CHAPTER FIVE

Regulated Deadenylation *In Vitro*

Aaron C. Goldstrohm,* Brad A. Hook,† *and* Marvin Wickens*

Contents

Abstract

The 3′-poly(A) tail, found on virtually all mRNAs, is enzymatically shortened by a process referred to as "deadenylation." Deadenylation is a widespread means of controlling mRNA stability and translation. The enzymes involved—so-called deadenylases—are surprisingly diverse. They are controlled by RNA sequences commonly found in 3′-untranslated regions (UTRs), which bind regulatory factors.

Both RNA-binding proteins and microRNAs accelerate deadenylation of specific mRNAs. In some cases, regulators enhance deadenylation by binding to and recruiting specific deadenylases to the target mRNA. The many hundreds of potential regulators encoded in mammalian genomes (both RNA-binding

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proteins and microRNAs) and the numerous deadenylases, coupled with the many potential regulatory sites represented in 3′ UTRs of mRNAs, provide fertile ground for regulated deadenylation. Recent global studies of poly(A) regulation support this conclusion. Biochemical and genetic approaches will be essential for exploring regulated deadenylation.

The methods we describe focus on the reconstruction *in vitro* of regulated deadenylation with purified components from yeast. We discuss broadly the strategies, problems, and history of *in vitro* deadenylation systems. We combine this with a more detailed discussion of the purification, activity, and regulation of the *Saccharomyces cerevisiae* Ccr4p-Pop2p deadenylase complex and its regulation by PUF (Pumilio and Fem-3 binding factor) RNA-binding proteins.

1. Introduction

Gene expression is extensively controlled by posttranscriptional mechanisms, including mRNA stability and translation (Garneau *et al.*, 2007, 2007b). Indeed, mRNA stabilities vary by four orders of magnitude, and protein levels vary by as much as six orders of magnitude (Beyer *et al.*, 2004; Ghaemmaghami *et al.*, 2003; Huh *et al.*, 2003; Lackner *et al.*, 2007; Vasudevan *et al.*, 2006; Yang *et al.*, 2003). These types of regulation permeate biology, contributing to quantitative, spatial, and temporal control of protein production. They are required for development and differentiation, cell growth, immunity, and memory formation (Bramham and Wells, 2007; Colegrove-Otero *et al.*, 2005, 2007b; Garneau *et al.*, 2007; Kimble and Crittenden, 2007; Khabar, 2007; Khabar and Young, 2007).

The poly(adenosine) (poly(A)) tail plays a central role in mRNA regulation (Gray and Wickens, 1998; Kuhn and Wahle, 2004). Poly(A) is added to the 3′-end of nascent mRNAs by the nuclear polyadenylation machinery (Zhao *et al.*, 1999). Once the mRNA is in the cytoplasm, poly(A) has at least two duties: to promote translation and to stabilize the mRNA. Both of these effects are mediated by poly(A) binding protein (PABP), which coats the poly(A) tail (Gorgoni and Gray, 2004; Kuhn and Wahle, 2004). PABP interacts with translation initiation factors, including the eIF4F complex that recognizes the 5′-7-methyl guanosine cap structure and promotes translation initiation (Gorgoni and Gray, 2004; Kuhn and Wahle, 2004). PABP wards off attack by cellular exoribonucleases (with some exceptions) by blocking their access to the mRNA (Goldstrohm and Wickens, 2008; Gorgoni and Gray, 2004; Kuhn and Wahle, 2004).

"Deadenylation" is the enzymatic shortening of the poly(A) tail over time. This process typically initiates, and is the rate-limiting step of, general mRNA decay (Parker and Song, 2004). Enzymes progressively degrade the poly(A) tail at a slow, basal rate, beginning at the 3′-end (Fig. 5.1). Once poly(A) is shortened to a certain length (typically 10 to 15 A's in yeast;

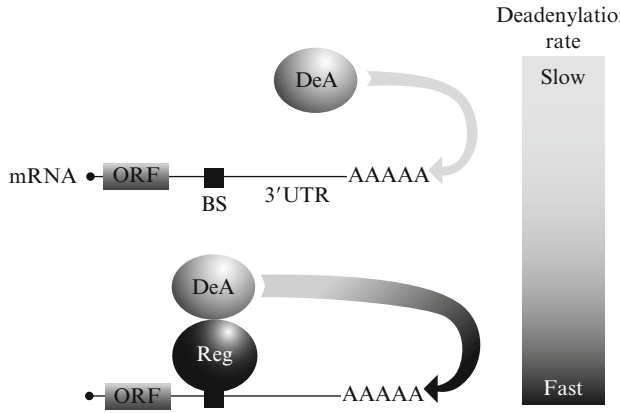

Figure 5.1 Regulators enhance deadenylation by recruiting a deadenylase to the mRNA. A deadenylase (DeA) slowly degrades the poly(A) tail of an mRNA that contains an open-reading frame (ORF) and a 3′-untranslated region (3′ UTR) with a binding site (BS) for a sequence-specific, RNA-binding regulatory factor (Reg). The regulator interacts with the deadenylase and recruits it to the mRNA, thereby accelerating the rate of deadenylation.

slightly longer in mammals), the mRNA is degraded by either of two pathways. The first is a 5′ to 3′-pathway wherein the triphosphate linkage of the 5′-cap is cleaved by a ''decapping'' enzyme (DCP2), and the mRNA body is subsequently degraded from the 5′-end by XRN1 exonuclease. The second pathway acts 3′ to 5′ and is catalyzed by a complex of exonucleases, the exosome (Garneau *et al.*, 2007; Parker and Song, 2004).

''Deadenylases'' are defined as exoribonucleases that degrade poly(A) in a 3′ to 5′-direction (Goldstrohm and Wickens, 2008; Parker and Song, 2004). In doing so, they release 5′-AMP (Astrom *et al.*, 1991, 1992; Lowell *et al.*, 1992). Many such enzymes exist (Goldstrohm and Wickens, 2008). In mammals, informatics suggest as many as 12 deadenylases (Dupressoir *et al.*, 2001; Goldstrohm and Wickens, 2008; Wagner *et al.*, 2007). Deadenylases have preference for 3′-poly(A), although in some cases can degrade non-adenosine homopolymers with reduced efficiency (Astrom *et al.*, 1991; Bianchin *et al.*, 2005; Chen *et al.*, 2002; Lowell *et al.*, 1992; Thore *et al.*, 2003; Tucker *et al.*, 2001; Uchida *et al.*, 2004). All known deadenylases are Mg^{2+}-dependent enzymes that belong to either of two superfamilies, defined by conserved nuclease sequence motifs that are necessary for catalysis (Goldstrohm and Wickens, 2008). The DEDD superfamily contains relatives of the POP2, PARN, and PAN2 deadenylases (Goldstrohm and Wickens, 2008; Thore *et al.*, 2003; Zuo and Deutscher, 2001). The second superfamily includes members related to a class of exonucleases, endonucleases, and phosphatases, known as the EEP superfamily (Dlakic, 2000;

Dupressoir *et al.*, 2001; Goldstrohm and Wickens, 2008). CCR4 and Nocturnin deadenylases belong to the EEP superfamily (Goldstrohm and Wickens, 2008).

Deadenylases function in general mRNA turnover, but also have specialized regulatory roles (Goldstrohm and Wickens, 2008; Parker and Song, 2004). Sequence-specific, RNA-binding regulators can target individual deadenylases to specific mRNAs (Fig. 5.1) (Chicoine *et al.*, 2007; Goldstrohm and Wickens, 2008; Goldstrohm *et al.*, 2006; Kim and Richter, 2006; Lykke-Andersen and Wagner, 2005; Moraes *et al.*, 2006). For example, the PUF family of RNA-binding regulatory proteins can repress specific mRNAs by interacting with and recruiting a deadenylase complex (Goldstrohm *et al.*, 2006, 2007; Hook *et al.*, 2007; Kadyrova *et al.*, 2007). Yeast PUF proteins directly bind the Pop2p subunit of the Ccr4p-Pop2p deadenylase complex and thereby accelerate deadenylation and decay and/or repress translation of target mRNAs. Regulation of the mRNA encoding *HO* mating-type switching endonuclease by two yeast PUF proteins is exemplary (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007).

