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Review

Impact of Methods on the Measurement of mRNA Turnover

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Abstract: The turnover of the RNA molecules is determined by the rates of transcription and RNA degradation. Several methods have been developed to study mRNA turnover since the beginnings of molecular biology. Here we summarize the main methods to measure RNA half-life: transcription inhibition, gene control and metabolic labelling. These methods were used to detect the cellular activity of the mRNAs degradation machinery, including the exo-ribonuclease Xrn1 and the exosome. Less progress has been made in the study of the differential stability of mature RNAs because the different methods have often yielded inconsistent results so that an mRNA considered to be stable can be classified as unstable by another method. Recent advances in the systematic comparison of different method variants in yeast have permitted the identification of the least invasive methodologies that reflect half-lives the most faithfully, which is expected to open the way for a consistent quantitative analysis of the determinants of mRNA stability.

Keywords: posttranscriptional regulation; *Saccharomyces cerevisiae*; nonsense mediated decay; NMD; splicing; 4-thiouracil; 4sU; rpb1-1; exponential decay

1. Introduction

mRNA turnover is determined by the rates of mRNA synthesis and degradation, which jointly adjust the level of gene expression. mRNA half-lives have been measured since the inceptions of molecular biology [1-3]. Three main classes of methods have been available to study mRNA degradation rates: transcriptional inhibition, gene control and metabolic *in vivo* labelling [4] (Figure 1). Transcriptional inhibition and *in vivo* labelling have been intensively used for the past decades for genome-wide measurement of mRNA half-lives. Despite the long history of mRNA half-life measurements, recent studies have revealed that different methods used to measure mRNA half-life often yield inconsistent data [5-7]. Here, we will discuss the advantages and critical points of the employed methods and how they can be compared and optimized.

The degradation of mRNAs occurs largely in the cytoplasm and begins with the removal of the poly(A) tail. There are two major pathways of the subsequent degradation [8]. The mRNAs are processed either by the Xrn1p-mediated 5′ to 3′ degradation pathway after decapping or by the exosome (3′ to 5′) without decapping [9]. The molecular mechanisms have been already extensively reviewed. Therefore, we will discuss in this review identification of the main components of the degradation machinery from a historical-methodological perspective, focusing on yeast.

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2. Methods for the measurement of RNA degradation rates

