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Multicopy Suppressors of Temperature-Sensitive Mutations of Yeast mRNA Capping Enzyme

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We have isolated three *Saccharomyces cerevisiae* genes—*CES1*, *CES2*, and *CES3*—that, when present in high copy, suppress the *ts* growth defect caused by mutations in the *CEG1* gene encoding mRNA guanylyltransferase (capping enzyme). Molecular characterization of the capping enzyme supressor genes reveals the following. *CES2* is identical to *ESP1*, a gene required for proper nuclear division. We show by deletion analysis that the 1573-amino acid ESP1 polypeptide is composed of distinct functional domains. The C-terminal portion of ESP1 is essential for cell growth, but dispensable for *CES2* activity. The N-terminal half of ESP1, which is sufficient for *CES2* function, displays local sequence similarity to the small subunit of the vaccinia virus RNA capping enzyme. This suggests a basis for suppression by physical or functional interaction between the CES2 domain of ESP1 and the yeast guanylyltransferase. *CES1* encodes a novel hydrophilic 915-amino acid protein. The amino acid sequence of CES1 is uninformative, except for its extensive similarity to another yeast gene product of unknown function. The *CES1* homologue (designated *CES4*) is also a multicopy suppressor of capping enzyme *ts* mutations. Neither *CES1* nor *CES4* is essential for cell growth, and a double deletion mutant is viable. *CES3* corresponds to *BUD5*, which encodes a putative guanine nucleotide exchange factor. We hypothesize that *CES1*, *CES4*, and *BUD5* may impact on RNA transactions downstream of cap synthesis that are cap dependent in vivo.

Yeast mRNA capping enzymes Multicopy suppressor Temperature-sensitive mutations

THE 5′ cap structure of eukaryotic mRNA consists of 7-methylguanosine linked to the end of the transcript via a 5′-5′ triphosphate bridge. Capping occurs by a series of three enzymatic reactions in which the 5′ triphosphate end of a primary transcript is hydrolyzed to a 5′ diphosphate by RNA triphosphatase, then capped with GMP by RNA guanylyltransferase, and methylated at N7 of guanine by RNA (guanine-7-)-methyltransferase (23). Although the importance of the cap in mRNA function has been well documented in vitro (5,7,11,17), there is relatively little genetic evidence regarding specific roles for the cap in vivo (i.e., which RNA transactions are affected when cap synthesis is blocked). The most straightforward way to address this issue is to genetically inactivate the enzymes involved in cap synthesis. This is feasible in the budding yeast *Saccharomyces cerevisiae* because the capping and methylating enzymes have been purified and the genes encoding them have been cloned (14,22).

Yeast mRNA guanylyltransferase (capping enzyme) is a 52-kDa protein encoded by the *CEG1* gene (22). The yeast capping reaction resembles that of the vaccinia virus guanylyltransferase. In both cases, GTP reacts with the enzyme to form a covalent enzyme-GMP intermediate with concomitant release of pyrophosphate. The enzyme then donates the GMP to a 5′ diphosphate-terminated RNA (23). Mechanistic similarities be-

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tween the cellular and viral capping enzymes are echoed at the structural level by regional amino acid sequence conservation (4,24,26). Sequence homology has guided structure-function analyses of the yeast capping enzyme by targeted mutagenesis (6,21,24). Through this approach, the guanylyltransferase activity of CEG1 was shown to be essential for yeast cell growth (i.e., mutations of *CEG1* that eliminated enzyme activity in vitro were invariably lethal in vivo).

In this article, we describe the isolation of a new collection of conditional lethal (*ts*) capping enzyme mutants and the use of this collection to identify multicopy suppressors of the *ceg1-ts* growth defect, the rationale being that such genes might encode proteins that either interact with CEG1 or impact on cap-dependent transactions in vivo. Three capping enzyme suppressor genes (*CES1, CES2, CES3*) were isolated by genetic selection from a multicopy genomic library. A fourth supressor gene, *CES4*, was identified by homology to *CES1*. A molecular characterization of the *CES* genes is presented.

MATERIALS AND METHODS

PCR Mutagenesis and Selection of ceg1-ts *Alleles*

The 1.4-kbp *CEG1* gene was amplified in vitro by Taq DNA polymerase using reaction conditions that promote nucleotide misincorporation (12). The standard PCR reaction mixture was modified to contain 0.5 mM $MnCl_2$ and a reduced concentration of dATP (0.2 mM) relative to the other three dNTPs (each at 1 mM). The PCR product was digested with *Eco*RI (which cleaves at nucleotide position +102 relative to the ATG translation initiation codon) and *Xho*I (which cleaves at +1189). The mutagenized *CEG1* DNA fragment was ligated into pGYCE-358 (*CEN, TRP1, CEG1*) that had been digested with *Eco*RI and *Xho*I and gel purified to separate the vector from the excised wild-type *CEG1* sequence. The ligation mixture was transformed into *E. coli*. A pooled plasmid library was prepared from approximately 10,000 ampicillin-resistant colonies harvested directly from the agar plates. This DNA library was transformed into YBS2 (MATa, *leu2, lys2, trp1, ceg1::hisG*, pGYCE-360), a yeast strain in which the chromosomal *CEG1* gene had been deleted (21). Growth of YBS2 depends on an extrachromosomal copy of *CEG1* on a *CEN, URA3* plasmid (pGYCE-360). Trp+ transformants (1500) were selected and replica-plated at 25°C on medium containing 5-FOA. Five percent of the isolates were unable to grow on 5-FOA. The surviving isolates, which had lost the *CEG1, URA3* plasmid, were replica-plated at 25°C (permissive temperature) and 37°C (nonpermissive temperature). Plasmid DNA isolated from 19 candidate *ts* mutants was amplified in vivo in *E. coli* and retested by plasmid shuffle for the *ts* growth phenotype in YBS2. The *ts* mutations of nine *ceg1-ts* plasmids were localized crudely by subcloning *Eco*RI/*Bam*HI (nucleotides 102–804) and *Bam*HI/*Xho*I (804–1189) fragments of the *ceg1-ts* genes back into pGYCE-358 to replace the corresponding fragment of the wild-type gene with that of the *ts* gene. These plasmids were tested for *ts* function by plasmid shuffle. After determining which gene fragment conferred the *ts* phenotype, the relevant segment of each of the nine *ceg-ts* plasmids was sequenced to identify the molecular lesions.

Isolation of Multicopy Suppressors of ceg1-ts *Mutations*

Isogenic strains containing different *ceg1-ts* alleles on a *CEN, TRP1* plasmid were transformed with a *2μ-URA3* genomic DNA library. An aliquot of the transformed cells was plated on Ura medium at 25°C to gauge transformation efficiency. The rest of the cells (about 25,000 transformants) were plated on Ura medium at 37°C. For each *ceg1-ts* mutant, we picked 10 colonies that grew at 37°C. Yeast plasmid DNA was isolated and transformed into *E. coli*. Plasmids were prepared from cultures of individual ampicillin-resistant transformants. The DNAs were digested with *Eco*RI and *Xho*I to weed out *2μ* plasmids containing a genomic insert of the wild-type *CEG1* gene. Candidate suppressor clones were retested by transformation into the *ceg1-ts* strain in which they were originally selected. Three capping enzyme suppressor genes—*CES1, CES2,* and *CES3*—were identified in this screen.

Cloning and Deletion Analysis of CES2

Limited sequencing of the 9.5-kbp insert of the *2μ-CES2* isolate indicated that it contained the *ESP1* gene (16). A subclone containing only *ESP1* was constructed by excising a 5.6-kbp DNA fragment obtained by partial restriction endonuclease digestion of the *CES2* clone with *Hin*dIII and *Eco*RI. This restriction fragment, which contained the entire *ESP1* coding region plus 416 bp upstream of the start codon and 500 bp downstream of the stop codon (16), was inserted into pBluescript KS+ to yield pKS-ESP1. The *ESP1* insert

was excised and inserted into YEP24 to yield pESP1. The C-terminal deletion clone pESP1(1-1033) was constructed by excising a 2121-bp *Spe*I restriction fragment from pKS-ESP1, and then cloning the truncated insert into YEP24. Deletion clone pESP1(1-887) was made by excising a 3071-bp *Hin*dIII fragment of pKS-ESP1 (containing *ESP1* sequence from −307 to +2655), cloning it back into pBluescript, and then transferring the insert into YEP24. Deletion clone pESP1(1-515) was made by deleting an internal *Bam*HI fragment from pESP1. The deletion clones are numbered according to the amino acid coordinates of the truncated ESP1 proteins they encode.

