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Yeast mRNA Cap Methyltransferase Is a 50-Kilodalton Protein Encoded by an Essential Gene

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RNA (guanine-7-)methyltransferase, the enzyme responsible for methylating the 5 cap structure of eukaryotic mRNA, was isolated from extracts of *Saccharomyces cerevisiae*. The yeast enzyme catalyzed methyl group transfer from *S*-adenosyl-L-methionine to the guanosine base of capped, unmethylated poly(A). Cap methylation was stimulated by low concentrations of salt and was inhibited by *S*-adenosyl-L-homocysteine, a presumptive product of the reaction, but not by *S*-adenosyl-D-homocysteine. The methyltransferase sedimented in a glycerol gradient as a single discrete component of 3.2S. A likely candidate for the gene encoding yeast cap methyltransferase was singled out on phylogenetic grounds. The *ABD1* gene, located on yeast chromosome II, encodes a 436-amino-acid (50-kDa) polypeptide that displays regional similarity to the catalytic domain of the vaccinia virus cap methyltransferase. That the *ABD1* gene product is indeed RNA (guanine-7-)methyltransferase was established by expressing the ABD1 protein in bacteria, purifying the protein to homogeneity, and characterizing the cap methyltransferase activity intrinsic to recombinant ABD1. The physical and biochemical properties of recombinant ABD1 methyltransferase were indistinguishable from those of the cap methyltransferase isolated and partially purified from whole-cell yeast extracts. Our finding that the *ABD1* gene is required for yeast growth provides the first genetic evidence that a cap methyltransferase (and, by inference, the cap methyl group) plays an essential role in cellular function in vivo.

Eukaryotic mRNAs contain a 5′-terminal cap structure consisting of 7-methylguanosine linked to the 5′ end of the transcript via a 5′-5′ triphosphate bridge (3). Capping occurs by a series of three enzymatic reactions in which the 5′ triphosphate terminus of a primary transcript is first cleaved to a diphosphate-terminated RNA by RNA triphosphatase, then capped with GMP by RNA guanylyltransferase, and finally methylated at the N-7 position of guanine by RNA (guanine-7-)methyltransferase:

$$\text{pppN(pN)}_n \rightarrow \text{ppN(pN)}_n + \text{P}_i$$

$$\text{ppN(pN)}_n + \text{pppG} \leftrightarrow \text{G(5′)pppN(pN)}_n + \text{PP}_i$$

$$\text{G(5′)pppN(pN)}_n + \text{AdoMet} \rightarrow {}^{m7}\text{G(5′)pppN(pN)}_n + \text{AdoHcy}$$

where AdoMet is *S*-adenosylmethionine and AdoHcy is *S*-adenosylhomocysteine. This synthetic pathway was defined originally during studies of vaccinia virus and reovirus mRNA synthesis (13, 31). Cellular transcripts are capped by the same series of reactions, and enzymes that catalyze these steps have been isolated from a number of cellular sources (30). A distinctive feature of the cellular enzymes vis-à-vis the well-characterized vaccinia virus capping enzyme is the lack of tight physical association between the guanylyltransferase and methyltransferase activities. Whereas the capping and methylation functions of the vaccinia virus protein are never dissociable during conventional purification (50), it has been found that these two activities readily separate during chromatography of cell extracts (29).

RNA guanylyltransferase has been purified from HeLa cells, rat liver, calf thymus, mouse myeloma, brine shrimp, wheat germ, and *Saccharomyces cerevisiae* (30, 43). The enzyme from *S. cerevisiae* has been purified as a bifunctional complex consisting of two polypeptides of 80 and 52 kDa (21). RNA triphosphatase activity is intrinsic to the 80-kDa subunit (21), whereas the 52-kDa subunit is the guanylyltransferase (21, 53). The *CEG1* gene encoding the 457-amino-acid guanylyltransferase subunit is essential for cell viability (40). The finding that the guanylyltransferase activity of CEG1 is essential provided the first genetic evidence that capping (and, by inference, the cap) is essential in vivo (11, 39).

Studies of cap methylation have focused, for the most part, on the vaccinia virus capping enzyme (6, 15, 16, 26, 28). Although an RNA (guanine-7-)methyltransferase has been isolated from rat liver (29) and partially purified from HeLa cells (9), in no case has the cellular cap methyltransferase been purified to homogeneity, and there has been no identification of a cellular gene encoding cap methyltransferase. The 7-methyl group of guanosine is essential for several RNA transactions for which cap dependence has been demonstrated in vitro (e.g., translation, pre-mRNA splicing) (1, 8, 23, 32, 33). Hence, a full understanding of the role of the cap in cellular RNA metabolism will require biochemical and genetic analysis of the RNA-methylating enzyme(s). In this paper, we report the partial purification of RNA (guanine-7-)methyltransferase from extracts of *S. cerevisiae* and the identification of the gene encoding this enzyme. Yeast cap methyltransferase is a 436-amino-acid polypeptide with regional sequence similarity to the cap methyltransferases encoded by DNA viruses. The cap methyltransferase gene is essential for yeast cell growth.

