The DCP2 protein is required for mRNA decapping in *Saccharomyces cerevisiae* and contains a functional MutT motif

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The major pathway of mRNA degradation in yeast occurs through deadenylation, decapping and subsequent 5' to 3' exonucleolytic decay of the transcript body. To identify proteins that control the activity of the decapping enzyme, which is encoded by the *DCP1* gene, we isolated a high-copy suppressor of the temperature-sensitive *dcp1-2* allele, termed DCP2. Overexpression of Dcp2p partially suppressed the *dcp1-2* decapping defect. Moreover, the Dcp2 protein was required for the decapping of both normal mRNAs and aberrant transcripts that are degraded by the mRNA surveillance pathway. The Dcp2 protein contains a MutT motif, which is found in a class of pyrophosphatases. Mutational analyses indicated that the region of Dcp2p containing the MutT motif is necessary and sufficient for Dcp2p's function in mRNA decapping. The Dcp2p also coimmunoprecipitates with the DCP1 decapping enzyme and is required for the production of enzymatically active decapping enzyme. These results suggest that direct or indirect interaction of Dcp1p with Dcp2p is required for the production of active decapping enzyme, perhaps in a process requiring the hydrolysis of a pyrophosphate bond.

Keywords: decapping/mRNA decay/MutT

Introduction

The stability of mRNA is an important determinant in gene expression (for reviews, see Ross, 1995; Caponigro and Parker, 1996; Jacobson and Peltz, 1996). An important goal will be to define the pathways of mRNA degradation and the mechanisms by which mRNA decay pathways are modulated. Recent results have indicated that two general pathways of mRNA decay function in the yeast *Saccharomyces cerevisiae*. The predominant decay pathway occurs via shortening of the poly(A) tail, followed by removal of the 5' cap structure by the Dcp1 decapping enzyme (Beelman *et al.*, 1996), which exposes the body of the transcript to Xrn1p-dependent 5' to 3' exonucleolytic degradation (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrads *et al.*, 1994, 1995). A second general mRNA decay pathway occurs by deadenylation of polyadenylated mRNA to an oligo(A) tail length followed by 3' to 5' exonucleolytic decay of the transcript body (Muhlrads *et al.*, 1995; Anderson and Parker, 1998).

Several lines of evidence indicate that these decay pathways identified in yeast are conserved in other

eukaryotes. For example, deadenylated, decapped, full-length mRNA species have been detected from murine liver cells (Couttet *et al.*, 1997). Intermediates in mRNA decay that are shortened at their 5' ends have also been identified in both plant and animal cells (Lim and Maquat, 1992; Higgs and Colbert, 1994; Gera and Baker, 1998). An important role for 3' to 5' mRNA decay in plants has also been demonstrated (Higgs and Colbert, 1994). Finally, the yeast proteins involved in these general mRNA decay pathways, such as Xrn1p, Ski2p, Ski6p/Rrp41p and Rrp4p, have homologs in higher eukaryotes (Dangel *et al.*, 1995; Lee *et al.*, 1995; Bashkirov *et al.*, 1997; Mitchell *et al.*, 1997).

Decapping is a key step in the 5' to 3' mRNA degradation pathway since it both precedes and permits the decay of the transcript body. Since individual mRNAs are decapped at different rates, decapping is also an important control point in mRNA decay (Muhlrads *et al.*, 1994, 1995). Decapping also plays a critical role in the process of mRNA surveillance wherein aberrant nonsense codon-containing mRNAs are very rapidly decapped and degraded in a 5' to 3' direction (Muhlrads and Parker, 1994; Hagan *et al.*, 1995; Beelman *et al.*, 1996). The product of the *DCP1* gene has been shown to be necessary for mRNA decapping *in vivo* and sufficient for decapping *in vitro*, suggesting that the *DCP1* gene encodes a yeast mRNA decapping enzyme (Beelman *et al.*, 1996; LaGrandeur and Parker, 1998). Understanding how the activity of the DCP1 decapping enzyme is regulated *in vivo* will be critical for understanding the basis for differential mRNA decay rates. However, to date little is known about the factors that modulate the activity of this key enzyme.

To identify proteins that affect the activity of the DCP1 decapping enzyme we have performed a genetic screen. The design of this screen exploits the observation that loss-of-function mutations in the Dcp1p decapping enzyme or the Xrn1p cytoplasmic 5' to 3' exoribonuclease are synthetically lethal in combination with mutations in proteins that are required for normal 3' to 5' mRNA degradation, such as Ski2p, Ski3p, Ski8p or Rrp41p/Ski6p (Johnson and Kolodner, 1995; Anderson and Parker, 1998). One implication of this finding is that mRNA decay is required for cell growth. We have made the synthetic lethality of *dcp1Δ ski8Δ* double mutants conditional using a temperature-sensitive allele of the DCP1 decapping enzyme, termed *dcp1-2* (Tharun and Parker, 1999). To then identify proteins involved in mRNA decapping, we isolated high-copy suppressors of the temperature-sensitive growth defect of the *dcp1-2 ski8Δ* strain. Here we describe the properties of one such suppressor, referred to as DCP2, which suppressed the growth defect of *dcp1-2 ski8Δ* mutants and is also required for decapping *in vivo*.

Fig. 1. *DCP2* overexpression rescues the growth defect of *dcp1-2 ski8Δ* mutants. Strains yRP840 and yRP1345, containing either a 2μ plasmid (yEP351) or *DCP2* expressed from a 2μ plasmid (pRP925), were grown at 24°C for 3 days on minimal media lacking leucine then replica plated and grown for an additional 2 days at either 24 or 34°C.

Results

Identification of high-copy suppressors of the conditional growth defect of *dcp1-2 ski8Δ* mutants

To identify proteins involved in mRNA decay, we performed a genetic screen for high-copy suppressors of *dcp1-2 ski8Δ* mutants. The *dcp1-2 ski8Δ* strain grows at 24°C, the permissive temperature for *dcp1-2* function (Tharun and Parker, 1999). However, the double mutant fails to grow at temperatures >30°C, which is the restrictive temperature for *dcp1-2* function (Tharun and Parker, 1999). The restrictive temperature for growth of the *dcp1-2 ski8Δ* mutant correlates with the temperature at which mRNA decay is severely inhibited (data not shown), suggesting that the inability of this mutant to grow results from a block to mRNA decay. We therefore reasoned that suppressors that restore the ability of the *dcp1-2 ski8Δ* double mutant to grow at the restrictive temperature would do so by restoring mRNA decay.

