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Genome-wide technology for determining RNA stability in mammalian cells

Historical perspective and recent advantages based on modified nucleotide labeling

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Changing the abundance of transcripts by regulated RNA degradation is a critical step in the control of various biological pathways. Recently, genome-wide inhibitor-free technologies for determining RNA stabilities in mammalian cells have been developed. In these methods, endogenous RNAs are pulse labeled by uridine analogs [e.g., 4-thiouridine (4sU), 5-etyniluridine (EU) and 5'-bromo-uridine (BrU)], followed by purification of labeled de novo RNAs. These technologies have revealed that the specific half-life of each mRNA is closely related to its physiological function. Genes with short-lived mRNAs are significantly enriched among regulatory genes, while genes with long-lived mRNAs are enriched among housekeeping genes. This review describes the recent progress of experimental procedures for measuring RNA stability.

Introduction

All cellular biochemical pathways rely on the regulated expression of genetic information. Hence, genome-wide gene expression profiling by measuring total transcript abundance at the whole transcriptome level is one of the most important issues in the field of molecular biology. Cellular RNA levels are determined by the interplay of tightly regulated processes for RNA transcription and degradation. Transcriptional regulation,[1] as well as regulated RNA degradation,[2-4] is a critical step in the control of the abundance of transcripts. It has been estimated that the mRNA abundances of 5–10% of human genes are controlled by the regulation of RNA stability.[5]

In mammals, the rate of RNA degradation is regulated by the interaction between cis-acting elements (specific RNA sequences) and trans-acting elements (RNA binding proteins or microRNAs).[6] For example, many short-lived mRNAs that encode proto-oncogenes, nuclear transcription factors and cytokines have AU-rich elements (AREs)[5,7] and GU-rich elements (GREs)[8] in their 3' untranslated regions (3' UTRs). These sequences are targets of many ARE- or GRE-binding proteins, some of which induce degradation, whereas others promote stabilization of the mRNA. Iron responsive elements (IREs)[9] are conserved stem-loops, which are bound by iron responsive proteins (IRPs), in the 5' UTRs and 3' UTRs of transcripts whose products are involved in iron metabolism. IRPs binding to IREs increase the stability of mRNA. MicroRNAs also regulate mRNA stability by forming imperfect hybrids with the 3' UTR sequences of target mRNAs during cell growth and development.[10,11]

RNA degradation pathways also play important roles in mRNA surveillance systems to ensure the fidelity of genetic information flow.[12,13] The mRNA surveillance system includes nonsense-mediated mRNA decay (NMD),[14,15] nonstop-mediated mRNA decay (NSD),[16-19] and no-go decay (NGD).[20] The mRNA surveillance systems monitor the integrity of transcripts and sort aberrant transcripts into degradation pathways. Thus, regulation of RNA stability is an important issue in terms of regulation of transcript abundance and RNA quality control. Moreover, changes in RNA stability plays a critical role in shaping the kinetics of gene induction in intricate gene networks in mammalian cells.[21-23] In particular, RNA stability significantly influences the induction kinetics of genes encoding inflammatory proteins.[24,25]

To unravel the underlying processes of the regulated RNA stability, determining RNA stability in genome-wide is indispensable. In this review, we will review chronologically the genome-wide technologies for investigating RNA stabilities in mammalian cells that have been conducted in the past decade and discuss the relationship between RNA stability and physiological function.

