An RNA 5′-Triphosphatase Related to the Protein Tyrosine Phosphatases

Toshimitsu Takagi, Christine R. Moore, Felix Diehn, and Stephen Buratowski
Department of Biological Chemistry and Molecular
  Pharmacology
Harvard Medical School
Boston, Massachusetts 02115

Summary

mRNA capping requires the sequential action of three enzymatic activities: RNA triphosphatase, guanylyltransferase, and methyltransferase. Here we characterize a gene (*CEL-1*) believed to encode the C. elegans capping enzyme. CEL-1 has a C-terminal domain containing motifs found in yeast and vaccinia virus capping enzyme guanylyltransferases. The N-terminal domain of CEL-1 has RNA triphosphatase activity. Surprisingly, this domain does not resemble the vaccinia virus capping enzyme but does have significant sequence similarity to the protein tyrosine phosphatase (PTP) enzyme family. However, CEL-1 has no detectable PTP activity. The mechanism of the RNA triphosphatase is similar to that of PTPs: the active site contains a conserved nucleophilic cysteine required for activity. These results broaden the superfamily of PTP-like phosphatases to include enzymes with RNA substrates.

Introduction

Transcripts produced by RNA polymerase II are modified at their 5′ end by the addition of a 7-methyl guanosine cap. Capping is the earliest modification of mRNA, occurring by the time the transcript is 25–30 nucleotides long (Jove and Manley, 1984; Rasmussen and Lis, 1993). The reaction occurs in three steps (see Figure 1): an RNA 5′-triphosphatase removes the gamma phosphate at the 5′ end of the transcript, a GTP::RNA guanylyltransferase adds a GMP residue to the 5′ diphosphate end in a 5′-to-5′ orientation, and an RNA (guanine-7-) methyltransferase adds the methyl group to the guanine (see Mizumoto and Kaziro, 1987, for review).

Biochemical purifications of capping enzymes from various organisms have revealed differences in subunit structure between organisms (Mizumoto and Kaziro, 1987). Vaccinia virus encodes its own capping enzyme, which consists of two subunits. The larger subunit has both triphosphatase and guanylyltransferase activities, while both subunits are required to reconstitute methyltransferase activity (Shuman, 1990). Biochemical characterization of capping enzymes from higher eukaryotes suggest that there is one protein carrying both triphosphatase and guanylyltransferase activities. In contrast to higher eukaryotes, the Saccharomyces cerevisiae capping enzyme has two subunits: a 53 kDa guanylyltransferase and an 80 kDa triphosphatase subunit (Itoh et al., 1984a, 1987). The yeast methyltransferase purifies as an unassociated enzyme and is encoded by the gene *ABD1* (Mao et al., 1995).

Only a few eukaryotic capping enzyme genes have been cloned to date, all from yeast. In addition to the yeast methyltransferase gene *ABD1*, three yeast guanylyltransferase genes have been described. The S. cerevisiae guanylyltransferase is encoded by *CEG1* (Shibagaki et al., 1992). By complementation of mutant *CEG1* alleles, guanylyltransferases for two other yeasts have been cloned (Shuman et al., 1994; Yamada-Okabe et al., 1996; L. D. Fresco et al., unpublished data). Both *CEG1* and *ABD1* are essential for viability. Sequence analysis, biochemical characterization, and genetic experiments have shown that the yeast guanylyltransferases are mechanistically related to DNA and RNA ligases (Shuman and Schwer, 1995, for review). The yeast guanylyltransferase proteins do not have RNA 5′-triphosphatase activity.

There are two major gaps in our understanding of capping enzymes. First, since the yeast triphosphatase subunit gene has not yet been identified, little is known about the enzymology of the eukaryotic triphosphatase reaction. Second, no genes for higher eukaryotic capping enzymes, which combine both triphosphatase and guanylyltransferase activities in one protein, have yet been described. In this report, we present the cloning and characterization of the putative C. elegans capping enzyme gene. Remarkably, this enzyme appears to consist of two domains: one that is closely related to the yeast guanylyltransferases and a second that is a member of the protein tyrosine phosphatase (PTP) superfamily. The PTP domain has no tyrosine phosphatase activity, yet apparently uses a mechanism similar to PTPs to remove the terminal phosphate of mRNA.

Results

To find higher eukaryotic capping enzymes, we carried out a search for genes with significant similarity to the yeast guanylyltransferases using the BLAST algorithm (Altschul et al., 1990). A strong match was found to a gene (C03D6.3, GenBank accession no. Z75525) that emerged from the C. elegans genome sequencing project. The predicted open reading frame of this gene is 573 amino acids. The C-terminal 340 amino acids of the protein exhibit very strong sequence similarity to the yeast guanylyltransferases (Figure 2A), particularly in regions previously noted to be conserved between eukaryotic and viral capping enzyme guanylyltransferases (Shuman and Schwer, 1995, for review). The C. elegans protein contains a motif containing the nucleophilic lysine required for formation of the enzyme-GMP intermediate of the capping reaction (marked by an asterisk in Figure 2A).

