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Development and Comparison of 4-Thiouridine to Cytidine Base Conversion Reaction

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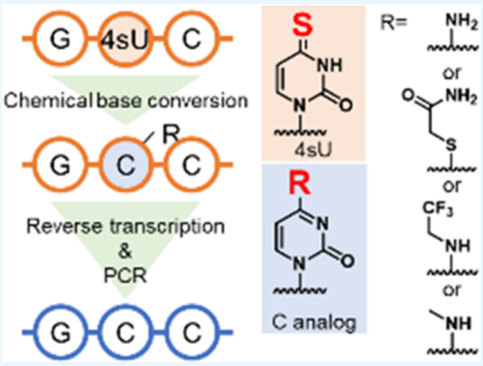
ABSTRACT: To study transcriptome dynamics without harming cells, it is essential to convert chemical bases. 4-Thiouridine (4sU) is a biocompatible uridine analogue that can be converted into a cytidine analogue. Although several reactions can convert 4sU into a cytidine analogue, few studies have compared the features of these reactions. In this study, we performed three reported base conversion reactions, including osmium tetroxide, iodoacetamide, and sodium periodate treatment, as well as a new reaction using 2,4-dinitrofluorobenzene. We compared the reaction time, conversion efficacy, and effects on reverse transcription. These reactions successfully converted 4sU into a cytidine analogue quantitatively using trinucleotides. However, the conversion efficacy and effect on reverse transcription vary depending on the reaction with the RNA transcript. OsO_4 treatment followed by NH_4Cl treatment showed the best base-conversion efficiency. Nevertheless, each reaction has its own advantages and disadvantages as a tool for studying the transcriptome. Therefore, it is crucial to select the appropriate reaction for the target of interest.



1. INTRODUCTION

Gene expression is strictly regulated, and cells fall into fatal conditions when they are out of control. As RNA turnover is essential for controlling gene expression in cells, tracing the biosynthesis and degradation of RNAs in cells is an attractive topic in cellular biology. Several methods have been developed for monitoring RNA turnover. Stalling transcription by chemical compounds or heating and measuring the amount of RNA are simple but effective methods to calculate the RNA degradation rate. Actinomycin D is a popular transcription inhibitor that is used to monitor RNA degradation.[1−5] Several studies have used other inhibitors, including 5,6-dichloro-1-β-D-ribofuranosylbenzimidazol (DRB) and α-amanitin, to examine the RNA decay rate.[5,6] These inhibitor-based methods are ready to conduct; however, applying the inhibitor is hazardous to cells and may affect the RNA half-life.[7−9]

Metabolic labeling with uridine (U) analogues is another effective way to examine RNA turnover. 4-Thiouridine (4sU) is a popular uridine analogue to label RNA because it is not found in eukaryotic cells but in prokaryotic tRNA.[10,11] 4sU is readily incorporated into RNA by exposing cells to it and has little influence on RNA function or cellular processes, depending on the treatment time and concentration.[12,13] RNA containing 4sU can be biotinylated via disulfide linkages and enriched with streptavidin beads. Biotin labeling distinguishes RNA containing 4sU from RNA transcribed before 4sU addition. These methods with metabolic labeling are often used to determine the RNA transcription and degradation rate.[14−21] While metabolic labeling has few effects on transcription, it has some problems with downstream analysis, including low sensitivity for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) because biotinylated RNA hampers reverse transcription.[7−9]

These biotinylation and enrichment methods provide rough information about the existence of 4sU in the RNA fragment and are not suitable for precisely locating the 4sU substituted position. Combining metabolic labeling with the base conversion reaction effectively circumvents this issue. In these methods, 4sU in RNA is converted into cytidine (C) or its analogue by chemical treatments, so that the sequence change from U to C distinguishes the labeled RNA from the nonlabeled one (Figure 1A). Base conversion can locate the 4sU substituted position at the nucleotide level resolution. Three base conversion methods have been reported previously. Thiouridine to cytidine-sequencing (TUC-seq) performs the conversion using osmium tetroxide (OsO_4) oxidation and adding an amine group by ammonium chloride (NH_4Cl) (Figure 1B).[22] The treatments generated natural C from 4sU and did not affect reverse transcription. Thiol(SH)-linked

