A Reverse Time-Course Method for Transcriptional Chase Analyses of mRNA Half-Lives in Cultured Cells

Osheiza Abdulmalik1, Alyssa A. Lombardi2, J. Eric Russell1,2

1 Department of Pediatrics (Hematology), University of Pennsylvania School of Medicine and The Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania, United States of America, 2 Department of Medicine (Hematology-Oncology), University of Pennsylvania School of Medicine and The Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania, United States of America

Abstract

Standard methods for assessing mRNA stabilities in intact cells are labor-intensive and can generate half-life ($t_{1/2}$) measures that are both imprecise and inaccurate. We describe modifications to a conventional tetracycline-conditional transcriptional chase method for analyzing mRNA stability that significantly simplify its conduct, while generating highly reproducible and accurate $t_{1/2}$ values. The revised method—which is conducted as a reverse time course, and which accounts for interval expansion in the number of cultured cells—is validated for the analyses of mRNAs with both short and long half-lives. This approach facilitates accurate assessment of mRNA metabolism, providing a user-friendly tool for detailed investigations into their structures and functions, as well as the processes that contribute to their post-transcriptional regulation.

Introduction

The biological importance of regulated mRNA stability is illustrated by pathological consequences that follow the abnormal metabolism of individual transcripts. A number of medical conditions have been linked to changes in the stabilities of specific mRNAs, including Type 2 Gaucher disease (glucocerebrosidase) [1], ichthyosis vulgaris (profilaggrin) [2], age-related macular degeneration (ARMS2) [3], systemic lupus erythematosis (TCR-$\gamma$) [4], and breast cancer (cyclin D1) [5]. The broader implications of post-transcriptional regulatory processes are illustrated by expression-profiling analyses of T-lymphocytic cells suggesting that nearly one-half of inductive changes in gene expression result from alterations in mRNA stability [6].

The general utilities of in vitro and cultured cell approaches that are commonly employed to estimate mRNA-specific $t_{1/2}$ values are limited by important methodological considerations. Assays conducted in cell-free systems—though convenient—fail to account for translation-coupled effects on mRNA metabolism [7] and are unlikely to faithfully reproduce stable and/or transient mRNA-protein interactions that specify the kinetics of mRNA decay in intact cells. Different concerns apply to cultured cell analyses that quantify the temporal decline in a study mRNA following transcriptional silencing of its cognate gene. Commonly used transcriptional inhibitors—actinomycin D and DRB (5,6-dichloro-beta-D-ribofuranosyl benzimidazole)—are global and nonspecific in effect, altering expression of the study mRNA, as well as the expression of mRNA-stabilizing and -decay factors that may be required for its constitutive regulation. Other methods that permit gene-specific transcriptional regulation in intact cells (e.g., serum stimulation of fos promoter-linked genes [8]) induce alterations in the cellular milieu that may have equally unpredictable effects on the observed half-lives of study mRNAs.

Tetracycline-conditional (‘on-off’) transcriptional chase approaches for assessing mRNA half-lives are widely employed because they are conducted in intact cells, are mRNA-specific, and do not alter cellular homeostasis. These analyses require cells that express a tetracycline trans-activator (tTA) fusion protein that promotes transcription of a second, exogenous study gene linked to a compound tetracycline response element (TRE) [9]. The transcription of TRE-linked genes is rapidly inhibited by exposure to antibiotics [tetracycline (tet) or doxycycline (dox)] that bind and inactivate tTA. Half-life analyses are conducted in tTA-transfected cells that express a TRE-linked study gene, by amending cultures with tet or dox and sacrificing aliquots at defined intervals. The level of study mRNA in each aliquot is then quantitated, relative to the level of an antibiotic-indifferent control mRNA, using any of several methods. When properly conducted, this transcriptional chase strategy additionally requires the accurate enumeration of cells in each individual aliquot, to permit correction for the expansion in cell number—and corresponding increase in the level of tet-indifferent control mRNA—that occurs during the interval when the study gene is transcriptionally silent. This concept is illustrated by considering that, in the absence of this correction, an infinitely stable study mRNA encoded by a transcriptionally-silenced gene would appear to have a $t_{1/2}$ value equal to the doubling time of the cell culture (Fig. 1). As a practical matter, the efforts required to mobilize and subsequently count cells in individual aliquots significantly limit the number of experimental replicates that can be conducted and/or the frequency with which data points can be collected. These low-throughput imperatives...
mandate data sets that are frequently too small to produce statistically robust estimates of mRNA half-life.

