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Isolation and characterization of Dcp1p, the yeast mRNA decapping enzyme

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A major mechanism of mRNA decay occurs by the process of deadenylation, decapping and 5′ → 3′ exonucleolytic degradation. Recently, the product of the *DCP1* gene has been shown to be required for decapping mRNAs *in vivo* and co-purifies with decapping activity *in vitro*. We have purified Dcp1p to homogeneity and shown that it is sufficient for decapping, thereby indicating that Dcp1p is the decapping enzyme. Characterization of Dcp1p activity *in vitro* indicated that the 7-methyl group of the cap structure contributes to the enzyme's substrate specificity. In addition, Dcp1p was effectively inhibited by uncapped mRNAs, and the enzyme efficiently cleaved substrates that were ⩾25 nucleotides in length, with a preference for longer mRNA substrates. These properties suggest that Dcp1p recognizes the mRNA substrate by interactions with both the cap and the RNA moiety. The Dcp1p is also a phosphoprotein, suggesting its activity may be regulated by post-transcriptional modification.

Keywords: decapping/decay/mRNA/turnover/yeast

Introduction

The regulation of gene expression is significantly influenced by differences in mRNA decay rates. To gain insight into the molecular basis of different decay rates, the pathways of mRNA turnover are being elucidated. Many eukaryotic mRNAs decay through a pathway of turnover that is initiated by shortening of the poly(A) tail, which is followed by degradation of the body of the transcript (Beelman and Parker, 1995). In *Saccharomyces cerevisiae*, mRNA deadenylation leads to decapping of the mRNA, which is followed by rapid 5′ → 3′ exonucleolytic degradation of the mRNA (Decker and Parker, 1993; Muhlrad *et al.*, 1994, 1995). Several observations indicate that decapping and 5′ → 3′ decay are also likely to occur in other eukaryotes. First, deadenylated, decapped, full-length mRNAs have been detected from murine liver cells (Couttet *et al.*, 1997). In addition, intermediates in mRNA decay that are shortened at their 5′ ends have been identified in both plant and animal cells (Lim and Maquat, 1992; Higgs and Colbert, 1994). Moreover, homologs of the yeast *Xrn*1p 5′ → 3′ exonuclease that degrades mRNA following decapping have been identified in several organisms (Heyer *et al.*, 1995; Bashkirov *et al.*, 1997).

Decapping is a key step in the turnover of yeast mRNAs, since it precedes the decay of the body of the transcript and is a site of control, as individual mRNAs are decapped at different rates (Muhlrad *et al.*, 1994, 1995). Decapping also occurs in nonsense-mediated decay in yeast, whereby mRNAs that contain a premature translation stop codon are rapidly decapped independent of deadenylation (Muhlrad and Parker, 1994; Hagan *et al.*, 1995). Given the central role of decapping in mRNA decay, resolution of the mechanisms by which mRNA decapping occurs and is controlled will be critical for understanding mRNA turnover.

Recent work has identified a protein, encoded by the yeast *DCP1* gene, which co-purifies with a decapping activity. That activity cleaves capped mRNAs within the cap 5′–5′ triphosphate linkage to release m^7GDP and a 5′-monophosphate mRNA (Beelman *et al.*, 1996). Importantly, in strains deleted for the *DCP1* gene, many mRNAs are more stable due to a block in mRNA decapping (Beelman *et al.*, 1996). These mRNAs include stable and unstable mRNAs that undergo deadenylation-dependent decapping and mRNAs with early nonsense codons that are degraded by deadenylation-independent decapping. These results indicate that a single decapping activity, which requires Dcp1p for function, is responsible for most, if not all, mRNA decapping *in vivo*. Elucidation of the function and regulation of Dcp1p is therefore fundamentally important for understanding decapping.

In this work, we affinity-purified an epitope-tagged Dcp1p to homogeneity from yeast. Dcp1p hydrolyzes capped mRNAs, indicating that Dcp1p is sufficient to function as the decapping enzyme without additional components. Several aspects of Dcp1p decapping activity were then examined *in vitro* towards elucidating the mechanism by which Dcp1p functions *in vivo*. These experiments suggest that Dcp1p recognizes its substrate through interaction with both the cap and RNA moieties. Decapping might therefore be regulated, in part, by the accessibility of Dcp1p to the mRNA 5′ end. Moreover, Dcp1p is a phosphoprotein, suggesting that its activity could be regulated by post-transcriptional modification.

Results

Highly purified Dcp1p has decapping activity

A central goal towards understanding decapping is to determine the protein(s) that are sufficient for decapping activity. Previously, a modified Dcp1p that contained a hexahistidine epitope at its N-terminus, His-Dcp1p, was purified from yeast cell extracts with nickel affinity chromatography (Beelman *et al.*, 1996). While decapping activity co-purified with the His-Dcp1p, stained SDS–PAGE gels of this preparation revealed the presence of several other proteins (data not shown). This observation raised the possibilities that Dcp1p might be sufficient for

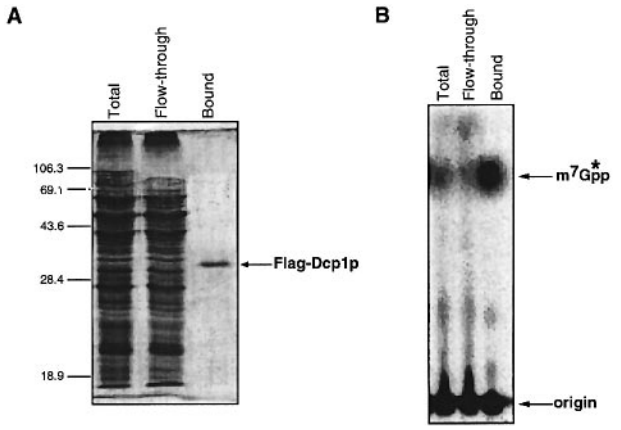


Fig. 1. Purified FLAG-Dcp1p has decapping activity. (**A**) Analysis of FLAG-Dcp1p purification by silver-stained SDS–PAGE. As described in Materials and methods, a cellular extract from yeast expressing FLAG-Dcp1p was chromatographed over heparin; the fraction that was not bound was then purified by anti-FLAG M2 immunoaffinity chromatography. Lanes: Total, the fraction that was loaded onto the anti-FLAG column; Flow-through, the fraction that did not bind to the anti-FLAG column; Bound, the eluate from the anti-FLAG column. The following amounts of each were resolved on the gel: Total, 3.6 μg; Flow-through, 3.6 μg; Bound, 140 ng. Protein size markers are indicated on the left in kDa. (**B**). Analysis of decapping activity from FLAG-Dcp1p purification fractions. Decapping was assayed as described in Materials and methods in 15 μl reactions which contained 0.01 pmol substrate and the following amounts of protein from each fraction of the anti-FLAG column: Total, 20 μg (of 6.5 mg total); Flow-through, 20 μg (of 6.4 mg total); Bound, 220 ng (of 50 μg total). The reaction products were separated by PEI-cellulose TLC. Lanes are as in (A). The * indicates the position of the radiolabeled phosphate.

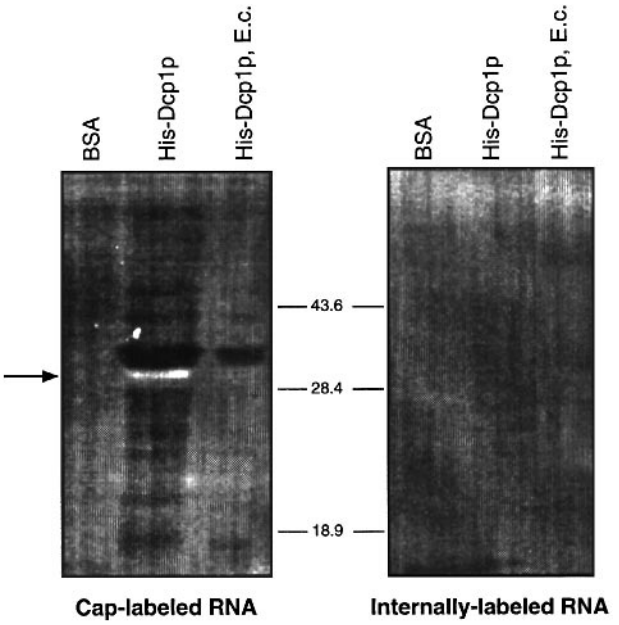


Fig. 2. Gel-isolated Dcp1p is enzymatically active. His-Dcp1p was resolved by denaturing SDS–PAGE in gels containing either cap-radiolabeled MFA2 mRNA or internally radiolabeled, uncapped MFA2 mRNA. The Dcp1p was then renatured in the gel, and assayed for decapping activity as described in Material and methods. Lanes: BSA, bovine serum albumin; His-Dcp1p, His-Dcp1p preparation from *S.cerevisiae*; His-Dcp1p, E.c., His-Dcp1p preparation from *E.coli*. 4 μg of each protein preparation was resolved on the gel. Protein size markers are indicated between the gels in kDa. The arrow indicates the position of clearing in the gel.

decapping or that it might function in a complex that requires additional components for activity. To distinguish between these two alternatives, we set out to purify Dcp1p to homogeneity and test its ability to decap mRNAs.