Recruitment of deadenylases by regulators is likely widespread (Beilharz and Preiss, 2007; Goldstrohm and Wickens, 2008; Grigull *et al.*, 2004; Lackner *et al.*, 2007; Meijer *et al.*, 2007). The potential for mRNA control is enormous, with hundreds of protein and microRNA regulators, a multitude of deadenylases, and the abundance of 3′ UTR controls (Chen and Rajewsky, 2007; Keene, 2007; Lander *et al.*, 2001). Biochemical approaches will be essential for dissecting mechanisms and control of deadenylation. Here we focus on *in vitro* methods for analyzing deadenylation, concentrating on the yeast, *S. cerevisiae*. With the PUF regulation of the Ccr4p-Pop2p deadenylase complex as an example, we describe an *in vitro* assay system that recapitulates regulated deadenylation, allowing mechanistic, enzymatic, and molecular genetic analysis.

2. *In vitro* Deadenylation Systems

In vitro systems have been indispensable for identifying deadenylases; indeed, the first deadenylases were discovered with *in vitro* assays (Astrom *et al.*, 1991, 1992; Lowell *et al.*, 1992; Sachs and Deardorff, 1992). Moreover, *in vitro* biochemical systems are important for identifying enzymatic properties and dissecting control mechanisms of mRNA deadenylation.

2.1. Advantages

The benefits of *in vitro* deadenylation analysis are manifold. First, this approach permits study of isolated, individual deadenylases out of the many in eukaryotic cells (Goldstrohm and Wickens, 2008). Several

deadenylase mutants are nonviable, complicating genetic analysis (Chiba *et al.*, 2004; Molin and Puisieux, 2005; Reverdatto *et al.*, 2004). Also, the interconnections and feedback among RNA-related steps, particularly translation and mRNA decay pathways, are circumvented by turning to the test tube (Jacobson and Peltz, 1996; Parker and Sheth, 2007; Schwartz and Parker, 1999, 2000).

In vitro analysis is particularly useful for examining the enzymatic properties of deadenylases, including reaction kinetics and cofactor requirements (Astrom *et al.*, 1991, 1992; Chen *et al.*, 2002; Korner and Wahle, 1997; Martinez *et al.*, 2000; Viswanathan *et al.*, 2003). The effects on substrate structures and RNA sequences on substrate recognition and catalysis can also be examined (Bianchin *et al.*, 2005; Dehlin *et al.*, 2000; Gao *et al.*, 2000; Martinez *et al.*, 2001; Viswanathan *et al.*, 2003, 2004). Importantly, these properties may influence regulatory outcomes: targeting an mRNA with a highly active, processive enzyme elicits more rapid repression than an enzyme with low activity.

Mechanistic analysis of deadenylation, and in particular of its regulation, also can be accomplished *in vitro*. For instance, the effects of regulatory factors on enzymatic activity can be determined (Balatsos *et al.*, 2006; Goldstrohm *et al.*, 2006; Moraes *et al.*, 2006). The detailed mechanisms of the interaction between regulator and deadenylase can be dissected.

Although the *in vitro* studies are powerful, molecular genetics and *in vivo* analysis clearly are a necessary complement (Chen *et al.*, 2002; Goldstrohm *et al.*, 2006, 2007; Hook *et al.*, 2007; Tucker *et al.*, 2001, 2002; Viswanathan *et al.*, 2004). Without them, it is difficult to know how faithfully an *in vitro* system recapitulates biologic regulation. The fact that many enzymes are capable of removing poly(A) is a serious complication and can send *in vitro* analysis down a misleading path.

2.2. A brief history

Multiple *in vitro* systems have been established over the past 15 years, beginning with the use of cell extracts (Astrom *et al.*, 1991; Sachs and Deardorff, 1992). Among the first deadenylases purified, Pan2p was initially identified as a PAB-stimulated deadenylase present in yeast extracts (Lowell *et al.*, 1992; Sachs and Deardorff, 1992). Vertebrate PARN was purified from HeLa cell nuclear and cytoplasmic extracts (Astrom *et al.*, 1991, 1992). These enzymes were subsequently purified by conventional chromatography, studied in reconstituted assays, identified, and cloned (Boeck *et al.*, 1996; Brown *et al.*, 1996; Copeland and Wormington, 2001; Dehlin *et al.*, 2000; Korner and Wahle, 1997; Korner *et al.*, 1998; Martinez *et al.*, 2000; Ren *et al.*, 2002, 2004; Sachs, 2000).

Extract systems also have revealed PARN-dependent deadenylation followed by ATP-dependent degradation of the mRNA body (Ford and

Wilusz, 1999; Ford *et al.*, 1999; Fritz *et al.*, 2000). The addition of poly(A) competitor, which likely alleviates PABP-mediated stabilization, was critical (Ford and Wilusz, 1999). Similar methods were later applied to insect cells and trypanosomes (Milone *et al.*, 2004; Opyrchal *et al.*, 2005). Regulated deadenylation in *Drosophila* extracts revealed ATP-dependent deadenylation and has only been observed in this system (Jeske *et al.*, 2006; Temme *et al.*, 2004).

Genetic analysis identified the heterodimeric Ccr4p and Pop2p complex as responsible for deadenylation activity in yeast, with Ccr4p predominating *in vivo* (Daugeron *et al.*, 2001; Tucker *et al.*, 2001, 2002). *In vitro* analysis with affinity tag–purified complexes, and later recombinant purified enzymes, verified their activities (Chen *et al.*, 2002; Tucker *et al.*, 2001; 2002). Orthologous proteins in mammals and flies were subsequently shown to be active in deadenylases *in vitro* (Baggs and Green, 2003; Bianchin *et al.*, 2005; Chen *et al.*, 2002; Morita *et al.*, 2007; Viswanathan *et al.*, 2004; Wagner *et al.*, 2007).

We studied the regulation of deadenylation by yeast PUF proteins, a family of sequence-specific repressor proteins found throughout eukarya (Wickens *et al.*, 2002). PUF repression correlates with deadenylation, and we found that PUFs make a direct protein contact with the Pop2p subunit of the Ccr4p-Pop2p complex (Goldstrohm *et al.*, 2006, 2007; Hook *et al.*, 2007).

Here we describe the *in vitro* system that we used to reconstitute regulated deadenylation with purified components. We consider each of the steps in developing an *in vitro* deadenylation system in detail. These are diagrammed in Fig. 5.2, which serves as a guide to the remainder of the chapter.

2.3. Optimizing deadenylation conditions

Developing optimal conditions to analyze the properties of a deadenylase is relatively straightforward. However, in reconstituting *regulated* deadenylation, the situation is complicated by the fact that at least three different biochemical events are required: the regulator needs to bind the RNA, the regulator needs to bind the deadenylase or deadenylase complex, and the enzyme has to be catalytically active (Fig. 5.2). Each of these biochemical events has its own distinct optima. With this in mind, we recommend first establishing functional deadenylation conditions, followed by analysis of regulator binding to the substrate under those conditions. If the RNA-binding regulator is functional, one can proceed to test regulated deadenylation *in vitro*. Otherwise, it may be necessary to adjust the deadenylation buffer conditions to suit both deadenylation and RNA binding.

A range of conditions has been used for *in vitro* studies and merits consideration here. These conditions vary with the enzymes and regulators.

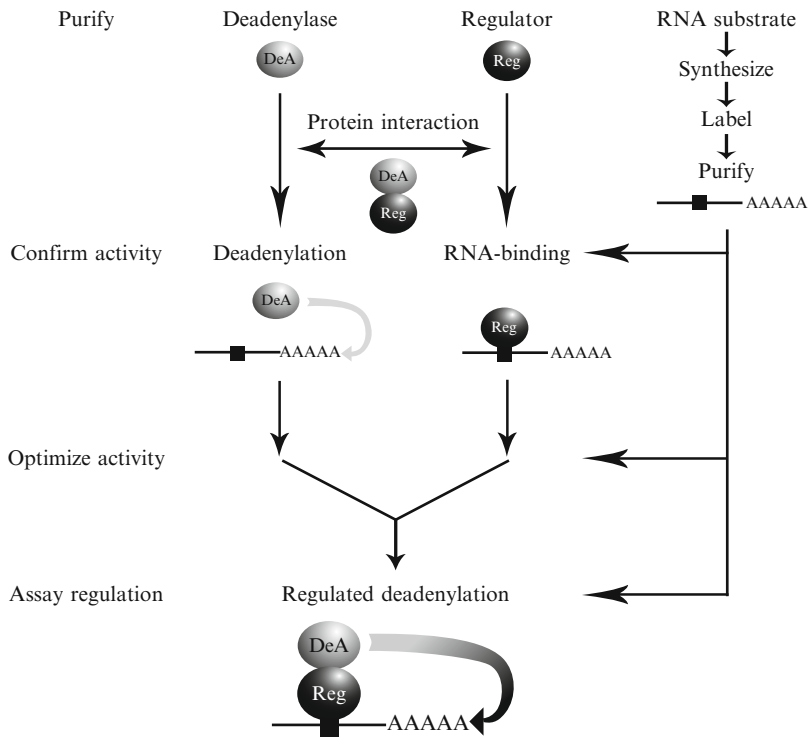

Figure 5.2 Strategy for analysis of regulated deadenylation *in vitro*. To analyze regulated deadenylation *in vitro*, begin by preparing purified deadenylase (DeA), regulator (Reg), and radioactively labeled RNA substrate. Confirm protein interactions between the regulator and deadenylase. Measure the deadenylase activity and RNA-binding activity of deadenylase and regulator, respectively. Titrate each component and optimize concentrations and reaction conditions. Finally, combine deadenylase and regulator with substrate RNA to observe enhancement of deadenylation.

