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A *Saccharomyces cerevisiae* RNA 5′-triphosphatase related to mRNA capping enzyme

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ABSTRACT

The *Saccharomyces cerevisiae* mRNA capping enzyme consists of two subunits: the RNA 5′-triphosphatase (Cet1) and the mRNA guanylyltransferase (Ceg1). Using computer homology searching, a *S.cerevisiae* gene was identified that encodes a protein resembling the C-terminal region of Cet1. Accordingly, we designated this gene *CTL1* (capping enzyme RNA triphosphatase-like 1). *CTL1* is not essential for cell viability and no genetic or physical interactions with the capping enzyme genes were observed. The protein is found in both the nucleus and cytoplasm. Recombinant Ctl1 protein releases γ-phosphate from the 5′-end of RNA to produce a diphosphate terminus. The enzyme is specific for polynucleotide RNA in the presence of magnesium, but becomes specific for nucleotide triphosphates in the presence of manganese. Ctl1 is the second member of the yeast RNA triphosphatase family, but is probably involved in an RNA processing event other than mRNA capping.

INTRODUCTION

RNA 5′-triphosphatase activity is widely distributed among prokaryotes, eukaryotes and viruses. Perhaps the most extensively characterized RNA triphosphatases are those required for the first step of eukaryotic and viral mRNA capping. The triphosphatase removes the γ-phosphate from the 5′-end of mRNA and guanylyltransferase adds GMP to the resulting mRNA diphosphate end in a 5′–5′ orientation (1,2). The cap structure is then further modified by one or more methyltransferases.

In most mRNA capping systems studied to date, the mRNA triphosphatase is associated with a GTP::mRNA guanylyltransferase to form mRNA capping enzyme. However, several configurations are seen. Capping enzyme from vaccinia virus purifies as a heterodimer complex carrying all three capping activities (1,2). The larger subunit has both triphosphatase and guanylyltransferase activities, while both subunits are required to reconstitute methyltransferase activity (1). Cellular capping enzymes are bifunctional, carrying RNA triphosphatase and guanylyltransferase activities. In higher eukaryotes, there is a single polypeptide with two separable domains that carry one or the other activity (3–8). A similar arrangement appears to be used by the baculovirus capping enzyme (9–11). The capping enzyme from trypanosomatid protozoa has also been proposed to be a bifunctional monomer, although RNA triphosphatase activity has not been demonstrated yet (12). In the yeasts *Saccharomyces cerevisiae* and *Candida albicans*, guanylyltransferase and triphosphatase activities are carried by distinct proteins that form a heterodimer complex (13–18). *Chlorella* virus encodes a monofunctional guanylyltransferase (19), but a mRNA triphosphatase protein has not yet been identified so it is unclear whether the two activities are complexed. In all eukaryotic systems studied to date, the mRNA methyltransferase is encoded by a different gene (20–22) and purifies as an unassociated enzyme.

Sequence analysis of the mRNA triphosphatases suggests that several distinct enzyme types exist. The higher eukaryotic mRNA triphosphatase domain contains the consensus active site motif (I/V)HCxxGxxR(S/T) found in protein tyrosine phosphatases (PTPs) (3–8). The conserved nucleophilic cysteine is required for activity (3,23–25), suggesting that these capping enzymes are mechanistically similar to PTPs (26,27). The vaccinia virus and baculovirus enzymes are not related to PTPs, but may have weak similarity to each other (9–11,28).

The *S.cerevisiae* RNA triphosphatase subunit is encoded by the essential *CET1* gene (14). This gene encodes a 62 kDa protein that is not obviously related to either PTPs or the viral enzymes, although some very short sequence similarities in the yeast and viral enzymes have been proposed to represent RNA triphosphatase motifs (11,28). Cet1 mRNA triphosphatase activity has been localized to its C-terminal region (14–16), but the reaction mechanism remains uncharacterized.

In this report, we describe the identification of a protein from *S.cerevisiae* that is significantly similar to the C-terminal portion of the capping enzyme RNA triphosphatase subunits from *S.cerevisiae* (Cet1) and *C.albicans* (CaCet1). We designated the gene encoding this protein *CTL1*, for capping enzyme triphosphatase-like protein 1. Enzymatic properties, cellular localization and ability to interact with the mRNA capping enzyme were determined for Ctl1. Like Cet1 and CaCet1, bacterially produced Ctl1 protein removes γ-phosphate from RNA to leave a diphosphate end. Genetic analyses show that *CTL1* is not essential for cell viability and apparently does not interact with the capping enzyme guanylyltransferase or triphosphatase. Although the physiological substrate of this enzyme is not yet known, it appears that Ctl1 is a yeast RNA 5′-triphosphatase that is not part of the mRNA capping apparatus.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

MATERIALS AND METHODS

Sequence analysis

Protein sequence similarity searching was carried out on the National Center for Biotechnology Information Web server using the BLAST algorithm (29). Sequence alignments were made using SEQVU.

DNA cloning methods

PCR was performed with Vent DNA polymerase (New England BioLabs) using oligonucleotide primers. A 1.9 kb fragment carrying the entire open reading frame (ORF) and flanking sequences of the *CTL1* gene (GenBank accession no. Z49808) was amplified from *S.cerevisiae* genomic DNA using oligonucleotide primers CTL1-A and CTL1-B, creating *Bgl*II sites at both ends of the fragment. A 1.4 kb fragment carrying the *CTL1* ORF was amplified using primers CTL1-B and CTL1-C, creating a *Nco*I site at the initiation codon and *Bgl*II sites at both ends of the fragment. The sequences of the PCR primers are as follows: CTL1-A, 5′-G**AGATCT**CCAGTTACAAATCTCCCAAACG-TATTCCT-3′ (*Bgl*II site in bold); CTL1-B, 5′-G**AGATCT**CAT-GAAGAAGTTTTGGATGAAGAGGAA-3′ (*Bgl*II site in bold); CTL1-C, 5′-GC**AGATCT**CCATGGCTGACCAACCCGAGAC-TCCTTCC-3′ (*Nco*I site underlined, *Bgl*II site in bold).

