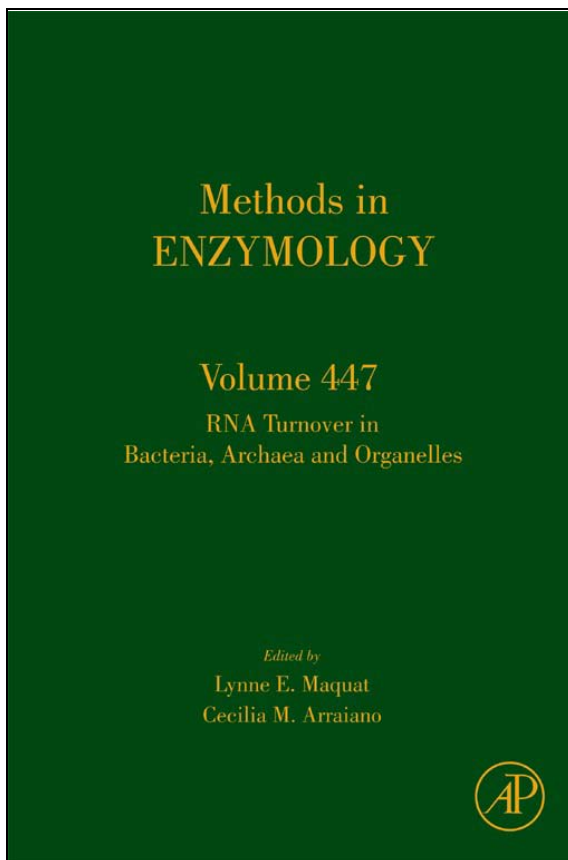


**Provided for non-commercial research and educational use only.  
Not for reproduction, distribution or commercial use.**

This chapter was originally published in the book *Methods in Enzymology*, Vol. 447, published by Elsevier, and the attached copy is provided by Elsevier for the author's benefit and for the benefit of the author's institution, for non-commercial research and educational use including without limitation use in instruction at your institution, sending it to specific colleagues who know you, and providing a copy to your institution's administrator.



All other uses, reproduction and distribution, including without limitation commercial reprints, selling or licensing copies or access, or posting on open internet sites, your personal or institution's website or repository, are prohibited. For exceptions, permission may be sought for such use through Elsevier's permissions site at: <http://www.elsevier.com/locate/permissionusematerial>

From: Shimyn Slomovic, Victoria Portnoy, and Gadi Schuster, Detection and Characterization of Polyadenylated RNA in Eukarya, Bacteria, Archaea, and Organelles. In Lynne E. Maquat and Cecilia M. Arraiano, editors: *Methods in Enzymology*, Vol. 447, Burlington: Academic Press, 2008, pp. 501-520.

ISBN: 978-0-12-374377-0

© Copyright 2008 Elsevier Inc.

Academic Press.

# DETECTION AND CHARACTERIZATION OF POLYADENYLATED RNA IN EUKARYA, BACTERIA, ARCHAEA, AND ORGANELLES

Shimyn Slomovic, Victoria Portnoy, *and* Gadi Schuster

## Contents

1. Introduction	502
2. Oligo(dT) RT-PCR Detection of Polyadenylated Degradation Intermediates	504
2.1. Steps 1 and 2: RNA purification and cDNA synthesis	505
2.2. Steps 3 to 6: PCR amplification and gel extraction	505
2.3. Steps 7 to 9: Cloning of the PCR products, selection, and sequencing analysis	508
3. Circularized Reverse Transcription (cRT)-PCR-Sequencing/Labeling Method for the Characterization of Polyadenylated RNA	509
3.1. Steps 1 and 2: RNA isolation, circularization, and RT	510
3.2. Steps 3 and 4: PCR amplification	512
3.3. Step 5a: Analysis by DNA sequencing	512
3.4. Step 5b: Analysis by radioactive labeling	513
4. cRT-PCR for the Detection of Truncated Polyadenylated RNA Degradation Intermediates	516
4.1. Polyadenylation analysis by 3'-end labeling and ribonuclease digestion	516
References	519

## Abstract

The posttranscriptional addition of poly(A) extensions to RNA is a phenomenon common to almost all organisms. In eukaryotes, a stable poly(A) tail is added to the 3'-end of most nucleus-encoded mRNAs, as well as to mitochondrion-encoded transcripts in animal cells. In prokaryotes and organelles, RNA molecules are polyadenylated as part of a polyadenylation-stimulated RNA degradation pathway. In addition, polyadenylation of nucleus-encoded transcripts in yeast and human cells was recently reported to promote RNA degradation. Not only

homopolymeric poly(A) tails, composed exclusively of adenosines, but also heteropolymeric poly(A)-rich extensions, which include the other three nucleotides as well, have been observed in bacteria, archaea, chloroplasts, and human cells. In most instances, the detection of nonabundant truncated transcripts with post-transcriptionally added poly(A) or poly(A)-rich extensions serves as a telltale sign of the presence of a polyadenylation-stimulated RNA degradation pathway. In this chapter, we describe several methods found to be efficient in detecting and characterizing polyadenylated transcripts in bacteria, archaea, organelles, and nucleus-encoded RNAs. Detailed protocols for the oligo(dT)- and circularized reverse transcription (cRT) PCR methods, as well as the ribonuclease digestion method, are outlined, along with examples of results obtained with these techniques.

## 1. INTRODUCTION

Polyadenylated RNA molecules are present in most organisms. In eukaryotes, a stable poly(A) tail is added to the 3'-end of most nucleus-encoded mRNAs. This process is important for mRNA stability, exit from the nucleus, and translation initiation. Stable poly(A) tails are also present at the 3'-ends of animal mitochondrial transcripts and are essential to establish functional translational stop codons for transcripts lacking such, as well as other postulated functions (Gagliardi *et al.*, 2004). In prokaryotes and organelles, RNA molecules are polyadenylated as part of a polyadenylation-stimulated RNA degradation pathway (Slomovic *et al.*, 2006b). This process consists of three sequential stages, initiating with endonucleolytic cleavage, followed by the addition of degradation-stimulating poly(A) or poly(A)-rich sequences to these cleavage products, and ending in exonucleolytic degradation. In addition, polyadenylation of nucleus-encoded transcripts in yeast and human cells was recently reported to promote RNA degradation, indicating that a form of polyadenylation-stimulated RNA degradation is present in the nucleus of eukaryotic cells as well, probably as part of a quality control mechanism (Doma and Parker, 2007; Houseley *et al.*, 2006; Isken and Maquat, 2007; Vanacova and Stef, 2007). In most instances, the detection of truncated transcripts harboring poly(A) extensions serves as initial evidence hinting at the presence of a polyadenylation-stimulated degradation RNA degradation pathway (Slomovic *et al.*, 2008).

Not only homopolymeric poly(A) tails, composed exclusively of adenosines, but also heteropolymeric poly(A)-rich extensions, which include the other three nucleotides as well, have been observed in bacteria, archaea, chloroplasts, and human cells (Slomovic *et al.*, 2006b, 2008). Polynucleotide phosphorylase (PNPase) and the archaeal exosome, which bear strong similarities to one another, both functionally and structurally, were found to polymerize the heteropolymeric tails in bacteria, spinach chloroplasts, and archaea.