Optimized *in vitro* conditions have been reported for vertebrate PARN (Astrom *et al.*, 1992; Korner and Wahle, 1997; Martinez *et al.*, 2000), yeast Pan2p (Lowell *et al.*, 1992), and yeast Ccr4p (Viswanathan *et al.*, 2003).

Magnesium ions are essential for all known deadenylases, and both magnesium chloride and acetate have been used successfully over a range of 0.01 to 3 m*M* (Astrom *et al.*, 1991; Baggs and Green, 2003; Chen *et al.*, 2002; Daugeron *et al.*, 2001; Lowell *et al.*, 1992; Tucker *et al.*, 2001; Wagner *et al.*, 2007). Excessive magnesium can be inhibitory (Astrom *et al.*, 1992; Viswanathan *et al.*, 2003). In general, other divalent cations cannot substitute (Astrom *et al.*, 1992; Korner and Wahle, 1997).

Common buffers include Tris-HCl, HEPES-KOH, and KPO_4, with a pH range of 6.8 to 8.5. Monovalent cations, sodium, or potassium in the form of NaCl, KCl, or potassium acetate, have been used, typically ranging

from 0 to 200 n*M*. In some instances, either no salt or excessive salt concentrations were inhibitory (Astrom *et al.*, 1992; Viswanathan *et al.*, 2003).

Detergents such as Nonidet P40/IGEPAL and Tween-20 have been included in some cases, with concentrations up to 0.2 and 0.02% being tolerated, respectively (Copeland and Wormington, 2001; Daugeron *et al.*, 2001; Tucker *et al.*, 2001; Wagner *et al.*, 2007). We recommend avoiding detergents unless they are necessary for protein solubility or for reducing spurious interactions.

Other common reaction additives include DTT, spermidine, BSA, polyvinyl alcohol, or glycerol. DTT is commonly used as a reducing agent, with concentrations up to 1 m*M*. Under certain conditions, the polyamine spermidine (up to 2 m*M*) can stimulate PARN or PAN2 (Korner and Wahle, 1997; Lowell *et al.*, 1992; Uchida *et al.*, 2004). BSA may help stabilize proteins and acts as a molecular crowding agent, with concentrations as high as 0.2 mg/ml. Other molecular crowding agents such as polyvinyl alcohol (MW 10000) have been used with concentrations up to 2.5% (Astrom *et al.*, 1991; Ford and Wilusz, 1999). Glycerol can be included up to at least 10%. The RNase A inhibitor RNasin has also been included to minimize spurious contamination.

Several extract systems have included ATP regeneration systems, such as 20 m*M* creatine phosphate, 80 ng/μl creatine kinase, and 800 μM ATP (Ford and Wilusz, 1999; Temme *et al.*, 2004). As a cautionary note, high levels of nucleotides can be inhibitory to several deadenylases, possibly by chelating Mg^{2+} (Astrom *et al.*, 1992). Likewise, inclusion of EDTA is not advisable, although very low levels have been tolerated in some systems (Astrom *et al.*, 1991; Martinez *et al.*, 2000).

Reactions are commonly performed at temperatures ranging from 25 to 37 °C. For instance, yeast Ccr4p activity is optimal at 37 °C, and nearly inactive at 0 °C and above 55 °C (Viswanathan *et al.*, 2003).

2.4. RNA substrates

Several methods can be used to create RNA substrates for deadenylation analysis. Minimally, RNA substrates must contain a high-affinity binding site for the sequence-specific regulator and a 3′-poly(A) tail. We have successfully observed regulated deadenylation in the yeast system with regulator RNA dissociation constants between 10 and 250 n*M*, as measured with purified protein and RNA (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007). Poly(A) tails may be as long as several hundred nucleotides or as short as a single adenosine (Sachs and Deardorff, 1992; Viswanathan *et al.*, 2003).

Substrates can be transcribed *in vitro* or chemically synthesized. Benefits of synthetic RNAs include a homogeneous starting material, high purity

and quality, and precise amount. Purity is commonly quite good, and gel purification is typically not necessary; however, costs may be considerable and lengths are limited. In our own experience, synthetic substrates as long as 54 nucleotides, including 14 nucleotide poly(A) tails, have yielded excellent results (Goldstrohm *et al.*, 2006, 2007; Hook *et al.*, 2007).

Transcripts can be produced by bacteriophage RNA polymerases, such as T7, from plasmid templates encoding poly(A) tracts (Kreig and Johnson, 1996). The plasmid template is linearized with a specific restriction enzyme to produce ''runoff transcripts'' with a defined 3′-end. Common restriction sites used to linearize the template produce RNAs with nonadenosine 3′-ends, arising from the restriction site 3′-overhangs. Because some deadenylases are inhibited by nonadenosine 3′-terminal residues, it is advisable to linearize the template with a restriction enzyme, such as Nsi1, which produces a 3′-terminus encoding only on a single additional adenosine nucleotide (Ford and Wilusz, 1999). Another common method is to generate PCR-amplified or double-stranded oligonucleotide templates that encode 3′-poly(A) tails as transcription templates (Ford and Wilusz, 1999). Longer poly(A) tails can also be added to RNAs by means of a tailing reaction with poly(A) polymerases, although it is difficult to control the length of the often heterogeneous tails (Brown *et al.*, 1996; Daugeron *et al.*, 2001; Ford *et al.*, 1999; Lowell *et al.*, 1992; Martin and Keller, 1998; Temme *et al.*, 2004).

2.4.1. Uniform labeling

Radioactive nucleotides, typically [α-^{32}P]-UTP, can be incorporated into the body of the RNA during transcription to produce substrates with high specific radioactivity (Kreig and Johnson, 1996). Transcription systems are available commercially for transcribing labeled RNAs *in vitro* (for example, from Promega or Ambion). Unincorporated nucleotides and truncated transcripts must be removed by size exclusion chromatography and/or gel purification (see later).

2.4.2. 5′-End labeling

RNA substrates can be labeled at the 5′-end by T4 polynucleotide kinase with [γ-^{32}P]-ATP. Deadenylation is then observed by progressive shortening from the 3′-end of the labeled RNA, in which all degradation products retain identical signal levels, thereby facilitating quantitation. Synthetic RNAs with a 5′-hydroxyl terminus, are ready to be phosphorylated, whereas transcribed RNAs, possessing 5′-triphosphate terminus, should be dephosphorylated with calf intestinal phosphatase before 5′-end labeling. Synthetic RNAs with 5′-fluorescent labels (for example, fluorescein derivatives) have also been used successfully to analyze deadenylation *in vitro* (Morita *et al.*, 2007).

2.4.3. 3′-End labeling

Labeled poly(A) tail substrates may also be created with poly(A) polymerases and [α-^{32}P]-ATP (Brown *et al.*, 1996; Daugeron *et al.*, 2001; Ford *et al.*, 1999; Lowell *et al.*, 1992; Martin and Keller, 1998; Temme *et al.*, 2004). This approach can be used to assay deadenylase activity by release of TCA-soluble AMP (Sachs and Deardorff, 1992).

