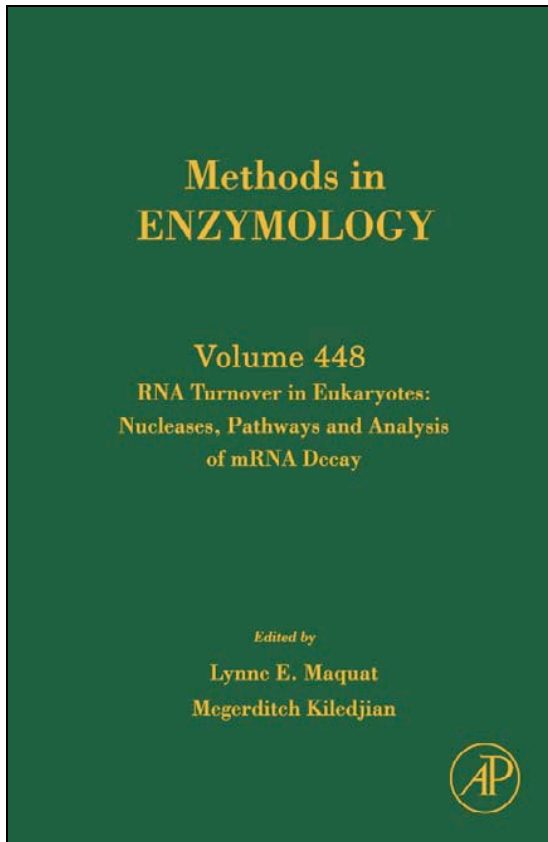


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METHODS TO DETERMINE MRNA HALF-LIFE IN *SACCHAROMYCES CEREVISIAE*

Jeff Coller

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Center for RNA Molecular Biology, Case Western Reserve University, School of Medicine, Cleveland
Ohio, USA

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Abstract

Much of our understanding of eukaryotic mRNA decay has come from studies in budding yeast, *Saccharomyces cerevisiae*. The facile nature of genetic and biochemical manipulations in yeast has allowed detailed investigations into the mRNA decay pathway and the identification of critical factors required for each step. Discussed herein are standard protocols for measuring mRNA half-lives in yeast. It should be noted, however, that variations of these assays are possible. In addition, a few *a priori* considerations are addressed.

1. INTRODUCTION

The degradation of mRNA is a vital aspect of gene regulation. Shutting off mRNA expression ensures that previously transcribed mRNAs do not translate *ad infinitum*. Moreover, mRNA degradation is used by the cell as a site of regulatory responses (reviewed in Collier and Parker, 2004). Last, specialized mRNA turnover systems exist that recognize and degrade aberrant mRNAs, thereby increasing the quality control of mRNA biogenesis (reviewed in Baker and Parker, 2004). Given these functions, it is important to understand how decay rates of different mRNAs are controlled and how aberrant mRNAs are targeted for destruction. Much can be learned, therefore, by determining the mechanism of how a specific transcript is degraded under various biologic conditions or in distinct genetic backgrounds.

Polyadenylated mRNAs can be degraded in eukaryotic cells by two general pathways (Fig. 14.1). In both cases, the degradation of the transcript begins with the shortening of the poly(A) tail at the 3'-end of the mRNA (reviewed in Collier and Parker, 2004). In yeast, shortening of the poly(A) tail primarily leads to removal of the 5'-cap structure (decapping), thereby exposing the transcript to digestion by a 5' to 3'-exonuclease (Fig. 14.1A; Decker and Parker, 1993; Hsu and Stevens 1993; Muhlrud *et al.*, 1994).

mRNAs can also be degraded in a 3' to 5'-direction after deadenylation (Fig. 14.1B) (Muhlrud *et al.*, 1995). 3' to 5'-degradation of mRNAs is catalyzed by the exosome (Anderson and Parker 1998; Mukherjee *et al.*, 2002; Rodger *et al.*, 2002), which is a large complex of 3' to 5'-exonucleases functioning in several RNA degradative and processing events (reviewed in van Hoof and Parker 2002). For the yeast mRNAs that have been studied, the process of 3' to 5'-decay is slower than decapping and 5' to 3'-decay (Cao and Parker, 2001). However, it is likely that for some yeast mRNAs, or in other eukaryotic cells, 3' to 5'-degradation will be the primary mechanism of mRNA degradation after shortening of the poly(A) tail (e.g., Higgs and Colbert 1994).