Inhibitor-Mediated Global Transcriptional Arrest

Historically, the most widely used method for genome-wide analysis of RNA degradation is based on transcriptional inhibitors such as actinomycin D (ActD), 5,6-dichloro-1–D-ribofuranosyl-benzimidazole (DRB) and α-amanitin (α-Am) (**Fig. 1**).[2,26] ActD inhibits transcription initiation broadly by intercalating into DNA, whereas DRB and α-Am specifically inhibit RNA Polymerase II-mediated transcription. Since 2001, genome-wide

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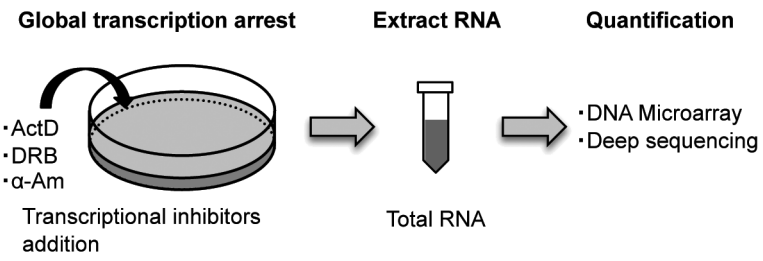

Figure 1. Schematic representation of the genome-wide method for RNA decay using transcriptional inhibitor-mediated global transcriptional arrest. Transcriptional inhibitors such as Actinomycin D (ActD), 5,6-dichloro-1–D-ribofuranosyl-benzimidazole (DRB) or -amanitin (α-Am) are added to cells, collect the cells at sequential time points after addition of the transcriptional inhibitors, and extract total RNA (blue).

RNA degradation has been assessed by blocking global transcription with transcriptional inhibitors, and subsequently monitoring ongoing RNA decay over time, using a DNA microarray.[21,24,27-33]

Although transcriptional inhibitors have been widely used for determining RNA stabilities, inhibitor-mediated global transcriptional arrest has a profoundly disruptive impact on cellular physiology, including splicing, polyA addition and other mRNA processing events; moreover, it interferes with the precise determination of the RNA degradation rate.[34-38] For instance, some transcripts are rapidly stabilized following ActD or DRB treatment.[39] Moreover, ActD alters the localizations and stabilities of a large number of long ncRNAs.[34,40] Thus, global inhibition of transcription by inhibitors is not considered suitable for measuring RNA stabilities.

Pulse Labeling by 4-Thiouridine (4sU)

In 1978, 4sU was first used to label endogenous RNAs in mammalian cells as a non-disruptive technology for measuring RNA decay.[41] First, similarly to other nucleosides, 4sU is rapidly taken up by cells. After entering a cell, 4sU is phosphorylated by cellular uridine kinases. In consequence, phosphorylated 4sU is continuously accumulated in cells over time. Additional steps, such as electroporation or lipofection, are not necessary for labeling RNA with this compound. This metabolic labeling of newly transcribed RNA with 4sU for short time has minimal adverse effects on gene expression, RNA decay, protein stability and cell viability.[41-43] However, prolonged culturing with 4sU causes inhibition of cell growth.[34] Although this technique includes the advantages of being able to isolate and analyze nascent RNA, the advantage was not broadly recognized during the next 30 years. In 2007, Kenzelmann et al. reported an integrated approach that combined the advantages of direct incorporation of 4sU following two hours of labeling, with the specific isolation of thiolated RNAs from total RNA using an agarose-based organomercurial affinity matrix.[43] Dölken et al. reported an improved approach using thiol-specific biotinylation and affinity purification with streptavidin-coated magnetic separation of total RNA into nascent and pre-existing untagged RNA, which achieved high purity following 10 to 60 min of labeling (**Fig. 2A and B**).[44]

Generally, 4sU (Catalog no. T4509, Sigma) and EZ-Link Biotin-HPDP (Catalog no. 21341, Pierce) are used for biotinylation of 4sU-labeled RNA, and the MACS streptavidin kit (Catalog no. 130-074-101, Miltenyi) is used for the specific isolation of biotinylated 4sU-RNA.[22,44,45] These methods allow microarray analysis of all three obtained RNA subsets (newly transcribed RNA, total RNA and pre-existing RNA) in parallel. RNA half-lives can then be determined, based on both newly transcribed RNA/total RNA ratios as well as pre-existing RNA/total RNA ratios.[44] Based on the newly transcribed RNA/total RNA ratios (R) and the duration of labeling (t_L), precise data on mRNA half-life ($t_{1/2}$) can be calculated according to the following equation:

