



5'-DEOXY-5'-HYDRAZINYLGUANOSINE AS AN INITIATOR OF T7 RNA POLYMERASE-CATALYZED TRANSCRIPTIONS FOR THE PREPARATION OF LABELING-READY RNAs

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- *5'-deoxy-5'-hydrazinylguanosine was incorporated into the 5'-termini of RNA transcripts using T7 RNA polymerase. Transcriptions provided 5'-hydrazinyl-RNA that was readily labeled and purified. The use of fluorophore-labeled material was validated in an endoribonuclease activity assay.*

Keywords T7 RNA polymerase initiator; fluorophore-labeled RNA

INTRODUCTION

Modified nucleic acid systems^[1–3] have facilitated mechanistic studies,^[4] structural studies,^[5,6] ribozyme selections,^[7] *in vivo* siRNA delivery,^[8] photochemical activity modulation,^[9] and the generation of new sensors^[10] and materials.^[11,12] RNA-based conjugates can be prepared *via* phosphoramidite chemistries or enzymatic methods,^[13] where enzymatic methods allow for the preparation of longer sequences. T7 RNA polymerase can incorporate modified 5'-initiating nucleosides based on guanosine^[14–19] or adenosine,^[11,20–22] which can introduce reactive chemical functionalities for bioconjugation. A limiting factor in the preparation of RNA conjugates is the time-consuming synthesis of the nucleoside-based precursors. The preparation of the 5'-deoxyguanosine derivatives is particularly challenging

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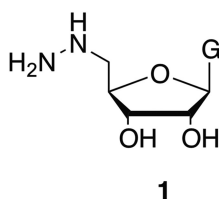


FIGURE 1 5'-deoxy-5'-hydrazinylguanosine.

because poor solubility often leads to difficult synthetic procedures and complex product mixtures that require time-consuming chromatography. The adoption of mechanochemical methods^[23,24] and ionic liquids^[25] has helped to overcome some of these difficulties, however, we have used aqueous approaches toward guanosine systems. These have included aqueous procedures for the phosphorylation of 5'-amino-5'-deoxyguanosine,^[26,27] and the preparation of 5'-deoxyguanosine derivatives, including 5'-deoxy-5'-hydrazinylguanosine **1** (Figure 1).^[28] We now report the use of 5'-deoxy-5'-hydrazinylguanosine **1** in T7 RNA polymerase-catalyzed transcriptions, subsequent biotinylation/fluorophore labeling of the transcripts, and the use of labeled materials in an endoribonuclease assay. The method is straightforward and offers nanomole quantities of labeled material that can be used as an alternative to radiolabeled materials.

EXPERIMENTAL

Determination of relative RNA yield and level of 5'-deoxy-5'-hydrazinylguanosine incorporation

Transcriptions were performed on a scale of 37.5 μ l where each experiment contained 40 mM Tris (pH 7.9), 10 mM DTT, 6 mM MgCl₂, 10 mM NaCl, 2 mM spermidine, 350–500 nM AtRib dsDNA template^[29] (see Supplemental file for details of template preparation), 1.25 mM of each NTP, [α -³²P]-UTP (3–5 μ l of 10 μ Ci/ μ l, 800 Ci/mmol stock) and 9.4 μ M T7 RNAP, prepared according to He *et al.*^[30] In addition, transcription experiments were supplemented with varying concentrations of the initiator 5'-deoxy-5'-hydrazinylguanosine **1**, where the preparation of 5'-deoxy-5'-hydrazinylguanosine **1** was reported earlier.^[28] The initiator 5'-deoxy-5'-hydrazinylguanosine **1** was added to transcription mixtures from a stock solution of 50 mM 5'-deoxy-5'-hydrazinylguanosine **1** dissolved in 50 mM NaOH solution. The final pHs of the transcription mixtures were 8.4–8.6. Transcription mixtures were incubated at 37.5°C for 2.5 h, before addition of RQ1 RNase-free DNase (1 μ l, 1 U) and further incubation at 37.5°C for 20 min. Unincorporated 5'-deoxy-5'-hydrazinylguanosine **1** was removed from each transcription mixture via gel filtration using spin-columns (ZEBATM Desalt, 0.5 ml). Each transcription mixture was then

