RNA (2002), *8*:959–965. Cambridge University Press. Printed in the USA.
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DOI: 10.1017.S1355838202025049

METHOD

Analysis of the products of mRNA decapping and 3′-to-5′ decay by denaturing gel electrophoresis

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ABSTRACT

The majority of mRNA turnover is mediated either by mRNA decapping/5′-to-3′ decay or exosome-mediated 3′-to-5′ exonucleolytic decay. Current assays to assess mRNA decapping in vitro using cap-labeled RNA substrates rely on one-dimensional thin layer chromatography. This approach does not, however, resolve free phosphate from ^{7me}GDP, the product of Dcp1p-mediated mRNA decapping. This can result in misinterpretation of the levels of mRNA decapping due to the generation of free phosphate following the action of the unrelated scavenger decapping activity on the products of exosome-mediated decay. In this report, we describe a simple denaturing acrylamide gel-based assay that faithfully resolves all of the possible products that can be generated from cap-labeled RNA substrates by turnover enzymes present in cell extracts. This approach allows a one-step assay to quantitatively assess the contributions of the exosome and DCP-1-type decapping on turnover of an RNA substrate in vitro. We have applied this assay to recalculate the effect of competition of cap-binding proteins on decapping in yeast. In addition, we have used the assay to confirm observations made on regulated mRNA decapping in mammalian extracts that contain much higher levels of exosome activity than yeast extracts.

Keywords: decapping; exonucleolytic decay; exosome; mRNA stability

INTRODUCTION

Messenger RNA turnover plays an important role in regulating both the level and accuracy of gene expression in eukaryotic cells. Differences in mRNA half-lives are used to control expression in a developmental and tissue-specific fashion for numerous genes involved in cell growth and cell–cell communication (Guhaniyogi & Brewer, 2001; Malter, 2001; Wilusz et al., 2001b). Accuracy of gene expression is maintained by activation of rapid turnover of mRNAs that contain premature stop codons due to mutation or inappropriate pre-mRNA processing (Frischmeyer & Dietz, 1999; Hentze & Kulozik, 1999; Hilleren & Parker, 1999; Maquat & Carmichael, 2001). A connection between mRNA turnover and translation is seen at other levels as well (Tharun & Parker, 2001), underscoring the significant networking of steps along the posttranscription pathway of gene expression (Keene, 2001; Wilkinson & Shyu, 2001).

The pathways and regulation of the enzymes involved in mRNA turnover are currently under investigation in a variety of systems. The shortening of the poly(A) tail by specific deadenylation enzymes initiates mRNA turnover in most cases (Dehlin et al., 2000; Gao et al., 2000; Daugeron et al., 2001; Tucker et al., 2001). Two general pathways appear to account for decay of the mRNA body in eukaryotic cells. First, deadenylation sends a signal that allows hydrolysis of the transcript's ^{7me}GTP cap by the enzyme Dcp1p to generate ^{7me}GDP (Tucker & Parker, 2000). The resulting decapped transcripts contain a 5′ phosphate and become susceptible to digestion by the 5′-to-3′ exonuclease Xrn1p (Hatfield et al., 1996; Johnson 1997). This decapping route is the major pathway of mRNA turnover in yeast (Muhlrad et al., 1994). In the alternative pathway, deadenylated mRNAs are rapidly degraded by the exosome, a complex of 3′-to-5′ exonucleases (Allmang et al., 1999; van Hoof & Parker, 1999; Butler,

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2002). Following exonucleolytic decay by the exosome, the remaining short 5′ fragments are decapped by the scavenger decapping activity to yield ^{7me}GMP (Nuss et al., 1975; Wang & Kiledjian, 2001) that can then be further recycled into metabolic pathways. Exosome-mediated mRNA turnover, rather than decapping, is the major pathway observed in vitro in mammalian cells (Chen et al., 2001; Wang & Kiledjian, 2001; Mukherjee et al., 2002). Whether this truly reflects the major in vivo pathway for mammalian mRNAs remains to be established. Finally, mRNAs that contain premature termination codons are rapidly decapped in a novel deadenylation-independent fashion in yeast (Hilleren & Parker, 1999).

The regulation of the enzymes involved in mRNA turnover is extensive. A variety of factors are required for the activity of Dcp1p, many of which can also be found associated with the enzyme. This growing list of factors includes Dcp2p (Dunckley & Parker, 1999), Pat1p/Mrt1p (He & Parker, 2001), Lsm proteins (Bouveret et al., 2000; Tharun et al., 2000), Edc1/2p (Dunckley et al., 2001), Vps16p (Zhang et al., 1999a), Dhh1p (Coller et al., 2001), and the Upf family of proteins (which are involved in nonsense-mediated decay; He & Jacobson, 2001; Lykke-Andersen et al., 2001; Pal et al., 2001; Wang et al., 2001). Furthermore, Dcp1p activity on an RNA substrate can be influenced by cap-binding proteins and other translation initiation factors (Schwartz & Parker, 2000; Vilela et al., 2000; Gao et al., 2001), as well as the poly(A) tail in association with poly(A) binding proteins (Wilusz et al., 2001a). Exosome activity can be regulated by Ski gene products, one of which is a helicase (Jacobs-Anderson & Parker, 1998; van Hoof et al., 2000). In addition, exosome-mediated degradation of an RNA substrate can be regulated by the AU-rich elements that are found in the 3′ UTR of many short-lived mammalian mRNAs (Chen et al., 2001; Mukherjee et al., 2002). Understanding the complex interplay of these factors and their relationship to mRNA turnover is a major goal of current research efforts.

