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Accelerated mRNA decay in conditional mutants of yeast mRNA capping enzyme

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ABSTRACT

Current models of mRNA decay in yeast posit that 3′ deadenylation precedes enzymatic removal of the 5′ cap, which then exposes the naked end to 5′ exonuclease action. Here, we analyzed gene expression in *Saccharomyces cerevisiae* cells bearing conditional mutations of Ceg1 (capping enzyme), a 52 kDa protein that transfers GMP from GTP to the 5′ end of mRNA to form the GpppN cap structure. Shift of *ceg1* mutants to restrictive temperature elicited a rapid decline in the rate of protein synthesis, which correlated with a sharp reduction in the steady-state levels of multiple individual mRNAs. *ceg1* mutations prevented the accumulation of *SSA1* and *SSA4* mRNAs that were newly synthesized at the restrictive temperature. Uncapped poly(A)$^+$ *SSA4* mRNA accumulated in cells lacking the 5′ exoribonuclease Xrn1. These findings provide genetic evidence for the long-held idea that the cap guanylate is critical for mRNA stability. The deadenylation–decapping–degradation pathway appears to be short-circuited when Ceg1 is inactivated.

INTRODUCTION

The m7GpppN cap structure of mRNA was elucidated in 1975 (1–3). Since then, the cap has been implicated in virtually every aspect of eukaryotic mRNA metabolism, including splicing, 3′ end formation, transport, translation and degradation (4–12). Specific mRNA transactions have been deemed cap-dependent on the basis of biochemical criteria, e.g. (i) preferential utilization of capped versus uncapped RNA substrates; (ii) inhibition by cap analogs or (iii) participation of a protein factor that binds to the cap. A genetic analysis would help to clarify whether the roles attributed to the cap on biochemical grounds are applicable *in vivo*. The budding yeast *Saccharomyces cerevisiae* is the obvious system of choice for this analysis. However, prior studies in yeast have dealt indirectly with the role of the monomethyl cap, i.e. most available genetic data concern the roles of cap binding proteins rather than the cap structure *per se*. To address the role of the m7G cap directly, one wishes to manipulate the cellular enzymes responsible for cap synthesis.

Capping occurs on nascent RNA chains and is carried out by three enzymes: (i) RNA triphosphatase, which converts the triphosphate end of the primary transcript to a diphosphate; (ii) RNA guanylyltransferase (capping enzyme), which catalyzes the transfer of GMP from GTP to the 5′ diphosphate end of RNA to form GpppN and (iii) RNA (guanine-7)-methyltransferase, which transfers a methyl group from S-adenosylmethionine to the cap guanosine to yield m7GpppN (13). The mRNA capping and methylating enzymes have been purified from *S.cerevisiae*, and the genes encoding them have been identified. Yeast mRNA guanylyltransferase is a 52 kDa protein encoded by the *CEG1* gene (14). Ceg1 reacts with GTP to form a covalent enzyme–GMP intermediate. The enzyme transfers the GMP to a 5′ diphosphate-terminated RNA to form the GpppN cap structure, which is then methylated at the N-7 of the cap guanosine, in a reaction catalyzed by the 50 kDa *S.cerevisiae* Abd1 protein (15). The guanylyltransferase and methyltransferase activities of Ceg1 and Abd1 are essential for yeast cell growth, i.e. mutations of *CEG1* or *ABD1* that eliminate enzyme activity *in vitro* are invariably lethal *in vivo* (16–21). The yeast RNA triphosphatase is a 62 kDa protein encoded by *CET1* (22). *CET1* gene disruption is lethal (22); however, it is not yet clear if the triphosphatase activity of Cet1 is essential for cell growth.

The most straightforward way to address cap function is to observe the phenotypes elicited by genetic or pharmacological inactivation of the capping and methylating enzymes. The pharmacological approach is precluded by the absence of any specific inhibitors of the triphosphatase, guanylyltransferase or (guanine-7) methyltransferase activities. A prerequisite for genetic analysis is the availability of conditional mutants. We and others have isolated collections of temperature-sensitive (ts) *ceg1* alleles (23–27). The preponderance of evidence argues that the *ceg1* conditional growth defect reflects a decrease in cap formation by mutant Ceg1 proteins to a sub-threshold level at the restrictive temperature. For example, the guanylyltransferase activity present in extracts prepared from *ceg1* cells grown at restrictive temperature is markedly thermolabile *in vitro*, whereas the activity of *CEG1* cell extracts is thermostable (24,26). In addition, when Fresco and Buratowski (25) analyzed the levels of immunoreactive Ceg1 protein in *ceg1* cells 4.5 h after shift from permissive to restrictive temperature, they found that the Ceg1-237 mutant protein was present at normal levels, but it was inactive in transguanylylation (25).

Two prior studies have focused on mRNA processing in *ceg1* cells after shift to the restrictive temperature, both showing that Ceg1

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inactivation can affect pre-mRNA splicing (24,25). Several *ceg1* mutations elicited an accumulation of unspliced mRNA precursors at restrictive temperature (24,25). Cap-affinity chromatography analysis of *CYH2* RNAs revealed the presence of uncapped precursors and uncapped mature transcripts in *ceg1* cells, indicating that the cap may facilitate, but is not required for, pre-mRNA splicing in yeast (25). We reached the same conclusions based on our findings that neither the cap structure nor the Ceg1 protein played any discernible role during yeast pre-mRNA splicing *in vitro* (24). Fresco and Buratowski found that the precursor and mature forms of *CYH2* RNA present in *ceg1* cells at the restrictive temperature contained poly(A), and that the total cellular poly(A) was normally distributed between the nucleus and cytoplasm (25). The inference is that the cap guanylate is not critical for polyadenylation and transport in yeast.

Here we report that *ceg1-3* and *ceg1-13* cells undergo a rapid and persistent decline in the rate of protein synthesis after shift to the non-permissive temperature, which correlates with a sharp reduction in the steady-state level of all mRNAs examined, be they spliced or unspliced. Transcripts induced at restrictive temperature fail to accumulate in *ceg1-3* and *ceg1-13* cells, but uncapped poly(A)$^+$ mRNAs do accumulate when the 5′ exoribonuclease Xrn1 is absent. We surmise that the conditional lethal *ceg1* phenotype stems primarily from accelerated decay of transcripts made at restrictive temperature. We discuss these findings in light of current models of mRNA turnover in yeast.