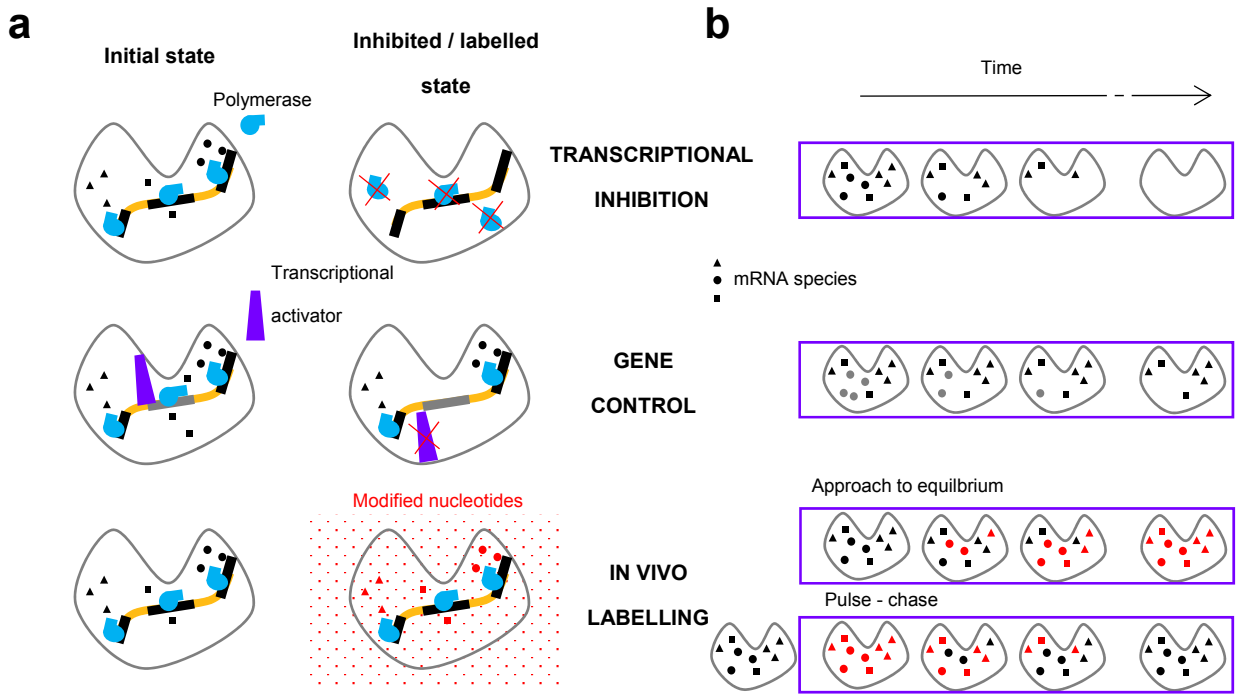

Figure 1. Main classes of methods to study mRNA stability. (a) Scheme of the molecular mechanism employed by the specific methods. In transcriptional inhibition, the RNA polymerase is inactivated reducing the expression of all genes. In the gene control method, a transcriptional activator dissociates from a promoter, shutting off the expression of the specific gene under the control of this promoter. For labelling of the RNAs, modified nucleotides introduced to the cell (red dots), which are incorporated into the RNA. (b) Time course of experiment to determine RNA half-lives. Inhibition of transcription of the gene(s) is triggered at t = 0 in transcriptional inhibition and gene control methods. The decline of the levels of the RNA(s) is then followed. For the in vivo labelling, there are two subclasses of measurements. In the approach to equilibrium method, a pulse of modified nucleotides is applied and the increase of the labelled mRNA is monitored. In the pulse-chase method, the mRNA is first labeled (pulse period). During the chase period starting at $t = 0$, the labeled nucleotides are washed and replaced with unlabeled nucleotides and the decline of the labelled mRNA is monitored.

2.1. In vivo metabolic labelling

Common to the variants of this method is that modified nucleotides are introduced into the cells to label the mRNA (Figure 2A, bottom panel). The mRNA half-life can be deduced by quantifying the rate at which the labelled RNA increases or declines after the introduction or removal of the labelled nucleotides, respectively (Figure 2b, bottom panel). The mRNA half-life can be deduced by quantifying the rate at which the labelled RNA increases or declines after the introduction or removal of the labelled nucleotides, respectively (Figure 2B, bottom panel). The labelling chemistry has changed over the five decades of the method's employment. Initially, radioactively labelled nucleotides were used, especially [^{3}H]-adenine and [^{32}P]-phosphate [1,3].

After these initial studies, the radioactive labelling has been less and less frequently used for several reasons. First, it cannot be scaled up to high-throughput measurements. Second, radioactivity may elicit cellular damage, including DNA double-strand breaks [10], and may trigger cellular signaling that alters transcription and mRNA stability. Third, the invention of qPCR and high-throughput RNA detection technologies paved the way for the spread of non-radioactively modified nucleotides so that the labelled RNA can be separated from the total RNA to be quantified. If the RNA contains bromouracil (BrU), the separation is performed by immunoprecipitation. If the RNAs

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incorporates 4-thiouracil, it can be biotinylated followed by binding to streptavidin beads [11,12]. The biotinylation-based separation has prevailed recently.

Interestingly, the uptake of 4-thiouracil and 4-thiouridin differs in mammalian and yeast cells. Uridine is a nucleoside containing uracil attached to a ribose. In yeast, only uracil and not uridine is imported into the cell efficiently. The uracil is then converted by the pyrimidine salvage pathway enzyme, the uracil phosphoribosyltransferase (*FUR1*) to uridine monophosphate, UMP [13]. In contrast, both compounds are transported into a mammalian cell but only uridine is incorporated into the RNA because the above salvage pathway is inactive [14]. For this reason, 4-thiouridine is mostly used in mammalian cells [11,12] and 4-thiouracil is used in yeast cells [15]. Since studies in mammalian cells preceded those in yeast cells, 4-thiouridine was attempted to be used also in yeast cells: to enable the uptake of 4-thiouridine (4sU) in yeast cells, the human equilibriative nucleoside transporter (hENT1) was expressed [16]. At the same, it was shown that the use of 4-thiouracil (4TU) permits a simpler method in yeast cells [15].

