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The Transcription Factor Associated Ccr4 and Caf1 Proteins Are Components of the Major Cytoplasmic mRNA Deadenylase in *Saccharomyces cerevisiae*

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Summary

The major pathways of mRNA turnover in eukaryotes initiate with shortening of the poly(A) tail. We demonstrate by several criteria that CCR4 and CAF1 encode critical components of the major cytoplasmic deadenylase in yeast. First, both Ccr4p and Caf1p are required for normal mRNA deadenylation in vivo. Second, both proteins localize to the cytoplasm. Third, purification of Caf1p copurifies with a Ccr4p-dependent poly(A)-specific exonuclease activity. We also provide evidence that the Pan2p/Pan3p nuclease complex encodes the predominant alternative deadenylase. These results, and previous work on Pan2p/Pan3p, define the mRNA deadenylases in yeast. The strong conservation of Ccr4p, Caf1p, Pan2p, and Pan3p indicates that they will function as deadenylases in other eukaryotes. Interestingly, because Ccr4p and Caf1p interact with transcription factors, these results suggest an unexpected link between mRNA synthesis and turnover.

Introduction

The process and regulation of mRNA turnover is a fundamental aspect of gene regulation. Two general pathways by which eukaryotic polyadenylated mRNAs can be degraded are now known (reviewed in Beelman and Parker, 1995). In each case, degradation of the transcripts is initiated by shortening of the poly(A) tail at the 3′ end of the transcript. This process, referred to as deadenylation, leads to a deadenylated transcript that is primarily a substrate for mRNA decapping, which exposes the mRNA to rapid 5′ to 3′ exonucleolytic digestion (Muhlrad and Parker, 1992; Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1994, 1995). Alternatively, transcripts can be degraded in a 3′ to 5′ direction following deadenylation (Muhlrad et al., 1995; Anderson and Parker, 1998).

Deadenylation is a critical step in the modulation of mRNA function and stability for several reasons. First, for both of the general decay pathways in yeast, deadenylation is required before degradation of the mRNA body (Muhlrad and Parker, 1992; Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1994, 1995). Second, deadenylation has been shown to be the initial step for mRNA turnover in a variety of eukaryotes for a number of transcripts (e.g., Wilson and Treisman, 1988; Shyu et al., 1991; Couttet et al., 1997). Third, individual mRNAs can have very different rates of deadenylation, and these variations can lead to overall differences in mRNA decay rates (e.g., Decker and Parker, 1993; Wreden et al., 1997; Grosset et al., 2000). Moreover, many known sequence elements that promote mRNA turnover do so, at least in part, by promoting deadenylation (e.g., Wilson and Treisman, 1988; Shyu et al., 1991; Muhlrad and Parker, 1992; Caponigro and Parker, 1996). Finally, because the presence of a 3′ poly(A) tail can enhance translation initiation, deadenylation of a transcript can lead also to a decreased rate of translation (reviewed in Sachs et al., 1997). The regulation of mRNA translation by both deadenylation and re-adenylation is a common strategy in early development wherein gene expression requires modulation by a variety of posttranscriptional mechanisms (for review, see Gray and Wickens, 1998).

An important step in understanding the mechanisms and control of deadenylation will be the identification and analysis of the mRNA deadenylases. Two possible deadenylases have been characterized previously. A poly(A)-specific exoribonuclease, referred to as PARN, was identified from calf thymus (Korner and Wahle, 1997) and subsequently shown to be a member of the RNaseD family of exonucleases (Korner et al., 1998). Immunodepletion or antibody inactivation experiments demonstrated that PARN is required for deadenylation during oocyte maturation in *Xenopus* (Korner et al., 1998; Dehlin et al., 2000), and for deadenylation in HeLa cell extracts (Martinez et al., 2000). Although there are PARN homologs in other mammals, the genomes of *Saccharomyces cerevisae* and *Drosophila melanogaster* do not appear to contain a similar enzyme. In yeast, a poly(A) nuclease, referred to as PAN, has been identified biochemically and shown to be an enzyme consisting of the Pan2 and Pan3 proteins (Lowell et al., 1992; Boeck et al., 1996; Brown et al., 1996). The Pan2p subunit is likely the catalytic subunit since this protein is a member of the RNaseD family of 3′ to 5′ exonucleases (Moser et al., 1997). However, *pan2*Δ and *pan3*Δ yeast strains show minimal effects on deadenylation in vivo (Boeck et al., 1996; Brown et al., 1996) and it has been suggested that the Pan2p/Pan3p exonuclease may play a role in initial trimming of the nuclear poly(A) tail (Brown and Sachs, 1998). These reports suggest that there are likely to be additional mRNA deadenylases.

Three observations led us to consider that the Ccr4 and Caf1 proteins might encode an mRNA deadenylase in yeast. First, both Ccr4p and its associated factor, Caf1p, have nuclease domains. Ccr4p belongs to a magnesium-dependent endonuclease-related family of nucleases (Dlakic, 2000; C. D., unpublished observations). Caf1p homologs in mammalian cells and *C. elegans* are members of the RNaseD family of 3′ to 5′ exonucleases, and

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the yeast Caf1p is related, albeit missing some residues thought to be critical for exonuclease function (Moser et al., 1997). Second, *ccr4*Δ and *caf1*Δ strains show increases and decreases in the steady state levels of mRNAs suggesting that these proteins somehow affect mRNA metabolism (e.g., Denis and Malvar, 1990; Sakai et al., 1992), although some of these effects may be at the level of transcriptional initiation (Denis and Malvar, 1990; Liu et al., 1998; Chang et al., 1999; Y. Cui and C. D., unpublished observations). Third, both Ccr4p and Caf1p show interactions with proteins functioning in mRNA degradation. For example, Caf1p coimmunoprecipitates with Dhh1p (Hata et al., 1998), which is a component of an mRNA decapping complex (J. Coller, M. T., and R. P., unpublished observations). Moreover, overexpression of Dhh1p suppresses some of the growth phenotypes of the *ccr4*Δ and *caf1*Δ strains (Hata et al., 1998). Similarly, overexpression of MPT5/PUF5, a member of the Puf family of proteins that regulate mRNA deadenylation (Olivas and Parker, 2000), can partially suppress growth phenotypes associated with *caf1*Δ mutants (Hata et al., 1998). Together, these observations suggested that the Ccr4p/Caf1p complex might be involved in mRNA turnover.

