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Conditional mutants of the yeast mRNA capping enzyme show that the cap enhances, but is not required for, mRNA splicing

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ABSTRACT

The 5′ end of eukaryotic mRNAs are modified by the addition of a 7-methyl guanosine (m^7G) cap. The role of the cap in translation has been well established. Additionally, studies conducted in vitro or in microinjected *Xenopus* oocytes have implicated the cap in RNA processing and transport. To determine the fate of uncapped mRNA in intact yeast cells, conditional alleles of the gene encoding the capping enzyme guanylyltransferase subunit (*CEG1*) were generated. RNA analysis of temperature-sensitive *ceg1* strains revealed an accumulation of unspliced pre-mRNAs and a corresponding decrease in spliced mRNAs at the restrictive temperature. A substantial proportion of spliced mRNA was also uncapped. Therefore, the cap appears to stimulate, but is not absolutely required for, splicing in vivo. In addition, steady-state levels of several mRNAs were decreased, perhaps due to increased degradation of uncapped mRNAs. In contrast to splicing, mRNA polyadenylation and transport to the cytoplasm were unaffected.

Keywords: cap; *CEG1*; guanylyltransferase; mRNA transport; m^7G cap; polyadenylation

INTRODUCTION

Eukaryotic mRNAs produced by RNA polymerase II are capped with an inverted 7-methyl-guanosine (m^7G) residue linked via a 5′–5′ triphosphate bridge to the first transcribed residue of the RNA (m^7GpppN) (Reddy et al., 1974; Furuichi & Miura, 1975; Shatkin, 1976). Capping proceeds by the action of three enzymatic activities: 5′ RNA triphosphatase, GTP:mRNA guanylyltransferase, and RNA (guanine-7-) methyltransferase. In short, these enzymes remove the terminal phosphate group on the pre-mRNA, transfer a GMP residue to the resulting diphosphate terminus, and methylate the newly attached guanine at the N_7 position, respectively. In yeast, the triphosphatase and guanylyltransferase subunits are associated as a heterodimer (Mizumoto & Kaziro, 1987; Shibagaki et al., 1992), and the methyltransferase is purified as a separate protein (Mao et al., 1995).

The cotranscriptional addition of the m^7G cap is the earliest detectable RNA processing event, apparently occurring soon after the 5′ end of the RNA emerges from the transcription apparatus (Weil et al., 1979; Proudfoot et al., 1980; Salditt-Georgieff et al., 1980; Hagler & Shuman, 1992; Rasmussen & Lis, 1993). Capping precedes other posttranscriptional processing events, including pre-mRNA splicing, polyadenylation, nuclear-cytoplasmic transport, poly(A) tail shortening, and, ultimately, degradation of the mRNA following its translation.

Many studies have implicated the m^7G cap in various stages of RNA metabolism. The cap promotes mRNA stability (Furuichi et al., 1977; Shimotohno et al., 1977; Green et al., 1983; Drummond et al., 1985; Gallie, 1991; Murthy et al., 1991), presumably because the cap provides a blocking structure to 5′→3′ exoribonucleases (Sachs, 1993). Also, nuclear export and import of capped RNAs may be mediated by the association of a cap-binding complex (Izaurralde & Mattaj, 1995 for review). Ultimately, the translation initiation factor eIF-4F binds to the cap structure and recruits mRNA to the ribosome (Sonenberg, 1988). Recruitment to the ribosome apparently involves interactions with both the cap and the poly(A) tail (Gallie, 1991).

In addition to the roles described above, the pre-mRNA cap may be important for mRNA splicing. Recognition of the cap has been proposed as an early step

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in spliceosome assembly on the pre-mRNA. Splicing is competitively inhibited by cap analogues or by capped RNAs lacking splice junctions in HeLa cell extracts (Konarska et al., 1984; Krainer et al., 1984; Edery & Sonenberg, 1985; Ohno et al., 1987; Patzelt et al., 1987; Izaurralde et al., 1994). Furthermore, splicing substrates capped with an ApppG dinucleotide rather than the m^7GpppG were less efficiently spliced in microinjected *Xenopus* oocytes (Inoue et al., 1989).

To date, all experiments addressing the role of the cap have been conducted in vitro or in microinjected oocytes. Although they have yielded a great deal of useful information, these systems necessitate disruption of the nuclear membrane as well as uncoupling of transcription from RNA processing and transport. Many of these studies used transcripts synthesized in vitro, a process that recently has been shown to produce significant amounts of transcripts with abnormal cap structures (Pasquinelli et al., 1995). To explore the role of the 5′ cap in mRNA metabolism in intact cells, temperature-sensitive alleles of the yeast capping enzyme guanylyltransferase subunit gene *CEG1* (Shibagaki et al., 1992) were generated and characterized. Analysis of conditional strains at the restrictive temperature revealed a lack of guanylyltransferase activity and that the majority of mRNA is uncapped. Northern blot analysis of intron-containing genes revealed a marked accumulation of unspliced transcripts, although some splicing still occurred. In contrast, uncapped mRNAs appeared to be polyadenylated normally and exported from the nucleus. Thus, the cap structure on mRNA stimulates specifically, but is not required for, pre-mRNA splicing.

RESULTS

Isolation of conditional mutants of the capping enzyme

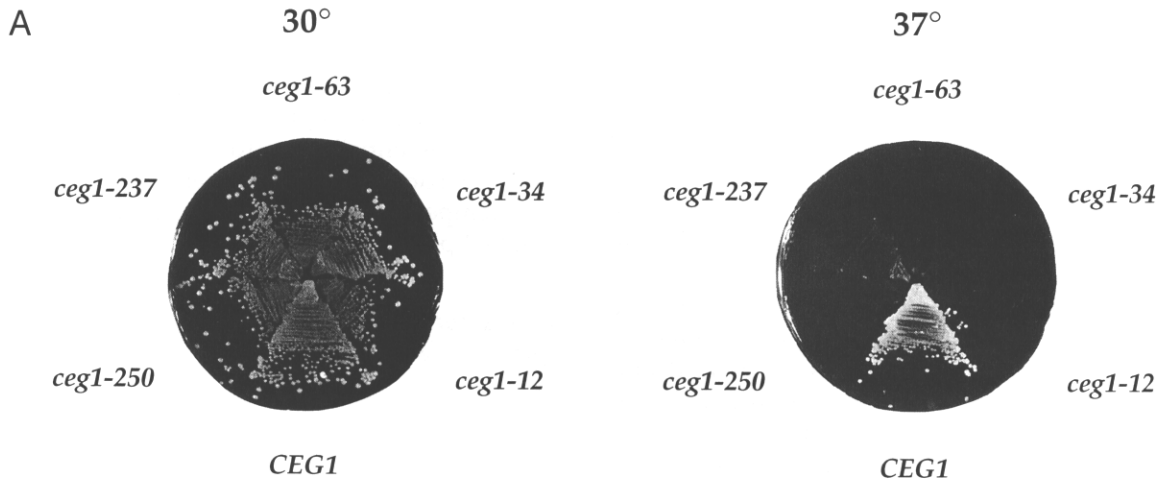
Plasmid shuffling (Boeke et al., 1987) was used to isolate temperature-sensitive alleles of the *Saccharomyces cerevisiae CEG1* gene. Five alleles were identified that grew at 30 °C, but not at 37 °C, and these were designated *ceg1-12*, *ceg1-34*, *ceg1-63*, *ceg1-237*, and *ceg1-250* (Fig. 1A). Sequencing of the mutant genes revealed that each contained a single point mutation. Four of five of the mutations are G to A transitions, as expected from hydroxylamine mutagenesis. Allele *ceg1-237* contains a G to C transversion mutation, and thus may have arisen spontaneously. It is interesting that several of the mutants isolated are similar or identical to those isolated independently by Yamagishi et al. (1995). The altered amino acid residues are conserved in the sequences of *CEG1* homologues from other yeasts (Shuman et al., 1994; L.D. Fresco, S.A. Woo, and S. Buratowski, in prep.), suggesting that they play important structural or functional roles in the capping enzyme (Fig. 1B).