Gene Disruptions

The *CES1* gene was disrupted by insertion of a *hisG-URA3-hisG* cassette (1). We first constructed plasmid pΔces1, in which the *hisG-URA3-hisG* casssete was flanked by a 500-bp PCR fragment derived from the 5′ end of the *CES1* gene and a 1058-bp XbaI/SpeI fragment from the 3′ portion of the gene. Linearized pΔces1 was transformed into the diploid strain YPH274 (MATa/MATα, *trp1Δ1/trp1Δ1*, *his3Δ200/his3Δ200*, *ura3-52/ura3-52*, *ade2-101/ade2-101*, *lys2-801/lys2-801*, *leu2-Δ1/leu2-Δ1*). Insertion of the *hisG-URA3-hisG* cassette at one *CES1* locus resulted in deletion of amino acids 13-712 of the CES1 protein; correct insertion was confirmed by Southern blotting of DNA from Ura+ transformants. The diploids were then sporulated. All four spores of each tetrad were viable and *URA3* segregated 2:2. *CES1* disuption was also performed in a separate experiment by introduction of pΔces1 into the haploid strain TR2 (MATa>, *trp1*, *his3*, *ura3*, *ade2*, *lys2*). Correct insertion into the *CES1* locus was confirmed by Southern blotting.

The entire *CES4* gene was deleted by replacing the sequence from nucleotides −13 to +2318 (relative to the ATG intiation codon) with the *hisG-URA3-hisG* cassette. To do this, we constructed plasmid pΔces4, in which the *hisG-URA3-hisG* casssete was flanked by a 929-bp *Eco*RI/*Dra*I 5′ of *CES4* and a PCR-amplified 1-kbp fragment beginning downstream of the *CES4* stop codon. Linearized pΔces4 DNA was transformed into strain YPH399 (MATa, *trp1*Δ1, *his3Δ200*, *ura3-52*, *ade2-101*, *lys2-801*, *leu2-Δ1*) and into strain YBS10 (MATa, *trp1*, *his3*, *ura3*, *ade2*, *lys2*, *ces1::hisG*) in which the chromosomal copy of *CES1* had been deleted (see above). Correct insertions were confirmed by Southern blotting.

ESP1 was disrupted by insertion of a *LEU2* marker. To do this we constructed plasmid pΔesp1 in which a 1673-bp *Eco*RV fragment of *ESP1* from nucleotide positions +414 to + 2087 (relative to the translation initiation codon) was replaced by a 2.2-kbp DNA fragment containing the *LEU2* gene. Linearized pΔesp1 was introduced into the diploid strain YPH274. Corrected insertion was confirmed by Southern blot analysis of DNA from Leu+ transformants.

RESULTS

Isolation of Temperature-Sensitive ceg1 *Mutants*

PCR amplification of the *CEG1* gene was performed using reaction conditions that promote nucleotide misincorporation (12). The PCR products were cloned into a *CEN, TRP1, CEG1* plasmid, to replace the wild-type *CEG1* gene fragment with the PCR-mutagenized DNA. After amplification in vivo in *E. coli*, a pooled plasmid library was transformed into a yeast *Δceg1* null strain containing wild-type *CEG1* on a *URA3* plasmid. Selection for Trp+ growth at 25°C was followed by counterselection on 5-FOA to eliminate the wild-type allele. Survivors were screened for growth at 37°C, defined hereafter as the nonpermissive condition. Nine *ceg1-ts* isolates that formed colonies at 25°C, but not at 37°C, were selected for further analysis. Plasmid DNA was recovered from the *ts* isolates and the relevant mutations were localized within the *ceg-ts* genes by replacing wild-type *CEG1* DNA with restriction fragments from each *ts* mutant clone, followed by rescreening for the conditional growth phenotype. The *ceg-ts* mutations were then mapped at the nucleotide level by DNA sequencing (Table 1). In every case, the thermosensitive phenotype was attributable to missense mutations. Several of the isolates had more than one amino acid substitu-

TABLE 1

ceg1-ts Allele	CEG1 Protein Mutations		
ceg1-1	Phe(197)Tyr	Met(201)Thr	
ceg1-3	Phe(51)Tyr	Asn(190)Tyr	Tyr(226)Phe
ceg1-5	Ser(216)Arg		
ceg1-6	Val(289)Ala	Glu(364)Gly	
ceg1-7	Asp(300)Gly		
ceg1-13	Ser(168)Pro	Asp(205)Gly	Lys(215)Asn
ceg1-17	Leu(123)Pro	Ile(135)Thr	Tyr(181)Ser
ceg1-25	Asn(283)Tyr	Asp(370)Ala	Thr(378)Ser
ceg1-27	Phe(195)Leu	Tyr(208)Asn	
ceg1-95	Asp(95)Ala	Arg(96)Ala	Glu(97)Ala

tion; no effort was made to assign the phenotype to a single missense change, as we were not interested in drawing inferences about structure-function relationships from conditional mutations. Each missense change was encountered only once, indicating that our initial round of selection and mapping did not exhaust the available pool of *ts* mutations.

The nine *ceg1-ts* mutants grew as well as the wild-type strain at 25°C (Fig. 1). Six mutants (*ceg1-3, ceg1-5, ceg1-6, ceg1-13, ceg1-17,* and *ceg1-25*) failed to form colonies at 37°C. Three mutants (*ceg1-1, ceg1-7,* and *ceg1-27*) were leaky and formed small colonies at the nonpermissive temperature (Fig. 1). We analyzed in parallel a 10th *ts* allele, *ceg1-95*, that we had identified during charge-cluster to alanine mutagenesis of CEG1 (24). The molecular lesion of *ceg1-95* is a triple alanine substitution at residues Asp^{95}, Arg^{96}, and Glu^{97}. The *ceg1-95* mutant cells grew as well as wild-type cells at 25°C, but did not form colonies at 37°C (Fig. 1). All of the *ceg1-ts* alleles were recessive to wild-type *CEG1* (not shown).

Isolation of Multicopy Suppressors of ceg1-ts *Mutations*

The suppressor screen entailed transformation of each of the *ceg1-ts* strains with a 2μ plasmid-based wild-type genomic DNA library and selection for Ura+ colonies that grew at 37°C. We analyzed 10 positives for each of the *ceg1-ts* strains that were transformed with the 2μ library. Plasmid DNA was recovered from individual yeast colonies and transformed into *E. coli*. Diagnostic restriction enzyme digestion revealed whether wild-type *CEG1* had been selected. Candidate suppressors that did not contain the *CEG1* gene were retransformed into the *ts* strain from which they were originally isolated and tested for growth at 37°C. Six genomic clones retested faithfully. Restriction mapping of the genomic inserts revealed that these six clones derived from three distinct genetic loci, which we named *CES1, CES2,* and *CES3* (*CES* = capping enzyme suppressor). *CES1* and *CES2* were recovered as single isolates in *ceg1-25*. *CES3* was recovered