2.4.4. 5′-Capping

A 5′-7-methyl guanosine cap structure can be added to RNA substrates if necessary (Kreig and Johnson, 1996). The 5′-cap can stabilize substrates in extract systems, presumably by protecting the RNA from 5′-exonucleolytic attack (Parker and Song, 2004). Furthermore, the 5′-cap activates PARN deadenylase activity and so may be desirable when studying PARN-like activities (Dehlin *et al.*, 2000; Gao *et al.*, 2000; Martinez *et al.*, 2001).

Cap analog can be incorporated by RNA polymerases during *in vitro* transcription; however, the incorporation is typically incomplete, and the cap analog can be incorporated in reverse orientation. To increase capping efficiency and maintain proper orientation, Vaccinia virus capping enzyme can be used. Capping enzyme can label RNAs with a [α-^{32}P]-GTP and *S*-adenosyl methionine (capping enzyme is available from Ambion) (See chapter 1 by Liu *et al* for details).

2.4.5. Gel purification

For transcribed substrates, gel purification of full-length product is recommended. Free nucleotides from the transcription reaction can inhibit deadenylases. Free cap analog inhibits PARN. Purification is easily accomplished by electrophoresis through denaturing urea-polyacrylamide gels (Gilman, 2000). For unlabeled RNAs, UV shadowing is used to visualize the RNA; for radioactively labeled RNAs, autoradiography can be used. A gel slice containing the RNA is excised, and the RNA is eluted and precipitated by standard methods (Gilman, 2000). For transcribed RNA substrates, we prefer to transcribe them without the radiolabel, gel purify the RNA, and measure its final concentration. Specific quantities of substrate RNA can then be 5′-labeled as needed.

2.5. Protocol: 5′-labeling of synthetic substrate RNA

Synthetic substrate RNAs (from Dharmacon or Integrated DNA Technologies) can be labeled at the 5′-end with the following protocol.

Assemble labeling reaction:

9 μl ddH_2O, sterile, RNase free
2 μl 10× kinase buffer (Promega)
4 μl 10 μM RNA (40 pmol total per reaction)

3 μl [γ-^{32}P]-ATP, 6000 Ci/mmol, 10 μCi/ml (Perkin-Elmer)
2 μL T4 polynucleotide kinase (Promega)
20 μl final volume, final concentration is 2 pmol/μl
Incubate at 37 °C for 1.5 h.
Heat inactivate at 70 °C for 20 min.
Remove free nucleotides and salts by size exclusion spin chromatography. Apply sample to spin columns, Nucaway (Ambion) or G25 Sepharose (GE Healthcare), according to the manufacturers' recommendations.
Store labeled RNA at −20 °C until ready to use.

2.6. Regulated deadenylation *in vitro*

2.6.1. Purified components

The general strategy requires multiple steps (Fig. 5.2). First, the deadenylase and sequence-specific regulator are purified, and their ability to interact confirmed. Second, the radioactively labeled, polyadenylated substrate RNAs are prepared. Third, the enzyme is titrated, and the RNA-binding activity of the regulator assayed. Finally, the effect of the regulator on the deadenylase is measured by combining the two assays.

Deadenylase complexes have been successfully purified for *in vitro* analysis from natural sources by conventional chromatography (Boeck *et al.*, 1996; Korner and Wahle, 1997; Korner *et al.*, 1998; Lowell *et al.*, 1992; Martinez *et al.*, 2000). Now, affinity purification techniques often are used, including epitope tags or tandem affinity purification (TAP) methods (Chen *et al.*, 2002; Tucker *et al.*, 2001, 2002; Viswanathan *et al.*, 2003). We purified Ccr4p-Pop2p deadenylase complexes from yeast strains expressing either chromosomally integrated or episomal, affinity-tagged proteins (Goldstrohm *et al.*, 2006, 2007; Hook *et al.*, 2007). The tags were engineered with tobacco etch virus (TEV) protease sites to allow specific elution of the bead bound, purified complexes by TEV cleavage (Puig *et al.*, 2001; Rigaut *et al.*, 1999).

Purification from natural sources offers several advantages. Native deadenylase complexes are isolated, maintaining potentially important posttranslational modifications and protein subunits. For instance, several subunits of the CCR4–POP2 complex function as adaptors that bridge regulators and the deadenylase subunits by means of protein interactions (TOB, NOT subunits) (Brown *et al.*, 1996; Chicoine *et al.*, 2007; Ezzeddine *et al.*, 2007; Goldstrohm *et al.*, 2007; Kadyrova *et al.*, 2007). However, there are disadvantages as well; native complexes are not completely defined without additional analysis and are likely heterogeneous in composition *in vivo*. (For instance, multiple forms of CCR4–POP2 complexes have been detected in yeast and mammals [Denis and Chen, 2003; Morel *et al.*, 2003]).

Deadenylation can also be reconstituted with recombinant deadenylases isolated from bacteria (Baggs and Green, 2003; Balatsos *et al.*, 2006;

Bianchin *et al.*, 2005; Korner *et al.*, 1998; Martinez *et al.*, 2001; Ren *et al.*, 2002, 2004; Viswanathan *et al.*, 2004; Wagner *et al.*, 2007). The obvious advantage is that recombinant enzymes are fully defined. However, it has proven difficult to obtain full-length forms of several deadenylases from bacterial expression systems. Active, truncated derivatives have successfully been purified as an alternative (Daugeron *et al.*, 2001; Simon and Seraphin, 2007). The purified protein may require other proteins to interact with the regulator (Brown *et al.*, 1996; Chicoine *et al.*, 2007; Kadyrova *et al.*, 2007).

Molecular genetics can be combined with biochemical purification to create and isolate complexes with specific mutations or with missing specific subunits. This powerful approach permits the structure-function analysis of enzymatic properties and the evaluation of protein domains and specific protein interactions (Chen *et al.*, 2002; Goldstrohm *et al.*, 2007; Hook *et al.*, 2007; Kim and Richter, 2006; Tucker *et al.*, 2002).

2.7. Purification of Pop2p deadenylase complexes

In the following protocol, we describe a method for purification of TAP-tagged Pop2p deadenylase complexes from yeast. We recommend collecting samples at each step of the purification to monitor purification efficiency (Fig. 5.3).

2.7.1. Yeast strain

The TAP-tagged POP2 haploid *Saccharomyces cerevisiae* strain (Open Biosystems) contains a C-terminal TAP tag integrated into the POP2 chromosomal locus by PCR-mediated gene modification. Expression of the POP2-TAP protein is driven by the natural POP2 promoter. For yeast culture techniques and media, refer to Guthrie and Fink (2002).

2.7.2. Grow yeast cultures

Grow a 50-ml starter culture in YPAD media overnight at 30 °C. The following morning, dilute the culture into 4 L of YPAD and grow to an optical density at 660 nm of 1.5 (4.5×10^7 cells/ml).

2.7.3. Harvest cells

Harvest cells at 4000*g* in a JLA8.1 rotor (Beckman-Coulter) at 4 °C. Resuspend cell pellets in ice-cold ddH_2O. Transfer the suspension to a 50-ml plastic conical tube. Collect cells by centrifugation in a refrigerated tabletop centrifuge at 4000*g*. Decant the supernatant from the cell pellets, which will be approximately 10 to 15 ml of packed cell volume. Cell pellets can be frozen and stored at −80 °C at this stage.

