METHOD

ePAT: A simple method to tag adenylated RNA to measure poly(A)-tail length and other 3' RACE applications

AMREI JÄNICKE, JOHN VANCUYLENBERG, PETER R. BOAG, ANA TRAVEN, and TRAUDE H. BEILHARZ¹

Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia

ABSTRACT

The addition of a poly(A)-tail to the 3' termini of RNA molecules influences stability, nuclear export, and efficiency of translation. In the cytoplasm, dynamic changes in the length of the poly(A)-tail have long been recognized as reflective of the switch between translational silence and activation. Thus, measurement of the poly(A)-tail associated with any given mRNA at steady-state can serve as a surrogate readout of its translation-state. Here, we describe a simple new method to 3'-tag adenylated RNA in total RNA samples using the intrinsic property of *Escherichia coli* DNA polymerase I to extend an RNA primer using a DNA template. This tag can serve as an anchor for cDNA synthesis and subsequent gene-specific PCR to assess poly(A)-tail length. We call this method **extension Poly(A) Test (ePAT)**. The ePAT approach is as efficient as traditional **Ligation-Mediated Poly(A) Test (LM-PAT)** assays, avoids problems of internal priming associated with oligo-dT-based methods, and allows for the accurate analysis of both the poly(A)-tail length and alternate 3' UTR usage in 3' RACE applications.

Keywords: mRNA polyadenylation; alternative polyadenylation; translational control; LM-PAT assay

INTRODUCTION

The poly(A)-tail is involved in all major functions of eukaryotic mRNA, and addition of this tail is the final quality control step that nascent mRNA undergo prior to nuclear export (Proudfoot et al. 2002). In this context, it is thought that the poly(A)-tail promotes circularization of the RNA molecule into a closed-loop configuration that promotes translation initiation (Sachs and Varani 2000). At synthesis, the length of the poly(A)-tail is generally uniform in any given system, with the absolute length being species-dependent; in yeast and mammalian cells, this is ~90 and 250 adenosine residues, respectively. However, in the cytoplasm, the steady-state length distribution of poly(A)-tails can vary dramatically for transcripts of different functional classes due to differential, transcript-specific deadenylation rates, a disconnect between deadenylation and decapping and/or stabilization during translation (Decker and Parker 1993; Brown and Sachs 1998; Beilharz and Preiss 2007). For example, targeted deadenylation is provoked by microRNA binding (Beilharz et al. 2009; Eulalio et al. 2009; Fabian et al. 2009) or RNA binding proteins. Compensatory and activating cytoplasmic adenylation can further modulate the polyadenylation state

of the transcriptomes of many eukaryotes (Rouhana and Wickens 2007; Burns et al. 2011). Finally, eukaryotic cells also employ the ancient, prokaryotic function of RNA adenylation to destabilize mitochondrial, structural, and noncoding RNA (Slomovic et al. 2010; for review, see Eckmann et al. 2011). We have previously shown that the adenylation state of the transcriptome is highly correlated with other gene expression parameters, such as ribosome occupancy and protein abundance (Beilharz and Preiss 2007; Lackner et al. 2007), which has led us to propose that the measurement of mRNA poly(A)-tail length can serve as a surrogate for translation-state measurements.

There are several well-characterized methods for measuring poly(A)-tail length. The most direct method is the combined use of RNase H digest ($\pm$ Oligo dT) and high-resolution Northern blot (Sippel et al. 1974; Decker and Parker 1993; Sheets et al. 1994). In our own laboratory, we have made extensive use of the **Ligation-Mediated Poly(A) Test (LM-PAT)** assay developed by the Strickland laboratory (Salles and Strickland 1995; Beilharz and Preiss 2007; Lackner et al. 2007; Beilharz et al. 2009; Traven et al. 2009; Dagley et al. 2011). Finally, several laboratories have used T4 RNA ligase approaches to either circularize mRNA (Couttet et al. 1997) or, more recently, to ligate adaptors to the 3' end of mRNA (Charlesworth et al. 2004). While each of these assays can serve to measure poly(A)-tail length, each has its own limitations. High-resolution Northern

