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An RNase P-based assay for accurate determination of the 5'-deoxy-5'-azidoguanosine-modified fraction of *in vitro* transcribed RNAs

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Abstract

Chemo-enzymatic approaches are important for generating site-specific, chemically-modified RNAs, a cornerstone for RNA structure-function correlation studies. T7 RNA polymerase (T7RNAP)-mediated *in vitro* transcription (IVT) of a DNA template containing the G-initiating class III $\Phi 6.5$ promoter is typically used to generate 5'-chemically-modified RNAs by including a guanosine analog (G-analog) initiator in the IVT. However, the yield of 5'-G-analog-initiated RNA is often poor and variable due to the high ratios of G-analog:GTP used in IVTs. We recently reported that a T7RNAP P266L mutant afforded a ~3-fold increase in fluorescent 5'-thienoguanosine-initiated pre-tRNA compared to the wild type. Here, we further explored the utility of T7RNAP P266L to generate 5'-deoxy-5'-azido-guanosine (^{az}G)-initiated RNA and found that the mutant generated ~4-fold more ^{az}G-initiated pre-tRNA^{Cys} than the wild type in an IVT containing a 10:1 ratio of ^{az}G:GTP. For accurate quantitation of the 5'-^{az}G-initiated RNA fraction, we employed RNase P, an endonuclease that catalyzes the removal of the 5'-leader in pre-tRNAs. Importantly, we show how RNase P can be leveraged for assessing 5'-G-analog incorporation in

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Author Contributions

SEL and VG prepared the manuscript, and all authors approved the final version of the manuscript.

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Supporting Information

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

Figure S7

Table S1

Table S2

any RNA by rendering the target, upon its binding to a customized external guide sequence RNA, into an unnatural substrate of RNase P. Such an approach in conjunction with T7RNAP P266L-based IVTs should aid chemo-enzymatic methods that are designed to generate 5'-chemically-modified RNAs.

Graphical Abstract

RNAs with site-specific chemical modifications are important tools in RNA structure-function studies.^[1] The chemo-enzymatic approach^[1–2] to site-specifically introduce a modified nucleoside/nucleotide monophosphate analog at the 5'-end of RNAs during *in vitro* transcription (IVT) is particularly attractive due to its low cost and its applicability to synthesize any full-length RNA (> 25 nt) at reasonable yield. T7 RNA polymerase (T7RNAP) has long been preferred in IVTs that have been employed to generate such 5'-chemically modified RNAs because of its tolerance to initiate RNAs with guanosine analogs^[3] (G-analogs; see Table S1 for a representative list of analogs used in previous IVTs with T7RNAP and the G-initiating class III $\Phi 6.5$ promoter).

The ease of T7RNAP-based IVTs to incorporate 5'-G-analogs in RNAs is counterweighed by the low yields that result from (i) use of high G-analog:GTP ratios in IVTs, and (ii) the propensity of T7RNAP to abort transcription when RNAs are initiated with G-analogs.^[4] We recently demonstrated^[4] that the decreased yield could be partly overcome by use of the Pro266Leu (P266L) mutant derivative of T7RNAP, which has a decreased propensity for abortive transcription.^[5] Use of the P266L mutant compared to the wild type (WT)^[4] afforded a 3-fold increase in the IVT yield of pre-tRNA^{Cys} initiated with thienoguanosine (thG), a fluorescent guanosine surrogate.^[6] When an IVT performed with this mutant was coupled with a one-pot multi-enzyme (OPME) method (Figure S1),^[4] which entails a post-transcriptional clean-up step using sequentially a pyrophosphohydrolase and an exoribonuclease to specifically degrade 5'-GTP-initiated pre-tRNA^{Cys}, we obtained near-homogeneous 5'-thG-initiated pre-tRNA^{Cys} at good yield (40 μ g/100 μ L IVT).

