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Candida albicans SSD1 can suppress multiple mutations in *Saccharomyces cerevisiae*

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The *SSD1* gene of *Saccharomyces* encodes a 160 kDa cytoplasmic protein that can suppress mutations in a number of other genes. A functional homologue of *SSD1* from the human pathogen *Candida albicans* was isolated on the basis of its ability to restore viability at the restrictive temperature in a *Saccharomyces cerevisiae swi4 ssd1-d* strain. The *C. albicans* gene, designated *CaSSD1*, encodes a 1262 aa protein which has 47% identity overall to *S. cerevisiae SSD1* as well as significant identity to *Schizosaccharomyces pombe dis3* and *sts5* products. It is shown that *CaSSD1* expression is constitutive through the mitotic cell cycle, which is consistent with a role for the protein in cell growth. *CaSSD1* rescues the *swi4*^{ts} defect in an *ssd1-d* background when expressed from its own promoter on a single-copy plasmid and under the same conditions can rescue mutations in genes encoding protein phosphatase type 2A catalytic subunits. These data suggest that *CaSSD1*, like its *S. cerevisiae* homologue, can limit the effect of mutations on a variety of cellular processes.

Keywords: *SSD1*, *Candida albicans*, cell cycle, protein phosphatase, RNase II

INTRODUCTION

Growth and division in eukaryotic cells are tightly regulated processes. Cell division in almost all cells comprises four distinct phases, G1, S, G2 and M. Changes in the DNA content of the cell occur exclusively during S phase, the period of DNA synthesis when the genome content is doubled, and M phase, the period of genome segregation and mitosis. Overall control of division is achieved principally by regulating the entry into one of these two phases, so that the major cell cycle controls operate at the G1–S-phase transition or at the G2–M-phase boundary. In budding yeast such as *Saccharomyces*, the major control occurs in late G1 with a process termed START (Pringle & Hartwell, 1981). Although START is molecularly complex, the key event is the activation of the Cdc28 protein kinase by phosphorylation and by association with labile G1 cyclins encoded by *CLN1*, *CLN2* and *CLN3* (Richardson *et al*., 1989; reviewed by Sherlock & Rosamond, 1993). Completion of START and activation of the Cdc28 kinase set in train the pathways needed to progress the cell from G1 to S phase.

One consequence of the assembly of Cdc28 protein kinase with Cln3 is the activation of the SBF transcription factor which is a heterodimer of Swi4 and Swi6 proteins (Nasmyth & Dirick, 1991; Ogas *et al*., 1991). Cells with mutations in *SWI4* fail to bud at the non-permissive temperature, are delayed for entry into G2 and are defective in the damage-induced transcription of *RNR2* and *RNR3* (Ho *et al*., 1997). Some *swi4* mutant haploids grow slowly with large, aberrantly shaped cells in which *CLN1*, *CLN2* and *PCL1* transcript levels are markedly reduced (Ogas *et al*., 1991). However, the specific phenotype of *swi4* cells is influenced by alleles of a second unrelated gene, *SSD1*, such that haploid cells carrying a *swi4*^{ts} mutation only exhibit a temperature-sensitive phenotype if the cells also carry a defective *SSD1* allele.

The *SSD1* gene was identified initially as a suppressor of mutations in the *SIT4* protein phosphatase. *SSD1* is naturally polymorphic within strains of *S. cerevisiae* and exists as either the *SSD1-v* (viable) allele which encodes a 160 kDa protein, or the *ssd1-d* (dead) allele which appears to encode an 83 kDa C-terminally truncated form of the protein (Uesono *et al*., 1994, 1997). Unlike *ssd1-d*, the *SSD1-v* allele can suppress lethality of mutations in a number of genes, including *CLN1*, *CLN2* (Fernandez-Sarabia *et al*., 1992), *BCY1*, *SLT2*, *RPC31*, *RPC53* (Chiannilkulchai *et al*., 1992; Mazzoni *et al*., 1993; Stettler *et al*., 1993; Watanabe *et al*., 1995; Wilson

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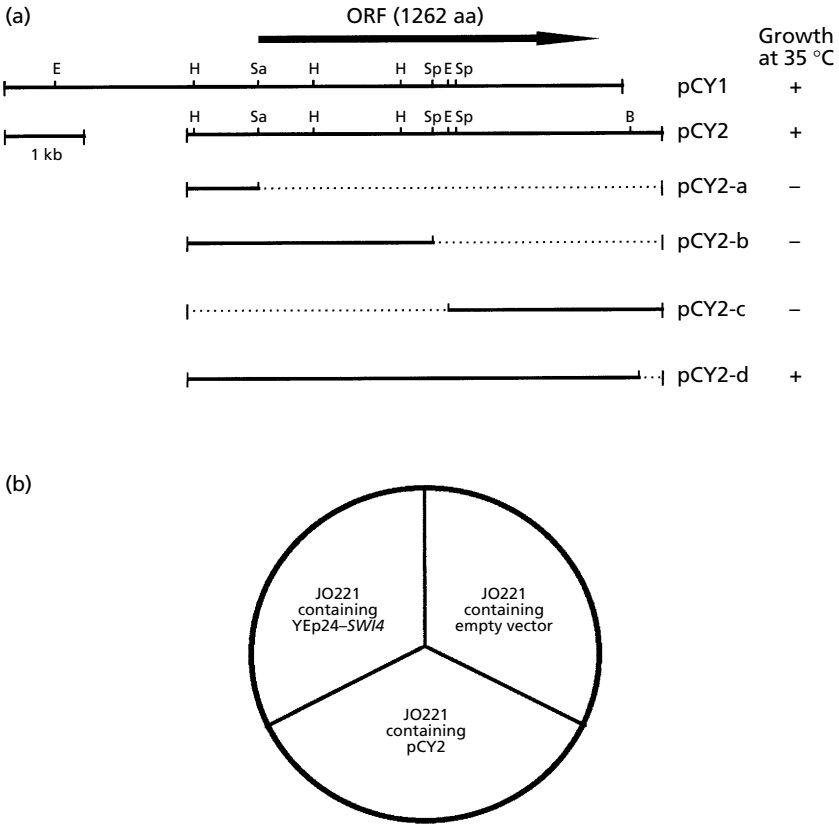

Fig. 1. Partial restriction maps and complementation analysis of *CaSSD1* subclones. (a) Complementation was assayed by the ability of subclones to restore growth of the JO221 strain at 35 °C; + indicates growth, – indicates no growth. The arrow shows the location, size and direction within the cloned DNA of the *CaSSD1* ORF. The deletion regions are indicated by dotted lines. B, *Bgl*II; E, *Eco*RI; H, *Hpa*I; Sa, *Sac*I; Sp, *Spe*I. (b) pCY2 suppresses the growth defect of JO221. The empty vector (pRS316) and YEp24 containing the *SWI4*$^{+}$ gene were introduced into JO221 as controls. The transformed strains were then streaked out on Ura dropout plates to select for the presence of the plasmids and incubated for 2 d at 25 or 35 °C.

et al., 1991) and *SIT4* (Sutton *et al*., 1991). The molecular mechanism by which *SSD1* suppresses any of these mutations is unclear although the protein has significant homology to a fungal protein phosphatase (Sutton *et al*., 1991) and weak but significant similarity to RNase II-related proteins (Uesono *et al*., 1997). Recently, *SSD1* has been shown to bind to RNA *in vitro* although there is as yet no evidence for RNase activity (Uesono *et al*., 1997).

