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The *Candida albicans* gene for mRNA 5′-cap methyltransferase: identification of additional residues essential for catalysis

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The 5′-cap structure of eukaryotic mRNA is methylated at the terminal guanosine by RNA (guanine-*N*^{7}-)-methyltransferase (cap MTase). *Saccharomyces cerevisiae ABD1* (*ScABD1*) and human *hMet* (also called *CMT1*) genes are responsible for this enzyme. The *ABD1* homologue was cloned from the pathogenic fungus *Candida albicans* and named *C. albicans ABD1* (*CaABD1*). When expressed as a fusion with glutathione *S*-transferase (GST), CaAbd1p displayed cap MTase activity *in vitro* and rescued *S. cerevisiae abd1*Δ null mutants, indicating that *CaABD1* specifies an active cap MTase. Although the human cap MTase binds to the human capping enzyme (Hce1p), which possesses both mRNA guanylyltransferase (mRNA GTase) and mRNA 5′-triphosphatase (mRNA TPase) activities, yeast two-hybrid analysis demonstrated that in yeast neither mRNA GTase nor mRNA TPase physically interacted with the Abd1 protein. Comparison of the amino acid sequences of known and putative cap MTases revealed a highly conserved amino acid sequence motif, Phe/Val-Leu-Asp/Glu-Leu/Met-Xaa-Cys-Gly-Lys-Gly-Gly-Asp-Leu-Xaa-Lys, which encompasses the sequence motif characteristic of divergent methyltransferases. Mutations in CaAbd1p of leucine at the second and the twelfth positions (so far uncharacterized) to alanine severely diminished the enzyme activity and the functionality *in vivo*, whereas those of leucine at the fourth, cysteine at the sixth, lysine at the eighth, and glycine at the tenth positions did not. Furthermore, valine substitution for the twelfth, but not for the second, leucine in that motif abolished the activity and functionality of CaAbd1p. Thus, it appears that leucine at the second and the twelfth positions in the motif, together with a previously identified acidic residue in the third, glycine at the sixth and glutamic acid at the eleventh positions, play important roles in the catalysis, and that side chain length is crucial for the activity at the twelfth position in the motif.

Keywords: mRNA 5′-capping, mRNA cap methyltransferase, human, cDNA, *Candida albicans*

INTRODUCTION

Almost all eukaryotic mRNAs harbour a cap structure at the 5′ terminus. Three enzymic activities are involved in mRNA capping. The triphosphorylated 5′ terminus of an RNA is converted to a diphosphate end by mRNA 5′-triphosphatase (mRNA TPase), then capped by mRNA guanylyltransferase (mRNA GTase) to create a GpppN-terminated RNA, and finally methylated at the N-7 position of the terminal guanosine by RNA (guanine-*N*^{7}-)-methyltransferase (cap MTase) (see Mizumoto & Kaziro, 1987; Shuman, 1995, for reviews).

In *Saccharomyces cerevisiae*, the *CET1*, *CEG1* (referred to as *CGT1* in *Candida albicans*), and *ABD1* genes are

Abbreviations: cap MTase, RNA (guanine-*N*^{7}-)-methyltransferase; 5-FOA, 5-fluoroorotic acid; GST, glutathione *S*-transferase; mRNA GTase, mRNA guanylyltransferase; mRNA TPase, mRNA triphosphatase; SAM, *S*-adenosyl-L-methionine.

The GenBank accession numbers for the nucleotide sequence of *CaABD1* and *hMet* are AB020965 and AB020966, respectively.

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responsible for mRNA TPase, mRNA GTase and cap MTase, respectively (Shibagaki *et al*., 1992; Mao *et al*., 1995; Tsukamoto *et al*., 1997). Because deletion of any one of these genes is lethal, every step of mRNA capping is essential for viability (Shibagaki *et al*., 1992; Mao *et al*., 1995; Tsukamoto *et al*., 1997). Furthermore, mRNA TPase and mRNA GTase are physically associated, forming a subunit structure: the association of these two enzymes is essential for the functionality of the enzyme *in vivo* (Ho *et al*., 1998).

In higher eukaryotes, both mRNA GTase and mRNA TPase activities are intrinsic to a single polypeptide called Hce1p (McCracken *et al*., 1997; Takagi *et al*., 1997; Yue *et al*., 1997; Tsukamoto *et al*., 1998a; Yamada-Okabe *et al*., 1998a). Hce1p binds to the hyperphosphorylated C-terminal domain of the largest subunit of RNA polymerase II, which accounts for the selective capping of RNA polymerase II transcripts (McCracken *et al*., 1997; Yue *et al*., 1997). The N-terminal mRNA TPase domain contains an amino acid sequence motif that is characteristic of the active site of protein-tyrosine-phosphatase families (Fauman & Shaper, 1996), suggesting that the catalytic mechanism of the higher eukaryotic mRNA TPase is similar to that of tyrosine phosphatases (Takagi *et al*., 1997; Wen *et al*., 1998). While the mRNA TPase domains of the higher eukaryotic capping enzyme show no sequence homology to the yeast TPases (McCracken *et al*., 1997; Takagi *et al*., 1997; Tsukamoto *et al*., 1997, 1998a; Yue *et al*., 1997; Yamada-Okabe *et al*., 1998a, b), the C-terminal mRNA GTase domains are rather conserved (Shibagaki *et al*., 1992; Shuman *et al*., 1994; McCracken *et al*., 1997; Yamada-Okabe *et al*., 1996, 1998a; Takagi *et al*., 1997; Yue *et al*., 1997; Tsukamoto *et al*., 1998a).

Recently, the capping enzyme cDNAs of *Crithidia fasciculata* and *Trypanosoma brucei* were isolated (Silva *et al*., 1998). The protozoan capping enzymes also have a domain structure similar to that of the mammalian homologues; although the TPase activity of the enzyme remains to be confirmed, the enzyme consists of a single polypeptide bearing both putative mRNA TPase and mRNA GTase domains. The amino acid sequences of the putative mRNA TPase domains of the protozoan enzymes are unrelated to the yeast and mammalian mRNA TPases (McCracken *et al*., 1997; Takagi *et al*., 1997; Tsukamoto *et al*., 1997, 1998a; Yue *et al*., 1997; Silva *et al*., 1998; Yamada-Okabe *et al*., 1998a, b), however, strongly suggesting a different phylogeny of mRNA TPase in yeast, protozoa and mammals.

A cap structure is important for the binding of mRNA to the ribosomes, although some mRNAs form a certain secondary structure that mimics the function of the cap structure (Pelletier *et al*., 1988). Methylation of the terminal guanosine of mRNAs (called cap methylation in this paper) is not an absolute requirement for mRNA translation *in vitro*. A certain level of translation occurs even from mRNAs with an unmethylated cap structure (Held *et al*., 1977); however, cap methylation significantly facilitates mRNA translation both *in vitro* and *in vivo* (Held *et al*., 1977). In fact, *ABD1*-deficient *S. cerevisiae* can not survive (Mao *et al*., 1995), and cap MTase activity is increased during *Xenopus* oocyte maturation, which stimulates translation of exogenous mRNAs bearing an unmethylated cap structure (Gillian-Daniel *et al*., 1998). The *S. cerevisiae ABD1* (*ScABD1*) gene on chromosome II and the human *hMet* (also called *CMT1*) cDNA both code for cap MTase (Pillutla *et al*., 1998; Tsukamoto *et al*., 1998b). By deletion and mutation analyses, the N-terminal 130 amino acids and C-terminal 10 amino acids of ScAbd1p were shown to be dispensable for the activity, and several residues important for the function have been identified (Mao *et al*., 1996; Wang & Shuman, 1997). In humans, the hMet/Cmt1p forms a ternary complex with Hce1p and the elongating form of the human RNA polymerase II (Pillutla *et al*., 1998).

