6

MicroRNAs and Messenger RNA Turnover

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Summary

Although initially believed to act exclusively as translational repressors, microRNAs (miRNAs) are now known to target complementary messenger RNA (mRNA) transcripts for either translational repression or cleavage via the RNA-induced silencing complex (RISC) (*[1]*, reviewed in **ref.** *2*). The current model postulates that mature miRNAs are incorporated into the RISC, bind target mRNAs based on complementarity, and guide cleavage of mRNA targets with perfect or nearly perfect complementarity and translational repression of targets with lower complementarity *(2)*. The translational repression mechanism of miRNA-mediated gene regulation, which is common in animals but also exists in plants, is not well understood mechanistically. Conversely, miRNA-directed mRNA cleavage by RISC is common in plants, but also occurs in animals *(3)*. This chapter focuses on the mRNA cleavage by miRNA-programmed RISC, and, specifically, on characterizing the products of such cleavage.

Key Words: RISC; miRNA; mRNA; 5'-cap; poly(A) tail; exoribonuclease.

1. Introduction

RNA-induced silencing complexes (RISCs) are multiple-turnover entities that direct many rounds of site-specific target mRNA cleavage *(1)*. A principal RISC component in all eukaryotes is a member of the Argonaute (AGO) protein family *(4)*. AGO contains the conserved PAZ and PIWI domains, and seems to be the sole protein required for RISC-mediated activities *(5)*. The discovery that the PIWI domain adopts a ribonuclease (RNase) H fold *(6–8)* has led to a concept of AGO as an "mRNA slicer" component of the miRNA-programmed or small interfering RNA (siRNA)-programmed RISC. The cleavage of the mRNA target occurs between the nucleotides that are complementary to positions 10 and 11 of the miRNA that guides the RISC to cleave its target mRNA, and is defined by the 5' end of the guide RNA strand *(9,10)*. This distance-dependent mechanism is now beginning to be understood in precise terms of structural constraints imposed on the target mRNA recognition by the molecular architecture of the AGO/guide RNA complex *(11,12)*.

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The characterization of the mRNA turnover events accompanying and following the initial endonucleolytic mRNA cleavage by RISC is an area of intense scrutiny *(13–16)*. That such cleavage products must be removed is dictated by several reasons. For instance, the 5' fragments, if translated, would result in truncated, and therefore, potentially toxic, polypeptides. Indeed, the existence of a specialized "nonstop" pathway of mRNA decay, which specifically targets mRNAs lacking stop codons *(17)*, is indicative of an evolutionary pressure to prevent such truncated mRNAs from being translated. In addition, the products of the mRNA cleavage by RISC that are not promptly removed may engage RNA-dependent RNA polymerases and, thus, initiate a chain of events resulting in gene silencing (e.g., **ref.** *14*). In this chapter, we outline procedures for characterizing the products of the mRNA cleavage by RISC, including:

a. Mapping the 5' end of the 3' fragment resulting from the RISC-mediated cleavage (**Subheading 3.1.**, **Fig. 1B**, top).
b. Mapping of the 3' end of the 5' fragment resulting from the RISC-mediated cleavage (**Subheading 3.2.**, **Fig. 1B**, bottom).
c. Assessing the 5'-cap status of the 5' fragment resulting from the RISC-mediated cleavage (**Subheading 4.1.**, **Fig. 2A**).
d. Assessing the poly(A) status and poly(A) tail length of the 3' fragment resulting from the RISC-mediated cleavage (**Subheadings 4.2.** and **4.3.**, **Fig. 2B**,**C**).

To facilitate downstream analyses, it is helpful to devise strategies to enrich for the products of mRNA cleavage by the RISC complex and/or stabilize them. Such strategies include overexpression of the mRNA whose RISC-mediated cleavage is being studied, to saturate the exonuclease systems that degrade them, and therefore increase the steady state level of the primary products of the endonucleolytic cleavage by RISC. Alternatively (or additionally), one might engineer a genetic depletion of the exonucleases that might be responsible for the degradation of the mRNA cleavage products. In either scenario, the relative abundance of the products of the endonucleolytic cleavage of mRNA by RISC will determine the degree of sensitivity that is required to detect them.