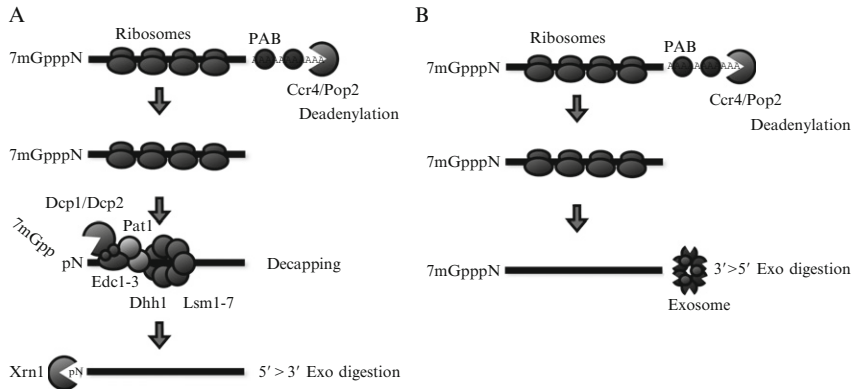


Figure 14.1 Pathways of mRNA decay in yeast. (A) Shows the major mRNA pathway that is initiated by deadenylation, followed by removal of the 5'-cap (decapping), and lastly exonucleolytic digestion of the mRNA body in a 5' > 3'-direction. (B) Degradation of mRNA can also occur by a minor pathway which is initiated by deadenylation, and then destruction of the mRNA body in a 3' > 5'-direction by the exosome.

In this review, I do not focus on the mechanisms of mRNA decay, but rather, how half-life measurements are made within the cell. The techniques I describe have proven useful in determining the steps of message decay. It should be noted, however, that it has been necessary to couple these assays with genetic manipulations and other techniques to powerfully demonstrate how a particular transcript is degraded (Mulhrad *et al.*, 1995). For the assays described here, the overarching theme is the same: RNA transcription is ceased in some manner, and the decay rates of specific transcripts are monitored. The method for stopping transcription depends on the questions asked and the information desired. I refer you to chapter 20 by Passos and Parker in this issue for details on how to determine the polarity of decay and questions related to the enzymatic activities of the decay machinery.

2. THE USE OF INDUCIBLE PROMOTERS

The use of reporter mRNAs that are expressed under the control of inducible promoters is the most common technique for measuring mRNA decay in yeast. The preferred choice is the galactose promoter; however, others systems have been recently exploited, especially the TET_{off} repressible promoter.

2.1. The GAL1 UAS

The *GAL1* upstream activating sequence (UAS) is also known as the GAL promoter or galactose inducible promoter. The GAL promoter mediates the expression of galactose metabolism genes, and it can confer galactose

inducibility to heterologous genes (reviewed in Lohr *et al.*, 1995). Simply put, the GAL promoter induces mRNA expression in the presence of the sugar galactose and rapidly shuts off transcription in the presence of glucose. This provides a powerful method for transcriptional pulse-chase and shut-off experiments in which mRNA decay kinetics is analyzed.

2.2. A primer on galactose metabolism

Before designing an RNA decay analysis with the GAL promoter, it is important to consider how yeasts use galactose. The GAL promoter serves as a binding site for the transcriptional regulator, Gal4p (reviewed in Lohr *et al.*, 1995). Gal4p has bipartite activities: a DNA binding domain, and a transcriptional activation domain. Gal4p binds strongly to the GAL UAS in the presence of galactose, inducing transcription through its activation domain. The addition of glucose causes a second gene, Gal80p, to bind to Gal4p and mask its activation domain, thereby immediately inhibiting further RNA synthesis.

The choice of yeast strain is important when the *GAL1* UAS is used to control gene expression. For example, the Gal4p protein is exploited for the yeast two-hybrid system (reviewed in Traven *et al.*, 2006). For this reason, yeast strains used in two-hybrid assays lack both Gal4p and Gal80p (*gal4Δ* and *gal80Δ*). It is not possible, therefore, to measure mRNA decay with the GAL promoter in a two-hybrid strain, because it lacks galactose-dependent regulation. It is advisable to test laboratory strains for galactose-dependent regulation before the GAL promoter is used to measure mRNA decay. This can be accomplished by Northern blot analysis for the *GAL1*, *GAL7*, or *GAL10* genes (see following). The yeast strains that we commonly use are BY4741 and BY4742; the WT backgrounds used in the *Saccharomyces* Genome Deletion Project (Winzeler *et al.*, 1999). Other strains are also commonly used, including S288C and W303 (He and Jacobson 1995; Muhlrad *et al.*, 1994).

In theory, mRNA decay of any gene can be measured with the GAL promoter. This requires that the gene of interest be engineered to be transcribed under GAL control, either by integrating the GAL promoter into the chromosomal gene (Longtine *et al.*, 1998) or placing the gene on a plasmid that contains the GAL promoter (Collier and Parker, 2005). Once established, the experiment can be performed in two distinct ways: either by transcriptional shut-off or by a transcriptional pulse-chase.

