Two transcripts, differing at their 3' ends, are produced from the *Candida albicans* SEC14 gene

Perry J. Riggle, Igor V. Slobodkin,[†] Douglas H. Brown Jr, Mark P. Hanson, Thomas L. Volkert and Carol A. Kumamoto

Author for correspondence: Carol A. Kumamoto. Tel: +1 617 636 0404. Fax: +1 617 636 0337.
e-mail: ckumamot@opal.tufts.edu

Department of Molecular
Biology and Microbiology,
Tufts University,
136 Harrison Ave, Boston,
MA 02111, USA

A search for *Candida albicans* mutants defective in filamentous growth led to the isolation of a mutant strain with an insertion mutation in the SEC14 gene. SEC14 encodes the phosphatidylinositol/phosphatidylcholine transfer protein, an essential protein in the yeast *Saccharomyces cerevisiae*. In the dimorphic yeast *Yarrowia lipolytica*, SEC14 is needed for growth only in the hyphal form and is not required for growth in the yeast form. However, unlike *Y. lipolytica* SEC14, *C. albicans* SEC14 is probably essential for growth. Northern blot analysis and PCR amplification of transcripts produced from the SEC14 gene demonstrated that two transcripts differing at their 3' ends were produced. The two transcripts may regulate the activity of SEC14 so that Sec14p can perform two functions in *C. albicans*. One function may be an essential function analogous to the function of Sec14p in *S. cerevisiae* and the second function may be important during filamentous growth, analogous to the function of Sec14p in *Y. lipolytica*.

Keywords: *Candida albicans*, SEC14, transcripts, restriction enzyme-mediated integration

INTRODUCTION

The opportunistic pathogen *Candida albicans* has become an increasingly important pathogen in recent years, particularly in hospital settings (Pfaller, 1994). Immune-compromised patients such as oncology patients and transplant recipients are susceptible to life-threatening infections by *C. albicans*. As a means of improving methods for treatment of such patients, increased understanding of the biology of *C. albicans* is important.

In this paper, an analysis of the *C. albicans* SEC14 gene is described. SEC14, encoding the phosphatidylinositol/phosphatidylcholine transfer protein, has been extensively studied in *Saccharomyces cerevisiae* (Bankaitis *et al.*, 1990). SEC14 is an essential gene in *S. cerevisiae* and its product is required for normal function of the protein

secretory pathway. Bankaitis and coworkers have proposed that SEC14 acts as a sensor of lipid composition in the Golgi (McGee *et al.*, 1994). Sec14p has been shown to regulate the activity of the enzyme choline-phosphate cytidyltransferase and, thereby, to modulate the activity of the CDP-choline pathway for phosphatidylcholine biosynthesis in *S. cerevisiae* (Skinner *et al.*, 1995). Recently, the *C. albicans* SEC14 gene was characterized (Monteoliva *et al.*, 1996). *C. albicans* SEC14 was shown to be highly homologous to the *S. cerevisiae* SEC14 gene. In addition, it was suggested that *C. albicans* SEC14 may be an essential gene (Monteoliva *et al.*, 1996).

In the dimorphic yeast *Yarrowia lipolytica*, SEC14 is not essential for growth when the cells are in the yeast form and it is not required for function of the protein secretory pathway (Lopez *et al.*, 1994). *Y. lipolytica* sec14 mutants are viable but exhibit defects in filamentous growth. Thus, in this organism, SEC14 has a function that appears to be unrelated to the protein secretory pathway.

C. albicans is also a dimorphic yeast, capable of growth by budding as a yeast cell or of growth in the filamentous

Abbreviations: REMI, restriction enzyme-mediated integration; UTR, untranslated region.

[†] Deceased.

The GenBank accession number for the sequence reported in this paper is AF005329.

forms, hyphae and pseudohyphae. These filamentous forms are preferentially observed in specimens taken from infected sites and it is generally believed that filamentous growth contributes to the virulence of *C. albicans* (Odds, 1994). In this study, a search for genes that were required for filamentous growth led to the isolation and study of the *SEC14* gene. An analysis of *SEC14* transcription revealed that two transcripts differing at their 3' ends are produced from the *SEC14* gene.

To isolate mutant strains defective in filamentous growth, restriction enzyme-mediated integration (REMI) was used as a means of mutagenizing the chromosome of *C. albicans*. REMI involves transformation of an organism with a linear fragment of DNA that is not homologous to its chromosome, in the presence of the enzyme used to produce the DNA fragment. In the presence of enzyme, the transforming DNA becomes integrated into the chromosome in an appropriate restriction site. This procedure, first used in *S. cerevisiae* (Schiestl & Petes, 1991), has been used as a method of insertion mutagenesis in *Dictyostelium discoideum* (Kuspa & Loomis, 1992) and *Ustilago maydis* (Bolker *et al.*, 1995). We have previously described stable integration of transforming DNA onto the chromosome of *C. albicans* cells using REMI (Brown *et al.*, 1996). In this study, REMI was used as a method for producing insertion mutants and a strain containing an insertion in the *SEC14* gene was isolated. However, further analysis showed that the insertion mutation is not responsible for the defect in filamentous growth exhibited by the mutant strain.

