Structure-Function Analysis of Yeast mRNA Cap Methyltransferase and High-Copy Suppression of Conditional Mutants by AdoMet Synthase and the Ubiquitin Conjugating Enzyme Cdc34p

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ABSTRACT

Here we present a genetic analysis of the yeast cap-methylating enzyme Abd1p. To identify individual amino acids required for Abd1p function, we introduced alanine mutations at 35 positions of the 436-amino acid yeast protein. Two new recessive lethal mutations, F256A and Y330A, were identified. Alleles F256L and Y256L were viable, suggesting that hydrophobic residues at these positions sufficed for Abd1p function. Conservative mutations of Asp-178 established that an acidic moiety is essential at this position (*i.e.*, D178E was viable whereas D178N was not). Phe-256, Tyr-330, and Asp-178 are conserved in all known cellular cap methyltransferases. We isolated temperature-sensitive *abd1* alleles and found that *abd1-ts* cells display a rapid shut-off of protein synthesis upon shift to the restrictive temperature, without wholesale reduction in steady-state mRNA levels. These *in vivo* results are consistent with classical biochemical studies showing a requirement for the cap methyl group in cap-dependent translation. We explored the issue of how cap methylation might be regulated *in vivo* by conducting a genetic screen for high-copy suppressors of the *ts* growth defect of *abd1* mutants. The identification of the yeast genes *SAM2* and *SAM1*, which encode AdoMet synthase, as *abd1* suppressors suggests that Abd1p function can be modulated by changes in the concentration of its substrate AdoMet. We also identified the ubiquitin conjugating enzyme Cdc34p as a high-copy *abd1* suppressor. We show that mutations of Cdc34p that affect its ubiquitin conjugation activity or its capacity to interact with the E3-SCF complex abrogate its *abd1* suppressor function. Moreover, the growth defect of *abd1* mutants is exacerbated by *cdc34-2*. These findings suggest a novel role for Cdc34p in gene expression and engender a model whereby cap methylation or cap utilization is negatively regulated by a factor that is degraded when Cdc34p is overexpressed.

THE m7GpppN cap of eukaryotic mRNA is synthesized by three enzymatic reactions: (i) the 5′ triphosphate end of nascent pre-mRNA is hydrolyzed to a diphosphate by RNA triphosphatase; (ii) the diphosphate end is capped with GMP by GTP:RNA guanylyltransferase; and (iii) the GpppN structure is methylated by AdoMet:RNA-(guanine-N7)-methyltransferase. The capping apparatus of the budding yeast *Saccharomyces cerevisiae* consists of separate triphosphatase (Cet1p), guanylyltransferase (Ceg1p), and methyltransferase (Abd1p) gene products (Shibagaki *et al.* 1992; Mao *et al.* 1995; Tsukamoto *et al.* 1997; Ho *et al.* 1998a). Cet1p and Ceg1p interact *in vivo* and *in vitro* to form a bifunctional triphosphatase-guanylyltransferase complex, whereas Abd1p is a monomeric methyltransferase (Itoh *et al.* 1987; Mao *et al.* 1995; Ho *et al.* 1998a; Lehman *et al.* 1999). RNA capping is essential for yeast cell growth; *i.e.*, mutations of the triphosphatase, guanylyltransferase, or methyltransferase components of the yeast capping apparatus that abrogate their catalytic activity are lethal *in vivo* (Fresco and Buratowski 1994; Schwer and Shuman 1994; Shuman *et al.* 1994; Mao *et al.* 1995, 1996; Shibagaki *et al.* 1995; Wang and Shuman 1997; Ho *et al.* 1998b).

The role of the cap in yeast mRNA metabolism has been addressed by studying the consequences of genetic inactivation of the cap guanylyltransferase using temperature-sensitive *ceg1* mutants. Failure to guanylate yeast mRNAs results in their accelerated decay through the agency of the 5′ exoribonuclease Xrn1p (Schwer *et al.* 1998a). Deletion of *XRN1* largely ameliorates the decay phenotype, but does not bypass the requirement for Ceg1p for cell growth. The implication is that stable unguanylated mRNAs are not functional *in vivo*. Available genetic evidence indicates that the cap is not required for 3′ polyadenylation of yeast mRNAs (Fresco and Buratowski 1996; Lo *et al.* 1998; Schwer *et al.* 1998a). Biochemical data show no role for the cap in yeast pre-mRNA splicing *in vitro*, and genetic evidence indicates that the cap facilitates, but is not required for, pre-mRNA splicing *in vivo* (Fresco and Buratowski 1996; Schwer and Shuman 1996a).

We have initiated a genetic analysis of the cap-methyl-

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ating enzyme Abd1p. Abd1p is a 436-amino acid polypeptide that catalyzes the transfer of a methyl group from AdoMet to the GpppN terminus of RNA to produce the m7GpppN-terminated RNA and AdoHcy. Deletion analysis has shown that the N-terminal 109 amino acids and the C-terminal 10 amino acids are dispensable for Abd1p function *in vivo*. Six individual amino acid side chains required for methyltransferase activity *in vitro* and *in vivo* have been identified through alanine scanning of 26 positions (Mao *et al.* 1995, 1996; Wang and Shuman 1997). All six essential Abd1p residues (Glu-170, Gly-174, Asp-178, Asp-194, Arg-206, and Tyr-254) are conserved in the cap methyltransferases of *Schizosaccharomyces pombe* (Pce1p), *Candida albicans* (Ccm1p), and humans (Hcm1p; Saha *et al.* 1999; see Figure 1). In this study, we extended the alanine scan to 35 new positions of Abd1p and have thereby identified two additional side chains (Phe-256 and Tyr-330) that are essential for Abd1p function *in vivo*.

Delineation of structure-activity relationships, although valuable, does not instruct us regarding the role of Abd1p in gene expression and whether cap methylation may be regulated *in vivo*. To address these issues, we have isolated a collection of temperature-sensitive (ts) *abd1* alleles and found that *abd1-ts* cells display a rapid shut-off of protein synthesis at the restrictive temperature, without a global decline in mRNA levels. These genetic findings are consistent with the classic biochemical studies of Shatkin and colleagues showing a requirement for the cap methyl group in cap-dependent translation *in vitro* (Muthukrishnan *et al.* 1975). We performed a genetic screen for high-copy suppressors of the *ts* growth defect of *abd1* mutants. We report that growth at the restrictive temperature can be restored by overexpression of AdoMet synthase (Sam2p or Sam1p) or the ubiquitin conjugating enzyme Cdc34p. Potential positive and negative regulatory mechanisms are discussed.

MATERIALS AND METHODS

Site-directed mutagenesis and test of *ABD1* function by plasmid shuffle: Missense mutations in the *ABD1* gene were programmed by synthetic oligonucleotides as described (Wang and Shuman 1997). Mutated *ABD1* genes were inserted into the yeast plasmid pSE358-5′ (*CEN TRP1*). Expression of *ABD1* in this context is driven by its natural promoter. The presence of the desired mutation was confirmed in every case by sequencing the entire *ABD1* insert; the occurrence of PCR-generated mutations outside the targeted region was thereby excluded. p358-ABD plasmids bearing missense mutations of *ABD1* were introduced into the *abd1*Δ yeast strain YBS10 (*MAT*α *leu2 ura3 lys2 trp1 his3 abd1::LEU2* p360-ABD1). YBS10 contains an extrachromosomal copy of *ABD1* on a *CEN URA3* plasmid (p360-ABD1). Trp^+ transformants were selected at 30° on medium lacking tryptophan. Individual colonies were patched on medium lacking tryptophan. Cells from each patch were then streaked on medium containing 0.75 mg/ml of 5-fluoroorotic acid (5-FOA). The plates were incubated at 30°. Alleles that supported the formation of wild-type-sized colonies after 3 days were scored as +++. Lethal mutations were those that formed no colonies after 7 days on 5-FOA (scored as −).

Isolation of *abd1-ts* mutants: The *ABD1* gene was amplified *in vitro* by *Taq* DNA polymerase. The standard PCR reaction mixture was modified to contain a reduced concentration of dATP (0.2 mm) relative to the other three dNTPs (each at 1 mm). Plasmid pET-His-ABD1 (Mao *et al.* 1995) was used as the template. The PCR product was digested with *Nde*I (which cleaves at the ATG translation initiation codon) and *Bam*HI (which cleaves downstream of the stop codon). The mutagenized *ABD1* DNA fragment was ligated into p358-ABD1-3′ (*CEN TRP1 ABD1*) that had been digested with *Nde*I and *Bam*HI and gel-purified to separate the vector from the excised wild-type *ABD1* sequence. (The p358-ABD1-3′ plasmid is a derivative of p358-ABD1 [described in Mao *et al.* 1995] that contains a 3′ flanking 530-bp *Bam*HI-*Xba*I fragment corresponding to the 3′ untranslated region of *ABD1* gene.) The ligation mixture was transformed into *Escherichia coli* DH5α. After amplification *in vivo*, a pooled plasmid library was prepared from ~24,000 ampicillin-resistant colonies harvested directly from the agar plates. This DNA library was transformed into the *abd1*Δ strain YBS6 (*MAT*α *leu2 ura3 lys2 trp1 his3 abd1::hisG* p360-ABD1; Mao *et al.* 1995). Trp^+ transformants ($n = 1300$) were selected at 25° and then patched on 5-FOA medium at 25° to eliminate the wild-type *ABD1* allele on the *URA3* plasmid. Of the transformants, 40% were unable to grow on 5-FOA. The surviving Ura^- isolates were replica-plated and incubated at 25° (permissive temperature) and 37° (nonpermissive temperature). Those able to grow at 25° but not at 37° were selected. Plasmid DNA was recovered from 31 candidate *ts* mutants, amplified *in vivo* in *E. coli* DH5α, and retested for the conditional growth phenotype by plasmid shuffle. In this way, we obtained a collection of 22 *abd1-ts* mutants. Of these mutant *abd1* clones, 10 were mapped at the nucleotide level by DNA sequencing. Multiple missense mutations in the *abd1* gene were noted in every case (see Figure 2). The 10 *CEN TRP1 abd1-ts* plasmids were transformed into YBS10. Trp^+ transformants were plated on 5-FOA to eliminate the *CEN URA3 ABD1* plasmid.