denatured at 95°C in 2 × urea loading buffer (8 M urea, 20 mM EDTA, 2 mM Tris, pH 7.5) before being subjected to urea-PAGE using 1.5 mm thickness 12% gels. Products were separated by electrophoresis at 21 W for 2 h before visualization by phosphorimaging and UV shadowing. Phosphorimaging data were used to determine relative RNA yields whereas shadowing was used to locate transcripts within gels to allow excision. The transcript bands were removed from the gel, and RNA was extracted by passive elution (>15 h) into 0.3 M NaCl (400 μ l) by means of an end-over-end rotator. Gel fragments were removed using a filter spin-column before precipitation of the RNA transcripts by addition of EtOH. The RNA pellet was washed with 70% EtOH (250 μ l) and dissolved in water.

Determination of Level of 5'-deoxy-5'-hydrazinylguanosine Incorporation

Biotinylations were performed using 67 mM HEPES (pH 8.1), 3.3 mM sulfo-NHS-biotin, and approximately 1 μ M RNA in a total volume of 15 μ l. Reaction mixtures were incubated at room temperature for 2 hours before addition of 3 M NaCl (4.5 μ l), H₂O (29 μ l), and precipitation with EtOH (92 μ l). The RNA precipitate was collected by centrifugation, washed with 70% EtOH (2 × 200 μ l) and re-suspended in DEPC water (9 μ l). Each RNA sample was divided in two, one sample being diluted in a 1:1 ratio with streptavidin loading buffer [1:1 streptavidin (2 mg/ml): 2 × urea loading buffer], the second half was diluted 1:1 with 2 × urea loading buffer. The products were resolved by urea PAGE and the gels were dried before quantification by phosphorimaging.

Preparation of FITC-Labeled Stem-Loop Substrate via 1 Millilitre-Scale Transcription

DNA template oligonucleotides for *in vitro* transcription were purchased from Eurogentec and were dissolved in 1 × TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer to give final concentrations of 100 μ M. Prior to use in transcriptions, 12.25 μ l of the template strand (5'-GGTCAAACCT-GACTGCGCTTCGGACAGTACAAGCTTGACCTCTCCTATAGTGAGTCG-TATTA-3') and 12.25 μ l of the promoter strand (5'-TAATACCGACT CACTATAG-3') solutions were mixed with 70 μ l of DEPC-treated water in a 1.5 ml tube, and the mixture was heated to 95°C (5 min). The mixture was removed from the heat, allowed to cool at RT over the course of 30 minutes and stored on ice until use.

In vitro transcription reactions were performed on a 1000 μ l scale using 40 mM Tris (pH 8.2), 0.01% Triton X-100, 10 mM DTT, 16 mM MgCl₂, 2 mM spermidine, 1.25 mM of each NTP (diluted from a 4 × 6.25 mM, pH 8.2 stock), 9 μ M T7 RNAP,^[30] 4 mM 5'-deoxy-5'-hydrazinylguanosine