Rapid degradation is the fate of the truncated RNA molecules, once polyadenylated, and, therefore, an efficient amplification and selection procedure is required to detect these molecules. The amplification power of the PCR method is essential. However, the combination of powerful amplification and the very low abundance of the intermediate degradation products results in nonspecific amplification of nonrelated sequences. To filter out these artifacts, the methods described in this chapter were developed and found to be highly efficient in detecting and/or isolating polyadenylated degradation intermediates derived from bacterial, archaeal, organellar, and nucleus-encoded RNAs (Lisitsky *et al.*, 1996; Portnoy *et al.*, 2005; Portnoy and Schuster, 2006; Rott *et al.*, 2003; Slomovic *et al.*, 2005, 2006a; see also chapters in RNA Turnover, Part B, ed. Maquat and Kiledjian).

Because the first method described here, oligo(dT)-primed RT-PCR, although highly efficient, is selective for relatively long polyadenylated tails, the picture obtained from this form of analysis does not always represent the entire mRNA population of a studied gene in terms of the ratio of adenylated versus nonadenylated transcripts and the span of tail lengths that is present. To obtain a more representative picture of the RNA population and tail (homo-hetero) consistency, a second, more quantitative, method should be used. Such a method is circularized reverse transcription (cRT)—RT-PCR. Actually this technique is usually applied to analyze stable poly(A) tails located at the mature 3'-ends of animal mitochondrial transcripts and nucleus-encoded mRNAs (Slomovic and Schuster, 2008). In many cases, applying this method to detect RNA degradation intermediates in various systems results in the isolation of molecules either lacking any adenosine tails or containing short extensions of only several adenosines (Slomovic *et al.*, 2006a; West *et al.*, 2006). This is most likely due to the rapid pace of degradation once the endo-cleavage products are tagged by polyadenylation but also shows that, in some systems, most of these posttranscriptional extensions are oligo(A) tails.

The third method described here is the 3'-end labeling and ribonuclease digestion analysis of poly(A) tails. This method is relatively simple and provides a global picture of the polyadenylation of all the gene transcripts in the tested organism at once. Likewise, organisms in which only unstable or no polyadenylation occurs, such as prokaryotes/organelles or certain archaea, respectively, can be analyzed and categorized with this method (Lisitsky *et al.*, 1996; Mohanty *et al.*, 2004; Portnoy *et al.*, 2005). In eukaryotes containing nucleus-encoded RNA with stable poly(A) tails, this method can disclose the lengths of these extensions (Bonisch *et al.*, 2007).

Additional methods for the detection and analysis of 3'-end extensions have been developed, including the oligonucleotide ligation RT-PCR technique, which provides results similar to those achieved with cRT-RT-PCR.

This method has the advantage that only sequences attached to the 3'-end are observed (Aravin and Tuschl, 2005; Elbashir *et al.*, 2001; Pfeffer *et al.*, 2003). However, additional uses of cRT-PCR, such as the analysis of 5'-processing, cannot be achieved with oligo ligation. See also protocols at [http://web.wimit.edu/bartel/pub/protocols\\_reagents.htm](http://web.wimit.edu/bartel/pub/protocols_reagents.htm) and <http://banjo.dartmouth.edu/lab/MicroRNAs/mir.html>.

The hybrid-selection RT-PCR method, which includes an additional selective step before circularization in the cRT-PCR, enables the detection of very low abundant and gene-specific extensions, for example, tails added to the pre-mRNA from the human  $\beta$ -globin gene during pre-mRNA processing in the nucleus (Risland *et al.*, 2007; West *et al.*, 2006). The smart RT-PCR protocol enables the detection of a population of adenylated RNA without the need to use a gene-specific oligonucleotide (Holec *et al.*, 2006). Once isolated and sequenced, the transcript is subsequently identified with a BLAST algorithm. This method is mostly useful for the identification of unknown polyadenylated transcripts found in prokaryotes and organelles.

## 2. OLIGO(dT) RT-PCR DETECTION OF POLYADENYLATED DEGRADATION INTERMEDIATES

The detection of nonabundant, truncated, polyadenylated RNA molecules corresponding to a studied gene sequence is considered to be a telltale sign of the presence of a polyadenylation-stimulated RNA degradation pathway, as witnessed in prokaryotes and organelles. These molecules, believed to be degradation intermediates between the sequential stages of endonucleolytic cleavage and polyadenylation, are rapidly 3' to 5'-exonucleolytically digested once polyadenylated. Their consequent low abundance makes them difficult to detect and isolate relative to the full-length transcript and the stable poly(A)-tails characterizing most eukaryotic mRNAs and animal mitochondria transcripts. Therefore, a powerful method composed of a number of stages of amplification and specification is necessary to isolate such molecules.

Oligo(dT)-adapter primed RT-PCR is a technique that allows the isolation and sequence identification of the degradation intermediates described previously. Therefore, the sites within the transcript sequence at which polyadenylation occurred can be detected. Furthermore, the nature of the posttranscriptionally added extensions, in terms of nucleoyide composition, can be determined as well. This, in turn, can serve as the first step in identifying the enzyme responsible for its polymerization (Mohanty and Kushner, 2000; Rott *et al.*, 2003; Slomovic *et al.*, 2008).

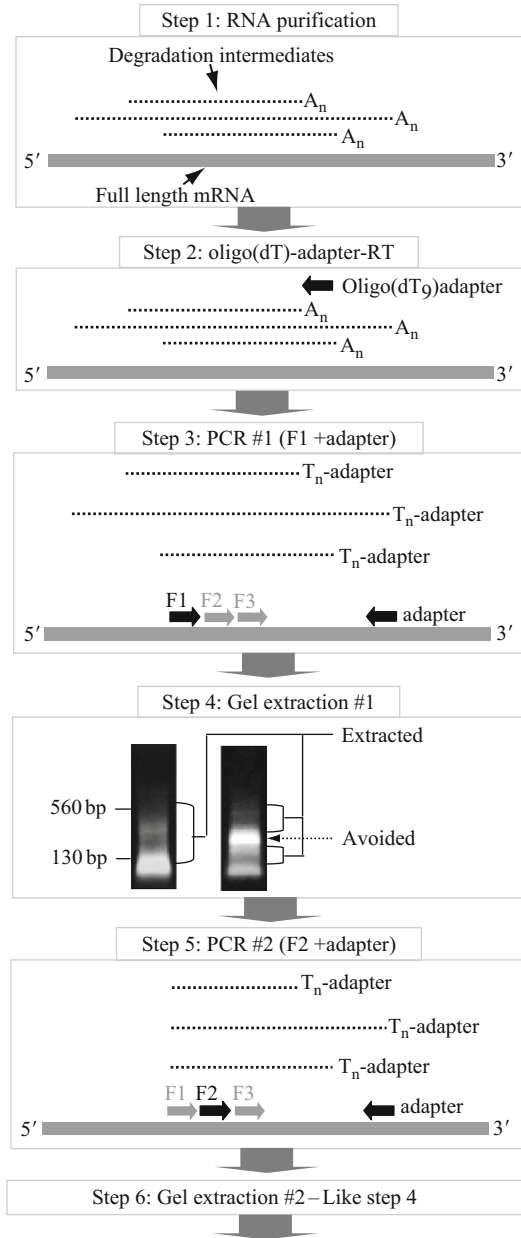
## 2.1. Steps 1 and 2: RNA purification and cDNA synthesis

Figure 24.1 shows a schematic description of the nine steps that comprise this method. When studying an organellar system, although organelles can be isolated before RNA purification, total-cellular RNA purification is usually adequate (step 1). RNA can be generated free of contaminating DNA by incubation with DNase. However, this is usually not necessary. Total-cell RNA is reverse transcribed, primed with an adapter oligo that includes a (dT) track at its 3'-end (step 2). The adapter sequence should be ~20 nt excluding the oligo(dT). The length of the (dT) stretch should be adequate to allow annealing at the temperature at which the RT reaction is performed; T<sub>9</sub> has been applied efficiently but longer oligos of up to T<sub>18</sub> can be used as well (Lisitsky *et al.*, 1996; Portnoy *et al.*, 2005; Rott *et al.*, 2003; Slomovic *et al.*, 2005).

