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Messenger RNA Half-Life Measurements in Mammalian Cells

Chyi-Ying A. Chen, **Nader Ezzeddine**, and **Ann-Bin Shyu**
Department of Biochemistry and Molecular Biology, The University of Texas Medical School at Houston, Houston, Texas, USA

Abstract

The recognition of the importance of mRNA turnover in regulating eukaryotic gene expression has mandated the development of reliable, rigorous, and "user-friendly" methods to accurately measure changes in mRNA stability in mammalian cells. Frequently, mRNA stability is studied indirectly by analyzing the steady-state level of mRNA in the cytoplasm; in this case, changes in mRNA abundance are assumed to reflect only mRNA degradation, an assumption that is not always correct. Although direct measurements of mRNA decay rate can be performed with kinetic labeling techniques and transcriptional inhibitors, these techniques often introduce significant changes in cell physiology. Furthermore, many critical mechanistic issues as to deadenylation kinetics, decay intermediates, and precursor-product relationships cannot be readily addressed by these methods. In light of these concerns, we have previously reported transcriptional pulsing methods based on the c-*fos* serum-inducible promoter and the tetracycline-regulated (Tet-off) promoter systems to better explain mechanisms of mRNA turnover in mammalian cells. In this chapter, we describe and discuss in detail different protocols that use these two transcriptional pulsing methods. The information described here also provides guidelines to help develop optimal protocols for studying mammalian mRNA turnover in different cell types under a wide range of physiologic conditions.

1. Introduction

Regulation of mRNA turnover in the cytoplasm is important for controlling the abundance of cellular transcripts and, in turn, the levels of protein expression (for reviews, see Parker and Song [2004] and Wilusz *et al*. [2001]). mRNA stability can be regulated at different levels. Under a given physiologic condition, mRNAs display a wide range of stability. For example, c-*fos* proto-oncogene transcript is degraded rapidly in the cytoplasm with a half-life of 10 to 15 min (Shyu *et al*., 1989; Treisman, 1985), whereas globin mRNA is rather stable and has a half-life of several hours in the same cells (Shyu *et al*., 1989). Although each individual mRNA has its intrinsic stability under a given condition, stability of an individual mRNA may change in response to a variety of extracellular stimuli. Examples include the autoregulated degradation of tubulin mRNA in response to changes in tubulin concentration (Yen *et al*., 1988), the iron-dependent destabilization of transferrin receptor mRNA (Casey *et al*., 1988; Muellner *et al*., 1989), the DNA synthesis-dependent destabilization of histone mRNA (Pandey and Marzluff, 1987), and the stabilization of lymphokine mRNAs by costimulatory molecules (Lindsten *et al*., 1989). Thus, modulation of mRNA stability provides a powerful means for controlling gene expression during the cell cycle, cell differentiation, the immune response, as well as many other physiologic transitions.

In mammalian cells, the first major step that triggers mRNA decay is deadenylation (i.e., removal of the 3′-poly(A) tail). All major mRNA decay pathways recognized in mammalian cells, including mRNA decay directed by AU-rich elements (AREs) in the 3′-untranslated region (UTR) (Chen and Shyu, 1995), decay mediated by destabilizing elements in protein-coding regions (Grosset *et al*., 2000), nonsense-mediated mRNA decay (NMD) (Chen and Shyu, 2003), decay directed by microRNAs (miRNAs) (Wu *et al*., 2006), and decay of stable

mRNAs such as *β*-globin mRNA (Loflin *et al*., 1999a; Shyu *et al*., 1991), are initiated with deadenylation. Mammalian deadenylation exhibits biphasic kinetics. During the first phase, PAN2 poly(A) nuclease, presumably complexed with PAN3, shortens the poly(A) tails to ~110 A nucleotides (nt) (Yamashita *et al*., 2005). In the second phase, the CCR4-CAF1 poly(A) nuclease complex further shortens the poly(A) tail to oligo(A) (Yamashita *et al*., 2005). Decapping mediated by the DCP1-DCP2 complex was found to occur after either the first or the second phase of deadenylation (Yamashita *et al*., 2005). The RNA body can be degraded by the exoribonuclease XRN1 from the 5′-end after decapping (Parker and Song, 2004). Alternately, the mRNA body can also be degraded from the 3′-end after deadenylation by a large protein complex termed the exosome (Parker and Song, 2004).

To unravel the underlying processes of regulated mRNA turnover, a detailed analysis of the major components involved in mRNA turnover is required. The observation that deadenylation is the major trigger for cytoplasmic mRNA degradation in mammalian cells underscores the necessity of explaining the deadenylation step in the process of mRNA turnover. Thus, determination and characterization of the decay mechanisms demand that mRNA decay kinetics and precursor-product relationships be accurately and readily monitored experimentally. The primary emphasis of this chapter is to describe two inducible promoter systems as examples to illustrate how mRNA turnover may be optimally investigated in mammalian tissue culture cells with transient transfection systems. Detailed step-by-step protocols are given so that the half-lives of mRNAs of interest can be determined with experimental systems described here.

2. General Considerations of mRNA Half-Life Measurements

Messenger RNA stability is often studied indirectly by monitoring changes in the steady-state level of mRNA in the cytoplasm. However, changes in mRNA abundance are not necessarily caused by alterations in mRNA stability. For example, mRNA biogenesis in the nucleus (such as transcription, RNA processing, and/or mRNA export) may be fortuitously altered because of changes of the physiologic condition or in response to the environmental stimuli. Thus, alterations in the steady-state level of mRNA may not reflect the changes in mRNA stability. Direct measurements of decay rates of endogenous mRNAs have been performed in a number of ways, including kinetic labeling techniques and the use of transcriptional inhibitors. However, these techniques often introduce significant changes in cell physiology, thereby perturbing the stability of many mRNAs that could potentially lead to secondary consequences on the stability of the tested mRNA (e.g., see Belasco and Brawerman [1993], Harrold *et al*. [1991], and Ross [1995]). As a result, decay rates obtained in experiments that use these methods may not reflect the true stability of the mRNAs.