An obvious benefit of the exonuclease depletion strategy is an immediate insight into the pathways of degradation of the primary mRNA cleavage products. Indeed, the key criterion in the identification of the factors responsible for the decay of the products of the RISC-mediated cleavage is their stabilization after the depletion of such factors. Based on the current literature, the list of possible factors includes (but is not limited to) the 5'–3' exonucleases of the XRN1 family and several 3'–5' exonucleases, such as the exosome (and its associated SKI2/3/8 complex), CCR4/POP2, and PARN (**Fig. 1A**). For instance, in the *Drosophila* S2 cells, the 5' and 3' products of the mRNA cleavage by the siRNA-programmed RISC are degraded exonucleolytically by exosome and XRN1, respectively, without previous decapping or deadenylation *(15)*. It remains to be determined whether this is also true in other systems, as well as in the case of the miRNA-programmed RISC. The choice of the exonuclease depletion method depends on particular features and constraints of a given model system. The range of possibilities includes the use of repressible promoters and specific enzyme inhibitors, as well as targeting the enzyme in question via RNA interference (reviewed in **ref.** *18*).

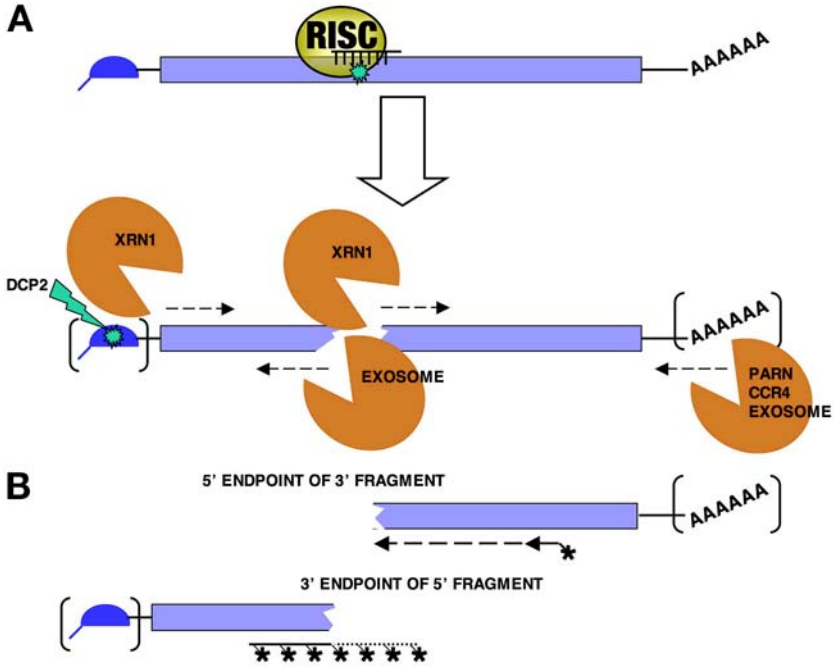

Fig. 1. Mapping the position of the messenger RNA (mRNA) cleavage by RNA-induced silencing complex (RISC). (**A**) A scheme illustrating the possible fates of the 5' and 3' mRNA fragments resulting from the RISC-mediated cleavage. The exonucleases that are known or suspected to degrade the 5' and 3' mRNA fragments are indicated. (**B**) A scheme illustrating the use of the primer extension and ribonuclease protection to map the 5' endpoint of the 3' fragment and the 3' endpoint of the 5' fragment, respectively.

2. Materials

2.1. Primer Extension

1. RNA extraction reagent (e.g., Trizol reagent, Invitrogen, Carlsbad, CA).
2. Chloroform.
3. Isopropanol.
4. 75% EtOH.
5. T4 polynucleotide kinase and 10X kinase buffer (New England Biolabs, Beverly, MA).
6. Primer design software Primer3.0 (web interface at http://frodo.wi.mit.edu).
7. Diethylpyrocarbonate (DEPC)-treated H_2O.
8. 6000 Ci/mmol [γ-^{32}P] adenosine triphosphate (ATP).
9. TE (pH 7.5).
10. AMV reverse transcriptase and 10X AMV buffer (Promega, Madison, WI).
11. Deoxyribonucleoside triphosphates (dNTPs) (100 m*M* stocks).
12. 1 *M* dithiothreitol (DTT).
13. 1 *M* $MgCl_2$.
14. Formamide loading dye: 95% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol FF, and 5 m*M* ethylenediaminetetraacetic acid (EDTA).