In this study, we examined the role of Ccr4p and Caf1p in mRNA turnover. We determined that both *CCR4* and *CAF1* gene products are required for normal rates of deadenylation in vivo. We demonstrate that both Ccr4p and Caf1p localize primarily to the cytoplasm where mRNA turnover occurs. Finally, we show that the Caf1p copurifies with deadenylase activity in vitro and does so in a Ccr4p-dependent manner. These observations indicate that the Ccr4p/Caf1p complex encodes the primary cytoplasmic deadenylase in yeast. These data also imply that the Ccr4p and Caf1p homologs that exist in other organisms will serve similar roles as cytoplasmic deadenylases.

Results

Ccr4p and Caf1p Are Required for Normal Rates of mRNA Turnover

To examine if Ccr4p and Caf1p had a role in mRNA turnover, we first determined if they affected the half-lives of yeast mRNAs. We examined the decay of the MFA2pG and PGK1pG reporter transcripts (Decker and Parker, 1993). These transcripts are under the control of the GAL1 UAS, thereby allowing the measurement of decay rates following the addition of glucose to repress transcription. In addition, these mRNAs contain a poly(G) tract in their 3′ UTR that inhibits 5′ to 3′ exonuclease digestion following decapping, allowing the detection of a decay intermediate that extends from the 5′ side of the poly(G) tract to the 3′ end of the mRNA.

We observed that the MFA2pG mRNA was stabilized approximately 2- to 3-fold in *ccr4*Δ and *caf1*Δ strains compared to wild type (Figure 1). In addition, the levels of the decay intermediate were reduced consistent with a block at or before the 5′ to 3′ decay of the transcript (Figure 1). We also observed that the PGK1pG, GAL1, GAL7, and GAL10 mRNAs were more stable in *ccr4*Δ and *caf1*Δ strains (Table 1) indicating that Ccr4p and Caf1p are required for the turnover of a number of yeast mRNAs.

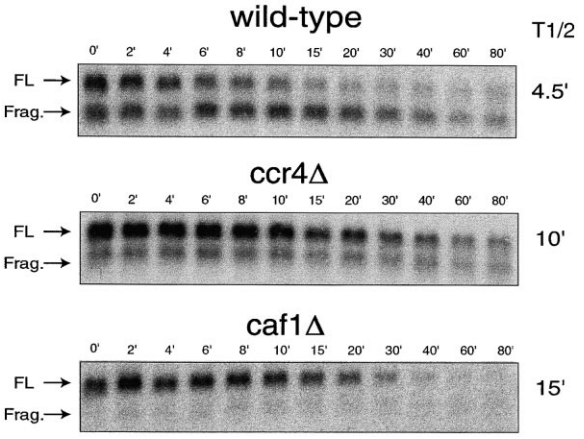

Figure 1. Measurement of the Decay Rate of the MFA2pG mRNA

Shown are transcriptional shutoff analyses of wild-type, *ccr4*Δ, and *caf1*Δ strains. Here, and in other figures, the full-length (FL) mRNA and decay fragment (Frag.) is indicated at the left. Time points are minutes after the addition of glucose.

Ccr4p and Caf1p Are Required for mRNA Deadenylation

To understand the role of Ccr4p and Caf1p in mRNA turnover, we determined whether the *ccr4*Δ and/or *caf1*Δ strains were altered in deadenylation, decapping, or 5′ to 3′ exonuclease digestion. To address this question, we analyzed the decay of the MFA2pG and PGK1pG mRNAs in the *ccr4*Δ and *caf1*Δ backgrounds by a transcriptional pulse chase (Decker and Parker, 1993). In this experiment, we utilize the carbon source regulation of the GAL UAS to rapidly induce and then repress transcription of these reporter mRNAs. This produces a pool of newly transcribed mRNAs whose metabolism can be followed over time to observe deadenylation and subsequent decay of the mRNA.

Comparison of transcriptional pulse chases for the MFA2pG and PGK1pG mRNAs in *ccr4*Δ and *caf1*Δ strains identified two differences from wild-type strains (Figure 2). First, in both *ccr4*Δ and *caf1*Δ strains, the rates of deadenylation for MFA2pG and PGK1pG were impaired. The poly(A) tail of the MFA2pG mRNA in wild-type shortened at a rate of approximately 13 nucleotides per minute, with a significant percentage of the population reaching an oligo(A) length of 10–12 nucleotides in 4 to 6 min (Figure 2A). In contrast, in both the *ccr4*Δ and *caf1*Δ mutant strains, the MFA2pG deadenylated at a rate of approximately 2–3 nucleotides per minute (Fig-

Table 1. mRNA Half-Lives in Wild-Type, *ccr4*Δ, and *caf1*Δ Strains

mRNA	WT	*ccr4*Δ	*caf1*Δ
MFA2pG	4.5′	10′	15′
PGK1pG	24′	38′	49′
GAL1	7.0′	24′	20′
GAL7	12′	27′	ND
GAL10	7.0′	18′	ND

Half-lives are based on multiple determinations and typically vary by less than 10% between individual experiments.