The dual nature of the pathways involved in mRNA turnover can complicate in vitro analysis of mRNA turnover, as both can contribute to the final results obtained. As outlined below, the current assay for analyzing the products of Dcp1p-mediated decapping can, in fact, be compromised by end products of the exosome pathway. Furthermore, analysis of the exosome pathway using internally labeled RNA substrates completely ignores the contribution of the decapping/Xrn1p pathway. In this study, we report the development of a denaturing gel electrophoresis assay that allows the clear identification of the products of mRNA decapping as well as the end products of 3′-to-5′ exonuclease decay in a single step. This method also affords a visualization of the extent of nonspecific nuclease activity, allowing the impact of these confounding activities on the results obtained to be evaluated. The application of this assay should improve the overall quality of the data generated by in vitro studies of mRNA turnover.

RESULTS AND DISCUSSION

Problems associated with conventional TLC assays of mRNA decapping

Following incubation of cap-labeled RNA substrates in cell extracts, the standard approach to analyze mRNA decapping is to apply reaction products to a PEI-F cellulose sheet and resolve them by thin layer chromatography (TLC) in a buffer containing 450 mM $(NH_4)_2SO_4$ (Zhang et al., 1999b). As seen in Figure 1, however, this assay has one very significant limitation. Although it can separate ^{7me}GMP, ^{7me}GDP, ^{7me}GTP, and ^{7me}GpppG, it does not allow one to distinguish between the main product of Dcp1p-mediated decapping, ^{7me}GDP, and free orthophosphate. Free phosphate can be generated in RNA turnover reactions as a major by-product of exosome-mediated mRNA turnover. Following 3′-to-5′ shortening by the exosome (Chen et al., 2001; Mukherjee et al., 2002), the remainder of the cap-labeled transcript (^{7me}G^{32}pppG) becomes a substrate for the scavenger decapping activity

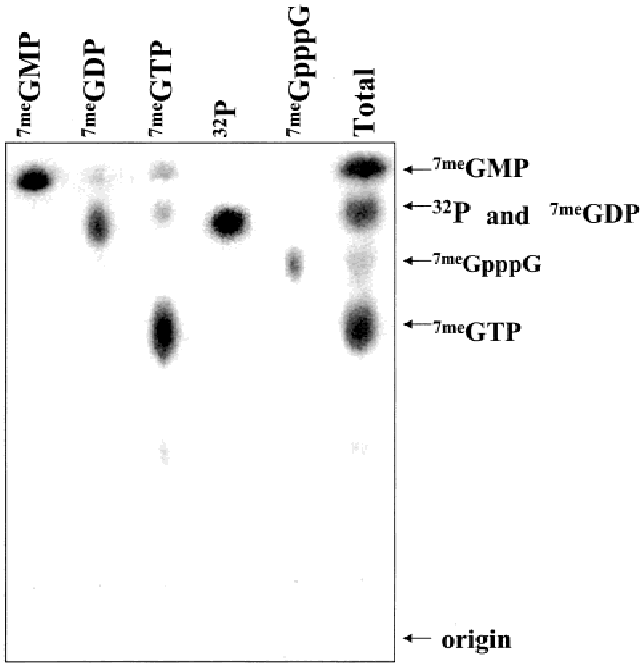

FIGURE 1. Limitations of analyzing the products of mRNA decapping by one-dimensional thin layer chromatography. ^{7me}GMP, ^{7me}GDP, ^{7me}GTP, ^{7me}GpppG, and ^{32}P orthophosphate were spotted onto a PEI-F cellulose sheet either individually or combined (total lane) and resolved in 450 mM $(NH_4)_2SO_4$. Nucleotide spots were visualized by UV shadowing and the ^{32}P position was visualized by phosphorimaging. Note that the phosphorimage has been overlaid onto the photograph of the UV shadowed gel so that the results of the entire experiment could be viewed together. The position of the origin and of the migration of individual molecules is indicated at the right.

first described by Shatkin and colleagues (Nuss et al., 1975). This scavenger enzyme generates ^{7me}GMP ($^{7me}G^{32}p$ in this case) that can then become a substrate for phosphatases in the extract to yield free ^{32}P (Wang & Kiledjian, 2001). In conclusion, the standard one-dimensional TLC assay cannot truly distinguish between the products of Dcp1p-mediated decapping and exosome-mediated decay when the assay is performed in the usual fashion using an RNA substrate labeled exclusively at the γ-phosphate of the cap.