2.3. Transcriptional shut-off

Transcriptional shut-off by use of the GAL promoter is perhaps the simplest and most straightforward approach to determine decay kinetics of an mRNA. Cells are grown in the presence of galactose until reaching mid-log phase.

Because galactose is always present, the mRNA reporter is constitutively expressed and at steady state when analysis is performed, which has certain ramifications on the information that is gathered (Section 2.5). Shut-off is achieved by concentrating the cells and resuspending in medium that contains glucose. Time points are then quickly taken, RNA extracted, and analysis by Northern blot ensues. The following is a detailed protocol for this analysis:

- An overnight culture is grown in 20 ml of SGS medium (Section 2.3.1).
- The next day, 200 ml of SGS medium is inoculated overnight to an $OD_{600} = 0.05$ U/1 ml (this number is variable depending on spectrophotometer but reflects approximately 1×10^6 cells/mL). Grow the cells in a 1-L flask to ensure appropriate aeration. The temperature for growth depends on the experiment. WT strains grow best at 30 °C; mutant strains may only grow at 24 °C.
- Cells are harvested when reaching an $OD_{600} = 0.400$ U/1 ml (3×10^7 cells/mL), by centrifugation in appropriately sized conical tubes.
- Working quickly, the galactose medium is poured off, and the cell pellet is resuspended in 20 ml of S medium (Section 2.3.2). Place the culture in a small 50-ml flask in a shaking waterbath.
- 1 ml of 40% glucose (2% final concentration) is added, and time points immediately taken.
- Use the following time course: 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, and 60 min.
- Harvest each time point quickly. We use a 5 ml pipet man to remove 2 ml of culture. Aliquots are placed in 2-ml tubes. The aliquot is quickly spun down in a small desktop picofuge, and the medium is removed by aspiration. The cell pellet is then quickly frozen on dry ice or liquid nitrogen. Ensure that the culture continues to shake in the waterbath between each time point.
- Once time course is complete, cell pellets can be stored at -80 °C indefinitely.
- Extract mRNA and analyze reporter by Northern blot analysis.

2.3.1. SGS Medium (1 L)

20 g D-galactose

10 g Sucrose

1.7 g Yeast nitrogen base without amino acids or ammonium acetate

5.0 g Ammonium acetate

2.0 g Amino acid dropout mix (variable dependent on selective requirements)¹

pH to 6.5 with 10 N NaOH

¹ See Burke *et al.* (2000) for recipe.

2.3.2. S-Medium (1 L)

1.7 g Nitrogen base without amino acids or ammonium acetate

5.0 g Ammonium acetate

2.0 g Amino acid drop-out mix (variable dependent on selective requirements)¹

pH to 6.5 with 10N NaOH

2.4. Transcriptional pulse-chase

Measuring mRNA decay with transcriptional pulse-chase is a bit more challenging, but the information obtained is exquisite. The use of a transcriptional pulse-chase allows for the analysis of the decay of a synchronized population of mRNA. This gives a wealth of information, because product-precursor relationships are visible (Fig. 14.2B). A synchronous pool of transcripts is made by keeping reporter expression off until just before analysis. The GAL promoter is repressed by growing cells in medium containing the nonfermentable carbon source raffinose. Once the appropriate cell density is reached, the mRNA is induced by adding galactose for a brief time, and then transcription is shut off by adding glucose. This procedure generates a burst of newly synthesized transcripts whose decay can be followed.

The following is a detailed protocol for transcriptional shut-offs:

- An overnight culture is grown in 20 ml of SR medium (Section 2.4.1).
- The next day, 200 ml of SR medium is inoculated overnight to an $OD_{600} = 0.05$ U/1 ml (1×10^6 cells/mL). Grow the cells in a 1-L flask to ensure appropriate aeration. The temperature for growth depends on the experiment.
- Cells are harvested when reaching an $OD_{600} = 0.300$ U/1 ml (2.5×10^7 cells/mL) by centrifugation in appropriately sized conicals. For this analysis, it is vital that the cell not be allowed to grow above an $OD_{600} = 0.300$ U/1 ml. Growth above this optical density can cause spontaneous induction of the GAL promoter (Section 2.4.1).
- Pour off medium and resuspend cell pellet in 20 ml of S medium (Section 2.3.2). Place the culture in a small 50-ml flask in a shaking waterbath.
- Harvest a 2-ml aliquot to use as a preinduction control. Treat this sample like a normal timepoint (i.e., remove medium by centrifugation and place tube on dry ice or in liquid nitrogen).
- Add 2 ml of 20% galactose (2% final concentration) and incubate culture for 8 min in shaking waterbath.
- Separate medium from cells by centrifugation (working quickly, a 1-min. spin at 4000 rpm is sufficient).