To purify Dcp1p, the *DCP1* gene was modified to encode a fusion protein containing the FLAG epitope at the Dcp1p N-terminus, thus enabling isolation of Dcp1p through anti-FLAG immunoaffinity chromatography. The gene encoding FLAG-Dcp1p was subcloned into a 2 μ plasmid under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter, to obtain high levels of Dcp1p expression, and transformed into a *dcp1*Δ strain. The FLAG-Dcp1p was functional *in vivo*, since it complemented the mRNA turnover and slow-growth phenotypes of the *dcp1*Δ strain (data not shown). Cells overexpressing the FLAG-Dcp1p contained significantly higher levels of Dcp1p than wild-type yeast cells, as judged by Western analysis. Despite this abundance of Dcp1p, the cells showed no growth defects and mRNA turnover rates for the PGK1 and MFA2 mRNAs were unchanged (data not shown). This observation suggests that the rates of mRNA decapping *in vivo* are not limited by the cellular abundance of Dcp1p.

The FLAG-Dcp1p was purified in two steps. First, a cellular extract was passed over a heparin–Sepharose column. FLAG-Dcp1p in the unbound fraction was then purified by anti-FLAG immunoaffinity chromatography. As shown in Figure 1A, the eluate from the anti-FLAG column contained only one protein that was detected in a silver-stained SDS–PAGE gel (even when greater levels of protein were examined). This protein was the FLAG-Dcp1p, as judged by its size of ~30 kDa and confirmed by Western blot analysis with antisera directed against the *DCP1* polypeptide (data not shown).

The FLAG-Dcp1p was next examined for decapping activity. Using an *in vitro* decapping assay, we tested the ability of the FLAG-Dcp1p to decap a synthetic mRNA that contained a radiolabeled cap. As shown in Figure 1B, the purified FLAG-Dcp1p preparation specifically decaps the substrate to yield the product m^7GDP. Isolation of the FLAG-Dcp1p to apparent homogeneity therefore results in purification of decapping activity.

Gel-isolated Dcp1p has enzymatic activity

The observation that purified Dcp1p had decapping activity suggested that Dcp1p is sufficient to function as a decapping enzyme *in vitro*. However, it was formally possible that the purified FLAG-Dcp1p contained an additional protein required for decapping that was present at low levels and not detected upon silver staining. To address this possibility we determined if Dcp1p, which was purified under denaturing conditions (to disrupt any protein–protein interactions), could be renatured to yield enzymatic activity.

For this experiment, we performed a SDS–PAGE activity assay, in which the His-Dcp1p polypeptide was first separated from other proteins on a SDS gel containing cap-radiolabeled substrate. Following electrophoresis, we renatured the Dcp1p in the gel and assayed for decapping activity. The in-gel Dcp1p was enzymatically active, as evidenced by clearing of signal from the gel in the region that contained Dcp1p (Figure 2). The clearing corresponds

to where radiolabeled m^7GDP, which was released from the substrate mRNA, diffused out of the gel, while the remainder of cap-radiolabeled mRNA primarily remained in the gel. Surprisingly, the clearing occurred slightly ahead of the majority of the Dcp1p in the gel, suggesting that only some of the Dcp1p was in an active form which migrates faster in the gel (see Discussion). The activity was specific to Dcp1p, as neither bovine serum albumin nor an inactive Dcp1p (that was produced in *Escherichia coli*) produced activity (Figure 2). While the clearing in the gel was suggestive of decapping, it could represent radiolabel released by a non-specific ribonuclease activity. To rule out this possibility, we also performed the in-gel assay with internally labeled, uncapped mRNA and failed to see any clearing (Figure 2). We therefore interpreted the activity observed in the gel containing cap-radiolabeled mRNA to result specifically from decapping by Dcp1p.

Based on the decapping activities observed with Dcp1p both in-gel and upon high purification of the protein, we concluded that Dcp1p was sufficient to function as a decapping enzyme *in vitro* and that it is the catalytic moiety of the decapping enzyme *in vivo*. However, given that decapping is a regulated process, Dcp1p may well interact with other proteins *in vivo*.

Optimization of Dcp1p decapping conditions

We optimized the conditions for decapping by FLAG-Dcp1p to facilitate further *in vitro* experiments. The FLAG-Dcp1p was tested in decapping assays as described in Materials and methods. The FLAG-Dcp1p required divalent cation for activity, and was optimally stimulated in the presence of 1 mM $MgCl_2$. The enzyme was 10-fold less active in the presence of $MnCl_2$, while no decapping activity was detected in the presence of $CaCl_2$. Also, FLAG-Dcp1p apparently does not require monovalent salt for activity, since decapping was not stimulated by the addition of KCl, NaCl, NH_4Cl or NH_4OAC. However, higher levels of monovalent salt affected decapping, as concentrations of 50, 75, 100 or 200 mM NaCl inhibited decapping ~1.8-, 4.4-, 5.4- or 12.6-fold, respectively. The FLAG-Dcp1p enzyme was most active at pH 7.0. The K_m for the synthetic, capped MFA2 mRNA substrate was determined (as described in Materials and methods) to be 18 nM.

Dcp1p recognizes the 7-methyl group of the cap structure

The ability of Dcp1p to decap mRNAs implied that it must be able to both recognize its substrate and cleave within the cap structure. To determine the substrate specificity of Dcp1p, we examined its activity with a number of substrates under varying conditions. The interaction of Dcp1p with its substrate could partially occur, in principle, through interaction with the cap structure 7-methyl group. The cap 7-methyl group has been implicated as being important for the cap-dependent processes of mRNA splicing and translation initiation (Adams *et al.*, 1978; Konarska *et al.*, 1984). We therefore addressed whether the 7-methyl group of the cap structure could also influence decapping by Dcp1p.

Capped mRNAs that either contained or lacked a 7-methyl group in their cap structures were prepared (see Materials and methods) and tested as substrates for decapping. The assays contained equal amounts of methylated or non-methylated substrates that were capped to the same extent. FLAG-Dcp1p is capable of cleaving the cap structure of the non-methylated substrate mRNA to release GDP, albeit less efficiently (Figure 3, compare left and middle panels). Based on the rates at which the cap was released from the two substrates, the non-methylated substrate was cleaved 10-fold less efficiently than the methylated substrate. To rule out the possibility that the non-methylated substrate contained an inhibitor of decapping, the methylated and non-methylated substrates were mixed and tested as decapping substrates. In this experiment, the methylated substrate was cleaved much more efficiently than the non-methylated substrate (Figure 3, right-hand panel), confirming that the enzyme prefers methylated substrates. The preference of Dcp1p for the methylated substrate indicated that the 7-methyl group of the cap structure contributes to the substrate specificity of the decapping enzyme.

