Identification of 2-methylthio cyclic N6-threonylcarbamoyladenosine (ms2ct6A) as a novel RNA modification at position 37 of tRNAs

Byeong-il Kang1,†, Kenjyo Miyauchi1,†, Michal Matuszewski2, Gabriel Silveira D’Almeida3, Mary Anne T. Rubio3, Juan D. Alfonzo3, Kazuki Inoue1, Yuriko Sakaguchi1, Takeo Suzuki1, Elżbieta Sochacka2,* and Tsutomu Suzuki1,†

1Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, Tokyo 113-8656, Japan, 2Institute of Organic Chemistry, Faculty of Chemistry, Lodz University of Technology, Lodz 90-924, Poland and 3Department of Microbiology and The Center for RNA Biology, The Ohio State University, Columbus, OH 43210, USA

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ABSTRACT

Transfer RNA modifications play pivotal roles in protein synthesis. N6-threonylcarbamoyladenosine (t6A) and its derivatives are modifications found at position 37, 3′-adjacent to the anticodon, in tRNAs responsible for ANN codons. These modifications are universally conserved in all domains of life. t6A and its derivatives have pleiotropic functions in protein synthesis including aminoacylation, decoding and translocation. We previously discovered a cyclic form of t6A (ct6A) as a chemically labile derivative of t6A in tRNAs from bacteria, fungi, plants and protists. Here, we report 2-methylthio cyclic t6A (ms2ct6A), a novel derivative of ct6A found in tRNAs from Bacillus subtilis, plants and Trypanosoma brucei. In B. subtilis and T. brucei, ms2ct6A disappeared and remained to be ms2t6A and ct6A by depletion of tcdA and mtaB homologs, respectively, demonstrating that TcdA and MtaB are responsible for biogenesis of ms2ct6A.

INTRODUCTION

RNA modifications are a type of qualitative information embedded in RNA molecules (1). To date, about 140 species of modified nucleosides have been identified in various RNAs from all domains of life (2). tRNAs contain a number of chemical modifications that are required for accurate translation of the genetic code and stabilization of the tRNA tertiary structure (3–5). In particular, a wide variety of modifications is present in the anticodon loop, especially at the first position of the anticodon (position 34) and position 37, which is 3′-adjacent to the anticodon. These modifications play critical roles in modulating codon recognition and ensuring accurate translation (6).

N6-threonylcarbamoyladenosine (t6A) (Supplementary Figure S1) and its derivatives are evolutionarily conserved essential modified bases at position 37 of tRNAs responsible for recognition of adenosine-starting codons (ANN codons) (7). The bulky side chain of t6A stabilizes the anticodon loop, promoting accurate decoding of ANN codons during protein synthesis (8,9). In addition, t6A is required for efficient aminoacylation of tRNA and efficient translocation, and it also prevents leaky scanning of initiation codons and read-through of stop codons. The biogenesis of t6A has been studied extensively. In E. coli, four enzymes, TsaC (YrdC), TsaD (YgjD), TsaB (YeaZ), and TsaE (YjeE), are required to synthesize t6A; L-threonine, adenosine triphosphate (ATP) and bicarbonate as substrates (8). In yeast, the YrdC homolog Sua5 and several components of the EKC-KEOPS complex, including Kae1, Pcc1, Gon7 and Bud32, are involved in t6A formation (10–12).

The presence of t6A in tRNAs from E. coli and yeast was first documented more than four decades ago (13). In 2013, however, we showed that most fraction of t6A in E. coli tRNAs is a hydrolyzed artifact of cyclic t6A (ct6A) (Supplementary Figure S1) (9). ct6A is an additional modification of t6A that enhances tRNA decoding activity. ct6A is present in tRNAs from certain groups of bacteria, fungi, plants and some protists, but not in tRNAs of mammals, archaea or other bacteria. In E. coli, little t6A is present in tRNAs because almost all t6A is converted to ct6A via an ATP-dependent dehydration catalyzed by TcdA. We also identified two additional factors, CsdA and CsdE, which are required for efficient ct6A formation. CsdA is a cysteine
desulfurase, and CsdE is a sulfur acceptor protein, implying that sulfur relay is involved in efficient formation of ct6A.

Initially, mass spectrometric and nuclear magnetic resonance (NMR) analyses determined the chemical structure of ct6A to be a cyclized active ester of the oxazolone ring (Supplementary Figure S1) (9,14). Very recently, however, X-ray crystallography of synthetic ct6A nucleoside revealed the existence of a distinct isomeric with a hydantoin structure (Supplementary Figure S1) (15). LC/MS co-injection analyses showed that chemically synthesized ct6A nucleoside and natural ct6A in Escherichia coli tRNAs co-elute as a single peak by both reverse-phase and hydrophilic interaction liquid chromatography. They also exhibit identical patterns of product ions in collision-induced dissociation (CID) and a characteristic UV spectrum with maximum absorption at 269 nm. These observations strongly suggest that the hydantoin isoform of ct6A is actually present in E. coli tRNAs.