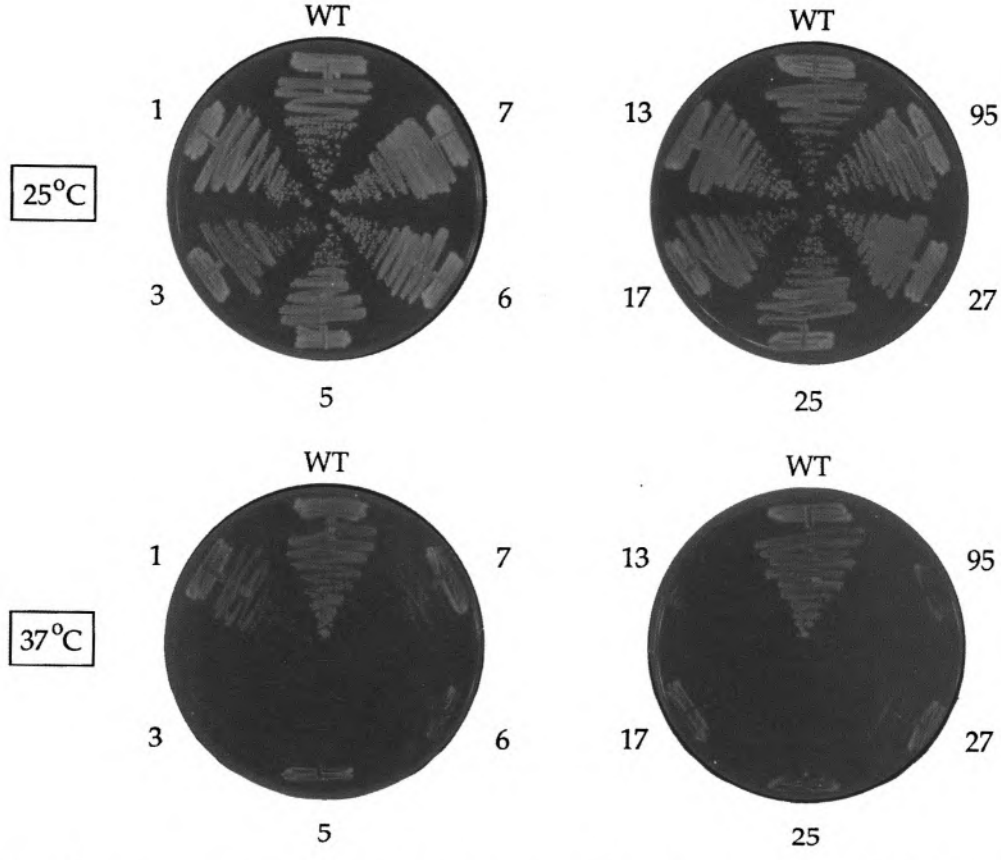

FIG. 1. Conditional growth phenotype of *ceg1-ts* mutants. Cells carrying the wild-type *CEG1* gene or *ceg1-ts* mutants (denoted by the *ts* allele number; see Table 1) on a *CEN* plasmid were plated on YPD medium and incubated for 3 days at either 25°C or 37°C. Photographs of the plates are shown.

four times, three times in *ceg1-5* and once in *ceg1-7*.

A side-by-side comparison of efficacy of multicopy suppression of the *ceg1-25* and *ceg1-5* mutations by the three *CES* genes is shown in Fig. 2. *ceg1-ts* mutants transformed with a 2μ-*CEG1* plasmid served as a positive control for wild-type growth at 37°C. *ceg1-ts* strains transformed with the YEP24 vector served as negative controls (i.e., the vector transformants grew at 25°C, but not at 37°C). *CES1* and *CES2* both restored growth of *ceg1-25* at 37°C. (This was expected given that *CES1* and *CES2* were both isolated in *ceg1-25*.) The size of the *CES1* and *CES2* colonies at 37°C was slightly smaller than that of the *CEG1* strain (Fig. 2). Defining the wild-type *CEG1* colony size as three-plus (+ + +), we designated the extent of suppression of *ceg1-25* by *CES1* and *CES2* as two-plus (+ +). *CES3* was less effective in suppressing *ceg1-25*, as judged by the even smaller colony size at 37°C. We classified this degree of suppression as one-plus (+). A different hierarchy of suppression was observed with the *ceg1-5* mutation. Here, *CES3* was the better suppressor (+ +), which was in keeping with the fact that *CES3* was isolated in the *ceg1-5* strain. *CES1* suppressed weakly (+), whereas CES2 did not suppress.

The *CES1, CES2*, and *CES3* multicopy plasmids were tested for their ability to rescue the conditional lethality of a *GAL-CEG1* strain, in which the chromosomal *CEG1* gene is deleted and a *CEN, TRP1* plasmid carries a wildtype *CEG1* gene under the control of a *GAL10* promoter. Growth of the *GAL-CEG1* strain is galactose dependent (21). Growth is precluded on medium containing glucose, which represses *GAL-CEG1* expression. Growth on glucose can be restored by wild-type *CEG1* expressed from a constitutive promoter, or by *PCE1*, a cDNA clone encoding the capping enzyme of *Schizosaccharomyces pombe* (24). We reasoned that if any of the multi-

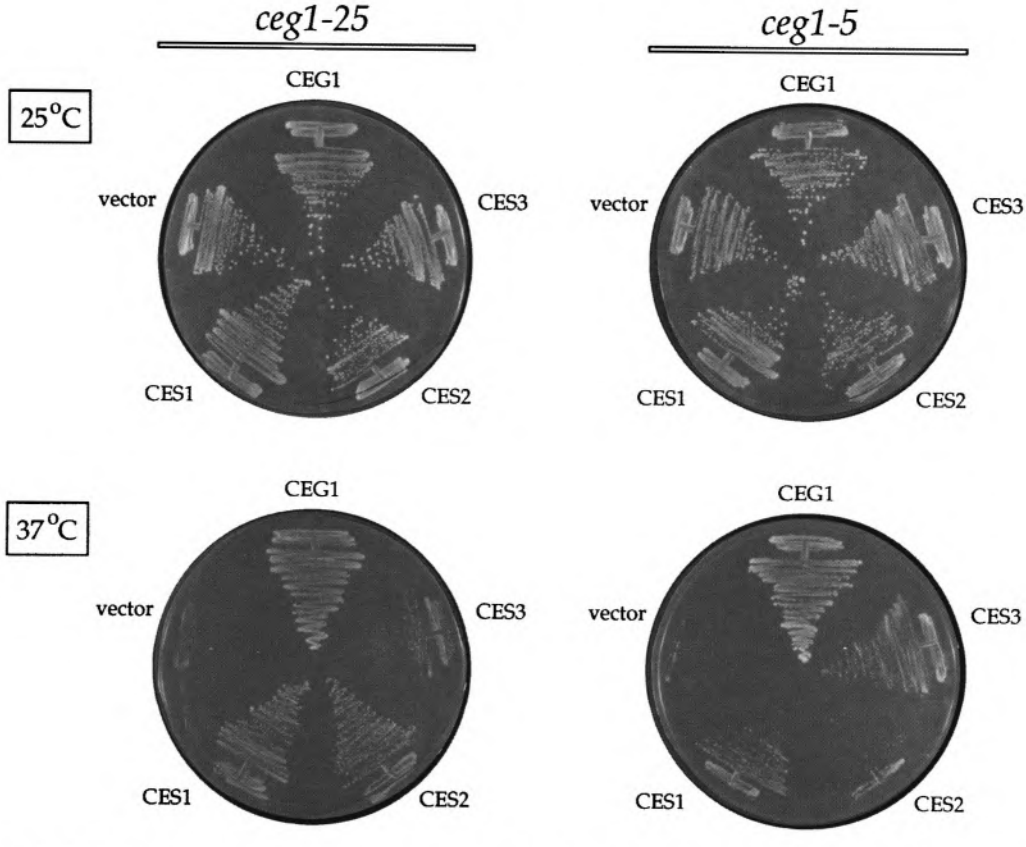

FIG. 2. Suppression of the *ts* growth defect of *ces1-25* and *ces1-5*. Strains *ceg1-25* and *ceg1-5* were transformed with a 2μ-*URA3* plasmid containing the wild-type *CEG1* gene, with the 2μ clones of *CES1, CES2,* and *CES3* that were isolated in the multicopy suppressor screen, and with the YEP24 vector without any insert. Ura+ transformants were selected and streaked on plates lacking uracil. The plates were photographed after incubation for 4 days at either 25°C or 37°C, as indicated.

copy capping enzyme suppressor genes were genetically bypassing *CEG1*, then the 2μ-*CES* plasmids should also permit growth of the *GAL-CEG1* strain on glucose. Neither *CES1*, *CES2*, nor *CES3* allowed any detectable growth of *GAL-CEG1* on glucose (not shown). We conclude that the *CES* genes are not bypassing the requirement for cap or capping enzyme for yeast cell growth.