$$t_{1/2} = t_L \times \ln(2) / \ln(1 - R)$$

Removal of newly transcribed RNA from total RNA offers a simple, novel approach for determining RNA decay rates without having to block transcription. Moreover, analysis of newly transcribed RNA allows the quantitative study of regulatory mechanisms governing transcription. This is of particular interest for subsequent promoter analyses. As no cellular stress response is provoked, the regulatory mechanisms that govern mRNA decay, such as the effects exerted by miRNAs, can be studied.

4sU is efficiently incorporated into RNA by broad range of cell types of human and murine origin, including fibroblasts, endothelial cells, dendritic cells, macrophages, B-cells and T-cells.[44] The median mRNA half-life in murine NIH3T3 fibroblasts and human B-cells were 4.9 and 5.3 h, respectively, as estimated by the 4sU-Microarray method.[38,44] Using the 4sU-seq method (massively parallel sequencing analysis of 4sU-containing mRNAs), the median mRNA half-life in NIH3T3 was estimated as 9.0 h (**Table 1**).[45] This discrepancy may be caused by inherent limitations in indirect estimation of RNA degradation rates when comparing purified 4sU-containing mRNAs with preexisting mRNAs. Indeed, the yield of purification of 4sU-containing mRNAs would dramatically alter the estimation.[22,38,45] In this regard, directly chasing decreasing levels of labeled RNA would be more suitable for determining RNA decay.

Pulse Labeling by 5-Ethynyluridine (EU)

In 2008, EU was first used to label endogenous RNAs in mammalian cells.[46] EU is efficiently incorporated into the nascent RNA in living cells, similarly to 4sU. Newly transcribed EU-labeled cellular RNAs are separated from total RNA by biotinylation of EU in a copper-catalyzed cycloaddition reaction (often referred to as click chemistry), followed by purification on streptavidin magnetic beads. The isolated RNAs are used as templates for reverse transcriptase-mediated cDNA synthesis for subsequent quantification of RNA (**Fig. 2C and D**).

This method is commercially available as the Click-iT Nascent RNA Capture Kit (Catalog no. C10365, Invitrogen).[47] The manufacturer's instruction of this kit recommends that the pulse labeling time is 30 to 60 min for a 0.5 mM EU dose, or