The C. elegans capping enzyme has not been previously studied. Extracts from C. elegans were incubated with [α-^{32}P]GTP in order to allow formation of the guanylyltransferase-GMP covalent intermediate (see Figure 1). The major labeled band was about 68 kDa (data not shown), in excellent agreement with the predicted molecular weight of C03D6.3. We attempted to
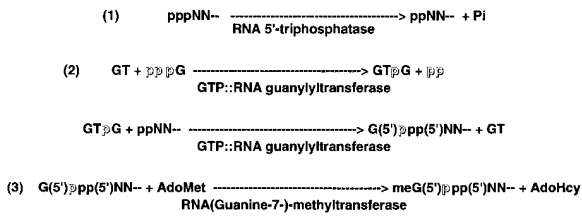
(1) pppNN-- ------------------------------------> ppNN-- + Pi
RNA 5'-triphosphatase

(2) GT + pppG --------------------------------------> GTpG + pp
GTP::RNA guanylyltransferase

GTpG + ppNN-- -------------------------------------> G(5')ppp(5')NN-- + GT
GTP::RNA guanylyltransferase

(3) G(5')ppp(5')NN-- + AdoMet --------------------------------------> meG(5')ppp(5')NN-- + AdoHcy
RNA(Guanine-7-)-methyltransferase

Figure 1. Enzymatic Steps in the mRNA Capping Reaction

pppNN- represents the 5′ end of the nascent RNA transcript; GT stands for the mRNA guanylyltransferase; AdoMet is S-adenosyl methionine; and AdoHcy is S-adenosyl homocysteine (Mizumoto and Kaziro, 1987, for review).

express recombinant protein in bacteria or reticulocyte lysates, but have so far been unable to detect guanylyltransferase activity. C03D6.3 may be toxic in bacteria, since sequencing of several independent clones revealed missense and premature termination mutations in the guanylyltransferase-like region. Based on the strong sequence alignment and data presented below, we believe that this protein encodes the C. elegans capping enzyme. Accordingly, we have tentatively designated this gene *CEL-1*, for Capping Enzyme from C. elegans. However, definitive proof of the guanylyltransferase activity remains to be shown.

CEL-1 Contains a PTP-like Domain

In addition to the region homologous to yeast guanylyltransferase, the C. elegans protein has an additional N-terminal region of approximately 230 amino acids. This region was of particular interest, since capping enzymes purified from some higher eukaryotes have been shown to carry both triphosphatase and guanylyltransferase activities on one peptide (Mizumoto and Kaziro, 1987, for review). A search of the sequence databases using amino acids 1–240 of CEL-1 led to the remarkable finding that this region of the protein was quite similar to the protein tyrosine phosphatase protein family (Figure 2B). In particular, the conserved active

Figure 2. CEL-1 Is Related to Guanylyltransferases and Protein Tyrosine Phosphatases

(A) Alignment between CEL-1 and yeast guanylyltransferases. The deduced amino acid sequence of CEL-1 is compared with guanylyltransferase subunits of the S. cerevisiae (CEG1; Shibagaki et al., 1992) and S. pombe (PCE1; Shuman et al., 1994) mRNA capping enzyme. Letters represent the single-letter amino acid code, and numbers represent the positions of the amino acid residues. The asterisk shows the guanylyltransferase active site lysine residue (Shuman and Schwer, 1995). Boxed residues denote identities, and shaded residues signify similarity to the CEL-1 amino acid sequence.

(B) Alignment between N-terminal region of CEL-1 and protein tyrosine phosphatases. The following sequences are shown: CEL-1 (residues 1–200 of C. elegans gene C03D6.3); BVP (Baculovirus PTP [Sheng and Charbonneau, 1993]); T23G7.5, F54C8.4 (two other C. elegans open reading frames, GenBank accession nos. Z68319 and Z22178). The active site consensus of PTPs is marked by a line while the asterisk shows the predicted phosphatase active site cysteine residue (Denu et al., 1996; Fauman and Saper, 1996).

(C) Schematic representation of the CEL-1 domain structure.