Another derivative of t6A, N6-methyl-N6-threonylcarbamoyladenosine (m4t6A) (Supplementary Figure S1), is present in tRNAs from bacteria, fly, plants and mammals. In E. coli, m4t6A is present at position 37 of two species of tRNAThr responsible for translating Lys codons. We identified TrmO, a member of a novel class of AdoMet-dependent RNA methyltransferase, as the enzyme responsible for N6 methylation of m4t6A37 of bacterial tRNAThr (16). Its human homolog, TRMO, is responsible for formation of m4t6A37 in cytoplasmic tRNASer. Lack of TrmO decreases attenuation activity of bacterial tRNAs. Mutations in MtaB (21,22) and human Cdkal1 (23) are associated with risk of type 2 diabetes (23). The presence of ct6A was previously detected. Indeed, a Tris-adduct of ms2t6A was detected in tRNAs co-injection (Figure 1B) from Trypanosoma brucei (19), indicating the presence of ms2t6A in natural tRNAs. Mutations in CDKAL1 are associated with risk of type 2 diabetes (24); consistent with this, pancreatic β-cell-specific knockout of mouse Cdkal1 results in a phenotype similar to that of type 2 diabetes (23). The presence of ct6A and TcdA homologs in B. subtilis, plants and Trypanosoma prompted us to speculate that the cyclic form of ms2t6A (ms2ct6A) (Figure 1A) would also be present in these organisms. As with ct6A, ms2ct6A must be hydrolyzed and converted to ms2t6A during tRNA preparation or conventional nucleoside analysis, which explains why it has not been previously detected. Indeed, a Tris-adduct of ms2t6A was detected in tRNA by LC/MS co-injection with the chemically synthesized authentic nucleoside. We also confirmed that orthologs of TcdA and MtaB are responsible for biogenesis of ms2ct6A in B. subtilis and T. brucei. In addition, we observed slower growth of T. brucei when the TcdA ortholog was down-regulated in the presence of cycloheximide, indicating that cyclic form of t6A is involved in integrity of protein synthesis.

**MATERIALS AND METHODS**

**Bacterial strains and cultivation**

*B. subtilis* str. 168 (WT) was kindly provided by Akiko Soma (Chiba University). *B. subtilis* strains were grown overnight in LB medium at 37°C. The ΔyqeV strain harboring the erythromycin resistance marker (Emr) was obtained from the National BioResource Project (National Institute of Genetics, Japan). The ΔyqeV ΔyrrM strain was constructed from the WT strain, and the ΔyqeV ΔyrrM double-deletion strain was constructed from the ΔyqeV strain by homologous recombination (26,27). The 5’ upstream and 3’ downstream regions (700–800 nt) of the yqeV gene were PCR-amplified from *B. subtilis* str. 168 genomic DNA with pairs of primers, 5’-ggacac tgttctgatcgtgat-3’ and 5’-aagcgagcttctgatcaagctggtttttttg-3’ and 5’-tggaggtgagcttctgatgaagccg-3’ and 5’-ggacac tgttgatccggaagaagc-3’, respectively. Chloramphenicol resistant gene (Cm r) gene was PCR-amplified from pCB31 (28) using a set of primers, 5’-gatcaagcttctgatcgtgat-3’ and 5’-tggaggtgagcttctgatgaagccg-3’ and ms2ct6A, subjected to the nested PCR amplification using a pair of primers, 5’-tcatgatcagcggagaagcc-3’ and 5’-ggacac tgttgatccggaagaagc-3’. The resultant PCR fragment was used for transformation.

**Total RNA extraction**

*B. subtilis* cells were suspended with 5 ml of RNA extraction buffer [50 mM NaOAc (pH 5.2) and 10 mM Mg(OAc)2 (pH 5.2)] and vigorously stirred for 10 min at room temperature. Next, 5 ml of water-saturated phenol was added and stirred for 10 min at room temperature. The mixture was frozen with liquid nitrogen and thawed in water; this process was repeated twice. The thawed solution was stirred for 50 min at room temperature. The aqueous phase was separated by centrifugation and washed once with chloroform, followed by re-extraction with 0.75 volumes of Trizol-LS (Life Technologies). Then, total RNA was precipitated by centrifugation with 2-propanol. The RNA pellet was dissolved in deionized water and subjected to ethanol precipitation; the resultant pellet was rinsed with 80% ethanol and dried. Thus, prepared RNA can be stored in pellet form for a long period of time without hydrolysis of ct6A and ms2ct6A. For use in all experiments, pellets were dissolved in ultrapure water.