We screened five genome equivalents of a yeast 2μ genomic DNA library for genes that, when overexpressed, would suppress the growth defect of the *dcp1-2 ski8Δ* double mutant. In cases where growth at 34°C was restored, we showed plasmid dependence of the growth phenotype through isolating the plasmid, retransforming the parental *dcp1-2 ski8Δ* double mutant, and retesting the growth phenotype. We identified 42 plasmid-dependent suppressors. Four of these plasmids were found to contain the *DCP1* gene and 34 were shown to contain *SKI8*. In addition, we identified three plasmids containing the *PSU1/DCP2* gene (ORF YNL118C) as the only common open reading frame. Subsequent testing of only the *PSU1/DCP2* gene demonstrated that this gene was sufficient to restore growth to the *dcp1-2 ski8Δ* mutant, although not to wild-type levels (Figure 1). Specifically, the *dcp1-2 ski8Δ* mutant overexpressing *DCP2/PSU1* has a growth rate of 190 min at 34°C compared with 90 min for an isogenic wild-type strain. Finally, one plasmid was found that contained six open reading frames. Of these, YGL222C was shown to be the gene responsible for suppression (data not shown). Characterization of YGL222C will be reported in future work. Here we define an important role for *PSU1/DCP2* in mRNA decapping. The *PSU1/DCP2* gene has been identified previously as a high-copy suppressor of a nuclear petite mutant (A.Tzagaloff, personal

communication). However, based on the identification of a critical role of this protein in mRNA decapping presented here, we will refer to the *PSU1* gene hereafter as the *DCP2* gene.

Overexpression of *Dcp2p* partially rescues the mRNA degradation defect of the *dcp1-2* mutation

In principle, the rescue of the *dcp1-2 ski8Δ* double mutant's growth defect by *DCP2* overexpression could be accompanied by some restoration of either 5' to 3' or 3' to 5' mRNA degradation. To determine whether *Dcp2p* overexpression restored mRNA decay through either of these pathways we made use of a galactose-inducible MFA2 mRNA that contains a poly(G) insertion in its 3' UTR, termed MFA2pG (Decker and Parker, 1993). By blocking the exonucleases that degrade mRNA, poly(G) insertions can be used to trap intermediates in the degradation of mRNA (Vreken and Raue, 1992; Decker and Parker, 1993; Anderson and Parker, 1998). Importantly, the specific degradation intermediates that are trapped can be used to infer the directionality of mRNA decay (Figure 2A; see also He and Parker, 1999). Therefore, we introduced the *DCP2* gene on a high-copy number plasmid into either *dcp1-2* or *ski8Δ* mutants that also contained the MFA2pG mRNA and determined the mRNA decay phenotype of the resulting strains (see Materials and methods).

Dcp2p overexpression partially rescues the mRNA degradation defect of the *dcp1-2* mutation at the restrictive temperature (Figure 2, compare C and D). This effect is seen in the *dcp1-2* mutant overexpressing *Dcp2p* as a small but clear increase in the level of mRNA degradation intermediate corresponding to that produced from 5' to 3' mRNA decay, referred to as the poly(G)→3' end fragment (Anderson and Parker, 1998). Consistent with only a partial restoration of 5' to 3' mRNA decay, *Dcp2p* overexpression did not restore production of the poly(G)→3' end fragment to wild-type levels. These results argue that only a low level of decapping is required for viability in combination with the *ski8Δ*, and are consistent with the observations that mutations in genes causing a partial block to decapping are not synthetically lethal with lesions preventing 3' to 5' decay (J.S.Anderson and R.Parker, unpublished observation). *Dcp2p* over-

Fig. 2. *DCP2* overexpression specifically suppresses the mRNA decay defect of the *dcp1-2* mutation. Shown schematically in (A) is the 5' to 3' decay pathway that produces the poly(G)→3' end fragment and the 3' to 5' decay pathway that produces the 5' end→poly(G) fragment. (B, C, D, E and F) Decay of the MFA2pG mRNA in either wild type (yRP840), *dcp1-2* (yRP1341), *dcp1-2* [*DCP2/LEU2*] (yRP1355), *dcp1Δ* (yRP1356) or *dcp1Δ* [*DCP2/LEU2*] (yRP1357), respectively. The experiment was performed as described in Materials and methods. Time points represent minutes after transcriptional repression.

expression did not restore 5' to 3' mRNA degradation in a *dcp1Δ* mutant (Figure 2, compare E and F). This observation indicated that Dcp2p overexpression was not a bypass suppressor of *dcp1* mutations. Finally, *DCP2* overexpression had no detectable effect on the 3' to 5' mRNA decay defect of the *ski8Δ* mutant (data not shown), suggesting that Dcp2p overexpression does not significantly affect 3' to 5' mRNA decay.

Dcp2p is required for 5' to 3' mRNA degradation

Since Dcp2p overexpression affects 5' to 3' decay in a Dcp1p-dependent fashion, we hypothesized that Dcp2p normally functions in the 5' to 3' mRNA decay pathway. To determine whether Dcp2p has a role in mRNA decay, we deleted the *DCP2* gene from the genome through homologous recombination and assayed the phenotypes resulting from loss of Dcp2p. The Dcp2p is not required for viability in our strain background at any temperature tested from 18 to 37°C, though *DCP2* has been reported to be an essential gene in other strain backgrounds

(A.Tzagaloff, personal communication). However, the *dcp2Δ* strain grows extremely slowly at all temperatures, a phenotype comparable to that resulting from loss of the Dcp1p decapping enzyme (Beelman *et al.*, 1996).

Analysis of mRNA degradation in the *dcp2Δ* mutant showed that Dcp2p was required for normal rates of decay of both stable and unstable transcripts (Table I). Specifically, loss of Dcp2p function resulted in an ~2-fold stabilization of the PGK1pG mRNA (Figure 3) and a >3-fold stabilization of the MFA2pG mRNA (Figure 4). The *dcp2Δ* also caused a 2-fold stabilization of the GAL1 and GAL7 mRNAs and a 4-fold stabilization of the GAL10 mRNA compared with wild type. Moreover, the poly(G)→3' end fragment that is normally produced upon 5' to 3' degradation of the PGK1pG mRNA is not detectable in *dcp2Δ* mutants, suggesting a complete block to 5' to 3' mRNA decay (Figure 3). Consistent with a complete block to 5' to 3' decay, the residual decay rates that we observed in the *dcp2Δ* strain were very similar to those seen in a *dcp1Δ* strain (Table I) and result from the

Table I. Comparison of mRNA half-lives (min) between wild-type (yRP840), *dcp1Δ* (yRP1070) and *dcp2Δ* (yRP1346) yeast strains

MRNA	wt	<i>dcp1Δ</i>	<i>dcp2Δ</i>
MFA2pG	3.5	12	14
PGK1pG	25	>40	>40
GAL1	6	10	13
GAL7	5	11	14
GAL10	4	10	16

mRNA half-lives were determined as described in Materials and methods. Numbers shown represent the average of three separate experiments.