initiator **1** (from a 50 mM stock in 50 mM NaOH), RNasin (6 μ l, 240 U), and 1.2 μ M annealed DNA template. The reagents were mixed, collected in the bottom of the tube by centrifugation, and then incubated at 37.5°C for 2.5 hours. The DNA template was degraded by addition of RQ1 RNase-free DNase (27 μ l, 27 U) followed by a further 20 min incubation at 37.5°C. The mixture was centrifuged (1500 g, 30 seconds) to remove precipitated inorganic pyrophosphate, the supernatant was pipetted into two 500 μ l portions, then LiCl solution (8 M, 55 μ l) and cold ethanol (1 ml) were added to each portion. The samples were frozen (-20°C freezer overnight) and centrifuged (16100 g, 10 minutes). The supernatants were removed and the pellets were allowed to air-dry for 6 to 7 minutes before each being dissolved in DEPC-treated water (120 μ l). LiCl solution (8 M, 14.5 μ l) and cold ethanol (3.5 v/v) were added to each sample, the samples were then frozen and centrifuged, and the pellets were collected, washed, and dried as above. The pellets were each dissolved in DEPC-treated water (120 μ l) and supplemented with $2 \times$ loading buffer ($2 \times 120 \mu$ l). The samples were heated to 95°C (5 minutes), cooled to RT (20 minutes), and divided equally across three preparative (1.5 mm thickness) 12% urea gels. The products were separated by electrophoresis (15 W for 3 hours), and the resolved bands were visualized by UV shadowing to allow excision from the gel. The RNA was extracted from each gel piece by passive elution (15 hours) into 0.8 M LiCl ($3 \times 300 \mu$ l) on an end-over-end rotator (4°C overnight). Gel fragments were removed using 0.5 ml spin filtration columns, and the RNA extracts were precipitated by addition of cold ethanol (3×1 ml) and cooling on CO₂-acetone (20 minutes). After centrifugation (16100 g, 10 minutes) and removal of the supernatant, the RNA pellets were washed with 70% ethanol, air-dried (6–7 minutes), dissolved in DEPC-treated water ($3 \times 20 \mu$ l), and the solutions were combined. The pH of the RNA solution was then increased by addition of 1 M sodium carbonate (7.5 μ l, pH 9). Fluorescein isothiocyanate (FITC, isomer I, 7.5 μ l of a 0.1 M stock dissolved in dry DMF) was then added, and the mixture was allowed to react for 4 hours at 4°C on an end-over-end rotator. The reaction was quenched by addition of 1 M sodium acetate (pH 4.5, 22.5 μ l), and then LiCl solution (8 M, 11.8 μ l) and cold ethanol (350 μ l) were added. After cooling on CO₂-acetone (20 minutes) and centrifugation (16100 g, 10 minutes), the precipitated pellet was washed with 70% ethanol, air-dried (6–7 minutes), and dissolved in DEPC-treated water (60 μ l). The solution was pipetted carefully onto a 7 K MWCO ZebaTM Spin desalting column, followed by DEPC-treated water (15 μ l). Centrifugation (1500 g, 2 minutes) eluted the purified fluorescently labeled RNA solution ($\sim 73 \mu$ l), which was stored in the dark at -20°C . The concentration of the FITC.RNA stock was determined to be 87 μ M by UV absorbance at 260 nm on a Thermo Scientific NanoDrop 2000 spectrophotometer (average of two readings), to give a total yield of 6.3 nmol FITC.RNA. To assess the purity of the material, a 1 μ l sample was removed and added to 4 μ l DEPC-H₂O to give a

5× dilution. Two further 5× dilutions were made, and to each sample (5×, 25×, and 125× dilutions), 5 μ l of 2 × loading buffer was added. The samples were heated to 95°C before being loaded onto an analytical (0.8 mm depth) denaturing (urea) 12% polyacrylamide gel and separated by electrophoresis (7 W, 2 hours). Gels were visualized on a Fujifilm FLA-3000 phosphorimager using a 473 nm laser and a yellow filter.

RNase Assays

RNase assays were prepared using 5 × RNA cleavage buffer (100 mM HEPES, 500 mM KOAc, pH 7.6, 6 μ l), fluorescently-tagged RNA (to final concentration of 4.1 μ M), DTT (100 mM, 0.3 μ l), ADP (10 mM, 6 μ l), and RNasin (0.5 μ l, 20 U) in a 1.5 ml tube. The mixture was heated to 95°C and allowed to cool to RT. Mg(OAc)₂ (20 mM, 3 μ l) was added and the mixture was allowed to stand for 5 minutes. The reaction mixture was collected in the bottom of the tube by centrifugation before addition of Ire1 (0.25 or 1 μ l, respectively, of a 4.1 mg/ml solution in PBS+5% glycerol, for initial rates and full time-course experiments) in a total volume of 30 μ l.

