Isolation of the mRNA-capping enzyme and ferric-reductase-related genes from *Candida albicans*

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The mRNA-capping enzyme (mRNA 5'-guanylyltransferase) gene was cloned from a *Candida albicans* genomic DNA library by functional complementation of a *Saccharomyces cerevisiae* *ceg1Δ* null mutation. This gene, referred to as *CGT1* (*C. albicans* guanylyltransferase 1), can encode a 52 kDa protein that is highly homologous to *S. cerevisiae* Ceg1p. *CGT1* in a single-copy plasmid complemented the lethality of the *S. cerevisiae* *ceg1Δ* null mutation and, like *S. cerevisiae* Ceg1p, bacterially expressed Cgt1p was able to form a stable complex with the GMP moiety of GTP and to synthesize the cap structure *in vitro*, demonstrating that *CGT1* is the *C. albicans* mRNA 5'-guanylyltransferase gene. *CGT1* seemed to exist as a single copy in the *C. albicans* genome and was actively transcribed into mRNA. Another ORF was found in an opposite strand very close to the *CGT1* locus. This gene shared significant sequence homology with *S. cerevisiae* *FRE1*, the gene encoding ferric reductase, and therefore was designated *CFL1* (*C. albicans* ferric-reductase-like gene 1). Despite its sequence homology with *S. cerevisiae* *FRE1*, *CFL1* mRNA was not induced by iron deprivation, and *CFL1* did not complement the slow growth of a *S. cerevisiae* *fre1Δ* null mutant in the absence of iron, suggesting that *CFL1* is functionally distinct from *S. cerevisiae* *FRE1*.

Keywords: mRNA-capping enzyme, guanylyltransferase, *Candida albicans*, stable GMP-enzyme complex, ferric reductase

INTRODUCTION

Almost all eukaryotic mRNAs are capped at their 5'-termini (Shatkin, 1976). Capping is required for the efficient processing (Edery & Sonenberg, 1985; Inoue *et al.*, 1989; Konarska *et al.*, 1984; Krainer *et al.*, 1984; Patzelt *et al.*, 1987; Ohno *et al.*, 1987), stabilization (Furuichi *et al.*, 1977; Shimotohno *et al.*, 1977; Murthy *et al.*, 1991), nuclear export (Hamm & Mattaj, 1990) and translation of mRNA (reviewed by Filipowicz, 1978; Shatkin, 1985). Capping occurs at an early step during transcription and is catalysed by the capping enzyme. At least three enzymic activities are required for the synthesis of the cap structure: (i) removal of the γ -phosphoryl

group of 5'-termini of mRNA by mRNA 5'-triphosphatase to produce diphosphate-end RNA; (ii) transfer of the GMP moiety of GTP to the resulting diphosphate end of RNA by mRNA 5'-guanylyltransferase; and (iii) methylation of position 7 of newly produced 5'-terminal guanosine by mRNA-cap methyltransferase. Among these three enzymes, mRNA 5'-guanylyltransferase has a unique feature: it covalently binds to the GMP moiety of GTP and forms a stable enzyme-GMP complex (for reviews see Mizumoto & Kaziro, 1987; Shuman, 1995).

In higher eukaryotes, both mRNA 5'-guanylyltransferase and mRNA 5'-triphosphatase activities are present in a single polypeptide (Yagi *et al.*, 1983), whereas the yeast capping enzyme consists of two subunits: the 52 kDa α subunit is responsible for mRNA 5'-guanylyltransferase activity and mRNA 5'-triphosphatase activity is intrinsic to the 80 kDa β subunit (Itoh *et al.*, 1987). Using the antibody raised against purified yeast capping enzyme, the

Abbreviations: 5-FOA, 5-fluoroorotic acid, GST, glutathione S-transferase.

The DDBJ accession numbers for the sequences reported in this paper are D83180 for *CGT1* and D83181 for *CFL1*.

CEG1 gene was cloned from a *Saccharomyces cerevisiae* genomic DNA library and was shown to encode a 52 kDa α subunit (Shibagaki *et al.*, 1992). In addition, disruption of *CEG1* was lethal, indicating that *CEG1* is essential for vegetative growth of *S. cerevisiae* (Shibagaki *et al.*, 1992). Mutational analyses of the *CEG1* gene revealed that Lys₇₀ is important for the covalent binding to GMP; amino acid substitution of Lys₇₀ results in the complete loss of enzyme activity and in the death of *S. cerevisiae* cells (Fresco & Buratowski, 1994; Schwer & Shuman, 1994). Furthermore, the amino acid sequence Lys₇₀-Thr-Asp-Gly of Ceg1p is highly conserved in mRNA 5'-guanylyltransferases from *Schizosaccharomyces pombe* and several viruses (Niles *et al.*, 1986; Pena *et al.*, 1993; Seliger *et al.*, 1987; Shuman *et al.*, 1994; Upton *et al.*, 1991), indicating that Lys₇₀ is located in the active site of the enzyme.

