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Isolation and Characterization of the Yeast mRNA Capping Enzyme β Subunit Gene Encoding RNA 5′-Triphosphatase, Which Is Essential for Cell Viability

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The yeast *Saccharomyces cerevisiae* mRNA capping enzyme is composed of two subunits of α (52 kDa, mRNA guanylyltransferase) and β (80 kDa, RNA 5′-triphosphatase). We have isolated the α subunit gene (*CEG1*) by immunological screening. In this report, with the aid of partial amino acid sequences of purified yeast capping enzyme, we isolated the gene, designated *CET1*, encoding the *S. cerevisiae* capping enzyme β subunit. Amino acid sequence analysis revealed that the gene encodes for 549 amino acids with a calculated M_r of 61,800 which is unexpectedly smaller than the size estimated by SDS-PAGE. Gene disruption experiment showed that *CET1* is essential for yeast cell growth. The purified recombinant *CET1* gene product, Cet1, exhibited an RNA 5′-triphosphatase activity which specifically removed the γ-phosphate from the triphosphate-terminated RNA substrate, but not from nucleoside triphosphates, confirming the identity of the gene. Interaction between the Cet1 and the Ceg1 was also studied by the West-Western procedure using recombinant Ceg1-[^{32}P]GMP as probe.

Eukaryotic mRNAs contain a 5′-terminal cap structure. The cap structure plays crucial roles in the various steps in gene expression including initiation of translation (1), RNA splicing (2,3), transport from the nucleus to cytoplasm (4), and. mRNA turnover (5). The cap structure is formed as an early cotranscriptional modification of nascent RNA chains transcribed by RNA polymerase II by a series of enzymatic activities (for reviews, see Refs. 6,7,8). The first step of cap formation is the removal of the γ-phosphate from RNA 5′-triphosphate end of newly-synthesized RNA to generate diphosphate end by RNA 5′-triphosphatase. Then the GMP moiety of GTP is transferred to the 5′-diphosphate terminus by mRNA guanylyltransferase. After these two consecutive reactions, methylation at the guanine-N7 position catalyzed by mRNA (guanine-7-)methyltransferase follows.

Capping enzyme from cellular sources is a bifunctional enzyme having the activities of mRNA guanylyltransferase and RNA 5′-triphosphatase (6). Capping enzyme from rat liver and *Artemia salina* consists of a single polypeptide chain of 69 kDa and 73 kDa, respectively, which contains catalytic domains for both mRNA guanylyltransferase and RNA 5′-triphosphtase (9,10). In contrast, yeast capping enzyme is composed of two subunits of a (52 kDa) and β (80 kDa), responsible for mRNA guanylyltransferase and RNA 5′-triphosphatase, respectively (11).

Using the antibody raised against the purified yeast capping enzyme we cloned the gene (*CEG1*) coding for mRNA guanylyltransferase (α subunit) and demonstrated that it is essential for growth of yeast cells (12). Mutational studies on *CEG1* indicated that the importance of a lysine residue (Lys70) which resides in a motif conserved among capping enzymes and polynucleotide ligases (13,14,15). mRNA guanylyltransferase genes from *Schiz. pombe* (*PCE1*) (16) and *C. albicans* (*CGT1*) (17) have also been cloned by complementation of mutant *CEG1* alleles and have been shown that their products are highly homologous to Ceg1. However, RNA 5′-triphosphatase gene has not been isolated so far. Although the isolation of yeast mRNA guanylyltransferase gene has enabled us to study de-

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Abbreviations used: *CET1*, capping enzyme triphosphatase subunit gene; *CEG1*, capping enzyme guanylyltransferase subunit gene; PVDF, polyvinylidene difluoride.

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tailed structure-function relationships of the enzyme, clearly, it is necessary to obtain the gene encoding its counterpart subunit, the RNA 5′-triphosphatase, to fully understand the biological roles of capping enzyme in RNA metabolism.

In this report, using the primers designed according to the partial amino acid sequences of purified β subunit protein from *S. cerevisiae* capping enzyme, we isolated the gene for RNA 5′-triphosphatase (*CET1*), and demonstrated that this gene was essential for cell viability. We also expressed recombinant Cet1 protein in *E. coli* and characterized its enzymatic activities, as well as its interaction with the α subunit.

MATERIALS AND METHODS

Partial amino acid sequences of the β subunit. The subunits of highly purified *S. cerevisiae* capping enzyme (40 μg) (Blue-Sepharose fraction of Ref. 11) was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The band corresponding to the β subunit was cut out and the protein was digested with lysylendopeptidase (18). The peptide fragments were sepatated by reverse-phase HPLC and sequenced using Beckman model LF3400D peptide sequencer.