A

B
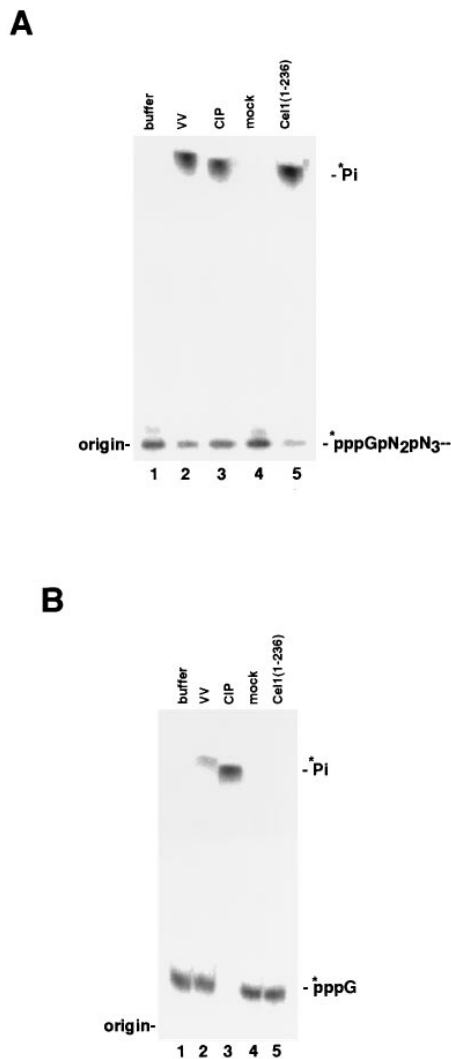
Figure 3. The CEL-1 Amino-Terminal Domain Is an RNA Triphosphatase

(A) 5′ RNA triphosphatase assay. [γ-^{32}P]GTP-terminated RNA was incubated with buffer (lane 1); 50 ng of vaccinia virus capping enzyme (VV, lane 2); 1 unit of calf intestinal phosphatase (CIP, lane 3); or 10 ng of his$_7$-Cel-1(1–236) (lane 5). Lane 4 (mock) is a negative control that has 10 ng of protein from an extract made from E. coli strain BL21(DE3) transformed with vector and chromatographed in parallel with the CEL-1 extract. Incubation was for 30 min at 30°C (lanes 1, 4, and 5) or 37°C (lanes 2 and 3). The reaction mixture was analyzed by thin-layer chromatography (TLC) on PEI-cellulose plates. ^{32}P label was detected by PhosphorImager.
(B) Nucleotide phosphohydrolase assay. Reactions in lanes 1–5 were identical to those in (A), except [γ-^{32}P]GTP-terminated RNA was replaced with [γ-^{32}P]GTP.

Figure 4. The CEL-1 Triphosphatase Leaves a Diphosphate End

A two-step reaction was performed as described in Experimental Procedures. In the first step, GTP-terminated RNA was incubated with the indicated enzyme preparations labeled as in Figure 3. After the first incubation, RNA was purified and subjected to a second incubation with [α-^{32}P]GTP and recombinant guanylyltransferase subunit of S. cerevisiae capping enzyme. RNA was extracted, treated with nuclease P1 and CIP, and the cap structure was analyzed by paper electrophoresis and PhosphorImager. The position of an authentic cap structure sample (GpppG) was detected by UV light.

site motif (I/V)HCxxGxxR(S/T)G of protein tyrosine phosphatases (Denu et al., 1996; Fauman and Saper, 1996) was matched by residues 122–132 (overlined in Figure 2B). Most alignments between CEL-1 and tyrosine phosphatases revealed some additional conserved residues flanking the active site region. Particularly extensive alignments (p < 10^{-15}) were found with two other C. elegans open reading frames (T23G7.5, GenBank accession no. Z68319 and F54C8.4, GenBank accession no.

Z22178). The closest non–C. elegans match was to a baculovirus tyrosine phosphatase that also has the ability to remove phosphate from serine and threonine (Sheng and Charbonneau, 1993).

The CEL-1 PTP-like Domain Is an RNA 5′-Triphosphatase but Not a Tyrosine Phosphatase

Based on the similarity to protein tyrosine phosphatases, two possible functions of the CEL-1 PTP-like domain were considered. Our primary hypothesis was that the PTP-like domain of CEL-1 was the mRNA triphosphatase necessary for the first step in capping. However, PTP domains often appear fused to other functional domains such as transmembrane receptors and SH2 domains (Denu et al., 1996; Fauman and Saper, 1996). Therefore, a second possibility was that the CEL-1 PTP-like domain was a true tyrosine phosphatase involved in regulation of capping enzyme or some other associated protein. To test these hypotheses, recombinant protein encompassing residues 1–236 was expressed in E. coli and purified to apparent homogeneity (data not shown).

