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Analysis of mRNA deadenylation by multi-protein complexes

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ABSTRACT

Poly(A) tails are found at the 3′ end of almost every eukaryotic mRNA and are important for the stability of mRNAs and their translation into proteins. Thus, removal of the poly(A) tail, a process called deadenylation, is critical for regulation of gene expression. Most deadenylation enzymes are components of large multi-protein complexes. Here, we describe an *in vitro* deadenylation assay developed to study the exonucleolytic activities of the multi-protein Ccr4-Not and Pan2-Pan3 complexes. We discuss how this assay can be used with short synthetic RNAs, as well as longer RNA substrates generated using *in vitro* transcription. Importantly, quantitation of the reactions allows detailed analyses of deadenylation in the presence and absence of accessory factors, leading to new insights into targeted mRNA decay.

1. Introduction

Gene expression levels depend on rates of mRNA turnover. Removal of the poly(A) tail from an mRNA, a process called deadenylation, is a critical and conserved process in eukaryotic mRNA decay [1]. It is the first step in general mRNA turnover, and is often rate-limiting in this pathway [2]. Deadenylation can also be triggered during mRNA quality control [3] and miRNA-mediated repression of gene expression [4]. The rate of poly(A) tail removal is a strong indicator of the half-life of an mRNA, with unstable transcripts undergoing more rapid deadenylation [2]. The rate that a particular mRNA undergoes decay can change and is regulated, for example, in response to environmental signals [5]. The regulation of deadenylation is therefore fundamental for cellular maintenance, but also allows responses to cellular cues. In addition to its role in RNA decay, the removal of the poly(A) tail is also linked to translation repression. This is partly because it coincides with the release of poly(A)-binding protein (Pab1/PABPC), which is required for efficient translation initiation [6].

The poly(A) tail is shortened in the 3′ to 5′ direction by the activity of adenosine-selective exonuclease enzymes. Several proteins have been identified that catalyze this reaction. The most highly conserved are Ccr4/CNOT6, Caf1/CNOT7, and Pan2 [7]. Importantly, these enzymes do not act as monomeric proteins, but are components of the larger multi-protein assemblies Ccr4-Not and Pan2-Pan3. Additionally, other factors, such as RNA-binding proteins and miRNAs, can recruit these deadenylase complexes to specific mRNA transcripts, promoting their deadenylation (e.g. [8–11]). By controlling mRNA stability in a transcript-specific manner, these proteins thereby regulate gene expression.

Given the central role of deadenylation in post-transcriptional regulation of gene expression, factors that control its rate are of great importance to many biological processes. For example, the control of the cell cycle, embryogenesis and the inflammatory response are known to depend on targeted deadenylation activity [8,12,13]. The network of proteins that orchestrate this activity is highly complex, and this has limited our understanding of its mechanism. Studies into the phenotypes of gene deletions have yielded many key insights, but genetic approaches are frequently hampered by compensatory mechanisms within the cell. For example, in the absence of efficient deadenylation, gene expression is repressed using alternative mechanisms, such as decreased transcription. This compensates for impaired mRNA decay, buffering the mRNA and protein quantities in the cell [14]. Therefore, even though deadenylation is the first step of general mRNA decay, deletion of genes encoding the nucleases of Ccr4-Not is not lethal in yeast [15], likely because overall mRNA levels remain relatively unaffected. In contrast, deletion of the poly(A) binding protein Pab1, or the Not1 subunit of Ccr4-Not is lethal [16,17]. This has made it challenging to dissect the specific functions of these

Abbreviations: 5- or 6-FAM, 5- or 6-carboxyfluorescein; ARE, AU-rich element; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; TBE, Tris-Borate-EDTA; UTR, untranslated region.

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proteins *in vivo*. Finally, the proteins involved in mRNA decay often play multiple roles in this process. For example, Ccr4-Not and its accessory factors can inhibit gene expression by catalyzing deadenylation, recruiting decapping stimulators [18,19], inhibiting translation [20,21], and regulating mRNA localization [22]. In many cell-based experiments, the contribution of each of these various mechanisms is consequently unclear.

For these reasons, it is advantageous to directly characterize deadenylation using an *in vitro* system reconstituted from purified factors. Previously, the activities of individual nuclease enzymes of Ccr4-Not have been examined [15,23–25]. Since both Ccr4-Not and Pan2-Pan3 are thought to be obligate multi-protein complexes in the cell, it is unclear whether the activities of isolated nucleases are representative of their activities within the context of their complexes. Furthermore, targeted deadenylation of specific mRNAs is typically a property of the intact complexes. This is because the RNA-binding adaptor proteins that link Ccr4-Not to a particular set of mRNAs (e.g. Tristetraprolin, Nanos, Roquin, and GW182) typically bind to the non-enzymatic subunits of Ccr4-Not [26–29]. The recruitment of Pan2-Pan3 to RNA is likewise believed to be mediated by interactions between the non-enzymatic Pan3 subunit and Pab1 [30]. Therefore, assays of deadenylation activity are best performed using intact complexes.

We recently reported methods for the purification of recombinant Pan2-Pan3 [31] and Ccr4-Not [32]. These complexes can also be prepared from endogenous sources [9], but lower yields prevent rigorous purification and subsequent quantitative *in vitro* analyses. The larger quantities obtained from a recombinant system permit more stringent purification, which minimizes co-purification with contaminating RNases and endogenous factors that may regulate the deadenylation activity. Furthermore, we have found that the constituent subunits of recombinant protein complexes are purified in stoichiometric ratios that likely match the physiological compositions [31,32].