The one-dimensional TLC assay has other limitations as well. First, the migration of reaction products can be significantly influenced by proteins in the extract. Smearing and slowed migration of reaction products can make unambiguous identification of spots by comigration with markers difficult, if not impossible. Second, inadequate or uneven drying of samples that are applied to the origin can result in significant variation in the degree of resolution that is obtained. Third, TLC analysis usually does not allow an overall evaluation of the quality of the experiment performed in terms of random nuclease degradation of the input RNA substrate. Due to their relatively large size, the oligonucleotide products that may be generated by contaminating nonspecific endonucleases unrelated to mRNA turnover will not migrate off the origin. Random RNA degradation, therefore, cannot be efficiently detected or evaluated by TLC. Finally, the relative expense of PEI cellulose sheets should not be overlooked.

A simple alteration of TLC running conditions was explored but did not satisfactorily address all of the problems outlined above. For example, resolving products in a saturated ammonium sulfate buffer (pH 3.5) will clearly separate ^{7me}GDP from ^{32}P, but resolution of ^{7me}GDP from other products is reduced (data not shown). In addition, the running time under these conditions is extended to 4 or more hours per sheet. Alternatively, a two-dimensional TLC approach can be employed (Bochner & Ames, 1982; J. Milone, J. Wilusz, & V. Bellofatto, submitted), but running times are now nearly 8 h per sheet and smearing can reduce sensitivity of detection. Furthermore, this two-dimensional TLC approach does not allow the application of multiple samples on a plate, making comparative analyses unwieldy. Unfortunately, all of these TLC approaches still suffer from the relatively minor but pesky issues of sample/lane variability and expense that also limit their usefulness. Finally, nucleoside di-phosphate kinases have been used to confirm the identification of ^{7me}GDP in one-dimensional TLC by altering its migration through the addition of a third phosphate (Wang & Kiledjian, 2001). This assay, however, is cumbersome to perform on numerous samples. Clearly there is a need for an alternative assay that allows effective one-step resolution of all of the relevant radiolabeled species that can be generated in an in vitro turnover assay with cap-labeled RNA substrates.

Development of a 20% denaturing gel assay for decapping and its application to yeast in vitro assays

Because the RNA substrate used in in vitro decapping reactions contains a single radioactive phosphate at the gamma position of the cap ($^{7me}G^{32}pppGN_n$), only ^{7me}GMP, ^{7me}GDP, ^{7me}GTP, ^{7me}GpppG, and ^{32}P orthophosphate could be routinely detected as end products of mRNA turnover pathways. We decided, therefore, to focus our efforts on the application of high-resolution gel electrophoresis technology that has the potential to effectively resolve these five species.

Cap-labeled RNA substrates were incubated with cytoplasmic extract at 30 °C as described in Materials and Methods. Following incubation, reaction mixtures were phenol extracted, adjusted to 2 M urea via the addition of a gel loading buffer, heat denatured, and loaded onto a 20% acrylamide gel containing 5 M urea. Gels were prerun to remove any salt front that might develop. The bromophenol blue tracking dye was allowed to migrate approximately halfway down the gel before electrophoresis was stopped and the gels were visualized by UV shadowing, autoradiography, or phosphorimaging.

As diagramed in Figure 2A, a 20% acrylamide gel containing urea is capable of clearly resolving ^{7me}GMP, ^{7me}GDP, ^{7me}GTP, ^{7me}GpppG, and ^{32}P orthophosphate. An application of the 20% gel assay to the study of mRNA decapping in yeast cytoplasmic extracts is shown in Figure 2B. Cap-labeled Gem-A0 RNA was incubated with low salt yeast cytoplasmic S100 extract prepared as described previously (Wilusz et al., 2001a) in the presence or absence of unlabeled cap analog as a competitor. In the absence of cap analog, the generation of both ^{7me}GMP, the end product of the 3′-to-5′ decay pathway, and ^{7me}GDP, the product of Dcp1p-mediated decapping, was observed. Free ^{32}P was generated throughout the time course by endogenous phosphatases that acted primarily upon the ^{7me}GMP reaction product. The addition of cap analog to the reaction had three effects: Dcp1p-mediated decapping was stimulated approximately sixfold, ^{7me}GpppG was detected instead of ^{7me}GMP, and the amount of free phosphate observed was significantly reduced.

These results confirm and extend previous findings of mRNA decapping in yeast extracts in several ways. First, Dcp1p-mediated decapping is a major pathway of mRNA turnover observed in yeast extracts. Second, the end products of the alternative 3′-to-5′ decay pathway can also be observed in vitro. Also note that a reduction in scavenger decapping through competition for the enzyme by the addition of unlabeled ^{7me}GpppG cap analog results in the detection of radioactive $^{7me}G^{32}$pppG generated from the RNA substrate primarily by 3′-to-5′ exonucleolytic decay (Fig. 2B, + lanes). These data confirm the presence of the scavenger de-

