Fig. 11A, fraction 3) and 4.8 S (Fig. 11A, fraction 15), but the distribution of the protein was biased toward the light component. Mixture with Ceg1p resulted in the formation of a 7.2 S Cet1(241–549)p-Ceg1p complex (Fig. 11B, lane 11) and the association of some Ceg1p with the heavy component.

The conclusions of the sedimentation analysis can be summarized as follows: (i) the C-terminal domain of Cet1p from residues 231 to 549 has an intrinsic capacity to self-associate to form a homodimer, which binds stably to Ceg1p; (ii) deletion of the segment from residues 231 to 245 unmasks a latent capacity to form a salt-stable 11–12 S oligomer consisting of at least 6 Ceg1p protomers; and (iii) deletion of the segment from residues 246 to 275 abolishes self-association and the ability of Cet1p to bind to Ceg1p.

A Putative Guanylyltransferase-binding Element Is Conserved and Essential in C. albicans RNA Triphosphatase—The C. albicans CaCET1 gene encodes a 520-amino acid RNA triphosphatase (15). The C-terminal segment of CaCet1p from residues 186 to 520 displays extensive sequence similarity to the carboxyl portion of S. cerevisiae Cet1p from positions 225 to 538 (95 identical residues and 64 positions with side chain similarity). In contrast, the N-terminal segments of CaCet1p and Cet1p are not conserved. We cloned wild type CaCET1 and a series of N-terminal deletion alleles of CaCET1 into CEN vectors under the control of the TPI1 promoter and then tested by plasmid shuffle for complementation of the S. cerevisiae Δcet1 mutant. The CaCET1(179–520), CaCET1(196–520), and CaCET1(203–520) mutants were viable (Fig. 12A, Δ178, Δ195, Δ202), whereas CaCET1(217–520) was lethal (Fig. 12A, Δ216). According to the sequence alignment shown in Fig. 12B, the viable Δ185 and Δ202 deletions of CaCet1p correspond to deletions of 235 and 242 amino acids from the N terminus of Cet1p, whereas the lethal Δ216 mutation corresponds to a deletion of 256 residues from Cet1p. These results show that the upstream functional borders of the two fungal RNA triphosphatases are quite similar.

The viable S. cerevisiae strains bearing deletion mutants of CaCET1 were tested for growth at 37 °C in rich medium. The CaCET1(179–520) and CaCET1(196–520) mutants grew at 37 °C, whereas CaCET1(203–520) cells did not (not shown). The ts growth phenotype of CaCET1(203–520) was suppressed by the introduction of a 2µ plasmid containing the S. cerevisiae guanylyltransferase gene CEG1 (not shown). Thus, the CaCET1 Δ202 mutant displayed the same phenotypes with respect to conditional growth and high copy suppression as did the Δ245 mutant of S. cerevisiae CET1.

The incremental deletions that result in lethality or conditional lethality of the TPI1-driven alleles highlight a conserved motif, PIWAQKWX, that may comprise part of the guanylyltransferase-binding site of yeast RNA triphosphatases (Fig. 12B). To begin to test this idea, we constructed two mutated versions of Cet1(201–549)p, P245A/I246A and K250A/W251A, in which pairs of neighboring amino acids within this conserved motif were replaced by alanine. Additional alanine cluster mutations, D225A/L226A, K237A/P238A, and K240A/Y241A, were introduced at conserved dipeptides just upstream of the PIWAQKWXP motif. The mutated residues are denoted by asterisks in Fig. 12B. The mutant genes were cloned into a CEN TRP1 vector under the control of the CET1 promoter and then tested by plasmid shuffle for complementation of a Δcet1 deletion strain. The D225A/L226A, K237A/P238A, K240A/Y241A, P245A/I246A, and K250A/W251A mutants were viable after selection on 5-FOA at 25 or 30 °C. D225A/L226A, K237A/P238A, K240A/Y241A, and P245A/I246A cells grew as well as wild type cells on rich medium at 25, 30, and 37 °C. In contrast, the K250A/W251A strain grew at 25 and 30 °C but not at 37 °C (Fig. 12B). The ts phenotype of the K250A/W251A mutant was suppressed by the introduction of CEG1 on a 2µ plasmid (Fig. 12B). These findings implicate the Lys250 and/or Trp251 side chains as constituents of a guanylyltransferase-binding site on yeast RNA triphosphatase.

