RNA Degradation in Cell Extracts: Real-Time Monitoring by Fluorescence Resonance Energy Transfer
Sarah A. Uhler, Dawen Cai, Yunfang Man, Carina Figge, and Nils G. Walter*
Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109-1055
Received June 23, 2003; E-mail: nwalter@umich.edu

The ability of an RNA molecule to persist in the cell among a plethora of ribonucleolytic activities is based on the tightly regulated relative rates of its synthesis and decay.1 Regulation of specific mRNA turnover has long been studied, but the inability to derive rate constants with a convenient technique for directly monitoring RNA degradation has limited the introduction of predictive mathematical models.2 In addition, the recent discovery of a multitude of short noncoding RNAs involved in gene regulation by RNA interference in eukaryotic genomes3 and the advent of synthetic small interfering RNAs (siRNAs) for manipulating gene expression and probing gene functions4 have made an understanding of the rates and pathways of the cellular degradation of small RNA molecules indispensable.

Toward this goal, we have developed assays based on steady-state fluorescence resonance energy transfer (FRET) between two RNA-coupled fluorophores to observe nucleolytic decay of short synthetic RNAs (more precisely, RNA/DNA chimera) in real time, thus providing the desired kinetic rate information (Figure 1). Fluorescence-based assays have previously been used to measure the activity of purified RNases in vitro,5 and fluorescent reporter proteins have been used to indirectly reflect cellular RNA abundance.6 Our FRET assays are specifically designed to continuously monitor the partitioning between intact and degraded RNA in complex cellular mixtures. In addition, labeling with two fluorophores allows us to test the relative contributions of 5’ to 3’ and 3’ to 5’ exonucleolytic activities to RNA decay.

To study the effects of secondary structure on RNA decay we designed two 16-nucleotide (16 nt) oligonucleotides, RNAs 1 and 2, that have similar base composition but have two very distinct secondary structures (Figure 1A). Additional design parameters included the incorporation of modified 2’-deoxy thymidines at nucleotide positions 3 and 13 for attachment of fluorescein and tetramethylrhodamine as a donor–acceptor FRET pair.7 While RNA 1, under our standard near-physiologic conditions (130 mM potassium glutamate, pH 7.5, 1 mM MgCl2, 10 mM DTT, at 37 °C)8 is predicted to reside >95% in the stem–loop secondary structure depicted in Figure 1A,9 RNA 2 is expected to be completely unstructured (Figure 1A). These predictions were confirmed by UV and FRET melting experiments,10 in which RNA 1 was found to melt at 64 and 61 °C without and with fluorophores attached, respectively, while RNA 2 showed no cooperative melting transition under these conditions.11 Thus, the attachment of two fluorophores only slightly lowers the melting temperature of RNA 1, indicating that it only insignificantly interferes with its secondary structure formation.

First, we determined the rate constants for in vitro degradation of RNAs 1 and 2 by RNase T1 (Figure 1b), which cleaves 3’ to G.12 RNA decay following addition of 0–250 nM RNase T1 (pH 7.5) to 50 nM RNA 1 or 2 was monitored as a decrease in steady-state FRET signal (i.e., acceptor:donor fluorescence ratio) under standard conditions, and rate constants k_{dec} were extracted by single-exponential decay fits as described.7,11 At all [RNase T1] k_{dec} is greater for the unstructured RNA 2 than for the stem–loop of RNA 1 (Figure 1b). Furthermore, k_{dec} for RNA 1 approaches an asymptotic limit of [RNase T1] reaches 4-fold excess over the RNA concentration, while k_{dec} for RNA 2 still increases. Fits of cooperative Hill binding equations to the data (Figure 1b) suggest stoichiometric (noncooperative) interaction of the enzyme with both RNAs, with rate constants at saturation of 3.9 min⁻¹ for RNA 1 and ≥25 min⁻¹ for the unstructured RNA 2 and apparent enzyme affinities K_{M} of 100 and 330 μM, respectively. Thus, the stem–loop structure of RNA 1 protects it from degradation by RNase T1 relative to the unstructured RNA 2 (especially given that RNA 1 has more G’s, potential RNase T1 targets, inserted between the fluorophores).