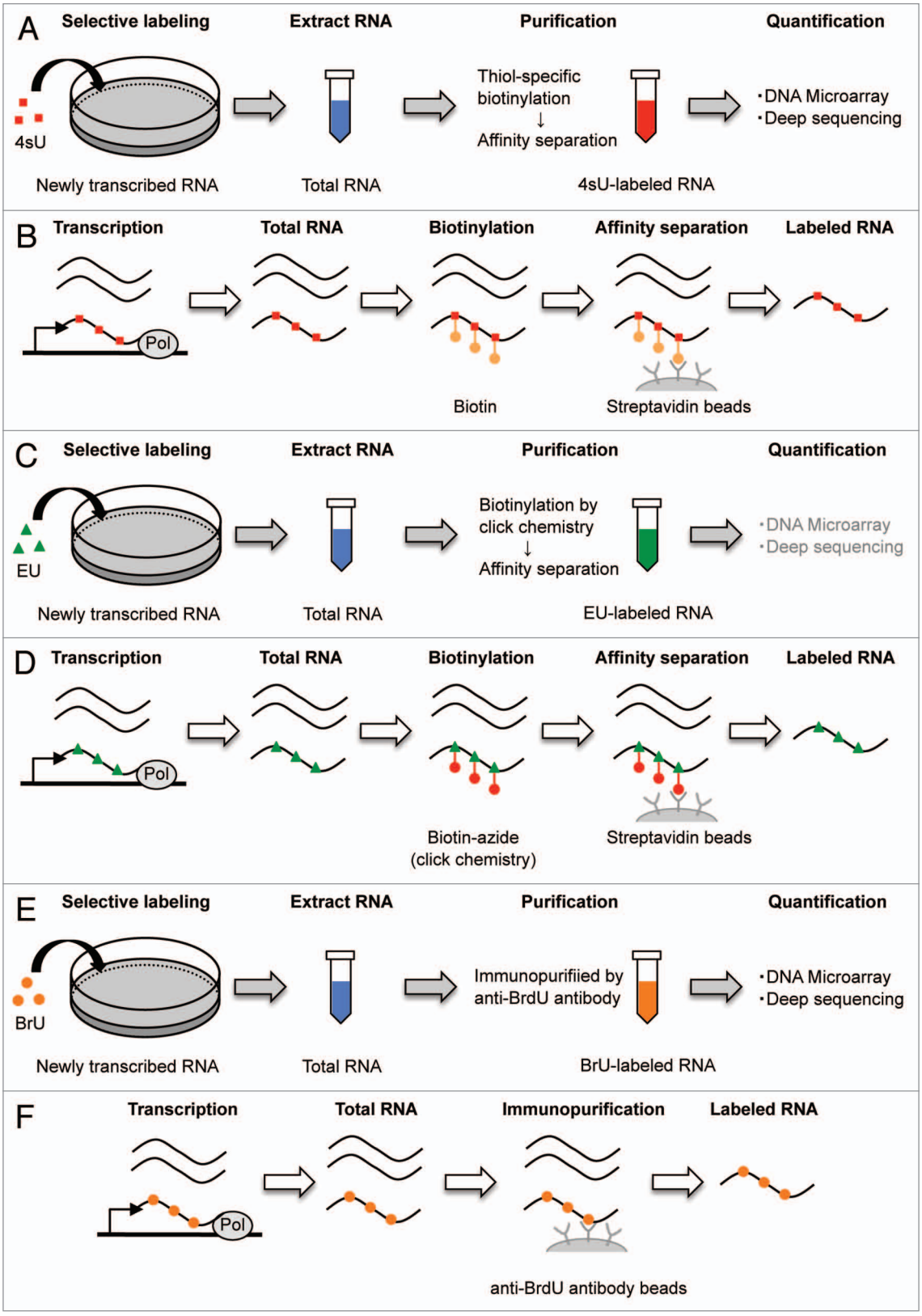

Figure 2. For figure legend, see page 1236.

Figure 2. Schematic representation of the genome-wide method for RNA decay using pulse labeling with uridine analogs. (**A**) 4sU is added to cells for a pre-defined time. Collecting the cells and extract total RNA (blue). Then, 4sU-labeled RNAs (red) are isolated by thiol-specific biotinylation and affinity separation. (**B**) 4sU is introduced into newly transcribed RNA in place of uridine. Total RNA extract is biotinylated by covalently linking biotin (orange) to 4sU, followed by binding streptavidin coated beads (gray). Biotinylated RNA is isolated, whereas unlabeled RNA is wash out. Then, cleaving the biotin-4sU disulfide bond releases the 4sU-labeled RNA from beads. (**C**) EU is added to cells for a pre-defined time. Collecting the cells and extract total RNA (blue). Then, EU-labeled RNAs (green) are isolated by biotinylation of EU in click chemistry and affinity separation. (**D**) EU is incorporated into newly transcribed RNA in place of uridine. Total RNA extract is biotinylated by copper-catalyzed cycloaddition reaction (click chemistry) (red) to EU, followed by isolation of EU-labeled RNA, similarly to 4sU method. (**E**) BrU is added to cells for a pre-defined time. Collecting the cells and extract total RNA (blue) at sequential time points after removal of surplus BrU from the culture medium. Then, BrU-labeled RNAs (orange) are isolated by immunopurification. (**F**) BrU is incorporated into newly transcribed RNA in place of uridine. BrU-labeled RNAs in total RNA extract are immunopurified using an anti-BrdU antibody coated beads (gray), which recognizes both BrdU and BrU.

