Supporting information

Transcription of 4'-thioDNA templates to natural RNA *in vitro* and in mammalian cells

Hideto Maruyama,[a] Kazuhiro Furukawa,[b] Hiroyuki Kamiya,[c] Noriaki Minakawa*[b] and Akira Matsuda*[a]

[a] *Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan*
[b] *Graduate School of Pharmaceutical Sciences, The University of Tokushima, Shomachi 1-78-1, Tokushima 770-8505, Japan*
[c] *Graduate School of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan*

PCR with d*S*NTP

PCR with d*S*NTPs were performed according to Kojima *et al.* (*ACS Synth. Biol.*, 2013, 2, 529-536). Briefly, the PCR mixture was prepared in a total volume of 20 µL by adding 2 µL of 10 × KOD DNA polymerase buffer, KOD Dash DNA polymerase (0.10 U/µL), 87 mer DNA template (5.0 nM), 200 µM each of dNTPs or d*S*NTPs, 0.5 µM of primers, and 2% DMSO. The reaction mixtures were gently mixed and then amplified using a thermal cycler (TaKaRa). PCR was performed under the condition [94 °C, 15 sec; (94 °C, 15 sec; 50 °C, 15 sec; 72 °C, 10 min) × 30 cycles; 72 °C, 5 min]. PCR products were analyzed by 6.4% native PAGE and purified by UV shadowing.

In vitro transcription of 4′-thioDNA

Transcription reactions using NTPs were performed for 2 h at 37 °C in 40 mM Tris-HCl (pH 8.0), 2 mM spermidine, 5 mM 1,4-dithiothreitol and 8 mM $MgCl_2$, with templates at 60 nM and T7 RNA polymerases (TaKaRa) at 0.42 U/µL final concentration. Reactions were stopped by adding an equal volume of gel loading buffer containing saturated urea. The transcripts were analyzed by 6.4% denaturing PAGE and visualized by ethidium bromide staining. The band intensities were quantified by a Molecular Imager FX Pro (Bio-Rad), and the relative transcription efficiencies compared to the template prepared with natural dNTPs were calculated for each template.

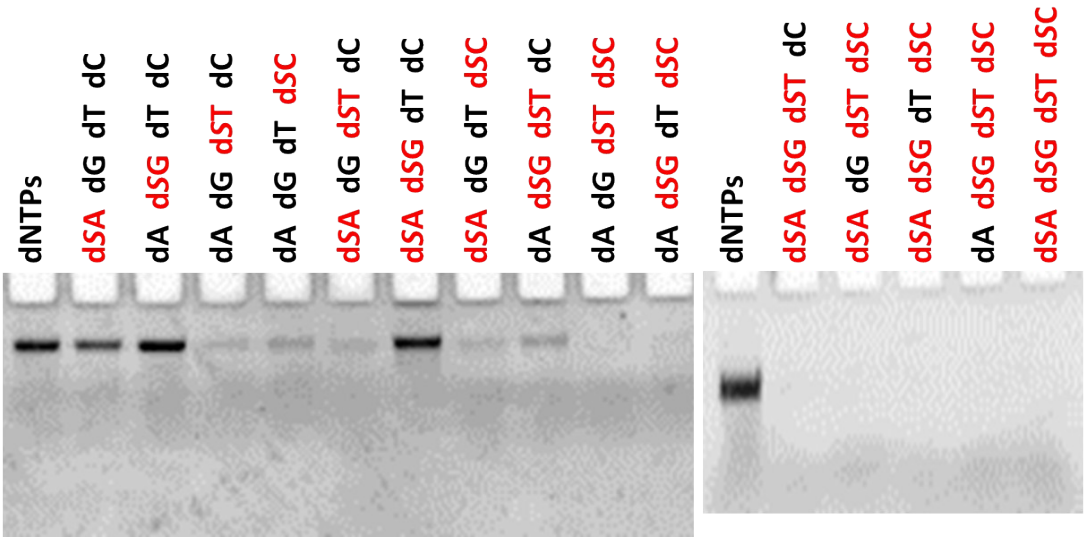
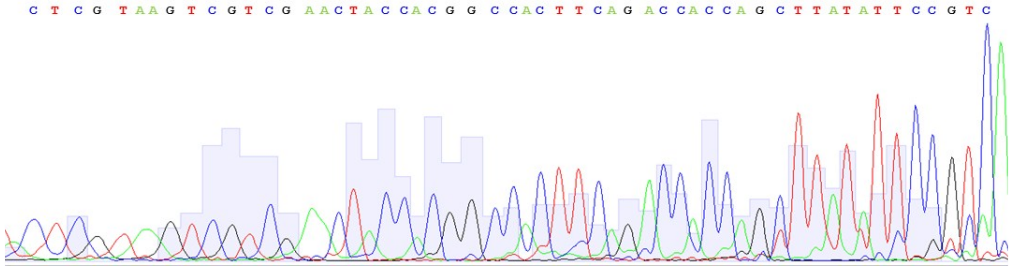


Fig. S1. Representative gel image of the *in vitro* transcription of natural RNA for various DNA templates containing 4′-thionucleotides by T7 RNA polymerase.