We are interested in the control of cell division in the related pathogen *Candida albicans* and have shown previously that many of the central elements responsible for cell cycle control during G1 phase are conserved in this organism (Sherlock *et al*., 1994; Nolan & Rosamond, 1996). We were interested to determine whether that conservation extends to factors which influence the periodic expression of a number of genes during G1 phase. To examine this, we sought to identify *C. albicans* genes that could restore viability in *S. cerevisiae swi4 ssd1-d* mutants under restrictive conditions. In this paper we describe the results of this work in which we have isolated and characterized the *C. albicans* homologue of *S. cerevisiae SSD1*.

METHODS

Microbial strains and methods. The *S. cerevisiae* strains used in this work were: JO221 (a/α *TRP*/*TRP swi4*Δ/*swi4-ts*

BAR1/bar1::LEU2 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 leu2Δ1/leu2Δ1 ssd1-d/ssd1-d) obtained from Jim Murray, University of Cambridge (Ogas *et al*., 1991); DEY102D (a/α *pph21Δ1::HIS3/pph21Δ1::HIS3 pph22-12/pph22-12 pph3Δ1::LYS2/pph3Δ1::LYS2 ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100 ssd1-d2/ssd1-d2*) and DEY1032-2C (*pph21Δ1::HIS3 pph22Δ1::URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 ssd1-d2 FUS3 KSS1*), both kindly provided by Mike Stark, University of Dundee (Evans & Stark, 1997); and W303 (*a/α ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 ssd1-d/ssd1-d*. *C. albicans* strain 126 was obtained from Richard Barton, University of Manchester. All strains were routinely grown on YEPD (complete) medium and supplemented synthetic minimal medium (YNB) with appropriate nutritional supplements was used for the selection and maintenance of plasmids in *S. cerevisiae* (Sherman *et al*., 1986). Standard yeast genetics and recombinant techniques were used (Sherman *et al*., 1986). Yeast transformations were performed by the lithium acetate procedure with single-strained carrier DNA (Schiestl & Gietz, 1989).

Escherichia coli HW87 was used routinely as the host for the propagation and preparation of plasmid DNA (Birnboim & Doly, 1979). Bacteria were transformed and DNA prepared using standard protocols (Dower *et al*., 1988).

Nucleic acid methods. Standard recombinant DNA techniques were used throughout (Sambrook *et al*., 1989). Restriction endonucleases, Klenow fragment of DNA polymerase I, *Taq* DNA polymerase and T4 DNA ligase were purchased from Boehringer Mannheim or Gibco-BRL and used according to the manufacturer's instructions. The nucleotide sequence of the cloned DNA was determined by the dideoxynucleotide sequencing technique (Sanger *et al*., 1977) using Sequenase version 2.0 (USB). The deduced sequence was analysed by using University of Wisconsin Genetics Computer Group (GCG) software on the Daresbury database facility. Two sets of oligonucleotide primers were used to amplify parts of the *CaSSD1* coding sequence by PCR. One pair of primers comprised CYC02 (5′ CAGAGAGCTCAAACGATACCAGAGGT 3′) and HPA1F (5′ TGTCGTCATTATCGTCAT 3′) which amplify the region encoding residues 208–652 within *Ca*Ssd1, with the engineered *Sac*I site in CYC02 shown by underlining. The other set was CYC03 (5′ TGATCACATAATATCTACCCCT 3′) and PV2 (5′ TGAAGAAGAGGAAATTA 3′) which amplify the region encoding residues 499–960 within *Ca*Ssd1, with the engineered *Bcl*I site in CYC03 shown by underlining.

Plasmid constructions for functional domain mapping. The serial deletion constructions for analysis of functional domains were designed using two methods. First, the 5·5 kb *Hin*dIII–*Not*I fragment containing the full-length genomic insert of pCY2 was cloned into the *Hin*dIII/*Not*I backbone of pCR2.1 (Invitrogen), yielding pCY2-0. The 1278 bp *Nsi*I–*Nsi*I, 525 bp *Nde*I–*Nde*I and 429 bp *Spe*I–*Spe*I fragments derived from *C. albicans* DNA were removed from pCY2-0; the residual genomic DNA in pCY2-0 was religated and the *Hin*dIII–*Not*I fragments carrying the modified *CaSSD1* coding sequences were cloned back into *Hin*dIII/*Not*I digested pRS316, yielding pCY2-2, pCY2-3 and pCY2-4, respectively. Second, a PCR method was used to create 5′ and 3′ deletion constructs. Plasmid pCY2-1 was constructed in this way by replacing the *Sac*I–*Asp*718 fragment within *CaSSD1* in pCY2-0 with a 1·3 kb PCR product double-digested with *Sac*I and *Asp*718. This PCR product was amplified with the primers CYC02 and HPA1F. This construct was then double-cleaved with *Hin*dIII and *Not*I, and cloned into the *Hin*dIII/*Not*I site of pRS316, yielding pCY2-1, a 5′ deletion construct. For the 3′ deletion construct, plasmid pCY2-5 was constructed by replacing the *Asp*718–*Bcl*I fragment of *CaSSD1* within pCY2-0 with a 1·3 kb PCR product double-digested with *Asp*718 and *Bcl*I. This PCR product was amplified with the primers CYC03 and PV2. The *Hin*dIII–*Not*I fragment of this construct was cloned back into the *Hin*dIII–*Not*I site of pRS316, yielding pCY2-5. All PCR products and constructs were confirmed by sequencing to avoid PCR artefacts and to confirm reading frames.