The 1.9 kb PCR product containing the entire gene was subcloned into pCR-Script SK+ (Stratagene) to generate pBS-CTL1. To generate a deletion construct for *CTL1*, the 3.9 kb *Afl*II–*Sna*BI backbone fragment from pBS-CTL1 was blunted with *Escherichia coli* DNA polymerase I Klenow fragment and ligated to a 3519 bp *Sma*I fragment carrying a *LEU2-KAN*^{R} cassette from plasmid pJA51 (30). The resulting plasmid, pBS-*ctl1*Δ*2*::*LEU2/KAN*^{R}, replaces all of the ORF sequence except for the last 40 bp. High copy plasmid pRS426-CTL1 was generated by ligating a 1.9 kb *Not*I–*Sal*I fragment from pBS-CTL1 into the *Not*I and *Sal*I sites of pRS426 (2μ, *URA3*, *AMP*^{R}).

The 1.4 kb fragment containing the *CTL1* ORF was also cloned into pCR-Script SK+ to generate pBS-CTL1orf. A 1.2 kb *Nco*I fragment was then excised and cloned into the *Nco*I site of pSBET-his$_7$, a modified version of pSBETa (31) that contains a cassette coding for seven histidines at the N-terminus of the protein. The sequence for the cassette was as follows: 5′-aga agg aga tat acC ATG CAT CAC CAT CAC CAT CAC CAT ATG G**cc atg g**ct-3′ (*Nco*I site in bold, *Nde*I site underlined, vector lower case, cassette upper case). The resulting plasmid, pSBET-his$_7$CTL1, produces polyhistidine-tagged Ctl1 protein.

A 1.3 kb *Bgl*II fragment from pBS-CTL1orf was subcloned into the *Bam*HI site of pPS809 (a gift from Pam Silver, Dana Farber Cancer Institute, Boston, MA). The resulting plasmid, pSP809-*CTL1*, encodes a fusion protein between green fluorescent protein (GFP) and Ctl1 controlled by a galactose-inducible promoter and also carries *URA3* and a 2μ origin of replication.

Yeast strains

YSB455 [*MAT***a**/*MAT*α, *ura3-52*/*ura3-52*, *leu2*Δ*1*/*leu2*Δ*1*, *trp1*Δ*63*/*trp1*Δ*63*, *his3*Δ*200*/*his3*Δ*200*, *lys2*Δ*202*/*lys2*Δ*202*] was transformed with the 4.3 kb *Sac*II–*Bam*HI fragment of pBS-*ctl1*Δ*2*::*LEU2/KAN*^{R} and Leu$^+$ transformants were selected and sporulated. Tetrads were dissected and Leu$^+$:Leu$^-$ phenotypes segregated 2:2. Leu$^+$ spores were viable: YSB612 [*MAT***a**, *ura3-52*, *leu2*Δ*1*, *trp1*Δ*63*, *his3*Δ*200*, *lys2*Δ*202*, *ctl1*Δ*2*::*LEU2/KAN*^{R}] and YSB613 [*MAT*α, *ura3-52*, *leu2*Δ*1*, *trp1*Δ*63*, *his3*Δ*200*, *lys2*Δ*202*, *ctl1*Δ*2*::*LEU2/KAN*^{R}]. YSB517 has been described previously [*MAT*α, *ura3*, *leu2*, *trp1*, *his3*, *ade2*, *ade3*, *can1*, *ceg1-250*] (32). YSB491 [*MAT***a**, *ura3*, *leu2*, *trp1*, *his3*, *ade2*, *ade3*, *can1*, *ceg1-250*] was generated in parallel with YSB517, except the mating type was not changed. Plasmids were introduced into yeast using the modified lithium acetate transformation protocol and media preparation and other yeast manipulations were performed by standard methods as described (33).

Expression of GFP fusion proteins

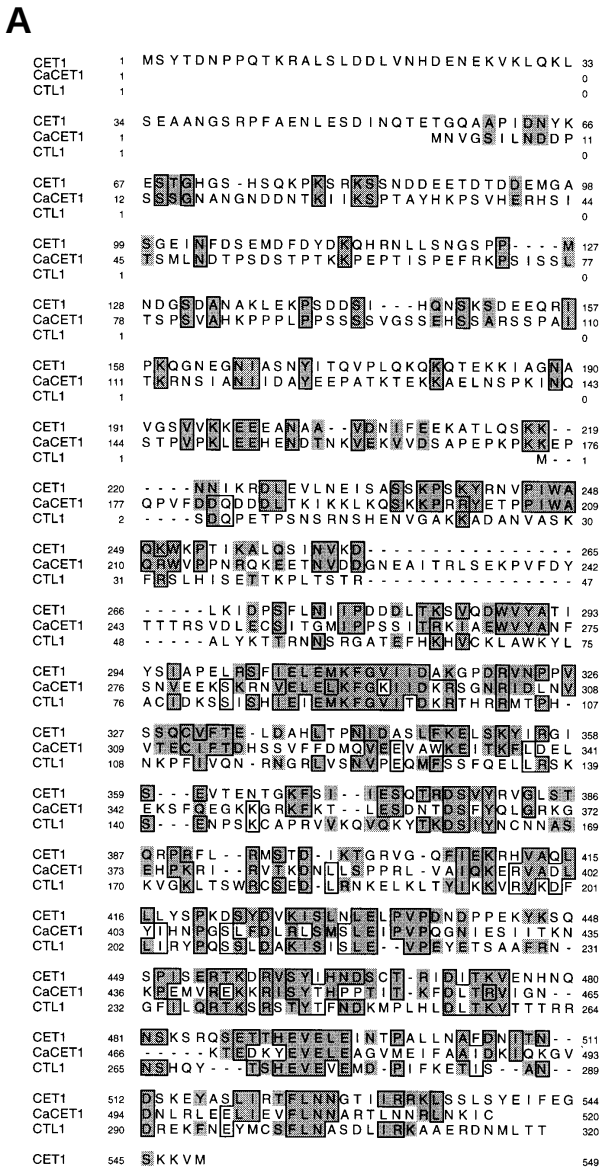
YSB613 was transformed with either pPS809 (expressing GFP), pPS809-*CTL1* (expressing a GFP–Ctl1 fusion) or pPS934 (a control expressing GFP fused to the nuclear spindle pole body protein Nuf2; 34). Cells were grown overnight at 30°C in selective synthetic complete media (lacking tryptophan for pPS934, lacking uracil for the others) with 2% raffinose. Cells were then washed and resuspended in 80 ml of medium plus 2% galactose. Aliquots of 20 ml were removed at 0, 2, 4 and 6 h post-induction and extracts were prepared as described (35). Immunoblot analysis was performed with 20 μg protein of whole cell extract per time point and fusion proteins were detected using anti-GFP polyclonal serum (a gift from P. Silver).

