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Mutational analysis of yeast mRNA capping enzyme

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ABSTRACT **RNA guanylyltransferase (capping enzyme) catalyzes the transfer of GMP from GTP to the 5′-diphosphate end of mRNA. The capping reaction proceeds via an enzyme-guanylate intermediate in which GMP is linked covalently to a lysine residue of the enzyme. In the capping enzyme of *Saccharomyces cerevisiae*, GMP is attached to a 52-kDa polypeptide, identified as the product of the essential *CEG1* gene. The amino acid sequence of the CEG1 protein includes a motif, Lys^{70}-Thr-Asp-Gly, that is conserved at the active site of vaccinia virus RNA guanylyltransferase and which is similar to the KXDG sequence found at the active sites of RNA and DNA ligases. To evaluate the role of this motif in the function of the yeast enzyme, we have expressed the CEG1 protein in active form in *Escherichia coli*. Replacement of Lys^{70} or Gly^{73} with alanine abrogated enzyme-guanylate formation *in vitro*; in contrast, alanine substitutions at Thr^{71} or Asp^{72} merely reduced activity relative to wild-type enzyme. The K70A and G73A mutations were lethal to yeast, whereas yeast carrying the T71A and D72A alleles of CEG1 were viable. These results implicate Lys^{70} as the active site of yeast guanylyltransferase and provide evidence that cap formation *per se* is an essential function in eukaryotic cells.**

mRNA capping occurs by three sequential enzymatic reactions in which the 5′-triphosphate terminus of a primary transcript is first cleaved to a diphosphate-terminated RNA by RNA triphosphatase, then capped with GMP by RNA guanylyltransferase, and finally methylated at the N7 position of guanine by RNA-(guanine-7) methyltransferase. A multifunctional capping enzyme which catalyzes all three reactions has been purified from vaccinia virus particles (1). The vaccinia enzyme is a heterodimer containing virus-encoded subunit polypeptides of 95 and 33 kDa. Of the three catalytic steps in cap formation, only the guanylyltransferase reaction has been dissected in detail. Transfer of GMP from GTP to the 5′-diphosphate terminus of RNA occurs in a two-stage reaction involving a covalent enzyme-GMP (EpG) intermediate (2).

$$E + pppG \rightleftharpoons EpG + PP_i$$

$$EpG + ppRNA \rightleftharpoons GpppRNA + E$$

The GMP residue is linked to the large subunit of the vaccinia capping enzyme through a phosphoamide bond to the ε-amino group of a lysine residue. The active site has been assigned to Lys^{260} of the 844-aa polypeptide (3, 4). This residue is situated within a sequence motif, Lys-Thr-Asp-Gly (KTDG), that is conserved between the poxvirus capping enzyme and the active-site regions of DNA and RNA ligases (3). This is remarkable because the ligase reaction entails formation of a 5′–5′ adenylylated polynucleotide via an enzyme-AMP intermediate consisting of AMP linked covalently to an active-site lysine (5).

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An equivalent mechanism of covalent catalysis applies to cellular mRNA guanylyltransferases, including enzymes isolated from human, mouse, rat, calf, wheat germ, brine shrimp, and yeast (6). The capping enzyme purified from *Saccharomyces cerevisiae* is a bifunctional complex consisting of two polypeptides of 80 kDa and 52 kDa. RNA triphosphatase activity is intrinsic to the 80-kDa subunit, whereas the 52-kDa subunit contains guanylyltransferase activity (7). Shibagaki *et al.* (8) isolated the *CEG1* gene encoding the 52-kDa guanylyltransferase subunit and demonstrated that this gene was essential for cell viability (8).

In this paper, we examine whether guanylyltransferase activity is an essential function of the *CEG1* gene *in vivo*. The goal was to create single missense mutations in the CEG1 protein that would abolish guanylyltransferase activity and to test whether these alleles could sustain cell growth. Comparison of amino acid sequences revealed that the KTDG motif at the active site of the vaccinia enzyme was conserved precisely in the yeast CEG1 protein and led to the prediction that Lys^{70} of the yeast capping enzyme would constitute the site of covalent adduct formation with GMP (3). By assessing the effects of single alanine substitutions in this motif on the activity of CEG1 expressed in *Escherichia coli*, we have found that Lys^{70} and Gly^{73} are indeed essential for covalent catalysis. Moreover, the K70A and G73A alleles are lethal to yeast growth, thus indicating that cap formation is an essential function *in vivo*.

