METHOD

A fluorescence-based assay for 3' → 5' exoribonucleases: Potential applications to the study of mRNA decay

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ABSTRACT

A cell-free mRNA decay assay has been adapted to permit the kinetics of 3' → 5' exoribonuclease activities to be monitored in real time. RNA probes containing 5' caps and 3' poly(A) tails generated by transcription *in vitro* are 3' labeled using fluorescein-N⁶-ATP and poly(A) polymerase. Release of fluorescein-conjugated adenosine residues from the 3' end of the RNA substrate is monitored by a time-dependent decrease in fluorescence anisotropy in the presence of cytosolic proteins. To demonstrate the utility of the assay, an RNA probe was constructed containing a fragment of the *c-myc* 3' untranslated region and an 85-base poly(A) tail. Following 3' fluorescein labeling, the rate of 3'-terminal adenosine excision was monitored in the presence of an S100 cytosolic extract prepared from K562 erythroleukemia cells. Removal of the fluorescein-tagged A residues resolved to a first-order decay function, allowing the rate constant and enzyme-specific activity to be determined in this extract. Further applications and advantages of this technology are discussed.

Keywords: enzyme kinetics; fluorescein; fluorescence anisotropy; RNA processing; RNA turnover

INTRODUCTION

In both prokaryotes and eukaryotes, 3' → 5' ribonuclease activities are essential for many aspects of RNA metabolism. For example, a number of 3' → 5' exoribonucleases are involved in the processing of transfer and ribosomal RNA precursors in bacteria (Reuven & Deutscher, 1993; Li et al., 1999), as well as the degradation of structured RNAs (Coburn et al., 1999). In eukaryotes, 3' → 5' exoribonucleases also participate in tRNA maturation (Papadimitriou & Gross, 1996) and pre-mRNA processing (Amrani et al., 1997; Brown & Sachs, 1998). Components of the exosome complex (Mitchell et al., 1997; Allmang et al., 1999) displaying 3' → 5' exonucleolytic activities are required for efficient rRNA processing (Zanchin & Goldfarb, 1999) and

contribute to the turnover of some mRNAs in yeast (Jacobs Anderson & Parker, 1998). Although relatively little is currently known about the nucleases participating in hydrolysis of mRNA substrates in higher eukaryotes, current evidence indicates that 3' → 5' ribonucleolytic activities often contribute to both deadenylation and subsequent decay of the mRNA body in these systems (Chen & Shyu, 1995; Couttet et al., 1997; Brewer, 1999).

Cell-free mRNA decay systems have been highly instrumental in the investigation of mechanisms regulating mRNA turnover (Brewer & Ross, 1990; Ross, 1993; Ford et al., 1997; Wilson & Brewer, 1999). In general, a substrate mRNA is prepared either by transcription *in vitro* or by purification of polysomal RNA from cytoplasm. The use of *in vitro*-transcribed RNA substrates allows incorporation of a radiolabeled nucleotide, thus permitting direct detection of decay products by autoradiography or phosphorimager scan. The RNA substrate is incubated with a cytoplasmic extract to initiate

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the decay process in a mixture that usually contains a buffer system, mono- and/or divalent cations, ATP, GTP, and an ATP-regenerating system (Brewer & Ross, 1990; Ford et al., 1997). At selected time points, aliquots are removed from the reaction and the decay products are analyzed. Although a number of variations have been reported for cell-free mRNA decay assays, they remain limited by time constraints, including the need for stopping reactions at each time point (denaturant or SDS), cleaning the RNA (phenol:chloroform extractions, ethanol precipitation), fractionation of decay products (gel electrophoresis), and detection. Furthermore, the heterogeneous nature of mRNA decay products hampers quantitative assessment of nucleolytic activity.