The growth phenotypes of the mutant strains indicated that the different *ceg1* alleles varied in their severity. At the restrictive temperature, *ceg1-12*, *-34*, and *-63* were relatively "tight" temperature-sensitive alleles, showing essentially no growth. In contrast, *ceg1-237* and *-250* continued to grow very slowly at the restrictive temperature. In particular, *ceg1-237* exhibited a "leaky" phenotype.

Characterization of mutant guanylyltransferase proteins

Ceg1 proteins from the mutant yeast strains were characterized biochemically. Cells were harvested after 4.5 h of growth at 30 °C or 37 °C for extract preparation. The steady-state level of Ceg1 protein in each extract was monitored by western blot analysis using anti-Ceg1 antibody. As shown in Figure 2A, levels of the mutant Ceg1 proteins in cells grown at the permissive temperature (lanes 3–7) were decreased only slightly relative that of the wild-type protein (lane 2). At the restrictive temperature, very little Ceg1 protein was detected in extracts prepared from *ceg1-12*, *ceg1-34*, *ceg1-63*, and *ceg1-250* strains (lanes 9, 10, 11, and 13, respectively), suggesting that these proteins were degraded. In contrast, nearly wild-type levels of *ceg1-237* protein were detected at the restrictive temperature (lane 12).

During the capping reaction, the guanylyltransferase forms a covalent intermediate with GMP (for review see Mizumoto & Kaziro, 1987). To assess the guanylylation activity of the mutant Ceg1 proteins, 10 μg of whole cell extract protein and [α-^{32}P]-GTP were used to assay formation of the covalent enzyme–GMP (E-GMP) complex (Fig. 2B). Levels of E-GMP complex formation did not necessarily correlate with levels of the Ceg1 proteins. E-GMP complex formation in extracts from *ceg1-12*, *ceg1-34*, *ceg1-63*, and *ceg1-237* cells grown at 30 °C (lanes 3, 4, 5, and 6, respectively) were all reduced significantly relative to that of the wild-type protein (lane 2), whereas that of *ceg1-250* (lane 7) was comparable to wild-type levels. Surprisingly, there is only a small effect of these reductions on growth rate at the permissive temperature, suggesting that levels of Ceg1 activity are in excess of the amount required for normal growth. In extracts from *ceg1-12*, *ceg1-34*, and *ceg1-63* cells grown at 37 °C, E-GMP complex formation was abolished. In contrast, some residual activity was evident in *ceg1-237* and *ceg1-250* strains (lanes 12 and 13, respectively). These findings correlated well with the growth phenotypes of the alleles. Identical results were obtained when the in vitro assay was conducted at 37 °C (data not shown). Thus, it appears that the mutant Ceg1 proteins are more thermolabile and/or less active than the wild-type protein. The *ceg1-63* allele exhibited the most severe growth phenotype and guanylylation defect at the restrictive temperature, and was therefore chosen for many of the analyses described below.



B

Allele	Codon change	Amino acid change
ceg1-237	GGT→GCT	$G_{45}A$
ceg1-63	GAT→AAT	$D_{152}N$
ceg1-250	CCA→TCA	$P_{288}S$
ceg1-34	CCA→CTA	$P_{346}L$
ceg1-12	TGC→TAC	$C_{354}Y$

MVLAMESRVAPEIPGLIQPGNVTQDLKMMVCKLLNSPKPTKTFPGSQPVS
A
FQHSDVEEKLLAHDYYVCEKTDGLRVLMFIVINPVTGEQGCFMIDRENNY
YLVNGFRFPRLPQKKKEELLETLQDGTLLDGELVIQTNPMTKLQELRYLM
FDCLAINGRCLTQSPTSSRLAHLGKEFFKPYFDLRAAYPNRCTTFPFKIS
N
MKHMDFSYQLVKVAKSLDKLPHLSDGLIFTPVKAPYTAGGKDSLLLKWKP
EQENTVDFKLILDIPMVEDPSLPKDDRNRWYYNYDVKPVFSLYVWQGGAD
S
VNSRLKHFDQPFDRKEFEILERTYRKFAELSVSDEEWQNLKNLEQPLNGR
L
IVECAKNQETGAWEMLRFRDDKLNGNHTSVVQKVLESINDSVSLEDLEEI
Y
VGDIKRCWDERRANMAGGSGRPLPSQSQNATLSTSKPVHSQPPSNDKEPK
YVDEDDWSD

FIGURE 1. **A:** Growth of wild-type and conditional *S. cerevisiae CEG1* strains. Strains carrying the indicated *CEG1* alleles were assayed for growth from single cells at 30 °C and 37 °C. **B:** Amino acid sequences of the *S. cerevisiae ceg1* temperature-sensitive alleles. Amino acid changes in the various *ceg1* temperature-sensitive alleles are depicted below the sequence of wild-type *CEG1*. Allele names, amino acid changes, and codon changes are indicated to the right. Amino acid residues are represented in the single-letter code. Residues whose identities are conserved in three yeast capping enzymes (*S. cerevisiae, Schizosaccharomyces pombe,* and *Candida albicans*) are shown in boldface type.

In contrast to the other mutants, the *ceg1-237* allele (G45A) gives rise to a fairly stable protein that exhibits markedly decreased E-GMP complex formation at 30 °C and 37 °C. This mutation also causes slow growth at both temperatures. These results suggest that the mutated glycine residue may function specifically in E-GMP complex formation rather than in protein folding.

Loss of capping inhibits mRNA splicing

To determine the fate of mRNAs in the absence of capping, mutant *ceg1* strains were shifted to the restrictive temperature and steady-state levels of individual RNA transcripts were monitored by northern blot analysis. Figure 3A shows the results using a probe for the *CYH2* mRNA, which contains a single intron. At the permissive temperature (odd numbered lanes), mRNA from the mutants closely resembled that of wild-type in both overall levels of transcript and in the extent of splicing. In contrast, RNA isolated at the restrictive temperature (even numbered lanes) showed significant differences between wild-type and mutant cells. The *ceg1* mutant cells exhibited significant accumulations of *CYH2* pre-mRNA, with a corresponding decrease in levels of ma-