We reasoned that technical barriers to the broader application of tet-conditional RNA half-life analyses could be resolved by altering both the design of the transcriptional chase, as well as the manner used to correct normalized study mRNA values for interval expansion in cell number. In the conventional method, identical aliquots are simultaneously amended with antibiotic, and individually processed (trypsinized, resuspended, counted, and transferred to storage) at defined intervals. Normalized RT-qPCR values for the study mRNA are then corrected for the corresponding aliquot-specific cell number, the data regressed to an exponential function, and a half-life value calculated. The new reverse-chase (RC) method uses a reverse time-course approach in which identical aliquots are individually amended with antibiotic at defined intervals, and simultaneously transferred to storage at the conclusion of the experiment. Normalized values for the study mRNA are first regressed to an exponential function, which is subsequently corrected to a formal t_{1/2} value using an experimentally determined culture-specific factor describing the rate of cell growth.

**Results**

The reverse-chase strategy for assessing mRNA stability was designed to mitigate the throughput-limiting characteristics of the conventional method that is commonly used for this purpose. The RC approach eliminates the effort required for conventional, iterative processing of individual aliquots, thus facilitating the generation of data sets with replicate and/or closely spaced time points. An additional benefit of the RC strategy is that it provides for equal numbers of cells in all aliquots at the time of sacrifice (unlike the conventional approach), obviating the need to individually adjust samples to maintain control mRNA values within the linear range of RT-qPCR or other quantitation assays.
We tested the RC method using tTA-expressing HeLa cells engineered to stably express TRE-linked genes encoding human β-globin mRNAs (not shown). The expansion of cultured HeLa cells was unaffected by supplemental dox, confirming the expectation that aliquots in RC analyses would each contain the same number of cells, regardless of antibiotic-exposure interval (Fig. 2).

Conventional and RC stability analyses were subsequently conducted in parallel in cells expressing wild-type β-globin mRNA (β⁴⁰⁴). For the conventional method, identical cell aliquots were simultaneously supplemented with dox (t = 0) and triplicate samples sacrificed, with cell counting, at defined intervals for 80 hours (Fig. 3A). For the RC method, identical cell aliquots were amended with dox at defined intervals, and simultaneously sacrificed, without cell counting, at 80 hours (Fig. 3B). In both experiments, β⁴⁰⁴ mRNA was quantitated by RT-qPCR relative to dox-indifferent control β-actin mRNA. Half-life values for the β⁴⁰⁴ mRNA were subsequently calculated from normalized RT-qPCR results using either aliquot-specific cell numbers (conventional method), or an experimentally determined factor (j, see Methods) that describes the rate at which each cell culture is expands (Fig. 4). Although both the conventional and RC studies generated equal numbers of data points, the conventional method specified a decay curve with a high level of uncertainty (R² = 0.23; Fig. 5A), while the RC results regressed to a highly reliable exponential decay function (R² = 0.98; Fig. 5B). Plotted separately, the three conventional replicates displayed low R² values (0.11–0.25; Fig. 5C), while the three RC replicates exhibited correspondingly high R² values (0.96–0.98; Fig. 5D). As might be anticipated, the conventional method produced t₁/₂ values for the study mRNA that were poorly reproducible (range 39.8–50.2 h), in contrast to t₁/₂ values calculated from the RC method (range 28.9–36.5 h).

Similar comparative analyses demonstrated the superiority of the RC method for analyses of mRNAs with shorter half-life values. The stability of a derivative β-globin mRNA (β⁴⁰⁴ARE) containing a destabilizing AU-rich element within its 3’UTR [12] was studied over a 120-minute chase interval in tTA-expressing HeLa cells using both the conventional and RC approaches. Cell aliquots were sacrificed with cell counting at 30-minute intervals (for the conventional method) or without counting at five-minute intervals (RC method). The conventional approach yielded four averaged time points, while the RC protocol permitted the accumulation of 21 averaged time points. The RC data regressed to a more reliable exponential decay function when replicates were analyzed in aggregate [R² = 0.99 (RC) v 0.35 (conventional); Fig. 6A and B] or individually [R² > 0.97 (RC) v 0.23–0.49 (conventional); Fig. 6C and D]. Likewise, mRNA t₁/₂ values calculated from the RC data were highly reproducible (range 19.0–20.7 min), in contrast to values determined by the conventional method (range 21.4–37.3 min). Conventional analyses displayed a high variability—even when uncorrected for cell number—emphasizing that simultaneous sacrifice can impart high reproducibility to data collected using the RC method (not shown).


