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Poly(A) tail dynamics: Measuring polyadenylation, deadenylation and poly(A) tail length

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Abstract

Transcription of mRNAs culminates in RNA cleavage and a coordinated polyadenylation event at the 3′ end. In its journey to be translated, the resulting transcript is under constant regulation by cap-binding proteins, miRNAs, and RNA binding proteins, including poly(A) binding proteins (PABPs). The interplay between all these factors determines whether nuclear or cytoplasmic exoribonucleases will gain access to and remove the poly(A) tail, which is so critical to the stability and translation capacity of the mRNA. In this chapter, we present an overview of two of the key features of the mRNA life-cycle: cleavage/polyadenylation and deadenylation, and describe biochemical assays that have been generated to study the activity of each of these enzymatic reactions. Finally, we also provide protocols to investigate mRNA's poly(A) length. The importance of these assays is highlighted by the dynamic and essential role the poly(A) tail length plays in controlling gene expression.

1. Introduction

Regulation of 3′ end processing is a crucial, dynamic post-transcriptional mechanism that alters transcript levels and/or translational potential in response to steady state as well as alterations in cellular homeostasis. Nearly all protein-coding mRNAs and long non-coding RNAs transcribed by RNA polymerase II (RNAP II) undergo a coupled co-transcriptional cleavage and polyadenylation (CpA) reaction, resulting in 200–300 adenosines synthesized on the 3′ end (Bentley & Coupling, 2014; Sheets & Wickens, 1989). More specifically, the newly transcribed precursor, which is complementary to the template DNA strand from which it was blueprinted, is catalytically cleaved in the phosphate backbone leaving a reactive 3′ OH group. Once endonucleolytic cleavage has occurred, a long string of non-templated adenosine residues is attached by a family of nucleotidyltransferases to the 3′ end of the transcript. For a more comprehensive overview of the process and history, the reader is directed to Proudfoot (2011). This two-part reaction is triggered by recognition of the poly(A) signal (PAS), the hexanucleotide sequence AAUAAA or one of its derivatives (Tian & Graber, 2012), which is required for both reactions to take place. The cleavage reaction takes place ~30 nucleotides downstream of the PAS and involves the recognition of less conserved sequence elements flanking either side of the poly(A) site. The poly(A) tail plays

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multiple roles in mRNA stability, translation regulation, nuclear export in an mRNA-specific fashion (Murphy & Kleiman, 2020a; Zhang, Virtanen, & Kleiman, 2010).

The CpA reaction is evolutionary conserved; while prokaryotes and archaea possess either oligonucleotide adenylate tracks or a CCA motif, multicellular eukaryotes have adopted polyadenosine (polyA) tails into the hundreds of bases. The universality of this two-step coupled process highlights its importance in normal cellular homeostasis. A myriad of factors from many different canonical pathways (Shi et al., 2009) are important for the efficacy of the CpA reaction, with a sizable repertoire of over 80 catalytic, structural, activating, repressing, upstream and downstream components in mammalian cells. A well-studied example of overlapping pathways is the role of transcription elongation/termination by RNAP II in CpA. RNAP II has a long C-terminal domain (CTD) of heptad repeats, which the presence and phosphorylation of are both key CpA requirements determined by structural, genome-wide and biochemical assays (Bentley & Coupling, 2014; Darnell, 2013; Ryan, Murthy, Kaneko, & Manley, 2002). An interesting early observation was that components of the cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF) complexes are deposited at the promoters of RNAP II-transcribed genes (Glover-Cutter, Kim, Espinosa, & Bentley, 2008; Hirose & Manley, 1998), allowing for co-transcriptional scanning of PAS. A consequence of such scanning and the selection of functional poly(A) hexamers is alternative polyadenylation within the same gene (Tian & Manley, 2017), a mechanism regulated by the core spliceosome component U1 snRNA genome-wide in a concentration dependent manner (Berg et al., 2012).

Once a transcript has been capped, polyadenylated, and released from the transcription machinery, several fail-safe methods have evolved to sense changes in cellular environment by changing the length of the poly(A) tail and controlling gene expression. Deadenylation is the physiological enzymatic removal of non-templated adenosine monophosphates primarily in mRNA and lncRNA, and this is accomplished by the action of deadenylase to trigger a cascade of events leading to regulation of mRNA transport, translation and degradation (reviewed in Goldstrohm & Wickens, 2008; Zhang et al., 2010). Deadenylases are categorized into two superfamilies based on their catalytic domains: DEDD-exonucleases, and exonuclease-endonuclease-phosphate (EEP)-nucleases. As there is a delay between deadenylation and mRNA decay (Decker & Parker, 1993) (Fig. 1A), deadenylation is commonly described as the initial and rate-limiting activity in mRNA turnover (Goldstrohm & Wickens, 2008; Zhang, Kleiman, & Devany, 2014). In fact, modeling predicts that deadenylation is by far the largest determinant of mRNA degradation, as both $5'-3'$ and $3'-5'$ nuclease activities are dependent upon poly(A) tail removal (Cao & Parker, 2001). Deadenylation allows fine-tuning of post-transcriptional regulation by having a number of decay intermediates with different length of poly(A) tails. The existence of a pathway that can control mRNA functions in response to stimuli allows the cell to adapt swiftly to a changing environment. For example, deadenylation-mediated mRNA decay not only prevents the expression of genes that are not necessary for stimulus response, but also allows the redirection of cellular resources to recover from the insult, such as during stress conditions (Zhang et al., 2014).

Several cis-acting sequence elements present in the 3′ untranslated region of target mRNAs have been shown to participate in the regulation of polyadenylation/deadenylation of specific mRNAs. Such cis-acting sequences are recognized by microRNAs, AU-rich element binding proteins, polyadenylation factors or other RNA binding factors. As the processing of the 3′ end of an mRNA can drastically affect gene expression, a number of genomic analysis have highlighted the relevance of PAS selection, cis-acting sequences and how the message is ended. However, direct analysis by biochemical approaches is necessary to have the mechanistic understanding required for the processing of specific genes.