Sequence analysis of the RNA transcript from 4′-thioDNA

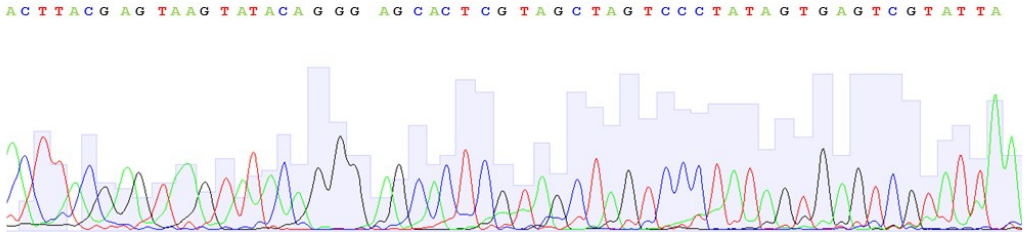
The fidelity of the transcription from 4′-thioDNA templates (containing d*S*A and d*S*C or d*S*T and d*S*G) with T7 RNA polymerase *in vitro* was confirmed by sequencing. RNA transcripts prepared in above were extracted with ISOGEN (Nippon Gene) according to the manufacturer's protocol. Then, to remove remaining DNA template, extracted RNA transcripts were treated with DNase (TaKaRa) at 1 U/μL final concentration for 12 h at 37 °C in 40 mM Tris-HCl (pH 7.5), 5 mM 1,4-dithiothreitol and 8 mM $MgCl_2$. The reaction was stopped by adding 2 μL of 0.5 M EDTA and the mixture was incubated for 2 min at 80 °C and the RNAs were purified by isopropanol precipitation. RNAs were reverse transcribed and amplified using an iScript One-Step RT-PCR Kit for Probes (Bio-Rad) and 0.5 μM of primers (Forward primer: 5′-TAATACGACTCACTATAGGGACTAGCTACGAGTGCTC-3′ and Reverse primer: 5′-GACGGAATATAAGCTGGTGG-3′). Amplification parameters were 50 °C for 10 min, followed by 30 cycles of 95 °C for 10 s, 55 °C for 30 s. The RT-PCR products were purified with NucleoSpin® Extract II (MACHEREY-NAGEL). The products were sequenced with ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) according to the manufacture's protocol.

(A) d*S*A and d*S*C-forward primer



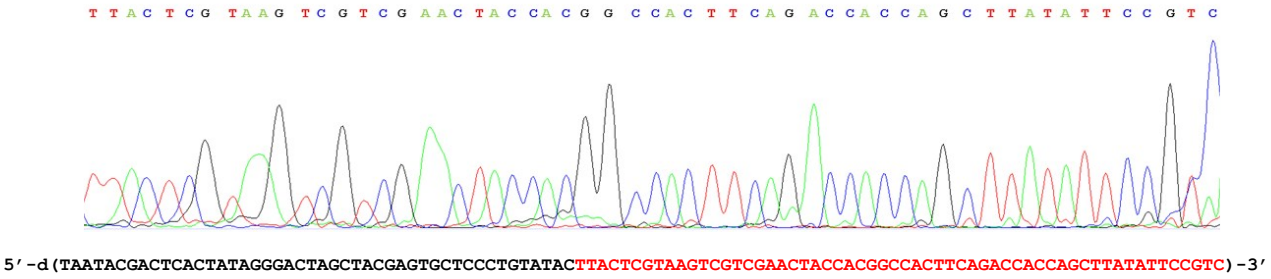
5′-d(TAATACGACTCACTATAGGGACTAGCTACGAGTGCTCCCTGTATACTTACTCGTAAGTCGTCGAACTACCACGGCCACTTCAGACCACCAGCTTATATTCCGTC)-3′

