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-N7)-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 abd1Δ 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-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′-terminal guanosine 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-N7)-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
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 Hcc1p (McCracken et al., 1997; Takagi et al., 1997; Yue et al., 1997; Tsukamoto et al., 1998a; Yamada-Okabe et al., 1998a). Hcc1p 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 Crichtid 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 Hcc1p 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 abd1Δ 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/CMT1 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′-GGATCCCGGGATATGCTACCTATCCAGCTAC- 3′ and 5′-GGATCCGGGACTATACCTTCTCCTGACAT- 3′, respectively. 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-primer method with [32P]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.
Expression and purification of the recombinant cap MTase. The coding regions of CaABD1, ScABD1 and the hMet/CMT1 cDNA were cloned into the SmaI 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-transferase (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), 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 15000 g for 45 min, the recombinant Abd1 and hMet/Cmt1 proteins in the supernatant were purified by glutathione–Sepharose CL-4B column chromatography as described previously (Yamada-Oka 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), GTPpppA-terminated RNA and various amounts of the purified proteins at 37 °C for 5 min, followed by incubation at 95 °C for 3 min. After adding sodium acetate (pH 5) to a final concentration of 50 mM and incubating with 10 U P1 nuclease (Fuji BAS 2000). The GTPpppA-terminated RNA was prepared by incubating 0.5 mg polyadenylic acid ml−1 in a reaction mixture which was analysed by TLC using polyethyleneimine cellulose plates (Mao et al., 1995), and the spots were visualized by an image analyser (Fuji BAS 2000). The GTPpppA-terminated RNA was produced by incubating 0.5 mg polyadenylic acid ml−1 in a reaction mixture which was incubated with 20 mM Tris/HCl (pH 7.5), 3 mM MgCl2, 10 mM DTT, 1 µM [32P]GTP (1000–5000 c.p.m. pmol−1), 0.1 mg purified GST–Cac1p ml−1 (Yamada-Oka et al., 1998b) and 0.1 mg purified GST–Cac2p ml−1 (Yamada-Oka et al., 1996) at 37 °C for 30 min. The produced GTPpppA-terminated RNA was extracted with phenol and chloroform, and separated from unincorporated [32P]GTP by Sephadex G-25 column chromatography.

GENERATION OF THE S. CEREVISIAE abd1 Δ NULL MUTANT STRAIN. To generate the S. cerevisiae abd1Δ null mutant strain, ScABD1 including its promoter and terminator was amplified by PCR from the S. cerevisiae genomic DNA extracted from strain A451 (MATa can1 aro7 can1 leu2 trpl 1 ura3) as a template, and cloned into the HindIII site of pUC19 and BamHI site of YEp24, generating pUC-ScABD1 and YEp-ScABD1, respectively. Primers used for amplifying ScABD1 were 5′-GGA- TCCGGAATCCATCATGGAAGTCGCCGGATATTTT-3′ and 5′-GATCCGGAATCTCATGGAAGTCGCCGGATATTTTT-3′. For the disruption of ScABD1, the 1.2 kb HindIII–BglII region of ScABD1 in pUC-ScABD1 was replaced with LEU2. Then, haploid S. cerevisiae YPH499 (MATa ade2 ura3 leu2 his3 trpl) was transformed with the ScABD1::LEU2 chimeric gene together with YEp-ScABD1 (Ito et al., 1983) and several Leu+ Ura+ transformants were selected. The corrected integration of LEU2 at the original ScABD1 locus was confirmed by PCR and by Southern blotting. The CAGG-ScABD1 null mutant (MATa ade2 ade3 leu2 his3 trpl) was transformed into the ScABD1::LEU2ABD1::URA3 strain in the S. cerevisiae abd1Δ 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 SalI 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 leu2-3 112 gal4-542 gal80-538 LYS2::GAL1-HIS3 URA3::(GAL4 17-MERS)−CYC1-LACZ) where the HIS3 gene expression was driven by the DNA-binding and transactivation domains of Gal4p (Feliot 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 SmaI site of pKF188 using a Smal linker, and hybridized with oligonucleotides containing the indicated mutations. The mutant CaABD1 was excised from the vector, ligated into the SmaI site of pGEX-2T and also between the HindIII 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-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 CaAbd1 is somewhat diverged, CaAbd1p shares significant sequence similarity with ScAbd1p (Mao et al., 1995) and hMet/Cmt1p (Pillutla et al., 1998).
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 Homo 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.
CaAbd1p and ScAbd1p are equally functional in presence of 5-FOA (Fig. 3a), demonstrating that mutation by CaABD1 antibody against the c-Myc tag sequences. Introduction detected by Western blotting with the monoclonal terminally fused with the triple c-Myc sequence tag and }

To assess the levels of the proteins expressed in yeast (Fig. 2b).

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 abd1Δ null mutant strain scabd1Δ::LEU2 carries a disrupted copy of AB1 in its chromosomal locus but harbours episomal copies of AB1 in YEp24, which contains URA3 (Yamada-Okabe et al., 1996). Thus, the scabd1Δ::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 abd1Δ::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 abd1Δ 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 scabd1Δ::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 abd1Δ 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; Pillutta et al., 1998), but their ways of associating with RNA polymerase II seem to be different. ScAbd1p is able to bind directly to the hyper-phosphorylated 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 Hec1p (Pillutta 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 H7c 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 H7c 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,
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.

**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 G32pppA–terminated RNA. After the RNA was digested with P1 nuclease, the released G32pppA and m7G32pppA were separated by TLC and visualized by an image analyser. The positions of G32pppA and m7G32pppA 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 abd1Δ null mutation by C. albicans ABD1. (a) Cells of S. cerevisiae abd1Δ::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 abd1Δ::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.
The Candida albicans mRNA cap methyltransferase

(a) C. albicans + His – His

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(b) S. cerevisiae + His – His

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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, A224442 for the H. sapiens hypothetical methyltransferase, P25627 for the S. cerevisiae hypothetical methyltransferase and U40419 for Cal. elegans hypothetical methyltransferase.

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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(a) Alanine mutation

(b) Conservative mutation

Fig. 7. Complementation of an S. cerevisiae abd1Δ null mutation by the mutant CaABD1. Cells of S. cerevisiae abd1Δ::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 abd1Δ::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.

Fig. 8. Levels of mutant CaAbd1 proteins expressed in S. cerevisiae cells. Cells of S. cerevisiae abd1Δ::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 abd1Δ::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.

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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 abd1Δ 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 bMet/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 bMet/CMT1 cDNA from a HeLa cDNA library. Unexpectedly, our bMet/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 bMet/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 bMet/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 Hec1p (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-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 comparing 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 Leu214 could be substituted by isoleucine but not by valine. In contrast, Leu204 (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 Leu204 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 Lys210 or Gly212 with Ala reduced the enzyme activity, and the growth of S. cerevisiae abd1Δ 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 Tyr182 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/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). Nevertheless, the physiological importance of other highly conserved amino acids in motif III and the role of motif III in catalysis await future study.

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