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Cleavage of the 5′ Cap Structure of mRNA by a Europium(III) Macrocyclic Complex with Pendant Alcohol Groups

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Abstract: Cellular and viral RNAs, synthesized by RNA polymerase II, have a unique chemical structure at their 5′ termini, 5′ m^{7}GpppNNN... 3′, referred to as the 5′ cap. This structure plays a pivotal role in several processes of mRNA metabolism, including translation and turnover. Consequently, the 5′ cap is a potential target for antisense directed chemistries. In this study the cyclen based lanthanide complexes, Eu(THED)$^{3+}$, Eu(s-THP)$^{3+}$, and La-(TCMC)$^{3+}$, were evaluated for their reactivity upon the 5′ cap structure. Only Eu(THED)$^{3+}$ showed significant reactivity upon a 5′ capped RNA:antisense substrate (86% decapitation after 4 h in the presence of a 0.10 mM complex at 37 °C, pH 7.4). Product analysis of the Eu(THED)$^{3+}$ reaction upon the capped monoribonucleotide, m^{7}GpppG, showed nucleotide diphosphates, GDP and m^{7}GDP, and nucleotide monophosphate THED adducts, GMP-THED and m^{7}GMP-THED, as major products. The isolated adducts were characterized as phosphodiesters comprising a hydroxyethyl pendant group attached to the phosphate group. Pseudo-first-order rate constants (k_0) were 5.3 × 10^{-5} and 4.5 × 10^{-5} s^{-1} for cleavage of GpppG and m^{7}GpppG, respectively, at a complex concentration of 0.31 mM and substrate concentration of 0.031 mM (pH 7.4, 37 °C). Cleavage of GpppG was first order in Eu(THED)$^{3+}$ for complex concentrations ranging from 0.158 to 1.26 mM. A plot of k_1 versus concentration gave a second-order rate constant of 0.17 M^{-1} s^{-1}. Cleavage of the 5′ cap structure by Eu(THED)$^{3+}$ is proposed to occur by a nucleophilic displacement reaction, where the nucleophile is the metal activated hydroxyethyl group of the THED ligand.

Introduction

Cellular and viral RNAs which are synthesized by RNA polymerase II have a unique chemical structure at their 5′ termini, referred to as the 5′ cap.[1] The 5′ cap of messenger RNA is a N7 methylated guanosine that is connected to the 5′ terminal nucleotide of the mRNA (transcription initiation site) via a triphosphate linkage between the 5′ hydroxyl groups of each sugar residue (Figure 1). The 5′ cap structure is added to nascent transcripts during transcription via a series of enzymatic reactions.[2] This structure facilitates several processes of mRNA metabolism, including splicing,[3] nucleocytoplasmic transport,[4] and the initiation of translation to protein.[5] Removal or lack of a 5′ cap may lead to nucleolytic degradation of a transcript inside the cell,[6] presumably through 5′ exonuclease activity.

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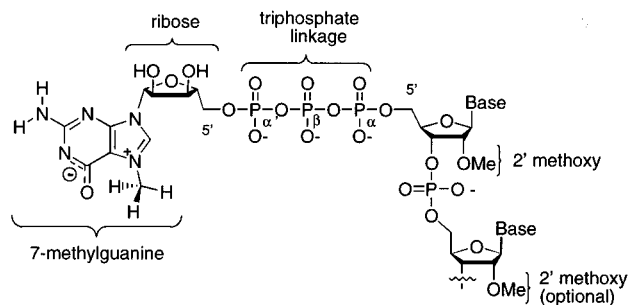
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Figure 1. 5′ cap structure of messenger RNA.

Figure 2. Cyclen based lanthanide complexes tested for reactivity upon the 5′ cap structure: (**1**) Eu(THED)$^{3+}$, where THED = 1,4,7,10-tetrakis-(2-hydroxyethyl)-1,4,7,10-tetraazacyclododecane; (**2**) Eu(s-THP)$^{3+}$, where s-THP = 1,4,7,10-tetrakis(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane; and (**3**) La(TCMC)$^{3+}$, where TCMC = 1,4,7,10-tetrakis(carbamoylmethyl)-1,4,7,10-tetraazacyclododecane.

Given these attributes it is believed that certain modifications of the 5′ cap structure, such as removal or chemical alteration, would impair or preclude the processes of mRNA metabolism that lead to the production of the encoded protein.[7]

Within the past two decades antisense oligonucleotides have emerged as a valid technology for sequence specific modulation of gene expression at the messenger RNA level, both *in vitro* and *in vivo*.[8] Development of new oligonucleotide chemistries has led to better analogs for antisense therapeutic applications,[9] but has also increased the opportunity and need for novel antisense termination events that are not directly dependent upon endogenous enzymes or factors such as RNase H. In this regard, a significant amount of research has been directed toward the development of antisense oligonucleotides that are equipped with functionalities or moieties that promote cleavage of the RNA backbone, *i.e.* artificial ribonucleases.[10,11] This strategy is based upon the 2′-OH group of the RNA target that attacks the adjacent phosphate ester to release the 5′ hydroxyl group under certain conditions.[12]

The biological function and inherent chemical properties of the 5′ cap structure indicate it as another unique component of mRNA available for application of antisense-directed technologies and chemistries.[7] Previous research demonstrated that certain copper complexes hydrolyze the triphosphate linkage of the 5′ cap structure at physiological temperature and pH.[13] Although these complexes demonstrated a productive reaction pathway under the specified conditions, their relatively low complex stabilities,[14] and sluggish reaction rates in relation to messenger RNA metabolism,[15] are considered insufficient for cell-based antisense applications.

