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Chemical reporters for monitoring RNA synthesis and poly(A) tail dynamics

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Understanding the mechanisms of gene expression requires a thorough knowledge of the balance between the biogenesis of an mRNA and its degradation. While advances in transcriptome profiling methods have enabled the large-scale analysis of RNA steady-state levels, monitoring the synthesis, processing and decay rates of specific transcripts remains technically challenging. The application of nucleoside analogs such as 4-thiouridine (4-sU) has recently enabled metabolic labeling and enrichment of nascent transcripts to monitor RNA dynamics in mammalian cells.^[1] Thiol-selective labeling allows the capture and enrichment of RNA but is incompatible with cellular imaging due to competing free thiols in cells. Alternatively, metabolic labeling with 5-ethynyluridine (EU) is compatible with fluorescence imaging of nascent transcripts in cells, tissues and model organisms following copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC or “click chemistry”) with azide-functionalized fluorescent dyes.^[2] Here we report N⁶-propargyl adenosine^[3] (N⁶pA; Figure 1a) is efficiently incorporated into RNA in mammalian cells by polyadenylate (poly(A)) polymerase and all three mammalian RNA polymerases: pol I, which transcribes ribosomal RNA in the nucleolus, pol II which transcribes mRNA and some noncoding RNAs, and pol III which transcribes specific small RNAs (tRNAs, *et al.*). We show that N⁶pA can be used for CuAAC-mediated fluorescence imaging and affinity enrichment of nascent transcripts. Finally, we demonstrate how “clickable” nucleosides can be employed to monitor poly(A) tail dynamics in cells.

To determine whether N⁶pA labels cellular RNA, we analyzed metabolic incorporation of N⁶pA into mammalian cells by fluorescence microscopy.^[2] HeLa cells were incubated in complete medium containing a range of N⁶pA concentrations (1.0 μ M - 1 mM). After 12 hours, the cells were washed, permeabilized, fixed, reacted with azido-rhodamine via CuAAC and visualized by fluorescence microscopy (Figure 1b). The majority of N⁶pA-specific fluorescence signal localized to distinct substructures of the nucleus, most likely nucleoli, in accordance with the dominant transcriptional activity of pol I. Incorporation of N⁶pA was observed with as little as 10 μ M of N⁶pA and appeared to reach its maximum between 10 μ M and 100 μ M. To distinguish between DNA and RNA labeling, we examined

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N⁶pA incorporation in the presence of hydroxyurea, a ribonucleotide reductase inhibitor, or actinomycin D, a transcription inhibitor (Figure 1c). While the addition of hydroxyurea had no effect, actinomycin D completely abolished fluorescence, demonstrating that N⁶pA is incorporated into RNA, but not DNA. We conclude that N⁶pA is efficiently incorporated into rRNA by pol I in cells.

In contrast to EU, N⁶pA is a potential substrate for incorporation into poly(A) tails by cellular poly(A) polymerases. To test this, we performed *in vitro* polyadenylation reactions with HeLa nuclear extracts, a uniformly ³²P-labeled RNA substrate and N⁶-propargyl adenosine-5'-triphosphate (N⁶pATP; Figure 1a). Incubation of the substrate in nuclear extract under polyadenylation conditions leads to the addition of a poly(A) tail of ~150–250 nt, as previously observed^[4] (Figure 2a, lanes 1 and 2). Inclusion of N⁶pATP in the reaction at various concentrations (lanes 3–5) had no apparent effect on polyadenylation efficiency, as judged by the intensity and lengths of the polyadenylated products (lanes 2–5). However, when ATP was replaced completely with N⁶pATP, no poly(A) extension was observed (lane 6). To show incorporation of N⁶pA into poly(A) tails, polyadenylation products were biotinylated using CuAAC with biotin-azide and captured on streptavidin beads. Polyadenylated RNAs generated in the presence of N⁶pATP were retained by streptavidin beads, whereas those generated in its absence were not (compare lanes 9–11 to lane 8). In principle, incorporation of only one N⁶pATP and its subsequent biotinylation should be sufficient for binding. However, the recovery of the polyadenylated substrates correlated with higher N⁶pATP:ATP ratios. This may indicate that N⁶pATP is not utilized by poly(A) polymerase as efficiently as ATP or that any one of the downstream steps (i.e. biotinylation, binding or retention on the column) requires more than one modified residue for maximal recovery. Interestingly, while N⁶pATP alone did not support processive polyadenylation, this substrate was recovered more efficiently than the control (lane 12 versus lane 7). It is therefore likely that a few N⁶pATP molecules were incorporated into the substrate, but the tail could not be further extended. Perhaps natural adenosines in the poly(A) tail are required to promote processive polyadenylation or ATP may be necessary for the activity of putative ATP-dependent enzymes (e.g. helicases) involved in polyadenylation in extract. In either case, these data clearly show that N⁶pA is utilized by a poly(A) polymerase *in vitro*.