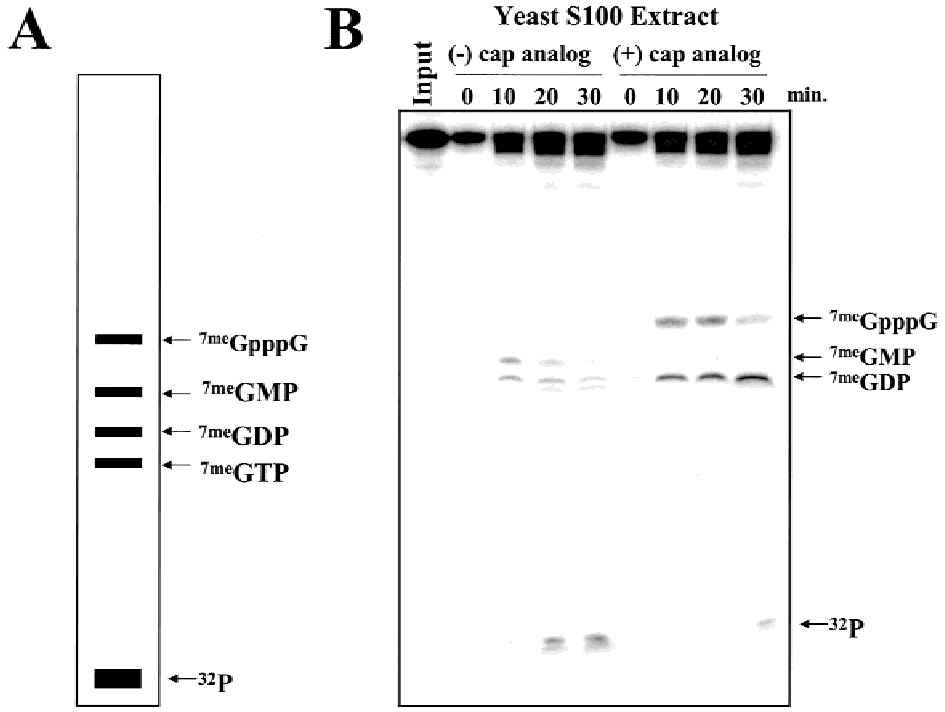

FIGURE 2. Analysis of the products of mRNA decapping by electrophoresis in a 20% denaturing acrylamide gel. **A:** Diagrammatic representation of the relative migration of ^{7me}GMP, ^{7me}GDP, ^{7me}GTP, ^{7me}GpppG, and ^{32}P orthophosphate on a 20% acrylamide gel containing 5 M urea. The relative migration of these species was determined by running each individually or in combination and visualizing bands by UV shadowing or phosphorimaging. **B:** Cap-labeled Gem-A0 RNA was incubated with yeast cytoplasmic S100 extract in the presence or absence of 20 μM ^{7me}GpppG cap analog for the times indicated. Reaction products were analyzed on a 20% denaturing acrylamide gel and results were visualized by autoradiography. The position of various relevant species is indicated at the right.

capping activity involved in metabolic recycling in *Saccharomyces cerevisiae* (Wang & Kiledjian, 2001). Third, previous reports indicated that the addition of cap analog to yeast extracts either had a minimal or at most two- to threefold effect on decapping, presumably by the sequestration of cap-binding proteins such as eIF4E from the RNA substrate (Schwartz & Parker, 2000; Wilusz et al., 2001a). The significantly higher stimulation by cap analog in the 20% gel assay shown in Figure 2B is likely due to an underestimation of decapping in previous studies resulting from the comigration of free radioactive phosphate with ^{7me}GDP that limits the sensitivity of TLC assays. Note, for example, the background level of free phosphate detected by the 20% gel assay in Figure 2B. If one adds the amount of free phosphate to the levels of ^{7me}GDP observed in Figure 2B, the apparent stimulation of Dcp1p-mediated decapping by cap analog is reduced to ~1.7-fold instead of the 6-fold effect when decapping is assayed using a 20% gel. Finally, the 20% gel assay allows for an evaluation of the impact of nonspecific nuclease degradation, the bane of all experiments performed using RNA. As seen in Figure 2B, only minor degradation of the RNA substrate occurred in the assay. Based on these observations, we believe that the 20% gel assay offers a significant improvement in the analysis of the products of mRNA turnover over conventional assays.

Use of the 20% denaturing gel assay to the study of mRNA turnover in mammalian cell extracts

We next applied the 20% denaturing gel assay to RNA turnover assays in mammalian extracts. Because exosome-mediated degradation clearly predominates in extracts from HeLa, Jurkat, and K562 cells (Chen et al., 2001; Wang & Kiledjian, 2001; Mukherjee et al., 2002), the possibility of misinterpretation of the results of mRNA decapping assays using the conventional TLC assay is high due to the potential generation of large amounts of ^{7me}GMP, ^{7me}GpppG, and free ^{32}P. Cytoplasmic extracts from some mammalian cell lines can, in fact, contain very high levels of phosphatases (Wang & Kiledjian, 2001). Using one-dimensional TLC assays, we have previously reported the identification of a Dcp1p-like decapping activity in HeLa cell extracts that is stimulated by the addition of unlabeled ^{7me}GpppG cap analog (Gao et al., 2001). To confirm this, we repeated the experiment and analyzed its products with the 20% gel assay. As seen in Figure 3A, lane −, ^{7me}GMP is the predominant product generated from