MATERIALS AND METHODS

Methyltransferase assay. RNA (guanine-7)methyltransferase was assayed by conversion of ^{32}P-cap-labeled poly(A), GpppA(pA)$_n$, to methylated capped poly(A), ^{m7}GpppA(pA)$_n$, in the presence of unlabeled AdoMet (41). Assay mixtures (10 μl) contained 50 mM Tris HCl, (pH 7.5), 5 mM dithiothreitol, AdoMet, ^{32}P-cap-labeled acceptor RNA, and enzyme. After incubation at 37°C for 5 min, the reaction mixtures were heated at 95°C for 3 min and then adjusted to 50 mM sodium acetate (pH 5.5). Samples were incubated with 5 μg of nuclease P1 for 30 to 60 min at 37°C. The digests were then spotted on polyeth-

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yleneimine-cellulose thin-layer chromatography (TLC) plates that were developed with 0.35 M $(NH_4)_2SO_4$. Labeled products were detected by autoradiography. The extent of methylation of the cap [as ^{m7}GpppA/(^{m7}GpppA + GpppA)] was determined either by excising the labeled species and counting in liquid scintillation fluid or by scanning the TLC plate with a FUJIX BAS1000 phosphorimager.

Partial purification of cap methyltransferase from yeast whole-cell extracts. Extract was prepared as described previously (25) from a mid-logarithmic culture of wild-type *S. cerevisiae* SS328 (51) grown in yeast-peptone-dextrose (YPD) medium at 30°C. The protein concentration of the extract (in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES-KOH; pH 7.9], 0.2 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, plus 20% glycerol) was 12.7 mg/ml. (Protein concentration was determined by using the Bio-Rad dye reagent with bovine serum albumin as a standard.) All operations during purification were performed at 4°C. A 1-ml aliquot of extract was diluted fivefold with buffer A (50 mM Tris HCl [pH 8.0], 1 mM EDTA, 2 mM dithiothreitol, 0.1% Triton X-100, 10% glycerol) and applied to a 2-ml column of phosphocellulose that had been equilibrated with buffer A. After being washed with buffer A, the column was eluted stepwise (6 ml per step) with buffer A containing 0.1, 0.2, 0.5, and 1 M NaCl. Methyltransferase activity was recovered primarily in the 0.5 M NaCl fraction, which contained 0.5 mg of protein. An aliquot (0.2 ml) of this fraction was analyzed by glycerol gradient sedimentation. The rest of the 0.5 M phosphocellulose preparation was dialyzed against buffer B (25 mM Tris HCl [pH 8.0], 0.5 mM EDTA, 1 mM dithiothreitol, 0.05% Triton X-100, 5% glycerol) and then applied to a 1-ml column of DEAE-cellulose equilibrated in buffer B. Methyltransferase activity was recovered in the DEAE flowthrough fraction; this material was then applied to a 1-ml column of heparin-agarose that had been equilibrated in buffer B. The column was eluted stepwise with 0.1, 0.2, 0.5, and 1 M NaCl in buffer A (4 ml per step). Methyltransferase activity was recovered predominantly in the 0.5 M NaCl eluate (30 μg of protein). The heparin-agarose fraction was dialyzed against buffer B. All fractions were stored at −80°C and thawed on ice prior to use. Methyltransferase activity was stable to multiple rounds of freezing and thawing.

Expression of yeast cap methyltransferase in bacteria. A DNA fragment containing the *ABD1* gene was isolated by PCR amplification of total genomic DNA from *S. cerevisiae* YPH274 with oligonucleotides corresponding to the 5′ end (5′-GGGGAAGTCATATGTCAACCAAACCAG) and to the 3′ end (3′-CGCATTTCGGGTTGACTCCTAGGCCC) of the *ABD1* gene (7). These oligonucleotides introduced an *Nde*I restriction site at the start codon and a *Bam*HI cleavage site just 3′ of the stop codon. The amplified DNA fragment was digested with *Nde*I and *Bam*HI and then ligated into the T7-based expression vector pET16b (Novagen) to generate plasmid pET-His-ABD1. The sequence of the *ABD1* insert in pET-His-ABD1 (determined by dideoxy sequencing) was identical to the *ABD1* sequence deposited in GenBank (7). pET-His-ABD1 was transformed into *Escherichia coli* BL21(DE3). Expression of the plasmid-encoded protein was induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to an exponentially growing 200-ml culture of BL21(DE3)/pET-His-ABD1 in Luria-Bertani medium (containing 0.1 mg of ampicillin per ml) at 37°C. Cells were harvested by centrifugation at 4 h postinduction and stored at −80°C.

Purification of recombinant cap methyltransferase. All procedures were performed at 4°C. Cell lysis was achieved by treatment of thawed, resuspended bacteria with lysozyme and Triton X-100, using modified lysis buffer containing 50 mM Tris HCl (pH 7.5), 0.15 M NaCl, and 10% sucrose (26). Insoluble material was removed by centrifugation at 18,000 rpm for 30 min in a Sorvall SS34 rotor. The supernatant was mixed with 2 ml of Ni-nitrilotriacetic acid-agarose resin (Qiagen) for 1 h. The slurry was poured into a column and then washed with lysis buffer. The column was eluted stepwise with IMAC buffer (20 mM Tris HCl [pH 7.9], 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) containing 25, 45, 200, and 500 mM imidazole. The polypeptide composition of the column fractions was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The His-ABD1 polypeptide eluted at 200 mM imidazole. This fraction was dialyzed against buffer B and then applied to a 2-ml column of phosphocellulose that had been equilibrated with buffer B. The column was developed stepwise with buffer A containing 0.05, 0.1, 0.2, 0.5, and 1 M NaCl. His-ABD1 was recovered in the 0.5 M NaCl fraction (0.62 mg of protein). An aliquot (62 μg) of the phosphocellulose preparation was applied to a 4.8-ml 15 to 30% glycerol gradient containing 0.5 M NaCl in buffer A. The gradient was centrifuged at 50,000 rpm in a Beckman SW50 rotor for 15 h at 4°C. Fractions (0.14 ml) were collected from the bottom of the tube. Marker proteins—catalase, bovine serum albumin, and cytochrome *c*—were sedimented in parallel gradients.