Candida albicans is a dimorphic fungus that commonly causes infection. It grows as a diploid and does not have a sexual cycle (Scherer & Magee, 1990). Upon stimuli such as serum addition or temperature shift, *C. albicans* cells undergo a morphogenetic transition from the yeast to the hyphal form. This morphogenetic transition seems to be somehow associated with virulence because clinical isolates are more efficiently converted into the hyphal form *in vitro* (Odds, 1987).

Several azole compounds such as fluconazole and itraconazole have been developed and used for anti-*Candida* chemotherapy. However, a recent increase in azole-resistant *C. albicans* requires the development of a new type of compound having a different mode of action. Capping enzyme seems to be a feasible antifungal target because mRNA capping is essential, even for fungal cells, and the structure of fungal capping enzyme differs from that of the mammalian counterpart (e.g. subunit vs single polypeptide) as described above.

In an attempt to search for a novel antifungal target, we isolated the mRNA-capping enzyme gene from *C. albicans*. The cloned gene shared significant sequence homology with the mRNA 5'-guanylyltransferase genes of *S. cerevisiae* (Shibagaki *et al.*, 1992) and *Schiz. pombe* (Shuman *et al.*, 1994), and the protein expressed in bacterial cells was fully active as mRNA 5'-guanylyltransferase.

METHODS

Generation of *ceg1Δ* and *fre1Δ* null mutant strains. In order to generate a *ceg1Δ* null mutant strain (*a ura3 leu2 his3 trp1 ceg1Δ::LEU2 CEG1-URA3*), the entire *S. cerevisiae* *CEG1* ORF that was amplified by PCR using primers possessing a *SmaI* cleavage site was subcloned at the *SmaI* site of pUC18, and *LEU2* was ligated at a unique *EcoRI* site of *CEG1* in an opposite transcriptional orientation to *CEG1*. *S. cerevisiae* diploid RAY3A-D (*a/α ura3/ura3 leu2/leu2 his3/his3 trp1/trp1*) was then transformed with the resulting *CEG1-LEU2* chimeric gene, and several Leu⁺ transformants were selected. After confirming the correct integration of the *CEG1-LEU2* gene at the *CEG1* locus by Southern blotting, the cells were further transformed with YEp24 (Botstein *et al.*, 1979) carrying intact *CEG1*, and dissected for tetrads. Thus, the resulting *ceg1Δ* cells could survive in the absence of 5-fluoroorotic acid (5-FOA) but died when it was present. A *S. cerevisiae* *fre1Δ* null mutant strain

(*a ura3 leu2 his3 trp1 fre1Δ::LEU2*) was also obtained by single-step gene disruption. The entire *S. cerevisiae* *FRE1* ORF, which was also amplified by PCR using primers possessing a *SmaI* cleavage site, was subcloned at the *SmaI* site of pUC19, and a 2.4 kb *XhoI-XhoI* fragment of *FRE1* was replaced by *LEU2*, which was inserted in an opposite orientation to *FRE1* transcription. *S. cerevisiae* RAY3A-D cells were then transformed with the resulting *FRE1-LEU2* chimeric gene, selected for leucine auxotrophy, and dissected for tetrads. Integration of the disrupted copy of *FRE1* was also confirmed by Southern blotting. Primers used for PCR were 5'-AATTACCCGGGAA-TGGTTTATGCAATG^{3'} and 5'-TCGTATCCCGGGCTTAG-CCTAATCCGA^{3'} for *CEG1*, and 5'-GGATCCCCGGGAA-TTCATATGGTTAGAACCCGTGTATTATTCTGC^{3'} and 5'-GTACCGAGCTCCCCGGGTACCATGTAAACTTTC-TTCTTCTAGT^{3'} for *FRE1*.

Construction of a genomic DNA library and screening for the *C. albicans* capping enzyme gene. Genomic DNA was extracted from *C. albicans* strain IFO 1060 as described by Lundblad (1989) and partially digested with *Sau3AI*. The resulting *Sau3AI*-digested DNA fragments, whose sizes were between 5 and 20 kb, were separated by agarose gel electrophoresis, isolated and ligated at the *Bam*HI cleavage site of YEp24T in which *URA3* (Botstein *et al.*, 1979) was replaced with *TRP1* (Struhl *et al.*, 1979). Then, *ceg1Δ* null mutant cells were transformed with a *C. albicans* genomic DNA library by the lithium acetate method (Ito *et al.*, 1983), selected for tryptophan auxotrophy, and further selected by 5-FOA resistance (Lundblad, 1989). Nine out of 3×10^4 Trp⁺ transformants were found to grow in the presence of 5-FOA, and plasmid DNA was recovered from the cells grown on the plates containing 5-FOA as described by Lundblad (1989). The inserted DNA fragments were excised by digesting the recovered plasmids with various restriction endonucleases and subcloned into YEp24T to test for 5-FOA resistance. The DNA fragment that allowed *ceg1Δ* null mutant cells to grow in the presence of 5-FOA was digested with several restriction endonucleases, further subcloned in the pUC19 vector, and sequenced using a Sequenase Version 2.0 kit (USB) and [α -³²S]dCTP (NEN).