Cell cycle synchronization of *C. albicans*. *C. albicans* strain 126 was grown overnight to mid-exponential phase (OD_{600} 0·4–0·6) in 1 l complete Edinburgh Minimal Medium (EMM) as described previously (Nolan & Rosamond, 1996). The cells were harvested and inoculated into 1 l EMM lacking ammonium sulphate at 23 °C overnight. The culture was examined microscopically to check for uniform G1 arrest, then harvested and inoculated into 250 ml YEPD at 23 °C. Synchrony of the culture was monitored by microscopic examination of the cells at various times after inoculation.

Budding index and FACScan analysis. The cells were sonicated for 5 s prior to determination of the budding index by microscopic examination of small budded cells. For FACScan analysis, samples were prepared as described by Hutter & Eipel (1979) and analysed by using a Becton Dickenson FACS analyser and FACSCAN software (Hewlett Packard).

Northern analysis and probes. Total RNA samples were prepared as described by Schmitt *et al*. (1990). Total RNA was loaded on to 1·2% (w/v) agarose containing 6% (v/v) formaldehyde, 0·02 M MOPS, 0·005 M sodium acetate and 0·001 M EDTA (final pH of 7·0). The gel was blotted on to Hybond-N nylon membranes (Amersham). Probes were the 1·3 kb *Pvu*II–*Eco*RI fragment from pCY2 and a 550 bp fragment corresponding to the 3′ end of the *C. albicans* actin gene which was amplified using the primers 5′ TCTGAACGTGGTTACAGTT 3′ and 5′ CTTAGAAACATTTGTGGTG 3′. The blot was washed once (10 min) at 25 °C using 2 × SSC, 0·5% SDS and twice (10 min each) at 60 °C using 0·1 × SSC, 0·1% SDS (1 × SSC is 0·15 M sodium chloride, 0·015 M sodium citrate).

RESULTS

Isolation of clones which rescue the *swi4 ssd1-d* mutations

To identify *C. albicans* clones which could rescue the *swi4 ssd1-d* temperature-sensitive lesion in *S. cerevisiae* strain JO221, we used CLS1 library DNA (Nolan & Rosamond, 1996). This library carries fragments of *C. albicans* genomic DNA in the vector pRS316 which is a centromeric vector carrying *URA3* (Sikorski & Hieter, 1989). *S. cerevisiae* JO221 was transformed to uracil prototrophy with CLS1 DNA and approximately 4×10^4 transformants were selected at 23 °C. These cells were recovered in pools of about 5×10^3 transformants and aliquots of each pool were replated on YPD agar at 35 °C. Plasmid DNA was recovered from the cells of two of the pools that grew at 35 °C and electroporated into *E. coli* for amplification. Plasmids isolated in this way

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CaSsd1 277  .....GLLEP PQVGFTPGHK PRNSSYGGGS SVSSLQQFLP NNGGSN.... ...NSGQQGG
ScSsd1 297  DYNSFNTLEP PAI.FQQGHK HRAS....NS SVHSF..... .......... .....SSQGN
SpSts5 252  .......... ....EGGGHR HRRS..TGSL SVGS...... .......... .....SGSGF
SpDis3 176  FASHLASLGI KIVLLTDDRE NARLAAEQGI QVSTLKDYVQ YLPDSEILLD MVSAIADAIA

CaSsd1 325  HQGGGNNGRK TLFAPYLPQS SLPELINEGR LVTGTLRVNK KNRSDAYVST DGLLDADIFI
ScSsd1 332  NNGG...GRK SLFAPYLPQA NIPELIQEGR LVAGILRVNK KNRSDAWVST DGALDADIYI
SpSts5 274  SSGGSGNPRK NLFSPYLPQS SIPALLAERR LVTGILIVSK KNRSDAFVSV DG.LDAEVFI
SpDis3 236  SKEQVESGTK NVYELHWSMS RLLACIKNGE VHKGLINIST YNYLEGSVVV PG.YNKPVLV

CaSsd1 385  CGSKDRNRAL EGDLVAVELL IVDEVWESKK EKEEKKRRKD NTLHSRPL.. ..TDDIHNDA
ScSsd1 389  CGSKDRNRAL EGDLVAVELL VVDDVWESKK EKEEKKRRKD ASMQHDLIPL NSSDDYHNDA
SpSts5 333  CGSKDRNRAL EGDVVAIELL DVDEVWAGKL EKEENRRRKD P......... ..........
SpDis3 295  SGRENLNRAV QGDIVCIQIL PQDQ...WKT EAEE...... .......... ..........

CaSsd1 441  TSAPNTAEGS VTGTSKEDGA GSNE...... .......... ....EETGGL ARRGSLKQRP
ScSsd1 449  SVTAATSNNF LSSPSSSDSL SKDDLSVRRK RSSTINNDSD SLSSPTKSGV RRRSSLKQRP
SpSts5 374  .......... ISTRGSFDNL RIDAVPF... .......... .......... ....EVPQRS
SpDis3 326  .IADDDEDVV VSTAAEPDSA RINDL..... .......... .......... ..........

CaSsd1 481  TMKKNDDVEV EGQSLLLVEE EEINDEIKPL YAGHVVAVVD RIPGQLFAGT LGLLRPAQAA
ScSsd1 509  TQKKNDDVEV EGQSLLLVEE EEINDKYKPL YAGHVVAVLD RIPGQLFSGT LGLLRPSQQA
SpSts5 399  AIKARDDEQV EGQTLFLLDQ KQLGADEKPK YAGHVVAVLQ RAPGQVFSGT LGILRPSSAA
SpDis3 350  .......... .......... .ELITKRNAH PTAKVVGILK RN....WRPY VGHVDNATIA

CaSsd1 541  QAARDKKNGK ESTVQNP..K APKIVWFKPT DKKVPLIAIP TEQAPKDFVE NHEKYADRLF
ScSsd1 569  NSDNNKP... ......P..Q SPKIAWFKPT DKKVPLIAIP TELAPKDFVE NADKYSEKLF
SpSts5 459  NKERQTSSGN QGSSNNSGND KPKIVWFKPS DKRVPLIAIP TEQAPTDFLG NDQAYAQRLF
SpDis3 385  QSK....... ........GG SQQTVLLTPM DRRVPKIRFR TRQAPR.... ....LVGRRI

CaSsd1 599  VASIKRWPIT SLHPFGTLVS NLGPIDSPET EIDSILRDNN FLCDEYPDD. .....DNDDI
ScSsd1 618  VASIKRWPIT SLHPFGILVS ELGQIHDPDT EIDSILRDNN FLSNEYLDQ. .....KNPQK
SpSts5 519  LASIKRWPVT SLHPFGMLVG ELGSMDSMSA QVSALLHDTG VHSEPWEGSA ATSAVTALNA
SpDis3 428  VVAIDLWDAS SRYPEGHFVR DLGEMETKEA ETEALLLEYD VQHRPFPKAV LDCLPEEGHN