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transcripts were spiked into total HeLa RNA to assess the accuracy of ePAT in length calling. By this approach, there was a good overall correlation between the average tail length of in vitro transcripts and the observed size of the ePAT amplification products. Specifically, in vitro transcripts with an estimated average poly(A)-tail length of ~ 15 , ~ 45 , ~ 65 , and ~ 115 adenosine residues (see Supplemental Fig. A) showed an estimated poly(A)-tail length of ~ 20 , ~ 40 , ~ 65 , and ~ 95 by ePAT. The estimation of the average tail length of both the in vitro transcripts and the PCR products is based on migration relative to RNA and DNA ladders, respectively, and is accurate to approximately ± 10 nt. Both assays reflect the length of the in vitro synthesized poly(A)-tail. However, the LM-PAT assay is slightly biased toward specific sizes, preferentially reporting the ~ 45 -A transcripts and artifactually reporting a tail of this length in mRNA having longer poly(A)-tails.

Next, we monitored the adenylation state of specific endogenous transcripts in response to a transcriptional pulse chase regimen in *Saccharomyces cerevisiae* involving activation of gene expression by galactose followed by repression by the addition of glucose (Decker and Parker 1993; Beilharz and Preiss 2007). Yeast cells were harvested 10 min after galactose induction and at the indicated time points in the pulse period after glucose repression (Fig. 2B). At the point of transcriptional shutdown (glucose addition), the two 3' UTR isoforms of the *GAL1* transcript had a long (~ 50 A) poly(A)-tail that shortened over time (Fig. 2C). Again, the two assays were equally efficient at generating cDNA that reflected the adenylation state of the transcripts. However, the ePAT assay provided a better reflection of the distribution of poly(A) tails in the sample. Because the LM-PAT assay depends on serial annealing of fixed length oligo-(pdT) oligonucleotides, only limited resolution can be achieved (see also Beilharz and Preiss [2009] for further discussion). We monitored the kinetics of deadenylation of both *GAL1* 3' UTR isoforms after transcriptional repression by densitometry and peak-identification (Fig. 1D). Linear deadenylation rates for both the *GAL1* transcript isoforms were calculated from ePAT data. Such decay curves were previously only possible by high-resolution Northern blots (Decker and Parker 1993). Therefore, compared to LM-PAT, the ePAT method provided a more accurate measurement of the poly(A)-tail length distribution and more detailed estimation of the deadenylation kinetics. It is important to note that the *GAL1* transcript has previously been shown to undergo an additional nonstandard post-transcriptional cleavage and adenylation step to generate the *GAL1*-(short) transcript ~ 10 min after glucose repression (Vodala et al. 2008). However, the similar initial length and decay kinetics of the poly(A)-tail at the start of the chase period in this experiment suggests that both forms were generated by the canonical transcript cleavage and adenylation machinery. Any additional cleavage products would remain invisible to the assay until they became adenylated.

FIGURE 2. (A) Four in vitro synthesized and adenylated transcripts with the indicated average poly(A)-tail length were spiked into a fixed amount of total HeLa RNA (800 ng) and analyzed by ePAT and LM-PAT (see Supplemental Material for details of in vitro transcripts). (B) Schematic representation of the time course of galactose induction and glucose repression. The arrows indicate the time points at which cells were harvested. (C) The 10-min time point represents newly synthesized *GAL1* transcript with a long poly(A)-tail. The *GAL1*-(long) and *GAL1*-(short) transcripts are generated by alternate poly(A) site usage. (D) The time-dependent shortening of the two *GAL1* amplicons was quantified relative to the 100-bp ladder and the migration of the TVN product. This is only really possible using the ePAT assay, since laddering in the LM-PAT assay results in two peaks of similar intensity at the last time point of this assay (20 min).

Alternate poly(A) site usage is revealed by ePAT

Recent evidence has shown that the use of alternate poly(A) sites is widespread and dynamic in eukaryotic transcriptomes (Mangone et al. 2010; Oszlak et al. 2010; Yoon and Brem 2010; Jan et al. 2011; Shepard et al. 2011; Wu et al. 2011). The use of shorter 3' UTRs often provides a mechanism to remove the influence of post-transcriptional regulatory modules within 3' UTRs, such as microRNA and regulatory protein binding sites (Sandberg et al. 2008). Since cDNA priming for ePAT requires extension of the 3' end of the RNA molecule, internal poly(A) stretches are avoided. *HXK1* is one of a number of genes, including *GAL1* above, that change their subnuclear position in response to activation by galactose in a 3' UTR-dependent manner, suggesting a role for 3' processing in gene activation and/or repression (Abruzzi et al. 2006; Taddei et al. 2006; Vodala et al. 2008). In this study, we performed a transcriptional pulse chase (Fig. 3A) and found evidence for alternative polyadenylation of the *HXK1* transcript. The *HXK1*-(long) transcript followed normal decay kinetics of