In this study, we isolated the cap MTase gene from the pathogenic fungus *C. albicans*. This gene, called *CaABD1*, encodes a 55 kDa protein, which exhibited cap MTase activity *in vitro* and functionally complemented an *S. cerevisiae abd1*Δ null mutant. Unlike the human cap MTase, the yeast Abd1 protein did not directly bind to mRNA TPase and mRNA GTase. By further exploring the highly conserved amino acid sequence motif that is characteristic of various cap MTases, additional residues important for catalysis were identified.

METHODS

Screening of a *C. albicans* DNA library and a human cDNA library. The partial sequence of the *C. albicans* DNA that resembles *ScABD1* was found in the *C. albicans* database (http://alces.med.umn.edu/candida.html). Using the above nucleotide sequence, a 448 bp DNA fragment of *CaABD1* was amplified by PCR from the genomic DNA of *C. albicans* (strain IFO1060). The full length *CaABD1* was cloned by screening a *C. albicans* genomic DNA library using the PCR-amplified fragment as a probe. The human *hMet/CMT* cDNA was also cloned by screening a HeLa cDNA library using the *hMet/CMT1* DNA fragment as a probe. Primers used to amplify the DNA fragments of *CaABD1* and *hMet/CMT1* were 5′-GGATCCCCGGGAATGTCTACCGATTCGTACACTCCC-3′ and 5′-GGATCCCCGGGACTATACCTTCTCAAATACAAATCC-3′ for *CaABD1*, and 5′-GGATCCCCGGGAATGGCAAATTCTGCAAAAGCAGAA-3′ and 5′-GGATCCCCGGGATCACTGCTGTTTCTCAAAGGCAAA-3′ for *hMet/CMT1*. Hybridization and washing of the filters were carried out under stringent conditions [20 mM sodium phosphate, pH 7·2, 5 × SSC (1 × SSC contains 150 mM NaCl and 15 mM sodium citrate), 5 × Denhardt's solution, 0·1 % SDS, 50 % formamide at 42 °C for hybridization; 0·1 × SSC and 0·1 % SDS at 50 °C for washing]. Plasmid DNA was extracted from bacterial clones that strongly hybridized with the probe DNA. The probe DNA was radiolabelled by the random-priming method with [α^{32}P]dCTP (Sambrook *et al*., 1989), and DNA sequencing was carried out as described elsewhere (Sambrook *et al*., 1989). Construction of the *C. albicans* genomic DNA library was described by Yamada-Okabe *et al*. (1996), and the HeLa cDNA library was purchased from Clontech.

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Expression and purification of the recombinant cap MTase. The coding regions of *CaABD1*, *ScABD1* and the *hMet/CMT1* cDNA were cloned into the *Sma*I site of pGEX2T (Smith & Johnson, 1988). The resulting plasmids were transfected into *Escherichia coli* JM109, and they were induced by IPTG to express CaAbd1p, ScAbd1p and hMet/Cmt1p as fusion proteins with glutathione *S*-tranferase (GST) (Smith & Johnson, 1988). Four hours after the addition of IPTG, the bacterial cells were harvested, suspended in a buffer 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 % NP-40 and 1 mM PMSF, and lysed by sonication. After the cell debris was removed by centrifugation at 15 000 ***g*** at 4 °C for 30 min, the recombinant Abd1 and hMet/Cmt1 proteins in the supernatant were purified by glutathione–Sepharose CL-4B column chromatography as described previously (Yamada-Okabe *et al*., 1996).

Assays for cap MTase. The assays for cap MTase were carried out in a buffer containing 50 mM Tris/HCl (pH 7·5), 5 mM DTT, 50 μM *S*-adenosyl-L-methionine (SAM), G^{32}pppA-terminated RNA and various amounts of the purified fusion proteins at 37 °C for 5 min, followed by incubation at 95 °C for 3 min. After adding sodium acetate (pH 5·5) to a final concentration of 50 mM and incubating with 10 U P1 nuclease (Seikagaku-kogyo) at 37 °C for 1 h, the reaction mixture was analysed by TLC using polyethylenimine cellulose plates (Mao *et al*., 1995), and the spots were visualized by an image analyser (Fuji BAS 2000). The G^{32}pppA-terminated RNA was prepared by incubating 0·5 mg polyadenylic acid ml^{-1} in a buffer containing 20 mM Tris/HCl (pH 7·5), 3 mM $MgCl_2$, 10 mM DTT, 1 μM [α-^{32}P]GTP (1000–5000 c.p.m. $pmol^{-1}$), 0·1mg purified GST–CaCet1p ml^{-1} (Yamada-Okabe *et al*., 1998b) and 0·1mg purified GST–CaCgt1p ml^{-1} (Yamada-Okabe *et al*., 1996) at 37 °C for 30 min. The produced G^{32}pppA-terminated RNA was extracted with phenol and chloroform, and separated from unincorporated [α-^{32}P]GTP by Sephadex G-25 column chromatography.

Generation of the *S. cerevisiae abd1* Δ null mutant strain. To generate the *S. cerevisiae abd1*Δ null mutant strain, *ScABD1* including its promoter and terminator was amplified by PCR from the *S. cerevisiae* genomic DNA extracted from strain A451 (*MATα can1 aro7 can1 leu2 trp1 ura3*) as a template, and cloned into the *Hinc*II site of pUC19 and *Bam*HI site of YEp24, generating pUC-ScABD1 and YEp-ScABD1, respectively. Primers used for amplifying *ScABD1* were 5′-GGATCCGGATCCATCACTGAAGTCGCCGGATATTTT-3′ and 5′-GGATCCGGATCCAATACTTTGCCGAGGACGAGAGTC-3′. For the disruption of *ScABD1*, the 1·2 kb *Hinc*II–*Bgl*II region of *ScABD1* in pUC-ScABD1 was replaced with *LEU2*. Then, haploid *S. cerevisiae* YPH499 (*MATa ade2 ura3 leu2 his3 trp1*) was transformed with the *ScABD1*::*LEU2* chimeric gene together with YEp-ScABD1 (Ito *et al*., 1983) and several Leu^+ Ura^+ transformants were selected. The correct integration of *LEU2* at the original *ScABD1* locus was confirmed by PCR and by Southern blotting. Thus, the resulting *abd1*Δ null mutant (*MATa ade2 ura3 leu2 his3 trp1 abd1*Δ::*LEU2 ABD1*::*URA3*) grew in the absence of 5-fluoroorotic acid (5-FOA) but died in the presence of 5-FOA. To test the ability of *CaABD1* and *hMet/CMT1* to complement an *S. cerevisiae abd1*Δ null mutation, the coding regions of *CaABD1*, *ScABD1* and *hMet/CMT1* were cloned between the *Hin*dIII and *Pst*I sites (between the *ADH1* promoter and terminator) of pGBT9 (Clontech) that carried *TRP1,* and the resulting plasmids were transfected into the above *S. cerevisiae abd1*Δ null mutant strain, in which the endogenous *ScABD1* gene was disrupted by *LEU2*, but where episomal copies of *ScABD1* cloned in YEp24 were maintained (Ito *et al*., 1983). To detect the proteins, the triple c-Myc sequence tag, which was excised from pMPY-3xMYC (Schneider *et al*., 1995), was introduced at the 3′ ends of the *ScABD1*, *CaABD1* and *hMet/CMT1* ORFs. The transformants were transferred to an agar plate containing 5-FOA and cultured at 30 °C for 3 d.