Southern and Northern blotting. Genomic DNA was extracted from *C. albicans* spheroplasts made by treating the cells with zymolyase and 0.1% SDS (Lundblad, 1989). After cell debris was removed by centrifugation, DNA was precipitated with ethanol, treated with pancreatic RNase and digested with the indicated endonucleases. For extracting RNA, *C. albicans* cells were cultured in minimum defined (MD) medium in the presence or absence of 2 mM FeCl₃ (Dancis *et al.*, 1990) until the OD₆₀₀ reached 1.0. RNA was then extracted by lysing the cells with glass beads in the presence of SDS, followed by phenol extraction and ethanol precipitation (Kasahara *et al.*, 1994). Poly(A)⁺ RNA was purified from total RNA with Oligotex-(dT)₃₀ as described by Kuribayashi-Ohta *et al.* (1993). Endonuclease-digested DNA and poly(A)⁺ RNA were fractionated by agarose gel electrophoresis, transferred to nylon membranes, hybridized with radiolabelled probes and visualized by autoradiography (Sambrook *et al.*, 1989). The probes used for blotting were the entire PCR-amplified ORFs of *CGT1* and *CFL1*. Radiolabelling of DNA probes was carried out by the random priming method with [α -³²P]dCTP (Sambrook *et al.*, 1989). Hybridization and washing of the filters were carried out under stringent conditions [$5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate), $1 \times$ Denhardt's solution (0.2 g Ficoll, 0.2 g polyvinylpyrrolidone, 0.2 g BSA; l⁻¹), 20 mM sodium phosphate buffer (pH 6.5), 0.1% SDS and 50% formamide at 42 °C for hybridization; $0.1 \times$ SSC and 0.1% SDS at 60 °C for washing] (Sambrook *et al.*, 1989).

Fig. 1. Restriction map of the *C. albicans* genomic DNA fragment that complemented the *S. cerevisiae ceg1Δ* null mutation. The 5.2 kb DNA fragment, isolated from a *C. albicans* genomic DNA library by functional complementation of the *S. cerevisiae ceg1Δ* null mutation, is illustrated with several restriction endonuclease sites. Two ORFs found in this DNA fragment are shown as ORF1 and ORF2. The ability of the deletion mutants to complement the *S. cerevisiae ceg1Δ* null mutation is indicated as - and +. For more details, see Methods.

Expression of *S. cerevisiae* and *C. albicans* capping enzymes

in *Escherichia coli*. The entire *S. cerevisiae CEG1* and *C. albicans CGT1* ORFs were amplified by PCR and ligated at the *SmaI* cleavage site of pGEX-2T, which would result in the expression of Ceg1p and Cgt1p as fusion proteins with glutathione *S*-transferase (GST) in bacterial cells (Smith & Johnson, 1988). Primers used for amplifying *CEG1* have already been described in this section, and those for *CGT1* were 5'-GGATCCCCG-GGAATTCTTATGATTCAATTAGAAGAAAGAGAAA TT⁸ and 5'-GTACCGAGCTCCCCGGGTTATTCGTCGTCA-CTATCTTCATAAGTG³. *E. coli* XL-1 Blue cells were transformed with the above plasmids and were induced to express Ceg1p or Cgt1p with 1 mM IPTG at 37 °C for 4 h. Bacterial cells were then lysed by sonication in buffer A containing 20 mM Tris/HCl (pH 7.5), 0.5 mM EDTA, 50 mM NaCl, 10 mM β -mercaptoethanol, 10% (v/v) glycerol, 0.05% (v/v) Nonidet P40 and 1 mM PMSF (Itoh *et al.*, 1987). After removing cell debris by centrifugation, GST-Ceg1p and GST-Cgt1p were purified by glutathione-Sepharose CL-4B column chromatography as described by Smith & Johnson (1988). The purified fusion proteins were dialysed against buffer A, assayed for enzyme-GMP complex formation in a standard reaction mixture containing 20 mM Tris/HCl (pH 7.5), 3 mM MgCl₂, 10 mM dithiothreitol, 0.4 mg bovine serum albumin ml⁻¹, 2 mg inorganic pyrophosphatase ml⁻¹, 20% (v/v) glycerol, 1 mM PMSF and 1 mM [α -³²P]GTP (specific activity 1 $\times$ 10⁵ c.p.m. pmol⁻¹) at 30 °C for 10 min (Itoh *et al.*, 1984), analysed by SDS-PAGE and visualized by autoradiography. *In vitro* cap formation was assayed according to the method of Mizumoto & Lipmann (1979) in a 25 μ l reaction mixture containing 10 mM Tris/HCl (pH 7.5), 3 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 20% (v/v) glycerol, 2.4 μ M [α -³²P]GTP (400 Ci mmol⁻¹; 14.8 TBq mmol⁻¹), 50 pmol ppG-terminated RNA and 1 μ g GST-Ceg1p or GST-Cgt1p protein at 30 °C for 60 min. After digesting the RNA with nuclease P1 and calf intestine alkaline phosphatase, the cap structure was identified by paper electrophoresis on DE81 paper and autoradiography.

