Optogenetics

Light-Activated Control of Translation by Enzymatic Covalent mRNA Labeling

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Abstract: Activation of cellular protein expression upon visible-light photocleavage of small-molecule caging groups covalently attached to the 5′ untranslated region (5′ UTR) of an mRNA was achieved. These photocleavable caging groups are conjugated to in vitro transcribed mRNA (IVT-mRNA) through RNA transglycosylation, an enzymatic process in which a bacterial tRNA guanine transglycosylase (TGT) exchanges a guanine nucleobase in a specific 17-nucleotide motif (Tag) for synthetic pre-queuosine1 (preQ1) derivatives. The caging groups severely reduce mRNA translation efficiency when strategically placed in the 5′ UTR. Using this method, we demonstrate the successful spatiotemporal photo-regulation of gene expression with single-cell precision. Our method can be applied to therapeutically relevant chemically modified mRNA (mod-mRNA) transcripts. This strategy provides a modular and efficient approach for developing synthetic gene regulatory circuits, biotechnological applications, and therapeutic discovery.

Tools that allow researchers to manipulate gene expression are critical for basic research, biotechnology, and therapeutic applications.[1,2] There is an extensive body of research dedicated to controlling gene expression, at both the transcriptional and translational level, using approaches such as DNA/RNA methylation, histone modification, and modulation of translation factors.[3-5] RNA biogenesis is a complex process that involves processing, transportation, and maturation, and this can lead to substantial lag time between stimulus and response in such transcriptional-based gene expression regulation systems.[6] On the other hand, directly controlling the translation of mature mRNA involves much quicker response times. The cell controls various cellular functions and cell fate decisions in this manner, especially at early development stages, when rapid changes in protein concentration are needed.[6-7] More recently, in vitro transcribed mRNA (IVT-mRNA) has been widely used to selectively differentiate induced pluripotent stem cells (iPSCs), as well as to treat genetic disorders.[8] In comparison to DNA-based therapeutics, mRNA can be utilized by cells immediately upon entering the cytoplasm, leading to rapid onset of protein expression.[9] Furthermore, the use of mRNA minimizes the risk of causing insertional mutagenesis.

Regulating mRNA translation in eukaryotic cells typically focuses on the initiation stage, when initiation factors (IFs) assemble to form the 80S initiation complex. In 5′-cap-dependent translation initiation, the initiation factors elf4B and elf4F bind to the mRNA 5′ cap and then recruit the 43S preinitiation complex.[10] This complex then scans through the 5′ untranslated region (5′ UTR) until it encounters the start codon, which triggers assembly of the ribosome and translation elongation. Accordingly, traditional strategies developed to tune the efficiency of translation include the use of 5′- and 3′-UTR-binding factors and microRNAs (miRNAs), which non-covalently interact with target mRNA.[11,12] Alternative strategies using covalent RNA modifications have also been developed to regulate gene expression. For example, by modifying oligonucleotides such as antisense oligonucleotides, short interfering RNAs (siRNAs), or miRNAs with photocleavable caging groups, optical control of translation can be achieved.[12-14] Apart from these nucleic acid based approaches, modifying mRNA transcripts directly with small-molecule effectors that are responsive to external stimuli can also lead to spatiotemporal regulation of gene expression. For example, Okamoto and his co-workers have developed strategies using diazocoumarins to cage mRNAs and photoregulate gene expression.[15-20] To achieve straightforward mRNA labeling, an RNA modification strategy capable of incorporating a diverse array of functional groups onto RNA in a single step would be ideal. Furthermore, sequence-specific modification can minimize over-modification, thereby resulting in precise control of gene expression.