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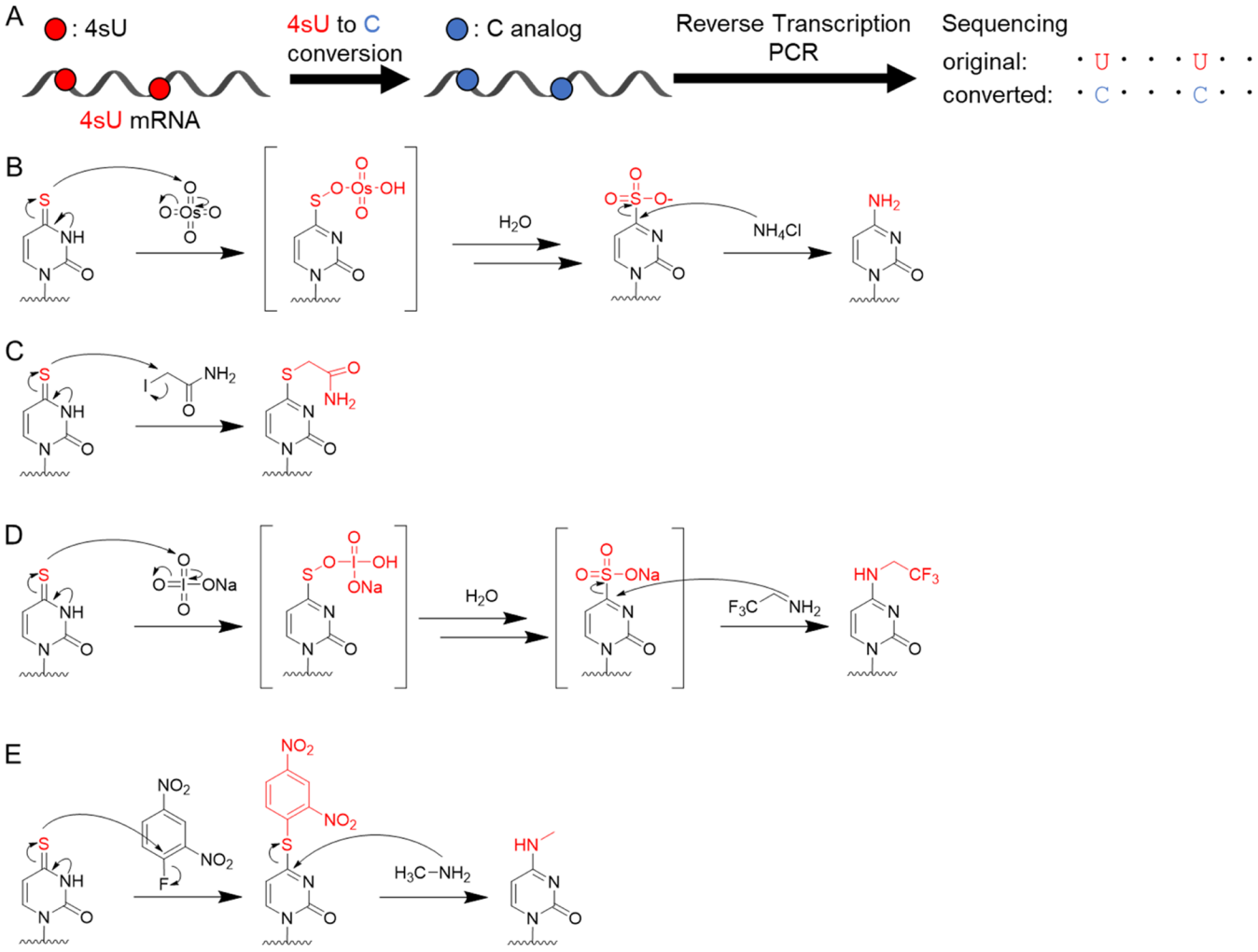

Figure 1. Reaction schemes for the base conversion from 4-thiouridine to cytidine analogues. (A) Schematic representation of the 4sU to C conversion experiment. (B) TUC-seq using osmium tetroxide and ammonium chloride. (C) SLAM-Seq using iodoacetamide. (D) TimeLapse-seq with sodium periodate and 2,2,2-trifluoroethylamine. (E) Novel method using 2,4-dinitrofluorobenzene (DNFB) and methylamine.

alkylation for metabolic sequencing (SLAM-seq) exploits the 4sU conversion to trace transcriptome dynamics.[23] In SLAM-seq, the 4sU group was alkylated with iodoacetamide (IAA) (Figure 1C). The alkylation product contains the amine group and is recognized as C. TimeLapse Chemistry is another method for locating 4sU. Sodium periodate ($NaIO_4$) oxidization and subsequent 2,2,2-trifluoroethylamine (TFEA) treatment converted 4sU to a cytidine analogue (Figure 1D).[24] However, the oxidation step with $NaIO_4$ causes a side reaction with diol at the 3′ end and cleaves it into aldehyde.[25]

In the present study, we developed a novel base conversion reaction in which 4sU is activated by 2,4-dinitrofluorobenzene (DNFB) and subsequently converted into a cytidine analogue by adding methylamine (Figure 1E). We also conducted three conversion reactions, as described above, to compare the optimized reaction times, conversion efficacies, and effects on reverse transcription.

2. MATERIALS AND METHODS

2.1. Solid-Phase Synthesis and Deprotection of 3 nt Oligonucleotide. Oligonucleotide synthesis was performed using an automated oligonucleotide synthesizer (nucleotide reactor NRs-4A10R7 from NIHON TECHNO SERVICE) with commercially available phosphoramidites (5′-DMT-2′-TOM-ribo-guanosine (*n*-acetyl) OP (ChemGenes), and 4-Thio-dU-CE Phosphoramidite (GLEN RESEARCH)) and 3′-TOM-ribo Cytidine (*n*-acetyl) 2′-lcaa CPG 1000 Å (ChemGenes). After synthesis, the solid support was treated with 1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in 2 mL of anhydrous acetonitrile for 2 h at room temperature and washed with dry acetonitrile to remove residual DBU and dry the support. Oligonucleotide cleavage from the solid support and base deprotection was performed with 1 mL of *tert*-butylamine/water (1:3, v/v) containing 50 mM NaSH for 9 h at 60 °C. The support was filtered using a Millex LCR 13 mm filter (Merck). A speed-vac evaporator (CVE-3000 from TOKYO RIKAKIKAI) removed the solvents, the oligonucleotides were desalted using a Sep-Pak C18 cartridge (Waters) eluted with water and acetonitrile, and the evaporator dried the collected fraction. Oligonucleotides were treated with 1 M tetrabutylammonium fluoride in tetrahydrofuran (THF) at room temperature overnight to remove the 2′-protecting group. The reaction was quenched by adding 1 M triethylammonium acetate (TEAA) (pH 7.0). The oligonucleotides were concentrated using a speed-vac evaporator; the solution was desalted with a Sep-Pak C18 cartridge eluted with water and acetonitrile, and the deprotected groups were filtered using a Millex LCR 13 mm filter. Crude oligonucleo-

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tides after deprotection were purified using a HITACHI Chromaster HPLC System (HITACHI) (260 nm, column: hydrosphere C18 250 × 4.6 mm^2 (YMC), column temperature: 50 °C, gradient: 0−15% solvent B for 15 min, solvent A: 50 mM TEAA, 5% acetonitrile (pH 7.0), solvent B: acetonitrile). The identity of the prepared oligonucleotides was confirmed by high-performance liquid chromatography (HPLC) and electrospray ionization-mass spectrometry (ESI-MS, negative wide mode).