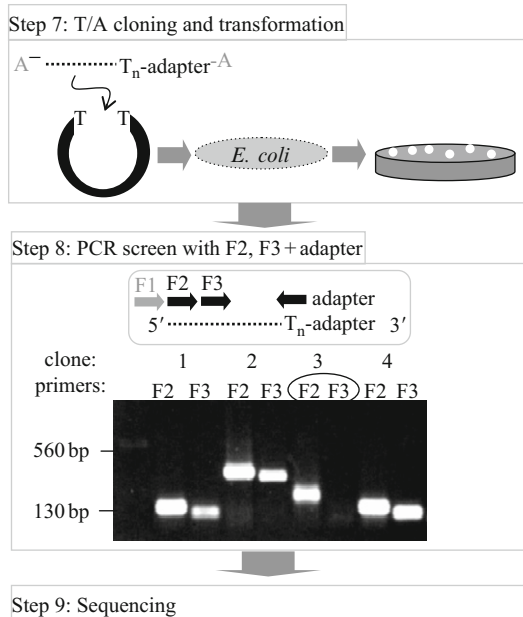
## 2.2. Steps 3 to 6: PCR amplification and gel extraction

During the RT reaction, the oligo(dT)-adapter can anneal at any point along the poly(A)-tails and, therefore, it is difficult to confirm its original length. Only transcripts with poly(A) extensions undergo reverse transcription, and the resulting first-strand cDNA contains the adapter sequence at its 5'-end (Fig. 24.1, step 3). An RT reaction with 5 µg of total-cell RNA should finally be diluted to 50 µl from which 1 µl will be used to template each PCR reaction in the next step. In this step, the cDNA is PCR-amplified with a forward primer, termed F1, specific to the chosen gene. The reverse primer is the adapter oligo lacking the oligo(dT) track (step 3). It is recommended that one apply multiple PCR reactions (up to 4 or a total volume of 80 µl) to increase the total amount of product. A Taq polymerase with A-tailing activity (in which an adenosine overhang is added to each end of the PCR product) is essential for the T/A cloning step (step 7) described at a later stage in the protocol.

In step 4, the PCR products can either be purified with an appropriate kit or electrophoresed in and eluted from a 1% agarose gel. In both cases, duplicate or triplicate reactions should be purified together. In all stages of this method, PCR templates should not be used as templates taken directly from the previous PCR step, because remnants of the F1 primer will have a negative effect on the specificity of subsequent stages. Indeed, the preferred method is to run the PCR products through an agarose gel, as shown in Fig. 24.1 (step 4), because this allows the exclusion of nonspecific bands visible on ethidium bromide staining. Because the PCR products are a mixture of molecules with 3'-ends terminating at different sites relative to the full-length mRNA, a smear, rather than a discrete band, is expected after electrophoresis (Fig. 24.1, step 4). Moreover, any discrete bands should be avoided, because they are most likely due to either nonspecific amplification



**Figure 24.1** (Continued)



**Figure 24.1** Schematic presentation of the oligo(dT) RT-PCR protocol developed for the detection and isolation of nonabundant, truncated, polyadenylated RNA molecules that are intermediates of the polyadenylation-stimulated RNA degradation pathway.

of undesired genes or even annealing of either the F1 primer or adapter in opposing directions (step 4). Such a product could dominate the T/A cloning stage described in the following. Likewise, during all stages of PCR, it is useful to include negative control reactions that use each of the primers alone and a reaction templated by the products from an RT reaction in which no reverse transcriptase has been added. These negative controls allow easy identification of unwanted or irrelevant gel bands that are due to nonspecific amplification and amplification of DNA contaminants.

The region between 100 and 600 bp should be excised from the gel and eluted with an appropriate kit at a final volume of 10 to 15  $\mu\text{l}$ . If the minimal elution volume exceeds this, the purified product volume can be decreased by use of a speed-vac. In step 5, PCR #2 is performed with the adapter oligo paired with F2, a second gene-specific primer, nested immediately downstream of the F1 primer. In PCR #2, 1  $\mu\text{l}$  of gel-purified product from PCR #1 should be used. This second PCR, which uses a nested gene-specific forward primer before the T/A cloning stage, is essential for product specificity as it excludes almost all unwanted products that were amplified during PCR #1 due to chance sequence similarity between the 3'-end of the F1 primer and unrelated cDNA. This simple step increases specificity as much as 500%. PCR #2 products should be purified as described for PCR #1 (step 6).



### 2.3. Steps 7 to 9: Cloning of the PCR products, selection, and sequencing analysis

In step 7, the entire volume of purified PCR #2 products is inserted into a T/A vector. The ligation reaction is precipitated and resuspended in 8  $\mu$ l H<sub>2</sub>O, of which 1  $\mu$ l is introduced into competent cells via electroporation. LB plates containing the appropriate selective antibiotic(s),  $\beta$ -galactosidase, and IPTG are then used to select colonies that potentially harbor a T/A vector with an insert (step 7). Ideally, each insert is expected to have been derived from a different truncated, polyadenylated RNA molecule corresponding to the studied gene.

In the eighth step, white, antibiotic-resistant colonies are screened with two consecutive PCR reactions after transferring each screened colony to a second LB plate. The first PCR reaction is identical to PCR #2, in which the F2 primer is paired with the adapter (step 8). The second PCR reaction, applied to the same colony, uses a third gene-specific forward primer, F3, which is nested immediately downstream of the F2 primer in the gene sequence, along with the adapter. The products of these two PCR reactions are electrophoresed side by side in a 1% agarose gel (step 8). In each pair, the first lane will ideally display one product band of any size within the range of 100 to 600 bps, according to the region excised from the gel in step 4. If the insert, indeed, corresponds to the studied gene, the second lane of the pair will display a single product band which is  $\sim$ 20 bp smaller than that seen in the first lane, assuming that the F3 primer is nested immediately downstream of the F2 oligo and is of 20 nt in length. In [Fig. 24.1](#), step 8, four PCR pairs from four screened colonies are shown. All but the third pair are positive, because the second lane of this pair shows no product.