Lanthanide ions are powerful Lewis acids which have previously been shown to promote cleavage of phosphodiesters (*e.g.* RNA, DNA, and model analogues)[16−19] and phosphoric anhydrides (*e.g.* NTPs).[20] Lanthanide ions are relatively toxic, however, and must be tightly complexed or encapsulated by ligands to be of use in therapeutic applications.[21] Several types of macrocyclic lanthanide complexes have been developed which are inert to dissociation of the metal ion, and hydrolyze the phosphodiester backbone of RNA as free complexes in solution[22] as well as covalently attached to antisense oligonucleotides.[11a,c,d]

In this study we have evaluated a set of three cyclen based lanthanide complexes (Figure 2) for their reactivity upon the 5′ cap structure of mRNA. These complexes, Eu(THED)$^{3+}$ (**1**), Eu(s-THP)$^{3+}$ (**2**), and La(TCMC)$^{3+}$ (**3**), were chosen for their range of known chemical and physical properties. Each complex has at least one coordination site available for binding a small molecule or substrate.[23−25] In solution, Eu(THED)$^{3+}$ and Eu(s-THP)$^{3+}$ have either a bound hydroxide or alkoxide ligand at neutral pH, whereas La(TCMC)$^{3+}$ does not. This type of ligand has proven to be necessary for cleavage of DNA phosphodiester analogs, but not for RNA and its phosphodiester analogs.[19a]

The three lanthanide complexes were first compared for their reactivity upon a 5′ capped RNA:antisense duplex in solution (Figure 3A). Utilization of a duplex tested the complexes in the context of antisense hybridization as well as protected the

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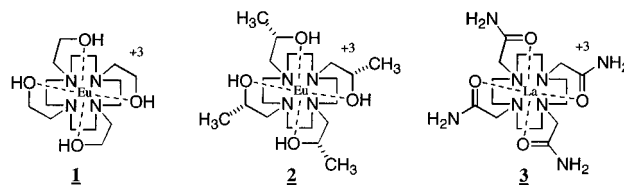
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A. 5′ Capped Radiolabeled Oligoribonucleotides

B. 5′ Capped Monoribonucleotides

$m^7GpppG \implies$ Derivatives of G
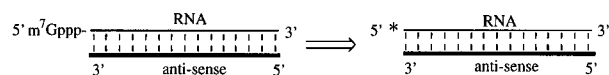
Figure 3. Model substrates for biochemical and chemical analysis of reactions upon the 5′ cap structure of mRNA:[13] (A) 5′ capped RNA: antisense substrate. (*, potential products include 5′ ppp-, pp-, p-, and HO-RNA) and (B) 5′ capped monoribonucleotide substrate, m^7GpppG or $GpppG$.

RNA substrate from backbone hydrolysis[26] during the course of the reaction. Product and kinetic analyses were performed on the most reactive complex of the set, $Eu(THED)^{3+}$, using the 5′ capped monoribonucleotide, m^7GpppG, and the un-methylated analog, $GpppG$, as substrates (Figure 3B). These studies have shown that lanthanide(III) complexes cleave the 5′ cap structure of messenger RNA, but require a metal activated nucleophile for cleavage to occur.

Materials and Methods

Reagents. Capped monoribonucleotides, m^7GpppG and $GpppG$, were purchased from Pharmacia. GTP was purchased from Clontech. Radiolabels were purchased from ICN and Amersham. T4 RNA ligase was purchased from Pharmacia. RNase T1 and CL3 were purchased from Boehringer Mannheim. T7 RNA polymerase was purchased from Ambion. Phosphoramidites were purchased from Glenn Research, ChemGene, and Cruachem. Reagents for buffers and nucleotide standards were purchased from Sigma.

Lanthanide Complexes. [La(TCMC)]$(CF_3SO_3)_3$ and [Eu(s-THP)]$(CF_3SO_3)_3$ were prepared as reported previously.[23,24] All solutions were made with Milli-Q water. [Eu(THED)]$(CF_3SO_3)_3$ was prepared as described previously[25] with the following modifications. THED was recrystallized from acetone and hexane. [Eu(THED)]$(CF_3SO_3)_3$ was prepared by addition of $Eu(CF_3SO_3)_3$ (0.544 g, 0.574 mmol) to an equimolar amount of THED (0.200 g, 0.574 mmol) in ethanol. The solution was refluxed for 1 h, and the solvent was removed in vacuo. A minimum amount of hot ethanol was added to dissolve the resulting white solid, and 2-propanol was added until cloudiness was observed. A white microcrystalline solid was obtained upon cooling the solution overnight to −20 °C.

Synthesis of RNA Substrates. The internally radiolabeled 5′ capped oligoribonucleotide, 5′ $m^7GpppGAGCUCCUCUGCUACUCAGA$ 3′, was enzymatically synthesized from a single stranded DNA template, using T7 RNA polymerase as previously described.[27] The 5′ capped 3′ [^{32}P]-radiolabeled RNA substrate, $m^7GpppGAGCUCCU^{32}pCp$ 3′, was synthesized by enzymatic ligation[28] of the chemically synthesized 3′ oligoribonucleotide, 5′ pUCCU 3′, to the enzymatically synthesized 5′ capped tetramer, 5′ $m^7GpppGAGC$ 3′, using T4 RNA ligase. The 5′ capped 8mer was purified by anion exchange chromatography and then radiolabeled by ligation of cytidine 3′,5′-[5′-^{32}P] bisphosphate to the 3′ terminus of the oligomer with T4 RNA ligase. The radiolabeled product was page purified, and its composition verified by base hydrolysis and RNase (T1 and Cl3) cleavage patterns. The 5′ uncapped 3′ [^{32}P]-radiolabeled RNA, 5′ HO-$GAGCUCCU^{32}pCp$ 3′ was synthesized by ligation of cytidine 3′,5′-[5′-^{32}P] bisphosphate to the 3′ terminus of the chemically synthesized oligoribonucleotide, 5′ $GAGCUCCU$ 3′, using T4 RNA ligase.