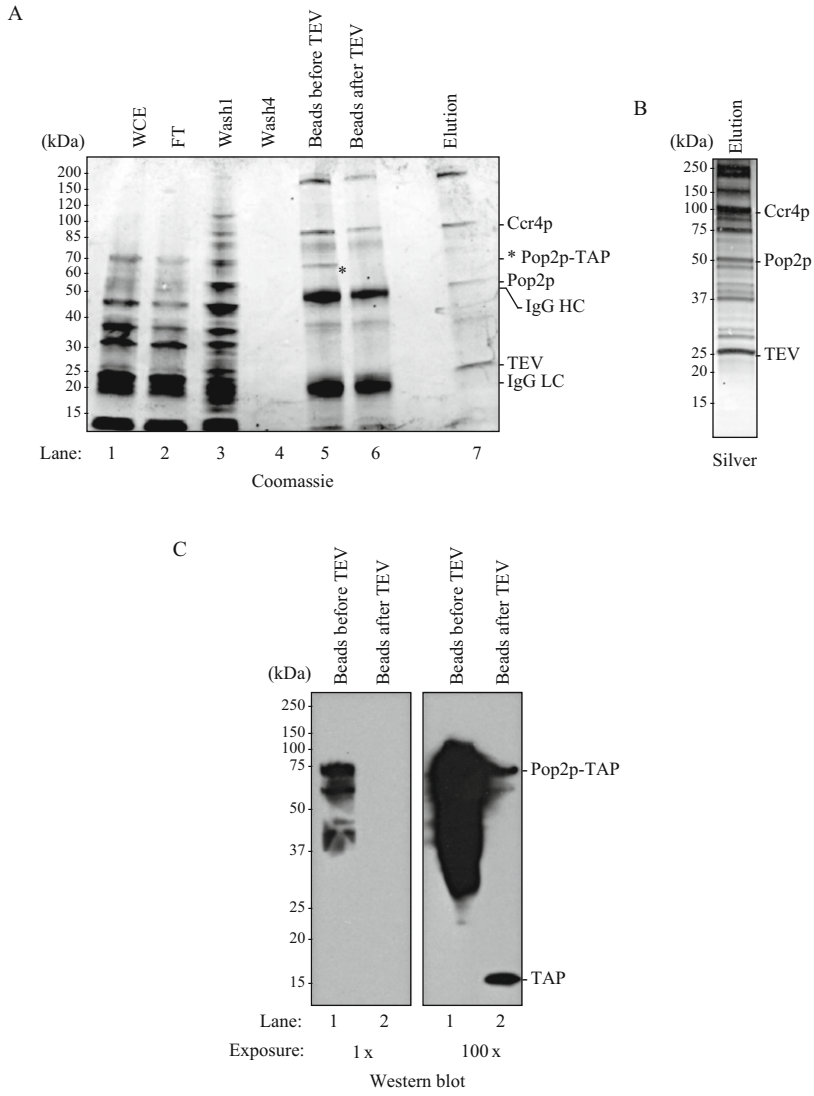

Figure 5.3 Purification of TAP-tagged Pop2p deadenylase complex. (A) Aliquots from the indicated purification steps of TAP-Pop2p were analyzed by SDS-PAGE and Coomassie blue protein stain, including: whole cell extract (WCE, 0.1% of the total), the flow through (FT, 0.1% of the total) material, and the first (Wash 1) and last (Wash 4) wash steps (0.3% of total of each). Purified material bound to the IgG agarose beads before and after elution with TEV protease (0.5% of total of each), as well as the eluted Pop2p complex (370 ng, representing approximately 8% of the total) were also analyzed. Molecular weights (in kilodaltons) are indicated on the left. On the right, the mobility of Pop2p-TAP, and Pop2p after TEV cleavage, in addition to TEV protease, is indicated. Also, the likely mobility of the Ccr4p deadenylase subunit is shown.

2.7.4. Lyse cells

Resuspend cell pellets, on ice, in 1 packed cell volume of TNEMN150 buffer (50 m*M* Tris-HCl (pH 8), 0.5% (v/v) Nonidet P40, 1 m*M* EDTA, 2 m*M* $MgCl_2$ and 150 m*M* NaCl) supplemented with 2× protease inhibitor cocktail (Complete, Roche). Dispense suspension into prechilled microfuge tubes containing 500 μl dry volume of sterile acid-washed glass beads (500 μm in diameter, Sigma). Mechanically lyse cells by "bead-bashing" for 15 min with a vortex genie with a bead-basher attachment (Disruptor Genie, Scientific Industries, Inc.). Clarify lysates at 16,000*g* at 4 °C for 10 min and then combine supernatants (the whole cell extract) in an ice-cold 15-ml conical tube.

2.7.5. Bind deadenylase to beads

Add rabbit IgG agarose beads (120 μl of a 1:1 slurry, binding capacity of 5 mg/ml of resin, Sigma) to the whole cell extract and allow binding to occur for 2 h at 4 °C on a rocking platform (for example, a Nutator).

2.7.6. Wash beads

Collect beads by centrifugation in a refrigerated tabletop centrifuge at 1000*g* for 5 min. Wash beads four times with (15 ml) 100-bed volumes of TNEMN150. The first wash will contain cellular protein, similar in pattern to that in the whole-cell extract or "flow-through" material (Fig 5.3A, lanes 1-3); the fourth should be virtually devoid of protein (Fig. 5.3A, lane 4).

2.7.7. Exchange buffer

To remove detergents and equilibrate purified protein in the downstream deadenylation buffer, wash the beads two times with 15 ml of deadenylation buffer (50 m*M* Tris-HCl, pH 8, 20 m*M* NaCl, 0.1% $MgCl_2$, 10% glycerol).

Major bands from IgG heavy and light chains, present on the IgG agarose beads, are indicated. (B) To further assess purity and composition, the TAP-purified Pop2p deadenylase complex (370 ng), after TEV elution, was analyzed by SDS-PAGE and silver staining. Approximately a dozen protein bands are abundant, with major bands of 50 kDa and 95 kDa corresponding to Pop2p and Ccr4p, respectively. (C) Western blot analysis, with peroxidase/antiperoxidase of purified Pop2-TAP material bound to IgG agarose beads before and after TEV cleave and elution (0.5% of total for each). Two exposures of the same blot are shown (a short exposure is on the right while a 100-fold longer exposure is on the right). TAP-tagged Pop2p migrates at approximately 71 kDa. Several minor degradation products are seen under the major 71-kDa band. TEV cleavage liberates the Pop2p protein from the IgG binding domains of the TAP tag. The TAP tag fragment remains bound to the IgG beads, seen in the longer exposure (right panel). A minor amount of uncleaved Pop2-TAP remains on the beads after TEV cleavage.

2.7.8. Elution of deadenylase complex

Elute purified deadenylase complex from the beads with TEV protease (AcTEV, Invitrogen). Cleavage can be performed for 12 h at 4 °C with 8 U of AcTEV in 1-bed volume (60 μl) of deadenylation buffer.

2.7.9. Analyze purified deadenylase complex

Measure the protein concentration of the eluted deadenylase by Bradford assay (Bio-Rad) or similar methods. Assess the purity and composition of the deadenylase complex by SDS-PAGE followed by Coomassie and silver staining (Fig. 5.3A and B). Western blotting of the deadenylase subunits is advisable (Fig. 5.3C). For TAP-tagged deadenylase subunits, a mixture of peroxidase/antiperoxidase (Sigma, at 1 to 5000-fold dilution), which recognizes the IgG binding domains of the TAP tag, is used to detect the purified Pop2p-TAP protein. Western analysis of a portion of the bead-bound material should reveal a strong signal at MW of 71 kDa (Fig. 5.3C). TEV cleavage efficiency can be monitored by Coomassie staining and Western blotting of the beads before and after cleavage (Fig. 5.3A, lanes 5 and 6, and Fig. 5.3C, lanes 1 and 2). After TEV treatment, the IgG binding domain of the TAP tag is detected on the beads (Fig. 5.3C, lane 2). Because the IgG binding domain is removed from Pop2p, the eluted Pop2p deadenylase is no longer detectable by peroxidase/antiperoxidase. When purifying Pop2p-Ccr4p complexes, we also coexpressed T7-tagged Ccr4p with the TAP-tagged Pop2p. Copurification of both deadenylase subunits was confirmed by Western blotting with anti-T7 (Novagen) and peroxidase/antiperoxidase antibodies, respectively (Goldstrohm *et al.*, 2006, 2007).

2.8. Test enzymatic activity of purified complex

The next step is to test the enzymatic activity of the purified deadenylase and determine the optimal amount of enzyme. To measure deadenylation rates, collect samples from the reactions over a time course, typically between 0 to 120 min. The relative rate can be adjusted by altering the enzyme concentration. An example of such a time course analysis of deadenylation activity versus protein concentration is shown in Fig. 5.4A for the yeast Pop2p deadenylase complex. At low concentrations or short times, very little activity is observed (Fig 5.4A, lanes 9 to 14); longer times and high concentrations result in degradation of all RNA molecules (Fig. 5.4A, lanes 3 to 8).