METHODS

Strains and plasmids. *C. albicans* strains SGY-243 (Δ ura3/ Δ ura3) (Kelly *et al.*, 1987) and CAI-4 (Δ ura3/ Δ ura3) (Fonzi & Irwin, 1993) were used as the parents of all strains in this study. Strain IVS9 was isolated as a mutant defective in filamentous growth after mutagenesis of SGY-243 by REMI and UV treatment. IVS9 carries an insertion of pCK61 DNA in one copy of *SEC14*. Strains IVS10 and IVS11 were constructed from CAI-4 using the Ura-blaster method (Alani *et al.*, 1987). CAI-4 was transformed by electroporation with pIS7 after digestion of the plasmid with *SpeI* and *HpaI*. pIS7 contains a deletion in the *SEC14* locus extending from a *BglII* site upstream of *SEC14* to the *HindIII* site within the *SEC14* structural gene (Fig. 1). A *BglII*–*HindIII* fragment from pMB-7 (Fonzi & Irwin, 1993), encoding *C. albicans* URA3 flanked by direct repeats of DNA encoding *hisG* from *Salmonella typhimurium* (the *hisG*-URA3-*hisG* Ura-blaster cassette), was cloned into this deletion. After transformation, Ura⁺ colonies were selected. Transformants were screened by Southern blot analysis and a strain designated IVS10 (*SEC14*⁺/ Δ *sec14*::*hisG*-URA3-*hisG*) was isolated. Ura⁺ derivatives of strain IVS10 were selected by plating cells on medium containing 5-fluoroorotic acid (1 mg ml⁻¹) (Ausubel *et al.*, 1989). The resulting Ura⁺, heterozygous *SEC14* deletion strain was designated IVS11 and verified by Southern blot analysis.

Plasmid pCK61 (pBR322 carrying *C. albicans* URA3) has been described previously (Brown *et al.*, 1996). pIS2, isolated from the chromosome of strain IVS9, is pCK61 containing addi-

Fig. 1. Restriction map of the *SEC14* locus. The map of the chromosome in the vicinity of the *SEC14* gene is shown. Only relevant restriction sites are illustrated. A polyA site determined from cDNA analysis is also shown. Open boxes denote probes used for hybridization in Figs 4 and 5. The DNA contained on plasmids used in this study is indicated below the restriction map.

tional DNA derived from the *SEC14* locus (Fig. 1). To create strain IVS9, pCK61 was digested with *Bam*HI and integrated into the *Bam*HI site of *SEC14* by REMI. Digestion of IVS9 chromosomal DNA with the enzyme *Hind*III, which does not cleave within pCK61, followed by ligation and transformation of *Escherichia coli*, allowed the isolation of a plasmid (pIS2) containing sequences derived from the chromosomal DNA flanking the site of insertion.

Clones encoding an intact copy of *SEC14* were obtained by hybridization of a probe derived from pIS2 to a plasmid library of SGY-243 genomic DNA fragments (D. Brown & C. Kumamoto, unpublished). Two of these clones, designated pIS4 and pIS6, contained inserts of approximately 3 and 5.5 kb, respectively, including the *SEC14* gene (Fig. 1).

E. coli strain XL1-Blue (Stratagene) was used for plasmid production.

Media. Media for growth of *C. albicans* strains were YPD rich medium and CM-ura minimal medium, as described by Ausubel *et al.* (1989). Liquid minimal medium was NC glucose [Yeast Nitrogen Base without amino acids (Difco), 0.1% Casamino acids (Difco), 2% glucose], as described previously (Brown *et al.*, 1996). To stimulate filamentous growth of strain SGY-243, cells were grown at 30 °C in YPD medium for 1–2 d until the cultures reached high culture density. The cells were washed and starved in water overnight. The cells were diluted 100-fold into GT1 medium (2% yeast extract, 2% glucose, 20 µg uridine ml⁻¹) or GT2 medium (0.4% yeast extract, 0.4% glucose, 4 µg uridine ml⁻¹) and incubated at 37 or 40 °C for 4 h. Both SGY-243 and CAI-4 produced germ tubes upon culturing in GT1 or GT2 medium at 37 or 40 °C.

For growth of *E. coli*, L broth (Miller, 1972) with or without 100 µg ampicillin ml⁻¹ was used.

Transformation by electroporation. Electroporation of *C. albicans* was performed essentially as described previously (Brown *et al.*, 1996), except that the first two washes of cells were performed using water rather than 10% (v/v) glycerol. The last two washes of cells were performed using 10% glycerol. Cells were frozen in 50 µl aliquots at –80 °C. For transformation by REMI, an aliquot of cells was mixed with 18 µg *Bam*HI-digested pCK61 DNA and 100 units *Bam*HI (New England Biolabs). The sample was electroporated at 2.0 kV, 25 µF, 200 Ω in a 0.2 cm gap cuvette (Bio-Rad). The electroporated cells were diluted with chilled YPD and plated on CM-ura plates. Integrative transformation by electro-

poration was performed similarly except that *Bam*HI was omitted and lower amounts of DNA were used.

Electroporation of *E. coli* was performed using a Bio-Rad Gene Pulser electroporator according to the manufacturer's recommendations.