Each nucleobase derivative has different impact on cell physiology. Bromouridine has been shown to be less toxic than 4-thiouridine in mammalian cells [17]. At elevated concentrations of 4-thiouridine (> 50 μM), which are usually used for mRNA labeling experiments, the production and processing of rRNA is inhibited [18]. Thus, *in vivo* labeling can trigger a nucleolar stress response, which can interfere with the RNA stability measurements.

The RNA half-lives can be determined by pulse-chase or by approach to equilibrium (Box 1). When the approach to equilibrium is followed, the increase of the labelled RNA upon addition of the modified nucleotides (pulse) is monitored. The rate of increase in the labelled RNA depends on the degradation rate [2], and not on the synthesis rate. In the pulse-chase method, a pulse of labelled nucleotide is added to the cells. In the subsequent chase period, the cells are washed with media containing unlabeled nucleotides and the decline of the labelled RNA is monitored as in the classical decay experiments.

It is important to note that the varying the duration of the pulse permits focusing on different time-scales [19]. Short pulses are particularly suitable to study fast processes, such as RNA splicing.

2.2. Transcriptional inhibitors

When RNA expression is inhibited, all mRNAs start to decay and by quantifying their change over time, their half-lives can be determined. In the earlier experiments, RNA expression was specifically inhibited only in the cytoplasm by blocking the export of RNAs into the cytoplasm; subsequently transcription was inhibited to block the expression of RNAs completely. The inhibition of RNA expression can be achieved by small-molecule inhibitors or by creating temperature-sensitive alleles. The *rna1* was one of the earliest examples of temperature-sensitive alleles that was used to determine mRNA half-lives [20]. The *RNA1* gene encodes a RanGAP, which generates the nucleocytoplasmic RanGTP gradient to drive the nucleocytoplasmic transport. Its inactivation causes a collapse of the gradient and transport [21]. By inhibiting RNA export, the level of cytoplasmic RNA and polyribosomes declines. Thus, the polyribosome fraction or instantly synthesized proteins can be measured to infer the amount of cytoplasmic mRNAs [22]. Later, inhibition of the polymerase became the most widely used technique, which was facilitated by the isolation of the *rpb1-1* allele. The standard name of *RPB1* is *RPO21* in budding yeast and it encodes the largest subunit of the RNA polymerase II [23].

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Transcriptional inhibition can be used for genome-wide measurements of RNA decay, which contributed to the popularity of this approach, even though potential disadvantages have been known. The stepwise increase of temperature required for the thermal inactivation of the polymerase and the rapid loss of labile factors upon transcriptional inhibition may have pleiotropic effects on cell physiology. A study using metabolic labelling has revealed that the *rpb1-1* allele alters the mRNA stability even at permissive temperature [6].

Chemicals, such as 1,10-phenanthroline and thiolutin, have been also used to inhibit the RNA polymerases. They, too, have their disadvantages. 1,10-phenanthroline, a heterocyclic compound, inhibits a large number of enzymes in addition to the RNA polymerase, especially zinc metalloproteases [24]. The action of thiolutin, an antibiotic isolated from the *Streptomycetes luteosporeus*, strongly depends on the applied concentration and different mRNA half-lives were obtained at different concentrations [25]. To inhibit transcription in mammalian cells, mostly actinomycin D has been used, which is an antibiotic isolated from the *Streptomyces parvulus*.

Box 1. Fitting of parameters to determine RNA half-lives

Just like radioactive decay, the decay of mRNA molecules is typically described by single exponential process.

$$R(t) = R_0 e^{-kt} \quad (1)$$

R_0 and $R(t)$ denote the RNA level at the initial and subsequent time points, respectively, and the decay rate constant is k = Ln [2]/$t_{1/2}$.

When the initial level of the RNA is zero then the time to reach the steady-state (equilibrium) is determined also by the decay rate, and not - as often incorrectly assumed - the synthesis rate, p.

$$R(t) = \frac{p}{k}\left(1 - e^{-kt}\right) \quad (2)$$

Using nonlinear regression, the half-life in the above equation can be fitted to the time series of (labelled) RNA levels upon induction of gene expression or upon addition of modified nucleotides. The time to reach the half-saturation corresponds exactly to the half-life [1,2]. If the data are transformed with R = 1- R^*/ $R^{*(\infty)}$ [3], where $R^{*(\infty)}$ denotes the steady-state level of the labelled mRNA, then equation (2) is converted to equation (1).