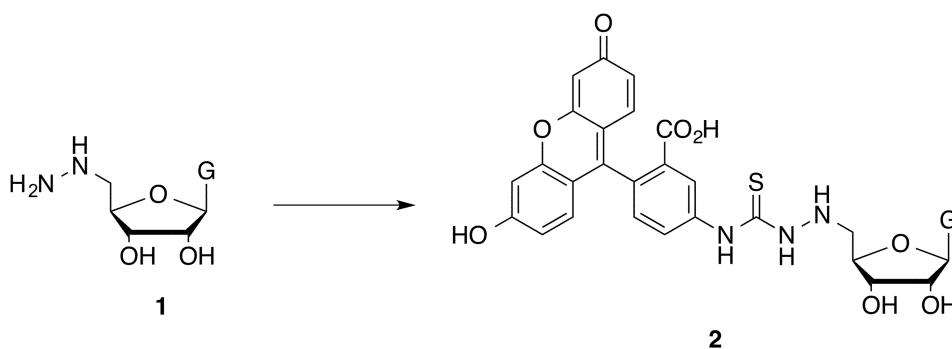
Immediately after the addition of Ire1, the tube was placed in a heat block set to 31°C. Samples of 5 μ l were removed from the reaction mixture at recorded time points and added directly to 2 × urea loading buffer (15 μ l). Each sample was flash frozen in CO₂-acetone before being stored at -20°C.

Once each time course was complete, the samples were rapidly defrosted before 10 μ l of each sample was removed and diluted with DEPC-treated water (7.5 μ l). The samples were heated to 95°C for 3 minutes, cooled slowly to RT before being resolved by 12% urea-PAGE on a 0.8 mm gel. Bands were separated by electrophoresis at 7 W for 2 hours. RNA bands were visualized on the phosphorimager (Fuji FLA 3000) via excitation at 473 nm and detection via a yellow filter. Band intensities were determined using the phosphorimager software, and the percentage of cleaved RNA was determined by calculating the relative intensities of the cleaved and uncleaved RNA bands for each time point.

Labeling 5'-deoxy-5'-hydrazinylguanosine 1 with Fluorescein Isothiocyanate (FITC) to Estimate Degree of RNA Labeling

5'-deoxy-5'-hydrazinylguanosine 1 (15 mg, 50 μ mol) was dissolved in triethylammonium bicarbonate (TEAB) solution (1 M, 22.5 ml, pH 8) and cooled in an ice bath. A solution of FITC (97 mg, 270 μ mol) in dry DMF (2.5 ml) was added to the reaction vessel dropwise over the course of 10 minutes. The reaction mixture was left to stir on ice for 4 hours before being lyophilized. The residue was then dissolved in ammonia solution (2.5 ml, 35% w/v), the mixture was stirred for 4 hours at room temperature, diluted with water (10 ml) and lyophilized. The resulting solid

residue was then purified by ion exchange chromatography on a DEAE Sepharose Fast FlowTM anion exchange resin (26 mm i.d. × 80 mm height) connected to an ÄKTApriTMplus chromatography system. The TEAB buffer gradient was constructed to rise from 0 M to 1 M over 200 minutes, with a flow rate of 4 ml/minute and detection at 254 nm. A control experiment was performed under identical conditions, in the absence of 5'-deoxy-5'-hydrazinylguanosine **1**. Residues from the control were also chromatographed, and the chromatograms for reactions with and without 5'-deoxy-5'-hydrazinylguanosine **1** were compared. In the presence of 5'-deoxy-5'-hydrazinylguanosine **1**, another species was apparent in the chromatogram, and this material was isolated by lyophilization, and analyzed by MS. The MS analysis confirmed the identity of this species as the adduct between FITC and 5'-deoxy-5'-hydrazinylguanosine **1**, namely thiosemicarbazide **2** (see Scheme 1 for details). We observed no evidence for the formation of *bis*-FITC-labeled products.



SCHEME 1 Model reaction to estimate the modification of the 5'-hydrazinyl functional group with FITC.

RESULTS

Transcriptions to assess relative RNA yields in the presence of 5'-deoxy-5'-hydrazinylguanosine initiator **1** and its level of incorporation were performed in the presence of varying concentrations of 5'-deoxy-5'-hydrazinylguanosine **1** and [α -³²P]-UTP using a known template dsDNA (ATRib, giving a 82 nt transcript).^[29] The choice of this template was based on our previous successful use of this system with modified guanosine initiators.^[26,27] Assessment of the total yield of RNA by phosphorimaging of a preparative urea-PAGE gel showed that increased concentrations of 5'-deoxy-5'-hydrazinylguanosine **1** did not deleteriously affect RNA yield (Figure 2).

