Messenger RNA

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# A Chemo-Enzymatic Approach for Site-Specific Modification of the RNA Cap**

*Daniela Schulz, Josephin Marie Holstein, and Andrea Rentmeister**

The attachment of labels to RNA is highly important to efficiently study the expression, transport, and dynamics of this biomolecule in a biological context. Within the different types of RNAs, mRNAs are particularly interesting for labeling, because they contain information about gene expression associated with development and/or disease. Moreover, a large fraction of mRNAs are localized to distinct sites within a cell, illustrating that the defined localization of different mRNAs to certain subcellular regions provides a mechanism for regulating gene expression with exquisite temporal and spatial control.[1] Both the study of gene expression with RNA-sequencing methods and the study of subcellular mRNA localization are currently of tremendous importance and mRNA-labeling approaches that could improve the overall process are urgently required.

Two main strategies have been developed to label RNA: a) the direct incorporation of small chemical reporter groups during chemical or enzymatic RNA synthesis followed by postsynthetic chemical modification[2] and b) the modification of native RNA molecules, usually in a chemo-enzymatic approach.[3] In an example following the first strategy, newly transcribed RNAs were labeled by 5-ethynyluridine and could be further modified by copper-catalyzed azide–alkyne cycloaddition (CuAAC).[4] This method was used to visualize the synthesis, turnover, and transport of endogenous RNAs. Azido-derivatized nucleotides could be incorporated into RNAs of interest using nucleotidyltransferases, notably poly(A)-polymerase, and later conjugated using copper-catalyzed or strain-promoted azide–alkyne cycloaddition.[5] Besides different versions of the azide–alkyne cycloaddition, the inverse electron-demand Diels–Alder reaction was successfully used to label RNA at the 5′-end.[6]

In the alternative chemo-enzymatic strategy, enzymes are used to modify native nucleic acids. Methyltransferases catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet or SAM) to their substrate but some of them show promiscuous activity on AdoMet analogues. Taking advantage of these side activities, DNA, RNA, and protein methyltransferases have been reported to utilize several different AdoMet analogues.[3,7]

In the field of RNAs, tRNAs could be modified with an extended alkynyl moiety by using a tRNA methyltransferase, followed by fluorescent labeling by means of CuAAC.[7d] Sequence-specific labeling was achieved by reconstituting a box C/D small nucleolar ribonucleoprotein (snoRNP), consisting of three different proteins (including an RNA methyltransferase) and a box C/D guide RNA. By using different guide RNAs it was possible to modify tRNA and in vitro transcribed pre-mRNA at targeted positions by incorporation of a terminal alkyne followed by CuAAC.[7e]

Despite these considerable achievements, none of the above approaches targets the characteristics of eukaryotic mRNAs which would allow specific modification of mRNAs within the large set of RNA types present within a cell. Labeling of eukaryotic mRNAs could open up new possibilities in the field of mRNA isolation and visualization.

We present here a strategy to specifically label the 5′-cap—a hallmark of eukaryotic mRNAs—using a chemo-enzymatic approach (Figure 1). To be able to selectively tag mRNAs within the pool of all RNAs present in the cell, we looked for enzymes capable of recognizing and modifying mRNA characteristics like the $N^7$-methylated cap and the poly(A) tail. Trimethylguanosinesynthases (Tgs) are suitable candidates because they specifically methylate the 5′-cap typical of mRNAs at position $N^2$ using AdoMet as the methyl donor. Human Tgs1 (hTgs1) is the enzyme that converts standard $m^7G$ caps to the 2,2,7-trimethylguanosine (TMG) caps characteristic of small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs).[8] Hypermethylation of the $m^7G$ cap of UsnRNAs leads to their concentration in nuclear foci, their colocalization with coilin, and the formation of canonical Cajal bodies.[9] In contrast to hTgs1, the trimethylguanosinesynthase from *Giardia lamblia* (GlaTgs2) transfers only a single methyl group to the $N^2$ position of $m^7GDP$, $m^7GTP$, and $m^7GpppA$.[10] The exact in vivo function of GlaTgs2 is unknown.