2. Equipment

- Thermal cycler (Thermo Fisher)
- Heat block set to 65 °C for RNA denaturation
- Water bath set to 37 °C for RNase H reactions
- Northern blotting equipment (gel electrophoresis, transfer, visualization)
- Common molecular biology tools (pipettes, micro centrifuge tubes, etc.)
- Equipment for polyacrylamide gel electrophoresis (glasses, chamber, Owl, Thermo Fisher)
- Phosphorimager (FLA7000IP Typhoon)
- Storage phosphor screen and exposure cassette (Thermo Fisher)
- Douncer (Thomas Scientific)
- Nanodrop 2000 (Thermo Scientific)
- ImageJ software (http://rsb.info.nih.gov/ij/)

3. Chemicals

3.1 Buffer A

- 10 mM Tris pH 7.9 (Sigma Aldrich)
- 1.5 mM $MgCl_2$ (Sigma Aldrich)
- 10 mM KCl (Sigma Aldrich)
- 0.5 mM *dithiothreitol* (DTT, Sigma Aldrich)
- 0.5 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Aldrich)
- 100 × Protease Inhibitor Cocktail (PIC, MilliporeSigma)

3.2 Buffer C

- 20 mM Tris pH 7.9
- 25% glycerol (Sigma Aldrich)
- 1.5 mM $MgCl_2$

- 0.45 M NaCl
- 0.5 mM DTT
- 0.5 mM PMSF
- 100 × PIC

3.3 RNA-labeling mix

- Linearized pG3SVL-A and pG3L3-A (1 μL of 1 μg/μL)
- SP6 RNA Polymerase (1 μL of 10 U/μL, Promega)
- [α-^{32}P]-GTP (0.6 μL of 10 μCi/μL, PerkinElmer)
- 5′Me (Shi et al., 2009) G(5′)ppp(5′)G-Cap analog (3 μL of 5 mM, Promega)
- 5 × transcription buffer (3 μL)
  - 200 mM Tris Cl, pH 7.9
  - 30 mM $MgCl_2$
  - 50 mM DTT
  - 50 mM NaCl
  - 10 mM spermidine (Sigma Aldrich)
- RNasin, 0.6 μL of 40 U/μL (Promega)
- DTT, 1.5 μL of 0.1 M
- rNTP mix (1.5 μL of 5 mM of ATP, CTP, UTP/1 mM GTP, Promega)
- RNase free water to 15 μL (Millipore)

3.4 RNA purification

- TRIzol (Thermo Scientific)
- Chloroform (Sigma Aldrich)
- Isopropanol (Sigma Aldrich)
- 75% Ethanol (Sigma Aldrich)
- Phosphate-buffered saline (PBS):

  137 mM NaCl (Sigma Aldrich)

  2.7 mM KCl

  10 mM Na_2HPO_4

  1.8 mM KH_2PO4

3.5 Urea-PAGE gels

- 5% Polyacrylamide (Sigma Aldrich)

- 8.3 M Urea (Sigma Aldrich)

3.6 Cleavage mix

- 0.2–0.5 ng of labeled RNA
- 250 ng of tRNA (Sigma Aldrich)
- 0.25 unit of RNasin
- 9.6 mM HEPES-NaOH pH 7.9 (Sigma Aldrich)
- 9.6% Glycerol (Sigma Aldrich)
- 24 mM $(NH_4)_2SO_4$ (Sigma Aldrich)
- 20 mM creatine phosphate (Sigma Aldrich)

3.7 Deadenylation mix

- 25 mM HEPES pH 7.0
- 100 mM NaCl
- 0.1 mM EDTA
- 1.5 mM $MgCl_2$
- 0.5 mM DTT
- 2.5% polyvinyl alcohol (Sigma Aldrich)
- 10% glycerol
- 0.25U RNasin
- 10 nM ^{7}MeGpppG capped in vitro transcribed L3(A_{30}) RNA substrate

3.8 Oligo(dT)/RNase H-Northern blot analysis

- Oligo$(dT)_{12–18}$ primer (Invitrogen)
- RNase H (Invitrogen) and 10 × RNase H reaction buffer (Invitrogen)
- RNA isolated from cells (at least 10 μg total RNA per condition)
- 0.25U RNasin

3.9 PAT assay

- Oligo(dT)-anchor primer (5′-GGGGATCCGCGGTTTTTTTTTT-3′)
- Gene-specific primer (located 200–400 bp upstream the poly(A) site)
- GoScript Reverse Transcriptase (Promega)
- PCR Nucleotide Mix, 10 mM of each dNTP (Promega)
- 25 mM $MgCl_2$
- 5 × GoScript Reaction Buffer (Promega)

- GoTaq PCR mix (Promega)
- 1 μg per condition RNA isolated from cells
- T4 Polynucleotide Kinase (New England Biolabs)
- T4 PNK 10 × Reaction Buffer (New England Biolabs)
- γ-^{32}P ATP (3000 Ci/mmol, 5 mCi/mL, PerkinElmer)
- DNA ladder (New England Biolabs)

3.10 Tris-EDTA (TE) buffer

- 15.759 g of Tris-Cl pH 8
- 2.92 g of EDTA pH 8
- Add distilled water to 1 L

3.11 2 × RNA loading buffer

50% Formamide (Sigma Aldrich)

20% Glycerol

6.5% Formaldehyde (sigma Aldrich)

1 × MOPS buffer pH 7

41.86 g of MOPS free acid (Sigma Aldrich)

4.1 g of sodium acetate (Sigma Aldrich)

3.72 g of Na_2 EDTA (Sigma Aldrich)

Adjust pH using NaOH

Add dH_2O until volume is 1 L

0.05% Bromophenol Blue (Fisher Scientific)

0.05% xylene cyanol (Fisher scientific)

3.12 Saline-sodium citrate (SSC) buffer

175.3 g of NaCl

77.4 g of sodium citrate (Sigma Aldrich)

Adjust to pH 7 with 14 N HCl

Add dH_2O until volume is 1 L

4. Cleavage and polyadenylation

4.1 Introduction

Most of our understanding of CpA has come from biochemical characterization of the process in mammals. Fractionation of HeLa cell nuclear extract resulted in the purification

of the CPSF and CstF protein complexes, which recognize a functional PAS to promote RNA cleavage in the phosphate backbone leaving a reactive 3′ OH group. While the subunit CPSF-160 directly binds to the RNA at the PAS, subunit CPSF-73 contains the enzymatic activity for endonucleolytic cleavage at the poly(A) site, several nucleotides downstream of the PAS. Helping to coordinate the architecture of CpA machinery is recognition of a GU-rich sequence downstream of the poly(A) site by CstF complex. Similarly, poly(A) polymerase (PAP) was also found to be recruited to this 3′ processing complex and shown to add a long string of non-templated adenosine residues on the 3′ to denote the end of the transcript and affect stability on the RNA. The CTD of RNAP II is also necessary for efficient CpA, partly through recruitment of CPSF and CstF components. It was found that CPSF is required to induce polymerase pausing once it encounters a functional PAS, suggesting that CstF and CPSF travel along with RNAP II during nascent gene transcription in search of the PAS. Other major components of the CpA machinery include cleavage factor (*CF*) required for the cleavage reaction, *CF* IIm involved in the binding to RNAP II CTD and other CpA factors (reviewed in Proudfoot, 2011).

The universality of this two-step coupled process highlights its importance in normal cellular homeostasis. Biochemical characterization of CpA was further achieved using genetic screens in yeast, with some factors homologous to their mammalian counterparts and others apparently unique to yeast. CpA has been shown to be a vital part in controlling gene expression by affecting the RNA's lifespan, regulating its stability, translational efficiency, and export from the nucleus. As the presence/absence of a poly(A) regulates these processes, the length of a poly(A) tail can itself be regulated for the same purposes.