In our recent study,^[4] we chose to study 5'-thG-initiated RNAs because the intrinsic fluorescence of thG together with Abs₂₆₀ measurements permitted an easy quantitation of the fraction of *in vitro* transcribed RNAs initiated with either thG or GTP. While exploring the potential of T7RNAP P266L to synthesize RNAs initiated with non-fluorescent G-analogs, we recognized the need for an accurate method to assess the fractional content of GTP- versus G-analog-initiated RNAs present at the end of an IVT. To this end, we describe the development and validation of a new assay to measure the fraction of RNAs initiated with a G-analog in IVTs by exploiting RNase P,^[7] an endonuclease that cleaves the 5'-leader in pre-tRNAs. Here, we demonstrate the utility of T7RNAP P266L in synthesizing 5'-deoxy-5'-azidoguanosine (^{az}G)-initiated RNAs. Additionally, we showcase the value of the OPME approach in generating near-homogeneous 5'-^{az}G-initiated RNA through targeted depletion of GTP-initiated RNAs.

This work was motivated by the fact that despite the increasing popularity of using click chemistry to conjugate spectroscopic or chemical (e.g., biotin) handles through the reaction between an alkyne and an azide to generate a 1,4-disubstituted 1,2,3-triazole,^[1–2, 8] there is an incomplete understanding of the variability in the fractional content and yield of 5'-modified RNAs generated during IVTs. In one study, 5'-azG was reported to be the exclusive initiator of a 66 nt RNA (IVT containing 4:1 azG:GTP), an inference based on the complete conversion of this RNA to a “clicked” end product;^[8a] in this instance, yields were not reported. In another study, increasing the azG:GTP ratio from 5:1 to 100:1 led to a ~2-fold increase (from 36% to 81%) in azG incorporation and a ~2-fold decrease (from 7.6 to 3.6 µg/200 µL IVT) in yield of a 76 nt-long 5'-azG-initiated RNA;^[8b] the latter was quantitated using the colorimetric reaction with 6-heptynyl p-nitroaniline. The high azG incorporation resulting from use of a 100:1 azG:GTP in the IVT was offset by a very low yield, although this is partly attributable to the use of 0.2 mM rNTPs in these specific IVTs.^[8b] In a comprehensive study that examined incorporation of O-(5'-guanosine)-O-propargyl monophosphate (GMP^{Prg}) as the initiator in five different RNAs (~60 nt),^[9] a 9:1 GMP^{Prg}:GTP ratio led to 21 to 38% G-analog incorporation and yields ~20 µg/300 µL IVT. These yields, which were calculated post-HPLC purification of the “fluor-clicked” end product,^[9] were likely enhanced by use of a fed-batch method^[10] that entailed periodic supplementation of GTP during the IVT.

Here, we focused on azG incorporation by T7RNAP P266L and developed a method to determine the fraction of RNAs initiated with either azG or GTP. We first considered using click chemistry with an alkyne-bearing fluorophore to detect the fraction of 5'-azG-initiated RNAs; however, we abandoned this idea because any non-covalently bound fluorophore that remains even after purification of the modified RNAs would render azide measurements inaccurate. We then explored a spectrophotometric assay that detects azides on the basis of a light-absorbing, ferric-azide complex ($\lambda_{\text{max}} = 450 \text{ nm}$);^[11] although we obtained a good standard curve for azG (data not shown), we found that reliable detection required high (> 0.1 mM) concentrations of azG-initiated RNAs. These roadblocks inspired us to design an alternative method, preferably with general applicability to studies of many G-analog initiators.

We chose to exploit the expected difference in electrophoretic mobility of small RNAs containing either 5'-triphosphate or a 5'-hydroxyl, since the latter lacks the negative charges present in GTP. Because IVTs are typically used to generate RNAs > 25 nt, any method that leverages electrophoretic mobility differences in standard denaturing PAGE requires an additional step to focus on a short (< 10 nt) segment corresponding to only the 5' region of the target RNA.^[12] We address this challenge using a two-phase approach: after confirming the expectation that small RNAs containing either a GTP or an uncharged G-analog at the 5'-end are resolvable by PAGE and easily quantitated, we customized an endonuclease to cleave longer RNAs and release short 5'-fragments for subsequent analysis by PAGE.

