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Letters

RNA Phosphorothioate Modification in Prokaryotes and Eukaryotes

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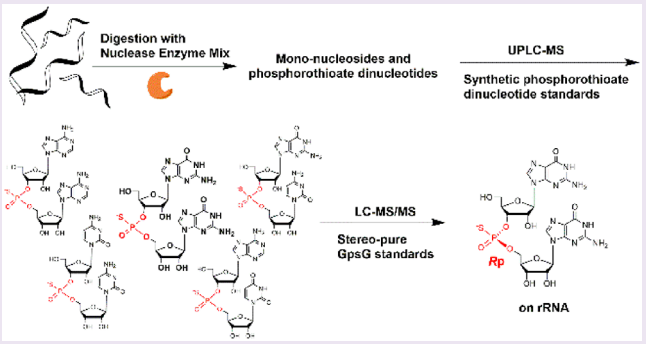
ABSTRACT: RNA modifications play important roles in RNA structures and regulation of gene expression and translation. We report the first RNA modification on the phosphate, the RNA phosphorothioate (PS) modification, discovered in both prokaryotes and eukaryotes. The PS modification is also first reported on nucleic acids of eukaryotes. The GpsG modification exists in the *Rp* configuration and was quantified with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). By knocking out the *Dnd*A gene in *E. coli*, we show the *Dnd* clusters that regulate DNA PS modification may also play roles in RNA PS modification. We also show that the GpsG modification locates on rRNA in *E. coli*, *L. lactis*, and HeLa cells, and it is not detected in rRNA-depleted total RNAs from these cells.



Post-transcriptional modifications of cellular RNAs play important roles in both normal and disease cells.[1,2] Over 150 distinct post-transcriptional RNA modifications have been identified. Beyond simply fine-tuning the structure and function of RNA, emerging studies have linked RNA modifications to various disease syndromes such as cancer and neurological disorder.[2] However, all the RNA modifications known to date are either on the ribose or on nucleobases. To the best of our knowledge, no natural modification has been reported on the phosphate backbone of RNA thus far.

The phosphorothioate (PS) modification on DNA has been discovered in bacteria,[3−6] and more recently in archaea.[7] In a phosphorothioate linkage, a sulfur atom replaces one of the nonbridging oxygen atoms and renders the stereogenic phosphorus center. The DNA phosphorothioate modifications in bacteria and archaea are known to be in the *Rp* configuration, with an occurrence of 4−31 PS modifications per 10^4 deoxynucleotides.[3,5] The DNA phosphorothioation is the product of the *Dnd* gene clusters *Dnd*A−E. Together with the DNA PS modification-based restriction enzymes by *Dnd*FGH, the *Dnd* genes constitute a restriction-modification system that protects bacteria from invading foreign DNA. The cellular functions of DNA PS modifications in bacteria and archaea also include the maintenance of cellular redox homeostasis,[3,8] cellular stress response,[4] and the epigenetic control of gene expression.[3] However, there is no report on PS modifications in eukaryotes to date.

In the rapidly growing molecular-based gene therapy field, phosphorothioate modified oligonucleotides are of central importance for therapeutic strategies that employ microRNA, small interfering RNA, and antisense oligonucleotides.[9] Most of the aforementioned therapeutic oligonucleotides approved by the U.S. Food and Drug Administration (FDA) or currently under clinical trials utilize phosphorothioate modifications to improve the metabolic stability and cellular uptake.[10,11] The traditional synthesis of phosphorothioate oligonucleotides based on phosphoramidites affords a mixture of stereoisomers. Recently, the stereogenic synthesis of phosphorothioate oligonucleotides has been significantly improved,[12−16] which will have huge impacts on future therapeutic developments.

Despite the wide investigations on DNA phosphorothioate modifications and synthetic phosphorothioate oligonucleotides, the existence of RNA phosphorothioate modification remains unknown. Since RNAs and RNA epitranscriptomics play central roles in numerous cellular processes,[17] research on RNA PS modification and related biological functions will also benefit drug development in gene therapeutics, in addition to expanding our understandings of RNA biochemistry and cell biology. For instance, if phosphorothioate modification occurs on RNA, the biological functions of RNA PS modification might impact pharmacology studies of phosphorothioate oligonucleotide-based gene therapies.[18] Encouraged by the significance of RNA phosphorothioate modification in both fundamental and health-related science, we set out to search the RNA PS modification with mass spectrometry.