The recombinant CEL-1 amino terminal domain was tested for tyrosine phosphatase activity. Two commonly used tyrosine phosphatase assays were used. The first was to test for release of phosphate from myelin basic protein phosphorylated on tyrosine. Although a bona fide T-cell PTP released phosphate from myelin basic protein, the CEL-1 protein was completely inactive in this assay (data not shown). The second assay tested the ability to hydrolyze 4-nitrophenyl phosphate (pNPP), a chromogenic substrate analog of phosphotyrosine. All true tyrosine phosphatases tested to date are able to

Figure 5. The CEL-1 RNA Triphosphatase Requires the PTP Active Site Cysteine

An RNA triphosphatase assay was performed with his$_7$-Cel-1(1–236) or his$_7$-Cel-1(1–236)C124S. After the incubation, the reaction mixture was processed by PEI cellulose thin-layer chroma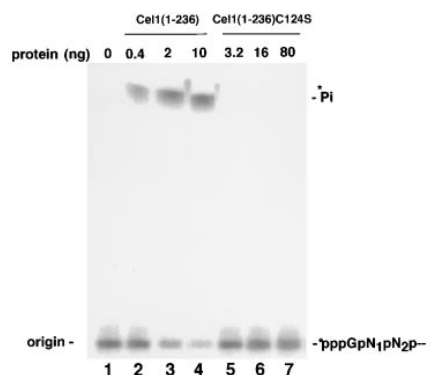tography. [γ-^{32}P]GTP-terminated RNA was incubated either with buffer (lane 1), the indicated amounts of wild-type his$_7$-Cel-1(1–236)(lanes 2–4), or mutant his$_7$-Cel-1(1–236)C124S (lanes 5–7). A mutant with the amino acid change C124A behaved identically to the C124S mutant (data not shown).

hydrolyze this substrate (Denu et al., 1996; Fauman and Saper, 1996). As in the first assay, the CEL-1 PTP-like domain exhibited no activity (data not shown). Therefore, it appears very unlikely that CEL-1 is a true protein tyrosine phosphatase.

To test the CEL-1 amino-terminal domain for RNA triphosphatase activity, the protein was incubated with a synthetic RNA terminated by a 5′ triphosphate end labeled with a [^{32}P]γ-phosphate. Release of monophosphate was assayed by thin layer chromatography (Figure 3A). Control reactions with calf intestinal phosphatase (CIP, lane 3) and vaccinia virus capping enzyme (VV, lane 2) released monophosphate from the RNA substrate. As would be expected for a bona fide capping enzyme, the CEL-1 amino-terminal domain also showed RNA triphosphatase activity (lane 5). The T-cell PTP used as a positive control in the tyrosine phosphatase assays had no detectable RNA triphosphatase activity (data not shown).

To see whether this triphosphatase activity was specific for polynucleotide RNA, the same experiment was carried out using [γ-^{32}P]GTP (Figure 3B) or ATP (data not shown). In this case, CIP (lane 3) completely released the labeled phosphate, while the vaccinia virus capping enzyme (VV, lane 2) had weak GTPase activity as previously described (Shuman et al., 1980; Myette and Niles, 1996). However, CEL-1 released no monophosphate (lane 5), demonstrating that the CEL-1 triphosphatase is specific for polynucleotide RNA as expected for a cellular mRNA capping enzyme (Yagi et al., 1983).

For the CEL-1 amino-terminal domain to function in the capping reaction, it must leave a diphosphate 5′ end to serve as the substrate for the subsequent guanylyltransferase. This was tested in a two step reaction. First, a 5′ triphosphate–ended RNA was incubated with CEL-1 triphosphatase, VV capping enzyme, CIP, or no enzyme. The reaction was then deproteinized and the resulting RNA was incubated with yeast guanylyltransferase

(which has no triphosphatase activity) and [α-^{32}P]GTP. The reactions were then assayed for the presence of the labeled GpppG cap structure. Only the VV capping enzyme and the CEL-1 triphosphatase produced RNA substrates with diphosphate ends that could be guanylylated (Figure 4, lanes 2 and 5). Therefore, the CEL-1 amino-terminal domain exhibits all the enzymatic activities necessary for a capping enzyme triphosphatase.

Conservation of Mechanism between the RNA Triphosphatase and PTPs

Because the sequence similarity between CEL-1 and PTPs spans the PTP active site, it seemed likely that the two types of enzymes would share some mechanistic features. PTPs have been extensively studied in terms of both enzyme mechanism and structure (Denu et al., 1996; Fauman and Saper, 1996). The conserved cysteine of the PTPs acts as a nucleophile to attack the phosphate, forming a transient phosphocysteine intermediate. The tyrosine and attached protein are the leaving group. A feature of PTPs that distinguishes them from many other phosphatases is that they do not require divalent cations (Denu et al., 1996; Fauman and Saper, 1996). The CEL-1 triphosphatase activity was tested at varying magnesium concentrations. No divalent cations were necessary for triphosphatase activity (data not shown), as would be predicted from a PTP-like mechanism.