RNase P is an endonuclease that catalyzes the removal of 5'-leaders from precursor tRNAs; it functions as either a ribonucleoprotein (powered by a catalytic RNA) or an RNA-free, protein-only form.^[13] For the studies described here, we have employed exclusively the biochemically well-characterized *Arabidopsis thaliana* protein-only RNase P 3 (A^PRORP3,

60 kDa).^[14] We chose pre-tRNAs for our initial tests because cleavage of the 5'-leader of a pre-tRNA, initiated with either ^{az}G or GTP, was expected to produce as cleavage products small RNAs bearing either 5'-^{az}G or 5'-GTP, which in turn could be resolved by denaturing PAGE and quantitated to obtain a reliable measurement of the percent of 5'-^{az}G-initiated RNA. To test this idea, we performed IVTs (which included or excluded ^{az}G) to generate *A. thaliana* pre-tRNA^{Cys} with a 5 nt leader and cleaved it with recombinant *A*PRORP3 (Figure 1A). Indeed, the two expected products (5'-^{az}GGUUU-3' and 5'-ppp-GGUUU-3') are easily separated (Figure 1). We included α -³²P-UTP in our IVTs to enable visualization and quantitation of the two 5 nt leaders. To establish size markers for the expected products, we used *A*PRORP3 to cleave pre-tRNA^{Cys}, which was generated in a standard IVT, to yield 5'-ppp-GGUUU-3' (Figure 1B). We then further processed this sample with a calf-intestinal phosphatase (CIP) treatment to dephosphorylate 5'-ppp-GGUUU-3' and generate 5'-OH-GGUUU-3', which, as expected, migrated similarly to 5'-^{az}GGUUU-3'; this migration pattern is expected since 5'-OH- and 5'-^{az}G-GGUUU-3' lack the 5'-triphosphate-associated negative charges (Figure 1B). We independently ascertained the presence of 5'-^{az}G in this RNA using the colorimetric ferric chloride-based assay^[11] (data not shown).

With the PRORP-based assay in hand, we investigated potential advantages afforded by T7RNAP P266L compared to the WT in generating 5'-^{az}G-initiated pre-tRNA^{Cys}. For pairwise comparisons, we used ~2 μ g of T7RNAP WT and P266L in our IVTs as described previously.^[4] First, we examined if aborted transcription with T7RNAP WT was indeed a roadblock in IVTs containing ^{az}G. For a sensitive visualization in this regard, we conducted "hot", small-scale transcriptions with α -³²P-UTP. With T7RNAP WT, we observed a substantial decrease in the full-length (likely due to an increase in aborted transcripts), when the ^{az}G:GTP ratio was increased progressively from 0:1 to 4:1 to 10:1 (Figure S2). In contrast, T7RNAP P266L produced more full-length pre-tRNA^{Cys} than T7RNAP WT regardless of the ^{az}G:GTP ratio used (Figure S2); this finding mirrors our previous observation with thG as the G-analog initiator in IVTs.^[4]

