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Engineered Viral RNA Decay Intermediates to Assess XRN1-mediated decay

Joseph Russo¹, Cary T. Mundell^{1,2}, Phillida A. Charley¹, Carol Wilusz^{1,2}, and Jeffrey Wilusz^{1,2}

¹Department of Microbiology, Immunology and Pathology

²Program in Cell & Molecular Biology, Colorado State University, Fort Collins, CO 80525

Abstract

Both RNA synthesis and decay must be balanced within a cell to achieve proper gene expression. Additionally, modulation of RNA decay specifically offers the cell an opportunity to rapidly reshape the transcriptome in response to specific stimuli or cues. Therefore, it is critical to understand the underlying mechanisms through which RNA decay contribute to gene expression homeostasis. Cell-free reconstitution approaches have been used successfully to reveal mechanisms associated with numerous post-transcriptional RNA processes. Historically, it has been difficult to examine all aspects of RNA decay in such an *in vitro* setting due, in part, to limitations on the ability to resolve larger RNAs through denaturing polyacrylamide gels. Thus, *in vitro* systems to study RNA decay rely on smaller, less biologically relevant RNA fragments. Herein, we present an approach to more confidently examine RNA decay parameters of large mRNA size transcripts through the inclusion of an engineered XRN1-resistant reporter RNA (xrRNA). By placing a 67 nucleotide xrRNA near the 3' end of any *in vitro* transcribed RNA with variable size or sequence context, investigators can observe the accumulation of the xrRNA as a readout of exoribonuclease-mediated 5'–3' decay. This approach may allow *in vitro* RNA decay assays to include full biologically relevant mRNA/mRNPs, extending their utility and allow improved experimental design considerations to promote biologically relevant outcomes.

Keywords

RNA decay; RNA stability; XRN1

1. Introduction

1.1. Importance of mRNA decay in gene expression

The balance between transcription (synthesis) and the stability (decay) of mRNAs must be tightly controlled to respond appropriately to cellular conditions. In fact, changes in both

Correspondence: jeffrey.wilusz@colostate.edu.

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transcription rate and stability of an mRNA contribute to responses to internal/external cellular stimuli and cues [1,2]. Understanding the precise role and underlying nuances of the contribution of mRNA stability remain challenging. We also need to better understand regulation of mRNA stability in the context of the mRNP to better put into context work done on individual RNA binding proteins and RNA-protein interactions.

1.2. The major 5' to 3' pathway involved in mRNA decay

Currently, it is appreciated that eukaryotic mRNAs are most commonly subjected to deadenylation and decapping-dependent 5' to 3' exonucleolytic decay. Once an mRNA is triggered for decay, shortening of the poly(A) tail commences which is considered a key rate-limiting step [3–6]. Several groups have identified mRNA deadenylases/deadenylase complexes including the CCR4-NOT complex [7,8], CAF1/POP2 [7–9], the PAN2/3 complex [10,11] and PARN [12,13]. After the poly(A) tail is shortened, removal of the protective 5' cap structure proceeds which then exposes the 5' end of an mRNA to cellular exoribonucleases [14,15]. Removal of the 5' cap is mediated in most cases by the decapping holoenzyme DCP1/2 [16] followed by XRN1-mediated 5' to 3' exonucleolytic decay [15,17,18].

Although we have many basic principles of mRNA decay understood, we still lack biochemical tools to fully tease out the how RNA sequences and structures in their biologically relevant context, as well as the plethora of associated RNA binding proteins that form the mRNP, contribute to these processes. The network of factors that coordinate the steps of mRNA decay are vast and highly compensatory/redundant in cell-based systems. Additionally, many factors have multiple roles including in other steps of RNA processing making knockdown/knockout studies even more difficult to interpret due to indirect/direct effects. Therefore, it has been difficult to understand the contribution of an individual factor(s) in the context of the *cell/in vivo*.