Aside from the methods described previously for measuring endogenous mRNA stability, one can determine the half-life of an mRNA of interest by transiently transfecting the reporter gene into mammalian tissue culture cells and using a transcriptional pulsing approach to monitor deadenylation and decay kinetics of the reporter transcript without the use of a global transcription inhibitor (Loflin *et al*., 1999b; Xu *et al*., 1998). We will detail the procedures and applications of two transcriptional pulsing systems and discuss the advantages of these systems over other methods in terms of investigating mRNA turnover in mammalian cells.

3. Determining mRNA Decay Constant

The turnover rate or stability of mRNA *in vivo* is usually reported as the time required for degrading 50% of the existing mRNA molecules (i.e., the half-life of mRNAs). Before the half-life of a given message can be calculated, the decay rate constant must be determined. Assuming an ideal *in vivo* situation, in which transcription of the mRNA of interest can be turned off completely (or at least to an undetectable level), mRNA decay follows first-order kinetics. The

rate of disappearance of mRNA concentration at a given time (dC/dt) is proportional to both the rate constant for decay (k_{decay}) and the cytoplasmic concentration of the mRNA (C). This relation is described by the following equation:

$$dC/dt = -k_{decay}C \tag{17.1}$$

The minus symbol indicates that the mRNA is being degraded rather than synthesized. This relationship leads to the derivation of the equation:

$$\ln(C/C_0) = -k_{decay}t \tag{17.2}$$

where C_0 is the concentration of the mRNA at time 0, before decay starts.

Because we want to determine the half-life ($t_{1/2}$), this means C/C_0 = 50%/100% = 1/2. Rearrangement of equation (17.2) leads to the following equation:

$$\ln 1/2 = -k_{decay}t_{1/2}$$

from where:

$$t_{1/2} = \ln 2/k_{decay} \tag{17.3}$$

It is important to note that the half-life of an mRNA ($t_{1/2}$) is inversely proportional to its decay rate constant (k_{decay}).

In a typical time-course experiment, determination of mRNA half-life begins with the analysis of several RNA samples collected at time intervals, and monitoring the loss of a particular message by analyzing equal amounts of these samples with two message-specific probes, one specific for the message of interest and the other specific for an internal control, used to normalize the data and calculating the half-life of the message of interest (Belasco and Brawerman, 1993). With linear regression (least-square) analysis to identify the line that best fits the data, the decay rate constant is obtained from the slope of a semilogarithmic plot of mRNA concentration (C) as a function of time (t). The half-life ($t_{1/2}$) can then be calculated with equation (17.3).

The congruity of mRNA decay to first-order kinetics implies that mRNA molecules of a given type that differ in age are recognized and targeted for degradation in the same way by cellular decay machinery. Although this generally seems to be the case in prokaryotes (Belasco and Higgins, 1988), it is now clear that the deadenylation step is the major step that triggers mRNA decay and determines the ultimate stability of many mRNAs in both yeast and mammalian cells (reviewed in Meyer *et al*. [2004] and Parker and Song [2004]). Therefore, in eukaryotic cells, many labile or stable mRNAs undergo a period of poly(A) tail shortening during which there is no apparent decay of the transcribed portion of mRNA (e.g., Chen and Shyu [1994] and Muhlrad *et al*. [1994]). Only after the poly(A) tail is shortened to a certain extent, usually between 10 and 60 nt, does first-order decay of the RNA body ensue. Consequently, eukaryotic mRNAs decay with biphasic kinetics, composed of an initial lag phase during which deadenylation occurs and a second phase during which the body of the mRNA is degraded. As a result, an RNA lifetime cannot meaningfully be described merely in terms of a half-life that obeys first-order kinetics. This caveat concerning decay kinetics further underscores the necessity of monitoring the deadenylation step with a transcriptional pulsing approach.

4. Methods for Measuring mRNA Half-Life

4.1. General inhibition of transcription

A relatively simple way of analyzing mRNA kinetics involves blocking cellular transcription with inhibitors that include actinomycin D (which interferes with transcription by intercalating into DNA) or 5,6-dichloro-1β-1-ribofuranosylbenzimidazole (DRB) (which interacts directly with the RNA polymerase II transcription apparatus) (Harrold *et al*., 1991). Typically, either actinomycin D (at a concentration of 5 to 10 μg/ml) or DRB (at a final concentration of 20 μg/ml) is added to cells, and the amount of a particular mRNA remaining at various times of treatment is used to calculate the mRNA decay rate. The advantage of this approach is that exogenous genes do not have to be constructed and introduced into cells, and it is an easy way of measuring stability changes of endogenous mRNAs. However, this method suffers the disadvantage that both drugs have a profound impact on cellular physiology and, in fact, have been shown through the years to alter the stability of many mRNAs (Chen *et al*., 1995; Harrold *et al*., 1991; Seiser *et al*., 1995; Shyu *et al*., 1989; Speth and Oberbaumer, 1993). Of course, this complicates interpretation of the data. In addition, monitoring mRNA decay after a constitutive block in transcription does not allow the determination of deadenylation rates or precursor-product relationships without the use of supplementary methods. Moreover, for experiments involving other factors (such as differentiating agents), transcription inhibitors can result in severe cytotoxicity. These disadvantages limit the application of the seemingly straightforward and convenient use of transcription inhibitors.

4.2. Use of inducible promoters to specifically promote transient transcription

The use of a regulatable promoter subject to transient induction represents a major improvement in the analysis of eukaryotic mRNA decay over other methods (Loflin *et al*., 1999b). This approach provides a tight and rapid genetic switch to control transcription as required for studying mRNA turnover without the use of transcription inhibitors. This ensures that mRNA degradation is investigated under physiologically undisturbed conditions. The rationale behind this approach, also termed transcriptional pulsing, is rather straightforward: provide a stimulus that activates transcription and leads to a burst of mRNA synthesis, then remove the stimulus to shut off transcription and monitor the decay of mRNA. The success of this method relies on stringent control of the inducible promoter so that induction and silencing of transcription is accomplished within a narrow window of time. In mammalian cells, the c-*fos* promoter has been valuable for this purpose, because it can be induced in response to serum addition quickly and transiently (Greenberg and Ziff, 1984; Treisman, 1985), thereby providing a reliable and simple way of achieving a transient burst in transcription. A more recently developed tetracycline (Tet) regulatory promoter system (Gossen *et al*., 1993) offers an alternate strategy that further broadens the application of the transcriptional pulsing approach to study mRNA turnover in mammalian cells. The two systems are presented in the following sections.