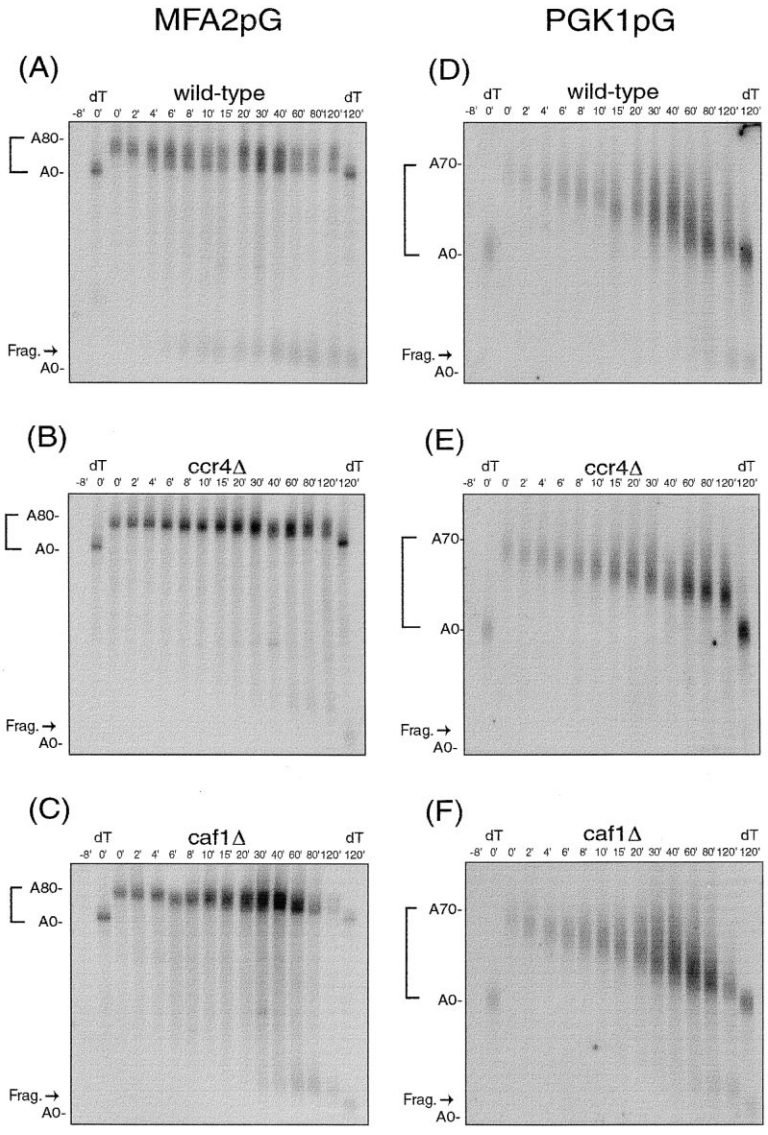

Figure 2. Transcriptional Pulse-Chase Analysis of the MFA2pG and PGK1pG Transcripts

Shown are polyacrylamide Northern gels examining the decay of MFA2pG (A, B, and C) and PGK1pG (D, E, and F) in wild-type, *ccr4*Δ, and *caf1*Δ strains. Numbers above the lanes are minutes after transcriptional repression by the addition of glucose following an 8 min induction of transcription (see Decker and Parker, 1993). The 0 and 120 min time points were treated with RNaseH and oligo(dT) to indicate the position of the deadenylated mRNA. Here, and in all subsequent figures, poly(A) tail lengths were determined by comparison of bands to size standards and the poly(A)-minus mRNA species generated by cleavage of RNaseH and oligo(dT) (data not shown). Here, and in other figures, to allow for size resolution of the poly(A) tail, the 3′ 319-nucleotides of the 1.4-kb PGK1pG mRNA were cleaved by hybridizing to oRP70 followed by cleavage with RNaseH prior to loading on the gel.

ures 2B and 2C). In this strain background, the MFA2pG reporter is not fully repressed by glucose and, for this reason, there is some residual transcription. This is the reason for the persistence of a distributed population of adenylated MFA2pG transcripts in wild-type cells. We also observed defects in deadenylation for the PGK1pG mRNA; however, the difference in rates was less dramatic. In wild-type strains, PGK1pG deadenylated at an approximate rate of 2–3 nucleotides per minute (Figure 2D). In *ccr4*Δ strains, the deadenylation rate of the PGK1pG mRNA was approximately 1 nucleotide per minute (Figure 2E). Interestingly, the *caf1*Δ had only a modest effect on the deadenylation rate of the PGK1pG mRNA (Figure 2F). This suggests that the change in half-life of the PGK1pG mRNA in the caf1Δ strain was due to a difference in the extent of deadenylation (see below). The difference in deadenylation rates between *ccr4*Δ and *caf1*Δ mutant strains also implies that Ccr4p has a more central role in deadenylation (see below).

A second difference in the *ccr4*Δ and *caf1*Δ strains was that the deadenylation of the MFA2pG and PGK1pG mRNAs was incomplete. In wild-type strains, the poly(A) tail shortens to an oligo(A) length of 10–12 nucleotides before the mRNA can become a substrate for decapping and subsequent 5′ to 3′ degradation (Decker and Parker, 1993). The MFA2pG and PGK1pG mRNAs at late time points in the *ccr4*Δ and *caf1*Δ strains had poly(A) tails slightly longer than observed in wild type (Figure 2). To measure this difference in the endpoint of deadenylation more clearly, we measured the distribution of poly(A) tails on MFA2pG and PGK1pG mRNA populations under steady state conditions from wild-type, *ccr4*Δ, and *caf1*Δ (Figures 3A and 3B). Treatment of samples with RNase H and oligo(dT) indicated that the size differences were due to the length of the poly(A) tail (Figure 3, lanes 8 to 13). Wild-type MFA2pG and PGK1pG mRNAs have a steady state poly(A) distribution from 75 to approximately 10–12 adenosine residues (Figure 3, lane 2; Decker and Parker, 1993). In *ccr4*Δ strains, the shortest poly(A) tails observed for both MFA2pG and PGK1pG were 20–26 adenosines (Figure 3, lane 3). Similarly, in *caf1*Δ strains, the shortest poly(A) tails observed for