By alignment of CEL-1 with the PTP active site consensus, we predicted that the CEL-1 RNA triphosphatase should use cysteine 124 as a nucleophile to attack the γ-phosphate at the mRNA 5′ end (marked by an asterisk in Figure 2B). To test whether C124 was essential for RNA triphosphatase activity, we mutated this residue to serine or alanine. The wild-type and mutant N-terminal domains were produced in bacteria and tested for RNA triphosphatase activity (Figure 5). The C124S mutant (lanes 5–7) and the C124A mutant (data not shown) proteins were not functional, suggesting that the capping enzyme RNA triphosphatase uses a mechanism very similar to that of protein tyrosine phosphatases.

Discussion

Surprisingly, the CEL-1 RNA triphosphatase is related to the family of protein tyrosine phosphatases. This enzyme family consists of both tyrosine-specific phosphatases as well as "dual specificity" tyrosine phosphatases that also hydrolyze phosphoserine and phosphothreonine to some extent (reviewed in Denu et al., 1996; Fauman and Saper, 1996). PTPs have been implicated in several processes, including signal transduction and cell cycle control. CEL-1 represents the first member of this family to recognize an RNA substrate. There are a number of genes (including CEL-1) emerging from genome sequencing projects that are listed in the databases as tyrosine phosphatases. The discovery that this family of enzymes has a wider than anticipated substrate range underscores the need for experimental confirmation of functional assignments based on sequence homology alone. Indeed, there are several receptor-like

PTP proteins that carry two PTP domains, one of which is often inactive on phosphotyrosine (reviewed in Denu et al., 1996; Fauman and Saper, 1996). These "inactive" domains may in fact have other substrates that have not yet been recognized.

The C. elegans capping enzyme triphosphatase is most closely related to the baculovirus protein BVP (Sheng and Charbonneau, 1993). This protein has been experimentally shown to have potent tyrosyl and seryl/threonyl phosphatase activities. Based on its sequence similarity, it would be interesting to test this protein for RNA triphosphatase activity as well. A T-cell PTP was tested and had no RNA triphosphatase activity (data not shown). Two other open reading frames with significant sequence similarity to CEL-1 and BVP have also emerged from the C. elegans genome sequencing project. The proteins encoded by these genes have not yet been tested for substrate specificity. These four proteins apparently represent a distinct branch of the PTP enzyme superfamily, as their similarity to other PTPs is largely confined to the active site consensus sequence.

Capping Enzyme Triphosphatases

It remains to be seen whether the CEL-1 triphosphatase is representative of all higher eukaryotic capping enzymes. Biochemical characterizations of capping enzymes from two higher eukaryotes (brine shrimp and rat) are consistent with a single protein with both triphosphatase and guanylyltransferase activity, although the genes for these proteins have not yet been cloned (reviewed in Mizumoto and Kaziro, 1987). In yeast, the capping enzyme mRNA triphosphatase is a separate polypeptide from the guanylyltransferase, although the two proteins are associated (Itoh et al., 1984a, 1987). A comparison of all yeast protein sequences with the CEL-1 triphosphatase domain revealed moderate matches to several proteins in the PTP family, particularly CDC14. However, none of the alignments were particularly extensive and the predicted sizes of the proteins were not in close agreement with the 80 kDa size of purified triphosphatase (Itoh et al., 1987).

It is possible that the yeast triphosphatase is not related to CEL-1 or the PTP enzymes. The yeast RNA triphosphatase requires divalent cations for activity (Itoh et al., 1984b), which is a property of many classes of phosphatases, but not of PTPs. CEL-1 triphosphatase was not inhibited by EDTA and was strongly inhibited (>90%) by the presence of 2 mM Mg^{2+} (data not shown). Similar results have been described for the rat liver capping enzyme (Yagi et al., 1983). In contrast, the vaccinia virus RNA triphosphatase requires divalent cations (Shuman, 1990; Myette and Niles, 1996). The vaccinia virus capping enzyme does not show obvious sequence similarity to tyrosine phosphatases (Niles et al., 1986; Yu and Shuman, 1996). Indeed, there are no obvious similarities to any of the known phosphatase families.

Therefore, it may be that distinct enzymatic mechanisms may carry out similar phosphatase reactions in different capping enzymes. This is in contrast to the guanylyltransferases, which appear to use a common enzymatic mechanism (Shuman and Schwer, 1995). Such major mechanistic differences suggest that inhibitors of the viral or yeast triphosphatases could act as antiviral or antifungal agents with limited effect on the host enzyme.

Experimental Procedures

DNA Cloning Methods

The CEL-1 open reading frame was amplified by AmpliTaq polymerase (Perkin Elmer) in a polymerase chain reaction (PCR) using a C. elegans oligo-dT-primed cDNA library kindly donated by the G. Ruvkun lab (Massachusetts General Hospital). Oligonucleotide primer sequences were as follows: top strand 5′GCGGATCCATGGG ACTGCCTGATAGATGGCTGCA3′, bottom strand 5′GCGGATCCT CACTCAATTAAATAGGTTTCAGTGAC3′.