2.2. Transcription of mRNA. Sequences of template DNA and transcribed RNA are shown in Supporting Table S1. Transcription was performed in the presence of 1× T7 RNA polymerase buffer (TaKaRa), 5 mM dithiothreitol (DTT), 5 ng/μL template DNA, 2 mM ATP (TCI), GTP (CHEM-IMPEX), CTP (Sigma-Aldrich), UTP (CHEM-IMPEX) (for natural RNA) or 4sUTP (for 4sU RNA) (Jena Bioscience), and 0.02 U/μL T7 RNA polymerase Y639F mutant[26] (prepared in our laboratory) for 2 h at 37 °C. Subsequently, 1× DNase I buffer (TaKaRa) and 0.25 U/μL recombinant DNase I were added and incubated for 1 h at 37 °C. The transcribed RNA was purified using phenol/chloroform extraction and alcohol precipitation. The existence of target mRNA was confirmed by denaturing polyacrylamide gel electrophoresis (PAGE) (7.5 M urea, 5% PAGE (acrylamide/*N*,*N*′-methylenebis(acrylamide) = 19:1)).

2.3. OsO_4 and NH_4Cl Treatment. Oligonucleotides (5 μM) were incubated with 180 mM ammonium chloride (pH 8.88) and 450 μM osmium tetroxide for 4 h at 25 °C. The oligonucleotides were purified by HPLC (260 nm, column: hydrosphere C18 250 × 4.6 mm^2 (YMC), column temperature: 50 °C, gradient: 0−15% solvent B for 15 min; solvent A, 50 mM TEAA, 5% acetonitrile (pH 7.0), solvent B: acetonitrile). UV spectra were obtained from the HPLC results. Molecular weights were confirmed using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS). The transcribed RNA (50 nM) was incubated with 180 mM ammonium chloride (pH 8.88) and 450 μM osmium tetroxide for 4 h at either 25 or 50 °C. The base-converted RNA was purified using an Amicon Ultra 10 K filter (MERCK). After base conversion, the transcribed RNA was analyzed by denaturing PAGE (7.5 M urea, 5% PAGE (acrylamide/*N*,*N*′-methylenebis(acrylamide) = 19:1)).

2.4. IAA Treatment. Oligonucleotides (5 μM) were incubated with 50 mM sodium phosphate (pH 8.0) and 10 mM iodoacetamide (dissolved in 100% ethanol) in 50% dimethyl sulfoxide (DMSO) for 15 min at 50 °C. Acetylated oligonucleotides were purified using an HPLC System (HITACHI) (260 nm, column: hydrosphere C18 250 × 4.6 mm^2 (YMC), column temperature: 50 °C, gradient: 0−15% solvent B for 15 min, solvent A: 50 mM TEAA, 5% acetonitrile (pH 7.0), solvent B: acetonitrile). UV spectra were obtained from the HPLC results. Molecular weights were confirmed using MALDI-TOF-MS. Transcribed RNA (50 nM) was incubated with 50 mM sodium phosphate (pH 8.0) and 10 mM IAA (dissolved in 100% ethanol) in 50% DMSO for 15 min at 50 °C. To quench residual IAA, two equal amounts of DTT were added. Alcohol precipitation was then performed for desalting. After base conversion, the transcribed RNA was analyzed by denaturing PAGE (7.5 M urea, 5% PAGE (acrylamide/*N*,*N*′-methylenebis(acrylamide) = 19:1)).

2.5. $NaIO_4$ and TFEA Treatment. Oligonucleotides (5 μM) were incubated with 600 mM 2,2,2-trifluoroethylamine, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM sodium acetate (pH 5.2), and 10 mM $NaIO_4$ for 1 h at 45 °C. After the reaction, 300 mM KCl and sodium acetate (pH 5.2) were added, and the reaction mixture was placed on ice for 10 min before centrifugation (10,000 rpm, 30 min, 4 °C) to precipitate residual $NaIO_4$. The supernatant was purified by HPLC (260 nm, column: hydrosphere C18 250 × 4.6 mm^2 (YMC), column temperature: 50 °C, gradient: 0−40% solvent B for 15 min, solvent A: 50 mM TEAA, 5% acetonitrile (pH 7.0), and solvent B: acetonitrile). UV spectra were obtained from the HPLC results. Molecular weights were confirmed using MALDI-TOF-MS. The transcribed RNA (50 nM) was incubated with 600 mM 2,2,2-trifluoroethylamine, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM sodium acetate (pH 5.2), and 10 mM $NaIO_4$ for 1−4 h at 45 °C. After the reaction, 300 mM KCl and sodium acetate (pH 5.2) were added, and the reaction mixture was placed on ice for 10 min before centrifugation (10,000 rpm, 30 min, 4 °C) to precipitate residual $NaIO_4$. Oligonucleotides in the supernatant were collected into a new tube. Then, 1:9 volume of 3 M sodium acetate (pH 5.2) and 10:9 volume of isopropanol were added and kept in −30 °C freezer for 1 h. After the centrifugation (15,000 rpm, 30 min, 4 °C), the oligonucleotide pellet was washed with ethanol. Transcribed RNA after base conversion was analyzed by denaturing PAGE (7.5 M urea, 5% PAGE (acrylamide/*N*,*N*′-methylenebis(acrylamide) = 19:1)).