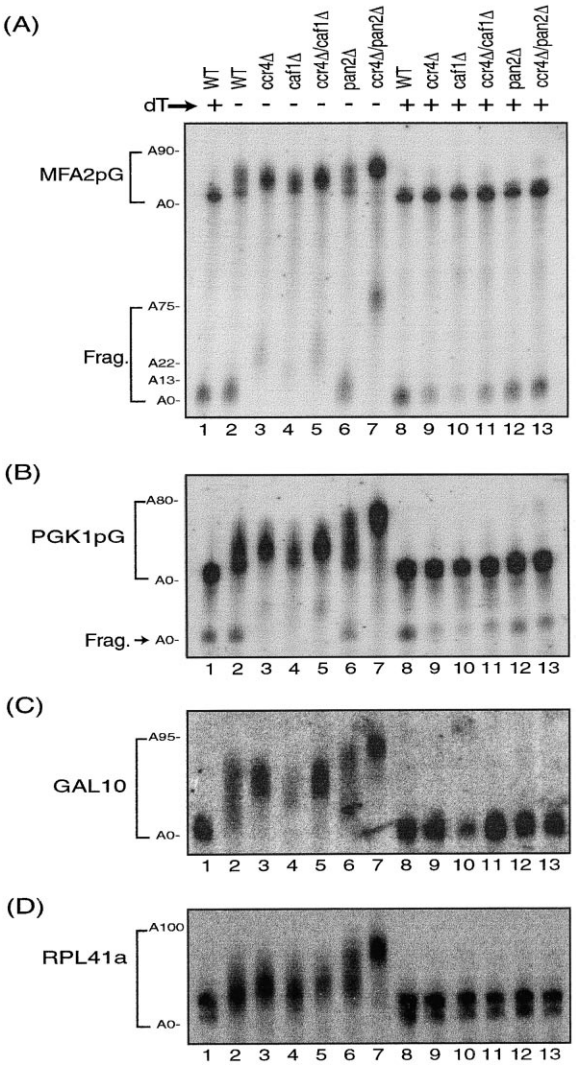

Figure 3. Comparison of Deadenylation End Points

Shown are the deadenylation end points for MFA2pG (A), PGK1pG (B), GAL10 (C), and RPL41a (D) transcripts in wild-type, *ccr4Δ*, *caf1Δ*, *ccr4Δ/caf1Δ*, *pan2Δ*, and *ccr4Δ/pan2Δ* strains. Steady state mRNA samples were resolved on 6% polyacrylamide/8 M urea Northern gels either with or without removal of poly(A) tails with RNaseH and oligo(dT) (as indicated). To allow for size resolution of the poly(A) tail, the 3′ 202-nucleodides of the 2.2-kb GAL10 mRNA were cleaved by hybridizing to oRP97 followed by cleavage with RNaseH prior to loading on the gel.

both MFA2pG and PGK1pG measured slightly shorter at 14–20 adenosines (Figure 3, lane 4). We also observed that in a *ccr4Δ/caf1Δ* double mutant, the end point of deadenylation was the same as in a ccr4Δ strain (Figure 3, lane 5), indicating that the *ccr4Δ* mutant had a stronger effect on the extent of deadenylation than the *caf1Δ* mutation. We observed similar effects of the *ccr4Δ* and caf1Δ on the GAL10 (Figure 3C), RPL41a (Figure 3D), and MRPL27 (data not shown) mRNAs. These observations indicate that the *CCR4* and *CAF1* gene products affect both the rate and extent of deadenylation for a variety of mRNAs.

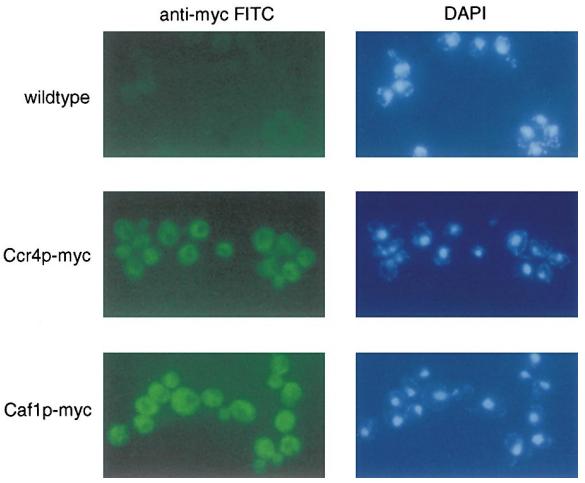

Figure 4. Localization of Ccr4p and Caf1p by Indirect Immunofluorescence

Strains expressing myc-tagged Ccr4p (yRP1621), myc-tagged Caf1p (yRP1622), or wild-type control (yRP841) were stained with anti-myc antibodies and anti-mouse (FITC) 2° antibody and DAPI as indicated.

Ccr4p and Caf1p Are Present in the Cytoplasm

The above experiments indicated that the Ccr4p and Caf1p had a role in cytoplasmic deadenylation. While prior work has suggested a nuclear, transcriptional role for these proteins, this may not be their sole function. If Ccr4p and Caf1p are directly involved in deadenylation, at least a portion of these polypeptides should be present within the cytoplasm. To determine the subcellular distribution of Ccr4p and Caf1p, we constructed chromosomal *CCR4* and *CAF1* genes with multiple Myc epitopes on their C termini and localized these proteins by immunofluorsecence (see Experimental Procedures). These epitope-tagged proteins were functional as assessed by both growth and mRNA turnover phenotypes (data not shown). Importantly, detection of the fusion protein by immunofluorescence indicated that the majority of Ccr4p-myc and Caf1p-myc are present in the cytoplasm (Figure 4). These data are consistent with the hypothesis that Ccr4p and Caf1p have a direct role in cytoplasmic deadenylation.

Caf1p Copurifies with a Ccr4p-Dependent, poly(A)-Specific Nuclease Activity

Since both Ccr4p and Caf1p were required for deadenylation in vivo, localized to the cytoplasm, and had significant homology to known nucleases, we hypothesized that a complex containing minimally Ccr4p and Caf1p would constitute the mRNA deadenylase. This possibility is supported by prior work demonstrating that Ccr4p and Caf1p interact (Sakai et al., 1992; Draper et al., 1995) and can be copurified along with several other proteins (Liu et al., 1997; Liu et al., 1998; Oberholzer and Collart, 1998). Given this, we purified a functional FLAG-Caf1 fusion protein from a wild-type strain under conditions that would copurify endogenous Ccr4p. Based on silver staining and Western analysis, this preparation contained FLAG-Caf1p, Ccr4p, and several additional proteins (data not shown, see Discussion).