Total RNA of spinach and Arabidopsis thaliana were extracted from plants as previously described (9). Nicotiana tabacum total RNA was obtained by the same procedure from tobacco BY-2 cells cultured for 1 week in modified Linsmaier and Skoog medium (29). Total RNA samples of spinach and tobacco were subjected to brief purification by weak anion exchange chromatography with DEAE Sepharose Fast Flow (GE Healthcare) to remove bulk contaminants and rRNA, as described (30).
Isolation of *B. subtilis* tRNA\(^{\text{Lys}}\)^{15}\(^{35}\)

*B. subtilis* tRNA\(^{\text{Lys}}\)\(^{15}\(^{35}\) was isolated from *B. subtilis* total RNA by reciprocal circulating chromatography, as described previously (9,31). The DNA probe, TGGTGAGC-CATGAAGGACTCGAACCTTCGA with 5'-terminal EC amino linker was covalently immobilized on NHS-activated Sepharose 4 Fast Flow (GE Healthcare). Fifty one micrograms of highly purified tRNA\(^{\text{Lys}}\)\(^{15}\(^{35}\) was obtained from 1.7 mg total RNA.

Nucleoside preparation

Before digestion, total RNA was pre-cleared by gel filtration on a Centri-Sep spin column (Princeton Separations) with deionized water or trimethylamine (TMA)-HCl (pH 7.0) buffer to remove contaminants that could interfere with the ionization efficiency of nucleosides. For this experiment, enzymes including nuclease P1 (Wako Pure Chemical Industries), phosphodiesterase I (PDase I, Worthington Biochemical Corporation) and bacterial alkaline phosphatase (BAP from *E. coli* C75, Wako Pure Chemical Industries) were dialyzed with deionized water and stored at −30°C until use. Phosphodiesterase II (PDase II, from bovine spleen, Sigma) was dissolved in 10 mM TMA-AcOH (pH 5.3) buffer and centrifuged. The supernatant was filtered through a 0.22 μm Ultrafree-MC unit (Merck-Millipore) and stored at −30°C.

For conventional digestion (32), 40 μg of total RNA was digested at 37°C for 1 h in a 25–50 μl reaction mixture consisting of 0.1 U nuclease P1 and 25 mM NH\(_4\)OAc (pH 5.3), followed by addition of 0.1 volume of 1 M ammonium bicarbonate (pH 8.2) and 0.08 U BAP and then incubated at 37°C for 3 h.

For neutral digestion (9), 40 μg of total RNA was digested at 37°C for 1 h in a 25–50 μl reaction mixture consisting of 0.1 U nuclease P1 and 25 mM NH\(_4\)OAc (pH 5.3), followed by addition of 0.1 volume of TMA-HCl (pH 7.0) and 0.127 U PDase I, and then incubated at 37°C for 1 h.
The prepared nucleotides were dephosphorylated with 0.08 U BAP at 37°C for 3 h at neutral pH. Digestion of plant and several other RNAs was carried out by one-step acidic digestion. Specifically, a solution (typically 40 μl) containing 1 μg/μl total RNA, 20 mM TMA acetate (pH 5.3), nuclease P1 (0.1 units for 40 μg of RNA), PDase II (0.1 units for 40 μg of RNA) and BAP (0.16 units for 40 μg of RNA) was incubated at 37°C for 1 h. In this procedure, PDase II was used for complete digestion of hypermodified adenosines under acidic conditions. We observed conservation of adenosine and N6-methyladenosine (mA) to inosine, indicating contamination by adenosine deaminase activity in the Sigma PDase II product (P9041). t6A derivatives remained intact under these conditions.

**LC/MS analyses of total nucleosides and isolated tRNA**

LC/MS analyses of total nucleosides were performed essentially as described previously (9.33–34), using an LCQ Advantage ion-trap (IT) mass spectrometer (Thermo Fisher Scientific) equipped with an ESI source and an HP1100 liquid chromatography system (Dionex). For RPC/ESI-MS with an LCQ Advantage instrument, nucleosides were separated on an Inertil ODS-3 column (2.1 mm × 250 mm, GL sciences) and analyzed as described previously (34). For RPC/ESI-MS with a Q Exactive instrument, digests were separated on a Sunshell C18 column (2.6 μm core-shell silica particle, 2.1 × 15 mm, ChromaNik Technologies). The mobile phase consisted of 5 mM ammonium acetate (pH 5.3) (solvent A) and acetonitrile (ACN) (solvent B). The gradient program was as follows: 0–40% B from 0 to 30 min, 40% B for 5 min and then 0% B at a flow rate of 75 μl/min. Nucleoside digest (8–12 μg) or synthetic ms2ct6A (100–500 pmol) dissolved in LC/MS grade ultrapure water (Wako) was injected.