Equation (1) is used to fit half-lives in transcriptional inhibition and gene control experiments, while both equations (1) and (2) can be used for in vivo labelling experiments.

2.3. Gene control

Gene control and transcriptional inhibition are related methods since gene expression is shut off, and the mRNA level starts to decline. The two methods differ with respect to the scale of inhibition. With transcriptional inhibition the expression of all genes is inhibited. On the other hand, gene (transcriptional) control is employed to shut off the expression of a single gene by placing a gene under the control of the regulatable promoters, such as the GAL or the TET promoter [4]. Despite the lesser probability of having side effect, the gene specific control has been rarely used because each experiment yields the half-life of a single mRNA only.

The advantage of the TET system is that it is of bacterial origin and thus orthogonal to the endogenous processes in eukaryotes. Doxycycline, which is often used to control the TET system, has no or minimal effect on the expression of the *S. cerevisiae* genome [26]. Doxycycline dissociates the tetracycline transactivator (tTA) activator from the promoter. It is important not to use a too strong promoter to express the tTA because high expression of the activator may cause cellular growth defects and major alterations in gene expression [27]. A moderately strong promoter (e. g. *CLN3*) generates sufficient expression of tTA without causing growth defects. Alternatively, a tetR-

repressor fusion protein can be recruited to *tet* operators inserted into promoters to shut off gene expression [7]. This strategy is even less invasive since the original promoter sequence is retained upon the insertion but it not all repressors have fast repression kinetics and not all promoters can be efficiently repressed.

The TET system can be also used in also mammalian cells [28]. Pre-exposure of cells to low concentration of doxycycline was shown to improve the decay kinetics, possibly because the reduced expression of some genes eliminated the side effects. Thus, the system may require prior optimization. Since most mammalian genes are very long and contains multiple introns, the cloning of mammalian genes into plasmids is limited technically, which can be a serious impediment to implement gene-specific shut-off of transcription. The cloning of the full length gene is desirable as it has been known that mRNA processing, including splicing is strongly dependent on the chromatin state [29].

The *GAL* promoters in yeast are activated in the presence of galactose and are repressed by glucose. When cells are grown in glycerol or the neutral sugar raffinose, the GAL promoters are inactive. The expression of most genes in the inactive state is similar to or slightly higher than the expression in the repressed state [30]. To shut off transcription driven by the GAL promoters in cells grown in galactose, most commonly glucose is added at high concentration [3]. It is important to note that glucose triggers a signal that can transiently increase the decay rate of some mRNAs [30]. Therefore, the decay rate may not reflect the steady-state turnover for these mRNAs. To avoid the transient signaling due to the shift from galactose glucose, galactose can be washed out and replaced by the neutral sugar, raffinose [7], which results in half-lives similar to those obtained with the TET system. In this case, it is recommended to use lower galactose concentration for induction to expedite its transport and removal from the cell [31]. In fact, the high concentrations (2%), used by most studies, are not needed because the GAL genes can be already induced by galactose at as low concentration as 0.05% [30].

2.4. Additional methods

The above three major classes of methods yield RNA half-lives directly. There are also methods that can be used to estimate half-lives indirectly. For example, the half-life can be calculated when the RNA synthesis rate constant and the mRNA concentration are known. The mRNA synthesis rates can be measured by genomic run-on experiments by stopping transcription and by resuming it in the presence of labelled nucleotides so that the nascent transcripts are extended [32].

In principle, it is also possible to deduce mRNA half-lives from steady-state expression of RNAs measured in single cells, provided the regulating transcription factor undergoes large-amplitude nucleocytoplasmic oscillations. [33]. Upon export of a transcription factor to the cytoplasm, the decline of mRNA can be observed in single cells and the RNA half-life can be in principle estimated. Such singe cell observations require the insertion of stem-loops into the RNA. Since stem-loops can affect RNA processing [34], the mRNA stability has to be assessed before and after insertion of stem-loops.

3. Comparison of the average mRNA half-lives

Since the beginnings of the studies on mRNA stability, arguments have been explicitly formulated that each method can affect the measured half-lives. Therefore, mRNA half-lives obtained by different methods have been compared. Two measures have been used for the comparison: the average half-life and the correlation of half-lives obtained by these methods.