MATERIALS AND METHODS

ceg1-ts mutants

The isolation of ts *ceg1* mutants was described previously (23). The wild-type *CEG1* strain and the *ceg1-3* and *ceg1-13* mutant strains used in this study are derivatives of YBS2 (*MAT**a** leu2 lys2 trp1 ceg1::hisG*), a strain in which the chromosomal *CEG1* gene has been deleted and viability is contingent on maintenance of the *CEG1* or *ceg1* alleles on a *CEN TRP1* plasmid (16). Strains deleted for *XRN1* and *CEG1* were constructed by mating RYK1483 (*MAT**a** ura3 leu2 xrn1::LEU2*) with YBS13A [*MAT*α *leu2 trp1 his3 ceg1::hisG* pGYCE-360 (*CEN URA3 CEG1*)]. Leu+ Ura+ diploids were sporulated. A Leu+ haploid strain that could not grow on 5-FOA [*xrn1::LEU2 ceg1::hisG* pGYCE-360 (*CEN URA3 CEG1*)] was transformed with *CEN TRP1* plasmids carrying *CEG1*, *ceg1-3* or *ceg1-13*. Strains that had lost the *CEN URA3 CEG1* plasmid were then selected by growth on 5-FOA.

Temperature shift and measurement of protein synthesis by pulse-labeling

Yeast cells were grown in supplemented minimal medium lacking methionine (SC–Met) at 25°C. One half of each culture was removed and shifted to 37°C by rapid addition of an equal volume of SC–Met medium preheated to 52°C. Incubation was continued thereafter at 37°C. The remaining half of each culture was mixed with an equal volume of SC–Met medium at 25°C. At 0, 20, 40, 60 and 120 min post-shift, aliquots of the cultures (2 ml) were removed, mixed with 8 µCi of [^{35}S]methionine (1175 Ci/mmol; Dupont NEN) and incubated for 10 min at 37 or 25°C. Incorporation of labeled methionine was measured by hot trichloroacetic acid (TCA) precipitation (28). The pulse-labeling was terminated by adding ice-cold 100% TCA to a final concentration of 10%, after which, the mixtures were placed on ice for 10 min and then heated at 70°C for 20 min. The samples were subsequently filtered through glass fiber filters (GF/C; 24 mm diameter, 1.5 µm pore size), which were washed with 20 ml of 5% TCA and with 10 ml of 95% ethanol, and then dried. The bound radioactivity was quantitated by liquid scintillation counting. The data were corrected for the background level of non-specific [^{35}S]methionine retention on the filters, which was determined by pulse-labeling cells that had been preincubated for 30 min at 25°C in cycloheximide (125 µg/ml) prior to adding [^{35}S]methionine.

SDS–PAGE analysis of pulse-labeled polypeptides

Pulse-labeling was performed as described above. After quenching the culture and heating in TCA, the acid-insoluble material was recovered by centrifugation at 13 000 r.p.m. for 20 min at 4°C. The pellets were washed with 1% TCA, followed by three cycles of washing with ethyl ether. The samples were dried in a vacuum centrifuge and then resuspended in SDS–PAGE sample buffer. The samples were heated for 5 min at 95°C, then electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. The gels were dried and autoradiographed.

RNA analysis

Yeast cells were grown in SC–Met medium at 25°C to an A_{600} of 0.2–0.4. After withdrawing an aliquot (time zero), an equal volume of SC–Met medium preheated to 52°C was added to the rest of the culture to adjust the temperature abruptly to 37°C. The cultures were transferred to a 37°C incubator. Aliquots were removed at 1, 2 and 3 h after temperature shift. The cells were harvested by centrifugation and stored as cell pellets at –80°C. RNA was isolated from thawed resuspended cells by extraction with hot phenol (29). The RNA was ethanol-precipitated and resuspended in TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA). RNA concentration was calculated on the basis of A_{260}. Aliquots (25–30 µg of total RNA) of each RNA preparation were mixed with sample buffer containing 50% formamide, 2.2 M formaldehyde, and 10 µg/ml ethidium bromide, then electrophoresed through a formaldehyde–1% agarose gel. The gels were photographed under short wave UV illumination to visualize ethidium bromide-stained RNA. The RNA was then transferred to a Hybond membrane (Amersham). Radiolabeled probes were prepared by using a random priming kit according to the manufacturer's instructions (Boehringer Mannheim). Hybridization was performed as described (29). Hybridized probe was visualized by autoradiography of the membrane. Where indicated, the strength of the hybridization signal was quantitated by scanning the membrane with a FUJIX BAS1000 Bio-imaging Analyzer.

Heat-shock mRNA induction

Yeast cells were grown in SC–Met or YPD media at 25°C. After withdrawing an aliquot (time zero), pre-heated 52°C medium was added to the culture so as to adjust the temperature abruptly to 42°C. The cultures were transferred to a 42°C shaking water-bath incubator. Aliquots were removed at 15, 30 and 60 min after heat shock. RNA isolation and northern blot analysis were performed as described above. Radiolabeled SSA4 and SSA1 probes were prepared with a random priming kit using purified PCR fragments of the *SSA4* and *SSA1* genes as templates. A 338 bp fragment of *SSA1* coding sequence was PCR-amplified from plasmid EC703 with primers 5′-GGAATCTCAAAGAATTGC and

5′-CTTCTTCAACGGTTGGAC. A 359 bp fragment of *SSA4* coding sequence was amplified from plasmid EC702 with primers 5′-GTTCAAGGCCGAAGATGAAC and 5′-CAACCT-CTTCAACCGTTG. EC702 and EC703 were obtained from Dr Charles Cole (Dartmouth Medical School).