Isolation of mutant IVS9. Strain SGY-243 was subjected to REMI mutagenesis with *Bam*HI-digested pCK61. Approximately 8000 transformants were obtained in 10 independent pools. Cells from each pool were grown in NC glucose liquid medium, cells were spread on YPD plates and the plates were irradiated with UV light for 40 s. After incubation, cells were scraped off the plates and grown in liquid culture in YPD at 30 °C. After 1–2 d of growth, when the cultures had reached high density, the cells were washed with water, resuspended in water and starved overnight. Cells were diluted 100 fold into GT1 medium and incubated at 37 °C for approximately 4 h. Following this treatment, the cultures were filtered through Whatman No. 1 filter paper. YPD was added to the filtrate and the cells were grown in YPD at 30 °C to high culture density. The mutagenized pools of cells were cycled through this enrichment procedure 4–6 times. After the last round of enrichment, cells were plated and allowed to form colonies on YPD plates. Individual colonies were tested for their ability to initiate filamentous growth. One colony from each independent pool was retained for further study.

DNA analysis. *C. albicans* chromosomal DNA extraction was performed either as described previously (Brown *et al.*, 1996) or using Qiagen columns, according to manufacturer's instructions. Plasmid DNA extractions, restriction digestion, agarose gel electrophoresis and Southern blotting were performed by standard methods (Sambrook *et al.*, 1989). DNA sequencing was performed using the Circumvent cycle sequencing kit (New England Biolabs) according to manufacturer's instructions or by the DNA Sequencing Core Facility at the Tufts University Protein Analysis Facility. Oligonucleotides were synthesized by Mike Berne of the Tufts University Protein Analysis Facility. DNA fragments were amplified by PCR (Mullis *et al.*, 1986) and PCR products were cloned using the Invitrogen TA cloning kit according to manufacturer's instructions.

RNA analysis. For RNA extraction, cells were lysed using glass beads (Ausubel *et al.*, 1989) and the lysate was extracted with phenol (Ausubel *et al.*, 1989) or Trizol (Gibco BRL). Ten or twenty micrograms of total RNA per sample was separated on a formaldehyde/agarose gel, transferred to a Nytran-plus membrane and probed by standard Northern blotting methods (Sambrook *et al.*, 1989). To prepare probe B (Fig. 1), pIS4 plasmid DNA encoding *SEC14*⁺ was amplified with primer MTTM (5' ATGACTACGATGACTACTGAAGA), corresponding to the first 8 codons of *SEC14*, and primer 1 (5' TGTGTGTGTTGGTGTATGTATGTT; see Fig. 5), complementary to sequences contained in the *SEC14* 3' untranslated region (UTR). A single PCR product was obtained. Inverse PCR was used to prepare probe C (Fig. 1) as follows. Plasmid pIS4 was digested with *Sau*3A and the fragments were ligated under dilute conditions. The ligated DNA was amplified with primers 5' CTTATTAACATTAGTTC and 5' CTAGGACATTACACC, corresponding to nt 127–143 (see Fig. 5) and the complement of 1222–1235 (Monteoliva *et al.*, 1996), respectively, to amplify DNA downstream of the known *SEC14* 3' end determined from cDNA analysis. The amplified DNA was cleaved with *Sau*3A to release sequences derived from the known 3' UTR that were joined to the downstream sequence by ligation. Probes A and D (Fig. 1) were generated by digestion of plasmids pIS4 and pIS2, respectively. PCR

products were purified using Qiagen spin columns. Fragments were labelled by random priming with [α -³²P]dATP. Size standards were visualized by staining with methylene blue (Wilkinson *et al.*, 1990). Blots were quantified using ImageQuant software (Molecular Dynamics) after either densitometric scanning of films or scanning of the blot using a Molecular Dynamics Phosphorimager. To calculate the ratio of the transcripts, the amount of the band representing the larger transcript was divided by the amount of the band representing the shorter transcript.

Analysis of cDNA. Total RNA was extracted from SGY-243 cells grown at 30 °C in YPD. PolyA⁺ RNA was purified from this RNA using the Invitrogen Fastrack Kit 2.0, according to manufacturer's instructions. The RNA was reverse transcribed using primer oligo-dT (NXT₁₈, where N is any of the four bases and X is A, C or G) as the primer (Ausubel *et al.*, 1989). To amplify the coding sequence and 3' UTR, the cDNA was amplified with primers MTTM and oligo-dT. Products of approximately the expected size, based on the amino acid sequence of Sec14p and expected sizes of the UTR, were cloned using the Invitrogen TA cloning kit. Clones containing *SEC14* cDNAs were identified by colony hybridization (Sambrook *et al.*, 1989). To amplify the 5' UTR, the cDNA was incubated with dCTP and terminal deoxynucleotide transferase and the products were amplified with the primers 5' AGGAATTCG₁₅ and either 5' TTTGATCGGTTGGAG-CGGTGAT or 5' AGGTGTTTAATTTGATGTGTAACC, corresponding to the complements of Sec14p residues 16–22 and 24–31, respectively. Amplified products were cloned as above.

To detect the second, longer *SEC14* transcript, cDNA was amplified using primer MTTM and either primer 1, primer 2 (5' ATTAAGGGAATTTCAAC) or primer 3 (5' GTATTTGCTATGTGGGTC), corresponding to the complement of nucleotides 24–47, 287–303 or 427–444 (see Fig. 5), respectively.