Understanding how RNA sequence and structural context impacts mRNA decay is critical. The RNA sequence itself may have the ability to modulate decay factor activity (e.g. exoribonuclease rate) in *cis*, independent of *trans*-acting RNA accessory factors. An example of this is the discovery of XRN1-resistant (xrRNA) structures encoded in flaviviral 3'UTRs that stall and repress XRN1 activity [19–21]. In addition to RNA sequence/structure, *trans*-acting factors (e.g. RNA binding proteins (RBPs) or small RNA regulators like miRNAs) that bind to a particular RNA sequence may dictate decay factor assembly or activity.

1.3. The Ultimate Goal: Reconstitution of biological systems to study mRNA decay

Reconstitution of biological systems *in vitro* have made important contributions to our understanding of many basic principles in mRNA decay in mammalian cells. Pioneering work by the Brewer lab [22], our laboratory [23] and others recapitulated several aspects of regulated mRNA decay using cytoplasmic extracts. More recently, elegant reconstitution of specific RNA decay processes such as deadenylation [24,25], P body formation [26], and exonucleolytic decay [27,28] have provided additional mechanistic insights. To fully understand the nuances of regulated mRNA decay, it would of course be optimal to develop

a fully reconstituted system that includes *bona fide* mRNP substrates. We propose that the approach presented herein is a step towards this lofty goal.

1.4. Major limitations exist with current *in vitro* RNA decay assays

A major limitation of current *in vitro* systems designed to study RNA decay kinetics is the use of small RNA fragments as substrates in order to maintain a “high level” resolution using denaturing PAGE. Therefore, studying large RNAs such as mammalian cellular mRNAs, that average over 2,700 bases, can be nearly impossible to interpret. In fact, in most systems, the RNA substrate, has been limited to specific size or context depending on the decay enzyme being studied [29–31,31]. Herein, we have developed an RNA reporter-type readout that allows the investigator to interrogate RNAs in their more relevant biological context (e.g. an entire transcript, isolated full size open reading frame, or full sized UTRs). As outlined below, by placing an engineered 67 nucleotide long xrRNA derived from the Dengue type 2 virus 3' UTR near the 3' end of any RNA substrate, the accumulation of this decay intermediate can be used as a very useful readout for decay of the RNA body. To demonstrate the feasibility of this approach, herein we outline the use of an open reading frame or 3' UTR of an mRNA can be engineered to contain a terminal reporter xrRNA fragment in reconstituted XRN1 decay assays to glean mechanistic perspectives on the 5' to 3' RNA decay pathway.

2. Materials and Methods

2.1. Overview

A basic *in vitro* RNA decay system requires an RNA substrate, enzyme(s) to be studied, and a buffer. Using this basic principle, we have designed an *in vitro* RNA decay system to examine 5' to 3' mediated decay of large RNA substrates. In short, the large 994 nucleotide RNA substrate (containing a reporter xrRNA fragment at the 3' end) is incubated with recombinant XRN1 (rXRN1) over a time course. The decay of the substrate and the accumulation of the xrRNA fragment are monitored via denaturing PAGE. Importantly, these studies are not limited to XRN1-mediated decay. Other 5'–3' exonucleases such as XRN2 could be examined, or other rate-limiting steps of RNA decay, including decapping and deadenylation, could be incorporated as prerequisite steps to exonucleolytic decay. In fact, recently Webster et al describe a method to purify the multicomponent deadenylation machinery for use in *in vitro* settings [29,32]. We believe our system could be combined with this approach or similar cytoplasmic extract based approaches [23] to reveal mechanistic insights into the regulation of RNA decay of biologically relevant substrates.