4.2.1. The c-*fos* serum-inducible promoter system—Our laboratory has extensively used the c-*fos* inducible promoter system to investigate mRNA degradation mediated by AREs in mouse fibroblast NIH3T3 cells (e.g., see Chen *et al*. [1994], Chen and Shyu [1994], and Grosset *et al*. [2000]). AREs are found within the 3′ UTRs of many unstable mRNAs and represent the most commonly found RNA destabilizing elements in mammals (Chen and Shyu, 1995; Wilusz *et al*., 2001). To explain the key functional features of AREs that constitute their destabilizing ability and to decipher the deadenylation and decay kinetics of ARE-containing mRNAs, we first made a chimeric reporter construct. This construct contains a rabbit β-globin gene under the transcriptional control of a 710-base pair (bp) promoter region from the human c-*fos* proto-oncogene (Shyu *et al*., 1989). The β-globin gene was chosen because it encodes a stable mRNA having a half-life of more than 8 h in NIH3T3 cells (Shyu *et al*., 1989; 1991).

This magnitude of stability facilitates detecting an mRNA destabilizing effect that is mediated by a potential destabilizing element that is experimentally introduced into the *β*-globin gene. After individually introducing test destabilizing sequences into the *β*-globin plasmid construct, the construct is delivered to cells by transient transfection. Cells are subsequently serum starved to make them quiescent. After >25 h, *β*-globin gene transcription driven by the c-*fos* promoter is transiently induced by the addition of serum, which returns to an inactive state after an additional 30 to 40 min (Greenberg and Ziff, 1984; Treisman, 1985). This results in a short burst of chimeric *β*-globin mRNA synthesis, after which chimeric *β*-globin mRNA decay can be monitored without the use of transcription inhibitors. To do so, RNA samples are collected at different time points after serum induction, and the decay rate of the chimeric *β*-globin mRNAs is determined by Northern blot analysis for example (Shyu *et al*., 1991).

During the time course experiments, in addition to mRNA decay rates, highly synchronized poly(A) shortening has been observed, which makes an unequivocal determination of deadenylation status possible (e.g., see Chen *et al*. [1994] and Shyu *et al*. [1991]). Therefore, many critical mechanistic issues, such as deadenylation kinetics, the existence of decay intermediates, and precursor-product relationships can also be readily addressed by this method (Yamashita *et al*., 2005). In addition, this system is efficient and reproducible, and by allowing the analysis of transcripts that derive from transiently introduced genes, it eliminates the need to establish stably transfected cell clones.

Here, we describe two different protocols for the time-course experiments that our laboratory has been using. The first one is highly cost-effective, which involves calcium phosphate-based transient transfection of NIH3T3 cells and RNA extraction without the use of a commercial RNA purification kit. The second protocol uses Lipofectamine 2000 (Invitrogen) for transfection and RNeasy RNA preparation kit (QIAGENE) for RNA purification.

4.2.1.1. Protocol I: Transient transfection and serum induction: ***4.2.1.1.1. Materials:*** All solutions should be prepared with extremely pure, glass-distilled water.

1. 2 *M* $CaCl_2$: Weigh 14.7 g of $CaCl_2$ in a 50-ml conical centrifuge tube and bring H_2O to 50 ml. Dissolve well and use Acrodisc (0.2 *μ*) to filter-sterilize it. Aliquot the solution to 1.5 ml microcentrifuge tubes and store at −20 °C.

   (*Note*: Different sources and lot numbers of calcium chloride may affect the timing and fineness of the calcium phosphate/DNA precipitate that results, thereby affecting transfection efficiency greatly. More efficient transfection is achieved with fine precipitates.)

2. 2× HBS: NaCl, 274 m*M*; KCl, 10 m*M*;Na_2HPO_4, 1.4 m*M*; glucose, 15 m*M*; HEPES, 42 m*M*. Adjust the pH of the solution with NaOH precisely to 7.02. Filter sterilize and aliquot the final solution into 15-ml conical centrifuge tubes and store at 4 °C.

   (*Note*: It is recommended that pH should be checked with two different meters. pH drift to the acidic end will, in subsequent steps, prevent the formation of the fine calcium phosphate/DNA precipitate that gives the solution an opaque look, whereas pH drift to the alkaline end will lead to quick formation of visible calcium phosphate/DNA clumps. Both situations can lower the transfection efficiency by an order of magnitude.)

3. DNA solution: The final concentration of DNA (including the test DNA, control DNA, and carrier DNA) should be 20 *μ*g/0.1 ml in H_2O for a 10-cm cell culture dish. This is made up by empirically determining the optimum amount of transfecting DNA and adding enough carrier DNA to make a total of 20 *μ*g.

(*Note*: We have been using plasmid vectors as carrier DNA [e.g., pUC18]. We usually use 2 to 3 μg of test DNA [e.g., pBBB + ARE] or its derivatives (Chen and Shyu, 1994; Chen *et al*., 1994). This amount of DNA seems sufficient to result in the production of an mRNA signal that can be readily detected by RNA blot analysis with an exposure time to X-ray film between 3 and 18 h. We have noticed that when more than 5 μg of test DNA is used in transient transfections, somehow mRNA decay is retarded. For example, instead of degrading with a half-life of 35 min, BBB + ARE mRNA displays a 2- to 3-h half-life. This might represent saturation of the decay machinery.)

4. Bottled Dulbecco's modified Eagle's medium (DMEM), calf serum, L-glutamine (1 m*M* final concentration), and 1% of penicillin-streptomycin (prepared with 10,000 units/ml penicillin G sodium and 10 mg/ml streptomycin sulfate in 0.85% saline). Note that all the reagents described here are purchased from GIBCO.

5. 1× Phosphate-buffered saline (PBS).