Complementation of *ceg1Δ* and *fre1Δ* null mutants by *CGT1* and *CFL1*. A 2.5 kb *ClaI*-*ClaI* fragment of *CGT1* and a 3.7 kb *SalI*-*XbaI* fragment of *CFL1* were subcloned in YE24T or pRS414 and transfected into the *ceg1Δ* null mutant or *fre1Δ* null mutant strains, respectively. The ability of *CGT1* or *CFL1* to complement the *ceg1Δ* null mutation or the *fre1Δ* null mutation were examined by culturing the above transfectants on agar plates containing 5-FOA (for *CGT1*) or on MD medium lacking iron (for *CFL1*) (Dancis *et al.*, 1990), respectively.

RESULTS

Cloning of the *C. albicans* mRNA-capping enzyme gene

We wanted to identify the mRNA-capping enzyme gene in *C. albicans*, one of the pathogenic fungi that causes human mycosis. Since we failed to detect a clear signal by Southern blotting of *C. albicans* genomic DNA using different regions of *S. cerevisiae CEG1* as probes, the approach we chose was functional complementation of the *S. cerevisiae ceg1Δ* null mutation. Since *CEG1* is an essential gene in *S. cerevisiae* (Shibagaki *et al.*, 1992), we generated an *S. cerevisiae* strain in which endogenous *CEG1* was disrupted but in which intact *CEG1* was retained in YE24 that harboured *URA3* as a selectable marker. This strain, designated *ceg1Δ*, grew in the absence of 5-FOA but died when it was present as a consequence of the elimination of the *URA3*-containing plasmid.

ceg1Δ cells were then transformed with a *C. albicans* genomic DNA library and selected by tryptophan auxotrophy and 5-FOA resistance. Nine out of 3 $\times$ 10⁴ Trp⁺ transformants were found to grow in the presence of 5-FOA, and all plasmids recovered from these 5-FOA-resistant clones reproducibly conferred resistance to 5-FOA when transfected into *ceg1Δ* cells. Restriction endonuclease digestion of the recovered plasmids revealed that all clones carried the same part of the *C. albicans* genomic DNA, while the size of the inserted DNA fragment was different in each clone. One clone carrying the shortest genomic DNA fragment was further digested with restriction endonucleases to make deletions, and a 5.2 kb *XbaI*-*Tth111I* fragment was found to be sufficient to complement the lethality of the *ceg1Δ* null mutant.

Sequencing of the cloned *C. albicans* genomic DNA fragment revealed that it contained two potential ORFs, designated ORF1 and ORF2 (Fig. 1). ORF1 could encode a protein with a molecular mass of 52 kDa that is highly homologous to mRNA 5'-guanylyltransferases of *S. cerevisiae* (Ceg1p) (Shibagaki *et al.*, 1992) and *Schiz. pombe* (Pce1p) (Shuman *et al.*, 1994) (39.3% identity and 76.5% homology with Ceg1p, and 38.2% identity and 75.9% homology with Pce1p, in the entire protein) (Figs 2a and

(a)