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There are two common measures of the average: the mean and the median. The mean half-life has been mostly reported in the earlier studies, while the median in the later ones. The median is typically larger than the mean because the distribution of the half-lives is often skewed to longer half-lives. The overwhelming majority of the studies yielded average mRNA half-lives between 10 and 25 minutes, with both in vivo labelling and transcriptional inhibition [7,22,35]. A recent study reported a median half-life of around 2 minutes, representing a substantial deviation from the above range [7].

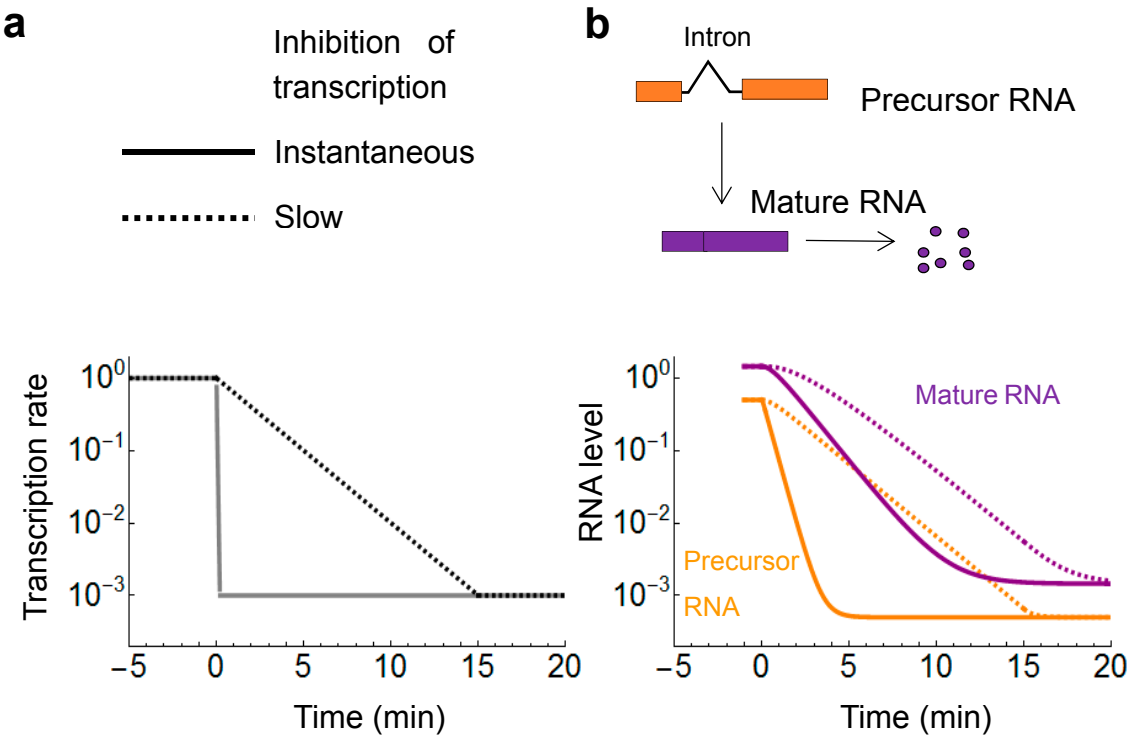

Figure 2. Impact of the speed of transcriptional inhibition on the measured mRNA half-lives. (a) Two different scenarios are shown for the inhibition of transcription. In the ideal case, the inhibition occurs instantaneously down to a baseline level (full line). In suboptimal cases, inhibition of transcription ensues with a slow kinetics. (b) The decay of RNA upon instantaneous (full lines) and slow (dashed lines) inhibition of transcription. The precursor mRNA, containing the intron, can be used to monitor the inhibition kinetics since the precursor is converted rapidly to mature mRNA by splicing. The half-life of the mature mRNA (purple) will appear much longer with the slow inhibition kinetics and there is also a longer lag period before the mRNA level starts to decline.

What may explain such considerable differences in the measured averages? In an ideal case, the inhibition transcription or the incorporation of labelled nucleotides occurs instantaneously after starting the experiment. In practice, these processes occur after a lag period or the change is not instantaneous but has a slower than expected kinetics. To estimate the lag time due to the incorporation of nucleotides, dual labelling experiments have been performed, which yielded an estimate of around 5 minutes in yeast [1].