1. 2×10^6 cells are plated in a 10-cm tissue culture dish 16 to 20 h before transfection and kept in a 5% CO_2 incubator.

2. Prepare calcium phosphate/DNA mixture by combining in the following order (for each plate):

100 μl	DNA (20 μg) in H_2O
100 μl	2× HBS
916 μl	1× HBS

Then add 84 μl of 2 *M* $CaCl_2$ solution and mix gently by inverting the tube. Let the mixture sit for 20 min at room temperature.

(*Note*: If more than one plate is to be transfected, simply multiply each component by the total number of plates and mix them in a 50-ml conical tube. The mixture should become a little opaque right after mixing, and 20 min later it should become fairly opaque, but no precipitation should been seen at this point. Otherwise, the transfection may not work, and we suggest that the pH of HBS solution should be rechecked.)

3. Add 1.2 ml DNA/calcium phosphate mixture directly to the cells that have been incubated in a 5% CO_2 incubator (from step 1) and gently swirl the plate to mix well and then immediately return the plate to the 5% CO_2 incubator.

(*Note*: Do not move the plates from the 5% CO_2 incubator to the tissue culture hood until ready to add the DNA/calcium phosphate mixture, because the pH of the culture medium will increase because of evaporation of CO_2 when plates are outside the incubator. In a high pH environment, the DNA/calcium phosphate mixture will form coarse precipitates that aggregate into large clumps in the medium. As a result, the transfection efficiency will be significantly reduced. Also, it is important to not swirl the plates again once they are put in the incubator. After a 2-h incubation when examined under the light microscope (20×), one should expect to see fine precipitate of DNA in the culture medium as many tiny black dots. If DNA precipitates can not be seen or form clumps, quit the experiment and check the quality of reagents used as described previously.)

4. Serum starvation

Twelve to 18 h after transfection, aspirate the medium from the dish and rinse the cells with 5 ml of 1× PBS gently but thoroughly twice. Add 10 ml of DMEM with only 0.5% calf serum and incubate cells in an 8.0% CO_2 incubator for 25 h.

(*Note*: It is normal to see some dead cells floating after 25 h of serum starvation.)

5. Serum induction

   After serum starvation for 25 h, remove culture medium from the dish and add 10 ml of fresh culture medium containing 20% calf serum. Harvest cells at desired time points for RNA purification.

   (*Note*: We routinely obtained 25 to 35% transfection efficiency with NIH3T3 cells following the preceding protocol.)

4.2.1.2. RNA extraction and Northern blot analysis: *4.2.1.2.1. Materials:*

1. NP-40 lysis buffer:	
10 m*M*	Tris-Cl, 7.4
10 m*M*	NaCl
3 m*M*	$MgCl_2$
0.5 %NP-40 (v/v) (may be sold under the trade name IGEPAL CA-630)	
2. 2× PK buffer:	
200 m*M*	Tris-Cl, 7.5
440 m*M*	NaCl
25 m*M*	EDTA
2%	SDS (w/v)

(*Note*: Both solutions should be autoclaved before use.)

A. On ice or at 4 °C

1. Chill culture dishes (6 to 7 × 10^6 cells per dish) on ice for 2 to 3 min. Aspirate the medium and wash cells twice with 3 ml of ice-cold 1× PBS.
2. Add 3 ml ice-cold 1× PBS and scrape off cells with a rubber policeman. Transfer cells to a 15-ml disposable conical tube. Salvage the remaining cells with 3 ml 1× PBS.
3. Pellet cells at 4 °C, 300*g* for 5 min.
4. Remove the supernatant, and loosen the cell pellet in the remaining PBS by finger vortexing gently.
5. Vortex at half-maximal speed as the 200 μl of NP-40 lysis buffer is added. Then vortex at the same speed for another 10 sec.
6. Incubate the lysed cells on ice for 5 min. Centrifuge at 4 °C, 300*g* for 5 min to pellet nuclei.
7. Transfer supernatant to a 1.5-ml microfuge tube containing 10 μl of ribonucleoside-vanadyl complex (200 m*M* stock green-black solution; New England BioLab). Mix well.

   (*Note*: Ribonucleoside-vanadyl complex is a potent inhibitor of various ribonucleases. Removal of the ribonucleoside-vanadyl complex from the RNA can be accomplished by adding 10 equivalents of EDTA before ethanol precipitation.)
8. Centrifuge at 4 °C, 300*g* for 5 min.

B. At room temperature:

1. Transfer supernatant to a new 1.5-ml microfuge tube containing 200 μl of 2× PK buffer. Then add 10 μl of protease K (20 mg/ml, Roche), mix well, and incubate at 37 °C for 30 min.

2. Extract once with phenol-chloroform (P/C) (containing 8-hydroxyquinoline). Precipitate the RNA with 2 vol of absolute ethanol. Store at −70 °C 10 to 15 min or at 20 °C overnight. (*Note*: It is not necessary to add extra salt for EtOH precipitation.)
3. Pellet RNA in a microfuge at 4 °C at maximal speed, remove supernatant as much as possible, and dry the pellet.
4. Redissolve the RNA pellet in 300 μl of TE (pH 7.4) buffer. Add 3 μl of 1 *M* $MgCl_2$. Mix well and add 1 μl of RNase-free DNaseI (10 mg/ml; Invitrogen).
5. Incubate at 37 °C for 20 min.
6. Add 6 μl of 10% SDS and 12 μl of 0.25 *M* EDTA. Extract twice with P/C (containing 8-hydroxyquinoline).
7. Ethanol precipitation, centrifugation, and desiccation of the RNA sample.
8. Resuspend RNA pellet in 100 μl of RNase-free ddH_2O. Take 5 μl to read OD_{260}. Yield of RNA should be approximately 30 to 50 μg per plate. RNA samples can be stored at −20 °C for several months to 1 y without any apparent degradation.

   (*Note*: It is extremely important to completely digest the transfected plasmid DNA that unavoidably exists in the RNA samples. Otherwise, residual plasmid DNA may be recognized during Northern blot hybridization, resulting in smeary black-looking lanes after autoradiography. Although various RNA purification kits are commercially available, we found often that DNase I-treatment is not very effective with the kits. Therefore, we highly recommend the protocol described previously for DNase I treatment.)