MATERIALS AND METHODS

Expression of Yeast Capping Enzyme in Bacteria. *CEG1* was isolated by PCR amplification of total genomic DNA from *S. cerevisiae* strain YPH274, using oligonucleotides corresponding to the 5′ end and the 3′ end of *CEG1* (8). These oligonucleotides introduced an *Nco* I restriction site at the start codon and a *Bgl* II cleavage site just 3′ of the stop codon. The amplified DNA fragment was digested with *Nco* I and *Bgl* II and then ligated into the phage T7-based expression vector pET14b. The resultant plasmid, pET-YCE, was transformed into *E. coli* BL21(DE3). Induced expression of the plasmid-encoded protein was achieved by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) to exponentially growing 35-ml cultures of BL21(DE3)/pET-YCE in LB medium with ampicillin (0.1 mg/ml) at 37°C. Cells were harvested by centrifugation 4 hr after induction. The bacteria were resuspended in 3 ml of lysis buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.5/10 mM dithiothreitol/10 mM EDTA/10% sucrose), then adjusted to 0.02% lysozyme and incubated on ice for 30–45 min. All subsequent operations were performed at 4°C. Suspensions were adjusted to 0.1% Triton X-100 and the lysates were sonicated briefly to reduce viscosity. Soluble and insoluble fractions were separated by centrifugation. The insoluble pellets were resuspended in 3 ml of lysis buffer. Aliquots of the protein samples were

Abbreviations: EpG, enzyme-GMP; FOA, 5-fluoroorotic acid; IPTG, isopropyl β-D-thiogalactopyranoside.

denatured and analyzed by SDS/PAGE to gauge the level of CEG1 expression and the extent of protein solubility.

Guanylyltransferase Assay. Reaction mixtures (20 μl) containing 50 mM Tris·HCl (pH 7.5 or pH 8.0, as indicated), divalent cation as indicated, 0.16 μM [α-^{32}P]GTP (1000–3000 Ci/mmol; 1 Ci = 37 GBq), and 1 μl of protein sample were incubated for 5 min at 37°C. Reactions were halted by the addition of SDS sample buffer, followed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS (3). Label transfer to protein was detected by autoradiographic exposure of the dried gel. The extent of covalent complex formation was quantitated by liquid scintillation counting of an excised gel slice.

Partial Purification of Yeast Guanylyltransferase Expressed in Bacteria. For enzyme purification, a soluble extract (30 ml) was prepared as described above from BL21(DE3)/pYCE cells (600- to 1000-ml cultures) grown with or without IPTG induction. Initial fractionation was performed by gradual addition of solid ammonium sulfate to the extract. After stirring for 30 min, the insoluble material was collected by centrifugation, and the pellet was suspended in buffer A [50 mM Tris·HCl, pH 8/2 mM dithiothreitol/1 mM EDTA/0.1% Triton X-100/10% (vol/vol) glycerol]. The clarified supernatant was then adjusted to a higher level of saturation with solid ammonium sulfate, and the suspension was again separated by centrifugation. Tandem fractionation schemes yielded protein preparations from 0–25%, 25–50%, and 50–85% saturation, or from 0–20%, 20–40%, and 40–85% saturation with ammonium sulfate. The 20–40% ammonium sulfate fraction (5.1 mg of protein) was dialyzed against buffer A and applied to a 3-ml column of heparin-agarose. Adsorbed material was eluted stepwise with buffer A containing 0.2 M, 0.5 M, and 1.0 M NaCl. Guanylyltransferase activity adsorbed to the column and was recovered in the 0.2 M NaCl eluate (containing 0.7 mg of protein).

***CEG1* Genomic Clones.** A plasmid containing *CEG1* and flanking sequences was isolated by colony hybridization screening of a yeast genomic library in YEP24 (2μ, *URA3*). A 3.6-kb *Pst* I–*Hin*dIII fragment was isolated and ligated into pBluescript KS(+) (Stratagene) to yield pBS-GYCE. From this plasmid, a 3.6 kb *Sac* I–*Hin*dIII and a 2.9-kb *Pst* I–*Eco*RI fragment were isolated and ligated into pSE360 (*CEN, URA3*) and pSE358 (*CEN, TRP1*), respectively, to yield pGYCE-360 and pGYCE-358.