Discussion

The reverse-chase strategy resolves critical limitations of conventional tetracycline chase experiments: substantial hands-on time that restricts data acquisition, and negatively impacts both data reliability and reproducibility. For analyses requiring a prolonged chase interval—where changes in cell number can be significant—conventional cell sacrifice and counting requires as much as 30 minutes of attention per time point, even for a limited number of samples. In contrast, the RC method requires 10–15 seconds of attention per time point (for the addition of antibiotic to individual aliquots) substantially increasing the number of samples that can be evaluated. Moreover, because the RC strategy permits simultaneous sacrifice of all aliquots at the conclusion of the chase interval, concentrations of mRNA (which correspond with cell number) are equal, and do not need to be individually adjusted to stay within the linear range of the mRNA quantitation method. Although not illustrated here, the denser data set obtained by the RC method also permits the application of statistical tools that account for the effect of background dox-indifferent transcription on the calculated mRNA half-life [13,14]. We have also observed that the RC method displays an almost negligible inter-operator variability, which is critically important for the conduct of prolonged chase experiments requiring investigators to work in two or more shifts (not shown).

One seemingly minor limitation to the RC method is that it requires study mRNAs to be expressed in steady state; i.e., transcribed from TRE-regulated genes that are stably integrated in tTA-expressing cells. We have not found this to be a major impediment, as a large number of tTA-expressing cell lines are commercially available [15] or have been previously generated by individual investigators [12,16]. Moreover, since all three components required for tet-regulated transcription are exogenous (tTA transactivator, TRE-linked study gene, and tetracycline), cell-homologous systems for studying the properties of a specific mRNA can easily be engineered from nearly any existing cultured cell line. Consequently, the method that we describe is not restricted to HeLa cells, but can be applied to cell lines that model many normal or diseased tissues.

We have also observed that half-life analyses conducted in cells that transiently express TRE-linked study genes are less reliable, and less reproducible, than similar analyses conducted in a corresponding cell line that stably expresses the study gene. Among other limitations, transient expression analyses utilize cells recovering from methodological insults (electroporation, lipofection) that alter cell homeostasis; do not account for cell-to-cell variation in the expression of study mRNA; and cannot guarantee a consistent baseline level of the study mRNA in independent experiments. In contrast, cell lines that stably express the test mRNA are not subject to any of these limitations; the test mRNA is expressed at a defined level, at steady-state, in healthy, clonal cells. As a matter of practice, we typically use transient assays for pilot, low-resolution analyses, and subsequently generate stable cell lines for definitive, reproducible and highly reliable measures of mRNA stability. This approach determines mRNA half-life values that are reliable and highly reproducible, and is likely to lower the ‘effort barrier’ for RNA biologists and other investigators who, until now, have avoided definitive mRNA stability analyses in stable cells because of the corresponding technical challenges.

Methods

Plasmids

The construction of parental pTRE-βVT, which contains the full-length human β-globin gene on a 3.3-kb fragment of genomic DNA, has been previously described [10]. pTRE-βARE was generated from pTRE-βVT by insertion of a 39-bp ΔU-rich mRNA instability element [11] at a position 15 bp 3′ to the translation stop codon [10]. A hygromycin-resistance gene, encompassed by a 1.5-kb XbaI fragment of pTRE2hyg (Clontech), was subsequently ligated into pTRE-βVT and -βARE at the corresponding position.

Cell Culture

HeLa cells expressing the tetracycline trans-activator (tTA) fusion protein (HeLa Tet-Off cell line, Clontech) [15] were maintained in DMEM/F12 media supplemented with 10% fetal bovine serum and antibiotics. Cells in log-phase growth were transfected with pTRE-βVT or -βARE using Superfect reagent according to the manufacturer’s recommendations (Qiagen). Hygromycin-resistant clones were isolated using standard cloning-disc methodology, and subsequently screened for steady-state levels of β-globin mRNA in tetracycline-free media.