We recombinantly produced Tgs from *Homo sapiens* (hTgs1) and *Giardia lamblia* (GlaTgs2) and tested these in vitro on a dinucleotide cap analogue ($m^7GpppA$, **3**) for promiscuous utilization of the AdoMet analogues Ado-

[*] D. Schulz, J. M. Holstein, Dr. A. Rentmeister
Universität Hamburg, Department Chemie
Institut für Biochemie und Molekularbiologie
Martin-Luther-King Platz 6, 20146 Hamburg (Germany)
E-mail: rentmeister@chemie.uni-hamburg.de

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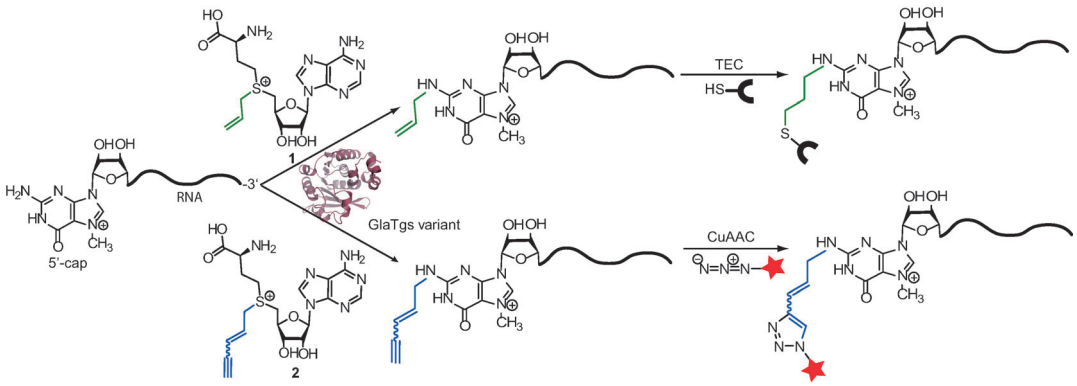


***Figure 1.*** Chemo-enzymatic labeling of cap-bearing RNAs. In a two-step procedure, the cap-specific enzyme GlaTgs2 or variants thereof are used to introduce a reactive handle which can be further modified using click chemistry. Top: GlaTgs2 or its variants produce an $N^2$-allyl-modified cap using **1** as cosubstrate. The alkene functionality can subsequently be labeled by thiol–ene click chemistry (TEC). Bottom: GlaTgs2 variants also allow modification of the cap with a terminal alkyne moiety using **2** as cosubstrate. This moiety is then amenable to CuAAC.

Propen (**1**) and AdoEnYn (**2**), which had been synthesized as previously described.[3a,7c] Activity with the natural cosubstrate AdoMet was tested as described by Monecke et al. (data not shown).[11] Both cofactor analogues (**1** and **2**) contain an unsaturated bond in β-position to the sulfur atom, which is supposed to stabilize the transition state of the transalkylation reaction and might thus make them favorable substrates for transfer.[3b] AdoPropen **1** should allow transfer of an allyl group to position $N^2$ of m$^7$GpppA **3**, resulting in $N^2$-allyl-m$^7$GpppA **4**. The use of (*E*/*Z*)-AdoEnYn (termed AdoEnYn in the following) should lead to formation of $N^2$-(pent-2-en-4-ynyl)-m$^7$GpppA **5** by transfer of a pentenynyl residue to the cap **3**. Both alkyl residues should be amenable to literature-known click chemistry.[12]

While *Homo sapiens* Tgs1 showed no activity on either of the AdoMet analogues tested (Figure S1 in the Supporting Information), GlaTgs2 showed low promiscuous activity on AdoPropen **1**, resulting in formation of a new peak at 10.6 min in the HPLC (Figure 2A), which could be assigned to $N^2$-allyl-m$^7$GpppA **4** by mass spectrometry (Figure S2 in the Supporting Information). As expected, we observed that GlaTgs2 transfered only a single alkyl chain to the $N^2$ position of the substrate.[13]

At 2.5 mol% GlaTgs2-WT, 4% of m$^7$GpppA was converted with **1** to the allyl-modified product **4**, corresponding to a total turnover number (TTN) of approximately 1 (Figure 2A). Under optimized conditions, GlaTgs2-WT reached a TTN of $3\pm2$ with AdoPropen **1**. The conversion of **3** with the bulkier cosubstrate AdoEnYn **2** by GlaTgs2-WT resulted in the formation of a very faint new peak that could not be assigned when initially observed (Figure 2B).

