THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 51, Issue of December 18, pp. 34151–34156, 1998
Printed in U.S.A.

Yeast and Viral RNA 5′ Triphosphatases Comprise a New Nucleoside Triphosphatase Family*

(Received for publication, September 4, 1998)

C. Kiong Ho, Yi Pei, and Stewart Shuman‡

From the Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021

Saccharomyces cerevisiae **Cet1p catalyzes the first step of mRNA capping, the hydrolysis of the γ phosphate of triphosphate-terminated RNA to form a 5′ diphosphate end. The RNA triphosphatase activity of Cet1p is magnesium-dependent and has a turnover number of 1 s^{-1}. Here we show that purified recombinant Cet1p possesses a robust ATPase activity (K_m = 2.8 μM; V_{max} = 25 s^{-1}) in the presence of manganese. Cobalt is also an effective cofactor, but magnesium, calcium, copper, and zinc are not. Cet1p displays broad specificity in converting ribonucleoside triphosphates and deoxynucleoside triphosphates to their respective diphosphates. The manganese- and cobalt-dependent nucleoside triphosphatase of Cet1p resembles the nucleoside triphosphatase activities of the baculovirus LEF-4 and vaccinia virus D1 capping enzymes. Cet1p, LEF-4, and D1 share three collinear sequence motifs. Mutational analysis establishes that conserved glutamate and arginine side chains within these motifs are essential for the RNA triphosphatase and ATPase activities of Cet1p *in vitro* and for Cet1p function *in vivo*. These findings are in accord with the effects of single alanine mutations at analogous positions of vaccinia capping enzyme. We suggest that the metal-dependent RNA triphosphatases encoded by yeast and DNA viruses comprise a novel family of phosphohydrolase enzymes with a common active site.**

The m7GpppN cap of eukaryotic mRNA is synthesized by three enzymatic reactions: (i) the 5′ triphosphate end of nascent pre-mRNA is hydrolyzed to a diphosphate by RNA triphosphatase, (ii) the diphosphate end is capped with GMP by GTP:RNA guanylyltransferase, and (iii) the GpppN structure is methylated by AdoMet:RNA-(guanine-N7)-methyltransferase (1). The architecture of the capping apparatus differs between metazoans, fungi, and DNA viruses. Metazoan species encode a two-component capping system consisting of a bifunctional triphosphatase-guanylyltransferase polypeptide and a methyltransferase polypeptide (2–10). The budding yeast *Saccharomyces cerevisiae* encodes a three-component system consisting of separate triphosphatase (Cet1p), guanylyltransferase (Ceg1p), and methyltransferase (Abd1p) gene products (11–14). Vaccinia virus capping enzyme is a multifunctional protein that catalyzes all three reactions. The triphosphatase, guanylyltransferase, and methyltransferase active sites are arranged in a modular fashion within a single polypeptide: the vaccinia D1 protein (15–21). Other DNA viruses encode a subset of the capping activities; *e.g.* baculoviruses encode a bifunctional triphosphatase-guanylyltransferase (LEF-4), and *Chlorella* virus PBCV-1 encodes a monofunctional guanylyltransferase (22–24). The guanylyltransferase and methyltransferase domains are conserved between DNA viruses, fungi, and metazoans. In contrast, the triphosphatase components are structurally and mechanistically divergent.

Metazoan capping enzymes consist of an amino-terminal RNA triphosphatase domain and a carboxyl-terminal guanylyltransferase domain. In the mammalian enzyme, the two catalytic domains are autonomous and nonoverlapping (6, 7). The metazoan RNA triphosphatase domains contain a (I/V)H-CxAGxGR(S/T)G signature motif initially described for the protein tyrosine phosphatase/dual-specificity protein phosphatase enzyme family. These enzymes catalyze phosphoryl transfer from a protein phosphomonoester substrate to the thiolate of the cysteine of the signature motif to form a covalent phosphocysteine intermediate, which is then hydrolyzed to liberate phosphate. The metazoan capping enzymes hydrolyze the phosphoanhydride bond between the β and γ phosphates of triphosphate-terminated RNA; they are not active on nucleoside triphosphates (2, 6, 25). The conserved cysteine of the signature motif is essential for RNA triphosphatase function (2, 14). A characteristic of the cysteine phosphatases is their lack of a requirement for a divalent cation cofactor.

The RNA triphosphatases of *S. cerevisiae* and DNA viruses are structurally and mechanistically unrelated to the cysteine phosphatases. The vaccinia RNA triphosphatase depends absolutely on a divalent cation cofactor. Vaccinia triphosphatase displays broad specificity in its ability to hydrolyze the γ phosphate of ribonucleoside triphosphates, deoxynucleoside triphosphates, and triphosphate-terminated RNAs (26). The NTPase[1] and RNA triphosphatase reactions occur at a single active site within a 545-amino acid amino-terminal domain of vaccinia capping enzyme that is distinct from the guanylyltransferase active site (17–21). Although the K_m of vaccinia triphosphatase for NTPs is 1000-fold higher than that for triphosphate-terminated RNA, the turnover number is 10-fold higher for NTP hydrolysis than for RNA triphosphate cleavage (20, 26). Also, the divalent cation cofactor specificities are distinct. The vaccinia RNA triphosphatase is optimal with magnesium, is 12% as active in manganese, and is inactive with cobalt. In contrast, the vaccinia NTPase is fully active with cobalt, manganese, or magnesium (26, 27). Baculovirus LEF-4 hydrolyzes the γ phosphate of RNA and NTPs; the LEF-4 NTPase is activated by manganese or cobalt, but not by magnesium (22, 23).