(B) d*S*A and d*S*C-reverse primer

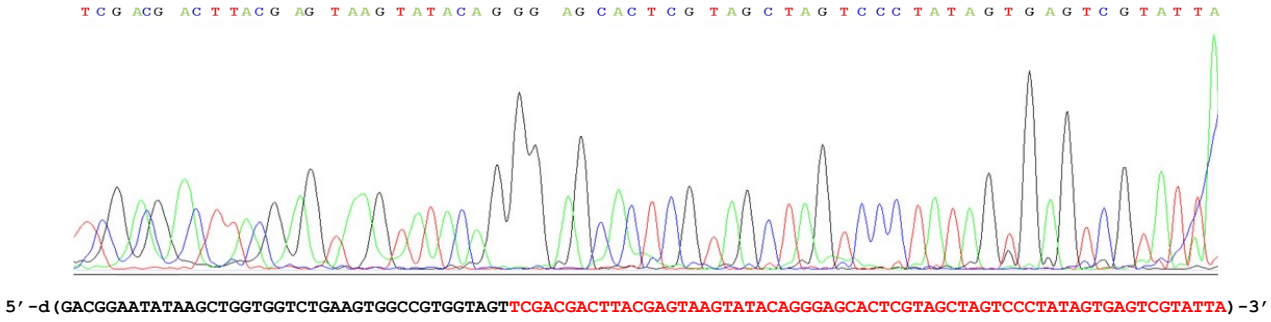


5′-d(GACGGAATATAAGCTGGTGGTCTGAAGTGGCCGTGGTAGTTCGACGACTTACGAGTAAGTATACAGGGAGCACTCGTAGCTAGTCCCTATAGTGAGTCGTATTA)-3′

(C) d*S*T and d*S*G-forward primer



(D) d*S*T and d*S*G-reverse primer



(E) Summary of the sequence analysis

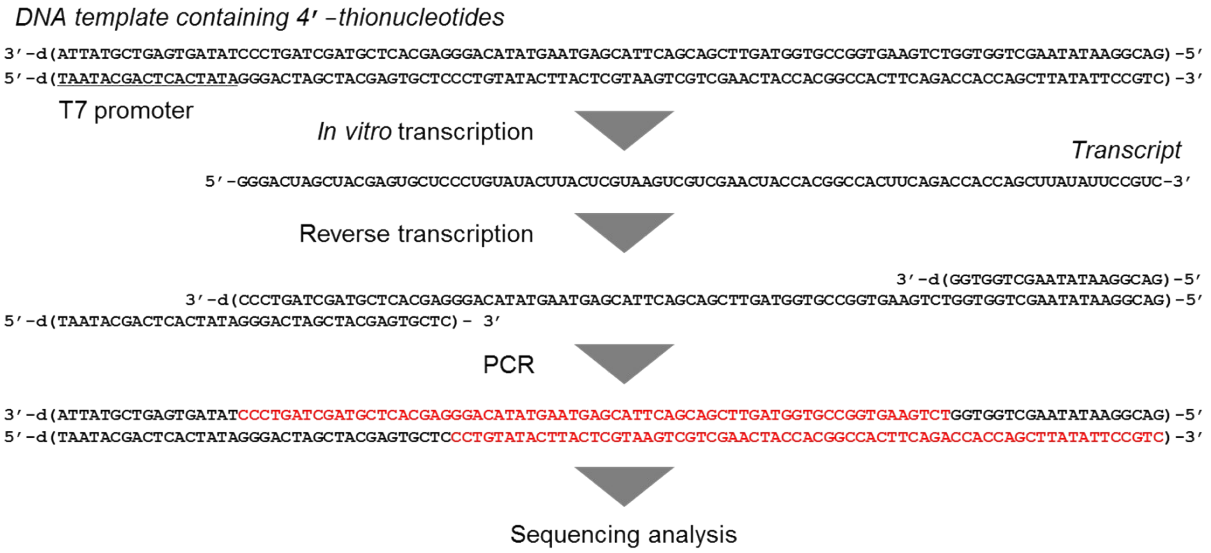


Fig. S2. Sequence analysis of the RNA transcripts from 4′-thioDNA templates containing d*S*A and d*S*G (A, B) or d*S*T and d*S*G (C, D) using forward (A, C) and reverse (B, D) primer. (E) Summary of the sequence analysis. As shown here, 4′-thioDNAs were transcribed into the natural RNA without losing the sequence information of the template.

