Research article

RNA triphosphatase is essential in *Schizosaccharomyces pombe* and *Candida albicans*

Yi Pei[1], Beate Schwer[2], Julia Saiz[1], Robert P Fisher[1] and Stewart Shuman*[1]

Address: [1]Molecular Biology and Cell Biology Programs, Sloan-Kettering Institute, New York, NY 10021, USA and [2]Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10021, USA

E-mail: Yi Pei - y-pei@ski.mskc.org; Beate Schwer - bschwer@med.cornell.edu; Julia Saiz - j-saiz@ski.mskccc.org; Robert P Fisher - r-fisherr@ski.mskcc.org; Stewart Shuman* - s-shuman@ski.mskcc.org

*Corresponding author

Abstract

Background: The first two steps in the capping of cellular mRNAs are catalyzed by the enzymes RNA triphosphatase and RNA guanylyltransferase. Although structural and mechanistic differences between fungal and mammalian RNA triphosphatases recommend this enzyme as a potential antifungal target, it has not been determined if RNA triphosphatase is essential for the growth of fungal species that cause human disease.

Results: We show by classical genetic methods that the triphosphatase (Pct1) and guanylyltransferase (Pce1) components of the capping apparatus in the fission yeast *Schizosaccharomyces pombe* are essential for growth. We were unable to disrupt both alleles of the *Candida albicans* RNA triphosphatase gene *CaCET1*, implying that the RNA triphosphatase enzyme is also essential for growth of *C. albicans*, a human fungal pathogen.

Conclusions: Our results provide the first genetic evidence that cap synthesis is essential for growth of an organism other than *Saccharomyces cerevisiae* and they validate RNA triphosphatase as a target for antifungal drug discovery.

Background

The m7GpppN cap structure is a defining feature of eukaryotic mRNA and is required for mRNA stability and efficient translation. The cap is formed by three enzymatic reactions: the 5' triphosphate end of the nascent pre-mRNA is hydrolyzed to a diphosphate by RNA triphosphatase; the diphosphate end is capped with GMP by RNA guanylyltransferase; and the GpppN cap is methylated by RNA (guanine-7-) methyltransferase [1].

Although the three capping reactions are universal in eukaryotes, there is a surprising diversity in the genetic organization of the capping enzymes as well as a complete divergence in the structure and catalytic mechanism of the RNA triphosphatase component in "lower" versus "higher" eukaryotic species [1]. Metazoans and plants have a two-component capping system consisting of a bifunctional triphosphatase-guanylyltransferase polypeptide and a separate methyltransferase polypeptide, whereas fungi contain a three-component system consisting of separate triphosphatase, guanylyltransferase, and methyltransferase gene products. The primary structures and biochemical mechanisms of the fungal and mammalian guanylyltransferases and cap methyl-

transferases are conserved. However, the atomic structures and catalytic mechanisms of the fungal and mammalian RNA triphosphatases are completely different [2,3]. Thus, it has been suggested that RNA triphosphatase is a promising target for antifungal drug discovery [2].

The triphosphatase (Cet1), guanylyltransferase (Ceg1), and methyltransferase (Abd1) components of the capping apparatus are essential for cell growth in the budding yeast *S. cerevisiae*[1,4–6]. Mutations of the RNA triphosphatase Cet1 that abrogate catalytic activity *in vitro* are lethal *in vivo*[7–9]; thus, it is reasonable to think that pharmacological inhibition of Cet1 function *in vivo* would impede cell growth. The key question is whether RNA triphosphatase is a valid drug target in other fungal species besides *Saccharomyces cerevisiae* (which is not a human pathogen) and whether a mechanism-based inhibitor of one fungal RNA triphosphatase could be expected to display broad spectrum activity against triphosphatases from other fungal species.