Second, for the purpose of assessing changes in the yield of RNAs from IVTs performed with the T7 RNAP P266L mutant compared to the WT, we set up "cold" IVTs using different ratios of ^{az}G:GTP. At 4:1 ^{az}G:GTP, we found that T7RNAP WT incorporated ^{az}G into $43 \pm 3\%$ of pre-tRNA^{Cys} transcripts and yielded $22 \pm 2 \mu$ g (total RNA)/100 μ L IVT; at 10:1 ^{az}G:GTP, there was only a modest increase in percent incorporation of ^{az}G ($52 \pm 1\%$) while the yield decreased by 2.2-fold to $10 \pm 1.5 \mu$ g/100 μ L IVT (Figures 1B, 1C, and S3). In contrast, when T7RNAP P266L was used in an IVT with a 4:1 ratio of ^{az}G:GTP, we observed $60 \pm 1\%$ incorporation of ^{az}G into pre-tRNA^{Cys} and a total RNA yield of $42 \pm 7 \mu$ g/100 μ L IVT; at 10:1 ^{az}G:GTP, percent incorporation of ^{az}G was $76 \pm 1\%$ and total RNA yield as $29 \pm 1 \mu$ g/100 μ L IVT (Figures 1B, 1C, and S4). Thus, the use of T7RNAP P266L compared to the WT in IVTs performed with either 4:1 or 10:1 ^{az}G:GTP led to ~2.5- or 4-fold, respectively, increased yield of 5'-^{az}G-initiated pre-tRNA^{Cys}. At least three independent replicates confirmed this trend.

Lastly, because GTP cannot be excluded from any IVT, some fraction of GTP-initiated transcripts is inescapable regardless of the ratio at which GTP is used with a G-analog initiator. This caveat motivated the design of the OPME approach (Figure S1),^[4] which we

tested here using the pre-tRNA^{Cys} generated in an IVT using T7RNAP P266L and a 10:1 ratio of ^{az}G:GTP. The OPME method led to 20 ± 1 µg/100 µL IVT of nearly homogeneous (93 ± 3%) 5'-^{az}G-initiated pre-tRNA^{Cys} (Figures 1B, 1C, and S5).

Building on the value of using *A*PRORP3 to accurately determine the fraction of 5'-^{az}G-initiated pre-tRNA^{Cys} (natural substrate of *APRORP*), we sought to extend the utility of this approach to any RNA generated in an IVT. To this end, we exploited the finding that any RNA could be tailored for targeted, site-specific cleavage by RNase P if binding of the target RNA to a customized external guide sequence (EGS) leads to a sequence- and structure-specific complex resembling the pre-tRNA (Figure 2A).^[15] While various aspects of substrate recognition by *A*PRORP3 have been studied,^[13a,13c] the ability of this enzyme to cleave bipartite substrates was unknown, a gap that we first addressed.

To render any target RNA·EGS complex an efficient non-natural substrate of *A*PRORP3, the EGS design must take into account the substrate-recognition determinants^[13a] of *A*PRORP3. Deletion of the anticodon (AC) stem-loop in a pre-tRNA^{Gly} had no effect on either the binding [$K_{M(STO)}$] or the rate of cleavage (k_{react}) by *A*PRORP3 under single-turnover (STO) conditions;^[13a] in contrast, deletion of the D stem-loop increased $K_{M(STO)}$ ~57-fold and decreased k_{react} 5-fold. However, substitution of the D stem-loop with a 9-nt loop in a model substrate (not a pre-tRNA) increased $K_{M(STO)}$ ~27-fold and had no effect on k_{react} . These findings inspired us to design an EGS that would result in a final substrate where the AC loop is disrupted and the D stem-loop is replaced by a 9-nt loop (Figure 2A).

To test our bipartite-substrate strategy, we designed a Model RNA I (MR1, 30 nt) and an External Guide Sequence 1 (EGS1) customized for MR1 (Figure 2A). Recognition and cleavage of the MR1·EGS1 complex by *A*PRORP3 was expected to produce 5 nt (5'-GGUUU-3') and 25 nt products. When 5'-[³²P]-MR1 was hybridized to EGS1, we observed ~90% cleavage by *A*PRORP3 under the appropriate assay conditions (Figure 2B). We then performed IVTs with T7RNAP P266L, α-³²P-UTP, and a 4:1 ratio of ^{az}G:GTP to generate internally radiolabeled MR1. Cleavage of the MR1·EGS1 complex by *A*PRORP3 and quantitation of the resulting products revealed that 58 ± 4% of the RNAs was initiated with 5'-^{az}G (Figure 2C; mean and standard deviation determined from three independent measurements).