Synthesis of DNAs

Controlled pore glass (CPG) support bound chemically modified ONs were synthesized on an Applied Biosystem 3400 DNA synthesizer using the corresponding phosphoroamidite units at a 1 μmol scale following the standard procedure described for ONs. Each of phosphoramidite units was used at a concentration of 0.12 M in dry acetonitrile or dichloromethane (for phosphoroamidite units of 2′-deoxy-4′-thioguanosine), and the coupling time was extended to 15 min for each step. After completion of the synthesis, the CPG support was treated with concentrated NH_4OH at 55 °C for 12 h, and the support was filtered off. The filtrate was concentrated, and the ONs protected by a DMTr group at the 5′-end were purified by reverse-phase HPLC. [Column: J′sphere ODS-M80 column (4.6 × 150 mm, YMC), Eluent A: 5% MeCN in 0.1 M TEAA buffer (pH 7.0), B: 50% MeCN in 0.1 M TEAA buffer (pH 7.0). B conc.: 0–100%/20 min. Flow rate: 1.0 mL/min.] The purified fractions containing the full-length DMTr-on ONs were concentrated. The residue was treated with aqueous 80% AcOH for 30 min at room temperature, and the solution was concentrated. The residue was partitioned between AcOEt and H_2O, and the separated aqueous layer was concentrated. The residue was purified by reverse-phase HPLC. [Column: J′sphere ODS-M80 column (4.6 × 150 mm, YMC). Eluent A: 5% MeCN in 0.1 M TEAA buffer (pH 7.0), B: 50% MeCN in 0.1 M TEAA buffer (pH 7.0). B conc.: 10-40 or 20–35%, 20–40%/20 min. Flow rate: 1.0 mL/min.] The structure of the ONs were confirmed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS) on an ultraflex TOF/TOF (Bruker Daltonics).

Table 1. MALDI-TOF mass analysis of DNAs inserted in the plasmid DNA.

sequence (underline means thioDNA modificatioin)		calculated	observed
5'-d(GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG)-3'	$C_{412}H_{516}N_{161}O_{229}P_{41}S_{18}$	13233.5 (M-H)	13260.5
5'-d(GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG)-3'	$C_{412}H_{516}N_{161}O_{233}P_{41}S_{14}$	13169.3 (M-H)	13224.7
5'-d(GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG)-3'	$C_{412}H_{516}N_{161}O_{230}P_{41}S_{17}$	13217.5 (M-H)	13214.7
5'-d(GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG)-3'	$C_{412}H_{516}N_{161}O_{239}P_{41}S_{17}$	13217.5 (M-H)	13233.3
5'-d(GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG)-3'	$C_{412}H_{516}N_{161}O_{232}P_{41}S_{15}$	13185.3 (M-H)	13044.0
5'-d(GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG)-3'	$C_{412}H_{516}N_{161}O_{232}P_{41}S_{15}$	13185.3 (M-H)	13281.2

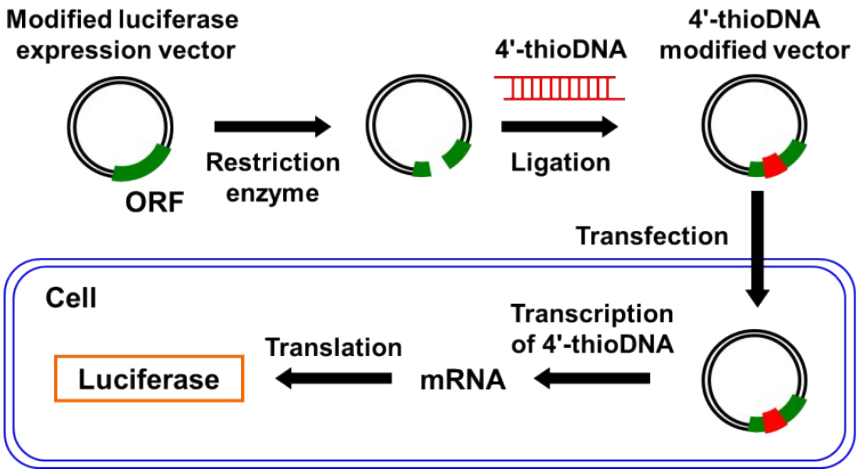


Fig. S3. Schematic illustration of this study to investigate transcription efficiency of 4′-thioDNA in mammalian cells. ORF; Open reading frame