To address these issues, we have characterized the RNA triphosphatases of two other fungi, including the human pathogen *Candida albicans* and the fission yeast *Schizosaccharomyces pombe*[10–12]. The fungal triphosphatases, *S. cerevisiae* Cet1, *C. albicans* CaCet1 and *S. pombe* Pct1, belong to a new family of metal-dependent phosphohydrolases that embraces the triphosphatase components of DNA virus and protozoan mRNA capping systems [1,7,13,14]. The defining features of the metal-dependent RNA triphosphatases are two glutamate-containing motifs that are required for catalysis and comprise the metal-binding site in the crystal structure of *S. cerevisiae* Cet1. The yeast triphosphatase has a novel tertiary structure in which the active site is situated within a topologically closed hydrophilic tunnel composed of 8 antiparallel β strands, which are conserved in CaCet1 and Pct1 [2]. Mutational analysis of Cet1 has identified 15 individual side chains within the tunnel that are important for Cet1 function *in vitro* and *in vivo*[7–9]. Each of the 8 strands contributes at least one functional group to the active site. Mutational analysis of the *Candida* triphosphatase suggested strongly that the tunnel fold and the constituents of the active site are similar, if not identical, in Cet1 and CaCet1 [10].

Here we address the critical question of whether RNA triphosphatase is essential for cell growth in fungal species other than *S. cerevisiae*. This is not a straw-man issue, given that *S. cerevisiae* encodes two homologous RNA triphosphatases (Cet1 and Cth1), of which only Cet1 is essential for capping and cell viability [8,15]. We use classical genetic approaches to show that the respective genes encoding RNA triphosphatase and RNA guanylyl-

transferase are essential in *S. pombe*. Using a novel method of Enloe et al. [16] to test gene function in diploid *C. albicans,* we were unable to disrupt both copies of the *CaCET1* gene, signifying that RNA triphosphatase is also essential in that species, a significant human pathogen. Based on these findings, and the presence of a Cet1 homolog in the *Apergillus fumigatus* proteome, we conclude that RNA triphosphatase is a valid target for antifungal drug development.

Results
RNA Triphosphatase and RNA Guanylyltransferase are Essential in S. pombe

S. pombe RNA triphosphatase Pct1 is a 303-amino acid polypeptide with a homodimeric quaternary structure [12]. The *pct1*+ gene contains a single intron within the open reading frame [12]. *S. pombe* RNA guanylyltransferase Pce1 is a 402-amino acid monomeric protein [20]; there are no introns within the *pce1*+ gene. Although recombinant Pct1 and Pce1 enzymes have been purified and characterized biochemically, and shown to function in cap formation when expressed in *S. cerevisiae*[12,20,21], there have been no antecedent genetic studies of the essentiality of Pct1 or Pce1 in fission yeast. Here we constructed *pct1Δ* and *pce1Δ* plasmids containing 5' and 3' flanking genomic sequences in which the entire triphosphatase or guanylyltransferase coding sequence was deleted and replaced by the kanamycin resistance gene [17]. The *pct1::kanMX* and *pce1::kanMX* constructs were transformed separately into a diploid strain of *S. pombe* and chromosomal integrants containing one copy of the wild-type gene and one of *pct1::kanMX* or *pce1::kanMX* were selected on medium containing G418. Correct integration was confirmed by diagnostic PCR amplification of genomic DNA from the heterozygotes. We then sporulated the heterozygotes, dissected tetrads, and scored for spore viability and the presence of the *kanMX* marker. We found for both knock-outs that 20 out of 20 tetrads yielded only 2 viable spores and all of the viable haploids were G418-sensitive, i.e., none contained the *pct1::kanMX* or *pce1::kanMX* alleles. We conclude that the RNA triphosphatase and RNA guanylyltransferase genes are essential for cell growth in *S. pombe*.

Plasmid-based complementation of pct1Δ and pct1Δ

The *pct1*+ and *pce1*+ cDNAs were cloned separately into the *S. pombe* expression vector pREP41X (*LEU2 ars1*+) so as to place them under the control of the *nmt1** promoter. We also cloned the intron-containing chromosomal *pct1*+ gene into the same expression vector. The plasmids were introduced into heterozygous *pct1*+/*pct1::kanMX* or *pce1*+/*pce1::kanMX* diploids. The Leu+ diploid transformants were selected and then sporulated. A random population of *Leu*+ haploids was tested for

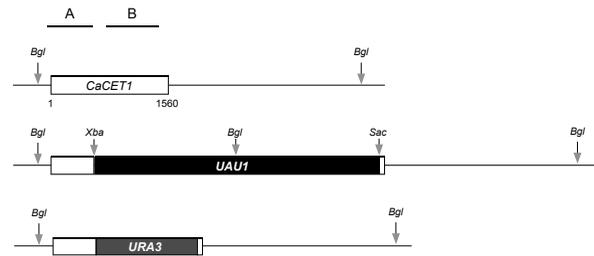**Table 1: Plasmid-based Complementation of *pct1Δ* and *pce1Δ***

