Disruption studies of a *Candida albicans* gene, *ELF1*: a member of the ATP-binding cassette family

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A 3.6 kb gene (*ELF1*) with homology to the ATP-binding cassette (ABC) gene family has been isolated from genomic libraries of *Candida albicans*. Members of this gene family include both membrane transport proteins which confer a drug-resistance phenotype, and proteins whose functions are associated with protein translation. *ELF1* (Elongation Like Factor) showed greatest homology with a *Saccharomyces cerevisiae* ORF (YPL226W), whose function is unknown, and lower homology with fungal elongation factor 3 (EF-3) genes. In comparison, homology with a gene conferring a drug-resistant phenotype (*CDR1*) was low. To understand the function of *ELF1* in *C. albicans*, gene-knockout experiments were conducted using the *hisG-URA3-hisG* disruption cassette. Both single-copy (heterozygote) and double-disrupted strains in *ELF1* were isolated. Phenotypically, the disrupted strains grew more slowly than wild-type and produced a mixture of large, irregular cells and apparently normal cells.

Keywords: *Candida albicans*, ATP-binding cassette gene, gene disruption

INTRODUCTION

Candida albicans is the most prevalent opportunistic fungal pathogen and is one of the major aetiological agents of nosocomial infections (Matthews, 1994). The clinical importance of candidiasis has increased recently due to the onset of AIDS and medical procedures including immunosuppressive therapy and organ transplants. Since *C. albicans* avidly binds to mucosal surfaces and catheters (Hawser & Douglas, 1994), this normally benign organism can rapidly become invasive in immunosuppressed patients. The mortality rate in systemic candidiasis is high (38–59%), due in part to difficulties in diagnosis and treatment (Matthews, 1994). Since yeasts are eukaryotic, many antifungal agents are also toxic to host cells. The azoles, a group of antifungal compounds, are effective against mucocutaneous infections but development of resistance in chronically infected patients is high (Sangeorzan *et al.*, 1994; Pfaller *et al.*, 1994; McCulloch & Hume, 1995). In one study, all strains isolated from patients developed resistance to

fluconazole, clotrimazole and itraconazole but not ketoconazole (McCulloch & Hume, 1995). Additionally, the high recurrence of oropharyngeal candidiasis in HIV-positive patients did not always correspond with increased azole resistance (Pfaller *et al.*, 1994). Consequently, there is an important need to identify new antifungal targets and develop new treatment strategies.

The ATP-binding cassette (ABC) transporter proteins are of interest because of their association with a drug-resistance phenotype and because they are potential antifungal target proteins. The genes encoding ABC proteins share significant sequence homology, including a conserved amino acid domain of approximately 200 bp which is associated with ATP binding. Many of the ABC proteins consist of two homologous halves with a nucleotide-binding-fold domain and six predicted membrane-spanning regions per half. Their known functions include the transport of small molecules or proteins bidirectionally across membranes, such as the efflux of cytotoxic compounds (reviewed by Higgins, 1992). Members of the ABC family include multi-drug resistance (MDR) proteins from tumour (P-glycoprotein) and bacterial cells. Overexpression of the P-glycoprotein, and consequently an ATP-dependent extrusion pump, increases the resistance of cancer cells to cytotoxic drugs (Gottesman & Pastan, 1993). Genes

Abbreviations: ABC, ATP-binding cassette; FOA, 5-fluoroorotic acid; MDR, multidrug resistance; pSK+, pBluescript SK+

The GenBank accession number for the sequence reported in this paper is AF038153.

conferring similar phenotypes have been reported for *Saccharomyces cerevisiae* (SNQ2 and PDR5) and *C. albicans* (CDR1) (reviewed by Balzi & Goffeau, 1994) and CDR2 (Sanglard *et al.*, 1997). In addition to genes encoding the MDR proteins, genes involved in translation are also members of the ABC gene family. GCN20 from *S. cerevisiae*, for example, is a positive effector of GCN2, and, thus, is indirectly involved in activation of translation initiation factor 2 (Vazquez de Aldana *et al.*, 1995). GCN20 shows significant sequence similarities to genes that encode elongation factor 3 (EF-3). EF-3 genes are found in several fungi including *C. albicans* (Colthurst *et al.*, 1992), *Pneumocystis carinii* (Ypma-Wong *et al.*, 1992) and *S. cerevisiae* (Qin *et al.*, 1990). The EF-3 proteins are attractive antifungal targets since they are not found in mammalian cells, but are essential for translation in fungal cells.

In this paper, we describe the identification and gene disruption of a new member of the ABC gene family in *C. albicans*, *ELF1*. Sequence analysis (Devereux *et al.*, 1984) identified two putative nucleotide-binding domains and an ABC transporter consensus sequence. Although the sequence of the *S. cerevisiae* homologue, ORF YPL226W, has been reported, no function or phenotype has yet been determined. Therefore, it is of interest to study this gene to determine whether it may play a role in drug resistance or growth of the organism.

METHODS

Strains and culture conditions. The *C. albicans* strains used in this study are listed in Table 1. Strains SC5314, CAF2-1 and CAI4 were donated by William Fonzi (Georgetown University). Strains were maintained on YPD medium (Sherman *et al.*, 1986) at 30 °C. Minimal medium (YNB) contained yeast nitrogen base with 0.5% (w/v) ammonium sulfate supplemented with 2% (w/v) dextrose. When required, 25 µg uridine ml⁻¹ was added. Ura⁻ auxotrophs were selected on YNB supplemented with 1 mg 5-fluoroorotic acid ml⁻¹ (FOA; Boeke *et al.*, 1984).

The yeast-to-hyphal transition was induced by either culture media or serum (Shepherd *et al.*, 1980). Tissue culture medium (M199) or 2.5% foetal calf serum was inoculated with 5–10 × 10⁵ cells ml⁻¹ from an overnight culture and incubated rocking at 37 °C for 30 min to 2 h. Germ-tube formation was monitored by light microscopy.