While the CpA reaction can be reconstituted in vitro using purified fractions from different cell nuclear extracts, it cannot be done using recombinant proteins due to the complexity of the factors needed in the CpA reaction and the extensive post-translational modifications they undergo. These modifications include: arginine methylation, lysine sumoylation, lysine acetylation, and the phosphorylation of serine, threonine and tyrosine residues. As these modifications occur in different cellular conditions, such as stress, proliferation, differentiation and development, it is important to study CpA in small-scale nuclear extracts from adherent cells. The use of these small-scale nuclear extracts allows for functional studies to be performed by depleting factors involved in the reaction. While CpA can be tested with endogenous substrates or tagged systems carrying functional PAS, purified fractions are generally tested with radiolabeled in vitro transcribed RNA carrying a PAS, such as simian virus 40 (SV40) late pre-mRNA and human adenovirus-2 L3 pre-RNA.

4.2 Nuclear extract preparation from adherent cells for CpA or deadenylation

1. Culture cells to ~80% confluency in a 10cm dish in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1% penicillin-streptomycin mix. Adherent cells such as human colon cancer HCT116 and cervical cancer HeLa cells have been used.
2. Remove media, wash once with *phosphate-buffered saline* (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.) and aspirate.

3. Treat cells with stressor or drug. For example, for UVC treatment, place plate in a Stratalinker 1800 (Stratagene) and deliver 2 pulses of 20J/m^2, replace media and incubate for 2h at 37 °C.

4. Harvest cells at ~80% confluency (~3 ×10^7 cells per 10cm plate).

5. Wash 2 × with 10 mL cold PBS, add 5 mL cold PBS and scrape cells into 15 ml falcon tube.

6. Spin 1000g for 5 min at 4 °C.

7. Resuspend the pellet in 4 mL Buffer A (10 mM Tris pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and 1 × PIC). Incubate for 10 min on ice.

8. Transfer resuspended cells into a 7 ml glass dounce tissue homogenizer, and dounce 20 times with a “tight” pestle (type B). While the hypotonic conditions will soften the cell membrane, douncing serves to break the cell membrane leaving the nucleus intact

   Tips:

   *Keep the douncer on ice as often as possible. Try to keep the douncer submerged the entire time, or be very gentle in removing the pestle from the liquid to avoid the introduction of bubbles.

   *Place the douncer on a hard surface at a slight angle. Press the pestle into the liquid at a slightly higher angle to create friction against the side of the tube.

   *When the bottom of the tube is reached, bring the pestle parallel with the tube and retract it slowly. Repeat 20 times.

   *Between samples treated in different conditions, rinse the douncer and pestle with a small amount of detergent and milliQ water.

9. Transfer the sample to a fresh falcon tube and spin at 4000 rpm for 10 min at 4 °C to pellet the nuclei. Non-nuclear fraction is in the supernatant

10. Resuspend the pellet in 200 μL Buffer C (20 mM Tris PH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.3 M NaCl, 0.5 mM DTT, 0.5 mM PMSF, and 1 × PIC) and transfer to a microcentrifuge tube, keeping the sample on ice. (The high salt conditions will rupture the nuclear membrane, while the glycerol aids in freezing and storing aliquots).

11. Incubate the sample for 30 min at 4 °C in a rotator.

12. Centrifuge the samples at 13,000 rpm for 15 min at 4 °C. Aliquot the extracts into chilled Eppendorf tubes (~50 μL per aliquot) and store at −80 °C for future use. The extracts are good to use for CpA and deadenylation reactions for 2 weeks.

13. Dialysis of the extracts is optional and might be needed for certain samples. Dialysis in buffer D (20 mM Tris pH 7.9, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 50 mM ammonium sulfate) might be required.

Tips:

*The resulting supernatant will contain the soluble nuclear fraction.

*Measure the concentration of soluble nuclear fraction with bicinchoninic acid (BCA) protein assay or equivalent.

*Fractionation of subcellular components can be tested by Western blot against Lamin A/C or topoisomerase II for the nuclear fraction, and GAPDH or actin for the cytoplasmic fraction.

4.3 RNA substrate for CpA or deadenylation

1. First, plasmids have to be linearized (Fig. 1B–C). For in vitro CpA reactions use either pG3SVL-A or pG3L3-A, which contain the SV40 late PAS and adenovirus 2 L3 PAS, respectively. pG3SVL-A and pG3L3-A plasmids are linearized at the *Dra*I and BamHI sites, respectively. For deadenylation, digest plasmid pT3L3(A_{30}), which contains L3 RNA body followed by 30 residues of adenosine, with *NsiI*. To further investigate the specificity of the deadenylation activity, RNA substrates from ML43(G_{30}), ML40(C_{32}) and ML54(U_{30}) plasmids can be used.
2. Linearized pG3SVL-A and pG3L3-A are transcribed in vitro by SP6 RNA polymerase. Linearized pT3L3(A_{30}) and control plasmids are transcribed using T3 RNA polymerase.
3. The conditions for in vitro transcription and radioactive labeling are the following: in a 15 μL total volume reaction, substrate RNA is uniformly labeled by including [α-^{32}P]-GTP (0.6 μL of 10 μCi/μL) and 5′ capped during transcription using the 5′Me (Shi et al., 2009) G(5′)-ppp(5′)G-Cap analog (3 μL of 5 mM). Add appropriate RNA polymerase (1 μL of 10 U/μL) and 5 × transcription buffer (3 μL), (RNasin, 0.6 μL of 40 U/μL), linearized DNA (1 μL of 1 μg/μL), DTT (1.5 μL of 0.1 M), and rNTP mix (1.5 μL of 5 mM of ATP, CTP, UTP/1 mM GTP). This rNTP mix favors the incorporation of radiolabeled GTP into the RNA. Add RNase free water to 15 μL. Make sure to use Perspex shield at all times when working with radioactive substrate.
4. Incubate at 37 °C for 1h. (Take 1 μL to assess transcription and RNA purification efficiency during step 18).
5. Add 1 volume of 2 × RNA Loading Dye to final concentration (47.5% formamide, 0.01% SDS, 0.01% bromophenol blue (BFB), 0.005% xylene cyanol and 0.5 mM EDTA). This ensures that the RNA in your sample does not reform secondary structures after the heat denaturation step.
6. Heat at 70 °C for 5 min to denature RNA and immediately transfer to ice. The use of a denaturing system after heating your sample maintains the denatured state of your RNA.
7. The transcript is then purified on a 0.4 mm denaturing polyacrylamide gel (6% polyacrylamide, 8.3 M urea) in 1 × Tris/Borate/EDTA (TBE) buffer. Use a 4-well

comb. While heating the samples, setup the gel box and flush urea out of the wells with running buffer using a large tip.