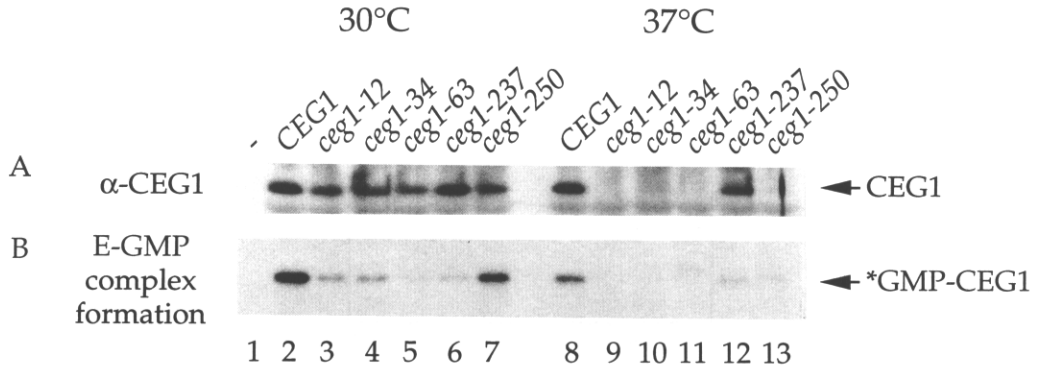


FIGURE 2. Analysis of proteins encoded by the temperature-sensitive *ceg1* alleles. Whole-cell extracts were made from wild-type or *ceg1* mutant cells grown for 4.5 h at 30 °C or 37 °C. The extracts were assayed using (**A**), western blot analysis with anti-CEG1 antibody, or (**B**) in vitro guanylylation of Ceg1 proteins. Lane 1, no extract; 10 μg of extract from cells containing: lanes 2, 8, wild-type *CEG1*; lanes 3, 9, *ceg1-12*; lanes 4, 10, *ceg1-34*; lanes 5, 11, *ceg1-63*; lanes 6, 12, *ceg1-237*; lanes 7, 13, *ceg1-250*. The migration of Ceg1 protein and E-GMP complex (*GMP-CEG1) are indicated.

ture *CYH2* mRNA. As a control, the same blot was also tested for levels of *CUP1* mRNA, which does not contain an intron (Fig. 3B). Except for a small increase seen at 37 °C in the wild-type, *ceg1-12*, and *ceg1-237* strains, no differences were seen between permissive and nonpermissive conditions. These results suggest that splicing was affected adversely in the *ceg1* mutant cells.

As observed previously for the growth phenotypes and biochemical analysis of the *ceg1* mutants, variation between alleles was evident. Alleles *ceg1-12*, *ceg1-34*, and *ceg1-63* exhibited the most severe inhibition of splicing, whereas *ceg1-250* was slightly less affected. Surprisingly, splicing in *ceg1-237* cells was unaffected at the restrictive temperature. This mutant produces guanylyltransferase that is relatively stable, but compromised in activity at both temperatures.

If the lack of capping affected splicing directly, there should be a close correlation between the splicing inhibition and loss of capping enzyme. To test this hypothesis, a time course of pre-mRNA accumulation after temperature shift was performed (Fig. 4). At early time points, both wild-type and mutant cells show a slight reduction in splicing, probably due to the effect of heat shock (Fig. 4B,C). However, clear differences are seen by 30 min after temperature shift, suggesting that pre-mRNA begins accumulating soon after inacti-

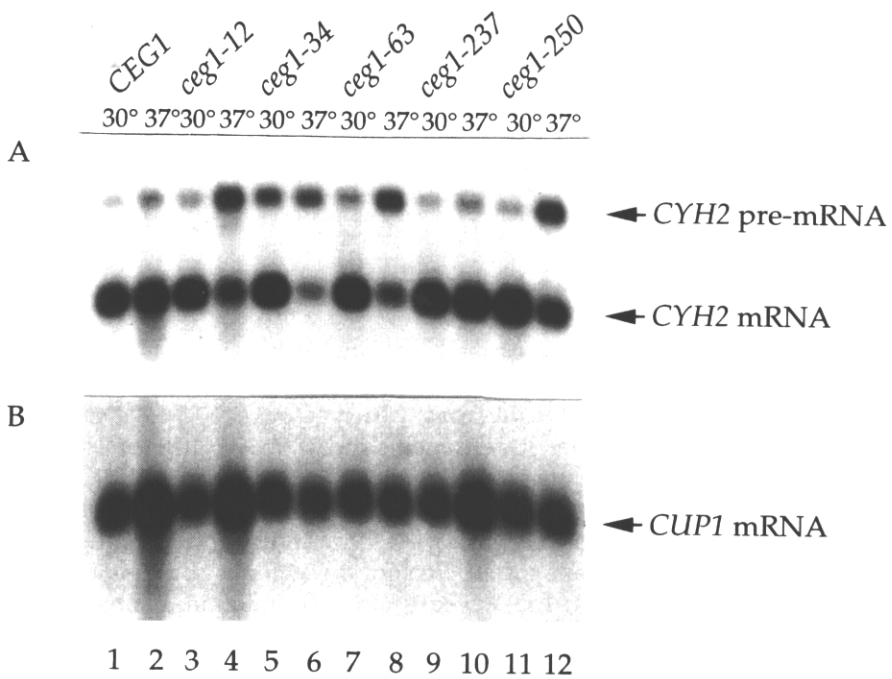


FIGURE 3. Pre-mRNA splicing is inhibited in capping enzyme mutants at the restrictive temperature. Total RNA was isolated from *ceg1* temperature-sensitive strains grown for 4.5 h at either 30 °C or 37 °C. RNA was analyzed by northern blot hybridization to a (**A**) *CYH2* or to a (**B**) CUP1 antisense probe. The *ceg1* allele in each strain and the temperature of growth is indicated above each lane. Positions of the pre-mRNAs and mRNAs are indicated.