For HILIC/ESI-MS, a ZIC-hILIC column (3 μm particle size, 2.1 × 150 mm, Merck-Millipore) was used on a Q Exactive instrument (33). The mobile phase consisted of 5 mM ammonium acetate (pH 5.3) (solvent A) and acetonitrile (ACN) (solvent B). The gradient program was as follows: 0–40% B from 0 to 30 min, 40% B for 5 min and then 0% B at a flow rate of 75 μl/min. Nucleoside digest (8–12 μg) or synthetic ms2ct6A (100–500 pmol) dissolved in LC/MS grade ultrapure water (Wako) was injected.

For RNA fragment analysis of isolated tRNA, *B. subtilis* tRNA was digested by RNase T1, followed by subjected to capillary liquid chromatography (LC) coupled to nano electrospray (ESI)/mass spectrometry (MS) on a linear ion trap-Orbitrap hybrid mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific) as described (9.34).

**Chemical synthesis of ms2ct6A**

The substrate nucleoside, 2-methylthio-N6-threonylcarbamoyladenosine (ms2t6A) was synthesized according to the procedures described previously (35–38). Cyclization of ms2t6A to form ms2ct6A was performed on the basis of carbodiimide chemistry. ms2t6A (10 mg, 0.022 mmol) was dissolved in anhydrous DMF (1 ml) and mixed with EDC·HCl (42 mg, 0.22 mmol). The reaction mixture was stirred at room temperature. After 3 h, consumption of all substrate was confirmed by TLC analysis (nBuOH/H2O, 85/15, v/v, RF values of ms2t6A and ms2ct6A are 0.15 and 0.43, respectively). The solvent was removed under reduced pressure, and the crude product was purified by reverse-phase chromatography (Ascentis C18 HPLC Column, 10 μm, 21.2 × 250 mm) at a flow rate of 7 ml/min with a linear gradient of acetonitrile in 0.1% acetic acid (B) and water (A) as follows: 2–30% B from 0 to 40 min, 30–50% from 40 to 45 min, 50–2% B from 45 to 47 min, 2% B for 3 min. The ms2ct6A fraction (22.35 min) was collected and evaporated to dryness. Yield of ms2ct6A nucleoside was 44% (4.2 mg).

The purity of ms2ct6A was checked by high performance liquid chromatography (HPLC) analysis using an XTerria® Waters column (MS C8, 5 μm, 4.6 × 150 mm, 100 A) (Supplementary Figure S2). The mobile phase consisted of 5 mM sodium acetate (pH 7) in water (solvent A) and ACN (solvent B). Chemically synthesized ms2t6A (A) and ms2ct6A (B) were chromatographed at a flow rate of 1 ml/min with a dual-step linear gradient: 0–20% B from 0 to 30 min, and 20–40% B from 30 to 45 min. Isolated ms2ct6A was characterized by UV spectroscopy (Supplementary Figure S3), IR spectroscopy (Supplementary Figure S4), 1H-NMR (Supplementary Figure S5), 13C-NMR (Supplementary Figure S6) and high resolution MS (Supplementary Figure S7).

**Cultivation and RNAi of T. brucei**

Partial segments of the coding sequences of the Tbid2tmp.02.2830 (*T. brucei*), and Tbid26.03.510 (*T. brucei*) were cloned into the tetracycline-inducible RNAi vector p2T7-177. These plasmids were then linearized by NotI digestion and introduced into procyclic *T. brucei* 29-13 cells for genomic integration; clonal lines were obtained by limiting dilution. The cell lines were grown in SDM-79 medium, and RNAi was induced by addition of 1 μg/ml tetracycline. Cell counts were taken every 24 h using a Beckman Z2 Coulter counter over the course of 12 days post-induction in the presence or absence of tetracycline (1 μg/ml) and cycloheximide (50 μg/ml).

Total RNA was extracted from uninduced and RNAi-induced *T. brucei* cells using a standard protocol (39). The steady-state levels of individual mRNAs were measured by RT-PCR. The cDNAs of *T. brucei* (677 bps) and TbTcdA (443 bps) were amplified using the following primers: 5′-aatcactattctctagcatcctc-3′ and 5′-ggtgtcagcactttcacaa-3′ for *TbMtaB*, and 5′-gtcactaacggaacctgcttg-3′ and 5′-gaeaggatgctagcagactga-3′ for *TbTcdA*.