Allele Specificity of Multicopy Suppression

The 2μ-*CES* clones were tested for suppression of each of the 10 *ceg-ts* mutations in our collection (Fig. 3). A hierarchy of suppression was apparent in that some *ts* mutations (*ceg1-1, ceg1-7, ceg1-27,* and *ceg1-25*) were suppressed by all three CES genes, whereas other mutations were not suppressed at all (e.g., *ceg1-3, ces1-13*). The former group includes the three *ts* mutants that display a partially leaky growth phenotype at 37°C (Fig. 1). We suspect that the least severe *ts* mutations were most prone to suppression because *CEG1* activity hovers about some threshold level when these strains are plated at the nonpermissive temperature, whereas the most severe *ceg1-ts* mutants could not be suppressed because they are functionally null at 37°C. Several examples of allele-specific suppression were encountered; *ceg1-6* was suppressed by *CES1* and *CES2*, but not by *CES3*, whereas *ceg1-17* and *ceg1-95* were suppressed only by *CES3* (Fig. 3).

CES1 Encodes a Novel 103-kDa Protein

The 2μ-*CES1* clone isolated in the suppressor screen contained a 9-kbp DNA insert. To narrow down the location of the *CES1* gene, we screened the 2μ genomic library by colony hybridization using a labeled probe specific for the *CES1* insert. We obtained a 2μ-*CES1* plasmid with a 5-kbp insert that was as effective as the original isolate in suppressing the *ceg1-25* mutation (Figs. 4 and 5). A physical map of the insert is shown in Fig. 4. An internal deletion of a 1-kbp *Bgl*II fragment from the 5-kbp insert abolished *CES1* function, whereas a 3.8-kbp *Hin*cII fragment including this *Bgl*II fragment was sufficient to suppress *ceg1-25* (Figs. 4 and 5). We completely sequenced both DNA strands of the insert and encountered a single long open reading frame (Fig. 4). Because this coding sequence was contained entirely within the *Hin*cII fragment sufficient for suppression, and because the predicted gene product would be inactivated by deletion of the *Bgl*II fragment, we concluded that this open reading frame corresponds to the *CES1* gene. (The *CES1* DNA sequence has been deposited in Genbank; U32580.) *CES1* has the capacity to encode a 103-kDa polypeptide (Fig. 4). The 915-amino acid CES1 protein is rich in serine, asparagine, and glutamic acid residues and is predicted to be strongly hydrophilic throughout its length. CES1 contains numerous clusters of repeating amino acids (e.g., a polyglutamine run at the C-terminus) and tandem repeats of short motifs (e.g., SSSP or SAPVQ, as underlined in Fig. 4).

The *CES1* gene was replaced by insertion of a *hisG-URA3-hisG* cassette (1). The disruption was performed in a diploid strain such that marker insertion eliminated the *CES1* coding sequence from amino acid positions 13–712. Correct insertion into one *CES1* locus was confirmed by Southern blotting of Ura+ transformants. After sporulation and tetrad dissection, we recovered viable Ura+ haploids in a 2:2 segregation pattern. *CES1* was also disrupted by insertion of the *hisG-URA3-hisG* cassette into the *CES1* locus of a haploid strain. The size of colonies formed by the Δ*ces1* null strains was indistinguishable from that of the

	ceg-ts allele									
	1	3	5	6	7	13	17	25	27	95
CES1	++	-	+	+	++	-	-	++	++	-
CES2	++	-	-	+	++	-	-	++	++	-
CES3	++	-	++	-	++	-	+	+	++	+

FIG. 3. Test of allele specificity of suppression. Each of the *ceg1-ts* strains (see Table 1) was transformed with 2μ-*URA3* clones of *CES1, CES2,* and *CES3* in parallel with 2μ-*CEG1* and YEP24 vector plasmids. Ura+ transformants were streaked to plates lacking uracil. The plates were incubated for 4 days at either 25°C or 37°C. The strength of suppression at 37°C was scored by comparison to the wild-type CEG1 transformant (+ + +) and the YEP24 vector (−), which were included as controls on every culture plate.

CES1 protein - 915 amino acids/103 kDa

MSNRDNESMLRTTSSDKAIASQRDKRKSEVLIAAQSLDNEIRSVKNLKRLSIGSMDL
LIDPELDIKFGGESSGRRSWSGTTSSSASMPSDTTTVNNTRYSDPTPLENLHGRGNS
GIESSNKTKQGNYLGIKKGVHSPSRKLNANVLKKNLLWVPANQHPNVKPDNFLELVQ
DTLQNIQLSDNGEDNDGNSNENNDIEDNGEDKESQSYENKENNTINLNRGLSRHGNA
SLIRRPSTLRRSYTEFDDNEDDDNKGDSASETVNKVEERISKIKERPVSLRDITEEL
TKISNSAGLTDNDAITLARTLSMAGSYSDKKDQPQPEGHYDEGDIGFSTSQANTLDD
GEFASNMPINNTMTWPERSSLRRSRFNTYRIRSQEQEKEVEQSVDEMKNDEEERLKL
TKNTIKVEIDPHKCPFRQQDEDSENMSSPGSIGDFQDIYNHYRQSSGEWEQEMGIEK
EAEEVPVKVRNDTVEQDLELREGTTDMVKPSATDDNKETKRHRRRNGWTWLNNKMSR
EDDNEENQGDDENEENVDSQRMELDNSKKHYISLFNGGEKTEVSNKEEMNNSSTSTA
TSQTRQKIEKTFANLFRRKPHHKHDASSSPSSSPSSSPSIPNNDAVHVRVRKSKKLG
NKSGREPVEPIVLRNRPRPHRHHHSRHGSQKISVKTLKDSQPQQQIPLQPQLEGAIE
IEKKEESDSESLPQLQPAVSVSSTKSNSRDREEEEAKKKNKKRSNTTEISNQQHSKH
VQKENTDEQKAQLQAPAQEQVQTSVPVQASAPVQNSAPVQTSAPVEASAQTQAPAAP
PLKHTSILPPRKLTFADVKKPDKPNSPVQFTDSAFGFPLPLLTVSTVIMFDHRLPIN
VERAIYRLSHLKLSNSKRGLREQVLLSNFMYAYLNLVNHTLYMEQVAHDKEQQQQQQ
QQP

FIG. 4. Physical map of the *CES1* locus and the amino acid sequence of the CES1 protein. Shown schematically is the 5-kbp insert of a 2μ-*CES1* clone. *Hin*cII and *Bgl*II restriction sites used in mapping *CES1* are indicated. The *CES1* gene is located within the 3.8-kbp *Hin*cII fragment, which was sequenced in its entirety. The location of a continuous open reading frame corresponding to the *CES1* gene is shown, with the orientation (N-terminus to C-terminus) indicated by an arrow. The sequence of the 915-amino acid CES1 polypeptide is shown in single-letter amino acid code. Short tandemly repeated sequence elements are underlined.

parental *CES1* strain at either 16°C, 25°C, or 37°C. We conclude that *CES1* is nonessential.

Multicopy Suppression of ceg1-ts *by a Yeast Homologue of* CES1

The CES1 protein is homologous to a 942-amino acid polypeptide encoded by open reading frame 8339.10 on yeast chromosome XIII that was identified during genomic sequencing (Genbank Z49210; blastp score = 419). CES1 and 8339.10 display high similarity to each other (50–70% identity) within a series of conserved sequence blocks (Fig. 6). 8339.10 lacks the C-terminal polyglutamate tail and the tandemly repeated motifs found in CES1. CES1 bears little resemblance to other proteins in the data base.

We found that a 2μ clone containing the 8339.10 gene (a gift of Desmond Clark and Dan Burke, University of Virginia) suppressed the *ts* growth phenotype of *ceg1-25* to the same extent as *CES1* (++ growth at 37°C). Because *CES1* and 8339.10 were functionally homologous with respect to capping enzyme suppression, we named the latter gene *CES4*. We deleted *CES4* in a haploid strain by insertion of a *hisG-URA-hisG* cassette. Correct insertion was confirmed by Southern blotting. The Δ*ces4* null strain grew at either 16°C, 25°C, or 37°C. Thus, *CES4* is nonessential, like its homologue *CES1*.

We examined whether these genes might carry out essential, albeit redundant, functions by constructing a double-knockout of *CES1* and *CES4*. This was done by disruption of the *CES4* gene in a Δ*ces1* haploid strain. We recovered viable haploids in which the *CES1* and *CES4* genes were both deleted. Thus, *CES1* and *CES4* are together dispensable for yeast cell growth.