The final step in the oligo(dT)-adapter primed RT-PCR method is the sequencing of each positive clone after plasmid purification (step 9). Although strict specificity measures are taken in all steps leading up to this stage, sequencing is essential to fully identify the plasmid insert. When aligned with the full-length mRNA sequence, sites at which the truncated molecules are polyadenylated can be determined. However, one must be aware that as few as six adenosines encoded in the gene sequence are sufficient to cause false annealing of the oligo(dT) during RT. Therefore, cases in which the polyadenylation sites of sequenced clones coincide with A-rich regions within the encoded mRNA sequence should be treated with suspicion. As stated previously, not only the polyadenylation sites but the nature of the extensions, in terms of homo/heterogeneity of nucleotide content, can be examined and used to identify the enzyme responsible for this posttranscriptional activity. Although homopolymeric poly(A) tails are produced by poly(A) polymerases, heteropolymeric tails, consisting of all four

nucleotides, are produced by polynucleotide-phosphorylase (PNPase) or the archaeal exosome (Mohanty and Kushner, 2000; Portnoy and Schuster, 2006; Portnoy *et al.*, 2005; Rott *et al.*, 2003; Yehudai-Resheff *et al.*, 2001). An example of poly(A) tails of heteropolymeric and homopolymeric nucleotide content, identified with the oligo(dT) RT-PCR method described previously, is presented in Fig. 24.2.

### 3. CIRCULARIZED REVERSE TRANSCRIPTION (CRT)-PCR-SEQUENCING/LABELING METHOD FOR THE CHARACTERIZATION OF POLYADENYLATED RNA

As described previously, oligo(dT) RT-PCR allows efficient isolation of nonabundant, truncated, polyadenylated RNA molecules. However, the nature by which this method selectively amplifies polyadenylated RNA is the very reason that it cannot be applied if the goal is to not only isolate such molecules, but also assess the unaltered length of the poly(A) extensions, because the oligo(dT) can anneal at any point along the tail during the RT reaction. Also, the oligo(dT) RT-PCR is a “fishing” method for isolating

#### Cyanobacteria; *Synechocystis: rbcL*

1. A<sub>2</sub>GA<sub>2</sub>GA<sub>4</sub>G<sub>2</sub>A<sub>3</sub>G<sub>2</sub>A<sub>3</sub>GAGAUA<sub>12</sub>GA<sub>9</sub>
2. GA<sub>9</sub>TA<sub>4</sub>UAAG<sub>2</sub>A<sub>8</sub>GUA<sub>4</sub>GU<sub>2</sub>A<sub>2</sub>UAGA<sub>6</sub>GUGCAGA<sub>4</sub>CA<sub>6</sub>GATA<sub>2</sub>CA<sub>2</sub>GACA<sub>2</sub>GUAUA<sub>3</sub>CGAUA<sub>12</sub>UA<sub>5</sub>CAGAU<sub>4</sub>UA<sub>9</sub>U<sub>3</sub>ACG<sub>3</sub>CACAGA<sub>2</sub>UAAGCA<sub>3</sub>GAGAUGA<sub>3</sub>UAGGACA<sub>6</sub>

#### Archaea; *Sulfolobus solfataricus: 16S rRNA*

1. A<sub>2</sub>GA<sub>2</sub>GA<sub>4</sub>G<sub>2</sub>A<sub>3</sub>G<sub>2</sub>A<sub>3</sub>GAGAUA<sub>12</sub>GA<sub>9</sub>
2. AGAUA<sub>3</sub>CUGA<sub>2</sub>GACAGA<sub>7</sub>G<sub>2</sub>A<sub>4</sub>GA<sub>2</sub>UA<sub>4</sub>GAUAGAGAU<sub>4</sub>UAGUAGAG<sub>3</sub>AUGA<sub>3</sub>GACUA<sub>12</sub>G<sub>2</sub>AUA<sub>17</sub>

#### Human Mitochondria: *CoxI*

1. A<sub>77</sub>
2. A<sub>45</sub>
3. A<sub>23</sub>

#### Human nucleus: *28S rRNA*

1. AGAUA<sub>3</sub>CUGA<sub>2</sub>GACAGA<sub>7</sub>G<sub>2</sub>A<sub>4</sub>GA<sub>2</sub>UA<sub>4</sub>GAUAGAGAU<sub>4</sub>UAGUAGAG<sub>3</sub>AUGA<sub>3</sub>GACUA<sub>12</sub>G<sub>2</sub>AUA<sub>17</sub>
2. A<sub>4</sub>G<sub>2</sub>A<sub>3</sub>GA<sub>3</sub>GAGA<sub>4</sub>GA<sub>16</sub>
3. A<sub>70</sub>
4. A<sub>45</sub>

**Figure 24.2** Examples of several homopolymeric and heteropolymeric tails isolated and sequenced with oligo(dT) RT-PCR. References describing the detection of these tails are: Cyanobacteria (Rott *et al.*, 2003), Archaea (Portnoy *et al.*, 2005), human mitochondria (Slomovic *et al.*, 2005), and human nucleus-encoded rRNA (Slomovic *et al.*, 2006a).

nonabundant polyadenylated molecules and, therefore, does not indicate which fraction of the transcript population is adenylated and to what extent. For this purpose, a method that is not based on poly(A) selectivity must be used.

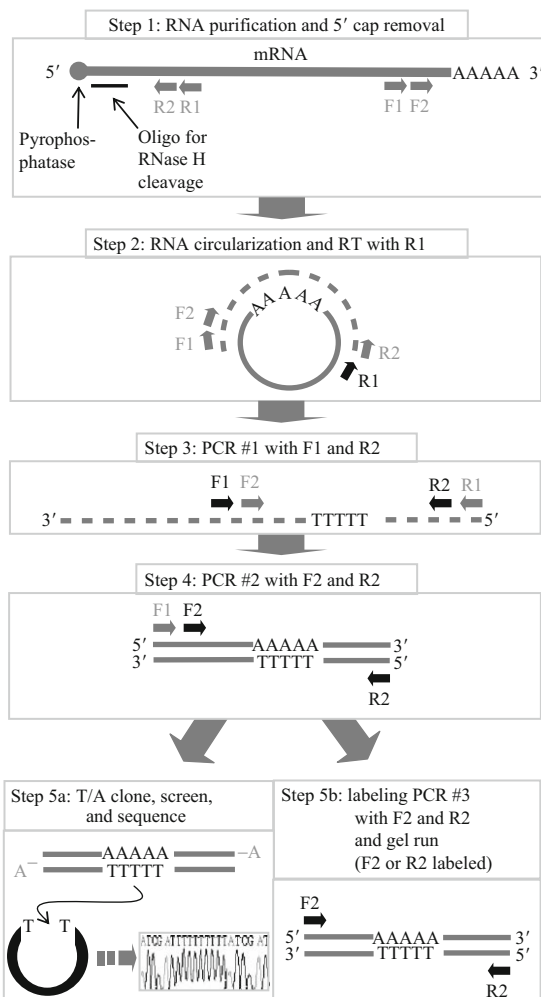
cRT-PCR is often used to assess the length and nature of stable post-transcriptional extensions at the mature 3'-ends of mRNA (Nagaike *et al.*, 2005; Slomovic and Schuster, 2008; Temperley *et al.*, 2003). Such stable poly(A) tails characterize most nucleus-encoded mRNA in eukaryotic cells and also mitochondrial RNA in animal cells.