The growth rates of heterozygote and homozygote disrupted strains were compared with the parental or the *URA3* heterozygote strain, CAF2-1. Strains were grown overnight in YPD at 30 °C. Equivalent numbers of yeast cells were inoculated into YPD or YNB so that the starting OD₅₅₀ of a 1:10 dilution was 0.03 (approx. 10⁶ cells). The OD was measured at 1 h intervals for 9 h in three separate experiments. Cells were also counted manually using an Improved Neubauer haemocytometer.

DNA manipulations and transformations. Plasmid isolation and enzymic reactions were performed either by standard methods (Sambrook *et al.*, 1989) or according to the manufacturer's instructions. Bacterial transformations were performed using *Escherichia coli* DH5α competent cells prepared in calcium chloride or by electroporation with *E. coli* XL1-Blue cells (Bio-Rad *E. coli* pulser; manufacturer's directions). *C. albicans* was transformed by lithium acetate following the protocol of Gietz *et al.* (1992). Selection of Ura⁺ transformants and FOA segregants was performed as described by Fonzi & Irwin (1993). Genomic DNA was isolated from *C. albicans* spheroplasts (Philippsen *et al.*, 1991) using Zymolyase-20T (ICN Biomedicals). An *EcoRI* SC5314 genomic library was constructed in Lambda Zap II following the manufacturer's instructions (Stratagene).

Isolation of *ELF1*. A *C. albicans* 4918 genomic DNA library constructed in λgt11 was screened using non-formamide hybridization conditions (Sambrook *et al.*, 1989) at 55 °C with λE41, the clone of the human B cell C3d receptor gene (Moore *et al.*, 1987). Library screening and phage purification were performed by routine methods (Titus, 1991). Positive plaques were purified and a 3.2 kb *EcoRI* fragment was isolated which hybridized with λE41 (Calderone *et al.*, 1992). The 5' *EcoRI* fragment was isolated by screening the Lambda Zap II library with pABC-4, a plasmid derived by inverse PCR (see below).

Table 1. *C. albicans* strains used in this study

Strain	Parental strain	Relevant genotype	Source/reference
4918		<i>ELF1/ELF1 URA3/URA3</i>	Manning & Mitchell (1980)
SC5314		<i>ELF1/ELF1 URA3/URA3</i>	Gillum (1984)
CAF2-1	SC5314	<i>ELF1/ELF1 Δura3/URA3</i>	Fonzi & Irwin (1993)
CAI4	CAF2-1	<i>ELF1/ELF1 Δura3/Δura3</i>	Fonzi & Irwin (1993)
CAJS1	CAI4	<i>Δelf1::hisG-URA3-hisG/ELF1 Δura3/Δura3</i>	This work
CAJS2	CAI4	<i>Δelf1::hisG-URA3-hisG/ELF1 Δura3/Δura3</i>	This work
CAJS1E	CAJS1	<i>Δelf1::hisG/ELF1 Δura3/Δura3</i>	This work
CAJS2D	CAJS2	<i>Δelf1::hisG/ELF1 Δura3/Δura3</i>	This work
CAJS5	CAJS2D	<i>Δelf1::hisG/Δelf1::hisG-URA3-hisG Δura3/Δura3</i>	This work

Fig. 1. Maps of plasmids containing portions of the *ELF1* genes constructed in this study, in relation to the *ELF1* locus (top line) and ORF (thick black line). B, *Bam*HI; C, *Cl*AI; H, *Hind*III; P, *Pst*I; R, *Eco*RI; RV, *Eco*RV; S, *Sac*I. The arrows indicate the location of primers used for inverse PCR.

Southern analysis. DNA was digested to completion, electrophoresed on 1% agarose gels and transferred to a nylon membrane by capillary action using a 'dry' method. Gels were soaked in 0.5 M NaOH, 1.5 M NaCl (2 × 15 min), then in 1 M ammonium acetate/0.02 M NaOH (2 × 30 min) and the DNA transferred to preincubated nylon membranes (GeneScreen Plus; Dupont, or Nytran; Schleicher and Schuell) without a buffer chamber for 1 h. DNA was fixed onto the membranes using UV or by baking at 80 °C. Prehybridization was performed at 65 °C for at least 5 min in 7% SDS, 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA (Monod *et al.*, 1994). DNA probes were labelled with α -³²P using Ready-to-Go DNA Labelling Beads (-dCTP) (Pharmacia) or Prime-It RmT Random Primer Labelling kit (Stratagene). Membranes were hybridized in the same buffer for 16–24 h at 65 °C. Blots were washed twice for 15 min in 0.1 × SSC, 1% SDS at 65 °C.

Plasmid construction. The plasmids constructed for this study are shown in Fig. 1. Plasmid pABC-1 was constructed by ligating the 3.2 kb *Eco*RI fragment isolated from the λ gt11 library into pBluescript SK+ (pSK+; Stratagene). pCa900 consists of an internal *Hind*III fragment from pABC-1, which hybridized with λ E41, ligated into pSK+. For sequencing purposes, two subclones of pABC-1 were constructed. pABC-1 was digested with *Eco*RI and *Bam*HI and the 3' 2.2 kb fragment was ligated into pSK+ to give pABC-2. The plasmid pABC-3 was constructed by ligating the 5' 1.1 kb *Eco*RI–*Hind*III fragment from pABC-1 into pSK+. The 3' end of the insert of pABC-3 overlaps with the 5' end of the insert of pABC-2.

To obtain sequence upstream of *ELF1*, inverse PCR was performed (Silver, 1992). SC5314 genomic DNA (20 μ g) was digested with *Cl*AI at a final concentration of 50 μ g DNA ml⁻¹ and 0.2 units *Cl*AI ml⁻¹; circularization of the genomic *Cl*AI fragments was performed at 10 μ g DNA ml⁻¹ with 400 units of T4 DNA ligase (New England Biolabs). Between 0.1 and 0.5 μ g of ligated DNA was PCR amplified with 1 μ M primer and *Taq* DNA polymerase according to the manufacturer's instructions (Life Technologies). Abc4b, the upstream primer was located

119 bp upstream of the *Eco*RI site. The downstream primer Abc3 was located 312 bp downstream of the *Eco*RI site. Locations of primers are shown in Fig. 1 and Table 2. The expected 800 bp product was subcloned by blunt ligation into pNoTA/T7 using a PCR cloning kit (5' prime → 3' prime) resulting in plasmid pABC-4. pELF1 in pSK+ phagemid was excised from a tertiary positive plaque from the Lambda Zap II SC5314 library probed with pABC-4.