Itoh *et al.* (28) isolated a yeast capping enzyme containing both RNA triphosphatase and guanylyltransferase activities.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Molecular Biology Program, Sloan-Kettering Institute, 1275 York Ave., New York, NY 10021. Tel.: 212-639-7145; Fax: 212-717-3623; E-mail: s-shuman@ski.mskcc.org.

[1] The abbreviations used are: NTPase, nucleoside triphosphatase; DTT, dithiothreitol; NTP, nucleoside triphosphate.

The yeast RNA triphosphatase, which was magnesium dependent, could not hydrolyze ATP or GTP (28). A highly purified yeast capping enzyme consisted of two major polypeptides: (i) a 52-kDa guanylyltransferase identified as the 459-amino acid Ceg1 protein (11), and (ii) an RNA triphosphatase component with an apparent electrophoretic mobility of 80 kDa (29). Tsukamoto *et al.* (13) identified the RNA triphosphatase polypeptide as the 549-amino acid product of the *CET1* gene. We independently isolated *CET1* as a multicopy suppressor of a temperature-sensitive mutation in the yeast guanylyltransferase Ceg1p (14). The *CET1* gene is essential for yeast cell growth (13, 14).

Tsukamoto *et al.* (13) reported that recombinant Cet1p catalyzed the hydrolysis of the γ phosphate of triphosphate-terminated RNA but did not hydrolyze GTP, prompting the conclusion that Cet1p acts only on RNA substrates. This would suggest that the yeast RNA triphosphatase has metazoan-like substrate specificity for RNA triphosphate ends yet exploits a virus-like catalytic mechanism that depends on metal-assisted cleavage of the phosphoanhydride bond. Here, we report that purified recombinant Cet1p is a highly active NTPase. Thus, it is not specific for RNA substrates. Cet1p resembles the vaccinia and baculovirus triphosphatases in that its NTPase function is activated by manganese and cobalt. We discerned three collinear sequence motifs that are shared by yeast Cet1p, vaccinia D1, and baculovirus LEF-4. Mutational analysis establishes that conserved glutamate and arginine side chains within these motifs are essential for Cet1p function *in vitro* and *in vivo*.

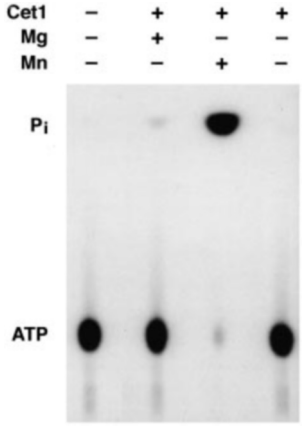


FIG. 1. **Hydrolysis of ATP by Cet1p.** Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 1 mM [γ-^{32}P]ATP, 250 ng of recombinant Cet1p (phosphocellulose fraction), and 1 mM divalent cation as specified were incubated for 15 min at 30 °C. An aliquot (4 μl) of the mixture was applied to a polyethyleneimine-cellulose TLC plate, which was developed with 0.5 M $LiCl_2$ and 1 M formic acid. An autoradiograph of the TLC plate is shown. The positions of P_i and ATP are indicated on the *left*.

MATERIALS AND METHODS

Protein Expression and Purification—Induced expression of the His-tagged Cet1p, Cet1(201–549)p, and Cet1(246–549)p in *Escherichia coli* BL21(DE3) cells was performed as described previously (14). The recombinant proteins were purified from soluble bacterial lysate by nickel-agarose and phosphocellulose column chromatography (14).

Mutational Analysis—Alanine substitution mutations were introduced into the *CET1(201–549)* gene by a polymerase chain reaction using the two-stage overlap extension method. The residues targeted for amino acid substitutions were Glu-305, Glu-307, Arg-454, Glu-492, Glu-494, and Glu-496. Plasmid p358-CES5(201–549) (14) was the template for the first-stage amplifications. The DNA products of the second-stage amplification were digested with *Nde*I and *Bam*HI and inserted into pET-16b (Novagen). The presence of the desired mutations was confirmed by DNA sequencing; the inserted restriction fragment was sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The His-tagged mutant proteins were purified from soluble bacterial lysates by nickel-agarose chromatography as described previously (14). The 0.2 M imidazole eluate fractions containing Cet1(201–549)p were dialyzed against Buffer C (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM DTT, 10% glycerol, and 0.05% Triton X-100). Protein concentration was determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard.

RESULTS

Cet1p Hydrolyzes the γ Phosphate of ATP—We have previously shown that purified recombinant Cet1p catalyzed the release of $^{32}P_i$ from γ-^{32}P-labeled triphosphate-terminated poly(A) with a turnover number of ~1 s^{-1} (14). RNA triphosphatase activity was strictly dependent on the inclusion of magnesium in the reaction mixture. The experiment shown in Fig. 1 demonstrates that Ceg1p also catalyzes the near-quantitative release of $^{32}P_i$ from 1 mM [γ-^{32}P]ATP in the presence of 1 mM manganese as the divalent cation cofactor. There was no detectable ATP hydrolysis in the absence of a divalent cation, and 1 mM magnesium was extremely feeble in supporting catalysis. The failure of previous investigators to appreciate the NTPase activity of the capping enzyme isolated from yeast extracts or recombinant Cet1p is most likely attributable to the reliance on magnesium as the divalent cation cofactor.