In this study, we have adapted a cell-free mRNA decay system to allow real-time assessment of 3' → 5' exoribonuclease activity *in vitro*. This technique requires the construction of RNA probes contiguous with a poly(A) tail. Fluorescein-conjugated adenosine analogs are then added to the 3' end of the RNA substrate using poly(A) polymerase (PAP). 3' → 5' ribonuclease activity is detected by monitoring the decrease in fluorescence anisotropy observed following hydrolytic release of the fluorescent nucleotides. This assay offers several advantages over those currently available, including real-time data presentation and sensitivity to very low levels of nuclease activity. Furthermore, automated data collection facilitates the accumulation of sufficient data for statistical analyses or mathematical modeling. This technology will greatly facilitate the functional identification of factors involved in RNA processing and turnover, as well as permit quantitative characterization of these activities in reconstituted *in vitro* systems.

RESULTS AND DISCUSSION

Generation of polyadenylated transcripts with 3' fluorescein labels

RNA probes containing a fragment of the *c-myc* 3' UTR spanning the *c-myc* A+U-rich element (ARE) were synthesized both lacking (*myc*) and containing (*myc*-pA) an 85-base poly(A) tail by *in vitro* transcription from pG7*myc*-pA digested with *Sma*I or *Hind*III, respectively (Fig. 1A). [α - 32 P]UTP was added to each transcription reaction to generate probe-specific activities of 1,000 cpm/fmol. Inclusion of a radiolabeled nucleotide was necessary for accurate quantitation of RNAs following subsequent steps in synthesis, as well as permitting probe integrity to be monitored by denaturing gel electrophoresis and autoradiography (Fig. 1B). The *myc* and *myc*-pA riboprobes resolved to single dominant bands of 196 and 293 nt, respectively.

The *myc*-pA riboprobe was 3' labeled with fluorescein-N⁶-ATP (FI-N⁶-ATP) and PAP to generate FI-*myc*-pA as described in Materials and Methods. Assuming that the quantum yield of the fluorescein-labeled adenosine was not significantly altered by polymerization at the 3' end of *myc*-pA RNA (discussed below), fluorescein was typically incorporated to a ratio of 0.3–0.5 mole fluorescein per mole of RNA under the conditions employed here. By denaturing gel electrophoresis, FI-*myc*-pA resolved to two principal species of roughly equal abundance; one comigrating with the nonfluorescent *myc*-pA probe (293 nt) and the other exhibiting retarded electrophoretic mobility (Fig. 1B). Given this binary distribution of RNA products and the calculated incorporation efficiency, it is likely that addition of FI-

FIGURE 1. A: A schematic of *c-myc* RNA substrates employed in this study. The probe *myc* encodes a fragment of the *c-myc* 3' UTR spanning the ARE, whereas *myc*-pA encompasses the same *c-myc* mRNA fragment appended to a poly(A) tail. A portion of the *myc*-pA synthesis was 3' tailed with FI-N⁶-ATP and poly(A) polymerase to generate FI-*myc*-pA. **B:** The integrity and product distribution of each RNA substrate was verified by denaturing gel electrophoresis.

N^6 -ATP to the 3' end of an RNA molecule inhibits further polymerization by PAP. Hence, the majority of fluorescein-labeled RNAs probably contain a single fluorescein-conjugated adenosine residue at the 3' terminus.

Incorporation of fluorescein N^6 -ATP does not impair 3' $\rightarrow$ 5' ribonucleolytic activity in vitro

To determine whether the presence of the fluorescein-conjugated adenosine residue at the 3' end of a poly(A) tail might influence 3' $\rightarrow$ 5' decay of an RNA substrate in vitro, cell-free mRNA decay assays were assembled in which 32 P-labeled *myc*-pA and FI-*myc*-pA RNAs were incubated with an S100 cytosolic extract from K562 cells. In accord with data published earlier (Brewer, 1998), the *myc*-pA substrate was degraded over time, generating a heterogeneous distribution of smaller products consistent with 3' $\rightarrow$ 5' shortening of the poly(A) tail (Fig. 2A). In addition, a single intermediate product was apparent within 20–30 min that comigrated with the nonpolyadenylated *myc* RNA. It must be noted that transcription from plasmid pG7*myc*-pA digested with

*Hind*III results in a nonameric sequence UAUUAAGCU being located at the extreme 3' end of *myc*-pA RNA, downstream of the A_{85} homopolymer. However, the generation of diffuse products leading to an intermediate consistent with poly(A-) RNA indicates that this 3' sequence does not prevent the 3' $\rightarrow$ 5' ribonucleolytic machinery from recognizing and catabolizing the RNA substrate in vitro.