Site-Directed Mutagenesis of *CEG1*. A 2-kb restriction fragment containing the entire *CEG1* expression cassette was excised from pET-YCE with *Bgl* II and *Hin*dIII and inserted into pBluescript KS(+) to yield pBS-YCE. Uracil-substituted single-stranded DNA was prepared for use as a template for oligonucleotide-directed mutagenesis (9). Mutagenic DNA primers were designed to create alanine substitutions at Lys^{70}, Thr^{71}, Asp^{72}, and Gly^{73}. Candidate mutant plasmids were screened by dideoxynucleotide sequencing. A 506-bp *Eco*RI–*Nde* I fragment containing each alanine mutation was exchanged for the corresponding wild-type region in pBS-YCE to generate the plasmid series pBS-Ala. The *Nco* I–*Hin*dIII fragment of each pBS-Ala plasmid was then transferred back into pET14b for bacterial expression of the mutated *CEG1* gene. A 702-bp *Eco*RI–*Bam*HI fragment of each pBS-Ala plasmid was also exchanged with the corresponding segment of pGYCE-358 to test the function of the mutated *CEG1* alleles *in vivo*.

Disruption of *CEG1*. *CEG1* was disrupted by insertion of a *hisG–URA3–hisG* cassette (10). The disruption was performed in the diploid strain TR1 (*MATa/MATα, ura3/ura3, lys2/lys2, his3/his3, ade2/ade2, trp1/trp1*). Integrative recombination at one chromosomal locus deleted *CEG1* nucleotides +102 to +1369, with replacement by the *URA3* cassette, thereby eliminating all *CEG1* coding sequence downstream of amino acid position 34. Correct insertion into one *CEG1* locus was confirmed by Southern blotting of Ura$^+$ transformants. This strain was then transformed with pGYCE-358. Ura$^+$/Trp$^+$ haploids were isolated after sporulation. Subsequent selection in the presence of 5-fluoroorotic acid (FOA) yielded a haploid strain, YBS1 (*MATα, ura3, lys2, his3, ade2, ceg1::hisG*, pGYCE-358) that had lost the *URA3* gene via excisive recombination between flanking *hisG* direct repeats (10). YBS1 was transformed with pGYCE-360. Ura$^+$ transformants were then screened for the loss of the *TRP* plasmid pGYCE-358. The resulting Trp$^-$/Ura$^+$ strain was mated with the Leu$^-$ strain A363A (11). Diploids were then sporulated and the Leu$^-$/Ura$^+$ haploid strain YBS2 (*MATa, leu2, lys2, trp1, ceg1::hisG*, pGYCE-360) was used to test the *in vivo* function of various *CEG1* alleles with the plasmid shuffle technique (12).

RESULTS

Yeast Guanylyltransferase Expressed in Bacteria. The *S. cerevisiae CEG1* coding region was amplified from total yeast DNA by PCR. Hybridization probes were then used to isolate a yeast genomic clone containing *CEG1* and flanking regions. The nucleotide sequence of the cloned "wild-type" genomic *CEG1* differed from the published sequence of Shibagaki *et al.* (8) at codons 29 and 30, such that methionine and valine residues were substituted for the isoleucine and leucine residues reported previously at these positions. Insertion of the PCR-amplified *CEG1* into a T7-based pET vector was performed to express the *CEG1* gene product in bacteria. IPTG induction of T7 RNA polymerase in cultures of *E. coli* BL21(DE3)/pET-YCE resulted in the accumulation of a 52-kDa polypeptide that was recovered predominantly in the insoluble protein fraction of crude cell lysates (Fig. 1*A*). This protein was absent when cells lacking *CEG1* were induced with IPTG (data not shown). The other polypeptides seen in Fig. 1*A* are endogenous bacterial proteins. The 52-kDa CEG1 polypeptide was also seen in the soluble protein fraction of IPTG-induced cells, albeit at a much lower level, amidst a background of bacterial polypeptides (data not shown). Incubation of the soluble extracts in the presence of [α-^{32}P]GTP and a divalent cation resulted in the formation of an SDS-stable nucleotide-protein adduct that migrated as a discrete 52-kDa species during SDS/PAGE (Fig. 1*B*). Labeling of this polypeptide was not detected in extracts prepared from