1. MOPS buffer: 0.2 *M* MOPS pH7.0, 50 m*M* Na acetate, 10 m*M* EDTA.
2. 10× sample loading buffer: 50% glycerol, 1 m*M* EDTA, 0.4% bromophenol blue (BPB), 0.4% xylene cyanol.
3. Dextran sulfate prehybridization buffer (approximately 20 ml).
   a. 10 ml formamide (deionized and molecular biology grade).
   b. 4 ml 5× P buffer (1% BSA, 1% poly-vinylpyrrolidone, 1% Ficoll, 250 m*M* Tris pH 7.5, 0.5% sodium pyrophosphate, 5% SDS).
   c. 4 ml 50% Dextran sulfate solution (w/v).
   d. Mix by inversion and then warm in 42 °C waterbath approximately 10 min to help dissolve.
   e. Add 1.16 g NaCl and mix by inversion until completely Replace in 42 °C waterbath.
   f. Add 2 ml of 1.5 mg/ml sheared salmon sperm DNA (boiled 10 min and cooled on ice for 5 min).
   g. Mix by inversion and use immediately for prehybridization. (*Note*: set aside 2 to 3 ml for mixing with the P-32 probe [see steps 12 and 13 of “Procedure” following].)

1. 1.4% agarose/2.2 *M* formaldehyde gel.

   a. For a 150 ml gel: boil 2.1 g agarose in 123 ml H_2O.

   b. Cool to 60 °C in waterbath.

   c. Add 15 ml 10× MOPS gel buffer and 12 ml formaldehyde.

   (*Note*: Make sure that the pH of formaldehyde is more than 4; pour in hood. Can put EtBr in gel [e.g., 0.66 μg/ml].)

2. Prepare RNA sample (20 μl per sample) by mixing:

   a. RNA (5 to 20 total RNA) in 4.5 μl H_2O.

10× MOPS	2.0 μl
Formaldehyde	3.5 μl
Formamide	10.0 μl

   b. Incubate 55 to 60 °C for 15 min.

   c. Add 2 μl of 10× loading buffer, and load each sample onto gel.

3. Run the gel in 1× MOPS buffer for approximately 3 to 6 h at 5 V/cm. (Approximately 100 to 150 V for 3 to 4 h) or until BPB completely runs into the gel. Be sure to circulate the buffer (e.g., with a peristaltic pump).

   (*Note*: To obtain a resolution sufficient for detecting changes in deadenylation, we recommend that the gel be run overnight by lowering the voltage accordingly.)

4. Remove the gel gently, cut out section to be transferred, and rinse it with water in a large tray two times (15 min each time) to remove formaldehyde.

5. Soak the gel in excess 50 m*M* NaOH/10 m*M* NaCl and shake for 30 min.

6. Neutralize the gel by shaking it in 0.1 *M* Tris (pH 7.5) twice for 15 min each time.

   (*Note*: If the RNA to be transferred is relatively small (<2.0 Kb), then omit steps 5 and 6.)

7. Soak the gel in 20× SSC for 1 to 2 h.

8. Blot the gel onto the Gene Screen Membrane (New England Nuclear/DuPont) with one of the commercial vacuum blotting devices (we use the Stratagene Posiblot) for up to 2 h.

9. Wash the membrane after transfer with 2× SSC. Illuminate the membrane and gel with UV to make sure the samples have been transferred. Mark the membrane for the position of rRNA markers or the orientation of the blot.

10. Place the wet membrane on 3MM and irradiate the side containing RNA with the UV Stratalinker (Stratagene) with 1 to 2 cycles to covalently cross-link RNA to the membrane. The wet membrane can directly be subjected to prehybridization.

11. Prehybridize the membrane in 20 ml of dextran sulfate prehybridization buffer with constant agitation for at least 6 h at 42 °C

12. Prepare probe solution by boiling the probe for 10 min and cooling it immediately on ice for 5 min before mixing it with 3 ml of prehybridization solution that has been set aside when preparing the dextran sulfate prehybridization buffer.

13. For hybridization, add the 3 ml of probe solution from step 12 into the prehybridization buffer, where the membrane has been incubated.

(*Note*: The final concentration of the probe should be less than 10 ng/ml in hybridization solution to avoid high nonspecific background.)

14. Incubate the membrane in hybridization solution with constant agitation for 12 to 16 h at 42 °C.

    (*Note*:Various hybridization buffers are commercially available. They may be used to shortened the prehybridization and hybridization steps from 18 to 22 h to 5 to 6 h, in which case the manufacturer's protocol should be followed for both steps.)

15. Remove hybridization solution and wash the membrane in the following solution and condition with constant agitation:

    a. Wash twice with 100 to 200 ml 2× SSC at room temperature for 10 min.

    b. Wash twice with 100 to 200 ml of 2× SSC/1.0% SDS at 65 °C for 15 min.

    c. Wash twice with 100 to 200 ml of 0.1× SSC at room temperature for 15 min.

16. If the membrane is to be rehybridized, do not allow the membrane to dry. Leave the membrane slightly damp and wrap it with plastic wrap before exposure to film for autoradiography. For long-term storage, the membrane should be stripped of probes (see step 17 below) and stored after drying at room temperature.

17. For stripping the membrane, incubate the membrane in approximately 200 ml of wash-off buffer (0.1× Denhardt's soln/5 m*M* Tris-Cl (pH8.0)/0.2 m*M* EDTA/0.05% sodium pyrophosphate) at 68 °C for 2 h. Change to a fresh buffer after the first hour of incubation.

1. 2.8×10^6 NIH 3T3 cells maintained in DMEM containing 10% calf serum (GIBCO) with 1% of l-glutamine (200 m*M* stock from GIBCO) are plated in a 10-cm tissue culture dish and kept in an 8.0% CO_2 incubator for 18 h, allowing the cells to reach 90 to 95% confluence.