CES2 *Is Identical to* ESP1

The 2μ-*CES2* clone contained a 9.5-kbp genomic insert. Limited sequencing of the insert revealed that the clone included *ESP1*, a previously

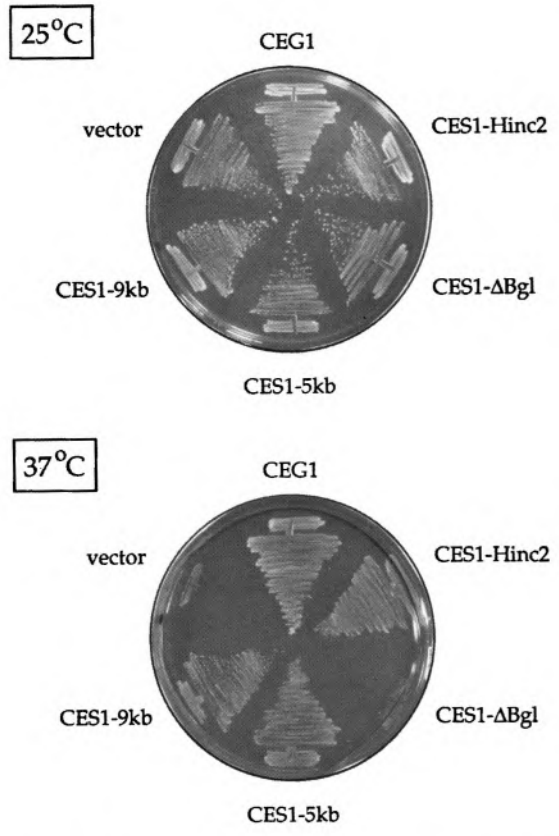

FIG. 5. Identification of the *CES1* gene. *ceg1-25* was transformed with the original 2μ-*CES1* isolate containing a ~9-kbp insert (CES1-9kb), with a genomic 2μ clone containing a 5-kbp insert that was isolated by colony hybridization (CES1-5kb), with a derivative of CES1-5kb in which the 1-kbp *Bgl*II fragment was deleted (CES1-ΔBgl), and with a YEP24-based plasmid containing a 3.8-kbp *Hinc*II fragment derived from CES1-5kb (CES1-Hinc2) (see Fig. 4). Ura+ transformants were selected and streaked on plates lacking uracil. Wild-type *CEG1* and YEP24 vector transformants were streaked on the same plates as controls. The plates were photographed after incubation for 4 days at either 25°C or 37°C.

identified essential gene involved in nuclear division (16). A 2μ subclone of the *CES2* insert containing only the *ESP1* gene was just as effective as the original *CES2* clone in suppressing the growth defect of *ceg1-25* (Fig. 7). We conclude, therefore, that *CES2* is identical to *ESP1*.

The ESP1 protein is a 1573-amino acid polypeptide; it contains a 371-amino acid region at its C-terminus that is homologous to the corresponding regions of *S. pombe cut1* and *Aspergillus nidulans bimB* (16). The latter genes, like *ESP1*, are involved in nuclear division. *esp1* mutations cause abnormal segregation of the nucleus during mitosis, such that the DNA and spindle poles are partitioned asymmetrically to the daughter cell (16). To examine the possible connection between the essential function of ESP1 in cell division and its action as a multicopy suppressor of capping enzyme mutants, we tested a series of 2μ-*URA3* clones encoding C-terminally truncated versions of ESP1 for both genetic functions (Fig. 8). *CES* activity was tested in the *ceg1-25* strain. We found that the *ESP1(1-1033)* and *ESP1(1-887)* clones were just as effective as the full-sized *ESP1* clone in supressing the *ceg1-ts* mutation (Fig. 7). However, further truncation of the gene in the case of *ESP1(1-515)* abolished *CES2* function (Fig. 7).

To assess *ESP1*-dependent cell growth, we constructed a diploid strain in which one copy of *ESP1* was deleted and replaced by a *LEU2* marker. This diploid was transformed with the full-length and truncated *ESP1* clones shown in Fig. 8. The Ura+ diploids were then sporulated. If the 2μ plasmid encoded a form of *ESP1* that was competent to carry out its essential function, then we expected the following: i) to recover Leu+ haploids in a 2:2 segregation pattern, and ii) that all Leu+ progeny would be 2μ plasmid dependent for growth and therefore unable to grow on 5-FOA. This is what we observed when diploids containing the full-length *ESP1* clone were sporulated and markers were subsequently tested (five tetrads analyzed). However, if the plasmid-encoded ESP1 protein was not able to perform its essential function, we predicted that no viable Leu+ haploids would be recovered. This is just what we observed for the *ESP1(1-1033), ESP1(1-887),* and *ESP1(1-515)* clones; that is, all tetrads yielded two viable spores and all haploids were Leu− (20 tetrads analyzed). Thirty-three of the 40 haploid Leu− progeny were Ura+, indicating that there was no problem with plasmid maintenace during meiosis and sporulation.

We conclude from this analysis that there are distinct functional domains of the ESP1 protein. ESP1 function during cell growth clearly requires the C-terminal protein segment that includes the 371-amino acid homology to cut1 and bimB. In contrast, *CES2* function is unaffected by removal of 686 residues from the carboxyl end.

The CES2 *Domain of ESP1: Similarity to the Small Subunit of Vaccinia Virus Capping Enzyme*

A search of the Genbank data base highlighted a resemblance between the ESP1 protein and the D12 subunit of the vaccinia virus mRNA capping enzyme. Four short segments of sequence similarity are arrayed in collinear fashion in the ESP1

```
ces4   25 SNNNIQMRRMRKTQLSKKELFEKRKSDVLIAAKSLDTEIQNVKNLKRLSIGSMDLVIDPELEFKVNSRNS 94
          . .:  : :   .  .     .::::.::::::::: ::..:::::::::::::::::::. :     :
ces1    3 NRDNESMLRTTSSDKAIASQRDKRKSEVLIAAQSLDNEIRSVKNLKRLSIGSMDLLIDPELDIKFGGESS 72

ces4  207 LTQNLLWVPADQHPNVKPENYLELIQDTLQNIQISTNQDIDENKLELGNN 256
          :  :::::::.::::::::.:.:::.:::::::::.: : . ..      :.
ces1  146 LKKNLLWVPANQHPNVKPDNFLELVQDTLQNIQLSDNGEDNDGNSNENND 195

ces4  261 NRKRTGSVVRRPSRLKTSYTKFDDEPPLADKPQEGEIQVDKRISSSDIKTIRSVSLKEIT 320
          .:    :..:::: :. :::.:::     .:       :.:          : :::..::
ces1  223 SRHGNASLIRRPSTLRRSYTEFDDNEDDDNKGDSASETVNKVEERISKIKERPVSLRDIT 282

ces4  321 EELTKISNNAGLTDSDAVTLARSLSMSGSFTNE 353
          :::::::::.:::::.::.:::::.:::....
ces1  283 EELTKISNSAGLTDNDAITLARTLSMAGSYSDK 315

ces4  361 HTENDNEFASNMFNETGLTIPERSSLRRSKFNTYKIR 397
          .: .: ::::::     .: ::::::::::.::::.::
ces1  338 NTLDDGEFASNMPINNTMTWPERSSLRRSRFNTYRIR 374

ces4  439 SLNDFHEIFDHYRRTSTDWSTE 460    532 HGWSWLNSSNGSLNGNEQ 549
          :. :: .:..::: .: .:  :          .::.:::.     . ::.
ces1  430 SIGDFQDIYNHYRQSSGEWEQE 451    502 NGWTWLNNKMSREDDNEE 519

ces4  799 DIGAEREDNTSPTAPQISTLPPRKLTFEDVVKPDYPNAPIKFTDSAFGFPLPMITNSTVI 858
          .  :. .   .:     : :::::::: :: ::: ::.:. :::::::::::..: ::::
ces1  787 EASAQTQAPAAPPLKHTSILPPRKLTFADVKKPDKPNSPVQFTDSAFGFPLPLLTVSTVI 846

ces4  859 MFDHRLGINVERAIYRLSHLKLSDPGRELRQQVLLSNFMYSYLNLVNHTLYMEQVGH 915
          :::::: :::::::::::::::.  : ::.:::::::::.:::::::::::::::: :
ces1  847 MFDHRLPINVERAIYRLSHLKLSNSKRGLREQVLLSNFMYAYLNLVNHTLYMEQVAH 903
```