*A*PRORP3 has been shown to use only N₋₁ and N₋₂ in guiding its cleavage between N₋₁ and N₊₁ (N₊₁ is the first position in the mature tRNA).^[14c] Thus, it is unlikely that the fraction of 5'-^{az}G-initiated RNA determined above could be influenced by a potential bias in the recognition/processing of 5'-^{az}G- versus 5'-GTP-initiated MR1 by *A*PRORP3 given that the modification is located at N₋₅. Regardless, we tested this potential bias by performing a time-course assay using MR1 RNA that was transcribed by T7RNAP P266L using either a 0:1 or 4:1 ratio of ^{az}G:GTP (Figures S6). Under single-turnover conditions, the rate of cleavage of MR1 transcribed under these two conditions was roughly the same (Figure S6). As expected, the percent of MR1 cleaved varied depending on the time of incubation (from 17 to 87% in this experiment), every time point yielded approximately the same quantitation with respect to the fraction of 5'-^{az}G-initiated MR1; averaging all time points resulted in a mean ± standard deviation of 62 ± 6%. Since even partial cleavage of a target RNA by

*A*PRORP3 suffices for a reliable measure of 5'-G-analog incorporation, there is no need to optimize the EGS and/or assay conditions to obtain near-complete cleavage of different target RNAs. Tweaking the annealing conditions, however, may be necessary for long target RNAs where the EGS binding site is not accessible.

We then used the *A*PRORP3- and EGS-based assay to determine the fraction of 5'-^{az}G-initiated RNAs in a second model RNA (MR2, 45 nt), which upon assembly with EGS1 and cleavage by *A*PRORP3 was expected to yield 8- nt (5'-GGUUUAUU-3') and 37-nt products (Figure S7). After obtaining MR2 RNA from IVTs containing a 4:1 ratio of ^{az}G:GTP, the PRORP-based assay revealed that 84% of MR2 RNA was 5'-^{az}G-initiated (Figure S7). While the electrophoretic mobility differences for the 8 nt 5'-^{az}G versus 5'-GTP were expectedly less pronounced than the 5 nt counterparts obtained from cleavage MR1, the two products from MR1 were easy to resolve and quantitate (Figure S7).

The PRORP (RNase P)-based assay described here provides a sensitive and easy method to calculate the fraction of 5'-G-analog-initiated RNAs generated in an IVT. Although we have provided proof of principle with 5'-^{az}G, we expect the method to be broadly applicable to any G-analog whose mass and charge difference with GTP will result in an electrophoretic mobility difference. In practice, the determination of the exact fraction of 5'-G-analog-initiated RNAs will require an EGS RNA and recombinant *A*PRORP3. The EGS can be either purchased or easily generated from an IVT using a pair of synthetic oligonucleotides, and the protein can be isolated using a single affinity purification step. This investment, however, will lead to various payoffs.