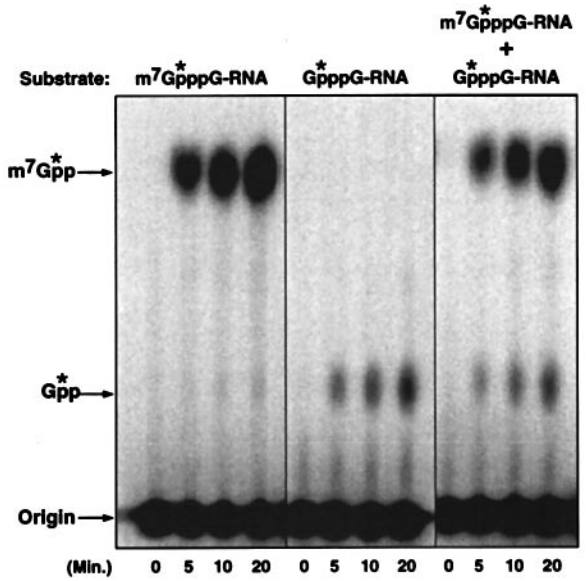


Fig. 3. Dcp1p prefers methylated substrates. Analysis of decapping activity by FLAG-Dcp1p on methylated (left-hand panel), non-methylated (middle panel) and mixed (right-hand panel) substrates. Decapping activity was assayed in 15 μl reactions containing 1.35 pmol of FLAG-Dcp1p and 0.07 pmol of substrate, as described in Materials and methods. Equal amounts (0.035 pmol each) of methylated and non-methylated RNAs were combined for the mixed substrate experiment. Decapping by FLAG-Dcp1p was examined over a 20 min time course, with aliquots of the decapping reactions removed at the indicated time points. The products of the reaction were separated by PEI-cellulose TLC. The * indicates the position of the radiolabeled phosphate. A small amount of GDP is produced from the reactions with the methylated substrate, due to incomplete methylation of the capped mRNAs by guanylyltransferase during substrate preparation.

Uncapped mRNA can inhibit the Dcp1p decapping enzyme

The role of Dcp1p in the turnover of yeast mRNAs is to decap full-length mRNAs. Dcp1p could, in principle, recognize its substrate primarily through interaction with the cap structure, through interaction with the mRNA moiety, or through interaction with both the cap structure and the mRNA portion of the substrate. To obtain addi-

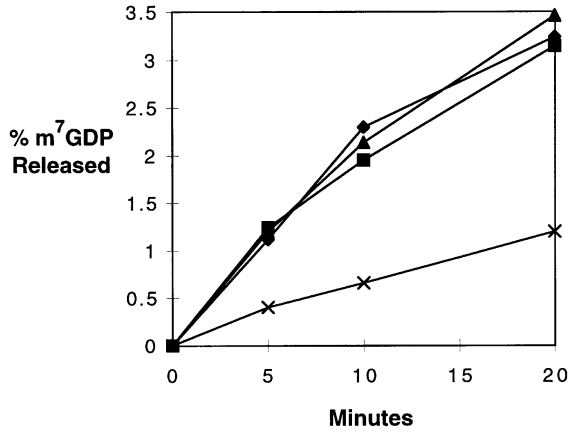


Fig. 4. Cap analog does not effectively inhibit decapping by Dcp1p. Analysis of decapping activity by FLAG-Dcp1p on MFA2 mRNA in the presence of 10-, 1000- or 200 000-fold molar excess of cap analog m^7GpppG_{OH} to capped mRNA. Decapping was performed in 15 μl reactions containing 1.35 pmol FLAG-Dcp1p, 0.06 pmol capped mRNA, and 0, 0.6 pmol, 60 pmol or 12 nmol cap analog, as described in Materials and methods. The reactions were carried out over a 20 min time course; aliquots of the decapping reactions were removed at 5, 10 and 20 min. The activity is expressed as a percentage of m^7GDP that is released from the starting mRNA that contains a radiolabeled cap. ◆, capped mRNA only; ▲, capped mRNA and 10-fold molar excess cap analog; ■, capped mRNA and 1000-fold molar excess cap analog; ×, capped MFA2 mRNA and 200 000-fold molar excess cap analog.

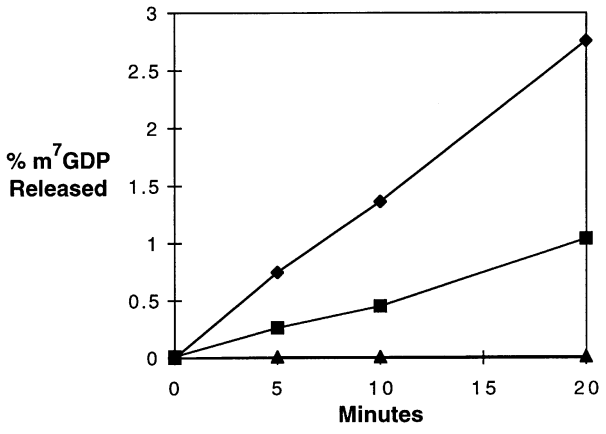


Fig. 5. Uncapped mRNA can inhibit decapping by Dcp1p. Analysis of decapping activity by Dcp1p on capped MFA2 mRNA in the presence of 14- or 104-fold molar excess uncapped MFA2 mRNA. Decapping was examined in 15 μl reactions containing 1.35 pmol FLAG-Dcp1p, 0.26 pmol capped mRNA, and 0, 3.6 pmol or 27 pmol uncapped mRNA, as described in Materials and methods. The reactions were conducted over a 20 min time course, with aliquots removed from the reactions at 5, 10 and 20 min. The activity is expressed as a percentage of m^7GDP that is released from the starting mRNA that contains a radiolabeled cap. ◆, capped MFA2 mRNA only; ■, capped MFA2 mRNA and 14-fold molar excess uncapped MFA2 mRNA; ▲, capped mRNA and 104-fold molar excess uncapped MFA2 mRNA.

tional information regarding the interactions between Dcp1p and its substrate, we examined how different molecules affect decapping in *trans*.

As shown above, Dcp1p activity was influenced by the cap structure 7-methyl group, demonstrating that the cap structure contributes to the interaction between Dcp1p and its substrate. We next tested the cap structure analog m^7GpppG_{OH} for its ability to inhibit the decapping of full-length, capped mRNAs by FLAG-Dcp1p. This was done by adding the cap analog to decapping reactions in molar excess amounts of 10-, 1000- or 200 000-fold relative to the capped mRNA substrate (Figure 4). Only the 200 000-fold molar excess of the cap analog to the capped mRNA substrate inhibited decapping by FLAG-Dcp1p, and then only to a modest degree. This observation suggests that Dcp1p might bind the cap analog very poorly, if at all. The inability of the cap analog to inhibit decapping is consistent with earlier observations that a partially purified decapping activity from yeast (that may reflect the Dcp1p decapping enzyme based on its biochemical properties) did not cleave m^7GpppG_{OH} or m^7GpppA_{OH} (Stevens, 1988).

The inability of the cap structure analog to effectively inhibit decapping suggests that substrate recognition by Dcp1p requires the cap to be attached to RNA. The RNA portion of the natural Dcp1p substrate might therefore be capable of inhibiting decapping *in vitro*. To address this possibility, decapping was performed on capped substrates to which uncapped mRNA was added in 14- or 104-fold molar excess amounts (Figure 5). A 14-fold molar excess of uncapped mRNA to capped mRNA caused a 5-fold decrease in the amount of m^7GDP that was released from the capped substrate, while a 104-fold molar excess of uncapped mRNA essentially blocks decapping of the substrate. These results suggest that Dcp1p interacts with the body of the mRNA during substrate recognition and have important implications for the control of decapping *in vivo* (see Discussion).

Dcp1p efficiently cleaves substrate RNAs that are ⩾25 nucleotides long

The above results suggested that both the cap structure and the mRNA body contribute to substrate recognition by Dcp1p. Preliminary characterization of a partially purified decapping enzyme from yeast indicated that the partially purified activity decapped longer mRNA substrates more efficiently (Stevens, 1988). Given this observation, and the fact that Dcp1p decaps full-length mRNAs *in vivo*, we examined whether the length of the transcript could influence decapping by the purified FLAG-Dcp1p *in vitro*.