8. Load samples and run gel at over 400V for ~1.5h until bromophenol blue reaches 1cm to the end of the gel.

9. Carefully open the gel glasses using a thin spatula and cover the gel with plastic wrap. Place the covered gel on a tray and expose on X-ray film for 2 min in a dark room. Remember to align the film and the gel well to allow the extraction of the labeled RNA.

10. Locate labeled RNA by the shadow on film and cut the region. Place the selected portion of the gel in an Eppendorf tube using a spatula. Carefully discard the rest of the gel into radioactive waste.

11. To gel purify the labeled RNA, add 500 μL of 0.3 M sodium acetate and 0.2% SDS. Elute by rotating for 3h at room temperature.

12. Centrifuge for 3 min at 3000 rpm. Take the solution and carefully discard the rest of the radioactive gel.

13. Add to the eluted RNA 1 μL of glycogen (20 mM) to facilitate the following precipitation.

14. Perform a phenol-chloroform extraction. Mix gently for 2–3 min and centrifuge at 10,000 rpm at room temperature for 10 min. Under acidic conditions, total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the interphase or in the lower organic phase.

15. Labeled RNA is then recovered by precipitation with 1 volume of isopropanol at −80 °C for 1h.

16. Centrifuge the sample at 13,000 rpm for 15 min at 4 °C. Wash the pellet with ethanol 70%. Let dry the pellet and then resuspend in 30 μL of nuclease-free water. Note: if the pellets dry out too much, the RNA crystallizes and is very difficult to solubilize. If it does not dry well, the RNA does not solubilize into solution. (Take 1 μL to assess transcription and RNA purification efficiency during step 18). Aliquot in tubes of 5 μL each and keep at −20 °C in a beta-box for emitting radioactive isotope samples.

17. To assess transcription and RNA purification efficiency, measure radioactivity from samples taken in step 4 and 17 (Fig. 2A). To the removed aliquots (1 μL), add 1 μL of glycogen (20 mM) and 100 μL of Tris-EDTA buffer. Add this mixture to an aqueous fluor solution and count in a scintillation counter to determine the total amount of radioactive nucleotide in the sample before and after purification of labeled RNA. The ratio of cpm of the sample after gel purification (step 17) to total cpm (step 4) is the fraction of labeled nucleotide incorporated into RNA. This value should generally be at least 30%. For more information on how to calculate RNA specific activity and yield please check https://www.thermofisher.com/us/en/

home/references/ambion-tech-support/nuclease-enzymes/tech-notes/determining-rna-probe-specific-activity-and-yield.html

4.4 In vitro 3′ cleavage reaction (Fig. 2B–C)

1. Master mix for a single reaction of total volume 12.5 μL contains 7.5 μL of the following: 2.5 μL of 12.5% polyvinyl alcohol (PVA), 1 μL of 0.25 M creatinine phosphate (CP), 1 μL of 0.5 mg/ml tRNA, 1 μL of 6.25 mM 3′ dATP (cordycepin), 0.25 μL of 20 U/μL nuclease-inhibitor, 1 μL of 25 mM EDTA pH 8, and 0.75 μL RNase-free water. As the nuclear extracts and the purified fractions might have PAP, 3′ dATP is added to facilitate observation of cleavage products without interfering with poly(A) addition. However, it has been shown that 3′ dATP can inhibit cleavage of some substrates, such as SV40 RNA. Extensive studies have shown that neither ATP nor hydrolysis of CP are essential for 3′ mRNA cleavage, suggesting that CP can function as a necessary cofactor or mimicking a phosphorylated one (Hirose & Manley, 1998; Khleborodova, Pan, Nagre, & Ryan, 2016). It is important to highlight that different RNA substrates might need slightly different conditions to reach maximum efficiency. For example, it has been shown that the requirements for magnesium concentrations can vary for different substrates (0–2.5 mM Khleborodova, Pan, Nagre, & Ryan, 2016).
2. Add to the 7.5 μL of master mix no more than 5 μL/reaction of nuclear extracts or purified fractions at concentrations no higher than 2 mg/mL. Sometimes dilution with buffer D is needed to compare nuclear extracts or purified fractions obtained from cells treated in different conditions.
3. Heat the labeled RNA to 80 °C for 2 min. Place on ice before adding 1 μL of it to the reaction mix in a concentration no higher than0.5 ng/μL. Alternatively, the RNA may be included in the master mix.
4. After addition of the RNA, the reaction mix is incubated at 30 °C for1.5h.
5. The reaction is stopped with the addition of 112.5 μL of 0.4 mg/mL of proteinase K, 20 mM Tris pH 7.9, 10 mM EDTA, 1% SDS, 100 mM NaCl. Incubate the sample (total volume of 125 μL) at 30 °C for 15 min.
6. Perform acid phenol-chloroform extraction. Mix gently for 2–3 min and centrifuge at 10,000 rpm at room temperature for 10 min.
7. To the upper aqueous phase, add 1 μL of glycogen (20 mM), 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice cold 100% ethanol. Mix thoroughly and precipitate at −80 °C for 1h.
8. Centrifuge the sample at 13,000 rpm for 15 min at 4 °C. Wash the pellet with ethanol 70%. Let the pellet dry and then resuspend in 10 μL of 90% formamide, 10 mM EDTA and BFB. Keep samples at −20 °C in a beta-box until analysis by gel electrophoresis.

9. Use 6% polyacrylamide/8.3 M urea gel in 1 × TBE buffer with a 32-well comb. Before loading, heat the sample to 60 °C for 2 min then place on ice. Remember to flush urea out of the wells with running buffer using a large tip before loading. Load the gel with 3 μL of each sample.

10. Run the gel at over 400V for ~1.5h until BFB reaches 1cm to the end of the gel.

11. Carefully open the gel glasses using a thin spatula and cover the gel with a piece of filter paper. Press the paper firmly onto the gel and carefully peel the paper, which should have the gel attached to it. Remember to label on the paper the directionality of the loaded samples. Cover the exposed gel side with plastic wrap.

12. Dry on a gel dryer with the paper side facing the vacuum. Dry for 15 min or until the gel is completely dry.

13. Expose to X-ray film in a light-tight cassette with intensifying screen at −80 °C. Alternatively, the gel can be visualized and quantitated using a Molecular Dynamics Storm Phosphorimager. Exposure time depends on strength of labeled RNA and efficiency of the reaction.

14. Determine the ratio between cleaved and un-cleaved RNA in each sample to quantitate cleavage efficiency. Relative cleavage is calculated as [cleaved fragment/(cleaved fragment+un-cleaved RNA substrate)] ×100. Quantifications can be done with ImageJ software (http://rsb.info.nih.gov/ij/).