Disruption of the *ABD1* gene. The *ABD1* gene was disrupted by insertion of a *hisG-URA3-hisG* cassette as described previously (2, 36). The disruption was performed with a diploid strain, YBS3 (*MAT***a**/*MAT*α *ura3*/*ura3 lys2*/*lys2 his3*/*his3 trp1*/*trp1 LEU2*/*leu2*). Integrative recombination at one chromosomal locus eliminated the *ABD1* coding sequence from amino acid positions 1 to 432 by replacement with the *URA3* cassette. Correct insertion into one *ABD1* locus was confirmed by Southern blotting of Ura$^+$ transformants (strain YBS4). After sporulation and dissection of 20 YBS4 tetrads, 32 viable haploids were recovered, all of which were Ura$^-$. Failure to recover Ura$^+$ haploids indicated that deletion of the *ABD1* gene was lethal.

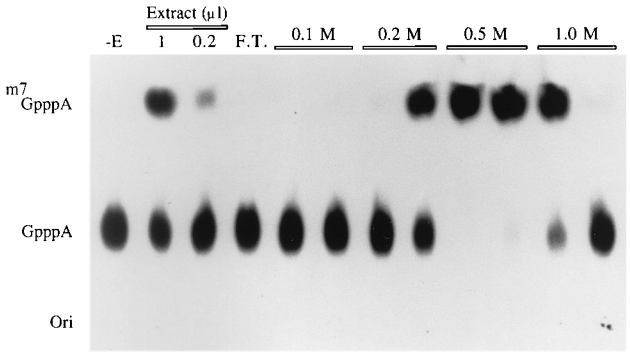

FIG. 1. Phosphocellulose chromatography of yeast cap methyltransferase. Extract preparation and phosphocellulose chromatography are described in Materials and Methods. Methyltransferase assay mixtures contained 9 fmol of ^{32}P-cap-labeled poly(A), 50 μM AdoMet, and either 1 or 0.2 μl of whole-cell extract, 1 μl of the phosphocellulose flowthrough fraction (F.T.), or 1 μl of fractions collected serially during step elution with 0.1, 0.2, 0.5, and 1 M NaCl. A control reaction mixture contained no enzyme (−E). An autoradiogram of the thin-layer chromatogram is shown. The origin (Ori) and the positions of methylated and unmethylated caps are indicated on the left.

Plasmid shuffle. A derivative of YBS4 that had lost the *URA3* gene by recombination between the *hisG* repeats was selected by growth on 5-fluoroorotic acid (5-FOA). Into this strain (YBS5) we introduced p360-ABD, a derivative of vector pSE-360 (*CEN URA3*) that contains a 1.84-kbp *ABD1* insert starting 530 bp upstream of the translation start codon of the ABD1 protein and ending at the ABD1 translation stop codon. After sporulation of YBS5/p360-ABD, Ura$^+$ haploid progeny were tested for growth on 5-FOA. We thereby obtained strain YBS6, which had a deletion at the chromosomal *ABD1* locus and whose viability was contingent on maintenance of p360-ABD. A second plasmid, p358-ABD (derived from vector pSE-358 [*CEN TRP1*] and containing the same 1.84-kbp *ABD1* insert as p360-ABD), was introduced into YBS6. A control transformation was performed with the pSE-358 vector. Trp$^+$ Ura$^+$ transformants were selected and subsequently streaked on medium containing 5-FOA (47).

RESULTS

Partial purification of cap methyltransferase from yeast cell extracts. RNA (guanine-7-)methyltransferase activity was demonstrated in yeast whole-cell extracts by a sensitive and specific assay that eschews detection of transmethylation reactions unrelated to capping. This assay measures the conversion of ^{32}P-cap-labeled poly(A) to methylated cap-labeled poly(A) in the presence of unlabeled AdoMet. The reaction products were digested to cap dinucleotides with nuclease P1 and then analyzed by polyethyleneimine-cellulose TLC, which resolved the GpppA cap from the methylated cap ^{m7}GpppA. Incubation of yeast extract (a whole-cell 100,000 × *g* supernatant fraction) with the cap-labeled substrate and AdoMet resulted in substantial conversion of GpppA ends to a less negatively charged species that comigrated with ^{m7}GpppA (Fig. 1). The extent of methylation was proportional to the amount of extract added (Fig. 1). Partial purification of the activity was achieved by phosphocellulose column chromatography. The methyltransferase activity was adsorbed quantitatively to phosphocellulose at low ionic strength and was recovered during step elution, predominantly in the 0.5 M NaCl fraction (Fig. 1). The 0.5 M phosphocellulose preparation contained approximately 4% of the protein applied to the column. The specific methyltransferase activity of this preparation was increased about 120-fold relative to the extract. The apparent increase in total methyltransferase activity probably reflected the removal of interfering activities during phosphocellulose chromatography.

The native size of the yeast methyltransferase was determined by sedimentation of the phosphocellulose preparation through a glycerol gradient. Given that prior estimates of the