For visualization of GFP fusion proteins *in vivo*, cells were grown in raffinose medium to OD_{600} = 1. Cells were washed with sterile water and then grown in selective medium plus 2% galactose for 6 h. To visualize DNA, 0.2 μg/ml of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and 30% ethanol were added. Cell suspensions (2 μl) were photographed using Nomarski optics and appropriate filters for DAPI and GFP signals.

Recombinant protein production and purification

his$_7$-Ctl1 was expressed using a T7 promoter/polymerase system in *E.coli* strain BL21(DE3) (36) transformed with pSBET-his$_7$*CTL1*. Induction was performed as described (37). All further operations were carried out at 0–4°C. Lysates were prepared by sonication in buffer A [20 mM HEPES–KOH, pH 7.9, 10% v/v glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF)] with 300 mM KCl and 0.5% (v/v) NP-40. Soluble extracts (100 000 *g* supernatant fraction) were incubated in batch with Ni^{2+}–NTA–agarose resin (Qiagen) overnight on a rotator. The resin was then poured into a column and extensively washed with buffer A with 20 mM KCl and 25 mM imidazole. Bound protein was eluted with buffer A containing 20 mM KCl and 600 mM imidazole. Fractions were dialyzed overnight against buffer B (20 mM HEPES–KOH, pH 7.9, 10% v/v glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) with 20 mM KCl and then loaded onto a column of CM-Sephadex C-50 (Pharmacia). The column was washed with buffer B containing 50 and then 250 mM KCl and proteins were eluted using a linear gradient of 250–750 mM KCl in buffer B. Fractions were assayed for RNA triphosphatase activity (19) and analyzed by immunoblotting with anti-polyhistidine (6×His) antibody (Clontech). Fractions with activity were dialyzed overnight against buffer B with 20 mM KCl and further purified by column chromatography over Mono S (HR 5/5; Pharmacia) using a gradient of 20–1000 mM KCl in buffer B.

Polyhistidine-tagged full-length (his$_7$-Cet1) and N-terminal truncated [his$_7$-Cet1(265–549)] proteins of *S.cerevisiae* mRNA capping enzyme RNA triphosphatase subunit were expressed and



partially purified through a Ni^{2+}–NTA–agarose column as described (12). For further purification of his_7-Ctl1 protein, column chromatography with Mono Q (HR 5/5) was performed with a linear gradient of 50–1000 mM KOAc in buffer B. his_7-Cet1(265–549) proteins were passed over a CM-Sephadex C-50 column with a linear gradient of 50–600 mM KCl in buffer C (20 mM MOPS–KOH, pH 7.0, 10% v/v glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF).

RNA triphosphatase assay and nucleotide phosphohydrolase assay

RNA 5′-triphosphatase activity was assayed using RNA synthesized with T7 primase as described (24). The standard reaction mixture contained in 10 µl: 50 mM Tris–HCl (pH 7.9), 5 mM $MgCl_2$, 5 mM DTT, 50 µg/ml BSA, 3–5 µM (of termini) of either [α-^{32}P]- or [γ-^{32}P]ATP-terminated trimeric RNA (500–2000 c.p.m./pmol) and the enzyme preparation to be assayed. Incubation was for 10 min at 30°C and the reaction was terminated by the addition of 1 M formic acid. RNA was chromatographed on polyethyleneimine (PEI)–cellulose thin-layer plates (J. T. Baker) with 0.5 M KH_2PO_4 (pH 3.5). Radioactivity was detected using a Fuji BasX Phosphor-Imager. For quantitation of the activity, released phosphate from the triphosphate end of RNA was detected by autoradiography of the PEI–cellulose plates. [^{32}P]P_i spots were cut out and the radioactivity was counted by liquid scintillation. Assays were performed in the linear range. To assay nucleotide phosphohydrolase activity, the RNA substrate was replaced with 5 µM [γ-^{32}P]ATP.

RESULTS

CTL1 is similar to the C-terminal region of RNA 5′-triphosphatase subunit of yeast mRNA capping enzyme

The BLAST algorithm (29) was used to search GenBank for genes with significant similarity to the *S.cerevisiae CET1* gene, which encodes the RNA 5′-triphosphatase subunit of mRNA capping enzyme (14). The highest score was to a hypothetical 37 kDa protein from *S.cerevisiae* with 320 amino acids, encoded by the ORF YMR180C (GenBank accession no. Z49808). More recently, Yamada-Okabe *et al.* (18) identified *CaCET1*, the *C.albicans* capping enzyme RNA triphosphatase subunit gene. The alignment of deduced amino acid sequences from these three genes is shown in Figure 1A. YMR180C aligns with the C-terminal region of Cet1 and CaCet1 and this same region has been shown to carry RNA triphosphatase activity in Cet1 (14–16; Fig. 1B). Accordingly, we designated this gene *CTL1*, for capping enzyme RNA triphosphatase-like 1.