Divalent cation specificity was tested in reaction mixtures containing 1 mM ATP and 2 mM divalent cation (Fig. 2*A*). Cobalt was at least as effective as manganese in activating the ATPase, whereas magnesium was one-twentieth as effective. Calcium, copper, and zinc did not activate the ATPase. Cofactor titration experiments showed that hydrolysis of 1 mM ATP was optimal at 1–3 mM $MnCl_2$ and declined slightly as $MnCl_2$ was

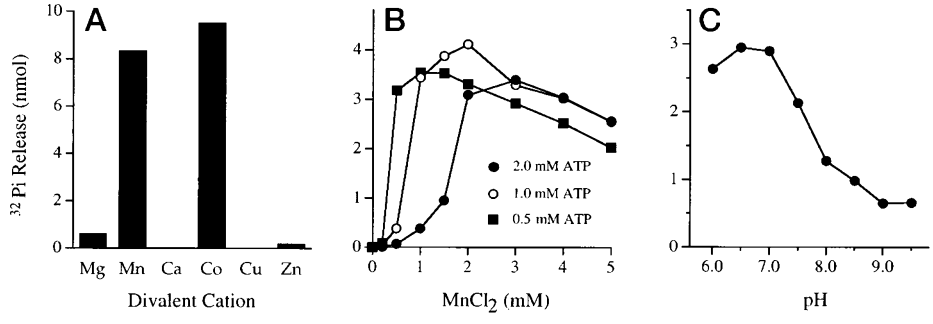


FIG. 2. **Divalent cation specificity and pH effect.** *A*, reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.0), 1 mM [γ-^{32}P]ATP, 100 ng of Cet1p, and 2 mM divalent cation as specified were incubated for 15 min at 30 °C. Mg, Mn, Ca, and Co were added as chloride salts; Cu and Zn were added as sulfates. The reaction products were analyzed by TLC. The release of $^{32}P_i$ from [γ-^{32}P]ATP was quantitated by scanning the TLC plate with a FUJIX BAS2000 Bio-Imaging Analyzer. *B*, reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.0), 20 ng of Cet1p, either 0.5, 1, or 2 mM [γ-^{32}P]ATP, and $MnCl_2$ as specified were incubated for 15 min at 30 °C. P_i release is plotted as a function of manganese concentration. *C*, effect of pH. Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH as indicated), 5 mM DTT, 2 mM $MnCl_2$, 1 mM [γ-^{32}P]ATP, and 20 ng of Cet1p were incubated for 15 min at 30 °C.