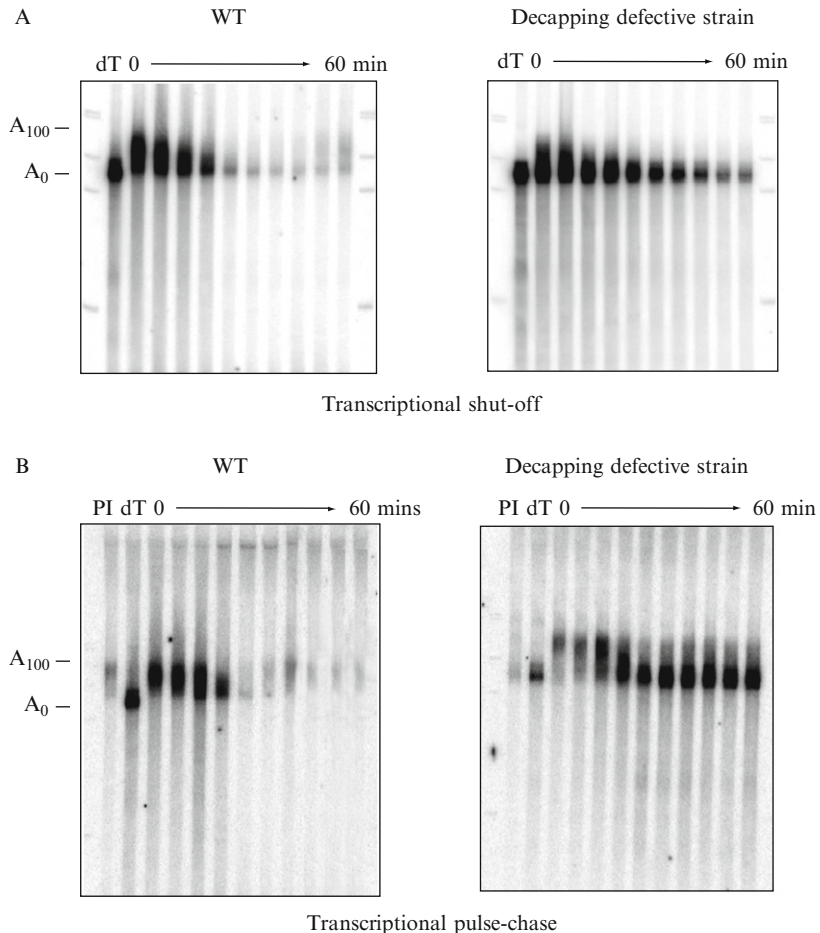


Figure 14.2 Comparison of a transcriptional shut-off (A) vs. a transcriptional pulse-chase (B). The specific reporter used is the MFA2 gene under control of the GAL1 UAS. dT represents a sample of the RNA treated with oligo dT and RNaseH in order to indicate the size of fully deadenylated RNA. PI indicates a pre-induction control. The left panels represent analysis in WT cells, while the right panel represents an experiment performed in a decapping defective strain (i.e. *dcp2* mutant).

- Resuspend pellet in 20 ml of S medium, place in a 50-ml flask, and place in shaking waterbath.
- Add 2 ml of 40% glucose (2% final concentration) and take time points immediately.
- Use the following time course: 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, and 60 min.
- Harvest each time point quickly. We use a 5-ml pipet man to remove 2 ml of culture. Aliquots are placed in 2-ml tubes. The aliquot is quickly

spun down in a small desktop picofuge, and the medium is removed by aspiration. The cell pellet is then quickly frozen on dry ice or liquid nitrogen. Ensure that the culture continues to shake in the waterbath between each time point.

- Once time course is complete, cell pellets can be stored at -80°C almost indefinitely.
- Extract mRNA and analyze reporter mRNA by Northern blot analysis (see following).