CaSsd1 653  VSVNAYDLPS IEPEFENTQR EEYLNDY.II AFTQN..... .......GEF VDHALHVKRI
ScSsd1 672  EKPSFQPLPL TAESLEYRRN FTDTNEYNIF AISEL..... .......GWV SEFALHVRNN
SpSts5 579  LSDNFLNVAG CADYRSEDVF LFVKNDVSKA AVSEVKQHES NINSSSATDF VSSAFHIRPT
SpDis3 488  WKV...PADK THPLWKNRKD FRDKLICSI. .......... ...DPPGCQD IDDALHACVL

CaSsd1 700  SNTKIELGFH VADIAYFIKP GSSLDRKSKK RSSSVFLPQK TVNLFPKQVN KIV.SFKENE
ScSsd1 720  GNGTLELGCH VVDVTSHIEE GSSVDRRARK RSSAVFMPQK LVNLLPQSFN DEL.SLAPGK
SpSts5 639  STG.YHVGIH VTDVSRVIEP GSPLDRELQR RSIAVNLCQK SVPLFPTTLG EAL.SLREDK
SpDis3 531  PNGNYEVGVH IADVTHFVKP NTSMDSEAAS RGTTVYLVDK RIDMLPMLLG TDLCSLRPYV

CaSsd1 759  KNLAVSVVFE IDTSNFEVED LYIHESVIIP KQLVTYDAFD TILLGQSVDS ISSATSDYVK
ScSsd1 779  ESATLSVVYT LDSSTLRIKS TWVGESTISP SNILSLEQLD EKL......S TGSPTS.YLS
SpSts5 697  DCYTMSLLLD V.SSTGKIRG TWIGWAVIRP RKAYTMKEAD ELLQTDARLR LFHTVSSRLR
SpDis3 591  ERFAFSCIWE MDENANIIKV HFT.KSVIAS KEAFSYADAQ ARIDDQ...K MQDPLTQGMR

CaSsd1 819  TFSLIAKEFR RHRLSNRSLG ITPNLTLLDQ LDDEKVRLDL NI..FKDSLA FDVISE.ISH
ScSsd1 832  TVQEIARSFY ARRINDPEAT LLPTLSLLES LDDEKVKVDL NI..LDRTLG FVVINE.IKR
SpSts5 756  THHL...... .....GTDVP LSRYCRLVRR WDEESCSFDP NETNLFISSA GEVLRETLLD
SpDis3 647  VLLKLSKILK QKRMDEGALN LASPEVRIQT DNETSDPMDV EIKQL...LE TNSLVEEFML

CaSsd1 876  KVNSAIAAKV HAGLGDQAIL RRHPLPTLQK METFVRKA.T SLGFKIDTTT SSTCKNSILK
ScSsd1 889  KVNSTVAEKI YTKLGDLALL RRQMQPIATK MASFRKKI.Q NFGYNFDTNT ADELIKGVLK
SpSts5 805  AANRAVASHL RQEFRENAFL RTQRLPSREN CRILQSMA.I QMGCVLDLSS TKSLLRSLSL
SpDis3 704  LANISVAQKI YDAFPQTAVL RRHAAPPLTN FDSLQDILRV CKGMHLKCDT SKSLAKSLDE

CaSsd1 935  IDDPVKR... KCVETLLYKC MSRGRY.... ...YVAGKQD TDSYAHYYFN LPLYTHFTAP
ScSsd1 948  IKDDDVR... VGIEILLFKT MPRARY.... ...FIAGKVD PDQYGHYALN LPIYTHFTAP
SpSts5 864  IEDDTVR... NILQLYYYKV TPRAVYEMQK YKGNLASQMM SLGIEDESDD L...THFTAP
SpDis3 764  CVDPKEPYFN TLLRILTTRC MLSAEY.... ...FCSGTFA PPDFRHYGLA SPIYTHFTSP

CaSsd1 985  LRRYADLIVH RQIKAVLNKQ ...VEDKDLD SLKAITEYCN FKKDCAANAQ EQAIHLLLSQ
ScSsd1 998  MRRYADHVVH RQIKAVIHDT ...PYTEDME ALKITSEYCN FKKDCAYQAQ EQAIHLLLCK
SpSts5 918  LERYGDIVVH YQLQLLLRGE L..ASEKRLR VWSQAANDAS RRLVISKFAQ ETSIHIKIFS
SpDis3 818  IRRYADVLAH RQIAAAIDYE TINPSLSDKS RLIEICNGIN YRHRMAQMAG RASIEYYVGQ

CaSsd1 1042 TINEMSETAG QLLCMGTVVQ VYESSFDVFI PEFGVEKRVH GDQLPLV... KAEFDKNERI
ScSsd1 1055 TINDMGNTTG QLLTMATVLQ VYESSFDVFI PEFGIEKRVH GDQLPLI... KAEFDGTNRV
SpSts5 976  DWAE.....S QVWQDGLVCF VAPSYFDVFF PSLGMEKRVH LDLLNLT... HVRFEEDQGI
SpDis3 878  ALKGGVAEED .....AYVIK VFKNGFVVFI ARFGLEGIVY TKSLSSVLEP NVEYVEDEYK

CaSsd1 1099 LEL.WWEKGV DSATYIPPDE KSSLSY.... .RNSIKNKVR TSALQAAKIQ SKTALEKSTT
ScSsd1 1112 LEL.HWQPGV DSATFIPADE KNPKSY.... .RNSIKNKFR STAAEIANIE LDKEAESEPL
SpSts5 1028 LSL.YDESGA VTVVKLLTSV KVKLFV.... .QLSTPPLIN VSNVEF.... ..........
SpDis3 933  LNIEIRDQPK PQTVQIQMFQ QVRVRVTTVR DEHSGKQKVQ ITLVY..... ..........
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Fig. 2. Comparison of the C terminus of *Ca*Ssd1, *S. cerevisiae* Ssd1 (*Sc*Ssd1), *Schizosaccharomyces pombe* Dis3 (*Sp*Dis3) and *Schizosaccharomyces pombe* Sts5 (*Sp*Sts5). Sequences were aligned using the GCG PILEUP program. Dots represent gaps introduced to maximize the alignment while the highly conserved regions within these proteins are boxed.

were rescreened for their ability to rescue the *swi4*^{ts} mutation in JO221 and individual clones were amplified again in *E. coli*. In this way we isolated two independent plasmids that were able to restore growth in *S. cerevisiae* JO221 at 35 °C (Fig. 1); these clones were designated pCY1 and pCY2.