Construction of plasmid DNA containing a *Bsm* BI site

To introduce a *Bsm* BI recognition site into the pGL2-Contol vector (Promega), 556 bp ds fragment containing *Bsm* BI recognition site was prepared by PCR amplification of pGL2-Control vector using a upper primer (5′-CTATCC**TCTAGA**GGATGGAA-3′ ; 309-328 of pGL2-Control vector, bold font indicate the *Xba* I recognition site) and a lower primer (5′-CCAGAG*GAATTC*ATTATCATCGTCTCGTTTTGTCCGATCAAAGGAC-3′ ; 866-819 of pGL2-Control vector except for the underlined sequence, italic and underline indicate the *Eco* RI and *Bsm* BI recognition site respectively). These primers (1 μM each), dNTPs (200 μM each), and Phusion® High-Fidelity DNA polymerase (0.02 U/μL, Finnzymes) were used in the PCR. Amplification was initiated by denaturation at 98 °C for 20 s, followed by a cycle of 98 °C for 10 s; 55 °C for 30 s; 72 °C for 30 s. After 20 cycles were completed, the sample was incubated at 72 °C for 5 min. After amplification, the fragment was purified by phenol and chloroform extractions, followed by ethanol precipitation. Both the 556 bp fragment and pGL2-Control vector were digested by *Xba* I and *Eco* RI at 37 °C for 1 h, and the digested DNAs were ligated with a Ligation High kit (TOYOBO) at 16 °C for 2 h. Target sequence insertion was confirmed by *Bsm* BI digestion and sequencing*. The constructed plasmid (pGL2-*Bsm* BI) was amplified in the *E. coli* DH5α strain, and was purified with a Qiagen Endofree Mega kit. Its UV spectrum was measured to confirm its purity and to calculate its yield. The concentration was determined by the molar absorption coefficient of DNA: 1.0 OD_{260} equals 50 μg of ds DNA.

* sequencing

primer: 5'-CTTTTTTGGAGGCCTAGGC-3' ; 211-229 or 5'-TGGCATGCGAGAATCTGAC-3' ; 916-934

3.2 μL (1μM)

template: plasmid, 100 ng

Reaction Buffer: Big Dye Therminator v3.1 Cycle Sequence Kit (Applied Biosystems), 0.5 μL

5 × Sequencing Buffer: Big Dye Therminator v3.1 Cycle Sequence Kit (Applied Biosystems), 3.75 μL

cycle: 96 ˚C, 2 min; (96 ˚C, 30 sec; 50 ˚C, 15 sec; 60 ˚C, 1 min) × 25 cycles

Preparation of 4'-thio DNA modified plasmid

pGL2-*Bsm* BI was digested with *Bsm* BI at 55 °C for 12 h and *Bst* EII at 60 °C for 3 h and purified by phenol and chloroform extractions, followed by ethanol precipitation. The digested DNA (7.5 μg) was ligated with ONs (each 1 μM, the upper strand 5′-AAAACAATTGCACTGATAATGAATTCCTCTGGATCTACTGG-3′ and the lower strand 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′, 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′, 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′, 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′, 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′, 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′, or 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′, thio DNA modification is underlined) by T4 DNA ligase (TaKaRa, 0.35 U/μL) at 16 °C for 6 h. The ligated mixtures were purified with NucleoSpin® Extract II (MACHEREY-NAGEL). Its UV spectra were measured to calculate their yields. The concentrations were determined by the molar absorption coefficient of DNA: 1.0 OD_{260} equals 50 μg of ds DNA.

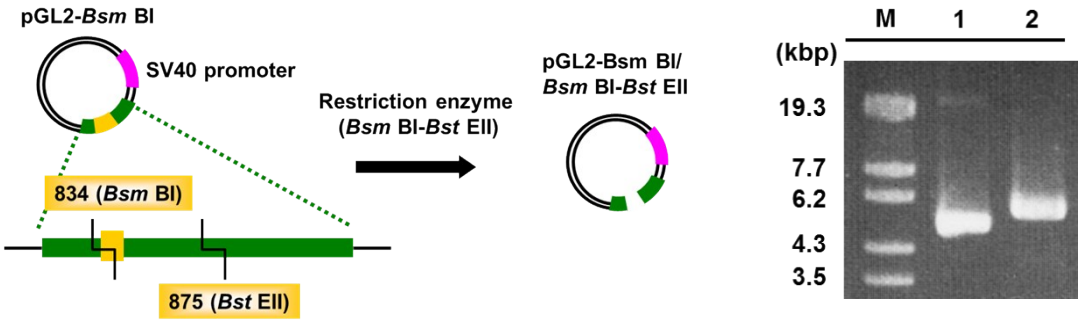


Fig. S4. Treatment of pGL2-*Bsm* BI with *Bsm* BI-*Bst* EII for insertion of 4′-thioDNA
Reactions were performed in a 400 μL reaction volume, containing 50 μg of pGL2-Control vector modified plasmid, each 10 μL of restriction enzyme (*Bsm* BI-*Bst* EII, each 10 U/μL), 40 μL of the reaction buffer at 10× concentration and 4 μL of BSA at 100× concentration. Reactions mixture was incubated for approximately 12 h at 57.5 °C. The treated sample and nontreated control were analyzed by electrophoresis on 1% agarose gel (1× TAE) and imaged by ethidium bromide staining. M; Marker, Lane 1; pGL2-Bsm BI, Lane 2; pGL2-Bsm BI treated with Bsm BI and Bst EII