Figure 1. *CTL1* encodes a non-essential protein similar to the yeast mRNA capping enzyme RNA triphosphatase subunit. (**A**) Alignment of Ctl1 with the RNA 5′-triphosphatase subunit of mRNA capping enzyme from *S.cerevisiae* (*CET1*; 14) and *C.albicans* (*CaCET1*; 18). 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. (**B**) Schematic representation of homology between Ctl1 and Cet1. Cet1 subdomains responsible for Ceg1 interaction and for RNA triphosphatase activity are labeled (16). (**C**) *CTL1* is not essential for viability. A diploid strain with the genotype *CTL1*/*ctl1Δ2*::*LEU2* was sporulated and dissected. Shown is one representative tetrad which has been streaked to YPD or synthetic complete medium lacking leucine at 30°C. *CTL1* and *ctl1Δ2*::*LEU2* spores are labeled.

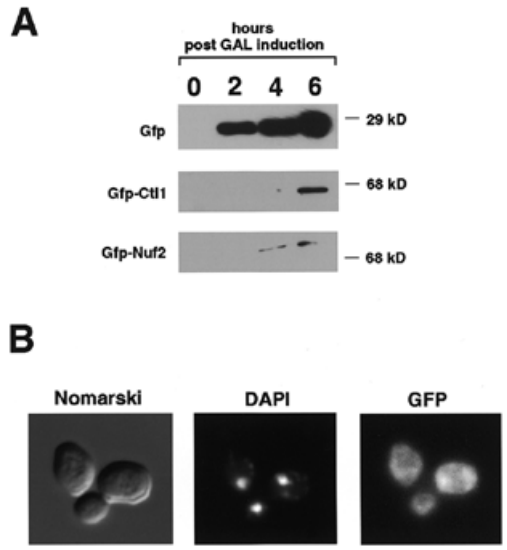


Figure 2. Ctl1 is found throughout the cell. A *ctl1Δ2*::*LEU2* strain was transformed with plasmids encoding GFP, GFP–Ctl1 or GFP–Nuf2. Expression of these proteins was induced by growth in galactose-containing medium. (**A**) Immunoblot analysis of whole cell extracts from strains bearing GFP fusion proteins. Cells were induced with galactose and aliquots were removed at the indicated time points. Aliquots of 20 µg of extract protein were analyzed by SDS–PAGE and immunoblotting using anti-GFP serum. Predicted sizes of proteins are: GFP, 28 kDa; GFP–Ctl1, 65 kDa; GFP–Nuf2, 81 kDa. (**B**) Cells bearing the GFP–Ctl1 fusion protein were induced with galactose for 6 h and treated with DAPI to stain DNA. Cells were photographed using Nomarski optics and DAPI and GFP filters.

CTL1 is not required for cell viability

To characterize the function of *CTL1* in yeast, we tested whether the gene is essential for viability. One copy of *CTL1* was replaced with *LEU2* in a diploid yeast strain (Fig. 1B). Upon sporulation, leucine auxotrophy segregated 2:2 in tetrads and all four spores of each tetrad were viable. A representative tetrad at 30°C is shown in Figure 1C. Disruption of the *CTL1* gene in Leu^+ haploids was confirmed by Southern blotting (not shown). Tetrads were further examined for mutant phenotypes at 15, 30 and 37°C and on media containing 15 mM caffeine or lacking inositol. Under all conditions tested, *ctl1*Δ spores were indistinguishable from *CTL1* spores. Therefore, *CTL1* is not essential for viability in yeast.

Ctl1 is found throughout the cell

To analyze the localization of Ctl1 protein, a fusion of GFP with full-length Ctl1 (GFP–Ctl1) was expressed in the *ctl1*Δ strain under the control of a galactose-inducible promoter. As controls, GFP and GFP–Nuf2 were expressed under the same conditions. Whole cell extracts were prepared at various times after induction and levels of GFP fusion proteins were monitored by immunoblotting (Fig. 2A). Extracts from strains transformed with vector alone were not recognized by the antiserum (not shown). All three GFP proteins were clearly present after 4–6 h induction. Bands were seen only at the predicted molecular weights (GFP, 28 kDa; GFP–Ctl1, 65 kDa; GFP–Nuf2, 81 kDa), indicating that these proteins are not significantly proteolyzed in cells.

Localization of the GFP signal was determined by fluorescence microscopy of cells that had been induced for 6 h. As expected, GFP was present throughout the cell (data not shown). Cells with GFP–Nuf2 displayed prominent nuclear fluorescence, which was expected for the nuclear spindle pole body protein (data not shown). Cells expressing the GFP–Ctl1 fusion fluoresced throughout the nucleus and cytoplasm (Fig. 2B). By comparing the GFP signal to DAPI staining of nuclei, it is clear that the fusion protein is distributed in both the nucleus and the cytoplasm.