2. Transfect the cells with Lipofectamine 2000 (Invitrogen)/DNA complexes as follows:

    a. Dilute 60 μl of Lipofectamine 2000 in 1.5 ml of Opti-MEM I medium (GIBCO), vortex for 1 sec, and incubate at room temperature for 5 min.

    b. Dilute 24 μg of DNA in 1.5 ml of Opti-MEM I medium; vortex for 1 sec.

    c. Combine the diluted DNA (from step b) with the diluted Lipofectamine 2000 (from step a), vortex for 10 sec, and incubate for 20 min at room temperature.

3. Add the DNA/Lipofectamine 2000 mixture (total volume 3 ml) gently directly to the cells. Gently rock the plate back and forth so that the DNA/Lipofectamine mixture distributes evenly in the dish, which is then incubated in a 5% CO_2 incubator.

4. 18 h later, aspirate the medium and add 2 ml of 0.25% trypsin-EDTA (GIBCO). Incubate the plate in a 5% CO_2 incubator at 37 °C for 5 min and then add 3 ml of DMEM/10% calf serum to stop trypsinization. Harvest the cells by centrifuging at 300*g* for 5 min.

5. Split 1.5×10^6 cells to several 6-cm tissue culture dishes (*Note*: the number of dishes depends on the time points required) in 5 ml of DMEM with 0.5% calf serum, 1% of l-glutamine (200 m*M* stock from GIBCO), 1% of penicillin-streptomycin (prepared with 10,000 U/ml penicillin G sodium (GIBCO), and 10 mg/ml streptomycin sulfate (GIBCO) in 0.85% saline (GIBCO).

6. Serum starvation: incubate cells in an 8.0% CO_2 incubator for 24 h, which will force cells to enter a quiescent (G0) state.

7. Serum induction: after serum starvation for 24 h, remove culture medium from the dish and add 5 ml of fresh culture medium containing 20% calf serum. Harvest cells at desired time points for RNA purification.

   (*Note*: After serum induction, transcription from the c-*fos* promoter returns to its original uninduced level in 30 to 40 min [Greenberg and Ziff, 1984].)

8. Refer to Protocol I for cytoplasmic RNA extraction. Alternately, cytoplasmic RNA may be extracted with RNeasy RNA preparation kit (QIAGENE) according to manufacturer's instruction. RNA samples are analyzed by Northern blotting as described previously.

4.2.2. The Tet-off regulatory promoter system—Although the c-*fos* promoter system has been used with success to investigate the decay kinetics and key features of AREs, the system has some limitations that prevent it from being used as a more general approach. Because activation of the c-*fos* promoter requires serum or growth factor induction of quiescent cells, this system has restricted the analysis of mRNA degradation to cells undergoing the G0 to G1 transition. In addition, many transformed cell lines cannot be readily forced to enter a quiescent state by serum starvation. Moreover, the use of serum induction complicates the analysis of regulatory mechanisms or signaling pathways, which may affect decay of mRNA. As an effort to develop a general approach that would be more suitable to study the regulatory aspects of differential and selective mRNA turnover in mammalian cells, we explored the Tet-regulatory promoter system as an alternative. In this system, a chimeric transcription activator termed tTA was generated by fusing the DNA-binding domain of the TN10-derived prokaryotic tetracycline repressor protein (tetR) to the transcription-enhancing domain of VP16 from herpes simplex virus, which binds and strongly activates a minimal promoter containing seven tetracycline operator (tetO) sequences in the absence of tetracycline (Gossen and Bujard, 1992; Gossen *et al*., 1993). Binding of the tTA to the tetO sequences can be quickly blocked by tetracycline, preventing the activation of the target promoter.

Although its potential application to address the cytoplasmic mRNA turnover seemed obvious, we found that simply blocking constitutive transcription of the Tet-regulatory promoter with tetracycline and then monitoring mRNA decay does not give an accurate measurement of mRNA half-life. This may be due to saturation of cellular decay machineries by the high level of constitutive mRNA expression. In mouse NIH3T3 or human K562 cells, steady-state level of the mRNA driven by the Tet-off promoter is 15- to 30-fold higher than that driven by SV-40 early promoter or CMV promoter (Xu, Loflin, Chen and Shyu, unpublished observations). Moreover, because of the size, heterogeneity of the poly(A) tails resulting from constitutive transcription, deadenylation and decay kinetics, and thus the relationship between deadenylation and decay of the mRNA, cannot be unequivocally determined. In light of these concerns, we have further optimized the systems by modulating the amount of tetracycline and the timing of its addition to or omission from culture medium. We were able to induce a short burst of mRNA synthesis from a reporter gene driven by the Tet-off promoter, which displays kinetics similar to those of the c-*fos* promoter system (Xu *et al*., 1998).

In this section, we describe a transcriptional pulsing approach developed by the tet-regulatory (Tet-off) system with mouse NIH3T3 cells as an example. With the new strategy, we have demonstrated that AREs can function in mouse NIH 3T3 fibroblasts under growth arrest and density arrest states (Xu *et al*., 1998). More recently, we have successfully used this new approach to study the destabilizing function of different AREs in various phases of the cell cycle and during blood cell differentiation (Chen *et al*., 2007). Furthermore, combining this approach with small interference RNA (siRNA)-mediated gene knockdown, we were able to trap different decay intermediates indicative of mechanistic steps of mRNA decay in mammalian cells (Chen *et al*., 2007; Yamashita *et al*., 2005). These *in vivo* studies demonstrate

the feasibility of this approach to investigate mechanisms underlying differential and selective mRNA turnover in mammalian cells.

4.2.2.1. Establishment of mammalian stable cell lines expressing the tTA: The first critical step toward the successful use of the transcriptional pulsing approach with the Tet-off promoter to monitor mRNA decay kinetics in mammalian cells is to identify or establish a stable line of interest that expresses tTA. In our laboratory, we have been using the stable *β*-globin mRNA as the reporter message for mRNA turnover study. To adapt this system to Tet regulation, we have constructed a new *β*-globin reporter plasmid, designated pTet-BBB (Loflin *et al*., 1999a; Xu *et al*., 1998). The plasmid pTet-BBB contains the *β*-globin gene under control of the Tet-off promoter, allowing transcription in the absence of tetracycline. The *Bgl*II site in the 3′ UTR of the *β*-globin gene remains unique in the pTet-BBB, providing a site into which RNA destabilizing elements, such as different AREs, can be introduced to test their ability to destabilize *β*-globin mRNA (Loflin *et al*., 1999a).