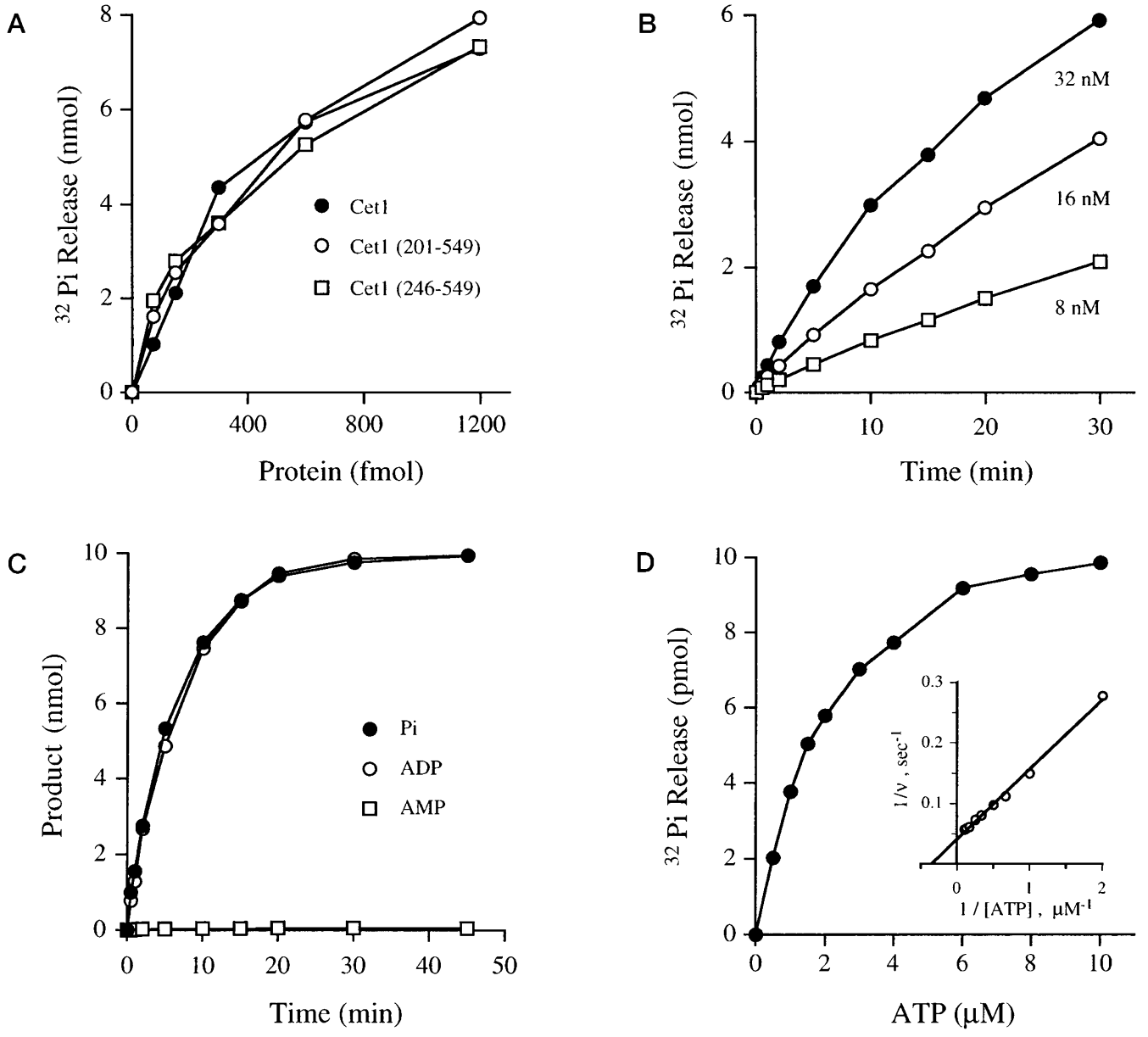


FIG. 3. **Kinetic analysis of ATP hydrolysis.** *A*, protein titration. Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.0), 5 mM DTT, 2 mM $MnCl_2$, 1 mM [γ-^{32}P]ATP, and either Cet1p, Cet1(201–549)p, or Cet1(246–549)p were incubated for 15 min at 30 °C. P_i release is plotted as a function of input protein. *B*, kinetics. Reaction mixtures (100 μl) containing 50 mM Tris-HCl (pH 7.0), 5 mM DTT, 2 mM $MnCl_2$, 1 mM [γ-^{32}P]ATP, and either 8, 16, or 32 nM Cet1p were incubated at 30 °C. Aliquots (10 μl) were withdrawn at the times indicated and quenched immediately by the addition of 2.5 μl of 5 M formic acid. P_i release is plotted as a function of time. *C*, hydrolysis of [α-^{32}P]ATP. Reaction mixtures (100 μl) containing 50 mM Tris-HCl (pH 7.0), 5 mM DTT, 2 mM $MnCl_2$, 1 mM [α-^{32}P]ATP, and 100 ng of Cet1p were incubated at 30 °C. Aliquots (10 μl) were withdrawn at the times indicated and quenched with formic acid. The products were analyzed by TLC. The levels of [α-^{32}P]ADP (○) and [α-^{32}P]AMP (□) are plotted as a function of time. An otherwise identical reaction containing 1 mM [γ-^{32}P]ATP was analyzed in parallel; P_i release, ●. *D*, ATP-dependence. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.0), 5 mM DTT, 2 mM $MnCl_2$, 40 pg of Cet1p, and [γ-^{32}P]ATP as indicated were incubated for 15 min at 30 °C. P_i release is plotted as a function of ATP concentration. *Inset*, a double-reciprocal plot of the data is shown.

increased to 5 mM (Fig. 2*B*). Lowering the ATP concentration to 0.5 mM elicited a shift to the left in the manganese titration curve, whereas increasing ATP to 2 mM caused a shift to the right (Fig. 2*B*). The titration curves were sharply sigmoidal at manganese concentrations below the level of input ATP. Cobalt-dependent hydrolysis of 1 mM ATP was optimal at 1–5 mM $CoCl_2$ (data not shown). ATP hydrolysis by an equivalent amount of Cet1p was 25% higher with cobalt than with manganese (data not shown). The activity with magnesium at the optimum concentration of 5–10 mM $MgCl_2$ was only 10% of the cobalt-dependent activity (data not shown).

ATP hydrolysis was measured in 50 mM Tris buffer from pH 6.0 to pH 9.5 (Fig. 2*C*). Activity was optimal from pH 6.5 to pH 7.0 and declined with increased alkalinity. Activity at pH 9.5 was 25% that at pH 7.0.

Kinetics of ATP Hydrolysis—The extent of $^{32}P_i$ release from [γ-^{32}P]ATP during a 15-min reaction was proportional to the amount of input Cet1p protein (Fig. 3*A*). $^{32}P_i$ accumulated with time over 30 min; the rate of reaction varied linearly with Cet1p concentration (Fig. 3*B*). From a plot of initial rate *versus* enzyme, we calculated a turnover number of 25 s^{-1}. Cet1p catalyzed the quantitative conversion of [α-^{32}P]ATP to [α-^{32}P]ADP. The rate of [α-^{32}P]ADP formation was identical to the rate of $^{32}P_i$ release from [γ-^{32}P]ATP assayed in a parallel reaction mixture (Fig. 3*C*). We detected no formation of [α-^{32}P]AMP from [α-^{32}P]ATP, even after 20–45 min of incuba-

```
          * *                *                    * * *
Cet1    SFIELEMKF  -140-  ISERTKDRVSYIHN  -25-  THEVELE
vvD1    INNELELVF   -31-  VKIRTKIPLSKVHG -102-  SLEIEFT
SFV     MNHEVELTF   -30-  VKIRNRINLSKIHG -103-  SLEFEII
MCV     VHHEVELIF   -33-  VKLRTRLPLATVHG -103-  TLEFEVL
ASF     STIELEIRF   -37-  NHCREKILPSENLY -102-  LYEIEIE
Lef4    FVIEKEISY   -33-  NGFRTRIPIQSACN -117-  RLEYEFD
180c    SHIEIEMKF  -141-  ILQRTKSRSTYTFN  -23-  SHEVEVE
          A                  B                  C
```

FIG. 4. **Conserved sequence elements of the metal-dependent RNA triphosphatases.** Three conserved motifs, designated A, B, and C in the RNA triphosphatases of *S. cerevisiae* (*Cet1*), vaccinia virus (*vvD1*), Shope fibroma virus (*SFV*), molluscum contagiosum virus (*MCV*), African swine fever virus (*ASF*), and baculovirus LEF-4 are aligned in the figure. Also included in the alignment is the predicted translation product of the *S. cerevisiae* YMR180c open reading frame (*180c*). Cet1p residues conserved in at least two other family members are *shaded*. The *numbers* of amino acids separating the motifs are indicated. The five amino acids in the vaccinia virus capping enzyme that are essential for triphosphatase activity are *underlined*. Cet1p residues Glu-305, Glu-307, Arg-454, Glu-492, Glu-494, and Glu-496 that were mutated to alanine in the present study are denoted by *asterisks*.