2.8.1. Control reactions

To control for potential ribonuclease contamination of reaction components, include a reaction in which substrate RNA is incubated with deadenylase buffer (Fig. 5.4B, lanes 3 to 8). To control for nonspecific ribonuclease contamination of the purified deadenylase preparation,

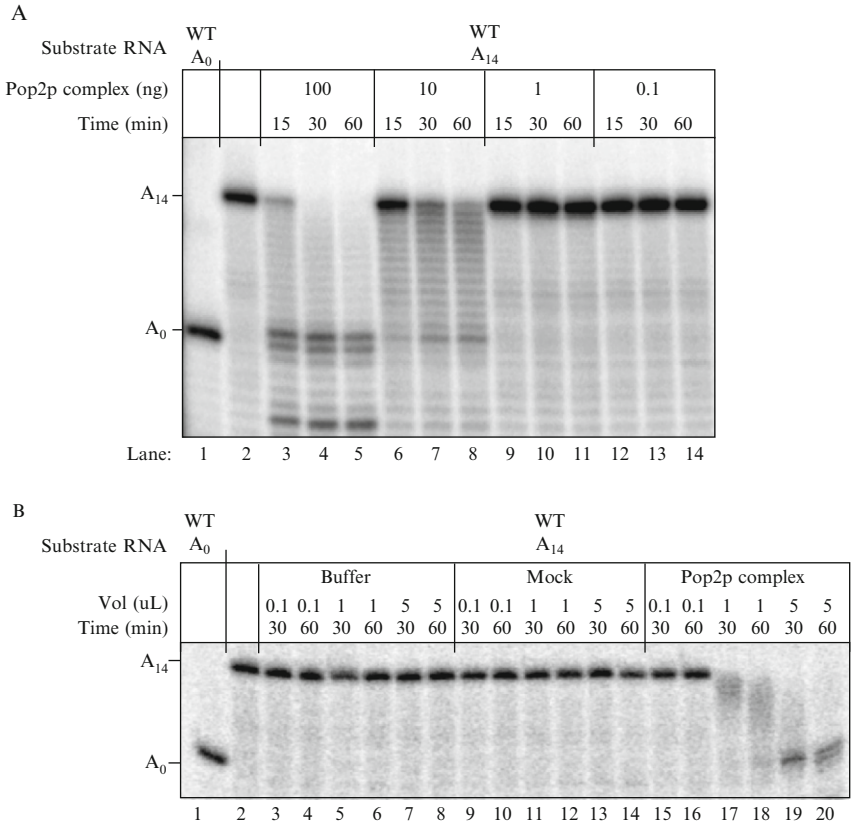

Figure 5.4 Assaying the enzymatic activity of the purified deadenylase complex. (A) The enzymatic activity of the TAP tag–purified Pop2p deadenylase complex was measured with *in vitro* deadenylation assays. Deadenylation was time and concentration dependent. Decreasing amounts of the Pop2p complex (indicated at the top) were incubated with radioactively labeled substrate RNA with 14 adenosine residues at its 3′ end (WT A_{14}). Reaction times are indicated at the top. Reaction products were then separated by denaturing polyacrylamide gel electrophoresis. The migration of marker RNAs without (WT A_0) or with poly(A) tail (WT A_{14}) are indicated on the left side; 100 ng of Pop2p complex was sufficient to rapidly degrade the poly(A) tail, and, in fact, degradation proceeded into the body of the RNA substrate. At 10 ng, the deadenylase complex slowly degraded the poly(A) tail, with fully deadenylated product accumulating at later time points. Lower concentrations of deadenylase were inactive. (B) Control deadenylation reactions were performed by incubating labeled substrate RNA with deadenylation buffer alone to control for ribonuclease contamination or with eluate from a mock TAP purification. As a positive control, purified Pop2p complex was included, resulting in deadenylation of the substrate.

perform a mock TAP purification from a wild-type yeast strain that lacks a TAP-tagged protein. No degradation of substrate should be observed with the mock-purified material (Fig. 5.4B, lanes 9 to 14), whereas an equal

volume of purified deadenylase is fully active (Fig. 5.4B, lanes 15 to 20). Another excellent control is to purify mutated, catalytically inactive versions of the deadenylase and test this material for the absence of deadenylation, thereby proving that the observed activity originates from the deadenylase enzyme and not a contaminant (Goldstrohm *et al.*, 2007).

2.8.2. Assemble deadenylation reactions

Use the following general method to test the activity of the purified deadenylase complex. Results for analysis of the Pop2p complex isolated from yeast are presented in Fig. 5.4.

Dilute substrate RNA to 200 fmol/μl concentration with deadenylation buffer.
Boil for 5 min; place on ice.

Assemble reactions on ice. Titrate the purified deadenylase complex over a wide range of concentrations (for instance, a 1000-fold range was tested in Fig. 5.4).

Purified deadenylase complex (0 to 100 ng of Pop2p-TAP complex) 200 fmol of substrate RNA (final concentration, 10 n*M* or 10 fmol/μl).
20 μl final reaction volume in deadenylation buffer.
Collect 5 μl from each sample. This is the zero time point sample. Transfer sample to ice-cold microfuge tube containing 5 μl of denaturing gel loading dye [95% (v/v) formamide, 18 m*M* EDTA, 0.025% (w/v) SDS, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue].
Incubate at 30 °C in a waterbath.
Collect 5-μl samples at 15-, 30-, and 60-min time points. Place samples in ice-cold microfuge tubes with 5 μl of denaturing gel loading dye, which effectively terminates the reaction.
Place on ice or −20 °C until ready to load gel.
Samples can be stored at −80 °C.
Boil the samples for 5 min before loading onto gel.

2.8.3. Analyze products on a denaturing polyacrylamide gel

Prepare 12% (w/v) acrylamide gel with 29:1 acrylamide/bis-acrylamide ratio, 7 *M* urea and 1× TBE buffer with a size of 15 × 25 cm × 0.5 mm. Note that the gel percentage will vary depending on the size range of RNAs being analyzed.

Prerun gel for 30 min at 1100 volts.
Flush urea and unpolymerized acrylamide from gel wells with running buffer before loading samples.
Load samples into gel wells.

Electrophorese gel at 1100 volts for approximately 1 h. Stop current when the bromophenol blue dye is approximately 2 inches from the bottom of the gel.
Carefully transfer the gel to 3MM Whatman paper and wrap in plastic.
Expose gel to a PhosphorImager screen.
Visualize data with a PhosphorImager.

2.8.4. Interpret data

Analysis of the *in vitro* deadenylation data should demonstrate concentration- and time-dependent shortening of the substrate RNA by the deadenylase (Fig. 5.4A). Deadenylation should halt, or substantially pause, once the 3′-adenosines are removed. Endonucleolytic cleavage products, with no evidence of progressive shortening, are telltale signs of *Exoribonucleolytic* contamination. No degradation should be observed in the control reactions, either mock or deadenylase buffer alone (Fig. 5.4B).

To detect enhancement of deadenylation by a regulator, the purified deadenylase must be carefully titrated so that its activity is rate limiting (for instance, see Fig. 5.4A, lanes 6, 7, and 8). For analysis of proteins that inhibit deadenylation, it will be desirable to use more enzyme, although not a vast excess, so that deadenylation is efficient in the absence the regulator (Fig. 5.4A, lanes 3 to 5). Very high amounts of enzyme will cause very rapid deadenylation, and in some cases, the enzyme will begin to degrade the body of the substrate RNA (Fig. 5.4A, lanes 3 to 5).

2.9. Purify recombinant regulator

Purify regulators, fused to an affinity tag such as GST, from *Escherichia coli* with standard methods (2007a). We purified PUF-GST fusion proteins for use in deadenylation assays (Goldstrohm et al., 2006). After binding to glutathione-agarose beads, wash the bead-bound regulator three times with high salt (500 m*M* NaCl) buffer and detergent (0.5% Nonidet P40). These stringent washes help remove contaminating ribonucleases. Next, wash the beads twice with deadenylation buffer to remove high salt and detergent. Finally, elute GST fusion protein in deadenylation buffer containing 10 m*M* glutathione. We elute in deadenylation buffer to match buffer conditions with downstream deadenylation and RNA-binding assays. Elution conditions should maintain solubility and activity of the regulator. For PUF proteins, like GST-Puf4p, we typically include up to 50% glycerol in the eluted stock to maintain protein solubility.

2.9.1. Analyze purified regulator

Measure the protein concentration of the purified regulator by Bradford assay (Bio-Rad) or similar methods. Assess the purity by Coomassie blue staining of protein separated by SDS-PAGE gel (Fig. 5.5A).