Synthetic Oligonucleotides. Oligonucleotides were synthesized by standard phosphoramidite synthetic procedures[29] on an ABI DNA synthesizer and purified by polyacrylamide gel electrophoresis techniques.

5′ Cap Cleavage Analysis. Radiolabeled 5′ capped RNA was prehybridized to complementary DNA in buffer by a heat denaturation (90 °C, 1 min) and room temperature cooling (22 °C, 10 min) process, after which metal complexes were added. Reaction conditions were as described in the figure legends. Reactions were analyzed by polyacrylamide gel electrophoresis [20% polyacrylamide (1:20 cross-linkage), 40% urea, Tris−Borate−EDTA running buffer]. RNA bands were visualized by autoradiography, using KODAK X-Omat film. Quantitation was performed by laser densitometry or phosphorimaging (Molecular Dynamics).

eIF-4E Gel Shift Analysis. A portion of each RNA reaction sample was ethanol precipitated and then resolubilized in buffer (40 mM Hepes, 100 mM KCl, pH 7.4) for incubation and gel shift analysis with eIF-4E at 1 μM as previously described.[30]

Analysis of Reaction Products by Anion Exchange Chromatography. Aliquots were removed at each time point and analyzed on a Pharmacia Mono Q HR 5/5 column with either a Waters 600E Multisolvent Delivery System with the Waters 991 photodiode array detector or the Pharmacia LKB FPLC system with the Pharmacia UV-M monitor. Program gradient: 0−40% B in 30 min, 40−100% B in 1 min, 100% B for 1 min, 100−0% B in 0.5 min; and 0% B for 7.5 min. Solvent A: 5 mM phosphate (pH 7.0−7.4). Solvent B: A + 1 M NaCl. Flow rate of 0.5 mL/min. Product ratios were measured at 274 nm, a wavelength at which absorbance by the m^7G chromophore was determined to be less than 10% (diphosphate) and 5% (monophosphate) variant to changes in ionic strength and pH. Ratios were measured on a molar basis by using the following extinctions coefficients (274 nm, $M^{-1} cm^{-1}$): m^7GpppG = 16 840; m^7GDP = 8110; m^7GMP = 9290; GDP = 8740; and GMP = 8740.[31] The covalent adduct measurements were based on the extinction coefficient of the respective nucleotide analog.

Analysis and Isolation of Reaction Products by Cation Exchange Chromatography. The same procedure as described for anion exchange chromatography, except with a Pharmacia Mono S HR 5/5 cation exchange column, was utilized with the following program gradient: 0−80% B in 30 min, 80−100% B in 1 min, 100% B for 1 min, 100−0% B in 0.5 min, 0% B for 7.5 min. Solvent A: double distilled water. Solvent B: 1 M ammonium acetate. Flow rate of 0.5 mL/min. Ammonium formate was utilized as solvent B to isolate the covalent THED products for NMR analysis.

MALDI-TOF Mass Spectral Analysis. Mass spectra were obtained with a Voyager-RP Biospectrometry Workstation (Perspective Biosystems) in the positive mode. The matrix was 100 mM 2,4,6-trihydroxy-acetophenone and 200 mM ammonium citrate.[32] The instrument was calibrated with myoglobin or insulin B, as an external standard.

NMR Studies. A Varian 500-MHz NMR was used for all ^{31}P NMR experiments as well as experiments with ^{31}P decoupled ^{1}H spectra at a probe temperature of 20 °C. ^{31}P NMR shifts are reported relative to phosphoric acid as a reference with resonances downfield of phosphoric acid reported as positive chemical shifts. A Bruker 400-MHz NMR was employed for DQF-COSY ^{1}H NMR experiments with a probe temperature of 30 °C. A spectral width of 400 Hz was used with 2048 data points in the t_2 dimension and 1024 t_1 increments each containing 16 transients. The data were processed on a SPARC 4/370 with NMR2/NMRZ software from New Methods Research Inc. (East Syracuse, NY) and Tripos Inc. (St. Louis, MO). The data were zero-filled to a 2 × 2 matrix, and a 90 °C shifted sine bell square function was used in both dimensions before fourier transformation.

Kinetics. Rate constants were determined by monitoring the disappearance of $GpppG$ or m^7GpppG by use of a Waters 600E HPLC equipped with a 490E UV−vis detector. The dinucleotide, ApA, was used as an internal standard. Reactons were analyzed on a C18 column (250 mm × 4.6 mm). Solvent A: 50 mM KH_2PO_4, 5 mM *tert*-butylammonium phosphate at pH 5.0. Solvent B contained 5 mM *tert*-

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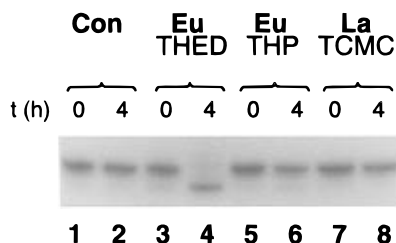(32) Pieles, U.; Zürcher, W.; Schär, M.; Moser, H. E. *Nucleic Acids Res.* **1993**, *21*, 3191−3196.