RNase protection analysis of poly(A)$^+$ *SSA4* RNA was performed using an RNase Protection Kit according to the manufacturer's instructions (Boehringer). [^{32}P]GMP-labeled antisense RNA probes were synthesized *in vitro* by T7 RNA polymerase from two plasmid templates: (i) pSSA4-5′ containing a 137 bp *SSA4* insert from nucleotides +223 to +359 (+1 being the start of the *SSA4* open reading frame) and (ii) pSSA4-3′ containing a 142 bp *SSA4* insert from nucleotides +1567 to +1708. Total RNA was isolated from *xrn1 CEG1* and *xrn1 ceg1-ts* cells harvested immediately prior to heat shock (time 0) and 15, 30, 45 and 60 min after heat shock. Poly(A)$^+$ RNA was isolated by oligo(dT) affinity chromatography using Dynabeads–Oligo(dT)$_{25}$ according to the manufacturer's instructions (Dynal). Poly(A)$^+$ RNA samples were annealed to a mixture of the 198 nucleotide 5′ *SSA4* probe and the 205 nucleotide 3′ *SSA4* probe. The RNase digests were analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 M urea in TBE. ^{32}P-labeled DNA restriction fragments (pBR322 *Msp*I digest) served as size markers.

Primer extension analysis of 5′ ends

Total RNA (20 µg) from heat-shocked *xrn1 CEG1* and *xrn1 ceg1-ts* cells was incubated in 90% DMSO for 20 min at 45°C, then ethanol-precipitated and resuspended in 30 µl of RT buffer (50 mM Tris–HCl, pH 8.5, 30 mM KCl, 8 mM $MgCl_2$, 1 mM DTT) containing 1 pmol of a 5′ ^{32}P-labeled 18mer DNA oligonucleotide primer (5′-CCAACAGCTTTTGACATG) complementary to the *SSA4* coding sequence from positions –1 to +17. The primer–RNA mixtures were incubated at 55°C for 30 min, then allowed to cool to 42°C. The samples were precipitated with 0.6 vol of isopropanol; the precipitates were washed with 80% ethanol, dried, and then resuspended in 30 µl of RT buffer containing 1 mM dNTPs and 10 U of AMV reverse transcriptase. The primer extension reaction mixtures were incubated for 2 h at 42°C. The samples were extracted with phenol–chloroform, ethanol precipitated, resuspended in formamide and then analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 M urea in TBE. ^{32}P-labeled DNA restriction fragments (pBR322 *Msp*I digest) served as size markers.

RESULTS

Conditional inactivation of *CEG1* inhibits protein synthesis *in vivo*

Yeast mRNA guanylyltransferase is encoded by the essential *CEG1* gene. Although the guanylyltransferase activity of the Ceg1 protein is required for cell viability, the specific roles played by the RNA cap *in vivo* are not fully known. We therefore undertook a phenotypic characterization of two mutants—*ceg1-3* and *ceg1-13*—both of which grow normally at 25°C, but display a tight growth arrest at 37°C. We focused first on protein synthesis, the rationale being that translation is the final step in the expression of protein coding information and that any gross defect in the processing, transport, translation or stability of unguanylated mRNA will be reflected in a decreased rate of protein synthesis.

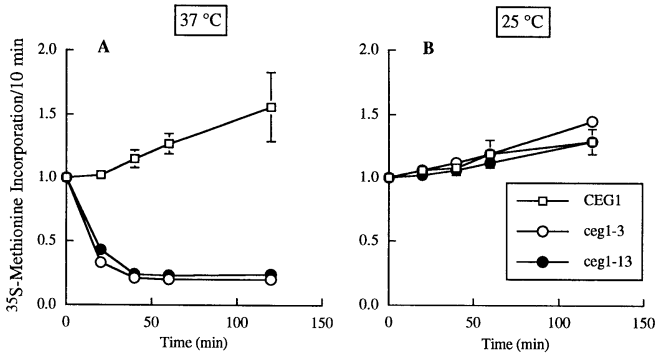

Figure 1. Effect of *ceg1-ts* mutations on protein synthesis. *CEG1*, *ceg1-3* and *ceg1-13* cells were grown at 25°C in synthetic medium lacking methionine. After growth to A_{600} of 0.2–0.4, the cultures were split and either shifted abruptly to 37°C (**A**) or maintained at 25°C (**B**). Aliquots (2 ml) were withdrawn at the times indicated and pulse-labeled for 10 min with [^{35}S]methionine. Incorporation of label into hot-TCA-insoluble material was determined by liquid scintillation counting. The extents of pulse-labeling were normalized to the value at time zero (defined as 1.0). The plotted data represent the average of three independent experiments; standard error bars are shown.

In the experiment shown in Figure 1, yeast cells were grown in methionine-free medium at 25°C. The cultures were then split such that one half was mixed with an equal volume of 25°C medium and maintained thereafter at permissive temperature, whereas the other half of the culture was adjusted immediately to 37°C and maintained thereafter at non-permissive temperature. Aliquots of the 25 and 37°C cultures were exposed to [^{35}S]methionine for a 10 min pulse at the time the cultures were split (time zero) and at various time intervals thereafter. Protein synthesis was quantitated by the incorporation of [^{35}S]methionine into hot-TCA-insoluble material during the 10 min pulse. The level of incorporation at each time point was normalized relative to the incorporation at time zero, and plotted as a function of the time of initiation of the pulse. The data shown in Figure 1 represent the average of three separate experiments (with error bars shown). It can be readily appreciated that the rates of protein synthesis by *CEG1*, *ceg1-3* and *ceg1-13* cells were unaffected by splitting the cultures at 25°C (Fig. 1B). Protein synthesis by the wild-type cells was unperturbed after shift to 37°C, whereas *ceg1-3* and *ceg1-13* cells displayed a sharp drop in the rate of translation (Fig. 1A). By 40 min post-shift, [^{35}S]methionine incorporation was reduced to 20% of the value at time zero. Translation persisted at this low rate for at least 2 h (Fig. 1A).