Fig. 3. DCP2 is required for 5' to 3' mRNA decay. The decay of the stable PGK1pG transcript in wild-type (yRP840) and *dcp2Δ* (yRP1346) yeast strains is shown. Strains were grown at 30°C to mid-log phase in YEP medium containing 2% galactose. Cells were harvested and resuspended in YEP medium. Glucose was added to a final concentration of 4% to repress transcription. Time points represent minutes after transcriptional repression. Hybridizations were performed with an oligonucleotide (oRP141; Caponigro and Parker, 1995) specific for the PGK1pG mRNA.

transcripts now being degraded by the alternative 3' to 5' decay pathway (see Anderson and Parker, 1998). These observations indicated that Dcp2p is required for the 5' to 3' degradation of a variety of transcripts.

Dcp2p is required for 5' to 3' decay following deadenylation

To define the role of Dcp2p in 5' to 3' mRNA decay, it was important to determine at which step 5' to 3' decay is blocked in the *dcp2Δ* mutant. In principle, any of the steps of deadenylation, decapping, or 5' to 3' exonucleolytic decay could be affected. To distinguish between these possibilities, we first examined the steady-state poly(A)-tail distribution of full-length MFA2pG mRNA. In wild-type cells, the MFA2pG transcripts exist with a distribution of poly(A) tails from ~75 nucleotides to an oligoadenylated length of ~10–12 nucleotides, which is the length at which the transcript becomes a substrate for decapping (Decker and Parker, 1993). In mutants such as *dcp1Δ* or *xrn1Δ*, which inhibit mRNA decapping or 5' to 3' exonucleolysis, oligoadenylated, full-length mRNA accumulates (Muhlrad *et al.*, 1994; Beelman *et al.*, 1996; Hatfield *et al.*, 1996; Boeck *et al.*, 1998). If loss of Dcp2p blocks decapping or 5' to 3' exonucleolysis, oligoadenylated mRNA species should be stabilized and therefore should accumulate in the *dcp2Δ* mutant.

To determine the steady-state poly(A)-tail distribution of full-length MFA2pG mRNA in the *dcp2Δ* mutant, we analyzed RNA from time points following glucose repression of the galactose-induced MFA2pG mRNA on polyacrylamide Northern gels to resolve polyadenylated and oligoadenylated mRNA species (Figure 4). Using this

Fig. 4. Dcp2p is required for 5' to 3' mRNA decay following deadenylation. The decay of the unstable MFA2pG mRNA in wild-type (yRP840) and *dcp2Δ* (yRP1346) strains is shown. Transcriptional repression was performed as for Figure 3. Hybridizations were performed with an oligonucleotide (oRP140; Caponigro and Parker, 1995) specific for the MFA2pG mRNA.

Fig. 5. Dcp2p is required for mRNA decapping *in vivo*. Immunoprecipitation of the MFA2pG mRNA with antisera directed against the 7-methyl cap structure is shown. Abbreviations used are T, total input RNA; P, immunoprecipitate pellet; S, supernatant. The strains used are wild-type (yRP840), *xrn1Δ* (yRP1359), *dcp1Δ* (yRP1070) and *dcp2Δ* (yRP1346). Hybridizations were performed as for Figure 4.

analysis, we found that the *dcp2Δ* mutant shifts the distribution of polyadenylated mRNA into the oligoadenylated pool at the early time points. Additionally, the oligoadenylated form of the mRNA persists at the later time points, suggesting that loss of Dcp2p function stabilizes the oligoadenylated form of the mRNA. The stabilization of oligoadenylated, full-length mRNA species indicated that 5' to 3' mRNA decay in the *dcp2Δ* mutant was blocked following deadenylation, either at the decapping or 5' to 3' decay steps. Further evidence that loss of Dcp2p function does not significantly affect deadenylation was obtained in transcriptional pulse-chase experiments wherein mRNA from the *dcp2Δ* mutant underwent poly(A) shortening with essentially wild-type kinetics (data not shown).

Oligoadenylated mRNA species are capped in *dcp2Δ* mutants

To distinguish between a role for Dcp2p in decapping or in 5' to 3' exonucleolysis, we performed immunoprecipitation experiments using antibody specific for the 5' cap structure of the mRNA (see Materials and methods). In *dcp1Δ* mutants, which block mRNA decapping, the mRNA is immunoprecipitable using these anti-cap antibodies (Figure 5; see also Beelman *et al.*, 1996). However, in *xrn1Δ* mutants, which block 5' to 3' exonucleolysis following decapping, only polyadenylated mRNAs are immunoprecipitable (Figure 5; see also Muhlrad *et al.*, 1994). Results for the *dcp2Δ* mutant showed that the

Fig. 6. Dcp2p is required for deadenylation-independent decapping. The degradation of the B55T-PGK1_{N103}pG mRNA, which contains a premature translation termination codon, is shown. Wild-type (yRP684) and *dcp2Δ* (yRP1358) strains containing the B55T-PGK1_{N103}pG transcript expressed from the GAL1 upstream activating sequence were grown at 30°C to mid-log phase in selective media containing 2% galactose. Transcriptional repression and hybridizations were performed as described for Figure 3.

oligoadenylated, full-length MFA2pG mRNA from a *dcp2Δ* strain was immunoprecipitable using these antibodies (Figure 5), indicating that the deadenylated mRNA that accumulated in *dcp2Δ* mutants was capped. This finding indicated that Dcp2p is required for mRNA decapping *in vivo*.

Dcp2p is required for deadenylation-independent decapping

Decapping of mRNA is a key step not only in the deadenylation-dependent decay pathway but also in the deadenylation-independent decay pathway, which acts to degrade aberrant mRNAs. In this pathway, aberrant mRNAs, such as those containing premature translation termination codons, are rapidly decapped and degraded independently of deadenylation (Muhlrad and Parker, 1994). The same DCP1 decapping enzyme that functions in the normal mRNA decay pathway is required for the decapping of aberrant nonsense codon-containing mRNAs through the nonsense-mediated decay pathway (Beelman *et al.*, 1996). However, a second class of proteins that have non-essential roles in deadenylation-dependent decapping, such as Mrt1p, Mrt3p and Spb8p are not required for deadenylation-independent decapping of aberrant mRNAs (Hatfield *et al.*, 1996; Boeck *et al.*, 1998). Since Dcp2p was essential for the decapping of mRNAs through the deadenylation-dependent decapping pathway, an important question was whether Dcp2p was also required for deadenylation-independent decapping of nonsense codon-containing mRNA.