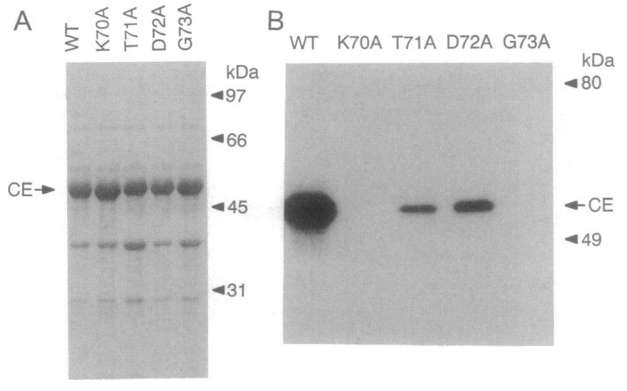

FIG. 1. Guanylyltransferase activity of wild-type (wt) and mutant CEG1 proteins expressed in *E. coli*. (*A*) The resuspended insoluble fraction of IPTG-induced bacteria carrying pET-based CEG1 expression plasmids was analyzed by SDS/PAGE followed by Coomassie blue staining. The *CEG1* allele expressed in each sample is indicated above the lanes. The polypeptide corresponding to the capping enzyme (CE) is indicated by the arrow. (*B*) Guanylyltransferase assay mixtures contained 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 0.16 μM [α-^{32}P]GTP, and 1 μl of soluble extract from uninduced bacteria. Label transfer to the capping enzyme (CE) was detected by autoradiography after SDS/PAGE.

bacteria that lacked *CEG1* (data not shown). These findings indicate that the expressed CEG1 protein was catalytically active.

Isolation of the Yeast Capping Enzyme from Bacterial Extracts. Preliminary experiments established that the level of guanylyltransferase activity recovered in the soluble protein fraction was not increased by exposure of the cells to IPTG. Rather, the effect of the induction step was to significantly enhance the accumulation of CEG1 in the insoluble fraction. Thus, for isolation of the guanylyltransferase from the soluble fraction, the IPTG-induction step was unnecessary. Initial fractionation was by ammonium sulfate precipitation. In one scheme, half the total protein, and nearly all the guanylyltransferase activity, was recovered in the 25–50% ammonium sulfate fraction. An alternative scheme yielded an active 20–40% ammonium sulfate fraction containing only 7% of the total soluble protein. This fraction was dialyzed and purified further by heparin-agarose column chromatography. Guanylyltransferase activity adsorbed to the column and was eluted with 0.2 M NaCl (Fig. 2*B*). SDS/PAGE showed that the active heparin fraction had been substantially enriched in a 52-kDa polypeptide, which now constituted the most abundant species in the preparation (Fig. 2*A*). When the heparin-agarose fraction was centrifuged through a 15–30% glycerol gradient in 0.5 M NaCl, a single peak of guanylyltransferase activity was detected (Fig. 3*A*). The sedimentation rate relative to marker proteins suggested a monomeric structure for the expressed yeast capping enzyme.

EpG Formation by Partially Purified CEG1 Protein. The amount of EpG complex formed by the glycerol gradient fraction of CEG1 increased with time during the first 2 min of incubation at 37°C and reached a plateau thereafter (Fig. 3*B*). The yield of labeled complex varied linearly with the amount of the glycerol enzyme fraction added (data not shown). Complex formation increased as a function of divalent cation concentration from 1 to 10 mM; Mn^{2+} was several-fold more effective than Mg^{2+} in supporting nucleotidyl transfer at equivalent concentrations over this range (Fig. 3*C*). Preference for Mn^{2+} over Mg^{2+} in EpG formation was noted previously for the human guanylyltransferase (13). CEG1 formed no detectable EpG in the presence of 5 mM Ca^{2+}, Co^{2+}, Cu^{2+}, or Zn^{2+}. Inclusion of 5 mM Co^{2+}, Cu^{2+}, or Zn^{2+} in reaction mixtures containing 5 mM Mn^{2+} abolished EpG formation; in contrast, 5 mM Ca^{2+} had no effect on activity when added in combination with Mn^{2+}. Activity in 0.1 M Tris·HCl buffer was optimal at pH 7.5–8.0 and declined sharply at lower or higher pH values (Fig. 3*D*). PP_i, a reaction product, inhibited formation of the covalent complex; in reactions constituted at pH 8.0 with 5 mM Mg^{2+}, the extent of EpG formation was inhibited by 42% at 10 μM PP_i (added as the Na^+ salt) and by 97% at 100 μM PP_i. Neither 100 μM nor 1 mM P_i had any effect on EpG formation.