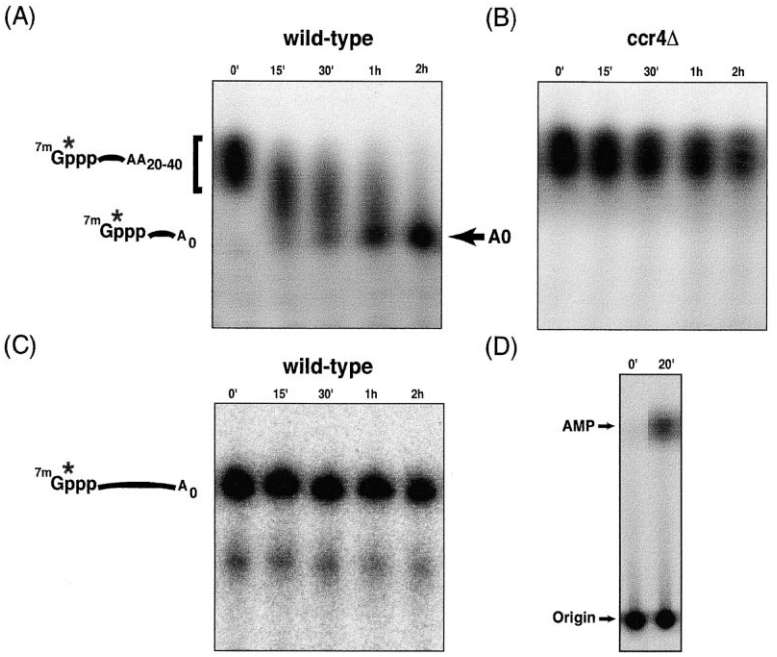

Figure 5. FLAG-Caf1p Copurifies with Deadenylase Activity

Analysis of deadenylation activity in FLAG-Caf1p elution from wild-type (A), and from *ccr4*Δ (B) strains on a capped, polyadenylated substrate, and analysis of nuclease activity in FLAG-Caf1p elution fractions from wild-type on capped, nonadenylated substrate (C). Numbers above the lanes indicate time points taken after addition of substrate to the reaction. The arrow in panel (A) represents the fully deadenylated form of the substrate based on migration of substrate prior to poly(A) addition (data not shown). The * indicates the position of the radiolableled phosphate. Deadenylation activity of FLAG-Caf1p elution fractions from wild-type on substrate 3′ end labeled with 32p adenosine (D). The products of the reaction were separated by PEI-cellulose TLC. Numbers above the lanes indicate time points taken after addition of substrate to the reaction. The migration of cold 5′ AMP standard is indicated on the left.

Purified FLAG-Caf1p/Ccr4p and associated proteins were then assayed for their ability to deadenylate an mRNA substrate in vitro. A 5′ labeled, capped mRNA, synthesized in vitro with a 49-nucleotide body and a poly(A) tail length of approximately 20 to 40 adenosines was incubated with purified FLAG-Caf1p extracts and analyzed at various times on 6% polyacrylamide gels. We observed that shorter species of the input RNA appeared over time. Since the substrate is 5′ labeled, these shorter species represent trimming from the 3′ end. We also observed that a new species accumulated of the correct length to represent a fully deadenylated substrate (Figure 5A). These conclusions were also supported by the observation that removal of the poly(A) tail with oligo(dT) and RNaseH shortens the input mRNA as expected but does not shorten the product produced in vitro (data not shown). The accumulation of the deadenylated RNA suggested that the nuclease activity present in these fractions is not active on non-poly(A) sequences. This conclusion is also supported by the observation that incubation of the purified FLAG-Caf1p extracts with a 5′ labeled, capped mRNA substrate of 134 nucleotides lacking a poly(A) tail failed to show any significant shortening from the 3′ end (Figure 5C). No nuclease activity was observed when we purified a FLAG-Cup1 fusion protein from a wild-type strain (data not shown) and performed deadenylation assays on these extracts. These observations indicate that a poly(A)-specific nuclease copurifies with Caf1p. Two observations argue that this activity is a 3′ to 5′ exonuclease. First, the gradual shortening observed over time of the polyadenylated substrate is consistent with an exonucleolytic mode of action. Second, use of a substrate with a ^{32}P-labeled adenosine tail led to the release of labeled product that comigrated with 5′ AMP by TLC analysis (Figure 5D).

Our analysis indicated that Ccr4p was required for deadenylation in vivo. Moreover, the stronger defects in deadenylation seen in a *ccr4*Δ strain as compared to a *caf1*Δ strain (see above) suggested that Ccr4p was critical to the action of the deadenylase. Given this, we determined if the nuclease activity that copurifies with Caf1p was dependent on Ccr4p by purifying FLAG-Caf1p from a *ccr4*Δ strain. In these fractions, we obtain Caf1p, and several other associated proteins, but not Ccr4p (data not shown, see Discussion). This FLAG-Caf1p preparation was no longer capable of deadenylation in vitro as shown in Figure 5B. This observation indicated that Ccr4p is essential for the FLAG-Caf1p-purified deadenylase activity. Together, the above results indicate that the Ccr4p and Caf1p are components of the major cytoplasmic deadenylase in yeast.

The Pan2p/Pan3p Exonuclease Represents a Second mRNA Deadenylase

Because *ccr4*Δ, *caf1*Δ, and *ccr4*Δ/*caf1*Δ strains show residual deadenylation (Figures 2 and 3), there must be one or more additional mRNA deadenylases. We hypothesized that either the exosome or the Pan2p/Pan3p nuclease carried out this residual deadenylation. The exosome is a multi-subunit enzyme responsible for a variety of 3′ to 5′ RNA processing and RNA degradation events including 3′ to 5′ decay of the mRNA body following deadenylation (reviewed in van Hoof and Parker, 1999). The Pan2p/Pan3p nuclease is a poly(A)-specific nuclease (PAN) thought to catalyze the initial trimming of the nascent poly(A) tail (Brown and Sachs, 1998).

To address whether the exosome or PAN activity was responsible for the observed deadenylation in *ccr4*Δ strains, we first created a *ccr4*Δ/*ski2*Δ double mutant strain. Ski2p has been shown previously to be required for cytoplasmic exosome function (Anderson and Parker, 1998) and is a reasonable marker for the potential involvement of the exosome in cytoplasmic functions. The *ccr4*Δ/*ski2*Δ strain grew at the same rate as a *ccr4*Δ strain and in vivo mRNA analysis indicated the same