2.6. DNFB and Methylamine Treatment. Oligonucleotides (5 μM) were incubated with 20 mM sodium phosphate (pH 6.0) and 20 mM DNFB in 40% DMSO for 30 min at 50 °C to activate 4sU. Subsequently, the chloroform extraction was repeated seven times to eliminate residual DNFB. The activated oligonucleotides were purified by HPLC (260 nm, column: hydrosphere C18 250 × 4.6 mm^2 (YMC), column temperature: 50 °C, gradient: 0−40% solvent B for 15 min, solvent A: 50 mM TEAA, 5% acetonitrile (pH 7.0), and solvent B: acetonitrile). The speed-vac evaporator concentrated the activated oligonucleotides and removed acetonitrile. To introduce amine groups, 20 mM sodium borate (pH 8.5) and 180 mM methylamine or ammonium chloride were added to the oligonucleotides, which were then incubated for 30 min at 50 °C. The amine-introduced oligonucleotides were prepared using an HPLC system (260 nm, column: hydrosphere C18 250 × 4.6 mm^2 (YMC), column temperature: 50 °C, gradient: 0−40% solvent B for 15 min, solvent A: 50 mM TEAA, 5% acetonitrile (pH 7.0), and solvent B: acetonitrile). UV spectra were obtained from the HPLC results. Activated and base-converted oligonucleotides were analyzed by using ESI-MS and MALDI-TOF-MS, respectively. The transcribed RNA (50 nM RNA) was incubated with 20 mM sodium borate (pH 8.5) and 20 mM DNFB in 40% DMSO for 30 min at 50 °C to activate 4sU. Subsequently, the chloroform extraction was repeated seven times to eliminate residual DNFB. To introduce an amine group, 180 mM methylamine and 20 mM sodium borate (pH 8.5) were added to the activated RNA and incubated for 30 min at 50 °C. Base-converted RNA was purified using alcohol precipitation. The transcribed RNA after base activation and conversion was analyzed by denaturing PAGE (7.5 M urea, 5% PAGE (acrylamide/*N*,*N*′-methylenebis(acrylamide) = 19:1)).

2.7. Digestion and Dephosphorylation of RNA Treated with DNFB and Methylamine. 4sUTP (5 μM) or transcribed RNA (50 nM) was treated with DNFB and methylamine following the protocol described above. The

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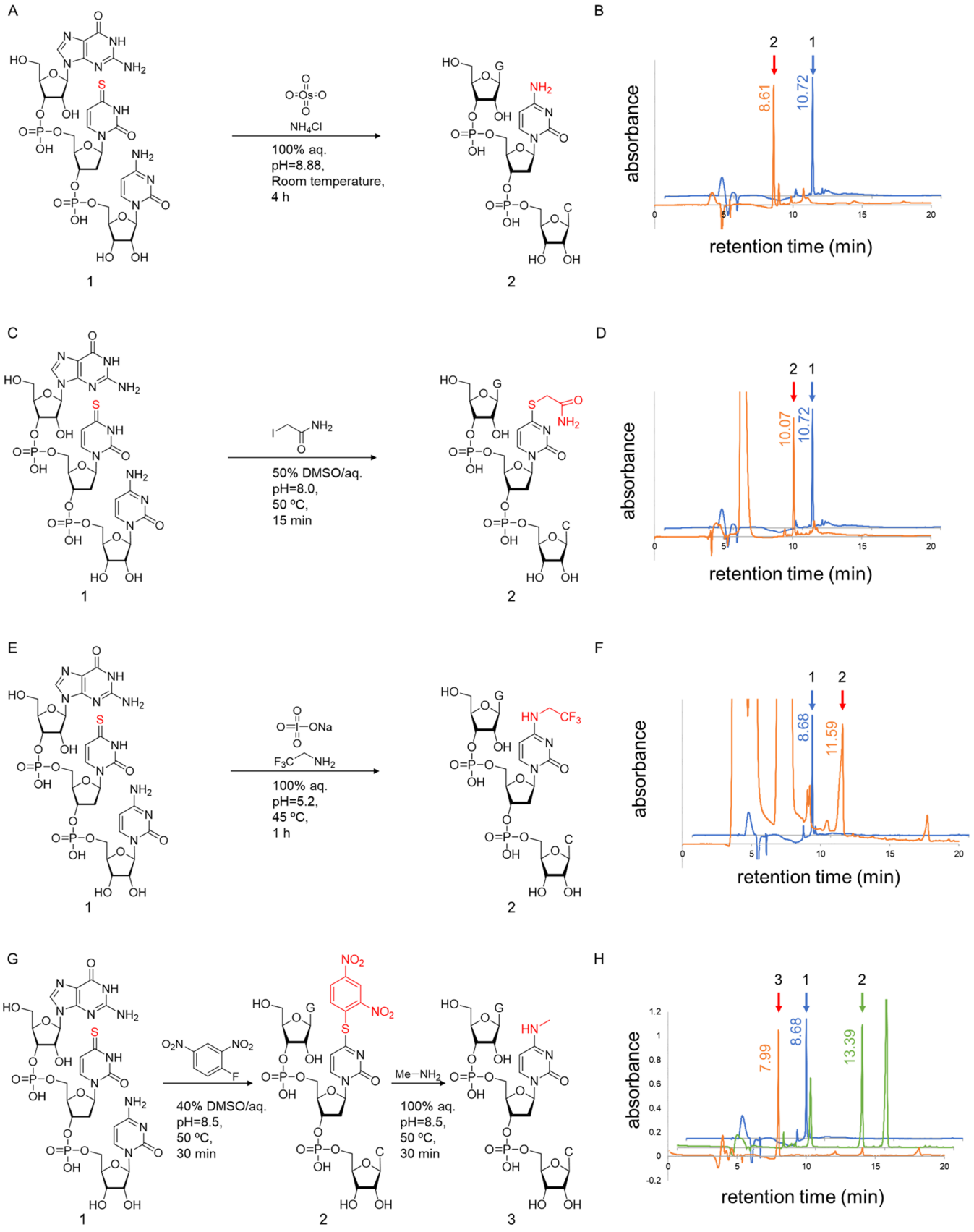

Figure 2. Base conversion of 4sdU with trinucleotides. (A) Reaction scheme of 4sdU with OsO_4·NH_4Cl treatment and (B) reaction progress followed by HPLC. (C) Reaction scheme of 4sdU with IAA treatment and (D) reaction progress followed by HPLC. (E) Reaction scheme of 4sdU with $NaIO_4$·TFEA treatment and (F) reaction progress followed by HPLC. (G) Reaction scheme of 4sdU with DNFB·methylamine treatment and (H) reaction progress followed by HPLC. HPLC profiles (260 nm) of the trinucleotides before, intermediate, and after conversion are indicated by blue, green, and orange lines, respectively.