2.4.1. SR Medium (1 L)

20 g Raffinose

10 g Sucrose

1.7 g Yeast nitrogen base without amino acids or ammonium acetate

5.0 g Ammonium acetate

2.0 g Amino acid dropout mix (variable dependent on selective requirements)¹

pH to 6.5 with 10 N NaOH

The biggest problem in obtaining a good transcriptional pulse-chase is preventing preinduction of the reporter. Raffinose is a trisaccharide composed of galactose, fructose, and glucose. Raffinose is spontaneously hydrolyzed at low pH to generate galactose and sucrose. It is important, therefore, to ensure the pH of the culture does not become acidic, because this would generate galactose and induce the GAL promoter. We minimize the likelihood of spontaneous raffinose hydrolysis by adjusting our medium to pH = 6.5 before use. Notably, yeast synthetic medium usually has a pH of 5.0 or lower. Second, we never allow the cell density to get above an OD_{600} of greater than 0.3 U/ml (2.5×10^7 cells/mL). Yeast cells secrete the enzymes *invertase* and α -*galactosidase*. *Invertase* will hydrolyze raffinose into melibiose and fructose. Melibiose is hydrolyzed by α -*galactosidase* into galactose and glucose, and this induces Gal4p-mediated gene expression (Johnston and Hooper, 1982). The higher the cell density, the higher the secreted *invertase* and α -*galactosidase* levels within the culture, and, therefore, the higher the probability of spontaneous induction of the GAL promoter. This has several implications for a pulse-chase experiment. First, cell density must be kept low enough to avoid significant conversion of raffinose but high enough to be able to obtain significant amounts of RNA from samples. Second, extracellular *invertase* and α -*galactosidase* accumulates in medium. Therefore, if cultures overgrow when conducting a pulse-chase, they cannot be simply diluted back to a lower OD. If diluting cultures back, it is advisable to pellet cells by centrifugation, wash with fresh medium, and then resuspend in new medium to an appropriate OD. Last, the choice of yeast strain can also have an impact on *invertase* levels in the medium. The *S. cerevisiae* genome contains six unlinked loci that encode *invertase*, *SUC1-5*, and *SUC7* (Carlson and Botstein 1982). Yeast strains can carry any number or combination of *invertase* genes. For example, S288C strains only

contain the *SUC2* gene (Carlson and Botstein, 1982). It is theoretically possible to avoid preinduction by use of sucrose as the carbon source rather than raffinose (Ambro Van Hoof 2008), although this has not been tested rigorously.

2.5. Differences between transcriptional shut-off and transcriptional pulse-chase

Both a transcriptional shut-off and a transcriptional pulse-chase will give important information about the half-life of an mRNA. The difference between these two analyses is that a shut-off is a decay from steady state, whereas a pulse-chase monitors the decay of a synchronized population (Fig. 14.2). There are times when one analysis is preferred over the other. Shut-off experiments are much easier than pulse-chase experiments, because cells grow better in galactose than in raffinose, and preinduction is not a concern. A transcriptional shut-off, however, gives only an approximation of how mRNA decay is impaired. In a transcriptional pulse-chase, the progression of a transcript through the steps of mRNA decay, (i.e., deadenylation, decapping, and exonucleolytic digestion) can be observed. This is especially important when trying to determine what step in decay is affected by an experimental condition (Fig. 14.2B).

2.6. A GAL promoter bonus

One particular advantage of choosing to use the GAL promoter for mRNA decay analysis is that, besides the reporter GAL UAS fusion, it is also possible to monitor the decay of three endogenous mRNAs: the *GAL1*, *GAL7*, and *GAL10* transcripts. All three mRNAs are naturally controlled by galactose and thus will be regulated transcriptionally like the reporter. These become powerful controls especially when determining transcript specific effects.

2.7. The TET-off system

Although not as common, mRNA decay has been successfully monitored by shutting off transcription with a tetracycline-dependent repressible promoter. Developed by Gari *et al.* (1997), the gene of interest is fused to the tetracycline operator (tetO). The plasmid must also express a second gene called the tTA transactivator. The tTA transactivator consists of the VP16 activator domain of herpes simplex virus fused to the tetracycline-inducible repressor (tetR) from the Tn10-encoded tetracycline-resistance operon (Gari *et al.*, 1997). Under these conditions, genes under tetO control are expressed when tetracycline is absent, but transcription is quickly shut-off when the antibiotic is added to media. Commonly, doxycycline, which is a derivative of tetracycline, is used. This system is useful when trying to

control expression of a single mRNA without altering carbon sources (Hilleren and Parker, 2003). However, this approach is limited, because only decay from the steady state can be performed.

3. MEASURING mRNA DECAY BY USE OF THERMALLY LABILE ALLELES OF RNA POLYMERASE II

Another useful method for analyzing mRNA decay takes advantage of the temperature-sensitive allele *rpb1-1* (Nonet *et al.*, 1987). The *rpb1-1* allele maps to the *RPO21* gene, which encodes the largest subunit of RNA polymerase II. At the permissive temperature, *rpb1-1* functions normally. Shifting to the restrictive temperature leads to a quick and synchronous disruption of all pol II transcription. The advantage of this approach is that the analysis of endogenous mRNAs can be measured. No prior cloning or alteration of promoter elements is required. In addition, decay analysis with *rpb1-1* is not dependent on nutrient conditions, allowing the effects of stress or starvation to be monitored (Hilgers *et al.*, 2006). Last, because *rpb1-1* stops all pol II transcription, decay of any mRNAs can be monitored. The disadvantage of *rpb1-1* is that the allele must be incorporated into different strains if distinct genetic backgrounds are desired. In addition, *rpb1-1* shut-off experiments analyze decay from the steady state. Therefore, product-precursor relationships cannot be determined as in transcriptional pulse-chase experiments. Nonetheless, it has proven advantageous to use *rpb1-1* to measure mRNA decay in some experimental settings. The following is a detailed protocol.