To determine whether Dcp2p is required for nonsense-mediated decay, we measured the half-life of a mutant PGK1 mRNA that contains an early nonsense codon, termed B55T-PGK1_{N103}pG (Muhlrad and Parker, 1994), in both wild-type and *dcp2Δ* yeast strains. Consistent with earlier work, in wild-type cells the B55T-PGK1_{N103}pG mRNA has a half-life of <2 min (Figure 6, top). The same mRNA in the *dcp2Δ* mutant was stabilized >20-fold (Figure 6, bottom). This observation indicated that Dcp2p was required for the rapid, deadenylation-independent decapping of nonsense codon-containing mRNA. Interestingly, the Dcp2 protein was also identified previously in a two-hybrid screen as interacting with the Upf1p (He and Jacobson, 1995; referred to as NMD1).

Since the Upf1p is a critical factor in triggering the degradation of aberrant mRNAs during mRNA surveillance (for review see Jacobson and Peltz, 1996), this identifies a set of interactions linking premature translation termination and the mRNA decapping machinery, with possible mechanistic implications (see Discussion).

Dcp2p contains a MutT motif that is required for mRNA decapping

The observations above indicated that Dcp2p was required for mRNA decapping *in vivo*. An important issue was the role of the Dcp2p in mRNA turnover. Insight into a possible function for Dcp2p came from an analysis of the protein sequence. The Dcp2 protein contains a conserved 23 amino acid MutT motif in its N-terminus (amino acids 134–156). This MutT motif is also conserved in several homologs of Dcp2p found in the databases (Figure 7). The MutT motif is found in a class of proteins that hydrolyze pyrophosphate linkages (for reviews see Koonin, 1993; Bessman *et al.*, 1996). Moreover, mutagenic studies have identified specific residues within the MutT motif that are required for the catalytic activity of these enzymes (Lin *et al.*, 1996; Safrany *et al.*, 1998). The presence of a conserved MutT motif in Dcp2p suggested that this protein might also be involved in the hydrolysis of pyrophosphate bonds, of which the mRNA cap structure contains two. For this reason, we determined if the MutT motif was important for Dcp2p's function in decapping.

To determine if the region of Dcp2p containing the MutT motif was sufficient for Dcp2p's function in mRNA decapping we constructed two N-terminally FLAG-tagged deletion mutants of Dcp2p (see Materials and methods). One deletion, *dcp2-ΔC*, consists of the FLAG peptide followed by the N-terminal 300 amino acids, which contains the MutT motif and is the most highly conserved region of the Dcp2p. The second deletion, *dcp2-ΔN*, consists of the FLAG peptide followed by the C-terminal 670 amino acids of Dcp2p. Both deletions were expressed from the GPD promoter in a *dcp2Δ* background. Expression of either a full-length FLAG-Dcp2p fusion protein or of the N-terminal 300 amino acids completely rescued both the slow growth (data not shown) and mRNA decapping defect of the *dcp2Δ* mutation, as measured by the production of wild-type levels of the poly(G)→3'

Fig. 7. The MutT motif is conserved in Dcp2p homologs. The most conserved region of an alignment of Dcp2p and its closest homologs are shown. Only amino acids that are conserved in at least four of the homologs are highlighted. The core residues of the MutT motif are in bold. The alignment was generated using the ClustalW alignment program with the default parameters. Accession numbers (followed by percentage identity and similarity to DCP2 in parentheses) for the sequences shown are: DCP2, L43065; *Schizosaccharomyces pombe*, 2388911 (39%, 59%); *Gossypium hirsutum*, AI054538 (39%, 58%); *Drosophila melanogaster*, AA802328 (33%, 58%); *Caenorhabditis elegans*, 3947559 (32%, 52%).

end fragment (Figure 8A). This result indicated that the N-terminal 300 amino acids of Dcp2p, which contain the MutT motif, are sufficient to perform Dcp2p's function in mRNA decapping. In contrast, the *dcp2-ΔN* construct failed to rescue either the slow growth (data not shown) or mRNA decay defect of the *dcp2Δ* mutant (Figure 8A). Consistent with the *dcp2-ΔN* protein being non-functional, this polypeptide fragment is not stably expressed *in vivo* (see below; Figure 9B) and suggests that the N-terminus of Dcp2p is required both for its decapping function and for the production of stable protein.

To determine if the MutT motif was required for mRNA decapping we introduced mutations into the Dcp2p MutT motif at key residues that have previously been shown to be essential for the catalytic activity of other MutT motif-containing proteins (Lin *et al.*, 1996; Safrany *et al.*, 1998). Specifically, changing the C-terminal-conserved glutamic acid in the MutT motif to glutamine has been shown to reduce greatly the enzymic activity of both the canonical *Escherichia coli* MutT protein and the rat diphosphoinositol polyphosphate phosphohydrolase (DIPP). We therefore constructed different combinations of mutations incorporating this conserved glutamic acid residue, which corresponds to glutamic acid 153 in the Dcp2 protein. In one allele, *dcp2-1*, we changed four of the conserved amino acids in the MutT motif to alanine (Arg148, Glu149, Glu152 and Glu153). As shown in Figure 8B, the *dcp2-1* allele was unable to promote the decapping of the MFA2pG mRNA as measured by the lack of production of the poly(G)→3' end fragment. Similarly, the *dcp2-2* allele, which contains both an E152Q mutation and the key E153Q mutation, was non-functional in mRNA decapping. The *dcp2-3* allele, containing the single E152Q mutation, was functional for decapping as shown by the production of near wild-type levels of the poly(G)→3' end fragment. Importantly, the single E153Q mutation in the *dcp2-4* allele completely eliminated Dcp2p's function in decapping. The effects of these mutations on decapping probably do not result from differences in the expression of the mutant proteins since epitope-tagged versions of these proteins show the same decapping phenotypes and are expressed

to similar levels (data not shown). Since the identical mutation in the homologous glutamic acid residue of *E. coli* MutT and rat DIPP greatly reduces the catalytic activity of those enzymes, this result with the E153Q mutation strongly suggested that the activity of Dcp2p's MutT motif is required for mRNA decapping. This implies that Dcp2p may function in some manner as a pyrophosphatase that is required for the decapping of mRNA (see Discussion).

Purified Dcp2p lacks mRNA decapping activity itself *in vitro* but coimmunoprecipitates with active Dcp1p

Since Dcp2p was required for decapping and contained a functional MutT motif, a simple possibility was that the Dcp2p was a second decapping enzyme. In order to test this possibility, we purified the FLAG-Dcp2p fusion proteins described above from a wild-type strain and assayed for their ability to decap a synthetic mRNA *in vitro* (Beelman *et al.*, 1996; LaGrandeur and Parker, 1998; see also Materials and methods). To avoid any possible contaminating decapping activity from the Dcp1p decapping enzyme, we also purified the FLAG-Dcp2p proteins from *dcp1Δ* strains.