Temperature shift and measurement of protein synthesis by pulse-labeling: Yeast cells were grown in supplemented minimal medium lacking methionine (SC-Met) at 25°. One-half of each culture was removed and shifted to 37° by rapid addition of an equal volume of SC-Met medium preheated to 52°. Incubation was continued thereafter at 37°. The remaining half of each culture was mixed with an equal volume of SC-Met medium at 25°. At 0, 20, 40, 60, and 120 min post-shift, 2-ml aliquots of the cultures were removed, mixed with 8 μCi of [^{35}S]methionine (1175 Ci/mmol; Dupont NEN), and incubated for 10 min at 37° or 25°. Incorporation of labeled methionine was measured by hot trichloroacetic acid (TCA) precipitation (Peltz *et al.* 1992). The pulse-labeling was terminated by adding ice-cold 100% TCA to a final concentration of 10%, after which the mixtures were placed on ice for 10 min and then heated at 70° for 20 min. The samples were subsequently filtered through glass fiber filters (GF/C; 24 mm in diameter, 1.5-μm pore size), which were washed with 20 ml of 5% TCA and with 10 ml of 95% ethanol and then dried. The bound radioactivity was quantitated by liquid scintillation counting. The data were corrected for the background level of nonspecific [^{35}S]methionine retention on the filters, which was determined by pulse-labeling cells that had been preincubated for 30 min at 25° in cycloheximide (125 μg/ml) prior to adding [^{35}S]methionine.

SDS-PAGE analysis of pulse-labeled polypeptides: Pulse-labeling was performed as described above. After quenching the culture and heating in TCA, the acid-insoluble material

was recovered by centrifugation at 13,000 rpm for 20 min at 4°. The pellets were washed with 1% TCA, followed by three cycles of washing with ethyl ether. The samples were dried in a vacuum centrifuge and then resuspended in SDS-PAGE sample buffer. The samples were heated for 5 min at 95° and then electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. The gels were dried and autoradiographed.

RNA analysis: Yeast cells were grown in SC-Met at 25° to an A_{600} of 0.2–0.4. After withdrawing an aliquot (time zero), an equal volume of SC-Met medium preheated to 52° was added to the rest of the culture to adjust the temperature abruptly to 37°. The cultures were transferred to a 37° incubator. Aliquots were removed at 1, 2, and 3 hr after temperature shift. The cells were harvested by centrifugation and stored as cell pellets at −80°. RNA was isolated from thawed resuspended cells by extraction with hot phenol (Herrick *et al.* 1990). The RNA was ethanol-precipitated and resuspended in TE (10 mm Tris-HCl, pH 7.5, 1 mm EDTA). RNA concentration was calculated on the basis of A_{260}. Aliquots (25–30 μg of total RNA) of each RNA preparation were electrophoresed through a formaldehyde/1% agarose gel. The gels were photographed under short-wave UV illumination to visualize ethidium bromide-stained RNA. The RNA was then transferred to a Hybond membrane (Amersham, Piscataway, NJ). Radiolabeled probes were prepared by using a random priming kit according to the instructions of the vendor (Boehringer Mannheim, Indianapolis). Hybridization was performed as described (Herrick *et al.* 1990). Hybridized probe was visualized by autoradiography of the membrane. Where indicated, the strength of the hybridization signal was quantitated by scanning the membrane with a FUJIX BAS1000 Bio-Imaging analyzer.

RNase protection: RNase protection analysis of poly(A)$^+$ *ABD1*, *SAM2*, and *CDC34* RNA was performed using an RNase protection kit according to the instructions of the vendor (Boehringer Mannheim). [^{32}P]GMP-labeled antisense RNA probes were synthesized *in vitro* by T7 or SP6 RNA polymerase from the following pTOPO-Blunt-based plasmid templates: (i) pAbd1 containing a 137-bp *ABD1* insert from nucleotides +44 to +151 [+1 being the start of the *ABD1* open reading frame (ORF)], (ii) pSam2 containing a 139-bp *SAM2* insert from nucleotides +39 to +169, and (iii) pCdc34 containing a 163-bp *CDC34* insert from nucleotides +51 to +213. Total RNA was isolated from cells grown at 30° in SC-Ura medium to an A_{600} of 0.7–0.8. Poly(A)$^+$ RNA was isolated by oligo(dT) affinity chromatography using Dynabeads-Oligo(dT)$_{25}$ according to the instructions of the vendor (Dynal). Poly(A)$^+$ RNA samples (1 μg) were annealed in separate reactions to the 207-nucleotide *ABD1* probe, the 242-nucleotide *SAM2* probe, or the 261-nucleotide *CDC34* probe. The RNase digests were analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 m urea in TBE. ^{32}P-Labeled DNA restriction fragments (pBR322 *Msp*I digest) served as size markers.

RESULTS

Alanine-scanning mutagenesis of yeast cap methyltransferase: Prior mutational analysis of *S. cerevisiae* Abd1p by alanine scanning led to the identification of six amino acid residues that are essential for Abd1p function *in vivo*; these are denoted by arrows above the Abd1p sequence in Figure 1. Here, we introduced alanine substitutions at 35 additional positions of Abd1p (indicated by dots in Figure 1). The residues to be mutated were chosen on the basis of one or more of the following criteria: (i) conservation in other cellular cap methyltransferases; (ii) proximity to residues known to be essential for Abd1p function, especially those within the putative AdoMet-binding motif I (Figure 1); and (iii) the potential for ionic, polar, or hydrophobic side-chain interactions with AdoMet or the GpppN cap substrates. We particularly targeted conserved aromatic residues (*e.g.*, Tyr-207, Phe-250, Phe-256, Phe-279, Trp-305, Phe-314, Tyr-330, Tyr-416, Phe-419, and Phe-421) in light of recent crystallographic findings for several proteins involved in cap formation or cap recognition that the protein-bound cap guanine is stabilized by base-stacking interactions with aromatic amino acids (Håkansson *et al.* 1997; Hodel *et al.* 1997; Marcotrigiano *et al.* 1997).

The 33 singly mutated *ABD1-Ala* alleles and 1 doubly mutated allele (*ABD1-N150A/N151A*) were cloned into *CEN TRP1* vectors under the control of the *ABD1* promoter, transformed into an *abd1*Δ strain containing a *CEN ABD1 URA3* plasmid, and then tested for function by plasmid shuffle. Of the mutants, 32 were functional *in vivo*; *i.e.*, the Trp$^+$ transformants formed wild-type-sized colonies on medium containing 5-FOA (Table 1). All of the 31 viable single Ala mutants formed wild-type-sized colonies on YPD medium at 18°, 25°, 30°, and 37° (not shown). The *N150A-N151A* double mutant displayed a slow growth phenotype at 18° (not shown). Two of the mutants, *F256A* and *Y330A*, failed to grow on 5-FOA (Table 1). The lethal *abd1-F256A* or *abd1-Y330A* alleles did not exert a dominant negative effect on cell growth when introduced into a wild-type *ABD1* strain. The F256A and Y330A proteins were completely insoluble when expressed in bacteria (not shown); thus, we were unable to assess the mutational effects on cap methyltransferase activity *in vitro*. The equivalent alanine mutants of human cap methyltransferase Hcm1p (F291A and F354A) are defective in cap methylation *in vitro* (Saha *et al.* 1999).

Structure-activity relationships at essential residues of Abd1p: Alanine substitution, which eliminates the side chain beyond the β-carbon, provides an indication of the importance of the side chain for protein function, but does not reveal the properties of the missing side chain that are important. This was addressed by introducing conservative substitutions at the newly defined essential residues, Phe-256 and Tyr-330, and at position Asp-178, which was shown previously by alanine scanning to be important for Abd1p function (Mao *et al.* 1996). Phe-256 was replaced by tyrosine or leucine and both mutant alleles were found to be functional *in vivo* (Table 2). Tyr-330 was substituted with phenylalanine or leucine and neither mutation resulted in loss of function *in vivo* (Table 2). Thus, an aromatic group is not critical at either position. Rather, a bulky aliphatic side chain at residues 256 or 330 suffices for Abd1p function *in vivo*. Replacement of Asp-178 by asparagine was lethal, whereas glutamate at this position had no apparent

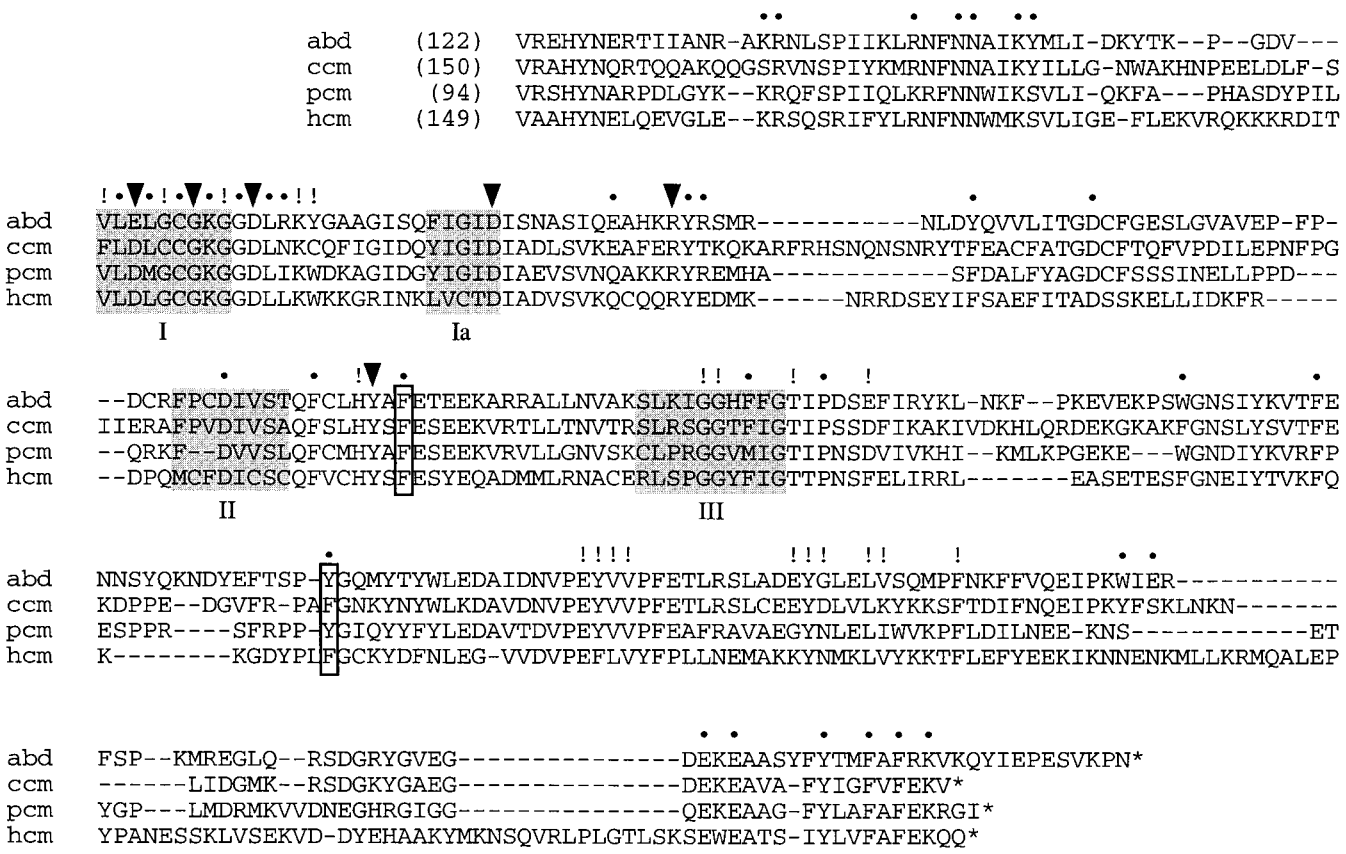

Figure 1.—Mutational analysis of Abd1p. The amino acid sequence of *S. cerevisiae* Abd1p from residues 110 to 436 is aligned with the homologous segments of the *C. albicans* (Ccm1p), human (Hcm1p), and *S. pombe* (Pcm1p) cap-methylating enzymes (Saha *et al.* 1999). Gaps in the sequences are indicated by dashes. The C termini are indicated by asterisks. The six amino acids in Abd1p deemed essential by prior mutational analyses are denoted by arrowheads. Nonessential amino acids in Abd1p are denoted by exclamation points. The Abd1p residues subjected to alanine scanning in the present study are indicated by dots. Newly defined essential residues Phe-256 and Tyr-330 are highlighted in boxes. Methyltransferase motifs I, Ia, II, and III are shown in shaded boxes.

effect on cell growth (Table 2). Hence, the acidic moiety is critical at this position.