Yeast two-hybrid analysis. The entire ORFs of the indicated yeast and human proteins were cloned into the *Sal*I site of pGBT9 and pGAD424 (Clontech) to express these products as fusion proteins with the DNA-binding domain of Gal4p or with the transactivation domain of Gal4p. Then, the resulting plasmids were transfected into *S. cerevisiae* strain HF7c (*MATa ura3-52 his3-200 lys2-801 ade2-101 trp1-901 leu2-3 112 gal4-542 gal80-538 LYS2*::*GAL1-HIS3 URA3*::(*GAL4 17-MERS*)$_3$–*CYC1-LACZ*) where the *HIS3* gene expression was driven by the DNA-binding and transactivation domains of Gal4p (Feilotter *et al*., 1994). After the transformation of HF7c (Ito *et al*., 1983), the Leu^+ Trp^+ transformants were collected and tested for the ability to grow in the absence of histidine.

Site-directed mutagenesis. A series of the *CaABD1* mutants were obtained by the oligonucleotide-directed dual amber method (Hashimoto-Gotoh *et al*., 1995). The entire *CaABD1* ORF was cloned into the *Sma*I site of pKF18k using a *Sma*I linker, and hybridized with oligonucleotides containing the indicated mutations. The mutant *CaABD1* was excised from the vector, ligated into the *Sma*I site of pGEX-2T and also between the *Hin*dIII and *Pst*I (between the *ADH1* promoter and terminator) sites of pGBT9. All mutations were confirmed by DNA sequencing as described by Sambrook *et al*. (1989).

Western blotting. The indicated amounts of proteins of the yeast cell extracts were separated on a 10 % SDS-polyacrylamide gel, transferred electrophoretically to a PVDF membrane (Sambrook *et al*., 1989) and reacted with the anti-c-Myc monoclonal antibody (clone 9E10; Santa Cruz Biotechnology) and then with horseradish-peroxidase-conjugated protein A (Amersham). The Abd1 proteins were detected using an ECL protein-detection kit (Amersham).

RESULTS

Cloning of the *C. albicans* MTase gene

A short nucleotide sequence of the *C. albicans* genome that resembles *ScABD1* was found in the *C. albicans* database. A full-length *CaABD1* was obtained by screening a *C. albicans* genomic DNA library using the 448 bp DNA fragment of *CaABD1* as a probe. The cloned *CaABD1* contained an ORF of 1446 nt, which can encode a protein of 55 kDa. Two possible TATA boxes were found 116 bp and 104 bp upstream of the first ATG in the *CaABD1* ORF (data not shown). We also cloned the human *hMet/CMT1* cDNA by screening a HeLa cDNA library using a 1·4 kb DNA fragment that was amplified by PCR. The expected product of the cloned cDNA differs by one amino acid from that of the reported hMet/CMT1p; methionine at position 179 is converted to isoleucine in our cDNA clone (Pillutla *et al*., 1998).The comparison of CaAbd1 and other cap MTases is shown in Fig. 1. Although the N-terminal region of CaAbd1p is somewhat diverged, CaAbd1p shares significant sequence similarity with ScAbd1p (Mao *et al*., 1995) and hMet/Cmt1p (Pillutla *et al*.,

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C. albicans       1:MSTDSYTPSQEPGSKRLKTGESVFARRGVSPST-GGVASAYGNESEKKPSWLQT-NKSDI: 58
S. cerevisiae     1:------------------------------MSTKPEKPIWMSQEDYDRQYGSITGDESST: 30
S. pombe          1:M--DS------HHM-ME------RSLT---F---E-RNYS--HL--------FIIIFIIR: 28
H. sapiens        1:M-ANSAKAEEYEKMSLEQAKASVNSETESSFNINENTTASGTGLSEKTSVCRQVDIARKR: 59
C. elegans        1:------------------------------------------------------------
D. melanogaster   1:------------------------------------------------------------

C. albicans      59:DGKYDKYGERRNAHTTTRDSRLDRLKRARQKSAEREDVGHEGDEGDEDEGILPYIHLQAA:118
S. cerevisiae    31:VSKKD-SKVTANAPGDGNGS-LPVLQSSSILTSKVSDLPIEAESGFKIQKRRHERYDQEE: 88
S. pombe         29:-----D---MSSS-----NS---R-VHEEQPPTE-NRRYA--RPTAQMNRVIEQQP----: 64
H. sapiens       60:KEFEDDLVKESSSCGKDTPS-KKRKLDPEIVPEEKDCGDAE-GNSKKRKRETEDVPKDKS:117
C. elegans        1:-----------M--MKE--VLD-AF-R--K---------S-GEAE-GFGHN---KMS-S-: 25
D. melanogaster   1:-----------MSLNYEQNAADEQFARAHKAVSLSDDEESEGQAETTSAPNQEPHVSKSP: 49

C. albicans     119:NPAIIHNEKQENYRTFQSRISNRENRDIN--SIVRAHYNQRTQQAKQQGSRVNSFIYKMR:176
S. cerevisiae    89:RLRKQRAQKLREEQLKRHEIEMTANRSINVDQIVREHYNERTIIA-NRAKRNLSFIIKLR:147
S. pombe         65:RRRDYFQN---NDNSGRRGYNRHEN-NGNAQDVVRSHYN--ARPDLGYKKRQFSFIIQLK:118
H. sapiens      118:STGDGTQNK--RKIALEDVPEKQKNLEEGHSSTVAAHYN--ELQEVGLEKRSQSRIFYLR:173
C. elegans       26:-------------S----------EVAS-HYN--KV---LQVGIE-GRK---ESRIFFMR: 52
D. melanogaster  50:REYYDEPGGKGNGSGADDQDEPETEAASGAANTHVVAHHYNELKEAGRKDRQKSKIFFMR:109

C. albicans     177:NFNNAIKYIL---LGNWAKHNPEELDLFSFLDLCCGKGGDLNKCQFIGIDQYIGIDIADL:233
S. cerevisiae   148:NFNNAIKYML---IDKYTK--P--GD--VVLELGCGKGGDLRKYGAAGISQFIGIDISNA:198
S. pombe        119:RFNNWIKSVLI--QKF---APHASDYPILVLDMGCGKGGDLIKWDKAGIDGYIGIDIAEV:173
H. sapiens      174:NFNNWIKSVLI--GEFLEKVRQKKKRDITVLDLGCGKGGDLLKWKKGRINKLVCTDIADV:231
C. elegans       53:NMNNWVKSQLIN-DAKQ-RVNDNGVNNPRVLDLACGKGGDLKKWDIAGAKDVVMADVADV:110
D. melanogaster 110:NFNNWIKSQLINEYMSQIKQNKRMGDALRVLDMCCGKGGDLLKWEKAAISHLICTDIAEV:169

C. albicans     234:SVKEAFERY-TKQKARFRHSNQNSNRYTFEACFATGDCFTQFVPDILEPNFPGIIERAFP:292
S. cerevisiae   199:SIQEA-------HK-RYR-SMRN-LDY--QVVLITGDCFGESLGVAVEP-FP--DCR-FP:242
S. pombe        174:SVNQAKKRY------REMHASFD---ALF--YAGDCFSSSINELL---PPD----QRKF-:214
H. sapiens      232:SVKQCQQRY------EDMKNRRDS-EYIF--SAEFITADSSKELLIDKFRDP---QMCF-:278
C. elegans      111:SIQQAEERY---KQ-MFGYKKNNIFTVQFIVADCTKENL-EDRIENKDPFDLVSCQFALH:165
D. melanogaster 170:SVEQCQRRYQDILQRSEKSKFANKFTAEFFACDSTLVRLRERYKDPSLQLNLVSCQFAFH:229