Characterization of cloned DNA in pCY1 and pCY2

The genomic fragments cloned in pCY1 and pCY2 were analysed after restriction enzyme digestion which demonstrated that pCY1 contained an insert of 7·1 kb while that of pCY2 was 5·6 kb. Although the genomic fragments were different in the two clones, analysis of the restriction maps showed that the two fragments were related and shared a common region of about 5·2 kb which we presumed carries the gene responsible for complementation of the *swi4 ssd1-d* mutations in JO221 (Fig. 1).

To define more precisely the region required for complementation, we constructed subclones of the cloned genomic fragment of pCY2 and tested each subclone for its ability to restore growth in *S. cerevisiae* JO221 at 35 °C. However, we were unable to construct any subclones from pCY2 that retained the capacity to rescue *swi4 ssd1-d*, with the exception of one subclone that had a deleted region of approximately 350 bp from one end of the genomic fragment (pCY2-d; Fig. 1a). From this we concluded that the gene responsible for complementation spanned most of the insert in pCY2.

We have determined the nucleotide sequence of the 5·2 kb genomic fragment contained within pCY2-d. This sequence contains a single significant ORF of 3786 nt encoding a predicted protein of 1262 aa. Comparison of this predicted *C. albicans* protein sequence with a database of other known and predicted protein sequences revealed that the protein was most similar to the *S. cerevisiae SSD1* gene product with 47 % identity over 1197 aa. Consequently, we have designated this gene *CaSSD1*. The predicted *Ca*Ssd1 protein also has 28·8 % identity over 667 aa with *Schizosaccharomyces pombe* Sts5 and 21·4 % identity over 636 aa with *Schizosaccharomyces pombe* Dis3 (Fig. 2). The highly conserved domains in these proteins are most obvious within amino acid residues 327–424, 472–643 and 975–1130 of CaSsd1. Within these domains, the proteins show up to 87 % identity in pairwise comparisons. Thus our data suggest that *CaSSD1* is a member of a conserved fungal gene family.

Analysis of the domain organization of *CaSSD1*

To investigate if these highly conserved domains are necessary for the function of *CaSSD1*, five *CaSSD1* derivatives were constructed. Each construct removes a DNA fragment which was predicted to encode a domain of about 200–400 aa while leaving the remainder of the *CaSSD1* ORF intact. In pCY2-1, the N-terminal 200 aa residues, which contain a poorly conserved domain, were deleted. In pCY2-2, a fragment encoding the predicted amino acid residues from 196 to 370, which contains the first conserved domain, was removed. In pCY2-3 and pCY2-5, fragments encoding predicted amino acid residues from 371 to 796 and 960 to 1241, which include the second and third conserved domains, were removed, respectively. The region between the second and third conserved domain was removed in the pCY2-4 subclone (Fig. 3). The functional ability of each clone was examined after transformation into JO221 cells. Remarkably, only pCY2-1, which is deleted for the N-terminal 207 aa, allowed JO221 cells to grow at the restrictive temperature. Even pCY2-4, which is deleted for a poorly conserved region, cannot rescue the *swi4 ssd1-d* defect in JO221 cells at the restrictive temperature, suggesting that this region is either essential for *CaSSD1* function in *S. cerevisiae* cells or that the spacing between the highly conserved domains in *CaSSD1* is critical for protein function. Significantly, none of the conserved domains can be deleted without loss of *CaSSD1* function.

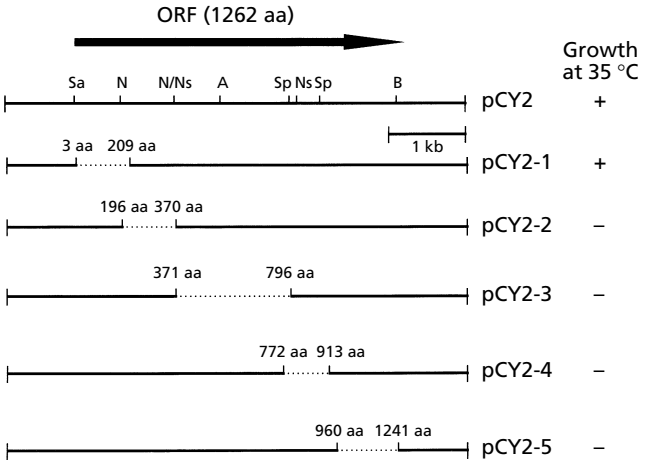

Fig. 3. Functional domains and complementation analysis of *CaSSD1* subclones. Complementation was assayed by the ability of subclones to restore growth of the JO221 strain at 35 °C; + indicates growth, – indicates no growth. The arrow shows the location, size and direction within the cloned DNA of the *CaSSD1* ORF. The deletion regions are indicated by dotted lines. B, *Bcl*I; E, *Eco*RI; N, *Nde*I; Ns, *Nsi*I; Sa, *Sac*I; Sp, *Spe*I.

Morphological analysis of *S. cerevisiae* expressing *CaSSD1*

Expressing *C. albicans* genes in *S. cerevisiae* can cause abnormal morphologies, while phenotypic changes in JO221 cells carrying pCY2 could be informative about the mechanism by which *CaSSD1* acts to restore viability at the restrictive temperature in this strain. To examine this, JO221 was independently transformed with pCY2 and YEp24 carrying *S. cerevisiae SWI4*. Cultures of these transformants together with untransformed cells and the original parental strain (W303) were grown in liquid medium to mid-exponential phase, then a sample of the culture was incubated at the restrictive temperature and the cells examined by phase-contrast microscopy. Over 250 cells were examined for each sample and representative examples of each are shown in Fig. 4.

S. cerevisiae W303 grows at 25 and 35 °C with a normal budded phenotype (Fig. 4a, e), whereas strain JO221 grows with an enlarged, slightly elongated morphology but without any major abnormalities at 25 °C and arrests division at 35 °C as large unbudded cells as observed previously by Ogas *et al.* (1991) (Fig. 4b, f).