C. albicans Cgt1p	1: MI-QLEEREIPVIPGKGLDEEETKEEELIMVAEET-GRNN-EPGSGPVSEERRHLEET	57
S. cerevisiae Ceg1p	1: MYLAMESRVAPEIPGLIQGNVTQDQIMVCKLLNSPKPKITPGSGPVSEHSDVEEKL	60
S. pombe Pce1p	1: MAPSEKIDIEEVSVPGLAPRODDVRVETRIAKLIGTSPD-EPGSGPVSESKGHL-QAL	57
C. albicans Cgt1p	58: MOKDQVCEETDQLHCLFLINDPDKGE--GVFLVITRENDYTPINIHFP-LSVNETRE-	113
S. cerevisiae Ceg1p	61: LAHDMVCEETDGLVIMFIVINVTGEQ-GCFMIDHNNYMLVNGFRFRLPQKKKEEL	119
S. pombe Pce1p	58: KKKMDFVCESEGLHLLYMTETFRYENRPSVYLFDHKNMFMHVEKIFTE-V--ENDKS-	113
C. albicans Cgt1p	114: KPTYHHCFLDGEIMLENRVSE-FVLRVVFDALETHCKCIDRPLPKETGYITENVM	172
S. cerevisiae Ceg1p	120: LETLDQGLFLDGEIMIQTNEMTKLOELRLMFDLAINRCRLTQSPSSRIARLGKEFFM	179
S. pombe Pce1p	114: GKRYHVDLFLDGEIMLDIYPGGK-KQLRYLVETDLACDGLVYMSRLDRKLGIFAKSIQ	172
C. albicans Cgt1p	173: EFDNFKKHNDIVNSPEEFPKVGFPNLTSTYHADDV-LSRMDKDFHSDGLIYCAETPY	231
S. cerevisiae Ceg1p	180: EYFDLRAAYPN--RCTTEPKISMHMDFSYQLVKV-AKSLDKLEHLSGLDIFPKVAPY	236
S. pombe Pce1p	173: E--LDEYTKHMETATEPELTSLKRMELGHGILKLFNEVIPRLHNGDGLIFCTETPY	230
C. albicans Cgt1p	232: VFTSDGT-LKWKBAERNIMDQDEFVFNVDQDLDERTPTSTYLDQAKENLIRK	290
S. cerevisiae Ceg1p	237: TAGKDSLLKWKPEQENTVDKILLDIPMVEDESLPKDRNRVYIMDVDP-VFSILVM	295
S. pombe Pce1p	231: VSTSDQS-LKWKPEKENTIDEML--KLEF-AGE--EEG-----IDLSAMEE-FQLGV	278
C. albicans Cgt1p	291: Q-SNV-----H-TD-PA-K-----L-----DL--SDDDERLEALEQFLOQTAEQR	327
S. cerevisiae Ceg1p	296: QGADVNSRLKHFDPDRKEFEILERTYRKFAELSVSDEENONLNLEQPLNGRUEVC	354
S. pombe Pce1p	279: E-S-R-N-M-Y--S-----F-----AF-MY-----VDEKENEKLSFVPLSERIVEC	314
C. albicans Cgt1p	328: QSTTKKGYEMLRFRNDRSNGNHISVEKIDVSTKIGKKEKEVLEWCPKISRAKKREND	387
S. cerevisiae Ceg1p	355: AYNDEGTGAKMLRFHDDKLNQNHISVQKLESTINDSLEDLEIVGDKKRC--D-E	410
S. pombe Pce1p	315: -YLDENNRFRFRHDDKRDANHISTVKSVLQELKSKEDLLKEMPTIREAYYNRKKP	373
C. albicans Cgt1p	388: RRQKHFNQVARD-ASVSHEEPRLKKIKTESNGNGHOTPPRQEQQQSQQTINDIPTYDS	446
S. cerevisiae Ceg1p	411: RRANMGAGSGRPLSPSQSNATLSTSKPVHSQPPSNDKEPKYVDEDDWS	459
S. pombe Pce1p	374: SVTRKRLDTSN-DDAPAIKKVAKSEKEI-----	402
C. albicans Cgt1p	447: DDE	449
S. cerevisiae Ceg1p	460: ---	
S. pombe Pce1p	403: ---	

(b)