A silver-stained SDS-polyacrylamide gel of the purified FLAG-Dcp2p fractions showed that this affinity selection yielded proteins of the expected size for both the wild-type Dcp2p and for the *dcp2-ΔC* mutant (Figure 9A). A Western blot using anti-FLAG antibody to detect the presence of the FLAG epitope confirmed that the proteins of the expected size were indeed the FLAG-Dcp2 fusion proteins. Two FLAG fusion proteins were present in the full-length Dcp2p preparation. One species corresponds to ~110 kDa, the predicted molecular weight of the full-length FLAG-Dcp2 fusion protein. The second fusion protein has a mobility of 44 kDa. At present it is unclear if the smaller molecular weight protein represents an artifactual *in vitro* degradation product, or if this form of the protein represents a relevant *in vivo* processing reaction. The protein detected by the FLAG antibodies in the *dcp2-ΔC* preparation corresponds to the predicted

Fig. 8. The MutT motif is necessary and sufficient for mRNA decapping *in vivo*. (A) Steady-state distribution of the full-length MFA2pG mRNA and the poly(G)→3' end fragment decay intermediate. Abbreviations used are: FL-Dcp2, full-length FLAG-Dcp2p fusion; dcp2-ΔC, N-terminally FLAG-tagged N-terminal 300 amino acids of Dcp2p; dcp2-ΔN, N-terminally FLAG-tagged C-terminal 670 amino acids of Dcp2p. FLAG fusions were constructed as described in Materials and methods. Strains used are wild-type (yRP840) and *dcp2Δ* (yRP1346) transformed with the indicated FLAG-Dcp2 construct. Strains were grown to mid-log phase at 30°C in selective media. Cells were harvested and RNA prepared as described in Materials and methods. The MFA2pG mRNA was detected as described for Figure 4. (B) Abbreviations used are: *dcp2-1*, an R148A, E149A, E152A, E153A quadruple mutant; *dcp2-2*, an E152Q, E153Q double mutant; *dcp2-3*, an E152Q single mutant; *dcp2-4*, an E153Q single mutant. The *dcp2* alleles shown were constructed as described in Materials and methods. The wild-type and *dcp2Δ* strains used are the same as for (A). RNA was prepared and detected as for (A).

molecular weight of this fusion protein. No specific proteins were detectable by Western analysis in the preparation from strains expressing the *dcp2-ΔN* fusion (Figure 9B). Additionally, we have been unable to detect expression of this fusion protein in crude cell extracts (data not shown). This indicates that the *dcp2-ΔN* fusion protein is not stably expressed *in vivo*.

To determine if Dcp1p interacts with Dcp2p, we performed Westerns on the partially purified FLAG-Dcp2p fractions using antibodies specific for the Dcp1 protein. As shown in Figure 9C, Dcp1p interacts with the full-length FLAG-Dcp2 fusion protein and with the *dcp2-ΔC* fusion protein. Dcp1p is not present in the *dcp2-ΔN* preparation, which contains no Dcp2p fusion protein (Figure 9B). The Dcp1p antibodies detect no band in either the full-length FLAG-Dcp2p or the *dcp2-ΔC* fractions from the *dcp1Δ* strain (Figure 9C). This result

indicated that the protein interacting with the full-length Dcp2p and with the *dcp2-ΔC* protein from the wild-type strain was Dcp1p. It should be noted that the Dcp1p is not present at stoichiometric amounts when compared with the levels of purified Dcp2p. In part, this may result from the fact that the Dcp2p is overexpressed in this experiment to allow the purification of a significant amount of this polypeptide for biochemical assays (see below). However, two observations argue that this interaction is significant. First, the presence of Dcp1p in the fractions requires the presence of the Dcp2 protein (Figure 9C). Secondly, the suppression of *dcp1-2* with overexpression of Dcp2p is consistent with some type of interaction between these proteins (Figure 2). These results suggest that Dcp1p and Dcp2p interact physically *in vivo*, either directly or through interactions with additional proteins.

We next assayed for decapping activity of the purified Dcp2p preparations using an *in vitro* decapping assay described previously (Beelman *et al.*, 1996; LaGrandeur and Parker, 1998). In this assay, the decapping of an *in vitro*-transcribed mRNA containing a radiolabeled cap is monitored over time using PEI-cellulose thin-layer chromatography (TLC) to separate reaction products. Using this assay we found that both the purified full-length Dcp2p and the purified *dcp2-ΔC*p from the wild-type strain were active for decapping, as shown by the production of the 7mGDP decapping product (Figure 9D). The purified *dcp2-ΔN*p fraction containing neither Dcp1p nor FLAG-Dcp2p protein lacked detectable decapping activity. Importantly, full-length Dcp2p and *dcp2-ΔC*p purified from the *dcp1Δ* strain lacked any detectable decapping activity. This finding shows that, although decapping activity copurifies with Dcp2p, the activity is dependent upon the presence of the Dcp1p decapping enzyme. Thus, under these *in vitro* conditions, Dcp2p is not itself capable of decapping an mRNA (see Discussion).

Dcp1 protein levels are unaffected by loss of Dcp2p

Since Dcp2p was essential for decapping *in vivo* but does not appear to be a decapping enzyme *in vitro*, we explored other possible functions for Dcp2p in decapping. One possibility was that Dcp2p was required for the expression of Dcp1p, and that the defect in decapping that we observed resulted from a decrease or a loss of Dcp1p. To test this hypothesis, we determined whether *dcp2Δ* affected the expression of the Dcp1p decapping enzyme by Western analysis using anti-Dcp1p antibodies. We found that Dcp1p expression was comparable between the wild-type strain and the *dcp2Δ* mutant (Figure 10A). This observation indicated that the block to decapping seen in the *dcp2Δ* mutant was not the result of an effect on the level of expression of Dcp1p.

Dcp2p is required for the production of active Dcp1p

The observation that Dcp1p was present at wild-type levels in *dcp2Δ* mutants suggested the possibility that the Dcp1 protein made in the *dcp2Δ* mutant was non-functional, possibly due to the lack of a required modification. This possibility was based on the prior observations that Dcp1p is modified and that only a

Fig. 9. Dcp1p, as well as decapping activity, copurifies with Dcp2p. (A) Silver-stained SDS-PAGE of the FLAG-Dcp2p preparations from both wild-type *DCP1* (yRP1346) and *dcp1Δ* (yRP1070) strains. Abbreviations used are FL, full-length FLAG-tagged Dcp2p; ΔC, dcp2-ΔCp; ΔN, dcp2-ΔNp. Numbers on the left correspond to molecular weight markers in kDa. (B) Western blot using antibody specific for the FLAG epitope. Lanes shown are the same as those for (A). (C) Western blot using antibody specific for Dcp1p of the same samples as in (B) is shown. The His-Dcp1p sample represents purified His₆-tagged Dcp1p from which the His₆ epitope has been cleaved (Beelman *et al.*, 1996). (D) *In vitro* decapping assays using the purified FLAG-Dcp2p fractions. Samples shown are identical to those shown in (C) with the exception of the first lane (neg), which represents a decapping assay performed with no protein sample added. The 20 min time points of the decapping assays are shown. The products of the reaction were separated using PEI-cellulose TLC.

fraction of the protein purified from yeast cells is enzymatically active (LaGrandeur and Parker, 1998). To test the possibility that Dcp2p is required for the production of active Dcp1 decapping enzyme, we purified a FLAG-tagged version of Dcp1p from wild-type *DCP2* and *dcp2Δ* strains and assayed for the ability of the purified Dcp1p to cleave a capped mRNA *in vitro* using the assay described above.