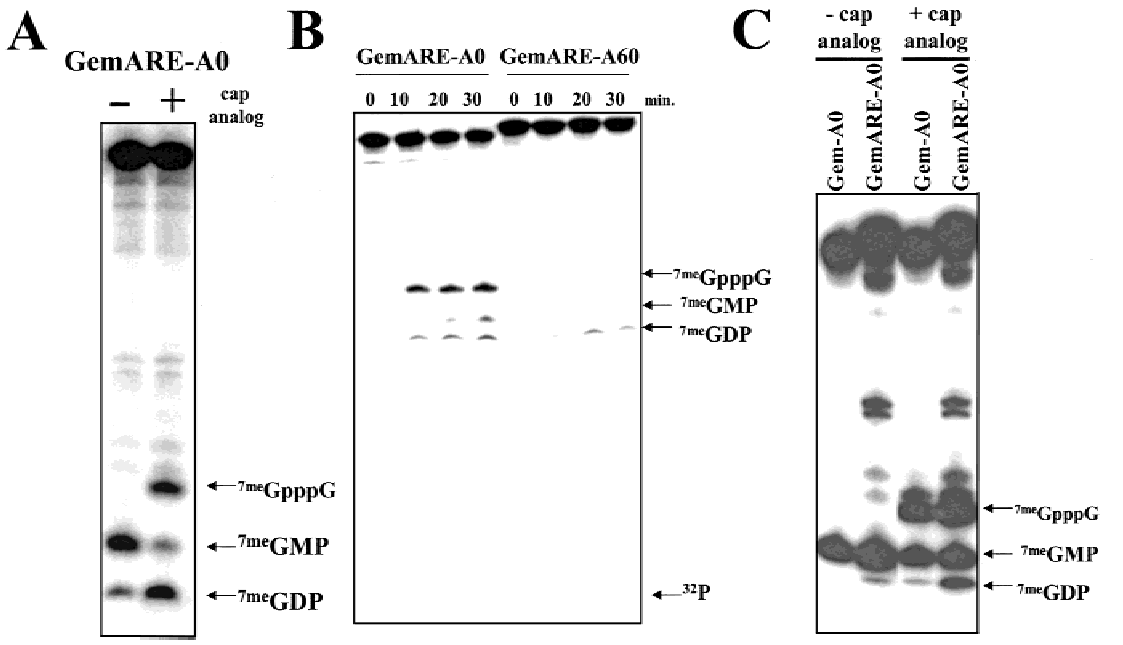

FIGURE 3. Analysis of regulated mRNA decapping in mammalian extracts using a 20% denaturing gel assay. **A:** Titration of cap-binding proteins stimulates mRNA decapping. Cap-labeled GemARE-A0 RNA was incubated in HeLa cytoplasmic S100 extract in the presence or absence of 20 μM ^{7me}GpppG cap analog for 30 min. **B:** Addition of a poly(A) tail represses mRNA decapping. Cap-labeled GemARE-A0 RNA or a variant that contains a 60-base poly(A) tail (GemARE-A60) were incubated in the presence of HeLa cytoplasmic S100 extract in the presence or absence of 20 μM ^{7me}GpppG cap analog for the times indicated. **C:** Presence of an AU-rich instability element stimulates decapping. Cap-labeled GemA0 RNA, or a variant that contains the 34-base AU-rich element from TNF-α (GemARE-A0 RNA) were incubated in HeLa cytoplasmic S100 extract in the presence or absence of 20 μM ^{7me}GpppG cap analog for 30 min. For all panels, reaction products were analyzed on a denaturing 20% acrylamide gel and results were visualized by autoradiography and/or phosphorimaging. The position of ^{7me}GMP, ^{7me}GDP, and ^{7me}GpppG markers is indicated at the right of each panel. Only low levels of free ^{32}P were generated in these experiments and orthophosphate was run off the gel to maximize resolution of the indicated species.

cap-labeled GemARE-A0 RNA upon incubation in HeLa extracts. This species is generated by the action of the scavenger decapping enzyme on the product of exosome mediated 3′-to-5′ decay (Nuss et al., 1975; Wang & Kiledjian, 2001). Only a low amount of the ^{7me}GDP product generated by Dcp1p-like decapping was observed. In the extracts tested for this study, the exosome degraded 21 to 48% of the input cap-labeled RNA, whereas Dcp1p-like decapping activity processed up to 4%. When cap analog was added to the extract to sequester cap-binding proteins, a dramatic 8–10-fold increase in the level of ^{7me}GDP was observed. The addition of cap analog also generated the appearance of ^{7me}GpppG, consistent with the analog inhibiting the action of the scavenger decapping enzyme on the final products of exosome decay. Finally, the Hela S100 extracts used in this study contained no significant levels of endogenous phosphatase activity, as no free phosphate was observed on gels (see Fig. 3B). We conclude that although the products derived from exosome-mediated degradation predominate in HeLa cytoplasmic extracts, the ^{7me}GDP product of Dcp1p-like decapping can still be observed. Furthermore, this HeLa cell Dcp1p-like decapping activity is stimulated by cap analog in a fashion similar to that observed in yeast extracts (Fig. 2B).