2.2. Materials and reaction tables

Template for *in vitro* transcription

- 1) Generated as a double-stranded DNA from PCR of cDNA
- 2) Designed as a gene-block (as described herein)

Custom primers for template generation—Fw Primer-

GATCATCGAATTTAGGTGACACTATAG + 20-30nt RNA-specific forward (underlined sequence is an SP6 bacteriophage promoter)

Rv Primer-

GGTGGAGCTACAGGCTGCACAGTTTACTCAAGCTGTGGCATTGTGATGGCCTGA
CTTCTTT + 20-30nt RNA-specific reverse complement

PCR reagents: PfuUltra II fusion HS DNA polymerase (Agilent, 600670)

dNTPs for PCR:

dATP (Thermo Fisher, R0141)

dCTP (Thermo Fisher, R0151)

dTTP (Thermo Fisher, R0171)

dGTP (Thermo Fisher, R0161)

Reaction table 1:

Standard PCR template reaction

PfuUltra II Fusion HS PCR Reaction Mix	Volume
Distilled Water	40µl
10× PfuUltra II reaction buffer	5.0µl
dNTPs (10mM each dNTP)	1µl
DNA Template (cDNA) 200ng/µl	1µl
Fw Primer (10µM) (see above)	1µl
Rv Primer (10µM) (see above)	1µl
PfuUltra II fusion HS DNA Polymerase	1µl
Total reaction volume	50µl

Note:

- (1) adjust cycle parameters based on cDNA sequence to be amplified using manufacturer suggestions
- (2) gel purify (via electroelution or use a kit [e.g. Promega Wizard SV Gel and PCR Clean-Up System, A9281]) to prepare the PCR product for use as template during *in vitro* transcription reactions

***In vitro* transcription reagents:** rNTPs for *in vitro* transcription reactions:

rUTP (Thermo Fisher, R0471)

rATP (Thermo Fisher, R0441)

rCTP (Thermo Fisher, R0451)

rGTP (Thermo Fisher, R0461)

rGMP (Millipore Sigma, G8377)

rUTP, [α - 32 P] (Perkin Elmer, BLU007X250UC)

SP6 transcription components and reaction:

SP6 polymerase (Thermo Fisher, EP0131)

5 $\times$ transcription reaction buffer (Thermo Fisher, EP0131)

Ribolock (Thermo Fisher, EO0381)

Reaction table 2:

Standard *in vitro* RNA transcription reaction

Template DNA (gene block or generated from PCR)	1 μ l (50-200ng)
SP6 Transcription Buffer	2 μ l
rNTPs- (5mM rATP & rCTP, .5mM rGTP and rUTP), 5mM GMP	1 μ l
RiboLock RNase Inhibitor	0.5 μ l
rUTP [α - 32 P]	4.5 μ l
SP6 RNA Polymerase	1 μ l
Total Volume	10 μ l

Components required for denaturing gel electrophoresis: Urea (Thermo Fisher, u15-3)

Bis-Acrylamide (VWR, VWRV0172)

Acrylamide (Millipore Sigma, A8887)

TEMED (Millipore Sigma, T22500)

Ammonium persulfate (APS) (Millipore Sigma, 248614)

TRIS (Millipore Sigma, T6066)

EDTA Millipore Sigma, E4884)

Boric Acid (Millipore Sigma B0394)

Urea gel mix: 240g Urea

50mL 10 $\times$ TBE

200mL ddH₂O

5% Polyacrylamide gel (scale as needed): 26.25mL Urea gel mix

3.75mL 40% polyacrylamide solution (19:1, acrylamide:bis-acrylamide)

300 μ L 10% APS

30 μ L TEMED

Urea RNA loading dye: 12g Urea

0.185g EDTA

12.5 μ l 1M Tris-HCl pH-7.6

.006g xylene cyanol (Millipore Sigma X4126)

.006g bromophenol blue (Acros 115-39-9)

Raise volume to 25mL with ddH₂O

Elution of RNA substrate: HSCB buffer

400mM NaCl (Thermo Fisher, S271-3)

25mM Tris-HCl (pH 7.6)

0.1% (w:v) SDS (Thermo Fisher, BP166)

Phenol:Chloroform:Isoamyl-Alcohol—25:24:1, make as needed

Components required for XRN1 decay assay: Recombinant XRN1 (New England Biolabs, M0338L)

Buffer 3 (New England Biolabs, B7003S)

Final Concentrations for Reaction:

100 mM NaCl

50 mM Tris-HCl (pH 7.9@25°C)

10 mM MgCl₂

1 mM DTT

2.3. Experimental Design

In this section we will discuss the required considerations for optimal experimental design using our *in vitro* xrRNA reporter readout system to assess 5' to 3' exoribonuclease-mediated RNA decay.