Parallel decay assays using the FI-*myc*-pA substrate yielded results similar to experiments with *myc*-pA, in which the levels of full-length FI-*myc*-pA RNA decreased concomitant with the appearance of heterogeneous products and a decay product comigrating with poly(A-) RNA (Fig. 2B). These data indicate that the presence of a fluorescein-conjugated adenosine residue at the 3' terminus of the *myc*-pA RNA substrate does not significantly affect its 3' $\rightarrow$ 5' decay in a cell-free assay system.

3' $\rightarrow$ 5' ribonuclease activity monitored in real time by fluorescence anisotropy

To monitor 3' $\rightarrow$ 5' decay kinetics in real time, the removal of fluorescein-conjugated adenosine from the 3' end of FI-*myc*-pA was monitored by changes in the fluorescence anisotropy of the fluorescein moiety. Under conditions of constant temperature and viscosity, the anisotropy value is related to the molecular volume (Checovich et al., 1995; Jameson & Sawyer, 1995; Heyduk et al., 1996). Thus, enzymatic excision of FI- N^6 -AMP from the FI-*myc*-pA substrate may be detected by a decrease in the anisotropy of fluorescein as its environment changes from a large molecule (FI-*myc*-pA) to a smaller one (fluorescein-conjugated nucleotides).

A preliminary experiment was performed to determine whether the quantum yield of the fluorescein moiety was altered by these nuclease-induced changes in its environment. A plot of fluorescence intensity versus reaction time demonstrated no significant change in total fluorescence during the decay reaction, indicating that the quantum yield of fluorescein was not altered following its release from FI-*myc*-pA (Fig. 3A). With quantum yield held constant, the relative concentrations of each fluorescent species may thus be determined by their fractional contributions to total anisotropy (Weber, 1952; Otto et al., 1994; Jameson & Sawyer, 1995). Furthermore, limiting the presence of the fluorescein group to one of two molecular species (FI-*myc*-pA or FI-nucleotides) allows a first-order reaction constant (k) to be estimated based on an exponential regression of anisotropy time course data as defined in Materials and Methods.

Incubation of the FI-*myc*-pA substrate (5 fmol) with K562 S100 (5 μ g protein) resulted in a time-dependent decrease in fluorescence anisotropy that was well resolved by the first-order decay function (Fig. 3B). Fol-

FIGURE 2. Cell-free RNA decay reactions were programmed with *myc*-pA (A) or FI-*myc*-pA (B) in the presence of 200 μ g K562 S100 extract. Aliquots of the reaction mixture were removed at indicated time points as described in Materials and Methods and decay products were visualized by denaturing gel electrophoresis and phosphorimager scan. The mobility of each full-length RNA substrate is indicated by brackets. An aliquot of the *myc* RNA substrate was also loaded on each gel (lane poly(A-)) to identify the mobility of deadenylated intermediates, noted by arrowheads.

lowing the 20-min time course, ^{32}P -uridylylate retention was quantified by anion exchange on DE81 filters to verify that nucleolytic activity was limited to 3' → 5' shortening of the poly(A) tail in these assays. Because the vast majority of the uridylylate residues are located upstream of the poly(A) tail, minimal ^{32}P -release was expected to result from deadenylation activity alone. After the 20-min incubation, <10% of

the incorporated ^{32}P was released in reactions containing 5 μg S100 (data not shown). By contrast, a parallel reaction containing 1 μg RNase A released >80% of the ^{32}P -nucleotide.

