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Amplification of 4′-ThioDNA in the Presence of 4′-Thio-dTTP and 4′-Thio-dCTP, and 4′-ThioDNA-Directed Transcription in Vitro and in Mammalian Cells

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A number of modified oligonucleotides (ONs), prepared by a general chemical approach using the corresponding phosphoramidite units, have been synthesized in order to evaluate their functions. An alternative enzymatic method using the corresponding nucleoside triphosphates could also be used. Since this approach affords long chain sequences from readily available natural DNA templates, if successful, it would be useful in a large number of biotechnologies.[1] Therefore, intense efforts are currently underway toward an efficient enzymatic synthesis of modified ONs.[2] However, this approach may have limited use because of the low-substrate specificity of the modified nucleoside triphosphates to DNA/RNA polymerases.

Our group synthesized 4′-thioDNA (*S*DNA), a modified DNA analogue which showed nuclease resistance and hybridization ability.[3] Prompted by these and other favorable properties, we decided to explore the enzymatic synthesis of *S*DNAs and their function. We now wish to report the amplification of double stranded *S*DNAs (ds-*S*DNAs)[4] by PCR in the presence of 4′-thio-dTTP (d*S*TTP; **1**) and 4′-thio-dCTP (d*S*CTP; **2**) (Figure 1). The ability of the resulting ds-*S*DNAs as templates to afford natural RNA in vitro and in mammalian cells will also be described.

The desired triphosphates **1** and **2** were prepared from the appropriately protected 2′-deoxy-4′-thio derivatives[5] in a manner similar to that described previously[6] (Supporting Information, Scheme S1). Prior to PCR amplification, primer extension reactions of **1** and **2** were examined using various thermophilic enzymes ((family A) Taq, Tth, and Tfl DNA polymerases; (family B) Vent, Deep Vent, Pfu, Tli, Therminator, and KOD dash DNA polymerases). Among the enzymes examined, the family B enzymes such as Therminator and KOD dash DNA polymerases were found to be the most favorable for incorporation of **1** and **2** (data not shown).[2a] With these results in hand, we examined the amplification of ds-*S*DNAs in the presence of **1** and/or **2** using Therminator and KOD dash DNA polymerases in addition to the Taq DNA polymerase for comparison. The sequences of the 87mer template and the appropriate primers, one of which contained the promoter sequence of the T7 RNA polymerase for a subsequent transcription experiment, to afford the 104mer amplified ds-DNA are presented in the Supporting Information (Figure S1).[4] The resulting ds-*S*DNAs were analyzed quantitatively, and the results are summarized in Figure 2 (and Table S1). When amplification was examined under the usual conditions (condition I; 72 °C for 15 s), neither Taq DNA polymerase nor Therminator and KOD dash DNA polymerases afforded the amplified product in sufficient yields compared with experiments carried out in the presence of the four natural dNTPs,

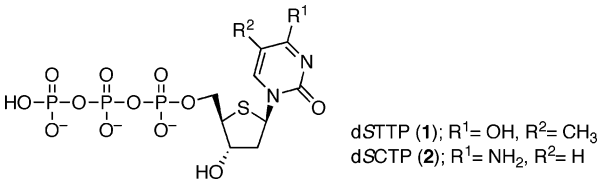

Figure 1. Structures of d*S*TTP (**1**) and d*S*CTP (**2**).

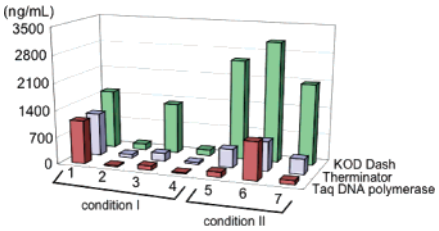

Figure 2. Yield of ds-*S*DNA amplified by PCR using Taq, Therminator, and KOD dash DNA polymerases in the presence of d*S*TTP (**1**) and/or d*S*CTP (**2**). The reaction mixture contains dTTP, dCTP, dATP, and dGTP (lane 1); **1**, dCTP, dATP, and dGTP (lanes 2 and 5); dTTP, **2**, dATP, and dGTP (lanes 3 and 6); and **1**, **2**, dATP, and dGTP (lanes 4 and 7). The reaction conditions (conditions I and II) are described in Supporting Information.

except for KOD dash in the presence of **2**. To improve efficiency, reaction conditions, such as temperature and reaction time of each PCR cycle, were examined. As a result, increasing the reaction time of each cycle from 15 s to 30 min (condition II; 65 °C for 30 min), produced a drastic improvement in amplification even in the presence of both **1** and **2**, especially when KOD dash DNA polymerase was used. Under these modified conditions, only the amplified ds-*S*DNAs,[4] whose lengths were confirmed by PAGE analysis (Figure S2), were obtained in the presence of **1** and/or **2** even under long reaction times such as these. Through this PCR study, it was shown that not only DNA-directed *S*DNA synthesis but also *S*DNA-directed *S*DNA synthesis occurred in the presence of **1** and/or **2**.

In subsequent investigations, we tested whether the resulting ds-*S*DNA acted as a template for transcription by RNA polymerases. In vitro transcription by the T7 RNA polymerase in the presence of the four natural NTPs using the aforementioned PCR products as templates was first examined to afford an 87mer RNA sequence (Figure S1). As can be seen in Figure 3 (and Figure S3), all ds-*S*DNAs[4] as templates afforded the same length of RNA, and the transcription efficiency was estimated in 38−49% yield relative to in the case of natural DNA template.