RESULTS

Restriction enzyme-mediated integration produces chromosomal insertions at a large number of sites

Previously, the ability of a restriction enzyme, *Bam*HI, to stimulate non-homologous transformation of DNA into *C. albicans* was reported (Brown *et al.*, 1996). Transformants were found to contain insertions of the transforming DNA at chromosomal *Bam*HI sites. Since such transformation events could disrupt structural genes, it was possible that REMI could be used to mutagenize the *C. albicans* genome, if the insertions occurred without preference for *Bam*HI sites. In a previous study, a small number of transformants were analysed and found to contain insertions of the transforming DNA at different *Bam*HI sites (Brown *et al.*, 1996).

To demonstrate that large numbers of sites could be used and that there were no preferred sites for integration, larger numbers of transformants were analysed. In this analysis, strain SGY-243 was transformed with *Bam*HI-digested pCK61 in the presence of *Bam*HI enzyme. Chromosomal DNA was prepared from two pools containing 50 Ura⁺ transformants and one pool containing 1000 Ura⁺ transformants. The DNA

Fig. 2. Analysis of the sites of plasmid integration in strains subjected to REMI. (a) A restriction map of the *URA3* locus of strain SGY-243 is shown, with the portion that hybridizes to the probe indicated by the black box. A diagram of a hypothetical insertion mutation is shown below. The open box indicates pCK61 sequences and the black portion indicates the fragment that hybridizes to the probe. B, *Bam*HI; C, *Cla*I. (b) Southern blot of chromosomal DNA isolated from transformants constructed by transforming SGY-243 cells with *Bam*HI-digested pCK61 in the presence of *Bam*HI enzyme. Chromosomal DNA was prepared from two pools of 50 transformants (lanes 1 and 2) and from one pool of 1000 transformants (lane 3). DNA was prepared as described in Methods and digested with *Cla*I. The fragments were separated by electrophoresis, transferred to a Nytran membrane and probed with a fragment containing the *Pst*I-*Sca*I fragment encoding *URA3*. The fragment marked '1' corresponds to the chromosomal *URA3* locus.

was digested with *Cla*I and analysed by Southern blot hybridization using the *URA3* gene as the probe. The chromosomal *URA3* locus in all strains contained a fragment that hybridized to the probe (Fig. 2a). This fragment, which is seen when chromosomal DNA from the starting strain SGY-243 is hybridized (data not shown), is prominent in the Southern blot of pools of transformants because each transformant contains this

band (Fig. 2b). In addition, each integrated plasmid gave rise to a hybridizing band corresponding to the junction between the transforming plasmid and chromosomal sequence (Fig. 2a). The size of this band is determined by the position of the first *Cla*I site in the chromosomal DNA adjacent to the insertion. In the two pools of 50 transformants, many bands appeared, corresponding to the junctions between plasmid and chromosome in the different transformants (Fig. 2b). The patterns of bands differed between the two pools, indicating that different sequences are adjacent to the transforming plasmids in the strains in the two pools of 50. The blot of the pool of 1000 transformants showed a continuous smear (Fig. 2b), indicating that a large number of different sequences are adjacent to the transforming plasmids in the different strains in the pool. There were no bands that were preferentially represented to a significant extent in the pool of 1000 transformants, demonstrating that there are no preferentially used sites. These results demonstrate that integration at a large number of chromosomal *Bam*HI sites can occur. Therefore, REMI should be useful as a means of mutagenizing the chromosome.

C. albicans is a diploid organism (Poulter, 1990) and the insertion mutations generated by REMI would be expected to be heterozygous. Since heterozygous mutants might not exhibit a phenotype, an additional event would be needed to reveal the mutation. For example, if homozygous insertion mutants could be isolated, such mutants could exhibit a phenotypic defect. Alternatively, since a heterozygous insertion mutation contains only one functional copy of the gene, a point mutation in the remaining functional copy could create a mutant strain with an observable phenotype. Thus, in conjunction with either of these processes, REMI insertion mutagenesis can be useful for mutant isolation in *C. albicans*.

Isolation of a mutant containing an insertion in the *SEC14* gene

To isolate *C. albicans* mutants defective in filamentous growth after REMI mutagenesis, pools of transformants were first irradiated with UV light to stimulate mitotic recombination and generate homozygous insertions (Poulter, 1990). Filtration enrichment, described in Methods, was then used to isolate mutant strains that were defective in filamentous growth. Populations of mutagenized cells were cultured under conditions that favour filamentous growth and then filtered. Cells from the filtrate were grown and then refiltered. After four to six rounds of filtration-enrichment, individual cells were plated and single colonies were screened for defects in filamentous growth. One of the mutants identified by this screen was strain IVS9.

Plasmid DNA was recovered from chromosomal DNA isolated from strain IVS9 to identify the gene that had sustained an insertion in this strain. The chromosomal DNA was digested with an enzyme that does not cleave within the transforming plasmid pCK61, so that the recovered DNA included portions of the *C. albicans*