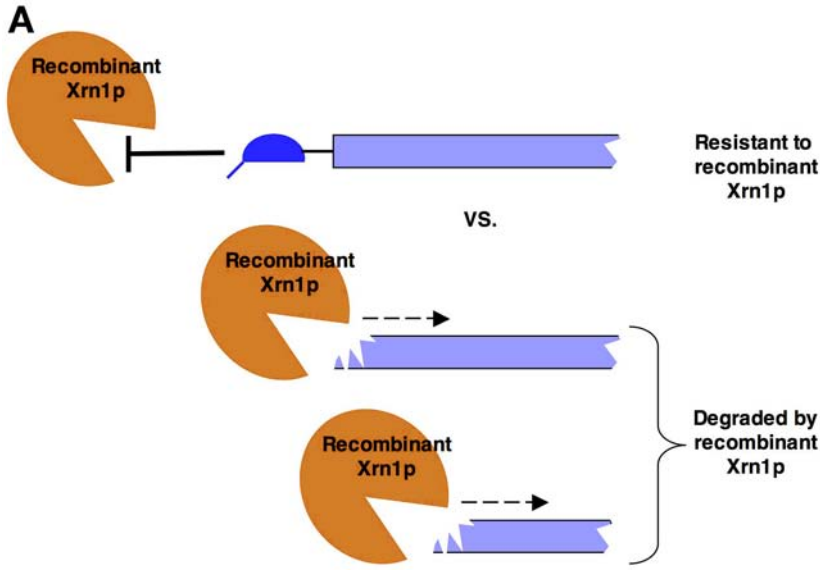

Fig. 2. Assessing the 5'-cap and 3'-poly(A) tail status of the products of the RNA-induced silencing complex (RISC)-mediated cleavage. **(A)** Assessing the 5'-cap status by XRN1 sensitivity.

15. Denaturing polyacrylamide gel (e.g., commercial SequaGel, National Diagnostics; www.nationaldiagnostics.com).
16. X-ray film or/and PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

2.2. Ribonuclease Protection

1. Reagents for in vitro transcription (Promega): 5X transcription buffer, DTT, RNasin, ribonucleoside triphosphates, and T7 RNA polymerase.
2. DEPC-treated H_2O.
3. 10 mCi/mL [α-^{32}P] uridine triphosphate (UTP) (3000 Ci/mmol).
4. RQ1 RNase-free deoxyribonuclease (DNase) (Promega).
5. 5 µg/µL glycogen carrier (Ambion, Austin, TX).
6. Phenol/chloroform/isoamyl alcohol.
7. 3 *M* Na acetate (pH 5.2).
8. 100% EtOH.
9. Cold 75% EtOH.
10. Dry ice.
11. Hybridization buffer: 80% deionized formamide, 10 m*M* Na citrate, 300 m*M* Na acetate, pH 6.4, and 1 m*M* EDTA.
12. 10X RNase ONE buffer and RNAse ONE (Promega).
13. 10% sodium dodecylsulfate (SDS).

2.3. XRN1 Assay

1. Recombinant Xrn1p. If the recombinant Xrn1p must be purified in-house, express and purify as described *(19)* on a Ni^{2+} column (we use the Ni-NTA kit from Qiagen [Valencia, CA] and follow the manufacturer's protocol).