Cloning by degenerate PCR. According to the amino acid sequences obtained degenerate primers were synthesized. Using these degenerate primers, *S. cerevisiase* genomic DNA was amplified by PCR. PCR was performed in a 50 μl volume of reaction mixture containing 10 mM Tris (pH 8.3), 50 mM KCl, 0.01% gelatin, 125 μM each of dGTP, dATP, dTTP, and dCTP, 2.5 units of *Taq* DNA polymerase, 100 pmol of each degenerate primer, and about 10 ng of *S. cerevisiae* genomic DNA. The amplification was carried out for 35 cycles, each consisting of a denaturing step at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. The last cycle was followed by a 7 minutes-extension at 72°C. The products were separated on a 2% agarose gel run in 0.5 × TBE buffer (1× TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA).

Library screening. The amplified band was purified from the gel and used as a probe for the screening of *S. cerevisiae* cDNA library (Clontech) and genomic library (Clontech). The inserts of positive clones were sequenced by dideoxy-chain termination method.

Disruption of the yeast gene. One-step gene disruption by fragment-mediated transformation of yeast was carried out as described (19). A *leu2* diploid strain (RAY3A-D) (19) was transformed with a 6-kb *Hin*dIII/*Pst*I fragment of *CET1* containing the coding region of RNA 5′-triphospatase which was interrupted by insertion of the 2.5-kb *Bam*HI fragment of *LEU2* gene at the *Bgl*II site. Leu$^+$ transformants were selected and the spores subjected to tetrad analysis (19).

Recombinant subunit proteins. For the recombinant Cet1 protein expression in *E. coli*, coding sequence of *CET1* was amplified from *S. cerevisiae* chromosomal DNA by PCR using sense primer corresponding to the upstream of initiating AUG generating 5′ *Nde*I site, and antisense primer located downstream of stop codon and of authentic *Hin*dIII site. The primers used were: SCF1, 5′-CCA GTG CAT ATG AGT TAC ACT GAC-3′(*Nde*I site underlined); SCR1, 5′-AAT GGC GAA AGA CTT GGT TAT TAT-3′. The amplified fragment was digested with *Nde*I and *Hin*dIII and ligated into pT7-7 (His) plasmid, a derivative of pT7-7 (21), containing seven consecutive histidine codons upstream of the unique *Nde*I site at the intiation codon (22). The recombinant Cet1 was expressed in a *E. coli* strain BL21(DE3)pLysS and purified on a Ni-nitrilo-triacetic acid (NTA) column. The recombinant Ceg1 without His-tag was expressed using T7-based expression vector as described (15). For the construction of deletion mutants of *CET1* gene, the primers SCBF (5′-GGG GAT CCC ACT ATG AGT TAC ACT GAC-3′) and SCR1 were used. The amplified fragment was digested with *Bam*HI (underlined) and *Bgl*II or *Pvu*II and *Hin*d III then ligated into pQE vector (Qiagen) to construct His-Cet1(1-265) and His-Cet1(205-549), respectively.

Western blotting. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking the blots were incubated with anti-capping enzyme antibodies (1:500) (11) and with horse radish peroxidase-conjugated protein A, and then detected by ECL (Amersham).

RNA 5′-triphosphatase activity. RNA 5′-triphosphatase acitivity was assayed by the procedure descried earlier (9) except that the RNA transcribed from *Hin*dIII-truncated pGEM-3Z by T7 RNA polymerase was used as the substrate.

West-western blotting. In order to analyze the interaction between Cet1 and Ceg1, the recombinant Ceg1 was incubated with [α-^{32}P]GTP to form a Ceg1-[^{32}P]pG intermediate (25) and used as a probe. *S. cerevisiae* cell extract, purified capping enzyme, and recombinant Cet1 were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with Ceg1-[^{32}P]pG complex in a solution containing 10 mM Tris-HCl (pH 7.9), 5 mM EDTA, and 100 mM NH_4Cl, for 2 hr at 30°C. The membrane was washed several times with a buffer containing 10 mM Tris-HCl (pH 7.9), 5 mM EDTA, and 150 M NaCl, then exposed to X-ray film.

RESULTS

The partial amino acid sequences obtained from the purified capping enzyme were; YIRGISEVTENTGK (peptide #9), EGNEGNIASNYIT(Q/E)VPLQ (peptide #15) and SXEAANGXXXX(F/A)ENLESDINQTX (peptide #17). From the amino acid sequences of peptides #9 and 15, following sense and antisense degenerate primers were synthesized: 9F, 5′-GAR GTN ACN GAR AAY ACN GG-3′, and 9R, 5′-CCN GTR TTY TCN GTN ACY TC-3′ for peptide #9; 15F, 5′-GAR GGN AAY GAR GGN AAY AT-3′, and 15R, 5′-ATR TTN CCY TCR TTN CCY TC-3′ for peptide #15. Amplification by PCR from *S. cerevisiae* chromosomal DNA using 2 different primer combinations (9F and 15R; 9R and 15F) yielded a 620-bp fragment only when the reaction was performed with primers 9R and 15F. Using this PCR fragment as a probe, we isolated one clone from a *S. cerevisiae* λgt11 cDNA library (λSCC 12) and 2 clones from λgt11 genomic library (λSCG 91 and λSCG92).