The distribution of pulse-labeled polypeptides was analyzed by SDS–PAGE. The polypeptide profile was unchanged when wild-type *CEG1* cells were shifted to 37°C (Fig. 2). In contrast, *ceg1-3* and *ceg1-13* cells experienced a progressive and nearly global inhibition of polypeptide synthesis over 2 h at 37°C (Fig. 2).

mRNA levels decline after inactivation of *CEG1*

Is the inhibition of protein synthesis in the *ceg1-ts* mutants at non-permissive temperature a direct effect or merely an indirect consequence of mRNA destabilization? To address this question, RNA was isolated from *CEG1*, *ceg1-3* and *ceg1-13* cells harvested immediately prior to temperature-shift, and at 1, 2 and 3 h after shift from 25 to 37°C. Northern blots were probed for RNAs derived from seven different protein-encoding genes:

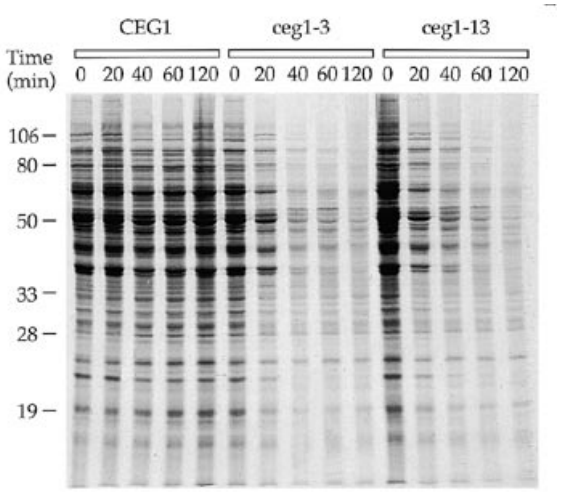

Figure 2. SDS–PAGE analysis of pulse-labeled polypeptides. Pulse-labeling of *CEG1*, *ceg1-3* and *ceg1-13* cells with [^{35}S]methionine was initiated at the indicated times post-shift to 37°C. Acid-insoluble material was denatured in SDS and then electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. An autoradiogram of the dried gel is shown. The positions and sizes (kDa) of co-electrophoresed marker polypeptides are indicated at the left.

PGK1, *CYH2*, *ACT1*, *AKY2*, *HTA1*, *GCN4* and *RP51A*. The steady-state levels of all seven mRNAs were apparently unchanged after wild-type *CEG1* cells were shifted to 37°C (Fig. 3). In contrast, the mRNA levels declined drastically in *ceg1-3* and *ceg1-13* cells within 1 h of transfer to the non-permissive temperature (Fig. 3).

PGK1 mRNA was quantitated by scanning northern blots using a phosphorimager. *PGK1* mRNA in *CEG1* cells at 1, 2 and 3 h post-shift was 108, 98 and 93% of the pre-shift level. In contrast, *PGK1* mRNA in *ceg1-3* cells at 1, 2 and 3 h was 23, 10 and 8% of the pre-shift value. In *ceg1-13* cells, the *PGK1* transcript decreased to 20, 8 and 4% of the initial value after 1, 2 and 3 h at 37°C. (The values cited are the averages of two separate temperature-shift experiments.) Comparable declines occurred in the other messages we analyzed. In *ceg1-3* cells, the levels of specific mRNAs at 3 h post-shift (expressed as the percent of the pre-shift value) were as follows: *AKY2* + *HTA1* (18%); *GCN4* (19%); mature *CYH2* (13%); *ACT1* (4%) and *RP51A* (16%). In *ceg1-13* cells, the 3 h mRNA levels (as percent of pre-shift values) were as follows: *AKY2* + *HTA1* (14%); *GCN4* (3%); mature *CYH2* (15%); *ACT1* (2%) and *RP51A* (5%).

Effects of *CEG1* inactivation on pre-mRNA splicing

We reported previously that two other ts *ceg1* alleles (*Y66A* and *C354Y*) conferred a pre-mRNA processing (*prp*) phenotype, characterized by an increase in the amount of unspliced pre-mRNA after shift to non-permissive temperature and a decrease in the amount of mature mRNA (24). Similar findings were made by Fresco and Buratowski (25) for three *ceg1-ts* alleles, one of which was *C354Y*. However, other *ceg1-ts* mutants in their and our collections did not demonstrate an increase in unspliced precursor at the restrictive temperature. This was the case for the two tight alleles *ceg1-3* and *ceg1-13* analyzed presently (Fig. 3). Low levels of unspliced *CYH2* RNA were detectable by northern analysis in *CEG1* cells; abundance of

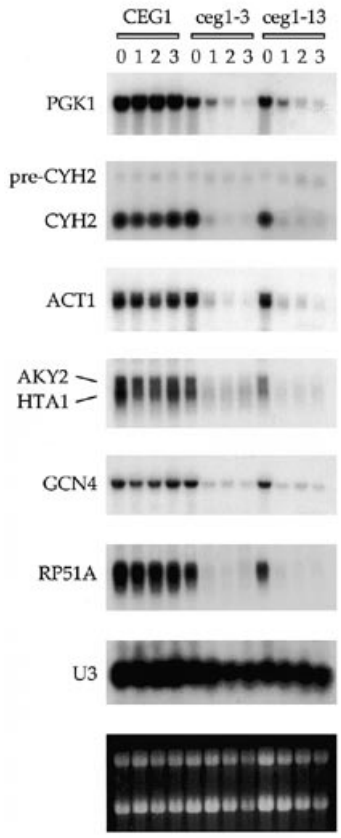

Figure 3. Effect of *ceg1-ts* mutations on steady-state RNA levels. *CEG1*, *ceg1-3* and *ceg1-13* cells were grown in liquid culture at 25°C, then shifted to 37°C. Cells from the same culture were harvested prior to shift (time 0) and at 1, 2 and 3 h after shift to 37°C. Total RNA was isolated for northern blot analysis. The agarose gel was photographed with short wave UV illumination (bottom panel) prior to transfer of the RNA to a membrane. Blots were probed for mRNAs derived from *PGK1*, *CYH2*, *ACT1*, *AKY2*, *HTA1*, *GCN4* and *RP51A* genes and for U3 snRNA. Hybridized ^{32}P-labeled probe was visualized by autoradiography of the membrane.

pre-*CYH2* did not vary after the shift to 37°C. In *ceg1-3* cells, the levels of pre-*CYH2* RNA remained constant for 3 h post-shift, despite the fact that the amount of mature *CYH2* RNA deceased by nearly an order of magnitude (Fig. 3). A similar pattern was observed for *CYH2* transcripts in *ceg1-13*. These results are not consistent with simple inhibition of splicing. Transcripts of the *ACT1* and *RP51A* genes are also spliced in yeast; we detected no increase in the unspliced precursors to these mRNAs in *ceg1-3* or *ceg1-13* cells after shift to 37°C. These findings argue that mutational effects on splicing are not a direct cause of the *ceg1-ts* growth phenotype. Rather, it appears that global mRNA destabilization is the likely culprit.