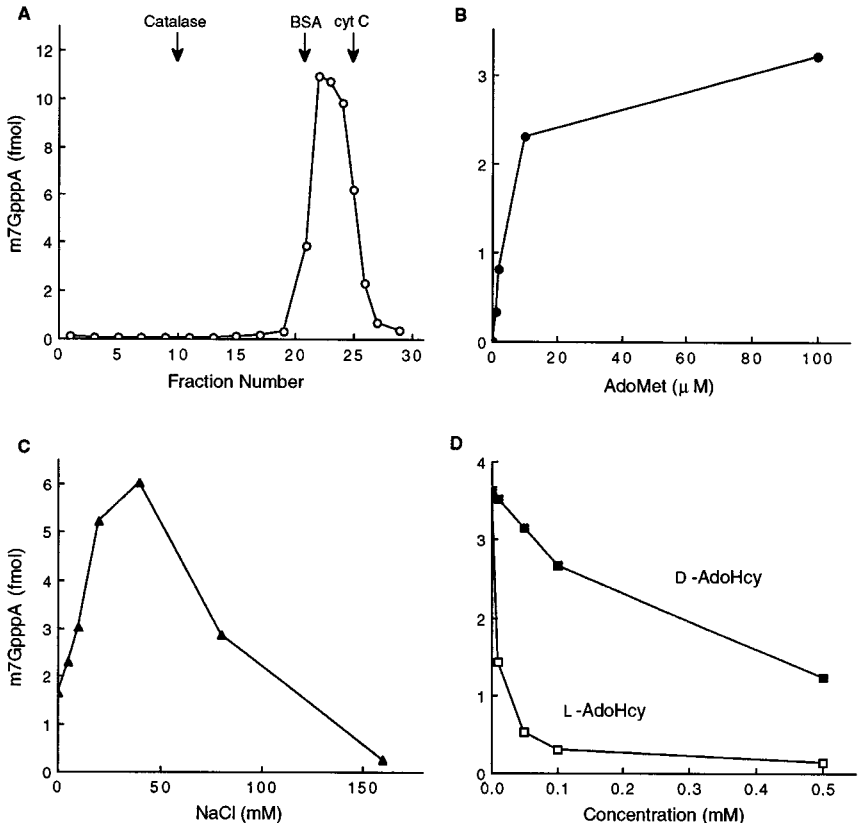

FIG. 2. Characterization of partially purified yeast cap methyltransferase. (A) Glycerol gradient sedimentation. An aliquot (0.2 ml) of the 0.5 M NaCl phosphocellulose fraction of yeast cap methyltransferase was applied to a 4.8-ml 15 to 30% glycerol gradient containing 0.5 M NaCl in buffer A. The gradient was centrifuged at 50,000 rpm in a Beckman SW50 rotor for 12 h at 4°C. Fractions (0.17 ml) were collected from the bottom of the tube. Methyltransferase reaction mixtures contained 18 fmol of cap-labeled poly(A), 50 μM AdoMet, and 1 μl of a 1:4 dilution of the indicated gradient fractions. The extent of cap methylation (in femtomoles) is plotted as a function of fraction number. Vertical arrows indicate the positions of marker proteins that were sedimented in a parallel gradient (BSA, bovine serum albumin; cyt C, cytochrome *c*). (B) AdoMet dependence of cap methylation. Reaction mixtures contained 10 fmol of cap-labeled poly(A), 50 mM NaCl, 0.5 μl of the dialyzed heparin-agarose fraction, and AdoMet as indicated. (C) Effect of NaCl. Reaction mixtures contained 10 fmol of cap-labeled poly(A), 50 μM AdoMet, 1 μl of heparin-agarose enzyme, and NaCl as indicated. (D) Stereospecific inhibition of yeast cap methyltransferase by AdoHcy. Reaction mixtures contained 23 fmol of cap-labeled poly(A), 10 μM AdoMet, 50 mM NaCl, 1 μl of heparin-agarose fraction, and either D- or L-AdoHcy at the concentrations indicated.

native size of mammalian cap methyltransferase have varied from 56 kDa (9) to 130 kDa (29), we performed this analysis under conditions that would resolve proteins as large as 350 kDa. A single peak of yeast methyltransferase activity was recovered; a sedimentation coefficient of 3.2S was estimated relative to marker proteins centrifuged in a parallel gradient (Fig. 2A).

Additional chromatography trials were performed after dialysis of the phosphocellulose fraction. We found that yeast cap methyltransferase did not adsorb to DEAE-cellulose but was retained on heparin-agarose and step eluted at 0.5 M NaCl (results not shown). Initial characterization of the partially purified enzyme was performed with the heparin-agarose preparation that had been dialyzed against buffer lacking salt. Formation of ^{m7}GpppA RNA ends depended absolutely on added AdoMet. Activity increased with increasing AdoMet concentration from 1 to 10 μM and plateaued thereafter (Fig. 2B). Cap methylation was inhibited in a concentration-dependent fashion by L-AdoHcy, a product of the methyl transfer reaction (Fig. 2D). D-AdoHcy was far less potent an inhibitor (Fig. 2D). Cap methylation was stimulated severalfold by low concentrations of salt but inhibited progressively as NaCl was added in excess of 100 mM (Fig. 2C).

A candidate yeast cap methyltransferase gene. Our intent in purifying the cap methyltransferase from yeast extracts was, in part, to identify the gene encoding this enzyme through reverse genetics. While this effort was under way, we identified on phylogenetic grounds a candidate gene by scanning the available sequence database for yeast proteins related to the catalytic domain of the vaccinia virus RNA (guanine-7-)methyltransferase. Although the genes encoding the two subunits of the vaccinia virus capping enzyme have been known for some time, only recently have the methyltransferase and guanylyltransferase activities been assigned to distinct autonomous domains (6, 15, 16, 26, 42, 46). For example, the guanylyltransferase is localized to an N-terminal 60-kDa segment of the 844-amino-acid (97-kDa) D1 subunit of vaccinia virus capping enzyme (15, 46). Several sequence motifs present in the vaccinia virus guanylyltransferase domain are conserved in the same order and with the same spacing in the guanylyltransferases encoded by *S. cerevisiae* and *Schizosaccharomyces pombe* (45). We presumed that this phylogenetic conservation would also apply to the cap-methylating enzymes.