A simple exponential model of decay assumes that the inhibition of transcription is instantaneous or occurs very rapidly. This may or may not be true and should be verified. It is not easy to monitor directly the change in the declining transcription because is faster than the change in decaying RNA levels. The level of the un-spliced precursor mRNA provides a good proxy for the precipitously changing transcription rate because splicing of the precursor mRNA is typically faster than the RNA decay (Figure 2). For the monitoring, RNAs have to be selected that undergo a fast, efficient splicing, which can be inferred from the ratio of the mature to the precursor RNA or related measures. The larger the mature-to-precursor ratio, the faster the splicing. If the mature-to-precursor ratio is 100

then the precursor amount should decline 100 times faster than the precursor mRNA. At lower splicing rate the formula has a correction terms that takes into account the escape of unspliced precursor mRNA to the cytoplasm [36,37]. Despite the importance of this control experiment, relatively few studies have reported the half-time of the precursor RNA [38,39]. The reported values range between 0.5 and 1 min, which is likely to be the lower limit of the half-times that can be reliably detected. If the experimental set-up is not optimal and the precursor declines slower, the experimental conditions can be modified to accelerate the transcriptional inhibition. For example, thermal inactivation of the polymerase at 39°C instead of 37°C accelerates the inhibition [39].

4. Correlation as a measure of method reliability

The similarity of the average half-lives measured by the majority of the studies may have led to the belief that the methods are consistent. However, recent studies assessed the correlation between the half-lives measured for a large number of mRNAs by these methods, which turned out to be surprisingly low. This means that different methods classify different mRNAs as stable and unstable.

There are two types of correlation coefficients: Pearson's product-moment and Spearman's rank correlation coefficient. When the distribution of the half-lives is not Gaussian but skewed then rather Spearman's rank correlation should be used. The rank correlation coefficient is less sensitive to outliers. The higher the correlation between two methods, the more likely that the same mRNAs will be classified as stable or unstable.

There is no uniform way to delimit the lower acceptable value of correlation. Generally, values between 0.3 and 0.5, and between 0.5 and 0.7 reflect low and moderate correlations, respectively. Values above 0.7 can be considered high correlation. To have a rule of thumb, it is instructive to consider a field in molecular biology where correlation is used to assess the robustness of predictions. For example, correlations have been used to predict biological age from unknown tissue samples based on epigenetic markers. Methylation of CG dinucleotides (CpG), a classical epigenetic mark, increases with age. Accordingly, the CpG methylation of appropriately selected promoters can be used to predict age based on biological samples. The correlation between total CpG methylation percentage of the genes and age can be then used to predict age from biological samples. Different gene selections yield different values for the correlations and thus have different predictive power. Some selections yield correlation coefficients of 0.7, while others surpass even 0.9, which is equivalent to a prediction error of ±3 years above 20 years of age [40,41]. Thus, a correlation above 0.7 can be expected for method to be of sufficient reliability to determine RNA half-lives faithfully and to identify RNA stability sequences.

5. Inter-method reliability: from simplicity to perplexity

In one of the first comparative studies, the half-lives of 11 mRNAs were measured with temperature sensitive polymerase (*rpb1-1*) and in vivo labelling with [^{32}P]-phosphate [3]. The two methods yielded similar decay rate constants for around half of the RNA species but 4 mRNAs displayed marked discrepancies. The authors analyzed also the poly(A)+ mRNA, and suggested that the kinetics of deadenylation may account for the discrepancy.

This study was followed by the first genome-wide comparison of two *rpb1-1* studies [5], which examined the consistency of a method. The internal consistency of a specific method can be assessed by calculating the correlation between two studies using the same method variant. The authors found that the correlation was nearly 3 times higher for highly expressed genes (R=0.48 at the 90th percentile) than for weakly expressed genes (R=0.15 at the 10th percentile), which may imply that weak signal intensity may introduce considerable measurement error.

A more recent study has reported that two variants of the metabolic labelling, employing different pulsing protocols, yielded uncorrelated half-lives [6]. The comparison of five different studies using the same variant of the same method (*rpb1-1* allele of the polymerase) indicated a low reproducibility of the method the Spearman's rank correlation coefficient between five different studies ranges from 0.28 to 0.77 [6]. These values are quite low and indicate a low reproducibility of

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the method. Similarly, there was no correlation between the transcriptional inhibition and metabolic labelling studies.