A series of truncated MFA2 mRNAs that were shortened from the 3′ end of the full-length transcript were tested as substrates for decapping by Dcp1p. Note that all of these substrates have the same 5′ sequence. Dcp1p apparently requires a minimal length of RNA for efficient substrate recognition, as the relative decapping activity by Dcp1p decreased significantly when the length of substrate was <25 nucleotides (Figure 6A). As the substrate was reduced from 25 to 20 nucleotides in length, the relative amount of decapping activity (compared with that observed for the full-length MFA2 substrate) decreased 6-fold from 36% to 5.7%, respectively (Figure 6B). The relative decapping activity further decreased to just 1.1% when the length of substrate was reduced from 20 to 15 nucleotides (Figure 6A and B). The decrease in decapping activity that occurred with substrates that were <25 nucleotides long indicated that Dcp1p requires a minimum length of RNA for efficient substrate recognition. Given this substrate preference, *in vivo* decapping rates could be

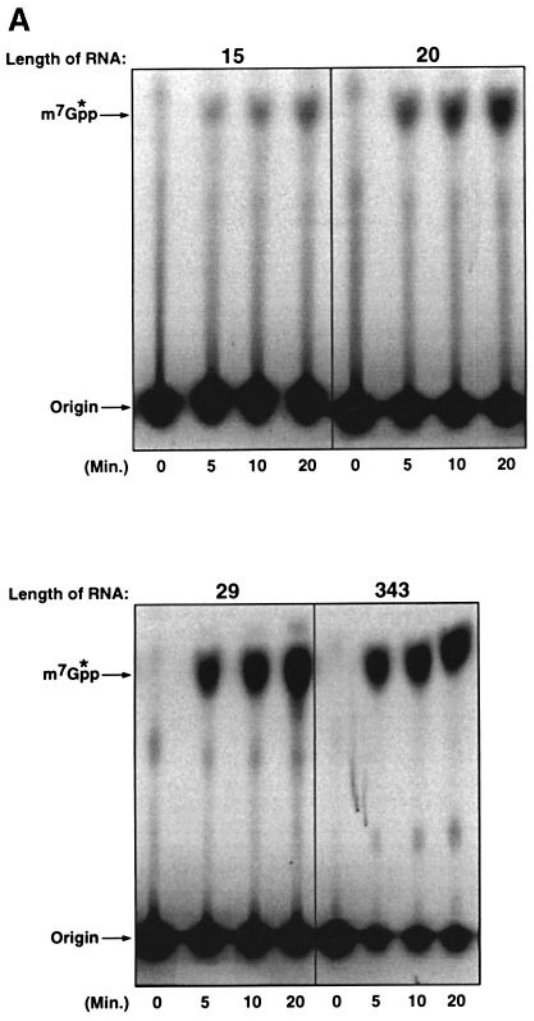
modulated by the amount of mRNA sequence that is accessible to the decapping enzyme (see Discussion).

While Dcp1p efficiently cleaved a substrate that was 25 nucleotides long, the enzyme displayed a minor preference for longer RNAs as substrates. As shown in Figure 6B, the relative decapping activity generally increased with RNA length up to an apparent plateau of ~300 nucleotides. The basis for this increase in activity is not yet apparent. One explanation is that greater RNA length might facilitate binding of Dcp1p to the RNA portion of the substrate. This binding to the longer RNA could in turn effectively increase the local concentration of the cap-RNA substrate for the enzyme, thereby yielding a higher rate of decapping.

The Dcp1p decapping enzyme releases m^{7}GDP from the PGK1 mRNA

In vitro studies of the decapping enzyme have shown that it cleaves the yeast MFA2 and several artificial mRNAs within the cap structure triphosphate linkage to release m^{7}GDP (Stevens, 1988; Beelman *et al.*, 1996). Consistent with this site of cleavage, cells that are deleted for the *Xrn*1p 5′ → 3′ exonuclease accumulate MFA2 transcripts that are deadenylated, decapped and full-length (Muhlrad *et al.*, 1994), suggesting that the site at which the decapping enzyme cleaves *in vivo* is the same as *in vitro*. However, several mRNAs that are known to require Dcp1p for decapping, such as the PGK1 transcript, accumulate in *xrn1*Δ strains as deadenylated, decapped species that lack two or more nucleotides from the 5′ end of the mRNA (Muhlrad *et al.*, 1995). Two general possibilities account for the accumulation of these decapped, shortened mRNAs. One is that they are decapped within the cap structure *in vivo*, and then undergo either exonucleolytic or endonucleolytic cleavage, to yield the slightly shortened species. The second possibility is that the decapping enzyme itself decaps these mRNAs by endonucleolytic cleavage of the RNA phosphodiester backbone, rather than cleaving within the cap structure triphosphate linkage. In this view, Dcp1p might cleave various substrates at different positions.

To help distinguish between these two possibilities, we examined the product released by Dcp1p upon decapping of PGK1 mRNA *in vitro*. *In vivo*, in an *xrn1*Δ strain, the PGK1 mRNA accumulates as a decapped species that lacks its 5′ first two nucleotides (Muhlrad and Parker, 1994; Muhlrad *et al.*, 1995). We therefore compared decapping of the MFA2 and PGK1 mRNAs in reactions containing limiting amounts of substrate. As shown in Figure 7, Dcp1p cleaves a truncated PGK1 mRNA within the cap structure 5′–5′ linkage to release m^{7}GDP. We interpret this result to argue that Dcp1p cleaves mRNAs within the cap structure, usually, if not always, to release m^{7}GDP. This observation implies that the decapped, truncated mRNAs that accumulate in an *xrn1*Δ strain are derived from a combination of Dcp1p decapping and other degradative activity(ies). However, it should be noted that



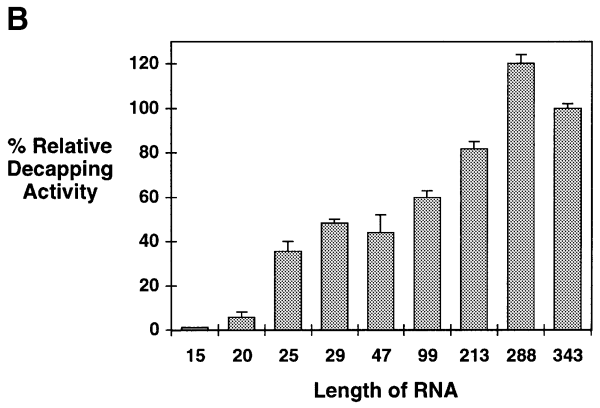


Fig. 6. Dcp1p prefers longer mRNA substrates. (**A**) Decapping activity by Dcp1p was analyzed as a function of substrate length. Representative assays are shown for substrates that were 15, 20 (upper panel), 29 and 343 (lower panel) nucleotides long. Decapping was assayed in 15 μl reactions containing 1.35 pmol FLAG-Dcp1p and 0.04 pmol substrate, as described in Materials and methods. The reactions were carried out over a 20 min time course, with aliquots of the decapping reactions removed at the indicated time points. The products of the reaction were analyzed by PEI-cellulose TLC. (**B**) The relative decapping activity by Dcp1p as a function of substrate length. MFA2 mRNAs that were truncated from the 3′ end were compared with full-length MFA2 mRNA as substrates for decapping *in vitro* by Dcp1p. The RNAs examined were 15, 20, 25, 29, 49, 99, 213, 288 and 343 (full-length) nucleotides long. Decapping by FLAG-Dcp1p was assayed as described above. The relative activity was determined by comparing the rates of decapping observed for the different substrates from three independent experiments, and is expressed as the percentage of activity relative to that observed for the full-length (343 nucleotide) MFA2 mRNA.

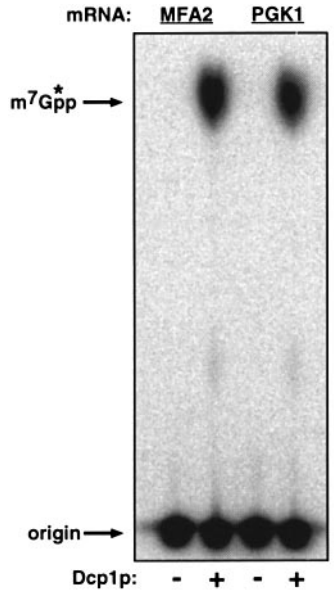


Fig. 7. Decapping of MFA2 and PGK1 mRNAs by Dcp1p releases m^7GDP. The MFA2 and PGK1 mRNA substrates were decapped *in vitro* by Dcp1p and analyzed by PEI-cellulose TLC as described in Materials and methods. The 15 μl decapping reactions contained 1.35 pmol FLAG-Dcp1p and 0.45 pmol substrate. The MFA2 mRNA was full-length (343 nucleotides), while the PGK1 mRNA corresponded to the 5′ 105 nucleotides of the 1376 nucleotide, full-length mRNA. The * indicates the position of the radiolabeled phosphate. The lower, faint spot corresponds to GDP that was probably released from capped mRNA that was not fully methylated during substrate preparation, due to incomplete methylation by guanylyltransferase.

