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

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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-
transfases 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 (Ceti), guanylyltransferase (Ceg1), and methyltransferase (Abd1) components of the capping apparatus are essential for cell growth in the budding yeast *Saccharomyces cerevisiae* [1,4–6]. Mutations of the RNA triphosphatase Ceti that abrogate catalytic activity *in vitro* are lethal *in vivo* [7–9]; thus, it is reasonable to think that pharmacological inhibition of Ceti 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 triphosphatasases 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* Ceti, *C. albicans* CaCeti and *S. pombe* Pett, 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* Ceti. 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 CaCeti and Pett [2]. Mutational analysis of Ceti has identified 15 individual side chains within the tunnel that are important for Ceti 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 Ceti and CaCeti [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 (Ceti and Cth1), of which only Ceti 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 guanylyltransferase 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 Ceti homolog in the *Aperigillus 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 Pett is a 303-amino acid polypeptide with a homodimeric quaternary structure [12]. The pett+ 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 pett+ gene. Although recombinant Pett 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 Pett or Pce1 in fission yeast. Here we constructed *pett*Δ and *pce1*Δ 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 *pett::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 *pett::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 *pett::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 *pett*Δ and *pce1*Δ**

The *pett*Δ and *pce1*Δ cDNAs were cloned separately into the *S. pombe* expression vector pREP41X (LEU2 2ARS1) so as to place them under the control of the nmt1* promoter. We also cloned the intron-containing chromosomal pett* gene into the same expression vector. The plasmids were introduced into heterozygous pett*/pett::kanMX or pce1*/pce1::kanMX diploids. The Leu* diploid transformants were selected and then sporulated. A random population of Leu* haploids was tested for...
These results show that the plasmid vector lacking an insert were G418-resistant. pce1 of the LeuCandida strains were Arg+ or Ura+ (Table 1). We found that the pct1::kanMX chromosomal allele and were resistant to G418. Similarly, half of the Leu+ haploids derived from a pce1::kanMX strain containing the plasmid vector lacking an insert were G418-resistant. In contrast, none of the Leu+ haploids derived from pce1::kanMX or pce1::kanMX strains containing the control LEU2 plasmid vector lacking an insert were G418-resistant. These results show that the pct1 and pce1 strains are viable if the chromosomal deletions are complemented by an extrachromosomal triphosphatase or guanylyltransferase gene. There was no apparent difference in complementation of pct1 by the intron-containing cDNA gene versus the pce1 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

Table 1: Plasmid-based Complementation of pct1 and pce1

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

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 pce1 cDNA or pce1 gene (with intron) also contained the pct1::kanMX chromosomal allele and were resistant to G418. Similarly, half of the Leu+ haploids derived from a pce1+/pce1::kanMX strain containing the pct1 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 guanylyltransferase gene. There was no apparent difference in complementation of pct1Δ by the intron-containing pct1 cDNA gene versus the pce1 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.

First we constructed a deletion allele plasmid containing 5' and 3' genomic sequence flanking the target CaCET1 gene and an intervening marker cassette (ura3Δ4'-ARG4-ura3Δ4', 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 cet1::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 BglII 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 BglII site located within the ARG4 component of the UAU1 insert of the disrupted cet1::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/cet1::UAU1 strain displayed normal growth and morphology (not shown).

Figure 1

**Genotype of the CaCET1/cet1::UAU1 heterozygote strain of C. albicans.** Illustrated in cartoon form are the configurations of the wild-type CaCET1 and the cet1::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 cet1::URA2 allele in the Arg+ Ura+ segregants.

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-
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 BglII 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 BglII fragment that hybridized to 5'-specific CaCET1 probe (Figure 2A and data not shown). The novel BglII fragment migrated identically in 53/54 of the strains analyzed. Recombination within UAU1 to regenerate URA3 eliminates the BglII site and results in a cacet1::URA3 locus that would yield an ~5-kbp fragment upon digestion with BglII (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 ScaI digests and EcoRI/PstI 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 Bacter et al.[25] had previously generated a single allele knockout in *C. albicans* of the guanyltransferase 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 guanyltransferase 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 human, 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 pct1Δ or pce1Δ; L2, a 40-mer in which 20 bases were identical to the 5' sequence of pFA6a-KanMX4 (GCTTCAGCT-GGCACGCCCAGGT) and 20 bases were identical to the antisense strand sequence immediately 5' of the translation start site of pct1Δ or pce1Δ; L3, a 40-mer in which 20 bases were identical to the 3' sequence of pFA6a-KanMX4 (AGTGCTATGCGGCCCAG) and 20 bases corresponded to the sense-strand sequence immediately 3' of the stop codon of pct1Δ or pce1Δ; L4, a 20-mer corresponding to the antisense-strand sequence of the 3'-flanking region ~1 kb downstream of stop codon of pct1Δ or pce1Δ. 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 NotI-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 AatII and NdeI. 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 leu1-32 ade6-M216 his3-D1I h*) and FY528(ura4-D18 leu1-32 ade6-M210 his3-D1I h*+) on ME plates at room temperature. After 24 h, the cells were streaked onto medium lacking adenine to select for dipsids. 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 pett::kanMX or pett::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 ascii and the spores were incubated at 30°C. All viable haploids were tested for growth on YES agar and G418 agar containing 200 μg/ml G418.

S. pombe expression vectors for RNA triphosphatase and guanylyltransferase

The cDNA encoding Pett was amplified from plasmid pET-PCT1 [12] using primers that introduced an XhoI site immediately upstream of the translation start codon and a BamHI site immediately downstream of the stop codon. The intron-containing chromosomal pett+ gene was amplified from total S. pombe genomic DNA. The intron-less pett+ gene was amplified from plasmid pJ32-PCE1 [12]. The PCR products were digested with XhoI and BamHI 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 pett+/pett::kanMX or pett+/pett::kanMX diploids using the lithium acetate method [18]. The Let+ 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-L(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 KpnI and XbaI 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 SacI and SacII sites of pBluescript KS+. The 3.8-kbp pUAU1 gene was excised from pBME101 with XbaI and SacII and inserted between the XbaI and SacII sites of pKS-5'3′CaCET1 to yield pCaCET1::UAU1. This DNA was linearized with KpnI and SacI 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 SacI (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 SacI 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 acet1::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 x 10-5 per division and Arg+ Ura+ segregants arose at rate of 8 x 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.

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