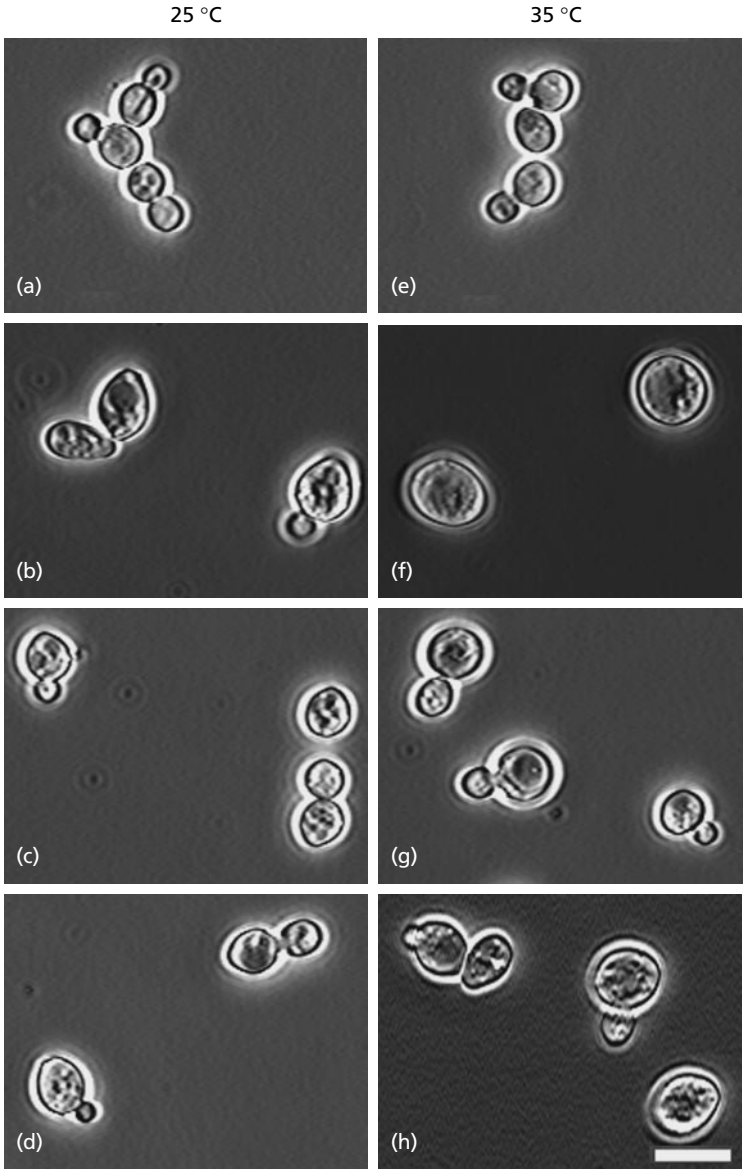

Fig. 4. Phenotypes of *S. cerevisiae* strains at 25 and 35 °C. Unfixed cells were photographed using phase-contrast microscopy. (a–d) Strains in exponential-phase growth at 25 °C; (e–h) the same strains 7 h after shifting to 35 °C. Strains: W303 (a, e), JO221 (*swi4*$^-$) (b, f), JO221 containing YEp24-*SWI4* (c, g) and JO221 containing pRS316-*CaSSD1* (d, h). Bar, 10 μm.

JO221 cells transformed with either YEp24-*SWI4* or pCY2 (*CaSSD1*) grow by budding with an apparently normal morphology at both 25 and 35 °C (Fig. 4c, d, g, h). In both cases the cells are smaller than untransformed JO221 cells although there is a small difference between the two in that cells expressing *CaSSD1* are slightly larger than cells carrying YEp24-*SWI4*. This probably reflects a small variation in the coordination of growth and division in these two cell types resulting from a difference in the effective Swi4 activity when the *swi4* mutation in JO221 is suppressed by *Ca*Ssd1 (on a single-copy plasmid) rather than complemented by expression of wild-type Swi4 protein from YEp24-*SWI4*.

Expression of the *CaSSD1* gene through the mitotic cell cycle

SWI4 is involved in G1 phase of the cell cycle and *CaSSD1* has a genetic relationship with *SWI4* because *CaSSD1* suppresses the *swi4* temperature-sensitive phenotype in JO221 cells. Since this implies that *CaSSD1* may have a periodic function during cell division, we examined the expression of *CaSSD1* during a synchronous round of mitotic division in *C. albicans*. A culture of *C. albicans* 126 was induced to undergo synchronous division (see Methods) that was monitored by microscopy (Fig. 5a) and by FACS analysis (Fig. 5b). Total RNA was prepared from aliquots of the culture, immobilized on to a Hybond-N filter and then probed for *C. albicans SSD1* and actin.

Although the results obtained by microscopy are ambiguous, the FACS analysis shows clearly that the first cell cycle begins about 100 min after release from nitrogen starvation and proceeds synchronously until its completion about 220 min later (Fig. 5a, b). Significantly, the *C. albicans SSD1* transcript is detectable throughout this period with changes in signal