The catalytic center of the vaccinia virus methyltransferase is contained within a 305-amino-acid C-terminal segment of the 844-amino-acid D1 subunit (16, 26). A search of GenBank with this region of the vaccinia virus enzyme readily identified the known homologs encoded by Shope fibroma virus (49) and African swine fever virus (35). The next highest score was to the predicted product of the *S. cerevisiae ABD1* gene, which is

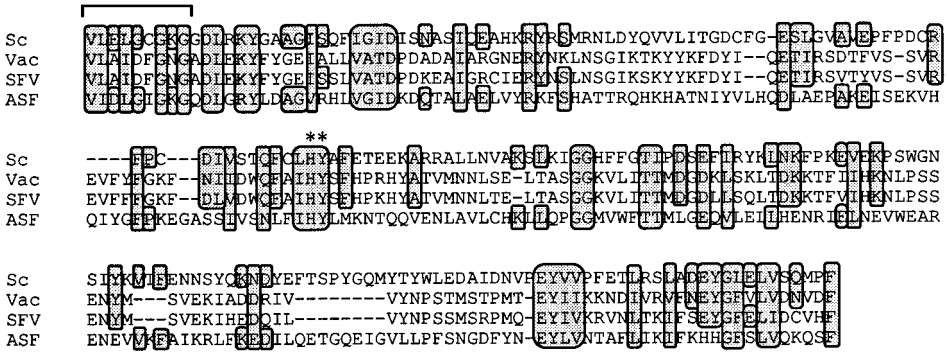

FIG. 3. Sequence similarity between yeast ABD1 and the methyltransferase domain of viral capping enzymes. Amino acid sequences from the C-terminal portions of the capping enzyme large subunits of vaccinia virus (Vac), Shope fibroma virus (SFV), and African swine fever virus (ASF) were aligned with a 205-amino-acid region of the polypeptide encoded by the *ABD1* gene of *S. cerevisiae* (Sc). Residues in the yeast protein that are conserved in one or more of the viral enzymes are highlighted in the shaded boxes. The bracketed region corresponds to a presumed AdoMet-binding motif found in numerous AdoMet-dependent methyltransferases. The residues indicated by asterisks have been defined by mutational analysis as important for the catalytic activity of the vaccinia virus cap methyltransferase.

located on chromosome II (10). Although the computer-based alignment was relatively weak (blast score = 58), it was remarkable for the presence in ABD1 (a 436-amino-acid polypeptide) of several sequence motifs conserved among the known viral methyltransferases, including a putative AdoMet-binding motif, **VLELGCGKG** (boldface type indicates conserved residues) (Fig. 3) (19). The apparent conservation of sequence between the *S. cerevisiae* protein and the vaccinia virus, Shope fibroma virus, and African swine fever virus capping enzymes was enhanced by manually aligning the proteins, as shown in Fig. 3. The region of sequence similarity spans a 205-amino-acid segment of ABD1 from residues 168 to 372. Most striking is the conservation in ABD1 of the sequence IHY (denoted by asterisks in Fig. 3) that we have shown is essential for catalysis by the vaccinia virus cap methyltransferase and which constitutes part of the enzyme active site (26, 27). The retention of functionally important residues suggested to us that *ABD1* might encode the yeast (guanine-7-)methyltransferase.

***ABD1* encodes yeast cap methyltransferase.** To test this hypothesis, we expressed the ABD1 protein in bacteria. An inducible T7 RNA polymerase-based vector in which a short histidine-rich amino-terminal leader segment was fused to the ABD1 polypeptide was constructed. The expression plasmid was introduced into *E. coli* BL21(DE3), a strain that contains the T7 RNA polymerase gene under the control of a *lac*UV5 promoter. A 52-kDa polypeptide corresponding to His-ABD1 was detectable by SDS-PAGE in soluble extracts of IPTG-induced bacteria (results not shown). The His tag allowed for rapid enrichment of ABD1 on the basis of the affinity of the tag for immobilized nickel. The bacterial lysate was applied to Ni-agarose, and adsorbed material was step-eluted with increasing concentrations of imidazole. SDS-PAGE analysis revealed a prominent 52-kDa Coomassie blue-stained species in the 200 mM imidazole eluate (Fig. 4). This polypeptide was not recovered when lysates of IPTG-induced BL21(DE3) carrying either the pET vector alone or pET plasmids expressing other His-tagged gene products were subjected to the same Ni-agarose chromatography procedure (results not shown).

His-ABD1 was purified further by adsorption to phosphocellulose and stepwise elution with salt. The 52-kDa polypeptide was recovered in the 0.5 M NaCl fraction (Fig. 5). Several low-molecular-weight contaminant polypeptides were eliminated at this step. Cap methyltransferase activity was eluted from the phosphocellulose column coincident with the His-ABD1 protein (results not shown). Given that bacterial RNAs are not capped and that bacterial extracts contain no endogenous RNA (guanine-7-)methyltransferase detectable by our standard assay (27), we concluded that the methyltransferase activity detected during phosphocellulose chromatography was intrinsic to His-ABD1.

The phosphocellulose preparation of His-ABD1 was centrifuged through a 15 to 30% glycerol gradient. Cap methyltransferase activity sedimented as a single peak coincident with the sedimentation profile of the His-ABD1 polypeptide (Fig. 5). At this stage, the preparation was homogeneous with respect to the His-ABD1. The apparent sedimentation coefficient of 3.2S (relative to markers analyzed in parallel) suggested that the recombinant yeast methyltransferase was a monomer. We emphasize that the sedimentation behavior of the recombinant ABD1 protein (Fig. 5) was identical to that of the cap methyltransferase isolated from *S. cerevisiae* (Fig. 2A).