Effect of *CEG1* inactivation on U3 RNA levels

The U1, U2, U3, U4 and U5 snRNAs are synthesized by yeast RNA polymerase II, but the mature snRNAs contain a 5′ trimethylated cap structure, m2,2,7GpppN. Amphibian and human snRNAs cotranscriptionally acquire a standard m7G cap, which is subsequently methylated by an AdoMet-dependent methyltransferase specific for snRNPs (30,31). U1, U2, U4 and U5 snRNAs are trimethylated in the cytoplasm and then

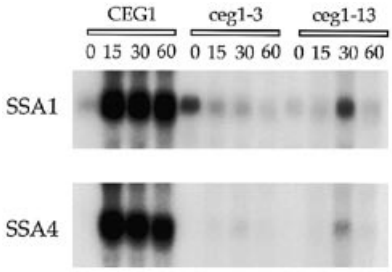

Figure 4. Effect of *ceg1-ts* mutations on the accumulation of *SSA1* and *SSA4* heat-shock mRNAs. *CEG1*, *ceg1-3* and *ceg1-13* cells grown in SC–Met at 25°C were shifted abruptly to 42°C. Cells from the same culture were harvested prior to shift (time 0) and at 15, 30 and 60 min after shift to 42°C. RNA was isolated for northern blot analysis. The membrane was probed for *SSA4* and *SSA1* transcripts. Hybridized ^{32}P-labeled probe was visualized by autoradiography.

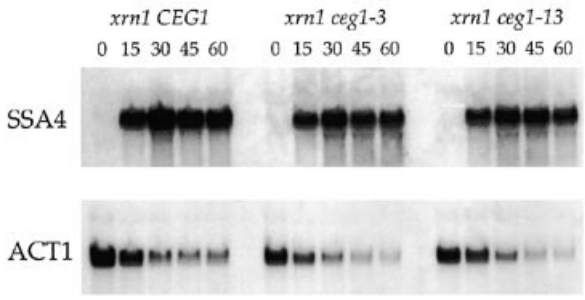

Figure 5. *XRN1* deletion ameliorates the effect of *ceg1* mutations on heat-shock induction of *SSA4*. *xrn1 CEG1*, *xrn1 ceg1-3* and *xrn1 ceg1-13* cells grown at 25°C were shifted abruptly to 42°C. Cells from the same culture were harvested prior to shift (time 0) and at 15, 30, 45 and 60 min after shift to 42°C. RNA was isolated for northern blot analysis. The membrane was probed for *SSA4* and *ACT1* transcripts. Hybridized ^{32}P-labeled probe was visualized by autoradiography.

transported back into the nucleus, whereas U3 snRNA is retained in the nucleus and undergoes trimethylation there (32). The pathway of U snRNA capping in yeast has not been defined; however, we presume that the same enzymes that cap and N-7 methylate mRNA also form the initial m7G cap on snRNAs. We found previously that the steady-state levels of U1, U4 and U5 snRNAs remained essentially constant for up to 4 h after *ceg1-ts* mutants *Y66A* and *C354Y* were shifted to 37°C (24).

In the present study of *ts* alleles *ceg1-3* and *ceg1-13*, we extended this analysis to U3 snRNA. *Saccharomyces cerevisiae* has two U3 genes (*SNR17A* and *SNR17B*), both of which contain mRNA-type introns (33). U3 snRNA levels did not change when wild-type *CEG1* cells were shifted from 25 to 37°C. In *ceg1-3* cells, the U3 level was unperturbed at 1 h post-shift, but declined by 3 h post-shift to 50% of the pre-shift level (Fig. 3). In *ceg1-13* cells, U3 levels were fairly stable over a 3 h after shift to 37°C (Fig. 3). We conclude that inactivation of Ceg1 elicited a specific decline in the steady-state level of mRNAs, which have half-lives on the order of 5–45 min in yeast (29), and had only a modest effect on the levels of snRNAs, which are extremely stable *in vivo*.

We propose that the decrease in mRNA levels post-shift *versus* pre-shift is the sequela of: (i) normal turnover of capped mRNAs that had been synthesized pre-shift and (ii) accelerated decay of uncapped transcripts synthesized after inactivation of Ceg1 at 37°C.

Effect of *ceg1* mutations on newly transcribed mRNAs

In order to focus on transcripts that were synthesized at the non-permissive temperature, we sought to simultaneously inactivate Ceg1 and to turn on transcription of previously quiescent genes. This was accomplished by abruptly shifting *ceg1* cells from 25 to 42°C rather than 37°C. Eukaryotic cells subjected to 42°C heat shock respond by rapidly redirecting their synthetic machinery to produce a set of evolutionarily conserved heat-shock proteins (34,35). We examined the induction of heat-shock mRNAs derived from *SSA1* and *SSA4* genes. *SSA1* and *SSA4* encode Hsp70 proteins. They display 82% identity at the amino acid level and 67% identity at the nucleotide level (36). To discriminate the transcripts derived from the *SSA1* and *SSA4* genes, we PCR-amplified 338 and 359 bp fragments encoding non-conserved carboxyl regions of the Ssa1 (amino acids 529–640) and Ssa4 (amino acids 523–641) proteins, respectively. These fragments were used as templates to prepare probes for northern analysis. The *SSA1* and *SSA4* probes did not cross-hybridize (not shown).