2.3.1. Considerations for RNA substrates used in XRN1-mediated decay

assay—Studies by our group and others have suggested that RNA structure can influence the ability of XRN1 to decay a substrate [19,20,30,31,33]. Additionally, global RNA structure analyses suggest that mRNAs tend to be more structured in their UTRs compared to their coding sequences [34–36]. With these observations in mind and as a proof-of-concept we designed 4 gene blocks for use as templates for RNA transcription (see table 1, supplementary figure 1). Using human GAPDH as our core sequence, we sought to

understand the influence of variable RNA sequence on 5' to 3' XRN1-mediated decay rates. To this end, we examined four 994 nucleotide long RNAs possessing (1) the GAPDH coding sequence (CDS) (GAPDH CDS), (2) the 3' half of the GAPDH CDS combined with the entire GAPDH 3' UTR sequence (GAPDH CDS-3' UTR), (3) the GAPDH CDS containing an internal artificial strong stem-loop ($\Delta G = -73.6$ kcal/mol) (GAPDH CDS-SL), and the GAPDH CDS containing an internal minimal BNYVV xrRNA known to stall the exonuclease [37] (GAPDH CDS-BNYVV). The internal artificial stem loop is 70% GC rich with a bulge every twelve bases due to constraints dictated by commercial DNA synthesis. For both the GAPDH-SL and GAPDH-BNYVV gene blocks, the structures were placed directly in the middle of the GAPDH CDS sequence. Using the PCR-based template construction outlined herein, investigators can look at any RNA sequence of any size to examine specific questions related to their interests. This approach is described in more detail below and illustrated in Figure 1.

While the protocol described herein involves radiolabeled RNAs, non-radioactive alternatives are available [38]. Modified nucleotides containing fluorophores for direct RNA detection can be co-transcriptionally incorporated into substrate RNAs or added enzymatically at the 3' end. Alternatively, nucleotides containing haptens such as biotin or digoxigenin can also be used for indirect detection using fluorophore-labeled streptavidin (for biotin) or antibodies (for digoxigenin). Finally, click chemistry approaches using smaller functional groups can be used to subsequently attach fluorophores or haptens [38]. The user must keep in mind that the addition of these labels can influence the behavior of these RNAs substrates in reactions due to their bulky nature. Collectively, non-radioactive RNA detection does broaden the applicability of the protocol below by allowing it to be performed in laboratories where the use of radiation is restricted or prohibited. Finally, in the absence of any label, one may be able to use ddPCR to assess the relative levels of upstream or downstream (of the xrRNA structure) products after the decay reaction.

Also note that the reaction conditions outlined in this protocol contain 10mM Mg^{2+} . Since magnesium ions can influence RNA structure [39], the reader may wish to assess different divalent ion concentrations to obtain optimal results for select RNA substrates.

2.4. Protocol

Herein, we describe the complete protocol for our *in vitro* xrRNA reporter read-out decay assay. We will describe approaches for template generation, RNA transcription and purification, substrate decay and visualization (Figure 1). Additional modifications to this approach are at the experimenter's discretion.

2.4.1. Template generation for *in vitro* RNA transcription—As a proof-of-concept we designed 4 gene blocks for use as templates for RNA transcription consisting of a total of 994 nucleotides (supplementary figure 1). Specifically we placed a 9 nucleotide spacer followed by a SP6 promoter at the extreme 5' end of the gene block to drive RNA transcription. We then used the last 911 nucleotides of human GAPDH CDS as our core sequence followed by the minimal 67 nucleotide engineered Dengue virus-derived xrRNA at the extreme 3' end.