tion, by which time all of the nucleotide had been converted to ADP. Thus, we conclude that Cet1p catalyzes the hydrolysis of ATP to ADP plus P_i and is unable to further hydrolyze the ADP reaction product. Kinetic parameters were determined by measuring ATPase activity as a function of input [γ-^{32}P]ATP concentration (Fig. 3*D*). From a double-reciprocal plot of the data, we calculated a K_m of 2.8 μM ATP and a V_{max} of 25 s^{-1} (Fig. 3*D*). The phosphohydrolase activity of Cet1p was not restricted to ATP. Cet1p also catalyzed manganese-dependent hydrolysis of [α-^{32}P]GTP to [α-^{32}P]GDP, [α-^{32}P]dCTP to [α-^{32}P]dCDP, and [α-^{32}P]dATP to [α-^{32}P]dADP (data not shown). Other nucleotides were not tested.

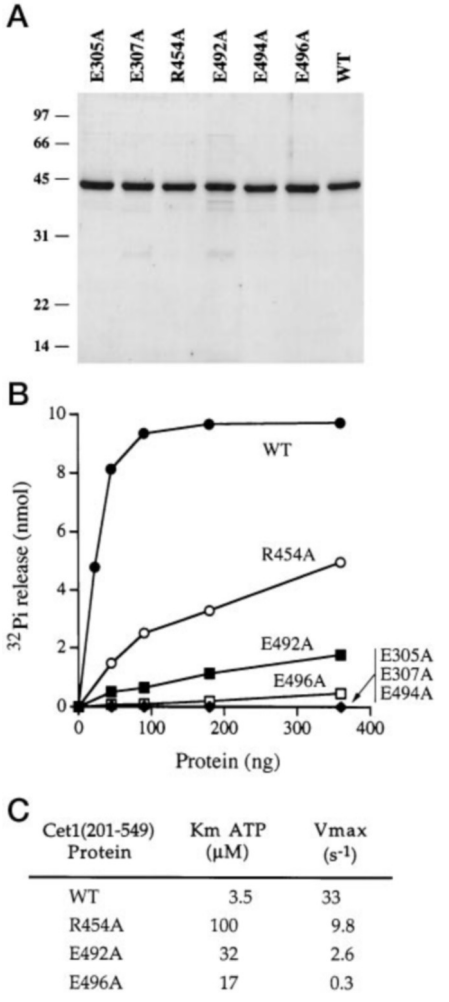
ATPase Activity of Cet1(201–549) and Cet1(246–549)—We previously purified and characterized two amino-terminal truncation mutants, Cet1(201–549)p and Cet1(246–549)p, that retained full activity in catalyzing the release of $^{32}P_i$ from γ-^{32}P-labeled triphosphate-terminated poly(A) (14). Similarly, the two truncated proteins were as active as full-length Cet1p in hydrolyzing ATP (Fig. 3*A*). We infer that both reactions are catalyzed by the same catalytic site within the carboxyl-terminal domain. The kinetic parameters determined for ATP hydrolysis by Cet1(201–549)p (K_m = 3.3 μM ATP; V_{max} = 33 s^{-1}) were similar to those of full-length Cet1p. These data suggest that the deleted amino-terminal 200-amino acid segment does not contribute to nucleotide binding or reaction chemistry.

Conserved Motifs in Metal-dependent RNA Triphosphatases—The yeast Cet1p, vaccinia D1, and baculovirus LEF-4 enzymes display remarkably similar biochemical characteristics in their hydrolysis of the β-γ phosphoanhydride linkage of RNA and NTP substrates. The activation of NTP hydrolysis by manganese and cobalt are the signature features of these enzymes (22, 27). Is there a common structural basis for metal-dependent catalysis? A Blastp search of the National Center for Biotechnology Information database with the catalytic domain of Cet1p revealed no similarity to vaccinia D1 or baculovirus LEF-4 or to any other known NTPases or phosphatases. A more instructive approach was to screen "by eye" for conservation of the several residues identified by mutational analysis of vaccinia D1 as being essential for the triphosphatase activity of the viral capping enzyme (18, 19). In this way, we found that the metal-dependent RNA triphosphatases share three collinear sequence motifs, designated A, B, and C (Fig. 4). These are present in yeast Cet1p, in the triphosphatase-guanylyltransferase domains of the vaccinia virus, Shope fibroma virus, molluscum contagiosum virus, and African swine fever virus capping enzymes, and in baculovirus LEF-4. The residues that are essential for the RNA triphosphatase and ATPase activities of vaccinia virus capping enzyme include two glutamates in motif A, an arginine in motif B, and two glutamates in motif C (19). Alanine substitutions at any of these positions in the vaccinia capping enzyme reduced phosphohydrolase specific activity by 2–3 orders of magnitude (51). Yu *et al.* (19) proposed that these 5 residues comprise part of the triphosphatase active site. All 5 residues essential for vaccinia triphosphatase activity are conserved in LEF-4 and Cet1p. Conceivably, the metal-dependent RNA triphosphatase/NTPases share a common active site, in which case mutations of the equivalent residues of Cet1p should result in a loss of triphosphatase activity.