FIG. 6. Amino acid sequence similarity between *CES1* and *CES4*. Segments of the CES1 protein were aligned with a homologous polypeptide encoded by an open reading frame on chromosome XIII (designated *CES4*, because this gene could function as a multicopy capping enzyme suppressor). The amino acid coordinates demarcating each segment are indicated. Amino acid identity is denoted by two dots (:); similarity is denoted by a single dot (.).

and D12 polypeptides (Fig. 8). Remarkably, these motifs are located in the N-terminal portion of ESP1 that constitutes the *CES2* domain. The vaccinia virus capping enzyme is a heterodimer of D1 (95 kDa) and D12 (33 kDa) subunits that catalyzes all three steps in cap synthesis (23). The three catalytic functions are organized in a modular fashion within the native enzyme. The RNA triphosphatase and RNA guanylyltranferase activities reside within a 60-kDa N-terminal region of the D1 subunit. The 287-amino acid D12 subunit, together with the C-terminal 305-amino acid portion of the D1 subunit, catalyzes the cap methylation reaction. The D12 protein itself has no catalytic activity; rather, it interacts tightly with the D1 subunit and stimulates its RNA (guanine-7)-methyltransferase activity by 50- to 100-fold (3,9,15). In light of our earlier findings regarding the structural and functional homology between the vaccinia virus and yeast capping enzyme systems (14,21,24), we regard it as significant that a genetic screen in yeast for suppression of *ts* capping enzyme function yielded a protein resembling a known subunit of the vaccinia capping enzyme. We consider this further in the Discussion.

CES3 *Corresponds to* BUD5

The four independent *CES3* isolates contained genomic DNA inserts of 6 to 9 kbp. We localized the *CES3* inserts by limited DNA sequencing to the vicinity of the *MAT* locus on yeast chromosome III. The four clones overlapped within an ~4-kbp region that included the entire *BUD5* gene. *BUD5* is a nonessential gene encoding a 537-amino acid polypeptide with sequence similarity to guanine nucleotide exchange factors (2,18). A 2.8-kbp *Pst*I-*Sca*I subclone containing sequence from nucleotides −568 to +2242 relative to the translation start site of the *BUD5* gene (which in-

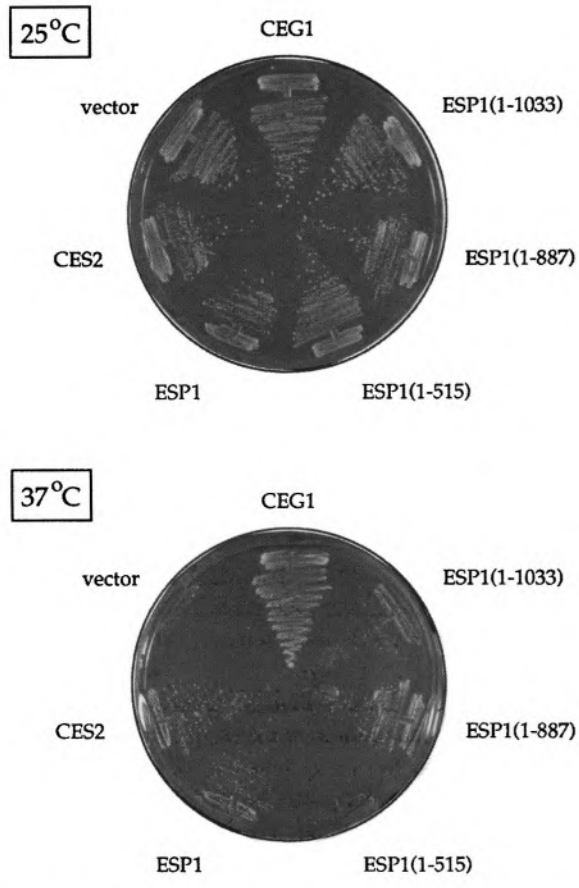

FIG. 7. *CES2* is identical to *ESP1*. *ceg1-5* was transformed with the original 2μ-*CES2* isolate (CES2), with a 2μ clone containing just the *ESP1* gene (ESP1), and with three deletion clones—ESP1(1-1033), ESP1(1-887), and ESP1(1-515)—numbered according to the amino acid coordinates of the truncated ESP1 proteins they encode (see Fig. 8). Ura+ transformants were selected and streaked on plates lacking uracil. Wild-type *CEG1* and *YEP24* vector transformants were streaked on the same plates as controls. The plates were photographed after incubation for 4 days at either 25°C or 37°C.

cludes the entire coding sequence and 631 bp of DNA 3′ of the translation stop codon) was capable of suppressing *ceg1-5*. However, a 2.9-kbp *Pvu*II subclone, extending from +681 within the *BUD5* open reading frame to position +3642 located 2031 bp downstream of the *BUD5* stop codon, did not suppress *ceg1-5*. We conclude that *CES3* corresponds to *BUD5*.

DISCUSSION

The RNA capping activity of the yeast CEG1 protein is required for cell growth (21). Yet the essential role played by the RNA cap in vivo remains unclear, and is likely to be complex, given that in vitro studies have implicated the cap in so many aspects of cellular RNA metabolism. As a first step toward analyzing cap function genetically, we isolated a collection of temperature-sensitive *ceg1* alleles from a pool of mutagenized *CEG1* clones. Conditional mutations constitute valuable tools for two levels of inquiry: i) what happens to RNA metabolism and gene expression when capping enzyme is inactivated? and ii) what other genes impact on *CEG1* function in vivo? Both areas are under investigation in our laboratories. Initial phenotypic analysis confirms the prediction that many aspects of gene expression are affected when *ceg1-ts* mutants are shifted to the nonpermissive temperature (e.g., we find that protein synthesis is shut off, pre-mRNA splicing is inhibited, and the steady-state level of mRNA declines sharply) (Schwer, Mao, and Shuman, unpublished).

The present report describes the use of the *ceg1-ts* mutants to identify interactions between *CEG1* and other yeast genes. We isolated and characterized three yeast *CES* genes that, when present in high copy, can suppress the conditional growth phenotype of specific mutations in the capping enzyme. *CES1* encodes a novel 103-kDa polypeptide. *CES2* corresponds to *ESP1*, a gene required for proper nuclear division during mitosis. *CES3* corresponds to *BUD5*, which encodes a putative guanine nucleotide exchange factor involved in bud site selection. An additional capping enzyme suppressor, *CES4*, was identified by homology to *CES1*.

Suppression of defects in mRNA capping could occur by increasing the dosage of a protein that interacts with *CEG1*. Alternatively, a high copy suppressor might act downstream of cap synthesis to enhance some essential cap-dependent RNA transaction that had become limiting for growth at the nonpermissive temperature. We consider below how these ideas might apply to multicopy suppression by the three CES genes identified in our screen.