Characterization of the ABD1 cap methyltransferase activity. The properties of the recombinant methyltransferase were

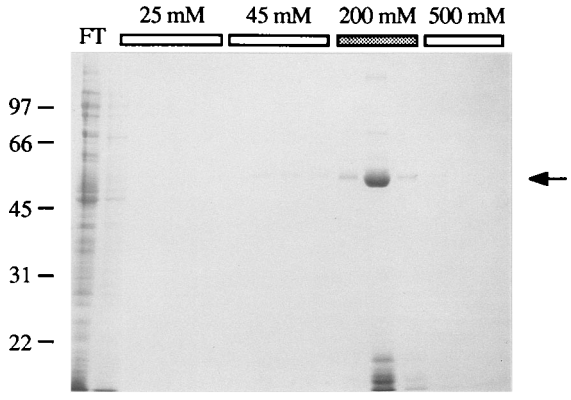

FIG. 4. Ni-agarose chromatography of His-ABD1. A soluble lysate from bacteria induced to express His-ABD1 was adsorbed to Ni-agarose. Aliquots (20 μl) of the flowthrough fraction (FT) and of fractions collected serially during step elution with 25, 45, 200, and 500 mM imidazole were denatured with SDS and analyzed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS. Polypeptides were visualized by staining with Coomassie blue dye. A photograph of the stained gel is shown. The positions and sizes (in kilodaltons) of coelectrophoresed marker proteins are indicated on the left. The His-ABD1 polypeptide, which eluted at 200 mM imidazole, is denoted by the arrow on the right.

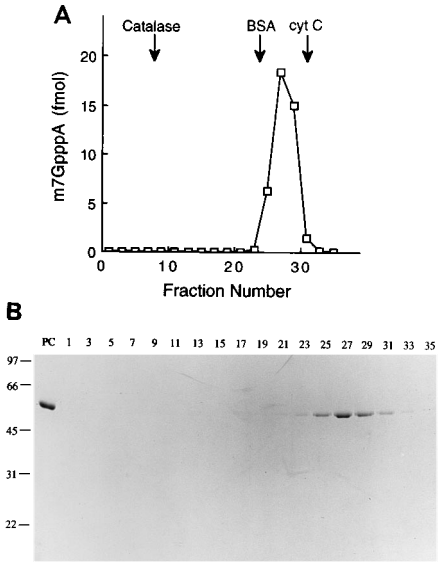

FIG. 5. Glycerol gradient sedimentation of His-ABD1. The phosphocellulose His-ABD1 preparation was sedimented in a glycerol gradient as described in Materials and Methods. (A) Methyltransferase reaction mixtures contained 18 fmol of cap-labeled poly(A), 50 μM AdoMet, and 1 μl of a 1:200 dilution of the indicated fractions. Vertical arrows indicate the positions of marker proteins that were sedimented in a parallel gradient. (B) Aliquots (30 μl) of the phosphocellulose fraction and the indicated glycerol fractions were made 1% in SDS and analyzed by SDS-PAGE. A photograph of the Coomassie blue-stained gel is shown. The positions and sizes (in kilodaltons) of coelectrophoresed marker proteins are indicated on the left.

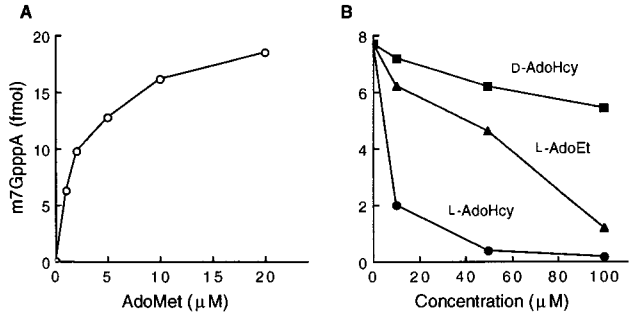

FIG. 6. Characterization of recombinant yeast cap methyltransferase. (A) AdoMet dependence. Reaction mixtures contained 23 fmol of cap-labeled poly(A), 40 mM NaCl, 9 fmol of His-ABD1 (glycerol fraction), and AdoMet as indicated. (B) Inhibition by AdoMet analogs. Reaction mixtures contained 23 fmol of cap-labeled poly(A), 10 μM AdoMet, 40 mM NaCl, 3.6 fmol of His-ABD1, and either L-AdoHcy, D-AdoHcy, or L-AdoEt as indicated.

determined from the peak glycerol gradient fraction of His-ABD1. Methylation of capped poly(A) varied with input enzyme and was quantitative at saturation. His-ABD1 formed 3.7 mol of methylated capped ends per mol of protein during a 5-min reaction. The extent of methylation did not increase with longer times of incubation (up to 20 min); thus, the standard assay measured the yield of product rather than reaction rate. The methyltransferase was active over a broad pH range (6.0 to 9.5) in Tris buffer. $MgCl_2$ strongly inhibited activity in a concentration-dependent fashion; methylation was reduced by an order of magnitude by 1 mM magnesium. (Similar inhibition by magnesium was noted for the vaccinia virus cap methyltransferase [16, 27].) The stimulatory effect of salt on His-ABD1 methyltransferase (optimal at 40 mM NaCl) was virtually identical to that observed for the methyltransferase activity isolated from yeast extract (data not shown).

The extent of methylation varied with AdoMet concentration (Fig. 6A). An apparent $K_{0.5}$ of 2 μM AdoMet was calculated from a double-reciprocal plot of the data. L-AdoHcy was a potent inhibitor, whereas D-AdoHcy was far less effective (Fig. 6B). *S*-Adenosyl-L-ethionine (L-AdoEt), an analog of AdoMet, also inhibited methyltransferase activity but was less potent than L-AdoHcy (Fig. 6B). (AdoEt was shown previously to inhibit cap methylation during reovirus mRNA synthesis [12].)