The *SSA4* transcript was undetectable in *CEG1* cells at 25°C, but accumulated to high levels 15–60 min after the shift to 42°C (Fig. 4). The amounts of *SSA4* mRNA detected in *ceg1-3* cells at 15, 30 and 60 min after heat shock were 2–4% of the wild-type levels (Fig. 4). *SSA4* mRNA levels in *ceg1-13* cells at 15, 30 and 60 min after heat shock were 3, 17 and 7% of the respective wild-type values.

SSA1 is 96% identical at the nucleotide level to *SSA2*. Hence, the *SSA1* probe detects both *SSA1* and *SSA2* mRNAs. *SSA1* is expressed under non-stress conditions, but is induced after heat-shock, whereas *SSA2* is expressed constitutively (37). We observed that *SSA1* mRNA in *CEG1* cells was increased 9-fold at 15 min after heat-shock, and remained at this level up to 60 min post-shift (Fig. 4). There was no increase in *SSA1* mRNA in heat-shocked *ceg1-3* cells above the basal level; indeed, the *SSA1* mRNA level decreased after heat shock. In *ceg1-13* cells, a transient increase in *SSA1* mRNA was observed at 30 min, which was severely attenuated with respect to the wild-type response. We surmise that inactivation of Ceg1 prevents the accumulation of newly synthesized Hsp70 transcripts.

Effect of *XRN1* deletion on the *ceg1* RNA phenotype

The yeast protein Xrn1 is a 5′ exoribonuclease that hydrolyzes uncapped RNAs to 5′ nucleoside monophosphates. Capped RNAs are protected from digestion (38). Evidence points to Xrn1 as the enzyme responsible for exonucleolytic digestion of yeast mRNAs that have undergone enzymatic decapping *in vivo* (39–41). We reasoned that if the failure to mount an effective transcriptional response to heat shock were caused by rapid turnover of newly synthesized transcripts lacking a blocking cap guanylate (as opposed to some direct effect of Ceg1 inactivation on transcription), then the RNA phenotype might be corrected by disruption of the *XRN1* gene.

We observed that the effects of *ceg1* mutations on the heat-shock mRNA response were indeed ameliorated in cells lacking Xrn1. Abrupt shift from 25 to 42°C elicited a strong accumulation of *SSA4* mRNA in *xrn1 ceg1* cells at 15–60 min post-shift, i.e. to levels that were 50–60% of those in heat-shocked *xrn1 CEG1* cells (Fig. 5). As a control, we probed the same blot for actin mRNA. The *ACT1* transcript was present prior

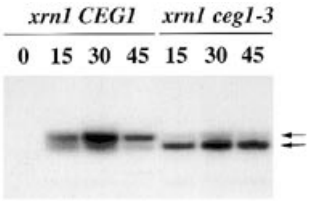

Figure 6. Primer extension analysis suggests that *SSA4* transcripts induced in *xrn1 ceg1* cell are uncapped. *xrn1 CEG1* and *xrn1 ceg1-3* cells grown at 25°C were shifted abruptly to 42°C. Cells were harvested immediately prior to (time 0) and 15, 30 and 45 min after heat shock, as noted above the lanes. Primer extension reaction products were analyzed by PAGE. The cDNAs synthesized on RNA template from *xrn1 CEG1* cells were 1 nt longer than the cDNA made on RNA from *xrn1 ceg1-3* cells (arrows at right).

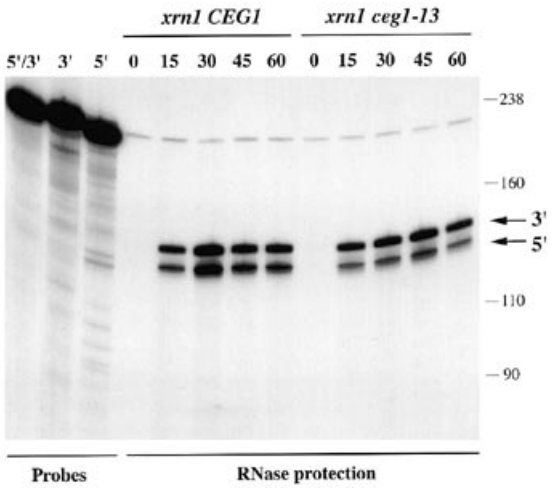

Figure 7. *SSA4* transcripts induced in *xrn1 ceg1* cells contain poly(A). Cells grown at 25°C were shifted abruptly to 42°C. Aliquots from the same culture were harvested prior to shift (time 0) and at 15, 30, 45 and 60 min after shift to 42°C, as specified above the lanes. Poly(A)$^+$ RNA was isolated for RNase protection analysis. RNase digests and aliquots of the antisense probes were resolved by PAGE. An autoradiogram of the gel is shown. The leftmost lane contains an aliquot of the probe mixture used in the protection assays (5′/3′); the flanking two lanes contain the 3′ probe alone and the 5′ probe alone. RNase-resistant fragments of the 3′ and 5′ probes protected from digestion by *SSA4* RNA are denoted by arrows to the right of the gel. The positions and sizes (nt) of co-electrophoresed DNA markers are also indicated on the right.

to heat shock, but its level declined significantly in *CEG1* and *ceg1* cells after the shift to 42°C. (The decrease in *ACT1* mRNA was also observed in heat-shocked *XRN1* cells; not shown.) Disappearance of pre-existing yeast transcripts after heat shock was noted previously and attributed to reduced transcription of the mRNAs during the stress response (42). Unlike Yost and Lindquist (42), we did not detect an increase in unspliced actin pre-mRNA after heat shock (Fig. 5). (This was also the case in heat-shocked *XRN1* cells.) The reasons for this difference are unclear, but may be a function of different strain backgrounds.