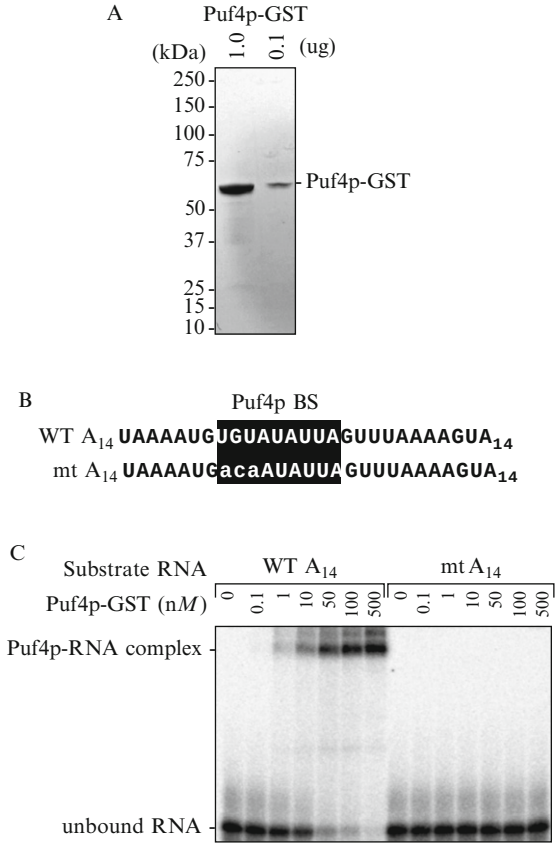

Figure 5.5 Measuring the RNA-binding affinity and specificity of purified RNA-binding protein, Puf4p. (A) Purified, recombinant yeast Puf4p, fused to the GST affinity tag, was purified from *E. coli* and analyzed by SDS-PAGE and Coomassie blue stain (1.0 and 0.1 μg of Puf4p-GST). (B) RNA substrates used in RNA binding and deadenylase assays. The wild-type synthetic substrate contains the Puf4p binding site from the yeast HO mRNA and 14 adenosines at its 3′-end (WT A_{14}). In the mutant RNA version (mt A_{14}), the UGU trinucleotide that is critical for Puf4p binding was changed to ACA. (C) The RNA-binding affinity and the specificity of the purified Puf4p-GST was determined by electrophoretic mobility shift assay. Increasing concentrations, indicated at the top of the gel, of Puf4p-GST were incubated with either the WT A_{14} or mt A_{14} radioactively labeled substrate RNAs. Puf4p-GST bound wild-type RNA with an approximate dissociation constraint of 10 ± 5 n*M* while no binding was detected with the mutant RNA (Hook *et al.*, 2007).

2.9.2. Test activity of purified regulator

Assess the RNA-binding activity of the regulator before proceeding to deadenylation assays. To measure RNA-binding activity and specificity, we perform electrophoretic mobility shift assays (EMSA) with radioactively labeled RNA substrates, both wild-type RNAs and versions with mutations

in the regulator's binding site (Hellman and Fried, 2007). For Puf4p, we used two substrate RNAs; the first RNA had a wild-type Puf4p binding site from *HO* mRNA, and the second substrate contained mutations in that binding site (Fig. 5.5B) (Hook *et al.*, 2007). It is important that the EMSAs be performed in deadenylation reaction buffer with a range of regulator concentrations. Results from this experiment are used to determine the amount of regulator used in subsequent deadenylation reactions.

2.9.3. Electrophoretic mobility shift assay (EMSA)

Determine the RNA-binding activity of purified regulatory protein with the following protocol. As an example, EMSA analysis of Puf4p binding to either wild-type or mutant RNA substrates is shown in Fig. 5.5.

Prepare native polyacrylamide gel. Typically we use a 15 × 15 × 1-mm gel composed of 6% polyacrylamide with 29:1 acrylamide/bis-acrylamide and 0.5× TBE running buffer.
Boil RNA for 5 min; place on ice.
Assemble RNA-binding reaction.
200 fmol of radioactively labeled substrate RNA (wild-type or mutant).
Purified regulator protein (0 to 500 n*M* GST-Puf4p).
20 μl final volume in deadenylation buffer.
Incubate the reactions at 25 °C for 30 min.
During the binding reaction, prerun gel at 300 volts for 30 min at 4 °C with 0.5× TBE running buffer.
Add 4 μl of 5× EMSA loading buffer (10% Ficoll 400, 5% DMSO, 0.1% bromophenol blue).
Flush gel wells thoroughly with running buffer.
Load samples onto gel.
Electrophorese for 3 h at 300 volts and 4 °C.
Transfer gel to Whatmann 3MM paper, wrap with plastic, expose to PhosphorImager screen.
Visualize data with a PhosphorImager.

2.9.4. Interpret data

Determine apparent binding constants (K_d) from the RNA-binding data. Binding must be specific, as judged by a comparison of apparent K_d for mutant and wild-type RNAs. For instance, we observed high-affinity, specific binding of Puf4p-GST to wild-type RNA substrate, but not a mutant version (Fig. 5.5B and C). If nonspecific binding to the mutant target is observed, alter conditions to establish specificity while maintaining high affinity. Titration of competing nucleic acids, such as tRNA or synthetic ribopolymers, is a common practice for optimizing binding specificity (Hellman and Fried, 2007).

2.10. Confirm protein interactions

Recruitment of the deadenylase obviously is dependent on protein–protein interactions between the regulator and deadenylase complex (Fig. 5.1). For example, Puf4p binds directly to the Pop2p subunit of the Ccr4p-Pop2p deadenylase complex, as do other PUF proteins (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007). It is essential that the reaction conditions used to assay deadenylation are compatible with those needed for the protein–protein contacts. These contacts can be assayed with purified regulator and deadenylase complex by means of assays such as coimmunoprecipitation or GST pull down (Goldstrohm *et al.*, 2006; Hook *et al.*, 2007).

2.11. Assay-regulated deadenylation *in vitro*

Measure the effect of a regulator on deadenylase activity by combining the regulator, a limiting amount of enzyme, and a substrate RNA with a high-affinity binding site for the regulator (Fig. 5.6). Regulation by recruitment depends on several factors, including the RNA-binding activity of the regulator, protein interactions between regulator and deadenylase, and the enzymatic activity of the deadenylase. Recruitment results in the acceleration of the deadenylation rate (Fig. 5.6).

To measure sequence-specific deadenylase recruitment by a regulator, both wild-type and mutant RNA substrates should be tested. The reaction rate in the presence of the regulator is compared with that of the deadenylase alone. Titration of the regulator may be necessary to observe maximum effect. Ideally, we use the minimal amount of regulator that binds all of the substrate RNA, as determined in the EMSA analysis. Note that excess regulator can potentially inhibit deadenylation, essentially squelching the recruitment of deadenylase. Finally, the order of addition of reaction components may affect the observations. We have found that preincubation of the regulator and deadenylase on ice, although not essential, can facilitate formation of the regulator–deadenylase complex. Subsequently, the substrate RNA is added, and the reactions are immediately shifted 30 °C to allow deadenylation to occur.

Control reactions should include substrate RNA incubated with deadenylase buffer alone and regulator alone (as controls for nuclease contamination of reaction components) and deadenylase alone (as a baseline level of activity). As mobility markers for the denaturing gel analysis, include labeled substrate RNA with and without a poly(A) tail (Fig. 5.6A, lanes 1 and 2).