Ca	MTTMTTEEI LASYPOI TAPTDQTYSTNLTPEQKTTLDI FRQQLTELGYK	50
Sc	VTQ- QEKEFLESYPONCPDLPPTGNL DSAQEKALAEKRLLEDAGFI	49
Kl	MV- SEQEI LESYPOVCPSSGLSGTPGNLDSEAEKLEFRELLESLGK	48
Yl	MTV- TEQFLASYPQKVPAGGPTGYPGNLTAEQEKLGELKMI LLTKGYE	49
Ca	DRLDDASLLRFLRARKFDI QKAI DMFVACEKWREDFGVNTLLKDEHYEEK	100
Sc	ERLDDSTLLRFLRARKFDVQLAKEMFENCEKWRKYDGTDTI LQDFHYDEK	99
Kl	ERLDDSTLLRFLRARKFDLEASKI MYENCEKWRKEFGVDTI FEDFYHEEN	98
Yl	DRTDDATLLRFLRARKFDVPLAQEMWENCEKWRKEFGTNTI LEDFWYKEK	99
Ca	PI VAKMYPTYYHKTDKDGRPVYFEELGKVDLVKML KI TTQERMLKNLVWE	150
Sc	PLI AKFYPPQYYHKTDKDGRPVYFEELGAVNLHEMNKVTSEERMLKNLVWE	149
Kl	TLVAKYPPQYYHKTNDGRPVYI EELGSVNLTKMYKI TTQERMLKNLVWE	148
Yl	KEVAKLYPPQYYHKTDKDGRPVYVENGVKNI HEMYKI TTQERMLRNLVWE	149
Ca	YEAMCOYRLPACSRKAGYLVETSTCTVLDLSGI SVTSAYNVI GYVREASKI	200
Sc	YESVVOYRLPACSRRAAGHLVETSTCTI MDLKGSI SI SSAYSVMYVREASVI	199
Kl	YEAFFRYRLPACSRKAGYLVETSTCTI LDLSGI SI SSAAQVLSYVREASNI	198
Yl	YESFVRHRLPACSRVVGHLI ETSTCTI LDLSGVSLSASQVYGFLLKASNI	199
Ca	QDQYYPERMKGKFI L NAPFGFSTAFKLFKFLDPVTVSKI HI LGSSYKKE	250
Sc	QNYYPERMKGKFI L NAPFGFSTAFRLKFLDPVTVSKI FI LGSSYQKE	249
Kl	QNYYPERMKGKFI L NAPFGFSTAFRLTKFLDPVTVSKI FI LGSSYQKD	248
Yl	QNYYPERMKGKFI L NAPFGFSTVFSVI KFLDPVTVSKI HVGSGNYKEK	249
	BamHI	
Ca	LLKQI PPONLPVKFGGMSDVS- - DDDLLKDVGPWRDPEFI GPE- - - - -	293
Sc	LLKQI PAENLPVKFGGSEVDSEKGLYLSI GPWRDPKYI GPE- - - - -	294
Kl	LLKQI PAENLPKKFGGSEVSEAEGLYLSI GPWRREEYI GPE- - - - -	293
Yl	LLAQVPAYNLPVKFGGSS- S- - KI GVELSDDGWRDPQFVGPFAWPPW	296
Ca	GECPRAYNI	301
Sc	GEAPEAFSNK	303
Kl	GEAPKAFQL	301
Yl	VSDPPVP	303

Fig. 3. Deduced amino acid sequence of *C. albicans* Sec14p aligned with the Sec14 gene products from other yeasts. Underlined amino acids indicate residues that are identical in all four sequences. Boxed residues indicate the 105 aa residues used to identify the *C. albicans* SEC14 gene. The BamHI site that sustained an insertion of pCK61 in strain IVS9 is shown. Ca, *C. albicans* sequence, accession nos X81937 and U61975 (GenBank); Sc, *S. cerevisiae* sequence, accession no. X15483 (GenBank); Kl, *K. lactis* sequence, accession no. A37766 (PIR); Yl, *Y. lipolytica* sequence, accession no. L20972 (GenBank). Only the N-terminal 303 aa are shown.

chromosome that flanked the site of insertion (see Methods). The plasmid generated by this cloning, pIS2, contained approximately 7 kb of chromosomal DNA.

Using primers complementary to the pCK61 sequence, the sequence of the chromosomal DNA flanking the insertion in strain IVS9 was determined from pIS2. The deduced 105 aa sequence was compared to the GenBank database and found to be homologous to the SEC14 gene product of *S. cerevisiae*, *Kluyveromyces lactis* and *Y. lipolytica* (Fig. 3). The *C. albicans* sequence (GenBank accession no. X81937) was highly similar to that of *S. cerevisiae* Sec14p with 75% identical amino acids in this region. The DNA sequence determined for pIS2 was found to be identical to a portion of the sequence of the *C. albicans* SEC14 locus. Based on these results, it was deduced that strain IVS9 contained an insertion of pCK61 in the *C. albicans* SEC14 gene.

We attempted to construct a SEC14 null mutant to determine whether SEC14 was essential for filamentous growth. Southern blot analysis revealed that strain IVS9 (*sec14::pCK61*) was heterozygous for the insertion mutation (data not shown). To determine whether the heterozygous mutation would cause a defect in filamentous growth, strain SGY-243 was transformed with pIS2 after linearization with HindIII. Transformants that were due to homologous recombination were obtained and verified by Southern blot analysis. All

Fig. 4. SEC14 transcripts. (a) Strain CAI-4 was cultured in YPD at room temperature until the culture reached high culture density. The cells were starved in water overnight and then diluted 1/10 or 1/100 into GT2 medium and incubated at room temperature or 40 °C for 4 h. Total RNA was extracted from each culture as described in Methods. RNA was separated by electrophoresis and transferred to Nytran-plus membranes. Blots were probed with probes A, B or C (Fig. 1). Cellular morphologies were determined by examining cells by phase-contrast microscopy. Transcript sizes, determined by comparison with the mobility of standards, are shown on the left. Lanes: 1, 40 °C, 1/100 dilution, GT2 medium (germ tubes), probe A; 2, 40 °C, 1/100 dilution, GT2 medium (germ tubes), probe B; 3, room temperature, 1/10 dilution, GT2 medium (yeast cells), probe B; 4, 40 °C, 1/100 dilution, GT2 medium (germ tubes), probe C. (b) Chromosomal DNA was prepared from cells of strain CAI-4 and digested with SpeI alone or SpeI and BamHI. Southern blots were probed with probes A, B or C as described in Methods. Lanes: 1, SpeI digest, probe A; 2, SpeI digest, probe B; 3, SpeI and BamHI digest, probe B; 4, SpeI digest, probe C.