The level of incorporation of 5'-deoxy-5'-hydrazinylguanosine **1** into transcripts was estimated using a biotinylation/streptavidin-dependent

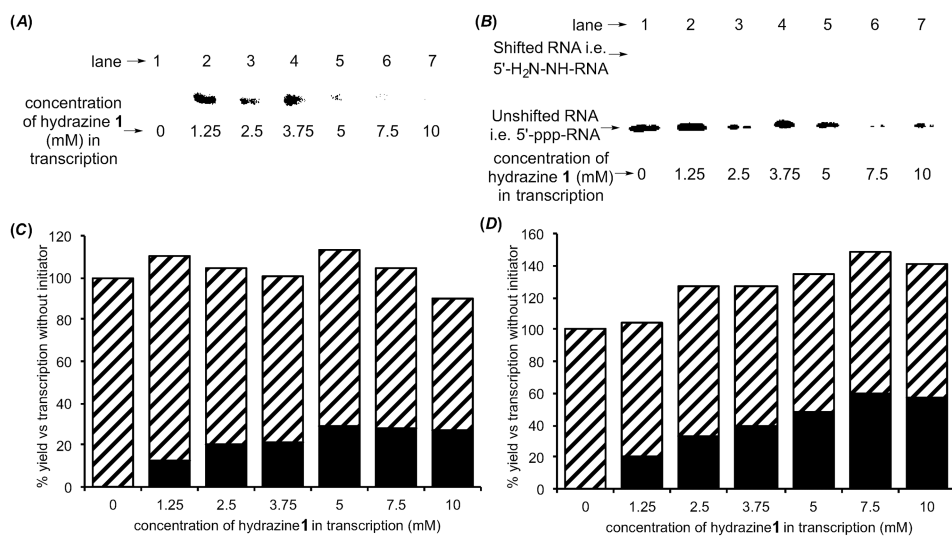


FIGURE 2 Results of transcription experiments using hydrazine 1 as initiator. (A) Phosphorimage of preparative gel for the isolation of transcripts from transcription reactions performed in the presence of equal quantities of [α - 32 P]-UTP and varying concentrations of hydrazine 1 with [GTP] = 1.25 mM. (B) Phosphorimage of streptavidin-dependent gel-shift assay for the determination of the level of incorporation of hydrazine 1 into transcripts from transcriptions with [GTP] = 1.25 mM. Prior to electrophoresis, transcripts were exposed to sulfo-NHS biotin. Approximately equal levels of radioactive material were loaded into each lane of the gel to facilitate quantitative analysis. (C) Combined yield and incorporation level data for transcriptions with hydrazine 1 using [GTP] = 1.25 mM where 100% represents the relative total RNA yield in the absence of 1 and the presence of 1.25 mM GTP. The proportion of hydrazine-initiated transcript is represented by the black area of each bar. (D) Combined yield and incorporation level data for transcriptions with hydrazine 1 using [GTP] = 0.32 mM where 100% represents the relative total RNA yield in the absence of 1 and the presence of 0.32 mM GTP. The proportion of hydrazine-initiated transcript within each experiment is represented by the black area.

gel-shift assay.^[31] On increasing the concentration of 5'-deoxy-5'-hydrazinylguanosine 1, increased incorporation was observed, with a maximum incorporation efficiency of 30% being measured for the transcriptions that contained 10 mM of 5'-deoxy-5'-hydrazinylguanosine 1.

To increase the incorporation level of 5'-deoxy-5'-hydrazinylguanosine 1, further transcriptions were performed using a four-fold-reduced GTP concentration. Higher incorporation levels of up to 40% were observed when using 10 mM of 5'-deoxy-5'-hydrazinylguanosine 1, however, RNA yields were reduced as a consequence of the lower GTP concentration.