C. albicans     293:VDVVSAQFSLHYSFESEEKVRTLLTNVTRSLRSGGTFIGTIPSSDFIKAKIVDKHLQRDE:352
S. cerevisiae   243:CDIVSTQFCLHYAFETEEKARRALLNVAKSLKIGGHFFGTIPDSEFIRYK-LNK--FPKE:299
S. pombe        215:-DVVSLQFCMHYAFESEEKVRVLLGNVSKCLPRGGVMIGTIPNSDVIVKHI--KMLKPGE:271
H. sapiens      279:-DICSCQFVCHYSFESYEQADMMLRNACERLSPGGYFIGTTPNS---FELI--RRLEASE:332
C. elegans      166:YSFVDEASARIFLKNAVGMLKPGGVFIGTLPDADRIVWSMRNGENGQFANEVCKITYENV:225
D. melanogaster 230:YCFESMAQADCMMRNAAECLKPGGFFIATMPDAYEI--IRRLRAAGPDARRFGNDVYSIE:287

C. albicans     353:KGKAKFGNSLYSVTFE--KDPPEDGVFRPAFGNKYNYWLKDAVDNVPEYVVPFETLRSLC:410
S. cerevisiae   300:VEKPSWGNSIYKVTFENNSYQKNDYEFTSPYGQMYTYWLEDAIDNVPEYVVPFETLRSLA:359
S. pombe        272:--KE-WGNDIYKVRF----PESPPRSFRPPYGIQYYFYLEDAVTDVPEYVVPFEAFRAVA:324
H. sapiens      333:--TESFGNEIYTVKF-------QKKGDYPLFGCKYDFNLE-GVVDVPEFLVYFPLLNEMA:382
C. elegans      226:EELAEGKVPLFGAKFHFSLDEQVNCPEFLAYFP-LVKHLLEELDMELLFVHNFAEAINKW:284
D. melanogaster 288:FDCETDPLPLFGAKYQFHLEGVVDCPEFLVHFPTLVK-LGRKYGLQLLKRSTFADYYKEN:346

C. albicans     411:EEYDLVLKYKKSFTDIFNQEIPKYFSKLNKNLIDGMKRSDGKYGAEGDEKE-AVAFYIGF:469
S. cerevisiae   360:DEYGLELVSQMPFNKFFVQEIPKWIERFSPKMREGLQRSDGRYGVEGDEKEAASYFYTMF:419
S. pombe        325:EGYNLELIWVKPFLDILNE-EKNSETYGPLMDRMKVVDNEGHRGIGGQEKEAAGFY-LAF:382
H. sapiens      383:KKYNMKLVYKKTFLEFYEEKIKNNE-NKMLLKRMQALEPYPANESSKLVSEKVDDYEHAA:441
C. elegans      285:LEPGRRLLESMTGLETYPNEKLSGKSDDEYLEAKAKLDAFPEDERIKTMGTLSKSEWEAI:344
D. melanogaster 347:LHHGRHLLQRMSGLE---SVQPQRCENDE--E-FAHVSNFQGAQRSRSVGTLSKSEWEA-:399

C. albicans     470:VFEKVDMSG-------------------------------------------------  :478
S. cerevisiae   420:AFRKVKQYIEPESVKPN-----------------------------------------  :436
S. pombe        383:AFEKRGI---------------------------------------------------  :389
H. sapiens      442:KYMKNSQVRLPLGTLSKSEWEATSIYLVFAFEKQQ-----------------------  :476
C. elegans      345:CMYLVFGFRKKKSEAEKTEEEPATTKPVAESESEQKEVTESEEKEDQEDCEHQEAQTN  :402
D. melanogaster 400:-ASEFTQHRLLNS-ITK-----------------------------------------  :414
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Fig. 1. Comparison of the amino acid sequences of CaAbd1p and other cap MTases. The amino acid sequence of CaAbd1p is compared with those of cap MTases of *Sacch. cerevisiae*, *Schiz. pombe*, *Homo sapiens*, *Cae. elegans* and *D. melanogaster*. Identical amino acids among these proteins are boxed. Amino acid alignment was carried out using the program GENETYX 8.0. GenBank accession numbers are AB020965 for the *Can. albicans* cap MTase, L12000 for the *Sacch. cerevisiae* cap MTase, AL031603 for the putative *Schiz. pombe* cap MTase, AF067791 for the *H. sapiens* cap MTase, Z81038 for the putative *Cae. elegans* cap MTase and AC002502 for the putative *D. melanogaster* cap MTase. According to the report by Santos & Tuite (1995), the CTG codon in *CaABD1* is decoded as serine instead of leucine.

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1998; Tsukamoto *et al*., 1998b), and also with probable cap MTases of *Schizosaccharomyces pombe*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Fig. 1), implying that cap MTase is evolutionarily conserved. Southern blotting of the *C. albicans* genomic DNA using the DNA fragment of the *CaABD1* ORF as a probe generated discrete bands, all of which coincided with the restriction map of *CaABD1* (not shown), indicating that one copy of *CaABD1* is present per haploid genome of *C. albicans*.

To examine its enzyme activity, CaAbd1p was expressed in *E. coli* as a fusion protein with GST and purified by affinity column chromatography (Fig. 2a). When incubated with G^{32}pppA-terminated RNA and SAM, all of the purified GST–CaAbd1, GST–ScAbd1, and GST–hMet/Cmt1 fusion proteins converted G^{32}pppA termini to $^{m7}G^{32}$pppA, whereas GST alone did not (Fig. 2b). For this reaction, SAM serves as a methyl donor, because cap methylation did not occur in the absence of SAM (data not shown). Furthermore, GST–hMet/Cmt1p, GST–CaAbd1p and GST–CaAbd1p all displayed the similar specific activities (Fig. 2c). From these results, we conclude that *CaABD1* specifies an active cap MTase of *C. albicans*.

Complementation of an *S. cerevisiae abd1*Δ null mutation by *CaABD1*

Because the recombinant CaAbd1 and hMet/Cmt1 proteins displayed the cap MTase activity *in vitro*, we addressed the functionality of *CaABD1* and *hMet/CMT1*. The *S. cerevisiae abd1*Δ null mutant strain *scabd1*Δ::*LEU2* carries a disrupted copy of *ABD1* in its chromosomal locus but harbours episomal copies of *ABD1* in YEp24, which contains *URA3* (Yamada-Okabe *et al*., 1996). Thus, the *scabd1*Δ::*LEU2* strain grew in the absence of 5-FOA but died in its presence, due to lack of the functional Abd1p. When expressed under the control of the *ADH1* promoter, both *ScABD1* and *CaABD1* rescued the *abd1*Δ::*LEU2* cells even in the presence of 5-FOA (Fig. 3a), demonstrating that CaAbd1p and ScAbd1p are equally functional in *S. cerevisiae* and CaAbd1p can substitute for ScAbd1p to keep yeast cells alive even without ScAbd1p. Unexpectedly, the *ADH1* promoter-driven expression of the *hMet/CMT1* cDNA did not support the growth of the *S. cerevisiae abd1*Δ null mutants in the presence of 5-FOA (Fig. 3a), despite the fact that the recombinant hMet/Cmt1 protein displayed MTase activity *in vitro* (Fig. 2b).