Identification of the methyltransferase reaction product. Additional characterization of the reaction product was performed with several different analytical reagents. In these experiments, the RNA methylation reaction was performed at saturating levels of His-ABD1. The product was analyzed directly (i.e., without nuclease digestion) by TLC on polyethyleneimine cellulose plates developed with 0.2 M ammonium sulfate. All the ^{32}P-labeled material remained at the origin, i.e., in polynucleotide form (Fig. 7). The product was refractory to digestion with alkaline phosphatase, indicating that the blocking guanosine moiety was still present at the 5′ end. Treatment of the reaction product with nuclease P1 liberated a species that comigrated with ^{m7}GpppA (as per the standard methyltransferase assay); the product of P1 digestion remained resistant to alkaline phosphatase (Fig. 7). When the reaction product was treated with nucleotide pyrophosphatase (NPPase) rather than nuclease P1, a different species was liberated; this species comigrated with ^{m7}GMP and was completely susceptible to alkaline phosphatase (which quantitatively released $^{32}P_i$ from ^{m7}GMP) (Fig. 7). Formation of ^{m7}GMP after NPPase digestion depended on prior reaction with His-ABD1. Digestion of the input cap-labeled poly(A) substrate with NPPase yielded only [^{32}P]GMP, which was converted to $^{32}P_i$ by alkaline

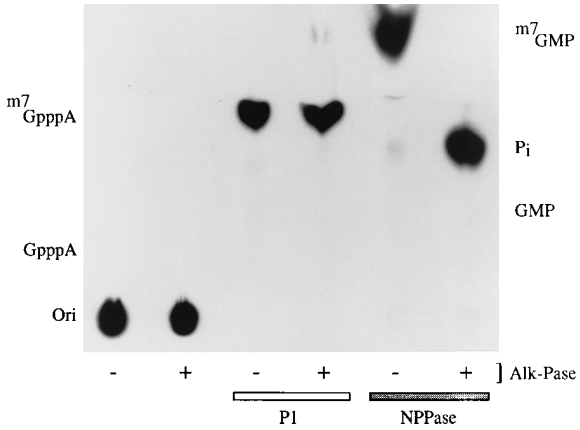

FIG. 7. Analysis of the methyltransferase reaction product. A methyltransferase reaction mixture (70 μl) containing 119 fmol of cap-labeled poly(A), 50 μM AdoMet, and 12.6 pmol of His-ABD1 (glycerol fraction) was incubated at 37°C for 5 min and then heated at 95°C for 6 min. Aliquots (22 μl) were distributed into three new tubes and treated as follows. Two samples were adjusted to 50 mM sodium acetate (pH 5.5). One of these two tubes was incubated for 60 min at 37°C without further addition, and the other was incubated for 60 min at 37°C with 5 μg of nuclease P1 (Boehringer). The third sample was adjusted to 5 mM $MgCl_2$ and incubated for 60 min at 37°C with 7.4 μg of NPPase from *Crotalus adamanteus* (type II; Sigma). Aliquots (10 μl) of each sample were distributed to new tubes, adjusted to 50 mM Tris HCl (pH 8.0), and incubated for 30 min at 37°C with (lanes +) or without (lanes −) addition of 1 U of alkaline phosphatase (Alk-Pase; from calf intestine; Boehringer). The samples were analyzed by TLC on polyethyleneimine-cellulose plates developed with 0.2 M ammonium sulfate. An autoradiogram of the TLC plate is shown. The positions of GMP, ^{m7}GMP, GpppA, ^{m7}GpppA, and P_i are indicated.

phosphatase (results not shown). The His-ABD1 reaction product was identical in all respects to methylated capped poly(A) synthesized by the vaccinia virus capping enzyme and analyzed in parallel (27). These results confirmed that ABD1 catalyzed the methylation of the N-7 position of the cap guanosine.

***ABD1* is an essential gene.** One copy of the *ABD1* gene in a Ura^- diploid yeast was deleted and replaced with a *URA3* gene flanked by direct repeats of *hisG* sequence (2). Inability to recover Ura^+ haploids after sporulation indicated that *ABD1* was essential for cell growth. A Ura^- diploid strain that lost the *URA3* gene by recombination between the *hisG* repeats was selected by growth on 5-FOA. Into this strain (YBS5) we introduced a centromeric plasmid marked with *URA3* and containing the *ABD1* gene under the control of its natural promoter. After sporulation, we selected for Ura^+ haploid progeny that could not grow on 5-FOA. We thereby obtained strain YBS6, which had a deletion at the chromosomal *ABD1* locus and whose viability was contingent on maintenance of the extrachromosomal *ABD1* allele. That the inability to grow on 5-FOA reflected a requirement for *ABD1* was demonstrated by the plasmid shuffle technique (47). A centromeric plasmid marked with *TRP1* and containing *ABD1* was introduced into YBS6 cells. The Trp^+ Ura^+ transformants were then plated on medium containing 5-FOA to select against retention of the *ABD1* gene on the *URA3* plasmid. Whereas cells containing the *TRP1* plasmid without any *ABD1* gene were incapable of growth on 5-FOA, cells bearing the *ABD1*/*TRP1* plasmid grew readily (results not shown). Thus, the yeast cap methyltransferase is encoded by an essential gene.

DISCUSSION

Although many studies suggest that the cap plays a role in RNA processing (8, 20, 23, 24, 33, 34), transport (14, 48), and translation (1, 3, 32), there have been few definitive genetic tests of cap function in vivo. The most direct approach to the problem entails manipulation of the genes that control cap formation. Identifying these genes is an obvious prerequisite.