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base-converted materials were digested at 37 °C for 16 h in 10 mM Tris−HCl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, 1.2 mU/μL phosphodiesterase 1 (Sigma-Aldrich), and 0.6 U/μL alkaline phosphatase (Calf intestine) (TaKaRa). Then, the enzymes were eliminated by collecting flow-through from an Amicon Ultra 10 K filter (MERCK). The degradants were analyzed by HPLC (260 nm, column: hydrosphere C18 250 × 4.6 mm^2 (YMC), column temperature: 50 °C, gradient: 10% solvent B for 5 min, 10−35% solvent B for 35 min, 80% solvent B for 5 min, 0% solvent B for 15 min, solvent A: 50 mM TEAA (pH 7.0), and solvent B: 50 mM TEAA (pH 7.0), 50% acetonitrile).

2.8. Reverse Transcription and PCR Amplification. Primer annealing to RNA was conducted in the presence of 0.5 mM dNTPs, 0.5 μM RT primer (5′-dTAACCGGTACGCG-TAGAATCTTTTTTTTTTTTTTTTTTT-3′), and 2 ng/μL base-converted RNA for 5 min at 65 °C and kept on ice. After annealing, 1× First Strand Buffer (Thermo SCIENTIFIC), 5 mM DTT, and 10 U/μL SuperScript III reverse transcriptase (Thermo SCIENTIFIC) were added. Reverse transcription was performed for 45 min at 50 °C, and reverse transcriptase was inactivated by heating for 15 min at 70 °C. Then, 1:20 volume of the reverse-transcribed sample was transferred to a PCR tube. 0.5 μM each forward (5′-dCATGGACTACAAGGACGACGAC-3′ for targeting 696 bp DNA or 5′-dGCCTGATCAACCCCGACGGC-3′ for targeting 175 bp DNA) and reverse primer (5′-dTAACCGG-TACGCGTAGAATC-3′), 1× KOD buffer (TOYOBO), 1.5 mM $MgSO_4$, 0.2 mM dNTPs, and 0.02 U/μL KOD plus neo (TOYOBO) were added, and the thermal cycle (95 °C for 2 min, (98 °C for 10 s, 68 °C for 30 s) × 25 cycles) was performed using a T100 thermal cycler (Bio-Rad). The PCR products were analyzed by using 1.2% agarose gel electrophoresis (100 V, 20 min).

2.9. Cloning and Sequencing Analysis. Plasmid cloning of the PCR-amplified samples was conducted using TArget Clone (TOYOBO). To generate sticky ends, 1× A-attachment Mix was added to the PCR products and incubated for 10 min at 60 °C. The PCR products with sticky ends were ligated into plasmids with the appropriate ratio of PCR products/pTA2 vector (1:6), 1× ligation buffer, and T4 DNA Ligase for 30 min at 16 °C. The ligated plasmid was transformed into *Escherichia coli* JM109 (Nippon Gene) via heat shock and spread on an LB plate containing 100 μg/mL ampicillin. Plasmids were extracted from 3 to 5 colonies with an objective insert. Plasmid sequencing was conducted using an ABI PRISM 3500xL Genetic Analyzer (Center for Gene Research at the University of Nagoya). The base conversion rates were determined as (number of U to C conversions)/(total number of U) × 100 for the desired 4sU to C conversion and (number of unwanted base conversions)/(total base number) × 100 for other mutations. *P* values were calculated using a two-tailed Student's *t* test with two groups.

3. RESULTS AND DISCUSSION

3.1. 4sdU Base Conversion with Trinucleotide. Initially, we performed four 4sdU conversion reactions, three reported and one novel, using a trinucleotide in which a single 4sdU was flanked with guanosine and cytidine (Figure S1). The reaction was analyzed by using HPLC, and the conversion rate was estimated from the profile to improve the reaction conditions. Mass spectrometry analysis was performed to identify the converted products.

3.1.1. Osmium Tetroxide (OsO_4) and Ammonium Chloride. Lusser and Micura performed the 4sU to C conversion using OsO_4 to evaluate 4sU in tRNA, and its conversion rate was 93%.[22] Following this, we treated the trinucleotides with OsO_4 and ammonium chloride at room temperature for 4 h (Figure 2A). The treatment shifted the oligonucleotide peak indicated by the blue arrow to the peak indicated by the red arrow in the HPLC chart (Figure 2B). Based on the peak areas, the conversion rate was calculated to be 73%. In addition, treatment altered the UV spectrum of the oligonucleotides. Before treatment, 330 nm absorbance derived from 4sdU was observed,[27] but it completely disappeared after the reaction (Figure S2A,B). Mass spectrometry analysis of the base-converted product matched the expected product's exact mass, the GdCC trinucleotide (Figure S2C). These results suggest that 4sdU is converted into dC by treatment with OsO_4 and ammonium chloride treatment.

3.1.2. Iodoacetamide (IAA). It has been reported that the base conversion rate of 4sU by IAA treatment is over 98%, calculated by a decrease in absorbance at 330 nm absorbance.[23] In the present study, we treated trinucleotides containing a single 4sdU with IAA at 50 °C for 15 min (Figure 2C). The peak shift in the HPLC profiles suggested a conversion rate of 95% (Figure 2D). The disappearance of the 330 nm absorbance and mass spectrometry analysis confirmed the consumption of 4sdU and generation of the expected alkylated trinucleotide product (Figure S3A−C). Consistent with the results of a previous study, quantitative alkylation of 4sdU by IAA was confirmed.