10 recombinants analysed exhibited a wild-type phenotype and were proficient in filamentous growth. These results show that the phenotype of strain IVS9 (*sec14::pCK61*) is not caused by the insertion mutation in SEC14. Although strain IVS9 was not a homozygous insertion mutant, as expected based on the mutant selection strategy, SEC14 was nevertheless of interest because it had been demonstrated that in *Yarrowia lipolytica*, a *sec14* mutant was defective in filamentous growth (Lopez *et al.*, 1994).

To determine the phenotype of a homozygous *sec14* null mutant, transformation was employed to attempt to construct a homozygous null mutant in strain CAI-4 using the Ura-blaster approach (Alani *et al.*, 1987; Fonzi & Irwin, 1993). As observed by Monteoliva *et al.* (1996), a $\Delta sec14::hisG/sec14^-$ mutant strain could not be isolated, suggesting that SEC14 is essential for growth.

A pop-in/pop-out recombination experiment (Scherer & Davis, 1979) was performed to determine if SEC14 is

Fig. 6. PCR amplification of *SEC14* cDNA. Plasmid pIS4 or cDNA, prepared as described in Methods, was subjected to PCR amplification with primer MTTM and primers 1, 2 or 3 (Fig. 5). The amplified DNA was purified, analysed by agarose gel electrophoresis and stained with ethidium bromide. Low levels of non-specific products were observed when primer 2 was used for amplification of either pIS4 and cDNA. Lanes: 1, size standards; 2, amplification of pIS4 with MTTM and primer 1; 3, amplification of pIS4 with MTTM and primer 2; 4, amplification of pIS4 with MTTM and primer 3; 5, amplification of cDNA with MTTM and primer 1; 6, amplification of cDNA with MTTM and primer 2; 7, amplification of cDNA with MTTM and primer 3.

Southern blots were probed with DNA fragments encoding DNA upstream and downstream of the 5' and 3' ends characterized above. Probe C (Fig. 1) is a PCR-generated fragment that extends approximately 500 bp beyond the polyadenylation site identified using the cDNA clones (Fig. 5). This probe hybridized specifically to *SEC14* in a genomic Southern blot (Fig. 4b). As shown in Fig. 4(a), probe C hybridized to only a 1600 nt transcript, suggesting that the longer transcript contains sequences extending beyond the 3' end of the shorter transcript. The failure to isolate a cDNA clone containing the larger 3' UTR sequence may reflect a bias in PCR for shorter products.

To test the 5' end, the blots were probed with probe A (Fig. 1), an approximately 800 bp *SpeI*–*AccI* fragment that ends 98 nt upstream of the 5' end characterized above. Monteoliva *et al.* (1996) demonstrated that a *C. albicans* *SEC14*⁺ clone extending from this upstream *SpeI* site to the downstream *SpeI* site complemented a *sec14ts* mutation in *S. cerevisiae*, suggesting that the 5' end of the gene is contained on this fragment. Although probe A hybridized well in a Southern blot (Fig. 4b), no hybridization was seen on the Northern blot (Fig. 4a), indicating that the sequences are not present in either transcript and that the two transcripts do not differ significantly at their 5' ends. Based on these results, we conclude that two transcripts are produced from the *SEC14* gene and they differ at their 3' ends.

PCR amplification of cDNA was performed to confirm that the 1600 nt transcript is a *SEC14* transcript with a

longer 3' end. To design primers for PCR, the sequence of chromosomal DNA downstream of the *SEC14* ORF was determined (Fig. 5). As shown in Fig. 5, a putative polyadenylation site corresponding to the site mapped above was observed and a second putative site, approximately 300 nt further downstream was observed. Sequences further downstream showed that the *Sau3A* site used to create probe C is probably located in an intergenic region because the sequence lacks ORFs of significant size (data not shown). PCR amplification was performed as described in Methods, using primer MTTM from the *SEC14* coding sequence and primers 1, 2 and 3, illustrated in Fig. 5. Primer 1 would be expected to amplify both transcripts, whereas primers 2 and 3 would only amplify the longer transcript. The results shown in Fig. 6 demonstrate that amplified products of the expected sizes were obtained when the cloned *SEC14* locus was amplified. Amplified fragments of the same sizes were obtained when cDNA was used as the template (Fig. 6). Therefore, we conclude that a transcript containing *SEC14* coding sequences and extending at least 444 nt beyond the coding sequence is present in the mRNA.