Foremost, an accurate determination of the extent of 5'-modification will decrease chemo-enzymatic synthesis costs by dialing down the stoichiometric excess of fluors or spin labels (or another probe) based on the exact amount of 5'-modified RNA rather than the total RNA as is customary. Second, our work reveals that empirical fine-tuning, based on accurate quantitation, is important for success of the chemo-enzymatic strategy. For example, our tests of 4:1 and 10:1 ratio of ^{az}G:GTP in IVTs revealed that T7RNAP P266L yields more 5'-^{az}G-initiated RNA than WT, with the 4:1 ratio engendering the best trade-off between RNA yield and percent of 5'-^{az}G-initiated RNA. Third, if a probe-tethered RNA is needed in large amounts for high-throughput enzyme assays or for structural studies, maximizing yield is critical. If the experimental work plan of the labeled RNA allows some latitude in terms of the 5' sequence in the target RNA, assessment of different 5'-leader sequences with the same EGS will help identify an initiating sequence that allows the highest fraction of G-analog-initiated RNA generated in an IVT. Such a recommendation is based on our finding that T7RNAP P266L generated 84% 5'-^{az}G-initiated MR2 compared to 58% with MR1 and pre-tRNA^{Cys}. While pre-tRNA^{Cys} and MR1 have Gs at +1, +2, +6, and +7, MR2 has Gs at only +1 and +2. Since G-analog-initiated RNAs are already more prone to result in aborted transcripts, the additional stress of multiple G residues in the first 8 nt of the nascent transcript probably accentuates aborted transcription due to the low concentrations of GTP used in these IVTs to favor initiation with the G-analog. Last, use of the OPME approach to enrich 5'-G-analog-initiated RNA is required only if there is a large fraction of 5'-GTP-initiated RNA, again lending importance to an accurate quantitation of that fraction.

In addition to validation of a customizable assay that affords a simple approach to calculate the fraction of 5'-G-analog-initiated RNAs at the end of an IVT, our results provide a second instance where (i) use of T7RNAP P266L in lieu of WT results in increased total RNA yield and percent of 5'-G-analog-initiated RNA, and (ii) the OPME approach can enrich the percentage of 5'-G-analog-initiated RNA to near homogeneity. These findings with ^{az}G parallel those obtained when thG was the G-analog, and underscore the tolerance of T7RNAP P266L to accept two different G-analogs. Our work should motivate studies to test other G-analogs (especially non-azide/alkyne initiators such as 5'-hydrazinyl-5'-deoxyguanosine^[16]), and determine if a common 10-nt leader/initiating sequence rich in A, C, U would afford high yields of RNAs initiated with different G analogs. Use of programmable RNases (e.g., Argonautes loaded with defined DNA guides^[17]) for site-specific cleavage of large RNAs and generation of smaller cleavage products bearing the 5'-G-analog initiator also merits consideration.

Experimental section

Overexpression and purification of recombinant proteins used in this study

Both WT and P266L T7RNAPs were overexpressed and purified as described earlier,^[4] while 9 A_{PRORP3} followed a previously published protocol but with only the first affinity chromatography step which sufficed to yield nearly homogeneous protein.^[14c]

Synthesis of ^{az}G (5'-deoxy-5'-azidoguanosine)

^{az}G was generated as described previously,^[18] by reacting 5'-deoxy-5'-iodoguanosine with sodium azide under aqueous conditions. The IR and ¹H-NMR data (not shown) confirmed the purity and identity of the final product. The ^{az}G stock used for IVTs was prepared in water; solubilization was facilitated by a 5 min incubation at 55°C.

In vitro transcription reactions

A PCR DNA template containing a class III Φ6.5 promoter (5'-TAA TAC GAC TCA CTA TA-3') and the gene encoding an *A. thaliana* mitochondrial pre-tRNA^{Cys} with a 5 nt 5'-leader and no trailer (Figure 1) was prepared as previously described.^[4, 19] DNA templates containing a class III Φ6.5 promoter and the genes for either MR1 or MR2 model RNAs were generated using a pair of DNA oligonucleotides for each RNA (Table S2; Sigma). These oligonucleotides were designed to be complementary at their respective 3'-termini; the 17-bp complement allowed fill-in with Phusion DNA Polymerase (NEB). The PCR amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN) and the amplicon concentration was determined using A₂₆₀ measurements on a NanoDrop 2000c spectrophotometer.