3.1.3. Sodium Periodate ($NaIO_4$) and Trifluoroethylamine (TFEA). Previously, M.D. Simon applied base conversion reaction for RNA containing single 4sU and obtained the base conversion rate with $NaIO_4$ and TFEA of 80%.[24] The conversion rate was determined by reverse transcription and PCR amplification, followed by restriction enzyme digestion with NotI, which can digest only the 4sU to C converted product.[25] In this study, oligonucleotides with a single 4sdU were treated with $NaIO_4$ and TFEA under optimal conditions (Figure 2E). HPLC analysis of the treated oligonucleotides showed a peak shift from blue to red and >99% conversion (Figure 2F). The UV spectrum change also provided evidence of base conversion (Figure S4A,B). Finally, mass spectrometry analysis of the treated oligonucleotide revealed the production of an oligonucleotide with an amine group (Figure S4C). These results indicated that 4sdU in the oligonucleotide was converted by $NaIO_4$ and TFEA treatment.

3.1.4. Dinitrofluorobenzene (DNFB) and Methylamine. In addition to the three conversion methods evaluated above, we developed a novel base conversion method. In this method, 4sdU reacts with DNFB to generate an activated form (Figure 2G). Mass spectrometry analysis revealed that the peak indicated by the black arrow was derived from activated trinucleotide. The activation yield was 27% when the activation was performed at 25 °C (Figure S5); however, increasing the reaction temperature to 50 °C improved the yield by 96% (Figures 2H and S6B). The activated trinucleotide was subsequently treated with ammonium chloride to produce natural cytosine; however, no reaction occurred (Figure S6).

The conversion reaction proceeded successfully when methylamine was added to the activated trinucleotide instead of ammonium chloride (Figure 2G). HPLC and mass spectrometry analyses of the product confirmed that almost 99% of the activated form was converted to N4 methylcytosine

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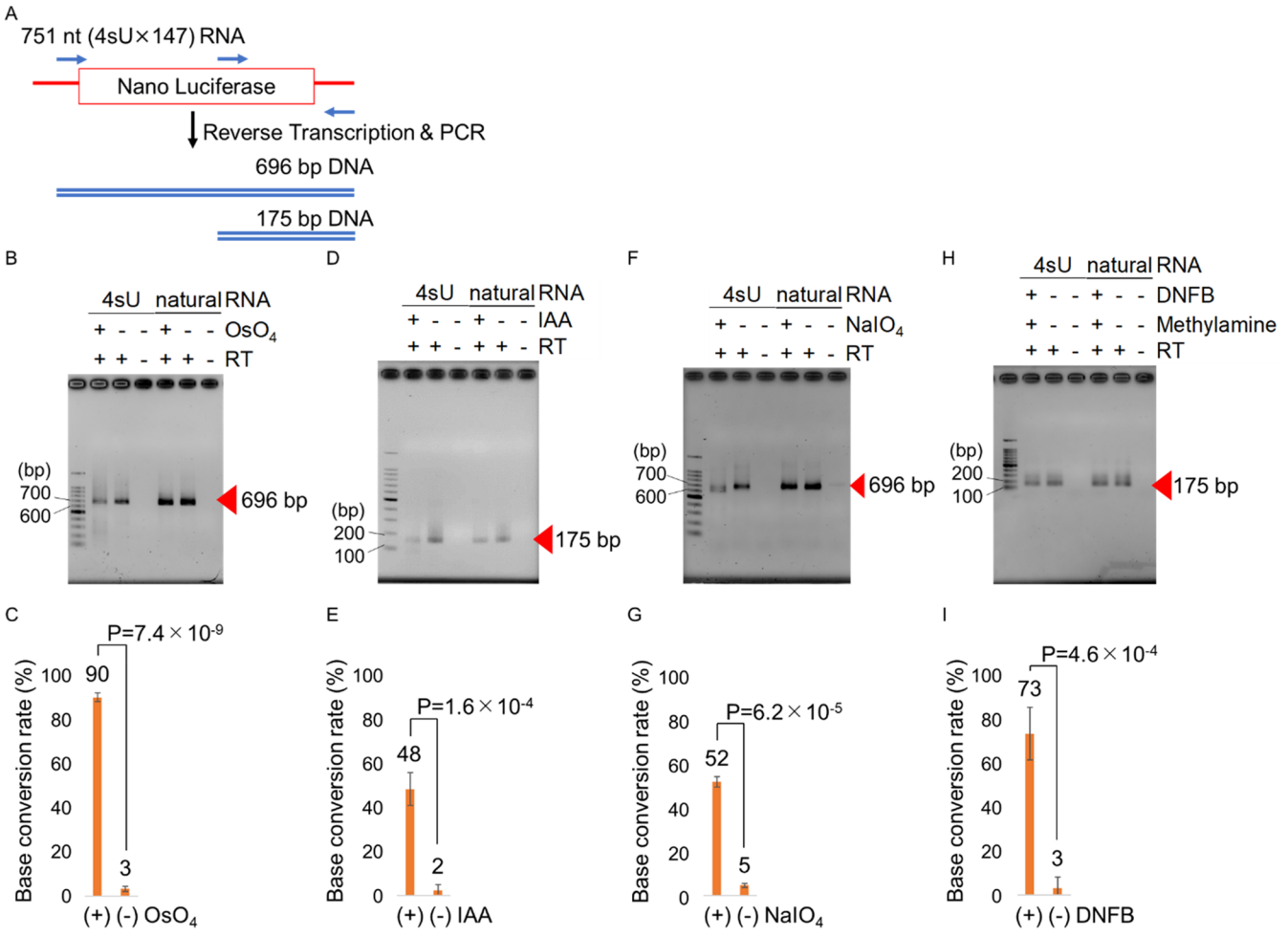

Figure 3. Base conversion of 4sU to RNA transcripts. (A) An overview of the base conversion reaction in the RNA transcript and primer design for 696 bp and 175 bp PCR products. (B) Gel analysis of long DNA after reverse transcription and PCR amplification and (C) base conversion rate calculated by sequencing change by OsO_4·NH_4Cl treatment at 50 °C for 4 h. (D) Gel analysis of long DNA after reverse transcription and PCR amplification and (E) base conversion rate calculated by sequencing change by IAA treatment. (F) Gel analysis of long DNA after reverse transcription and PCR amplification and (G) base conversion rate calculated by sequencing change by $NaIO_4$·TFEA treatment. (H) Gel analysis of long DNA after reverse transcription and PCR amplification and (I) base conversion rate calculated by sequencing change by DNFB-methylamine treatment.