An important observation was that the purified FLAG-Dcp1p from *dcp2Δ* strains was inactive for decapping *in vitro* (Figure 10C). In contrast, the Dcp1p purified from the *DCP2* strain was active *in vitro*. This suggested either that Dcp2p was required for the activation of Dcp1p, or that the purified FLAG-Dcp1p fraction from the *dcp2Δ* strain contained an inhibitor of decapping. The presence of a diffusible inhibitory molecule in the preparations from the *dcp2Δ* strain is unlikely, since the addition of active Dcp1p to these preparations yielded decapping activity (Figure 10C, last lane). It should be noted that, although additional proteins copurified with Dcp1p in these preparations, the additional proteins from the *DCP2* and *dcp2Δ* strains were indistinguishable between Dcp1p preparations. This argues that the inactivity of Dcp1p from the *dcp2Δ* strain does not result from copurification of a polypeptide that is inhibitory to decapping. Therefore,

these results suggested that the Dcp1p made in the *dcp2Δ* strain is inactive for decapping and imply that Dcp2p is required for the production of active Dcp1p decapping enzyme (see Discussion). Interestingly, addition of partially purified FLAG-Dcp2p to the inactive FLAG-Dcp1p did not restore decapping activity *in vitro* (data not shown), indicating that purified Dcp2p is not sufficient to reconstitute decapping activity with the inactive Dcp1p. This observation suggests either that additional proteins are required for Dcp2p to activate Dcp1p or that the purified fractions do not contain the substrate for Dcp2p, the hydrolysis of which may activate Dcp1p (see Discussion).

Discussion

Dcp2p is required for mRNA decapping

Several observations showed that Dcp2p was required for mRNA decapping *in vivo*. First, both stable and unstable mRNAs were stabilized in *dcp2* mutants (Table I). Secondly, *dcp2Δ* mutants accumulated oligoadenylated, full-length mRNAs (Figure 4), indicating that the block to decay occurs following deadenylation. Thirdly, immunoprecipitation experiments with anti-cap antibody showed that these deadenylated mRNAs accumulating in

Fig. 10. Dcp2p is required for the production of active Dcp1p. (A) Western blots using anti-Dcp1p antibodies are shown. Cell extracts from wild-type (yRP840), *dcp1Δ* (yRP1070) and *dcp2Δ* (yRP1346) strains were prepared as previously described (Beelman *et al.*, 1996). The FLAG-Dcp1p control used in this experiment was purified previously (LaGrandeur and Parker, 1998). The lower panel shows a silver-stained gel of the same samples to demonstrate consistent protein levels. (B) Silver-stained SDS-PAGE of purified FLAG-Dcp1p preparations from wild-type *DCP2* (yRP1070) and *dcp2Δ* (yRP1346) strains. Abbreviations used are: NO FLAG, a mock purification from a wild-type strain (yRP840) that does not express a FLAG-Dcp1p construct; wt, FLAG-Dcp1p purified from a *DCP2* strain (yRP1070); *dcp2Δ*, FLAG-Dcp1p purified from a *dcp2Δ* strain (yRP1346). Numbers on the left indicate molecular weight standards in kilodaltons. (C) Decapping assays using the purified FLAG-Dcp1p preparations from (B) are shown. Lanes shown correspond to those from (B) with the addition of the negative (neg) and positive (His-Dcp1p) controls. The products at the end of a 20 min time course are shown.

dcp2Δ mutants were capped (Figure 5). Fourthly, the Dcp2 protein was required for the decapping of mRNAs that contain premature translation termination codons (Figure 6). Since Dcp2p was required for both deadenylation-independent and -dependent decapping, and no 5' to 3' decay products were observed in *dcp2Δ* strains (Figure 3), we conclude that Dcp2p, like the DCP1 decapping enzyme (Beelman *et al.*, 1996), is required for all mRNA decapping *in vivo* and is therefore likely to be a critical component of the mRNA decay machinery. This is in contrast to other proteins such as Mrt1p, Mrt3p and Spb8p, which affect the efficiency of decapping, but are not absolutely required for decapping (Hatfield *et al.*, 1996; Boeck *et al.*, 1998).

Dcp2p is required for the production of active Dcp1p

The decapping defect seen in the *dcp2Δ* strains can be attributed, at least in part, to the absence of active Dcp1p. The critical observation was that although Dcp1p is present at normal levels in a *dcp2Δ* mutant (Figure 10A), when purified this Dcp1p is inactive for decapping *in vitro* (Figure 10C). This suggests that the inactive Dcp1p made in the absence of Dcp2p may be significantly altered in some way from the active Dcp1 protein made in the wild-type strain (see below). The simplest interpretation of these observations is that Dcp2p is required to produce active Dcp1p enzyme and that this is the basis for the decapping defect seen in the *dcp2Δ* strains. However, it should be noted that we cannot rule out the possibility that Dcp2p has additional roles in mRNA decapping.

Dcp2p is likely to function as a pyrophosphatase

Consideration of the specific role of Dcp2p should include the inference that Dcp2p is likely to function as a pyrophosphatase. This was first suggested by the fact that Dcp2p contains a MutT motif, which has been described previously in a variety of pyrophosphatases (Koonin, 1993; Bessman *et al.*, 1996; Safrany *et al.*, 1998). Three observations suggest that this MutT motif is central to the function of Dcp2p in decapping. First, the region of Dcp2p containing the MutT motif and the MutT motif itself are conserved in several Dcp2p homologs in other species (Figure 7). Secondly, deletion analysis of the Dcp2 protein showed that the region of the protein containing the MutT motif was sufficient to rescue the decapping defect of the *dcp2Δ* mutation. Thirdly, based on prior work on other MutT-like enzymes, mutation of the key glutamic acid residue in the Dcp2p MutT motif eliminated the function of Dcp2p in mRNA decapping (Figure 8). The simplest interpretation of these observations is that Dcp2p's role in decapping is to hydrolyze a specific pyrophosphate bond, which must be cleaved to allow decapping to occur. However, it should be noted that we cannot rule out other possible functions of the MutT motif, perhaps in a novel role as a protein phosphatase.