ESP1

The suppressor gene identified initially as *CES2* is identical to *ESP1*, a gene required for proper nuclear division (16). *ESP1* encodes a fairly large polypeptide (181 kDa). No specific role of the ESP1 protein in nuclear division has been described. The C-terminal homology between the *ESP1*, *cut1*, and *bimB* gene products has been suggested to confer a common function in nuclear

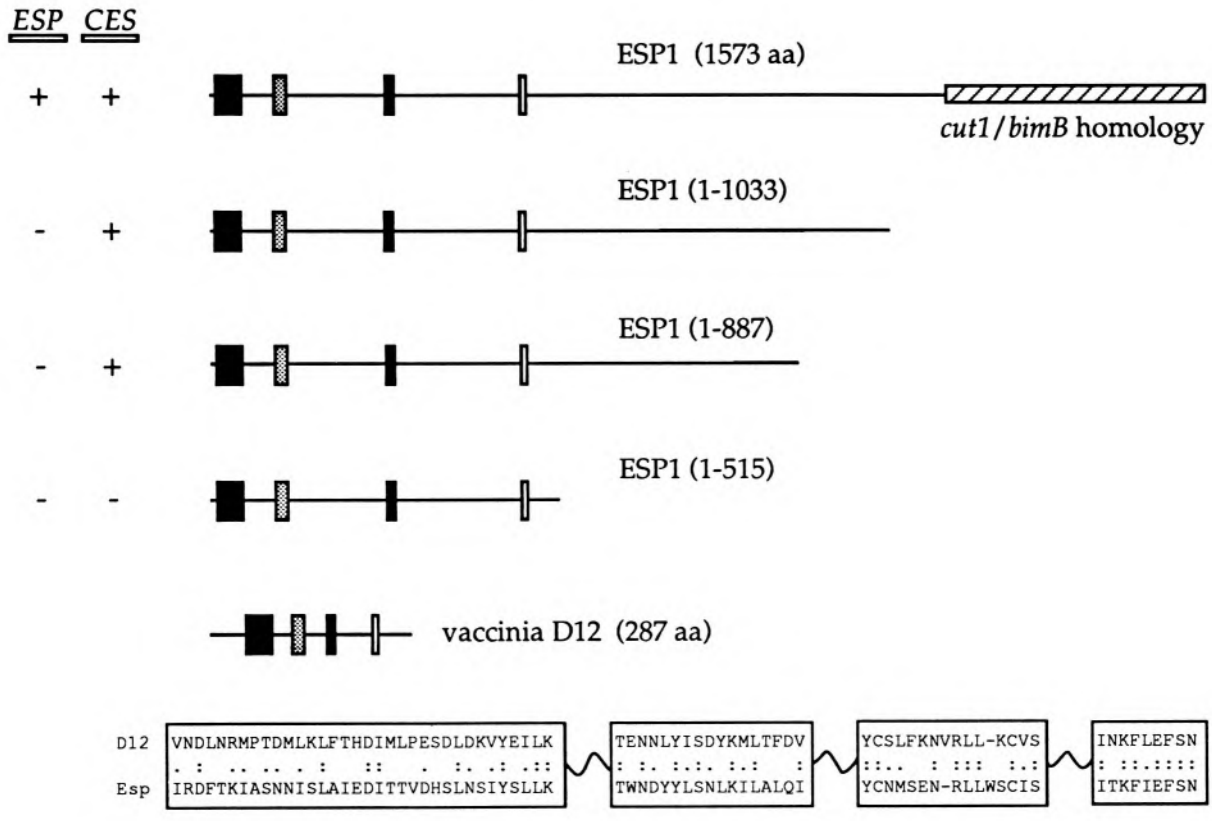

FIG. 8. Deletion analysis of CES2/ESP1 and sequence similarity to a subunit of the vaccinia virus capping enzyme. 2μ plasmid clones containing the complete *ESP1* gene and deleted versions of *ESP1* were constructed as described in Materials and Methods. The polypeptides encoded by these constructs are shown schematically in the figure. The clones are numbered according to the amino acid coordinates of the truncated ESP1 proteins they encode. The C-terminal 371-amino acid region homologous to nuclear division proteins cut1 and bimB is shown as a hatched box. Regions of sequence similarity between the N-terminus of ESP1 and the D12 subunit of the vaccinia virus capping enzyme are depicted as bars. The sequences of the D12 and ESP1 proteins in these regions are aligned at the bottom. Amino acid identity is indicated by two dots (:); similarity is denoted by a single dot (.). The results of functional analyses of the clones are summarized on the left. ESP function refers to the ability of the plasmid-encoded gene to complement an *esp1::LEU2* mutation. *CES* activity refers to the experiment of Fig. 7, in which the clones were tested for multicopy suppression of *ceg1-25*.

division (16). Our findings support this model, insofar as deletion of the C-terminal segment of ESP1 that includes the cut1/bimB domain inactivates the essential *ESP1* function. [This extends an earlier finding that *ESP1* was inactivated by a transposon insertion near the C-terminus of the open reading frame (16).] The capping enzyme suppressor activity of ESP1 is clearly distinct from its function in nuclear division, because nearly half of the protein can be deleted from the C-terminus without affecting *CES2* activity. It remains to be determined if the capping enzyme suppressor and nuclear division functions are carried out by autonomous or overlapping protein domains (i.e., whether the N-terminal half of the protein that is sufficient for *CES2* function is necessary for *ESP1* activity in nuclear division).

The N-terminal *CES2* domain of ESP1 includes segments of homology with the D12 subunit of the vaccinia virus capping enzyme. The extent of the homology is modest, but is likely to be functionally significant. Indeed, the extent of sequence similarity in this case is not much different than what has been noted for other vaccinia and yeast capping proteins that perform the same biochemical function [e.g., the CEG1 protein versus the guanylyltransferase domain of the vaccinia capping enzyme D1 subunit (24,26), or the yeast ABD1 cap methyltransferase versus the methyltransferase domain of the vaccinia D1 protein (14)]. The D12 subunit of the vaccinia capping enzyme binds tightly to the D1 subunit and stimulates the intrinsic D1 methyltransferase activity (9,15). The stimulation of catalysis by D12 depends on the ability of the two subunits to heterodimerize (3,15). The D12 subunit is also essential

for the action of the vaccinia capping enzyme as a transcription termination factor (13). A potentially relevant property of the D12 subunit is its ability to protect the D1 subunit from proteolysis in vivo (8,25). Nothing is known about the domain structure of the D12 subunit or about specific structural requirements for subunit association. Hence, it is not obvious what function might be conferred by the D12 motifs found in ESP1. We speculate, by analogy to the vaccinia system, that increased dosage of CES2/ESP1 drives its physical interaction with CEG1, either directly or through a third protein (such as ABD1), the effect of which is to either stabilize CEG1 against denaturation or proteolysis at the nonpermissive temperature or else to enhance its catalytic activity above a threshold level required for viability. Attempts to directly test the effects of ESP1 on CEG1 guanylyltransferase activity in vitro have been hampered at the stage of expressing the ESP1 protein in bacteria.

CES1

CES1 encodes a novel hydrophilic 103-kDa polypeptide. The sequence of the CES1 protein is uninformative, except for its extensive similarity to the product of *CES4*. *CES4* is a functional homolog of *CES1* in that it too acts as a multicopy suppressor of *ts* mutations in the capping enzyme. We found that neither gene is essential and that a double-knockout is viable.

CES4 has been isolated as a multicopy suppressor of mutations in *cdc20* and *sin4* (D. Burke, personal communication; Yu et al., Genbank U32938), two genes that have no apparent connection to *CEG1*. The fact that *CES1* and/or *CES4* have been identified in diverse high copy suppressor screens suggests that they may affect some aspect of gene expression. For example, multiple defects might be suppressed by genes that enhance protein stability or upregulate gene expression at the transcriptional or posttranscriptional level. Given the unusual amino acid composition of CES1, it is conceivable that it interacts physically with a variety of other proteins and that this is relevant to its suppressor activity. Yet, we favor the idea that *CES1* affects gene expression. Of the various proteins that are suppressed by *CES1* or *CES4*, the capping enzyme seems most closely tied to gene expression. Our preliminary analysis of the *ceg1-ts* phenotype reveals, among other effects, a significant reduction in the steady-state level of mRNA after shift of *ceg1-ts* cells to the nonpermissive temperature (unpublished). We speculate that *CES1* may suppress the capping enzyme defect either by stimulating the utilization of residual mRNAs that are capped or by enhancing the use of uncapped RNAs. This effect could occur at the level of RNA stability or translation or both. A possible role for CES1 in translation is suggested by the independent isolation of *CES1* as a multicopy supressor of a *ts* mutation in the translation initiation factor eIF4A (P. Linder, personal communication).

BUD5

BUD5 is not essential for cell growth; however, a Δ*bud5* deletion elicits a random budding pattern in lieu of the axial or bipolar patterns seen in wild-type cells (2). *BUD5* encodes a putative guanine nucleotide exchange factor, which is believed to regulate the activity of the BUD1 and CDC42 proteins; the latter are Ras-like GTPases involved in bud site selection and bud formation, respectively (2). *BUD5*, when overexpressed from a high copy plasmid, can also interact with RAS2 and suppress the growth defect of a dominant-negative RAS2 mutation (18).