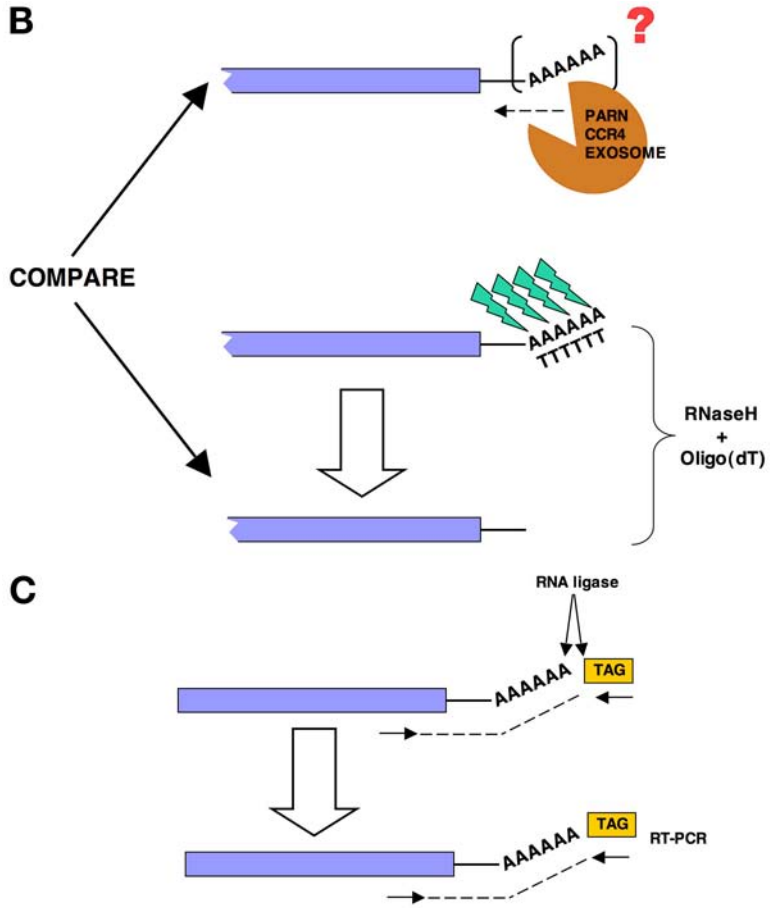

Fig. 2. **(B)** Analysis of the poly(A) tail status of the 3' fragment by oligo(dT)/ribonuclease H cleavage. **(C)** Analysis of the poly(A) tail status of the 3' fragment using ligation-mediated reverse transcriptase polymerase chain reaction.

2. Xrn1p reaction buffer: 33 m*M* Tris-HCl (pH 8.0), 50 m*M* NaCl, 2.5 m*M* $MgCl_2$, 0.2 m*M* DTT, with and without 5 m*M* EDTA.
3. Phenol/chloroform/isoamyl alcohol.
4. 100% EtOH.

2.4. RNase H Assay

1. $Oligo(dT)_{15}$.
2. 10X RNase H buffer and RNase H (Promega).
3. Hybridization membrane (BioTrans Plus, MP Biomedicals, Irvine, CA).
4. Phenol/chloroform/isoamyl alcohol.
5. 100% EtOH.
6. Formamide loading dye.

7. Electrophoresis buffer: 1X MOPS buffer for 1.2% agarose/6% formaldehyde gels or 0.5X TBE for high-resolution polyacrylamide gels (*see* **Subheading 4.2.3.**).
8. Transfer solution: 1X TAE (for polyacrylamide gels) or 10X standard sodium citrate (SSC) (for agarose gels).
9. Hybridization solution: 6X SSC, 5X Denhardt solution, 0.5% SDS, and 100 µg/mL of salmon sperm DNA.
10. Wash solutions: 2X SSC, 0.1% SDS plus 0.2X SSC, and 0.1% SDS.

2.5. Ligation-Mediated Reverse Transcriptase Polymerase Chain Reaction (PCR)

1. 5'-Phosphorylated adaptor oligoribonucleotide with the inverted deoxynucleotide in the 3' position (Dharmacon Inc, Lafayette, CO).
2. Phenol/chloroform/isoamyl alcohol.
3. 100% EtOH.
4. Cold 75% EtOH.
5. DEPC-treated H_2O.
6. 10X T4 RNA ligase buffer and RNA ligase (20 U/µL, New England Biolabs).
7. MMLV reverse transcriptase Superscript II and 10X Superscript II buffer (Invitrogen).
8. Appropriate DNA oligos, any commercial supplier (e.g., IDT, Coralville, IA or Sigma-Genosys, St. Louis, MO).

3. Methods

3.1. Mapping of the 5' Endpoint of the 3' Fragment by Primer Extension (see Note 1)

3.1.1. Prepare Total RNA

Procedures for total RNA extraction vary depending on the model system; an example protocol using Trizol (Life Technologies) follows (Qiagen Rneasy and BD Biosciences NucleoSpin kits are also commonly used in many laboratories). Homogenize 100 mg of tissue in 1 mL Trizol reagent and let samples stand for 3 min at room temperature (if necessary, remove the insoluble material from the homogenate by centrifugation at 12,000*g* for 10 min at 4ºC). Add 0.2 mL chloroform per 1 mL Trizol, mix well, let stand for 10 min at room temperature. Centrifuge at 12,000*g* for 15 min at 4ºC. Transfer upper aqueous phase into new tubes. Add 0.5 mL isopropanol per 1 mL Trizol, mix, and incubate at room temperature for 10 min. Centrifuge at 12,000*g* for 10 min at 4ºC. Wash with 75% EtOH (use at least 1 mL of 75% EtOH per 1 mL of Trizol used for homogenization). Air-dry for 10 min. Dissolve in DEPC-treated H_2O and determine the optical density of the samples (high-purity RNA should have an $A_{260/280}$ ratio of approx 2:1).