DNA sequence analysis of the isolated clones showed that the gene contained an open reading frame of 1,647 nucleotides coding for 549 amino acids with a calculated M_r of 61,821 (Fig. 1). We designated this gene as *CET1.* The predicted amino acid sequence of the open reading frame contained the amino acid sequences identical to those of all three peptide fragments (underlined in Fig. 1) except for one N-terminal Q instead of E of peptide #15. DNA sequence database search revealed that the sequence is identical with the gene P1433 in GenBank (GenBank accession number X94561) located in the left arm of chromosome XVI of *S. cerevisiae* except for 3 nucleotides in the coding region one of which resulted in an amino acid substitu-

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-120  AAAGATCTGCTGGGAATACTATACTGATATTTCCAAAATATCCCTTATAAATTGAATCTG  -61
 -60  GAATAGCACTTCTTTTTTTAAAAAACCTTGAATTGTTGGTAGCATTTCTATCCTCCCACT   -1
   1  ATGAGTTACACTGACAACCCTCCTCAAACAAAAAGAGCTTTATCGTTAGACGATCTGGTG   60
   1  M  S  Y  T  D  N  P  P  Q  T  K  R  A  L  S  L  D  D  L  V    20
  61  AATCACGATGAAAATGAAAAGGTTAAATTACAAAAATTAAGTGAGGCGGCTAATGGCAGC  120
  21  N  H  D  E  N  E  K  V  K  L  Q  K  L  S  E  A  A  N  G  S    40
 121  AGACCTTTTGCCGAAAATTTAGAATCTGATATAAATCAAACGGAAACGGGCCAAGCTGCT  180
  41  R  P  F  A  E  N  L  E  S  D  I  N  Q  T  E  T  G  Q  A  A    60
 181  CCGATTGACAATTACAAGGAGAGTACTGGTCATGGCTCGCACTCACAAAAACCTAAATCA  240
  61  P  I  D  N  Y  K  E  S  T  G  H  G  S  H  S  Q  K  P  K  S    80
 241  CGCAAGTCATCTAATGATGATGAAGAAACCGATACGGATGACGAAATGGGTGCAAGTGGA  300
  81  R  K  S  S  N  D  D  E  E  T  D  T  D  D  E  M  G  A  S  G   100
 301  GAAATTAATTTTGATTCAGAAATGGACTTTGACTATGATAAACAACATAGAAATTTACTA  360
 101  E  I  N  F  D  S  E  M  D  F  D  Y  D  K  Q  H  R  N  L  L   120
 361  TCCAACGGATCACCTCCTATGAATGATGGTAGTGATGCCAATGCGAAGTTAGAAAAGCCT  420
 121  S  N  G  S  P  P  M  N  D  G  S  D  A  N  A  K  L  E  K  P   140
 421  TCTGATGATTCAATTCATCAGAATAGCAAGAGTGATGAAGAACAGAGAATACCGAAACAA  480
 141  S  D  D  S  I  H  Q  N  S  K  S  D  E  E  Q  R  I  P  K  Q   160
 481  GGTAATGAAGGGAACATTGCCAGCAACTATATAACCCAAGTACCTCTGCAAAAGCAGAAG  540
 161  G  N  E  G  N  I  A  S  N  Y  I  T  Q  V  P  L  Q  K  Q  K   180
 541  CAAACTGAGAAGAAGATAGCGGGAAATGCAGTAGGAAGCGTGGTCAAGAAGGAAGAAGAA  600
 181  Q  T  E  K  K  I  A  G  N  A  V  G  S  V  V  K  K  E  E  E   200
 601  GCGAATGCAGCTGTAGATAATATTTTTGAAGAAAAAGCTACTTTACAATCAAAAAGAAT  660
 201  A  N  A  A  V  D  N  I  F  E  E  K  A  T  L  Q  S  K  K  N   220
 661  AATATCAAGAGAGATTTGGAGGTTCTGAATGAAATATCTGCGTCTTCCAAGCCCAGTAAG  720
 221  N  I  K  R  D  L  E  V  L  N  E  I  S  A  S  S  K  P  S  K   240
 721  TACAAGAATGTTCCAATTTGGGCACAAAAATGGAAACCTACTATCAAAGCTCTTCAAAGT  780
 241  Y  K  N  V  P  I  W  A  Q  K  W  K  P  T  I  K  A  L  Q  S   260
 781  ATAAATGTGAAAGATCTCAAAATTGACCCATCTTTTTTAAACATTATTCCCGATGATGAC  840
 261  I  N  V  K  D  L  K  I  D  P  S  F  L  N  I  I  P  D  D  D   280
 841  TTAACAAAGTCAGTACAGGACTGGGTTTATGCTACAATATACTCAATTGCTCCTGAACTA  900