To obtain disrupted strains, a transforming plasmid was constructed. Plasmid pMB7 (Fonzi & Irwin, 1993) was kindly donated by William Fonzi (Georgetown University). pSK+ was digested with *Sac*I, blunt-ended with T4 DNA polymerase and religated to give pSK+ deleted of the *Sac*I restriction site (pSK+ Δ *Sac*I). pABC-1 was cut with *Bam*HI and *Eco*RV and ligated into *Bam*HI/*Eco*RV-digested pSK+ Δ *Sac*I to give plasmid pJSCa2. The internal 1.2 kb *Pst*I–*Sac*I fragment of pJSCa2 was removed and replaced with the *hisG*(I–*Sce*I)–*URA3*–*hisG*(I–*Sce*I) insert from pMB7 to give pJSCa4ura.

DNA Sequencing. Double-stranded and single-stranded DNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase version 2.0 kit (Amersham) and [³⁵S]dATP α S, 1 μ g single-stranded M13 or 3–5 μ g denatured plasmid and M13 commercial sequencing primers or synthetic oligonucleotide primers. For initial comparison with databases, pCa900 was sequenced with T3 and T7 primers. pABC-2 was cut with *Bam*HI and *Eco*RI and ligated into M13mp18 and M13mp19 (Life Technologies) to sequence both strands. The Cyclone I Biosystem (IBI) was used to produce sequentially overlapping clones. M13 clones were sequenced with M13 forward and reverse commercial primers. pABC-3 was sequenced using the T3 and T7 primers. Additional sequence was obtained with the synthetic oligonucleotide, Abc3 (see above). pABC-4 was sequenced with T7 and with the commercial M13–40 primer included in the Sequenase kit. pELF1 was sequenced with the T3 and T7 primers, to identify overlapping sequence with pABC-4. The rest of the ORF was sequenced with the specific internal primers (Life Technologies) ElfR1, ElfR2, ElfR3, ElfR4 and

Table 2. Primer sequences used in this study

Primer name	Position	Sequence (5'-3')
Elf121	-228--208	CGGTTATCGGGAAACGTTGTC
Elf318	-22--9	CACACACACTCGACTATTGACAATGTCCAAG
ElfR4	301-272	GTGGAACCTTTGGTTAGTGTATTATTATAG
ElfR1	559-538	ATTTACTTCAATTTCCATTTCAT
ElfR2	595-575	CTAAGTCAGGACTTTTAGGTTTC
ElfR3	625-602	TGAATGATTAAATAAGCTCCTTCT
ElfF1	602-625	AGAAGGAGCTTTATTAATCATTCA
Abc4b	951-935	ATAAATCAGTCAATACC
Abc3	1154-1173	ATTAGTCCCCATTTTAGATA
495R	1173-1154	TATCTAAAATGGGGACTAAT
ElfDR2	3006-2988	TCACTGATGGAGTTAATTC
ElfDR1	3598-3579	TCATACGCGTCTATATAACTCGATCCTCATC
3929R	3928-3909	ATGAGATATTGTCGTTGTTG
Oligo(dT)	-	GACAACTAGTCTCGA(T) ₁₇

1	MSKSFDFFIK QKQKQSKPK GFGYRNNQ GYNYNQAYGN VPTFQPGNQ FQYQGGYNQ	60
61	FNQQQYQYG QGYNQGYNS NNRYNNSNNY NYNNNTNQSS TISTPVDSL TSGRSTPNPN	120
121	ASTTSLTSLN TALAKLNVS IPFEENLSNI EKAGKIAEIR PEVETIVKII DEQEDLCIIN	180
181	EWKLNEILKS LLKPKSPALV KEGALLIQQ LATKFGGQTP KEAYLLQFLS TAYDMFTDKD	240
241	KNVVKAAKSA TDALFGIYPV EALGSIVLDE YLTIFKSGAK WNSKVAALVN FDKLIDDVPA	300
301	DILEMKFIDV VPVLTDLSTD FKPELAKAGL STLKKEVVKV DNLDLQNKYD LIVDTLADPK	360
361	KVTDCIKNLS SVTFVAEVE PALSLLVPIL DKSLKMSSSS NDELQRTVMV TKNLTRLVNN	420
421	KREIEQFIPI LLPGVKEVNV NASLPEVREL AGKALKVLKD AENEQTDGKF HGRITLQAE	480
481	KFYTEDIPDE QHQQPVLQK DDILKKYLAT VLQSLMSML GTLQEYLMMA TNNNETSSQL	540
541	VVTHVKNLFN PESAADNEND DGAVVIVDAD FSLAYGTRML LNKTKLRLK GHRVGLCGRN	600
601	GAGKSTLMRA ISKGQLEGFP TADELKTFCV EHKLGSEAD MDLVSFIGSD PELANVGREE	660
661	IEQALKDVGF PDERLQQVVG SLSGGWTKL ELARAMIMKA DVLLDEPTN HLDVANVKWL	720
721	QDYLVNTES LICSHDSGFL DAVCTDIIHY ENKKLAYYKG NLSEFVKIKP EGKSYTTLTD	780
781	SNVKMAFPFP GILTVKSNST RAVARMSNVT FAYPGADKPS LKNVSCSLSL SSRVAILGPN	840
841	GAGKSTLIKL LTAELVPQEG KVEKHPNLRI GYIAQHALQH VEQHKETCQ SIFTWRYRFG	900
901	DDREVLLKES RKVSDEEQEM MKKEIDIGDG RGKRAIEALV GRQKLKKSFO YEVKWKYMLP	960
961	KYNSWVPKEV LLEHGFDKLI QKFFDHEASR EGLGYRELTP SVIRKHFEDV GLDGDIDHT	1020
1021	PMDHYLGGQL VKVVIAGAMW NNPHLVLDE PTNYLDRDSL GGLAMAREW NGGVVMISHN	1080
1081	NEFVGALCPE QWHVENGEVI QKGTLSMLK DLKIKVVKH LLLKKVHSCQ NQLKRESMM	1140
1141	ILSLILSRTR KKKMTRNEKK QAERRRLRY IEWLSSPKGT PKPVDTDDEE D	1192

Fig. 2. Deduced amino acid sequence of *ELF1*. The nucleotide-binding domains A and B are boxed. The ATP transporter consensus sequence is in bold. The region deleted from the CAJ55 mutant is italicized (amino acids 851-1192).