How might BUD5, a component of a GTPase signaling pathway, act in high dosage to suppress mutations in the capping enzyme? Although capping enzyme binds GTP, it is an unlikely target for a guanine nucleotide exchange factor, because GDP is neither an intermediate nor a product in the guanylyltransferase reaction (23). We speculate that BUD5 overexpression may impact on a downstream phase of gene expression. Yeast RNA processing and RNA transport are affected by mutations in genes that encode guanine nucleotide signaling proteins (10,19,20). In addition, GTPases and guanine nucleotide exchange factors play key roles in translation. The cap structure is thought to facilitate each of these RNA events.

Capping and Budding

A strange nexus between cap synthesis and cell polarity is emerging that involves at least four yeast genes: *CEG1, ABD1, CES1*, and *BUD5*. For example: i) *ABD1*, which encodes RNA (guanine-7)-methyltransferase, the enzyme that catalyzes RNA cap methylation (14), has been isolated in a genetic screen for mutational synergy with *BEM1*, a gene involved in bud emergence (Corrado and Pringle, Genbank L12000); ii) *bem1* mutations display synergy with *bud5* mutations (2); iii) *BUD5* in high copy partially suppresses *ts* mutations in mRNA guanylyltransferase (this study);

iv) *ceg1-ts* mutations are suppressed by *CES1* in high copy (this study); v) *CES1* has been isolated in an indepedent high copy screen as a negative regulator of cell polarity (*NRC1*) (Bi and Pringle, Genbank L42821). What does 5′ end modification of RNA have to do with cell polarity? Budding entails the recruitment of numerous structural components and regulatory factors to a site on the cell cortex. It is possible that synthesis of some of these components or factors might be especially cap dependent in vivo. It is even conceivable that mRNA encoding one of the proteins involved in budding is itself localized within the cell (e.g., at the bud site) and that this process is cap dependent.

Conclusions

We have identified a genetic connection between capping enzyme and four other yeast proteins. CES2/ESP1 displays sequence similarity to the small subunit of the vaccinia capping enzyme and is a candidate to interact physically or functionally with yeast RNA guanylyltransferase. CES1 and CES4, which are novel proteins, and CES3/BUD5, a putative guanine-nucleotide exchange factor, have no obvious connection to capping, but may impact on downstream transactions that are cap dependent in vivo.

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REFERENCES

1. Alani, E.; Cao, L.; Kleckner, N. A method for gene disruption that allows repeated use of *URA3* selection in the construction of mutiply dirupted yeast strains. Genetics 116:541–545; 1987.
2. Chant, J.; Corrado, K.; Pringle, J. R.; Herskowitz, I. Yeast BUD5, encoding a putative GDP-GTP exchange factor, is necessary for bud site selection and interacts with bud formation gene BEM1. Cell 65: 1213–1224; 1991.
3. Cong, P.; Shuman, S. Methyltransferase and subunit association domains of vaccinia virus mRNA capping enzyme. J. Biol. Chem. 267:16424–16429; 1992.
4. Cong, P.; Shuman, S. Covalent catalysis in nucleotidyl transfer: A KTDG motif essential for enzyme-GMP complex formation by mRNA capping enzyme is conserved at the active sites of RNA and DNA ligases. J. Biol. Chem. 268:7256–7260; 1993.
5. Edery, I.; Sonenberg, N. Cap-dependent RNA splicing in a HeLa nuclear extract. Proc. Natl. Acad. Sci. USA 82:7590–7594; 1985.
6. Fresco, L. D.; Buratowski, S. Active site of the mRNA capping enzyme guanylyltransferase from *Saccharomyces cerevisiae* resembles the nucleotidyl transfer motif of DNA and RNA ligases. Proc. Natl. Acad. Sci. USA 91:6624–6628; 1994.
7. Furuichi, Y.; LaFiandra, A.; Shatkin, A. J. 5′-Terminal structure and mRNA stability. Nature 266:235–239; 1977.
8. Guo, P.; Moss, B. Interaction and mutual stabilization of the two subunits of vaccinia virus mRNA capping enzyme coexpressed in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 87:4023–4027; 1990.
9. Higman, M. A.; Christen, L. A.; Niles, E. G. The mRNA (guanine-7-) methyltranferase domain of the vaccinia virus mRNA capping enzyme: Expression in *Escherichia coli* and structural and kinetic comparison to the intact capping enzyme. J. Biol. Chem. 269:14974–14981; 1994.
10. Kadowaki, T.; Goldfarb, D.; Spitz, L. M.; Tartakoff, A. M.; Ohno, M. Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily. EMBO J. 12:2929–2937; 1993.
11. Konarska, M. M.; Padgett, R. A.; Sharp, P. A. Recognition of cap structure in splicing in vitro of mRNA precursors. Cell 38:731–736; 1984.
12. Leung, D. W.; Chen, E.; Goeddel, D. V. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. Technique J. Methods Cell Mol. Biol. 1:11–15; 1989.
13. Luo, Y.; Mao, X.; Deng, L.; Cong, P.; Shuman, S. The D1 and D12 subunits are both essential for the transcription termination factor activity of vaccinia capping enzyme. J. Virol. 69:3852–3856; 1995.
14. Mao, X.; Schwer, B.; Shuman, S. Yeast mRNA cap methyltransferase is a 50-kilodalton protein encoded by an essential gene. Mol. Cell. Biol. 15:4167–4174; 1995.
15. Mao, X.; Shuman, S. Intrinsic RNA (guanine-7) methyltransferase activity of the vaccinia virus capping enzyme D1 subunit is stimulated by the D12 subunit: Identification of amino acid residues in the D1 protein required for subunit association and methyl group transfer. J. Biol. Chem. 269:24472–24479; 1994.
16. McGrew, J. T.; Goetsch, L.; Beyers, B.; Baum, P. Requirment for *ESP1* in the nuclear division of *Saccharomyces cerevisiae*. Mol. Biol. Cell 3:1443–1454; 1992.

17. Muthukrishnan, S.; Both, G. W.; Furuichi, Y.; Shatkin, A. J. 5′-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. Nature 255:33–37; 1975.
18. Powers, S.; Gonzales, E.; Christensen, T.; Cubert, J.; Broek, D. Functional cloning of *BUD5*, a *CDC25*-related gene from *S. cerevisiae* that can suppress a dominant-negative RAS2 mutant. Cell 65: 1225–1231; 1991.
19. Schlenstedt, G.; Saavedra, C.; Loeb, J. D.; Cole, C. N.; Silver, P. A. The GTP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly(A)$^+$ RNA in the cytoplasm. Proc. Natl. Acad. Sci. USA 92:225–229; 1995.
20. Schneiter, R.; Kadowaki, T.; Tartakoff, A. M. mRNA transport in yeast: Time to reinvestigate the role of the nucleolus. Mol. Biol. Cell 6:357–370; 1995.
21. Schwer, B.; Shuman, S. Mutational analaysis of yeast mRNA capping enzyme. Proc. Natl. Acad. Sci. USA 91:4328–4332; 1994.
22. Shibagaki, Y.; Itoh, N.; Yamada, H.; Hagata, S.; Mizumoto, K. mRNA capping enzyme: Isolation and characterization of the gene encoding mRNA guanylyltransferase subunit from *Saccharomyces cerevisiae*. J. Biol. Chem. 267:9521–9528; 1992.
23. Shuman, S. Capping enzyme in eukaryotic mRNA synthesis. Prog. Nucleic Acid Res. Mol. Biol. 50: 101–129; 1995.
24. Shuman, S.; Liu, Y.; Schwer, B. Covalent catalysis in nucleotidyl transfer reactions: Essential motifs in *Saccharomyces cerevisiae* RNA capping enzyme are conserved in *Schizosaccharomyces pombe* and viral capping enzymes and among polynucleotide ligases. Proc. Natl. Acad. Sci. USA 91:12046–12050; 1994.
25. Shuman, S.; Morham, S. G. Domain structure of vaccinia virus mRNA capping enzyme: Activity of the Mr 95,000 subunit expressed in *Escherichia coli*. J. Biol. Chem. 265:11967–11972; 1990.
26. Shuman, S.; Schwer, B. RNA capping enzyme and DNA ligase—a superfamily of covalent nucleotidyl transferases. Mol. Microbiol. 17:405–410; 1995.