Our previous work reported two additional factors that regulate the Dcp1p-like decapping activity. First, the presence of a poly(A) tail was concluded to repress Dcp1p-like decapping by one-dimensional TLC assays (Gao et al., 2001). Based on the limitations of this assay, however, it is possible that the presence of a poly(A) tail was instead decreasing the generation of the ^{32}P spot that comigrates with ^{7me}GDP by repressing 3′-to-5′ exonucleolytic digestion of the RNA substrate. As seen in Figure 3B, the 20% gel assay confirms our original conclusion that the presence of a poly(A) tail does indeed repress decapping. As estimated from the level of released ^{7me}GDP, GemARE RNA substrates that contained a 60-base poly(A) tail were decapped approximately fourfold less efficiently than a matched substrate that lacks a poly(A) tail. Also note that the low level of background nonspecific nuclease activity allows conclusions to be drawn from these data with confidence.

Second, the presence of an AU-rich element was previously concluded to stimulate decapping by one-dimensional TLC (Gao et al., 2001). Based on the limitations of this assay, however, it is possible that the presence of an AU-rich element was instead solely increasing the level of the ^{32}P spot that comigrates with ^{7me}GDP by stimulating exosome-mediated 3′-to-5′ exo-

nucleolytic digestion of the RNA substrate (Chen et al., 2001; Mukherjee et al., 2002). As seen in Figure 3C, the 20% gel assay confirms our original conclusion that the addition of an ARE stimulates decapping, although not to the same 10-fold extent as we concluded previously with the one-dimensional TLC assay. Decapping was stimulated approximately fourfold by the addition of an AU-rich element into the GemA0 RNA background as assayed using a 20% gel. The difference from our previous result is likely due to the fact that the AU-rich also stimulates exosome activity. Due to the high baseline exosome activity in the extracts used in this study and the 30-min time point chosen, the presence of AU-rich elements only stimulated exosome activity 25–30% in Figure 3. Because both decapping and exosome activity are occurring simultaneously in this assay, it is impossible to rule out that one process is not contributing to the other. Conditions that fully separate these two pathways need to be elucidated before the true contribution of ARE elements to the regulation of decapping can be investigated.

In summary, we used a 20% denaturing acrylamide gel for the identification of products of mRNA turnover generated in in vitro assays using cap-labeled RNA substrates. This approach allows the definitive identification of all of the relevant species generated from cap-labeled transcripts with the added bonus of allowing an assessment of nonspecific RNA degradation that occurred during the reaction. This assay should allow the results of mRNA decapping assays to be interpreted with confidence and represents an important tool in the experimental arsenal to unlock the mechanism of regulated mRNA turnover in eukaryotic cells.

MATERIALS AND METHODS

RNAs

GemA0 RNA is transcribed from pGem4 that was linearized with *Hin*dIII. GemARE-A0 RNA is a derivative of GemA0 that contains the 34-base AU-rich element found in the 3′ UTR of TNF-α mRNA inserted between the *Pst*I and *Hin*dIII sites (Ford et al., 1999). GemARE-A60 RNA is a derivative of GemARE-A0 that contains a 60-base poly(A) tail at its 3′ end. Templates for transcribing this RNA were generated by ligating poly(A)-containing oligonucleotides onto *Hin*dIII-cut pGemARE by a ligation-PCR approach (Ford et al., 1999).

All RNAs were transcribed using SP6 RNA polymerase in the presence of 0.5 mM rNTPs. Transcripts were collected by ethanol precipitation and capped in vitro using recombinant vaccinia virus capping enzyme in the presence of radiolabeled [α-^{32}P]GTP as described previously (Zhang et al., 1999b). ^{7me}GpppG was purchased from Amersham-Pharmacia Biotech. ^{7me}GMP, ^{7me}GDP, and ^{7me}GTP markers were purchased from Sigma. For a typical lane, 1 μL of a 100-mM solution of a marker was loaded. Following electrophoresis, positions of these markers were visualized by UV shadowing. ^{32}P orthophosphate was obtained from Perkin-Elmer and visualized in gels by autoradiography or phosphorimaging.

Extracts

S100 cytoplasmic extracts were made from *S. cerevisiae* grown in YPD media by zymolase treatment and lysis in low salt as described previously (Wilusz et al., 2001a). S100 cytoplasmic extracts were made from HeLa spinner cells grown in JMEM supplemented with 10% horse serum as previously described (Dignam et al., 1983; Ford et al., 1999).