ElfF1 (Table 2) using [³⁵S]dATPαS and the Sequenase 2.0 or ThermoSequenase cycle sequencing kit (Amersham). Analysis of sequence data was performed using the University of Wisconsin GCG sequence analysis software programs (Devereux *et al.*, 1984). Nucleotide database searches were performed using BLAST (Altschul *et al.*, 1990).

PCR Analysis. To confirm the *ELF1* deletion in the genome, 0.1 µg genomic DNA was PCR amplified with reverse primers located in the deleted region (ElfDR1), 5' of the deletion (495R) or 3' of the deletion (3929R). In all three amplifications the forward primer was Elf318.

RT-PCR. One microgram of RNA isolated from SC5314, CAJ52 or CAJ55 was treated with DNaseI (amplification grade; Life Technologies) according to the manufacturer's instructions, in a volume of 10 µl. The reaction was stopped and 1 µl saved to check for contamination with DNA. The remainder was annealed to 495R or an oligo(dT) primer and reverse transcribed in a final volume of 20 µl with Superscript II RNase H-Reverse Transcriptase (Life Technologies) according to the manufacturer's instructions. Two microlitres of this reaction was directly amplified with 495R and ElfF1, or with a reverse primer located in the deleted region ElfDR2 and the

forward primer Abc3. PCR was performed with the upstream forward primer Elf121 to verify the absence of DNA. PCR amplifications were performed with *Taq* DNA polymerase (Life Technologies); annealing was at 52 °C.

PFGE and I-SceI digestion. PFGE was performed with the CHEF-DRII pulsed-field electrophoresis system and Pulse-wave 760 Switcher (Bio-Rad). Agarose plugs of chromosomal DNA from desired strains were prepared as described by Wickes *et al.* (1991a). Plugs were pre-equilibrated in 0.5 × TBE prior to loading in a 0.75 % agarose gel. Gels were run at 75 V with a linear ramping of the switching interval from 300 s to 1400 s for 144 h at 13 °C. Fresh running buffer was added after 72 h. Southern transfer of pulsed field gels was performed as suggested by the manufacturer (Bio-Rad). To determine the site of integration of transforming DNA, chromosome plugs were digested with the meganuclease *I-SceI* (Boehringer Mannheim Biochemicals) as described by Fonzi & Irwin (1993).

RESULTS

Isolation of *ELF1*

A protein which binds the human complement breakdown product C3d, and therefore has binding functions similar to the mammalian complement receptor 2 (CR2),

has been described (Saxena & Calderone, 1990). To isolate the corresponding gene from *C. albicans*, genomic DNA from strain 4918 was digested with *EcoRI* and *HindIII* and probed with a 1.8 kb internal *EcoRI* fragment of *λE41*, which is a cDNA clone of the human CR2 gene (Moore *et al.*, 1987). The 1.8 kb probe hybridized with *EcoRI* fragments of 11, 8, 3.2 and 1.3 kb and *HindIII* fragments of 9.5 and 0.7 kb (data not shown). The 3.2 kb *EcoRI* fragment was isolated by plaque hybridization from an *EcoRI* genomic library constructed in *λgt11*. Further analysis showed that *λE41* hybridized with a *HindIII* fragment of approximately 900 bp, contained within the *EcoRI* fragment (Calderone *et al.*, 1992). This *HindIII* fragment was subcloned and partially sequenced. At the nucleotide level, overall homology between the human CR2 gene and *ELF1* was not significant. However, a 36 bp stretch demonstrated a 76 % identity, which may be the region that was recognized in Southern and library screens. No homology with the human CR2 gene was identified in a BLAST search (Altschul *et al.*, 1990). On the other hand, significant homology existed along the entire sequence at both the nucleotide and amino acid levels with the ABC genes. Consequently, it was of interest to continue