4.5 In vitro polyadenylation reaction (Fig. 2B–C)

1. Master mix is similar to the one for cleavage reaction, with the replacement of 3′ dATP for ATP to allow the CpA reaction to proceed (2.5% PVA, 20 mM CP, 40 ng/mL tRNA, 0.25 mM ATP, 0.4 U/μL nuclease-inhibitor, 2 mM EDTA pH 8, and RNase-free water).

2. Samples are analyzed as in Section 4.4, steps 2–14.

5. Deadenylation

5.1 Introduction

Early experiments into the role of the mRNA poly(A) tail in translation utilized 3′ OH exonucleases isolated from nuclei to remove the tail. Later studies identified that both poly(A) specific endo- and exonucleases can remove the poly(A) tail from target mRNAs (Goldstrohm & Wickens, 2008; Webster, Stowell, Tang, & Passmore, 2017; Zhang et al., 2014). Deadenylation can lead to either the removal of the cap structure at the 5′ end, exposing the transcripts to digestion by a 5′-to-3′ exonuclease, or transcript degradation in the 3′-to-5′ direction. Thus, mechanisms involving poly(A) tail length control and degradation represent an additional check-point for regulation of gene expression (reviewed in Goldstrohm & Wickens, 2008; Zhang et al., 2014). One of the most characterized deadenylases so far is the CCR4-POP2-NOT complex, which belongs to the family of EEP nucleases. The most studied deadenylases in the DEDD-exonucleases family are poly(A)-

specific ribonuclease (PARN), which is a major deadenylase in mammals, and poly(A) nuclease (PAN), which is involved in early steps of poly(A) tail metabolism. Deadenylases localize both in the nucleus and the cytoplasm, and are involved in different cellular processes such as DNA damage response, non-sense mediated decay, cell differentiation, etc. Deadenylation results in either a decrease in mRNA stability followed by degradation by the exosome, or the transient modulation of the length of the poly(A) tail allowing a new round of poly(A) tail extension and translation.

As opposed to the CpA reaction, deadenylation can be reconstituted in vitro by both using recombinant proteins and purified fractions from different cell nuclear extracts. The in vitro reaction can be tested using an artificial radiolabeled substrate that is 5′ capped and includes sequences preceding the adenovirus-2 L3 PAS followed by 30 residues of adenosine. A construct containing a longer poly(A) is suitable for analyzing poly(A) intermediate regulation and the reader is directed to an in-depth protocol to that end (Webster et al., 2017). RNA substrates from ML43(G_{30}), ML40(C_{32}) and ML54(U_{30}) constructs can be used to further investigate the specificity of the deadenylating activity. Functional studies on deadenylases have been performed using reporter genes with modified cis-acting sequence elements or RNA structures or depleting factors involved in the reaction, allowing to test the effect of ribonuclease activities on mRNA stability of individual genes and on a global scale, respectively.

5.2 Deadenylation reaction (Fig. 2B–C)

1. The preparation of nuclear extracts from adherent cells and of RNA substrate is described in Sections 4.2 and 4.3.
2. Conditions for in vitro deadenylation in reaction volume of 15 μL:1.5 mM $MgCl_2$, 2.5% PVA (molecular weight, 10,000), 100 mM KCl, 0.15U ribonuclease inhibitor, 10 mM HEPES-KOH, pH 7, 0.1 mM EDTA, 0.25 mM DTT, and 10% glycerol. Heat the MeGpppG capped in vitro transcribed/radiolabeled L3(A_{30}) RNA substrate to 80 °C for 2 min, place on ice before adding 1 μL of it to the reaction mix in a concentration no higher than 0.5 ng/μL. To test the specificity of the deadenylating activity for poly(A), RNA substrates from ML43(G_{30}), ML40(C_{32}) and ML54(U_{30}) plasmids can be used.
3. Add no more than 5 μL/reaction of nuclear extracts to the master mix, purified fractions or recombinant factors at concentrations no higher than 2 mg/mL.

   Tips:

   *Sometimes dilution with buffer D is needed to compare different samples. When testing the influence of factors on recombinant ribonuclease activity, the in vitro reconstituted deadenylation reactions have to be done using a limiting amount of deadenylase and in the absence or presence of increasing amounts of the regulatory factor.
4. After addition of the RNA, the reaction mix is incubated at 30 °C for 1h.
5. Reactions are terminated and analyzed as in Section 4.4, steps 5–13.

6. Determine the ratio between deadenylated and non-deadenylated RNA in each sample to quantitate deadenylation efficiency. Relative deadenylation is calculated as [L3 fragment/(L3 fragment + L3(A_{30}) substrate)] ×100. Quantifications can be done with ImageJ software (http://rsb.info.nih.gov/ij/).

7. Alternatively, the products formed by the deadenylation reaction of L3(A_{30}), ML43(G_{30}), ML40(C_{32}), or ML54(U_{30}) RNA substrates can be analyzed by one-dimensional thin-layer chromatography (TLC). Release of 5′ AMP mononucleotides is expected only if the ribonuclease activity is specific for poly(A) and is a 3′ exonuclease and not endonuclease. Deadenylation reactions are analyzed in TLC using 0.75 M KH_2PO_4, pH 3.5 (H_3PO_4), as the solvent and polyethylenimine-cellulose F plates. The liberated [^{32}P]AMP product is detected after TLC plates are dried and scanned by a 400 S Phosphorimager (Molecular Dynamics). The amount of released AMP is calculated through the specific activity of [^{32}P]AMP in the RNA substrate.

6. Poly(A) tail length measurements

6.1 Introduction

The length of the poly(A) tail has been implicated in mRNA transcripts transport to cytoplasm, stability, as well as translational efficiency (Garneau, Wilusz, & Wilusz, 2007; Schoenberg & Maquat, 2012). Several methods have been developed in order to measure poly(A) tail length, including the oligo(dT)/RNase H-Northern blot analysis (Murray & Schoenberg, 2008), the PCR poly(A) test (PAT) (Sallés & Strickland, 1999), and more recently, genome-wide sequencing techniques such as TAIL-seq (Chang, Lim, Ha, & Kim, 2014) and FLAM-seq (Legnini et al., 2019).

The oligo(dT)/RNase H-Northern blot assay allows for the direct, comparative analysis of poly(A) tail length. In brief, RNA with putative poly(A) tails are incubated with oligo(dT) primers and digested with RNase H, an endoribonuclease that cleaves phosphodiester bonds of RNA bound to DNA. Both the original RNA and the oligo(dT)-incorporated RNA are visualized using Northern blot analysis and their sizes are compared. A change in migration between samples treated with oligo(dT) and those not treated indicates the presence of a poly(A)tail. When visualized alongside a molecular weight marker, the distance of the shift indicates the length of the poly(A) tail. Several protocols have been developed for this technique (Murray & Schoenberg, 2008), as we describe it briefly in this chapter. While this technique allows for quick and direct analysis of poly(A) tail length, there are several drawbacks. One issue is the utilization of oligo(dT) primers and the possibility of short tracts of A-residues existing in the middle of transcript, thereby preventing the exclusive study of poly(A) tails. Another concern is the large starting material requirements of the assay. If the amount of starting RNA is low, it would be better to perform the PAT assay.