In addition to our concept templates purchased as gene blocks, essentially any double stranded template representing any RNA can be made using PCR-based approaches. We have provided template forward and reverse primers for use in high fidelity PCR reactions using cDNA as template (Reaction table 1). After optimization of PCR, the double-stranded PCR product can be purified and used as template for *in vitro* transcription reactions. Included in our primers are the SP6 promoter on the forward oligonucleotide in combination with an investigator-designed region for specific amplification of their mRNA of interest. The reverse primer also contains the reporter xrRNA sequence in combination with a variable region for RNA-specific amplification.

2.4.2. In vitro transcription reaction and purification of RNA substrate—To perform an *in vitro* transcription reaction, first set up reactions in accordance with Reaction Table 2 in 1.5mL microcentrifuge tubes. Next, incubate the reactions at 37°C for 1-3 hours. Following incubation, raise the volume of each reaction to 200µl with nuclease free water and perform phenol:chloroform:isoamyl alcohol extraction. Adjust the solution to 2.5 M ammonium acetate using a 10M stock ammonium acetate solution and add 2.5 volumes of 100% ethanol. The use of ammonium acetate is preferred over sodium acetate to more effectively prevent unincorporated nucleotides from precipitating. Glycogen or tRNA can be added as a carrier here to ensure quantitative precipitation of the RNA. Chill tubes at –80°C for at least 20 minutes to aid in RNA precipitation and recovery. While the RNA is chilling, pour a 5% denaturing (7M urea) PAGE gel using the recipe described in the Materials and Methods section above.

Once the gel has set, pre-run the gel at 600v in 1× TBE buffer for at least 30 minutes to ensure that the ion front has moved down the gel for optimal resolution of small fragments. Meanwhile, precipitate the RNA by microcentrifugation and then wash the precipitated RNA with 80% ethanol. Resuspend the precipitated RNA in 10µl of urea RNA loading dye (see materials and methods above), load into gel wells and run for ~30 min to 1 hour at 600v. Remove the gel from the apparatus, wrap it in plastic wrap, and expose to X-Ray film. Next, develop the film and use the image as a positional guide to excise the band at the appropriate size of the RNA of interest. One approach that we use to align the gel and the film is to place a cassette over the wrapped gel plate and briefly preflash the film by turning the room light on. This will give you an image of the wells and gel border on your film for easy alignment. Place the excised gel slice containing your RNA in 400µl of High Salt Column Buffer (HSCB, materials and methods section) and allow the RNA to elute overnight at room temperature. The following day, perform phenol:chloroform:isoamyl alcohol extraction as described above. Remove the aqueous phase into a new tube and directly add 2.5 volumes of ethanol. Note that you do not need to add additional salt due to the constituents of the HSCB and the small amount of linear acrylamide that will extract with the RNA from the gel serves as an excellent carrier to ensure full precipitation of your RNA. After washing with 80% ethanol, resuspend the RNA pellet in 11µl nuclease free water. Remove 1µl of your resuspended RNA and place in 3ml of ScintiSafe™ Econo 1 LSC Cocktail. Quantify the RNA using a Liquid Scintillation Counter and dilute to desired counts per minute. We routinely use 100-200,000 cpms per experiment. Note that the molar amount of the RNA can

be readily determined from the specific activity of the ^{32}P -UTP, the ratio of radioactive to cold UTP in the transcription, and the number of uridine residues in the final product.

2.4.3. XRN1 decay assay—Prepare a set of microcentrifuge tubes based on the desired time points to be interrogated over the decay time course. In a single tube, prepare the decay assay reaction in accordance with Table 2. In the additional tubes, place 400 μl of HSCB for quenching of each time point. As soon as possible, take 5 μl of the reaction and place in the 0 minute time point tube (input RNA). Then add Xrn1, mix and incubate at 37°C for the duration of the time course, taking 5 μl samples per time point and placing it into the appropriate tube containing 400 μl HSCB to stop the reaction.