xrn1 ceg1 cells accumulate uncapped poly(A)$^+$ mRNA

The cap status of *SSA4* mRNA synthesized in *xrn1 CEG1* and *xrn1 ceg1* cells was analyzed by primer extension. Davison and Moss showed that the presence of a 5′ cap promotes the addition of an extra 3′ nucleotide during cDNA synthesis by reverse transcriptase; hence, primer extension products formed on otherwise identical capped *versus* uncapped transcripts differ in size by one nucleotide (58). In the experiment shown in Figure 6, a ^{32}P-labeled 18mer oligonucleotide complementary to the translation start site of *SSA4* mRNA was annealed to total RNA isolated from *xrn1 CEG1* cells and extended with reverse transcriptase. No primer extension product was made in reactions containing RNA from cells harvested prior to heat shock. However, a discrete cDNA product with an apparent chain length of 73 nucleotides (nt) was synthesized on template RNA isolated from heat-shocked cells (Fig. 6). Trace levels of a second extension product, shorter by one nucleotide step, were also formed. This experiment maps the 5′ end of the *SSA4* transcript to the same position reported by Boorstein and Craig (36). The salient finding was that the cDNA formed on RNA isolated from heat-shocked *xrn1 ceg1-3* cells was 1 nt shorter (lower arrow in Fig. 6). The most reasonable and likely explanation for this difference is that *SSA4* mRNA synthesized in *ceg1* cells at the non-permissive temperature lacks a 5′ cap. The observation that virtually all of the *SSA4* mRNA present 15 min after shift to restrictive temperature is uncapped attests to the rapid inactivation of the mutant Ceg1-3 protein *in vivo*. *xrn1 ceg1-13* cells also accumulated predominantly uncapped *SSA4* transcripts at 15 min post-heat-shock (not shown).

We prepared poly(A)$^+$ RNA from *xrn1 CEG1* and *xrn1 ceg1-13* cells harvested prior to, and 15, 30, 45 and 60 min after, heat shock. The presence of *SSA4* transcripts in the poly(A)$^+$ RNA fraction was then assayed by RNase protection using a mixture of a radiolabeled 198 nt probe complementary to a 5′ segment of the *SSA4* mRNA and a 205 nt probe complementary to a 3′ segment (Fig. 7). No RNase-resistant fragments were observed prior to heat shock. However, poly(A)$^+$ RNA from heat-shocked *xrn1 CEG1* cells protected two fragments: an ~142 nt fragment from the 3′ probe and an ~137 nt species derived from the 5′ probe (Fig. 7). The sizes of the protected fragments were consistent with the length of the antisense portions of the probes. [The assignment of the 5′ and 3′ protected fragments was confirmed by control RNase protection assays in which poly(A)$^+$ RNA was annealed separately to each probe rather than to a mixture of the probes.] Poly(A)$^+$ *SSA4* transcripts were induced in *xrn1 ceg1-13* cells to nearly the same levels as in *xrn1 CEG1* cells (Fig. 7). Similar results were obtained with poly(A)$^+$ RNA from heat-shocked *xrn1 ceg1-3* cells (not shown).

Elimination of Xrn1 does not bypass the requirement for Ceg1 for cell growth

We surmise from the suppressive effects of *xrn1* deletion on the *ceg1* RNA phenotype that the mRNA decrement accompanying Ceg1 inactivation is the result of enhanced turnover of newly made uncapped transcripts, which is mediated, in a large part, by Xrn1. Although disruption of *XRN1* is not lethal, *xrn1* cells are slow-growing and accumulate mRNA and rRNA processing intermediates that are normally not found in wild-type cells (39,40,43). We reasoned that if the cap structure served only to protect mRNAs from digestion by Xrn1, then an *xrn1* gene disruption might suppress the lethal phenotype of a *ceg1* null mutation. We found that a Δ*xrn1* Δ*ceg1* strain carrying a *CEN URA3 CEG1* plasmid could not grow on 5-FOA. We conclude that the lethality of Δ*ceg1* mutants is not Xrn1-dependent.

To test if *xrn1* deletion would mitigate the ts growth phenotype of the *ceg1* mutants, the Δ*xrn1 ceg1* and Δ*xrn1 CEG1* strains were tested for growth at 25, 30, 34 and 37°C in parallel with *XRN1* strains. We observed that Δ*xrn1 CEG1* cells were unable to from colonies at 37°C, i.e. the *xrn1* deletion was *ts* itself in this strain background (not shown). Moreover, the growth of *ceg1-3* and *ceg1-13* cells at semi-permissive temperatures was slowed rather than improved by deletion of *xrn1* (not shown), suggesting that: (i) the pleiotropic negative effects of *xrn1* disruption overrode any potential salutary effects on the stability of uncapped mRNA in *ceg1-ts* cells and/or (ii) stabilized mRNAs lacking a cap guanylate and a methyl group were still not capable of supporting cell growth.

DISCUSSION

This study provides genetic evidence that the cap structure protects mRNA from untimely degradation. Current models of mRNA decay in yeast posit that 3′ deadenylation precedes enzymatic removal of the cap, which then exposes the naked end to 5′ exonuclease action (40,41). mRNA can also be degraded without prior deadenylation; this occurs during accelerated turnover of mRNAs containing premature translation stop codons (nonsense-mediated decay) and in yeast *pab1* strains bearing mutations in the poly(A) binding protein (44–46). In these cases, too, it is envisioned that decapping precedes RNA degradation. Consistent with this idea, it has been found that mutations which inactivate a component of the yeast decapping enzyme result in an increase in mRNA half-life (47).

Our studies suggest that the deadenylation–decapping pathway is short-circuited when the yeast RNA guanylyltransferase Ceg1 is genetically inactivated. Steady-state mRNA levels drop precipitously after shift of *ceg1-ts* mutants to the restrictive temperature. As mentioned above, we presume that this reflects the combined effects of the 'normal' decay of capped transcripts made prior to the shift plus accelerated exonucleolytic degradation of uncapped mRNAs synthesized after the shift. We believe that the RNA phenotype of *ceg1* cells is not caused by a cessation of transcription by RNA polymerase II, because *xrn1 ceg1* cells were capable of synthesizing *SSA4* mRNA from a regulated promoter that was quiescent at permissive temperature and activated at the restrictive temperature. Primer extension analysis indicates that transcription initiation occurs accurately in *xrn1 ceg1* cells at restrictive temperature.