The PAT assay allows for the measurement of poly(A) tail length of specific mRNA targets in a population through real-time (RT)-PCR amplification (Sallés & Strickland, 1999). Variations of the PAT assay each include reverse transcription of a pool of RNA using oligo(dT)-anchor primer, followed by PCR amplification of the cDNA using a gene-specific

primer and oligo(dT)-anchor primer again (to prevent shortening of the poly(A) sequence after each cycle of PCR). A “smear” of products indicates the heterogeneous pool of poly(A) tail lengths of a target RNA (Fig. 2C). This assay has several variations, outlined in Rio, Ares, Hannon, and Nilsen (2018), of which we describe the RACE-PAT protocol here.

While a myriad of genomic analysis has highlighted the relevance of poly(A) length, this chapter focuses on direct analysis by biochemical approaches.

6.2 RNA purification from adherent cells

1. Aspirate media from a cell culture in 10cm plate (~10^6 cells) and wash once with ice cold PBS.
2. Aspirate the PBS and add 1 mL TRIzol, scrape the plate briefly and transfer TRIzol/cell lysate into a 1.5 mL Eppendorf tube. (TRIzol followed by chloroform extraction can be used to isolate a large amount of starting material while maintaining RNA purity).
3. Leave the cell lysates at room temperature for 5 min.
4. Add 250 μL chloroform and shake the tube vigorously. Leave at room temperature for 5 min.
5. Centrifuge at 10,000 rpm for 5 min. Remove the aqueous phase, avoiding the organic phase.

   Tips:

   *It may be easier to avoid contamination by tilting the Eppendorf tube, pipetting along the side of the tube, and by leaving a small amount of aqueous phase behind.
6. Add 550 μL isopropanol to the aqueous phase and mix gently. Leave at room temperature for 5 min.
7. Centrifuge at 14,000 rpm for 30 min at 4 °C.
8. Place samples on ice. Wash the pellet with 1 mL 75% ethanol. Mix gently.
9. Centrifuge at 9500 rpm for 5 min at 4 °C.
10. Remove supernatant and let the pellets air-dry. Centrifuge the tubes briefly to force remaining fluid on the side of the tube to the bottom, then pipette off as much of the ethanol as is feasible.
11. Add approximately 15–25 μL (depending on yield) of either Tris-EDTA (TE) buffer or water to the RNA pellet. Determine the concentration of a 1/40 dilution of the purified RNA by measuring absorbance at 260 and 280 nm. The 260/280 ratio should be greater than 1.8. If the ration is <1.8, the RNA is likely partially degraded or there is DNA contamination. The equivalent of the OD at 260 nm (in μg/μL) is the concentration.

6.3 Oligo(dT)/RNase H-northern blot analysis

1. RNA is purified as described in Section 6.2. Importantly, the oligo(dT)/RNase H assay requires a large amount of starting material (> 10 μg).

   Tips:

   *It is also important to make sure any ribonuclease inhibitors added during or after extraction do not interfere with RNase H activity.

2. Set up the reaction by combining 10 μg of denatured RNA, 6 μL of oligo(dT)$_{12–18}$ primer (0.5 μg/μL), and 2 μL of 10 × RNase H buffer (Invitrogen) for a total volume of 18 μL. Set up another reaction without oligo(dT) primer for comparison (adding an equal amount of RNase-free water instead). If the RNA transcript being studied is greater than 2kb and/or it will be difficult to visualize a shift in size between the oligo(dT)± lanes, 3 μg of a gene-specific primer antisense to a 3′ sequence near the poly(A) site can be included. This will produce a truncated RNA transcript and allow for easier visualization of a transcript otherwise too large to discern differences when its tail is digested.

3. Denature at 65 °C for 5–10 min.

4. Add 1U of RNase H (Invitrogen), and 0.2U of ribonuclease inhibitor (RNasin) into each tube. Incubate reaction for 1h at 37 °C.

5. Inactivate by heating at 65 °C for 10 min, or by adding 1 μL of 0.5 M EDTA.

6. Digested RNA at this stage can be extracted through a variety of different purification protocols. Ethanol precipitation is a quick and efficient technique to purify RNA, and suitable at this point (Rio et al., 2018). Add 1/10 volume of 3 M sodium acetate to the reaction and include 2.5 volumes of cold 100% EtOH. Mix briefly, and keep at −80 °C for 1h. Recover precipitated RNA by centrifugation at 12,000 × *g* for 10 min at 4 °C, and decant supernatant. Wash the pellet twice, by adding cold 70% ethanol, centrifuging at 12,000 × *g* for 10 min at 4 °C, and removing the supernatant. Let the pellet air dry for 5 min, and dissolve RNA pellet in RNase-free water

7. Continue to visualize the two reactions (oligo (dT)±) alongside starting RNA material through Northern blot analysis. In brief, RNAs are separated using gel electrophoresis in either agarose or polyacrylamide in the presence of formaldehyde. Mix 15 μg RNA sample with equal volume of 2 × RNA loading buffer (50% formamide, 20% glycerol, 6.5% form-aldehyde, 1 × MOPS buffer, 0.05% BFB, 0.05% xylene cyanol). Incubate at 65 °C for 12–15 min and put samples on ice immediately. The RNA is transferred by overnight capillary transfer onto a nylon membrane (HYBOND N+, GE Healthcare). All other manipulations are essentially as described in Sambrook (1989).

   Tips:

   *After the blotting, mark the gel wells on the nylon membrane with a needle or blade.

8. Prepare radiolabeled RNA probes complementary to RNA transcript of interest using the protocol described in Section 4.3, steps 3–18. Use either T7, T3, or SP6 RNA polymerases accordingly.

9. Wash the membrane with 0.1% SDS, 0.1 SSC. Then pre-hybridize for 1h at 65 °C with ULTRAHyb ultrasensitive hybridization buffer (Thermo Fisher Scientific).

   Tips:

   *Pre-warm ULTRAHyb to 68 °C until the SDS fully dissolves.

10. The labeled probes are then hybridized to the nylon membrane overnight in ULTRAHyb ultrasensitive hybridization buffer (Thermo Fisher Scientific) at 65 °C overnight.

11. Nonspecifically bound probes are washed away after hybridization first by rinsing with 6 × SSC, then with 6 × SSC at 65 °C for at least 20 min, followed by two washes with 0.1 × SSC 0.1% SDS at 65 °C for 20 min.