C. albicans Cfl1p	1: M--TE--SK---P--H-AK-Y-DK--IQAE---FKING-TE-YAKMTTKSSSGERTSTA	41
S. cerevisiae Fre1p	1: -----MVRTRVLCIF--TSF-PAT-VQSSATLISIS-CISQAALYQFGCSSEKSCYCK	50
S. cerevisiae Ykw0p	1: MHMTSLSAILLCLSGARASPAKTIVIRNKVPLLVDNACTRIFQKVTWEYTSKSKRSPV	60
C. albicans Cfl1p	42: SKSSKSTGSSNASKSST-NAHG-SNSSTSTSSSSSKS-GK-GNS-GTSTETITITPILI	96
S. cerevisiae Fre1p	51: NIMLGSVTCAYENSK-SNKTLSALMKLA-HQCSSI-KVYTLEDMMKINYLNASNYLRA	107
S. cerevisiae Ykw0p	61: CSYEPAPQSMLYCIYETLDEKGYSNKTLEKTFHTIKKNCASYSDALQNTNSEFYOVINN	120
C. albicans Cfl1p	97: DYKKTPTPY-KDAYMS-N-N-NFNLSNMGSGILGWAGI-LA-I--AIF-ANMIKRM-F	146
S. cerevisiae Fre1p	108: PEKSDKKTVVSQPLMANETAYHYEENMGIL-NLM-RSQWCA-WGLVF--FWAVLTA	162
S. cerevisiae Ykw0p	121: GTRMTPTVYKGSANLTPVEMDTQLRKAHYAHGFIYANLDVGNIIYGIIICAYFVIMAF	180
C. albicans Cfl1p	147: PSLTNLNS-GSISN-LFRKHL--FLPA--TFRK-KKAQEFSGVYGFPGGLIPFRLTII	199
S. cerevisiae Fre1p	163: ATILNLKRVFGKNIM-ANS-VKSLIYPSVYKDYNETIFYLWKLEF--NFTTSGGLV	218
S. cerevisiae Ykw0p	181: AGVLHGMNYPFKTVLLKQKLVGVIRGYLTPTIGSKRASDFYFRITGYLPELLEGI	240
C. albicans Cfl1p	200: VVIFVVTGLFSALHIIHVKNDFQYATKNAELGHLIADRTGILGTFLIPLILFGGRNF	259
S. cerevisiae Fre1p	219: VLIFVITLISLSFGHNIKLPHPYDRPWRMSAFVSRHADLMALFVAVYLIGIRNRP	278
S. cerevisiae Ykw0p	241: ILGLVMTHTPLAYGYEYDPENIIFKSRRVQVARYVADHSGVLAFARHETLVLEACNN	300
C. albicans Cfl1p	260: LQWLTPWDFATIMYFPAISRVDVLLIIVHAITFSVSDKATGKYKNNRKRDFMIRG-TGS	318
S. cerevisiae Fre1p	279: FIPITLSFSTENYHKMSAYVCFMLAVHSIVMTASGVKRGVQSIVRKYFRNGI-VA	337
S. cerevisiae Ykw0p	301: LEYISVYKTSJIMFTHKILGRMFLDAMHSGAYSITYVANKTWATSKNR--LYKQGLA	358
C. albicans Cfl1p	319: TIC-GGFIPLQAMLFRRKCYVDFELIRIVLVVFVVGGYHLESQCYGDFMMAAIAVWA	377
S. cerevisiae Fre1p	338: TI-LMSIILPQSEKVFNRGYHILLINKANNIMLIAMYYHCHTLGWMGIWSMAGILC	396
S. cerevisiae Ykw0p	359: ALCLAGTMVFESEAVFKYVYEAFLHIVLGAMFYACWELHVSLSGIEWIYTAIAWI	418
C. albicans Cfl1p	378: FDRVRLGRIFFFARATVSIGDITLKIEV-PRP-KYKWSVAQGHAFIHFKE--TLF	433
S. cerevisiae Fre1p	397: FDFRCHIVRLIMNGLKTATLSTTDSNVIKISVKGKFFKYQVGFAPAYMYFLSKSAMF	456
S. cerevisiae Ykw0p	419: VDRITLIIKASYGFFNASLQIGDLIRLTIV-KFAPRWRKPKQYVVSFELH--LYB	475
C. albicans Cfl1p	434: ---LDSPPFT---FITTESDKIV-LYARINQISNIAKLSPLPGNTATIRVLVEGFPY	486
S. cerevisiae Fre1p	457: YSFDSPHPTVLSERHRDPNPDLTMYVANKHIDVLLSKVLSPNHTVDCIKFLGEPY	516
S. cerevisiae Ykw0p	476: ---WDSHPTV---LDSVSKNGELV-IITLKKKIDRLVKKYVCR-NGGKTSMLRLAEGPY	528
C. albicans Cfl1p	487: EEPSSAGRN-CKRVPFVAGNIPGIYSECVDLAKKS--K-NISIRLINIIRHMKSDSWF	542
S. cerevisiae Fre1p	517: IVTVPHIAKLKRLVGVAAILVAAYPHFVECLRLPSTQGL-HFYVTVNDLSHLKWF	575
S. cerevisiae Ykw0p	529: SSSFPVNN-YNVLTLTGTLPGPIAHAIKLGKTSAAAGCSVSLVIAVIRGFDVLEAY	586
C. albicans Cfl1p	543: TEETEVTKKTVOSTIYVLPQDCSGLECFEHDVSFEKRSDEKDSVSSQYSLSNIIQ	601
S. cerevisiae Fre1p	576: ENELQWLEKSCSEVSIYH--GSSVEDTNSDESTKGFDDH-SE-SEITVECLNKRDL--	629
S. cerevisiae Ykw0p	587: KPELMCLNVLNVLHIYNNVEVSLTPSDSLDISQ-DEADKGTGVATLEKSN-PL	644
C. albicans Cfl1p	602: GLSHQEPFEGCPDSTIQVEQVKAQDGAIGFVTCHPAMDELFAVTO-QLNVSXKRVB	660
S. cerevisiae Fre1p	630: KELM-----SEIKLSP-LENNNITTY-SCGPATFNDDFNNAVVGDSLSKIDVB	678
S. cerevisiae Ykw0p	645: GFDGVVFHCGFPNV-KELLHAAELSGSLSVVCCGPIFVYKVNDETAKIVLDSKAKAIB	703
C. albicans Cfl1p	661: YHPCLOTA	669
S. cerevisiae Fre1p	679: LESEFTN	686
S. cerevisiae Ykw0p	704: YFEPYQCH	711

Fig. 3. Comparison of the amino acid sequences of Cgt1p and Cfl1p with homologous proteins in other organisms. (a) The amino acid sequence of *C. albicans* Cgt1p is compared to those of mRNA 5'-guanylyltransferases of *S. cerevisiae* (Ceg1p) and *Schiz. pombe* (Pce1p). Identical amino acids among these three proteins are boxed. (b) The amino acid sequence of *C. albicans* Cfl1p is compared to those of *S. cerevisiae* ferric reductase (Fre1p) and Ykw0p, whose function is unknown. Identical amino acids in these three proteins are boxed. Amino acid alignment was carried out using the program in Genetyx version 7.3.

3). Experiments involving the deletion of the cloned DNA fragment revealed that ORF1 was essential for complementation of the *ceg1Δ* null mutation (Fig. 1).

Furthermore, a 2.5 kb *ClaI*–*ClaI* fragment that contained only ORF1 rescued the *ceg1Δ* null mutant in the presence of 5-FOA, even in a single-copy plasmid, whereas a 3.7 kb

Fig. 4. Complementation of the *S. cerevisiae* *ceg1Δ* null mutation by *C. albicans* *CGT1*. *S. cerevisiae* *ceg1Δ::LEU2* cells that harboured intact *CEG1* in *Yep24* were further transfected with *YEpcGT1*, *YEpcFL1*, *pRSCGT1* or *pRSCFL1*. The transfectants were then seeded on agar plates with or without 5-FOA and incubated at 30 °C for 3 d. For more details, see Methods.