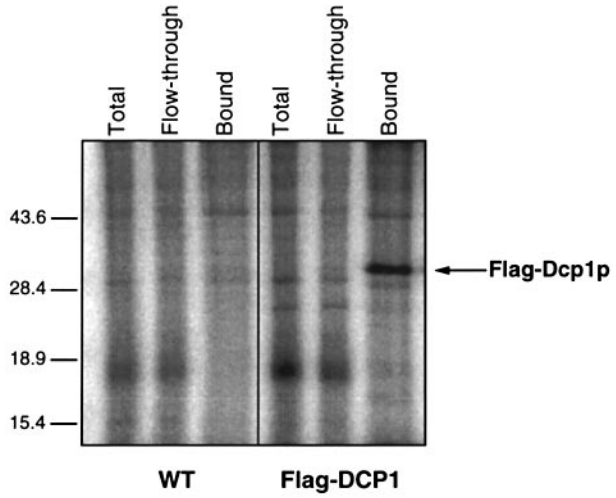


Fig. 8. Dcp1p is phosphorylated. Analysis of phosphorylated FLAG-Dcp1p by SDS–PAGE. As described in Materials and methods, cellular extracts from either wild-type or FLAG-Dcp1p overexpressing yeast were made from isotopically labeled cultures and purified by anti-FLAG M2 immunoaffinity chromatography. Lanes: Total, the fraction that was loaded onto the anti-FLAG column; Flow-through, the fraction that did not bind to the anti-Flag column; Bound, the eluate from the anti-FLAG column. WT (left half of panel), preparation from wild-type strain yRP840; FLAG-Dcp1p (right half of panel), preparation from *dcp1*Δ strain yRP1070 expressing FLAG-Dcp1p from pRP801. Protein size markers are indicated on the left in kDa.

we cannot rule out the formal possibilities that additional proteins *in vivo* could change the site at which the Dcp1p enzyme cleaves, or that Dcp1p might be required to activate a different nuclease.

Dcp1p is a phosphoprotein

Decapping is a regulated process as unstable and stable yeast mRNAs are decapped at different rates by a single decapping enzyme (Beelman *et al.*, 1996). The regulation of decapping may well occur through modulation of Dcp1p, its substrate, or both. Dcp1p has previously been observed to occur as two different migrating forms on SDS–PAGE (Beelman *et al.*, 1996), and the in-gel activity assay presented here suggests that Dcp1p might have alternative active and inactive conformations. These different forms of Dcp1p might represent differences in post-translational modification. Given that protein functions are commonly regulated through phosphorylation, we examined whether Dcp1p might be a phosphoprotein.

To determine if Dcp1p might be phosphorylated, we isotopically labeled a culture of yeast expressing FLAG-Dcp1p with radiolabeled orthophosphate. FLAG-Dcp1p was then isolated from a whole-cell extract with anti-FLAG immunoaffinity chromatography and analyzed by SDS–PAGE. The results of this analysis, shown in Figure 8, demonstrate that Dcp1p was phosphorylated. Similar results were obtained when Dcp1p was immunoprecipitated with Dcp1p-specific antibodies following phospholabeling of yeast (data not shown). The extent and location(s) of phosphorylation on Dcp1p remain to be determined, as does the role of phosphorylation of Dcp1p for decapping activity. However, these results suggest that decapping might be modulated, in part, through modification of Dcp1p.

Discussion

The Dcp1p is the decapping enzyme

Two experiments indicated that Dcp1p is the catalytic moiety of the yeast decapping enzyme. First, purification of FLAG-Dcp1p from yeast to apparent homogeneity gave a preparation that was sufficient for decapping *in vitro*. Second, separation of Dcp1p from other components in the purified preparation on a SDS gel yielded a band of Dcp1p that could be renatured into an active enzyme. However, it should be noted that *in vivo* Dcp1p's activity is likely to be modulated by additional proteins. Given that decapping is a regulated process, such proteins associated with Dcp1p could partially be responsible for control of decapping rates.

Our work demonstrated that Dcp1p is a phosphoprotein (Figure 8). Although we do not know the role of this phosphorylation, two observations suggest that post-translational modifications will be important in Dcp1p activity. First, the band of active enzyme ran slightly faster in the SDS gel than the majority of the Dcp1p polypeptide. This observation was consistent with a modified, faster-migrating form of Dcp1p being the active form of the enzyme. In addition, recombinant Dcp1p produced in *E.coli* did not possess catalytic activity (data not shown). A simple possibility is that the bacterially expressed protein is lacking a post-translational modification that Dcp1p requires for activity. It will be of interest in the future to determine the role of post-translational

modification in the function and control of the decapping enzyme.

Interaction of the mRNA substrate with the decapping enzyme

The characterization of the Dcp1p decapping enzyme *in vitro* suggested that both the mRNA moiety and the cap structure contribute to its interaction with the substrate. Although we have not demonstrated a direct interaction between Dcp1p and the cap structure guanosine in this study, such an interaction can be inferred from the effect of the cap structure 7-methyl group on decapping. As shown above, Dcp1p preferentially cleaves methylated, capped substrates compared with non-methylated, capped substrates, suggesting that Dcp1p specifically binds the cap 7-methyl group. Studies with the cap binding protein eIF-4E, which recognizes and binds the cap during an early step in the initiation of protein synthesis (reviewed in Merrick and Hershey, 1996), provide precedent for a specific influence of the cap 7-methyl group, as eIF-4E binds m^7GpppG ~2.5-fold more strongly than its non-methylated counterpart (McCubbin *et al.*, 1988). Moreover, eIF-4E has recently been shown to make contacts to the cap 7-methyl group through a tryptophan residue (Marcotrigianol *et al.*, 1997).

Comparison of Dcp1p with other proteins that interact with the mRNA 5′ cap suggests possible means by which Dcp1p could bind the cap. Co-crystallization studies of eIF-4E and m^7GDP reveal that the recognition of 7-methylguanine is mediated by two conserved tryptophans in eIF-4E that sandwich the base (Marcotrigianol *et al.*, 1997). The binding of the vaccinia virus VP-39 protein, which is a 2′-*O*-methyltransferase (Schnierle *et al.*, 1992), to the cap 7-methylguanine has similarly been observed to occur through sandwiching of the guanine by the aromatic side chains of a tyrosine and phenylalanine (Hodel *et al.*, 1997). Comparison of Dcp1p with several putative homologs from other eukaryotes (A.v.Hoof, S.Mian and R.Parker, unpublished data) suggests that Dcp1p contains two conserved tryptophans and a conserved tyrosine in Dcp1p that could serve to bind the cap in a similar fashion.

Several lines of evidence suggest that recognition of capped substrates by the decapping enzyme occurs, in part, through interaction with the mRNA portion of the substrate. As shown above, Dcp1p was inhibited *in vitro* by uncapped mRNAs, implying that Dcp1p can bind uncapped mRNA at some level. In contrast, the cap analog m^7GpppG_{OH} did not inhibit Dcp1p, suggesting that Dcp1p does not efficiently bind an isolated cap structure. This notion is supported by the observation that a partially purified yeast decapping enzyme did not cleave m^7GpppG_{OH} or m^7GpppA_{OH} (Stevens, 1988). Finally, Dcp1p did not efficiently decap mRNAs that were <25 nucleotides in length. Thus, the interaction of Dcp1p with its substrate might be analogous to that observed for the viral VP-39 RNA methyltransferase. Structural analysis of VP-39 demonstrates that it has a cap binding site and an RNA binding domain, since mutations in VP-39 have been isolated that affect binding of either the cap or RNA (Schnierle *et al.*, 1994; Shi *et al.*, 1996). Dcp1p might interact with its substrate in a similar manner, with domains that are specific for interaction with either the cap or RNA.