12. Pull the membrane out of the hybridization bottle, leave semidry, wrap the membrane in Saran Wrap, and expose the membrane to a phosphor screen in a cassette for different times

    Tips:

    *Use Perspex shield while handling the probe and the membrane a substantial amount of radioactivity is used.

    *The phosphor screen should be blanked just before use.

6.4 PAT assay (Fig. 2C)

1. RNA is purified as described in Section 6.2. cDNAs are synthesized from purified RNA using oligo(dT)-anchor primers (5′-GGGGATCCGCGGTTTTTTTTTT-3′), which can anneal to poly(A) transcripts and then extended in a cDNA synthesis reaction. cDNAs with a uniform size are obtained from RNAs with short poly(A) tails as they have limited number of sites for oligo(dT) binding. cDNA products of heterogeneous sizes are obtained from RNAs with long poly(A) tails that have multiple sites for binding the oligo(dT) primer/adapter.

2. Incubate 1 μg (or more) of RNA with 0.5 μg oligo(dT)-anchor primer at 80 °C for 5 min (in a total reaction volume of 5 μL), quick-spin centrifugation and then keep at 4 °C for 10 min.

3. Combine the RNA/oligo(dT)-anchor primer mixture (5 μL) with 15 μL of master mix. For a single reaction of 15 μL total volume add 3 μL of 5 × GoScript reaction buffer, 2 μL of 25 mM $MgCl_2$, 2 μL 10 mM dNTPs, 1 μL of GoScript reverse transcriptase, 20U RNase inhibitor and 7 μL of RNase-free water.

4. Reactions are left at 25 °C (anneal) for 5 min, 42 °C (extend) for 1.5h, and lastly at 70 °C for 15 min (inactivation of reverse transcriptase). The cDNA may be used immediately as the template for PCR or stored at −20 °C.

5. The PCR reaction is performed using 1 μL of cDNA, 1.25 μL of oligo(dT)-anchor primer and 1.25 μL of gene-specific primer located 200–400 bp upstream the poly(A) site. Using this strategy, the PCR products will be between 100 and 300 nucleotides, a size easily resolved on a 6% polyacrylamide gel. Combine the cDNA/primers mixture(3.5 μL) with PCR mix: 2 μL 10 × PCR buffer, 3 μL 50 mM $MgCl_2$, 3 μL 1.25 mM dNTPs, 8 μL of RNase-free water and 0.5 μL Taq DNA polymerase. The reactions are incubated in a thermal cycler first at 95 °C for 2 min, and then for 30cycles, which comprises of a denaturation step at 95 °C for 30s, an annealing step at a temperature 5 °C below the melting temperature of the gene-specific primer for 45s, and an extension step at 72 °C for 1.5 min. After the cycles, a final extension at 72 °C is performed for 5 min

   Tips:

   *PCR steps may be optimized by adjusting the annealing temperature and length of time and/or adjusting primer/cDNA concentrations.

6. Reactions are kept at 4 °C until gel electrophoresis analysis or stored at −20 °C.

7. For better detection of PCR products, alternatively, the PAT assay can be performed using the upstream gene-specific primer radiolabeled at the 5′ end with [γ-^{32}P]-ATP. It is convenient to also radiolabel a size marker. For a reaction volume of 20 μL add 250 ng primer or 1 μg of DNA ladder, 2 μL 10 × polynucleotide kinase buffer (supplied with the enzyme), 0.5 μL of 10 μCi/μL of [γ−^{32}P]-ATP, 1 μL of T4 polynucleotide kinase, and RNase-free water to 20 μL.

8. Incubate reaction at 37 °C for 30 min, and then inactivate the T4 polynucleotide kinase by keeping reaction at 65 °C for 5 min.

9. Purification to eliminate any unincorporated radioactivity is not required as it does not interfere with the analysis of PCR products in the gel. The labeled oligo may be purified on a silica spin-column for nucleotide removal. The labeled oligo may be used immediately for PCR or stored at −80 °C.

10. Repeat PCR as described in step 5 using 1 μL of cDNA, 1.25 μL of oligo(dT)-anchor primers and 1.25 μL of radiolabeled gene-specific primer.

11. PCR products are then resolved through gel electrophoresis. Combine 3 μL of each PCR reaction with 3 μL formamide loading buffer (95% formamide, 0.025% xylene cyanol and BFB, 18 mM EDTA and 0.025% SDS), heat to 95 °C for 5 min and resolve on a 6% polyacrylamide gel or 1% agarose gel. To differentiate PCR products of different length the gel has to be long enough to allow the resolution. Run the gel in TBE buffer at 60W until the lower dye front is at the bottom of the gel. Remove one glass plate and treat as in Section 4.4, steps 11–13. While mRNAs with a short poly(A) tail will appear as a discrete

peak, mRNAs with long tails will appear as a range of PCR products of different sizes that appear as a smear of bands. Sometimes peaks are observed at 25 nucleotides intervals, which corresponds in size to the distribution of PABP.

7. Summary

The intricate network of factors impacting poly(A) tail metabolism highlights the relevance of these pathways in cellular homeostasis. In recent years, numerous studies have described genome-wide changes in different cellular conditions, such as stress, proliferation, differentiation and development. Direct connections between the mRNA 3′ end processing machinery and factors involved in those cellular pathways provide an obvious link to different diseases, such as cancer and other clinical conditions (Murphy & Kleiman, 2020b). In this chapter, we provide an overview of two key steps in the mRNA life-cycle, CpA and deadenylation, and biochemical assays generated to study each of these enzymatic reactions and their effect on mRNA's poly(A) length. While bioinformatic studies facilitate our understanding of the dynamics of these reactions and their effect on cellular functions, this research will benefit from a more mechanistic approach that offers details on the factors and sequences involved in this complex process of gene expression.