To assess the levels of the proteins expressed in yeast cells, ScAbd1p, CaAbd1p and hMet/Cmt1p were C-terminally fused with the triple c-Myc sequence tag and detected by Western blotting with the monoclonal antibody against the c-Myc tag sequences. Introduction of the triple c-Myc sequence tag to *ScABD1* and *CaABD1* did not affect their abilities to rescue the *scabd1*Δ::*LEU2* cells in the presence of 5-FOA (Fig. 3a). As shown in Fig. 3(b), a significant amount of CaAbd1p was expressed in the yeast cells, whereas hMet/Cmt1p was barely detectable even when expressed by the same promoter. This result accounts for the inability of *hMet/CMT1* to complement an *S. cerevisiae abd1*Δ null mutation. The low level of hMet/Cmt1p in the yeast cells was not due to a lower level of *hMet/CMT1* mRNA, because the *CaABD1* and *Met/CMT1* mRNAs were equally expressed compared to that of actin as judged by Northern blotting (data not shown).

No direct interaction of CaAbd1p with mRNA GTase and mRNA TPase

Both ScAbd1p and hMet/Cmt1p are associated with the elongating form of RNA polymerase II (McCracken *et al*., 1997; Pillutla *et al*., 1998), but their ways of associating with RNA polymerase II seem to be different. ScAbd1p is able to bind directly to the hyperphosphorylated C-terminal domain of RNA polymerase II (McCracken *et al*., 1997), whereas hMet/Cmt1p is not; it is recruited to a RNA polymerase II complex through the interaction with Hce1p (Pillutla *et al*., 1998). Although the yeast cap MTase activity was separated from those of mRNA GTase and mRNA TPase at an early stage of the purification (Mizumoto & Lipmann, 1979), the above fact prompted us to examine whether the yeast cap MTase also interacts with mRNA GTase and mRNA TPase of yeast. Yeast two-hybrid analysis demonstrated that the co-transfection into the yeast HF7c cells of pGAD424-CaCET1 and pGBT9-CaCGT1 suppressed histidine auxotrophy, whereas those of pGAD424-CaABD1 and pGBT9-CaCGT1, and pGAD424-CaABD1 and pGBT9-CaCET1 did not (Fig. 4a). Furthermore, pGAD424-hMet/CMT1 also did not support the growth of HF7c in the absence of histidine even when co-transfected with pGBT9-CaCGT1 or pGBT9-CaCET1 (Fig. 4a). The same results were obtained with the *S. cerevisiae* homologues (Fig. 4b). These results demonstrated that, unlike hMet/Cmt1p, the yeast Abd1 proteins are unable to directly interact with mRNA GTases and mRNA TPases.

Essential residues of CaAbd1p for catalysis

Comparison of the amino acid sequence of CaAbd1p with other known and putative cap MTases revealed the highly conserved amino-acid sequence motif, Phe/Val-Leu-Asp/Glu-Leu/Met-Xaa-Cys-Gly-Lys-Gly-Gly-Asp-Leu-Xaa-Lys, where Xaa represents some variations (Fig. 5). This motif locates in the region between amino acid positions 204 and 216 of CaAbd1p and encompasses the known amino acid sequence motif that is characteristic of divergent methyltransferases, Val-Leu-Asp/Glu-Xaa-Gly-Xaa-Gly-Xaa-Gly, and is considered to be a part of the SAM-binding pocket (Koonin, 1993; Kagan & Clarke, 1994). Using ScAbd1p, Mao *et al*. (1996) and Wang & Shuman (1997) examined the importance of some of the amino acids within this motif,

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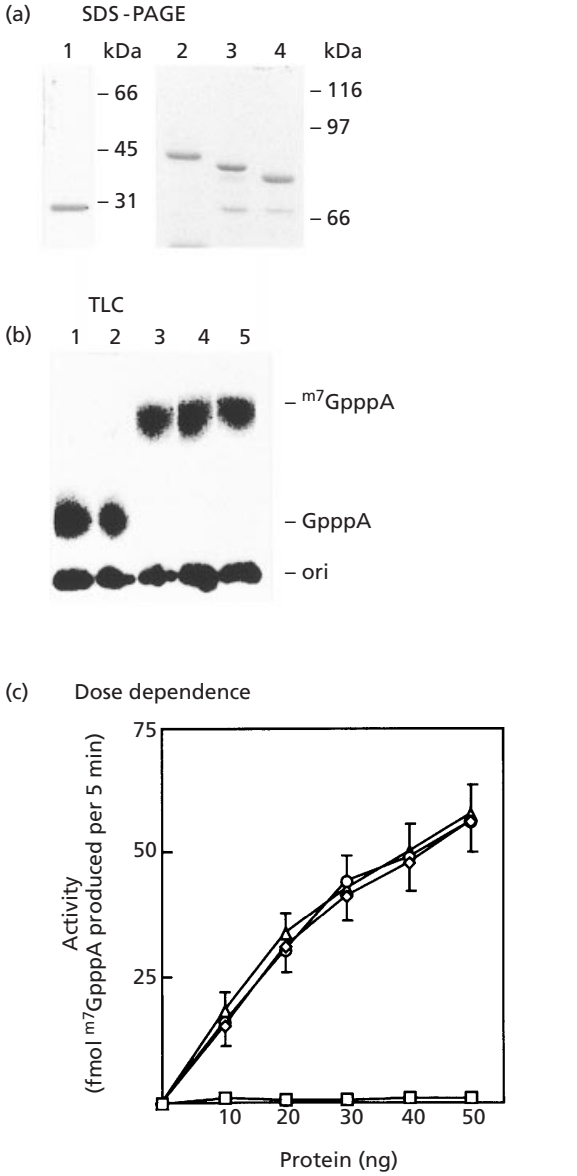

Fig. 2. Enzyme activities of the *C. albicans* cap MTase. The CaAbd1p was expressed in bacteria as a fusion with GST and purified by glutathione–Sepharose affinity column chromatography. (a) Approximately 1 μg of the purified GST, GST–hMet/Cmt1p, GST–CaAbd1p and GST–ScAbd1p were separated on a 12·5 % (for GST) and a 10 % (for others) SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Lane 1, GST; lane 2, GST–hMet/Cmt1p; lane 3, GST–CaAbd1p; lane 4, GST–ScAbd1p. (b) Approximately 0·4 μg of the purified proteins were incubated with G^{32}pppA-terminated RNA. After the RNA was digested with P1 nuclease, the released G^{32}pppA and $^{m7}G^{32}$pppA were separated by TLC and visualized by an image analyser. The positions of G^{32}pppA and $^{m7}G^{32}$pppA on the TLC plates are indicated. Lane 1, no protein added; lane 2, GST; lane 3, GST–hMet/Cmt1p; lane 4, GST–CaAbd1p; lane 5, GST–ScAbd1p. (c) The indicated amounts of GST (□), GST–hMet/Cmt1p (◇), GST–CaAbd1p (○) and GST–ScAbd1p (△) were assayed as in (b) and their cap methylating activities are shown.