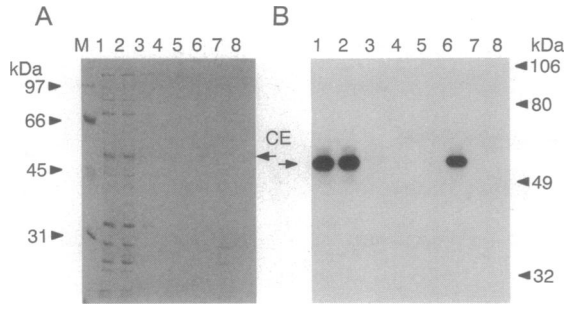

FIG. 2. Heparin-agarose chromatography of yeast capping enzyme. The polypeptide composition (*A*) and guanylyltransferase activity (*B*) of the 20–40% ammonium sulfate fraction (lane 1), dialyzed 20–40% ammonium sulfate fraction (lane 2), heparin-agarose flowthrough (lane 3), and wash fractions (lanes 4 and 5), 0.2 M NaCl eluate (lane 6), 0.5 M NaCl eluate (lane 7), and 1.0 M NaCl eluate (lane 8) are shown. An equal volume (30 μl) of each fraction was applied to the gel in *A*. Positions and sizes of marker proteins (lane M) are indicated at left. For *B*, reaction mixtures contained 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 0.16 μM [α-^{32}P]GTP, and 1 μl of each fraction. Positions of the capping enzyme (CE) (*A*) and EpG (*B*) are indicated by arrows.

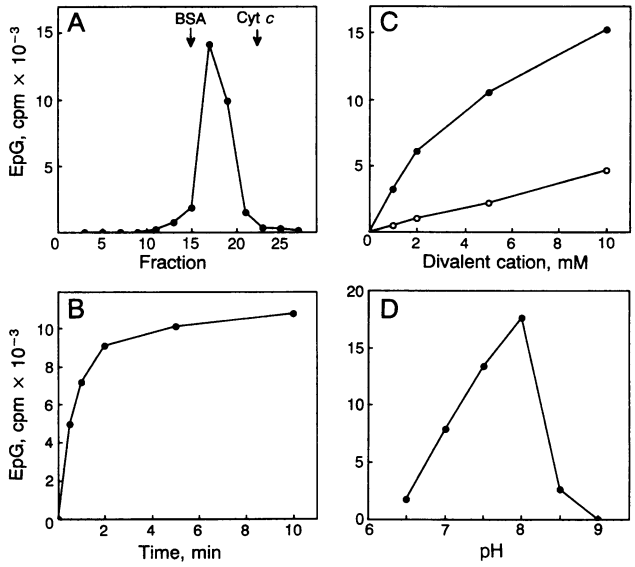

FIG. 3. Characterization of yeast capping enzyme. (*A*) Glycerol gradient sedimentation. An aliquot (0.3 ml) of the heparin-agarose 0.2 M NaCl eluate was applied to a 4.7-ml 15–30% glycerol gradient in buffer A containing 0.5 M NaCl. The gradient was centrifuged for 18 hr at 4°C in a Beckman SW55 rotor at 55,000 rpm. Fractions (0.17 ml) were collected from the bottom of the tube. Guanylyltransferase reactions contained 50 mM Tris·HCl (pH 8.0), 10 mM $MnCl_2$, 0.16 μM [α-^{32}P]GTP, and 1 μl of glycerol gradient fraction. Positions of marker proteins [bovine serum albumin (BSA) and cytochrome *c* (Cyt *c*)] sedimented in a parallel gradient are indicated. (*B*) Kinetics of EpG formation. A reaction mixture (100 μl) containing 50 mM Tris·HCl (pH 7.5), 5 mM $MnCl_2$, 0.16 μM [α-^{32}P]GTP, and 5 μl of glycerol fraction 18 was incubated at 37°C. Aliquots (18 μl) were removed at the indicated times, denatured, and analyzed by SDS/PAGE. (*C*) Divalent cation requirement. Reaction mixtures containing 50 mM Tris·HCl (pH 7.5), 0.16 μM [α-^{32}P]GTP, $MgCl_2$ (○) or $MnCl_2$ (●) as indicated, and 1 μl of glycerol fraction 18 were incubated for 5 min. (*D*) Effect of pH. Reaction mixtures contained 0.1 M Tris·HCl (pH as indicated), 5 mM $MnCl_2$, 0.16 μM [α-^{32}P]GTP, and 1 μl of glycerol fraction 18. In all experiments, the extent of EpG formation was determined by liquid scintillation counting of excised gel slices.