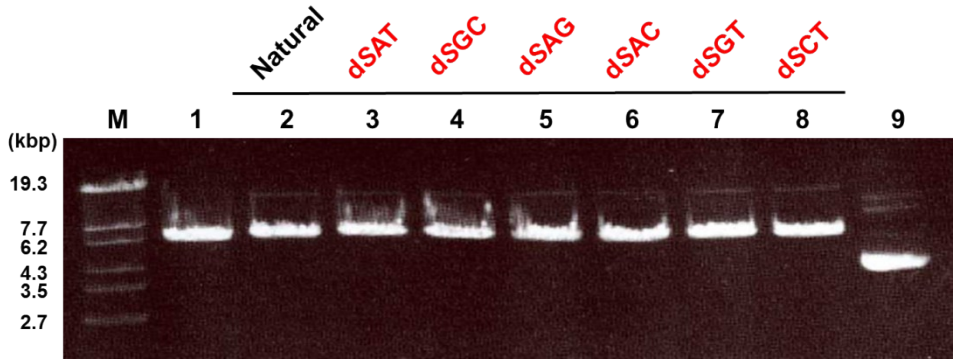


Fig. S5. The insertion of 4′-thioDNA into pGL2-*Bsm* BI/ *Bsm* BI-*Bst* EII
Reactions were performed in a 100 μL reaction volume, containing 7.5 μg of restriction enzyme treated pGL2-*Bsm* BI, each 1 μL of annealed ONs (each 100 μM), 1 μL of T4 DNA ligase (350 U/μL), 10 μL of the reaction buffer at 10× concentration. Reactions mixtures were incubated for approximately 6 h at 16 °C. The treated samples were analyzed by electrophoresis on 1% agarose gel (1× TAE) and imaged by ethidium bromide staining. M; Marker, Lane 1; pGL2-*Bsm* BI treated with *Bsm* BI-*Bst* EII (pGL2-*Bsm* BI/*Bsm* BI-*Bst* EII), Lane 2-8; pGL2-*Bsm* BI/*Bsm* BI-*Bst* EII inserted each natural DNA or 4′-thioDNA (d*S*AT, d*S*GC, dSAG, d*S*AC, d*S*GT, d*S*CT), Lane 9; pGL2-*Bsm* BI

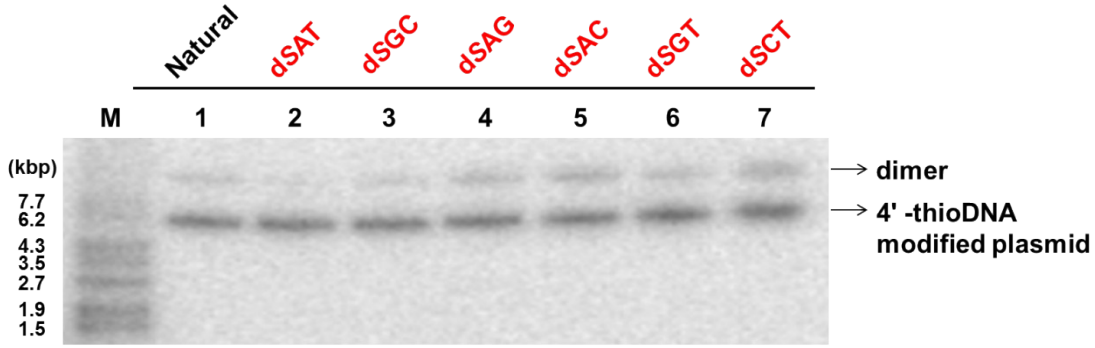


Fig. S6. Confirmation of 4′-thioDNA insertion into pGL2-*Bsm* BI/ *Bsm* BI-*Bst* EII
Reactions were performed in a 10 μL reaction volume, containing 750 ng of restriction enzyme (*Bsm* BI-*Bst* EII) treated pGL2-*Bsm* BI, each 1 μL of annealed ONs (5'-OH of mRNA non-coding strands are ^{32}P-labeled, each 10 μM), 1 μL of T4 DNA ligase (35 U/μL), 1 μL of the reaction buffer at 10× concentration. Reaction mixtures were incubated for approximately at 16 °C 6 h. The treated samples were analyzed by electrophoresis on 1% agarose gel (1× TAE) and imaged by BAS-2500. M; Marker, Each lane shows inserted DNA into pGL2-*Bsm* BI/ *Bsm* BI-*Bst* EII. Lane 1; natural DNA, Lane 2-7; 4′-thioDNA (d*S*AT, d*S*GC, dSAG, d*S*AC, d*S*GT, d*S*CT)