To explain the discrepancy between the transcriptional inhibition and in vivo labelling, the study using a variant of the metabolic labelling, the cDTA, found that the *rpb1-1* allele increases mRNA stability even at permissive temperature [42]. Similar stabilization was found with gene control measurements when decay was measured in cells with mutant polymerases with reduced elongation rates [43]. Furthermore, there may be a global interdependence between the transcription and degradation machinery [7,44].

It is somewhat surprising that there is no correlation between two variants of the metabolic labelling [6]. What may explain this discrepancy? This inconsistency cannot be explained unequivocally because the applied protocols differ at multiple points. The study by Munchel et al employs the pulse-chase approach, the RNA is labelled with low nucleotide concentration (0.2 mM TU for 3 hours in a log-phase culture) and the RNA is quantified by high-throughput sequencing. On the other hand, cDTA uses the approach to equilibrium method after introducing high concentrations of nucleotides (5 mM TU for 6 minutes in a log-phase culture), and the RNA was quantified by microarray.

At the same time, it has been difficult to appreciate the validity of conclusions of the cDTA study concerning the mRNA stabilization due to transcriptional inhibition, because it did not correlate with the other variant of the metabolic labelling [6].

This conundrum was in part resolved by a recent study using a multiplexed version of the gene control method using the TET system, which yielded half-lives with a high correlation to one version of the metabolic labelling, the cDTA (rank correlation coefficient of 0.77) [7]. This high correlation can be particularly appreciated from the perspective that the two studies are methodologically independent, since they introduce different perturbations into the cells. Despite the large positive correlation between the gene control and the cDTA variant of metabolic labelling there is a significant difference in the median half-lives. The five times longer half-life in the cDTA study may be explained by the slow incorporation of the labelled nucleotides into the RNA. The cellular uptake of uracil is subject to a complex regulatory circuit: the uracil inhibits its own import by the uracil permease *FUR4.* The half-life of the *FUR4* RNA and protein is reduced upon exposure to uracil [45]. Thus, the high concentration pulse of labelled nucleotides may slow down the uptake and incorporation of labelled uracil.

The methods applied to measure the half-life can perturb the measured cell, and each method can have a differential effect on the stability of individual mRNAs. Therefore, it is possible that a sequence motif identified with a particular method reflects rather the interaction of perturbation with the cell and not the original half-life. Thus, using two independent methods or cross-validated methods can ensure that a motif identified reflects the parameter to be measured. This is particularly important because RNA decay may be determined not only by simple sequence motifs but also by RNA secondary structure [46] and by the translation efficiency of the mRNA [47].

6. Comparison of half-lives in mammalian cells

A systematic comparison of mRNA stabilities in mammalian cells is hampered by the large variety of cell lines and differentiation states. A study using global transcriptional inhibition with actinomycin D reported a median mRNA half-life of 10 h [48]. Two studies used 4sU labeling in the same cell line (NIH3T3) [11,49]. The correlation between the two data set was moderately high (r = 0.59 to 0.64), having median half-lives of 7.6 and 4.6 h. A metabolic labelling study with 4sU labelling reported considerably shorter mRNA half-lives in dendritic cells, in the range between 10 and 70 minutes, depending on the type of parameter fitting [38]. Thus, cross-validation of methods will be important for mammalian cells too.

7. Identification of the RNA degradation machinery: Xrn1, exosome and the nonsense mediated decay

Next, we will review the identification of the major components of the mRNA degradation machinery from a historical-methodological perspective. We address the Xrn1, the exosome and the mediators of the nonsense mediated decay.

The Xrn1 protein was identified based on its biochemical activity, a 5′-to-3′ ribonuclease activity on uncapped mRNA. Capped mRNAs were shown to be quite resistant to degradation by the purified Xrn1 [50]. Upon identifying the encoding gene, it was shown to be a non-essential gene but its deletion causes a marked growth defect [51]. To link the *in vivo* activity of Xrn1 to mRNA decay, a gene control experiments was performed. The GAL promoter was used to study how *XRN1* affects the half-life of the unstable *MFA2* and stable *PGK1* mRNAs [52]. Soon afterward, the *in vivo* involvement of Xrn1 in decay was confirmed by a *rpb1-1* study, showing that the glucose-dependent stability of an mRNA is Xrn1-dependent [53]. Xrn1 also controls the levels of sense-antisense mRNA pairs, which can arise as a result of convergent gene expression [54].