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In the present work, we discovered RNA PS modifications in both prokaryotes and eukaryotes. We first digested synthetic RNA oligonucleotides containing PS modification on certain locations with a mixture of nuclease enzymes to study the kinetics of the RNA oligonucleotide digestion in the presence of phosphorothioate and phosphodiester linkages (Supporting Information Figure S8). After identifying the optimal reaction conditions in which the normal phosphodiester bonds are hydrolyzed while the phosphorothioate bonds are intact, we then digested the total RNAs extracted from various types of cells including bacteria *E. coli* and *L. lactis*, model systems such as *Drosophila melanogaster* S2 cells, and mouse liver tissues, as well as human cancer cell lines HeLa cells and HCT116 cells. After digesting the total RNA with a nuclease enzyme mixture, the resulting mononucleosides and anticipated phosphorothioate dinucleotides, together with 16 synthetic RNA dinucleotide standards linked by phosphorothioate bond, are subjected to ultraperformance liquid chromatography coupled with mass spectrometry (UPLC-MS) for identifying the RNA PS modifications. As a result, we found the RNA PS modifications, just like the DNA counterpart, occur in a sequence-specific manner. For instance, CpsA, GpsC, and GpsG are all found in the total RNA of *L. lactis* (Supporting Information Chart S1).

Next, we chose to further study the GpsG modification (shown in Figure 1) as this dinucleotide occurs at the highest

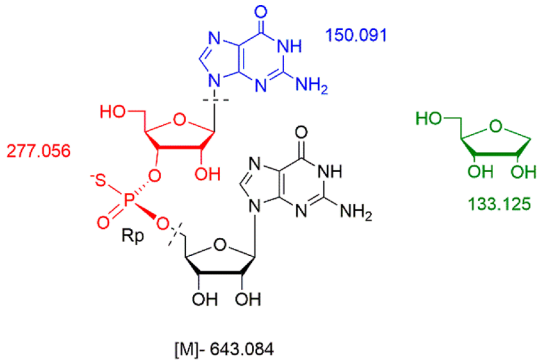

Figure 1. Mass fragmentations of GpsG.

frequencies in most of the samples we tested and exists across prokaryotes and eukaryotes (Supporting Information Chart S1). The stereo pure GpsG dinucleotides were prepared by HPLC separation after phosphoramidite-based synthesis with 3-((N,N-dimethylaminomethylidene)amino)-3H-1,2,4-dithiazole-5-thione (DDTT) as the sulfurizing reagent on an automated solid-phase oligonucleotide synthesizer. The stereo configurations of GpsG-*Rp* and GpsG-*Sp* were assigned with ^{31}P NMR (Supporting Information Figures S3 and S4). The extinction coefficients at 254 nm of the GpsG-*Rp* and GpsG-*Sp* were determined by UV spectroscopy to be 6.46×10^3 (AU cm^{-1} M^{-1}) and 5.71×10^3 (AU cm^{-1} M^{-1}), respectively. LC-MS/MS has been widely used as a sensitive technique for the detection and quantification of nucleic acid modifications.[19−24] Synthetic GpsG-*Rp* was used to develop the LC-MS/MS method. As expected, the LC retention time, the isotopic pattern, ion fragments, and ratios in the digested total RNA samples match with the data from our synthetic GpsG-*Rp* 643.084 > 227.056, 643.084 > 150.091, 643.084 > 133.125 in a negative electrospray ionization (ESI) mode (Supporting Information Chart S2, Figures S8 and S9)

We found that the nuclease enzyme mixture, purchased from New England Biolab, inhibits the hydrolysis of both GpsG-*Rp* and GpsG-*Sp* in synthetic oligonucleotides, resulting in both GpsG-*Rp* and GpsG-*Sp* being detected with LC-MS/MS (Supporting Information Figure S7). We observed GpsG-*Rp* modifications in *E. coli*, *L. lactis*, *Drosophila melanogaster* S2 cells (DM S2), mouse liver, as well as in human cancer cell lines HeLa and HCT116 cells. No GpsG-*Sp* modification was detected in these samples. The observation of only GpsG-*Rp* and no GpsG-*Sp* RNA PS modification in our study is consistent with the stereo configuration of DNA PS modification reported in bacteria and archaea.[3,7] Next, for the quantification of GpsG-*Rp* in cells, we digested the extracted total RNAs, subjected the samples for LC-MS/MS analysis, and obtained the concentrations of GpsG-*Rp* and guanosine, respectively. The ratios of GpsG-*Rp*/G are shown in Figure 2.