Upper band in the gel might be considered the dimer of 4′-thioDNA modified plasmid, but we do not have any more detailed data.

Confirmation of the insertion of 4'-thio DNA into pGL2-*Bsm* BI/*Bsm* BI-*Bst* EII

Reactions were performed in a 20 μL reaction volume, containing 1.0 μg of DNA, 1 μL of *Bam* HI (20 U/μL), 1 μL of the reaction buffer at 10× concentration. After the reaction at 37 °C for 2 h, 1 μL of *Bsm* BI (10 U/μL), 1 μL of BSA (10 mg/mL) were added and incubated at 55 °C for further 2 h. The treated samples were analyzed by electrophoresis on 1% agarose gel (1× TAE) and imaged by ethidium bromide staining. The band intensity was quantified by LAS-4000.

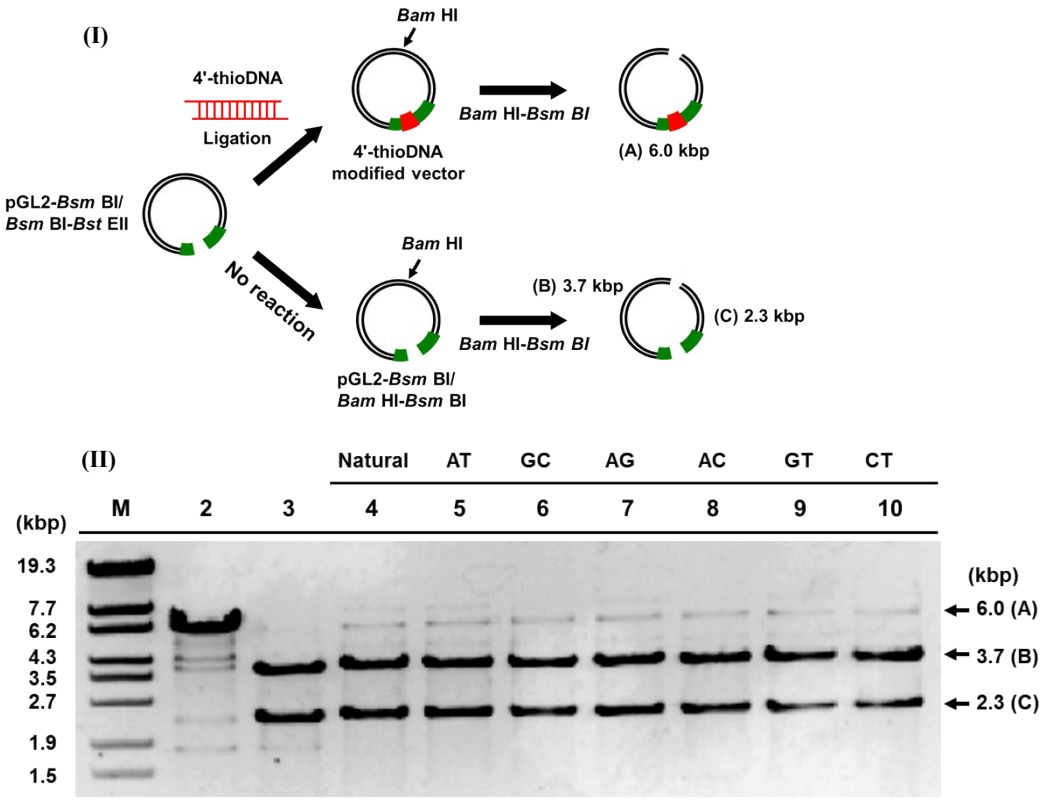


Fig. S7. Confirmation of the insertion of 4′-thioDNA into pGL2-Bsm BI/Bsm BI-Bst
(I) Schematic representation of the treatment of 4′-thioDNA modified plasmid with restriction enzyme to investigate insertion efficiency of 4′-thioDNA into pGL2-*Bsm* BI/*Bsm* BI-*Bst* EII. The desired plasmid should give a 6.0 kbp product due to the absence of Bsm BI recognition site (Fig. S6 (I)-A) whereas non-reacted pGL2-Bsm BI/Bsm BI-Bst EII should yield 3.7 and 2.3 kbp products (Fig. S6 (I)-B). (II) 1% Agarose gel electrophoresis analysis of the reaction. M; Marker, Each lane shows restriction enzyme (*Bam* HI-*Bsm* BI) treated samples. Lane 1; pGL2-Control, Lane 2; pGL2-*Bsm* BI/*Bsm* BI-*Bst* EII, Lane 3-9; 4′-thioDNA modified plasmid inserted each 4′-thioDNA (d*S*AT, d*S*GC, d*S*AG, d*S*AC, d*S*GT, d*S*CT)

Dual-luciferase reporter assay

NIH3T3 cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 10% fetal bovine serum (FBS), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cells were regularly passaged to maintain exponential growth. 24 hours before transfection, cells were diluted with fresh medium (4×10^4 cells per 500 μL) and transferred to 24-well plates. Co-transfection of reporter plasmids and modified pGL2-Control plasmids was carried out with Lipofectamine 2000 (invitrogen) as described by the manufacturer. Before co-transfection, the culture medium was removed. To each well, 