In vitro RNA turnover reactions

In vitro turnover reactions were performed as previously described (Zhang et al., 1999b; Gao et al., 2001; Wilusz et al., 2001a). Briefly, 100,000 cpm of cap-labeled RNAs were incubated in a 10-μL reaction containing 50 mM Tris, pH 7.9, 33 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 4 μL S100 cytoplasmic extract. Reactions were supplemented with 20 μM ^{7me}GpppG cap analog where indicated. Mixtures were incubated at 30 °C for 30 min.

Analysis of in vitro turnover reactions

Thin layer chromatography was performed as described previously (Zhang et al., 1999b). Briefly, following the addition of 1 μL of 500 mM EDTA, reaction mixtures were spotted onto PEI-F thin layer chromatography sheets. Products were resolved in 450 mM $(NH_4)_2SO_4$ and visualized by UV shadowing, autoradiography, or phosphorimaging.

The 20% denaturing gel assay for analyzing the products of in vitro turnover reactions was performed as follows. Following incubation at 30 °C, the volume was increased to 20 μL by the addition of H_2O. Each reaction was extracted using 20 μL of Tris-equilibrated phenol and 10 μL of the aqueous phase was removed. Four microliters of gel loading buffer (20 mM Tris, pH 7.6, 7 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol, 1 mM EDTA) were added to the aqueous phase. Tubes were heated to 90 °C for 30 s and samples were loaded onto a 20% acrylamide gel (37.5:1 acrylamide to bis-acrylamide) containing 5 M urea. Gels were 20 × 19 cm in size, cast using 0.75 mm spacers, and prerun prior to loading to remove any salt fronts that might interfere with the resolution of small molecules. The bromophenol blue dye was allowed to migrate approximately halfway down the gel before electrophoresis was stopped. Gels were exposed to X-ray film at −80 °C or dried and visualized by phosphorimaging. Quantitation was performed using ImageQuant software.

ACKNOWLEDGMENTS

We thank Phil Zamore for very helpful discussions, C.J. Wilusz for yeast extracts, and members of the Wilusz laboratory for critical comments. This work was supported by National Institutes of Health Grants CA80062 and GM63832 to J.W.

Received February 11, 2002; returned for revision February 28, 2002; revised manuscript received May 6, 2002

REFERENCES

Allmang C, Petfalski E, Podtelejnikov A, Mann M, Tollervey D, Mitchell P. 1999. The yeast exosome and human PM-Scl are related complexes of 3′ → 5′ exonucleases. *Genes & Dev 13*: 2148–2158.

Bochner BR, Ames BN. 1982. Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. *J Biol Chem 257*:9759–9769.

Bouveret E, Rigaut G, Shevchenko A, Wilm M, Seraphin B. 2000. A Sm-like protein complex that participates in mRNA degradation. *EMBO J 19*:1661–1671.

Butler JS. 2002. The yin and yang of the exosome. *Trends Cell Biol 12*:90–96.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, Karin M. 2001. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell 107*:451–464.

Coller JM, Tucker M, Sheth U, Valencia-Sanchez MA, Parker R. 2001. The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA 7*:1717–1727.

Daugeron, MC, Mauxion F, Seraphin B. 2001. The yeast POP2 gene encodes a nuclease involved in mRNA deadenylation. *Nucleic Acids Res* 29:2448–2455.

Dehlin E, Wormington M, Korner CG, Wahle E. 2000. Cap-dependent deadenylation of mRNA. *EMBO J 19*:1079–1086.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res 1*:1475–1489.

Dunckley T, Parker R. 1999. The DCP2 protein is required for mRNA decapping in *Saccharomyces cerevisiae* and contains a functional MutT motif. *EMBO J 18*:5411–5422.

Dunckley T, Tucker M, Parker R. 2001. Two related proteins, Edc1p and Edc2p, stimulate mRNA decapping in *Saccharomyces cerevisiae*. *Genetics 157*:27–37.

Ford LP, Watson J, Keene JD, Wilusz J. 1999. ELAV proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system. *Genes & Dev 13*:188–201.

Frischmeyer PA, Dietz HC. 1999. Nonsense-mediated mRNA decay in health and disease. *Hum Mol Genet 8*:1893–1900.

Gao M, Fritz DT, Ford LP, Wilusz J. 2000. Interaction between a poly(A)-specific ribonuclease and the 5′ cap influences mRNA deadenylation rates in vitro. *Mol Cell 5*:479–488.

Gao M, Wilusz CJ, Peltz SW, Wilusz J. 2001. A novel mRNA-decapping activity in HeLa cytoplasmic extracts is regulated by AU-rich elements. *EMBO J 20*:1134–1143.

Guhaniyogi J, Brewer G. 2001. Regulation of mRNA stability in mammalian cells. *Gene* 265:11–23.

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

He F, Jacobson A. 2001. Upf1p, Nmd2p, and Upf3p regulate the decapping and exonucleolytic degradation of both nonsense-containing mRNAs and wild-type mRNAs. *Mol Cell Biol 21*:1515–1530.

He W, Parker R. 2001. The yeast cytoplasmic LsmI/Pat1p complex protects mRNA 3′ termini from partial degradation. *Genetics 158*:1445–1455.