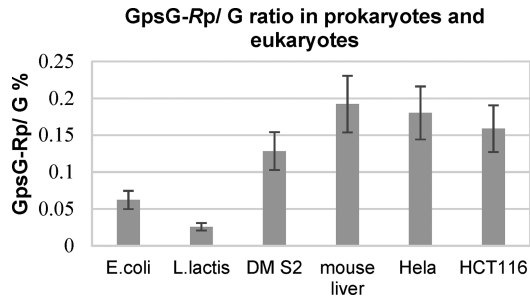

Figure 2. Frequency of the GpsG-*Rp* modification, shown as a GpsG-*Rp*/G ratio, in *E. coli*, *L. lactis*, *Drosophila melanogaster* S2 cells (DM S2), mouse liver, as well as in human cancer cell lines HeLa and HCT116 cells.

Unlike the DNA PS modification, which is reported only in bacteria and archaea, we discovered the RNA PS modification also exist in eukaryotes. This discovery expands the knowledge of the phosphorothioate modification in nucleic acids and attracted us to further investigate the enzymes responsible for the regulation of RNA PS modification. Since the PS modification GpsG also exists only in the *Rp* form and the abundance is similar to that of DNA PS modifications found in bacteria, it might be possible that this modification is regulated by the same set of enzymes. The *Dnd* gene clusters that regulate archaeal and bacterial DNA PS modifications exhibit conservation in all domains of life. For instance, *Dnd*A shares significant nucleotide sequence identity and protein structural similarity with the pyridoxal 5-phosphate (PLP)-dependent cysteine desulphurases IscS and NifS in eukaryotes.[4] Therefore, we set out to knock out the *Dnd*A gene, which is reported to provide the sulfur source in the biosynthesis of DNA PS bonds in bacteria, to test whether *Dnd*A is also involved in RNA PS modifications.[3]

The lambda red homologous recombination method was chosen to manipulate the *Dnd*A gene in the *E. coli* BUN21/pML300 strain. The pML300 carries the genes encoding the exo, beta, and gam recombination functions of bacteriophage lambda, as well as RecA, to render the *E. coli* strain recombination proficient.[25] A bioinformatic search and DNA sequencing of the anticipated PCR products confirmed that the BUN21/Pml300 strain harbors the *Dnd*A gene (GenBank accession number ZP 00714230).[6] After recombineering,[26] the ampicillin-resistant gene replaces the *Dnd*A gene with 50-

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nucleotide homologous arms to delete the *Dnd*A gene and serve as the selection marker. The *Dnd*A-knocked out colony was confirmed by DNA sequencing of the PCR amplified gene of interest. The BUN21/pML300 and DndA-knocked out strains were grown; the total RNAs were extracted and digested for GpsG quantification with LC-MS/MS.

The LC-MS/MS analysis of the GpsG (Figure 3) shows that the modification level of the *Dnd*A-knocked out strain is less

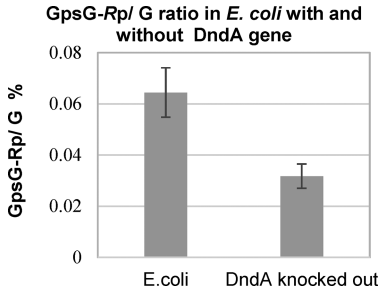

Figure 3. Frequency of GpsG-*Rp* modification before and after *Dnd*A gene was knocked out in *E. coli*.

than that of the BUN21 strain by half. The result suggests *Dnd*A is also involved in the production of RNA PS modification. Previously, it was reported that DNA PS modification was also detected in bacteria strains lacking the DndA gene, and this backbone thiolation was performed by other sulfur-providing enzymes such as cysteine desulphurase IscS.[27,28] Similarly in the RNA PS modification case, other enzymes in the *Dnd* gene cluster as well as the evolutionarily conserved eukaryotic counterparts will need to be investigated, probably guided by bioinformatic studies.