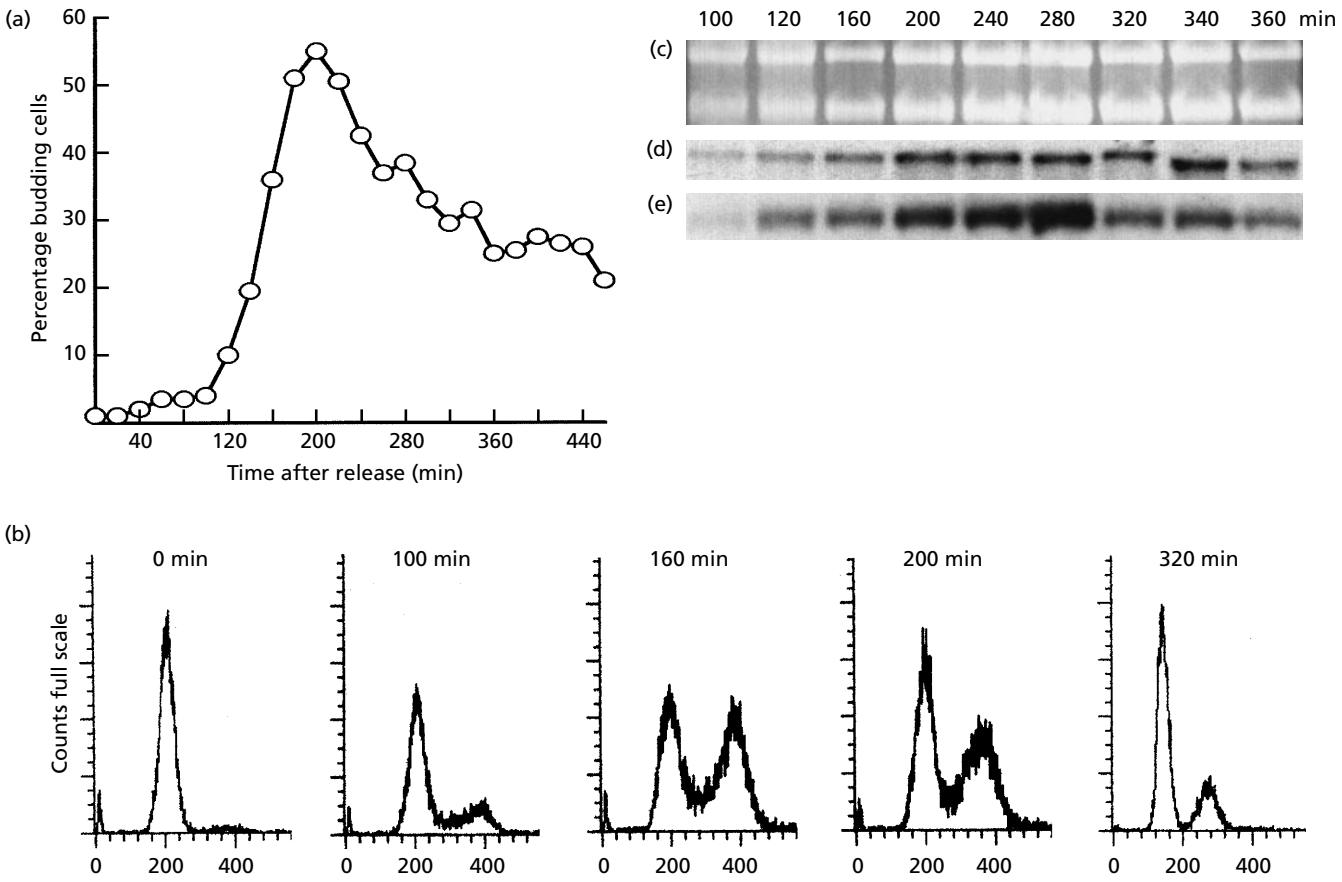

Fig. 5. Expression of *C. albicans SSD1* through the mitotic cell cycle. Samples were taken at 20 min intervals after release from nitrogen starvation. Samples were examined by microscopy for the appearance of small buds, which were scored as spherical outgrowths with a diameter less than a quarter of that of the mother cell (a) and analysed by FACS (b). Total RNA was prepared from each sample and visualized by (c) staining with ethidium bromide, (d) probed for *CaSSD1* and (e) probed for *C. albicans* actin. For *CaSSD1*, the probe corresponded to a 1294 bp region of the 3′ end of *CaSSD1* which was prepared by *Eco*RI and *Pvu*II double digestion. For *C. albicans* actin, the probe was prepared by amplifying a 500 bp region of the 3′ end of the actin gene by PCR.

intensity largely paralleling the variation in total RNA, as reflected in the ethidium-bromide-stained gel (Fig. 5c, d). When compared with actin, the *CaSSD1* transcript level largely parallels that of the actin mRNA transcript signal level, which suggests that *C. albicans SSD1* transcription is not periodically regulated during cell cycle progression. However, this clearly does not preclude the possibility that Ssd1 function is regulated periodically in the cell cycle by translational or post-translational mechanisms, a possibility supported by the demonstration that the Ssd1 protein in *S. cerevisiae* is phosphorylated (Uesono *et al*., 1994).

Effect of *CaSSD1* on protein phosphatase regulation

S. cerevisiae contains two genes, *PPH21* and *PPH22*, that encode protein phosphatase 2A (PP2A) catalytic subunits (Ronne *et al*., 1991). Strains deleted for both *PPH21* and *PPH22* retain viability that is both temperature-sensitive and dependent on *PPH3* which encodes another protein phosphatase catalytic subunit that is presumed to have some overlapping functions with PP2A (Ronne *et al*., 1991; Evans & Stark, 1997).

SSD1-v suppresses mutations in genes required for PP2A activity (Evans & Stark, 1997; Sutton *et al*., 1991) and we have examined whether *CaSSD1* can perform the same function in *S. cerevisiae*.

Initially we used *S. cerevisiae* strain DEY102D which is deleted for *PPH21* and *PPH3* and carries the temperature-sensitive *pph22-12* allele as well as *ssd1-d* (Evans & Stark, 1997). Expressing *CaSSD1* in these cells restored growth at 37 °C (Fig. 6b), indicating that *Ca*Ssd1 can suppress temperature-sensitive mutations in PP2A. We then examined the effect of expressing *CaSSD1* in *S. cerevisiae* DEY1032-2C cells, which are deleted for both *PPH21* and *PPH22* and which show temperature-sensitive, *PPH3*-dependent growth. In these cells, *CaSSD1* restored weak growth at 35 °C (Fig. 6a) but not at 37 °C (not shown), probably because these cells lyse at the higher temperature (Evans & Stark, 1997). Thus these data show that *Ca*Ssd1 can apparently