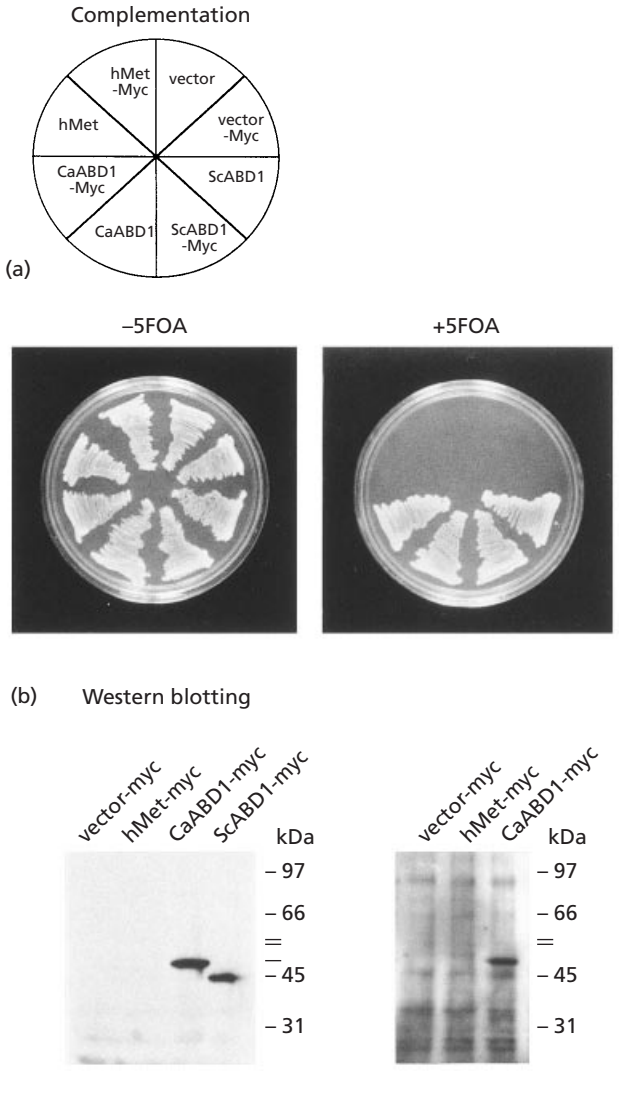

Fig. 3. Complementation of an *S. cerevisiae abd1*Δ null mutation by *C. albicans ABD1*. (a) Cells of *S. cerevisiae abd1*Δ::*LEU2*, which carried the disrupted *ABD1* at its chromosomal locus but harboured intact *ABD1* in YEp24, were further transfected with the c-Myc sequence-tagged or untagged pGBT9 (vector and vector-myc), or with plasmids bearing *ScABD1* (ScABD1, ScABD1-myc), *CaABD1* (CaABD1, CaABD1-myc), or *hMet/CMT1* (hMet, hMet-myc). The transfectants were seeded onto agar plates with (+) or without (−) 5-FOA and incubated at 30 °C for 3 d. (b) The *S. cerevisiae abd1*Δ::*LEU2* cells transformed with the c-Myc sequence-tagged pGBT9 (vector-myc), or those carrying *hMet/CMT1 (hMet-myc) or CaABD1* (CaABD1-myc) were cultured to stationary phase and harvested. Twenty (left panel) or fifty (right panel) micrograms of proteins from total cell extracts of the indicated strains were fractionated on 10 % SDS-polyacrylamide gels and hybridized with the anti-c-Myc monoclonal antibody. Positions of the hMet and CaAbd1 proteins are indicated by the double bars.

and showed that alanine substitutions for glutamic acid at the third position and glycine at the seventh position abolished the enzyme activity and the ability of the cell to overcome the *ScABD1* defect. Conversely, substitutions of the first valine, non-conserved glycine at the fifth (corresponds to Xaa), glycine at the ninth, glutamic acid at the eleventh, and lysine at the fourteenth positions did not abolish the enzyme activity.

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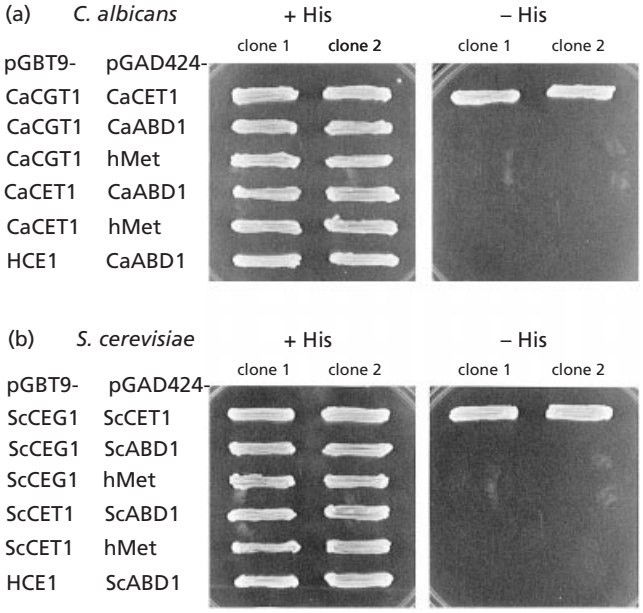

Fig. 4. There is no direct interaction of Abd1p with GTase or TPase. Yeast HF7c cells that were transformed with pGBT9 bearing the GTase or TPase gene of *C. albicans* (a) or that of *S. cerevisiae* (b) were further transfected with pGAD424 carrying the indicated genes. The ability of the human capping enzyme (HCE1) to interact with the Abd1 protein of *C. albicans* (CaAbd1p) (a) and *S. cerevisiae* (ScABD1) (b) is also tested in the yeast two hybrid analysis. Two independent colonies from each transformation were collected and cultured on agar plates with (+) or without (−) histidine for 3 d.

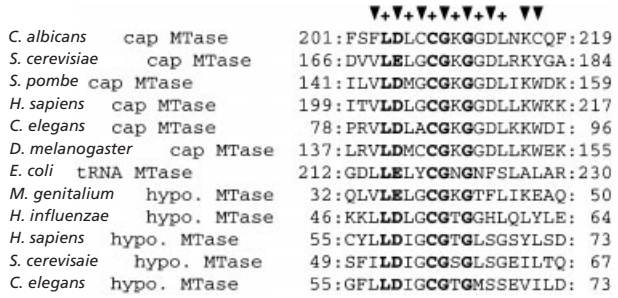

Fig. 5. The conserved amino acid sequence motif in cap MTases and other methyltransferases. The amino acid sequences that are highly conserved in cap MTases are compared with those of known and putative methyltransferases. Identical amino acids from among all the proteins listed here are shown in bold. Amino acids that were previously characterized in *S. cerevisiae* cap MTase are marked by arrowheads and those of *C. albicans* cap MTase, which were mutated to alanine in this study, are indicated by +. GenBank accession numbers are 1790403 for the *E. coli* tRNA methyltransferase, P47589 for the *Mycoplasma genitalium* hypothetical methyltransferase, P44074 for the *Haemophilus influenzae* hypothetical methyltransferase, AJ224442 for the *H. sapiens* hypothetical methyltransferase, P25627 for the *S. cerevisiae* hypothetical methyltransferase and U40419 for *Cal. elegans* hypothetical methyltransferase.