- An overnight culture is grown at 24 °C in 20 ml of medium (medium choice depends on need).
- The next day, 200 ml of medium is inoculated with the overnight, and cells are grown to an $OD_{600} = 0.05$ U/1 ml (1×10^6 cells/mL). In a 1-L flask to ensure appropriate aeration. Cultures must be grown at 24 °C.
- Ten milliliters of medium is preheated to 56 °C at least 1 h before performing the shut-off and, a 50-ml flask is preheated to 37 °C in a shaking waterbath.
- Cells are harvested when reaching an $OD_{600} = 0.400$ U/1 ml (3×10^7 cells/mL), by centrifugation in appropriately sized conicals. Resuspend cell pellet in 10 ml of room temperature medium.
- Working quickly, the preheated medium is poured into the freshly harvested 10-ml culture. The combination of 10 ml of medium at 56 °C and 10 ml of culture at 24 °C gives a rapid shift to 37 °C.
- Place the culture in the preheated 50-ml flask in a shaking waterbath set at 37 °C.
- Immediately take time points at 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, and 60 min.

- Harvest each time point quickly as previously described.
- Cell pellets can be stored at -80°C indefinitely.

Extract mRNA and analyze reporter by Northern blot analysis.

4. MEASURING mRNA DECAY WITH THIOLUTIN

Chemical means can also be used to measure mRNA decay in yeast (Herrick *et al.*, 1990). Thiolutin is a sulfur-containing antibiotic from *Streptomyces laterosporus* that inhibits RNA synthesis directed by all three yeast RNA polymerase. Adding thiolutin at low concentrations is sufficient to completely block RNA synthesis, thereby allowing mRNA decay analysis. Like the use of the *rpb1-1* allele, this approach allows the analysis of endogenous mRNAs. Moreover, the effects of stress or nutrient starvation on mRNA decay can be monitored, because carbon source choice is irrelevant. Last, the decay of *any* RNA can be monitored because thiolutin blocks the activity of all three RNA polymerases. Unlike an *rpb1-1* mutant, however, a thiolutin shut-off can be conducted on any preexisting strain; genetic engineering is not required. The disadvantage of thiolutin, like *rpb1-1*, is that shut-off experiments limit the analysis to decay from the steady state. Last, new evidence suggests that some mRNAs may be stabilized by the response of cells to the drug (Pelechano and Pérez-Ortín, 2007). Nonetheless, for many mRNAs, thiolutin has proven useful in monitoring decay kinetics. The protocol is analogous to the protocols we have previously described for transcriptional shut-off experiments except that $3\ \mu\text{g/ml}$ (final concentration) of thiolutin is added before taking time points.

5. RNA EXTRACTIONS

Irrespective of how mRNA decay analysis is performed, once samples are collected, it is necessary to extract the mRNA and analyze decay rates. The following is our protocol for extracting total-cell mRNA from yeast. We analyze *total* RNA by Northern blotting. Because many mutations and conditions can affect the decapping step exclusively, it is important to not poly(A)-select mRNA by use of oligo(dT) columns before analysis. Alterations in decapping stabilize poly(A)-minus mRNAs. Therefore oligo(dT) selection would result in the loss of information.

- Resuspend frozen yeast cell pellets (in 2-ml tube) in $150\ \mu\text{l}$ LET (Section 5.1).
- Add $150\ \mu\text{l}$ phenol equilibrated with LET.

- Add equal volume (approximately 300 to 400 μl) of glass beads (bead size = 500 μm ; available from Sigma-Aldrich; cat #G8772).
- Vortex in MultiMixer (VWR cat# 58816-115) for 5 min at top speed.
- Add 250 μl DEPC H_2O and 250 μl 1:1 phenol/chloroform equilibrated with LET.
- Vortex in MultiMixer for an additional 5 min followed by centrifugation for 5 min at 14,000 rpm.
- Transfer aqueous phase (approximately 450 μl , top layer) to new 1.5-ml tube.
- Add 450 μl of 1:1 phenol/chloroform equilibrated with LET. Vortex 60 sec and spin 3 min at 14,000 rpm.
- Transfer aqueous phase to new 1.5-ml tube.
- Add 400 μl of chloroform. Vortex 60 sec and spin 3 min at 14,000 rpm.
- Transfer aqueous phase to a new 1.5-ml tube. Add 40 μl of 3 M sodium acetate and 800 μl of 100% cold ethanol. Mix well and place at -20°C for 1 h.
- Collect RNA by centrifugation at room temperature for 10 min at 14,000 rpm. Wash pellet with 500 μl 70% EtOH and recentrifuge for 5 min. Drain supernatant and dry pellet (either air-dry or dry in SpeedVac [no heat]).
- Resuspend pellet in 50 to 150 μl DEPC dH_2O .
- Quantify each RNA sample by measuring the absorbance at 260 nm with spectrophotometer. Assay 4 μl of RNA in 996 μl of dH_2O .
- Determine concentration of each sample on the basis of the extinction coefficient for RNA of 40 $\mu\text{g}/\text{ml}$. (*Note:* if the preceding dilution is used, simply multiply the OD_{260} by 10; this equals the concentration of each sample in $\mu\text{g}/\mu\text{l}$).
- Analyze 10 to 30 μg of each sample by Northern blot.