 281  L  T  K  S  V  Q  D  W  V  Y  A  T  I  Y  S  I  A  P  E  L   300
 901  AGATCCTTCATTGAGTTAGAAATGAAATTTGGTGTTATTATTGATGCGAAAGGCCCAGAT  960
 301  R  S  F  I  E  L  E  M  K  F  G  V  I  I  D  A  K  G  P  D   320
 961  CGTGTAAATCCACCAGTTTCTTCACAATGTGTTTTCACTGAGCTTGATGCCCATCTAACG 1020
 321  R  V  N  P  P  V  S  S  Q  C  V  F  T  E  L  D  A  H  L  T   340
1021  CCTAATATTGATGCATCTTTGTTCAAAGAGTTGAGCAAATATATTCGTGGTATTAGCGAA 1080
 341  P  N  I  D  A  S  L  F  K  E  L  S  K  Y  I  R  G  I  S  E   360
1081  GTCACTGAAAATACAGGTAAATTCAGTATTATTGAATCCCAGACAAGAGATTCCGTCTAT 1140
 361  V  T  E  N  T  G  K  F  S  I  I  E  S  Q  T  R  D  S  V  Y   380
1141  AGAGTCGGACTATCCACGCAAAGACCAAGGTTTTGAGAATGAGTACAGATATTAAGACT 1200
 381  R  V  G  L  S  T  Q  R  P  R  F  L  R  M  S  T  D  I  K  T   400
1201  GGGAGGGTAGGACAATTTATAGAGAAAAGACATGTAGCCCAACTACTATTATATTCACCA 1260
 401  G  R  V  G  Q  F  I  E  K  R  H  V  A  Q  L  L  L  Y  S  P   420
1261  AAAGATAGTTACGACGTTAAAATCTCCCTAAACTTGGAATTACCTGTACCTGACAACGAT 1320
 421  K  D  S  Y  D  V  K  I  S  L  N  L  E  L  P  V  P  D  N  D   440
1321  CCGCCAGAAAAATATAAATCTCAAAGCCCAATTAGTGAAAGGACGAAAGACCGTGTTAGT 1380
 441  P  P  E  K  Y  K  S  Q  S  P  I  S  E  R  T  K  D  R  V  S   460
1381  TACATTCATAATGATTCCTGTACCAGAATTGATATTACAAAAGTCGAAAATCATAACCAA 1440
 461  Y  I  H  N  D  S  C  T  R  I  D  I  T  K  V  E  N  H  N  Q   480
1441  AATTCAAAAAGTAGACAATCAGAGACCACTCACGAAGTGGAACTAGAAATCAACACGCCT 1500
 481  N  S  K  S  R  Q  S  E  T  T  H  E  V  E  L  E  I  N  T  P   500
1501  GCACTGTTAAACGCCTTTGATAACATAACGAACGATAGTAAAGAATATGCATCTCTTATT 1560
 501  A  L  L  N  A  F  D  N  I  T  N  D  S  K  E  Y  A  S  L  I   520
1561  AGAACATTTCTGAATAATGGTACAATTATTAGAAGAAAGTTATCGTCTTTATCATATGAA 1620
 521  R  T  F  L  N  N  G  T  I  I  R  R  K  L  S  S  L  S  Y  E   540
1621  ATTTTTGAAGGTTCAAAGAAAGTCATGTAATATTTGAATCATTTCAAAAAAAAATAAGCA 1680
 541  I  F  E  G  S  K  K  V  M  *
1681  AATGCCCTTGAGCGAGAAATTTTTTTTGTTTACTCAAATGCTGTTATGAAAGCTTATGTA 1740
1741  ATAATAATAATAATGATAATAATAACATAAAAATAATTACTCTAACATTTCTTATTATCT 1800
1801  CTATATATCCCTAATAAATAGGCCATTCTTATAATAACCAAGTCTTTCGCCATTTTGATA 1860
1861  CTATCAATAGCTAAAGCCATCTTAGAGCCATCAACCTCATGCCAGGAAATTGGTATCTCC 1920
1921  TCAATTTGGATTCTTTTTCTGATGGCTAAGATCAAAATTTCAACGTCAAAGATCCAACCC 1980
1981  TCTGTATGCAAATACGGAAAAATTTTCAATATAGCGGCTCTGTTGAACAATTTGAATCCA 2040
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FIG. 1. Nucleotide and deduced amino acid sequences of *S. cerevisiase* RNA 5′-triphosphatase (β subunit) gene (*CET1*). The coding region has been translated and the one letter code is presented. Putative TATA is boxed. Amino acid sequences of peptide fragments obtained from the purified β subunit are underlined. In-frame termination codons in the 5′ flanking region and a putative polyadenylation signal are underlined. Nucleotides and amino acid sequences differing from the database sequences are indicated by double underlines. Wavy underline indicates the location of termintion codon of the downstream flanking gene on the complementary strand.