Regression of anisotropy data to a first-order decay function allowed rate constants for excision of the fluorescein-conjugated nucleotides to be estimated. Averaged rate constants and calculated enzyme-specific activities from triplicate experiments are listed in Table 1. Increasing the S100 extract fourfold led to a threefold increase in reaction rate; however, the resulting enzyme specific activities were not significantly different between experiments using either 5 μg or 20 μg S100 protein. The activity responsible for removal of the fluorescent nucleotides was contained within the S100 extract, as reactions substituting bovine serum albumin for S100 did not generate changes in anisotropy values for fluorescein (Fig. 3C). Substitution of FI- βG -pA RNA for the fluorescent *myc* RNA substrate did not inhibit excision of fluorescent nucleotides from the RNA 3' end (Table 1), indicating that the measured nuclease activity likely reflects a number of 3' → 5' nucleolytic activities present in the cytosolic extract, rather than specific ARE-induced deadenylation per se (Xu et al., 1997; Brewer, 1998). Alternatively, initial hydrolytic events in poly(A) tail shortening might be uncoupled from overall mRNA decay rates. For example, the poly(A) tails of yeast *CYH2* and *PGK1* mRNAs are rapidly shortened following inhibition of transcription, yet the body of each mRNA is stable (Herrick et al., 1990), indicating that initiation of deadenylation is not rate limiting with respect to their turnover.

In our 3' → 5' ribonuclease assays using unfractionated S100 extracts, reaction rates may be slightly underestimated because of the presence of low levels of endogenous cytoplasmic RNAs that may compete for enzymatic activity. This effect would be accentuated in reactions containing higher concentrations of S100, but may be minimized by additional fractionation steps. A further limitation is the protein mass included in the reaction, as protein concentrations above 0.2 $\mu\text{g}/\mu\text{L}$

FIGURE 3. Fluorescence-based 3' → 5' RNA decay reactions were programmed with FI-*myc*-pA in the presence of 5 μg K562 S100 extract. Removal of fluorescein-labeled adenosine residues from the 3' end of FI-*myc*-pA RNA was monitored by fluorescence anisotropy as described in Materials and Methods. **A:** A plot of total fluorescence intensity versus reaction time was prepared to verify that significant changes in quantum yield did not occur as a result of fluorescein-AMP release from the FI-*myc*-pA RNA substrate. **B:** Time-dependent changes in fluorescence anisotropy following addition of FI-*myc*-pA to the cell-free decay reaction (top panel). Total measured anisotropy (A_t) was fitted to the equation $A_t = A_M + (A_P - A_M)e^{-kt}$ describing a first-order reaction as described in Materials and Methods (solid line). A residual plot was also prepared to detect any bias for subsets of the kinetic data (bottom panel). **C:** A control RNA decay assay was prepared in which 5 μg bovine serum albumin was substituted for the K562 S100 extract and monitored by fluorescence anisotropy.

TABLE 1. 3' → 5' ribonuclease activity in K562 S100 extracts monitored by fluorescence anisotropy.

RNA	Protein (μg)	$k(\text{min}^{-1})^a$	Specific activity ^b (pmol AMP released · min ⁻¹ · μg protein ⁻¹)
Fl-myc-pA	5	0.11 ± 0.02	1.1 ± 0.2
Fl-myc-pA	20	0.33 ± 0.04	0.8 ± 0.1
Fl-βG-pA	5	0.17 ± 0.02	1.7 ± 0.2

^aFirst-order reaction constants were determined by nonlinear regression of anisotropy time courses as described in Materials and Methods. Results are presented as the mean ± standard deviation of triplicate experiments except for Fl-βG-pA, which reflects duplicate experiments.

^bInitial velocity of the nuclease reaction was calculated from the first-order decay constant as $V_0 = k[\text{RNA}]_0$ with $[\text{RNA}]_0 = 5 \times 10^{-11}$ M. Specific enzyme activity was then determined by normalization to reaction volume and total protein mass.

result in nonspecific increases in anisotropy values, likely due to increased sample viscosity (data not shown). This also limits the use of some stabilizing additives such as glycerol in crude protein preparations. However, because enzymatic activity is measured by excision of the 3' terminal adenosine residue only, ribonuclease activity may be observed in much smaller protein samples than is possible using gel-based assay systems, where a larger portion of the RNA substrate must be removed for detection. Furthermore, the anisotropy-based detection system allows reaction progress to be monitored in real time, and permits quantitation of reaction parameters.