2.4.5. Denaturing gel electrophoresis and visualization—Once the time course is completed, add 400 μl of 25:24:1 phenol:chloroform:isoamyl-alcohol to each tube, vortex and centrifuge. Remove the top aqueous layer, add 1 μg of glycogen as a carrier, and place in 1ml of 100% ethanol and freeze at -80°C for at least 20 minutes. While the RNA is at -80°C , pour a 5% denaturing polyacrylamide gel, allow to set, and pre-run at 600v for at least 30 minutes. Next, precipitate the RNA by centrifugation for 20 minutes, wash with 160 μl 80% ethanol, and resuspend in 5 μl RNA loading dye. Then, load the RNA into the gel, run for 30 minutes- 1 hour and dry the gel on Whatman paper for 1hr using a gel dryer. Expose the dried gel to a phosphor screen for the desired time and then scan phosphor screen to visualize RNA decay.

3. Results

In order to verify the approach, we first examined our GAPDH CDS RNA substrate as a control. As described above, we *in vitro* transcribed RNA using our gene-block as a template. The RNA substrate was then purified and subjected to an XRN1 decay assay using time points of 0, 5, 10, 20, and 30 minutes post rXRN1 addition. The samples were then resolved via denaturing PAGE, dried, exposed to a phosphor screen, and viewed via an Azure Sapphire biomolecular imager providing sufficient sensitivity to observe all required bands with one exposure (Figure 3A, left gel image). Decay of the full length RNA can be observed over the time course with nearly all of the available rXRN1 substrate (5' monophosphate) decayed within 5 minutes (Figure 3A, top right lightened inset image). Additionally, accumulation of the reporter xrRNA read-out fragment is observed over the time course and increases with time (Figure 3A, bottom right darkened inset image). These data support that the observed RNA decay is indeed specifically mediated by rXRN1 as it is occurring in the proper 5' to 3' direction and not random as would be observed by contaminating endonucleases. With read-outs depicting the decay of the input RNA substrate and the accumulation of the reporter xrRNA available, you can be certain that (1) the decay being observed is specific for rXRN1 and (2) the parameters of rXRN1 activity can change depending on substrate context (input RNA vs reporter xrRNA).

To begin to understand the influence of RNA sequence on XRN1 activity, we made substrate RNA from the 3' UTR of GAPDH. It has been suggested that 3' UTRs may surprisingly be more structured than coding sequences [34–36], therefore it is possible that rXRN1 may behave differently towards a potentially structured substrate. As seen in Fig. 3B, rXRN1 was

able to decay the full length GAPDH CDS-3' UTR RNA within 5 minutes (Figure 3B, top right, lightened inset image) and accumulation of the reporter xrRNA was observed over the entire time course (Figure 3B, bottom right, darkened inset image). These data suggest that rXRN1 is able to decay this 3' UTR sequence with no observable differences from the GAPDH-CDS transcript.

We then sought to challenge rXRN1 with potent RNA structures. To this end, we designed a gene-block containing a very strong stem loop (84 nucleotides, bulges every 12th position (due to manufacturer's dictated constraints for efficient DNA synthesis)) (diagrammed in Fig. 3C, left panel) and placed it directly in the middle of the GAPDH CDS (GAPDH CDS-SL). The GAPDH CDS-SL RNA was then subjected to rXRN1-mediated decay.

Surprisingly, the synthetic 84 nucleotide SL structure effectively stalled rXRN1 activity (Figure 3C, band ~450bp). Interestingly, although rXRN1 clearly stalled at this structure, accumulation of the reporter xrRNA was still observed (Figure 3C, bottom right, darkened inset image) suggesting that rXRN1 can eventually get through stem loop structures through structural pliability or 'breathing'.

Finally, to further interrogate the influence of structured RNA on XRN1-mediated decay, we designed a gene-block with the well-characterized minimal xrRNA sequence from BNYVV [37] and placed it directly in the middle of the GAPDH CDS sequence (GAPDH CDS-BNYVV RNA). Again, rapid XRN1-mediated decay of the GAPDH CDS-BNYVV RNA was seen (Figure 3D, top band, top right, lightened image) along with the accumulation of the BNYVV xrRNA (~500nt) (Figure 3D, middle band ~500nt). Importantly, accumulation of the reporter xrRNA can also be resolved (Figure 3D, lower right image), again suggesting that rXRN1 can eventually degrade through repressive structures perhaps because the BNYVV RNA structure may exist in a pliable state.