These results prompted us to further investigate whether the transcription occurred in cells. Accordingly, we constructed a certain

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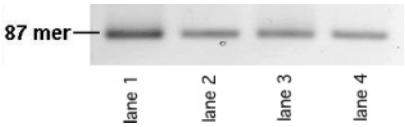

Figure 3. Ultraviolet image from ethidium bromide-stained 2.5% agarose gel of the transcriptional products by T7 RNA polymerase (Full image of the gel was presented in the Supporting Information). The reaction mixture contains DNA104 as a template and NTPs (lane 1); *S*DNA104+**1** as a template and NTPs (lane 2); *S*DNA104+**2** as a template and NTPs (lane 3); and *S*DNA104+**1,2** as a template and NTPs (lane 4). The transcriptional products were quantified by RiboGreen as 0.47 μg/mL (lane 1, 100%), 0.23 μg/mL (lane 2, 49%), 0.21 μg/mL (lane 3, 45%), and 0.18 μg/mL (lane 4, 38%).

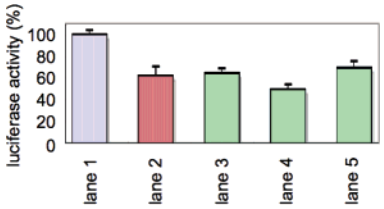

Figure 4. Gene silencing of the expression of pGL3 luciferase gene using scramble ds-DNA (lane 1), natural ds-DNA (lane 2), *S*DNA+**1** (lane 3), *S*DNA+**2** (lane 4), and *S*DNA+**1,2** (lane 5). Data are averages of three independent experiments.

sequence of ds-DNA (or ds-*S*DNAs) by PCR, which was expected to act as templates for transcription in cells by RNA polymerase III (pol III), with the resulting short-hairpin RNA (shRNA) showing a gene-silencing effect via RNAi machinery.[7]

Thus, only specific parts of the shRNA expression vector (pSIREN-Shuttle-Luc) consisting of a U6 promoter and a gene of interest (pGL3 luciferase) were amplified by PCR in the presence of **1** and/or **2** (approximately 300 bp in length). The efficiency of amplification of the ds-*S*DNAs[4] relative to the natural ds-DNA was as follows: *S*DNA+**1**, 51%; *S*DNA+**2**, 99%; *S*DNA+**1**,**2**, 11%. The resulting ds-*S*DNAs were then used for gene-silencing experiments. Cotransfection of individually amplified ds-*S*DNA (or ds-DNA) and reporter plasmids (pGL3 control vector) into NIH/3T3 cells was carried out, and the potency of gene-silencing, RNAi activity, after 24 h was evaluated on the basis of luminescence activity by luciferase. The reporter plasmids included secreted alkaline phosphatase (SEAP) gene as an internal standard, and the luminescence activity was normalized on the basis of SEAP activity to correct the transcription efficiency of each experiment (see the Supporting Information). As shown in Figure 4, the natural ds-DNA inhibited gene expression by approximately 40% relative to conditions using a scramble ds-DNA. Since the gene-silencing that occurred was sequence specific, we postulated that it is due to RNAi arising from the formation of shRNA in cells from the transfected ds-DNA. Either *S*DNA+**1** and *S*DNA+**2** or *S*DNA+**1**,**2** showed the RNAi activity with similar potency. Since *S*DNA+**1**,**2** consists of a 50% ratio of 2′-deoxy-4′-thionucleoside units in the whole sequence, including the U6 promoter and target sequence parts, the fact that transcription occurred in the cells in this modified ds-DNA is worth noting. In addition, *S*DNA+**2** has a higher RNAi activity than that of unmodified ds-DNA. Although the transcription efficiency in vitro from *S*DNA104+**2** was lower than the natural DNA template (Figure 3), the susceptibility toward pol III in cells may differ from the in vitro results. Alternatively, this trend may come from nuclease resistance of ds-*S*DNA.[3] With more research, ds-*S*DNA could be developed as a new DNA device for gene-silencing.

In conclusion, we prepared 4′-thio-dTTP and 4′-thio-dCTP and investigated their susceptibility for PCR amplification. Ds-*S*DNAs were amplified sufficiently by KOD dash DNA polymerase under appropriate conditions. The resulting 4′-thioDNAs acted as templates for transcription not only in vitro but also in mammalian cells to afford RNA which showed gene-silencing effects via RNAi machinery. Unlike nucleoside triphosphates modified on their nucleobase and phosphorus positions,[8,9] no example of PCR amplification using sugar-modified nucleoside triphosphates is known. While pioneering investigations of PCR amplification using αS-dNTPs and in vitro transcription has been carried out using the resulting modified DNA,[10] to the best of our knowledge, the potential of ds-*S*DNA for transcription in cells as reported in this study is the first reported application with modified nucleic acids. Thus, these results suggested the importance of *S*DNA when implementing an enzymatic approach to produce not only aptamers by SELEX but also new DNA devices for gene-silencing via RNAi machinery.

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Supporting Information Available: Synthesis of **1** and **2**, PCR amplification, in vitro transcription, and transcription in NIH/3T3 cells resulting gene-silencing. This material is available free of charge via the Internet at http://pubs.acs.org.

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(4) In this paper, double-stranded DNA products containing 4′-thio-dT and/or 4′-thio-dC were refered to ds-*S*DNAs. Furthermore, ds-*S*DNAs were classified into *S*DNA104+**1** (containing 4′-thio-dT), *S*DNA104+**2** (containing 4′-thio-dC), and *S*DNA104+**1**,**2** (containing 4′-thio-dT and 4′-thio-dC) in the text and Figures 2 and 3. Ds-*S*DNAs described in Figure 4 were classified into *S*DNA+**1**, *S*DNA+**2**, and *S*DNA+**1**,**2**.

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