1	MSKSFDDFIK	QQKQQSQKPK	GFGYRNNQGG	GYNYNQAYGN	VPTFPQGNQ	FQYQGGYNQ	FNQQQYQYQG
Elf	~MPPKKFKD	LNSFLDDQPK	DPNLVASPFG	GYFKNPAADA	GSNNASKKSS	YQQQRNWKQG	GNYQQGGYQS
71	*	*	*	*	*	*	*
Elf	QGYNNQGYNS	NNRYNNSNNY	.NYYNNNTNQS	STISTPVDLS	PTSGRS.TPN	PNASTTSLTS	LNTALAKLNV
SC	YNSNYNNYNN	YNNYNNYNNY	NNYNNYNGQG	YQKSTYKQSA	VTPNQSGTPT	PSASTTSLTS	LNEKLSNLEL
141	*	*	*	*	*	*	*
Elf	SNIPFEENLS	NIEKAGKIAE	IRPEVETIVK	IIDEQEDLC.	.IINEWKLINE	ILKSLKPKPS	PALVKEGALL
SC	T..PISQFLS	KIPECQSTID	CKNQKLIIE	EFGEKGNSTG	EKIEEWKIVD	VLSKFIKPKN	PSLVRESAML
211	*	*	*	*	*	*	*
Elf	IIQQLATKFG	GQTPKEAYLL	QFLSTAYDMF	TDKDKNVVKA	AKSATDALFG	IYPVEALGSI	VLDEYLTIFK
SC	IIISNIAQFFS	GKPPQEAAYL	PFFNVALDCI	SDKENTVKRA	AQHAIKSLN	CFPMEALTCF	VLPTILDYLS
281	*	*	*	*	*	*	*
Elf	SGAKWNSKVA	ALVNFDKLID	DVPADILEMK	FIDVVPVLTD	LSTDFKPELA	KAGLSTLKKF	VKVLNDLQDQ
SC	SGAKWQAKMA	ALSVVDRIE	DSANDLLELT	FKDAVPVLTD	VATDFKPELA	KQGYKTLLDY	VSILDNDLDS
351	*	*	*	*	*	*	*
Elf	NKYDLIVDTL	ADPKKVTDCI	KNLSSVTFVA	EVTEPALSL	VPILDKSLKM	SSSSNDELQ	TMVMTKNLTR
SC	PRYKLIVDTL	QDPKVPESV	KSLSSVTFVA	EVTEPSLSLL	VPILNRSLL	SSSSQEQRLQ	TVIVVENLTR
421	*	*	*	*	*	*	*
Elf	LVNNKREIEQ	FIPILLPGVE	KVVNNASLPE	VRELAGKALK	VLKDAENQET	DGKFHGRITL	EQAEKF...Y
SC	LVNNRNEIES	FIPLLLPGLQ	KVVDVTSALPE	VRELAEKALN	VLKEDDEADK	ENKFSGRRLT	EEGRDFLLDH
491	*	*	*	*	*	*	*
Elf	TEDIPDEHQ	QPVQLLKDDI	LKKYLATVLQ	.SMLMSMLGT	LQEYLMMATN	NNETSSQLV.	...VTHVKNL
SC	LKDIKADDS	FKVPYMNDET	VKKYMSKILT	VDSNVNDWKR	LEDFLTAFTG	GSDSQREFTV	QDFIHNLRAL
561	*	*	*	*	*	*	*
Elf	FNPEAAADNE	NDDGAVVIVD	ADFSLAYGTR	MLLNKTKLRL	LKGHRYGLCG	RNGAGKSTLM	RAISKQGLEG
SC	FYQEK..ERA	DEDEGIEIVN	TDFSLAYGSR	MLLNKTNLRL	LKGHRYGLCG	RNGAGKSTLM	RAIANGQLDG
631	*	*	*	*	*	*	*
Elf	FPTADELKT	FVEHKLQGS	ADMOLVSFIG	SDPELANVGR	EEIEQALKDV	GFPDERLQQ	VGSLSGGWKM
SC	FPPDKTLRT	FVEHKLQGS	GDLDLVSFIA	LDEELQSTSR	EEIAAALESV	GFDEERRAQ	VGSLSGGWKM
701	*	*	*	*	*	*	*
Elf	KLELARAMLM	KADVLLLEDP	TNHLDSNVK	WLQDYLVENT	E..SLICSHD	SGFLDAVCTD	IIHYENKKLA
SC	KLELARAMLQ	KADILLLEDP	TNHLDSNVK	WLEEYLLLEHT	DITSLIVSHD	SGFLDTVCTD	IIHYENKKLA
771	*	*	*	*	*	*	*
Elf	YKGNLSEFV	KIKPEGKSY	TLTDSNVKMA	FPPPGILTGV	KSNTAVARM	SNVTFAYPGA	DKPSLKNVSC
SC	YKGNLAFAV	EQKPEAKSY	TLTDSNAQMR	FPPPGILTGV	KSNTAVAKM	TDVTFSPYGA	QKPSLSHVSC
841	*	*	*	*	*	*	*
Elf	SLSLSSRVAI	LGPNGAGKST	LILKLTAEIV	PQEGKVEKHP	NLRIGYIAQH	ALQHVQEHKE	KTCQSIFTWR
SC	SLSLSSRVAC	LGPNGAGKST	LILKLTGELV	PNEGKVEKHP	NLRIGYIAQH	ALQHVNEHKE	KTANQYLQWR
911	*	*	*	*	*	*	*
Elf	YRFGDDREVL	LKESRRKISD	EQEMMKKEID	IGDGRGKRAI	EALVGRQKLG	KSFQYEVKWK	YWLPKYNSWV
SC	YQFGDDREVL	LKESRRKISD	EDMMTKKEID	IDDGRGKRAI	EALVGRQKLG	KSFQYEVKWK	YWLPKYNSWV
981	*	*	*	*	*	*	*
Elf	PKEVLLHGF	DKLIQKFDH	EASREGLGYR	ELTPSVIRKH	FEDVGLDGD	ADHTPMDHYL	GGQLVKVIVIA
SC	PKDVLVEHGF	EKLIVQKFDH	EASREGLGYR	ELIPSVITKH	FEDVGLDSEI	ANHTPLGSL	GGQLVKVIVIA
1051	*	*	*	*	*	*	*
Elf	GAMMNNPHLL	VLDEPTNYLD	RDSLGGLAMA	IREWNGGVVM	ISHNNEFVGA	LCPEQWHVEN	GEVIQKGTLS
SC	GAMMNNPHLL	VLDEPTNYLD	RDSLGGLAMA	IRDWSGGVVM	ISHNNEFVGA	LCPEQWHVEN	GKMQVKGSAQ
1121	*	*	*	*	*	*	*
Elf	LMLKDLKIKV	VTKHLKLVK	HSCQNLKRE	SMMILSLIL	SRTKKKKMTR	NEKKAQAERR	RLRYIEWLSS
SC	VDQSKFEDGG	NADAVGLGS	NLAKPSVDD	DSPANIKV..	.KQRKKRLR	NEKKAQAERR	RLRYIEWLSS
1191	*	*	*	*	*	*	*
Elf	PKGTTPKVD	DDEED					
SC	PKGTTPKVD	DDEED					
	*****	*****					

Fig. 3. Comparison of *ELF1* (Elf) with the *S. cerevisiae* ORF, SCYPL226W (SC; Genbank accession no. Z73582) using the GCG PILEUP program. Amino acid residues which are identical between *ELF1* and SCYPL226W are denoted by asterisks. Walker A and B regions are in bold.