DISCUSSION

The original objective of this study was to use insertion mutagenesis by REMI as a method for identifying genes that are important for filamentous growth. Insertion mutagenesis has the advantage of producing mutations that are physically marked. Thus, it is straightforward to recover the DNA from the site of the insertion for analysis. Insertion by the REMI technique has been shown to occur without preference for insertion sites, demonstrating that it could be used for random mutagenesis. Because all insertion mutations were expected to be heterozygous, UV irradiation was used in this study to induce mitotic recombination and mutants defective in filamentous growth were selected using an enrichment strategy. A heterozygous *SEC14* insertion mutant was isolated but the insertion mutation was found not to be responsible for the defect in filamentous growth. Therefore, the phenotype of mutant IVS9 was caused by another mutation and it was not possible to determine the nature of the second mutation. It is possible that a *sec14* point mutation arose in the structurally normal copy of *SEC14*, conferring the selected phenotype. Alternatively, it is possible that the UV irradiation induced a mutation in another gene and this second mutation was responsible for the phenotype of IVS9. To permit the use of REMI as a technique for mutagenesis, it appears that a method allowing selection of homozygous insertions will be necessary.

Nevertheless, *SEC14* is an interesting gene because of its role in protein secretion and filamentous growth in other yeasts. In *S. cerevisiae*, *SEC14* is essential for growth and is required for proper function of the protein secretory pathway. Bankaitis and coworkers have demonstrated that Sec14p is localized in the Golgi where it plays a role in modulating lipid biosynthesis and may sense the lipid

composition of the Golgi (McGee *et al.*, 1994). Sec14p modulates the activity of the enzyme choline-phosphate cytidylyltransferase (Skinner *et al.*, 1995). It is likely that Sec14p performs a similar function in the *C. albicans* Golgi and that protein secretion would become blocked in the absence of *SEC14* function.

In contrast, in the dimorphic yeast *Y. lipolytica*, *SEC14* is not an essential gene. Inactivation of *Y. lipolytica SEC14* leads to cells with undetectable phosphatidyl-inositol/phosphatidylcholine transfer activity, yet such cells are viable. Furthermore, the protein secretory pathway functions normally in a *sec14* null mutant. The only observable defect caused by the *sec14* mutation was a defect in the ability to undergo filamentous growth. One interpretation of these results is that Sec14p participates in a cellular function that is unrelated to the protein secretory pathway during filamentous growth. Alternatively, it is possible that there is another molecule that performs the essential function of Sec14p in the protein secretion pathway in yeast cells but not in hyphae.

Based on these results, it is possible that Sec14p plays two roles in *C. albicans*. Sec14p may perform an essential function in the protein secretory pathway and a second function that is needed during filamentous growth. The demonstration that two transcripts are produced from *SEC14* suggests that the activity of Sec14p could be regulated. The two transcripts may allow Sec14p activity to respond to different environmental conditions, so that Sec14p can perform different functions. In *S. cerevisiae*, where Sec14p may perform only one major function, transcription of *SEC14* was found to be essentially constitutive and was unaffected by temperature shifts or low phosphate conditions (Vahlensieck *et al.*, 1995). In *C. albicans*, alteration of Sec14p activity could occur in response to environmental conditions as cells prepare for producing germ tubes.

The UTRs of mRNAs affect gene function in a variety of ways. The 3' sequences affect translational efficiency, message stability and message localization (Decker & Parker, 1995). The 5' UTRs play a role in translational regulation (Kozak, 1991). In *S. cerevisiae*, examples of differential polyadenylation have been described. The *CBP1* gene, encoding a protein that stabilizes pre-mRNA encoding cytochrome *b*, utilizes two different polyadenylation sites to produce a long and short transcript (Mayer & Dieckmann, 1989). Only the long transcript encodes the entire Cbp1p coding sequence and this transcript is required for growth of cells by respiration. The levels of the two transcripts are affected by the carbon source used for growth of the cells, providing a mechanism for regulation of Cbp1p production. In the case of *SEC14*, both transcripts encode the Sec14p coding sequence. However, the two transcripts could differ in their efficiency of translation.

Localization of mRNA transcripts is common in *Drosophila* development and contributes to the establishment of embryonic polarity (St Johnston, 1995). For example, the *bicoid* mRNA is localized to the

anterior pole of the oocyte. Upon translation, the *bicoid* gene product establishes a gradient that contributes to the definition of the head, thorax and abdomen of the embryo. Sequences in the 3' end have been shown to be responsible for mRNA localization (Macdonald & Struhl, 1988). It is possible that the different 3' ends of *SEC14* may lead to localization of the mRNA in different locations in the cell.

Alternatively, the 3' ends of *C. albicans SEC14* transcripts may permit efficient expression under different conditions of growth. In the mouse, alternative splicing in the 5' UTR of HSP47 permits efficient translation under heat shock conditions (Takechi *et al.*, 1994).

ACKNOWLEDGEMENTS

This paper is dedicated to the memory of Igor V. Slobodkin, our colleague and friend. We are grateful to all who interacted with Igor and assisted him in his work, both with stimulating discussions and by providing materials. We especially thank Claire Moore and Andrew Wright for extensive discussion and careful reading of the manuscript. We thank Heather Cook for her many contributions. We also thank Dean Dawson, Ralph Isberg, Concha Gil, Vytas Bankaitis and Elliot Androphy for key suggestions and helpful discussion. We are grateful to Paula Sundstrom, Bill Fonzi and Jessica Gorman for strains and plasmids. This work was supported by grants 92009660 from the American Heart Association (to C.A.K.) and AI38591 from the National Institutes of Health (to C.A.K.). Pilot studies that initiated the project were supported by the GRASP Digestive Disease Center, NIDDK P30 DK34928. This work was performed during the tenure of an American Heart Association Established Investigator Award (to C.A.K.).