The PCR reaction introduced an NcoI site at the initiation codon and BamHI sites at both ends of the fragment. The 1.775 kb fragment was cloned into pCR-Script SK+ (Stratagene) to generate pBS-CEL-1. A 1.75 kb NcoI-BamHI fragment was then excised and cloned into the NcoI–BamHI sites of pET11-his$_7$, a modified version of pET11d (Novagen) that contains a cassette coding for seven histidines at the N-terminus of the protein. The resulting plasmid was cut with BstBI and BamHI, blunted, and religated to remove the C-terminal BstBI-BamHI fragment. The resulting plasmid encodes the N-terminal 236 amino acids of CEL-1.

Oligonucleotide-directed mutagenesis of the the cysteine 124 codon was carried out on single-stranded phagemid from pBS-CEL-1 as described (Kunkel et al., 1987; Vieira and Messing, 1987). The oligo was designed to change the cysteine codon to that for either serine or alanine. Mutations were identified by dideoxy-DNA sequencing. The NcoI-BstB1 fragments from the mutant plasmids were then cloned into the NcoI and ClaI sites of the pET11-his$_7$ to produce bacterial expression plasmids.

Recombinant Protein Production and Purification

Polyhistidine-tagged N-terminal portion of CEL-1 protein [his$_7$-Cel-1(1–236)] was expressed using a T7 promoter/polymerase system in E. coli strain BL21(DE3) (Studier et al., 1990). Strains carrying bacterial expression plasmids were grown at 30°C to an $OD_{600} = 0.5$ and induced by the addition of 0.4 mM IPTG. Cells were further cultured for 3 hr at 30°C and harvested. Lysates were prepared by sonication, and soluble extracts were incubated in batch with Ni^{2+}-NTA-agarose resin (Qiagen) overnight on a rotator at 4°C. The resin was extensively washed with buffer A (20 mM HEPES–KOH [pH 7.9], 100 mM KCl, 10% (v/v) glycerol, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride [PMSF]) containing 15 mM imidazole and then was poured into a column. Bound protein was eluted with buffer A containing 600 mM imidazole. Fractions were collected and dialyzed overnight at 4°C against 500 vol of buffer B (20 mM HEPES–KOH [pH 7.9], 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, 1 mM PMSF) containing 30 mM KCl. his$_7$-Cel-1(1–236) was further purified by column chromatography over Heparin Sepharose CL-6B (Pharmacia) using a gradient of 30–600 mM KCl in buffer B. Proteins were monitored by SDS–PAGE and Coomassie blue staining. his$_7$-Cel-1(1–236) eluted at 100 mM KCl. To provide a "mock" negative control, soluble extract was prepared from BL21(DE3) harboring pET11-his$_7$ and was chromatographed as described above.

Recombinant vaccinia virus capping enzyme was produced as follows. E. coli strain BL21(DE3) (Studier et al., 1990) was transformed with plasmid pETD12D1 (Guo and Moss, 1990; a gift from Dr. B. Moss, NIH), which expresses both subunits of the enzyme. Recombinant VV capping enzyme was partially purified from the soluble fraction through successive columns of DE52 (Whatman), P11 (Whatman), single-stranded DNA cellulose (Pharmacia), and Heparin Sepharose CL-6B. Activity was assayed by formation of enzyme-GMP complex as described (Guo and Moss, 1990).

Tyrosine Phosphatase Assays

Protein tyrosine phosphatase activity was assayed using a commercially available assay kit (New England Biolabs). ^{32}P-tyrosine-labeled myelin basic protein was prepared with [γ-^{32}P]ATP (NEN/DuPont) and release of phosphate was monitored as suggested by the manufacturer. For the hydrolysis of 4-nitrophenyl phosphate (pNPP), 100 ng of the indicated protein was mixed with 10 mM pNPP, 50 mM

Tris–HCl (pH 7.0), 1 mM EDTA, 5 mM DTT, 0.01% Brij 35, and 1 mg/
ml bovine serum albumin (BSA). The reaction was incubated for up
to 1 hr before the reaction was terminated by an equal volume of
0.1 M NaOH. The production of the colored product was quantitated
by monitoring absorbance of the reaction mix at 405 nm. For both
assays, the positive control was a sample of T-cell PTP provided
in the kit.