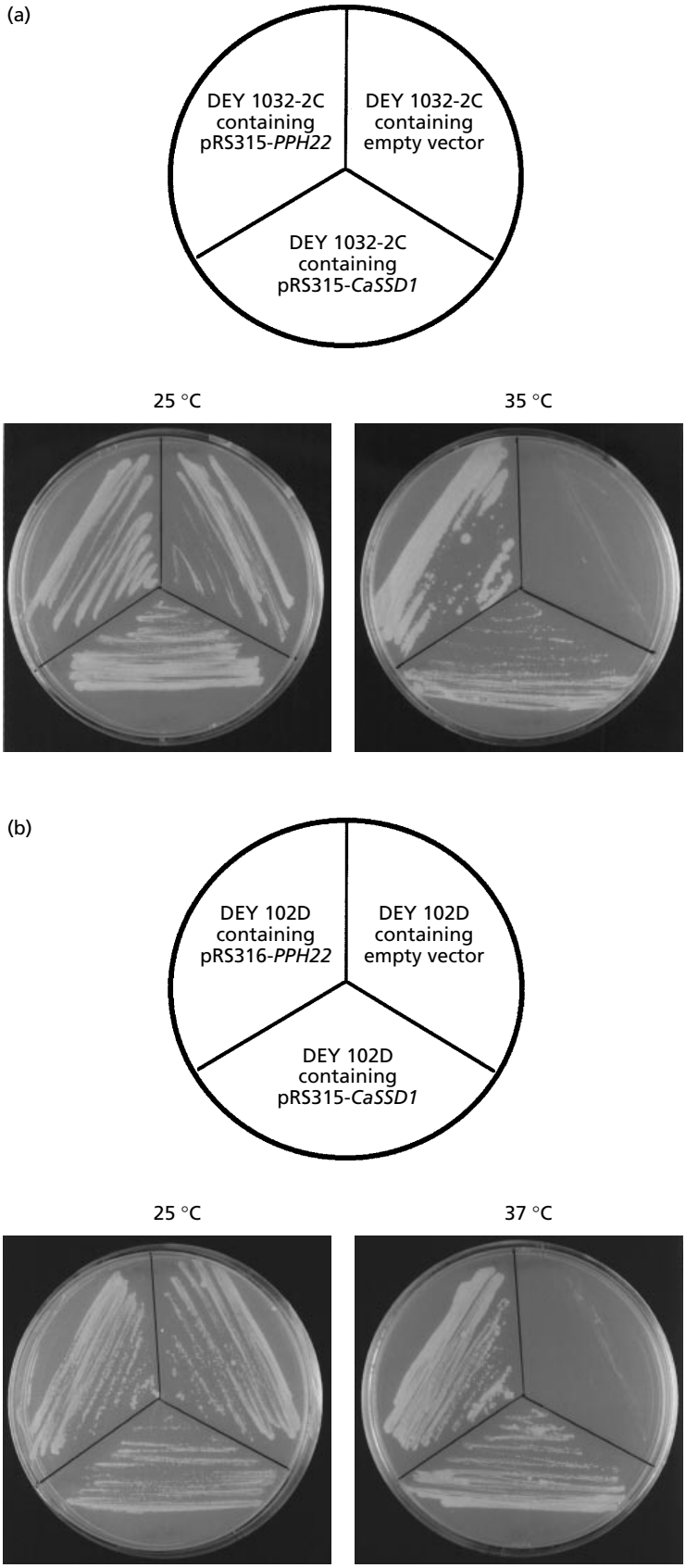

Fig. 6. Complementation analysis of *CaSSD1* on *pph21*$^{-}$ *pph22*$^{-}$ strains. *CaSSD1* suppresses the growth defect of (a) DEY1032-2C and (b) DEY102D strains. The empty vectors (pRS316 and pRS315) and vectors containing *PPH22*$^{+}$ were introduced into DEY102D or DEY1032-2C as controls. The transformed strains were then streaked out on dropout plates lacking (a) Ura or (b) Leu to select for the presence of the plasmids and incubated for 3 d at 25, 35 or 37 °C.

elevate both PP2A and PP2A-related protein phosphatase activity in these strains and so it is functioning in a manner similar to *S. cerevisiae SSD1*.

DISCUSSION

We have described here the isolation and characterization of the *C. albicans* homologue of *S. cerevisiae SSD1* which was isolated by complementation of the *swi4*^{ts} *ssd1-d* mutations. The *CaSSD1* gene encodes a potential 140 kDa protein that has 47 % identity to its *S. cerevisiae* counterpart. Both proteins show homology to *Schizosaccharomyces pombe dis3* which encodes a protein phosphatase involved in the control of mitosis (Kinoshita *et al*., 1991). Since we have shown that *CaSSD1*, like *S. cerevisiae SSD1*, can suppress mutations in the genes encoding PP2A protein phosphatase catalytic subunits, then one possibility is that, like *dis3*, *SSD1* encodes a protein phosphatase whose activity at least partially overlaps the functions of *PPH21*, *PPH22* and *PPH3*. However, this is difficult to reconcile with the recent demonstration that Ssd1 can bind RNA, with a preference for rRNA, and has sequence similarity with exoribonucleases from a number of micro-organisms (Dmochowska *et al*., 1995; Uesono *et al*., 1997). This would suggest that the ability of *SSD1* to suppress mutations in a number of genes whose products have various molecular and cellular functions might be a consequence of post-transcriptional or translational regulation. Whichever of these possibilities is the case, the clear structural and functional relationship between the *S. cerevisiae* and *C. albicans* proteins indicates a common molecular activity for the two proteins.

Although *SSD1* influences some functions specifically associated with the G1 phase of the cell cycle, it also has a relationship with genes needed for cell growth. Consequently, it is perhaps not surprising that the *CaSSD1* transcript is present throughout the mitotic cell cycle at an approximately constant level, implying a lack of critical regulatory motifs within the *CaSSD1* promoter. However, our results also suggest that the level of *CaSSD1* transcription is important and cannot exceed a relatively low threshold. In all cases, we have cloned *CaSSD1* into *S. cerevisiae* on a single-copy plasmid and expressed the gene from its own promoter, which we would expect to produce a relatively low level of *CaSSD1* expression. This view is reinforced by our observation that we can remove all upstream sequences from *CaSSD1* such that its expression in *S. cerevisiae* is driven from adjacent cryptic promoter elements within the vector and that such constructs are still capable of complementation (data not shown). Furthermore, all attempts to increase *CaSSD1* expression by increasing the vector copy number or expressing *CaSSD1* under the control of the *S. cerevisiae* GAL1 promoter have been unsuccessful. It seems likely then that excess *CaSSD1* protein might be toxic to the cell and that the level of expression of *CaSSD1* (and presumably *S. cerevisiae SSD1*) is specifically maintained at a low level.

Finally, we note that we were only able to isolate *CaSSD1* from our original screen when we might also have expected to isolate plasmids carrying a homologue of *S. cerevisiae SWI4*. The fact that we were unable to isolate such a clone is probably not due to the parameters of the CLS1 library that we used since we have failed to identify such a gene in two other independent libraries that we have also screened. One possible explanation for this is that *C. albicans* does not regulate gene expression during G1 by a mechanism that involves a homologue of *SWI4*, although this is unlikely given the overall general conservation of function between *C. albicans* and *S. cerevisiae* and particularly the conservation of cell cycle components (Odds, 1988; Scherer & Magee, 1990; Smith *et al*., 1988). However, we note that there is currently no obvious *SWI4* homologue that has been identified in the *C. albicans* genome sequencing project. Another possibility is that a putative *C. albicans SWI4* contains CTG codons that are differentially decoded in the two yeasts (Leuker & Ernst, 1994; Santos *et al*., 1993), thus leading to a loss of function. A further possibility is that *C. albicans SWI4* interacts with a different specific sequence such that it cannot function at the SBF in *S. cerevisiae*. This is consistent with the absence of SBF motifs in the upstream regions of all currently known *C. albicans* cyclin genes (Sherlock *et al*., 1994; Whiteway *et al*., 1992).

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