Alanine Substitutions in the Conserved KTDG Motif. Previous studies indicated that GMP was linked to the yeast capping enzyme via a phosphoamide bond, apparently to a lysine residue (8). A strong clue to the location of the active-site lysine within CEG1 was provided by studies of the vaccinia enzyme, whose active site has been assigned by mutational analysis (3) and by peptide mapping (4) to Lys^{260}. This lysine is situated in a motif, KTDG, that is conserved among the guanylyltransferases from yeast (KTDG), vaccinia virus (KTDG), Shope fibroma virus (KTDG), and African swine fever virus (KADG) (3, 14, 15). A similar motif occurs at the active sites of T4 RNA ligase (KEDG), mammalian DNA ligase (KYDG), and yeast tRNA ligase (KANG) (16–18).

To evaluate the hypothesis that Lys^{70} of CEG1 is the site of covalent guanylylation, this residue was changed to alanine via oligonucleotide-directed mutagenesis of the cloned *CEG1* gene. Single alanine mutations were also created at conserved residues Thr^{71}, Asp^{72}, and Gly^{73}. These mutated alleles were expressed in bacteria in parallel with the wild-

type gene. IPTG-induced expression resulted in the accumulation of full-sized 52-kDa polypeptide in each case (Fig. 1*A*). Assay of EpG formation by soluble protein extracts from uninduced cells indicated that the K70A and G73A mutations completely abolished enzyme activity (Fig. 1*B*). In contrast, the T71A and D72A mutant proteins retained EpG-forming activity, albeit at a reduced level (7–15%) compared with the wild-type protein (Fig. 1*B*). Substitution of Mn^{2+} for Mg^{2+} in the reaction mixtures did not alter the relative activities of the alanine-substituted proteins (data not shown). EpG assays of the soluble extracts of cells induced with IPTG to express the mutant polypeptides gave the same results as shown in Fig. 1 for uninduced extracts. Thus, Lys^{70} and Gly^{73} of the yeast capping enzyme were essential for transguanylylation *in vitro*. The concordance of these findings with previous studies of nucleotidyl transfer by mutated versions of vaccinia capping enzyme (3) and of mammalian DNA ligase (19) suggests that Lys^{70} is indeed the active site of the yeast capping enzyme. This assignment is supported by peptide-mapping studies of the yeast protein–GMP adduct (unpublished data).

Effect of Alanine Substitutions on CEG1 Function *in Vivo*. One copy of *CEG1* in a diploid yeast was deleted. After introduction of a centromeric plasmid marked with *URA3* and containing *CEG1* under the control of its natural promoter, we obtained haploid cells deleted at the chromosomal locus (strain YBS2), whose viability was contingent on maintenance of the extrachromosomal allele. A plasmid-shuffle strategy (12) was then employed to assess the ability of mutated *CEG1* alleles to support cell growth. Wild-type and alanine-substituted *CEG1* alleles on centromeric plasmids marked with *TRP1* were introduced into YBS2 cells. The Trp^+/Ura^+ strains were then plated on medium containing FOA to select against retention of the wild-type *CEG1* on the *URA3* plasmid. Whereas cells containing the TRP1 plasmid without any *CEG1* gene were incapable of growth on FOA, cells bearing the wild-type *CEG1/TRP1* plasmid grew readily (Fig. 4). The T71A and D72A alleles, whose protein products retained partial activity *in vitro*, also supported growth on FOA. In contrast, the K70A and G73A alleles, which encoded catalytically inert guanylyltransferases, were unable to sustain growth under counterselective conditions (Fig. 4). These results indicated that guanylyltransferase activity was essential for yeast viability.

A conditional lethal growth phenotype was elicited by placing the *CEG1* open reading frame under the transcriptional control of a galactose-inducible promoter in a 2μ plasmid vector marked with *LEU2*. Exchange of the wild-type *GAL–CEG1* plasmid for the centromeric *CEG1/URA3* plasmid was accomplished by counterselection on FOA in the presence of galactose. These cells, which grew well on galactose, were unable to grow in the presence of glucose—i.e., when *CEG1* expression was transcriptionally repressed (Fig. 5). The T71A and D72A mutant alleles in the same 2μ vector also supported growth on galactose-containing medium but not on medium containing glucose (Fig. 5). The K70A and G73A alleles were unable to sustain growth on FOA/galactose (data not shown), confirming the findings above that these mutations were lethal and suggesting that the lethality was not surmountable by increased gene dosage and enhanced expression of the mutant alleles. In addition, there was no evidence for a dominant negative effect on cell growth in the presence of galactose when the inducible K70A and G73A alleles were introduced into yeast cells which had an unperturbed chromosomal *CEG1* gene (data not shown).