SalI–*XbaI* fragment that contained only ORF2 did not (Fig. 4). All these results demonstrate that ORF1 encodes *C. albicans* mRNA 5'-guanylyltransferase, and therefore ORF1 was designated *CGT1* (*C. albicans* guanylyltransferase gene 1).

***CGT1* encodes mRNA 5'-guanylyltransferase**

The expected size of Cgt1p deduced from the nucleotide sequence was identical to that of mRNA 5'-guanylyltransferase detected by the enzyme–GMP complex formation using crude nuclear extracts of *C. albicans* (data not shown). In *S. cerevisiae* Ceg1p, Lys₇₀ is expected to be the GMP-binding site, and the neighbouring sequence including this lysine, Lys-X-Asp-Gly, in which X can be any amino acid, is widely conserved in proteins that form a stable nucleotide monophosphate–enzyme complex (Cong & Shuman, 1993; Fresco & Buratowski, 1994; Schwer & Shuman, 1994). Cgt1p also contained the Lys-Thr-Asp-Gly motif starting at position 67 (Fig. 2a).

To further confirm that *CGT1* encodes mRNA 5'-guanylyltransferase, we tested whether or not Cgt1p could form a stable complex with the GMP moiety of GTP. To address this possibility, *C. albicans* Cgt1p and *S. cerevisiae* Ceg1p were expressed in *E. coli* as fusion proteins with GST, and were purified by affinity column chromatography using glutathione-Sepharose. As shown in Fig. 5, purified GST–Ceg1p and GST–Cgt1p were specifically

Fig. 5. Expression of Cgt1p in *E. coli* and stable complex formation with the GMP moiety of GTP. *S. cerevisiae* Ceg1p and *C. albicans* Cgt1p were expressed in *E. coli* as fusion proteins with GST, and purified by glutathione affinity column chromatography. One microgram of purified GST (lane 1), GST–Ceg1p (lane 2) or GST–Cgt1p (lane 3) was incubated with [α - 32 P]GTP, separated on 10% SDS-PAGE gels and visualized by staining with Coomassie Brilliant Blue (left panel) or by autoradiography (right panel). Positions of size markers are indicated in kDa. For more details, see Methods.

labelled with 32 P when incubated with [α - 32 P]GTP, whereas GST alone was not. The GMP–enzyme complexes were stable, even in the presence of SDS, and were never dissociated through SDS-PAGE (Fig. 5). Furthermore, Cgt1p synthesized GpppG when incubated with GTP and ppG-terminated RNA (Fig. 6). Two bands were labelled with 32 P in the purified GST–Cgt1p fraction (Fig. 5). Because Coomassie Brilliant Blue staining of the purified Cgt1p also showed two bands whose sizes were identical to those detected by enzyme–GMP complex formation (Fig. 5), the faster-migrating band might be the degraded product of Cgt1p. Binding of Cgt1p to nucleotides was specific to the GMP moiety of GTP, and it had little affinity to ATP, CTP and UTP (data not shown).

Southern blot analysis demonstrated that digestion of *C. albicans* genomic DNA with *Bgl*II, *Eco*RI or *Xba*I produced bands of 1.2 kb, 0.6 and 1.2 kb, or 1.9 kb, respectively. This pattern was coincident with the expected size from the restriction map of *CGT1*, suggesting that *CGT1* was a single-copy gene in the *C. albicans* haploid genome (Fig. 7).

Another gene present near the *CGT1* locus

We found another ORF (indicated as ORF2 in Fig. 1) very close to the *CGT1* locus in the opposite strand of the *CGT1* ORF. ORF2 shared significant sequence homology

Fig. 6. Cap formation by Cgt1p *in vitro*. One microgram each of purified GST (lane 1), GST-Ceg1p (lane 2) or GST-Cgt1p (lane 3) was incubated with [α - 32 P]GTP and ppG-terminated RNA. After incubation, the RNA was digested with nuclease P1 and calf intestine alkaline phosphatase, and analysed by paper electrophoresis. Positions of GpppG and m^7 GpppG are indicated. For more details, see Methods.

Fig. 8. Northern blotting of *CGT1* and *CFL1* mRNA. Indicated amounts of poly(A)⁺ RNA extracted from *C. albicans* cells that were cultured in MD medium in the absence (–) or presence (+) of 2 mM FeCl₃ were separated on agarose gels and hybridized with radiolabelled *CGT1* (left panel) or *CFL1* (middle and right panels) probes. Positions of 25S and 18S rRNA are indicated. For more details, see Methods.