Conserved Residues in Motifs A, B, and C Are Essential for the ATPase Activity of Cet1(201–549)p—Cet1p residues Glu-305 and Glu-307 (motif A), Arg-454 (motif B), and Glu-492, Glu-494, and Glu-496 (motif C) were replaced individually by alanine. The Ala mutations were introduced into Cet1(201–549)p, which is fully active *in vitro* and *in vivo* (14). The six mutated proteins were expressed as His-tagged fusions and purified from soluble lysates by nickel-agarose column chromatography in parallel with wild type Cet1(201–549)p (Fig. 5*A*). Mutational effects on manganese-dependent ATP hydrolysis were gauged by enzyme titration under conditions of substrate excess (1 mM ATP). The E305A and E307A mutants of motif A and the E494A mutant of motif C were unable to hydrolyze ATP at levels of input enzyme sufficient for the quantitative release of $^{32}P_i$ by wild type Cet1(201–549)p (Fig. 5*B*). From these data, we calculated that the specific activities of E305A, E307A, and E494A were <0.5% of the activity of the wild type enzyme. Alanine substitutions for two other glutamate residues in motif C, E492A and E496A, reduced specific activity to 2.8 and 0.6% of the wild type value, respectively. The specific activity of the R454A mutant of motif B was 15% of the wild type value. Similar mutational effects were noted when the ATPase assays were performed in the presence of 2 mM cobalt (data not shown).

Kinetic parameters for ATP hydrolysis were determined for three of the mutants (R454A, E492A, and E496A) that displayed reduced ATPase activity (Fig. 5*C*). The R454A mutation increased the K_m for ATP to 100 μM (nearly 30-fold higher than the wild type K_m), whereas the V_{max} was 30% of the wild type value. The E492A mutation increased the K_m for ATP 9-fold to 32 μM and lowered the V_{max} to 8% of the wild type value. The ratios of V_{max} to K_m for R454A and E492A were 2 orders of magnitude lower than the wild type ratio. The E496A mutation increased the K_m for ATP 5-fold and decreased the V_{max} to 0.9% of the wild type value; the ratio of V_{max} to K_m for E496A was 0.2% of the wild type ratio.

Mutational Effects on RNA Triphosphatase—The RNA triphosphatase activities of the wild type and six mutant Cet1(201–549)p proteins were assayed by the release of $^{32}P_i$ from 2 μM [γ-^{32}P]-labeled poly(A). Specific enzyme activity was determined from the slopes of the protein titration curves in the linear range of enzyme dependence (Fig. 6). The specific activity of the wild type enzyme (16.5 nmol of P_i released per microgram of protein in 15 min) corresponds to a turnover number of ~0.8 s^{-1}. Analysis of RNA triphosphate cleavage by wild type Cet1(201–549)p as a function of RNA substrate concentration revealed a K_m of 1 μM for poly(A) triphosphate termini and a V_{max} of 1 s^{-1} (data not shown). The specific activities of the alanine mutants, calculated from the data in Fig. 6 and expressed as a percentage of the wild type value, were as follows: E305A, 0.03%; E307A, 0.03%; R454A, 0.2%; E492A, 1.7%; E494A, 0.03%; and E496A, 0.8%. Kinetic parameters were not determined for these catalytically defective enzymes. The effects of the single Glu-to-Ala mutations on RNA triphosphatase specific activity were similar in magnitude to



C

Cet1(201-549) Protein	Km ATP (μM)	Vmax (s^{-1})
WT	3.5	33
R454A	100	9.8
E492A	32	2.6
E496A	17	0.3

FIG. 5. **Effects of alanine mutations on the ATPase activity of Cet1(201–549)p.** *A*, protein purification. Aliquots (3 μg) of the nickel-agarose preparations of recombinant wild type (*WT*) Cet1(201–549)p and the indicated Cet1(201–549)p-Ala mutants were electrophoresed through a 12% 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*, ATP hydrolysis. Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.0), 5 mM DTT, 2 mM $MnCl_2$, 1 mM [γ-^{32}P]ATP, and either WT or mutant proteins as specified were incubated for 15 min at 30 °C. P_i release is plotted as a function of input protein. *C*, kinetic parameters. Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.0), 5 mM DTT, 2 mM $MnCl_2$, varying concentrations of [γ-^{32}P]ATP, and wild type or mutant enzymes as specified were incubated for 15 min at 30 °C. The amount of input enzyme was adjusted to attain roughly similar levels of ATP hydrolysis at saturating ATP concentrations. The extent of P_i release at the lowest ATP concentrations tested was less than than 20% of the input substrate. Kinetic parameters were determined from double-reciprocal plots of the data.

their effects on ATPase specific activity. The R454A mutation appeared to exert a more drastic effect on RNA triphosphate cleavage than on ATP hydrolysis. The overall concordance of the mutational effects suggests that both reactions are catalyzed at a single active site.

Conserved Residues in Motifs A, B, and C Are Essential for CET1 Function in Vivo—Mutant alleles of *CET1(201–549)* encoding triphosphatase-defective enzymes were tested for their function *in vivo* using the plasmid shuffle assay described by Ho *et al.* (14). The wild type and mutants coding sequences were cloned into a *CEN TRP1* vector so as to place the *CET1(201–549)* gene under the control of the natural *CET1* promoter. The plasmids were transformed into the Δ*cet1* strain YBS20, in which the chromosomal *CET1* locus has been deleted