Table 1. Median mRNA half-lives in various studies

Reagent	Platform	Species	Cell line	Median half-life (h)	Reference
Actinomycin D	Microarray	Human	Hepatocellular carcinoma cell (HepG2) / Primary fibroblast cell (Bud8)	10.0	30
Actinomycin D	Microarray	Mouse	Embryonic stem (ES) cell (MC1/MC2-B6)	7.1	24
Actinomycin D	Microarray	Mouse	Neuro-2a neuroblastoma cell (N2A)	5.1	27
Actinomycin D	Microarray	Mouse	Fibroblast cell (NIH3T3)	4.9	45
4-Thio-Uridine	Microarray	Human	B-cell (BL41)	5.3	39
4-Thio-Uridine	RNA-seq	Mouse	Fibroblast cell (NIH3T3)	9.0	25
5′-Bromo-Uridine	RNA-seq	Human	Cervical cancer cell (HeLa)	3.4	26

1 to 24 h for a 0.1 or 0.2 mM EU dose. This instructions state that Jurkat cells were pulsed with EU for 24 h, after which the medium was replaced with growth medium without EU. Then, total RNA was isolated and subjected to nascent RNA capture using the Click-iT Nascent RNA Capture Kit. RT-qPCR analysis was performed and the RNA stabilities of several RNAs were determined. Ideue et al. also reported that several histone mRNAs stabilities were determined by measuring the half-life of EU pulse-labeled histone mRNAs.[48] HeLa cells were incubated with 0.5 mM EU for 30 min, and total RNAs were isolated from cells at sequential time points after removal of surplus EU from the culture medium. EU-labeled RNAs were biotinylated and captured using the Click-iT Nascent RNA Capture Kit, followed by RT-qPCR analysis.

EU has been shown to be non-toxic to the cells, as evidenced by propidium iodide/Annexin staining and it does not affect the global transcriptome of the cell.[47] However, prolonged culture with EU causes inhibition of cell growth.[34] Moreover, the EU-labeled RNA can be used for DNA microarrays or deep sequencing on a genome-wide scale; however, this method has been mainly used for RT-qPCR, so far.

Pulse-Labeling by 5'-Bromo-Uridine (BrU)

In 1959, BrU was first used to label endogenous RNAs in mammalian cells.[49] During the next 50 years, BrU was widely used for immunocytochemical detection of nascent RNA in a broad range of cell types to determine the number of RNA polymerases that are active at any moment, the number of transcription sites and the number of polymerases associated with one transcription unit.[50-58] An anti-bromodeoxyuridine (BrdU) antibody was used to immunoprecipitate this BrU-labeled nascent RNA because the antibody recognizes both BrdU and BrU. In 2008, Ohtsu et al. reported an integrated method that combined immunopurification of anti-BrdU antibody-coated magnetically separated BrU-RNAs from total RNA with a DNA microarray method for transcriptional profiling of nascent mRNAs.[59] They applied this method to mouse FM3A cells. Moreover, Core et al. reported an improved approach that combined nuclear run-on assays (NRO) using BrU-labeling of nascent RNA during the run-on step, with deep sequencing for mapping the position, amount and orientation of transcriptionally engaged RNA polymerases on a genome-wide scale.[60] They used this method, which is called global run-on sequencing (GRO-seq), on human IMR90 cells.