RNA Triphosphatase Assay

[γ-^{32}P]GTP-terminated RNA for RNA 5′ triphosphatase assay was
synthesized by in vitro transcription of linearized plasmid DNA tem-
plate by T7 RNA polymerase. The template was Bluescript II KS(+)
linearized at the EcoRI restriction site, which produced an RNA
transcript of 65 nucleotides in length. The in vitro transcription was
carried out for 5 hr at 37°C in a 500 μl reaction mixture containing
40 mM Tris–HCl (pH 7.5), 10 mM NaCl, 6 mM MgCl$_2$, 2 mM spermi-
dine, 10 mM DTT, 20 units RNase inhibitor (Promega), 100 μg/ml
BSA, 1 mM each of ATP, CTP, and UTP, 250 μM [γ-^{32}P]GTP (2,000–
5,000 cpm/pmol) (NEN/DuPont), 60–100 μg linearized DNA template,
and 200 units T7 RNA polymerase (New England Biolabs). Template
DNA was digested with 5 units of RNase-free DNase I (Promega).
After 60 min, the reaction mixture was treated with 250 μg/ml of
proteinase K for an additional 30 min at 37°C. Transcripts were
extracted with phenol-chloroform (1:1) and precipitated with etha-
nol. The product was further purified through a column of Sephadex
G-25 (Pharmacia) to eliminate labeled GTP.

RNA 5′-triphosphatase activity was assayed by the liberation of
[^{32}P]Pi from [γ-^{32}P]GTP-terminated RNA. The standard reaction mix-
ture contained in 10 μl: 50 mM Tris–HCl (pH 7.9), 5 mM DTT, 10 mM
KCl, 100 μg/ml BSA, 2–5 μM (of termini) of [γ-^{32}P]GTP-terminated
RNA (2,000–5,000 cpm/pmol), and the enzyme preparation to be
assayed. MgCl$_2$ (5 mM) was included in the incubation with recombi-
nant vaccinia virus (VV) capping enzyme (Guo and Moss, 1990), and
50 mM Tris–HCl (pH 9.0) instead of Tris–HCl (pH 7.9), and 5 mM
Mg(OAc)$_2$ were included for calf intestine alkaline phosphatase (CIP)
(Boehringer Mannheim). Incubation was for 30 min at 37°C for VV
capping enzyme and CIP, and at 30°C for his$_7$-Cel-1(1–236). The
reaction was terminated by the addition of 1 M HCOOH. The reaction
mixture was applied to the polyethyleneimine (PEI) cellulose thin-
layer plate (J. T. Baker) and chromatographed with 0.5 M KH$_2$PO$_4$
(pH 3.4). Radioactivity was detected using a Fuji BasX PhosphorIm-
ager and autoradiography. After autoradiography, [^{32}P]Pi spots were
cut out and the radioactivity was counted. For the nucleotide phos-
phohydrolase assay, [γ-^{32}P]GTP-terminated RNA was replaced with
5 μM [γ-^{32}P]GTP or ATP (NEN/DuPont).

Cap Formation Assay

To confirm that 5′-diphosphate end was produced, 20 pmol (of
termini) of GTP-terminated RNA were preincubated with the enzyme
to be assayed in the same conditions as the RNA 5′-triphosphatase
reaction. RNA was extracted with phenol-chloroform after treatment
with proteinase K, precipitated with ethanol, dissolved in water, and
added to the guanylylation reaction. Guanylylation reactions were
performed in 20 μl and contained 50 mM Tris–HCl (pH 7.9), 5 mM
MgCl$_2$, 5 mM DTT, 100 μg/ml BSA, 5 μM [α-^{32}P]GTP (30,000–60,000
cpm/pmol) (NEN/DuPont), 60 ng his$_7$-CEG1 (Fresco and Buratowski,
1994), and pretreated RNA. After incubation for 60 min at 30°C,
RNA was extracted with phenol-chloroform and precipitated with
ethanol. RNA was then digested with 6 μg nuclease P1 (Boehringer
Mannheim) for 60 min at 37°C in 30 mM ammonium acetate (pH 5.8)
to liberate the cap structure, GpppG. The sample was additionally
incubated for 60 min at 37°C with 1.6 units CIP in 100 mM Tris–HCl
(pH 9.0), 5 mM Mg(OAc)$_2$ to release [^{32}P]Pi from residual [α-^{32}P]GTP,
and the cap structure was analyzed by paper-electrophoresis at
500 volts using DE81 paper (Whatman) at pH 3.4 with pyridine-
acetic acid-water (10:100:899).

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J.
(1990). Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Denu, J.M., Stuckey, J.A., Saper, M.A., and Dixon, J.E. (1996). Form
and function in protein dephosphorylation. Cell 87, 361–364.

Fauman, E.B., and Saper, M.A. (1996). Structure and function of the
protein tyrosine phosphatases. Trends Biochem. Sci. 21, 413–417.

Fresco, L.D., and Buratowski, S.B. (1994). Active site of the mRNA-
capping enzyme guanylyltransferase from Saccharomyces cerevisi-
iae: similarity to the nucleotidyl attachment motif of DNA and RNA
ligases. Proc. Natl. Acad. Sci. USA 91, 6624–6628.

Guo, P., and Moss, B. (1990). Interaction and mutual stabilization
of the two subunits of vaccinia virus mRNA capping enzyme coex-
pressed in Escherichia coli. Proc. Natl. Acad. Sci. USA 87, 4023–
4027.