(Figures 2H and S7). These results suggest that DNFB and subsequent methylamine treatments quantitatively activated and converted 4sdU into a cytidine analogue in trinucleotides.

3.2. 4sU Base Conversion in RNA Transcript. The four conversion reactions optimized above quantitatively converted 4sdU into dC or dC analogues in trinucleotides. Next, we performed the conversion reaction for a 751 nt RNA transcript encoding Nano Luciferase. The transcript was prepared using T7 RNA polymerase Y639F mutant in the presence of 4sUTP, and all 147 uracil bases in the sequence were substituted with 4sU (Figure 3A). After the base conversion reaction, the RNA transcript was purified, reverse-transcribed, and amplified by using PCR. PCR primers were designed to amplify the 175 bp and 696 bp regions of the reverse-transcribed product (Figure 3A). The PCR product was cloned into plasmids and sequenced to locate 4sU and convert C or C analogues in the RNA transcript. The 4sU base conversion rate of the RNA transcript was calculated from the obtained sequencing results.

3.2.1. Osmium Tetroxide and Ammonium Chloride. Following the base conversion reaction performed with trinucleotides, the RNA transcript was treated with OsO_4 at 25 °C. OsO_4 and ammonium chloride treatments did not affect the band intensity of RNA transcripts (Figure S8A). After base conversion, the RNAs were reverse-transcribed and amplified by PCR (Figures 3B and S8B,C). Because the reaction converts 4sU into natural C, it does not affect the reverse transcription or PCR amplification. Sequencing analysis of the amplified DNA revealed that the base conversion rate was 72% for the 696 bp or 71% for the 175 bp fragment (Figure S8E–H). The reaction was also performed at 50 °C to improve the conversion rate (Figure S8A,D). The rates were calculated as 90% for the 695 bp fragment or 86% for the 175 bp fragment (Figures 3C and S8I,J).

3.2.2. Iodoacetamide. Next, 4sU conversion of the RNA transcript was performed using IAA. Similar to that of OsO_4 and ammonium chloride, IAA treatment did not affect the band intensity of RNA transcripts (Figure S9A). Although reverse transcription and subsequent PCR barely amplified the 175 bp fragment, 696 bp amplification from the IAA-treated 4sU RNA transcript failed. Interestingly, IAA treatment of natural RNA also diminished the level of amplification (Figures 3D and S9B). These results suggest that IAA treatment affects natural bases. The base conversion rate of reverse-transcribed RNA was 48%, which was lower than that of the

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oligonucleotides (Figure 3E). This treatment resulted in a negligible number of unwanted mutations (Figure S9C).

3.2.3. Sodium Periodate and Trifluoroethylamine. Compared with other conversion methods, $NaIO_4$ and subsequent TFEA treatment resulted in a notable change in the band pattern of the 4sU RNA transcript in PAGE. The treatment caused an upshift above 1000 nt but did not have an effect on natural RNA (Figure S10A). Despite generating an unnatural C analogue through conversion, the impact on reverse transcription and PCR targeting both 696 and 175 bp was relatively minor (Figures 3F and S10B,C). The calculated conversion rates were 52 and 57% for the 696 bp and 175 bp fragments, respectively (Figures 3G and S10D,E). Increasing the reaction time from 1 to 4 h did not improve the rate but slightly increased unwanted base conversion (Figure S10D,E). Similar to base conversion using IAA, the conversion rate with RNA transcripts was relatively low, considering that quantitative conversion proceeded with trinucleotide.

3.2.4. Dinitrofluorobenzene and Methylamine. Base conversion using dinitrofluorobenzene and methylamine significantly reduced the band intensity of the RNA transcripts (Figure S11A). Although the treatment reduced the band intensity, reverse transcription and PCR successfully amplified a 696 bp product from a natural RNA transcript. In contrast, this failed in the 4sU RNA transcript (Figure S11B). To clarify whether the cause of failure is the intermediate or N4-methylcytosine, we applied DNFB and methylamine treatment to 4sUTP, transcribed natural RNA transcript, and 4sU containing RNA transcript and digested into nucleoside. The produced degradants were analyzed by HPLC (Figure S11C–E). From the HPLC profile, the peak at 14.58 min was determined to be 4sU because of its peculiar absorbance at 331 nm (Figure S11D). After the DNFB activation, two novel peaks at 45 and 52 min were observed (Figure S11C,E). We expect that the former one is the intermediate because the intermediate peak of the trimer experiment almost completely disappeared after methylamine introduction, and 4sUTP should show the same behavior with trimer. In other words, the intermediate was almost gone even with the long RNA. Also, N4-methylcytidine was observed after 12 min (Figure S11C,D). It has been reported that reverse transcriptase can recognize N4-methylcytidine, the cytidine analogue produced by DNFB and methylamine treatment, with identical efficacy as cytidine.[28,29] Considering that the base conversion treatment reduced the band intensity (Figure S11A), the treatment altered the base and other parts, including the sugar backbone, and hindered reverse transcription. Similar to the IAA treatment, the 175 bp region was successfully amplified for both natural and 4sU-containing RNA (Figure 3H). The base conversion rate of the 175 bp fragment was calculated to be 73% with negligible unwanted mutations (Figures 3I and S11F).