Figure 4. Reaction of Eu(THED)$^{3+}$, Eu(s-THP)$^{3+}$, and La(TCMC)$^{3+}$ upon a 5′ capped [^{32}P]RNA (antisense duplex). Autoradiogram of the analytical gel (20% acrylamide, 1:20 cross-linkage, 40% urea): (Lane 1) control, no complex, 0 h; (Lane 2) control, no complex, 4 h; (Lane 3) +0.1 mM Eu(THED)$^{3+}$, 0 h; (Lane 4) +0.1 mM Eu(THED)$^{3+}$, 4 h; (Lane 5) +0.1 mM Eu(s-THP)$^{3+}$, 0 h; (Lane 6) +0.1 mM Eu(s-THP)$^{3+}$, 4 h; (Lane 7) +0.1 mM La(TCMC)$^{3+}$, 0 h; and (Lane 8) +0.1 mM La(TCMC)$^{3+}$, 4 h. All reactions included the internal ^{32}P-radiolabeled RNA (5′ m^{7}GpppGAGCUCCUUGCUACUCAGA 3′) and 10 μM antisense DNA (5′ TCTGAGTAGCAGAGGAGCTC 3′). RNA and antisense DNA were hybridized prior to addition of the metal complexes. Solutions were buffered in 20 mM Hepes (pH 7.4), 50 mM KCl, and 100 mM NaCl. $T = 37$ °C.

butylphosphate in a 50:50 methanol water mixture. For experiments with GpppG, an isocratic flow of 50% solvent A and 50% solvent B was used with an isocratic flow of 1.5 mL/min over 30 min. A typical reaction solution contained 0.031 mM m^{7}GpppG or GpppG and 0.31 mM Eu(THED)$^{3+}$ with 50 mM Hepes buffer (pH 7.4). Control experiments containing GpppG in buffer only showed negligible cleavage over the time period where cleavage was monitored for the Eu(III) complex.

Results

The lanthanide complexes, Eu(THED)$^{3+}$, Eu(s-THP)$^{3+}$, and La(TCMC)$^{3+}$, were tested and compared for their reactivity upon the 5′ cap structure of mRNA utilizing a 5′ capped internally radiolabeled oligoribonucleotide, 5′ m^{7}GpppGAGCUCCUUG-CUACUCAGA 3′, hybridized to its DNA complement. The radiolabeled RNA was analyzed by polyacrylamide gel electrophoresis (PAGE) after a 4 h reaction period at 37 °C, pH 7.1 (Figure 4). Under these conditions no reaction was observed in the absence of metal complex (lane 2) or in the presence of Eu(s-THP)$^{3+}$ (lane 6) or La(TCMC)$^{3+}$ (lane 8). In striking contrast, 86% of the 5′ capped RNA was converted to a product with faster gel mobility by Eu(THED)$^{3+}$ (lane 4).

Additional experiments were performed to verify that the Eu-(THED)$^{3+}$ reaction occurred upon the 5′ cap structure, and not at the 5′ or 3′ terminal phosphodiester linkage of the oligoribonucleotide substrate, utilizing the 3′ radiolabeled capped and uncapped RNA, 5′ m^{7}Gppp- and HO-GAGCUCCU^{32}pCp 3′. PAGE analysis showed that no reaction occurred upon the uncapped RNA substrate in the presence of the Eu(THED)$^{3+}$ complex (Figure 5A, lane 2). And, as observed with the internally labeled RNA, a product of faster gel mobility was generated in the 5′ capped RNA reaction (Figure 5A, lane 4). The faster gel mobility of the RNA cleavage product in comparison to the uncapped 5′ HO-RNA is characteristic of a 5′ phosphorylated RNA of equivalent length. The 5′ mono-, di-, and triphosphorylated derivatives were not resolved under the given PAGE conditions.[13] Further analysis of these reaction samples showed that the RNA product resulting from the reaction of Eu(THED)$^{3+}$ upon the 5′ capped RNA was not a substrate for the human recombinant 5′ cap specific binding protein, eIF-4E,[33] as demonstrated by the comparable reduction in RNA shifted or complexed by eIF-4E (Figure 5B, lane 4). Together, these results verified that the Eu(THED)$^{3+}$ reaction
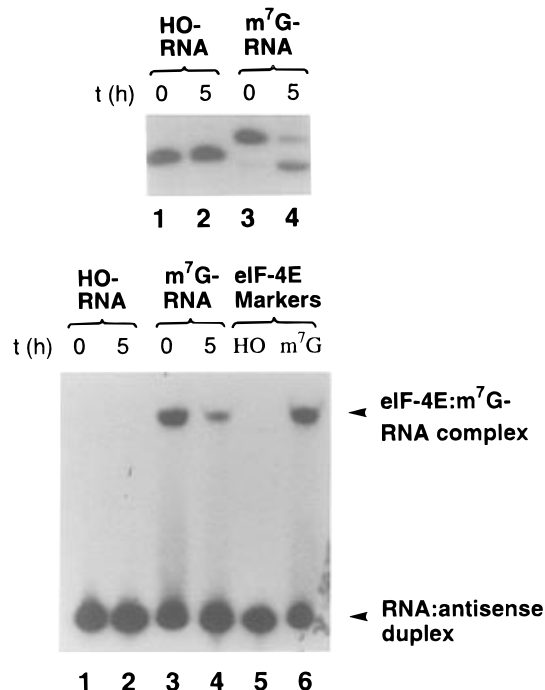
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Figure 5. Verification of 5′ cap specificity: (A) uncapped RNA duplex not a substrate for Eu(THED)$^{3+}$. Autoradiogram of the cleavage analysis gel (20% acrylamide, 1:20 cross-linkage, 40% urea): (Lane 1) uncapped HO-RNA, 0 h; (Lane 2) uncapped HO-RNA, 5 h; (Lane 3) capped m^{7}Gppp-RNA, 0 h; and (Lane 4) capped m^{7}Gppp-RNA, 5 h. (B) RNA cleavage product not a substrate for the 5′ cap specific binding protein, eIF-4E. Autoradiogram of the 6% (1:25) nondenaturing acrylamide gel: (Lane 1) uncapped HO-RNA, 0 h; (Lane 2) uncapped HO-RNA, 5 h; (Lane 3) capped m^{7}Gppp-RNA, 0 h; and (Lane 4) capped m^7-Gppp-RNA, 5 h. All reactions included 0.10 mM Eu(THED)$^{3+}$, 3′ radiolabeled RNA (5′ GAGCUCCU^{32}pCp 3′),[28] and 1 μM antisense (5′ AGGAGCUC 3′). The antisense oligonucleotide was 2′OMe modified. RNA was prehybridzed to the antisense oligonucleotide prior to addition of the metal complex. Reaction buffer was 10 mM Hepes (pH 7.4), 100 mM KCl, and 1 mM MgCl$_2$. $T = 37$ °C.

occurred specifically upon the 5′ cap structure of the RNA: antisense duplex.