Temperature-sensitive mutants of yeast cap methyltransferase: To examine the role of the cap methyl group *in vivo*, we isolated a collection of temperature-sensitive *abd1* alleles from a pool of mutagenized *ABD1* clones. To do this, we performed PCR amplification of the *ABD1* gene under reaction conditions designed to promote nucleotide misincorporation by *Taq* polymerase. The PCR products were restricted and cloned into a *CEN TRP1* expression plasmid, so as to replace the wild-type *ABD1* gene (under the control of its natural promoter) with the PCR-mutagenized DNA. After amplification *in vivo* in *E. coli*, a pooled library of *CEN TRP1* DNAs was transformed into yeast containing wild-type *ABD1* on a *URA3* plasmid. After selection for Trp^+ growth at 25°, followed by selection on 5-FOA to eliminate the wild-type allele, individual isolates were screened for growth at 37°. We obtained a collection of *abd1-ts* isolates that were temperature sensitive, *i.e.*, they readily formed colonies at 25°, but not at 37°. Plasmid DNA was recovered from all conditional isolates and retested by plasmid shuffle for the *ts* phenotype.

A total of 10 *abd1* mutant DNA clones were sequenced. Multiple missense mutations in the *abd1* gene were noted in every case (Figure 2A). Although several of the missense changes were encountered more than once, the 10 *abd1-ts* alleles represented independent isolates, insofar as each mutant clone contains unique mutations not found in the other mutant clones. The 67 missense mutations were unevenly distributed within the 436-amino acid Abd1 polypeptide (Figure 2B). Mutations were underrepresented in the N-terminal segment, which is nonessential for cap methyltransferase activity. Mutations were absent from the essential AdoMet binding motif (Figure 2B). Most of the mutations were located in the carboxyl portion of the enzyme. We surmise that none of the 60 amino acids that were mutated can be considered strictly essential for Abd1p function *in vivo*, because every allele supported cell growth at 25°. No effort was made to determine which, if any, of the many mutations might be singly responsible for the conditional phenotypes. All 10 *abd1-ts* alleles were recessive to wild-type *ABD1*; *i.e.*, growth at 37° could be restored to the *abd1-ts* strains by transforming them with a *CEN URA3* plasmid bearing the wild-type *ABD1* gene

TABLE 1

Effect of alanine mutations on *ABD1* function *in vivo*

Mutation	Growth on FOA
K137A	+++
R138A	+++
R147A	+++
N150A-N151A	+++
K154A	+++
Y155A	+++
L169A	+++
L171A	+++
C173A	+++
K175A	+++
G177A	+++
L179A	+++
R180A	+++
E202A	+++
Y207A	+++
R208A	+++
Y215A	+++
D223A	+++
D244A	+++
F250A	+++
F256A	–
F279A	+++
P284A	+++
W305A	+++
F314A	+++
Y330A	–
W383A	+++
E385A	+++
E408A	+++
E410A	+++
Y416A	+++
F419A	+++
F421A	+++
K423A	+++

(not shown). We observed that the *abd1-ts* alleles had different thresholds for thermal inactivation *in vivo*. Most of the mutants were sick or dead even at 34° (see Figure 6).

Conditional inactivation of *ABD1* inhibits protein synthesis *in vivo*: Although the cap methyltransferase activity of Abd1p is required for cell viability, the specific roles played by the cap methyl group *in vivo* are still unclear. We therefore conducted a preliminary phenotypic characterization of two *abd1-ts* mutants, *abd1-8* and *abd1-5*, both of which grow normally at 25°, but display a tight growth arrest at 37°. We first analyzed the rate of protein synthesis. In the experiment shown in Figure 3, yeast cells were grown in methionine-free medium at 25°. The cultures were then split such that one-half was mixed with an equal volume of 25° medium and maintained thereafter at permissive temperature, whereas the other half of the culture was adjusted immediately to 37° and maintained thereafter at nonpermissive temperature. Aliquots of the 25° and 37° cultures were exposed to [^{35}S]methionine for a 10-min pulse at the time the cultures were split (time zero) and at various time intervals thereafter. Protein synthesis was quantitated by the incorporation of [^{35}S]methionine into hot-TCA-insoluble material during the 10-min pulse. The level of incorporation at each time point was normalized relative to the incorporation at time zero and plotted as a function of the time of initiation of the pulse. The data shown in the figure represent the average of three separate experiments (with error bars shown). It can be readily appreciated that the rates of protein synthesis by *ABD1*, *abd1-8*, and *abd1-5* cells were unaffected by splitting the cultures at 25° (Figure 3B). Protein synthesis by the wild-type cells was unperturbed after shift to 37°, whereas the rates of protein synthesis by *abd1-8* and *abd1-5* cells declined sharply to 28% of the time zero values by 20 min post-shift to 37°. At 40–120 min post-shift, methionine incorporation was reduced to 17% of the time zero value (or 12% of the incorporation of *ABD1* cells after 2 hr at 37°; Figure 3A). The distribution of pulse-labeled polypeptides was analyzed by SDS-PAGE (Figure 3C). The polypeptide profile was qualitatively and quantitatively unchanged when wild-type *ABD1* cells were shifted to 37°. In contrast, *abd1-8* and *abd1-5* cells experienced a progressive and general inhibition of polypeptide synthesis over 2 hr at 37°.

We next performed a Northern analysis of RNA isolated from *ABD1* and *abd1-5* cells before and after shift to 37°. The results indicated that steady-state levels of four different mRNAs were either unaffected or modestly reduced at the restrictive temperature (Figure 4). *GCN4* mRNA abundance was unaffected; *i.e.*, the level of *GCN4* transcript at 3 hr post-shift (as percentage of the initial level at time zero) was 83% for wild-type *ABD1* cells compared to 93% for *abd1-5* cells. *ACT1* and *PGK1* mRNA levels were stable after shift of *ABD1* cells to 37°, whereas in *abd1-5* cells, *ACT1* and *PGK1* RNA levels at 3 hr post-shift were 39 and 31%, respectively, of the pre-shift values. U3 snRNA levels were not altered significantly in *abd1-5* cells after the shift to 37°.

With respect to pre-mRNA processing, we did not detect an increase in the level of unspliced *ACT1* precursor in the *abd1-5* mutant after shift to 37°. However, we noted consistently that the relative amounts of unspliced pre-CYH2 transcript *vs.* mature *CYH2* mRNA [expressed as pre-CYH2/(pre-CYH2 + CYH2)] was increased in

TABLE 2

Effect of conservative substitutions on *ABD1* function *in vivo*

Mutation	Growth on FOA
D178E	+++
D178N	–
F256Y	+++
F256L	+++
Y330F	+++
Y330L	+++

A

Allele	Missense Changes
abd1-15	K75R, I158T, D166G, V246A, D340G, K390I
abd1-16	V52A, L98S, L252S, F256L
abd1-35	K78E, R105G, I158V, K160E, M210K, K271E, I289V, T327S
abd1-8	N41S, F190L, V231A, D285E, F296S, I309V, K382E, D407V
abd1-23	N307S, Y334H
abd1-5	Q189L, C243G, N317S, R356G, K390I, D407V
abd1-42	Q96R, I143V, E227G, F241S, R263G, N316D, Y348F, M370T, R401S, K409R, M418T
abd1-20	T30G, Q216R, N294S, I309T, Y334C, E431G
abd1-41	S32P, E69A, D119Y, A185P, I289V, I309V, V312A, E353G, E385V
abd1-37	E14G, I195V, C239R, D343G, N344S, K374R, E408K

B

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            G               G P       S         A
MSTKPEKPIWMSQEDYDRQYGSITGDESSTVSKKDSKVTANAPGDGNGSLPVLQSSSILT  60

        A     R  E                  R S      G             Y
SKVSDLPIEAESGFKIQKRRHERYDQEERLRKQRAQKLREEQLKRHEIEMTANRSINVDQ  120

                                      T
                         V            V E     G  .  .  .
IVREHYNERTIIANRAKRNLSPIIKLRNFNNAIKYMLIDKYTKPGDVVLELGCGKGGDLR  180

    P   LL   •V          •   K     R          G   A       R
KYGAAGISQFIGIDISNASIQEAHKRYRSMRNLDYQVVLITGDCFGESLGVAVEPFPDCR  240

                 L
S G  A     S • •      G       E             E   V    S S
FPCDIVSTQFCLHYAFETEEKARRALLNVAKSLKIGGHFFGTIPDSEFIRYKLNKFPKEV  300

       T                              C
     S V  A   DS          S  •    H     G  GS   F    G  G
EKPSWGNSIYKVTFENNSYQKNDYEFTSPYGQMYTYWLEDAIDNVPEYVVPFETLRSLAD  360

          T   R       E  V    I          S     VKR         T
EYGLELVSQMPFNKFFVQEIPKWIERFSPKMREGLQRSDGRYGVEGDEKEAASYFYTMFA  420
0

           G
FRKVKQYIEPESVKPN  436
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Figure 2.—*abd1-ts* mutants. (A) The coding changes detected in each of 10 *abd1-ts* alleles are listed. (B) The mutations are annotated above the sequence of the wild-type Abd1 protein. Amino acids that were altered more than once in the *ts* collection are underlined. Amino acids identified by alanine scanning as essential for Abd1p function are denoted by dots. The putative AdoMet binding motif is shaded.

abd1-5 cells at the permissive temperature. In *ABD1* cells grown at 25°, pre-CYH2 RNA constituted 9% of the total, whereas in *abd1-5* cells at 25°, the unspliced precursor accounted for 27% of total *CYH2* transcript (Figure 4). The distribution of spliced *vs.* unspliced *CYH2* RNA did not change significantly after shift to 37°. In *ABD1* cells at 37°, pre-CYH2 was 12% of the total. In *abd1-5* cells at 1, 2, and 3 hr post-shift to 37°, pre-CYH2 was 28–32% of the total. Similar increases in pre-CYH2 at permissive and restrictive temperatures were observed for *abd1-8* cells (not shown). Thus, the increase in pre-CYH2 in *abd1* cells at permissive temperature (relative to *ABD1* cells) was not exacerbated by the shift to the restrictive temperature. This suggests that defective splicing of *CYH2* RNA is not the cause of the *ts* growth defect in the *abd1* mutants. Rather, our experiments point to defective protein synthesis as the limiting transaction. This is consistent with long-held views about the requirement for the cap methyl group in cap-dependent translation initiation (Muthukrishnan *et al.* 1975).