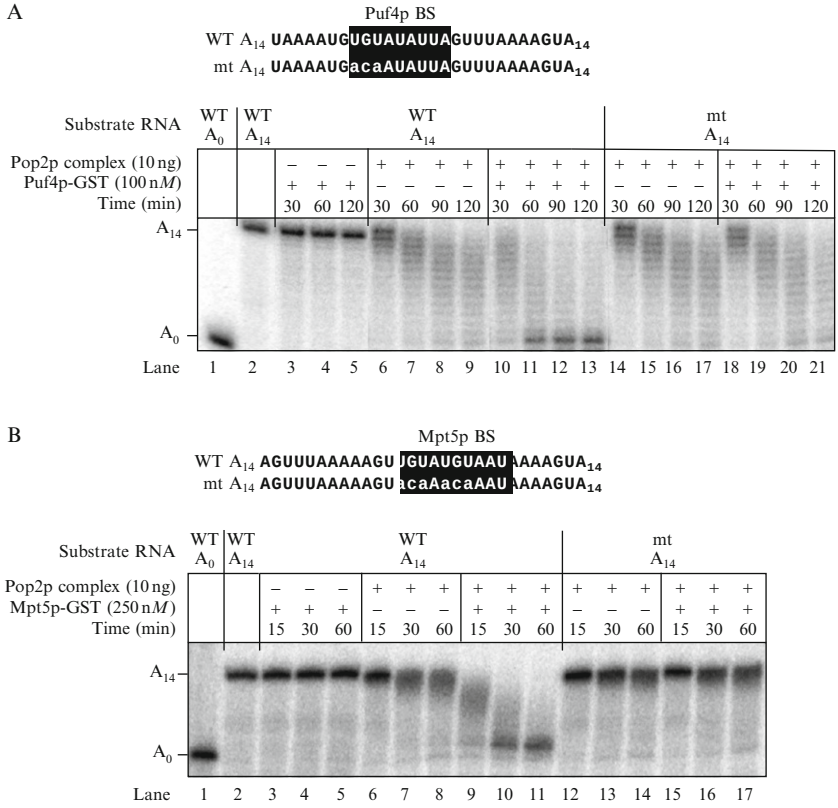

Figure 5.6 PUF proteins recruit and enhance deadenylation by the Pop2p deadenylase complex. (A) Puf4p enhances deadenylation. Deadenylation of wild-type RNA (WT A_{14}) containing a Puf4p binding site and, at the 3′-end, 14 adenosines, was analyzed in the presence of purified Pop2p deadenylase complex (10 ng) without (lanes 6 to 9) or with Puf4p-GST (100 n*M*, lanes 10 to 13) for the indicated times. As a negative control, Puf4p-GSTwas incubated alone with the substrate RNA (lanes 3 to 5). The Pop2p complex slowly deadenylated wild-type RNA, and when Puf4p was combined with the Pop2p complex, deadenylation was enhanced, resulting in the accumulation of fully deadenylated RNA (A_0). Mutant substrate RNA (mt A_{14}), which Puf4p could no longer bind, was deadenylated by the Pop2p complex at the same rate as wild-type RNA (lanes 14 to 17); however, addition of Puf4p had no effect on deadenylation of the mutant RNA (lanes 18 to 21). Sequences of the synthetic substrate RNAs are shown at the top. The migration of marker RNAs without (WT A_0) or with poly(A) tail (WT A_{14}) are indicated on the left side. (B) Mpt5p accelerates deadenylation. A similar, but separate, analysis with the PUF protein Mpt5p and RNA substrates (shown at the top) with either wild-type or mutant Mpt5p binding sites (BS) demonstrates regulated deadenylation with a limiting amount of Pop2p complex deadenylase activity.

2.11.1. Assemble deadenylation reactions

Dilute substrate RNA to 200 fmol/μl concentration with deadenylation buffer.
Boil for 5 min; place on ice.
Assemble reactions on ice. First add deadenylase buffer, then enzyme, then regulator protein.
Purified deadenylase complex (10 ng of Pop2p complex).
Purified regulator (100 n*M* of GST-Puf4p).
20 μl final reaction volume in deadenylation buffer.
Preincubate on ice up to 15 min.
Add 200 fmol of substrate RNA (final concentration, 10 n*M* or 10 fmol/μl).
Collect 5 μl from each sample. This is the zero time point sample. Transfer sample to ice cold microfuge tube containing 5 μl of denaturing gel loading dye.
Incubate at 30 °C.
Collect 5-μl samples at 15-, 30-, and 60-min time points. Place samples in ice-cold microfuge tubes and add 5 μl of denaturing gel loading dye.
Store samples on ice or −20 °C until ready to load gel.
Boil the samples for 5 min before loading onto gel.
Analyze samples by denaturing polyacrylamide gel as described earlier.

2.11.2. Interpret data

As an example of data analysis, consider Fig. 5.6A, in which we analyzed regulation of Ccr4p–Pop2p deadenylase complex by Puf4p. The RNA substrate in the control reaction, containing only the regulator (Puf4p), was unaffected (Fig. 5.6A, lanes 3 to 5). If degradation is observed, this is indicative of nuclease contamination (see ''Troubleshooting'' section). RNA incubated with the limiting amount of purified deadenylase was slowly deadenylated, with very little fully deadenylated product seen at the longest time point (Fig. 5.6A, lanes 6 to 9). If the regulator enhances deadenylation by recruiting the enzyme to the substrate, deadenylation of wild-type substrate will be accelerated when both are combined, resulting in the accumulation of fully deadenylated RNA over time (Fig. 5.6A, lanes 10 to 13). In some instances, the regulator may hyperactivate the deadenylase, causing degradation to proceed beyond the poly(A) tail and into the body of the RNA substrate (Hook *et al.*, 2007). With deadenylase alone, the rate of poly(A) shortening of the mutant substrate should be identical to wild-type RNA (Fig. 5.6A, lanes 14 to 17); however, in the presence of both regulator and deadenylase, deadenylation should not be enhanced, because RNA-binding and recruitment cannot occur (Fig. 5.6A, lanes 18 to 21). Mutations that disrupt the interaction between the two proteins are ideal additional controls. An equivalent analysis with a different PUF protein, Mpt5p, is included for comparison (Fig 5.6B). Like Puf4p,

Mpt5p enhances deadenylation by the Ccr4p-Pop2p complex (Goldstrohm *et al.*, 2006). In the example shown in Fig. 5.6B, the activity of Pop2p complex added was lower than Fig. 5.6A, and, hence, very little deadenylation was observed in the absence of the PUF protein or its binding site (Fig. 5.6B, lanes 6 to 8 and 12 to 14). The addition of Mpt5p greatly accelerated deadenylation of the wild-type RNA substrate, with completely deadenylated RNA observed at 60 min (Fig. 5.6B, lanes 9 to 11), whereas the RNA substrate with mutated Mpt5p binding site was unaffected (Fig. 5.6B, lanes 15 to 17).

3. Troubleshooting

3.1. Enzyme concentration

Establishing the right amount of purified deadenylase complex is essential to the success of these assays. Too much enzyme leads to rapid degradation of the substrate, likely obscuring any enhancement. Too little enzyme, or enzyme with poor activity, obviously also prevents analysis. For this reason, proper storage of the enzyme is necessary. For short-term storage, we maintain small aliquots of enzyme in deadenylation buffer at −20 °C. Long-term storage is done at −80 °C. After a month or so, we prefer to prepare freshly purified deadenylase. Because the specific activity of purified enzyme can vary over time and between preparations, it is essential to titrate the deadenylase before assaying regulated deadenylation.

Comparison of Fig. 5.6 A and B, both of which lead to readily interpretable results, reveals the effect of differences in Pop2p complex concentration and activity. The higher activity used in Fig. 5.6A leads to more deadenylation in the absence of the PUF protein (compare Fig. 5.6A lanes 6 to 8 with 5.6B lanes 6 to 8).

3.2. Regulator concentration

The amount of RNA-binding regulator used in the *in vitro* deadenylation reactions is critical. Carefully determine the RNA-binding activity by titration, and use this information to guide the amount of regulator used in deadenylation assays. Still, it may be necessary to also titrate the regulator in deadenylation assays. If too little regulator is used, then enhanced deadenylation cannot occur because most of the RNA is not bound. Too much regulator inhibits sequence-specific deadenylation, either because most regulator-deadenylase complexes are not engaged with the RNA or because the RNA-binding protein nonspecifically binds the RNA, blocking deadenylase access.

3.3. Contaminating ribonuclease

These assays are extremely sensitive to contaminating nucleases, and all precautions should be taken at all steps to ensure RNase-free procedures, reagents, glassware, and plastic ware. Addition of RNase A inhibitors, such as RNAsin (Promega), may help diminish common sources of contamination, but we recommend avoiding other chemical RNase inhibitors or chelating agents that may inhibit deadenylases. The addition of competing nucleic acids may help diminish the effect of low levels of contaminants but may require subsequent adjustment of deadenylase concentration. In our experience, recombinant proteins from bacterial sources are a common source of contamination. To reduce this problem, increase the stringency of washing steps during the purification procedure, including with high salt (1 *M* NaCl), more detergent, additional washes with more volume, and longer duration.

3.4. Buffer conditions

High demands are placed on the buffer conditions used in these assays. The buffer must be compatible with deadenylase enzymatic activity, regulator-deadenylase protein interaction and, high affinity, specific RNA-binding by the regulator. Arriving at these conditions can require considerable effort. In some cases, compromising one or more activities may be necessary. Conditions that are suboptimal for one or more parameters may be required.

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