A detailed product analysis was performed on the Eu-(THED)$^{3+}$ reaction with the 5′ capped monoribonucleotides, m^7-GpppG and GpppG, as substrates. A combination of techniques were implemented for this purpose, including anion and cation exchange chromatography, MALDI-TOF mass spectrometry,[34] and ^{1}H and ^{31}P NMR. Initial analysis of the Eu(THED)$^{3+}$ 5′ cap reaction was performed by anion exchange chromatography as previously described for OP:Cu(II).[13] Incubation of the m^7-GpppG substrate with Eu(THED)$^{3+}$ at 37 °C showed a disappearance of substrate over time (0 to 8 h) with concomitant appearance of products. Coinjection experiments, using commercially available standards, showed that the four resolved anion exchange products were m^{7}GMP, GMP, m^{7}GDP, and GDP (Figure 6A). Interestingly, the diphosphates were produced in greater amounts than their monophosphate counterpart, *i.e.* m^{7}GDP to GMP and GDP to m^{7}GMP. After a 4 h reaction time the m^{7}GDP/GMP ratio was 4.5:1.0 and the GDP/ m^{7}GMP ratio was 3.0:1.0. At 8 h these ratios had decreased to 3.9:1.0 and 2.4:1.0, respectively. The decrease in ratios over time implied that secondary reactions had occurred to result in a redistribution of products. The fact that the ratios were not 1:1

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(35) The 544.6 (*m/z*) signal has been assigned as Eu(THED)·Na$_2$ based on analysis of spectra not shown. This signal may represent a positive charged aggregate. Sodium ion originated from the m^{7}GpppG starting material, which is provided by the manufacturer as the sodium salt.
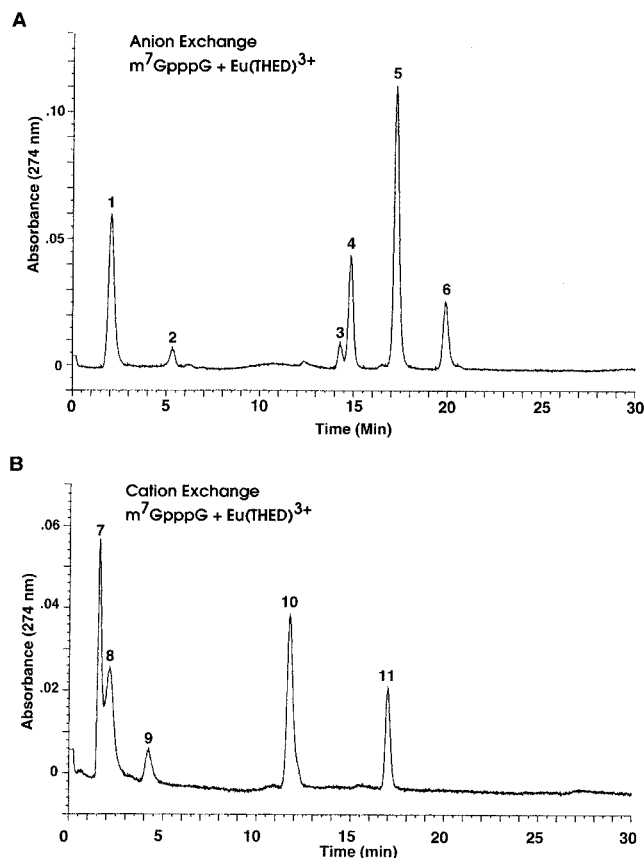
Figure 6. Product analysis of the m⁷GpppG + Eu(THED)³⁺ reaction by anion and cation exchange chromatography. (A) Anion exchange chromatogram at 4 h with peak assignments by coinjection of commercial standards: (1) void, (2) m⁷GMP, (3) GMP, (4) m⁷GDP, (5) m⁷GpppG, and (6) GDP. (B) Cation exchange chromatogram at 8 h with peak assignments by MALDI-TOF mass spectrometry (positive mode): (7) void, (8) m⁷GMP, m⁷GDP, and GDP-THED, (9) m⁷GDP-THED (and PO₃-THED), (10) GMP-THED, and (11) m⁷GMP-THED. Reaction conditions: 0.20 mM Eu(THED)³⁺ and 0.10 mM m⁷GpppG in 20 mM Hepes (pH 7.4), 50 mM KCl, and 100 mM NaCl. $T = 37$ °C.

indicated that chemistry other than direct hydrolysis of the phosphoanhydride linkage had occurred. Other guanine based products were observed, by UV spectral analysis, in the void volume, which indicated formation of neutral or net positive reaction products, *e.g.* nucleosides or metal complex adducts. The area of this void volume peak measured 0.92 (±0.14) relative to that of the sum of the mono- and diphosphate product peaks on a per mole basis over the course of the reaction.

Cation exchange chromatography was employed to further characterize the unidentified reaction products present in the anion exchange void volume. By this method two major peaks were observed, each showing UV spectra characteristic of a guanine-like residue (Figure 6B, peaks 10 and 11). Coinjection with commercial 7-methylguanosine showed that these products had unique elution times, and excluded the m⁷G nucleoside as one of the products. Usage of a volatile elution buffer allowed for final peak assignments to be made by MALDI-TOF analysis of the collected peak fractions (peaks 8−11). These assignments were further supported by analysis of the complete reaction mix as detailed in the following paragraph.