Initially, we attempted to test whether tight regulation of transcription from the Tet-off promoter may be obtained by transiently cotransfecting both the pTet-BBB + ARE and the pUHD15-1 plasmid encoding the tTA (Gossen and Bujard, 1992). We observed high basal-level expression of *β*-globin mRNA bearing the c-*fos* ARE (BBB + ARE) in the presence of tetracycline, indicating inefficient repression when tetracycline was added back to turn off transcription. In addition, the level of BBB + ARE mRNA expression from the Tet-off promoter varies considerably depending on the relative amount of pTet-BBB + ARE and the pUHD15-1 plasmids used in the transient transfections. Therefore, we do not recommend transient cotransfection of the Tet-off promoter-driven reporter plasmid and tTA-encoding plasmid. We refer readers to our previous publications (Chen *et al*., 2007; Loflin *et al*., 1999b) for details regarding establishing and characterizing tTA-expressing stable lines that are suitable for the transcriptional pulsing approach.

4.2.2.2. Transcriptional pulse strategy by modulating the amount of tetracycline in culture medium: To obtain a homogeneous population of mRNA, transcription should proceed for a period sufficient to produce a detectable signal by Northern blot analysis, yet brief enough to limit heterogeneity of poly(A) tails. The optimal condition can be determined by a titration experiment. Various concentrations of tetracycline are included in the culture medium to test at which concentration the steady-state level of *β*-globin mRNA transcribed from the transiently transfected pTet-BBB plasmid will be inhibited >99% compared with that in the absence of tetracycline (Chen *et al*., 2007; Loflin *et al*., 1999b; Xu *et al*., 1998). Depending on the cell lines and sources of tetracycline, the appropriate concentration of tetracycline ranges from 25 ng/ml to 100 ng/ml. After transient transfection of the Tet-off promoter-driven reporter plasmid, the transfected cells should be kept in culture medium containing the identified concentration of tetracycline until transcription induction (transcriptional pulsing). It is striking that all three different mammalian stable lines we established, including mouse NIH3T3 B2A2, human K562 III-2, and human BEAS-2B-19, show the same transcriptional pulsing kinetics in Northern blot analysis (Chen *et al*., 2007; Loflin *et al*., 1999b; Xu *et al*., 1998). A population of mRNA homogenous in size appears in the cytoplasm after removal of tetracycline for 100 to 110 min, making it possible to study deadenylation and decay kinetics and precursor and product relationships during mRNA decay. Typically, the reporter mRNA becomes detectable 90 min after the transfected cells are moved to fresh medium without tetracycline. After another 20 min (i.e., 110 min pulse), a descent signal representing a population of mRNAs with poly(A) tails homogeneous in size is produced. Because this time period is highly reproducible and is also observed in all different stable clones we have tested, this period has been used in our studies that use the Tet-off promoter-driven transcriptional pulsing approach and detailed as follows.

1. After transfection, the cells are grown in 25 to 40 ng/ml tetracycline for a period of 36 to 48 h.
2. Induction of a detectable homogeneous population of β-globin mRNA is accomplished by shifting the cells to fresh culture medium for 110 min.
3. The pulse is quickly terminated by adding 500 ng/ml tetracycline, and RNA samples are isolated at various times afterward.
4. Conduct RNA blot analysis as described previously.

Notes: Several experimental details and caveats are important for the success of transcriptional pulsing strategy. First, the amount of transfected plasmid DNA coding for the reporter mRNA must be empirically optimized with a positive control coding for an unstable message to avoid saturation of the decay machinery with excess mRNA. Second, the optimal amount of transfected DNA may vary when different transient transfection reagents or cells are used and must be optimized empirically. Third, to detect the mRNA synthesized after the removal of tetracycline for 110 min (transcriptional pulsing), it is crucial to select a stable tTA-expressing clone that gives the maximally induced expression level of the reporter gene. Both the NIH3T3 B2A2 and the K562 III-2 clones we selected for our mRNA decay study give more than 10^9 RLU/mg of protein by luciferase assay in transient transfection experiments (Loflin *et al*., 1999b; Xu *et al*., 1998). Fourth, to accurately determine deadenylation and decay kinetics, a robust, yet brief, transcription is necessary. Thus, the resumption kinetics of transcription on the removal of tetracycline displayed by the selected stable line is a crucial factor. For example, although we were able to identify a few stable clones that overexpress the transfected reporter genes at an equally high level in the absence of tetracycline, we noticed that not all of them exhibited fast resumption kinetics of transcription after tetracycline removal (data not shown). This difference may be derived from variations in copy number and integration site of the tTA cDNA in stable clones. Because slow resumption kinetics of transcription requires a prolonged pulse, and thus leads to heterogeneity in the size of mRNA molecules, one should pick a clone that is able to give at least 10% of the maximal level of expression after the 110-min pulse period without tetracycline so that a sufficient signal representing an mRNA population homogeneous in size can be generated for kinetic studies. Last, cells should be continually maintained in the presence of tetracycline, except for the short period of induction, because production of tTA for a longer period seems cytotoxic to the cells, and cells that have lost tTA expression may gradually take over the population during prolonged incubation without tetracycline. However, this problem may be avoided if one establishes stable lines with a modified tTA that seems to show little cytotoxicity (Clontech). Also, medium with tetracycline should always be prepared fresh and kept in the dark at 4 °C. A variety of mammalian cell lines harboring the tTA gene have been established, and they are commercially available. They may be tested for appropriateness for mRNA decay kinetic studies as described previously.

1. 2.6 to 2.8 × 10^6 NIH 3T3 B2A2 cells maintained in DMEM containing 10% calf serum (Gibco), 1% of l-glutamine (200 m*M* stock from Gibco), and 100 ng/ml tetracycline (CalBiochem) are plated in a 10-cm tissue culture dish and incubated in an 8% CO_2 incubator.