Viral genes encoding the capping and methylating activities have been known for some time, yet viral systems, which have been powerful in defining the biochemistry of capping, have not been as useful for genetic analysis. Vaccinia virus, a DNA virus, is genetically tractable in principle, yet the multifunctional nature of the vaccinia virus capping enzyme, which serves as a transcription factor as well as an RNA-processing enzyme (44, 52), makes it difficult to correlate in vivo mutational effects (i.e., temperature-sensitive replication) with a specific biochemical defect (4). Many RNA viruses encode their own capping and methylating enzymes, yet these viruses (especially the negative-strand viruses) are not easily engineered genetically. Nevertheless, RNA viruses have provided insights into the role of the cap in vivo. For example, evidence that vesicular stomatitis virus (a negative-strand rhabdovirus) encodes its own cap-methylating enzyme(s) stems from the characterization of a mutant virus that synthesizes mRNA with capped but unmethylated 5′ ends (18). Such RNAs are stable but untranslatable during a nonpermissive infection by the vesicular stomatitis virus mutant strain. Replication of the vesicular stomatitis virus mutant is rescued by coinfection with vaccinia virus, which allows for methylation of vesicular stomatitis virus transcripts in *trans* by the vaccinia virus-encoded methyltransferase (17). This elegant study provides some of the best evidence to date that the cap methyl group is required for translation in vivo. Genetic evidence for an essential role of cap methylation has also emerged from studies of Sindbis virus (a positive-strand alphavirus), for which a mutation in the nsP1 protein (the virus-encoded cap methyltransferase) results in acquisition of the ability to grow under conditions of methionine deprivation and is correlated biochemically with enhanced affinity of the mutant methyltransferase for AdoMet (37).

S. cerevisiae is the obvious system of choice for genetic studies of capping and its role in cellular RNA metabolism. Mutational analysis of the *CEG1* gene encoding RNA guanylyltransferase has established that cap formation is essential for yeast cell growth (11, 39, 40, 45). The present study reports the first identification of a cellular gene encoding a cap methyltransferase. The *ABD1* gene was singled out as a likely candidate for the gene encoding yeast cap methyltransferase on the basis of its sequence similarity to the catalytic domain of the vaccinia virus cap methyltransferase. That the *ABD1* gene product is indeed RNA (guanine-7-)methyltransferase was established by expressing the ABD1 protein in bacteria, purifying the protein to homogeneity, and characterizing the cap methyltransferase activity intrinsic to recombinant ABD1. Most important, the physical and biochemical properties of recombinant ABD1 methyltransferase were indistinguishable (by all criteria tested) from those of the cap methyltransferase isolated and partially purified from whole-cell yeast extracts. We infer from these results that ABD1 is the enzyme responsible for cap methylation in yeast cells.

The sequence of the 436-amino-acid ABD1 protein is well conserved with respect to the methyltransferase catalytic domain of vaccinia virus capping enzyme and to homologous regions of the capping enzymes of Shope fibroma virus (49) and African swine fever virus (35). The vaccinia virus cap methyltransferase domain is a heterodimeric protein consisting of the C-terminal region of the D1 subunit (the catalytic site) complexed with the 33-kDa D12 subunit (6, 16, 26). The D12 subunit, which has no methyltransferase activity by itself, stimulates the methyltransferase activity of the D1 protein by a factor of 50 to 100 (16, 26). We were unable to find any obvious sequence similarity between ABD1 and the stimulatory D12 subunit of the vaccinia virus methyltransferase. The specific activity of recombinant His-ABD1 in methylation of capped poly(A) was only slightly lower than what we observed for the heterodimeric vaccinia virus methyltransferase domain (also His tagged) with the same assay procedures (26); thus, ABD1 may suffice for cap methylation in yeast cells without the necessity for a stimulatory factor. Given that the native size of the cap methyltransferase from yeast extract was identical to that of recombinant ABD1, we consider it likely that the yeast methyltransferase is an ABD1 monomer. The existence of ancillary factors that might regulate the ABD1 methyltransferase is in no way excluded, but such factors, if present, would appear on the basis of the sedimentation analysis not to be tightly associated with the methyltransferase during purification from yeast cells. The size of the ABD1 methyltransferase (50.3 kDa) is similar to the native size of 56 kDa estimated by gel filtration and sucrose gradient sedimentation for the cap methyltransferase from HeLa cells (9).

The finding that the *ABD1* gene is required for yeast growth is the first genetic evidence that a cap methyltransferase (and, by inference, the cap methyl group) plays an essential role in cellular function. It is intriguing, then, that *ABD1* was identified initially by virtue of mutational synergism with *BEM1*, a yeast gene involved in bud emergence (7). The BEM1 protein includes two SH3 domains, which may facilitate protein-protein interactions (5). It has been suggested that BEM1 plays a role in positioning or assembling other proteins at sites of bud formation; indeed, BEM1 binds directly to the CDC24 protein,

which has guanine nucleotide exchange factor activity and which is required for bud formation (54). Bud emergence and bud site selection are regulated by multiple gene products, many of which resemble components of GTPase signaling pathways. How *ABD1* fits into this picture is not yet clear to us, nor is it immediately obvious how cap methyltransferase would be relevant to GTPase regulation of cell polarity. Although it cannot be excluded that *ABD1* has functions above and beyond its activity in cap methylation, we note that other investigators have established a connection between yeast RNA processing, RNA transport, and genes that encode putative guanine nucleotide-signaling proteins (22, 38). More extensive genetic and biochemical studies of *ABD1* are needed to understand the intersection of these superficially unrelated cellular processes.

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