Tetracycline-conditional Gene Silencing

Conventional method. HeLa cells (1×10⁶) were aliquoted in 450 µL media in 24-well format (for 120-minute chase experiments), or 225 µL in a 48-well format (for 80-hr chase experiments) 24 hours prior to the start of the transcriptional chase. At t = 0, all aliquots were supplemented with a 10X stock solution of doxycycline (10 µg/µL in culture media).

Reverse Chase method. HeLa cells (5×10⁶) were aliquoted in 180 µL media in a 96-well format 24 hours prior to the start of the transcriptional chase. Starting at t = 0, and continuing for the duration of the experiment, serial aliquots were amended with a 10X doxycycline stock at defined intervals. For both the 80-hr conventional and reverse-chase methods, doxycycline was refreshed at 48 hours with a second volume of 10X stock.

Cell Sacrifice

Conventional method. Cells were washed twice with excess phosphate-buffered saline (PBS), mobilized with trypsin, washed, and re-suspended in 30 µL of PBS. Ten µL of the suspension was reserved for cell counting, which was conducted in triplicate using a hemocytometer, and reported as a mean value. Whole-cell lysates were prepared from the remaining 20 µL using the Cells-to-Ct kit (Applied Biosystems).

Simplified Analyses of mRNA Half-Lives in Culture

Figure 4. HeLa cell expansion under transcriptional chase conditions. Data from dox-supplementation experiments in Fig. 2 (1 µg/mL) was regressed to an exponential function, and expansion factors defined as $j = 0.0224 (hr)$ or 0.0004 (min). doi:10.1371/journal.pone.0040827.g004
Reverse-chase method. Wells were washed twice with excess PBS and lysed in situ using Cells-to-Ct reagents.

Analysis of cell growth rate. Approximately $5 \times 10^4$ HeLa cells were cultured in 12-well plates in the presence or absence of dox, then manually counted at defined intervals. 96-hour aliquots were refreshed with dox-supplemented media at $t = 48$ hr.

RT-qPCR. Equal volumes of prepared RNA were subjected to RT using Cells-to-Ct reagent. First-strand cDNA was subjected to qPCR amplification using Taqman assays specific for $\beta$-globin (#00747223_g1) or endogenous control $\beta$-actin (#99999903_m1) mRNAs, according to the manufacturer’s recommendations (Applied Biosystems). Samples were analyzed using an ABI 7500 Real-Time PCR system (Applied Biosystems). Each RT-qPCR assay was conducted in triplicate using a previously validated multiplexed format (not shown); samples exhibiting outlier Ct values (defined by Applied Biosystems) were censored.

**Data Analysis**

**Conventional method.** For each sample, the level of globin mRNA was first normalized to the level of control $\beta$-actin mRNA, and subsequently to the aliquot-specific cell number. These data were regressed to an experimental decay function, and half-life values determined.

**Reverse-chase method.** Levels of globin mRNA were normalized to the level of control $\beta$-actin mRNA, then regressed to an exponential decay function. Decay factors were adjusted for increases in cell number using an experimentally determined expansion factor (see below).

**Correction of RT-qPCR Data for Expansion of Cell Number**

Results from RT-qPCR analyses are fit by nonlinear regression to the general equation for exponential decay:
\[ Q_t = Q_0 e^{-kt} \]  
\[ Q_i = Q_0 e^{-kt} \]

where \( Q_t \) and \( Q_0 \) are observed quantities of subject mRNA normalized to levels of \( \beta \)-actin mRNA at time \( t \) and time \( 0 \), and \( k \) is the decay constant. A new term \( Q_i \) accounts for the increase in cell number during the interval when TRE-regulated genes are transcriptionally silent. \( Q_i \) is derived from \( Q_t \) by multiplying by \( \frac{N_0}{N_t} \), where \( N_0 \) and \( N_t \) are the number of cells present at time \( 0 \) and time \( t \), respectively:

\[ Q_i = \frac{N_0}{N_t} Q_t = Q_0 e^{-kt} \]

The equation can be solved by manual determination of \( N_t \) at each individual time \( t \) (as required by the conventional method), or by separately determining the expansion constant \( j \) that describes the growth of specific cells under defined culture conditions (as practiced in the RC method). Specifically,

\[ N_t = N_0 e^{jt} \]  

Substituting for \( \frac{N_0}{N_t} \):

Table 1. Values used in the current report.