An intriguing question is why Dcp1p requires substrates to be longer than 20 nucleotides for efficient decapping. The simplest possibility is that this length approaches the size of the RNA binding site on the Dcp1p enzyme and that shorter RNA lengths would not be bound efficiently. An alternative possibility is that the RNA binding site is distinct and physically separated from the active site into which the cap structure must bind. In this view, a minimal RNA length would be needed to allow the cap structure to reach the active site when the RNA portion is bound. Resolution of the physical interaction of Dcp1p and its substrate will require further structural studies.

The apparent interaction of Dcp1p with the RNA portion of its substrate has important implications. First, it suggests that sequence specific differences at the 5′ end may modulate the rate of decapping by directly affecting the manner in which Dcp1p interacts with the substrate. In this light, it is striking to note that in at least three cases, specific sequences which promote rapid mRNA decay in yeast have been mapped to the 5′ UTR (Pierrat *et al.*, 1993; Cereghino and Scheffler, 1995; Gonzalez and Martin, 1996). Also, the means by which Dcp1p recognizes its substrate *in vitro* implies that Dcp1p might require access to the cap and a minimal length of RNA before it can decap mRNAs *in vivo*. This access could be influenced by a number of proteins implicated in controlling decapping. For example, inhibition of decapping by the poly(A) tail is mediated by the poly(A)-binding protein (Caponigro and Parker, 1995), while deadenylation-independent decapping in nonsense-decay requires the products of the *UPF1*, *UPF2* and *UPF3* genes (Leeds *et al.*, 1991, 1992; Cui *et al.*, 1995; He and Jacobson, 1995). Additionally, the *MRT1* and *MRT3* gene products appear to promote decapping following deadenylation (Hatfield *et al.*, 1996). While these proteins may directly interact with Dcp1p to alter its activity toward different mRNAs, they alternatively might function to alter the state of the mRNP complex. The latter is attractive, since it would presumably affect the access of the decapping enzyme to the cap structure and the 5′ end of the mRNA.

The Dcp1p preference for longer substrates may have biological significance, since the 5′ cap structure is often complexed with a set of proteins involved in translation initiation, referred to as the cap-binding complex or eIF-4F. A requirement for both a significant amount of RNA and the cap structure for substrate recognition by Dcp1p could prevent decapping of mRNAs on which translation initiation complexes are assembled. The cap binding complex might therefore effectively compete with Dcp1p for the 5′ cap structure. Alternatively, the cap binding complex might recruit Dcp1p to the cap structure, to promote decapping following release of the cap binding complex and associated translation initiation factors. This alternative is appealing, since release of the cap binding complex might coincide with loss of translational competence of the mRNA, whereupon it would be advantageous for the cell to readily decap and subsequently degrade the mRNA.

Materials and methods

Construction of the FLAG-Dcp1p overexpression vector

The *FLAG-DCP1* gene was created by PCR amplification of the *DCP1* gene from pRP718 (Beelman *et al.*, 1996) with the oligonucleotide

GCAGCACCGGATCCATGGACTACAAGGACGACGATGACAAGA-TGACCGGAGCAGCAAC (oRP311), which places the *FLAG* gene 5′ of the *DCP1* gene, and the oligonucleotide GCAGCACCGTCGACTTC-TCACTTGGGCATCTC (oRP312). This fragment was digested with *Bam*HI and *Sal*I and ligated into the yeast expression vector pG-1 (Schena *et al.*, 1991) between the *Bam*HI and *Sal*I sites, to yield pRP801. This 2μ, TRP1 plasmid expresses FLAG-Dcp1p from the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter.

Purification of Dcp1p

An 800 ml culture of the *dcp1*Δ strain yRP1070 (Beelman *et al.*, 1996) harboring pRP801 was grown in selective media containing 2% dextrose to late log phase and harvested by centrifugation. Cells were washed in buffer L (10 mM Tris–HCl, pH 7.6, 100 mM KCl, 2 mM magnesium acetate, 1 mM DTT) and then suspended in an equal volume of buffer L containing Complete (Boehringer-Mannheim) protease inhibitors. Acid-washed beads equal in volume to the cell suspension were added, and cells were lysed by six cycles of vortexing for 15 s and 45 s of cooling in iced water. The lysate was centrifuged at 18 000 *g* for 15 min at 4°C, and the supernatant (~3.5 ml) was chromatographed at 4°C by gravity flow on a 1 ml Heparin Hi-Trap (Pharmacia) column that was equilibrated with buffer L. The unbound fraction was then chromatographed at 4°C by gravity flow on a 1 ml Anti-FLAG M2 monoclonal antibody immunoaffinity column (Kodak) equilibrated with buffer L. The flow-through was collected and re-applied to the column. The column was washed with 10 ml buffer L, 10 ml buffer LH (buffer L containing 700 mM KCl), and then 10 ml buffer L. FLAG-Dcp1p was eluted with 200 μg/ml FLAG peptide in buffer L in a total of ~2.5 ml. The FLAG peptide was separated from the FLAG-Dcp1p by dialysis into buffer containing 50 mM Tris, pH 7.6 and 1 mM DTT. The enzyme was aliquoted and stored at –80°C.

The FLAG-Dcp1p preparation was analyzed by standard SDS–PAGE methods on a 12% gel (Laemmli, 1970). Protein size markers were purchased from Gibco-BRL. The gel was silver-stained using Silver Stain Plus reagents (Biorad).

Substrate preparation

Uncapped mRNAs lacking poly(A) tails were synthesized *in vitro* by T7 RNA polymerase run-off transcriptions. Full-length MFA2 mRNA was transcribed from plasmid RP802 which was linearized with *Eco*RI, to produce a 343 nucleotide mRNA consisting of the natural MFA2 325 nucleotides plus an additional 3′ 18 nucleotides encoded by the plasmid polylinker. The *MFA2* gene in this plasmid was created by PCR amplification of pRP264 (Muhlrad and Parker, 1992) with the oligonucleotide TAATACGACTCACTATAGCGAGCTATCATCTTCATAC-AACAATAAC (oRP202), which places the T7 promoter 5′ of the *MFA2* gene, and the oligonucleotide CATGAAAAAATCTGTTAAAGTG (oRP250). This fragment was then cloned by blunt-ended ligation into *Sma*I linearized pUC18 (Yanisch-Perron *et al.*, 1985) to yield pRP802. Truncated RNAs consisting of the *MFA2* 5′ 49, 99, 213 and 288 nucleotides were synthesized from pRP802 that was linearized with *Sau*3AI, *Mnl*I, *Afl*III or *Nde*I, respectively. The transcription templates for the truncated RNAs consisting of the *MFA2* 5′ 15, 20, 25 or 29 nucleotides were created by respectively annealing the oligonucleotides AAGATGATAGCTCGCTATAGTGAGTCGTATTA (oRP463), GTATG-AAGATGATAGCTCGCTATAGTGAGTCGTATTA (oRP464), TTGT-TGTATGAAGATGATAGCTCGCTATAGTGAGTCGTATTA (oRP465) or GTTATTGTTGTATGAAGATGATAGCTCGCTATAGTGAGTCGT-ATTA (oRP203) to the oligonucleotide TAATACGACTCACTATAG (oRP207), which contained the T7 promoter sequence, following the procedure described by Milligan and Uhlenbeck (1989). The truncated mRNA that corresponded to the 5′ 105 nucleotides of the PGK1 transcript was synthesized from *Hinc*II linearized pRP803. The *PGK1* gene in this plasmid was created by PCR amplification of pRP129 (Heaton *et al.*, 1992) with the oligonucleotide GCAGCACCGG-ATCCTAATACGACTCACTATAAAGTAATTATCTACTTTTTACAAC (oRP444), which places the T7 promoter 5′ of the *PGK1* gene, and the oligonucleotide GCAGCACCAAGCTTGGCATTAAAAAGAGGGACG (oRP445). This fragment was digested with *Bam*HI and *Hin*dIII and cloned into pUC18 to yield pRP803.

T7 transcriptions were done in 100 μl reactions containing 1–2 μg of template DNA, 5 mM NTPs, 40 mM Tris–HCl, pH 8.0, 1 mM spermidine, 5 mM DTT, 50 μg/ml BSA, 0.01% Triton X-100, 20 mM $MgCl_2$, 5 U yeast inorganic pyrophosphatase (Sigma) and 40 U T7 RNA polymerase (Boehringer-Mannheim) at 37°C for ~15 h (Harris *et al.*, 1994). The uncapped transcripts were purified by polyacrylamide electrophoresis (LaGrandeur *et al.*, 1994).