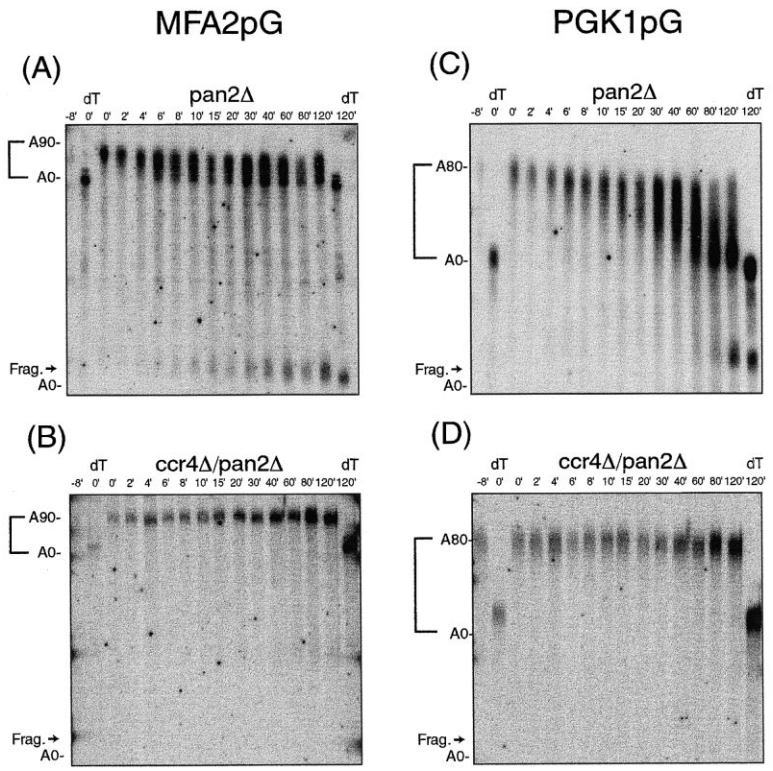

Figure 6. Transcriptional Pulse-Chase Analyses of the MFA2pG and PGK1pG Transcripts

Shown are polyacrylamide Northern gels of transcriptional pulse-chase experiments examining the decay of MFA2pG (A and B) and PGK1pG (C and D) in *pan2*Δ and *ccr4*Δ/*pan2*Δ strains. Numbers above the lanes are minutes after transcriptional repression by the addition of glucose following an 8 min induction of transcription (see Decker and Parker, 1993). The 0 and 120 min time points were treated with RNaseH and oligo(dT) to indicate the position of deadenylated mRNA.

defect in deadenylation as a *ccr4*Δ strain (data not shown). This suggested that the cytoplasmic exosome was not responsible for the residual deadenylation seen in *ccr4*Δ and *caf1*Δ strains. However, these results do not rule out the formal possibility that the exosome may be involved in the deadenylation process in a Ski2p-independent manner.

To examine the role of the Pan2p/Pan3p nuclease, we constructed a *ccr4*Δ/*pan2*Δ double mutant. This *ccr4*Δ/*pan2*Δ double mutant strain grew very slowly suggesting some overlap in function (data not shown). Moreover, analysis of the decay of the MFA2pG and PGK1pG mRNAs in the *ccr4*Δ/*pan2*Δ double mutant strain by a transcriptional pulse chase showed that the transcripts failed to undergo any significant shortening of their poly(A) tails (Figure 6). This demonstrated that the residual deadenylation seen in a *ccr4*Δ strain requires Pan2p. The simplest interpretation of these observations is that PAN is the only other significant mRNA deadenylase in yeast. In addition, it should be noted that the MFA2pG and PGK1pG mRNAs are extremely stable in the *ccr4*Δ/*pan2*Δ strain since they do not appreciably decay and no decay fragments are produced over a period of two hours (Figure 6). This provides additional evidence that deadenylation is a prerequisite for later steps in mRNA turnover.

*ccr4*Δ, *caf1*Δ, and *ccr4*Δ/*pan2*Δ Strains Aberrantly Degrade mRNAs Prior to Complete Deadenylation

Since *ccr4*Δ, *caf1*Δ, and even *ccr4*Δ/*pan2*Δ strains were viable, mRNA turnover must be occurring by some mechanism. This is based on the observation that inactivation of both decapping and 3′ to 5′ exonucleolytic degradation of the mRNA body leads to cell death (Anderson and Parker, 1998). Several observations indicate that strains defective in deadenylation eventually degrade their mRNAs by a slow decapping step that bypasses the need for deadenylation to an oligo(A) tail of 10–12 residues. First, even with defects in poly(A) shortening, these strains still produced the poly(G) decay intermediate that normally arises due to decapping and 5′ to 3′ exonuclease digestion (Figure 3). Second, the decay intermediates produced in these strains had substantial poly(A) tails as indicated by the size change when samples were treated with RNaseH and oligo d(T) (Figure 3, e.g., lane 3 versus lane 9). We observed that the poly(A) tails on the mRNA decay fragment were approximately 14–22 nucleotides in the *ccr4*Δ and *caf1*Δ strains (Figure 3, lane 3 and 4), and are roughly 70–75 nucleotides long in the *ccr4*Δ/*pan2*Δ double mutant (Figure 3, lane 5). This is similar to the shortest poly(A) tail length observed on the full-length mRNA and suggests that the decay fragment is being generated after partial deadenylation of the mRNA. Finally, both the *ccr4*Δ and *caf1*Δ deletions were synthetically lethal with dcp1Δ mutants (data not shown). Since DCP1 encodes the decapping enzyme, this argues that decapping is required for the residual mRNA decay seen in these strains. However, we cannot rule out the formal possibility that mRNA decay is occurring by a novel general endonucleolytic mechanism.

It is important to note that these decay intermediates were produced in the *ccr4*Δ, *caf1*Δ, and *ccr4*Δ/*pan2*Δ strains at significantly lower levels and at much later time points relative to wild type (Figures 2 and 6). This provides additional evidence that deadenylation is nor-

mally a prerequisite for decapping, although when the normal pathway is perturbed, some slow decapping can occur on adenylated mRNAs.

Discussion

Ccr4p and Caf1p Are Components of a Cytoplasmic Deadenylase

Three observations indicate that Ccr4p and Caf1p encode critical components of the major cytoplasmic deadenylase in yeast. First, *ccr4*Δ and *caf1*Δ strains show defects in both the rate and extent of deadenylation for the MFA2pG, PGK1pG, GAL10, and RPL41a mRNAs (Figures 2 and 3). Second, based on indirect immunoflourescence, the majority of epitope-tagged Ccr4p and Caf1p are present in the cytoplasm, the anticipated subcellular location for the deadenylation process (Figure 4). Third, FLAG-Caf1p copurifies with a poly(A)-specific deadenylase activity (Figure 5). This activity is independent of the *PAN2*/*PAN3* encoded 3′ to 5′ poly(A) nuclease as it copurifies with FLAG-Caf1p in a *pan2*Δ strain (data not shown). However, this activity does require the presence of the Ccr4p since activity is absent in purified FLAG-Caf1p extracts made from *ccr4*Δ strains (Figure 5). These observations demonstrate that Ccr4p and Caf1p are components of the major cytoplasmic mRNA deadenylase in yeast.