Next we tested whether N⁶pA is incorporated into poly(A) tails in mammalian cells. To do this, we labeled HEK293 cells with 200 μM EU or N⁶pA for 12 hr, harvested cells, and extracted RNA. To degrade total RNA but leave poly(A) tails intact, we treated the RNA with RNase T1, a G-specific endonuclease, prior to biotinylation and selection. Only the poly(A) tails from cells labeled with N⁶pA bound the streptavidin column (Figure 2b), while the EU-treated or control (DMSO) poly(A) tails did not. These results demonstrate that N⁶pA is incorporated into poly(A) tails in cells. While several poly(A) polymerases are expressed in cells, our results are consistent with the conclusion that we are monitoring incorporation by the “canonical” poly(A) polymerase, PAPα.^[5] PAPα is responsible for the polyadenylation of cellular mRNAs that contain a typical AAUAAA poly(A) signal. Both our *in vitro* substrate (Figure 2a) and the majority of the “bulk” poly(A) tails (Figure 2b) fall into this category, so it seems likely that this is the poly(A) polymerase responsible for the activity we are monitoring. Further studies are necessary to determine whether the “noncanonical” poly(A) polymerases involved in a variety of alternative polyadenylation pathways^[6] can also utilize N⁶pA.

We next examined whether pol II and pol III utilize N⁶pA. For pol II, we needed to experimentally distinguish between pol II and PAPα activity in cells. To do so, we tested whether the histone mRNAs, which are non-polyadenylated pol II transcripts^[7], were labeled with N⁶pA. Total RNA from N⁶pA-treated cells was biotinylated, selected, and recovered transcripts were analyzed by semi-quantitative RT-PCR (Figure 3, top). H2A

mRNA was efficiently recovered from N⁶pA-treated cells as well as from positive control (EU) cells. In contrast, minimal H2A mRNA was observed in the negative control (DMSO). Similar results were obtained for histone HIST3H2BB mRNA (data not shown). Additionally, the pol III-transcribed 7SK RNA was detected among the selected transcripts from EU and N⁶pA-treated cells (Figure 3, bottom). From these results and those presented above, we conclude that N⁶pA is incorporated into RNA by pol I, II, III and PAPα.

The general pathway for destruction of cytoplasmic mRNAs begins with a slow distributive deadenylation of the transcript, which is followed by nearly coincident decapping, processive deadenylation, and transcript decay^[8]. In most cases, distributive deadenylation has been observed using specific reporter transcripts driven from inducible promoters, but has not been demonstrated at the bulk level. Changes in bulk poly(A) lengths have been limited to steady-state observations primarily due to the lack of simple approaches for global pulse-chase analysis of poly(A) tail lengths. While steady-state analysis can be informative (e.g.^[9]), interpreting poly(A) tail length differences in a pool of transcripts that contains both newly synthesized transcripts and older transcripts can be difficult. We therefore developed a pulse-chase strategy using EU or N⁶pA to study bulk poly(A) tail dynamics in cells. HEK293 cells were pulse-labeled with 200 μM EU or N⁶pA for 2 hr, washed, and chased with media lacking modified nucleoside. Cytoplasmic RNA was extracted from cells at given time intervals following the chase. Metabolically labeled transcripts were biotinylated by CuAAC, captured on streptavidin columns, and digested with RNase T1. Northern blot analysis of the resulting poly(A) tails showed that after a 2-hr pulse (t=0), the cytoplasmic poly(A) tails were ~150–200 nt long (Figure 4). Importantly, the tails shortened over time and decreased in their abundance, consistent with the model that most cytoplasmic mRNAs are gradually deadenylated prior to their destruction. This assay has the advantage of observing RNAs transcribed from endogenous promoters in the absence of general transcription inhibitors, which often have pleiotropic effects.^[10] The ability to detect bulk poly(A) tails after a short labeling pulse allows us to examine poly(A) dynamics of transcripts that were synthesized within a 2-hr window, thereby limiting the heterogeneity of poly(A) tail length that arises over time. We conclude that alkyne-nucleosides and click chemistry can be employed to examine poly(A) tail dynamics in cells. In principle, this approach could be extended to examine poly(A) tail dynamics of specific cellular mRNAs using previously described RT-PCR techniques to detect poly(A) tail length.^[11]