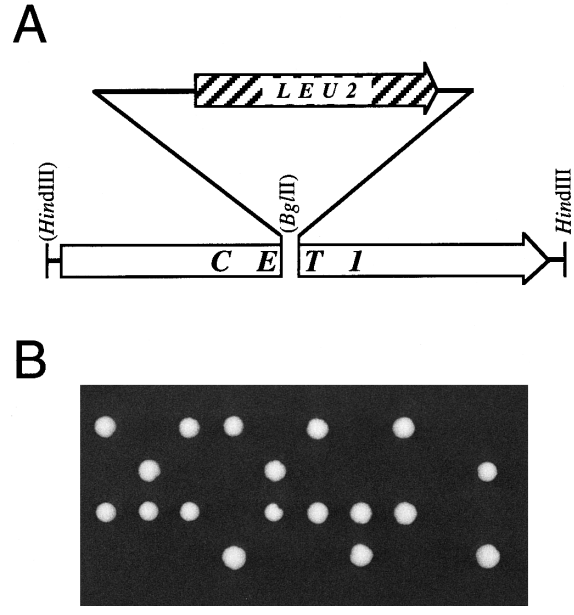

FIG. 2. Disruption of *S. cerevisiase* RNA 5′-triphosphatase gene (*CET1*). (A) Restriction sites of *CET1* and *LEU2* locus. The hollow arrows indicate open reading frames. A 2.5-kb *Bam*HI fragment containing the *LEU2* gene was inserted in the *Bgl*II site of *CET1*. (B) Tetrad analysis. Spores from 9 individual asci of a Leu$^+$ transformant were allowed to germinate on a YPD plate. All viable spores were found to be leucine auxotrophs.

tion in codon 242 from arginine to lysine (double underlined in Fig. 1). Seventy-five base pairs upstream from the predicted initiator methionine codon a putative TATA box is observed. The gene is flanked by the *ALG5* locus encoding the UDP-glucose:dolichyl-phosphate glucosyltransferase (23) tail to tail separeted by only 131 bp. The location of termination codon of *ALG5* on the complementary strand is indicated by wavy underline in Fig.1.

Comparison of the predicted amino acid sequence of *CET1* with GenBank data base showed no apparent homologies to known nucleases and phosphatases. Furthermore, no significant similarity to vaccinia virus capping enzyme containing RNA 5′ triphosphatase activity (26) was found.

In order to elucidate whether *CET1* is essential for yeast growth, we disrupted *CET1* gene using fragment mediated transformation (20). A *leu2* diploid strain was transformed with a 6-kb *Hin*dIII/*Pst*I restriction fragment containing *CET1* which had been disrupted by the 2.5-kb yeast *LEU2* gene as a genetic marker (Fig. 2A). Leu$^+$ transformants were selected and are subjected to tetrad analysis. Fig. 2B shows the spores of 9 asci from one of the transformants. Only two of the four spores from each ascus were viable, and all the growing cells were Leu$^-$. That is, *LEU2* segregates with lethality, thus suggesting that *CET1* is essential for growth of haploid yeast cells.

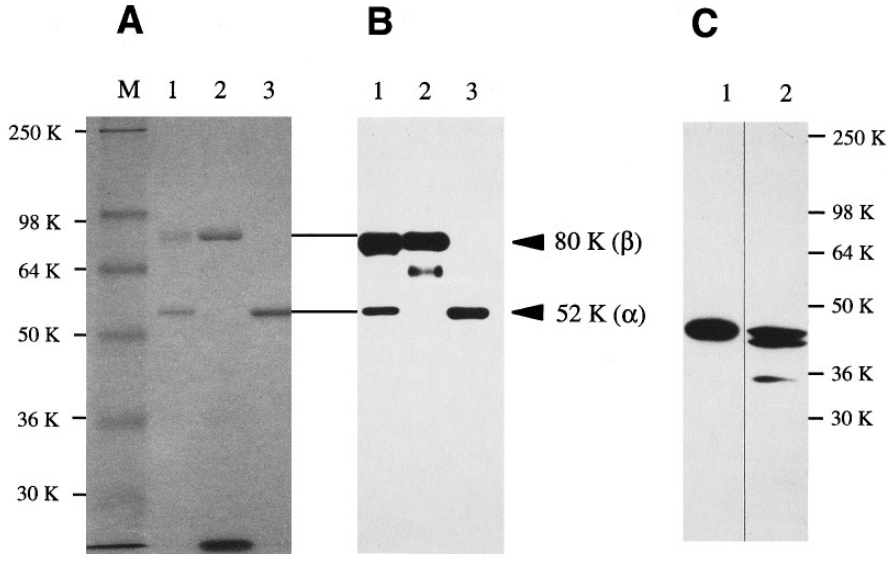

FIG. 3. Expression and purification of recombinant β subunit (Cet1) proteins. (A) Purified yeast capping enzyme and recombinant proteins were prepared as descried in Materials and Methods and were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, 0.32 μg of purified capping enzyme from *S. cerevisiae;* lane 2, 0.6 μg of recombinant His-Cet1; and lane 3, 0.9 μg of recombinant α subunit (Ceg1). M, molecular mass markers. (B) Western blot analysis. The same protein samples were separated by SDS-PAGE and transferred to a PVDF membrane, then probed with anti-capping enzyme antibody as described in Materials and Methods. Lane 1, purified capping enzyme; lane 2, His-Cet1; and lane 3, Ceg1. (C) Western blot analysis of truncated Cet1 proteins. His-tagged C- and N-terminal deletion mutant proteins,His-Cet1 (1-265) and His-Cet1 (205-549), respectively, were expressed in *E. coli* as described in Materials and Methods and subjected to western blot analysis with anti-capping enzyme antibody as the probe. Lane 1, His-Cet1 (1-265); lane 2, His-Cet1 (205-549).