CTL1 has no obvious role in 5′ mRNA cap formation

In yeast, the capping enzyme triphosphatase Cet1 forms a heterodimer with the guanylyltransferase Ceg1 (14–16,38). To determine if Ctl1 might be partially redundant with Cet1 and have a role in 5′ mRNA cap formation, several genetic experiments were performed. First, a *CTL1* deletion was crossed with mutant alleles of *CEG1*, *CET1* or the RNA polymerase II C-terminal domain (pol II CTD) to see if the mutant phenotype was enhanced. As a precedent, we previously observed that combining a partial deletion (*rpb1-101*) of the pol II CTD with the guanylyltransferase conditional mutant *ceg1-250* results in a synthetic lethal phenotype, confirming their interaction *in vivo* (32). A *ctl1*Δ haploid strain was crossed with a *ceg1-250* haploid strain and diploids were sporulated and dissected. Spores were identified that were *ctl1*Δ *ceg1-250*. These spores were viable and indistinguishable from *ceg1-250* cells (data not shown). Similarly, when a *ctl1*Δ haploid was crossed with a temperature-sensitive allele of *CET1* (*cet1-4*; C.R.Rodriguez, unpublished data), *ctl1*Δ *cet1-4* strains were recovered which had growth characteristics identical to a *cet1-4* strain (data not shown). Double mutant strains carrying *ctl1*Δ and the *rpb1-101* partial truncation of the pol II CTD were viable and behaved similarly to strains with *rpb1-101* alone (data not shown).

Overexpression of *CET1* can suppress the temperature sensitivity of certain *ceg1* alleles (15,16). To test whether *CTL1* overexpression could rescue capping enzyme mutants, a high copy plasmid bearing *CTL1* was transformed into strains bearing conditional alleles of either *ceg1* or *cet1* or partial truncations of the pol II CTD (32,39; unpublished data). No improvement in growth of any of the capping enzyme mutants was seen (data not shown). Therefore, genetic experiments fail to suggest a role for *CTL1* in 5′ mRNA cap formation.

In addition to the genetic experiments, *in vitro* protein interaction experiments were performed. Under conditions where the capping enzyme subunits Ceg1 and Cet1 bind, co-immunoprecipitation experiments failed to show any interaction between Ctl1 and the capping enzyme guanylyltransferase (data not shown).

Ctl1 has RNA triphosphatase activity that leaves a diphosphate terminus

To determine whether the Ctl1 protein has RNA triphosphatase activity, it was expressed in *E.coli* as a polyhistidine-tagged protein (his_7-Ctl1) and purified by column chromatography over Ni^{2+}–NTA–agarose, CM-Sephadex C-50 and MonoS-FPLC. The nickel column eluate contained a 37 kDa protein that was not produced by cells carrying vector alone (Fig. 3A, lanes 1 and 2). In two subsequent chromatography steps, his_7-Ctl1 co-purified exactly with 5′ RNA triphosphatase activity (Fig. 3B shows the CM-Sephadex elution). Endogenous RNA triphosphatase activity

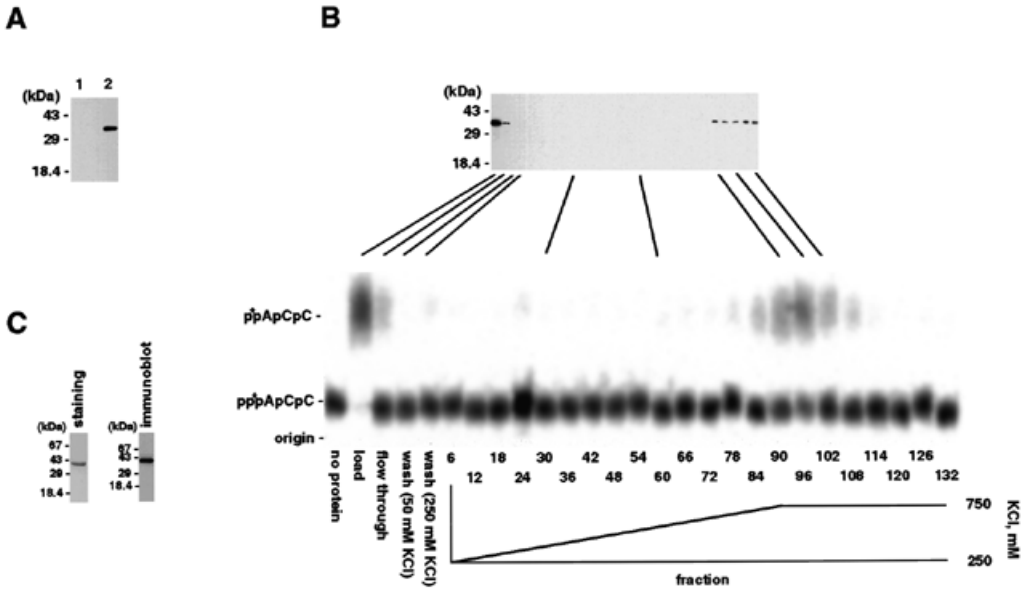


Figure 3. Purification of polyhistidine-tagged Ctl1 protein (his$_7$-Ctl1). (**A**) Ni^{2+}–NTA–agarose column chromatography. Soluble extract from *E.coli* strain BL21(DE3) transformed with either pSBET-his$_7$ (lane 1) or pSBET-his$_7$ Ctl1 (lane 2) was incubated overnight with Ni^{2+}–NTA–agarose. Bound proteins were then eluted with 600 mM imidazole and 10 µg protein was analyzed by immunoblotting with anti-6×His monoclonal antibody. (**B**) CM-Sephadex C-50 column chromatography. Nickle column eluate was loaded onto a CM-Sephadex C-50 column and eluted with a linear gradient of 250–750 mM KCl. Fractions were assayed by immunoblotting with anti-6×His antibody (upper panel) and for RNA triphosphatase activity (lower panel). For the RNA triphosphatase assay, [α-^{32}P]ATP-terminated trinucleotide RNA (pp**p**ApCpC) was used as a substrate and the reaction mixtures were analyzed by thin layer chromatography (TLC) on PEI–cellulose plates. ^{32}P label (asterisk) was detected by PhosphorImager. The position of ADP-terminated trinucleotides (ppACC) was determined using ADP-terminated trimers as standard (24; not shown). (**C**) Mono S column chromatography. Aliquots of 1 µg of protein from the peak fraction of the Mono S column were analyzed by SDS–PAGE and visualized by Coomassie brilliant blue staining (left) and immunoblotting with anti-6×His antibody (right). This preparation was used for subsequent enzymological analysis.

from *E.coli* (40) was completely separated from his$_7$-Ctl1 by these two columns.