3.1.2. Prepare the Labeled Oligo for Primer Extension

Combine 0.5 µL oligo (1 µg/µL), 1 µL of 10X kinase buffer, 2 µL of ^{32}P γ-ATP (6000 Ci/mmol), 5.5 µL of DEPC-treated H_2O, and 1 µL of T4 polynucleotide kinase (sufficient for 10 extension reactions). Incubate at 37ºC for 30 to 60 min. To stop the reaction, add 40 µL of TE and heat at 95ºC for 3 min.

3.1.3. Anneal the Labeled Oligonucleotide

Combine 10 to 20 µg of total RNA with 2 µL of the 5X AMV reverse transcriptase buffer (Promega) and 0.5 µL of the labeled oligonucleotide (*see* **Note 2**), and bring the total reaction volume to 8 µL with DEPC-treated H_2O. Incubate at 65ϒC for 10 min, transfer to a 42ϒC water bath, and incubate for 30 to 60 min to allow annealing.

3.1.4. Conduct the Primer Extension Reaction

For 10 primer extensions, make up a cocktail containing 5 µL of 0.2 *M* $MgCl_2$, 5 µL of 20 m*M* DTT, 2.5 µL of dNTPs (2.5 m*M* each), 5.5 µL of DEPC-treated H_2O, and 2 µL of AMV reverse transcriptase (Promega). Add 2 µL of this cocktail to the 8 µL annealing mix (**Subheading 3.1.3.**) and incubate at 42ϒC for 30 to 60 min. Add 10 µL of formamide loading dye to terminate the reaction, heat at 75ϒC, and resolve by electrophoresis on a denaturing polyacrylamide gel (6–20%, depending on the size of the expected extended product). Expose to a X-ray film or, for quantitation, to a Phosphorscreen, scan on a PhosphorImager, and analyze with ImageQuant software (Molecular Dynamics).

3.2. Mapping the 3' Endpoint of the 5' Fragment by Ribonuclease Protection

A detailed protocol using RNase ONE (Promega) is provided next (*see* **Notes 3** and **4** for general considerations regarding the use of ribonuclease protection). Alternatively, the RNase ONE kit (Promega) or the RPA III kit (Ambion) could be used, as described by the respective manufacturers (*see* **Note 5** on differences in the properties of the RNase A/T1 that is used in the RPA III kit compared with the RNase ONE kit).

3.2.1. Prepare the Appropriate Body-Labeled Probes by In Vitro Transcription

For example, in the case of a T7 promoter-driven run-off probe, combine 1 µL of linearized template DNA (at 1 µg/µL) with 4 µL of 5X in vitro transcription buffer (Promega); 2 µL of 100 m*M* DTT; 1 µL of 40 U/µL RNasin; 1 µL each of 10 m*M* ATP, GTP, and CTP; 0.6 µL of 100 µ*M* UTP; 5 µL of 10 mCi/mL [α-^{32}P] UTP (3000 Ci/mmol); 2.4 µL of DEPC-treated H_2O; and 1 µL of T7 RNA polymerase. Incubate the reaction for 60 min at 37°C. Add 1 µL of RQ1 RNase-free DNase and incubate for another 15 min at 37°C to digest away the template DNA.

3.2.2. Purify the RNA Probe

To each RQ1 DNase-treated sample, add 100 µL of DEPC-treated H_2O, 2 µL of 5 µg/µL glycogen as a carrier, and extract with phenol/chloroform/isoamyl alcohol (*see* **Note 6**). To the aqueous phase, add 1/10 volume of 3 *M* Na acetate (pH 5.2) and 2.5 volumes of EtOH and incubate for 15 min on dry ice, followed by centrifugation for 15 min at 12,000*g*. Carefully wash the pellet with cold 75% EtOH, air-dry, and resuspend in the DEPC-treated H_2O at $1 \leftrightarrow 10^6$ cpm/µL (for the specific probe against the RISC-cleaved mRNA fragment), or at $2 \leftrightarrow 10^5$ cpm (for the internal control probe directed against a suitable housekeeping message, such as actin or a ribosomal protein).