Possible mechanisms of Dcp2p function

Based on the points discussed above there are two critical, and possibly related, issues for understanding the role of Dcp2p in mRNA decay. First, what is the actual bond, pyrophosphate or other, cleaved by Dcp2p? Secondly, why is Dcp1p produced in an inactive form in *dcp2Δ* strains?

Table II. Strains used in this study

Strain	Genotype	Source
yRP684	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52</i>	Hatfield <i>et al.</i> (1996)
yRP840	MAT α <i>his4-539 leu2-3112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG</i>	Hatfield <i>et al.</i> (1996)
yRP841	MAT α <i>his4-539 leu2-3112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG</i>	Hatfield <i>et al.</i> (1996)
yRP1070	MAT α <i>his4-539 leu2-3112 trp1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG dcp1::URA3</i>	Beelman <i>et al.</i> (1996)
yRP1340	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52 dcp1-2::TRP1</i>	this study
yRP1341	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52 dcp1-2::TRP1 [MFA2pG/LYS2]</i>	this study
yRP1345	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52 dcp1-2::TRP1 ski8::URA3</i>	this study
yRP1346	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52 dcp2::TRP1 cup1::LEU2/PGK1pG/MFA2pG</i>	this study
yRP1355	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52 dcp1-2::TRP1 [MFA2pG/LYS2] [DCP2/LEU2]</i>	this study
yRP1356	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52 dcp1::URA3 cup1::URA3 [MFA2pG/LYS2]</i>	this study
yRP1357	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52 dcp1::URA3 cup1::URA3 [MFA2pG/LYS2] [DCP2/LEU2]</i>	this study
yRP1358	MAT α <i>his4-539 leu2-3112 lys2-201 trp1 ura3-52 dcp2::TRP1</i>	this study
yRP1359	MAT α <i>his4-539 leu2-3112 lys2-201 ura3-52 xrn1::URA3 cup1::LEU2/PGK1pG/MFA2pG</i>	this study

One formal possibility was that Dcp2p is the decapping enzyme and that all Dcp1p preparations previously tested for decapping activity were contaminated with Dcp2p. Three observations make this possibility unlikely. First, separation of Dcp1p from other proteins by denaturing gel electrophoresis followed by renaturation of Dcp1p yields a protein capable of decapping an mRNA substrate (LaGrandeur and Parker, 1998). Secondly, fractionation of purified Dcp1p by size exclusion chromatography yielded a peak of active enzyme at 30 kDa, the expected size for Dcp1p (T.E.LaGrandeur and R.Parker, unpublished observation). Thirdly, we have not been able to demonstrate cleavage of an mRNA substrate by purified Dcp2p under standard (Figure 9) or a variety of other conditions (data not shown), wherein highly purified Dcp1p does have decapping activity (LaGrandeur and Parker, 1998). We interpret these observations to indicate that Dcp1p is a decapping enzyme and that Dcp2p is somehow required for its function.

Given the possible pyrophosphatase function of Dcp2p, a working hypothesis is that Dcp2p activates Dcp1p by cleaving a pyrophosphate bond that either relieves a specific inhibitory effect or provides an activation of Dcp1p function. For example, the Dcp2 protein could be required either for an activating covalent modification of Dcp1p or to prevent an inactivating modification of Dcp1p. In this view, the Dcp1p that is produced in *dcp2 Δ* strains would never be properly modified and would therefore be inactive for decapping. However, we have been unable to observe any difference in mobility between wild-type Dcp1p and Dcp1p purified from *dcp2 Δ* strains by SDS-PAGE (data not shown), suggesting that if there is a relevant modification it is of low molecular weight. The Dcp1 protein is a phosphoprotein (LaGrandeur and Parker, 1998). However, the form of the phosphorylation is unknown and could consist of covalent linkage of a small pyrophosphate-containing molecule. It may be that both active and inactive forms of Dcp1p are phosphorylated and that the enzymic activity of Dcp2p's MutT motif functions to alter the form of the phosphorylation, perhaps by releasing the covalently bound small molecule, thereby converting inactive Dcp1p to active Dcp1p. Alternatively, Dcp2p could be required for the hydrolysis and subsequent inactivation of a small inhibitor of Dcp1p, or for the production of a small molecule that activates Dcp1p. This model differs from the first only in that the pyrophosphate-

containing molecule need not be covalently linked to Dcp1p. Future work should resolve these issues.

Could Dcp2p be an important site for control of mRNA surveillance?

Several observations suggest that Dcp2p may be a critical site for the control of mRNA surveillance. First, Dcp2p is required for the rapid degradation of nonsense codon-containing mRNAs. Secondly, Dcp2p is required for the production of active Dcp1p. Thirdly, Dcp2p was identified several years ago as a protein that showed two-hybrid interactions with Upf1p (He and Jacobson, 1995; referred to as NMD1). The Upf1p is required for mRNA surveillance (Leeds *et al.*, 1991) and part of its role is to function as an ATPase, whose ability to hydrolyze ATP is required for activation of decapping (Weng *et al.*, 1996). Since we have shown an interaction between Dcp2p and the DCP1 decapping enzyme (Figure 9), the interaction between Dcp2p and Upf1p, which has been shown to associate with translation termination release factors (Czapinski *et al.*, 1998), provides a continuous set of interactions from the site of translation termination to the decapping enzyme. This suggests a possible mechanism wherein the hydrolysis of ATP by Upf1p would trigger Dcp2p to hydrolyze its as yet unknown substrate, which would then lead to activation of Dcp1p and decapping of the mRNA. Future experiments should be able to test the relevance of this specific hypothesis.

Is Dcp2p a multifunctional protein?

Recent results raise the possibility that Dcp2p/Psu1p may also have a function in transcription. The key observation is that small regions of Dcp2/Psu1p can activate transcription when tethered to DNA by fusion to a DNA binding domain (Gaudon *et al.*, 1999). Moreover, depletion of Dcp2p/Psu1p led to a loss of ligand-stimulated transcription. However, the *in vivo* significance of this finding remains unclear, and is probably unrelated to the role of Dcp2p in decapping, since the MutT motif of Dcp2p is required for decay and is not included within the regions that activate transcription when tethered to DNA.

Materials and methods

Plasmids and strains

Strains used in this study are listed in Table II. Strain yRP1340 was constructed in three steps. First, a portion of genomic DNA 3' of the

DCP1 gene was PCR amplified from plasmid pRP872 and cloned into the pBluescriptII KS+ vector using the *Bam*HI and *Sac*II sites. Next, the *TRP1* gene was cloned into the *Bam*HI site of this plasmid to generate plasmid pRP926. The *dcp1-2* allele was then PCR amplified from plasmid pRP872 and cloned into pRP926 using the *Apa*I and *Clal* sites. This plasmid was then digested with *Apa*I and *Sac*I to release the region of DNA containing the *dcp1-2* allele, *TRP1* and the genomic DNA from the region 3' of *DCP1*. This fragment was then transformed into yRP1070 and TRP+ transformants were screened at 24°C for loss of the *URA3* marker. Genomic integration of the *dcp1-2* allele was confirmed by Southern analysis.