Strain	Plasmid	G418 resistant	G418 sensitive
pct1+/pct1::kanMX	pREP41X	0	24
	pREP41X-*pct1+* (cDNA)	21	19
	pREP41X-*pct1+*	22	18
pce1+/pce1::kanMX	pREP41X	0	24
	pREP41X-*pce1+*	22	18

Figure 1
Genotype of the CaCET1/cacet1::UAU1 heterozygote strain of C. albicans. Illustrated in cartoon form are the configurations of the wild-type *CaCET1* and the *cacet1::UAU1* chromosomal loci in the Arg+ heterozygous diploids. The positions of pertinent restriction sites and the *CaCET1* 5'-specific (A) and 3'-specific (B) hybridization probes are shown. Also shown is the configuration of the triplicated *cacet1::URA2* allele in the Arg+ Ura+ segregants.

G418-resistance or sensitivity (Table 1). We found that half of the Leu+ haploids derived from a *pct1+/pct1::kanMX* strains containing a plasmid with either the *pct1+* cDNA or *pct1+* gene (with intron) also contained the *pct1::kanMX* chromosomal allele and were resistant to G418. Similarly, half of the Leu+ haploids derived from a *pce+/pce1::kanMX* strain containing the *pce1+* plasmid were resistant to G418. In contrast, none of the Leu+ haploids derived from *pct1+/pct1::kanMX* or *pce1+/pce1::kanMX* strains containing the control *LEU2* plasmid vector lacking an insert were G418-resistant. These results show that the *pct1Δ* and *pce1Δ* strains are viable if the chromosomal deletions are complemented by an extrachromosomal triphosphatase or guanylyl-transferase gene. There was no apparent difference in complementation of *pct1Δ* by the intron-containing *pct1+* gene versus the *pct1+* cDNA.

Although the plasmid-encoded capping enzyme genes are under the control of a regulated *nmt1** promoter, which can be repressed by inclusion of 5 μg/ml thiamine in the growth medium [19], we observed that the growth of the plasmid-dependent strains was not affected by exogenous thiamine. We suspect that expression levels of the Pct1 or Pce1 enzymes in these strains exceeded a threshold required for cell viability.

Test of CaCET1 Essentiality in C. albicans
Candida albicans strains are diploid and do not undergo meiotic division. Thus, the classical approach of allelic disruption in diploid cells followed by sporulation and segregation analysis of haploids is not applicable to the analysis of gene function in *C. albicans*. Tests of gene essentiality in *Candida* necessitate serial disruption of both alleles using two different selection markers. If the gene of interest is nonessential, a homozygous diploid disruptant can be isolated. However, if the gene is essential, it will be impossible to disrupt both alleles. Mitchell and colleagues [16] have developed a single-transformation method to test gene function in diploid *C. albicans* that entails the following steps, which we have applied to

First we constructed a deletion allele plasmid containing 5' and 3' genomic sequence flanking the target *CaCET1* gene and an intervening marker cassette (*ura3Δ3'-ARG4-ura3Δ5'*, referred to as *UAU1*) composed of the *C. albicans ARG4* gene flanked by overlapping 5' and 3' fragments of the *URA3* gene. This construct deletes the coding sequence for amino acids 206 to 506 of the 520-aa CaCet1 polypeptide. The deleted segment contains the catalytic domain essential for triphosphatase activity *in vitro* and for complementation of the *cet1Δ* strain of *S. cerevisiae*[10,11]. Second, we introduced the linearized deletion allele into a diploid *C. albicans ura3/ura3 arg4/arg4* strain and selected for Arg+ transformants. Correct insertion via homologous recombination into one copy of the *CaCET1* gene, resulting in *cacet1::UAU1* (Figure 1), was confirmed by Southern blotting of genomic DNA digested with diagnostic restriction endonucleases. For example, a probe specific for the 5' end of the *CaCET1* gene (probe A in Figure 1) hybridized to a single 4.4 kbp *Bgl*II fragment after restriction digestion of total DNA from the parental diploid strain, whereas the heterozygote contained an additional 2.7-kbp fragment derived from scission at a novel *Bgl*II site located within the *ARG4* component of the *UAU1* insert of the disrupted *cacet1::UAU1* allele (Figure 2A, lane P versus lane H). The 2.7-kbp fragment was also detected with an *ARG4*-specific probe (not shown). We found that the heterozygous *CaCET1/cacet1::UAU1* strain displayed normal growth and morphology (not shown).