Isolation of multicopy suppressors of *ceg1-ts* mutations: We exploited the newly isolated conditional mutants of yeast cap methyltransferase to identify multicopy suppressors of the *abd1-ts* growth defect, the rationale being that such suppressor genes might encode proteins that either interact physically or functionally with the cap methyltransferase or else impact on cap-dependent RNA transactions *in vivo*.

We tested whether the *CEG1* gene encoding the guanylyltransferase component of the yeast capping apparatus could act as a dosage-dependent *abd1* suppressor. A 2μ *URA3* plasmid containing *CEG1* was transformed into three *abd1* mutant strains (*abd1-42*, *abd1-16*, and *abd1-37*) and the Ura$^+$ cells selected at 25° were tested for growth at 34° and 37°. We found that none of these *abd1-ts* alleles was suppressed by 2μ *CEG1* (not shown). These findings are consistent with the earlier observations that Abd1p isolated from yeast cell extracts is a monomeric enzyme (Mao *et al.* 1995) that is not associated physically with the guanylyltransferase. Two other genes, *CES1* and *CES4*, that act as high-copy suppressors of *ts* guanylyltransferase mutations (Schwer and Shuman 1996b; Schwer *et al.* 1998b) were tested in the same three *abd1-ts* strains and found not to be capable of suppressing the *ts* methyltransferase mutations (not shown). The *CET1* gene encoding the RNA triphosphatase component of the capping apparatus, which we had isolated as a high-copy suppressor of conditional

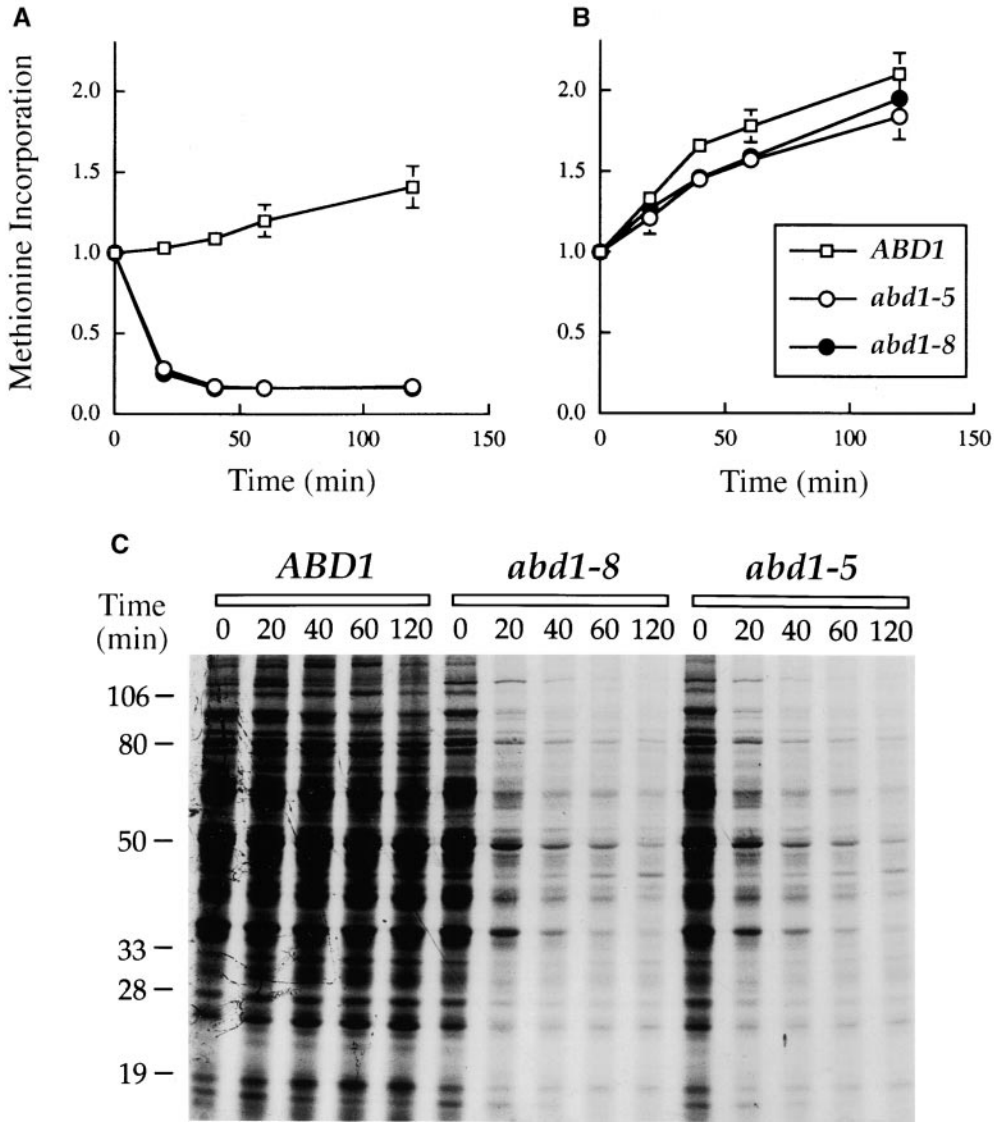

Figure 3.—Effect of *abd1-ts* mutations on protein synthesis. *ABD1*, *abd1-8*, and *abd1-5* cells were grown at 25° in synthetic medium lacking methionine. After growth to A_{600} of 0.2–0.4, the cultures were split and either shifted abruptly to 37° (A) or maintained at 25° (B). Aliquots were withdrawn at the times indicated and pulse-labeled for 10 min with [^{35}S]methionine. Incorporation of label into hot-TCA-insoluble material was determined by liquid scintillation counting. The extents of pulse-labeling were normalized to the value at time zero (defined as 1.0). The plotted data represent the average of three independent experiments; standard error bars are shown. (C) SDS-PAGE analysis of pulse-labeled polypeptides. Pulse-labeling of *ABD1*, *abd1-8*, and *abd1-5* cells with [^{35}S]methionine was initiated at the indicated times post-shift to 37°. Acid-insoluble material was denatured in SDS and then electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. An autoradiogram of the dried gel is shown. The positions and sizes (kilodaltons) of coelectrophoresed marker polypeptides are indicated at the left.

guanylyltransferase mutants (Ho *et al.* 1998a), also failed to suppress the *abd1-ts* mutants after transformation with a 2μ *CET1* plasmid (not shown).

In light of the above findings suggesting that genetic interactions of the triphosphatase and guanylyltransferase do not overlap with the cap methyltransferase, we initiated a screen for novel genes that would act as high-copy suppressors of conditional *abd1* mutations. The screen entailed transformation of several of the *abd1-ts* strains with a 2μ plasmid-based wild-type genomic DNA library and selection for Ura$^+$ colonies that grew at 34°. We analyzed at least 10 positives for each of the *abd1-ts* strains that were transformed with the 2μ library. Plasmid DNA was recovered from individual yeast colonies and transformed into *E. coli*. Diagnostic restriction enzyme digestion of the plasmids recovered from bacteria revealed whether wild-type *ABD1* had been selected. Candidate suppressors that did not contain the *ABD1* gene were retransformed into the *ts* strain from which they were originally isolated and tested for growth at 34°. Four genomic clones retested faithfully. Restriction mapping of the genomic inserts revealed that these four clones derived from two distinct genetic loci, which we provisionally named *CMS1* and *CMS2* (*CMS* = cap methyltransferase suppressor). *CMS1* was isolated in *abd1-15*. *CMS2* was recovered three times, twice in *abd1-37* and once in *abd1-35*. Analysis of the two suppressor genes is presented below.

Suppression of cap methyltransferase mutations by AdoMet synthase: DNA sequencing of the borders of the genomic insert in the 2μ *CMS1* plasmid revealed that the suppressor gene was located on chromosome IV between coordinates 1452295 and 1461657. This 9.4-kbp region includes three known genes, *SAM2* (AdoMet synthase), *LPP1* (lipid phosphate synthase), and *PSP1* (a high-copy suppressor of mutations in DNA polymerase), along with two ORFs of unknown function (YDR504C and YDR506C) and a partial ORF of unknown function (YDR501W). Our attention focused immediately on *SAM2*, which is one of two yeast genes that encodes

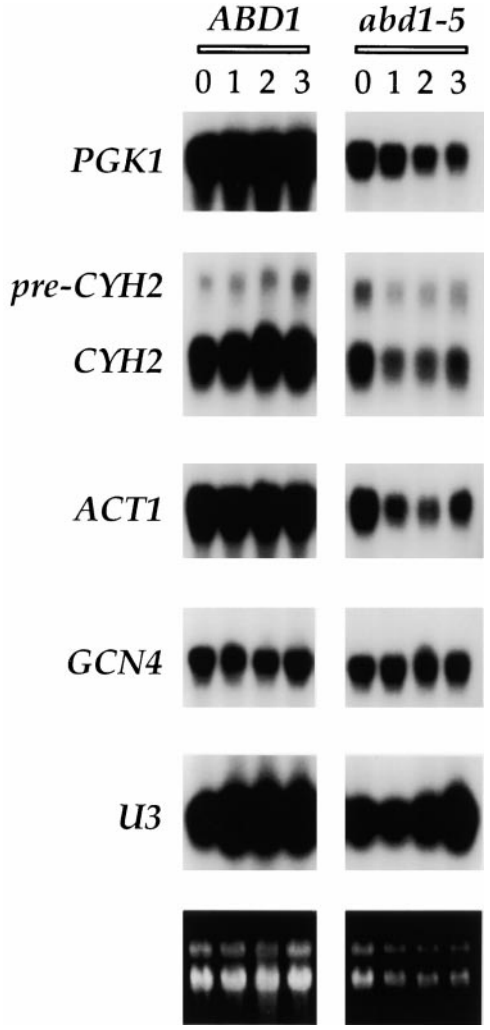

Figure 4.—Effect of *abd1-5* on steady-state RNA levels. *ABD1* and *abd1-5* cells were grown in liquid culture at 25° and then shifted to 37°. Cells from the same culture were harvested prior to shift (time zero) and at 1, 2, and 3 hr after shift to 37°. Total RNA was isolated for Northern blot analysis. The agarose gel was stained with ethidium bromide (bottom) prior to transfer of the RNA to a membrane. Blots were probed for mRNAs derived from *PGK1*, *CYH2*, *ACT1*, and *GCN4* genes and for U3 snRNA. Hybridized ^{32}P-labeled probe was visualized by autoradiography of the membrane.