In summary, we report N⁶pA is incorporated by all three cellular RNA polymerases (pol I, pol II, pol III) in mammalian cells. Moreover, N⁶pA is added to poly(A) tails *in vitro* and *in vivo*, demonstrating that this chemical reporter is utilized by poly(A) polymerase. Furthermore, we showed that clickable nucleosides can be employed to monitor cellular poly(A) tail dynamics. These alkyne-adenosine reporters should provide new tools to monitor RNA metabolism *in vitro* and *in vivo* using bioorthogonal chemistry^[12] and vibrational spectroscopy.^[13]

Methods

Detailed methods are given in the supplementary information.

Acknowledgments

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Figure 1.

N^6pA is incorporated by pol I in cells. (a) Structure of N^6pA and N^6pATP . (b) Cells were incubated for 12 hours in media containing the indicated concentrations of N^6pA . After fixation and permeabilization, rhodamine-azide was conjugated via click chemistry. Top panels are rhodamine signal and the bottom panels are nuclei counterstained with TO-PRO-3. (c) Cells were incubated with 1 mM N^6pA in the presence of actinomycin D or hydroxyurea as indicated; fluorescent assay performed as in (b).

Figure 2.

N⁶pA is incorporated into poly(A) tails in vitro and in vivo. (a) In vitro polyadenylation assays with a ³²P-labeled “pre-cleaved” substrate in HeLa nuclear extract.^[4] The substrate is a 247-nt in vitro transcribed RNA derived from the 3′ end of PANΔ79 RNA^[14] that contains the AAUAAA polyadenylation signal and terminates at the natural cleavage site. As a result, polyadenylation is uncoupled from the endonucleolytic cleavage step of 3′-end formation. The concentrations of N⁶pATP and ATP in the reaction are shown above each lane. Lanes 1–6 contain the products of the polyadenylation reaction (20% of the total reaction), while lanes 7–12 are the streptavidin-bound transcripts. (b) N⁶pA is incorporated into poly(A) tails in vivo. 10% of the RNase T1-treated RNA (Input) or 100% of the streptavidin-bound RNA was analyzed by northern blot using an oligo dT probe to detect poly(A) tails. The experimental flow is shown to the right.

Figure 3.

N⁶pA is incorporated into pol II and pol III transcripts in vivo. RNA harvested from cells treated with DMSO, 200 μ M EU, or 200 μ M N⁶pA for 12 hr was subject to click chemistry with biotin-azide and selected on a streptavidin column. 10% of the input and 100% of the bound RNA was analyzed by semi-quantitative RT-PCR using primers specific for the histone H2A mRNA or the 7SK small noncoding RNA. Negative controls lacking reverse transcriptase are also shown (-RT).

Figure 4. Pulse-chase assay to analyze poly(A) tail dynamics. (A) Experimental flow. Cells were labeled for two hours with EU or N⁶pA and chased for 2, 4, or 6 hr prior to cytoplasmic RNA extraction. Poly(A) tails of captured transcripts were then analyzed by northern blot using an oligo dT probe. (B) Data from an in vivo poly(A) tail dynamics assay.