Third, we grew 54 independent liquid cultures of the heterozygotes in nonselective medium and then selected for cells that were Arg+ and Ura+. Uracil prototrophy re-
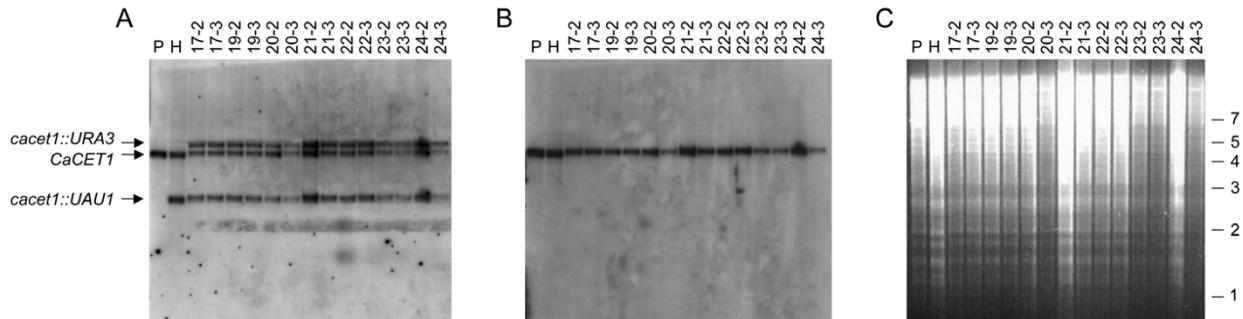
Figure 2
Southern blot analysis. DNA isolated from the parental diploid strain (lane P), one of the Arg⁺ heterozygotes (isolate #19; lane H), and fourteen independent Arg⁺ Ura⁺ segregants were digested with *Bgl*II and resolved by agarose gel electrophoresis. A photograph of the ethidium bromide-stained gel is shown in *panel C*. The positions and sizes (kbp) of DNA size markers are indicated on the *right*. The DNA was transferred to a Hybond membrane, which was serially hybridized to ³²P-labeled DNA probes A (*panel A*) and B (*panel B*) derived from the 5' and 3' segments of the *CaCET1* gene, respectively.

quires restitution of the integrity of the disrupted *ura3* gene of the *UAU1* cassette by recombination between the overlapping regions of the *ura3Δ3'* and *ura3Δ5'* fragments with excision of the intervening *ARG4* gene [16]. If *CaCET1* were nonessential, then recombination of *UAU1* into the second copy of *CaCET1* (to generate *cacet1::UAU1/cacet1::UAU1*) followed by excisional recombination of *ARG4* in one allele to restore *URA3* (generating *cacet1::UAU1/cacet1::URA3*) would result in the selected Arg⁺ Ura⁺ phenotype with complete loss of the wild-type *CaCET1* locus. However, if *CaCET1* is essential for growth, then all of the Arg⁺ Ura⁺ isolates will have three copies of the *CaCET1* locus (*cacet1::UAU1/cacet1:: URA3/CaCET1*).

We used Southern blotting to determine the genotype of one randomly selected Arg⁺ Ura⁺ derivative from each of the 54 separate cultures of the heterozygote diploids. The blots were probed with a 5' specific *CaCET1* fragment (probe A in Figure 1), which detects both the wild-type *CaCET1* allele and the *cacet1::UAU1* allele, and with a 3'-specific *CaCET1* fragment (probe B in Figure 1) derived from the segment deleted during construction of the *cacet1::UAU1* disruption cassette. Note that probe A hybridized to a single 4.4-kbp *Bgl*II fragment in both the parental diploid strain and the heterozygote (Figure 2B, lanes P and H), thereby verifying that it did not detect the disrupted allele. We found that 54/54 Arg⁺ Ura⁺ isolates retained the wild-type *CaCET1* locus (Figure 2 and data not shown), implying that *CaCET1* is an essential gene. All 54 isolates also retained the *cacet1::UAU1* allele that was present in the heterozygote (Fig. 2A and data not shown) and they acquired a new ~5-kbp *Bgl*II fragment that hybridized to 5'-specific *CaCET1* probe (Figure 2A