Fusion of the Catalytic Domain of Yeast Triphosphatase (Δ275) to the Guanylyltransferase Domain of Mouse Capping
Enzyme Bypasses the in Vivo Requirement for a Ceg1p Interaction Domain—The experiment in Fig. 13 shows that expression of mammalian triphosphatase-guanylyltransferase in yeast can complement the growth of a Δcet1 Δceg1 strain (YBS50) in a plasmid shuffle assay. Growth of YBS50 depends on maintenance of a CEN URA3 CET1 CEG1 plasmid. Transformation of YBS50 with a CEN TRP1 plasmid containing MCE1 (the gene encoding the bifunctional mouse capping enzyme) under control of the yeast TRP1 promoter allowed growth of YBS50 on medium containing 5-FOA. A control transformation showed that MCE1(211–597), which encodes only the guanylyltransferase domain of mouse capping enzyme, was incapable of complementing Δcet1 Δceg1 (Fig. 13). We then tested the activity of two novel genes, CET1(201–547)-MCE1(211–597) and CET1(276–547)-MCE1(211–597), encoding chimeric capping enzymes in which segments of the yeast RNA triphosphatase [Cet1(201–547)p and Cet1(276–547)p] are fused to the mouse guanylyltransferase domain. Both chimeric genes were perfectly capable of supporting growth of the Δcet1 Δceg1 strain on 5-FOA (Fig. 13). Both chimeric strains grew normally on YPD medium at 37 °C (data not shown). The salient point of this experiment is that the in vivo requirement for Cet1p residues 241–275 (containing the putative binding site for yeast guanylyltransferase) can be obviated by linking the triphosphatase catalytic domain in cis to a heterologous guanylyltransferase.

Additional experiments showed that the chimeric gene CET1(301–547)-MCE1(211–597) was incapable of complementing Δcet1 Δceg1 (not shown). We infer from this result that the segment from residues 276 to 300 is important for Cet1p activity in a capacity other than guanylyltransferase binding. Efforts to test whether Cet1(301–549)p has catalytic activity have been hampered by the insolubility of the recombinant protein expressed in bacteria (2).

DISCUSSION

The work presented here contributes to an emerging model for the structural and functional organization of the yeast capping apparatus. Physical and genetic evidence shows that the triphosphatase component Cet1p consists of three domains as follows: (i) a 230-amino acid N-terminal segment that makes no discernible contribution to catalysis and is dispensable for Cet1p function in vivo; (ii) a protease-sensitive segment from residues 230 to 275 that is essential for Cet1p function in vivo and that mediates both Cet1p self-association and Cet1p binding to the yeast guanylyltransferase Ceg1p; (iii) an essential C-terminal catalytic domain that includes the conserved metal-dependent triphosphatase motifs A, B, and C.

Two lines of evidence argue strongly that the interaction of Cet1p with Ceg1p is essential. First, the complete loss of in vivo function with incremental N-terminal deletion of Cet1p to position 275 correlates with the loss of Ceg1p binding in vitro. Second, the in vivo function of Cet1(276–549)p can be restored by fusing this protein in cis to mouse guanylyltransferase, effectively bypassing the need for the guanylyltransferase interaction domain. A requirement for the assembly of a bifunctional triphosphatase-guanylyltransferase enzyme (whether in trans as in yeast or in cis as in mammals and the yeast-mouse chimeras) highlights an evolutionarily conserved strategy for targeting the cellular capping apparatus to nascent pre-mRNAs via the binding of the guanylyltransferase component to the phospho-
rlylated C-terminal domain (CTD) of elongating RNA polymerase II (9-11, 17). Both full-length mouse capping enzyme Mce1p and the C-terminal guanylyltransferase domain Mce1(121–597)p bind directly to the phosphorylated CTD, whereas the isolated N-terminal mouse triphosphatase domain Mce1(1–211)p has no capacity to bind the CTD (11, 18). In yeast, the guanylyltransferase per se also binds to CTD-PO44 (10), whereas the yeast triphosphatase alone does not (14).2 In effect, the guanylyltransferase chaperones the triphosphatase to the transcription complex. It is noteworthy that the mammalian guanylyltransferase can act as chaperone for the yeast triphosphatase, even though Cet1p is completely divergent in structure and catalytic mechanism from the mammalian RNA triphosphatase domain. This result implies that there is no vital functional interaction between the mammalian triphosphatase and guanylyltransferase domains that is unique or specific to the mammalian triphosphatase. It also suggests that the mammalian guanylyltransferase can act as a convenient vehicle to direct heterologous proteins to the transcription complex in vivo.