To show that this kinetic FRET assay also works in complex cellular mixtures, we studied the degradation of RNAs 1 and 2 in S100 cytosolic extract from HeLa cells, a common human epithelial cell line derived from cervix carcinoma. Again, we incubated the RNA under our standard near-physiologic conditions (130 mM potassium glutamate, pH 7.5, 1 mM MgCl2, 10 mM DTT, at 37 °C),8 then added increasing volume fractions of protease inhibitor treated cell extract (pH 7.6) and analyzed the resultant FRET decay as described above.11 As with RNase T1, the decay rate constant k_{dec} for RNA 1 approaches an asymptotic limit as the content of cell extract is raised to 30% (v/v), while that of RNA 2 does not (Figure 1c). Fits of the cooperative Hill binding equation to the data indicate noncooperative binding of one RNase enzyme in the cell extract to either RNA (Figure 1c). However, RNA 1 is degraded faster than RNA 2 at <10% (v/v) cell extract, yet slower at >10% (v/v). This results in rate constants at saturation of 0.90 min⁻¹ and 1.76 min⁻¹ for RNAs 1 and 2, respectively, and apparent extract affinities of 6.4% (v/v) and 19% (v/v), respectively. Thus, the stem–loop structure of RNA 1 confers some RNase protection, but only at conditions close to the cellular environment.
assays, we tested their ability to report on known and potential essential degradative pathway component (data not shown).

Commercially available RNase T1 inhibitor, was able to inhibit with unlabeled RNA show that the addition of RNA

Degradation of 50 nM RNA

fold excess (5×) with the degradation of RNA

Similarly, poor competition is observed when adding excess RNA

that the major nuclease activity found in S100 cytosolic HeLa cell extract. However, in aurin tetramethylrhodamine-labeled (yellow) 15 n and 14 nt cleavage bands, followed by a size gap and release of a short fluorescein-only labeled (green) band, as indicated.

To further characterize the utility of our FRET-based RNA decay assays, we tested their ability to report on known and potential RNase inhibitors. Divalent cations, such as Mg2+, inhibit RNase T1. As expected, addition of increasing [Mg2+] to 50 nM RNA under otherwise standard conditions gradually decreases the rate constant of degradation by 75 nM RNase T1 from 1.54 min−1 at 1 mM Mg2+ to 0.73 min−1 (that is, by 53%) at 500 mM Mg2+, an effect that is prevented when Mg2+ is chelated by EDTA. We also tested other potential inhibitors and found that addition of 10% Contrad70 (Decon Labs, Inc.), a strong, alkaline laboratory exonuclease, as evident from the fluorescein′-labeled RNA degradation by both RNase T1 and S100 HeLa cell extract. (a) Contrad70 inhibits degradation by S100 HeLa cell extract, stopped at the indicated times and analyzed by gelFRET. Primary nuclease activity is that of a 5′ to 3′ exonuclease, as evident from the fluorescein−tetramethylrhodamine-labeled RNA loop RNA.

Here, we have demonstrated the utility of a novel FRET assay to monitor in real-time the degradation kinetics of short RNAs by a purified RNase and in S100 cytosolic HeLa cell extract. We find that single-stranded RNA 2 is degraded more rapidly than the stem−loop RNA 1 under all conditions tested except for low concentrations of cell extract. Furthermore, our assay allows for the observation of in-assay inhibition of the RNase activity using inhibitors such as Contrad70 and ATA. Observation of the exact sites of cleavage using gelFRET confirmed that the change in FRET was a result of nuclease activity. Extension of these methods to living cells to probe cellular processes involving short RNAs, such as siRNAs, is under active investigation.

Acknowledgment. This work was supported by NIH Grant GM62357, ACS-PRF Grant 37728-G7, and a Dow Corning endowment. We thank Tom Kerppola for use of his FluorImager, and David Engelke and Danny Reinberg for HeLa cell extract.

Supporting Information Available: Details of RNA synthesis, fluorescence methods, and additional figures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

Figure 2. Degradation of 50 nM RNA 1 by 5% (v/v) S100 HeLa cell extract. (a) Contrad70 inhibits degradation by S100 HeLa cell extract, as evident from the lack of a FRET decrease after inhibitor addition, while Superselzn has minimal effects. (b) ATA is the most potent inhibitor of RNA 1 degradation by both RNase T1 and S100 HeLa cell extract. (c) RNA 1 degradation by S100 HeLa cell extract, stopped at the indicated times and analyzed by gelFRET. Primary nuclease activity is that of a 5′ to 3′ exonuclease, as evident from the fluorescein−tetramethylrhodamine-labeled (yellow) 15 n and 14 nt cleavage bands, followed by a size gap and release of a short fluorescein-only labeled (green) band, as indicated.