### 3.1. Steps 1 and 2: RNA isolation, circularization, and RT

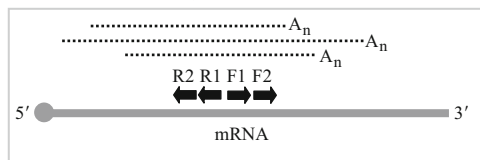
Figure 24.3A presents a schematic description of the application of cRT-PCR for the evaluation of stable poly(A) tails. The same guidelines for RNA purification as described for the oligo(dT) RT-PCR method should be applied here. Once purified,  $\sim 5 \mu\text{g}$  of total RNA is circularized by ligation of the 5'- and 3'-ends by T4 RNA ligase. When studying nucleus-encoded mRNA, tobacco acid pyrophosphatase (TAP) must be applied before this stage, because ligation is hampered by the 5'-cap. TAP hydrolyzes the phosphoric acid anhydride bonds in the triphosphate bridge of the cap structure, releasing the cap nucleoside and generating a 5'-phosphorylated terminus. Alternately, oligonucleotide-directed RNase H cleavage can be applied to remove the 5'-region of the transcript, thereby producing a 5'-phosphorylated terminus ready for the ligation step. When studying mRNA generated from polycistronic RNA from organelles or prokaryotes, this step is not necessary, because such molecules lack a 5'-cap or three phosphates of the transcription initiation. If the ligation reaction included DNase to digest contaminant DNA, a phenol/chloroform purification step should be included to avoid DNase contamination at later stages.

In the next step, the entire resuspension of the ligation reaction, after precipitation, is subjected to reverse transcription (RT) and should, therefore, be resuspended in an appropriate volume. As shown in Fig. 24.3A, the reverse oligo used to prime the RT reaction, termed R1, should be designed to anneal to the mRNA sense strand  $\sim 100$  nt downstream of the predicted 5'-end (if RNase H cleavage was applied, the "new" 5 end). During RT, the enzyme reverse transcribes, initiating at the R1 primer, crossing the adjoined 5'- and 3'-ends, including any posttranscriptional extensions, until it eventually detaches. Once completed, the RT reaction should be diluted to a final volume of  $50 \mu\text{l}$  from which  $1 \mu\text{l}$  will be taken for each subsequent PCR reaction, as described in the following.

A cRT-PCR sequencing/labeling for stable polyadenylation



B cRT-PCR sequencing for unstable polyadenylation



**Figure 24.3** Schematic presentation of the circularized (c)RT-PCR protocol for the quantitative analysis of both stable (A) and unstable (B) poly(A)-tails.

### 3.2. Steps 3 and 4: PCR amplification

In the first PCR reaction, PCR #1, a reverse primer (relative to the sense directionality of the original mRNA sequence) termed R2, which is nested upstream of the R1 primer and  $\sim 50$  nt downstream of the 5'-end, is used. This oligo is paired with a forward primer, termed F1, which is designed to anneal to the mRNA  $\sim 100$  nt upstream of the 3'-end (Fig. 24.3A). As described in the previous paragraph for oligo(dT) RT-PCR, duplicate or triplicate reactions should be either run through 1% agarose gel, excised, and eluted or cleaned with an appropriate PCR purification kit. Unlike the oligo(dT) isolation of truncated polyadenylated RNA molecules, when examining mature 3'-end stable polyadenylation with cRT-PCR, the expected product size can be estimated. In the case described here, the expected product size from PCR #1 includes 50 nt from the R2 primer to the 5'-end and 100 nt from the F1 primer to the 3'-end. The product size is increased by any posttranscriptional extensions at the 3' (or 5') end. Therefore, when excising the PCR #1 products from the agarose gel, the excised gel fragment should start from the expected product size and include higher molecular weight to accommodate any 3'-tails. These products are then cleaned and eluted to a volume of  $\sim 10 \mu\text{l}$ .

In the next PCR stage, PCR #2,  $1 \mu\text{l}$  from the gel elution is used to template each reaction. The same R2 primer from the PCR #1 step is used, along with a second forward primer, F2, which is nested downstream of the F1 primer and 50 nt upstream of the 3'-end (Fig. 24.3A). The products from the PCR #2 reactions are cleaned by electrophoresis through 1% agarose gel, like the PCR #1 products, but the expected product size is now  $\sim 100$  bp, plus any increase in size because of the posttranscriptional tail. The elution volume, as before, is  $\sim 10 \mu\text{l}$ .

### 3.3. Step 5a: Analysis by DNA sequencing

At this point, two different approaches can be chosen: cloning and sequencing or radioactive labeling and detection by gel fractionation and autoradiography. For the sequencing alternative (step 5a), half of the elution volume is used for a cloning reaction with a T/A vector system, as in the case of oligo(dT) RT-PCR, and the other half is reserved for the labeling option, described later. The steps that include T/A cloning, precipitation, and transformation to competent cells are as described previously for oligo(dT) RT-PCR. In contrast to the oligo(dT) RT-PCR colony screening, here, individual resistant white bacterial colonies are PCR screened with a single PCR reaction, identical to PCR #2, with the R2 and F2 primers. Ideally, each positive colony contains a T/A vector with an insert derived from the adjoined 5'- and 3'-ends of the studied mRNA, including any 3' (or 5') extensions. The difference in molecular weight of PCR products between

different colonies is due only to variations in poly(A)-tail lengths, assuming that the RNA molecules they originated from were properly processed and, therefore, initiated and terminated at the same points (Slomovic and Schuster, 2008).

After plasmid purification from positive colonies and sequencing, the processing of the RNA molecules and the nature and length of the 3'-extensions can be assessed. It must be noted that after sequencing, when applying this cRT-PCR method, it is not possible to determine whether a posttranscriptional extension was originally added to the 5' or 3'-end of the RNA molecule. If, in the given system, it is yet unknown whether 5'-extensions exist and it is, therefore, unclear if the observed tail is 5' or 3', it is possible to apply a similar procedure in which, instead of RNA circularization, an oligonucleotide is ligated to the 3'-end of the transcript and serves as a platform for the annealing of a reverse primer (coupled with a gene-specific forward primer) during RT and subsequent PCR reactions (Elbashir *et al.*, 2001).

### 3.4. Step 5b: Analysis by radioactive labeling

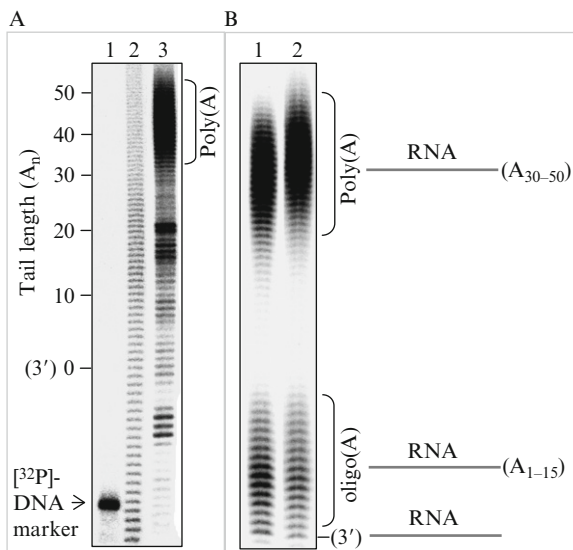
The second approach that can be applied after the PCR #2 gel excision and elution stage is radioactive labeling (step 5b). Unlike the sequencing approach, which involves analysis of individual clones and, in turn, the assessment of 5'- and 3'-processing and the homo/hetero composition of the poly(A) tail, the labeling approach reveals a more global view of the entire mRNA population of the studied gene at the time of RNA purification. Therefore, trends of lengthening or shortening of the poly(A) tails in the RNA population of the studied gene can be easily detected, whereas with the sequencing approach, a large (statistical) amount of clones need to be sequenced to gain a general picture of the polyadenylation status of a studied gene (Nagaïke *et al.*, 2005; Slomovic and Schuster, 2008). The labeling approach is based on the radioactive labeling of the products from PCR #2, during a third PCR reaction, termed PCR #3. To achieve this, either the R2 or F2 oligos must be labeled with [ $\gamma$ - $^{32}$ P]-ATP and polynucleotide kinase (PNK).