Fig. 4. Disruption of the *ELF1* gene. (a) Restriction map of the *ELF1* locus and the *hisG*-*URA3*-*hisG* insertion positions. Position of *Cla*I (C) sites are shown in parental allele, disrupted allele and FOA segregant. An internal 2.5 kb *Cla*I-*Eco*RI fragment was used as a hybridization probe (indicated by hatched rectangle). Genomic DNA expected to hybridize to the probe is shown as a hatched rectangle. Thick black lines indicate the *Cla*I fragments recognized by the probe on a Southern blot. C=*Cla*I sites, R=*Eco*RI sites, S=*Sac*I sites. (b) Southern blot analysis of *Cla*I-digested genomic DNA. DNA was isolated from CAJ55 (lane 1), CAJ52 (lane 2) and CAI4 (lane 3). The size of the fragments in kilobases is indicated on the right and migration of standards are noted on the left. Since there is a *Cla*I site in the *hisG* gene, digestion of disrupted alleles results in two fragments of 1.8 kb and 2.3 kb that hybridize with the *elf* probe, instead of 4.1 kb as seen in the non-disrupted allele; see (a).

characterizing this gene. The strategy for the complete sequence analysis of the 3.2 kb *Eco*RI fragment and the upstream sequence is described in Methods. To obtain the full gene, a section between *Eco*RI sites was obtained by inverse PCR (pABC-4). Southern analysis of *Eco*RI-digested genomic DNA with ³²P-labelled pABC-4 showed two bands, one at 3.2 kb as expected and a second at 2.8 kb (data not shown). pABC-4 was used to screen an *Eco*RI-digested SCS314 genomic DNA library constructed in Lambda Zap II. Positive plaques were excised, plasmids isolated and digested with *Eco*RI. The insert of one clone, pELF-1, migrated at 2.8 kb, as predicted. The remainder of pELF-1 (1.2 kb) was sequenced with the T3 and T7 primers. The T7 sequence matched pABC-4. 1.2 kb of pELF-1 was then sequenced with specific internal primers to obtain the full ORF (see Methods).

ELF1 encodes a putative protein of 1192 amino acids. GCG analysis of the deduced amino acid sequence (Fig. 2) demonstrated the presence of two ATP/GTP-binding

site motifs and an ABC transporter consensus sequence located between the Walker A and B regions of the first ATP-binding site. BLAST analysis (Altschul *et al.*, 1990) indicates that *ELF1* shows significant homology to the ABC gene family, and in particular, the *S. cerevisiae* ORF, SCYPL226W (63 % identity, 70 % similarity), the EF-3 genes (36 % identity, 46 % similarity) and GCN20. The homology between *ELF1*, SCYPL226W and the EF-3 genes is more extensive than with the MDR-like genes. Comparison of the deduced protein sequences of *ELF1* and SCYPL226W is shown in Fig. 3. In addition to the length of the gene and spacing between Walker A and B regions, the majority of the identity is seen in the ATP-binding domains. The 5' ends of the genes are the least similar. However, the final 30 amino acids at the 3' ends are identical in *ELF1* and SCYPL226W but not in *C. albicans* EF-3 (not shown). This region in EF-3 is proposed to be responsible for ribosomal interaction (Chakraborty *et al.*, 1995). Northern analysis confirmed that a 3.6 kb RNA was produced in yeast, pseudohyphal and hyphal forms. The level of expression did not vary

significantly during serum-induced morphogenesis (data not shown).

Construction of disrupted strains

The function of the *S. cerevisiae* ORF, SCYPL226W, has not yet been determined. To study the function of the gene product of *ELF1*, a disrupted mutant was constructed using the methodology described by Fonzi & Irwin (1993). pABC2, which contains 70% of the open reading frame of *ELF1* and the 3' downstream sequence, was cloned into pSK+ (see Fig. 1). pJSCa4ura was digested with *Not*I and *Xho*I (located in the polylinker), to release the *ELF1* disruption cassette, and 15 µg was used to transform CAI4. *Ura*⁺ transformants (12 out of 80) were analysed for integration by Southern hybridization (Fig. 4b, lane 2). Three transformants underwent the desired recombination event. Spontaneous *Ura*⁻ derivatives of two of the resulting heterozygotes, CAJS1 and CAJS2, were selected on YNB containing 1 mg FOA ml⁻¹ (Fonzi & Irwin, 1993). FOA is toxic for strains containing *URA3* genes. Therefore, only those colonies which have lost the *URA3* gene by intrachromosomal recombination will grow on YNB+FOA. Segregants were analysed by Southern hybridization. Of 15 FOA segregants analysed, four lost the *URA3* gene and one copy of the *hisG* gene (see Fig. 4a). Two segregants, CAJS1E and CAJS2D, were transformed with pJSCa4ura in five separate experiments. Out of a total of 36 *Ura*⁺ transformants, one had lost the second wild-type allele and was designated CAJS5 (Fig. 4b, lane 1).

PCR analysis of genomic DNA confirmed the presence of a disrupted allele in CAJS5. Genomic DNA isolated from strains SC5314, CAJS2 and CAJS5 was PCR amplified with a forward primer, ElfF1, and a primer located in the deleted region, ElfDR1. No product was seen in CAJS5. However, PCR with reverse primers 5' (495R) or 3' (3929R) of the deletion did result in the expected products (data not shown). Since only the 3' end of the gene was deleted, RT-PCR was performed to determine whether a truncated form of the gene was expressed (Fig. 5). RNA isolated from SC5314, CAJS2 and CAJS5 was reverse transcribed with an oligo(dT) primer. This product was then PCR amplified with a forward primer, ElfF1, and the reverse primer 495R. A 0.5 kb product was observed in both the parent and disrupted strains (Fig. 5). To confirm that this product did not result from genomic DNA, PCR amplification with a primer located in the promoter region (Elf121) and at 1173 bp (495R) resulted in no product in CAJS5 (lane 7) or the other strains (not shown), confirming the absence of contaminating genomic DNA. PCR of genomic DNA with the same primers acted as a control. To confirm that a full-length transcript was not present in CAJS5, PCR of the cDNA from the three strains was also performed with a reverse primer located in the deleted region, ElfDR2, and Abc3 (Fig. 5). The expected 2.2 kb product was seen in the parental and single disruptant, but not the double disruptant (lane 6).