AdoMet synthase, the enzyme that forms AdoMet from methionine and ATP (Thomas *et al.* 1988). AdoMet is the substrate for cap methylation by Abd1p. Restriction fragments of the insert were subcloned into YEp24 and retested for *abd1* suppression. Suppression was observed with a 2.86-kbp fragment and a 1.75-kbp fragment, both of which contained *SAM2* (not shown).

Final identification of the suppressor locus was achieved by cloning the *SAM2* ORF into a 2μ *URA3* yeast vector in which *SAM2* expression is driven by the constitutive *TPI1* promoter. Suppression of the *abd1-8* mutation by the 2μ *TPI-SAM2* construct is shown in Figure 5. Serial 10-fold dilutions of *abd1-8* cells were plated at 25° and 34°. *abd1-8* cells transformed with the

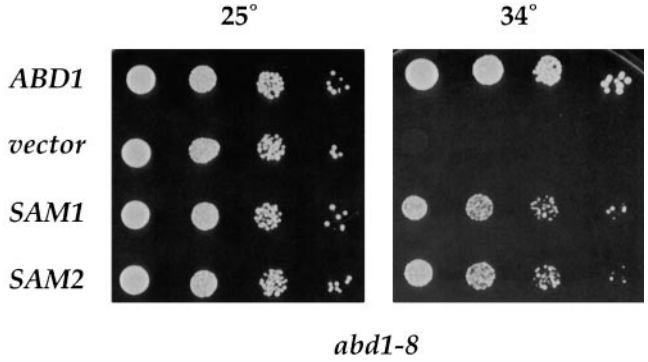

Figure 5.—Dosage suppression of *abd1-8* by AdoMet synthase. *abd1-8* cells were transformed with 2μ *URA3* plasmids containing either *ABD1*, *SAM2*, or *SAM1* under the transcriptional control of the *TPI1* promoter. Control *abd1-8* cells were transformed with the 2μ *URA3* vector. Ura$^+$ transformants were selected and grown in liquid SC-Ura medium at 25°. The A_{600} was adjusted to 0.1. Aliquots (5 μl) of serial 10-fold dilutions of the cell suspensions were spotted onto SC-Ura agar medium. The plates were photographed after incubation for 3 days at 25° or 34°.

2μ vector grew at 25°, but not at 34°; cells transformed with a wild-type *ABD1* gene grew well at both temperatures. The 2μ *SAM2* clone restored growth at 34° (Figure 5) and also improved growth at 37° (not shown). The efficacy of high-copy suppression by *SAM2* was somewhat greater when expression was driven by the *TPI1* promoter than when its expression was under the control of its natural promoter (not shown); we presume this reflects the strength of the *TPI1* promoter.

The 2μ *URA3 TPI-SAM2* plasmid did not display strict allele specificity, but rather was capable of suppressing all of the *abd1-ts* alleles, albeit not to the same extent. Some of the *abd1* alleles were restored to growth at 34° and 37°, whereas others (*e.g.*, the most severely affected allele *abd1-20*) were suppressed only at the lower restrictive temperatures of 30°, 32°, or 34° (not shown). Thus, the strength of suppression correlated roughly with the severity of the *ts* phenotype.

To exclude the possibility that overexpression of AdoMet synthase was genetically bypassing *ABD1*, we constructed a 2μ *TRP1 TPI-SAM2* plasmid and transformed it into a yeast strain in which the chromosomal *ABD1* gene is deleted and growth is contingent on maintenance of a *CEN URA3 ABD1* plasmid. The 2μ *TRP1 TPI-SAM2* transformants were unable to form colonies on medium containing 5-FOA (not shown); hence *SAM2* overexpression is not bypassing the requirement for cap methyltransferase for yeast cell growth.

The *S. cerevisiae SAM1* gene encodes a second isozyme of AdoMet synthase; the amino acid sequence of Sam1p is 92% identical to that of Sam2p (Thomas *et al.* 1988). A 2μ plasmid containing *SAM1* under the control of the *TPI1* promoter was just as effective as the *SAM2* clone in restoring growth of *abd1-8* at the restrictive temperature (Figure 5). That we did not actually isolate

SAM1 in the suppressor screen may reflect either its scarcity in the genomic library used or lack of saturation of the screen.

Suppression of cap methyltransferase mutations by exogenous AdoMet: The simplest explanation for suppression of *abd1* mutations by overexpression of AdoMet synthase is that elevated intracellular levels of the methyl donor either stimulate the activity of the mutant Abd1p enzymes or stabilize the mutant Abd1p proteins at the restrictive temperature. A more elaborate explanation would be that AdoMet synthase interacts directly with Abd1p. If the former model is correct, then it should be possible to suppress the *abd1-ts* mutations by an alternative route that provides AdoMet without increasing the levels of AdoMet synthase. *S. cerevisiae* can actively take up AdoMet from the medium; moreover, it has been reported that exogenous AdoMet lowers the expression of *SAM1* and *SAM2* in strains that are wild type for both AdoMet synthase loci (Thomas and Surdin-Kurjan 1991). In light of this information, we tested whether exogenous AdoMet would enhance the growth of the *abd1-ts* strains at the restrictive temperature. In the experiment shown in Figure 6, serial dilutions of wild-type *ABD1* cells and the 10 *abd1-ts* mutants were plated in parallel at 34° on control agar medium and on agar plates that had been overlaid with AdoMet (Bailis and Rothstein 1990). The growth of the *abd1-23*, *abd1-37*, *abd1-41*, *abd1-5*, *abd1-8*, *abd1-15*, and *abd1-16* strains was clearly improved in the presence of exogenous AdoMet (Figure 6). Growth of *abd1-20*, which is the most severe of the *ts* mutations, was not enhanced by AdoMet at 34° (Figure 6), but was improved when growth was tested at 30° (not shown). *abd1-42*, the mildest conditional mutant, which grows well at 34°, was accordingly unaffected by AdoMet at this temperature; however, the growth defect of *abd1-42* at 37° was partially ameliorated by AdoMet (not shown). These findings suggest that an increase in the available AdoMet substrate is likely responsible for the suppression of *abd1-ts* mutations by *SAM2* or *SAM1* overexpression. Only *abd1-35* was not appreciably suppressed by exogenous AdoMet.

Suppression of cap methyltransferase mutations by *CDC34*: *CMS2* was isolated three times in the high-copy suppressor screen. Sequencing of the margins of the genomic inserts in the three different 2μ *CMS2* plasmids revealed that the suppressor gene was located on chromosome IV. The three inserts extended from genome coordinates 554800 to 567450, 560860 to 567450, and 551880 to 567081, respectively. The 6.2-kbp overlap region includes one known gene, *CDC34* (an essential E2 ubiquitin conjugating enzyme), along with two ORFs of unknown function (YDR055W and YDR056C) and a partial ORF of unknown function (YDR057W). Restriction fragments of the inserts were subcloned into YEp24 and retested for *abd1* suppression. Suppression was observed with a 2.44-kbp fragment that contained *CDC34* (not shown).

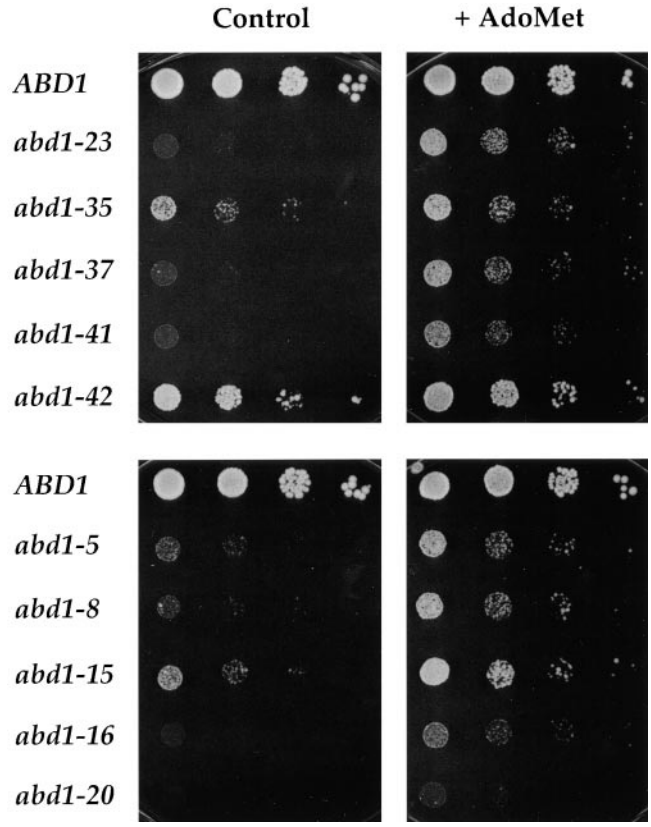

Figure 6.—Suppression of *abd1-ts* mutations by exogenous AdoMet. *ABD1* and *abd1-ts* cells were grown in liquid YPD medium at 25°. The A_{600} was adjusted to 0.1. Aliquots (3 μl) of serial 10-fold dilutions of the cell suspensions were spotted onto YPD agar medium (control) and YPD agar medium overlaid with 0.25 ml of a 10-mg/ml solution of AdoMet to achieve a final concentration of ~0.1 mg/ml (+AdoMet). The plates were photographed after incubation for 4 days at 34°.

Definitive identification of the suppressor locus was accomplished by cloning the 295-amino acid *CDC34* ORF into a 2μ *URA3* vector in which *CDC34* expression is driven by the *TPI1* promoter. Suppression of the *abd1-8* mutation by *CDC34(1-295)* is shown in Figure 7. *abd1-8* cells transformed with the 2μ vector grew at 25°, but not at 34°; cells transformed with a wild-type *ABD1* gene grew well at both temperatures. The 2μ *CDC34(1-295)* clone restored growth at 34° (Figure 7) and also at 37° (not shown).

CDC34 was capable in high copy of suppressing all of the *abd1-ts* alleles. As with *SAM2*, the strength of suppression by *CDC34* correlated with the severity of the *ts* phenotype of the individual mutants. A 2μ *TRP1 TPI-CDC34* plasmid was unable to confer growth of an *abd1*Δ strain on medium containing 5-FOA. Thus, *CDC34* overexpression is not bypassing the requirement for *ABD1*.