The molecular weight of yeast capping enzyme β subunit was estimated to be about 80 kDa by SDS-PAGE (11). The predicted molecular weight of about 62 kDa calculated from *CET1* sequence was unexpectedly smaller than 80 kDa. To characterize the encoded protein from *CET1*, the coding region of the gene was amplified from the yeast chromosomal DNA by PCR using the primers synthesized according to the sequence and was ligated to the expression vector to produce a His-tagged recombinant protein and expressed in *E. coli*. When the purified recombinant Cet1 protein was subjected to SDS-PAGE (Fig. 3), it migrated at the position of about 80 kDa (Fig. 3A, lane 2) which corresponded well to the size of the β subunit of purified capping enzyme from *S. cerevisiae* (Fig. 3A, lane 1). Immunoblot analysis showed that the anti-capping enzyme antibody raised against purified yeast capping enzyme reacted with the recombinant Cet1 (Fig. 3B, lane 2), as well as the α subunit of the enzyme (Fig. 3B, lane 1) or the recombinant Ceg1 (Fig. 3B, lane 3). In order to investigate the discrepancy between the calculated M_r and the migration pattern on the polyacrylamide gel, we constructed His-tagged C- and N-terminal deletion mutants, His-Cet1(1-265) and His-Cet1(205-549), respectively, and expressed in *E. coli*. Western blot analysis of these truncated proteins showed that the His-Cet1(1-265) having a calculated M_r of 31,468 run abnormally slowly (as 44 kDa) on SDS-PAGE (Fig. 3C, lane 1), whereas His-Cet1(205-549) with a calculated M_r of 40,971 migrated to the position of about 42 kDa, albeit with some degradation products with smaller sizes (Fig. 3C, lane 2). Thus, the anomalous electrophoretic movility of Cet1 may be ascribed to the nature of its N-terminal portion.

To demonstrate the enzymatic activity of RNA 5′-triphosphatase of Cet1, [γ-^{32}P]triphosphate-terminated RNA was prepared and tested for the release of ^{32}P-radioactivity (Fig. 4). The recombinant Cet1 released [^{32}P]Pi from [γ-^{32}P]triphosphate-terminated RNA (Fig. 4, lane 9) but not from [γ-^{32}P]GTP (Fig. 4, lane 3) indicating that it retains the specificity to the RNA chain that was observed with the intact capping enzyme (26). The activity of RNA 5′-triphosphatase of recombinant Cet1 was comparable to the purified capping enzyme from *S. cerevisiae* (Fig. 4, lane 10). To assign the functional domain for RNA 5′-triphosphatase, His-tagged C- and N-terminal deletion mutants, His-Cet1(1-265) and His-Cet1(205-549) were assayed for RNA 5′-triphosphatase activity. When His-Cet1(205-549) was incubated with 5′-[γ-^{32}P]GTP-terminated RNA, [^{32}P]Pi was released (Fig. 4, lane 12), However, His-Cet1(1-265) did not (Fig. 4, lane 11), indicating that the active site resides in the C-terminal 345 amino acid fragment.

We developed a novel procedure to detect α-β subunit interactions using the α subunit Ceg1-[^{32}P]pG covalent reaction intermediate as the probe for west-western