CEG1 Truncation Mutants. *CEG1* was altered by site-directed mutagenesis, so that the mutated alleles would, upon insertion into the pET vector, express amino- and carboxyl-truncated versions [CEG1-(29–459) and CEG1-(1–431)] of the yeast capping enzyme in *E. coli*. Polypeptides of lower molecular weight than the wild-type CEG1 were seen prominently in the insoluble protein fraction of IPTG-induced cells (Fig. 6*A*). Deletion of 28 aa from the amino terminus abrogated EpG formation *in vitro* (Fig. 6*B*). In contrast, the enzyme retained activity after removal of 28 aa from the carboxyl end (Fig. 6*B*). When tested for *in vivo* function in yeast, the catalytically impaired CEG1-(29–459) allele was lethal, whereas the active CEG1-(1–431) allele was viable (data not shown). A more extensive carboxyl-deletion allele, CEG1-(1–366), was inactive *in vitro* and *in vivo* (data not shown). Finally, although an amino deletion was deleterious to the yeast capping enzyme, a histidine-rich amino-terminal extension, MGSHHHHHHSGH-, was well tolerated. When expressed in *E. coli*, the histidine-tagged CEG1 was catalytically active; its electrophoretic mobility was, as expected, slower than that of the wild-type enzyme (Fig. 6*B*, compare His-YCE with WT). The "histidine-tagged" *CEG1* gene was active *in vivo* (data not shown).

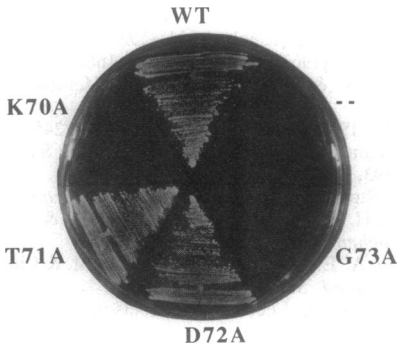

FIG. 4. Effect of alanine substitutions on CEG1 function *in vivo*. YBS2 was transformed with pGYCE-358 [containing the wild-type (wt) *CEG1*], with plasmid derivatives containing the indicated alanine-substitution mutants of *CEG1*, or with the plasmid vector pSE358 (- -), which lacks *CEG1*. Trp^+ transformants were plated on medium containing FOA (0.75 mg/ml) and incubated at 30°C for 4 days. A photograph of the plate is shown.

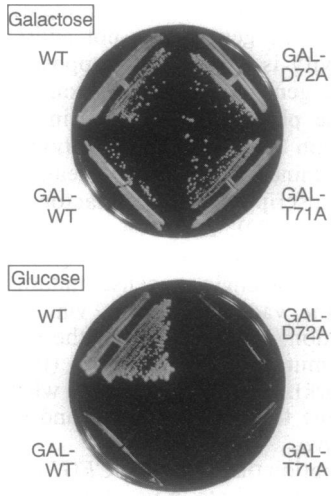

FIG. 5. Conditional lethal phenotype. YBS2 was transformed with pGal-YCE (2μ, *LEU2*, *GAL10–CEG1*), which contains the wild-type (wt) *CEG1* gene driven by a *GAL10* promoter, or with plasmid derivatives containing the indicated alanine-substitution mutants of CEG1. Leu^+/Ura^- transformants were selected by growth on plates containing FOA and galactose. Cells from single colonies were restreaked on YP/galactose or YB/glucose plates and incubated at 30°C for 4 days. YBS2 (wt) was plated in parallel as a control.

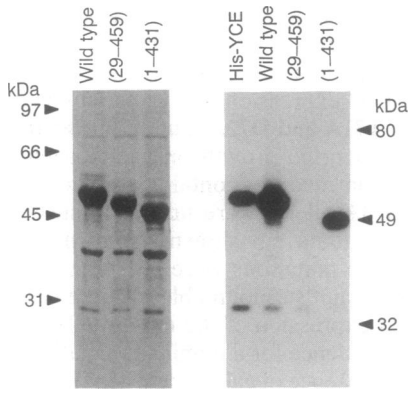

FIG. 6. Truncation mutants of yeast capping enzyme. Truncated *CEG1* genes encoding CEG1-(29–459) and CEG1-(1–431) were inserted into pET14b. (*A*) Polypeptide composition of the resuspended insoluble fraction of IPTG-induced bacteria carrying the indicated *CEG1* allele was analyzed by SDS/PAGE. A Coomassie Blue-stained gel is shown. (*B*) Guanylyltransferase assay mixtures contained 50 mM Tris·HCl (pH 8.0), 10 mM $MnCl_2$, 0.16 μM [α-^{32}P]GTP, and 1 μl of soluble extract from uninduced bacteria containing the indicated allele of *CEG1*. The His-YCE allele encodes an amino-terminal leader peptide (histidine tag) as described in the text.