The genetic interaction whereby Cet1p overexpression suppresses a conditional mutation in Ceg1p (2, 14) is now reciprocated by the observation that Ceg1p overexpression suppresses the conditional phenotype of the 2p Cet1(246–549) strain. The simplest interpretation of this result is that Cet1(246–549)p is defective in its interaction with Ceg1p. However, our analysis of the sedimentation behavior of recombinant Cet1(246–549)p hints that the situation is more complex. The predominant form of the Δ245 protein sediments as discrete salt-stable multimer that does not interact in a stoichiometric fashion with Ceg1p. Yet, the minor “light” component of Δ245 does interact with Ceg1p in vitro. Hence, the in vivo phenotype of the Δ245 mutation might arise as follows: (i) at low gene dosage and under the control of its own promoter, there is insufficient light Δ245 to function in concert with Ceg1p and therefore yeast cells cannot grow; (ii) increased expression of Δ245 at 25 or 30 °C increases the level of triphosphatase available to interact with Ceg1p above the threshold required for viability; (iii) at 37 °C, the light-heavy equilibrium might be skewed toward the multimeric state such that the level of functional Δ245 dips below the threshold for viability; and (iv) increased expression of Ceg1p in vivo drives the equilibrium toward Δ245-Ceg1p heteromerization (and away from Δ245 multimerization) by mass action, thus restoring a suprathereshold level of active capping enzyme complex at 37 °C. The in vivo phenotype is apparently sensitive to changes in the multimerization equilibrium of the triphosphatase, given that the viable Δ240 mutant also forms a stable multimer in vitro, albeit to a lesser extent that does Δ245.

Overexpression of Ceg1p also suppressed the ts phenotype elicited when the K250A/W251A mutant of Cet1p is present in single copy from the native CET1 promoter. Preliminary characterization of recombinant Cet1p(201–549)-K250A/W251A protein revealed the following: (i) as expected, the mutant enzyme displayed full activity in manganese-dependent ATP hydrolysis in vitro, and (ii) the mutant protein sedimented as a discrete peak coincident with BSA (this peak comprised 94% of the ATPase activity), and only trace amounts of a heavy component (comprising 6% of the ATPase activity) were detected in the 11–12 S size range.3 Thus, the in vivo effects of the K250A/W251A mutation are unlikely to be mediated by the overzealous multimerization observed for the Δ245 deletion mutant. Instead, we hypothesize that elimination of

\[ Lys^{250} \text{ and Trp}^{251} \] side chains simply lowers the affinity of Cet1p for Ceg1p at restrictive temperature and that Ceg1p overexpression can compensate to restore a threshold level of the heteromeric complex. In preliminary sedimentation analysis, we found that Cet1(201–549)-K250A/W251A interacted normally with Ceg1p; this is not surprising given that the sedimentation is performed at low temperature. Our working model is that Lys^{250} and/or Trp^{251} comprise part of the guanylyltransferase-binding site on S. cerevisiae RNA triphosphatase. Sequence similarity between S. cerevisiae and C. al-

bicans Cet1p suggests that the putative guanylyltransferase-binding site is conserved in other fungal RNA triphosphatases.

There has been uncertainty in the literature concerning the native size and subunit stoichiometry of the yeast triphosphatase-guanylyltransferase complex. Itoh et al. (19) initially reported the purification of a bifunctional complex from yeast cell extracts that consisted of a 45-kDa guanylyltransferase α-subunit and a 39-kDa RNA triphosphatase β-subunit. This enzyme sedimented at 7.3 S in a glycero gradient calibrated with internal standards. From this datum, the authors estimated a native size of 140-kDa and proposed that the enzyme was a α2β2 heterotetramer (19). An improved purification from yeast extracts yielded an enzyme, composed of a 52-kDa guanylyltransferase α-subunit and a 80-kDa RNA triphosphatase β-subunit, that sedimented at 9.0 S in a glycero gradient (20). It is now known that the 80-kDa triphosphatase subunit corresponds to full-length Cet1p, which has an actual size of 62-kDa, but migrates anomalously slowly during SDS-PAGE (1, 2). We surmise that the 39-kDa triphosphatase subunit present in the enzyme purified initially is a proteolytic fragment of Cet1p comprising the carboxyl catalytic domain. The 45-kDa form of guanylyltransferase is probably a proteolytic fragment lacking a nonessential segment at the C terminus (21). Yamada-Okabe et al. (15) recently suggested that the 9.0 S enzyme capping enzyme isolated from yeast cells (with an estimated size of 180 kDa) is either an α2β1 or α1β2 trimer. Based on the sedimentation analysis in the present study, we suggest that the enzyme complex reconstituted in vitro from separately expressed Cet1(201–549)p and Ceg1p proteins is a Ceg1p-[Cet1(201–549)p]2 trimer and that Cet1(201–549)p by itself is a homodimer. An α1β2 trimer structure for the capping enzyme complex raises the prospects that either (i) the binding of one Ceg1p to one of the Cet1(201–549)p protomers of the dimer occludes the Ceg1p-binding surface of the other Cet1(201–549)p protomer or (ii) moieties on both triphosphatase protomers contribute to form a single guanylyltransferase-binding site.

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