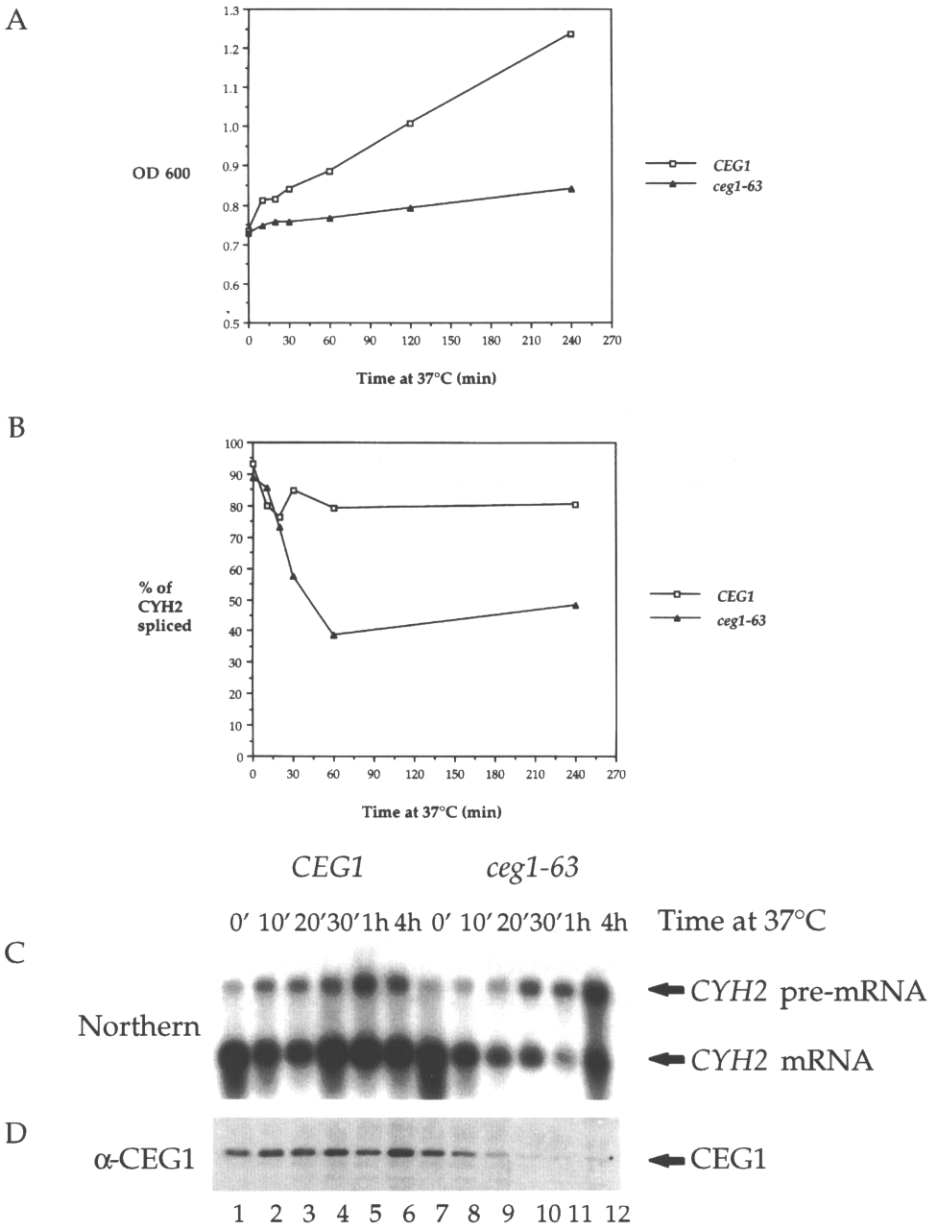


FIGURE 4. Accumulation of pre-mRNA correlates with loss of Ceg1 protein. Wild-type (*CEG1*) and mutant cells (*ceg1-63*) were shifted to 37 °C for the indicated amount of time. Cell division was monitored by light scattering (OD_{600}, **A**). RNA from each time point was tested for splicing of *CEG1* mRNA by northern blot analysis (**C**) and the fraction of spliced mRNA was quantitated and plotted as a function of time (**B**). Protein extracts from the cells were also assayed for the presence of Ceg1 protein by immunoblotting (**D**). Positions of the pre-mRNAs and mRNAs are indicated.

vation of the capping enzyme. This correlates well with cessation of cell division (Fig. 4A) and loss of Ceg1 protein (Fig. 4D). Enzymatic assays showed similar loss of the CEG1–GMP complex (data not shown). Therefore, the loss of capping affects splicing rapidly, arguing for a direct connection between the two processes. For most experiments, RNA was harvested after 4 h.

Splicing of the *CYH2* transcript is relatively inefficient in wild-type cells, and so it may not represent a typical mRNA. In order to determine whether the inhibition of splicing was specific to *CYH2*, or was in fact more general, splicing of other intron-containing mRNAs was examined. Figure 5 shows northern blots of total RNA from wild-type or *ceg1-63* cells after 4 h at either 30 °C (lanes 1 and 3) or 37 °C (lanes 2 and 4). The blots were hybridized to probes for the *CYH2* transcript (top panel), *RPS13*, a ribosomal protein transcript (middle panel), or *ACT1*, a transcript encoding actin (bottom panel). Each of these genes contains a single intron. In each case, an accumulation of pre-mRNA was observed in the *ceg1* mutant at the restrictive temperature (lane 4) compared to the wild-type (lane 2).

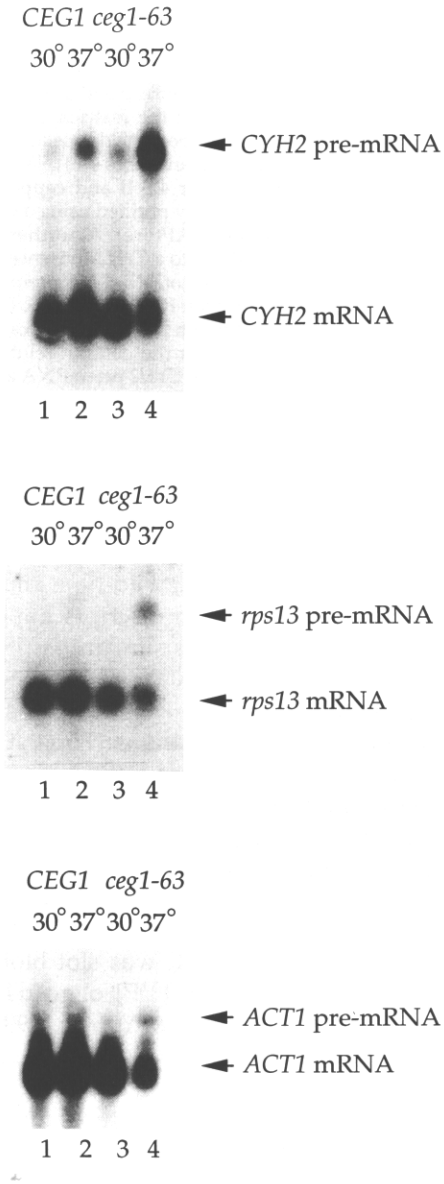


FIGURE 5. Splicing of several mRNAs is inhibited in the capping enzyme mutants. Wild-type (lanes 1, 2) or *ceg1-63* (lanes 3, 4) strains were grown at either 30 °C (lanes 1, 3) or 37 °C (lanes 2, 4) for 4 h. Total RNA was isolated and a northern blot was hybridized to *CYH2* (top), *rps13* (middle), or *ACT1* (bottom) probes. Temperature of growth and the positions of the pre-mRNAs and mRNAs are indicated. Similar results were observed using a *rp51* probe (not shown).

The increase in pre-mRNA was accompanied by a corresponding decrease in mature mRNA levels. Blots were also hybridized to an *rp51* (ribosomal protein 51) intron probe. Intron-containing RNA was observed in the mutant strain at the restrictive temperature, but was not evident at the permissive temperature. No intron-containing RNA was observed in wild-type cells at either temperature (data not shown). Therefore, the inhibition of mRNA splicing appears to be a general phenomenon.

In addition to the inhibition of mRNA splicing, the total levels of mRNA transcripts (i.e., spliced plus unspliced) were slightly decreased in the *ceg1* mutant cells. The severity of the reduction increased with time and appeared to differ between mRNAs. *CUP1* mRNA, which lacks an intron, also displayed this reduction (Fig. 3B). Therefore, the decrease in steady-state transcript levels is probably not directly linked to splicing. A more likely explanation is provided by the finding that uncapped mRNA is degraded faster than capped mRNA (Sachs, 1993; Beelman & Parker, 1995 for review). Experiments to measure degradation rates in the capping enzyme mutants have not yet been done.

To determine whether loss of capping affected U snRNA stability, which could in turn affect pre-mRNA splicing, levels of several snRNAs were assayed by northern blotting. No significant differences between levels of U1, U2, U4, U5, or U6 snRNAs were seen at the permissive and nonpermissive temperatures at several points up to 4-h post-shift (data not shown). This is consistent with genetic depletion results demonstrating that U snRNAs have a low turnover rate relative to mRNAs (reviewed in Rymond & Rosbash, 1992). These results argue against a model in which the inhibition of splicing is mediated through an effect on the snRNAs (see the Discussion).