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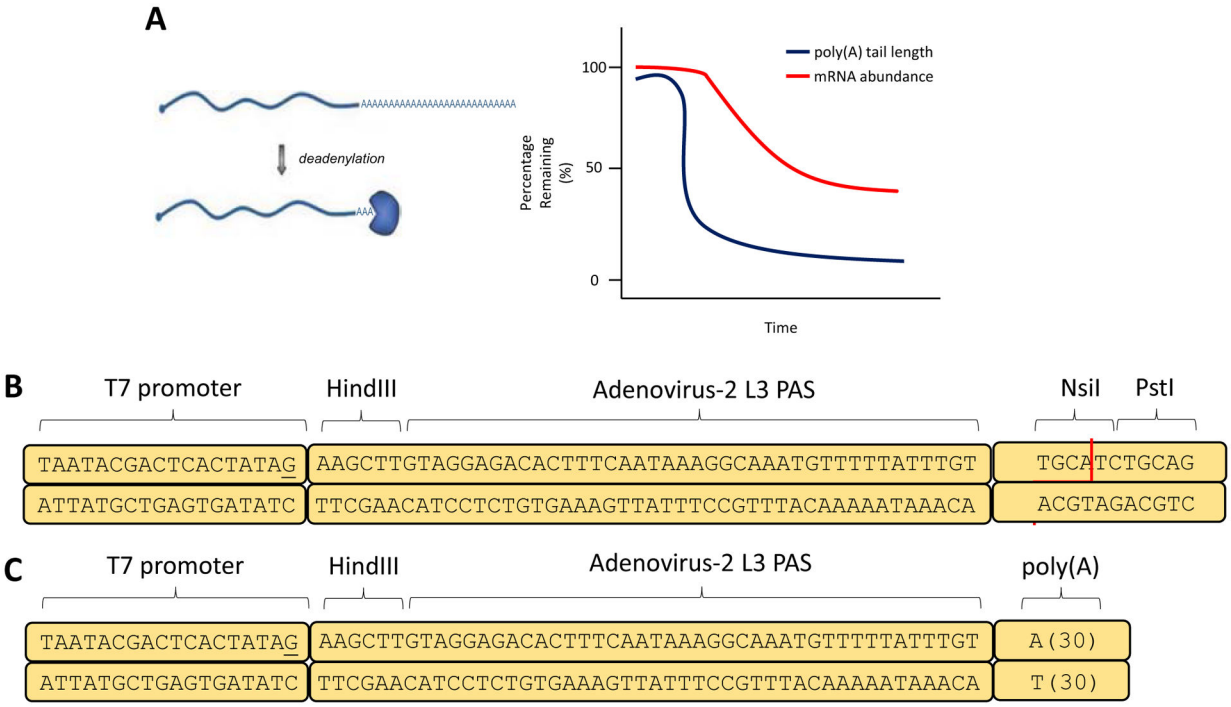

Fig. 1.

(A) A simplified model of mRNA decay lagging behind deadenylation. Enzymatic deadenylation leads to a negligible level of substrate. Further enzymatic reactions of decapping and mRNA decay by exonucleases results in lag to qualify an mRNA as "decayed." (B-C) Constructs used to test cleavage and polyadenylation (A) and for deadenylation (B).

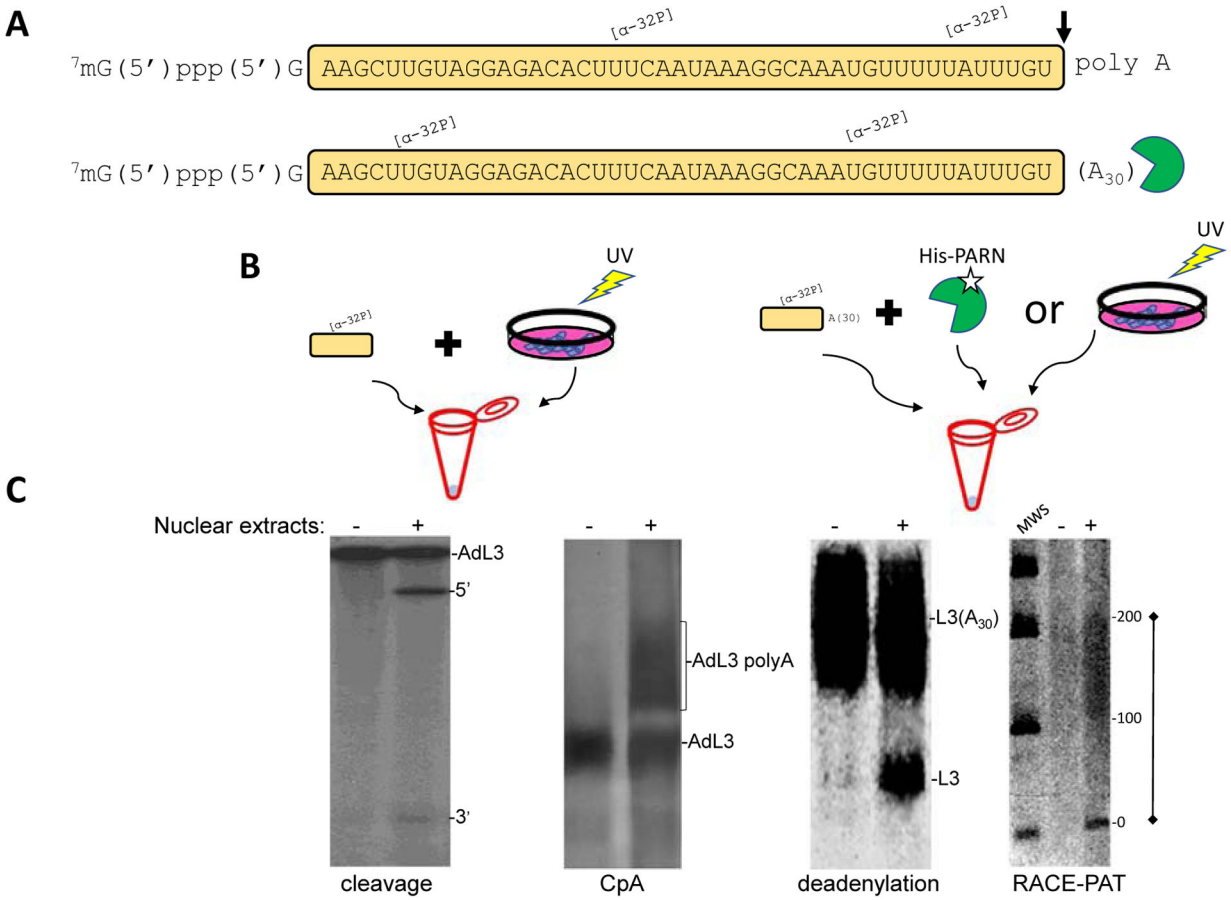

Fig. 2.

(A) Capped radiolabeled substrates used in CpA and deadenylation reactions. (B) General workflow for CpA and deadenylation reactions with radiolabeled RNA and recombinant protein or cell lysates. (C) Representative cleavage, CpA, deadenylation and RACE-poly(A) test reactions. For CpA and deadenylation reactions, different capped radiolabeled substrates are incubated with nuclear extracts purified from human colorectal carcinoma HCT116 cells. For in vitro CpA reactions pG3L3-A, which contains adenovirus 2 L3 PAS, is used. For deadenylation, plasmid pT3L3 (A_{30}), which contain L3 RNA body followed by 30 residues of adenosine, is used. Samples were then analyzed in denaturing urea gels followed by autoradiography. For RACE-PAT assays, nuclear RNA from HCT116 cells are reverse transcribed using oligo(dT)-anchor primer and amplified using an oligonucleotide that hybridizes TP53 transcript. The products were separated on a non-denaturing PAGE and detected by ethidium bromide staining. Molecular weight standard (MWS, 100 base pair ladder) is also included.