An important issue is the actual composition of the Ccr4p/Caf1p deadenylase both in terms of the catalytic subunit and other factors involved in deadenylation. Ccr4p is the most likely candidate for a catalytic subunit because the nuclease domain of this protein contains all the critical residues predicted to be required for function (Dlakic, 2000; C. D., unpublished observations). Consistent with this view, the *ccr4*Δ has a stronger effect on deadenylation than the *caf1*Δ (Figures 2 and 3). In addition, overexpression of Ccr4p can suppress some of the growth defects of a *caf1*Δ strain (Hata et al., 1998). In contrast, the yeast Caf1p is missing amino acids thought to be required for catalysis by members of the RNaseD family of proteins (Moser et al., 1997). Nevertheless, the conservation of the exonuclease motifs in *CAF1* homologs in other eukaryotes raises the possibility that the Caf1p may also be a functional nuclease. Interestingly, both Ccr4p and Caf1p have been shown to exist in two large complexes of ~1.0 MDa and ~1.9 MDa (Liu et al., 1998; Bai et al., 1999; Lie et al., 2001). The larger complex includes the Not proteins (Not1p to Not5p) and several other proteins (Liu et al., 1998; Bai et al., 1999). The smaller complex contains only Ccr4p, Caf1p, the five Not proteins, and two newly identified proteins, Caf40p and Caf130p (C. D., unpublished observations). Thus, one possibility is that one or both of these complexes constitutes a deadenylase complex. Additional experiments to define the catalytic subunit and the diversity of proteins that can affect deadenylase activity should resolve these issues.

An important implication of the work presented here is that Ccr4p and Caf1p in other organisms will serve a function similar to their yeast counterparts. The Ccr4p is a member of a broader family of nucleases (Dlakic, 2000). Within this family, true *CCR4* orthologs have been identified that contain both the leucine-rich repeat domain used for binding *CAF1* (Bai et al., 1999) and the C-terminal putative nuclease domain (Dlakic, 2000; J. C. and C. D., unpublished observations). The human (h) *CCR4* retains its ability to interact with *hCAF1* (C. D., unpublished observations) and both show multiple interactions with *hNOTs* suggesting the existence of a human CCR4-NOT complex (Albert et al., 2000; Y. Chiang and C. D., unpublished observations). This suggests that that these protein interactions, and by implication their functions, have been conserved in vertebrates. Interestingly, since both the mammalian and yeast *CAF1* have been implicated in cell-cycle regulation (Liu et al., 1997; Ikematsu et al., 1999; Puisieux and Magaud, 1999), it is possible that this control is attained through changes in mRNA deadenylation rates of key cell-cycle genes.

Possible Connections between Transcription and mRNA Turnover

Several previous reports demonstrate a clear interaction of Ccr4p and Caf1p with components of the transcription machinery. First, Ccr4p has been identified in a Paf1p-containing RNA polymerase II transcription complex (Chang et al., 1999). Second, the CCR4-NOT complex components display physical interaction with TFIID and components of SAGA (Benson et al., 1998; Badarinarayana et al., 2000; Lemaire and Collart, 2000). Third, components of the 1.9 mDa CCR4-NOT complex interact with the SRB9-11 proteins of the RNA polymerase II holoenzyme (Liu et al., 2001). Fourth, CCR4 and CAF1 appear to affect the initiation of transcription of several genes (Denis and Malvar, 1990; Sakai et al., 1992; Liu et al., 1998; Chang et al., 1999). There are three types of explanations for the role of Ccr4p/Caf1p in both transcription and mRNA degradation. First, the Ccr4p/Caf1p complex may function both as a cytoplasmic deadenylase and have an alternative role in the initiation of transcription. Alternatively, the Ccr4p/Caf1p may serve a similar function in the nucleus deadenylating nuclear RNAs, and its association with the transcription machinery is a consequence of that nuclear deadenylation function. Finally, it is possible that the connection to transcriptional machinery represents a requirement for an initial interaction of the Ccr4p/Caf1p cytoplasmic deadenylase with the mRNA in a cotranscriptional manner. In this model, the Ccr4p/Caf1p deadenylase would require a direct interaction with the RNA polymerase II holoenzyme to be assembled on to the growing mRNP complex. An important goal in future work will be to determine the nature of this link between the transcriptional and mRNA degradative machinery.

The Pan2p/Pan3p Ribonuclease Can Function as an mRNA Deadenylase

Our data indicate that the Pan2p/Pan3p poly(A)-specific exonuclease can also function as an mRNA deadenylase in vivo. The critical observation is that although *ccr4*Δ and *caf1*Δ strains show slow residual deadenylation, a *ccr4*Δ/*pan2*Δ strain is completely blocked for deadenylation (Figure 6). Interestingly, prior work has shown that the Pan2p/Pan3p deadenylase functions in an initial trimming of the poly(A) tail. The critical observations are that the initial shortening of nascent poly(A) tails from ~90 residues to ~55–70 in a mRNA-specific manner is

lacking in *pan2*Δ and *pan3*Δ strains (Brown and Sachs, 1998). We also observed a longer nascent poly(A) tail length in *pan2*Δ, and *pan2*Δ/*ccr4*Δ strains (data not shown). This suggests that in the normal metabolism of yeast transcripts, the nascent poly(A) tail is trimmed by the Pan2p/Pan3p nuclease, and then subsequently passed to the Ccr4p/Caf1p for the majority of cytoplasmic deadenylation. Thus, normal deadenylation requires the sequential action of two different nucleases. How, or why, this transition in nucleases occurs is unknown, but it may coincide with transport of the mRNA into the cytoplasm, or changes in mRNP organization, perhaps including entry into translation.