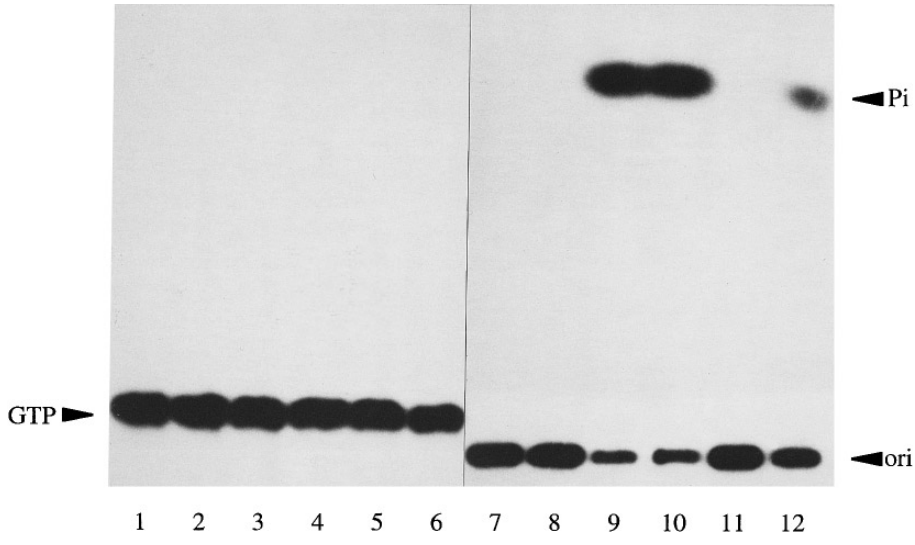

FIG. 4. Substrate specificity of RNA 5′-triphosphatase. Purified yeast capping enzyme and recombinant proteins were assayed for phosphohydrolase activity using [γ-^{32}P]GTP (lanes 1-6) or [γ-^{32}P]triphosphate-terminated RNA (lanes 7-12) as substrate. Lanes 1 and 7, no protein added. Lanes 2 and 8, 10 ng of recombinant α subunit (Ceg1). Lanes 3 and 9, 10 ng of recombinant β subunit (His-Cet1). Lanes 4 and 10, 20 ng of purified capping enzyme from *S. cerevisiae.* Lanes 5 and 11, 100 ng of C-terminal deletion mutant, His-Cet1 (1-265). Lanes 6 and 12, 100 ng N-terminal deletion mutant, His-Cet1(205-509).

analysis (Fig. 5). When crude yeast cell extract was electrophoresed and proteins were probed with Ceg1-[^{32}P]pG, a single labeled band of 80 kDa was observed (Fig. 5, lane 1), indicating that this procedure provides the specific detection of α-β interaction. To confirm that the recombinant Cet1 expressed in *E. coli* interacts similarly with Ceg1-[^{32}P]pG. The His-Cet1 was subjected to this procedure. The labeled probe reacted with the recombinant Cet1 immobilized on the filter to give a 80 kDa signal (Fig. 5, lane 3) as well as the β subunit of purified capping enzyme (Fig. 5, lane 2). It should be noted that His-Cet1 (205-549) retained the binding activity to the α subunit (data not shown). We are now extending this procedure to map the region of Cet1 protein responsible for its subunit-subunit interaction using various deletion mutants.

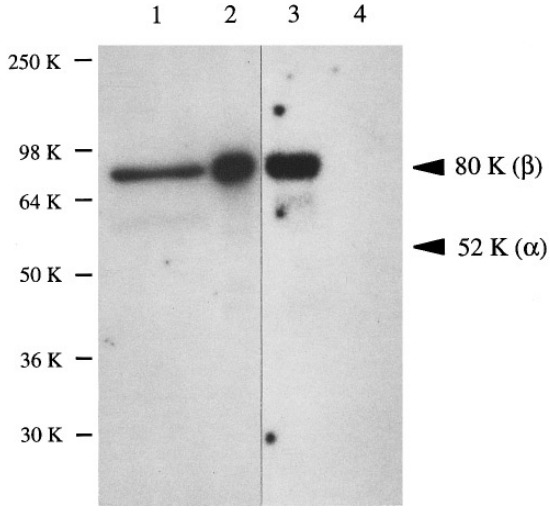

FIG. 5. West-western analysis. *S. cerevisiae* S-100 (10 μg) (lane 1), purified capping enzyme from *S. cerevisiae* (0.32 μg) (lane 2), recombinant Cet1 (0.6 μg) (lane 3), and recombinant Ceg1 (0.9 μg) (lane 4) were subjected to SDS-PAGE and transferred to a PVDF membrane and then probed with Ceg1-[^{32}P]pG complex (specific activity, 2×10^5 cpm/pmol).

DISCUSSION

In the present study, we have cloned the gene (*CET1*) encoding the β subunit (RNA 5′-triphosphatae) of yeast mRNA capping enzyme and demonstrted that it is essential for the growth of yeast cells. We have also shown that the active site of the triphosphatase resides in the C-terminal 60% of the Cet1 molecule. Moreover, a novel procedure to study subunit-subunit interactions in the yeast mRNA capping enzyme has been presented.

Our previous studies demonstrated that the purified yeast capping enzyme is composed of two separate chains of 52 (α) and 80 (β) kDa when analyzed by SDS-PAGE (11). Although the cloned *CET1* gene contains an open reading frame encoding 549 amino acids with a calculated M_r of 61,849, the recombinant protein expressed in *E. coli* migrated as a 80 kDa protein (Fig. 3). The reason for this discrepancy, however, remains unknown at present. Nevertheless, the migration pattern of a N-terminus-deletion mutant roughly coin-

cided with the calculated M_r, while a C-terminus-deletion mutant migrated much slower than expected from its calculated M_r, implicating that the N-terminus portion of Cet1 molecule is responsible for its aberrant migration in the acrylamide gel. It is possible that, for instance, the N-terminal region of Cet1 is rich in acidic amino acids resulting in the reduced affinity for SDS molecules, and indeed the N-terminal region (residues 1-200) contains 40 acidic amino acids which give rise to a total net charge of −16, whilst the rest of the molecule (residues 201-549) has a net charge of +2. However, these considerations do not rule out possibilities such as special features of higher order structures of the Cet1 molecule, and covalent modifications that might occur even in *E. coli*. The phosphorylation of Cet1 in Cet1 in *E. coli* cells is improbable, since the treatment of Cet1 with alkaline phosphatase did not affect the migration pattern (data not shown). Similar observations of anomalous electrohoretic mobility have been reported for nuclear proteins (27,28) and CANP inhibitor (29).