Spliced and unspliced mRNAs are not capped in *ceg1* mutant cells

To demonstrate directly that mRNAs produced in the *ceg1* mutant cells were not capped, we made use of an affinity purification procedure for isolating capped RNAs. In the "CAPture" protocol (Edery et al., 1995), the ribosomal cap-binding protein eIF-4E is immobilized as a fusion protein on a column matrix. Capped RNA is specifically retained on the column and can be eluted using the cap analogue m^7GDP. Although quantitative binding of capped RNA is not obtained routinely, comparison of RNA produced in wild-type and mutant cells can be useful.

Total RNA from wild-type or *ceg1-63* mutant cells grown at 37 °C was isolated and 200 μg were loaded onto GST-eIF-4E columns. The input RNA (Σ), flow-through (FT), and eluted fractions were analyzed by northern blotting using the *CYH2* probe (Fig. 6). The proportion of spliced and unspliced *CYH2* mRNA from wild-type cells that was bound to the column was identical to that in the input fraction (compare lanes 1 and 3). Although most of the input mRNA is presumably capped, only about 10–30% of the input was retained on the column.

Affinity purification of capped RNAs from the *ceg1-63* cells revealed a pattern radically different from that in wild-type cells. Very little RNA from the mutant cells was retained on the cap affinity column (lane 6). If one assumes that all wild-type mRNA is capped, quantita-

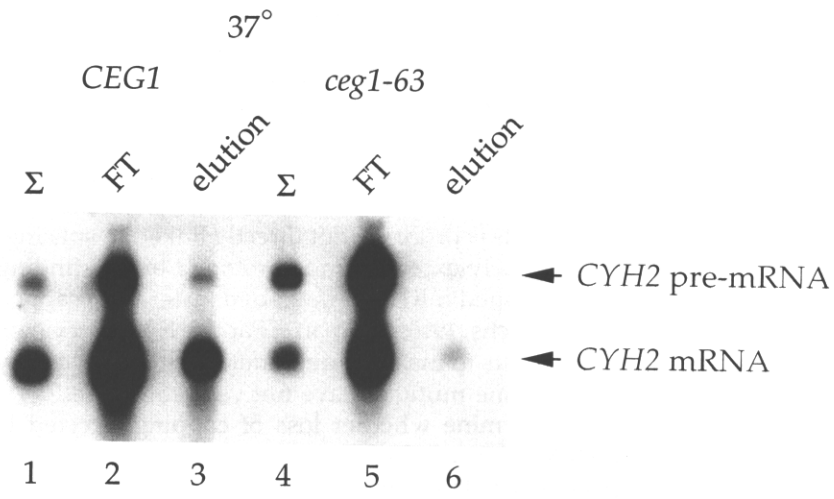


FIGURE 6. Spliced and unspliced mRNAs produced at the restrictive temperature are not capped. Wild-type (lanes 1–3) and *ceg1-63* mutant (lanes 4–6) cells were grown at 37 °C for 4.5 h and capped mRNAs were affinity purified using a GST-eIF-4E column (CAPture). A northern blot was hybridized to a *CYH2* antisense transcript. Lanes 1, 4, total (Σ) RNA representing 5% of the input RNA; lanes 2, 5, RNA in the flowthrough (FT); lanes 3, 6, capped RNA retained on the column (elution). Positions of the *CYH2* pre-mRNA and mRNA are indicated.

tion of this and other trials suggests that no more than 5–10% of mRNA in the *ceg1-63* cells is capped. Furthermore, despite the fact that the input RNA from the *ceg1-63* cells contained more unspliced than spliced mRNA (lane 4), the small amount of RNA that was retained was almost exclusively spliced (lane 6). It is unclear whether this capped mRNA reflects capping enzyme activity below the levels of detectability (Fig. 2), or represents residual mRNA processed prior to the shift to the restrictive temperature. In either case, mRNA capping appears to be largely absent in the *ceg1-63* mutant at the restrictive temperature. Similar results were obtained with all other mRNAs assayed in several experiments (data not shown).

Clearly, the unspliced mRNAs in the mutant cells are not capped. Surprisingly, comparison of levels of capped RNA from the wild-type and mutant cells suggests that a significant proportion of the spliced mRNA in the mutant cells is also uncapped. A far smaller percentage of spliced RNA from the mutant cells (1–2%) bound reproducibly to the affinity column than from the wild-type spliced RNA (10–15%). Thus, the cap appears to enhance, but is not absolutely required, for splicing. This finding is consistent with results of others obtained with in vitro splicing extracts and in microinjected *Xenopus* nuclei (see the Discussion).

Uncapped and unspliced mRNAs are polyadenylated in *ceg1* mutants

To determine whether polyadenylation occurs in the absence of capping, the polyadenylation state of mRNA in the *ceg1* mutant at the restrictive temperature was examined (Fig. 7). Total RNA from the wild-type (lanes 1–3) or *ceg1-63* (lanes 4–6) strains grown at 37 °C for 4 h was passed twice over an oligo(dT) cellulose column. Proportional amounts of total (Σ, lanes 1 and 4), poly(A)$^+$ (lanes 2 and 5), and poly(A)$^-$ (lanes 3 and 6) RNA were subjected to northern analysis and hybridized to the *CYH2* probe. In both wild-type and mutant cells, nearly all of the *CYH2* transcripts appeared to have poly(A) tails. Moreover, a similar distribution of spliced and unspliced mRNA was observed in the poly(A)$^+$, poly(A)$^-$, and total RNA samples in both the wild-type and the mutant strains. Thus, it appears that the uncapped, unspliced pre-mRNA that accumulates following the loss of capping is polyadenylated normally. Therefore, a capped transcript is not a prerequisite for polyadenylation in yeast.

To quantitate the total amount of poly(A)$^+$ RNA in cells, RNA isolated from the wild-type or *ceg1* mutant strains grown at 30 °C or 37 °C was slot blotted to a membrane and hybridized to [^{32}P] oligo(dT) as described previously (Thompson & Young, 1995). Each slot contained an equivalent amount of rRNA as judged by visualization of RNAs following agarose gel electrophoresis. At the restrictive temperature, the *ceg1* mutant cells displayed slightly reduced steady-state levels of total poly(A)$^+$ RNA, whereas the wild-type cells exhibited slightly increased levels of poly(A)$^+$ RNA relative to the permissive temperature (data not shown). This was consistent with earlier experiments (Figs. 3, 4, 5) showing reductions in levels of specific messages, and may reflect increased degradation rates of uncapped mRNAs.