To obtain GlaTgs2 variants with improved promiscuous activity on AdoMet analogues **1** and **2**, we built a homology model of GlaTgs2 based on the hTgs1 structure (pdb: 3GDH[11]) using SWISS-MODEL[14] and tried to identify amino acid positions affecting accommodation of the bulkier cosubstrates (Figure S3A in the Supporting Information). We searched positions within 8 Å of the sulfur atom of the cocrystallized *S*-adenosylhomocysteine (SAH), the coproduct of the reaction, for residues that could facilitate binding of AdoMet analogues when replaced, for example by less bulky amino acids. Position V34 in GlaTgs2 was chosen for mutation after disregarding residues that were 100% conserved as well as residues known to be detrimental to folding or activity.[8,11,15]

For the best variant, GlaTgs2-Var1 (V34A), the total turnover number on AdoPropen **1** was more than three times higher than that of the wild-type enzyme (TTN $10\pm2$ vs. $3\pm2$ for WT) under the same reaction conditions (Figure 2A and Table S1 in the Supporting Information). Kinetic analysis revealed that both $k_{cat}$ and $K_M$ values were improved for AdoPropen. GlaTgs2-Var1 exhibited a $K_M$ value for AdoPropen of $57\pm29$ μM corresponding to almost threefold higher substrate affinity ($K_M = 151\pm19$ μM for WT) and a $k_{cat}$ value of $0.18\pm0.08$ min$^{-1}$ (compared to $k_{cat} = 0.09\pm0.08$ min$^{-1}$ for WT). In reactions with the native cosubstrate AdoMet, the TTN of Var1 was reduced ($108\pm6$ vs. $296\pm38$), whereas the $k_{cat}$ value was not affected by the mutation ($7.6\pm4.7$ min$^{-1}$ vs. $8.5\pm5.7$ min$^{-1}$).

We determined $T_{50}$ values to characterize the thermostability of the enzymes. $T_{50}$ is the temperature at which an enzyme loses 50% of its activity after 15 min of incubation.[16] The $T_{50}$ values of Tgs enzymes were low (hTgs1: $38.5\pm0.5$ °C, GlaTgs2: $39.9\pm0.2$ °C) suggesting that their evolvability is limited.[17] The thermostability of the most active variant, GlaTgs2-Var1, was $40.4\pm0.2$ °C and thus not compromised by the introduced mutations, which is in accordance with the observed higher TTN. Importantly, the expression of GlaTgs2-Var1 was up to 30-fold greater than that of the WT. Therefore, the enzyme concentration in the reaction could be increased and 91% conversion of m$^7$GpppA **3** was easily obtained with cofactor AdoPropen **1** (Figure 2A).

We next tested whether Var1 also exhibited improved characteristics on **2** and thus allowed efficient transfer of the comparatively large pentenynyl group. Indeed, conversion of m$^7$GpppA **3** with **2** was doubled when GlaTgs2-Var1 was used instead of WT under identical reaction conditions (Figure 2B). Due to the higher expression level of the variant, the enzyme concentration could be increased and 25% conversion could be reached (Figure 2B).

The enzymatic modification of m$^7$GpppA **3** by the cosubstrates **1** and **2** using GlaTgs2-Var1 was confirmed to

  