To demonstrate the use of the hydrazine bioconjugation handle, we used a 5'-hydrazine-labeled RNA prepared using our method as a substrate for the site-specific endoribonuclease Ire1 *in vitro*. Ire1 cleaves conserved CXGXXG sequences in stem-loop structures after the first guanosine in sequences such as the *Saccharomyces cerevisiae* HAC1^u mRNA.^[32] A stem loop structure containing this conserved motif was designed (Figure 3), and corresponding 5'-hydrazinyl RNA was transcribed by using ssDNA template annealed to a

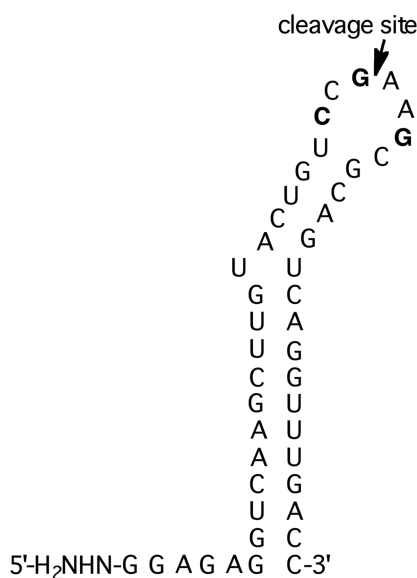


FIGURE 3 Predicted structure of the HAC1^Δ-based 3'-stem-loop of the Ire1p endoribonuclease substrate prior to fluorophore labeling. Emboldened letters represent conserved nucleotides in the CXGXXG site recognized by Ire1p. The arrow highlights the Ire1p cleavage site.

T7 promoter oligonucleotide. To allow detection of the cleaved/uncleaved RNA, the 5'-hydrazine RNA was labeled with fluorescein isothiocyanate (FITC). The labeled RNA was then separated from excess FITC by preliminary ethanol precipitation followed by size-exclusion/desalting spin column, and was used without further purification in Ire1p-based endoribonuclease assays. Overall, from 1.2 nmol of each of the un-annealed ssDNA template/promoter oligos, this process yielded 6.3 nmol of FITC-labeled RNA. The level of FITC modification of the 5'-hydrazinyl RNA was estimated using 5'-deoxy-5'-hydrazinylguanosine **1** as a model that allowed for the preparation of significant amounts of material for expedient analysis of the products (Scheme 1). Triethylammonium bicarbonate solution was used as the buffer for this process owing to its volatile nature under freeze-drying conditions. This afforded similar conditions of pH to our standard RNA conjugation protocol, and identical conditions in terms of FITC concentration (10 mM) were also employed. After ion exchange chromatography, MS and ¹H NMR analyses of the products demonstrated complete mono-modification of the 5'-hydrazinyl group to produce mono-FITC-labeled nucleoside **2** (Supplemental file).

RNase assays were performed under two distinct sets of conditions in order to (i) show that the stem-loop substrate was completely consumed, and (ii) to demonstrate the possibility of using the labeled RNA in initial rate kinetic assays. When using a higher Ire1p concentration, an extended time-course revealed complete consumption of the labeled substrate

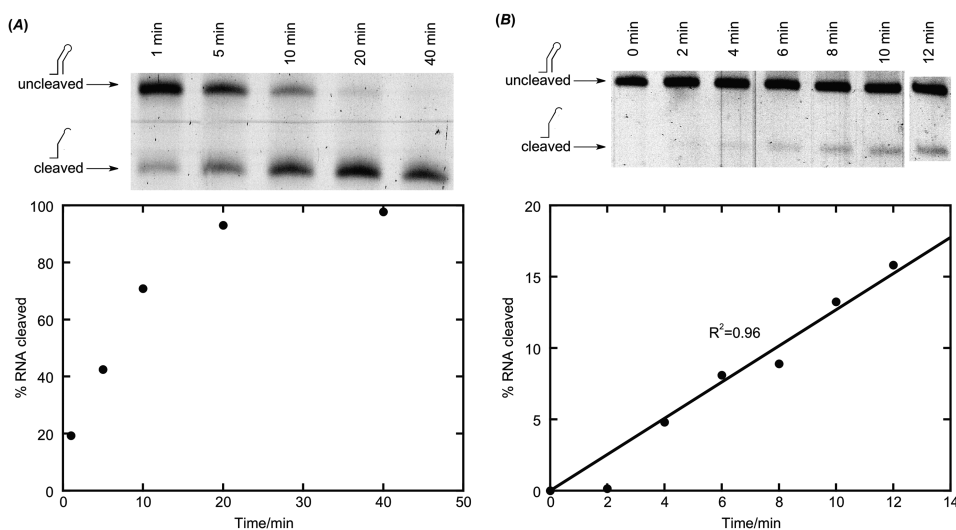


FIGURE 4 Endoribonuclease activity assays at 31°C using FITC-labeled 5'-hydrazinyl-RNA transcripts based on the HAC1^U-based 3'-stem-loop of the Ire1p substrate. (A) Extended time-course demonstrating complete cleavage of the RNA substrate with [Ire1p] = 1 μ M. (B) Initial rates time-course with [Ire1p] = 0.25 μ M.