The poly(A) tails of mRNAs are synthesized in the nucleus and are shortened slightly upon transport to the cytoplasm (Sachs & Davis, 1989). Some RNA processing and transport mutants exhibit defects in this reaction. The average length of poly(A) tails in the capping enzyme mutants was assayed by digestion of total RNA with RNase A followed by radioactive labeling of the resistant RNAs. Because poly(A) tails are resistant to RNase A, electrophoresis of the labeled RNAs shows their length distribution (Sachs & Davis, 1989). No significant differences in average poly(A) tail length were observed between the wild-type and mutant *ceg1* strains at either permissive or restrictive temperature using

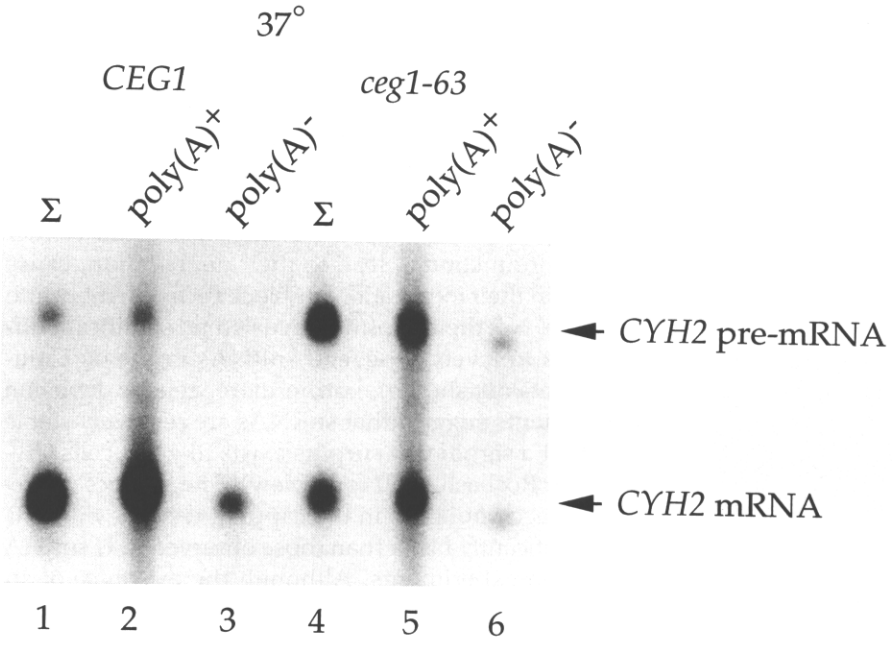


FIGURE 7. Spliced and unspliced mRNA is polyadenylated in the absence of capping. RNA was isolated from wild-type (lanes 1–3) and *ceg1-63* mutant (lanes 4–6) cells that were grown at 37 °C for 4 h. RNA was separated into poly(A)$^+$ and poly(A)$^-$ fractions by oligo(dT) chromatography and northern blot analysis of the *CYH2* transcript was performed. Lanes 1, 4, total (Σ) RNA; lanes 2, 5, poly(A)$^+$ RNA; lanes 3, 6, poly(A)$^-$ RNA. Positions of the *CYH2* pre-mRNA and mRNA are indicated. It should be noted that all RNAs exhibit a slightly faster mobility in the poly(A)$^-$ RNA lane; this reflects the molecular weight difference caused by the lack of the poly(A) tail.

this assay (data not shown). Therefore, the cap is not required for polyadenylation or for the cytoplasmic shortening of poly(A) tails.

Nuclear export of RNA does not require the cap

To determine whether capping plays a role in nuclear export of mRNAs, in situ poly(A)$^+$ RNA localization was examined in *ceg1* mutant cells and wild-type cells. Cells were grown at 30 °C and half of the culture was shifted to 37 °C for 2, 4, or 9 h. Figure 8 shows one representative mutant (*ceg1-63*) grown at 37 °C. Localization of poly(A)$^+$ RNA was examined by *in situ* hybridization using a digoxygenin-labeled poly(dT)$_{50}$ probe and visualized by FITC (fluorescein isothiocyanate) fluorescence microscopy (Fig. 8B). The same field of cells was observed by Nomarski optics microscopy (Fig. 8A) and nuclei were localized using DAPI (4′,6-diamidino-2-phenylindole-dihydrochloride) fluorescence (Fig. 8C).

In both wild-type and all mutant *ceg1* strains, poly(A)-dependent fluorescence was distributed diffusely throughout the cell, consistent with rapid nuclear export of RNA. Wild-type and mutant cells appeared essentially the same, except that many of the mutant cells contained large cytoplasmic vacuoles. No differences were observed between the permissive and the restrictive temperatures (data not shown). The capping enzyme mutants differ from RNA transport mutants, which exhibit bright nuclear fluorescence due to nuclear retention of poly(A)+ RNA.

Specific mRNAs were not localized, so it is possible that a small subset of transcripts (for example, transcripts containing introns) might be affected by the lack of capping. However, because poly(A)+ RNA was localized properly in the *ceg1* mutants, we conclude that capping is not generally required for mRNA export from the nucleus.

DISCUSSION

Although the role of the mRNA cap in translation has been established firmly, no clear consensus has been reached on what function the cap may play in RNA processing and transport. Using conditional alleles of the capping enzyme, we have been able to probe the fate of uncapped mRNA in the normal context of transcription, mRNA processing, and nuclear export. We conclude that mRNA capping is not linked to polyadenylation or nuclear export of mRNA. In contrast, we find that capping increases the efficiency of mRNA splicing significantly. However, it appears that splicing in vivo is not absolutely dependent upon the presence of the cap.

Within 30 min after shifting the capping enzyme mutant strains to the restrictive temperature, the level of unspliced transcripts began to increase. The accumulation of unspliced mRNA was accompanied by a corresponding decrease in the level of spliced mRNA. In this respect, the capping enzyme mutants differ from several genes described previously that are involved in mRNA transport (*MTR, RNA1, PRP20*: see Izaurralde & Mattaj, 1995 for review) and degradation (the *NMD* and *UPF* genes, reviewed in Peltz et al., 1994). Mutants in these genes exhibit increased levels of unspliced transcripts because the pre-mRNA is not degraded rapidly (Sachs, 1993; Beelman & Parker, 1995 for review). However, they do not typically show a reduction in

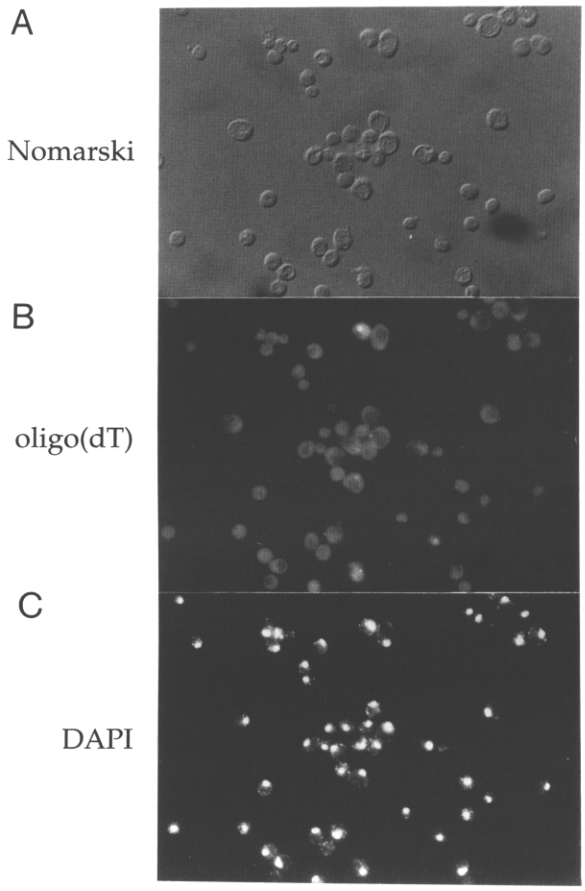


FIGURE 8. Localization of poly(A)$^+$ RNA is not affected by the absence of capping. Wild-type (not shown) and *ceg1-63* mutant yeast strains were grown at 37 °C and tested for localization of poly(A) RNA. Because no differences were observed, only the mutant strain is shown. The same field of cells is shown in three views: (**A**) Nomarski optics to show cell structure; (**B**) fluorescence microscopy of FITC-linked oligo(dT), which hybridizes to poly(A)$^+$ RNA; and (**C**) DAPI staining of nuclei.

spliced mRNA. Therefore, it is highly unlikely that the capping enzyme mutations cause selective stabilization of unspliced mRNA.