The RNAs were capped in reactions typically containing 23 pmol RNA, 45 pmol [α-^{32}P]GTP, 40 U RNasin (Promega), 0.67 mM *S*-adenosylmethionine, 50 mM Tris–HCl, pH 7.6, 2 mM $MgCl_2$, 6 mM KCl, 1 mM DTT and 25 U guanylyltransferase (Gibco-BRL) at 37°C for 2 h (Beelman *et al.*, 1996). These conditions typically capped only 15–20% of the RNA in the reaction, due to the qualitative nature of capping by guanylyltransferase (Shuman and Moss, 1990). Transcripts containing non-methylated caps were synthesized in similar reactions without a methylating reagent and in the presence of 1 mM *S*-adenosyl-homocysteine, to inhibit methylation by guanylyltransferase (Shuman and Moss, 1990). These conditions typically capped only 4–6% of the RNA in the reaction. For the assays comparing methylated and non-methylated substrates, the relative amounts of capped to uncapped RNA were adjusted to be the same to compensate for the differences in capping efficiencies. Cap-labeled RNAs were separated from unincorporated label by Sepharose-CL6B (Pharmacia) spin chromatography, with the exception of the 15, 20, 25 and 29 nucleotide RNAs, which were purified by polyacrylamide electrophoresis (LaGrandeur *et al.*, 1994).

The cap analog m^7GpppG_{OH} used for the decapping inhibition studies was purchased from Gibco-BRL.

Decapping assays

Decapping assays used in this study were similar to those previously described (Beelman *et al.*, 1996). Decapping was assayed at 30°C for 20 min or over a time course, as noted in the text or figure legends. All time course assays were averaged from at least two independent experiments. The reactions generally contained 40 ng Dcp1p (1.35 pmol), 0.01–0.2 pmol of $m^7G[^{32}P]$pppMFA2 mRNA, 50 mM HEPES, pH 7.0, 1 mM $MgCl_2$, 1 U RNasin (Promega) and 1 mM DTT in a volume of 15 μl. Time courses were carried out by withdrawing an aliquot of the reaction at the time points indicated for each experiment. Decapping in the aliquot was stopped by adding 1 μl of 0.25 M EDTA to the aliquot and placing it on ice. The products of the reaction were separated by PEI-cellulose thin layer chromatography developed in 0.45 M $(NH_4)_2SO_4$ and detected with a Molecular Dynamics PhosphorImager.

Optimization of Dcp1p reaction conditions

The effect of monovalent salt on decapping was assayed by adding 10, 25, 50, 75, 100 or 200 mM of either NaCl, KCl, NH_4Cl or NH_4OAC to the decapping assay. The optimal $MgCl_2$ concentration for decapping by Dcp1p was determined by varying the amount of $MgCl_2$ (1, 2.5, 5, 10 or 25 mM) in the decapping assay. Alternative divalent cations were tested by substituting 1 mM $MgCl_2$ with either 1 mM $MnCl_2$ or 1 mM $CaCl_2$ in the decapping assay. The pH optimum for decapping was determined by varying the pH in the decapping assay with a series of different buffers. These included: 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 5.5, 6.0 or 6.5; piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) at pH 6.5, 7.0 or 7.5; and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7, 7.5 or 8.0. All buffers were titrated to pH with KOH.

The K_m was determined under standard conditions by assaying the activity of ~40 ng Dcp1p (~1.35 pmol) in a series of 15 μl reactions (as described above) containing the substrate $m^7G[^{32}P]$pppMFA2 RNA in amounts ranging from 0.67 to 28.75 pmol. Reactions were sampled at 0, 2.5, 5, 7.5 and 10 min, and stopped and analyzed as described above. The K_m was determined from a Lineweaver–Burk plot.

SDS–PAGE activity assay

His-Dcp1p was prepared from *S.cerevisiae* as previously described (Beelman *et al.*, 1996). His-Dcp1p was expressed in *E.coli* from plasmid RP785, a derivative of pProEX-1 (Gibco-BRL), and purified by chromatography on a nickel-NTA column (Qiagen/Gibco-BRL) according to the supplier's protocol. The His tag was subsequently removed by cleavage with TEV protease (Gibco-BRL). Proteins were resolved by SDS–PAGE on a 12% gel containing ~0.8 nm $m^7G[^{32}P]$pppMFA2 mRNA. After electrophoresis, proteins were renatured in the gel essentially according to the procedure of Blank *et al.* (1982) [as modified by Dr Rune-Paer Nilsson in the laboratory of Prof. Alexander von Gabain, Vienna Biocenter, Institute of Microbiology and Genetics, A-1030 Vienna, Austria (unpublished)]. The gel was sequentially incubated at 4°C, with gentle shaking, in the following for the indicated times: 25% isopropanol, overnight; 25% isopropanol, 30 min; 25 mM HEPES–KOH, pH 7.7, 25 mM NaCl, 5 mM $MgCl_2$ (HBB buffer), twice, 10 min each; 6 M guanidine-HCl in HBB (GuHCl/HBB), twice, 20 min each; 3 M GuHCl/HBB, 15 min; 1.5 M GuHCl/HBB, 15 min; 0.75 M GuHCl/HBB, 15 min; 0.375 M GuHCl/HBB, 15 min; 0.187 M GuHCl/HBB, 15 min; and HBB, 15 min. The gel was then incubated in decapping

reaction buffer (50 mM HEPES, pH 7.0, 1 mM $MgCl_2$, 1 mM DTT) for 3–4 h at 30°C. The radiolabeled gel was then visualized with a Molecular Dynamics PhosphorImager.

The SDS–PAGE activity assay was also performed as above with a gel that contained a similar amount of uncapped, internally labeled MFA2 mRNA. This RNA was synthesized as a radiolabeled transcript using T7 RNA polymerase in a reaction (similar to that described above) containing 1–2 μg of template DNA, 100 μCi of [α-^{32}P]UTP, 1 mM UTP, 5 mM (each) ATP, CTP and GTP, 40 mM Tris–HCl, pH 8.0, 1 mM spermidine, 5 mM DTT, 50 μg/ml BSA, 0.01% Triton X-100, 20 mM $MgCl_2$ and 40 U T7 RNA polymerase at 37°C for ~1.5 h. The internally labeled RNAs were separated from unincorporated label by Sepharose-CL6B (Pharmacia) spin chromatography.

Protein phosphorylation analysis

To obtain radiolabeled FLAG-Dcp1p, a 200 ml culture of the *dcp1*Δ strain yRP1070 harboring pRP801 was grown in inorganic phosphate-depleted, selective media containing 2% dextrose to late log phase at 30°C (Rubin, 1973). As a control, the parent strain yRP840 (Hatfield *et al.*, 1996) was similarly grown in complete media. To label cellular proteins, carrier-free ^{32}P-orthophosphate was then added to 140 μCi/ml, and the cultures were further grown at 32°C for 2 h. The cells were then harvested and lysed as described above. The radiolabeled FLAG-Dcp1p was then purified from the whole-cell lysate by anti-FLAG immunoaffinity, as described above. After elution from the anti-FLAG column, radiolabeled proteins were analyzed by SDS–PAGE on a 12% gel. Following electrophoresis, the gel was dried and visualized with a Molecular Dynamics PhosphorImager.

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References

Adams,B.L., Morgan,M., Muthukrishnan,S., Hecht,S.M. and Shatkin,A.J. (1978) The effect of cap analogs on reovirus mRNA binding to wheat germ ribosomes. *J. Biol. Chem.*, **253**, 2589–2595.

Bashkirov,V.I., Scherthan,H., Solinger,J.A., Buerstedde,J.-M. and Heyer,W.-D. (1997) A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. *J. Cell Biol.*, **136**, 761–773.

Beelman,C.A. and Parker,R. (1995) Degradation of mRNA in eukaryotes. *Cell*, **81**, 179–183.