To covalently modify mRNA in a site-specific manner, we applied our previously developed technology, RNA transglycosylation at guanosine (RNA-TAG), to an mRNA of interest.[21] RNA-TAG utilizes a bacterial (E. coli) tRNA guanine transglycosylase (TGT) to recognize a specific genetically encoded 17-nucleotide RNA sequence (Tag) and exchange the original guanine for a synthetic derivative of pre-queuosine1 (preQ1), the natural substrate of TGT (Figure 1). Previously, we demonstrated that a variety of preQ1 derivatives bearing fluorophores or affinity tags could be covalently conjugated to an mRNA in vitro with high yield.[21-22] We hypothesized that the incorporation of bulky preQ1 derivatives (caging groups) onto the 5′ UTR of an mRNA would significantly hinder translation initiation, and that subsequent cleavage of the caging groups would activate translation. Therefore, by designing caging groups that are cleaved upon light irradiation, photoregulation of gene expression can be achieved through enzymatic mRNA labeling. Photoregulation would offer rapid and non-invasive
manipulation of target mRNA, and enable the selective activation of protein expression with high spatiotemporal resolution. Furthermore, the ability to precisely control the laser wavelength and illumination time allows minimization of cell death (Figure S11).[23] Thus, a light-activated mRNA translation system would be an extremely useful method to regulate gene expression.

An ideal photocleavable caging group would show high photolysis efficiency and be cleaved using visible light (>400 nm) to limit cytotoxicity. Therefore, we chose 6-bromo-7-aminoethoxycoumarin-4-ylmethoxycarbonyl (Bac), which shows high cleavage efficiency at a wavelength of 405 nm in vivo, as the photocleavable linker.[24,25] We synthesized the photocleavable preQ1 substrate (7) using a strategy similar that used to previous reports (Scheme 1).[21] Briefly, compound 4 was obtained through alkylation of the exocyclic amine of preQ1. The generated preQ1 derivative was then coupled to the Bac linker building block (3) to yield a Boc-protected preQ1-Bac conjugate (5).[26] To append an affinity biotin handle for purification of the labeled mRNAs, the preQ1-Bac conjugate (5) was deprotected and further coupled to a biotin-NHS ester to obtain the final TGT substrate (7), which can be photocleaved by 405 nm laser light (Scheme S7).[24,25]

To test whether covalent labeling of mRNA with the bulky biotin-Bac groups affected the efficiency of translation, three Tag sequences were inserted at various locations within the 5′UTR of an IVT-mRNA encoding EGFP (Figure 1, RNA-Tag1/2/3). Tag-1 is located immediately adjacent to the 5′cap, beginning at the third base of the IVT-mRNA. Tag-2 is located in the middle of the 5′UTR. Tag-3 is located immediately before the start codon of the EGFP open reading frame. Following in vitro transcription, the EGFP mRNA transcript (RNA-Tag1/2/3) containing all three Tags was capped and polyadenylated to enable expression in mammalian cells. The RNA-Tag1/2/3 was then labeled with photocleavable biotin-Bac-PreQ1 (7) by following standard TGT labeling conditions. TGT-labeled RNA-Tag1/2/3 was isolated through streptavidin affinity purification (57% recovery). The caged RNA-Tag1/2/3 was then transfected into cultured HeLa cells. Transfected cells were incubated overnight and imaged by fluorescence confocal microscopy. Significantly lower EGFP expression (ca. 90%) decrease was observed in cells transfected with photocaged RNA-Tag1/2/3 compared to cells transfected with unlabeled RNA-Tag1/2/3 (Figure S8A), thus suggesting that the introduction of the bulky biotin-Bac groups perturbs mRNA translation initiation.