(Figure 4A). The initial rates experiment, with lower Ire1p concentration and a shorter time-course, revealed the expected zero-order consumption of the substrate over time (Figure 4B). The stem-loop structure of the substrate RNA is expected to hold the 5'-fluorophore-labeled end of the RNA away from the Ire1p endoribonuclease active site, where it should not interfere with catalytic activity. Further kinetic studies (Supplemental file) revealed a K_M value of 1.5 μ M and a k_{cat} value of $1.2 \times 10^{-3} \text{ s}^{-1}$.

DISCUSSION

5'-Deoxy-5'-hydrazinylguanosine **1** was effective as a 5'-initiator of transcription with T7 RNA polymerase, showing $\sim 40\%$ incorporation of the 5'-hydrazine group, which compares favorably with other amine-based initiator systems, which have afforded incorporation levels of 49% to 65%.^[15,16] Unlike phosphorylated initiators, increased 5'-deoxy-5'-hydrazinylguanosine **1** concentrations did not reduce total RNA yields. We believe this is because 5'-deoxy-5'-hydrazinylguanosine **1** competes against GTP for its place at the 5'-terminus during initiation,^[33] however, owing to its lack of charge, it does not compete against GTP during elongation.^[14]

A key factor that aids successful biotinylation and fluorophore-labeling of the 5'-hydrazinyl-RNA is the hyper- or α -effect nucleophilicity and the low basicity of the hydrazine group^[34,35] compared to simple alkylamines. This enhanced reactivity facilitates the use of widely available succinimide esters and isothiocyanates, and overcomes difficulties with competing hydrolysis

processes when using less nucleophilic amines.^[3] An alkylhydrazine, such as 5'-deoxy-5'-hydrazinylguanosine **1**, can be expected to be at least one order of magnitude more nucleophilic than a simple alkylamine. The reaction rates of amines with a range of conjugated isothiocyanates have been measured by Martvon and Sura^[36] with the slowest reaction having a bimolecular rate constant of $9.81 \text{ M}^{-1} \text{ min}^{-1}$. In addition, owing to the lower basicity of hydrazines, lower pHs can be employed, and this reduces the possibility of competing hydroxide-based hydrolysis of the NHS-ester or isothiocyanate reagent.

The clean, rapid, and quantitative mono-fluorophore labeling of the 5'-hydrazine functional group was proven using a preparative scale model reaction with 5'-deoxy-5'-hydrazinylguanosine **1** as the substrate. The procedure used the same concentration of FITC (10 mM) as in the RNA conjugations. Anion exchange chromatograms (Supplemental file) of 5'-deoxy-5'-hydrazinylguanosine **1** that had been exposed to FITC showed that a single new entity was formed and all 5'-deoxy-5'-hydrazinylguanosine **1** was consumed. This new peak was collected and analyzed by MS and ¹H NMR methods, and was confirmed to be the mono-FITC-labeled nucleoside **2**. These results demonstrate the nucleophilicity of the hydrazine group that we were expecting.

RNase assays demonstrated clean kinetics while avoiding the use of ³²P-labeling. A K_M value of $1.5 \mu\text{M}$ and a k_{cat} value of $1.2 \times 10^{-3} \text{ s}^{-1}$ were observed, which align with the values observed for closely related substrate/enzyme pairings.^[31,36,37] These results suggest that the FITC modification at its distal point from the scissile phosphodiester has no effect on substrate binding and cleavage.

In summary, 5'-deoxy-5'-hydrazinoguanosine **1** is an effective initiator of T7 RNA polymerase catalyzed transcriptions that allows for the preparation of nanomole quantities of labeled RNAs. We have demonstrated the utility of the hydrazine-initiated transcripts in an endoribonuclease assay, where the fluorophore-labeled material presented a convenient alternative to a radiolabeled system.

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