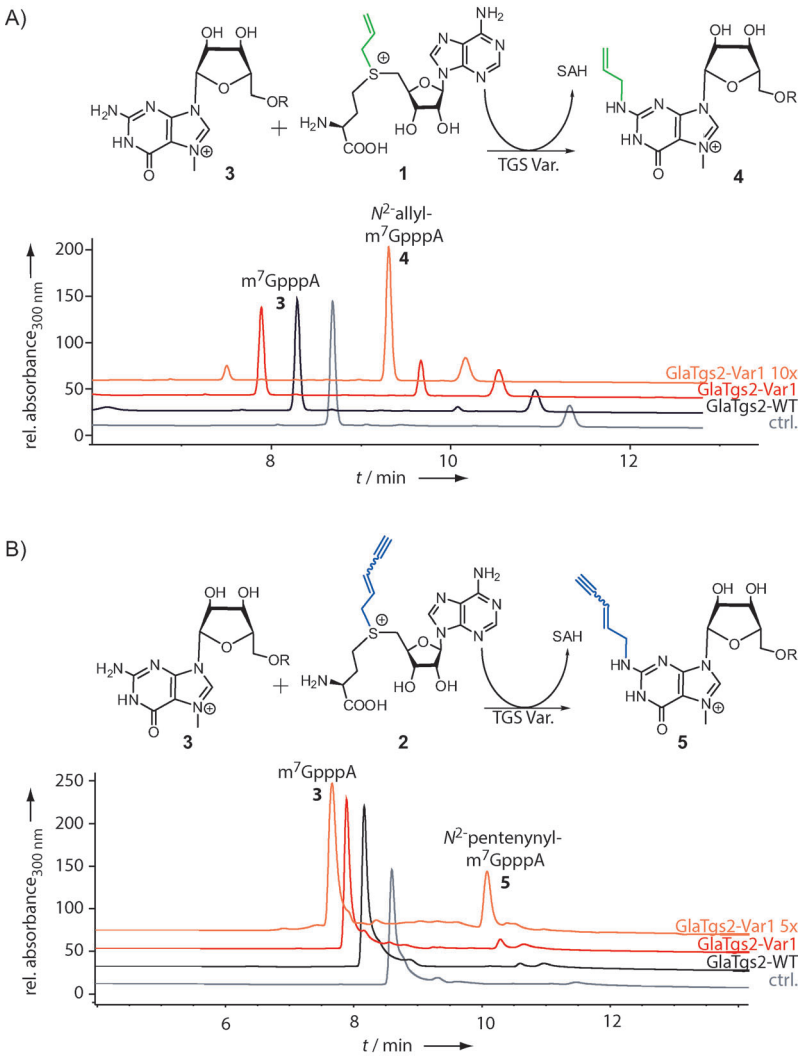


***Figure 2.*** Enzymatic modification of the mRNA cap analogue m$^7$GpppA **3** by GlaTgs2-WT and Var1 using cosubstrates **1** and **2**. HPLC analysis of indicated reactions is shown. A) Reactions with **1**. GlaTgs2-WT (6 μM) generates 11 μM **4** ($t_R$ = 10.6 min) in 3 h (black), GlaTgs2-Var1 (12 μM) produces 83 μM **4** (red). Due to its higher expression level (up to 30×), Var1 could be used in higher concentrations, leading to the formation of 250 μM **4** (91% conversion) at 120 μM Var1 (orange). Control shows the Var1-catalyzed reaction at $t$ = 0 (ctrl., gray). B) Reactions with **2**. GlaTgs2-WT (18 μM) generates 5 μM **5** ($t_R$ = 11.3 min) in 3 h (black). Var1 (26 μM) generates 18 μM **5** (red). Five times higher concentration of Var1 allowed 25% conversion (orange). Control shows Var1-catalyzed reaction at $t$ = 0 (ctrl., gray). R = pppA.

occur regiospecifically at position $N^2$ (Figures S4 and S5 in the Supporting Information). We can thus efficiently produce the modified caps **4** and **5** with excellent regiospecificity using an engineered GlaTgs2 variant.

AdoMet analogues **1** and **2** were chosen because they lead to cap structures modified with a terminal alkene and alkyne group, respectively, allowing further modification by different types of click chemistry. The thiol–ene reaction can be used to "click" a thiol group to a terminal alkene by means of a free radical mechanism upon initiation by a chemical radical initiator or by light.[12b] It can be started photochemically or thermally and leads to the formation of a stable thioether bond.[12b,18] We used the thermally activated catalyst VA-044 (2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride), and the biotin-carrying thiol **6** to convert $N^2$-allyl-m$^7$GpppA **4** to the $N^2$-biotinylated cap derivative **7** under mild conditions (44 °C, aqueous buffer) (Figure 3A). The reaction was monitored by HPLC and resulted in the formation of a new peak at 13.6 min (Figure 3B). The identity of the biotinylated cap could be confirmed by MS (Figure 3C). The thiol–ene reaction is highly specific for olefins and robust in aqueous buffer.[18a] Nonetheless, it is not applicable to reactions within the cellular environment due to competing free thiols.