Our data indicate that in *ccr4*Δ strains, the slow residual deadenylation that occurs requires Pan2p. One intriguing feature of the residual deadenylation catalyzed by Pan2p/Pan3p deadenylase is that it appears to stop at the last 20 to 26 adenosines. This stall could be due to the inability of the Pan2p/Pan3p nuclease to shorten the mRNA further. Support for this possibility comes from observations in vitro wherein PAN requires the poly(A) binding protein (Pab1p) as a cofactor (Lowell et al., 1992). Since the footprint of a single bound Pab1p is approximately 25 residues (Sachs et al., 1987), this would suggest that the last Pab1p bound to the poly(A) tail might be insufficient to promote Pan2p/Pan3p deadenylation in vivo. The observation that the Ccr4p/Caf1p complex has the unique ability to process the last phase of deadenylation indicates a fundamental difference in substrate specificity between these two enzymes.

Implications for Regulation of Deadenylation Rate

There are now three distinct enzymes that have been shown to function as mRNA deadenylases, Ccr4p/Caf1p and Pan2p/Pan3p in yeast and PARN in vertebrates. Although there does not appear to be a direct homolog of PARN in yeast, Ccr4p/Caf1p and Pan2p/Pan3p are all highly conserved among eukaryotes. This implies that eukaryotic cells will contain at least two, and likely multiple, distinct deadenylases. This raises the possibility that deadenylation of different mRNAs will occur through the modulation of different nucleases. For example, there may be yeast mRNAs whose primary deadenylation is carried out by the Pan2/Pan3 deadenylase. Consistent with the view that specific deadenylases may target different mRNAs or respond to different regulatory elements, in vitro work has suggested that the enhanced deadenylation promoted by 3′ UTR, AU-rich instability elements requires PARN (Gao et al., 2000). Similarly, the rapid deadenylation of the MFA2 mRNA is also promoted by specific 3′ UTR elements (Muhlrad and Parker, 1992), and this rapid deadenylation is lost in *ccr4*Δ and *caf1*Δ strains. This indicates that the elements within the MFA2 3′ UTR that promote deadenylation do so by accelerating the action of the Ccr4p/Caf1p deadenylase. This implies that both PARN as well as the Ccr4p/Caf1p deadenylases in other eukaryotes will be targets of mRNA-specific, regulated deadenylation rates. An important goal of future work will be to determine how the rates of specific deadenylases are controlled and how 3′ UTR elements influence the deadenylases acting on individual mRNAs.

Experimental Procedures

Strains

All strains used in this study are: *trp1 ura3-52 leu2-3, 112 cup1*Δ*::LEU2PM*. Strains differ as follows: yRP840 (Hatfield et al., 1996) *MATa his4-539,* yRP841 (Hatfield et al., 1996) *MAT*α *lys2-201,* yRP1616 *MATa his4-539 ccr4*Δ*::NEO,* yRP1617 *MAT*α *caf1*Δ*::URA3,* yRP1618 *MATa his4-539 ccr4*Δ*::NEO caf1*Δ*::URA3,* yRP1619 *MATa his4-539 pan2*Δ*::URA3,* yRP1620 *MATa his4-539 ccr4*Δ*::NEO pan2*Δ*::URA3,* yRP1621 *MAT*α *his4-539 lys2-201 CCR4-myc::NEO,* yRP1622 *MAT*α *his4-539 CAF1-myc::NEO.*

The constructions of the ccr4Δ, caf1Δ, and pan2Δ were made by standard methods of transformation and yeast genetics. All of the above gene disruptions were verified by genomic Southern analysis. Double mutant strains were obtained by mating combinations of single deletion haploid strains, (yRP1618 and yRP1620). *myc*-tagged versions of Ccr4p and Caf1p were generated as described in Longtine et al. (1998) and confirmed both by Southern and Western blots.

Plasmids

The *FLAG-CAF1* plasmid (pRP1042) was created by PCR amplification of the *CAF1* gene from yRP840, placing the FLAG epitope 5′ of the *CAF1* gene, and ligated into the yeast expression vector pG-1 (Schena et al., 1991) and confirmed by sequencing.

RNA Isolation and Analysis

All procedures with RNA were done as previously described; transcriptional shutoff experiments, RNA isolations and normalization (Caponigro et al., 1993), transcriptional pulse-chase experiments (with the exception that all growth media was supplemented with 1% sucrose/2% raffinose) (Decker and Parker, 1993), and RNaseH reactions (Muhlrad and Parker, 1992).

Localization of Epitope-Tagged Ccr4p and Caf1p

yRP841, yRP1621, and yRP1622 were grown to midlog, fixed, and analyzed by standard methods using anti-myc-FITC 9E10 antibody (CoVance) at a dilution of 1:200 and goat anti-mouse IgG-FITC (Roche) was used at a dilution of 1:300.

In Vitro Deadenylation Assays

FLAG-Caf1p proteins were purified from wild-type and *ccr4*Δ strains (yRP840 and yRP1616) harboring the plasmid pRP1042 following similar procedures (Tharun and Parker, 1999). Fractions were analyzed for activity prior to storage of samples at −80°C in 20% (v/v) glycerol.

To prepare substrate, uncapped RNA was transcribed as previously described from plasmid pRP802 (LaGrandeur and Parker, 1998), which was partially digested with Sau3AI, to produce both a 49- and a 134-nucleotide transcript. The RNAs were capped (LaGrandeur and Parker, 1998) and poly(A) tails were added to capped substrate using Poly(A) polymerase and 650 nM of ATP (Barkoff et al., 1998). 3′ end labeling of substrate was achieved by addition of 500 nM of α32p ATP to the Poly(A) polymerase reactions.

FLAG-Caf1p elution fractions were assayed for deadenylation activity at 37°C. 100 μl deadenylation reactions contained 70 μl of FLAG-Caf1p elution, 0.01 pmol cap labeled RNA, 20 mM HEPES, pH 7.0, 1 mM MgOAc, 2 mM spermidine, 1 mM DTT, 0.2 μg/μl BSA, 0.2% NP-40, and 1 U/μl RNasin. 10 μl time points were stopped with 300 μl of stop mix (20 mM EDTA, 300 mM NaOAc, 3 ng/μl glycogen). Products from the reaction with 3′ labeled, poly(A) RNA substrates were separated by PEI-cellulose thin layer chromatography developed in 0.45 M $(NH_4)_2SO_4$.

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