Analysis of the complete Eu(THED)³⁺ + m⁷GpppG reaction mixture by mass spectrometry was achieved by changing the reaction buffer from HEPES to ammonium acetate. Usage of a volatile buffer, which could be removed by lyophilization, provided a means to minimize salt suppression of signals. The MALDI-TOF MS spectra (Figure 7) identified two nucleotide-

THED covalent adducts (mono- and diphosphate) as products after a 8 h reaction period at 37 °C, and substantiated the other product assignments made by anion exchange chromatography. The most predominant signals were the monophosphate-THED adducts without the europium ion. The metalated forms of these adducts were observed in increasing amounts with reduction of the laser intensity. A Eu(phosphoryl-THED) sodium salt was also identified in the spectra, believed to be generated from a secondary hydrolysis reaction of the diphosphate adducts, Eu-(GDP-THED) and Eu(m⁷GDP-THED). Nucleosides were not observed, confirming the absence of m⁷-guanosine in the cation chromatogram of the reaction mix. Analysis of the reaction mix by electrospray mass spectrometry (positive mode, 1% acetic acid:isopropyl alcohol at 1:1) showed signals at 694.5, 708.4, and 858.6 (*m/z*) for the GMP-THED, GDP-THED, and Eu(GMP-THED) covalent adducts respectively, as well as the two isotope derivatives of Eu(THED) at 497.3 and 499.3 (*m/z*) and Eu(THED)·CF₃SO₃ at 647.5 and 649.4 (*m/z*).

The two products observed by cation exchange chromatography (at 8 h, 37 °C, pH 7.1) were isolated in sufficient quantities for ³¹P and ¹H NMR analysis, again using a volatile elution buffer (ammonium formate). Preliminary MALDI-TOF analysis of the isolates showed masses equivalent to the GMP- and m⁷GMP-THED covalent adducts (peaks 10 and 11, respectively, in Figure 5B). A ³¹P NMR resonance at 0.382 ppm was observed for the GMP-THED adduct. The ¹H NMR spectrum of the GMP-THED adduct was assigned by use of HMQC-COSY experiments. A proton NMR resonance at 7.90 ppm was assigned to the base H8 proton with ribose resonances at 5.80 ppm (H1′), 4.65 (H2′), 4.35 (H3′), 4.15 (H4′), and 3.93 ppm (H5′ and H5″). A multiplet at 3.75 ppm was assigned to the hydroxyethyl pendent group attached to the phosphate diester; this assignment was confirmed by use of ³¹P−¹H NMR decoupling experiments. This resonance showed a change in its pattern from a pseudotriplet to a doublet when the phosphorus resonance was decoupled. A similar ¹H NMR spectrum was obtained for the m⁷GMP-THED adduct with the exception that no resonance was observed for the guanosine H8 due to rapid exchange of this proton with D₂O.

Eu(THED)³⁺ promoted cleavage of both the N7 methylated and unmethylated 5′ capped monoribonucleotide substrates. Pseudo-first-order rate constants (k_0) were 5.3×10^{-5} and 4.5×10^{-5} s⁻¹ for cleavage of GpppG and m⁷GpppG, respectively, at a complex concentration of 0.31 mM and substrate concentration of 0.031 mM in 50 mM Hepes (pH 7.4) at 37 °C. Cleavage of GpppG was first order in Eu(THED)³⁺ for complex concentrations ranging from 0.158 to 1.26 mM. A plot of k_1 versus concentration gave a second-order rate constant of 0.17 M⁻¹ s⁻¹. Rate constants were nearly constant upon addition of 10% or 20% EDTA, suggesting that the reaction upon the 5′ cap substrate was not due to trace amounts of Eu³⁺ ion. No reaction was observed in the presence of the THED ligand alone. Saturation kinetics were not observed, up to complex concentrations of 1.26 mM, suggesting a relatively low binding constant for the 5′ capped monoribonucleotide, GpppG. However, line-broadening and shifting of ³¹P NMR resonances of GpppG were observed upon addition of Ln(THED)³⁺, where Ln = La or Eu, consistent with direct coordination of these complexes to the substrate. Cleavage of GpppG was inhibited by addition of phosphate anion to the reaction. The pseudo-first-order rate constant for cleavage of GpppG was 3.3×10^{-5} s⁻¹ in 50 mM Hepes (pH 7.4) containing 0.31 mM Eu(THED)³⁺, 0.031 mM GpppG, and 0.031 mM phosphate at 37 °C. At 0.150 mM phosphate anion the rate of the reaction was further reduced to 1.47×10^{-5} s⁻¹.
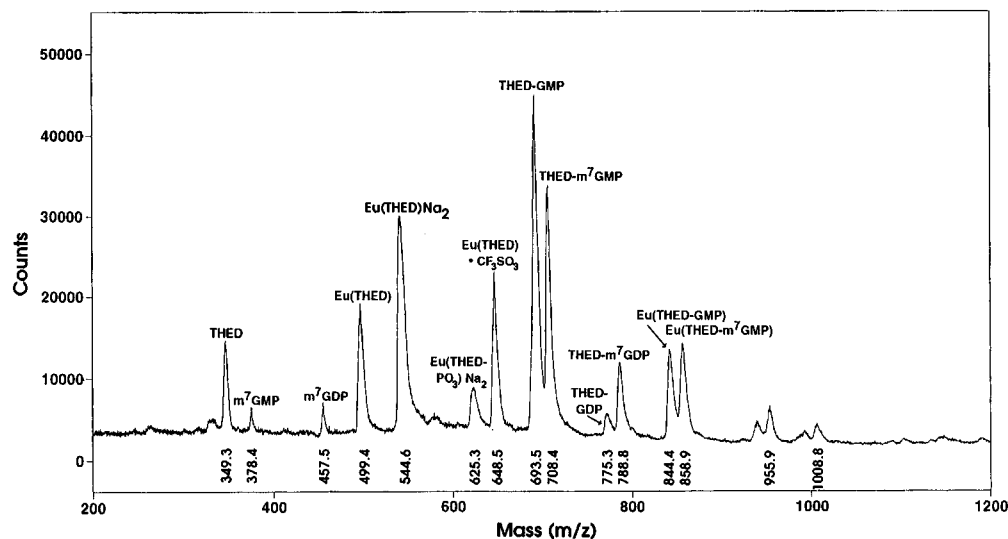
Figure 7. MALDI-TOF mass spectrum of the complete m^{7}GpppG + Eu(THED)$^{3+}$ reaction mix after 8 h at 37 °C in 10 mM NH$_4$OAc (pH 7.1). Positive mode.