DISCUSSION

It is postulated that the cap plays a critical role in mRNA synthesis and function, by facilitating posttranscriptional processing, nucleocytoplasmic transport, and ultimately the recognition of mature mRNA by the translation machinery. Capping is also implicated in protecting mRNA from nucleolytic degradation. Elucidation of the mechanism and potential regulation of cap formation is thus pertinent to our understanding of eukaryotic gene expression.

The presumption that RNA guanylyltransferase is a biologically essential activity had not previously been substantiated in either cellular or viral systems. Genes encoding guanylyltransferases have been identified in several eukaryotic viruses, including reovirus (20) as well as vaccinia and African swine fever virus (14). Conventional evidence for the essentiality of these genes for virus replication—e.g., by inability to delete the gene or by mapping of a thermosensitive mutation to the gene—does not address whether the enzymatic activity is essential, as opposed to some other function of the gene product. For example, the vaccinia capping enzyme plays multiple roles in mRNA synthesis beyond its action in 5′ end modification. It serves as a transcription termination factor during early gene expression (21) and as a transcription initiation factor during intermediate gene expression (22). The RNA capping functions can be genetically distinguished from transcriptional roles of this enzyme, insofar as a single missense mutation at the active-site lysine of the vaccinia protein, which abolishes EpG complex formation, has no effect on the termination factor activity of the mutant enzyme *in vitro* (P. Cong and S.S., unpublished work). It remains to be seen whether this mutant allele can replace the wild-type gene and support vaccinia replication *in vivo*.

The demonstration that the yeast *CEG1* gene was essential (8) prompted us to evaluate in yeast whether guanylyltransferase activity was an essential function of this protein *in vivo*. This was accomplished by mutating single residues within the motif KTDG, which is conserved at the active site of the vaccinia guanylyltransferase and which resembles the active sites of RNA and DNA ligases. Alanine substitutions at Lys^{70} and Gly^{73} rendered the enzyme inactive in nucleotidyl transfer, whereas substitutions at Thr^{71} and Asp^{72} only partially diminished EpG formation *in vitro* (Fig. 1). Our screening of the alanine-substituted proteins for EpG formation was performed with crude bacterial extracts. To exclude the possibility that the apparent activity decrement of the T71A or D72A proteins was caused by decreased solubility (i.e., less CEG1 protein in the extract), we cloned the alanine-substituted alleles into the histidine-tag expression vector and then purified the wild-type and mutant histidine-tagged proteins from bacterial lysates. This was done by ammonium sulfate precipitation followed by affinity chromatography using an immobilized Ni^{2+} resin. Elution of the histidine-tagged proteins with imidazole yielded preparations in which CEG1 was the most abundant polypeptide (unpublished data). Titration of wild-type and mutant enzymes using equivalent amounts of the histidine-tagged CEG1 polypeptide showed that the histidine-tagged T71A protein was 19% and the histidine-tagged D72A protein 14%, as active as the histidine-tagged wild-type enzyme in EpG formation; these results confirm that these two substitutions affect the intrinsic activity of the capping enzyme, as suggested in Fig. 1. As expected, the purified histidine-tagged K70A protein was inert in EpG formation.

Significantly, the ability of singly mutated *CEG1* alleles to sustain yeast growth depended on competence in EpG formation. A similar correlation between *in vivo* function and *in vitro* guanylyltransferase activity applied to truncation mutants of the yeast capping enzyme. The most straightforward interpretation of these results is that guanylyltransferase activity *per se* (and, hence, cap formation) is essential for cell growth. The viability of the T71A and D72A mutants may indicate that the level of guanylyltransferase activity is not normally limiting *in vivo*. The conditional growth phenotype of the *GAL–CEG1* strain should facilitate studies aimed at defining which aspects of RNA biogenesis and function are cap-dependent *in vivo*. Of course, cap formation may not be the *only* essential function of CEG1; additional roles for the capping enzyme in RNA synthesis or processing are in no way excluded.

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