and data not shown). The novel *Bgl*II fragment migrated identically in 53/54 of the strains analyzed. Recombination within *UAU1* to regenerate *URA3* eliminates the *Bgl*II site and results in a *cacet1::URA3* locus that would yield an ~5-kbp fragment upon digestion with *Bgl*II (Figure 1). Therefore, we surmise that the vast majority of the events leading to the Arg⁺ Ura⁺ phenotype entailed allelic triplications. This conclusion is supported by additional Southern analyses of *Sca*I digests and *Eco*RI/*Pst*I digests of genomic DNA from the parental diploid, the heterozygotes, and the 54 Arg⁺ Ura⁺ segregants (data not shown).

We conducted in parallel an analysis of the function of the *C. albicans CES1/ZDS1* gene, which encodes a protein homologous to the product of the *S. cerevisiae CES1/ZDS1* gene isolated by us and others in various suppressor screens [[23] and references therein]. We found that 3/26 Arg⁺ Ura⁺ segregants emanating from a *CES1/ces1::UAU1* heterozygote were homozygous for disruption at both loci (*ces1::UAU1/ces1::URA3*) and had lost the wild-type *CES1/ZDS1* allele [B. Schwer, unpublished]. This frequency of homozygosity at a nonessential locus is similar to that reported by Mitchell's group (2 out of 30) for homozygous disruption of the *C. albicans CDC25* gene [16]. These results confirm that the single-transformation test can, in our hands, be used to identify a nonessential gene and they underscore the inference from the data presented here that RNA triphosphatase is essential for growth of *C. albicans*.

Discussion
Previous genetic analyses establishing the essentiality of cap formation were performed in the budding yeast *S.*

cerevisiae. It remained to be seen whether the homologs from other fungal species are also essential for viability. It is not a foregone conclusion that essentiality or dispensability of a gene product in *S. cerevisiae* can be extrapolated to pathogenic fungi. For example, Cdc25 is not essential in *C. albicans* whereas its homolog is essential in *S. cerevisiae*[16]. Conversely, the enzyme DNA topoisomerase I is nonessential in *S. cerevisiae* and *S. pombe,* but essential for viability in the pathogenic fungus *Cryptococcus neoformans*[24].

Here we have shown that the RNA triphosphatases Pct1 and CaCet1 are essential for viability of *S. pombe* and *C. albicans,* respectively The conclusion that CaCet1 is essential is based on a finding that none of the 54 independent isolates in the single-transformation test were homozygous for *cacet1Δ;* our interpretation is consistent with criteria established by Mitchell and colleagues for inference of essentiality using this genetic approach. De Backer et al. [25] had previously generated a single allele knockout in *C. albicans* of the guanylyltransferase component of the capping apparatus (Cgt1) using the URA-blaster technique and noted a variety of pleiotrophic effects on stress response, hygromycin sensitivity, and colony morphology in the *CGT1/cgt1Δ* heterozygote, but they found that the heterozygote was just as virulent as the wild-type strain in animal models of systemic candidiasis. They were unable to recover a homozygous *cgt1Δ/ cgt1Δ* isolate after a second transformation with the *URA3* disruption cassette after testing 13 transformants. Although their sample size was not large, their data, together with the present findings, indicate that the triphosphatase and guanylyltransferase are both essential for viability of *C. albicans*.

Conclusions

RNA triphosphatase is an attractive therapeutic target for fungal infections because: (i) the active site structure and catalytic mechanism of fungal RNA triphosphatase are completely different from the RNA triphosphatase domain of the metazoan capping enzyme and (ii) metazoans encode no identifiable homologs of the fungal RNA triphosphatases. Thus, a mechanism-based inhibitor of fungal RNA triphosphatase should be highly selective for the fungal pathogen and have minimal effect on the human or animal host. This scenario is plausible *only* if RNA triphosphatase is essential for growth of pathogenic fungi that cause human disease (e.g., *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans, Pneumocystis carinii,* etc.). The finding that the RNA triphosphatase CaCet1 is essential in the pathogenic fungus *C. albicans* provides impetus for the discovery of compounds that inhibit CaCet1 activity. Searches of public genome databases indicate that *Aspergillus fumigatus* (a major invasive pathogen in hu-

mans, with severe morbidity and mortality) encodes a homolog of Cet1, as does *Neurospora crassa.* Thus, we suspect that all fungal species will have metal-dependent RNA triphosphatases resembling those of *S. cerevisiae, C. albicans* and *S. pombe.*