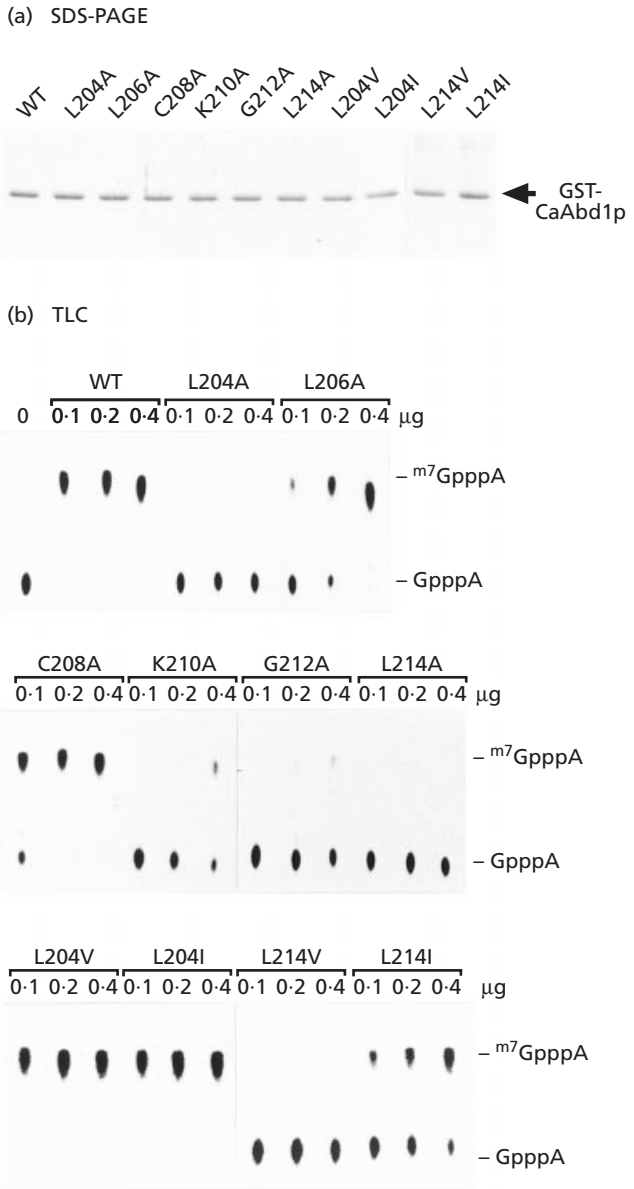

Fig. 6. Effects on CaAbd1p activity of alanine substitution for the conserved amino acids within the motif. Mutant CaAbd1 proteins bearing an alanine substitution for each of the amino acids that are marked by + in Fig. 5 or a conservative mutation at Leu^{204} or Leu^{214} were expressed as fusion proteins with GST and purified by the glutathione–Sepharose affinity column chromatography. (a) The wild-type and indicated mutant proteins (approx. 1 μg) were separated on 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. The position of GST-CaAbd1p is indicated. (b) The indicated amounts of the mutant proteins were incubated with G^{32}pppA-terminated RNA. After the RNA was digested with P1 nuclease, the released G^{32}pppA and $^{m7}G^{32}$pppA were separated by TLC and visualized by an image analyser. The positions of G^{32}pppA and $^{m7}G^{32}$pppA on the TLC plates are indicated.

The conserved amino acids at positions 2, 4 (leucine in CaAbd1p), 6, 8, 10 and 12 in this motif remain uncharacterized, although some of them are also conserved in *E. coli* tRNA (uracil-5-)-methyltransferase and several putative methyltransferases (Fig. 5). Therefore, we mutated these conserved amino acids of CaAbd1p to alanine. All the mutant enzymes were expressed as a

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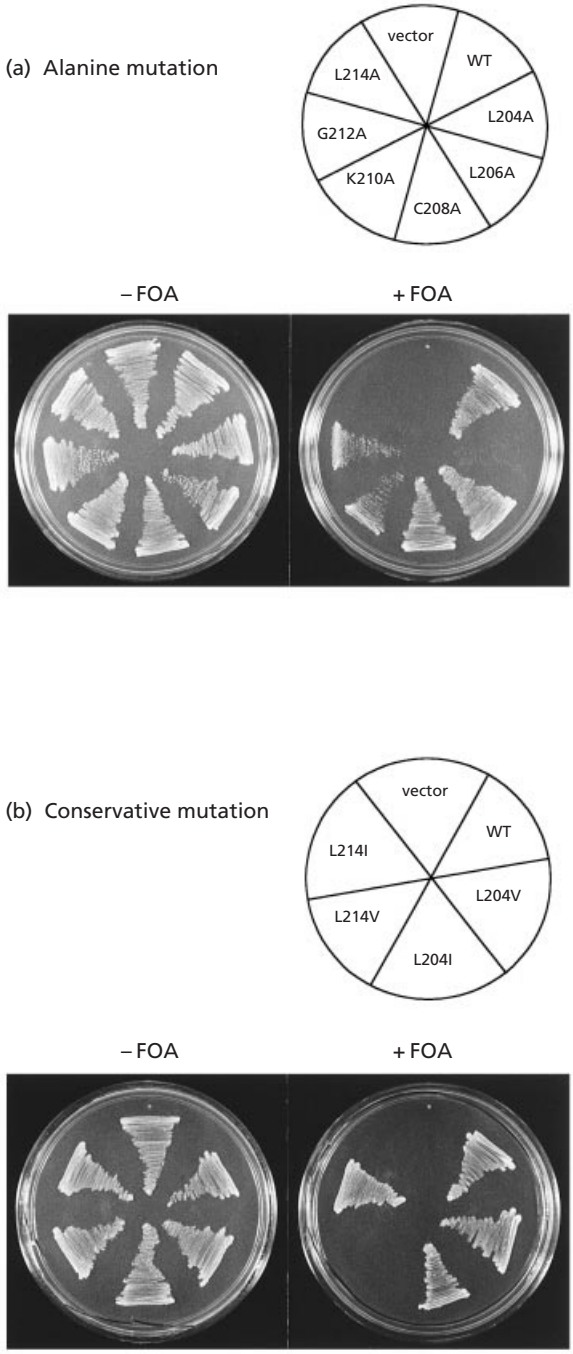

Fig. 7. Complementation of an *S. cerevisiae abd1*Δ null mutation by the mutant *CaABD1*. Cells of *S. cerevisiae abd1*Δ::*LEU2*, which carried the disrupted *ABD1* in its chromosomal locus but harboured intact *ABD1* in YEp24, were further transfected with the c-Myc sequence-tagged pGBT9 bearing the indicated mutant *CaABD1*. The abilities of the *CaABD1* mutant carrying an alanine substitution for the conserved amino acid (a) or a conserved mutation at position 204 and 214 (b) of CaAbd1p are shown. The transfectants were seeded onto agar plates with (+) or without (−) 5-FOA and incubated at 30 °C for 3 d.

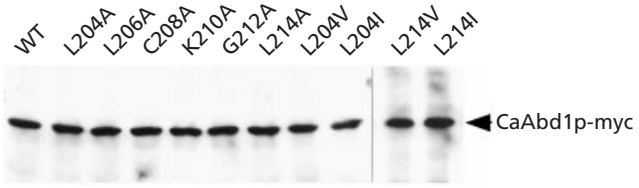

Fig. 8. Levels of mutant CaAbd1 proteins expressed in *S. cerevisiae* cells. Cells of *S. cerevisiae abd1*Δ::*LEU2*, which carried the disrupted *ABD1* in its chromosomal locus but harboured intact *ABD1* in YEp24, were further transfected with the c-Myc sequence-tagged pGBT9. The *S. cerevisiae abd1*Δ::*LEU2* cells transformed with the c-Myc sequence-tagged pGBT9 bearing the indicated mutant *CaABD1* were cultured to stationary phase and harvested. Twenty micrograms of proteins from total yeast cell extracts were fractionated on 10 % SDS-polyacrylamide gels and hybridized with the anti-c-Myc monoclonal antibody.