We advise first performing two negative control PCR reactions to determine which of the two oligos, R2 or F2, is more suitable to be used in PCR #3. In each of these negative control reactions, 1- $\mu$ l template from the PCR #2 elution is used, and only one of the primers is present. On examination by ethidium bromide agarose gel staining, the primer to be labeled will be that which does not display any dominant gel bands when reacted with itself, which could interfere with the final analysis. Once either the R2 or F2 primer is chosen, it is labeled in a standard labeling reaction with PNK and [ $\gamma$ - $^{32}$ P]-ATP. 100 ng of the oligo is recommended to be used in the labeling reaction, which, after precipitation, is resuspended in  $\sim$ 10  $\mu$ l H<sub>2</sub>O. For the PCR #3 labeling reaction, 1- $\mu$ l template from the PCR #2 elution is applied per total 20- $\mu$ l

reaction; 5 ng of the unlabeled oligo and 5 ng of the labeled oligo (either R2 or F2) are added to the reaction. In the case of the labeled primer, it is possible to simply add  $\sim 0.5 \mu\text{l}$  from the  $10 \mu\text{l}$  of labeled oligo, because this is approximately 5 ng (50 nM in the total PCR volume of  $20 \mu\text{l}$ ). Between 6 and 10 cycles are sufficient to achieve labeled PCR products.

In the final stage, the  $[^{32}\text{P}]$ -labeled PCR #3 products are fractionated through denaturing 10% acryl amide gel; 5 to  $10 \mu\text{l}$  of the  $20 \mu\text{l}$  are sufficient and should be denatured in a formamide sample buffer for 2 min at  $90^\circ\text{C}$  before loading. The gel run should be long enough to achieve high resolution and then exposed in a radiography cassette. A molecular weight marker can be achieved by producing an  $[\alpha^{32}\text{P}]$ -UTP-labeled synthetic RNA (transcribed with T7, SP6, or T3 RNA polymerases and a transcription plasmid) or by labeling a DNA nucleotide sequence of known length with PNK and  $[\gamma^{32}\text{P}]$ -ATP. These markers supply a point of reference from which band length (in nucleotides) can be counted. Because nucleotide-nucleotide resolution can be reached with 10% acryl amide gel, in the PCR #3 products lane, every possible length is usually represented by a gel band, because of natural detachment of the Taq polymerase during PCR. If a clear nucleotide-nucleotide representation is not apparent in the PCR product lane, such a ladder can be achieved by producing a second  $[\alpha^{32}\text{P}]$ -UTP-labeled synthetic RNA and fragmenting it by alkaline hydrolysis, by incubating it at  $90^\circ\text{C}$  in buffer carbonate (pH = 11) for various time points. From the point of reference supplied by the  $[^{32}\text{P}]$ -DNA marker or the  $[\alpha^{32}\text{P}]$ -UTP-labeled synthetic RNA, one can identify the  $\sim 100$  nt region, which is the expected size of the PCR product lacking any additional extensions (poly(A) tail), by counting the bands either in the PCR product lane or the  $[^{32}\text{P}]$ -labeled RNA fragmented by alkaline hydrolysis. From this “zero” point, one can climb the gel bands in the PCR product lane like a ladder while counting, until reaching a point at which the gel bands drastically intensify. The distance in nucleotide units (gel bands) from the “zero” point ( $3'$  in Fig. 24.4) to the intense gel bands is the length of the poly(A) extensions. Because the PCR #3 products originated from a population of mRNA molecules of the studied gene with poly(A) tails of varying lengths, a discrete band is not expected to be observed, rather, a number of bands comprising an average length (Fig. 24.4).

Figure 24.4 shows an example of two gel runs of the human mitochondrial mRNAs, COX1 (A) and ND3 (B) (Slomovic and Schuster, 2008). In the COX1 gel (A), the first lane displays a  $[^{32}\text{P}]$ -DNA marker, the second lane shows a  $[^{32}\text{P}]$ -labeled RNA, fragmented by alkaline hydrolysis, and the third lane is the PCR #3 products lane. The “zero” point is marked as ( $3'$ ), and a polyadenylated fraction with tails ranging from  $\sim 35$  to 55 adenosines in length is apparent. In the ND3 gel (B), two fractions of the studied mRNA population can be observed in each of the two lanes; an oligoadenylated fraction, with tails of up to 15 nucleotides in length, and a



**Figure 24.4** Example of results obtained with cRT-PCR labeling. (A) The stable poly (A)-tails located at the 3'-end of the human mitochondrial COX1 transcript were analyzed as described in the text and Fig. 24.3. Lane 1, [<sup>32</sup>P]-DNA marker; Lane 2, *in vitro*-transcribed [<sup>32</sup>P]-labeled RNA ladder after alkaline hydrolysis; Lane 3, total RNA from HeLa cells. The DNA marker is used, along with the alkaline hydrolysis ladder, to identify the band, in Lane 3, that represents the COX1 mRNA lacking any 3'-extensions (position marked as 3'-0). From this "zero" point, the rungs of the ladder are counted until reaching the polyadenylated fraction (here, between 35 and 55 adenosines). (B) Human mitochondrion-encoded ND3 mRNA was analyzed by cRT-PCR. Total-RNA purified from HeLa cells (Lane 1) and from a HeLa cell line with stable RNAi-mediated silencing of polynucleotide phosphorylase (PNPase) (Lane 2) was subjected to the protocol described in Fig. 24.3 (Slomovic and Schuster, 2008). Aside from two distinct RNA fractions observed in both lanes, one oligoadenylated and the other polyadenylated, a slight increase in the lengths of the poly(A)-tails of the polyadenylated RNA fraction in lane 2 is apparent. To the right of the gel picture, the examples of RNA molecules are schematically shown: a bare mRNA, oligoadenylated mRNA, and polyadenylated mRNA.

polyadenylated fraction with extensions of ~30 to 50 adenosines in length. In lane 2, in which the process was applied to RNA isolated from a cell line with constitutive RNAi silencing of the enzyme, polynucleotide phosphorylase (PNPase), an increase in the average length of the polyadenylated fraction is apparent compared with the poly(A) fraction of the nonsilenced cell line in lane 1 (Slomovic and Schuster, 2008). Although the difference is clear, it is nonetheless slight and, therefore, would be difficult to detect on sequencing of even a large number of clones, without applying the labeling assay (Nagaike *et al.*, 2005; Slomovic and Schuster, 2008).