3.2.3. Hybridize the RNase Protection Probe With Target RNA

For each reaction, combine the RNA sample to be analyzed (5–20 µg RNA in a total volume of 8 µL or less) with 20 µL of the hybridization buffer, 1 µL of a specific probe directed against the RISC-cleaved mRNA fragment ($1 \leftrightarrow 10^6$ cpm), and 1 µL of an internal control probe for normalization purposes ($2 \leftrightarrow 10^5$ cpm). For each specific probe, also prepare two control reactions, via substituting transfer RNA (tRNA) for the target sample RNA. Mix, denature at 75°C to 80°C for 3 min (avoid prolonged incubation at 75°C to 80°C, because it may lead to nonspecific RNA fragmentation). Incubate at 45°C overnight.

3.2.4. Perform the RNase Digestion

To each hybridization reaction, add 270 µL of DEPC-treated H_2O, 30 µL of 10X RNase ONE buffer, and 5 U of RNAse ONE (Promega). Incubate at 37°C for 60 min. To one of the two "tRNA only" control reactions, add 270 µL of DEPC-treated H_2O and 30 µL of 10X RNase ONE buffer ("no RNase" sample). Add all three components to the second "tRNA only" control reaction.

3.2.5. Terminate the RNase Digestion

To each sample, add 5 µL of 10% SDS, 800 µL EtOH, and 1 µL glycogen (5 µg/µL), mix, incubate for 15 min on dry ice, and centrifuge at 12,000*g* for 15 min. Carefully wash the RNA pellet with cold 75% EtOH and air-dry the pellet for several minutes. Carefully resuspend the RNA in the 8 µL of the formamide loading dye, denature at 75°C to 80°C for 3 min, tap-spin, and load onto the denaturing polyacrylamide gel (6–20% acrylamide, depending on the size of the expected extended product). Expose the dried gel to a Phosphorscreen, scan on a PhosphorImager, and analyze with ImageQuant software (Molecular Dynamics).

4. Assessing the 5'-Cap and 3'-Poly(A) Tail Status of the Products of the RISC-Mediated Cleavage

4.1. Assessing the 5'-Cap Status by XRN1 Sensitivity

In this approach, the cap status of the 5' cleavage product is revealed by testing its sensitivity to the action of the recombinant 5'–3' exonuclease Xrn1p that is purified from yeast. Only the uncapped, but not the capped, RNA species are degraded by Xrn1p (*see* **Note 7**).

4.1.1. Purification of Xrn1p

Express the His-tagged Xrn1p encoded by the construct pAJ95 (2 µ/*LEU2*/p*GAL10-XRN1*-HA-His_6) in the yeast strain BJ5464, as described ***(19)***, and purify on a Ni^{2+} column (Ni-NTA, Qiagen) as suggested by the manufacturer.

4.1.2. Digest RNA With Xrn1p

Digest the total RNA samples containing the RISC-mediated mRNA cleavage products of interest with the purified Xrn1p ***(20)***. Combine 5 to 10 µg of total RNA with 400 ng of purified Xrn1p in a final volume of 10 µL of 33 m*M* Tris-HCl (pH 8.0), 50 m*M*

NaCl, 2.5 m*M* $MgCl_2$, and 0.2 m*M* DTT, in the presence or absence of 5 m*M* EDTA (*see* **Note 8**). Incubate the Xrn1p reactions for 30 min at 37°C. Terminate the reactions by phenol extraction followed by EtOH precipitation (*see* **Note 9**).

4.1.3. Analysis of Xrn1p Reaction Products

Visualize the 5' RISC cleavage product by ribonuclease protection as described in **Subheading 3.2.** or by Northern blotting (**Subheading 4.2.**). Disappearance of the 5' cleavage products after the Xrn1p treatment serves as an indication that they are uncapped and, thus, conversely, their resistance to this treatment attests to their capped status (*see* **Note 10**).

4.2. Analysis of the Poly(A) Tail Status of the 3' Fragment by Oligo(dT)/RNase H Cleavage

A straightforward way to detect the presence, as well as to assess the size, of the poly(A) tail on the 3'-fragment involves oligo(dT)/RNase H cleavage, followed by the Northern blot analysis (obviously, this is possible only if the 3' fragment is abundant enough to be detected by Northern blot; alternatively, one must use ligation-mediated PCR, as described in **Subheading 4.3.**). A characteristic increase in the electrophoretic mobility of the 3' cleavage fragment as a result of RNase H treatment indicates that it is polyadenylated. Furthermore, judicious choice of the molecular weight markers allows determination of its poly(A) tail length distribution and comparison with the poly(A) tail length of intact, full-length mRNA, and, thereby, allows inference of whether a significant amount of deadenylation precedes the RISC-mediated cleavage.