REFERENCES

- Alani, E., Cao, L. & Kleckner, N. (1987). A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics* **116**, 541–545.
- Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. & Struhl, K. (1989). *Current Protocols in Molecular Biology*. New York: Wiley, Inc.
- Bankaitis, V. A., Aitken, J. R., Cleves, A. E. & Dowhan, W. (1990). An essential role for a phospholipid transfer protein in yeast Golgi function. *Nature* **347**, 561–562.
- Bolker, M., Bohnert, H. U., Braun, K. H., Gori, J. & Kahmann, R. (1995). Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Mol Gen Genet* **248**, 547–552.
- Brown, D. H., Jr, Slobodkin, I. V. & Kumamoto, C. A. (1996). Stable transformation and regulated expression of an inducible reporter construct in *Candida albicans* using restriction enzyme-mediated integration. *Mol Gen Genet* **251**, 75–80.
- Decker, C. J. & Parker, R. (1995). Diversity of cytoplasmic functions for the 3' untranslated region of eukaryotic transcripts. *Curr Opin Cell Biol* **7**, 386–392.
- Fonzi, W. A. & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**, 717–728.
- Guo, Z. & Sherman, F. (1995). 3'-end-forming signals of yeast mRNA. *Mol Cell Biol* **15**, 5983–5990.

- Kelly, R., Miller, S. M., Kurtz, M. B. & Kirsch, D. R. (1987). Directed mutagenesis in *Candida albicans*: one-step gene disruption to isolate *ura3* mutants. *Mol Cell Biol* 7, 199–207.
- Kozak, M. (1991). An analysis of vertebrate mRNA sequences: intimations of translational control. *J Cell Biol* 115, 887–903.
- Kuspa, A. & Loomis, W. F. (1992). Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc Natl Acad Sci USA* 89, 8803–8807.
- Lopez, M. C., Nicaud, J.-M., Skinner, H. B., Vergnolle, C., Kader, J. C., Bankaitis, V. A. & Gaillardin, C. (1994). A phosphatidylinositol/phosphatidylcholine transfer protein is required for differentiation of the dimorphic yeast *Yarrowia lipolytica* from the yeast to the mycelial form. *J Cell Biol* 124, 113–127.
- Macdonald, P. M. & Struhl, G. (1988). Cis-acting sequences responsible for anterior localization of bicoid mRNA in *Drosophila* embryos. *Nature* 336, 595–598.
- McGee, T. P., Skinner, H. B., Whitters, E. A., Henry, S. A. & Bankaitis, V. A. (1994). A phosphatidylinositol transfer protein controls the phosphatidylcholine content of yeast Golgi membranes. *J Cell Biol* 124, 273–287.
- Mayer, S. A. & Dieckmann, C. L. (1989). The yeast *CBP1* gene produces two differentially regulated transcripts by alternative 3'-end formation. *Mol Cell Biol* 9, 4161–4169.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Monteoliva, L., Sanchez, M., Pla, J., Gil, C. & Nombela, C. (1996). Cloning of *Candida albicans* SEC14 homologue coding for a putative essential function. *Yeast* 12, 1097–1105.
- Mullis, K., Faloona, F., Scharf, S., Daiki, R., Horn, G. & Erlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harbor Symp Quant Biol* 51, 263–273.
- Odds, F. C. (1994). *Candida* species and virulence. *ASM News* 60, 313–318.
- Pfaller, M. A. (1994). Epidemiology and control of fungal infections. *Clin Infect Dis* 19 (suppl. 1), S8–S13.
- Poulter, R. (1990). Classical methods for the genetic analysis of *Candida albicans*. In *The Genetics of Candida*, pp. 75–123. Edited by D. R. Kirsch, R. Kelly & M. B. Kurtz. Boca Raton, FL: CRC Press.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scherer, S. & Davis, R. W. (1979). Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. *Proc Natl Acad Sci USA* 76, 4951–4955.
- Schiestl, R. B. & Petes, T. D. (1991). Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 88, 7585–7589.
- Skinner, H. B., McGee, T. P., McMaster, C. R., Fry, M. R., Bell, R. M. & Bankaitis, V. A. (1995). The *Saccharomyces cerevisiae* phosphatidylinositol-transfer protein effects a ligand-dependent inhibition of choline-phosphate cytidylyltransferase activity. *Proc Natl Acad Sci USA* 92, 112–116.
- St Johnston, D. (1995). The intracellular localization of messenger RNAs. *Cell* 81, 161–170.
- Takechi, H., Hosokawa, N., Hirayoshi, K. & Nagata, K. (1994). Alternative 5' splice site selection induced by heat shock. *Mol Cell Biol* 14, 567–575.
- Vahlensieck, Y., Riezman, H. & Meyhack, B. (1995). Transcriptional studies on yeast SEC genes provide no evidence for regulation at the transcriptional level. *Yeast* 11, 901–911.
- Wilkinson, M., Daskow, J. & Lindsey, S. (1990). RNA blots: staining procedures and optimization of conditions. *Nucleic Acids Res* 19, 679.

Received 28 May 1997; accepted 22 July 1997.