Here, we describe a method for analyzing the deadenylation activities of the Ccr4-Not and Pan2-Pan3 deadenylation complexes *in vitro*. By examining reactions performed with polyadenylated RNA substrates of uniform length, the sequential removal of adenosine nucleotides can be visualized at high resolution by denaturing polyacrylamide gel electrophoresis. This allows quantitation of the reaction rate. We provide a detailed rationale for the experimental setup, as well as key considerations necessary to obtain reliable and accurate results. While the deadenylation activity of Ccr4-Not is shown here as an example, the techniques described could aid in high-resolution analysis of the RNase or DNase activities of a variety of other enzymes.

2. Assay overview

A minimal deadenylation reaction contains three components: polyadenylated RNA substrate, purified deadenylase enzyme, and a buffer solution. We designed such a system to examine the deadenylation activities of Ccr4-Not and Pan2-Pan3 *in vitro* (Fig. 1) [31,32]. Briefly, deadenylation enzymes are incubated with a purified RNA substrate containing a poly(A) tail of defined length. Samples are taken at regular intervals and the poly(A) tail length is analyzed by denaturing gel electrophoresis. Quantitation of the gel allows evaluation of the rate of deadenylation, the processive vs. distributive nature of the enzyme, and the effect of accessory proteins on activity. Details of each of these steps are discussed below.

3. Design of RNA substrates for deadenylation

A variety of RNA substrates can be used in assays to investigate the rates and patterns of deadenylation. Here we evaluate the types of RNAs used in previous studies, and highlight the advantages of each.

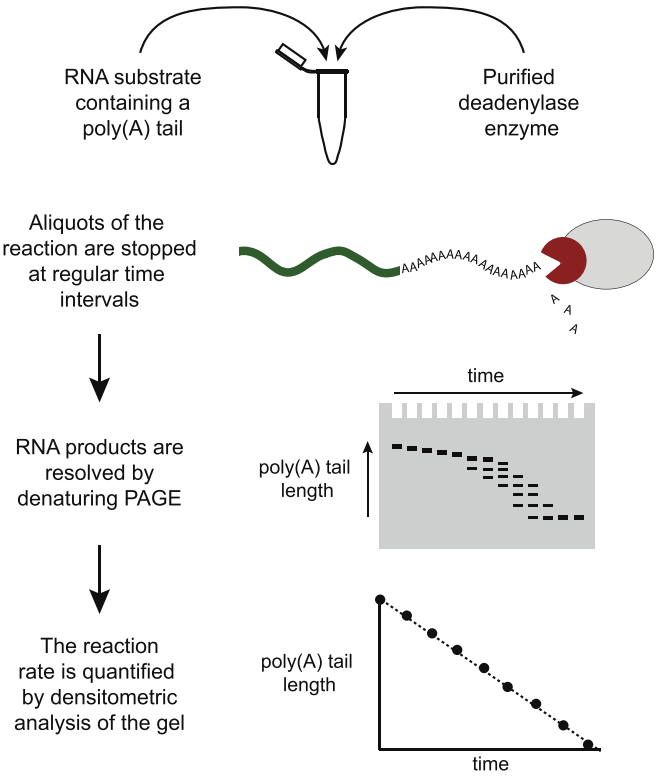

Fig. 1. Workflow for the *in vitro* measurement of deadenylation activity of purified proteins. Deadenylation assays are initiated by addition of purified deadenylase protein to an RNA substrate containing a poly(A) tail. The RNA can be generated by chemical synthesis or *in vitro* transcription. During incubation, aliquots of the reaction are removed at regular time intervals and stopped by the addition of denaturing loading dye. The RNA products are resolved by denaturing polyacrylamide gel electrophoresis. The reaction is quantified by densitometric analysis of the gel lanes.

3.1. Short synthetic RNAs

Since deadenylation is the exonucleolytic removal of adenosines from the 3′ end of RNA, the simplest substrate is a short polyadenosine RNA such as A10 or A15 [24,33]. Specificity for the removal of adenosines can be assessed by comparing these reactions with those performed with other RNA homopolymers: poly (C), poly(U) and poly(G) [24,34]. RNA substrates containing poly (G) tracts can form G-quadruplexes, which may hinder or complicate analysis of deadenylase specificity [35].

To examine whether exonuclease activity is limited to the poly (A) tail, substrates containing ~20 non-poly(A) nucleotides upstream of 10–30 contiguous adenosines have been used [23,25,36–38]. These studies revealed that deadenylase enzymes display specificity for nucleotides on both sides of the scissile bond [36,39], and as a result, the adenosine proximal to a 3′ UTR may not be rapidly removed [23,37]. In addition, the use of longer RNAs may promote binding of the deadenylase enzymes to the RNA substrate [23]. We have found this type of model substrate to be highly versatile. As they are shorter than ~50 nucleotides in length, they can be commercially synthesized with reasonable yield, purity and affordability [40]. The products of the deadenylation reaction can be separated by polyacrylamide gel electrophoresis at single-nucleotide resolution due to their small size (see Section 6).

Furthermore, sequence motifs can be incorporated into the upstream non-poly(A) sequence. RNA-binding proteins that regulate deadenylation typically bind to short sequence motifs 4–10