The ubiquitin conjugating activity of Cdc34p is essential for *abd1* suppression: Cdc34p is an E2 ubiquitin conjugating enzyme that is critical for proper cell cycle progression. Cdc34p catalyzes the ubiquitination of

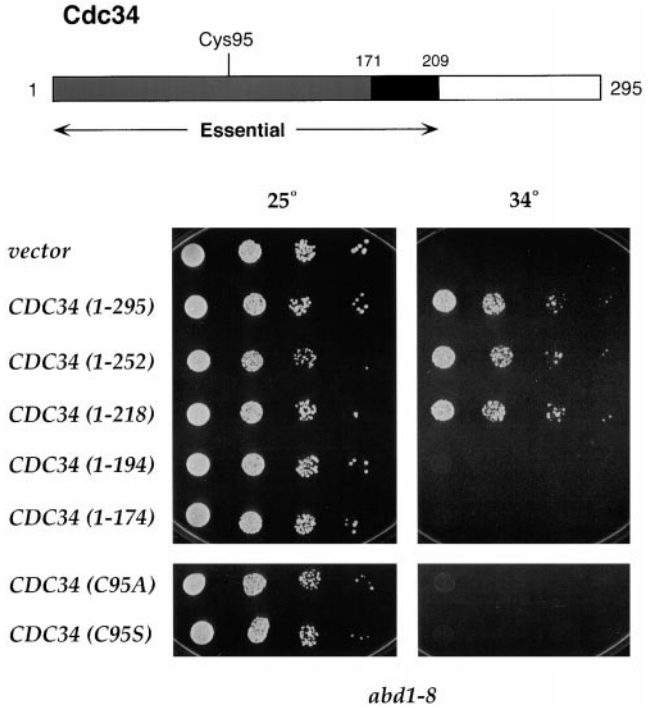

Figure 7.—Dosage suppression of *abd1-8* by Cdc34p. The domain structure of the 295-amino acid ubiquitin conjugating enzyme is shown (see text for details). *abd1-8* cells were transformed with 2μ *URA3* plasmids containing the indicated *CDC34* alleles under the transcriptional control of the *TPI1* promoter. Control *abd1-8* cells were transformed with the 2μ *URA3* vector. Ura^+ transformants were selected and grown in liquid SC-Ura medium at 25°. The A_{600} was adjusted to 0.1. Aliquots (5 μl) of serial 10-fold dilutions of the cell suspensions were spotted onto SC-Ura agar medium. The plates were photographed after incubation for 3 days at 25° or 34°.

Sic1p, which is an inhibitor of the Cdc28p-cyclin protein kinase complexes that are required for the G1-S transition. Sic1p ubiquitination results in its degradation and consequent activation of the Cdc28p-cyclin protein kinase. Cdc34p functions in conjunction with an E3 complex, SCF, containing Cdc53p, Skp1p, and one of several F-box proteins (*e.g.*, Cdc4p, Grr1p, or Met30p; Bai *et al.* 1996; Feldmann *et al.* 1997; Skowyra *et al.* 1997; Kaiser *et al.* 1998; Patton *et al.* 1998). The F-box component is implicated in recognizing the protein target for Cdc34p-mediated ubiquitination. Yeast proteins that are targeted for decay via a Cdc34p/SCF ubiquitination pathway include Sic1p, Far1p, Cln2p, Gcn4p, Swe1p, Cdc6p, Cdc4p, and Grr1p (Kornitzer *et al.* 1994; Deshaies *et al.* 1995; Drury *et al.* 1997; Feldmann *et al.* 1997; Henchoz *et al.* 1997; Skowyra *et al.* 1997; Kaiser *et al.* 1998; Zhou and Howley 1998).

The biochemical properties and domain structure of Cdc34p have been analyzed in considerable detail. Ubiquitin transfer by E2 enzymes entails the formation of a covalent thiol ester intermediate between an E2 cysteine (Cys-95 in the case of Cdc34p) and the C-terminal carboxyl moiety of ubiquitin. Replacement of the cysteine by alanine abolishes activity, whereas replacement by serine permits the formation of an E3 ubiquitin acyl intermediate, but inhibits subsequent transfer of ubiquitin to the target protein (Banerjee *et al.* 1995). The C95S and C95A mutants of *CDC34* are both nonfunctional *in vivo* (Banerjee *et al.* 1995). To gauge whether the action of *CDC34* as a high-copy cap methyltransferase suppressor depended on its catalytic function in ubiquitin transfer, we engineered Cys95Ser and Cys95Ala mutations in the *CDC34* gene and cloned these alleles into a 2μ *URA3* vector under the control of a *TPI1* promoter. The C95S and C95A changes abolished the capacity of *CDC34* to suppress *abd1-8* (Figure 7). Hence, we conclude that formation of the Cdc34p-Ub intermediate and its transfer to an idoneous acceptor are essential for *abd1* suppression.

Cdc34p catalyzes both intramolecular and intermolecular ubiquitin transfer reactions (Banerjee *et al.* 1993; Goebl *et al.* 1994). In the intramolecular autoubiquitination reaction, Ub is transferred from Cys-95 to a lysine residue to form a branched multiubiquitin conjugate. The sites of autoubiquitination have been localized to any of four lysines (Lys-273, Lys-277, Lys-293, and Lys-294) in the C-terminal region of Cdc34p (Banerjee *et al.* 1993). To test if Cdc34p autoubiquitination was relevant to *abd1* suppression, we constructed a series of C-terminal truncation mutants of *CDC34* and tested their suppressor function. *CDC34(1-252)* and *CDC34(1-218)* were just as effective as full-length *CDC34* in suppressing *abd1-8*, even though the potential sites of autoubiquitination were eliminated from the truncated gene products (Figure 7). Thus, it is the intermolecular ubiquitin conjugating activity of Cdc34p, not the autoubiquitination function, that is essential for *abd1* suppression.

An N-terminal segment from residues 1 to 209 is sufficient for Cdc34p function *in vivo* (Mathias *et al.* 1998), whereas the protein segment from residues 1 to 170, which comprises a catalytic domain conserved among E2 enzyme, cannot sustain cell growth (Kolman *et al.* 1992; Silver *et al.* 1992; Figure 7). Mathias *et al.* (1998) have shown that the essential region of Cdc34p from residues 171 to 209 mediates the interaction of Cdc34p with Cdc53p and the F-box protein Cdc4p *in vivo*. We find that overexpression of *CDC34(1-218)*, which includes the Cdc53p/Cdc4p binding domain, is active in *abd1* suppression, whereas *CDC34(1-174)*, which lacks the interaction domain, is nonfunctional (Figure 7). The implication of these data is that the Cdc34p exerts its *abd1* suppressor function in the context of an E2-E3 complex. The intervening truncation mutant *CDC34(1-194)* is nonfunctional in *abd1* suppression (Figure 7), presumably because residues 195–209 are critical for the interaction of Cdc34p with E3 components.

Suppression of *abd1* by AdoMet synthase and Cdc34p is independent of the *ABD1* promoter: Analyses of mutational effects on Abd1p function *in vivo* and *in vitro*

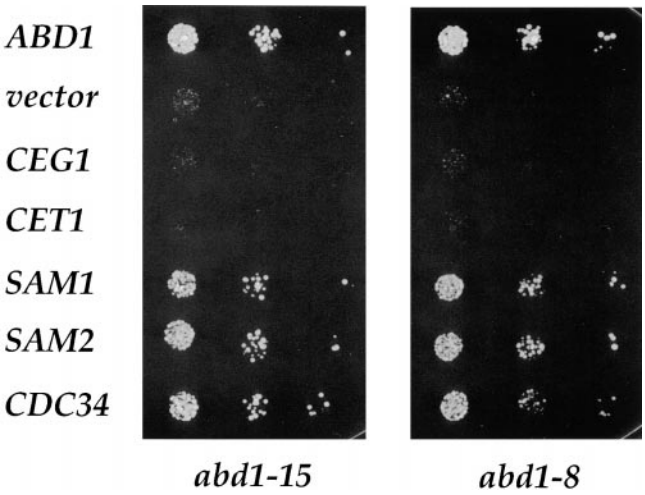

Figure 8.—Dosage suppression of *abd1-8* by AdoMet synthase and Cdc34p is independent of the *ABD1* promoter. *TPI-abd1-8* and *TPI-abd1-15* cells were transformed with a 2μ *URA3* vector or 2μ *URA3* plasmids containing the indicated genes driven by the *TPI1* promoter. Ura$^+$ transformants were selected and grown in liquid SC-Ura medium at 25°. The A_{600} was adjusted to 0.1. Aliquots (5 μl) of serial 10-fold dilutions of the cell suspensions were spotted onto SC-Ura agar medium. The plates were photographed after incubation for 2.5 days at 37°.

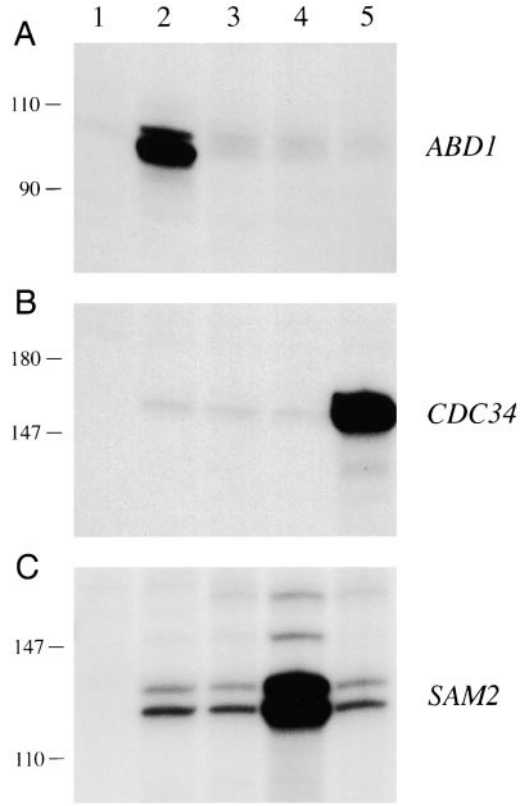

Figure 9.—Lack of transcriptional crosstalk between Ado Met synthase and Cdc34p. Poly(A)$^+$ RNA isolated from yeast cells bearing 2μ *URA3* plasmids that contained either *ABD1* (lane 2), no gene insert (lane 3), *TPI1-SAM2* (lane 4), or *TPI1-CDC34* (lane 5) was annealed to radiolabeled antisense *ABD1*, *CDC34*, or *SAM2* probes (as specified to the right of each panel). Labeled RNAs protected from digestion from RNase were resolved by PAGE and visualized by autoradiography. A control RNase protection reaction lacking yeast RNA is shown in lane 1 of each panel. The positions and sizes (in nucleotides) of radiolabeled size markers are indicated on the left of each panel.