In fact, the capping enzyme mutant cells exhibit a reduction in the steady-state level of total poly(A)$^+$ RNA relative to that of rRNA. Although we cannot rule out that this decrease is due to a decrease in transcription rates at the elevated temperature, it seems much more likely that the reduction was due to increased degradation of uncapped RNAs. Decapping appears to be an essential step in the mRNA degradation pathway and uncapped mRNAs may be targeted preferentially for decay by 5′→3′ exonucleases (reviewed in Sachs, 1993, and Beelman & Parker, 1995).

Mechanism of splicing inhibition in *CEG1* deficient cells

Why does the cessation of capping lead to a reduction in the level of pre-mRNA splicing? There are several possible explanations: (1) Functional spliceosomes are not assembled because the essential snRNA components are not capped. (2) The capping enzyme has a second function in splicing. (3) The cap on mRNA helps recruit the transcript to the spliceosome. Several lines of evidence argue against the first two ideas, and instead support the third hypothesis.

Production of uncapped U snRNAs could render them nonfunctional, lead to their degradation, cause defects in their localization, or block their assembly into snRNPs. Northern blotting revealed no significant differences in levels of several snRNAs in the *ceg1* mutants (data not shown). Furthermore, genetic depletion experiments suggest that snRNAs are relatively stable and that a significant surplus exists in yeast cells (Rymond & Rosbash, 1992 for review). The kinetics of pre-mRNA accumulation in the capping enzyme mutants are significantly faster than those observed in U snRNA depletion experiments. Although the existence of an unknown U snRNA with a very short half-life cannot be ruled out, it appears unlikely that the inhibition of splicing in the capping enzyme mutants is mediated by the U snRNAs. A second line of evidence arguing against the first two models is the finding that yeast extracts derived from *ceg1* mutant cells are fully competent for in vitro splicing (B. Schwer & S. Shuman, pers. comm., see paper this issue). If the mutant capping enzyme was a component of (or caused the assembly of) defective spliceosomes, it is likely that such defects would be apparent in the in vitro splicing system.

The simplest explanation for our results is that uncapped mRNA is not efficiently recognized by the spliceosome. Our results strongly support earlier models invoking recognition of the cap structure by the splicing machinery. The earliest evidence for this model came from experiments showing that capped mRNA was spliced more efficiently than uncapped mRNA in vitro or in vivo, and that splicing could be partially inhibited by the addition of cap analogues or capped RNA lacking introns (Konarska et al., 1984; Krainer et al., 1984; Edery & Sonenberg, 1985; Ohno et al., 1987; Patzelt et al., 1987; Inoue et al., 1989; Izaurralde et al., 1994, 1995). More recently, a mammalian cap binding complex (CBC) has been identified that appears to be involved in both pre-mRNA splicing and nuclear export of U snRNAs (Izaurralde et al., 1994, 1995). The CBC is a dimer consisting of the cap-binding proteins CBP80/NCBP (Ohno et al., 1990; Izaurralde et al., 1994) and CBP20/NIP1 (Izaurralde et al., 1995; Kataoka et al., 1995). Antibodies against these proteins inhibit pre-mRNA splicing prior to the first cleavage reaction both in HeLa cell extracts and when microinjected into *Xenopus* oocytes (Izaurralde et al., 1994, 1995). Furthermore, a yeast homologue of CBP20 (*MUD13*) encodes a non-snRNP splicing factor (H. Colot, F. Stutz, & M. Rosbash, pers. comm.). Our work-

ing hypothesis is that uncapped pre-mRNAs are not recruited to the spliceosome by the CBC.

Coupling of pre-mRNA splicing to the presence of the cap offers several potential benefits to the cell. First, it provides a mechanism for the splicing machinery to discriminate RNA polymerase II transcripts from those produced by the other polymerases. RNAs synthesized by RNA polymerases I or III do not possess m^7G caps, are not typically substrates for the splicing machinery, and are not translated into protein. Therefore, the m^7G cap provides a specific marker for RNAs produced by RNA polymerase II. A second related benefit is that the splicing machinery will not assemble on partially degraded mRNAs lacking a m^7G cap.

Another rationale for linking the cap to mRNA splicing is provided by the "exon definition" model (Robberson et al., 1990; Berget, 1995). Although the unit of spliceosome recognition was traditionally considered to be the intron, the very large size of some introns makes it difficult to understand how the splicing machinery can identify correct splice sites. The exon definition model redefines the unit of recognition as the (typically smaller) exon flanked by a 3′ and a 5′ splice site. In this model, the 3′ splice site of one intron could influence recognition of the 5′ splice site in the next intron downstream. In the unique case of the first exon of an mRNA, there is no 3′ splice site to identify the 5′ end of the exon. It has been proposed that the cap serves to define the 5′ border of the first exon. In support of this idea, Shimura and coworkers demonstrated that the cap structure was most important for splicing of the 5′ proximal intron from a two-intron-containing mRNA in mammalian extracts or *Xenopus* oocytes (Ohno et al., 1987; Inoue et al., 1989). Although yeast introns are relatively small in number and size, splicing in yeast appears very similar to that in higher eukaryotes. It would be interesting to test the effects of the *ceg1* mutations on the splicing of an mRNA containing two introns.

Having described the advantages of linking splicing to the presence of the cap, one may ask why this and other studies do not demonstrate an absolute requirement for the cap in splicing. Results presented in this and earlier reports indicate that the cap acts to enhance splicing. In fact, an intron engineered into a tRNA can be excised by the mRNA splicing machinery (Kohrer et al., 1990). A strict requirement might necessitate a high-affinity interaction between the spliceosome and the mRNA cap. In yeast, where most genes lack introns, such a high-affinity interaction could cause the splicing machinery to bind and be inhibited by the large excess of mRNA-lacking introns. In addition, the subsequent release of spliced mRNA might be slowed such that it becomes a rate-limiting step for splicing. Therefore, one can speculate that stimulation of splicing by the cap represents a compromise between the advantages and disadvantages of cap binding by the spliceosome.

MATERIALS AND METHODS

General methods

Plasmid cloning, PCR, and bacterial transformation were performed using standard methods (Ausubel et al., 1991). Dideoxy sequencing was performed using the Sequenase v. 2.0 kit (United States Biochemicals). pRS315-CEG1 and pRS316-CEG1 were created as described (Fresco & Buratowski, 1994). Solutions used for RNA work were treated with diethylpyrocarbonate (DEPC) to inactivate RNases.

Yeast methods

Plasmids were introduced into yeast using modified lithium acetate transformation protocol (Gietz et al., 1992). Media preparation, genomic DNA isolation from spheroplasts, plasmid shuffling, and all other yeast manipulations were performed by standard methods as described (Ausubel et al., 1991; Guthrie & Fink, 1991). 5-Fluoro-orotic acid (5-FOA) was purchased from PCR Incorporated.