In epitranscriptomic research, the distribution and abundance of post-transcriptional modifications in mRNA, rRNA, and other noncoding RNAs in different cellular environments shed light on its biological functions in both normal and diseased cells. Therefore, it is important to investigate the distribution and frequency of the newly discovered RNA PS modification in different types of RNAs. We hypothesized that localizing on rRNA may account for the high frequency of the GpsG modification relative to other dinucleotide RNA PS modification. We purified rRNA and rRNA-depleted total RNA from *E. coli*, *L. lactis*, and HeLa cells to study the GpsG frequency with the LC-MS/MS method. The rRNA from *E. coli*, *L. lactis*, and HeLa cells were purified with sucrose cushion centrifugation[30] followed by the acid phenol extraction or TRIzol extraction. The rRNA-depleted total RNA samples were obtained by treating the total RNA extracted from bacteria *E. coli* and *L. lactis* with the RiboMinus Transcriptome Isolation Kit for bacteria from Invitrogen. For HeLa cells, the mRNA was first pulled down with GenElute mRNA Miniprep Kit from Sigma-Aldrich then treated with RiboMinus Eukaryote Kit from Invitrogen to obtain the HeLa mRNA. The rRNA and rRNA-depleted total RNAs were then digested and subjected to LC-MS/MS analysis for measuring the GpsG frequency. In Table 1, we show that the GpsG modification was found in rRNA but not detected in the rRNA-depleted samples from *E. coli*, *L. lactis*, and HeLa cells. The same trend of GpsG distribution was also found in the *DndA*-deleted mutant, with the frequency reduced by half relative to that of the wild type *E. coli* cell.

Table 1. Frequency of the GpsG-*Rp* Modification, Shown As a GpsG-*Rp*/G % Ratio, in Total RNA, Purified rRNA, and rRNA-Depleted Total RNA from Wild Type *E. coli*, *Dnd*A-*E. coli*, *L. lactis*, and HeLa Cells

type of RNA	GpsG-*Rp*/G%
E. coli total RNA	0.046 ± 0.009
E. coli rRNA	0.033 ± 0.007
E. coli rRNA-depleted	0.00048 ± 0.0001
E. coli DndA- total RNA	0.020 ± 0.004
E. coli DndA- rRNA	0.017 ± 0.004
E. coli DndA- rRNA-depleted	0
L. lactis total RNA	0.021 ± 0.004
L. lactis rRNA	0.022 ± 0.005
L. lactis rRNA-depleted	0.00068 ± 0.0001
HeLa total RNA	0.25 ± 0.05
HeLa rRNA	0.39 ± 0.08
HeLa mRNA	0.0022 ± 0.0005

We show that the GpsG-*Rp* modification, which is the most frequently occurring dinucleotide RNA PS modification in total RNAs from the samples we tested, locates on the rRNA in *E. coli*, *L. lactis*, and HeLa cells. Similarly, other PS dinucleotide modification, albeit less frequent in total RNA, may be enriched in certain types of cellular RNAs. Our discovery opens new opportunities to investigate the RNA PS modification epitranscriptomic-wide and reveal their significance in chemical biology. It is anticipated that other closely related research topics, such as whether the RNA PS modification is permanent or reversible, its dynamic regulation enzymes and mechanisms, its potential epigenetic functions, and the existence of complex PS modification combined with base or ribose modifications, will attract further investigations and the developments of other detection techniques and molecular tools[29] focusing on RNA PS modification. Meanwhile, the recently developed stereocontrolled synthesis of phosphorothioate oligonucleotides[16] could benefit the synthesis of longer sterically pure phosphorothioate standards to further investigate the PS modifications in forms of trinucleotides and beyond.

In summary, RNA phosphorothioate modification exists in both prokaryotes and eukaryotes. Our discovery expands the library of nucleic acid modifications and provides new opportunities to investigate the regulation of genetic information by the phosphorothioate modification. Because the phosphorothioate oligonucleotides are common in gene therapy, it also raises the significance of the cellular function of RNA PS modification for developing future gene therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00163.

Description of the synthesis and purification of oligonucleotide, phosphorothioate dinucleotide standards, extinction coefficient data, UPLC-MS and LC-MS/MS analysis, DndA gene knock out with lambda-red homologous recombineering, and purification of cellular RNAs (PDF)

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Notes

Part of this work has been published as a preprint in *ChemRxiv*. The authors declare no competing financial interest.

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