have indicated that yeast cell growth is dependent on a threshold level of cap methyltransferase activity (Mao *et al.* 1996; Wang and Shuman 1997). In this light, we questioned whether the conditional phenotype of our mutants might be sensitive to the levels of *abd1* expression. Accordingly, we placed several of the *abd1-ts* alleles under the control of the strong constitutive *TPI1* promoter and introduced these genes on *CEN TRP1* plasmids into yeast cells in lieu of the resident *CEN URA3 ABD1* plasmid. Each of the *TPI1-abd1* mutants remained temperature sensitive (*i.e.*, unable to grow at 37°, the original restrictive temperature in the mutant selection scheme), but cell growth was improved at lower temperatures (30° and 34°) that were either semipermissive or nonpermissive for the *abd1* alleles when driven by their natural promoter (not shown). If, as implied by this result, the *ts* phenotype is sensitive to *abd1* gene expression levels, then the key question is whether high-copy *abd1* suppression by AdoMet synthase and Cdc34p is mediated through transcriptional effects on the *ABD1* promoter. If so, then one predicts that AdoMet synthase and Cdc34p will be unable to suppress the *TPI-abd1-ts* phenotype. In fact, the opposite scenario was observed; *i.e.*, 2μ plasmids containing *SAM2*, *SAM1*, or *CDC34* clearly did suppress the growth defect of *TPI-abd1-8* and *TPI-abd1-15* cells at 37° (Figure 8). The *CDC34* mutants *CDC34(C95S)* and *CDC34(1-174)* were inactive in *TPI-abd1-8* suppression (not shown), just as they were in suppressing *abd1-8* driven by its own promoter. 2μ plasmids containing the *CET1* or *CEG1* genes were also inactive in *TPI-abd1* suppression (Figure 8). We surmise that the salutary effects of increased AdoMet and Cdc34p do not occur through the *ABD1* promoter, but are more likely to reflect posttranscriptional effects on Abd1p itself or downstream transactions involving the cap methyl group.

This hypothesis is supported by RNase protection analysis of the steady-state levels of poly(A)$^+$ *ABD1* mRNA in cells that overexpress AdoMet synthase or Cdc34p (Figure 9). Whereas the basal level of *ABD1* transcript (Figure 9A, lane 3) was increased dramatically by the introduction of *ABD1* on a multicopy plasmid (Figure 9A, lane 2), the introduction of multicopy plasmids containing *SAM2* (Figure 9A, lane 4) or *CDC34* (Figure 9A, lane 5) elicited no change in the abundance of *ABD1* mRNA. Control RNase protection experiments confirmed that the steady-state levels of *CDC34* and *SAM2* mRNAs were themselves increased by increased *CDC34* and *SAM2* gene dosage (Figure 9B, lane 5, and Figure 9C, lane 4).

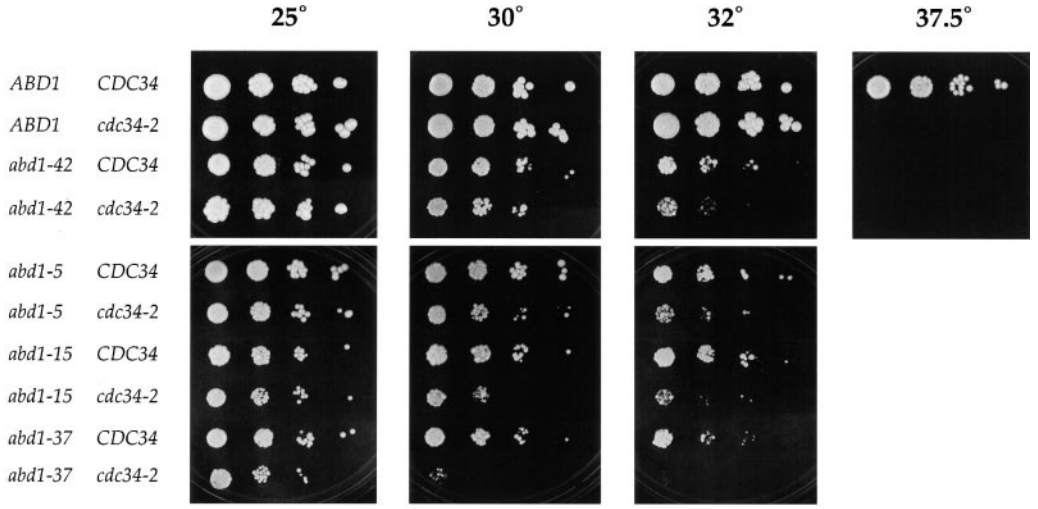

Figure 10.—Phenotypic synergy of *abd1* and *cdc34* mutations. Isogenic *abd1::KAN CDC34* and *abd1::KAN cdc34-2* strains containing *ABD1* or the indicated *abd1* alleles on *CEN TRP1* plasmids were grown in liquid YPD medium at 25°. The A_{600} was adjusted to 0.1. Aliquots (3 μl) of serial 10-fold dilutions of the cell suspensions were spotted onto YPD agar medium. The plates were photographed after incubation at 25° (for 4.5 days), 30° (for 3.5 days), and 32° or 37° (for 3 days).

The results in Figure 9 also reveal a lack of crosstalk at the transcriptional level between AdoMet synthase and Cdc34p. Overexpression of AdoMet synthase had no effect on the steady-state level of *CDC34* mRNA (Figure 9B, compare lanes 3 and 4). More important, the overexpression of *CDC34* did not increase the level of mRNA encoding AdoMet synthase (Figure 9C, compare lanes 3 and 5). (The *SAM2* antisense probe will detect both *SAM1* and *SAM2* mRNAs.) We infer that the suppression of *abd1* mutations by overexpression of *CDC34* does not occur via upregulation of *SAM* gene expression.

Phenotypic synergy of *abd1* and *cdc34* mutations: If *abd1* suppression by Cdc34p overexpression reflects a role of Cdc34p in cap methylation or m7G cap-dependent pathways, then we might expect a partial loss-of-function mutation of Cdc34p to exacerbate the growth phenotype of *abd1* cells. To test for mutational synergy, we constructed isogenic *abd1*Δ *CDC34* and *abd1*Δ *cdc34-2* strains containing *CEN* plasmid-borne *ABD1* or *abd1* alleles. *cdc34-2* cells display a temperature-sensitive growth phenotype caused by a single Cdc34p missense mutation of Gly-58 to Arg (Liu *et al.* 1995). *ABD1 cdc34-2* cells grew normally at 25° to 32° but failed to grow at 37.5° (Figure 10). The *cdc34-2* mutation clearly enhanced the conditional lethality of the *abd1-37* strain, such that it was no longer able to grow at 30° and 32° (Figure 10). (Note that 2μ *CDC34* was isolated originally as a suppressor of *abd1-37*.) The *cdc34-2* mutation also enhanced the growth defect of *abd1-15* cells at 30° and 32° (Figure 10). Mutational synergy between *cdc34-2* and *abd1-42* or *abd1-5* was apparent at 32° (Figure 10). These results suggest that Abd1p and Cdc34p act in a common pathway.

DISCUSSION

Structure-function relationships in cellular cap methyltransferases: Alanine mutations have now been targeted to 61 positions of Abd1p located within the C-terminal catalytic domain. The eight residues found to be essential for Abd1p function (Glu-170, Gly-174, Asp-178, Asp-194, Arg-206, Tyr-254, Phe-256, and Tyr-330) are conserved in the cap methyltransferases from other fungi, including *S. pombe* Pcm1p and *C. albicans* Ccm1p (Figure 1). cDNAs encoding the human cap methyltransferase have been identified recently (Pillutla *et al.* 1998; Tsukamoto *et al.* 1998; Saha *et al.* 1999). The 476-amino acid human enzyme (Hcm1p) displays extensive sequence conservation with the 436-amino acid yeast Abd1p protein. Expression of the *HCM1* gene in yeast complements the *abd1*Δ mutation (Saha *et al.* 1999). On the basis of the mutational analysis of Abd1p, Saha *et al.* (1999) identified by alanine-scanning mutagenesis 8 amino acids of Hcm1p (Asp-203, Gly-207, Asp-211, Asp-227, Arg-239, Tyr-289, Phe-291, and Phe-354) that are essential for human cap methyltransferase function *in vivo* in yeast. These are the equivalents of the 8 positions essential for Abd1p.

What does mutational analysis tell us about cap methyltransferase function? It is remarkable that alanine substitutions at 53/61 positions had no effect on Abd1p function *in vivo*, even though most of the nonessential residues are conserved among the cellular cap methyltransferases (Figure 1). The eight essential amino acid residues are candidates to comprise the active site. But what is their role in substrate recognition or catalysis? Essential residues Glu-170, Gly-174, and Asp-178 are located within or immediately flanking a putative AdoMet binding motif VL(D/E) × G × G × G, which is conserved among AdoMet-requiring methyltransferases (Kagan and Clarke 1994). This sequence element is referred to as motif I (Figure 1).

A database search by Wang and Shuman (1997) with the segment of Abd1p from residues 168 to 194 illuminated a distinct subfamily of 31 methyltransferases with diverse substrate specificity that display strict conservation at positions corresponding to essential residues

Glu-170, Gly-174, and Asp-194 of Abd1p. They discerned a second conserved element, motif Ia (FIGID in Abd1p), located just downstream of motif I (Figure 1). Motif Ia includes Asp-194, which is required for Abd1p function. In other methyltransferases, motif Ia is separated from motif I by a nonconserved spacer of 12–15 amino acids. A recent survey of the *S. cerevisiae* proteome by Niewmierzycka and Clarke (1999) identified 33 Abd1p-like methyltransferases in yeast that contain motifs I and Ia with appropriate spacing. The fact that essential Abd1p residues Glu-170, Gly-174, and Asp-194 are broadly conserved in methyltransferases with diverse methyl acceptor specificities argues that they serve a common role, *i.e.*, interaction with the methyl donor AdoMet.

Niewmierzycka and Clarke (1999) also discerned other methyltransferase motifs, including motif II (FPCDIVST in Abd1p) and motif III (SLKIGGHFFG in Abd1p). Motifs II and III are present in the cellular cap methyltransferases (Figure 1). Although several of the conserved side chains that define motifs II and III have been subjected to alanine substitution in Abd1p, none of the residues analyzed are essential for Abd1p function *in vivo*. Thus motifs II and III are unlikely to contribute directly to catalysis by Abd1p.