The substrate specificity of Ctl1 was analyzed using the Mono S fraction (Fig. 3C) and compared to that of Cet1 (Fig. 4). C-terminal fragments containing amino acids 205–549 or 246–549 of Cet1 have been shown to have RNA triphosphatase activity (14,15). We constructed and purified a shorter C-terminal fragment of Cet1 (amino acids 265–549) that corresponds to the region with similarity to Ctl1 (Fig. 1). All three proteins [his$_7$-Cet1, his$_7$-Cet1(265–549) and his$_7$-Ctl1] released phosphate from [γ-^{32}P]ATP-terminated trimeric RNA (**p**ppApCpC) (Fig. 4A), but not from [γ-^{32}P]ATP (**p**ppA) (Fig. 4C). Using [α-^{32}P]ATP-terminated trimeric RNA (pp**p**ApCpC) as substrate, we found that both Cet1 and Ctl1 left diphosphate-ended RNA (p**p**ApCpC; Fig. 4B). The specific activities of his$_7$-Cet1 and his$_7$-Ctl1 under standard reaction conditions were 25 000 and 1500 nmol γ-phosphate released/min/mg protein, respectively.

It has been shown that full-length Cet1 RNA triphosphatase depends on magnesium (14,28,41). The effects of changing pH and divalent cation concentration on the RNA triphosphatase activities of Cet1(265–549) and Ctl1 proteins were determined (Fig. 5). The activities of these proteins are significantly inhibited below pH 7.0 and showed an optimum between 7.5 and 8.0 (Fig. 5A). Both enzymes require divalent cations for activity (Fig. 5B), but Cet1(265–549) activity was optimal at 5 mM Mg^{2+} and inhibited at higher concentrations. In contrast, the activity of Ctl1 was stimulated by up to 30 mM Mg^{2+}. Also, neither Cet1 nor Ctl1 was inhibited by 10 mM sodium vanadate (data not shown).

Cet1 RNA triphosphatase is specific for polynucleotide RNA in reactions containing magnesium (14,28,41), as is Ctl1 (Fig. 4). Ho *et al.* (28) recently showed that Cet1 exhibits nucleotide phosphohydrolase (NTPase) activity in the presence of manganese or cobalt. We confirmed this result, finding that Cet1 releases γ-phosphate from ATP (**p**ppA) in the presence of 0.001–30 mM $MnCl_2$. RNA triphosphatase activity of Cet1 on an RNA trimer (**p**ppACC) was comparable using either magnesium or manganese (data not shown). For comparison, we tested the effects of different divalent cations on the RNA triphosphatase and ATPase activities of Ctl1 (Fig. 6). Interestingly, Ctl1 behaved quite differently from Cet1. Whereas the capping enzyme triphosphatase could use either cation to support RNA triphosphatase activity, Ctl1 was completely inactive on trimer RNA in the presence of manganese (Fig. 6A). Like Cet1, Ctl1 exhibited ATPase activity only in the presence of manganese (Fig. 6B).

DISCUSSION

We have characterized a previously unidentified *S.cerevisiae* ORF, YMR180C. We designated this gene *CTL1* because it shares considerable sequence similarity and catalytic properties with the *S.cerevisiae* and *C.albicans* capping enzymes (14–16,18). Mutational analysis of the Cet1 protein (28) identified several catalytically essential glutamate and arginine sidechains that are conserved in Ctl1 (Fig. 1). All of these observations suggest that the reaction mechanisms of Cet1 and Ctl1 are similar.

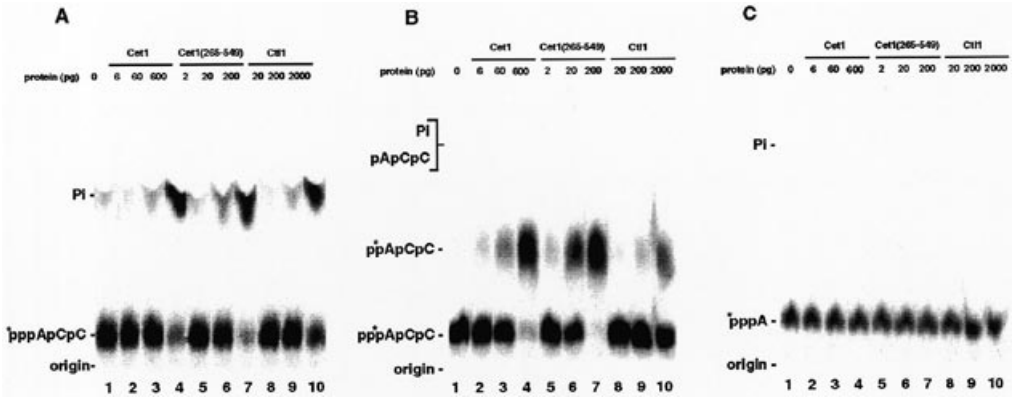


Figure 4. Substrate specificity of *S.cerevisiae* RNA triphosphatases. Reactions were analyzed as in Figure 3. The positions of ppACC and pACC were determined using ADP- and AMP-terminated trimers as standards (24; not shown) and the position of phosphate was determined by treating the substrate with calf intestinal phosphate (CIP) (not shown). (**A**) RNA triphosphatase activity. The indicated amounts of full-length (Cet1; lanes 2–4) or C-terminal fragment [Cet1(265–549); lanes 5–7] of mRNA capping enzyme RNA triphosphatase subunit and Ctl1 (lanes 8–10) were assayed with 5 μM (of termini) [γ-^{32}P]ATP-terminated trinucleotide RNA (**p**ppApCpC) as substrate. (**B**) Ctl1 and Cet1 leave a 5′ diphosphate end. Reactions in lanes 1–10 were identical to those in (A), except [γ-^{32}P]ATP-terminated trinucleotide RNAs were replaced with [α-^{32}P]ATP-terminated trinucleotide RNAs (pp**p**ApCpC). (**C**) Nucleotide phosphohydrolase activity. Reactions in lanes 1–10 were identical to those in (A), except [γ-^{32}P]ATP-terminated trinucleotide RNAs were replaced with 5 μM [γ-^{32}P]ATP (**p**ppA).