Further applications of this technology may include its use as a functional assay for the purification of novel factors participating in RNA processing or turnover, including nucleases and essential or inducible cofactors. Although multiple ribonucleolytic activities will undoubtedly be detected using this assay, additional experiments using purified fractions and/or alternative RNA targets will allow assessment of substrate specificity. In addition, this assay may be particularly suited for characterizing the enzymology of purified 3' → 5' exoribonucleases, as well as mutant or posttranslationally modified recombinant proteins. In some cases, these studies would be enhanced by placement of fluorophores throughout an RNA substrate, as the decay of uniformly labeled or double-labeled RNAs would allow additional mechanistic issues such as enzyme processivity to be evaluated. This presents a challenge using current technology, however, because most of the fluorescein-conjugated nucleotides available are linked to the fluorophore by long (10–12 atom) spacer arms. Although easily incorporated using bacteriophage RNA polymerases, the change in fluorescein anisotropy upon polymerization is likely to be minimal, owing to the rotational freedom afforded the fluorescein moiety by segmental motion throughout the spacer. However, as new fluorescent nucleotide analogs and labeling techniques

develop, alternative placement of fluorophores may allow this assay system to be extrapolated to the study of other nucleolytic events in real time, including decapping and 5' → 3' exonucleases.

MATERIALS AND METHODS

Construction of poly(A)-selectable in vitro transcription cassette

A DNA fragment containing an adenylate homopolymeric sequence was assembled by annealing oligomers 5'-GGG A₈₅TATTAAGCTTGGC-3' and 5'-GCCAAGCTTAATATT-3' (Operon) and extending with Klenow. This fragment was digested with *Hind*III and subcloned into pGEM7Z(+) (Promega) digested with *Sma*I + *Hind*III to generate the plasmid pG7-pA. A 152-bp fragment of the human *c-myc* 3' UTR including the *c-myc* ARE and the first polyadenylation hexamer was amplified from primers 5'-GCGAGCTAGCTAAAAG AACTTTTTTATGCTTACC-3' and 5'-GCACGGTACCACTG CTATAAACGTTTTATTAAAG-3' by polymerase chain reaction using *Pfu* DNA polymerase (Stratagene). This product was digested with *Nhe*I + *Acc*65I and subcloned into pG7-pA digested with *Xba*I + *Acc*65I to generate the plasmid pG7myc-pA. The fidelity of all constructs was verified by restriction mapping and automated DNA sequencing.

Synthesis of riboprobes

Templates for synthesis of *c-myc* RNA substrates were prepared by digesting pG7myc-pA with *Hind*III or *Sma*I to generate *c-myc* riboprobes containing or lacking the 3'-A₈₅ sequence, respectively. The template for a polyadenylated RNA probe encoding human β-globin mRNA was prepared by digestion of pSPκβ/c (Ross & Kobs, 1986) with *Hind*III. 5'-capped RNAs were generated by transcription in vitro in 40-μL reactions containing 1× optimized transcription buffer (Promega), 10 mM dithiothreitol, 80 U RNasin (Promega), 0.5 mM ATP, CTP, and UTP, 50 μM GTP, 0.5 mM 7-methyl-GTP (Sigma), and 0.5 μg linearized template DNA. T7 RNA polymerase (40 U; Promega) was added to initiate transcription of the *c-myc* riboprobes, whereas SP6 RNA polymerase (40 U; Promega) was used to generate the human β-globin RNA. [α -³²P]GTP (ICN) was included to give a final specific activity of 1,000 cpm/fmol RNA in each case. Reactions were incubated at 37 °C for 30 min before adding a further 20 U of the appropriate RNA polymerase. After a further 30 min at 37 °C, DNA templates were digested by addition of 2 U RQ1 DNase (Promega) and incubation at 37 °C for 30 min. [³²P]-labeled RNA probes were extracted twice with phenol: chloroform:isoamyl alcohol (25:24:1) and unincorporated nucleotides were removed using Sephadex G-50 Quick Spin columns (Roche Molecular Biochemicals). RNA yield was determined by liquid scintillation counting. The integrity of each RNA probe was verified by electrophoresis through 5% acrylamide:bis-acrylamide (40:1) gels containing 7 M urea and autoradiography.