Table 1. Calculated and Observed Mass Values for the m^{7}GpppG + Eu(THED)$^{3+}$ Reaction[a]

compd	formula	calc mass	obsd mass
GMP	C$_{10}$H$_{15}$N$_5$O$_8$P	364.069	n.o.
m^{7}GMP	C$_{11}$H$_{17}$N$_5$O$_8$P	379.085	378.4
GDP	C$_{10}$H$_{16}$N$_5$O$_{11}$P$_2$	444.036	n.o
m^{7}GDP	C$_{11}$H$_{18}$N$_5$O$_{11}$P$_2$	458.052	457.5
Eu(THED-PO$_4$)Na$_2$	C$_{16}$H$_{36}$N$_4$O$_7$PEuNa$_2$	625.139	625.3
THED-GMP	C$_{26}$H$_{49}$N$_9$O$_{11}$P	695.338	693.5
THED-m^{7}GMP	C$_{27}$H$_{51}$N$_9$O$_{11}$P	708.354	708.4
Eu(THED-GMP)	C$_{26}$H$_{46}$N$_9$O$_{11}$PEu	843.234	844.4
Eu(THED-m^{7}GMP)	C$_{27}$H$_{48}$N$_9$O$_{11}$PEu	857.250	858.9
THED-GDP	C$_{26}$H$_{50}$N$_9$O$_{14}$P$_2$	774.305	775.3
THED-m^{7}GDP	C$_{27}$H$_{52}$N$_9$O$_{14}$P$_2$	788.321	788.8
Eu(THED-GDP)	C$_{26}$H$_{47}$N$_9$O$_{14}$P$_2$Eu	923.201	n.o.
Eu(THED-m^{7}GDP)	C$_{27}$H$_{50}$N$_9$O$_{14}$P$_2$Eu	938.225	n.o.
THED	C$_{16}$H$_{37}$N$_4$O$_4$	349.288	349.3
Eu(THED)	C$_{16}$H$_{34}$N$_4$O$_4$Eu	498.184	499.4
Eu(THED)Na$_2$	C$_{16}$H$_{32}$N$_4$O$_4$EuNa$_2$	542.148	544.6
Eu(THED)CF$_3$SO$_3$	C$_{17}$H$_{35}$N$_4$O$_7$SF$_3$Eu	648.134	648.5

[a] Compound formulas describe the [M + H]$^+$ or [M − (n − 1)H + I^n]$^+$ species, where M is the neutral compound, I is europium or sodium ion, and n is the charge of the ion. Calculated masses for compounds containing europium were derived by using the average mass of the two major europium isotopes (151 and 153). THED was utilized as an internal reference for the observed mass values. Not observed is indicated by n.o. Assignments were supported by analysis of commercially available standards (*e.g.* m^{7}GDP and m^{7}GMP), Eu(THED)$^{3+}$, and m^{7}GpppG separately, and analysis of other reaction samples.[35]

Discussion

Identification and quantitation of the THED-nucleotide adducts established that cleavage of the 5′ cap triphosphate linkage occurs by a nucleophilic displacement reaction, where the nucleophile is assigned as the metal activated hydroxyethyl group of the THED ligand (Figure 8). The kinetic data further support the proposed mechanism. A first-order dependence on both the lanthanide complex and the 5′ capped monoribonucleotide substrate was observed. Cleavage of the 5′ cap substrate was not catalyzed by trace amounts of Eu(THED)$^{3+}$, but required a stoichiometric amount of complex. This latter result is consistent with the formation of the covalent adducts.

The propensity for formation of the monophosphate adduct with release of a nucleotide diphosphate explains the production of greater amounts of the nucleotide diphosphates in comparison to the monophosphates, which was observed by anion exchange analysis of the m^{7}GpppG reaction. An unequivocal understanding of the basis of this regioselectivity is beyond the scope of

this study.[36] However, it is inferred that this selectivity reflects differences in metal coordination chemistry at the two sites, in relation to the electrophilicity of the α and β phosphorus atoms and the leaving group ability of the nucleotide monophosphate and diphosphate.

A subtle degree of selectivity between the two α-phosphates of the asymmetric capped monoribonucleotide, m^{7}GpppG, was observed. The α-phosphate proximal to the guanine residue was preferred over that of the m^{7}G residue, where the relevant product ratios were GMP-THED/m^{7}GMP-THED = 1.6 and m^7-GDP/GDP = 1.5. This preference is also reflected by the higher pseudo-first-order rate constant of GpppG (5.3 × 10^{-5} s^{-1}) versus that of m^{7}GpppG (4.5 × 10^{-5} s^{-1}). An interaction between the α-phosphate group and the electropositive aromatic ring of m^{7}GMP, m^{7}GDP, and m^{7}GTP has been described based on proton NMR data.[37] The electropositive charge of the m^{7}G and its interaction with the proximal phosphate group may interfere with coordination of the metal complex to the m^{7}G α-phosphoryl oxygens to result in a reduction in cleavage at this site.