Methods and materials
Gene disruption in S. pombe

We used a modified version of the long flanking homology PCR technique [17] to produce *pct1Δ* and *pce1Δ* gene disruption cassettes in which the open reading frames were replaced by the *kanMX* gene. For each gene, a set of four primers was synthesized: LI, a 20-mer corresponding to the sense-strand sequence of the 5'-flanking region ~1.2 kb upstream of the translation start codon of *pct1+* or *pce1+*; L2, a 40-mer in which 20 bases were identical to the 5' sequence of pFA6a-KanMX4 (GCTTCAGCTGGCGGCCGCGT) and 20 bases were identical to the antisense strand sequence immediately 5' of the translation start site of *pct1+* or *pce1+*; L3, a 40-mer in which 20 bases were identical to the 3' sequence of pFA6a-KanMX4 (AGTGGCCTATGCGGCCGCGG) and 20 bases corresponded to the sense-strand sequence immediately 3' of the stop codon of *pct1+* or *pce1+*; L4, a 20-mer corresponding to the antisense-strand sequence of the 3'-flanking region ~1 kb downstream of stop codon of *pct1+* or *pce1+*. In the first-stage PCR, a 5'-flanking fragment was synthesized using *S. pombe* genomic DNA as the template and LI plus L2 as primers. The 3' flanking fragment was synthesized using primers L3 and L4. In the second-stage PCR, aliquots of the purified products from the first amplification (0.1–0.2 μg) were mixed with 0.5 μg of *Not*I-digested pFA6a-kanMX4 and amplification synthesis was primed with the LI and L4 oligonucleotides. The products of the second PCR amplification were gel-purified and subcloned into pGEM-T (Promega). The recombinants were selected on LB agar medium containing 100 μg/ml ampicillin and 60 μg/ml kanamycin. The pPCT1Δ and pPCE1Δ plasmid constructs were confirmed by restriction enzyme digestion and partial sequencing. The *pct1::kanMX* cassette was PCR-amplified from the pPCT1Δ plasmid using primers LI and L4. The *pce1::kanMX* cassette was excised from PCE1Δ by digestion with *Aat*II and *Nde*I. The cassette fragments were gel-purified and then used to transform diploid *S. pombe.*

The *S. pombe* diploid strain was generated by crossing two heterothallic strains FY527(ura4-D18 *leuI-32 ade6-M216 his3-DI h⁻*) and *FY528(ura4-D18 leuI-32 ade6-M210 his3-D1 h⁺*) on ME plates at room temperature. After 24 h, the cells were streaked onto medium lacking adenine to select for diploids. The Ade⁺ diploids were verified by staining with phloxin B and a single diploid colony was picked and incubated in 100 ml of YE medi-

um to prepare competent *S. pombe* cells. The transformations were performed using the lithium acetate method [18]. The integrants were selected at 30°C on YE plates containing 200 µg/ml G418. Single colonies were restreaked on YE agar containing G418. Genomic DNA was prepared from individual isolates and the integration of the *pct1::kanMX* or *pce1::kanMX* cassettes into the correct locus was tested by PCR using diagnostic primers. The heterozygous diploids were sporulated on ME plates at room temperature. Tetrads were dissected from single asci and the spores were incubated at 30°C. All viable haploids were tested for growth on YES agar and YES agar containing 200 µg/ml G418.

S. pombe expression vectors for RNA triphosphatase and guanylyltransferase

The cDNA encoding Pct1 was amplified from plasmid pET-PCT1 [12] using primers that introduced an *Xho*I site immediately upstream of the translation start codon and a *Bam*HI site immediately downstream of the stop codon. The intron-containing chromosomal *pct1⁺* gene was amplified from total *S. pombe* genomic DNA. The intron-less *pce1⁺* gene was amplified from plasmid pl32-PCE1 [12]. The PCR products were digested with *Xho*I and *Bam*HI and then inserted into the *S. pombe* expression vector pREP41X (*LEU2 ars1⁺*) [19]. The inserts were sequenced to exclude the acquisition of unwanted mutations during the amplification and cloning steps. Expression of the capping enzymes from these plasmids is driven by the *nmt1** promoter [19]. The plasmids were transformed into heterozygous *pct1⁺/pct1::kanMX* or *pce1⁺/pct1::kanMX* diploids using the lithium acetate method [18]. The *Leu⁺* diploid transformants were then sporulated on ME plates at room temperature. A loopful of cells was inoculated into 500 µl of sterile water and the mixture was incubated overnight at 28°C with 10 µl of β-glucuronidase (Sigma G7770). The spores were plated on EMM(-Leu) agar medium and incubated at 30°C. Individual colonies were then restreaked onto YES agar and on YES agar containing 200 µg/ml G418. Growth was scored after incubation for 5 to 7 days at 30°C.