4.2.1. Assemble the RNase H Cleavage Reaction

Combine 25 µg of total RNA with 500 ng of oligo$(dT)_{15}$, denature at 75°C for 5 min, and allow to anneal for 20 min. Add the 10X RNase H buffer to achieve final concentrations of 20 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$, 0.5 m*M* EDTA, 50 m*M* NaCl, 1 m*M* DTT, and 30 µg of bovine serum albumin per milliliter. Add 0.25 U of RNase H (Promega) and digest for 1 h at 37°C.

4.2.2. Terminate the RNase H Cleavage

Stop the RNase H reaction by conducting phenol extraction followed by EtOH precipitation. Alternatively, stop the reaction by directly adding an equal volume of the formamide loading dye (but *see* **Note 9**).

4.2.3. Conduct Northern Blotting

Denature the RNA samples for 3 min in the formamide loading dye at 75°C to 80°C. Separate the RNA cleavage products via polyacrylamide or agarose gel electrophoresis, depending on the expected product size. For analysis by polyacrylamide gel electrophoresis, aliquots (⊕10 µg) of treated and control (no RNase H and oligo$[dT]_{15}$) samples are loaded onto 6 to 20% (depending on the expected product sizes) polyacrylamide/8 *M* urea gel in 0.5X TBE. For agarose gel electrophoresis, at least 10 µg of total RNA is loaded onto 1.2% agarose/6% formaldehyde gel in 1X MOPS buffer (20 m*M* MOPS, 8 m*M* sodium acetate, and 1 m*M* EDTA, pH 7.0). RNA is then transferred

either electrophoretically in 1X TAE (for high-resolution polyacrylamide gels) or with a vacuum blotter in 10X SSC (for formaldehyde/agarose gels) onto a BioTrans Plus membrane (ICN). In the case of formaldehyde/agarose gels, reverse the formaldehyde modifications by baking the membrane at 80°C for 1 hr. To fix the RNA, crosslink the membrane, when it is still slightly damp, for 1 min on an ultraviolet transilluminator (254 nm), or using a commercial ultraviolet crosslinker set at 120 mJ/cm^2.

4.2.4. Hybridize the Membrane

Hybridize the membrane in 6X SSC, 5X Denhardt solution, and 0.5% SDS plus 100 µg/mL of salmon sperm DNA at 65°C (if a random hexamer-labeled DNA fragment is used as a probe) or at 40°C to 48°C (if an oligonucleotide probe is used, the hybridization temperature should be calculated according to **ref. *21***). After overnight hybridization, wash the membranes twice in 2X SSC plus 0.5% SDS for 10 min at the temperature of hybridization, followed by high-stringency washes in 0.2X SSC plus 0.5% SDS. Expose the membranes to a Phosphor storage screen, scan on a PhosphorImager, and process and quantitate images using ImageQuant software (Molecular Dynamics).

4.3. Poly(A) Tail Status Analysis Via Ligation-Mediated Reverse Transcriptase-PCR

This method, modified from the ligation-mediated poly(A) tail assay ***(22)***, was developed to address the need for a more sensitive alternative method for analyzing the poly(A) tail length distribution. In **Subheading 4.3.**, we describe a modified ligation-mediated poly(A) tail assay that we have developed ***(23)*** from the published protocol for the identification of the 3' ends of siRNAs ***(24)***. In this method, the mRNA is first tagged at its 3' end via ligation of a RNA oligonucleotide of arbitrary sequence. The resulting tag is used as a priming site for reverse transcription, which is then followed by PCR across the poly(A) tail with a tag-specific and a gene-specific primer pair. The length distribution of the resulting population of PCR products reflects the poly(A) tail length distribution of the 3' cleavage product.

4.3.1. Separate the mRNA 3' Cleavage Products

Separate the mRNA 3' cleavage products away from the full-length (uncleaved) transcripts by size fractionation on a denaturing (polyacrylamide ***[25]*** or agarose ***[26]***, depending on the expected size) gel. Elute the RNA in the target size range, extract with phenol/chloroform/isoamyl alcohol, precipitate with EtOH, wash with ice-cold 75% EtOH, air-dry, and dissolve in DEPC-treated H_2O at approx 1 µg/µL.