The IVT reaction conditions were identical to our previous study^[4] with respect to buffer, enzyme amounts, etc. IVTs containing ^{az}G were performed using 4.8 mM ^{az}G and either 1.2 mM GTP (4:1 ratio) or 0.48 mM GTP (10:1 ratio). To determine the total RNA yields of pre-tRNA^{Cys}, 100-μL IVTs were performed at 37°C for 5 h. Post-transcription, the DNA template was digested by addition of 10 U of DNase I (Roche) and the reaction was then subjected to phenol-chloroform extraction. Next, the samples were extensively dialyzed

against water at 4°C to remove unincorporated ribonucleotides; we used a 3,500-Da cut-off dialysis membrane. The RNA samples were recovered and then precipitated by ethanol precipitation. The resulting pellets were re-suspended in water and total RNA yield was determined using Abs₂₆₀ measurements on a NanoDrop 2000c spectrophotometer and the extinction coefficient of pre-tRNA^{Cys}.

To internally label pre-tRNA^{Cys}, MR1, and MR2 with ³²P, 20-μL IVTs were performed as described above, except with the inclusion of 20 μCi of α-[³²P]-UTP [3000 mCi/mmol; PerkinElmer, Waltham, MA]. We chose α-[³²P]-UTP because each of the expected cleavage products contained multiple U residues, thus enabling visualization of the 5'-leader post-PAGE regardless of whether the RNA had been initiated with ^{az}G or GTP. IVTs containing α-[³²P]-UTP were performed at 37°C for 3 h and subsequently quenched with an equal volume of loading dye containing 7 M urea, 10 mM EDTA, and 20% (v/v) phenol. The samples were then electrophoresed on an 8 or 12% (w/v) polyacrylamide/7M urea gel at 30 mA for 1 h. After identification by autoradiography, the full-length RNAs were excised and eluted in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.01% SDS (w/v). The elution was performed in two steps: 37°C for 3 h, and overnight at 4°C. The eluents were filtered through 0.22-μm cellulose acetate Spin-X centrifuge tube filters (Corning Costar, Corning, NY), and then subjected to ethanol precipitation. The radioactivity of the resulting RNA pellets was counted using a Bioscan QC-4000 XER radioisotope counter (Bioscan, Inc., Washington, D.C.) and the pellets were then typically resuspended in 10 to 20 μL of water.

Post-transcriptional removal of 5'-ppp-G-initiated RNAs

The OPME approach was performed essentially as described earlier^[4] but with two differences: the Xrn-1 incubation was performed exclusively at 37°C for 1.5 h, and the reactions were performed in a heat block.

PRORP-based assays to determine percent incorporation of ^{az}G in pre-tRNA^{Cys}

To determine the fraction of pre-tRNA^{Cys} initiated with either ^{az}G or GTP, we first performed “hot” IVTs and purified the full-length RNA as described above. Then, the RNAs were cleaved with recombinant *A*PRORP3. The cleavage assays (10 μL) were performed by incubating internally-labeled pre-tRNA^{Cys} RNA (~2000 dpm) with 2 μM *A*PRORP3 in 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM MgCl₂, 4 mM DTT, and 5% (v/v) glycerol for 45 min at 25°C. Reactions were quenched by adding an equal volume of quenching [7 M urea, 20% (v/v) phenol, 0.2% (w/v) SDS, 10 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] and then electrophoresed at 30 mA on a 20% (w/v) polyacrylamide/7 M urea gel (40 cm length x 20 cm width) for 1 h. The gel was then exposed overnight to a phosphorimager screen (at -80°C), and subsequently visualized using an Amersham Typhoon 5 Biomolecular Imager (GE Healthcare). Percent incorporation of ^{az}G was determined using ImageQuant 5.1 software-based quantitation of the slower (RNAs initiated with 5'-^{az}G) and faster (RNAs initiated with 5'-triphosphate) migrating RNAs; these two RNA products were generated by *A*PRORP3-mediated cleavage of pre-tRNA^{Cys}.