| Replicate | \( j \)  | \( k \)  | \( k-j \) | \( t_{1/2} \) |
|-----------|---------|---------|---------|-----------|
| ARE 1     | .0004   | .0338   | .0334   | 20.7 m    |
| ARE 2     | .0004   | .0353   | .0349   | 19.9 m    |
| ARE 3     | .0004   | .0369   | .0365   | 19.0 m    |
| WT 1      | .0224   | .0414   | .0190   | 36.5 h    |
| WT 2      | .0224   | .0425   | .0201   | 34.5 h    |
| WT 3      | .0224   | .0464   | .0240   | 28.9 h    |

doi:10.1371/journal.pone.0040827.t001
\[ Q'_t = Q_0 e^{(j/k)t} \] (4)

A \( t_{1/2} \) value for the test mRNA is calculated as \( \frac{0.693}{k-j} \) using experimentally determined values for both \( k \) and \( j \). Values for \( k \) and \( j \) derived from our experiments are included in Table 1.

**Acknowledgments**

The authors thank S. van Zalen for helpful discussions. This work was funded by grants R01-HL082754 and R01-HL061399 (JER), K01-HL103186 (OA), and through an award from the UNICO Foundation (OA).

**Author Contributions**

Conceived and designed the experiments: OA JER. Performed the experiments: OA AL JER. Analyzed the data: OA AL JER. Contributed reagents/materials/analysis tools: OA JER. Wrote the paper: OA JER.

**References**

1. Pasmanik-Chor M, Madar-Shapiro L, Stein OE, Aerts H, Gatt S, et al. (1997) Expression of mutated glucocerebrosidase alleles in human cells. Hum Mol Genet 6: 867–875.
2. Nirumukiari W, Zhang SH, Fleckman P (1998) Reduced stability ans bi-allelic, coequal expression of profilaggrin mRNA in keratinocytes cultured from subjects with ichthyosis vulgaris. J Invest Dermatol 110: 854–861.
3. Fritsche LG, Loo nhardt T, Janssen A, Fisher SA, Rivera A, et al. (2008) Age-related macular degeneration is associated with an unstable ARMS2 (LOC387715) mRNA. Nat Genet 40: 892–896.
4. Chowdhury B, Tsokos CG, Krishnan S, Robertson J, Fisher CU, et al. (2005) Decreased stability and translation of T cell receptor \( \zeta \) mRNA with an alternately spliced 3'-untranslated region contribute to \( \zeta \) chain down-regulation in patients with systemic lupus erythematosus. J Biol Chem 280: 18959–18966.
5. Lebwohl DE, Muise-Helmericks R, Sepp-Lorenzino L, Serve S, Timaul M, et al. (1994) A truncated cyclin D1 gene encodes a stable mRNA in a human breast cancer cell line. Oncogene 9: 1925–1929.
6. Cheadle C, Fan J, Cho-Chung YS, Werner T, Ray J, et al. (2005) Stability regulation of mRNA and the control of gene expression. Ann N Y Acad Sci 1058: 196–204.
7. Wisdom R, Lee W (1991) The protein-coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. Gene Dev: 232–243.
8. Greenberg ME, Ziff EB (1984) Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature 311: 433–437.
9. Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline responsive promoters. Proc Natl Acad Sci USA 89: 5547–5551.
10. Jiang Y, Xu X, Russell JE (2006) A nucleolin-binding 3'UTR element stabilizes \( \beta\)-globin mRNA in vivo. Mol Cell Biol 26: 2419–2429.
11. Shaw G, Kamen R (1986) A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46: 659–667.
12. Liu X, Russell JE (2010) Cytoplasmic stabilities of 3'UTR-polymorphic prothrombin mRNAs. J Thromb Haemost 8: 2580–2583.
13. Levenberg K (1944) A method for the solution of certain non-linear problems in least squares. Q Appl Math 2: 164–168.
14. Marquardt DW (1963) An algorithm for the least-squares estimation of nonlinear parameters. SIAM J Appl Math 11: 431–441.
15. Chrontech. Available: www.clontech.com/US/Products/Inducible_Systems/Tetracycline-Inducible_Expression/Tet-Off_Cell_Lines?site=x=10020:22372:US. Accessed 2012 June 19.
16. Xu N, Loflin P, Chen C-YA, Shyu A-B (1998) A broader role for AU-rich element-mediated mRNA turnover revealed by a new transcriptional pulse strategy. Nucleic Acids Res 26: 558–565.