The steady-state levels of Hsp70 mRNAs were very low in heat-shocked *ceg1* cells. This and other technical obstacles make it difficult to measure Hsp70 mRNA half-life in *ceg1* cells using standard methodologies (41) because: (i) the strategy of following mRNA decay by measuring steady-state transcript levels after arresting transcription by a ts version of RNA polymerase II is obviously not suitable for analysis of RNA metabolism under restrictive conditions in ts *ceg1* mutants and (ii) the approach of following RNA decay after addition of transcription inhibitors (thiolutin or 1,10 phenanthroline) is not applicable to studying heat-shock RNAs, because both drugs actually induce, rather than block, the heat-shock response (48). The finding that Hsp70 RNA levels were restored in *xrn1 ceg1* cells implicates Xrn1 in the degradation of newly transcribed mRNAs that never received a 5′ cap. Xrn1 is regarded as the principal enzyme responsible for exonucleolytic digestion of cytoplasmic mRNAs that have undergone deadenylation and enzymatic decapping (39–41). Xrn1 constitutes 0.2–0.3% of the total soluble protein in yeast (49). At least 90% of the Xrn1 is localized in the cytoplasm (49) and its action *in vivo* is believed to be restricted to the cytoplasmic compartment (50). Hsp70 transcripts are selectively transported and accumulate in the cytoplasm after heat shock (51). Hence, we suspect that Hsp70 transcripts synthesized in *ceg1* cells at restrictive temperature are turned over by Xrn1 in the cytoplasm. This does not exclude a contribution of nuclear RNA decay to the *ceg1* RNA phenotype (discussed below). Future experiments to address this question will be contingent on the trapping of uncapped Hsp70 mRNA degradation intermediates, e.g. by inserting a stable secondary structure that arrests exonuclease action (41) and localizing the decay intermediates within the cell.

Cap function *in vivo* is clearly not limited to the protection of mRNAs from digestion by Xrn1, insofar as an *xrn1* gene disruption did not suppress the lethal phenotype of a *ceg1* deletion. Uncapped mRNAs that are stable in the *xrn1* background would presumably not be translated efficiently enough to sustain cell growth. Uncapped poly(A)$^+$ mRNA is translated ~15% as well as capped poly(A)$^+$ mRNA in yeast extracts (52).

The steady-state level of Hsp70 mRNA in heat-shocked *xrn1 ceg1* cells was 50–60% of the level in *xrn1 CEG1* cells. Thus, Xrn1 might not be the only nuclease contributing to the *ceg1* RNA phenotype. Yeast cells contain a second 5′ exoribonuclease, Rat1, which is structurally related to Xrn1 (38,53,54). Because the *RAT1* gene is essential (unlike *XRN1*) (53,54), its contribution to the *ceg1* phenotype cannot be tested by simple gene disruption in a *ceg1* background. Rat1 protein localizes to the nucleus *in vivo* and its function is normally restricted to the nuclear compartment (50). A conditional *rat1* mutant is not complemented by *XRN1* overexpression (54), yet it can be rescued when the Xrn1 protein is targeted artificially to the nucleus (50). Conversely, disruption of the Rat1 nuclear localization signal allows cytoplasmic Rat1 to rescue the pleiotropic phenotypes resulting from deletion of *XRN1* (50). A conditional *rat1* mutant exhibits a 5.8S ribosomal RNA processing defect and accumulates poly(A) in the nucleus (53). It is not known if Rat1 plays any role in nuclear mRNA decay.

Studies of *ceg1* conditional mutants presented here and by Fresco and Buratowski (25) underscore that polyadenylation in *S.cerevisiae* is not contingent on capping of the nascent pre-mRNA. Donahue and colleagues recently arrived at the same conclusion by showing that yeast *HIS4* mRNA is accurately polyadenylated, but apparently not capped, when it is transcribed in an *xrn1* strain by RNA polymerase I instead of RNA polymerase II (57).

Previous studies indicated that some *ceg1* alleles conferred a pre-mRNA processing phenotype at the restrictive temperature, seen as an increase in the level of unspliced pre-mRNAs and a decrease in the abundance of mature spliced RNAs (24,25). Because uncapped transcripts were detected in the precursor and mature species, it was surmised that the cap cannot be required for yeast splicing *in vivo* (25). We found that the cap structure also plays no discernible role in yeast pre-mRNA splicing *in vitro* (24). Based on the *ceg1* phenotype noted previously, it was suggested that the cap enhances splicing *in vivo* (24,25). The identification of a subunit of the nuclear cap binding complex (Mud13/CBP20) in a genetic screen for synthetic lethality with a viable U1 snRNA mutation lends further support to a connection between the cap and splicing in yeast (55). The fact that Mud13/CBP20 is non-essential for growth (55) underscores the point that the cap and cap-binding complex may enhance, but are not required for, yeast splicing *in vivo*.

The present findings suggest that the conditional lethality of *ceg1-3* and *ceg1-13* is not caused by defective pre-mRNA splicing *per se*, but rather by global decline in mRNA levels and a resulting decrease in protein synthesis. We noted that *ceg1-3* and *ceg1-13* cells did not exhibit a general increase in the steady-state level of unspliced precursors; however, they did show an increased ratio of pre-*CYH2* to mature *CYH2* after shift to restrictive temperature. The level of pre-*CYH2* remained unchanged even after 3 h at 37°C. This could result if a modest reduction in the efficiency of splicing of uncapped pre-*CYH2* RNAs at 37°C is offset by a modestly increased rate of pre-*CYH2* decay. Mature *CYH2* declines precipitously in *ceg1-3* and *ceg1-13* cells, presumably because uncapped mature *CYH2* is degraded by cytoplasmic Xrn1.

In summary, our findings provide genetic evidence that the cap protects mRNA *in vivo* from untimely decay catalyzed by Xrn1. In the absence of Xrn1, *ceg1* cells accumulate accurately initiated uncapped transcripts that contain poly(A). A positive role for the cap in yeast pre-mRNA splicing remains plausible. Additional roles for the cap in yeast RNA metabolism may be revealed by genetic analysis of the other essential enzymatic components of the capping apparatus—the methyltransferase Abd1 and the triphosphatase Cet1.

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