Recently, BrU was used for pulse labeling endogenous transcripts by adding it to cell culture media as non-disruptive technology for directly measuring RNA decay (**Fig. 2E and F**).[34] BrU is incorporated into cells, which converted it to BrUTP, similarly to 4sU. BrUTP is recognized as the same substrate as UTP; therefore, nascent RNA is labeled by BrU. Total RNAs are isolated from cells at sequential time points after removal of surplus BrU from the culture medium, and BrU-labeled RNAs (BrU-RNAs) are recovered by immunopurification. Generally, BrU (Catalog no. 320–34741, Wako), anti-Bromodeoxyuridine (Clone. 2B1, Catalog no. MI-11–3, MBL), and Protein G Sepharose [In our experience, Protein G Immobilized Protein G (Catalog no. 20399, Thermo Scientific) is better than Protein G Sepharose 4 FF (GE Healthcare)] are used for immunopurification of BrU-labeled RNA.[34] The ongoing decrease in BrU-RNA levels over time is measured directly using deep sequencing. The half-lives of RNAs are determined by calculating the time when the RNA-seq value reached half of the initial RNA-seq value. This method is called 5'-bromo-uridine immunoprecipitation chase-deep sequencing analysis (BRIC-seq). The isolated BrU-RNAs can be used as templates for reverse transcriptase-mediated cDNA synthesis for subsequent quantification of RNA, because the BrU-RNA template does not cause misincorporation by reverse transcriptase.[60]

By contrast, 4sU permits base-paring with guanine instead of adenine, which causes base-changes in the RNA sequence. This is a serious disadvantage for determination of RNA abundance based on RNA-sequencing. Moreover, unlike ActD treatment, BrU does not alter RNA localization of long ncRNAs.[34] Thus, BrU is also a suitable uridine analog for labeling RNAs in the monitoring of RNA degradation under physiological conditions. BrU does not cause harmful effects compared with other uridine analogs, such as 4sU and EU; however, BrU-labeling takes 24 h for effective incorporation,[34] as compared with 4sU-labeling, which takes about 1 h.[22,44]

The median mRNA and ncRNA half-lives in HeLa cells were 3.4 and 3.4 h, respectively, as estimated by BRIC-seq.[34] We found that the relative distribution of ncRNA half-lives was similar to that of mRNA half-lives, suggesting that the stabilities of ncRNAs are regulated, as is the case with mRNAs.

Relationship Between RNA Stability and Physiological Function

Genome-wide technologies for investigating mRNA stability have revealed that the specific half-life of each mRNA is closely related to its physiological function.[22,27,34,45] Genes with short-lived mRNAs are significantly enriched among regulatory genes, which encode proteins that are required for only a limited time in the cell, such as transcription factors, signaling genes, chromatin modifying enzymes and cell cycle regulators. Conversely, genes with long-lived mRNAs are enriched among housekeeping genes, such as those involved in translation, respiration, energy metabolism and protein degradation. Recently, two independent research groups reported that non-coding RNA (ncRNA) half-lives vary over a wide range that is comparable with that of mRNAs. ncRNAs with short half-lives included known regulatory ncRNAs, while those with long half-lives contain a significant proportion of ncRNAs involved in housekeeping functions.[28,34] These results suggest that the half-lives of ncRNAs is indicative of functionality. The median mRNA half-lives in *E. coli* and *S. cerevisiae* are in the range of 5–21 min;[61,62] however, the median mRNA half-lives in mouse and human cells are reported to be 5–10 h (**Table 1**).[27–29] The half-life of the mRNA pool of a cell is roughly in proportion to the length of its cell cycle.

Concluding Remarks

Genome-wide technology for determining RNA stability has been employed to examine the response of mouse fibroblasts to type I and II interferons (IFN)[44] or the response of mouse dendritic cells to lipopolysaccharide (LPS).[22] Dölken et al. identified a previously undisclosed, highly connected network of short-lived transcripts that are selectively downregulated by IFN, between 30 and 60 min after IFN treatment, which showed strong associations with cell cycle and apoptosis. Rabani et al. found that changes in transcription rates determine the majority of temporal changes in RNA levels, but that changes in degradation rates are important for shaping sharp 'peaked' responses to LPS.[22]

We believe that the genome-wide technology for determining RNA stability, which is becoming more sensitive, represents an increasingly important facet in the characterization of the regulatory circuitry of biological systems in mammalian cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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