Fig. 5. PCR analysis of the *ELF1* gene. (a) Line diagram showing the 4.4 kb of sequenced genomic DNA at the *ELF1* locus. The location of the ORF (1–3573 bp) (heavy line) and deleted region (2559–3763 bp) (grey rectangular box) and Walker A (grey square boxes) and B (white square boxes) regions are shown. PCR amplification of genomic DNA to confirm the deletion was done with Elf318 and a primer in the deleted region, ElfDR1, 5' of the deleted region, Abc3, or 3' of the deleted region, 3929R. To determine whether a truncated form of the *ELF1* gene was expressed, RT-PCR was performed with the oligo(dT) primer and DNase-treated RNA isolated from SC5314 (lanes 1 and 4), CAJS2 (lanes 2 and 5) and CAJS5 (lanes 3, 6 and 7). The cDNA was PCR amplified with a forward primer, ElfF1, and a reverse primer, 495R, (lanes 1–3), or a forward primer, Abc3, and a reverse primer in the deleted region, ElfDR2 (lanes 4–6). To confirm that genomic DNA was not amplified, cDNA was PCR amplified with a forward (Elf121) and reverse (495R) primer. No product was seen in CAJS5 (lane 7) but the expected 1.3 kb product was seen when genomic DNA was used as the template (lane 8).

RT-PCR was also performed with specific elf primers. The same results were obtained: a truncated product was observed with RNA isolated from CAJS5 when RT-PCR was performed with the reverse primer at 1154 bp but no product with the reverse primer at 3006 bp.

PFGE confirmed that disruption of the gene was not a result of gross chromosome rearrangements (Fig. 6a). Southern analysis of the pulsed-field gel localized *ELF1* to chromosome R (Fig. 6b). Since *I-SceI* is present in the *hisG* gene of the disruption cassette but not elsewhere in the *Candida* genome, digestion with *I-SceI* results in cleavage at the site of the disrupted gene. Therefore, if the gene is correctly disrupted, the chromosome will be restricted with *I-SceI*. Chromosome plugs of CAJS2 and CAJS5 were digested with *I-SceI*, subjected to PFGE, Southern blotted and probed with the *EcoRI* insert of

Fig. 6. PFGE of transformed and I-SceI-digested strains. Chromosomal DNA was prepared from the following strains: CAI4 (lane 1), CAJS5 (lanes 2 and 3) and CAJS2 (lanes 4 and 5). DNA was incubated with (+) or without (–) I-SceI. (a) The chromosome bands were visualized with ethidium bromide. Chromosomes are numbered on the left. The position of the R chromosome (R) or digested R chromosome (r^*) is indicated. (b) Southern blot of the gel in (a) hybridized with the 3.2 kb *EcoRI* fragment from pABC-1.

pABC-1. Fig. 6(b) shows that integration corresponds with localization of the *ELF1* gene on chromosome R. Fig. 6 shows that chromosome R migrates differently in the three strains, and in CAJS5, appears to comigrate with chromosome 1. It has been reported that chromosome R and chromosome 1 comigrate in some strains (Wickes *et al.*, 1991b). However, digestion with I-SceI and hybridization with *ELF1* clearly shows the faster migration of one digested chromosome in CAJS2 and both chromosomes in CAJS5.

Phenotypal characterization of CAJS5

CAJS5 was grown in YPD and YNB media supplemented with uridine. Growth was slower compared to CAF2-1 (the *URA3* heterozygote), and CAJS2 (*ELF1::elf1 URA3:ura3*), in both liquid (Fig. 7) and solid media (data not shown) but was comparable to the Ura^- strain, CAI4. The intermediate growth rate of CAJS2 (*URA3/ura3*) appears to be due to a dosage effect of the *ELF1* gene and not the *URA3* gene, since in a similar experiment, the growth rate of CAF2-1 (*URA3* heterozygote) was the same as SC5314 (data not shown). Cell growth based on OD_{550} resulted in the same differences between growth curves (data not shown).

There is a significant difference in colony growth between CAJS5 and CAF2-1; CAJS5 colonies are smaller and more heterogeneous in size. Microscopic examination of both small and normal-sized colonies revealed that yeast forms of CAJS5 are variable in shape: while normal budding cells were observed, many cells were large and misshapen, and multiple buds were common (Fig. 8). There was no obvious difference in the proportion of cell types in small and normal-sized colonies. However, the small colonies normally contained 80% fewer cells. Aggregated forms were also observed which could contribute to the slower growth rates of CAJS5 (see Fig. 7). Aberrant yeast forms made up between 50–60% of the population in CAJS5, compared to 20–30% in CAJS2 and less than 10% in SC5314, during the first 6 h growth in YPD. During the next 3 h, aberrant forms in CAJS5 and CAJS2 decreased to 15% and 8%, respectively. Aberrant yeast forms were viable as determined by staining with methylene blue. An equivalent number of cells from 2 and 6 h cultures of CAJS5 and CAF2-1 were plated on YPD. Again, the viability of the cultures was equivalent but the CAJS5 colonies were significantly smaller. Aberrant cells were no more sensitive than normal cells to 1% SDS or high salt (data not shown).

Fig. 7. Growth of *C. albicans* strains in YPD. Cultures (4 ml) were inoculated with 1×10^6 cells from overnight cultures. Samples were taken and cells counted with a haemocytometer at the times indicated. These data are representative of three separate experiments. Similar results were obtained when cells were grown in YNB, or in the presence of uridine, or when cell concentration was determined by OD₅₅₀ measurement. The growth curve of SC5314 was identical to that of CAF2-1.