In contrast to the capped monoribonucleotide, m^{7}GpppG, a high degree of selectivity for the m^{7}G α-phosphate was observed with the 5′ capped oligoribonucleotide:antisense substrate, as indicated by the production of a single RNA band with a gel mobility similar to a 5′ phosphorylated RNA.[13] A nucleophilic substitution reaction at the 5′ α-phosphate of the oligoribonucleotide would generate an RNA-THED adduct and m^{7}GDP. One would expect that the gel mobility of the RNA-THED product would be slow. In other experiments (data not shown) an RNA product of slower gel mobility than the 5′ capped starting material was observed. This product was observed under the same reaction conditions, but at a higher concentration of 1 mM Eu(THED)$^{3+}$.

Alcohol groups bound to Lewis acidic metal ions may be readily deprotonated.[19,38] Such metal ion bound alkoxide ligands have previously been shown to act as nucleophiles in displacement reactions of phosphate diesters[19,38] and nucleotide tri-

(36) For examples in nature, *i.e.* regioselective cleavage of 5′ capped RNA, ApppA, and ATP by enzymes, see: (a) Stevens, A. *Mol. Cell. Biol.* **1988**, *8*, 2005−2010. (b) Guranowski, A.; Brown, P.; Ashton, P. A.; Blackburn, G. M. *Biochemistry* **1994**, *33*, 235−240. (c) Mildvan, A. S. *NMR and Biochemistry*; Opella, S. J., Lu, P., Eds.; Marcel Dekker; New York, 1979; pp 301−321.

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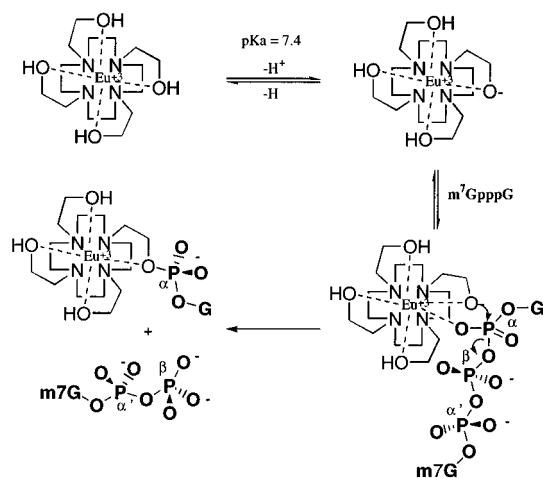
Figure 8. Proposed reaction mechanism for Eu(THED)$^{3+}$ + m^{7}GpppG.

phosphates.[39] Previous studies have shown that Eu(THED)$^{3+}$ promotes cleavage of the activated phosphodiesters, BNPP or ENPP, by a nucleophilic displacement reaction similar to that observed here for the 5′ cap substrate, m^{7}GpppG, wherein a covalent THED phosphodiester adduct is formed.[19]

The two other lanthanide(III) complexes, Eu(s-THP)$^{3+}$ or La-(TCMC)$^{3+}$, did not promote cleavage of the 5′ capped oligoribonucleotide. The Eu(s-THP)$^{3+}$ complex is similar to Eu-(THED)$^{3+}$ in that it contains an acidic group which is either a metal bound hydroxide or alkoxide ligand with a pK_a of 7.7, and it has a single coordination site available for binding substrate. That this complex is not active in promoting cleavage of the 5′ cap substrate may be attributed to the bulkier hydroxyl propyl group, which may affect ternary complex formation as well as restrict nucleophilic attack. The low reactivity of Eu-(s-THP)$^{3+}$ toward the 5′ cap parallels that observed for phosphate diesters. The La(TCMC)$^{3+}$ complex has more available coordination sites than does either of the Eu(III) complexes. However, at pH 7.4, it does not form a hydroxide species and therefore lacks a potent nucleophile for the reaction.

The second-order rate constant for cleavage of the triphosphate linkage of the 5′ cap structure by Eu(THED)$^{3+}$ under similar conditions (37 °C, pH 7.4) is 4-fold slower than that of the sugar phosphodiester backbone of the RNA substrate A$_{12-18}$ (k_2 = 0.63 M^{-1} s^{-1}).[22a] Both reactions are the result of nucleophilic attack of phosphorus by an activated hydroxyl

nucleophile and may be categorized as transesterifications. However, in the 5′ cap reaction the metal complex provides or imports the nucleophile, whereas in the RNA reaction the nucleophile is provided by the 2′-OH group of the substrate. As a consequence of the difference in source of nucleophile, the products of the two reactions are different. The 5′ cap reaction results in the formation of a covalent adduct with the ligand of the metal complex, and the RNA phosphodiester reaction results in the formation of an intramolecular adduct, a 2′3′ cyclic phosphodiester. The stability of the covalent 5′ cap adduct results in deactivation of the metal complex for additional cleavage reactions upon the substrate, thus the reaction is stoichiometric. In contrast, the RNA reaction is catalytic without formation of such an adduct. In the case of the 5′ cap reaction, adjustment or replacement of the nucleophile, such that an unstable covalent intermediate is formed, presumably would yield a catalytic reaction.

Conclusion

The rapid rate of cleavage of the 5′ cap structure by Eu-(THED)$^{3+}$, as well as its stability in comparison to other metal complexes, *e.g.* OP:Cu(II), has led to the development of THED analogs for attachment to oligonucleotides. Future research will focus on the design, synthesis, and reactivity of these novel oligonucleotide conjugates upon the 5′ cap structure of a target mRNA in solution and in cell-based assays.

Supporting Information Available: Anion exchange chromatograms that show appearance of products over time for m^7-GpppG + Eu(THED)$^{3+}$ at 0, 2, 4, 6, and 8 h; anion exchange chromatograms for assignment of products by coinjection of commercial standards; cation exchange chromatogram with coinjection of m^{7}guanosine standard; and ^{1}H NMR spectra (^{31}P coupled and decoupled) of the isolated GMP-THED adduct (7 pages). See any current masthead page for ordering and Internet access instructions.

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