We therefore used the copper-catalyzed azide–alkyne cycloaddition (CuAAC), a click reaction which is bioorthogonal as neither azide nor terminal alkyne functional groups are generally present in natural systems.[2,18a,19] The reaction has been used for the postsynthetic modification of DNA[20] and RNA.[7d,e] We used the reaction to "click" a commercially available fluorophore-carrying azide (Eterneon 480/635 azide) onto $N^2$-pentenynyl-m$^7$GpppA **5** (Figure 3D) to produce the $N^2$-fluorescently labeled cap **8**. Formation of **8** was analyzed on a PAA gel using in-gel fluorescence (Figure 3E). Only reactions starting from enzymatically modified cap **5** produced a fluorescent band upon reaction with Eterneon azide in the presence of the copper catalyst (Figure 3E).

Importantly, the formation of **5** and subsequent CuAAC could also be carried out successfully in bacterial and eukaryotic cell lysates (Figure 3E and Figure S7 in the Supporting Information). This result suggests that chemo-enzymatic cap modification is possible in the complex environment of bacterial and notably eukaryotic cells, which contain cap interacting proteins.

We next asked whether our approach could be applied to modify longer capped RNAs which match eukaryotic mRNAs more closely (see Figure 1) than the minimalistic m$^7$GpppA used so far. For this purpose we produced a 106 nt long, capped RNA by in vitro transcription and capping, and tested our chemo-enzymatic labeling approach. Reaction of RNA with **2** catalyzed by GlaTgs2-Var1 and subsequent CuAAC yielded fluorescently labeled RNA that could be detected by in-gel fluorescence (Figure 3F). The overlay showed the fluorescent band at the same position as the band found by ethidiumbromide staining, indicating that the fluorescently labeled RNA was still intact.

To test whether the RNA modification is specific for the modified cap, we also produced a control RNA of the same length but without addition of AdoMet in the capping reaction. The control RNA should thus contain the guanosine linked by a 5′-5′-triphosphate bridge but without the methylation at position $N^7$. Since $N^7$-methylation is a prerequisite

  