Gene disruption in C. albicans

The *CaCET1* gene was disrupted by insertion of a *UAU1* cassette [16]. We first constructed plasmid pKS-5'3'CaCET1, in which a 665-bp PCR fragment derived from the 5' end of the *CaCET1* gene (from nucleotides -50 to +615 of the open reading frame, with the A residue of the ATG translation start codon defined as position +1) was cloned between the *Kpn*I and *Xba*I sites of pBluescript *KS⁺* and a 720-bp fragment extending from position +1518 of the 1560-nt *CaCET1* coding sequence into the 3' flanking genomic region was inserted between the *Sac*I and *Sac*II sites of pBluescript KS⁺ The 3.8-kbp *UAU1* gene was excised from pBME101 with *Xba*I and

*Sac*II and inserted between the *Xba*I and *Sac*II sites of pKS-5'3'CaCET1 to yield pCaCET1::UAU1. This DNA was linearized with *Kpn*I and *Sac*I and then transformed into the diploid *C. albicans* strain BWP17 using the lithium acetate method. We selected 25 *Arg⁺* transformants and analyzed them by Southern blotting for integration of the *UAU1* cassette into one of the two *CaCET1* chromosomal loci to yield the heterozygote *CaCET1/cacet1::UAU1* configuration depicted in Figure 1. Briefly, genomic DNA was isolated from the 25 *Arg⁺* strains, then digested with *Sca*I (which cuts neither *CaCET1* nor *UAU1*). The digests were resolved by agarose gel-electrophoresis and transferred to membranes, which were probed with a radiolabeled DNA corresponding to the 5' segment of *CaCET1* (probe A in Figure 1). Whereas probe A hybridized to a single 3.8-kbp *Sca*I fragment in the parental BWP17 strain, the probe detected two fragments in the heterozygote − a 3.8-kbp fragment corresponding to the wild-type *CaCET1* locus and an ~7.5-kbp fragment corresponding to *cacet1::UAU1* (data not shown). This analysis identified 16/25 of the Arg⁺ transformants as *CaCET1/cacet1::UAU1* heterozygotes. Recombination rates at the *UAU1* gene in the heterozygote were determined as described by Enloe et al [16]. Ura⁺ segregants arose at a rate of 5×10^{-5} per division and Arg⁺ Ura⁺ segregants arose at rate of 8×10^{-9} per division.

The sixteen *CaCET1/cacet1::UAU1* heterozygotes were streaked to YPD agar and grown for 3 days at 30°C. A total of 54 single colonies derived from the 16 heterozygotes were inoculated into separate YPD liquid cultures. After growth to saturation, aliquots of the cultures were plated on SD(-Arg-Ura) agar medium. Genomic DNA was prepared from one Arg⁺ Ura⁺ segregant from each culture and subjected to restriction digestion and Southern analysis.

References

1. Shuman S: **Structure, mechanism, and evolution of the mRNA capping apparatus.** *Prog. Nucleic Acid Res. Mol. Biol* 2000, **66**:1-40
2. Lima CD, Wang LK, Shuman S: **Structure and mechanism of yeast RNA triphosphatase: an essential component of the mRNA capping apparatus.** *Cell* 1999, **99**:533-543
3. Changela A, Ho CK, Martin A, Shuman S, Mondragon A: **Structure and mechanism of the RNA triphosphatase component of mammalian mRNA capping enzyme.** *EMBO J* 2001, **20**:2575-2586
4. 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* 1992, **267**:9521-9528
5. Tsukamoto T, Shibagaki Y, Imajoh-Ohmi S, Murakoshi T, Suzuki M, Nakamura A, Gotoh H, Mizumoto K: **Isolation and characterization of the yeast mRNA capping enzyme □ subunit gene encoding RNA 5'-triphosphatase, which is essential for cell viability.** *Biochem. Biophys. Res. Comm* 1997, **239**:116-122