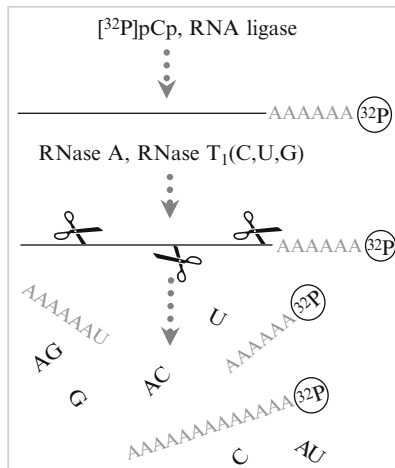
## 4. cRT-PCR FOR THE DETECTION OF TRUNCATED POLYADENYLATED RNA DEGRADATION INTERMEDIATES

cRT-PCR assays can be easily applied for the analysis of stable 3'-polyadenylation, because the PCR product is of a predetermined size, depending on the locations along the gene sequence at which the R2 and F2 primers are designed to anneal, relative to the 5'- and 3' of the studied transcript. However, when the goal is to isolate and analyze truncated polyadenylated degradation intermediates with cRT-PCR, the product size cannot be anticipated, because there is no knowledge of where the truncated molecules begin or end. Therefore, the design of the F1, F2, R1, and R2 primers is different than described previously. In this case, the four primers should be grouped as close as possible to one another, as shown in Fig. 24.3B. The closer the primers are to one another, the higher the chance that a truncated molecule will span the entire region covered by the primers. Otherwise, its isolation would not be possible with this primer set.

When applying cRT-PCR to truncated molecules, the labeling approach is much less conclusive than its application during the analysis of stable 3'-polyadenylation. However, the cRT-PCR sequencing assay can be applied and yield useful results. Other than the primer design described previously, there are no major differences in the protocol for isolating truncated molecules. One disadvantage of this technique is that a high percentage of isolated molecules, although truncated, lack poly(A) tails (Slomovic and Schuster, 2008; Slomovic *et al.*, 2006a). Such molecules could be between the stages of endonucleolytic cleavage and polyadenylation or, alternately, may have been polyadenylated but already partially exonucleolytically digested from 3' to 5'. This is a consequence of the low abundance and rapid digestion of these degradation intermediates and the fact that this assay was especially designed to lack a bias toward polyadenylated RNA to avoid altering the natural poly(A) tail length or report a false ratio between polyadenylated and nonadenylated RNA, as occurs when applying oligo(dT) RT-PCR.

### 4.1. Polyadenylation analysis by 3'-end labeling and ribonuclease digestion

The method of 3'-end labeling, followed by ribonucleolytic digestion, is an efficient way to assess the general polyadenylation status of a particular organism or isolated organelle (Bonisch *et al.*, 2007; Hajnsdorf *et al.*, 1995; Lisitsky *et al.*, 1996; Mohanty *et al.*, 2004; Portnoy and Schuster, 2006; Portnoy *et al.*, 2005; Rott *et al.*, 2003). The principle of this method is presented in Fig. 24.5. First, total RNA is isolated and [<sup>32</sup>P]-labeled at



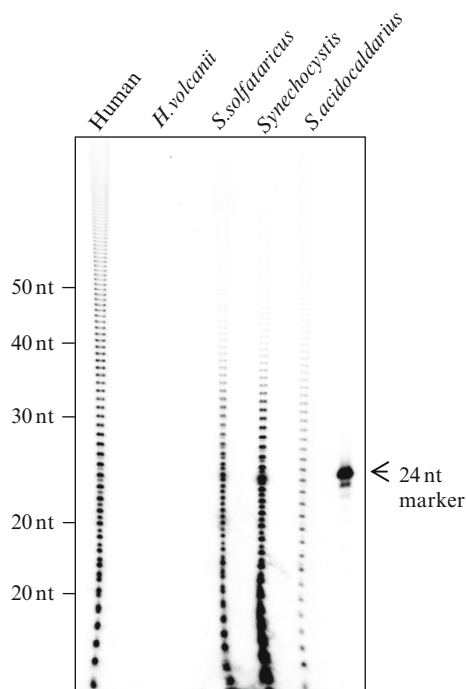
**Figure 24.5** Schematic presentation of the 3'-end-labeling and ribonuclease digestion method used for the general assessment of poly(A)-tail length in a given organism or organelle. Total-cell RNA is purified and [ $^{32}\text{P}$ ]-labeled at the 3'-ends, followed by complete digestion with RNase A and RNase T<sub>1</sub>. Adenosine tracks remain intact, and those from the 3'-end are labeled and, therefore, can be visualized when resolving the digestion products with denaturing polyacrylamide gel electrophoresis and autoradiography.

the 3'-end. This is usually performed by use of [ $^{32}\text{P}$ ]-pCp and T4 RNA ligase. Special care should be taken when choosing the T4 RNA ligase, because some of the vendors' preparations are contaminated with RNA fragments that can interfere when analyzing RNA preparations. Such contamination can cause an organism lacking polyadenylation to appear as if polyadenylation actually exists. Therefore, we advise performing a negative control reaction, in which no RNA is added, before analyzing the tested RNA. From our experience, the T4 RNA ligase of New England Biolabs was contaminated with a small amount of RNA molecules, whereas that obtained from Ambion was found to be clean of any residual polyadenylated transcripts. An alternative to [ $^{32}\text{P}$ ]-pCp labeling is [ $^{32}\text{P}$ ]-labeling with [ $\gamma$ - $^{32}\text{P}$ ] 3'-dATP by the enzyme, poly(A)-polymerase (Wahle, 1991).

The next step is the complete digestion of the RNA by simultaneously applying RNase A and RNase T1. These enzymes efficiently cleave ribonucleic acids after the G, U, and C residues but not after A. Therefore, only adenosine stretches remain intact after digestion, and only those situated at the 3'-end are radioactively labeled. In a typical reaction, digestion of 20  $\mu\text{g}$  RNA with 25  $\mu\text{g}$  of RNase A and 300 units of RNase T1 (both obtained from Sigma) for 1 h at 37 °C is sufficient. After digestion, the RNA is purified by phenol extraction and ethanol precipitation.

Next, the RNA is fractionated on denaturing polyacrylamide gel. Usually, a long (40 cm) 14% acrylamide/1.5% bisacrylamide gel is used and of adequate

acrylamide (AA) percentage to observe tails as short as 4 to 5 adenosines (Mohanty *et al.*, 2004; Portnoy *et al.*, 2005). If shorter tails, of 2 to 3 adenosines, are to be detected, a 20% acrylamide gel can be used. Likewise, if significantly longer tails are of interest, gels of lower percentage should be used. Oligonucleotides of known length, labeled with [ $\gamma$ - $^{32}$ P]-ATP and polynucleotide kinase (PNK), are used as size markers to count the number of adenosines in the tails, similar to the description of markers used in the cRT-PCR labeling procedure. When such a marker is clearly observed in the autoradiogram, the number of adenosines can easily be counted along the ladder of labeled tails that remain intact after the RNase digestion (Fig. 24.6). Alternately, RNA molecules of known length that are synthesized *in vitro* with bacteriophage RNA polymerase and [ $^{32}$ P]-labeled can be applied.



**Figure 24.6** Detection of poly(A)-tails with the 3'-end labeling and ribonuclease digestion method. RNA, purified from several resources, as shown at the top of the figure, was [ $^{32}$ P]-labeled at the 3'-ends, digested with RNase A and RNase T<sub>1</sub>, and resolved by denaturing polyacrylamide gel electrophoresis and autoradiography. The lengths of the tails were determined with a 24-nt oligonucleotide size marker as a point of reference. Long poly(A)-tails were observed in the human RNA preparation, reflecting the stable polyadenylation of the mRNAs. Shorter tails were observed in the cases of hyperthermophilic Archaea and Cyanobacteria, reflecting the unstable poly(A)-tails associated with a polyadenylation-stimulated RNA degradation pathway. No adenosine extensions were detected in the RNA from the halophilic archaea, *H. volcanii*, indicating that RNA does not undergo any form of polyadenylation in this organism (Portnoy *et al.*, 2005). (Reproduced with permission from Portnoy *et al.* [2005].)