FIGURE 3. Alternate polyadenylation sites are revealed by ePAT. (A) Schematic representation of the time course of galactose induction and glucose repression. The arrows indicate the time points at which cells were harvested. (B) *HXK1* transcription is rapidly repressed by glucose. During this repression phase, the ratio between *HXK1*-(long) and *HXK1*-(short) changes as the short 3' UTR form is transiently induced without a significant increase in poly(A)-tail length. *GCV1* is transiently induced with a long poly(A)-tail by glucose addition. The faster migrating *GCV1* amplicon, indicated by **, is likely a product of internal priming in the TVN reaction. The *GAL10* and *APQ12* panels are included as pulse-chase and ePAT assay controls, respectively.

deadenylation followed by decay of the transcript body after repression by glucose. The compaction of the PCR amplicons from a smear to a tight, short-tailed band explains the apparent increase of the *HXK1*-(long) transcript at 12.5 and 15 min. In contrast, the abundance of *HXK1*-(short) transcript transiently increased at 17.5 min. This was similar to the observation of *GAL1*-(short) accumulation by a nonstandard cleavage and polyadenylation mechanism reported by Vodala et al. (2008). Sequencing of the two *HXK1* PCR amplicons allowed for precise mapping of the alternate poly(A)-sites, located 65 and 175 bases after the stop codon for the short and long UTRs, respectively. This result suggests that a switch to the shorter UTR removes two of three canonical UGUA pumilio consensus sites previously identified as candidate Puf1 sites in the 3' UTR of *HXK1* (Ulbricht and Olivas 2008). However, how this might influence Hxk1 protein expression remains unexplored. It is important to note that, in contrast to *GAL1* and *HXK1*, the apparent shorter alternate 3' UTR of *GCV1* suggested by the TVN-PAT reaction (see ** in Fig 3B) is likely an artifact, having a size consistent with internal priming to a 7-adenosine internal stretch within the *GCV1* transcript. Thus, in contrast to oligo-dT-based priming methods, the ePAT approach allows identification of bona fide alternate poly(A) sites.

3'-tagging by ePAT is efficient

An alternative method for measuring poly(A)-tail length has been the use of T4-ligase to append preadenylated linkers to the 3' end of mRNA using methods analogous to those applied to the cloning of microRNA (Charlesworth et al. 2004; Kim et al. 2009) or circularization of mRNA

(Couttet et al. 1997; Rissland and Norbury 2009). These approaches are conceptually appealing in that they do not require knowledge of the bases that terminate the RNA of interest and can, therefore, be used to identify 3' uridilation and other heteropolymeric 3' extensions on RNA. The disadvantage of these methods is that they are inefficient and often require high PCR cycle numbers (commonly 40 cycles) and/or the use of radiolabeling to detect a product. To assess the performance of ePAT in applications where a T4-RNA ligation-based assay had been previously applied, we replicated the results presented by Kim et al. (2009), who elegantly demonstrated the dependence of several transcripts on the cytoplasmic deadenylase Gld-2 in the *Caenorhabditis elegans* germline. Using 10-fold less RNA input, twofold less cDNA, and 12 fewer cycles of PCR amplification (28 versus 40), the ePAT assay replicated and extended the data provided by Kim et al. (2009) (Fig. 4). RNA was isolated from wild-type Bristol Normal (N2) and *gld-2* mutant worms. Two hundred ng of this was spiked into yeast total RNA and subject to ePAT. The adenylation state of *egg-1*, *pup-2*, and *oma-2* were confirmed to depend on Gld-2, as does the germline specific *gpd-4* (GAPDH). The steady-state poly(A)-tail length of somatic *gpd-2* (GAPDH) and *eft-3* were not significantly altered (data not shown).