The finding that a C-terminal ~39 kDa fragment (redidues 205-549) exhibits RNA triphosphatase activity (Fig. 4) recalls our earlier observations that the yeast capping enzyme purified after treatment of cell with Zymolyase consists of the 45 kDa (α) with mRNA guanylyltransferase activity and the 39 kDa (β) subunits with RNA 5′-triphosphatase activity (25). It is considered that the 45 (α) and the 39 kDa (β) polypeptides are derived from the intact 52 (α) and 80 kDa (β) polypeptides, respectively, due to the proteolytic degradation during preparation of spheroplasts (11). The 39 kDa C-terminal fragment in the present study may, by chance, roughly correspond to our earlier 39 kDa β subunit. Indeed, the computer analysis of secondary structure of Cet1 according to Chow and Fasman (30) predicted that there exist a hinge region spaning residues 170 and 200, between the N-terminal and the C-terminal mostly folded regions in the Cet1 molecule.

We have already shown that *CEG1* is essential for yeast cell growth (12). Recently, Mao *et al.* (31) have demonstrated that the disruption of *S. cerevisiae ABD1* encoding mRNA (guanine-7-)methyltransferse is lethal to yeast. Thus, together with our present finding that *CET1* is required for yeast cell growth, all three capping-related genes so far cloned, and by inference, methylated cap structure formation, are indispensable in cellular function *in vivo*. It may be noted that *CEG1*, *CET1*, and *ABD1*, encoding three enzymes that catalyzes three consecutive reactions in cap formation, are located on different yeast chromosomes, VII L, XVIL, and II R, respectively. Although the cap structure of yeast mRNA has been considered cap0 structure (m^7GpppN), earlier studies does not rule out the presence of cap1 (m^7GpppNm) in some yeast mRNAs. In this regard, it is interesting to examine whether mRNA (ribose-2′-*O*-)methyltransferase gene, which has not cloned yet from any sources, is present in yeast.

The RNA 5′-triphosphtase activity of yeast (11) and rat liver (9) specifically removes the γ-phosphate from pppN-RNA, but not from nucleotide triphosphates. It is noteworthy that the recombinant Cet1 still retains the same substrate specificity in the absence of he Ceg1, suggesting that Cet1 itself must have an RNA recognition site. Similarly, the recombinant Ceg1 requires RNA chain for its activity (unpublished observation). The important question still remains how these RNA recognition sites are related to each other in capping reaction within the intact enzyme. The substrate specificity of RNA 5′-triphosphtase of cellular capping enzyme represent a striking contrast to vaccinina virus triphosphatase activity which also has high NTPase activity (32).

In vaccinia virus capping enzyme, regions responsible for mRNA guanylyltransferase and RNA 5′-triphosphatase activities are overlapping in modular fashion (32). Comparison of newly identified *CET1* with vaccinia virus capping enzyme showed no apparent sequence homologies. Recently, Takagi *et al.* (33) identified *C. elegans* capping enzyme gene (*CEL-1*) and showed that its RNA 5′-triphosphatase domain resembles the protein tyrosine phosphatase (PTP) family. More recently, we have isolated human and murine cDNAs encoding mRNA capping enyme (manuscript in preparation). Predicted amino acid sequence of *CET1* bears no significant sequence similarities to these cellular RNA 5′-triphosphatase domains including the PTP active site motif. In this regard, the vaccinia virus capping enzyme also does not show obvious sequence homology to PTP motfs (33). It is curious to mention that yeast Cet1 has a much larger size (62 kDa, calculated) compared to the sizes of the RNA triphosphatase domains of animal capping enzymes: for exmple, a 20 kD domain can be isolated in the active state after limited proteolysis of *A. salina* enzyme (10), and an N-terminal 236 amino acid-fragment of *C. elegans* enzyme expressed in *E. coli* exhibits an RNA triphosphatase activity. The facts that our C-terminal recombinant fragment (residues 205-549) possesses RNA triphosphatase activity, and the 39 kDa β subunit of purified capping enzyme from Zymolyase-treated yeast cells, as discussed above, catalyzes the same reaction (26,11) imply that N-terminal region (1-205) of Cet1 may not be essential for the RNA triphosphatase *per se*. Furthermore, since the 39 kDa β subunit is tightly associated with the α subunit, the 39 kDa C-terminal fragment of Cet1 must have the binding site to the α subunit together with the RNA chain recognition site which has been discussed in the previous section. Then, the important question arises with respect to the function of the N-terminal acidic region of Cet1 molecules, that at present remain unknown. Clearly, introduction

of various mutations into *CET1* and functional analyses of the mutant proteins *in vitro* as well as *in vivo* should help our understanding about the functional domains for RNA phosphohydrolytic activity, binding to the α subunit, and other unknown functions in RNA metabolism, if exist.

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