In systems in which RNA undergoes polyadenylation, a ladder of poly(A) that is almost equally distributed between all the lengths (from a certain minimum to a certain maximum length) is usually observed, indicating that approximately the same amount of each tail length is present at any given time. The reason for this is not completely clear, but it indicates the level of synchronization between the adenylation and degradation rates.

Not only can this technique be efficiently applied to detect changes in the lengths of the stable poly(A) tail population in eukaryotic cells, but it can also be used to analyze unstable poly(A) extensions involved in polyadenylation-stimulated RNA degradation pathways in prokaryotes and organelles. In addition, this method has been used to reveal the few currently known organisms that metabolize RNA without polyadenylation (Fig. 24.6) (Portnoy and Schuster, 2006; Portnoy *et al.*, 2005; Slomovic *et al.*, 2008).

## REFERENCES

- Aravin, A., and Tuschl, T. (2005). Identification and characterization of small RNAs involved in RNA silencing. *FEBS Lett.* **579**, 5830–5840.
- Bonisch, C., Temme, C., Moritz, B., and Wahle, E. (2007). Degradation of hsp70 and other mRNAs in *Drosophila* via the 5' to 3'-pathway and its regulation by heat shock. *J. Biol. Chem.* **282**, 21818–21828.
- Doma, M. K., and Parker, R. (2007). RNA quality control in eukaryotes. *Cell* **131**, 660–668.
- Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188–200.
- Gagliardi, D., Stepien, P. P., Temperley, R. J., Lightowers, R. N., and Chrzanowska-Lightowers, Z. M. (2004). Messenger RNA stability in mitochondria: Different means to an end. *Trends Genet.* **20**, 260–267.
- Hajnsdorf, E., Braun, F., Haugel-Nielsen, J., and Regnier, P. (1995). Polyadenylation destabilizes the *rpsO* mRNA of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **92**, 3973–3977.
- Holec, S., Lange, H., Kuhn, K., Alioua, M., Borner, T., and Gagliardi, D. (2006). Relaxed transcription in *Arabidopsis* mitochondria is counterbalanced by RNA stability control mediated by polyadenylation and polynucleotide phosphorylase. *Mol. Cell. Biol.* **26**, 2869–2876.
- Houseley, J., LaCava, J., and Tollervey, D. (2006). RNA-quality control by the exosome. *Nat. Rev. Mol. Cell. Biol.* **7**, 529–539.
- Isken, O., and Maquat, L. E. (2007). Quality control of eukaryotic mRNA: Safeguarding cells from abnormal mRNA function. *Genes Dev.* **21**, 1833–1856.
- Lisitsky, I., Klaff, P., and Schuster, G. (1996). Addition of poly(A)-rich sequences to endonucleolytic cleavage sites in the degradation of spinach chloroplast mRNA. *Proc. Natl. Acad. Sci. USA* **93**, 13398–13403.
- Mohanty, B. K., and Kushner, S. R. (2000). Polynucleotide phosphorylase functions both as a 3' to 5'-exonuclease and a poly(A) polymerase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **97**, 11966–11971.
- Mohanty, B. K., Maples, V. F., and Kushner, S. R. (2004). The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol. Microbiol.* **54**, 905–920.

- Nagaike, T., Suzuki, T., Katoh, T., and Ueda, T. (2005). Human mitochondrial mRNAs are stabilized with polyadenylation regulated by mitochondria-specific poly(A) polymerase and polynucleotide phosphorylase. *J. Biol. Chem.* **280**, 19721–19727.
- Pfeffer, S., Lagos-Quintana, M., and Tuschl, T. (2003). Cloning of small RNA molecules. In “Current Protocols in Molecular Biology” (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidmann, J. A. Smith, and K. Struhl, eds.), pp. 26.24.16–26.24.21. Wiley, New York.
- Portnoy, V., Evguenieva-Hackenberg, E., Klein, F., Walter, P., Lorentzen, E., Klug, G., and Schuster, G. (2005). RNA polyadenylation in Archaea: Not observed in *Haloflex* while the exosome polyadenylates RNA in *Sulfolobus*. *EMBO Rep.* **6**, 1188–1193.
- Portnoy, V., and Schuster, G. (2006). RNA polyadenylation and degradation in different Archaea; roles of the exosome and RNase R. *Nucleic Acids Res.* **34**, 5923–5931.
- Rissland, O. S., Mikulasova, A., and Norbury, C. J. (2007). Efficient RNA polyuridylation by noncanonical poly(A) polymerases. *Mol. Cell. Biol.* **27**, 3612–3624.
- Rott, R., Zipor, G., Portnoy, V., Liveanu, V., and Schuster, G. (2003). RNA polyadenylation and degradation in cyanobacteria are similar to the chloroplast but different from *Escherichia coli*. *J. Biol. Chem.* **278**, 15771–15777.
- Slomovic, S., Laufer, D., Geiger, D., and Schuster, G. (2005). Polyadenylation and degradation of human mitochondrial RNA: The prokaryotic past leaves its mark. *Mol. Cell. Biol.* **25**, 6427–6435.
- Slomovic, S., Laufer, D., Geiger, D., and Schuster, G. (2006a). Polyadenylation of ribosomal RNA in human cells. *Nucleic Acids Res.* **34**, 2966–2975.
- Slomovic, S., Portnoy, V., Yehudai-Resheff, S., Bronshtein, E., and Schuster, G. (2008). Polynucleotide phosphorylase and the archaeal exosome as poly(A)-polymerases. *Biochem. Biophys. Acta* **1779**, 247–255.
- Slomovic, S., Portnoy, V., Liveanu, V., and Schuster, G. (2006b). RNA polyadenylation in prokaryotes and organelles; different tails tell different tales. *Crit. Rev. Plant Sci.* **25**, 65–77.
- Slomovic, S., and Schuster, G. (2008). Stable PNPase RNAi silencing; its effect on the processing and adenylation of human mitochondrial RNA. *RNA* **14**, 310–323.
- Temperley, R. J., Seneca, S. H., Tonska, K., Bartnik, E., Bindoff, L. A., Lightowlers, R. N., and Chrzanowska-Lightowlers, Z. M. (2003). Investigation of a pathogenic mtDNA microdeletion reveals a translation-dependent deadenylation decay pathway in human mitochondria. *Hum. Mol. Genet.* **12**, 2341–2348.
- Vanacova, S., and Stef, R. (2007). The exosome and RNA quality control in the nucleus. *EMBO Rep.* **8**, 651–657.
- Wahle, E. (1991). Purification and characterization of a mammalian polyadenylate polymerase involved in the 3′-end processing of messenger RNA precursors. *J. Biol. Chem.* **266**, 3131–3139.
- West, S., Gromak, N., Norbury, C. J., and Proudfoot, N. J. (2006). Adenylation and exosome-mediated degradation of cotranscriptionally cleaved pre-messenger RNA in human cells. *Mol. Cell* **21**, 437–443.
- Yehudai-Resheff, S., Hirsh, M., and Schuster, G. (2001). Polynucleotide phosphorylase functions as both an exonuclease and a poly(A) polymerase in spinach chloroplasts. *Mol. Cell. Biol.* **21**, 5408–5416.