The capping enzyme triphosphatase Cet1 is an essential nuclear protein (14,38). In contrast, the Ctl1 protein is found throughout the cell and is not essential for viability (Figs 1C and 2B). Since Cet1 is tightly associated and co-purifies with Ceg1 (14–16,41), we tested whether Ctl1 interacts with yeast capping enzyme, both physically and functionally. Neither co-immunoprecipitation nor genetic interaction tests suggested any connection. Consistent with these results, the minimal region of Cet1 required for heterodimerization with Ceg1 is amino acids 205–265 (16), which is not conserved in Ctl1 (Fig. 1A and B).

The *S.cerevisiae* RNA triphosphatase proteins are distinct from the PTP-like RNA triphosphatase domains of higher eukaryotic capping enzymes (3–8). This study (Fig. 4B) and others (15,28,41) have shown that the two classes of RNA phosphatases can be distinguished by their dependence on divalent cations: both Cet1 and Ctl1 require magnesium for activity, while the mouse and *C.elegans* RNA triphosphatases are metal independent (23,25; T.Takagi, unpublished observation). Another difference is sensitivity to vanadate. Vanadate inhibits PTPs by acting as a transition state mimic (42) and also inhibits the mouse and *C.elegans* RNA triphosphatases (25; T.Takagi, unpublished observation). However, vanadate does not affect the Cet1 and Ctl1 triphosphatase activities (data not shown).

In the presence of magnesium, Cet1 and Ctl1 remove the γ-phosphate from polynucleotide RNA, but not NTPs. The PTP-like RNA triphosphatase of higher eukaryotic capping enzymes is also specific for polynucleotide RNA substrates (3,23,43). However, Ho *et al*. (28) showed that Cet1 is highly active as a nucleotide phosphohydrolase in the presence of manganese or cobalt. We similarly found that replacement of magnesium with manganese uncovered ATPase activities of Ctl1 (Fig. 6B) and Cet1 (data not shown). Ctl1 and Cet1 differ in one aspect: in the presence of manganese the RNA triphosphatase activity of Cet1 was unaffected (data not shown) but that of Ctl1 was inhibited (Fig. 6A). The optimal concentration of manganese for Ctl1 ATPase activity is ~1 mM (Fig. 6B), lower than that of magnesium for RNA triphosphatase activity (Figs 5B and 6A). The specific activity of ATPase in the presence of 10 mM $MnCl_2$ is of the same order as that of RNA triphosphatase with magnesium (Figs 5B and 6A). Although we suspect that Ctl1 functions in processing of some RNA 5′-ends, the fact that it has ATPase activity in the presence of manganese raises the formal possibility that the *in vivo* substrate of Ctl1 is NTP.

Analysis of the *S.cerevisiae* genome suggests that a large scale duplication event occurred during yeast evolution (44). A region on chromosome XIII that includes *CTL1* is clearly related to a region on chromosome XVI containing *CET1*. It is interesting to speculate upon two possible scenarios. Perhaps Cet1 evolved from Ctl1 and subsequently developed the ability to bind to the capping enzyme guanylyltransferase subunit. Alternatively, Ctl1 may be derived from Cet1 but has lost the ability to associate with the capping machinery. It is striking that Ctl1 has retained functional RNA triphosphatase activity despite the fact that it is not essential. Genomic expression analysis shows that the *CTL1* gene is in fact transcribed in yeast cells (45,46). This suggests that Ctl1 has some important function that remains to be discovered, perhaps in RNA degradation or in processing an RNA other than mRNA.

The idea that RNA triphosphatases are important for processes other than capping is supported by their widespread occurrence over evolution. Maitra and Hurwitz (40) reported the characterization of an enzyme from *E.coli* which releases a γ-phosphate from the 5′-end of RNA. Prokaryotic RNA is not capped, so this enzyme must have some other function. Recently, we (24) and others (47) characterized an RNA phosphatase (known as BVP, BVH1 or BV-PTP) carried by the baculovirus *Autographa californica*. This viral enzyme is a member of the PTP family and has significant sequence similarity to the RNA triphosphatase domains of higher eukaryotic capping enzymes (3,4). However, the BVP RNA phosphatase is unlikely to be a baculovirus capping enzyme, because: (i) it removes both γ- and β-phosphates to leave a 5′-monophosphate end (24); (ii) the viral RNA polymerase subunit LEF-4 is a bifunctional capping enzyme with both RNA triphosphatase and guanylyltransferase activities (9–11).