To infer the efficiency of ePAT, we designed a PCR calculator that uses the PCR cycle number, an estimated PCR efficiency, and the amplicon length and mass in combination with the molecular weight of a DNA base pair to

FIGURE 4. The cytoplasmic poly(A)-polymerase Gld-2 is required for normal adenylation of transcripts in the *C. elegans* germline. The adenylation state of four maternal mRNA *egg-1*, *Pup-2*, *oma-2*, and *gpd-4* was analyzed in Bristol Normal (N2) and *gld-2* mutant worms. In each case, the mRNA is short-tailed in the mutant, which reflects the inactive state. The adenylation state of the somatic transcript *gpd-2* is not affected in a major way by the loss of Gld-2. Yeast total RNA (800 ng) from a deadenylase-deficient mutant (*pan2Δ/ccr4-1*) was spiked into the assay as ballast for the low concentration of worm RNA (200 ng). The yeast *APQ12* panel is included to demonstrate equal ePAT efficiency across samples.

estimate the number of cDNA input molecules that contribute to the final PCR product (Supplemental File 2). For example, at least 5000 template *egg-1* cDNA molecules in the PCR reaction mix were required to generate 110 ng of the 120-bp PCR amplicon as measured by densitometry (Fig. 4, *gld-2*). If 40 cycles of amplification had been required to generate this amount of product, then the number of contributing cDNA molecules would reduce to approximately two. Thus, with every additional PCR cycle necessary to produce detectable product, there is an increased risk of biased sampling. Interestingly, using ePAT, we observed a tail-length distribution of ~12–100 A-residues for most transcripts in wild-type (N2) worms, which was a striking difference to that observed by Kim et al. (2009). Using the ligation approach and 40 cycles of amplification, Kim et al. (2009) reported a uniform tail length of ~40 bases for *oma-2*. The length was significantly reduced in *gld-2* mutant worms as measured by both studies. Taken together, we have shown that the ePAT approach returns similar and possibly more detailed information on poly(A) status than T4-ligase-based approaches and at significantly higher efficiency.

CONCLUSION

In this study, we describe a simple method to tag adenylated RNA within a population and demonstrate its use for the identification of alternate 3' UTRs and measurement of the poly(A)-tail length of mRNA. The method is quantitative within the linear range of amplification and can be applied to detect transcripts from very low concentrations of RNA. This approach is the closest method described to date that matches the sensitivity observed by high-resolution Northern blot but avoids radiolabeled probes, requires a fraction of the time and RNA input, and generates enough cDNA for analysis of over 20 specific transcripts from a single ePAT reaction. Moreover, all of the necessary components are standard in any molecular biology laboratory.

MATERIALS AND METHODS

Saccharomyces cerevisiae

The By4741 yeast strain (*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) was grown to exponential phase (OD₆₀₀ ~0.6) in rich medium (2% peptone and 1% yeast extract) with 2% raffinose as the sole carbon source. Transcription of *GAL* genes were transiently induced by addition of 2% galactose and then repressed after 10 min by the addition of 2% glucose. At each indicated time point, 5 mL of culture was removed into 15 mL tubes containing 50 μL 10% sodium azide prechilled in an ice bath. Once all samples were collected, the cells were harvested by centrifugation (4000g for 2 min), washed once in ice-cold water containing sodium azide (0.1%), snap frozen, and stored at –80°C.

Caenorhabditis elegans

C. elegans wild-type Bristol N2 and *gld-2(q497)* strains were maintained at 20°C using standard methods (Brenner 1974).

RNA extraction

Total RNA from yeast cells was prepared according to the hot phenol method. Total *C. elegans* RNA was prepared by suspending between 50 and 100 snap frozen worms in 1 mL of Trizol (Life Technologies). After addition of ~200 μL ziconia beads (Biospec Products), the sample was homogenized for 30 sec using a Mini-Beadbeater 8 (Biospec Products). Trizol extraction was performed according to the manufacturer's instructions, except that 2 μL glycogen was added prior to precipitation with isopropanol. To improve the generally poor Nano-drop QC A260/A230 ratios that result from Trizol purifications, we resuspended the resulting pellet in 100 μL dH₂O and reprecipitated the RNA with 1/10 volume of 3 M NaOAc [pH 5.2] and 2.5 volumes of ethanol. RNA quantification was performed with a Nano-Drop 1000 (Thermo Scientific).