PCR products were then analyzed by agarose gel electrophoresis. The same reaction performed without reverse transcriptase was used as a negative control (RT-).
RESULTS
Identification of ms<sup>2</sup>ct<sup>6</sup>A in B. subtilis tRNAs

We previously detected ct<sup>6</sup>A in total nucleosides of B. subtilis tRNAs digested under neutral conditions (9). Consistent with this finding, the B. subtilis YrvM is an ortholog of TcdA, which catalyzes ATP-dependent dehydration of t<sup>6</sup>A to form ct<sup>6</sup>A (9). In addition, ms<sup>2</sup>t<sup>6</sup>A is present in tRNA<sup>Lys</sup> from B. subtilis (21,22). These observations strongly suggest that, in B. subtilis, ms<sup>2</sup>t<sup>6</sup>A in tRNA<sup>Lys</sup> is converted to ms<sup>2</sup>ct<sup>6</sup>A by the TcdA homolog.

To confirm the presence of ms<sup>2</sup>ct<sup>6</sup>A, total RNA of B. subtilis was digested into nucleosides under conventional or neutral conditions, and then subjected to LC/MS analysis (Figure 2A). In the conventional conditions, we observed proton adducts of t<sup>6</sup>A and ms<sup>2</sup>t<sup>6</sup>A, but no ct<sup>6</sup>A, as reported previously (9). On the other hand, in the neutral conditions, ct<sup>6</sup>A was clearly detected. In addition, we detected a dehydrated form of ms<sup>2</sup>t<sup>6</sup>A (m/z 441). This modified species, tentatively named N<sup>440</sup>, was not detected in total nucleosides digested in the conventional conditions, indicating that it was susceptible to hydrolysis (Figure 2A). This observation strongly suggested that N<sup>440</sup> is ms<sup>2</sup>ct<sup>6</sup>A.

Considering that B. subtilis tRNA<sup>Lys</sup> has ms<sup>2</sup>t<sup>6</sup>A at position 37 (Figure 1B) (17), ms<sup>2</sup>ct<sup>6</sup>A should be found in this tRNA. B. subtilis tRNA<sup>Lys</sup> was isolated by the reciprocal circulating chromatography (9,31), digested by RNase T<sub>1</sub> and subjected to capillary LC coupled to ESI/MS. The 12 mer-fragments containing anticodon region were clearly detected. Judging from the chloroplast values of triply-charged negative ions of this fragment (Figure 2B), we clearly detected three fragments having different modifications at position 37, namely ms<sup>2</sup>A, ms<sup>2</sup>t<sup>6</sup>A and ms<sup>2</sup>ct<sup>6</sup>A. Although ms<sup>2</sup>t<sup>6</sup>A37 was present more abundant than ms<sup>2</sup>ct<sup>6</sup>A37 in the isolated tRNA<sup>Lys</sup>, it is likely that a certain population of ms<sup>2</sup>t<sup>6</sup>A37 in this tRNA originates from ms<sup>2</sup>ct<sup>6</sup>A37 hydrolyzed during tRNA isolation by RCC. The 12 mer-fragment with ms<sup>2</sup>ct<sup>6</sup>A was further probed by collision-induced dissociation to map the modified residues (Figure 2C). By assignment of product ions in the CID spectrum, we unequivocally mapped cmnm<sup>5</sup>s<sup>2</sup>U at position 34 and ms<sup>2</sup>ct<sup>6</sup>A at position 37 (Figure 2C).

To determine the structure of N<sup>440</sup>, we chemically synthesized ms<sup>2</sup>ct<sup>6</sup>A from ms<sup>2</sup>t<sup>6</sup>A (Supplementary Figure S2B). As reported in the accompanying paper (15), activation of the carboxyl group of t<sup>6</sup>A by water-soluble carbodiimide (EDC), which facilitates cyclization of the side chain, predominantly generates the hydantoin isoform of ct<sup>6</sup>A. Hence, we employed the same procedure as for ct<sup>6</sup>A synthesis to cyclize the side chain of ms<sup>2</sup>t<sup>6</sup>A, the hydantoin isoform should be the predominant form in chemically synthesized ms<sup>2</sup>ct<sup>6</sup>A. Detailed spectroscopic analyses of the synthesized ms<sup>2</sup>ct<sup>6</sup>A using UV (Supplementary Figure S3B), IR (Supplementary Figure S4B), <sup>1</sup>H NMR (Supplementary Figure S5) and <sup>13</sup>C NMR (Supplementary Figure S6) supported the hydantoin isoform as observed for ct<sup>6</sup>A in the accompanying paper (15). Especially, in the IR spectrum (Supplementary Figure S4B), two characteristic absorption bands at 1720 cm<sup>−1</sup> and 1788 cm<sup>−1</sup> are associated with C = O bond stretching in the hydantoin ring.