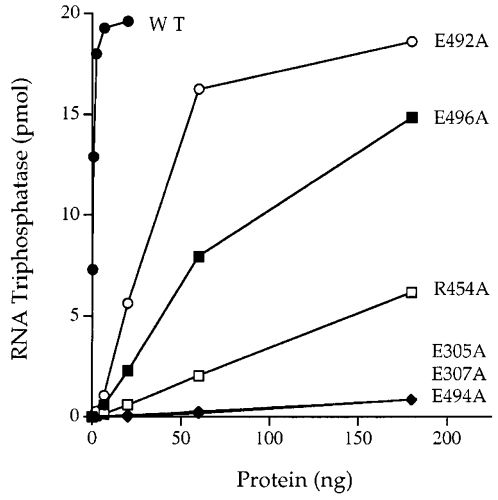


FIG. 6. **Mutational effects on the RNA triphosphatase activity of Cet1(201–549)p.** Reaction mixtures (10 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 1 mM $MgCl_2$, 20 pmol (of triphosphate termini) of γ-^{32}P-labeled poly(A), and either wild type or mutant proteins as specified were incubated for 15 min at 30 °C. Aliquots of the mixtures were applied to a polyethyleneimine-cellulose TLC plate, which was developed with 0.75 M potassium phosphate (pH 4.3). $^{32}P_i$ release is plotted as a function of input protein.

and replaced by *LEU2*. Growth of YBS20 is contingent on the maintenance of a wild type *CET1* allele on a *CEN URA3* plasmid. Hence, YBS20 is unable to grow in agar medium containing 0.75 mg/ml 5-fluoroorotic acid, which selects against the *URA3* plasmid, unless it is transformed with a biologically active *CET1* allele or a functional homologue from another organism (8, 14). We found that growth on 5-fluoroorotic acid was complemented by *CET1(201–549)*, but not by mutant alleles *E305A*, *E307A*, *R454A*, *E492A*, *E494A*, or *E496A* (data not shown). The correlation of *in vitro* and *in vivo* mutational effects suggests that the triphosphatase activity of Cet1p is essential for yeast cell growth.

DISCUSSION

We have shown that the yeast RNA 5′-triphosphatase Cet1p hydrolyzes the γ phosphate of nucleoside triphosphates. The NTPase of Cet1p is activated by manganese and cobalt. This is a property shared with the triphosphatase components of the vaccinia D1 and baculovirus LEF-4 capping enzymes. The turnover number of the yeast enzyme in ATP hydrolysis (25 s^{-1}) is similar to the values reported for the baculovirus (30 s^{-1}) and vaccinia virus (10 s^{-1}) triphosphatases, although the affinity of the yeast enzyme for ATP (K_m = 2.8 μM) is significantly higher than that of either LEF-4 (K_m = 43 μM) or vaccinia triphosphatase (K_m = 800 μM) (21, 22).

We propose that Cet1p is the prototype of a previously unrecognized family of metal-dependent phosphohydrolases that includes the DNA virus and yeast RNA triphosphatases involved in cap formation. A common structural basis for catalysis by members of this enzyme family is suggested by the conservation of collinear motifs A, B, and C. Conserved amino acid residues within the motifs likely comprise part of the active site. As shown here and by Yu *et al.* (19), single alanine substitutions within the three motifs abrogate or severely diminish the triphosphatase activity of yeast Cet1p and vaccinia D1. The essential residues identified by alanine scanning include the two alternating glutamates in motif A, an arginine residue in motif B, and either two (in the poxvirus capping enzymes) or three (in Cet1p and African swine fever virus capping enzyme) alternating glutamates in motif C. The effects of alanine cluster mutations in baculovirus LEF-4 are concord-

ant with the findings for Cet1p and D1, to wit, manganese-dependent NTPase and RNA triphosphatase activities are eliminated or drastically reduced by simultaneous replacement of both motif A glutamates by alanine, by single alanine substitution for the motif B arginine, or by simultaneous replacement of the two glutamates of motif C (EYEFD) by alanine (23).

The marked decrease in affinity for ATP elicited by the R454A mutation of Cet1(201–549)p suggests a role for this motif B residue in substrate binding. We speculate that the arginine side chain interacts with the negatively charged 5′ triphosphate moiety. Elimination of the glutamate side chains in motifs A and C profoundly inhibited ATPase activity, even at very high substrate concentrations, which suggests a role for these residues in phosphohydrolase reaction chemistry. The glutamates in motif A and C may coordinate the essential divalent cation(s). An additional role for the proximal glutamate of motif C in substrate binding is inferred from the 9-fold increase in K_m for ATP caused by the E492A mutation of Cet1(201–549)p.

The sequence similarity between Cet1p and the DNA virus-encoded RNA triphosphatase/NTPases appears to be limited to motifs A, B, and C. Cet1p possesses triphosphatase activity only, whereas LEF-4 and D1 also have guanylyltransferase activities. In the baculovirus and vaccinia capping enzymes, the triphosphatase motifs are located amino-terminal to the six conserved motifs that comprise the guanylyltransferase active site (19, 22, 23). The vaccinia D1 protein cannot be truncated to less than 545 amino acids without loss of its triphosphatase and guanylyltransferase activities. The minimum functional domains of the 464-amino acid baculovirus LEF-4 triphosphatase/guanylyltransferase have not yet been defined. The 305-amino acid Cet1p truncation mutant, Cet1(245–549)p, which retains full triphosphatase activity *in vitro*, is the smallest functional domain that has been identified for the metal-dependent RNA triphosphatase family. Additional candidates for membership in this family may emerge as genome sequencing uncovers new polypeptides containing motifs A, B, and C. For example, the 320-amino acid polypeptide encoded by the *S. cerevisiae* YMR180C open reading frame is homologous to the carboxyl-terminal domain of Cet1p and includes all six of the amino acids in motifs A, B, and C that are important for NTP hydrolysis (Fig. 4). Preliminary characterization of recombinant YM180C protein indicates that it possesses magnesium- and cobalt-dependent ATPase activity.[2] The function of YMR180C is unknown; this enzyme may well catalyze phosphohydrolase reactions unrelated to mRNA capping.