The ePAT method and product detection

The ePAT approach uses the same PAT-anchor primer that has been described for the LM-PAT method (PAT anchor 5'-GCGAGCTCCGCGGCCGCGTTTTTTTTTTT), which was stored as small 100-μM aliquots at –20°C. Of note, alternative anchor sequences were equally efficient in our laboratory. The incubation steps of the assay were most conveniently performed in a thermocycler with an accessible lid programmed with a series of temperature hold/pause settings, where the pause maintains temperature while allowing access to the tubes.

To assemble the ePAT reaction, 1 μg of total RNA (or less) and 1 μL of PAT-anchor primer were combined and brought to a volume of 8 μL with dH₂O in a 200-μL PCR tube. The mixture was incubated at 80°C for 5 min and then cooled to room temperature. Once cooled, the sample was flash-centrifuged, and 12 μL of a master mix was added that contained 4 μL dH₂O, 4 μL 5× Superscript III buffer (Life Technologies), 1 μL 100 mM DTT, 1 μL 10 mM dNTPs, 1 μL RNaseOUT (Life Technologies), and 1 μL (5 U) Klenow polymerase (New England Biolabs) per reaction. The sample was mixed thoroughly by inversion, flash-centrifuged, and then incubated at 25°C for 1 h. Importantly, 37°C worked equally well in our laboratory and may improve selectivity. The polymerase was then inactivated by increasing the temperature to 80°C for 10 min prior to cooling the reaction to 55°C for 1 min. While maintaining the tubes at that temperature in the block, 1 μL (200 U) of Superscript III (Life Technologies) was added to the tubes. The tubes were closed and mixed rapidly by flick-inversion. Incubation was then resumed at 55°C for 1 h, followed by inactivation of the reverse transcriptase by increasing the temperature to 80°C for 10 min. It is critical to maintain the temperature during the reverse transcription step because internal priming can occur if the temperature drops. At 55°C, only priming from an end-extended RNA molecule is possible. It can also be useful to include spiked-in RNA from an unrelated organism as ballast for dilute RNA reactions and to control for equal assay efficiency across samples. We routinely spike total HeLa RNA in yeast samples and RNA from a deadenylase deficient yeast strain in metazoan RNA samples.

For the PCR reactions, cDNA was diluted 1:6 by the addition of 120 μ L dH₂O. The PCR reactions were typically conducted in a total volume of 20 μ L using 5 μ L of diluted cDNA input and a fast-start polymerase, such as Fast-Start (Roche) or Amplitaq Gold 360 master (Life Technologies). It can also be useful to include a TVN-PAT reaction as a size control for the size of the amplicon with a fixed A12 poly(A)-tail. Both the LM-PAT and the TVN-PAT reactions were performed exactly as described previously (Beilharz and Preiss 2009). The cycle number was dependent on the abundance of the transcript of interest in the sample but normally ranges between 23–33 cycles.

To detect the PCR amplicons from TVN-PAT, ePAT, and LM-PAT PCR reactions, 50% of a 20- μ L PCR reaction was loaded per lane into a 2% high-resolution agarose gel (Ultra pure 1000; Life Technologies) that was prestained with SYBR safe (Life Technologies). The primers used in this study are supplied (Supplemental File 1). To estimate the PCR product sizes and to quantify the mass of PCR amplicons from such gels, the band intensity and migration was determined relative to a 100-bp ladder (New England Biolabs) using an LAS 3000 imager and multigaue software (Fujifilm). To track deadenylation kinetics, the migration of the highest peak intensity of each band was determined for each lane and expressed relative to the migration of the TVN-PAT peak. The length of poly(A) at time zero was then normalized to 100%, and the peaks of subsequent time points were expressed relative to the normalized control. Using this approach in Figure 2, highly linear [$R^2 = 0.9997$ and 0.9682 for *GALI*-(long) and *GALI*-(short), respectively] deadenylation curves were possible from ePAT samples but not LM-PAT samples [$R^2 = 0.1751$ and 0.4049 for *GALI*-(long) and *GALI*-(short), respectively]. The graphs and statistical analyses were prepared using GraphPad Prism software.

Efficiency calculations used to estimate the number of cDNA input molecules were based on a calculated mass of 110 ng and an amplicon length of 120-bp at an estimated efficiency of 98% as user inputs for *oma-2* in the PCR calculator (Supplemental File 2).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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