   (*Note*: In previous publications, we used 25 to 30 ng/ml of tetracycline that was purchased from Sigma. We found that higher concentration of tetracycline was needed to achieve the desired transcriptional pulsing when we used the tetracycline purchased from CalBiochem. Thus, the optimal amount of each critical reagent used in the experiment should be tested empirically when it is ordered from different companies or when a new lot is ordered from the same company.)

2. After 18 to 24 h, when cells reach 90 to 95% confluence, prepare the transfect cells and Lipofectamine 2000 (Invitrogen)/DNA complexes and harvest cells as described in Protocol II sections 2 to 4.
3. Split 1.5×10^6 cells to several 6-cm tissue culture dishes (*Note*: the number of dishes depends on the need of each experiment) in 5 ml of DMEM with 10% calf serum, 1% of L-glutamine (200 m*M* stock from Gibco), 1% of penicillin-streptomycin [prepared with 10,000 U/ml penicillin G sodium (Gibco) and 10 mg/ml streptomycin sulfate (Gibco) in 0.85% saline (Gibco), and 100 ng/ml tetracycline (CalBiochem)]. Incubate cells in an 8.0% CO_2 incubator for 24 h.
4. Remove medium and add 5 ml of complete medium without tetracycline to allow transcription of the reporter gene driven by the Tet-off promoter. Incubate the cells in an 8.0% CO_2 incubator immediately for 110 min.
5. To stop transcription, add complete medium containing tetracycline (500 ng/ml) to each plate.
6. Refer to Protocol I for cytoplasmic RNA extraction. Alternately, cytoplasmic RNA may be extracted with RNeasy RNA preparation kit (Qiagen) according to the manufacture's instruction. RNA samples are analyzed by Northern blotting as described previously.

Notes: The same procedure can also be used to introduce DNA and siRNA simultaneously into NIH 3T3 B2A2 cells. When introducing siRNA together with DNA, up to 320 pmol siRNA should be diluted in the same tube with 6.5 μg of plasmid DNA and 40 μl of Lipofectamine 2000. The ratio between siRNA and DNA is critical, as increasing siRNA amount may reduce the transfection efficiency or expression of the reporter DNA. We also refer readers to one of our previous publications (Chen *et al*., 2007), in which we describe another protocol that combines a consecutive siRNA knockdown procedure with plasmid DNA transfection with transfectants from Qiagen.

4.2.2.4. Directly measuring mRNA half-life with the Tet-off promoter system without transcriptional pulsing: For studies that do not directly deal with mRNA deadenylation and decay kinetics or mechanistic steps (e.g., if one wants to investigate whether a known signaling pathway may alter the stability of the transcript of interest), time-course experiments for measuring mRNA stability can be done without the use of the transcriptional pulsing approach. After transiently transfecting the tTA-expressing cells with the Tet-off promoter-driven reporter plasmid, cells are kept in culture medium without tetracycline to allow constitutive expression of the reporter mRNA. After 48 h, tetracycline is added to the medium to a final concentration of 500 ng/ml to stop transcription from the Tet-off promoter and total RNA is extracted at different time intervals. This approach has been used successfully to study the stability of mRNAs of interest in the presence or absence of a stimulus known to activate a desired signaling pathway (Winzen *et al*., 1999), as well as to identify stimuli and signaling pathways that modulate stabilities of several cytokine and chemokine mRNAs during various immune responses (reviewed in Stoecklin and Anderson [2006]).

One potential caveat when the aforementioned approach is used to directly measure mRNA half-life is that highly robust and constitutive transcription of the reporter mRNA driven by the Tet-off promoter may saturate the system, thereby impeding proper decay of the transcript. We recommend that a titration experiment should be carried out first to identify the optimal amount of transfecting DNA that does not force the mRNA stabilization but still results in a signal detectable by Northern blot analysis (e.g., by an overnight exposure of an RNA blot to X-ray film).

5. Concluding Remarks

We have described two transcriptional pulsing methods that result in the synthesis of an mRNA population nearly homogeneous in size. These methods have several advantages over other approaches used to measure mRNA half-life. They offer the opportunity to determine deadenylation and decay kinetics, as well as the precursor-product relationship of mRNA turnover (Yamashita *et al*., 2005). Although the c-*fos* promoter system is convenient to quickly address the mechanistic steps involved and characterize the *cis*-acting sequences, the Tet-regulatory system provides the opportunity to study the regulation under physiologically relevant and undisturbed conditions. For example, the role of mRNA turnover in controlling the cell cycle can also be studied with the protocol developed with cells arrested at individual phases of the cell cycle (Chen *et al*., 2007). In addition, with an increasing collection of tTA-expressing cell lines, the Tet-regulatory promoter system combined with our strategy allows the analysis of mRNA turnover and its regulation under various physiologic conditions including, but not limited to, during cell growth and differentiation, the immune response, and tissue repair. For instance, with the establishment of the Tet-regulatory promoter system in lymphoid cell lines or hematopoietic cell lines representing different hematopoietic stages, explanation of the mechanisms responsible for the regulation of cytokine and chemokine mRNAs are already underway (Chen *et al*., 2007).

Recently, we have successfully combined the siRNA-mediated gene knockdown and the transcription pulsing systems to decipher mechanistic steps in mammalian mRNA turnover (Yamashita *et al*., 2005). We showed that deadenylation is the major trigger of mRNA decay in mammalian cells and that decapping does not occur until or after the second phase of deadenylation (Yamashita *et al*., 2005). By use of the approaches and protocols described here, the applications of transcription pulsing systems can be further expanded to study not only the mechanistic steps of mRNA decay but also their regulation by *trans*-acting factors. For instance, the transcriptional pulsing approach will facilitate our understanding as to how miRNAs tie into the mRNA decay machinery to accomplish gene silencing in mammalian cells, because miRNA-mediated mRNA decay also starts with deadenylation (Wu *et al*., 2006). The protocols described in this chapter and our approaches to optimize these protocols provide guidelines for more widespread development of new protocols with transcriptional pulsing strategies and their applications to study mRNA turnover under various physiologic conditions.

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