The synthetic ms<sup>2</sup>ct<sup>6</sup>A was mixed with total nucleosides of B. subtilis and subjected to LC/MS analyses using reverse-phase chromatography (Figure 3A), as well as hydrophilic interaction chromatography (Figure 3B). The synthetic ms<sup>2</sup>ct<sup>6</sup>A co-eluted with N<sup>440</sup> as a single peak under both conditions. Next, we used CID to further probe the base-related ions (BH<sup>2+</sup>, m/z 309) of N<sup>440</sup> and the synthetic ms<sup>2</sup>ct<sup>6</sup>A (Figure 3C). The product ions of these two compounds exhibited identical patterns (Figure 3D), and assigned in the chemical structures of the ms<sup>2</sup>ct<sup>6</sup>A base (Figure 3E). Collectively, these observations indicated that N<sup>440</sup> is ms<sup>2</sup>ct<sup>6</sup>A.

Identification of ms<sup>2</sup>ct<sup>6</sup>A in plant tRNAs

According to phylogenetic analyses (9,40), homologs of mtaB and tcdA, respectively (9,22), are encoded in plant genomes, indicating the presence of ms<sup>2</sup>ct<sup>6</sup>A in plant tRNAs. Indeed, ct<sup>6</sup>A and ms<sup>2</sup>t<sup>6</sup>A were previously detected in Spinach (9) and Elaeis coracana (20), respectively. Therefore, we prepared total RNA from Nicotiana tabacum, Spinacia oleracea and Arabidopsis thaliana, and enzymatically digested these samples into nucleosides under acidic conditions. LC/MS analyses clearly detected ms<sup>2</sup>ct<sup>6</sup>A along with other t<sup>6</sup>A derivatives in all three species of plant (Figure 5). Next, total nucleosides of spinach RNA were co-injected along with synthetic ms<sup>2</sup>ct<sup>6</sup>A by LC/MS using hydrophilic interaction chromatography (Supplementary Figure S8A), as well as reverse-phase chromatography (Supplementary Figure S8B). Plant ms<sup>2</sup>ct<sup>6</sup>A co-eluted with the synthetic molecule as a single peak under both conditions, demonstrating that ms<sup>2</sup>ct<sup>6</sup>A is present in plant tRNAs.

Presence of ms<sup>2</sup>ct<sup>6</sup>A in T. brucei tRNAs

We previously speculated that ms<sup>2</sup>ct<sup>6</sup>A is present in T. brucei tRNA<sup>Lys</sup> (9). Total RNA from T. brucei was digested into nucleosides under neutral conditions and subjected to
Figure 2. Mass spectrometric analyses of total RNA and isolated tRNA<sup>37</sup> from <i>B. subtilis</i>. (A) Nucleoside analyses of total RNA from <i>B. subtilis</i>. Total nucleosides were prepared under conventional conditions (left panels) and neutral conditions (right panels). The panels second from the bottom show mass chromatograms corresponding to the proton adducts of t<sup>6</sup>A (m/z 413), ms<sup>2</sup>t<sup>6</sup>A (m/z 459), ct<sup>6</sup>A (m/z 395) and ms<sup>2</sup>ct<sup>6</sup>A (m/z 441), respectively. n.d., not detected. (B) Mass spectrum of the 12 mer-fragments containing anticodon region. Three peaks for the triply-charged negative ions of the RNA fragments having ms<sup>2</sup>A, ms<sup>2</sup>t<sup>6</sup>A and ms<sup>2</sup>ct<sup>6</sup>A at position 37 are indicated. (C) A collision-induced dissociation (CID) spectrum of the 12 mer-fragment of <i>B. subtilis</i> tRNA<sup>37</sup> digested by RNase T<sub>1</sub>. The triply-charged negative ion of the ms<sup>2</sup>ct<sup>6</sup>A<sub>37</sub>-containing fragment (m/z 1364.492) was used as a precursor ion for CID. The product ions were assigned according to the literature (45). Sequences of parent ion and assigned product ions are described upper side in this panel.
Figure 3. Structural confirmation of ms2ct6A from B. subtilis. Co-injection analyses of synthetic ms2ct6A and total RNA from B. subtilis by (A) RPC/ESI-MS and (B) HILIC/ESI-MS. UV trace at 254 nm and mass chromatograms of the synthetic ms2ct6A, total nucleosides of B. subtilis and co-injection are shown in the top, middle and bottom panels, respectively. Conversion of adenosine to inosine is due to the contamination of PDase II (Sigma P9041) with adenosine deaminase activity. (C) The mass spectrum for the proton adduct of natural ms2ct6A (MH+, m/z 440.9) from B. subtilis. The base-related ion (BH2+, m/z 309.0) was also detected. (D) The CID spectra for natural (upper panel) and synthetic (lower panel) BH2+ of ms2ct6A. Parent ions for CID are indicated by arrows. (E) Assignment of the product ions in the CID spectrum of the natural ms2ct6A BH2+ ion.
LC/MS analysis (Figure 6A). Along with ct6A, N^440 was clearly detectable. Next, the total nucleosides of T. brucei and B. subtilis were co-injected to LC/MS, revealing that T. brucei N^440 co-eluted with B. subtilis ms2ct6A as a single peak (Figure 6B). Finally, we probed BH^2^- of T. brucei N^440 by CID (Figure 6C). The product ions exhibited a pattern identical to those of synthetic ms2ct6A (Figure 3D). These results demonstrated that ms2ct6A is also present in T. brucei.