PRORP-based assays to determine percent incorporation of ^{az}G in MR1 and MR2

To determine if *At*PRORP3 would cleave an RNA-EGS hybrid (Figure 2B), MR1 and EGS1 mimicking part of mitochondrial *At* pre-tRNA^{Cys} were first assembled to form a non-covalent bipartite substrate. These RNAs were synthesized by Sigma Aldrich. A mixture of 400 nM MR1 (spiked with 2,000 dpm 5'-³²P-MR1 that was gel purified post-labeling with T4 polynucleotide kinase and γ -[³²P] ATP) and 4 μ M EGS1 were denatured at 95°C for 3 min, followed by annealing at 37°C for 10 min. Next, an equal volume of reaction buffer [for a final concentration of 20 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 10 mM MgCl₂ and 4 mM DTT] was added and the substrates were incubated for an additional 30 min at 25°C. The cleavage reaction was initiated by adding an equal volume of 8 μ M *At*PRORP3 in reaction buffer supplemented with a final concentration of 5% (v/v) glycerol. The cleavage assays (10 μ L) were performed using final concentrations of 100 nM MR1, 1 μ M EGS1, and 4 μ M *At*PRORP3. After defined periods of incubation at 25°C, the reaction was quenched using 1.5 volumes of quenching solution [7 M urea, 20% (v/v) phenol, 0.2% (w/v) SDS, 10 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol] and products were electrophoresed on a 20% polyacrylamide/7 M urea gel (20 cm length x 20 cm width) at 30 mA for 1 h. The product sizes were confirmed using for reference the product generated by 100 nM recombinant *E. coli* RNase P;^[20] this reaction was performed at 37°C for 20 min in 10 mM HEPES-KOH (pH 7.5), 400 mM NH₄OAc, 10 mM Mg(OAc)₂, 5% (v/v) glycerol and 0.01% (v/v) Igepal-40.

To test if the PRORP-based assay could be used to determine the fraction of 5'-^{az}G or 5'-GTP-initiated MR1/MR2 (Figure 2C and S7), we performed “hot” IVTs and purified the full-length RNAs as described above. The PRORP-based assay was performed as described above one change: cold MR1 or MR2 supplements were not included.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

T7RNAP:	T7 RNA polymerase
G-analog:	guanosine analog
nt:	nucleotide

OPME:	one-pot multi-enzyme
IVT:	<i>in vitro</i> transcription
azG:	5'-deoxy-5'-azidoguanosine
PRORP:	proteinaceous RNase P

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or using the OPME method with P266L. Mean and standard deviation values were calculated based on results from at least three independent measurements.

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Figure 2.

A) Schematic of the *AtPRORP3*- and EGS-based assay to assess the percent incorporation of 5'-azG in MR1. Upon *AtPRORP3*-mediated cleavage of MR1-EGS1, two different 5 nt leaders are generated: one having 5'-ppp and the other 5'-azG ("X" indicates different initiators). Open arrow indicates site of cleavage by RNase P. **B)** Cleavage of a bipartite substrate by *AtPRORP3*. A synthetic MR1 RNA oligo was 5'-³²P-labeled and annealed to EGS1. The lane designated "PC" indicates cleavage of MR1-EGS1 hybrid when *Escherichia coli* RNase P was used to generate an independent size marker. The lane designated "-" contains MR1-EGS1 hybrid but was not treated with any enzyme. **C)** Representative results from assays of MR1-EGS1 that was transcribed by T7RNAP P266L with 4:1 ratio of azG:GTP (lane labeled 4:1). The lane designated "-" contains MR1-EGS1 that was not treated with *AtPRORP3*, and the lane designated "5'ppp" reflects *AtPRORP3*-mediated processing of MR1-EGS1 generated by an IVT in which azG was excluded; *AtPRORP3* cleavage in this instance is expected to yield a 5 nt leader that has a 5'-ppp (due to initiation with GTP). The lane labeled "5'OH" mirrors the sample in "5'ppp" except that it was treated with CIP after incubation with *AtPRORP3*. Cleavage reactions were quenched after a 2-h incubation.