Beelman,C.A., Stevens,A., Caponigro,G., LaGrandeur,T.E., Hatfield,L., Fortner,D.M. and Parker,R. (1996) An essential component of the decapping enzyme required for normal rates of mRNA turnover. *Nature*, **382**, 642–646.

Blank,A., Sugiyama,R.H. and Dekker,C.A. (1982) Activity staining of nucleolytic enzymes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis: use of aqueous isopropanol to remove detergent from gels. *Anal. Biochem.*, **120**, 267–275.

Caponigro,G. and Parker,R. (1995) Multiple functions for the poly(A)-binding protein in mRNA decapping and deadenylation in yeast. *Genes Dev.*, **9**, 2421–2432.

Cereghino,G.P. and Scheffler,I.E. (1995) Glucose-dependent turnover of the mRNAs encoding succinate dehydrogenase peptides in *Saccharomyces cerevisiae*: sequence elements in the 5′ untranslated region of the Ip mRNA play a dominant role. *Mol. Biol. Cell*, **6**, 1125–1143.

Couttet,P., Fromont-Racine,M., Steel,D., Pictet,R. and Grange,T. (1997) Messenger RNA deadenylation precedes decapping in mammalian cells. *Proc. Natl Acad. Sci. USA*, **94**, 5628–5633.

Cui,Y., Hagan,K.W., Zhang,S. and Peltz,S.W. (1995) Identification and characterization of genes that are required for the accelerated degradation of mRNAs containing a premature translation termination codon. *Genes Dev.*, **9**, 423–436.

Decker,C.J. and Parker,R. (1993) A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. *Genes Dev.*, **7**, 1632–1643.

Gonzalez,C.I. and Martin,C.E. (1996) Fatty acid-responsive control of mRNA stability. *J. Biol. Chem.*, **271**, 25801–25809.

Hagan,K.W., Ruiz-Echevarria,M.J., Quan,Y. and Peltz,S.W. (1995) Characterization of *cis*-acting sequences and decay intermediates involved in nonsense-mediated mRNA turnover. *Mol. Cell. Biol.*, **15**, 809–823.

Harris,M.E., Nolan,J.M., Malhotra,A., Brown,J.W., Harvey,S.C. and Pace,N.R. (1994) Use of photoaffinity crosslinking and molecular modeling to analyze the global architecture of ribonuclease P RNA. *EMBO J.*, **13**, 3953–3963.

Hatfield,L., Beelman,C.A., Stevens,A. and Parker,R. (1996) Mutations in *trans*-acting factors affecting mRNA decapping in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **16**, 5830–5838.

He,F. and Jacobson,A. (1995) Identification of a novel component of the nonsense-mediated mRNA decay pathway by use of an interacting protein screen. *Genes Dev.*, **9**, 437–454.

Heaton,B., Decker,C., Muhlrad,D., Donahue,J., Jacobson,A. and Parker,R. (1992) Analysis of chimeric mRNAs derived from the STE3 mRNA identifies multiple regions within yeast mRNAs that modulate mRNA decay. *Nucleic Acids Res.*, **20**, 5365–5373.

Heyer,W.D., Johnson,A.W., Reinhart,U. and Kolodner,R.D. (1995) Regulation and intracellular localization of *Saccharomyces cerevisiae* strand exchange protein 1 (Sep1/Xrn1/Kem1), a multifunctional exonuclease. *Mol. Biol. Cell*, **15**, 2728–2736.

Higgs,D.C. and Colbert,J.T. (1994) Oat phytochrome A mRNA degradation appears to occur via two distinct pathways. *Plant Cell*, **6**, 1007–1019.

Hodel,A., Shi,X. and Gershon,P.D. (1997) Specific protein recognition of an mRNA cap through its alkylated base. *Nature Struct. Biol.*, **4**, 350–354.

Konarska,M.M., Padgett,R.A. and Sharp,P.A. (1984) Recognition of cap structure in splicing *in vitro* of mRNA precursors. *Cell*, **38**, 731–736.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

LaGrandeur,T.E., Hüttenhofer,A., Noller,H.F. and Pace,N.R. (1994) Phylogenetic comparative chemical footprint analysis of the interaction between ribonuclease P RNA and tRNA. *EMBO J.*, **13**, 3945–3952.

Leeds,P., Peltz,S.W., Jacobson,A. and Culbertson,M.R. (1991) The product of the yeast UPF1 gene is required for rapid turnover of mRNAs containing a premature translational termination codon. *Genes Dev.*, **5**, 2303–2314.

Leeds,P., Wood,J.M., Lee,B.-S. and Culbertson,M.R. (1992) Gene products that promote mRNA turnover in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **12**, 2165–2177.

Lim,S.K. and Maquat,L.E. (1992) Human β-globin mRNAs that harbor a nonsense codon are degraded in murine erythroid tissues to intermediates lacking regions of exon I or exons I and II that have a cap-like structure at the 5′ termini. *EMBO J.*, **11**, 3271–3278.

Marcotrigianol,J., Gingras,A.-C., Sonenberg,N., and Burley,S.K. (1997) Cocrystal structure of the messenger RNA 5′ cap-binding protein (eIF-4E) bound to 7-methyl GDP. *Cell*, **89**, 951–961.

McCubbin,W.D., Edery,I., Altmann,M., Sonenberg,N. and Kay,C.M. (1988) Circular dichroism and fluorescence studies on protein synthesis initiation factor eIF-4E and two mutant forms from the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **263**, 17663–17671.

Merrick,W.C. and Hershey,J.W.B. (1996) The pathway and mechanism of eukaryotic protein synthesis. In Hershey,J.W.B., Mathews,M.B. and Sonenberg,N. (eds), *Translational Control*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Milligan,J.F. and Uhlenbeck,O.C. (1989) Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol.*, **180**, 51–62.

Muhlrad,D. and Parker,R. (1992) Mutations affecting stability and deadenylation of the yeast MFA2 transcript. *Genes Dev.*, **6**, 2100–2111.

Muhlrad,D. and Parker,R. (1994) Premature translational termination triggers mRNA decapping. *Nature*, **370**, 578–581.

Muhlrad,D., Decker,C.J. and Parker,R. (1994) Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5′ to 3′ digestion of the transcript. *Genes Dev.*, **8**, 855–866.

Muhlrad,D., Decker,C.J. and Parker,R. (1995) Turnover mechanisms of the stable yeast PGK1 mRNA. *Mol. Cell. Biol.*, **15**, 2145–2156.

Pierrat,B., Lacroute,F. and Losson,R. (1993) The 5′ untranslated region of the PPR1 regulatory gene dictates rapid mRNA decay in yeast. *Gene*, **131**, 43–51.

Rubin,G.M. (1973) The nucleotide sequence of *Saccharomyces cerevisiae* 5.8 S ribosomal ribonucleic acid. *J. Biol. Chem.*, **248**, 3860–3875.

Schena,M., Picard,D. and Yamamoto,K.R. (1991) Vectors for constitutive and inducible gene expression in yeast. *Methods Enzymol.*, **194**, 389–398.

Schnierle,B.S., Gershon,P.D. and Moss,B. (1992) Cap-specific mRNA (nucleoside-O2′)-methyltransferase and poly(A) polymerase stimulatory activities of vaccinia virus are mediated by a single protein. *Proc. Natl Acad. Sci. USA*, **89**, 2897–2901.

Schnierle,B.S., Gershon,P.D. and Moss,B. (1994) Mutational analysis of a multifunctional protein, with mRNA 5′ cap-specific (nucleoside-O_2)-methyltransferase and 3′- adenylyltransferase stimulatory activities encoded by vaccinia virus. *J. Biol. Chem.*, **269**, 20700–20708.

Shi,X., Yao,P., Jose,T. and Gershon,P.D. (1996) Methyltransferase-specific domains within VP-39, a bifunctional protein which participates in the modification of both mRNA ends. *RNA*, **2**, 88–101.

Shuman,S. and Moss,B. (1990) Purification and use of vaccinia virus messenger RNA capping enzyme. *Methods Enzymol.*, **181**, 170–180.

Stevens,A. (1988) mRNA-decapping enzyme from *Saccharomyces cerevisiae*: purification and unique specificity for long RNA chains. *Mol. Cell. Biol.*, **8**, 2005–2010.

Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) Improved m13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103–119.

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