In summary, there is now clear evidence for at least two mechanistically and structurally distinct classes of RNA 5′ triphosphatases: (i) the divalent cation-dependent RNA triphosphatase/NTPase family (exemplified by yeast Cet1p, baculovirus LEF-4, and vaccinia D1), which require motifs A, B, and C for activity, and (ii) the divalent cation-independent RNA triphosphatases, *e.g.* the metazoan cellular enzymes and the baculovirus enzyme BVP (30, 31), which require the HCx-AGxGR(S/T)G phosphate-binding motif. The existence of additional classes of RNA 5′-triphosphatases is likely, given that the candidate capping enzymes of several RNA viruses and trypanosomatid protozoa lack the defining motifs of the two known RNA triphosphatase families (32, 33). Hence, the triphosphatase components of the capping apparatus provide attractive targets for the identification of specific antifungal, antiviral, and antiprotozoal drugs that will block capping of pathogen mRNAs but spare the mammalian host enzyme.

[2] C. K. Ho, unpublished observations.

REFERENCES

1. Shuman, S. (1995) *Prog. Nucleic Acid Res. Mol. Biol.* **50,** 101–129
2. Takagi, T, Moore, C. R., Diehn, F., and Buratowski, S. (1997) *Cell* **89,** 867–873
3. Wang, S. P., Deng, L., Ho, C. K., and Shuman, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 9573–9578
4. McCracken, S., Fong, N., Rosonina, E., Yankulov, K., Brothers, G., Siderovski, D., Hessel, A., Foster, S., Shuman, S., and Bentley, D. L. (1997) *Genes Dev.* **11,** 3306–3318
5. Yue, Z., Maldonado, E., Pillutla, R., Cho, H., Reinberg, D., and Shatkin, A. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 12898–12903
6. Ho, C. K., Sriskanda, V., McCracken, S., Bentley, D., Schwer, B., and Shuman, S. (1998) *J. Biol. Chem.* **273,** 9577–9585
7. Tsukamoto, T., Shibagaki, Y., Murakoshi, T., Suzuki, M., Nakamura, A., Gotoh, H., and Mizumoto, K. (1998) *Biochem. Biophys. Res. Commun.* **243,** 101–108
8. Yamada-Okabe, T., Doi, R., Shimmi, O., Arisawa, M., and Yamada-Okabe, H. (1998) *Nucleic Acids Res.* **26,** 1700–1706
9. Wang, S. P., and Shuman, S. (1997) *J. Biol. Chem.* **272,** 14683–14689
10. Pillutla, R. C, Yue, Z., Maldonado, E., and Shatkin, A. J. (1998) *J. Biol. Chem.* **273,** 21443–21446
11. Shibagaki, Y., Itoh, N., Yamada, H., Hagata, S., and Mizumoto, K. (1992) *J. Biol. Chem.* **267,** 9521–9528
12. Mao, X., Schwer, B., and Shuman, S. (1996) *Mol. Cell. Biol.* **16,** 475–480
13. Tsukamoto, T., Shibagaki, Y., Imajoh-Ohmi, S., Murakoshi, T., Suzuki, M., Nakamura, A., Gotoh, H., and Mizumoto, K. (1997) *Biochem. Biophys. Res. Commun.* **239,** 116–122
14. Ho, C. K., Schwer, B., and Shuman, S. (1998) *Mol. Cell. Biol.* **18,** 5189–5198
15. Mao, X., and Shuman, S. (1994) *J. Biol. Chem.* **269,** 24472–24479
16. Higman, M. A., Christen, L. A., and Niles, E. G. (1994) *J. Biol. Chem.* **269,** 14974–14981
17. Cong. P., and Shuman, S. (1995) *Mol. Cell. Biol.* **15,** 6222–6231
18. Yu, L., and Shuman, S. (1996) *J. Virol.* **70,** 6162–6168
19. Yu, L., Martins, A., Deng, L., and Shuman, S. (1997) *J. Virol.* **71,** 9837–9843
20. Myette, J. R., and Niles, E. G. (1996) *J. Biol. Chem.* **271,** 11936–11944
21. Myette, J. R., and Niles, E. G. (1996) *J. Biol. Chem.* **271,** 11945–11952
22. Gross, C. H., and Shuman, S. (1998) *J. Virol.,* in press
23. Jin, J., Dong, W., and Guarino, L. A. (1998) *J. Virol.,* in press
24. Ho, C. K., Van Etten, J. L., and Shuman, S. (1996) *J. Virol.* **70,** 6658–6664
25. Yagi, Y., Mizumoto, K., and Kaziro, Y. (1983) *EMBO J.* **2,** 611–615
26. Shuman, S., Surks, M., Furneaux, H., and Hurwitz, J. (1980) *J. Biol. Chem.* **255,** 11588–11598
27. Shuman, S. (1990) *J. Biol. Chem.* **265,** 11960–11966
28. Itoh, N., Mizumoto, K., and Kaziro, Y. (1984) *J. Biol. Chem.* **259,** 13930–13936
29. Itoh, N., Yamada, H., Kaziro, Y., and Mizumoto, K. (1987) *J. Biol. Chem.* **262,** 1989–1995
30. Gross, C. H., and Shuman, S. (1998) *J. Virol.* **72,** 7057–7063
31. Takagi, T., Taylor, G. S., Kusakabe, T., Charbonneau, H., and Buratowski, S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9808–9812
32. Bisaillon, M., and Lemay, G. (1997) *Virology* **236,** 1–7
33. Silva, E., Ullu, E., Kobayashi, R., and Tschudi, C. (1998) *Mol. Cell. Biol.* **18,** 4612–4619