4. DISCUSSION

We evaluated four types of chemical reactions to conduct 4sU to C base conversion, which help to study transcriptome dynamics. All four reactions achieved quantitative base conversion using trinucleotides. However, the base conversion rate and its effect on reverse transcription and subsequent PCR amplification varied with the RNA transcript (Table 1). Our study showed no significant differences in unwanted mutations among the four reactions. Each reaction has its advantages and drawbacks as a tool for studying the transcriptome. OsO_4 and ammonium chloride showed the highest base conversion rate and did not affect PCR amplification. Although there is a report that OsO_4 and ammonium chloride treatment can affect modified pyrimidine bases including 5-methylcytidine (5mC), the OsO_4 concentration we used is unlikely to react with those modifications.[29] In this experiment, a long incubation time of 4 h was applied for OsO_4 treatment, but it also reported that a shorter reaction time could work well.[30] However, this method requires toxic heavy-metal reagents. The 4sU base conversion exploiting IAA had the lowest conversion rate but took the shortest time among the four reactions. Additionally, the conversion reaction altered the natural RNA strand that failed the PCR amplification, targeting 696 bp. $NaIO_4$ and subsequent TFEA treatment resulted in a moderate conversion rate within 1 h of reaction, and the reaction did not affect PCR amplification. Notably, $NaIO_4$ cleaves the *cis*-diol group on the RNA 3′ terminus and makes 2′,3′-dialdehydes.[25] Therefore, this method is unsuitable for some experiments, including RNA adapter ligation of the 3′ terminal. Furthermore, OsO_4 and ammonium chloride treatment and $NaIO_4$ and TFEA treatment are practical tools for converting 6-thioguanosine to adenosine analogues.[31,32] Our novel conversion method, DNFB, and subsequent methylamine treatment were completed within 1 h and converted 73% of 4sU in the RNA transcript. Similar to IAA treatment, the method could not amplify 696 bp target fragments from the 4sU RNA transcript. In our study, the converted nucleotide samples were purified at each step to exclude the carryover of the unreacted reagents. Thus, cDNA synthesis by reverse transcriptase failed, resulting in a failure of PCR amplification. In other words, IAA, DNFB, and methylamine treatment modified RNA to inhibit the long reverse transcription. In conventional short-read high-throughput RNA sequencing, reverse transcriptase synthesizes cDNA fragments shorter than 200 nt using random primers, and 150–200 nt regions are analyzed by the sequencer.[32,33] The PCR amplified a 175 bp fragment from IAA or DNFB and methylamine-treated RNA transcript, suggesting that these conversion methods are applicable for high-throughput sequencing. As mentioned above, the four base conversion methods evaluated in this study have advantages and drawbacks. Therefore, selecting an appropriate method for the target of interest is crucial for studying transcriptome dynamics.

Table 1. Comparison of 4sU Conversion Rates

	base conversion rate (%)			
	3 nt	175 bp	696 bp	conditions
OsO_4·ammonium chloride	73	86	90	50 °C, 4 h
IAA	95	48		50 °C, 15 min
$NaIO_4$·TFEA	>99	57	52	45 °C, 1 h
DNFB·methylamine	96	73		50 °C, 30 min for activation
	>99			50 °C, 30 min for amine addition

5. CONCLUSIONS

In this study, we developed a new 4sU base conversion reaction and compared it to three reported conversion reactions against trinucleotides or RNA transcripts. All four reactions achieved quantitative base conversion using trinucleotides. However, the required reaction time, base

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conversion rate, and effect on reverse transcription vary in the reaction with RNA transcripts. The OsO_4 treatment had the highest conversion rate but required toxic heavy metal. The IAA treatment showed the lowest conversion rate, but the reaction time was the shortest among the four reactions. Each reaction presents advantages and drawbacks for studying transcriptome dynamics; therefore, selecting a suitable conversion method for the target sample is essential.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08516.

(Table S1) Sequences of DNA template and transcribed RNA; (Figure S1) synthesis of 4sU trinucleotide; (Figure S2) OsO_4 and ammonium chloride treatment of 4sU trinucleotide; (Figure S3) IAA treatment of 4sU trinucleotide; (Figure S4) $NaIO_4$ and TFEA treatment of 4sU trinucleotide; (Figure S5) DNFB activation of 4sU trinucleotide; (Figure S6) DNFB and ammonium chloride treatment of 4sU trinucleotide; (Figure S7) DNFB and methylamine treatment of 4sU trinucleotide; (Figure S8) OsO_4 and ammonium chloride treatment of 4sU mRNA; (Figure S9) IAA treatment of 4sU mRNA; (Figure S10) $NaIO_4$ and TFEA treatment of 4sU mRNA; (Figure S11) DNFB and methylamine treatment of 4sU mRNA; (Figure S12) raw data of Figures 1B (OsO_4(−)), 1D (IAA(−)), and S1; (Figure S13) raw data of Figure 1B (OsO_4(+)); (Figure S14) raw data of Figure 1D (IAA(+)); (Figure S15) raw data of Figures 1F ($NaIO_4$(−)), 1H (DNFB (−)), and S6 (DNFB(−)); (Figure S16) raw data of Figure 1F ($NaIO_4$(+)); (Figure S17) raw data of Figures 1H (DNFB(+)) and S6 (DNFB(+)); (Figure S18) raw data of Figure 1H (DNFB(+), methylamine(+)); (Figure S19) raw data of Figure S5 (DNFB(+)); (Figure S20) raw data of Figure S6 (DNFB(+), ammonium chloride(+)) (PDF)

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Author Contributions

S.O., F.H., and H.A. conceptualized and designed the study. M.N., S.A., and M.I. synthesized nucleotide monomer. S.O. performed the experiments. S.O., N.A., Y.K., and F.H. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

4sU, 4-thiouridine; 4sdU, 4-thio-2′-deoxyuridine; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazol; OsO_4, osmium tetroxide; IAA, iodoacetamide; $NaIO_4$, sodium periodate; TFEA, 2,2,2-trifluoroethylamine; DNFB, 2,4-dinitrofluorobenzene; DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene; TEAA, triethylammonium acetate; DMSO, dimethyl sulfoxide; 5mC, 5-methylcytidine

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