The *dcp2Δ* strain was constructed by PCR amplification of the regions flanking the *DCP2* gene from plasmid pRP925. The PCR products were subcloned on either side of the *TRP1* gene in a pBluescript vector. The resulting plasmid was digested to release the *dcp2Δ* cassette and transformed into a wild-type diploid (yRP840 crossed to yRP841). Heterozygous transformants were identified using Southern analysis and dissected to generate strain yRP1346.

The FLAG-*DCP2* fusions were constructed using *in vivo* recombination between a FLAG-tagged *DCP2* PCR product and *Bam*HI-*Sal*I digested pRP935 in strain yRP1346. Plasmids were rescued from the resulting transformants and sequenced to confirm that no mutations were introduced during PCR. The full-length FLAG-*DCP2* fusion PCR product was constructed through amplification of the *DCP2* gene from pRP925 using universal PCR primers from Research Genetics (YNL118C). This product was then reamplified using oRP798 and oRP799. The *dcp2-ΔC* allele was constructed through amplification of the N-terminal 900 nucleotides of *DCP2* from pRP925 using the Research Genetics YNL118C forward primer and oRP752. This PCR product was then reamplified using oRP798 and oRP799. The *dcp2-ΔN* allele was constructed through amplification of the C-terminal 2010 nucleotides of *DCP2* from pRP925 using the Research Genetics YNL118C reverse primer and oRP751 followed by reamplification with oRP798 and oRP799.

The FLAG-Dcp1p fusion was constructed in two steps. The FLAG-*DCP1* fusion was PCR amplified from plasmid pRP801 using primers oRP311 and oRP312. This PCR product was then cotransformed into strain yRP1070 with *Bam*HI-*Sal*I digested pRP935. Plasmids resulting from *in vivo* recombination that complemented the *dcp1Δ* were rescued to *E.coli* using a yeast DNA isolation system (Stratagene), sequenced and subsequently transformed into yRP1346.

DCP2 missense mutations were constructed using oligonucleotide-directed mutagenesis as previously described (Tharun and Parker, 1999). The template was the *DCP2* gene, containing its native promoter and terminator elements in pRS416.

Genetic screening procedures

To screen for high-copy suppressors of the *dcp1-2 ski8Δ* mutant, strain yRP1345 was transformed with a yeast 2μ genomic DNA library (ATCC 37323). Transformants were selected at 24°C. Positive transformants were then replica plated and screened for growth at 24, 34 and 37°C. Plasmids were isolated from clones that supported growth at either 34 or 37°C, retransformed into yRP1345 and retested for growth at the restrictive temperature. The inserts from plasmids that conferred growth at high temperature were then sequenced.

RNA procedures

RNA samples were prepared and isolated as previously described (Caponigro *et al.*, 1993). Half-lives were determined by quantitation of blots using a Molecular Dynamics PhosphorImager. Loading corrections for quantitation were determined by hybridization to the 7S RNA, a stable RNA polymerase III transcript.

Determination of *dcp1-2* suppression

Strains yRP840, yRP1341, yRP1355, yRP1356 and yRP1357 were grown at 24°C to mid-log phase in selective media containing 2% galactose. Cultures were then shifted to 34°C and grown for an additional 1 h. Cultures were then pelleted and resuspended in 1/10 volume YEP medium. Dextrose was then added to 4% to repress transcription from the *GAL1* upstream activating sequence. Aliquots of cells were harvested at various time points following repression of transcription. RNA was prepared as previously described (Caponigro *et al.*, 1993).

Immunoprecipitation using anti-cap antibodies

Anti-cap immunoprecipitations were performed as described by F.He and A.Jacobson (manuscript in preparation). Briefly, 10 mg protein A-Sepharose CL4B was swelled in IPPH buffer (500 mM NaCl, 10 mM

Tris pH 7.5, 0.1% NP-40). The swelled protein A was washed three times and resuspended in 1 ml IPPH. Anti-cap antibody (10 μl) (Munns *et al.*, 1982) was then added to the beads and allowed to bind overnight at 4°C in the presence of 0.1 U/μl RNasin. The antibody-coupled beads were washed five times with IPPH buffer (150 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.05% NP-40). A 10 μl aliquot of total RNA was added to 100 μl antibody-coupled beads and incubated overnight at 4°C with 40 U RNasin. The supernatant was recovered and bound to fresh antibody-coupled beads overnight. The two pellets were combined and resuspended in 180 μl IPPH. RNA was eluted during a 1 h incubation at room temperature using SDS at a final concentration of 1%. The solution was centrifuged to pellet the protein A-Sepharose and the resulting supernatant extracted with phenol/chloroform. Fractions were analyzed on a 6% polyacrylamide Northern gel.

Purification of FLAG fusion proteins

A 500 ml culture containing the appropriate FLAG fusion was grown to late log phase in selective medium containing 2% dextrose. Cells were washed once with 30 ml ddH₂O, once with 30 ml TMN-150 (10 mM Tris-HCl pH 7.7, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM PMSE, 0.5 mM DTT), and resuspended in 5 ml TMN-150. A 2.5 ml aliquot of acid-washed beads was added to the cell suspension and cells were lysed by 10 cycles of vortexing for 30 s and 45 s in ice water. The lysate was centrifuged at 18 000 g for 15 min at 4°C and the supernatant removed. This supernatant was spun at 36 000 g for an additional 45 min at 4°C. The clear lysate was then removed and glycerol added to 20%. For purification of the FLAG construct, 500 μl of the lysate was added to 100 μl anti-FLAG M2 affinity gel (Kodak) pre-equilibrated in TMN-150. Binding was performed in batch overnight at 4°C. The affinity gel was washed five times with 1 ml TMN-150 and the bound FLAG fusion protein eluted at 4°C in TMN-150 containing 200 μg/ml FLAG peptide (Sigma).

The FLAG fusion protein preparations were analyzed by standard SDS-PAGE on a 10% gel (Laemmli, 1970). Protein size markers were purchased from Gibco-BRL. The gels were silver-stained using Silver Stain Plus reagents (Bio-Rad).

Determination of Dcp1p expression

Strains yRP840, yRP1070 and yRP1346 were grown to an OD₆₀₀ of 1.0. Whole cell extracts were prepared as previously described (Beelman *et al.*, 1996). One hundred micrograms of total protein was run on a standard 10% SDS-polyacrylamide gel. Expression of Dcp1p was detected by Western analysis using polyclonal antibodies to the full-length Dcp1 protein.

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