Sequence gazing indicates that essential Abd1p residue Asp-178 immediately flanking motif I is found in only 3 out of 31 Abd1p-like proteins (Wang and Shuman 1997). Furthermore, none of the 25 different *E. coli* methyltransferases aligned by Koonin *et al.* (1995) contains an acidic residue at this position. However, Asp-178 is strictly conserved among the cap methyltransferases. In the same vein, the 250-FCLHYAF-256 segment of Abd1p, which contains two functionally important residues and is well-conserved in cellular and viral cap methyltransferases (Figure 1), is not widely encountered in other methyltransferases. This suggests that the Asp flanking motif I and the two distal aromatic residues in FCLHYAF contribute uniquely to cap methylation, perhaps by interacting with the GpppN cap structure of the RNA methyl acceptor. Crystal structures of two enzymes involved in cap formation, *Chlorella* virus guanylyltransferase (Håkansson *et al.* 1997) and vaccinia virus cap-specific nucleoside 2′ *O*-methyltransferase (Hodel *et al.* 1997; Hu *et al.* 1999), and the crystal structure of the cap-binding translation initiation factor eIF4E (Marcotrigiano *et al.* 1997) reveal that the protein-bound cap guanine is stabilized by base-stacking interactions with aromatic amino acids. Efforts to crystallize a yeast cap methyltransferase are underway.

Inactivation of Abd1p affects yeast protein synthesis: The shut-off of protein synthesis in *abd1-ts* cells is detectable at 20 min post-shift to 37° and fully manifest at 40 min post-shift. Inactivation of Ceg1p elicits the same decrement in the rate of yeast protein synthesis (Schwer *et al.* 1998a), yet the *abd1* and *ceg1* RNA phenotypes are different. Whereas the cap guanylate protects mRNA from premature exonucleolytic decay, the methyl group appears to be less critical for mRNA stability, as inferred from steady-state mRNA levels after shift to restrictive temperature. For example, *GCN4* mRNA levels in *abd1-5* cells were unaffected 3 hr after shift to 37°; this contrasts with the decline in *GCN4* mRNA to 3% of the pre-shift level in *ceg1-13* cells after 3 hr at 37° (Schwer *et al.* 1998a,b). Other specific transcripts in *abd1-5* cells were reduced modestly after the shift to 37°.

The *in vivo* half-lives of "stable" yeast mRNAs such as *PGK1*, *ACT1*, and *CYH2* are on the order of 25–45 min at 37° (Herrick *et al.* 1990; Beelman *et al.* 1996); hence, we can infer that the majority of mRNA detected in *abd1* cells at 3 hr post-shift was transcribed at the restrictive temperature. In principle, the mRNAs made at 1–3 hr post-shift in *abd1* cells should be 5′ guanylylated, but not methylated at N7. Confirmation of this prediction is hampered by the lack of straightforward methodology to distinguish GpppN-cap *vs.* m7GpppN-cap structures on specific yeast mRNAs. It is possible, even likely, that inactivation of Abd1p also affects mRNA guanylylation, because concomitant methylation, or lack thereof, influences the yield of guanylylated RNA synthesized by RNA guanylyltransferase. In the absence of cap methylation, the yield of blocked 5′ RNA termini formed by RNA guanylyltransferase is reduced by 60% (Martin and Moss 1975; Shuman 1990). This occurs because: (i) the cap guanylate of GpppRNA is easily transferred from GpppRNA back to the enzyme and then to PPi in a reversal of the capping reaction (*i.e.*, capping enzyme has decapping activity in the absence of cap methylation) and (ii) N7 methylation of the cap guanylate renders the reaction irreversible and therefore drives the overall equilibrium toward cap formation (Martin and Moss 1976; Shuman 1982; Ho *et al.* 1996). The variable effects of *abd1* inactivation on steady-state levels of individual transcripts may be an indirect consequence of different efficiencies of cap guanylylation in the absence of cap methylation, *i.e.*, because unguanylylated yeast mRNAs are rapidly turned over *in vivo* (Schwer *et al.* 1998a).

We consider it unlikely that defective mRNA transport was responsible for the protein synthesis defect seen in *abd1* cells at the restrictive temperature, insofar as *abd1-8* and *abd1-5* cells did not accumulate poly(A)$^+$ RNA in the nucleus after shift to 37°; the intracellular distribution of poly(A)$^+$ RNA in the *abd1* mutants at the restrictive temperature was indistinguishable from that of *ABD1* cells (C. Saavedra and C. Cole, personal communication). Note also that Fresco and Buratowski (1996) did not detect poly(A) accumulation in the nucleus of *ceg1-ts* cells at the restrictive temperature. A reasonable inference from these collective data is that neither the cap guanylate nor the cap methyl group is required for mRNA transport in yeast. Thus, a parsimonious explanation for the growth defect is that mRNAs made at the restrictive temperature are not efficiently utilized by the

translation apparatus. A primary defect in translation in *abd1-ts* cells seems plausible, given that the cap methyl group is important for cap-dependent translation *in vitro*. The phenotype of *abd1-ts* cells *vis a vis* protein synthesis is similar to that observed with conditional mutations in the essential 24-kD cap-binding translation initiation factor eIF-4E. Yeast eIF-4E *ts* mutants, when shifted to the restrictive temperature, synthesize proteins at 15–30% of the rate of wild-type cells (Altmann *et al.* 1989). Additional experiments are necessary to pinpoint whether the translation defect in *abd1-ts* cells is caused by a specific block at the initiation step. We cannot exclude a model in which Abd1p might perform an essential methyl transfer function unrelated to mRNA cap formation, but we regard such a scenario as unduly complicated.

Potential for regulation of cap methylation and cap-dependent transactions: The issue of whether (and how) cap methylation might be regulated has received scant attention. Potential regulatory events include: (i) those that affect the transcription or stability of the mRNA encoding cap methyltransferase, (ii) those that influence the targeting of the cap methyltransferase to the transcription apparatus, and (iii) those that affect the activity of the cap methyltransferase.

Genome-wide analyses of mRNA transcript levels in *S. cerevisiae* cells have shown that *ABD1* mRNA does not vary during the mitotic cell cycle (Cho *et al.* 1998; Spellman *et al.* 1998). *ABD1* transcription in mitotic cells is affected by mutations in RNA polymerase II itself (as expected) and by mutations in transcription factor components Kin28p, Srb4p, and Taf17p. *ABD1* expression is unaffected by mutations in Srb5p, Srb10p, Swi2p, Taf145p, and Gcn5p (Holstege *et al.* 1998).

Cap methyltransferase is targeted to nascent pre-mRNAs by direct binding of Abd1p to the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II (McCracken *et al.* 1997). The Abd1p-CTD interaction depends on CTD phosphorylation; therefore cap methylation might be regulated by the dynamics of CTD phosphorylation and dephosphorylation during transcription elongation.

The catalytic activity of Abd1p could conceivably be modulated by: (i) changes in the concentrations of the substrate AdoMet or the inhibitory reaction product AdoHcy or (ii) protein-protein interactions that affect enzyme activity. There is precedent for protein-mediated stimulation of cap methylation by vaccinia capping enzyme, whereby the low basal methyltransferase activity of the catalytic D1 subunit is stimulated 50- to 100-fold by its association with the regulatory D12 subunit (Higman *et al.* 1994; Mao and Shuman 1994).

This study suggests the plausibility of regulation by substrate concentration, *i.e.*, via our finding that *abd1-ts* mutations are suppressed by overexpression of AdoMet synthase and by provision of exogenous AdoMet. Suppression by AdoMet synthase in turn suggests plausible explanations for the conditional defects of the Abd1-ts proteins: that they either have a decreased binding affinity for AdoMet at the restrictive temperature or that AdoMet stabilizes the mutant proteins against thermal inactivation. Studies of the vaccinia capping enzyme indicate that AdoMet allosterically stimulates binding of the RNA cap to the methyl acceptor site of the enzyme (Mao *et al.* 1996). Thus, increased AdoMet might also suppress Abd1p-ts mutants that are conditionally defective in cap binding. Purification and biochemical characterization of the 10 *ts* mutants of Abd1p will be pursued in the context of future studies of the role of AdoMet in RNA binding and catalysis by Abd1p. Note that the assays of these parameters have not yet been established for the wild-type Abd1p; hence, a complete characterization of the *ts* mutants is beyond the scope of the present genetic study.

A novel mode of regulation is suggested by the findings that *abd1-ts* mutations are suppressed by overexpression of the ubiquitin conjugating enzyme Cdc34p and that Cdc34p overexpression has no effect on the steady-state levels of mRNAs encoding Abd1p or AdoMet synthase. Given that Cdc34p-mediated ubiquitination is known to target yeast proteins for intracellular proteolysis, we posit a model whereby cap methylation or cap utilization is negatively regulated by a factor that is degraded when Cdc34p is overexpressed. This implies a balance between the normal function of Abd1p and a putative antagonist of cap methylation or m7G-dependent transactions that is deranged in favor of the antagonist in *abd1-ts* cells at the restrictive temperature. The *ts* growth defect of *abd1* cells is exacerbated in a *cdc34-2* background, presumably because the antagonist is stabilized when Cdc34p function is diminished. A more elaborate alternative model, which we do not exclude, is that ubiquitination by Cdc34p positively regulates Abd1p function, either by ubiquitination of Abd1p itself or ubiquitination of a putative Abd1p activator, such that the Ub modification leads to a gain of function for the target protein rather than proteolysis.

The key issues raised by the inhibitor model are: (i) the identity of the target protein that, when ubiquitinated by Cdc34p, relieves the *abd1-ts* phenotype and (ii) whether it is cap methylation or m7G cap utilization that is affected by the target protein. We initially considered the possibility that alterations in the level or function of certain translation initiation factors might affect the *abd1-ts* phenotype. Yeast p20 (Caf20p) is a repressor of cap-dependent translation initiation that competes with eIF4G for binding to the m7G cap-binding protein eIF4E (Cdc33p; Altmann *et al.* 1997; de la Cruz *et al.* 1997; Ptushkina *et al.* 1998). We found that: (i) disruption of the *CAF20* gene did not ameliorate the *abd1-ts* growth phenotype; (ii) introduction of *CAF20* on a 2μ plasmid did not exacerbate the *abd1-ts* growth phenotype; and (iii) transformation of *abd1-ts* cells with a 2μ plasmid bearing *CDC33* under the control of the

TPI1 promoter also did not ameliorate the conditional growth defect (B. Schwer, unpublished results). Thus, we suspect that suppression of the *abd1-ts* phenotype (and by inference Cdc34p suppression) is not mediated through the eIF4E-p20 axis. Future experiments to address potential regulators of Abd1p will include: (i) biochemical identification of proteins in yeast extracts that interact with purified recombinant Abd1p and (ii) a genetic screen for chromosomal suppressors of *abd1-ts* mutations, with the expectation that recessive extragenic suppressors might identify the putative inhibitor targeted by Cdc34p.

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