Fig. 7. Southern blotting using *CGT1* and *CFL1* as probes. *C. albicans* genomic DNA (25 µg) was digested with *Bgl*II (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3) or *Xba*I (lane 4), separated on agarose gels, and hybridized with radiolabelled *CGT1* (left panel) or *CFL1* (right panel) probe. Positions of size markers are indicated in kb. For more details, see Methods.

with *S. cerevisiae* ferric reductase (Fre1p) (Dancis *et al.*, 1992) and a protein of unknown function (Ykw0p) (28.1% identity and 69.4% homology with Fre1p, and 30.7% identity and 71.5% homology with Ykw0p, in the entire protein) (Fig. 3b). Therefore, ORF2 was designated *CFL1* (*C. albicans* ferric-reductase-like gene 1).

The above facts raise the possibility that *CFL1* is the *C. albicans* ferric reductase gene. However, *CFL1* in either a single-copy plasmid or a multicopy plasmid did not complement the slow growth of the *S. cerevisiae* *fre1Δ* null mutant in the absence of iron (data not shown) and the ferric reductase activity was not increased when *CFL1* was overexpressed in *S. cerevisiae* cells using the alcohol dehydrogenase promoter. Moreover, the *CFL1* mRNA level was extremely low compared to that of *CGT1*, and this level of *CFL1* expression was not increased, even when cells were cultured in iron-depleted medium (Fig. 8). Two bands of *CFL1* mRNA appeared by Northern blotting (Fig. 8). Since *CFL1* did not contain introns, there seemed to be several transcription start sites in the *CFL1* promoter.

DISCUSSION

We have demonstrated here that *CGT1* encodes mRNA 5'-guanylyltransferase, and that the GST-Cgt1p fusion protein expressed in bacterial cells was fully active in forming a stable enzyme-GMP complex and in synthesizing the cap structure *in vitro*. *Schiz. pombe* *PCE1* was also cloned by functional complementation of the *S. cerevisiae* *ceg1Δ* null mutation (Shuman *et al.*, 1994).

Although the expected size of Pce1p (47 kDa) is smaller than that of Ceg1p and Cgt1p (52 kDa) (Shuman *et al.*, 1994), the fact that Ceg1p, Pce1p and Cgt1p share a high degree of sequence homology suggests that mRNA 5'-guanylyltransferase is highly conserved in various yeasts. In addition, we did not detect mRNA 5'-triphosphatase activity in the recombinant Cgt1p, indicating that like other yeasts, *C. albicans* CGT1 encodes only mRNA 5'-guanylyltransferase and that mRNA 5'-triphosphatase is encoded by another gene.

Lys₇₀ was shown to be the important residue for covalent binding of GMP in *S. cerevisiae* Ceg1p (Fresco & Buratowski, 1994; Schwer & Shuman, 1994), and Lys-X-Asp-Gly in which Lys is expected to be the GMP-binding site was widely conserved among the enzymes that form a stable enzyme-nucleotide monophosphate complex (Shuman *et al.*, 1994). Cgt1p also contained the Lys-X-Asp-Gly motif near the N-terminus. However, comparison of the *S. cerevisiae*, *Schiz. pombe* and *C. albicans* sequences revealed that there were several completely conserved areas (Fig. 3a). Since mRNA 5'-guanylyltransferase should recognize and may bind to mRNA, some of these conserved sequences may be responsible for RNA recognition.

Recently, *S. cerevisiae* ABD1 was identified as the mRNA-cap methyltransferase gene (Mao *et al.*, 1995). ABD1 encodes a protein with a molecular mass of 50 kDa, and disruption of this gene was lethal in *S. cerevisiae* cells. Since CEG1 is also an essential gene, it seems likely that every step of mRNA capping is essential for vegetative growth of yeasts, and that yeasts have no isozyme for enzymes synthesizing the mRNA-cap structure.

In *S. cerevisiae*, ORF2 was found adjacent to the CEG1 locus (Shibagaki *et al.*, 1992). As in the *S. cerevisiae* CEG1 locus, we also identified the CFL1 gene located very close to the *C. albicans* CGT1 locus. Despite its sequence homology with *S. cerevisiae* FRE1, the results that CFL1 expression was not induced by iron deprivation and that CFL1 did not complement the *fre1Δ* null mutation of *S. cerevisiae* suggest that CFL1 is functionally distinct from *S. cerevisiae* FRE1. Although the physiological function of *S. cerevisiae* YKW0 remains to be established, CFL1 may be functionally related to *S. cerevisiae* YKW0 rather than FRE1. However, we cannot rule out the possibility that the failure of CFL1 to substitute for *S. cerevisiae* FRE1 was the consequence of the different usage of the CUG codon between *C. albicans* and *S. cerevisiae*: it is decoded as serine and not leucine in *C. albicans* (Santos & Tuite, 1995). In fact, there is one CTG in the CFL1 ORF (at position 548 in Fig. 2b). In *S. cerevisiae*, ferric reductase is coupled to the ferrous transporter to convert biologically inaccessible ferric (Fe³⁺) ion into the ferrous (Fe²⁺) state (Dancis *et al.*, 1990), and at least two ferric reductases are present and encoded by the distinct genes FRE1 and FRE2 (Georgatsou & Alexandraki, 1994). We do not know if *C. albicans* incorporates iron by a similar mechanism to *S. cerevisiae*, but identification of the ferric reductase genes in *C. albicans* would answer this question.

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