Itoh, N., Mizumoto, K., and Kaziro, Y. (1984a). Messenger RNA gua-
nylyltransferase from Saccharomyces cerevisiae I. Purification and
subunit structure. J. Biol. Chem. 259, 13923–13929.

Itoh, N., Mizumoto, K., and Kaziro, Y. (1984b). Messenger RNA gua-
nylyltransferase from Saccharomyces cerevisiae II. Catalytic proper-
ties. J. Biol. Chem. 259, 13930–13936.

Itoh, N., Yamada, H., Kaziro, Y., and Mizumoto, K. (1987). Messenger
RNA guanylyltransferase from Saccharomyces cerevisiae: large
scale purification, subunit functions, and subcellular localization. J.
Biol. Chem. 262, 1989–1995.

Jove, R., and Manley, J.L. (1984). In vitro transcription from the
adenovirus 2 major late promoter utilizing templates truncated at
promoter-proximal sites. J. Biol. Chem. 259, 8513–8521.

Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987). Rapid and effi-
cient site-specific mutagenesis without phenotypic selection. Meth.
Enzymol. 154, 367–382.

Mao, X., Schwer, B., and Shuman, S. (1995). Yeast mRNA cap
methyltransferase is a 50-kilodalton protein encoded by an essential
gene. Mol. Cell. Biol. 15, 4167–4174.

Mizumoto, K., and Kaziro, Y. (1987). Messenger RNA capping en-
zymes from eukaryotic cells. Prog. Nucleic Acid Res. Mol. Biol. 34,
1–28.

Myette, J.R., and Niles, E.G. (1996). Characterization of the vaccinia
virus RNA 5′-triphosphatase and nucleoside triphosphate phospho-
hydrolase activities: demonstration that both activities are carried
out at the same active site. J. Biol. Chem. 271, 11945–11952.

Niles, E.G., Condit, R.C., Caro, P., Davidson, K., Matusick, L., and
Seto, J. (1986). Nucleotide sequence and genetic map of the 16-kb
vaccinia virus HindIII D fragment. Virology 153, 96–112.

Rasmussen, E.B., and Lis, J.T. (1993). In vivo transcriptional pausing
and cap formation on three Drosophila heat shock genes. Proc.
Natl. Acad. Sci. USA 90, 7923–7927.

Sheng, Z., and Charbonneau, H. (1993). The Baculovirus Autographa
californica encodes a protein tyrosine phosphatase. J. Biol. Chem.
268, 4728–4733.

Shibagaki, Y., Itoh, N., Yamada, H., Nagata, S., and Mizumoto, K.
(1992). mRNA capping enzyme: Isolation and characterization of the
gene encoding mRNA guanylyltransferase subunit from Saccharo-
myces cerevisiae. J. Biol. Chem. 267, 9521–9528.

Shuman, S. (1990). Catalytic activity of vaccinia mRNA capping en-
zyme subunits coexpressed in Escherichia coli. J. Biol. Chem. 265,
11960–11966.

Shuman, S., and Schwer, B. (1995). RNA capping enzyme and DNA
ligase: a superfamily of covalent nucleotidyl transferases. Mol. Mi-
crobiol. 17, 405–410.

Shuman, S., Surks, M., Furneaux, H., and Hurwitz, J. (1980). Purification and characterization of a GTP-pyrophosphate exchange activity from vaccinia virions: association of the GTP-pyrophosphate exchange activity with vaccinia mRNA guanylyltransferase-RNA (guanine-7-)methyltransferase complex (capping enzyme). J. Biol. Chem. *255*, 11588–11598.

Shuman, S., Liu, Y., and Schwer, B. (1994). Covalent catalysis in nucleotidyl transfer reactions: essential motifs in *Saccharomyces cerevisiae* RNA capping enzyme are conserved in *Schizosaccharomyces pombe* and viral capping enzymes and among polynucleotide ligases. Proc. Natl. Acad. Sci. USA *91*, 12046–12050.

Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. Meth. Enzymol. *185*, 60–89.

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. Meth. Enzymol. *153*, 3–11.

Yagi, Y., Mizumoto, K., and Kaziro, Y. (1983). Association of an RNA 5′-triphosphatase activity with RNA guanylyltransferase partially purified from rat liver nuclei. EMBO J. *2*, 611–615.

Yamada-Okabe, T., Shimmi, O., Doi, R., Mizumoto, K., Arisawa, M., and Yamada-Okabe, H. (1996). Isolation of the mRNA capping enzyme and ferric-reductase-related genes from *Candida albicans*. Microbiology *142*, 2515–2523.

Yu, L., and Shuman, S. (1996). Mutational analysis of the RNA triphosphatase component of vaccinia virus mRNA capping enzyme. J. Virol. *70*, 6162–6168.