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

7. Ho CK, Pei Y, Shuman S: **Yeast and viral RNA 5' triphosphatases comprise a new nucleoside triphosphatase family.** *J. Biol. Chem* 1998, **273**:34151-34156

8. Pei YHCK, Schwer B, Shuman S: **Mutational analyses of yeast RNA triphosphatases highlight a common mechanism of metal-dependent NTP hydrolysis and a means of targeting enzymes to pre-mRNAs in vivo by fusion to the guanylyltransferase component of the capping apparatus.** *J. Biol. Chem* 1999, **274**:28865-28874

9. Bisaillon M, Shuman S: **Structure-function analysis of the active site tunnel of yeast RNA triphosphatase.** *J. Biol. Chem* 2001, **276**:17261-17266

10. Pei Y, Lehman K, Tian L, Shuman S: **Characterization of Candida albicans RNA triphosphatase and mutational analysis of its active site.** *Nucleic Acids Res* 2000, **28**:1885-1892

11. Schwer B, Lehman K, Saha N, Shuman S: **Characterization of the mRNA capping apparatus of Candida albicans.** *J. Biol. Chem* 2001, **276**:1857-1864

12. Pei Y, Schwer B, Hausmann S, Shuman S: **Characterization of Schizosaccharomyces pombe RNA triphosphatase.** *Nucleic Acids Res* 2001, **29**:387-396

13. Ho CK, Shuman S: **A yeast-like mRNA capping apparatus in Plasmodium falciparum.** *Proc. Natl. Acad. Sci. USA* 2001, **98**:3050-3055

14. Ho CK, Gong C, Shuman S: **RNA triphosphatase component of the mRNA capping apparatus of Paramecium bursaria Chlorella virus 1.** *J. Virol* 2001, **75**:1744-1750

15. Rodriguez CR, Takagi T, Cho E, Buratowski S: **A Saccharomyces cerevisiae RNA 5'-triphosphatase related to mRNA capping enzyme.** *Nucleic Acids. Res* 1999, **27**:2182-2188

16. Enloe B, Diamond A, Mitchell AP: **A single-transformation gene function test in diploid Candida albicans.** *J. Bacterial* 2000, **182**:5730-5736

17. Wach A: **PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in S. cerevisiae.** *Yeast* 1996, **12**:259-265

18. Gietz D, St Jean A, Woods RA, Schiestl RH: **Improved method for high efficiency transformation of intact yeast cells.** *Nucleic Acids Res* 1992, **20**:1425

19. Forsburg SL: **Comparison of Schizosaccharomyces pombe expression systems.** *Nucleic Acids Res* 1993, **21**:2955-2956

20. 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* 1994, **91**:12046-12050

21. Hausmann S, Ho CK, Schwer B, Shuman S: **An essential function of Saccharomyces cerevisiae RNA triphosphatase Cet1 is to stabilize RNA guanylyltransferase Ceg1 against thermal inactivation.** *J. Biol. Chem* 2001, **276**:36116-36124

22. Yamada-Okabe T, Mio T, Matsui M, Kashim Y, Arisawa M, Yamada-Okabe H: **Isolation and characterization of the Candida albicans gene for mRNA 5' triphosphatase: association of mRNA 5' triphosphatase and mRNA 5' guanylyltransferase activities is essential for the function of mRNA 5' capping enzyme in vivo.** *FEBS Lett* 1998, **435**:49-54

23. Schwer B, Linder P, Shuman S: **Effects of deletion mutations in the yeast Ces1 protein on cell growth and morphology and on high copy suppression of mutations in mRNA capping enzyme and translation initiation factor 4A.** *Nucleic Acids Res* 1998, **26**:803-809

24. Del Poeta M, Tofaletti DL, Rude TH, Dykstra CC, Heitman J, Perfect JR: **Topoisomerase 1 is essential in Cryptococcus neoformans: role in pathobiology and as an antifungal target.** *Genetics* 1999, **152**:167-178

25. DeBacker MD, de Hoogt RA, Froyen G, Odds FC, Simons F, Contreras R, Luyten WHM: **Single allele knock-out of Candida albicans CGT1 leads to unexpected resistance to hygromycin B and elevated temperature.** *Microbiology* 2000, **146**:353-365