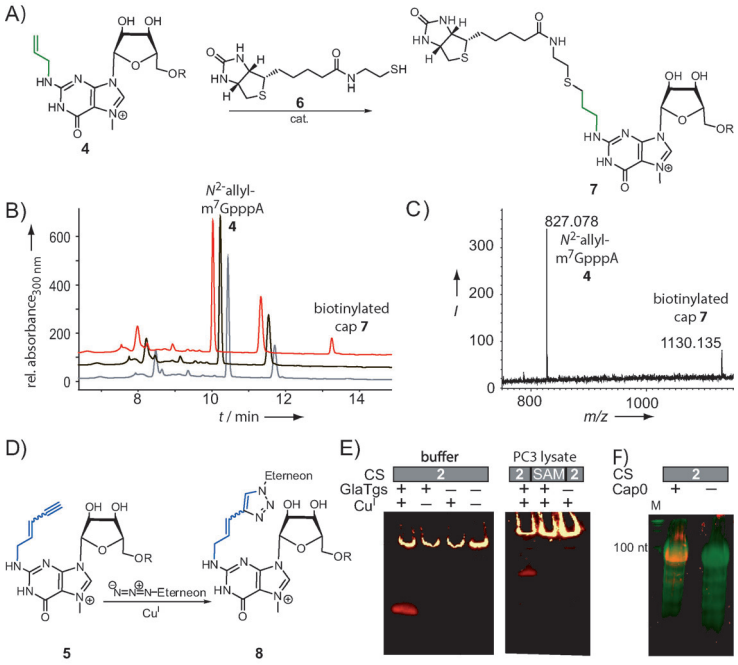


***Figure 3.*** Labeling of enzymatically modified mRNA caps using click chemistry. A) Labeling of **4** by thiol–ene click chemistry with biotin-thiol **6**. B) HPLC analysis of the thiol–ene click reaction. Formation of a new peak ($t_R = 13.6$ min) for biotinylated $N^2$-allyl-$m^7$GpppA **7** was observed after an incubation period of 4 h (red). No product was formed in negative controls ($t = 0$ h (gray) or without catalyst (black)), respectively. C) MALDI-TOF-MS analysis of a thiol–ene click reaction. Click product **7** ($m/z$ 1130), $N^2$-allyl-modified cap **4** ($m/z$ 827). D) Labeling of **5** by CuAAC with Eterneon 480/635 azide should lead to the modified cap **8**. E) CuAAC was analyzed by PAGE and Eterneon 480/635 fluorescence. A fluorescent band was detected in samples containing $N^2$-pentenynyl-$m^7$GpppA as well as $Cu^I$ after incubation at 37 °C for 1 h, but not in any of the negative controls . The chemo-enzymatic labeling of $m^7$GpppA using **2** followed by CuAAC was also performed in a lysate of PC3 cells (right gel). CS: cosubstrate in enzymatic reaction, **2** or SAM as control. Unreacted Eterneon dye causes the fluorescing signal at the top of all lanes. F) Chemo-enzymatic labeling of capped mRNA (+) by CuAAC with Eterneon 480/635 azide. In vitro transcribed capped RNA was enzymatically modified using **2** and GlaTgs2-Var1 followed by CuAAC. PAA gels were analyzed for Eterneon 480/635 fluorescence (red) and also stained with ethidiumbromide (shown in green). An overlay of obtained images is shown. The negative control (−) contained RNA of the same length but capped with an unmethylated guanosine . Marker (M), the band for 100 nt RNA, is indicated.

for further modification by GlaTgs2, the control RNA will not contain the $N^2$-pentenynyl-modified 5′-cap. As expected, the control RNA did not lead to a fluorescent band in the gel after chemo-enzymatic modification under the same conditions, indicating that the modification is specific to the 5′-cap (Figure 3F). Hence, our approach can be used to specifically label capped RNAs.

In conclusion, we have developed a novel approach for the site-specific modification of 5′-capped RNA. Since the cap is a hallmark of mRNAs, our method has a wide range of potential applications, such as the isolation and enrichment of mRNAs from total eukaryotic RNA and the selective labeling of mRNAs. We harness the ability of trimethylguanosinesynthases to recognize an $m^7$G-triphosphate at the 5′-end of the biopolymer RNA and introduce bioorthogonal reporter groups that allow attachment of functional moieties of choice—as demonstrated by introduction of a biotin or a fluorophore. Using CuAAC we could fluorescently label the cap even in the complex environment of a cellular lysate.

Our strategy consists of the enzymatic modification of mRNA cap by an alkene or alkyne residue followed by a typical click reaction, such as TEC and CuAAC. While numerous enzymes are known for the modification of DNA and noncoding RNAs (especially tRNAs and rRNAs),[21] the spectrum of mRNA-specific methyltransferases is limited, restricting the set of candidate enzymes for this purpose. The promiscuous activity of Tgs enzymes for AdoMet analogues seems to be low compared to that of other methyltransferases used previously for the chemo-enzymatic modification of proteins,[7c,f] DNA,[3,7a] tRNA,[7d,e] and pre-mRNA.[7e] We had to engineer GlaTgs2 variants to obtain good activity on AdoMet analogues. The best variant, GlaTgs2-Var1, showed not only higher specific activity for **1** and **2** but also a remarkably higher expression level. GlaTgs2-Var1 was able to convert up to 91% of $m^7$GpppA **3** using AdoPropen **1** as substrate and up to 25% using **2**.

Importantly, our approach can also be used to label longer capped RNA as we demonstrated by labeling an in vitro transcribed and capped 106 nt long RNA. This result could pave the way for useful applications in mRNA isolation and labeling.

By further extending the cosubstrate scope using protein engineering it is conceivable to achieve chemo-enzymatic modifications of the cap suitable for copper-free click reactions. For intracellular applications, potential problems such as interference of the modification with the snRNA/snoRNA machineries and 5′-tagging-induced changes in the cellular localization will have to be investigated and overcome.

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