Little is known about the regulation of Xrn1; subcellular sequestration of Xrn1 may play an important role. The protein is diffusely distributed in the cells in exponentially growing cultures but becomes localized to the cytoplasmic P-bodies at the diauxic shift. Subsequently, at the post-diauxic stage, Xrn1 is localized to the plasma membrane, which prevents mRNA degradation, as shown by the reduced decay of the MFα2 mRNA using a TET-based gene control method [55].

Xrn1 is primarily an exoribonuclease but has other functions in the cell, which are often denoted by different aliases. Of practical importance is the *KEM1* alias, which refers to Kar-Enhancing Mutations: haploid cells with Xrn1 deletion have a very low mating efficiency due to a defective karyogamy [56]. Indeed, the defective fusion of two nuclei makes the construction of xrn1 null diploid cells difficult. It has been shown that the Xrn1p undergoes rapid evolution, as evidenced by the large sequence divergence of *XRN1* among related yeast species [57]. This may in part underlie the multiple functions associated with Xrn1p.

The main component of the second pathway of mRNA decay is the exosome, which is a multi-protein complex with 3′-to-5′ ribonuclease activity [58]. The exosome has also a nuclear function It was isolated and identified by mass-spectrometry [59]. It is more difficult to study the effect of the exosome because the deletion of its components is lethal. It is however possible to delete the SKI genes; the Ski proteins bridge the exosome and the RNA. The in vivo activity of the exosome was studied by how the inactivation of the decapping protein Dcp1, which is a prerequisite for the 3′-to-5′ decay to be initiated, and Ski8 deletion interact [60]. Individual deletion of each of the genes had little effect on the decay rates of the *GAL* and *MFA2* RNAs as studied by the gene controlled method, using the *GAL* promoter. However, when both of these genes were deleted the half-life of the mRNAs increased up to 6 – 7 times.

The above examples indicate that having stable and unstable mRNAs, *MFA2* and *PGK1*, was instrumental in the identification of the components of the RNA degradation machinery. Yet, very few stability sequence motifs were identified that could change the stability of mRNAs when transferred to other mRNAs. A simple sequence that consistently changes the half-life of the mRNAs is the premature stop codon [61-63]. Using radioactive labelling, it has been shown that the premature stop codons – in a position dependent manner - alter the stability of mRNAs without affecting the transcription rate [62]. This phenomenon known as nonsense mediated decay (NMD) was shown to be mediated by the Upf1p, as studied with transcriptional inhibition using the *rpb1-1* mutant [61]. The closer the stop codon to the 5′end of the stable PGK1 mRNAs was, the more strongly the mRNA got destabilized [63]. The NMD was also confirmed with gene control experiments [36]. A recent study revealed that NMD affects many mRNAs that lack premature termination codons but are prone to out-of-frame translation [64].

8. Conclusions

In vivo labelling and inhibition of RNA expression have been used for at least four decades to measure RNA half-lives. Surprisingly, only recent studies unveiled that the two methods yield half-

lives with no or minimal correlation. A new study showed that there is a high correlation between specific variants of the *in vivo* metabolic labelling and gene control methods. The consistency between two independent methods indicates high inter-method reliability. Thus, some variants of the genetic control and metabolic labelling may reflect the half-lives the most faithfully. However, there is no guarantee that using a method class, let it be metabolic labelling or gene control, will deliver the half-lives faithfully. If the detailed conditions are not optimal, such as the concentration of the labelled nucleotide or the expression level of the activator that controls the gene, the measured half-lives and RNA synthesis rates may be inconsistent.

The most robust methods are expected to have played a major role in the identification of the molecular machinery of mRNA degradation. *In vitro* biochemical studies were often first steps, followed by gene control studies. *In vivo* radioactive labelling played also an important role in the discovery of the degradation pathways involving Xrn1 and the exosome. The nonsense mediated decay has been reproduced by all three major methods. Thus, all methods capture at least some forms of the RNA decay, but current evidence indicates that specific variants of the gene control and metabolic labelling methods can be used reliably to study the differential stability of mature mRNAs. We expect that the conformation of mRNA stabilities by two different methodologies and further developments in the assessment of inter-method reliability will help to identify mRNA stability determinants.

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