Fluorescein labels were added to the 3' end of poly(A) tracts using a modification of a protocol by Martin and Keller (1998). Briefly, 1 pmol RNA was incubated in a 50-μL reac-

tion containing 100 pmol FI-N⁶-ATP (NEN), 40 U RNasin, and 700 U yeast PAP (Amersham Pharmacia) in the buffer provided by the manufacturer for 30 min at 37 °C. Following the fluorescein-labeling reaction, RNAs were purified by organic extraction and spin column chromatography as described above. The yield and integrity of fluorescein-labeled RNAs were also monitored as described above. The efficiency of fluorescein incorporation was determined by measurement of total fluorescence intensity using the Beacon 2000 Variable Temperature Fluorescence Polarization System (Panvera) equipped with fluorescein excitation (490 nm) and emission (535 nm) filters and comparison to a standard curve of FI-N⁶-ATP.

In vitro 3' → 5' ribonuclease assays

A cytoplasmic S100 extract was prepared from K562 erythroleukemia cells as described previously (Brewer, 1998) and total protein concentration was determined (Bradford, 1976). Cell-free RNA decay reactions were assembled in a final volume of 100 μL containing 10 mM Tris HCl, pH 7.5, 5 mM magnesium acetate, 100 mM potassium acetate, 2 mM dithiothreitol, 10 mM creatine phosphate, 1.4 U creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine, and 200 μg S100 extract. Reactions were prewarmed at 37 °C for 2 min prior to probe addition. At $t = 0$, RNA probe was added (20 fmol). Aliquots (15 μL) were removed at various time points and RNA decay terminated by addition of 400 μL ULB (10 mM Tris HCl, 7 M urea, 2% SDS, 350 mM NaCl, 1 mM EDTA, pH 8.0) containing 50 μg/mL tRNA. RNA samples were first extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform:isoamyl alcohol (24:1) before precipitation with ethanol. Recovered RNAs were then fractionated through 5% acrylamide gels (40:1 acrylamide:bis-acrylamide) containing 7 M urea. Gels were dried and radio-labeled decay products visualized using a PhosphorImager (Molecular Dynamics).

Real-time detection of 3' → 5' ribonuclease activity by fluorescence anisotropy

Fluorescence anisotropy measurements of mRNA decay reactions were made using the Beacon 2000 System (Panvera) equipped with fluorescein filters as described above. Reaction components (minus RNA) were assembled as described above in 6 × 50 mm glass tubes. To minimize changes in reaction viscosity, S100 protein was limited to 5–20 μg per reaction. The reaction tubes were prewarmed to 37 °C for 2 min in the polarimeter sample chamber before reading as blank. Following addition of the RNA probe (5 fmol), fluorescence anisotropy was measured in 15-s intervals, with five readings taken at each time point. All nonlinear regression fitting of anisotropic data was performed using PRISM software version 2.0 (GraphPad).

Under conditions of constant quantum yield, the measured fluorescence anisotropy A_t of the decay reaction may be interpreted by the additive function

$$A_t = \sum_i A_i f_i \quad (1)$$

where A_i represents the intrinsic anisotropy value of each fluorescent species and f_i its fractional concentration (Weber, 1952; Otto et al., 1994; Jameson & Sawyer, 1995). Thus, in the described decay reactions

$$A_t = A_P \frac{[P]_t}{[P]_0} + A_M \frac{[M]_t}{[P]_0} \quad (2)$$

where the intrinsic anisotropy values of the full-length FI-RNA and released fluorescein-conjugated nucleotides are given by A_P and A_M , respectively. Here, $[P]$ represents the concentration of fluorescein associated with RNA polymers and $[M]$ the concentration of released fluorescent nucleotides. Substituting the conservation of mass equation $[P]_0 = [P]_t + [M]_t$ and the first-order rate expression $[P]_t = [P]_0 e^{-kt}$ allows equation 2 to be simplified to

$$A_t = A_M + (A_P - A_M) e^{-kt}. \quad (3)$$

A plot of A_t versus t thus resolves the first-order reaction constant k , which is independent of concentration. Assuming that the 3' → 5' ribonuclease activity does not discriminate between fluorescein-labeled versus native A residues (Fig. 2), initial reaction velocity may thus be interpreted in terms of the total number of 3' A residues as $V_0 = k[\text{RNA}]_0$.

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