5.1. Recipe for LET

25 mM Tris, pH 8.0

100 mM LiCl

20 mM EDTA

(All reagents should be made in DEPC-treated distilled H_2O).

5.2. RNase H cleavages of 3' UTRs

mRNA decay in yeast is initiated by deadenylation (reviewed in [Collier and Parker, 2004](#)). The rate of poly(A) shortening, therefore, is an important consideration in decay analysis. The poly(A) tail typically ranges in size from 10 to 100 adenosines. Because the tail is small, the size difference between adenylated and deadenylated transcripts is negligible for most large mRNAs. It is necessary, therefore, to first cleave an mRNA within its 3' UTR with

an antisense oligonucleotide and RNase H to generate a smaller RNA fragment that can be analyzed by high-resolution Northern blotting after electrophoresis in polyacrylamide gels. The antisense oligo that we typically use is 20 nucleotides in size and binds near the stop codon of the gene of interest. The following is the protocol for generating a smaller mRNA fragment from full-length mRNA.

- Dry down in SpeedVac 10 μg of total-cell RNA and 300 ng antisense oligo (can dry down as much as 40 μg of RNA and still maintain the 300 ng of oligo; do not overdry, because it will be difficult to resuspend). To control for poly(A) tail lengths, also treat a sample with antisense oligo and 300 ng of oligo d(T).
- Resuspend pellet in 10 μl of 1 \times hybridization mix (Section 5.3).
- Heat sample at 68 $^{\circ}\text{C}$ for 10 min. Cool slowly to 30 $^{\circ}\text{C}$. Pulse spin down.
- Add 9.5 μl of 2 \times RNase H buffer and 0.5 μl RNase H. Mix well.
- Incubate sample at 30 $^{\circ}\text{C}$ for 60 min.
- Add 180 μl stop mix. Extract sample with 200 μl of phenol/chloroform. Remove aqueous phase and extract with 200 μl of chloroform.
- Precipitate RNA by adding 500 μl of 100% ethanol. Freeze at -20°C for 60 min.
- Spin down sample for 10 min at room temperature. Wash RNA pellet with 300 μl of 70% ethanol. Dry either at room temperature or under vacuum in Speedvac (once again, do not overdry).
- Resuspend sample in 10 μl of DEPC-treated water and add 10 μl of loading dye. Heat sample at 100 $^{\circ}\text{C}$ for 5 min before loading in 6 to 8% denaturing acrylamide gel. After resolving by PAGE, perform a Northern Blot analysis and detect RNA fragment with an mRNA-specific probe.
- Prepare solutions with DEPC-treated distilled water and store in aliquots at -20°C .

5.3. 10 \times Hybridization mix

0.25 M Tris-HCl, pH 7.5

10 mM EDTA

0.5 M NaCl

5.4. 2 \times RNase H buffer

40 mM Tris-HCl, pH 7.5

20 mM MgCl_2

100 mM NaCl

2 mM DTT

60 $\mu\text{g}/\text{ml}$ BSA

5.5. Stop mix

0.04 mg/ml tRNA
20 mM EDTA
300 mM NaOAc

6. NORTHERN BLOT ANALYSIS

After performing an mRNA decay analysis, it is possible to obtain a half-life measurement by resolving mRNA in a formaldehyde agarose gel followed by Northern blot. To obtain information about the deadenylation rate, it is necessary to analyze mRNA or RNase H fragments by resolution in a 6% polyacrylamide gel followed by Northern blotting. Protocols for these techniques are described elsewhere ([Sambrook *et al.*, 1989](#) and Chapter 17 by *Chen et al.* in this volume).