5. Conclusions and perspectives

5.1. Conclusions

In this article, we describe a PAGE-based approach for examining RNA decay *in vitro*. Importantly, we use an engineered XRN1-resistant RNA structure as a read-out for examining 5' to 3' decay. Using our approach investigators may now be able to interrogate the decay of large, biologically relevant RNAs on denaturing polyacrylamide gels in a defined *in vitro* setting.

5.2. Perspectives

By using our universal forward and reverse primers with RNA-specific amended sequences investigators can amplify a template of any size for use during *in vitro* RNA transcription. This substrate RNA can then be purified and subjected to directional (5' to 3') *in vitro* RNA decay assays with absolute confidence through visualization of the accumulation of a reporter xrRNA read-out. As an added control to ensure that the observed decay is indeed due to Xrn1, the user could include 5' capped RNA substrates in the assay. Capped RNAs are totally resistant to Xrn1 decay and thus should not generate any degradation intermediates in the assay. An application of the XRN1 assays described here, for example,

could readily be used to characterize the effect of RBPs or helicases on XRN1 activity. Finally, we believe the overall reporter xrRNA approach may offer a unique opportunity to understand RNA decay kinetics in the context of biologically relevant RNAs.

Several potentially interesting observations can be discerned from the demonstration gels presented in Fig. 3. First, the apparent decay kinetics of the input RNA are faster and do not match the accumulation pattern of the reporter xrRNA in all four panels (see Fig. 4). Similar data were obtained for the other two RNA substrates shown Fig. 3 (data not shown). It is important to keep in mind that disappearance of the input band does not always indicate complete degradation of the substrate. Partially degraded RNA fragments are difficult to detect due to their range of sizes which results in variable migration down the gel and decreased detection sensitivity over background. This suggests that the assessing the accumulation of the xrRNA reporter may be an optimal way of determining exonuclease enzymatic parameters on an RNA substrate rather than assuming disappearance of input bands equates with full degradation of the transcript. Second, we note that based on the quantification that we have done to date, the reporter xrRNA does not generally accumulate in a 1:1 molar ratio with the input RNA. This suggests pliability and a degree of XRN1 sensitivity even in the knot-like three helix junction structure of the Dengue virus xrRNA. This parameter may need to be assessed for individual constructs if quantitative kinetic data are needed. One must also consider that surrounding RNA sequences can interfere with folding of local regions (a phenomenon that has been noted with a variety of inserted RNA aptamers) and therefore could interfere with the formation of xrRNA. Finally, the data in Fig. 3C indicate that the 87 base synthetic stem loop structure we designed can be added to the growing list of RNA structures capable of stalling XRN1. This may prove to be a useful addition to the molecular toolbox available for assessing directionality of RNA decay pathways in mammalian cells, particularly because the poly(G) block that has been instrumental in assessing decay directionality in yeast [14] does not work particularly well if at all when placed in the context of many mammalian mRNAs.

One final note of caution needs to be considered when using xrRNAs in the fashion described here or in in vivo contexts as has been recently described [40]. Several studies have demonstrated that the stalling of Xrn1 on these RNAs is associated with a measurable, reversible inhibition of enzymatic activity [20,21,30,41]. Therefore high concentrations of xrRNA-containing transcripts relative to Xrn1 levels could result in enzymatic repression and impact the results obtained. Therefore titration studies and time courses are advisable to ensure that the associated temporary repression of Xrn1 activity when the enzyme stalls at these structures does not significantly influence data interpretation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Abbreviations:

xrRNA	XRN1-resistant reporter RNA
UTR	untranslated region
mRNP	messenger ribonucleoprotein
RBP	RNA binding protein

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Highlights

- The insertion of a structure that stalls XRN1 at the end of an RNA substrate generates a convenient read out for RNA decay in the form of a small stable decay intermediate
- We demonstrate a synthetic stem loop structure that can stall XRN1
- The approach outlined herein may allow convenient PAGE analysis of *In vitro* RNA decay assays using larger, more biologically relevant mRNA/mRNPs