Yeast strains

YSB104 *MATa, ura3-52, leu2-3,112, his3Δ200*
YSB228 *MATa, ura3-52, leu2-3,112, his3Δ200, ceg1Δ1::HIS3,* [pRS315-ceg1-12]
YSB229 *MATa, ura3-52, leu2-3,112, his3Δ200, ceg1Δ1::HIS3,* [pRS315-ceg1-34]
YSB230 *MATa, ura3-52, leu2-3,112, his3Δ200, ceg1Δ1::HIS3,* [pRS315-ceg1-63]
YSB231 *MATa, ura3-52, leu2-3,112, his3Δ200, ceg1Δ1::HIS3,* [pRS315-ceg1-237]
YSB232 *MATa, ura3-52, leu2-3,112, his3Δ200, ceg1Δ1::HIS3,* [pRS315-ceg1-250]
YSB242 *MATa, ura3-52, leu2-3,112, his3Δ200, ceg1Δ1::HIS3,* [pRS315-CEG1]
YSB245 *MATα, ura3-52, leu2-3,112, his3Δ200, ceg1Δ1::HIS3,* [pRS316-CEG1].

Construction of the *S. cerevisiae* strain for plasmid shuffling was described previously (Fresco & Buratowski, 1994).

Isolation of conditional *CEG1* alleles

Thirty micrograms of pRS315-CEG1 were mutagenized with hydroxylamine and transformed into YSB245. Transformants were selected at 30 °C on 44 SC plates lacking histidine and leucine at a density of ~300 colonies/plate. Colonies were replica plated to SC-his-leu plates containing 5-FOA to select for loss of the wild-type *CEG1* (pRS316-CEG1). FOA-containing plates were replica plated to 3 SC-his-leu plates and incubated at 16 °C, 37 °C, and 30 °C.

Conditional mutants were isolated and the plasmids rescreened to confirm the phenotypes. Five temperature-sensitive mutations and no cold-sensitive mutations were isolated. All the mutations were complemented by transformation with a plasmid carrying a wild-type copy of *CEG1*.

Yeast whole-cell extract preparation for analysis of Ceg1 proteins

For characterization of Ceg1 proteins, yeast cells were grown overnight at 30 °C in SC-his-leu medium to an OD_{600} of 1, pelleted, and diluted to an OD of 0.5. An equal volume of YPD medium at either 30 °C or 46 °C was added and cells were grown at either 30 °C or 37 °C. At 4-h post-temperature shift, the cells were harvested and whole-cell extracts were prepared as described (Eisenmann et al., 1992).

Antibody production and western blot analysis

Antibodies were raised in rabbits against soluble his_7CEG1 protein that had been purified by nickel chelate chromatography (Qiagen) and polyacrylamide gel purification, as described (Harlow & Lane, 1988). For western blot analysis, 38 μg of each whole cell extract was electrophoresed on an SDS-10% polyacrylamide gel and electroblotted onto nitrocellulose using a Trans-blot apparatus (Bio-Rad). Immunoreactive proteins were detected using rabbit anti-his_7CEG1 antibody and HRP anti-rabbit IgG secondary antibody using the ECL Western Blotting Kit (Amersham). To confirm that equivalent amounts of extract were loaded in each lane, nitrocellulose filters were stained following transfer using Ponceau S (data not shown).

Assay for capping enzyme guanylylation

Enzyme–GMP complex formation was assayed by incubation of 10 μg of protein from each whole cell extract with [α-^{32}P] GTP at either 30 °C or 37 °C followed by SDS-gel electrophoresis as described previously (Fresco & Buratowski, 1994). Proteins were electroblotted onto nitrocellulose using a Trans-Blot apparatus (Bio-Rad) and labeled proteins were visualized by autoradiography.

RNA isolation and northern blot analysis

Total yeast RNA was isolated as described (Ausubel et al., 1991). Poly(A)$^+$ RNA was isolated using oligo(dT) cellulose as described (Guthrie & Fink, 1991). RNAs were electrophoresed on 0.75, 1.2, or 2% agarose formaldehyde gels, RNAs were transferred to GeneScreen (Dupont), and northern blotting was conducted as described (Ausubel et al., 1991). Lanes contained an equivalent amount of ribosomal RNA as judged by ethidium bromide staining. Antisense RNA probes were labeled using [α-^{32}P] UTP (3,000 Ci/mmol) (NEN Dupont) and SP6 or T7 RNA polymerase (Ambion) by in vitro transcription as recommended by the manufacturer. DNA fragments were labeled radioactively by random priming using [α-^{32}P] dCTP (3,000 Ci/mmol) (NEN Dupont) and the Oligolabelling Kit (Pharmacia). Hybridizations using the *rps13* and *rp51* DNA probes (Cheng & Abelson, 1987) and the *CYH2* and *ACT1* riboprobes (Ausubel et al., 1991) were conducted as described. Relative amounts of RNA were quantitated using a Fuji BasX 2000 PhosphoImager.

pDH34 containing the *Act1* cDNA was provided by Rick Young (Whitehead Institute) and used to generate an antisense riboprobe. pWF1 (*CUP1*) and pWF6 (*CYH2*) were generous gifts from Wayne Forrester (University of Wisconsin) and were used to generate antisense riboprobes as described (Forrester et al., 1992). An *rps13* DNA probe was generated from a plasmid provided by Charles Klein (Harvard Medical School). An DNA probe specific for the *rp51* intron was generated from a plasmid provided by Michael Rosbash (Brandeis University).

Affinity purification of capped RNAs

Affinity purification of capped mRNA was performed essentially as described (Edery et al., 1995) except that the protocol was modified to use a GST-eIF-4E fusion protein rather than a protein A-eIF-4E fusion protein. *E. coli* carrying the expression plasmid were grown in superbroth (25 g bactotryptone, 15 g bacto-yeast extract, 5 g NaCl/L supplemented with 100 μg/mL ampicillin) to OD_{600} 0.7–0.9. Expression was induced with 1 mM IPTG for 3 h at 30 °C and cell extract was prepared as described (Edery et al., 1995). GST-eIF-4E was purified by passing the clarified extract over a cap analogue (m^7GDP) column, washing with 50 μM GDP, and subsequent elution with 500 μM m^7GDP (Edery et al., 1995). Approximately 1.8 mg of protein was obtained from 2 L of cells. One milligram of the purified fusion protein was linked to 0.5 mL of glutathione agarose beads (Sigma) for 2 h at 4 °C. The beads were poured into a column, washed with 15 mL HCB (1 M KCl, 20 mM Hepes, pH 7.5, 0.2 M EDTA, 0.5 mM PMSF), and 100 mL of LCB (0.1 M KCl, 20 mM Hepes, pH 7.5, 0.2 M EDTA, 0.5 mM PMSF) to remove the cap analogue. The column was equilibrated with binding buffer (10 mM K_2PO_4, $KHPO_4$ pH 8, 0.1 M KCl, 2 mM EDTA, 5% glycerol) and split into two equal volumes.

Two-hundred micrograms total RNA from wild-type or mutant cells (in 425 μL 0.5 × BB, 100 U RNasin) were passed twice over the columns at room temperature. The columns were washed with 2 mL 1× BB, with 0.5 mL 50 μM GDP in 1× BB, and capped RNAs were eluted with 1 mL 1× BB containing 500 μM m^7GDP. Twenty micrograms of glycogen were added to each sample and RNAs were ethanol precipitated using NaOAc.

Analyses of polyA$^+$ RNA

In situ poly(A) localization, microscopy, and photography was conducted as described (Amberg et al., 1992). Total poly(A) quantitation was performed by slot-blot analysis as described (Thompson & Young, 1995). Average poly(A) tail length was determined as described (Sachs & Davis, 1989).

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