7. LOADING CONTROLS

It is important to consider several controls in an mRNA decay analysis. First, if monitoring deadenylation rate, a sample of mRNA (usually from the first time point) should be treated with RNase H and oligo (dT) to generate a nonadenylated marker that indicates the size of the fully deadenylated mRNA ([Figs. 14.2 and 14.3](#)). Second, the Northern blot should be reprobbed with a loading control. *SCR1* RNA is ideal for this purpose. *SCR1* RNA is the 7S signal recognition particle RNA used by the cell for cotranslational protein targeting to the endoplasmic reticulum. Importantly, this small 522-nt RNA is noncoding and transcribed by RNA polymerase III and is unaffected by changes in *mRNA* transcription except in the case of thiolutin. Thiolutin inhibits all three RNA polymerases, including *SCR1* RNA levels, albeit only slightly within the recommended 1 h time course. *SCR1* RNA is abundant and can be easily detected with a gamma ^{32}P -ATP kinase-labeled oligonucleotide probe.

8. DETERMINATION OF mRNA HALF-LIVES

Yeast mRNAs generally decay with first-order kinetics ([Herrick *et al.*, 1990](#)). Half-lives, therefore, can be represented by the equation $t_{1/2} = 0.693/k$, where k = the rate constant for mRNA decay (i.e., percent change over time). Half-life calculations for a particular mRNA transcript

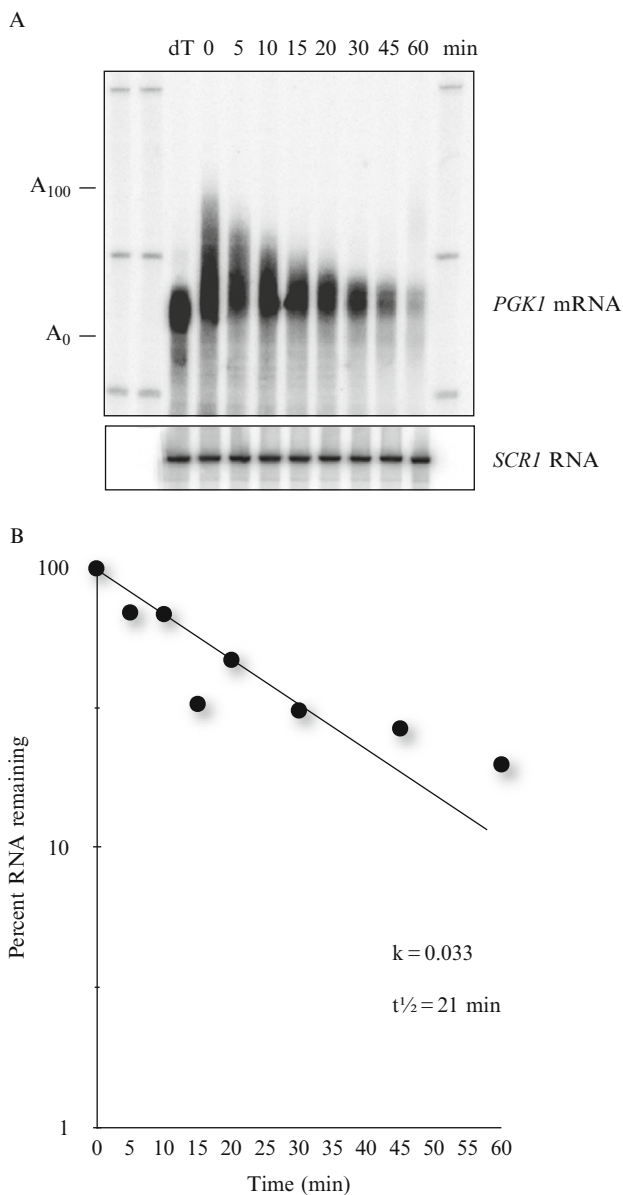


Figure 14.3 Transcriptional shut-off analyzes of the *PGK1* mRNA (A) and calculation of *PGK1* half-life (B). In (A) the mRNA reporter was cleaved by RNaseH using an anti-sense oligo and then resolved by PAGE. dT represents a sample that was also cleaved with oligo d(T) and RNaseH to indicate size of fully deadenylated mRNA. Below is a Northern of the *SCR1* RNA which is used as a loading control. (B) is the quantitation of the experiment shown in (A) and representation of these data on a semi-log plot. This was used to calculate the half-life based on the protocol outlined in the text.

are made by quantitating the amount of mRNA present at each time point with a PhosphorImager. This value should be normalized for loading variations with a control like *SCR1* RNA (Fig. 14.3A). The value for k can be determined with a semilogarithmic plot of the concentration of mRNA over time and determining the slope of a best-fit line (slope= k ; Fig. 14.3B). Once the rate constant is determined, the half-life can be calculated. It is strongly advised to measure mRNA half-lives at least three times to ensure reproducibility.

9. CONCLUDING REMARKS

In summary, the techniques described in this review provide useful methods for measuring mRNA decay rates in yeast. The most important consideration in choosing a particular technique is to determine the type of information that is desired. If simple half-life measurements are needed, then a transcriptional shut-off experiment will suffice. If information is sought about the sub-step of decay affected, then a transcriptional pulse-chase is required.

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