Growth phenotype of T. brucei with hypomodified ms2ct6A

We hypothesized that TbMtaB and TbTcdA are responsible for the synthesis of ms2ct6A in T. brucei. To test this speculation, we generated a transgenic RNAi line for each gene and confirmed knockdown efficiency by RT-PCR. No transcript of each gene was detected in either strain, even 7 days after knockdown induced by tetracycline (Supplementary Figure S9). Upon knockdown of TbMtaB, ms2ct6A and ms2t6A disappeared and remained to be ct6A and t6A, respectively (Figure 7A). In fact, ct6A accumulated slightly more than non-treated T. brucei (WT). Likewise, when TbTcdA was down-regulated, ms2ct6A and ct6A disappeared and remained to be ms2t6A and t6A, respectively (Figure 7A).

To determine the physiological importance of this modification, we measured the growth rates of these strains after induction of RNAi (Figure 7B). Down-regulation of expression of either gene alone did not cause a major growth defect (data not shown). However, in the presence of non-inhibitory concentrations of cycloheximide (Chx), growth of cells with down-regulated TbTcdA was slowed (Figure 7B), whereas little growth phenotype was observed when TbMtaB was knocked down. Thus, hypomodification of ms2ct6A and ct6A results in sensitivity to an antibiotic that targets the ribosome, suggesting that cyclization of these modifications contributes to efficient cell growth and protein synthesis.

DISCUSSION

Because ct6A is a chemically labile derivative of t6A, it had never been detected by conventional nucleoside analysis for more than 40 years since t6A’s discovery. ct6A can be detected only if total RNA is extracted from the cell under acidic conditions and digested into nucleosides under neutral conditions, explaining why ms2ct6A has not been detected previously. In this study, we performed nucleoside analysis under neutral or acidic conditions and successfully...
identified ms²ct⁶A in total RNA samples from B. subtilis, three plants and T. brucei. Because we also detected t⁶A and ms²t⁶A, we assume that ct⁶A and ms²ct⁶A are partial modifications in these organisms, indicating that cyclization of t⁶A and ms²t⁶A might be regulated under certain physiological conditions.

By referring to the synthetic nucleoside as a reference, the chemical structure of ms²ct⁶A was determined to be a hydantoin isoform, rather than an oxazolone isoform previously predicted. According to the structural analysis of ct⁶A nucleoside (15), two carbonyl oxygen atoms of the hydantoin ring are repulsive to the nitrogen atoms (N1 and N7) of the adenine base (Supplementary Figure S1). Consistent with this, the C6-N6 bond length is longer than the normal C-N bond length. Therefore, the hydantoin ring adopts a twisted position against the adenine base with a torsion angle of −52.7°. In light of this observation, it is difficult to speculate how the hydantoin ring of ct⁶A contributes to the efficient decoding of tRNA on the ribosome. Structural studies of ribosomes in complex with tRNA containing ct⁶A or ms²ct⁶A will be necessary to reveal the functional and structural roles of these modifications in protein synthesis.

We now know of five species of t⁶A derivatives (Figure 1A and S1), of which ms²ct⁶A is the most chemically complex. Initially, t⁶A is formed at position 37 on tRNAs with NNU anticodons (i.e. those responsible for ANN codons) (Figure 8). This process is catalyzed by multiple enzymes (TsaB, TsaC, TsaD and TsaE in bacteria) using L-threonine, bicarbonate and ATP as substrates. In tRNAThr from certain species of γ-proteobacteria, m⁶t⁶A37 is formed via the methylation of t⁶A37 by TrmO using AdoMet as a substrate. In some species of bacteria, fungi, plants and protists, ct⁶A37 is formed via the cyclization of t⁶A37 by ATP-dependent dehydration by TcdA. In organisms that harbor an MtaB homolog, t⁶A37 and ct⁶A37 are 2-thiomethylated to form ms²t⁶A and ms²ct⁶A, respectively. According to the nucleoside analyses of B. subtilis knockout strains, 2-thiomethylation and cyclization of t⁶A are independent reactions involved in synthesis of ms²t⁶A (Figure 8).