fusion protein with GST in *E. coli* (Fig. 6a) and tested for the enzyme activity *in vitro*. As shown in Fig. 6b, the cap-methylating activity of L204A and L214A was undetectable, but those of L206A and C208A were almost equivalent to that of the wild-type enzyme when assayed with 0·4 μg each protein. The activities of K210A and G212A were impaired and were about 20 % and 12 % of the wild-type enzyme, respectively, at the same protein dose of 0·4 μg (Fig. 6b). The ability of the mutant *CaABD1* genes to complement an *S. cerevisiae abd1*Δ null mutation was also examined to confirm the functionality *in vivo*. When expressed under the control of the *ADH1* promoter, L206A and C208A fully supported the growth of the *S. cerevisiae abd1*Δ::*LEU2* cells in the presence of 5-FOA, while L204A and L214A failed. K210A and G212A also restored the growth of the *ABD1*-deficient *S. cerevisiae*, but the growth of the cells, which was conferred by K210A or G212A, was apparently retarded compared with that produced by L206A or C208A (Fig. 7a). We also carried out Western blotting to determine the level of the mutant CaAbd1 proteins expressed in yeast cells and found that the levels of the mutant CaAbd1 proteins in yeast cells were more or less the same as that of wild-type CaAbd1p (Fig. 8). Hence, the abilities of the mutant CaAbd1 proteins to complement an *S. cerevisiae abd1*Δ null mutation were well correlated with the enzyme activities. To further validate the importance of Leu^{204} and Leu^{214}, we introduced conservative mutations at these positions. As shown in Figs 6 and 7, valine substitution for Leu^{214} diminished the enzyme activity and *in vivo* functionality, whereas that for Leu^{204} did not. In contrast, L204I and L214I still sustained the significant activity and functionality (Figs 6b and 7b). Together, these results indicate that the leucines at the second and twelfth positions in the motif are additional residues essential for the activity, and that length of the side chain at position 214 is important for catalysis. Moreover, although cysteine at the fifth position in the motif is highly conserved even in a different class of methyltransferases, such as the tRNA (uracil-5-)-methyltransferase of *E. coli*, it turned out not to be crucial for catalysis.

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DISCUSSION

In this paper, we have identified *CaABD1*, the *C. albicans* gene for cap MTase. The *C. albicans* Abd1 protein shared significant sequence homology with *S. cerevisiae* Abd1p and functionally complemented an *S. cerevisiae abd1*Δ null mutation. Recently, Pillutla *et al.* (1998) and Tsukamoto *et al.* (1998b) independently cloned the human cap MTase cDNA and called it *hMet* and *CMT1*, respectively. Three types of *hMet/CMT1* cDNAs called *CMT1a*, *CMT1b* and *CMT1c* were generated, possibly due to alternative splicing. *CMT1a* and *CMT1c* differed only in the 3′ noncoding region and, therefore, encoded an identical protein, which displayed the cap MTase activity *in vitro* (Tsukamoto *et al.*, 1998b). In contrast, the protein specified by *CMT1b* possesses a completely different C-terminal portion (the region after amino acid position 465), and showed no enzyme activity *in vitro* (Tsukamoto *et al.*, 1998b). We have also isolated the *hMet/CMT1* cDNA from a HeLa cDNA library. Unexpectedly, our *hMet/CMT1* cDNA did not support the growth of the *ABD1*-deficient yeast cells even when expressed under the control of the strong constitutive promoter *ADH1*. The inability of *hMet/CMT1* to functionally substitute for the yeast *ABD1* is presumably due to its low level of expression in yeast cells: *S. cerevisiae* cells expressed only a barely detectable level of hMet/Cmt1p as judged by Western blotting. Because similar levels of *hMet/CMT1* and *CaABD1* mRNAs were expressed in yeast cells, the low level of hMet/Cmt1p expression in yeast may be the consequence of a post-transcriptional event.

Although ScAbd1p directly interacts with the hyperphosphorylated C-terminal domain of RNA polymerase II (McCracken *et al.*, 1997), hMet/Cmt1p forms a complex with the elongating form of RNA polymerase II not by directly binding to RNA polymerase II but by interacting with Hce1p (Pillutla *et al.*, 1998). By yeast two-hybrid analysis, we found that neither mRNA GTase nor mRNA TPase physically associates with the Abd1 proteins. Thus, yeast and humans apparently utilize a different mechanism to recruit cap MTase to an RNA polymerase II complex. Although cap MTases seem to be evolutionarily conserved proteins, their N-terminal regions are rather divergent. This may imply that the N-terminal regions of cap MTases are involved in protein–protein interaction and determine the specificity of the interaction.

In ScAbd1p, the catalytic site should reside within the region between the amino acid positions 130 and 426, because the short ScAbd1p fragment encompassing the above region was active as cap MTase *in vitro* (Wang & Shuman, 1997). By comparing the amino acids of CaAbd1p and other cap MTases, we were able to define the amino acid sequence motif for cap MTase as Phe/Val-Leu-Asp/Glu-Leu/Met-Xaa-Cys-Gly-Lys-Gly-Gly-Asp-Leu-Xaa-Lys. In this motif, mutations of uncharacterized amino acids to alanine identified the additional residues essential for the activity. By combining the previous results of Mao *et al.* (1996) and Wang & Shuman (1997), we concluded that the important amino acids in the motif are leucine at the second, aspartic acid or glutamic acid at the third, glycine at the seventh, glutamic acid at the eleventh and leucine at the twelfth positions. Moreover, Abd1p requires a hydrophobic amino acid with a certain length of side chain at the position 214 (corresponding to the leucine at the twelfth position within the motif) for its activity, because Leu^{214} could be substituted by isoleucine but not by valine. In contrast, Leu^{204} (corresponding to the leucine at the second position within the motif) was replaced either by isoleucine or valine without loss of activity and functionality. Thus, it seems that just the presence of a hydrophobic amino acid at this position is sufficient for activity. Hydrophobic amino acids are also conserved at the fourth position in the motif (see Fig. 5). However, because the mutation of Leu^{206} of CaAbd1p to Ala did not affect the activity and functionality, a hydrophobic moiety at the fourth position in the motif may not be essential for the catalytic reactions by methyltransferases. Replacement of Lys^{210} or Gly^{212} with Ala reduced the enzyme activity, and the growth of *S. cerevisiae abd1*Δ null mutants, which was conferred by K210A or G212A, was retarded compared with that of the wild-type. This result coincides with the previous reports by Mao *et al.* (1996) and Wang & Shuman (1997) that the viability of yeast cells is contingent on a threshold level of MTase activity. Tyrosine at position 182 of ScAbd1p, which is adjacent to the conserved sequence motif of cap MTase, is converted to cysteine in CaAbd1p but is conserved as tryptophan in other organisms (Fig. 5); however, alanine substitution for Tyr^{182} of ScAbd1p did not affect the activity and functionality of the enzyme (Wang & Shuman, 1997).

Pillutla *et al.* (1998) also pointed out two other conserved amino acid sequence motifs, Leu-Ser/Lys-Pro/Ile-Gly-Gly-Xaa-Phe-Ile/Phe-Gly/Ala-Thr and Gly-Thr-Leu-Ser-Lys-Ser-Glu-Trp-Glu-Ala, which are called motif III and motif X, respectively (Pillutla *et al.*, 1998). Motif III is preserved in a wide variety of methyltransferases, including CaAbd1p, but motif X is missing in the fungal cap MTases (Pillutla *et al.*, 1998). An amino acid sequence that fits motif III is present and located at amino acid position 323 to 332 of CaAbd1p. In ScAbd1p, glycines at the second and the third positions and the last threonine in this motif were shown to be replaced by alanine without loss of the functionality (Wang & Shuman, 1997). Neverthless, the physiological importance of other highly conserved amino acids in motif III and the role of motif III in catalysis awaits future study.

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