Hentze MW, Kulozik AE. 1999. A perfect message: RNA surveillance and nonsense-mediated decay. *Cell 96*:307–310.

Hilleren P, Parker R. 1999. Mechanisms of mRNA surveillance in eukaryotes. *Annu Rev Genet 33*:229–260.

Jacobs-Anderson JS, Parker R. 1998. The 3′ to 5′ degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3′ to 5′exonucleases of the exosome complex. *EMBO J 17*:1497–1506.

Johnson AW. 1997. Rat1p and Xrn1p are functionally interchangeable exoribonucleases that are restricted to and required in the nucleus and cytoplasm, respectively. *Mol Cell Biol 17*:6122–6130.

Keene JD. 2001. Ribonucleoprotein infrastructure regulating the flow of genetic information between the genome and the proteome. *Proc Natl Acad Sci USA 98*:7018–7024.

Lykke-Andersen J, Shu MD, Steitz JA. 2001. Communication of the position of exon–exon junctions to the mRNA surveillance machinery by the protein RNPS1. *Science 293*:1836–1839.

Malter JS. 2001. Regulation of mRNA stability in the nervous system and beyond. *J Neurosci Res 66*:311–316.

Maquat LE, Carmichael GG. 2001. Quality control of mRNA function. *Cell 104*:173–176.

Muhlrad D, Decker CJ, Parker R. 1994. Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5′ → 3′ digestion of the transcript. *Genes & Dev 8*:855–866.

Mukherjee D, Gao M, O'Connor JP, Raijmakers R, Pruijn G, Lutz CS, Wilusz J. 2002. The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *EMBO J 21*:165–174.

Nuss DL, Furuichi Y, Koch G, Shatkin AJ. 1975. Detection in HeLa cell extracts of a 7-methyl guanosine specific enzyme activity that cleaves m7GpppNm. *Cell 6*:21–27.

Pal M, Ishigaki Y, Nagy E, Maquat LE. 2001. Evidence that phosphorylation of human Upfl protein varies with intracellular location and is mediated by a wortmannin-sensitive and rapamycin-sensitive PI 3-kinase-related kinase signaling pathway. *RNA 7*:5–15.

Schwartz DC, Parker R. 2000. mRNA decapping in yeast requires dissociation of the cap binding protein, eukaryotic translation initiation factor 4E. *Mol Cell Biol 20*:7933–7942.

Tharun S, He W, Mayes AE, Lennertz P, Beggs JD, Parker R. 2000. Yeast Sm-like proteins function in mRNA decapping and decay. *Nature 404*:515–518.

Tharun S, Parker R. 2001. Targeting an mRNA for decapping: Displacement of translation factors and association of the Lsm1p-7p complex on deadenylated yeast mRNAs. *Mol Cell 8*:1075–1083.

Tucker M, Parker R. 2000. Mechanisms and control of mRNA decapping in *Saccharomyces cerevisiae*. *Annu Rev Biochem 69*: 571–595.

Tucker M, Valencia-Sanchez MA, Staples RR, Chen J, Denis CL, Parker R. 2001. The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in *Saccharomyces cerevisiae*. *Cell 104*:377–386.

van Hoof A, Parker R. 1999. The exosome: A proteasome for RNA? *Cell 99*:347–350.

van Hoof A, Staples RR, Baker RE, Parker R. 2000. Function of the Ski4p (Csl4p) and Ski7p proteins in 3′-to-5′ degradation of mRNA. *Mol Cell Biol 20*:8230–8243.

Vilela C, Velasco C, Ptushkina M, McCarthy JE. 2000. The eukaryotic mRNA decapping protein Dcp1 interacts physically and functionally with the eIF4F translation initiation complex. *EMBO J 19*:4372–4382.

Wang W, Czaplinski K, Rao Y, Peltz SW. 2001. The role of Upf proteins in modulating the translation read-through of nonsense-containing transcripts. *EMBO J 20*:880–890.

Wang Z, Kiledjian M. 2001. Functional link between the mammalian exosome and mRNA decapping. *Cell 107*:751–762.

Wilkinson MF, Shyu AB. 2001. Multifunctional regulatory proteins that control gene expression in both the nucleus and the cytoplasm. *Bioessays 23*:775–787.

Wilusz CJ, Gao M, Jones CL, Wilusz J, Peltz SW. 2001a. Poly(A) binding proteins regulate both mRNA deadenylation and decapping in yeast cytoplasmic extracts. *RNA 7*:1416–1424.

Wilusz CJ, Wormington M, Peltz SW. 2001b. The cap-to-tail guide to mRNA turnover. *Nat Rev Mol Cell Biol 2*:237–246.

Zhang S, Williams CJ, Hagen K, Peltz SW. 1999a. Mutations in VPS16 and MRT1 stabilize mRNAs by activating an inhibitor of the decapping enzyme. *Mol Cell Biol 19*:7568–7576.

Zhang S, Williams CJ, Wormington M, Stevens A, Peltz SW. 1999b. Monitoring mRNA decapping activity. *Methods 17*:46–51.