In terms of phylogenetic distribution, ms²ct⁶A should be present in organisms with MtaB and TcdA orthologs.
Figure 6. Detection of ms²ct⁶A in T. brucei tRNAs. (A) RPC/ESI-MS nucleoside analyses of total RNAs from T. brucei. Top panel shows a UV trace at 254 nm. Panels second from the bottom show mass chromatograms corresponding to the proton adducts of t⁶A (m/z 413), ms²t⁶A (m/z 459), ct⁶A (m/z 395) and ms²ct⁶A (m/z 441), respectively. (B) Mass chromatograms showing the proton adduct of ms²ct⁶A (m/z 441) in total nucleosides of B. subtilis (top panel), T. brucei (middle panel) and the co-injection fraction (bottom panel) obtained by RPC/ESI-MS. (C) CID spectrum of BH₂⁺ of ms²ct⁶A from T. brucei. The parent ion for CID is indicated by an arrow.

Among bacteria, δ-proteobacteria and approximately half of the species that compose Firmicutes and Bacteroidetes encode these two enzymes in their genomes. In protists, ms²ct⁶A is found in Trypanosoma and Tetrahymena. In Archaeplastida, both MtaB and TcdA are present in Plants and Chlorophyta, but not in Rhodophyta or Glaucoophyta.

In E. coli, ct⁶A37 is involved in the decoding activity of tRNA⁰lys (9). tcdA engages in a genetic interaction with mnmA, as demonstrated by the observation that a synthetic growth reduction occurs when both genes are deleted simultaneously. Considering that mnmA encodes a 2-thiouridylase to form mnm²s²U at the wobble position of tRNA⁰lys, which also contains ct⁶A37, cyclization of ct⁶A37 must play a functional role in the decoding activity of tRNA⁰lys.

The functional role of the 2-methylthio modification was first studied in an in vitro E. coli translation system using tRNA containing ms²ct⁶A37. E. coli tRNA⁰phe containing the ms²-modification was more active in poly(U)-dependent poly(Phe) synthesis than tRNA lacking the ms²-modification (41), indicating that 2-methylthiolation of ms²ct⁶A37 plays a critical role in maintaining the reading frame during elongation. The structure of ms²t⁶A37 in tRNA has revealed that the 2-methylthio group stabilizes the codon–anticodon interaction through cross-strand stacking with the first base of the P-site codon (43). This stabilization effect might contribute to the prevention of +1 frameshifting.

In the case of ms²ct⁶A modification in B. subtilis, the decoding ability of tRNA⁰lys with or without the ms²-modification was examined using a luciferase reporter. The results showed that the ms²-modification is required to decode AAA and AAG codons efficiently (23). Given that a large fraction of ms²ct⁶A37 must be converted to ms²ct⁶A37 in B. subtilis tRNA⁰lys, this finding throws light on the function of the ms²-modification of ms²ct⁶A37 (but not of ms²t⁶A37). However, no significant growth or temperature-sensitivity phenotypes have been observed to date in ΔyqeV, ΔyrvM or the double-deletion strains (data not shown). Future studies should investigate whether phenotypes emerge under various stress or culture conditions.
Figure 7. Biogenesis of ms\(^2\)ct\(^6\)A and growth phenotype of T. brucei with hypomodified tRNAs. (A) RPC/ESI-MS nucleoside analyses of total RNAs from T. brucei wild type (left panels) and transgenic RNAi lines of TbMtaB (middle panels) and TbTcdA (right panels). Mass chromatograms of \(^i\)\(^6\)A derivatives were normalized by that of \(^i\)\(^6\)A, and abundance of each peak is displayed relative to the highest peak (100\%) among them. Top panels show the UV traces at 254 nm. Panels second from the bottom show mass chromatograms detecting the proton adducts of ms\(^2\)ct\(^6\)A (m/z 441), ct\(^6\)A (m/z 395), ms\(^2\)t\(^6\)A (m/z 459), t\(^6\)A (m/z 413) and \(^i\)\(^6\)A (m/z 336), respectively. n.d., not detected. (B) Growth curves of T. brucei transgenic RNAi lines of TbMtaB (left) and TbTcdA (right). Cumulative cell counts indicated by logarithmic cell number (Log of cells/ml) were measured in the presence of non-inhibitory concentration of cycloheximide. RNAi was induced by adding (black circle) or not adding (black square) tetracycline (Tet) at 7 days (as indicated by arrow) after inoculation.
of cycloheximide, indicating defective decoding activity of tRNA125 with hypomodified ms2ct6A. To obtain a deeper understanding of the physiological role of this modification, future studies should be directed toward analyzing the phenotypic features of T. brucei knockdown lines.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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