DISCUSSION

ABC membrane pump proteins are found in a variety of micro-organisms including *C. albicans*, as well as in mammalian cells. Structurally, these proteins are composed of two repeated halves, each of which includes a hydrophilic, cytoplasmic domain and a hydrophobic domain containing six transmembrane regions. Each

half consists of one Walker A (P-loop), one Walker B and an ATP-binding domain, typical of the ATP-binding cassette transporters (Higgins, 1992). Sequence analysis of the *ELF1* gene revealed a similar structural arrangement. Two ATP/GTP-binding site domains and an ABC transporter consensus sequence located between the Walker A and B domains in the first half were identified in *ELF1*. The homology of *ELF1* described in this report was most extensive to SCYPL226W, an ORF of unknown function recently reported from *S. cerevisiae*; a lower level of homology was seen to EF-3. Homology with the MDR-like genes was low, but still significant.

To resolve questions about the function of *ELF1*, in the light of its homology with other genes, we constructed disrupted strains and examined the role of *ELF1* in growth. Since the disruption is at the 3' end of the gene and some ABC transporters can function as half molecules, the double-disruptant may not be a true null. We found that disrupted strains grew less well than the wild-type, and heterozygotes demonstrated an intermediate growth rate. The growth impairment was observed on agar media as the presence of smaller-than-normal and pin-point-sized colonies. In liquid media, cells were pleiotropic in size and morphology. While some budding cells were normal in size, a significant number were larger and formed elongated buds. Some cells were swollen and elongated with a slight constriction in the middle, resembling a peanut shell, appearing as if they could not form a bud wall. The swollen size could result from a growth control defect or, alternatively, a deregulation of efflux/influx proper-

Fig. 8. Photomicrographs of CAJ55 and SC5314 (125× magnification). YPD+uridine was inoculated with an overnight culture of SC5314 (a) or CAJ55 (b) to a starting density of 10^6 cells ml⁻¹ and incubated at 30 °C for 3 h. The variability in the morphology of CAJ55 can be seen here.

ties. The aberrant cells made up 50% of the population early in culture (2–6 h). The lower percentage of aberrant cells at 7–9 h is probably due to the division of 'normal' cells. The pleiotropy may be due to the absence of the *ELF1* gene or alternatively, because only the 3' end of the gene is deleted in CAJS5. The normal cells seen in the population could be the result of the action of the truncated *ELF1* gene. However, the truncated activity must be insufficient for the entire population. Cassettes which will disrupt the 5' region of the gene are now being constructed. At this point, it is impossible to determine whether the abnormal growth in *ELF1* mutants is due to an altered permeability in the cell, or whether the ELF gene product affects the expression of specific genes required for optimal growth. It is not yet known whether a single compound can restore a normal growth pattern to CAJS5, or whether the absence of this gene results in a general deficiency in the transport of required metabolites to the correct components of the cell or export of toxic compounds from the cell. A gene-dosage effect is apparent, in that strains heterozygous for *ELF1* show an intermediate growth rate and percentage of abnormally shaped yeast cells. Consequently, this gene may be a potential antifungal target, since its reduced expression may affect viability in the host.

Drug resistance and sensitivity in yeast have been linked to altered permeability (Fling *et al.*, 1991). We compared strains CAJS2 and CAJS5 to SC5314 for sensitivity to amphotericin B, and three azoles, ketoconazole, fluconazole and miconazole, by the broth microdilution method for drug susceptibility (data not shown) (National Committee for Clinical Laboratory Standards, 1995). No significant differences in susceptibilities were seen. However, when the small colonies of CAJS5 were compared with the normal-sized colonies, preliminary data indicated that small colonies have a 100-fold or greater resistance to miconazole and amphotericin B. The MIC₅₀ of normal sized and small colonies for miconazole was 0.008 µg ml⁻¹ and 0.4 µg ml⁻¹, respectively, and for amphotericin B, 0.06 µg ml⁻¹ and >1 µg ml⁻¹, respectively. No difference was seen with fluconazole and only a slight difference with ketoconazole. Others have shown that resistance to one azole does not necessarily confer resistance to azoles in general (McCullough & Hume, 1995). The increased resistance in the small colonies to miconazole and amphotericin B, but not the other azoles could be due to their slower growth rate, associated with changes in transport (influx or efflux). In this case, *ELF1* would have an effect opposite that of the *Candida* ABC genes, *CDR1* and *CDR2*, in that increased expression of *CDR1* and *CDR2* is seen in fluconazole-resistant clinical isolates (Sanglard *et al.*, 1995, 1997; White, 1997). *CDR1* but not *CDR2* knockout mutants in *C. albicans* demonstrate hypersensitivity to a variety of antifungal agents, including azoles; strains deleted in both *CDR* genes show increased sensitivity over *cdr1* null mutants (Sanglard *et al.*, 1996, 1997). It is interesting to note that both *ELF1* and *CDR1* lack an ABC signature sequence between the second set of Walker domains.

Although most of the ABC superfamily are transport proteins, a few are involved in UV repair and protein translation (Higgins, 1992). BLAST analysis identified significant homology between *ELF1* and the fungal EF-3 genes. EF-3 is involved in the ATP-dependent interaction between aminoacylated tRNA and the ribosome A-site (Chakraborty *et al.*, 1995). After SCYPL226W and the EF-3 genes, *GCN20* was the most homologous to *ELF1*, particularly at the ATP-binding sites. *GCN20* is a positive regulator of the transcriptional activator protein *GCN4*, which is involved in general amino acid control. One function proposed for *GCN20*, like that for EF-3, is to facilitate the release of uncharged tRNA from the ribosome E site (Vasquez de Aldana *et al.*, 1995). Therefore, *ELF1* may play a role in polypeptide elongation. The abnormal morphology and increased resistance to some drugs may indicate an altered permeability of the cell. On the other hand, if the translation mechanism is altered, there may be a subsequent effect on the expression of genes for optimal growth. In either scenario, drug transport or translational control, the importance of *ELF1* is apparent.

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