Figure 1: Graphical representation of PCR based amplification of *in vitro* transcription template. A forward primer containing the SP6 promoter and an investigator-designed RNA specific sequence is annealed to first strand cDNA, synthesizing the second strand. A reverse primer containing the engineered reporter xrRNA (reverse complement) and an investigator designed RNA specific sequence anneals to the new strand. PCR proceeds for many cycles creating a sufficient amount of dsDNA template for use as a specific template in *in vitro* transcription reactions. Upon cleaning of the dsDNA product, an *in vitro* transcription reaction is set up using SP6 polymerase promoting to the production of an investigator-defined substrate RNA containing the reporter xrRNA at the 3' end for use in downstream decay assays.

Figure 2: Typical workflow for interrogating 5' to 3' decay using reporter xrRNA read-out.

In a single reaction vessel, an *in vitro* decay assay is assembled including the RNA decay factor(s) as well as the RNA substrate to be investigated. Aliquots of RNA are collected over a time course, precipitated, washed, and resolved by denaturing PAGE. The gels are then exposed to a phosphor screen and viewed. Images can be visually inspected for 5' to 3' resistant structures as well as relative changes in reporter xrRNA accumulation via densitometric quantification.

Figure 3: *In vitro* decay assay and xrRNA read-out using different RNA substrates.

Shown are 4 representative denaturing PAGE gels for *in vitro* decay assays over a time course using 4 different RNA substrates. (A) The terminal 994 nucleotides of the human GAPDH CDS are used as a control RNA substrate. Left side gel image represents the entire scanned image with a RNA ladder for reference. The top band is the full length RNA substrate (994nts) and the bottom band is the reporter xrRNA read-out (67nts). The top right (lightened image) is shown to visualize decay of the full length RNA, while the bottom right (darkened image) is shown to visualize the reporter xrRNA read-out. (B) The end of human

GAPDH CDS was combined with the entire 3' UTR sequence to generate GAPDH CDS-3' UTR RNA substrate. (C) An engineered stem-loop was placed directly in the middle of the GAPDH-CDS 994nt sequence. A novel xrRNA structure can be observed (including the downstream sequence) between 400 and 500 nucleotides on the image, supporting the idea that structures can impact XRN1 activity. (D) The minimal 55nt BNYVV xrRNA sequence was placed in the middle of the GAPDH-CDS 994nt sequence. An expected xrRNA consisting of the BNYVV sequence and downstream sequence is observed as predicted (~500nt band).

Figure 4: Quantification/graphical representation of gels presented in vitro decay assays and xrRNA readouts of selected RNA substrates.
Using the representative data obtained in Fig. 3, the relative levels of the full length input RNA (Input circles) or the xrRNA reporter decay intermediate (xrRNA squares) for GAPDH CDS and GAPDH CDS-BNYVV were quantified by phosphorimaging using pixel counting. Results are presented as the amount of the indicated RNA species relative to the band/lane of the gel with the maximal pixel count for each RNA.

Table 1:

RNA substrates to demonstrate changes in XRN1 activity

Substrate RNA	Potential impact on XRN1 activity
GAPDH CDS	Model RNA with presumably rapid decay
GAPDH CDS-3' UTR	Uncharacterized structure encoded in the UTR could slow decay rates
GAPDH CDS-SL	The large stem-loop may slow or stall XRN1, perhaps forming detectable decay intermediates
GAPDH CDS-BNYVV	Internal XRN1 stalling by a known xrRNA structural element should result in detectable decay intermediates

Table 2:*In vitro* decay assay reaction

Decay Assay Component	Volume (scale as required)
RNA Construct	~6fmol RNA in 1μl
NEB Buffer 3	2μl
RiboLock RNase Inhibitor	1μl
NEB Recombinant XRN1	1μl
Nuclease Free ddH ₂ O	15μl
Total Volume	20μl