To investigate whether the site of covalent modification within the 5′UTR affects translation efficiency, mRNA transcripts with mutations at one or more Tag sites were assayed for activity. To minimize changes among these transcripts, a guanine to cytosine (G-to-C) mutation was introduced to the key U-G-U site of the Tag, thereby rendering it unreactive for TGT-mediated labeling while maintaining the secondary structure of the RNA (Figure S1 in the Supporting Information).[21] In addition to the RNA-Tag1/2/3 with three fully functional Tags, a single G-to-C mutation was introduced to the first, second, or third Tag sequence of RNA-Tag1/2/3 to obtain RNA-Tag2/3, RNA-Tag1/3 and RNA-Tag1/2 transcripts, respectively. RNA-Tag1, RNA-Tag2, and RNA-Tag3 were obtained by introducing mutations...
at two of the three RNA-Tag1/2/3 Tags. RNA-Tag0 contains the G-to-C mutation at all three Tags and thus cannot be labeled with a photocleavable caging group. All synthetic mRNA transcripts were treated with TGT enzyme and compound 7. The enzymatically labeled and purified mature mRNAs were transfected into cultured HeLa cells. EGFP expression of these cells was quantified using fluorescence microscopy (Figure 2). A substantial decrease in EGFP expression was observed for the transcripts with only one functional Tag (RNA-Tag1, RNA-Tag2, and RNA-Tag3) compared to the unlabeled mRNA (RNA-Tag0). Notably, RNA-Tag3 demonstrated severely impaired translation activity, resulting in an EGFP expression level of 18.7% (± 0.97% SEM) compared to RNA-Tag0 (Figure 2), thus indicating that the photocaging group closest to the start codon (Tag-3) has the strongest effect on reducing translation activity. Lower expression level (15%–20%) was observed with transcripts containing two caging groups (RNA-Tag2/3, RNA-Tag1/3 and RNA-Tag1/2). Minimal EGFP expression was observed in cells transfected with labeled RNA-Tag1/2/3, with only 9.2% (± 0.64% SEM) fluorescence intensity relative to the unlabeled RNA-Tag0. Together, these data show a clear trend indicating that labeling mRNA with more photocaging groups leads to lower translation activity, with modification at Tag-3 showing the greatest impact on translation. EGFP expression of the unlabeled and labeled RNA-Tag1/2/3 was also examined with immunoblotting (Figure 3, line 2 and line 3). Upon in vitro UV cleavage of the labeled photocaging groups, translation activity of RNA-Tag1/2/3 was restored (Figure 3, line 4). However, we didn’t observe full translation reactivation of photocaged RNA-Tag1/2/3 upon in vitro UV uncaging, which might be due to the remaining residues left on the mRNA after uncaging and/or UV damage to the mRNA.

Having demonstrated that covalent labeling of three Tags on an mRNA transcript significantly hinders its translation efficiency, we next sought to explore whether protein expression could be restored by photo-uncaging. First, we demonstrated that upon 5000 μJ of in vitro UV irradiation, more than 90% of the labeled photocaging groups can be cleaved from the Tag (Figure S4, Figure S7). To demonstrate live-cell photo-uncaging of labeled IVT-mRNA, cultured HeLa cells were transfected with either unlabeled RNA-Tag0 or labeled RNA-Tag1/2/3. In order to activate protein expression while limiting cytotoxicity, we performed a laser toxicity test, demonstrating that less than 15 seconds of laser irradiation was nontoxic to HeLa cells (Figure S11). Thus, the cells transfected with photocaged RNA-Tag1/2/3 were irradiated with a 405 nm laser for 10 seconds to trigger photo-uncaging. To allow time for EGFP expression and maturation, cells were imaged 8 hours after the uncaging event (Figure 4A). To our delight, recovered EGFP expression was observed in previously “dark” cells transfected with TGT labeled RNA-Tag1/2/3, thus suggesting successful photoaactivation of gene expression (Figure 4B). Moreover, the expressed EGFP was found within the laser-illuminated cells but not in adjacent cells, thus demonstrating spatiotemporal regulation of gene expression with single-cell precision.
mRNA and may facilitate applications of mod-mRNA as an important therapeutic RNA in degenerative diseases, as well as in the treatment of genetic disorders.

In conclusion, we have demonstrated a strategy to achieve light-activated control of mRNA translation in mammalian cells through the removal of photocleavable caging groups within the 5’ UTR of an mRNA transcript. Using the RNA-TAG technology, we are able to incorporate photocleavable caging groups at site-specific positions within the 5’ UTR of an mRNA transcript, which significantly diminishes protein expression compared to the unlabeled mRNA. Translation was activated using a 405 nm laser with a single-cell precision. Through careful design of orthogonal photocleavable probes, our approach should be amenable to the activation of multiple mRNAs using different wavelengths of light. We believe that the ability to spatiotemporally regulate gene expression through enzymatic, site-specific labeling of RNA will serve as a powerful and versatile tool with applications from basic research in developmental biology and genetics to the development of novel mRNA-based therapeutics.

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**Conflict of interest**

The authors declare no conflict of interest.

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