4.3.2. Ligate the Oligonucleotide Tag to the 3' End of RNA

Ligate the appropriate RNA size fractions with the 5'-phosphorylated adaptor oligoribonucleotide of arbitrary sequence that contains an inverted deoxynucleotide at its 3' end (e.g., 5'-UACUCAUCAUACGUUGUAGAGUACCUUGUAidT; *see* **Note 11**). Combine 1 to 5 µg of size-fractionated RNA with 100 pmol of the adaptor oligo in 17 µL of DEPC-treated H_2O, denature for 3 min at 75°C to 80°C, and chill on ice. Add 2 µL of 10X T4 RNA ligase buffer, mix, and add 1 µL (20 U) of T4 RNA ligase (New England Biolabs). Incubate for 1 h at 37°C.

4.3.3. Conduct Reverse Transcriptase Reaction

Reverse-transcribe 1 to 5 µL of the ligated RNA using a primer complementary to the adaptor oligonucleotide and SuperScript II reverse transcriptase (Life Technologies), according to the manufacturer's protocol.

4.3.4. Carry Out the PCR

Dilute the complementary DNA 10-fold with DEPC-treated H_2O. Use 1 µL of the diluted complementary DNA as a template for the PCR, using the oligo that is complementary to the adaptor oligonucleotide as an antisense primer, and using an appropriate gene-specific primer as a sense primer (*see* **Note 12**). If necessary, diluted first-round products can be subjected to nested PCR under the same conditions.

4.3.5. Analyze the PCR Products

Resolve the products on nondenaturing agarose or polyacrylamide gel and authenticate by Southern hybridization with an internal, gene-specific oligonucleotide as a probe (*see* **Note 13**).

5. Notes

1. Mapping the endpoints of the mRNA cleavage products by Northern blotting is also possible provided that the products are sufficiently abundant (e.g., **ref.** ***24***), however, the precision that is afforded by even high-resolution polyacrylamide Northern blots is inferior.
2. For normalization in quantitative assays, include a second-labeled oligo targeted against a transcript that is not subject to RISC-mediated cleavage.
3. This protocol is also suitable for mapping the 5' endpoint of the 3' fragment, in addition to the primer extension protocol.
4. Nuclease S1 and DNA probes can be used for this purpose, as well. However, S1 nuclease is prone to nonspecific cleavage in AU-rich regions, as well as to nibbling (i.e., acting as a double-stranded exonuclease) artifacts.
5. RNAses ONE (Promega) and A/T1 (Ambion) have different properties. RNase ONE cleaves at a position immediately 3' to either C, A, U, or G, whereas RNAse A and RNAse T1 cleave at positions immediately 3' to pyrimidines and to G residues, respectively. Also, RNase ONE is easily inactivated by 0.1% SDS.
6. If premature polymerase stops are observed, purify the full-length RNA probe away from the truncated species by denaturing gel electrophoresis ***(25)***.
7. Alternatively, one can assess the cap status of the 5' cleavage product by immunoprecipitation with anticap antibody. The monoclonal antibody H20 (Biodesign International, Saco, ME; www.biodesign.com) was originally generated against the 2,2,7-trimethylguanosine cap, but it also crossreacts with the 7-methylguanosine-cap ***(27)***. However, we find the Xrn1p resistance assay to be more quantitative and reproducible.
8. EDTA chelates Mg^{2+}, which is absolutely required for the Xrn1p activity. Therefore, target RNA species should remain intact in the control reactions containing EDTA.
9. We prefer to purify RNA by phenol extraction because carryover of EDTA and Mg^{2+} from the reactions tend to affect the electrophoretic mobility of the RNA fragments, especially on high-resolution polyacrylamide gels.
10. Completeness of the Xrn1p digestion can be verified by Northern hybridization or RNase protection using a probe directed against an RNA species that is known to be naturally uncapped, such as the 7S rRNA precursor species.

11. The inverted residue at the 3' end prevents formation of oligonucleotide multimers.
12. We prefer to use the touchdown profile (initial annealing at 59ϒC, then decreasing by 1ϒC per cycle until the annealing temperature of 48°C is reached, followed by another 25 annealing cycles at 48°C).
13. With appropriate oligos, the ligation-mediated PCR protocol can also be applied to mapping the 3' endpoint of the 5'-fragment produced by RISC. In this case, the poly(A) RNA fraction must be first isolated (e.g., using an Oligotex kit from Qiagen), to remove the polyadenylated full-length species and the cleavage 3' fragments.

Acknowledgments

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