In addition to these viral and prokaryotic RNA triphosphatases, higher eukaryotes also contain non-capping enzyme RNA phosphatases. A human BVP-like protein has been identified that removes two phosphates from the 5′-end of RNA (48). The

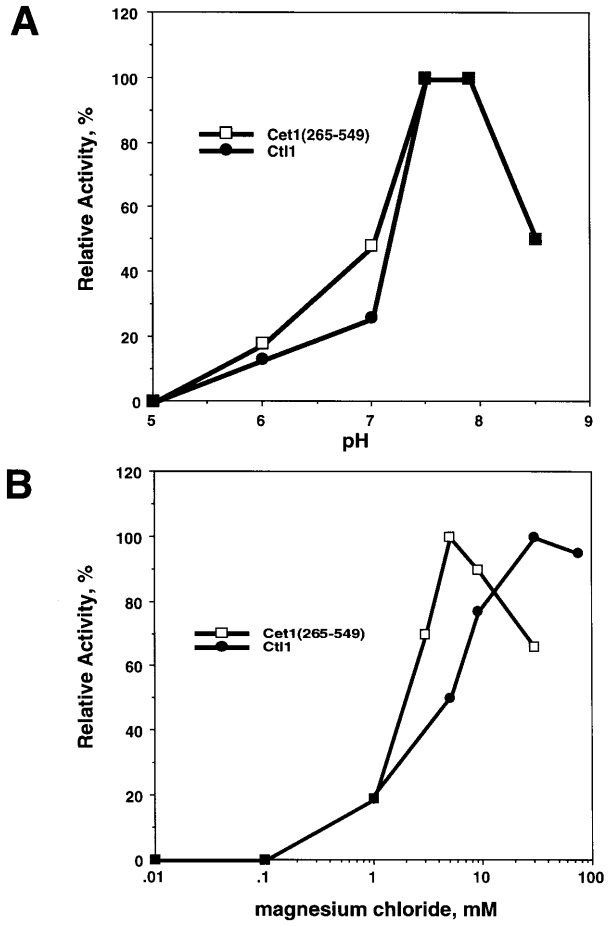


Figure 5. Catalytic properties of Ctl1 and Cet1 RNA triphosphatases. Aliquots of 120 pg his$_7$-Cet1(265–549) or 400 pg his$_7$-Ctl1 were used for the assay. Reaction mixtures contained 3 µM (of termini) [γ-^{32}P]ATP-terminated trinucleotide RNA (**p**ppApCpC). (**A**) Effect of varying pH. Reactions contained 5 mM $MgCl_2$ and 50 mM MES–KOH (pH 5–7) or Tris–HCl (pH 7.5–8.5) at the indicated pH. (**B**) Effect of varying magnesium concentration. Reactions contained 50 mM Tris–HCl (pH 7.9) and the indicated concentration of $MgCl_2$. After incubation, the reaction mixtures were analyzed by TLC on PEI–cellulose plates and autoradiography and radioactive spots were cut out and counted by liquid scintillation.

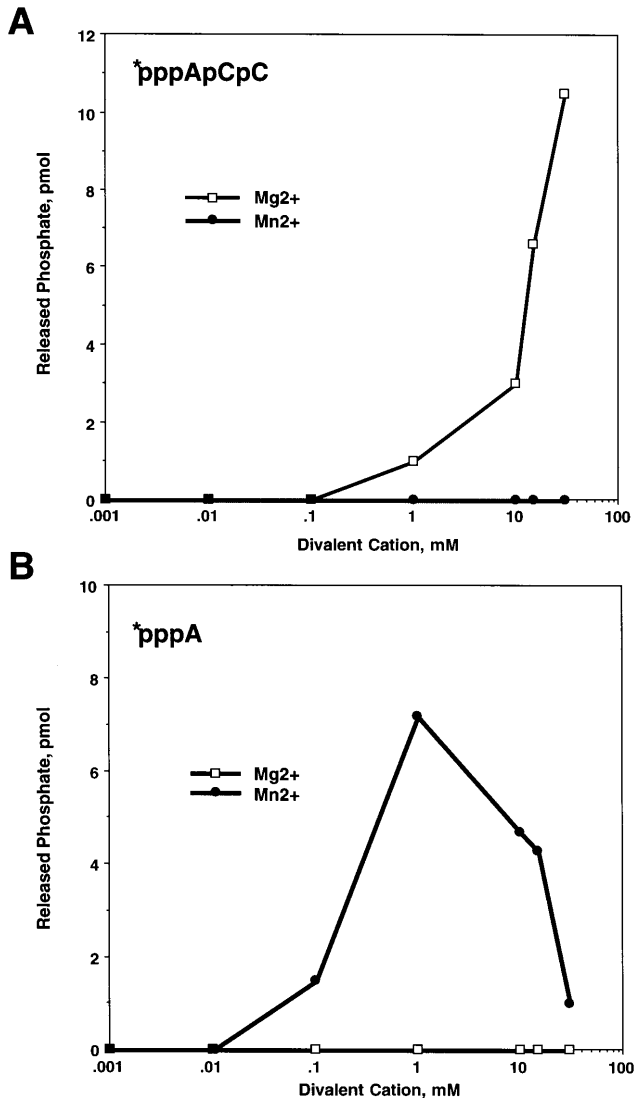


Figure 6. The substrate specificity of Ctl1 is strongly affected by the choice of divalent cation. Reactions were performed at pH 7.9 with 400 pg his$_7$-Ctl1 and the indicated concentration of $MgCl_2$ or $MnCl_2$. Substrates were either (**A**) 3 µM (of termini) [γ-^{32}P]ATP-terminated trinucleotide RNA (**p**ppApCpC) or (**B**) 5 µM [γ-^{32}P]ATP. After incubation, the reaction mixtures were analyzed by TLC on PEI–cellulose plates and autoradiography. Radioactive spots were cut out and counted by liquid scintillation.

human capping enzyme gene (*HCE1*) encodes a bifunctional protein with both RNA triphosphatase and guanylyltransferase domains. However, two additional variant cDNAs (*HCE1A* and *HCE1B*) thought to arise by alternative splicing have been isolated (8). Interestingly, the Hce1a and Hce1b proteins do not carry the guanylyltransferase domain and have only RNA triphosphatase activity (8). Although they have not been characterized as to subcellular location or *in vivo* function, it is interesting to speculate that they might be functionally analogous to the Ctl1 protein. In order to elucidate the biological functions of these non-capping RNA triphosphatases, it will be essential to identify their physiological substrates.

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