0.1 μg pRL-TK (Promega), 1.0 μg pGL2-Control (Promega) or 4′-thioDNA modified pGL2-Control plasmids formulated into liposomes in Opti-MEM medium without serum and antibiotics were sequentially applied; the final volume was 500 μL per well. After 6 hours incubation at 37 °C, the culture medium was replaced with 500 μL of fresh medium supplemented with 10% FBS and antibiotics, and further incubated at 37 °C. After 24, 48 or 72 hours transfection, the cell lysate was prepared and luciferase expression was subsequently monitored with the Dual-Luciferase assay system (Promega).

Cytotoxicity assay of 4′-thioDNAs

NIH3T3 cells were plated in 96-well plates (1×10^4 cells/100 μL per well) one day prior to transfection. To each well, 12.5 pmol of double-stranded DNA (upper strand; 5′-AAAACAATTGCACTGATAATGAATTCCTCTGGAT CTACTGG-3′ and the lower strand; natural: 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATT G-3′, d*S*AG: 5′-GTAACCCAGTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′ or d*S*AC: 5′-GTAACCCA GTAGATCCAGAGGAATTCATTATCAGTGCAATTG-3′, thio DNA modification is underlined) formulated into liposomes (Lipofectamine 2000) in Opti-MEM medium without serum and antibiotics were sequentially applied; the final volume was 50 μL per well. After 4 hours incubation at 37 °C, the culture medium was replaced with 100 μL of fresh medium supplemented with 10% FBS, and further incubated at 37 °C. After 24, 48 or 72 hours transfection, cell viability was measured with WST-8 (Cell Counting Kit-8; Dojin Kagaku) according to the manufacturer's protocol.

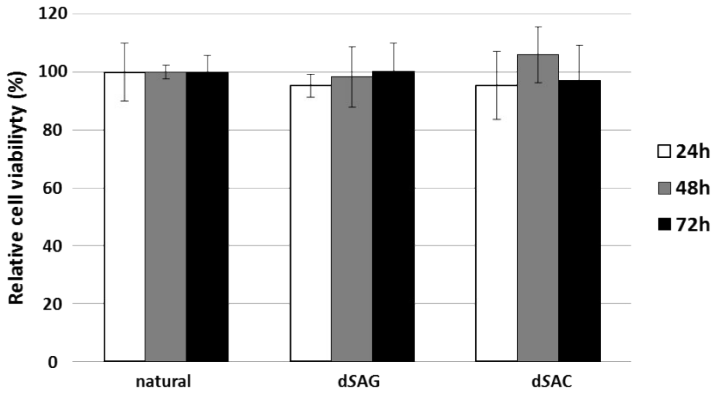


Fig. S8. Cytotoxicity assay of 4′-thioDNAs
The cytotoxicity of 4′-thioDNAs (d*S*AG and d*S*AC) transfected into NIH3T3 cells were evaluated with WST-8 cell counting kit. Each 4′-thioDNA (d*S*AG and d*S*AC) was complexed with natural DNA and the cell viability was compared with natural DNA duplex at 24, 48 or 72 h post transfection. The relative cell viability of natural DNA duplex transfected cells was set as 100%. The plotted data are the means ± standard deviation of three independent experiments.