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Synthesis of DNA fragments containing 2'-deoxy-4'-selenonucleoside units using DNA polymerases: comparison of dNTPs with O, S and Se at the 4'-position in replication

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4'-SelenoDNA fragments were synthesized for the first time using 4'-selenothymidine triphosphate (SeTTP) by taking advantage of its bioequivalence against DNA polymerases. DNA fragments each with homologous element (O, S or Se) at the 4'-position of the thymidine units were effectively amplified using KOD Dash DNA polymerase.

Over the last two decades, a huge number of chemically modified oligonucleotides (ONs) have been designed for use in biotechnologies and nucleic acids-based therapeutics. In addition to solid phase synthesis using the phosphoramidite method, enzymatic synthesis of modified ONs using polymerases overcomes some of the issues confronting automated chemical synthesis on solid supports, e.g., size limitation and chemical stabilities.

DNA polymerases can incorporate modified 2'-deoxynucleoside triphosphates (dNTPs) into growing oligodeoxynucleotides (ODNs) in primer extension reactions and even in PCR. Since many DNA polymerases tolerate modifications of the C5 position of the pyrimidine base or C7 of the 7-deazapurine base of dNTPs, a variety of chemically modified ODNs with functional moieties at those positions have been prepared.[1] However, a limited number of dNTPs with modified sugar moieties have been incorporated into ODNs using DNA polymerases, which often must be specifically engineered for each modified triphosphate, since sugar-modified dNTPs are poor substrates.[2] As an example of sugar-modified ODN synthesis using enzymatic methods, we have reported syntheses of the 4'-thioDNAs, which have sulfur atoms at the 4'-position instead of oxygen atoms (Fig. 1C).[3] Since 4'-thioDNA is effectively amplified in PCR, we became interested in substitution of O4' in dNTPs with homologous, selenium. Selenium belongs to the same group in the periodic table as oxygen and sulfur do and is known to be a useful element in structural elucidation using X-ray crystallography when it is incorporated into ON structures.[4] However, there are no reports concerning 4'-selenoDNA (Fig. 1B) synthesis either by using chemical or enzymatic method, although there is a report about 2'-deoxy-4'-selenonucleoside synthesis.[5,6]

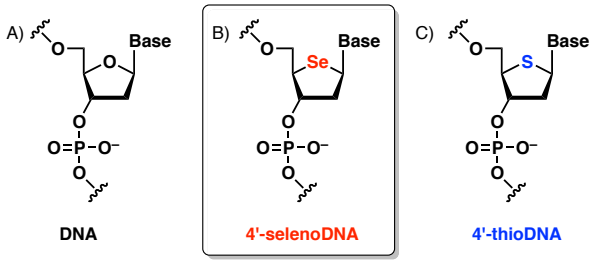

Fig. 1 Structures of natural DNA (A), 4'-selennoDNA (B) and 4'-thioDNA (C).

Herein, we report the first synthesis of DNA fragments containing 4'-selenothymidine (SeT) units via enzymatic replication. In addition, PCR successively proceeded in the presence of 4'-selenothymidine triphosphate (SeTTP). On the basis of these studies, we discuss the effects of substitution at the 4'-position in a dNTPs with homologous elements in enzymatic replication.

Starting with SeT,[5] we first prepared the corresponding 5'-triphosphate SeTTP, which is a substrate for DNA polymerase (Scheme S1†). Then, a primer extension reaction in the presence of SeTTP was carried out using various DNA polymerases. As shown in Fig. 2, all of the polymerases afforded full-length products (30 mer) when natural TTP was used (lanes 2–8). Under the same conditions, DNA polymerases belonging to family B afforded elongated products in the presence of SeTTP (lanes 11–15), whereas those belonging to family A did not (lanes 9 and 10). To the best of our knowledge, these are the first examples of 4'-selenoDNA syntheses, although the stalling of the polymerization was observed because of a homo-polymeric template sequence.[7] When a

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† Electronic Supplementary Information (ESI) available: All information of the experimental details, one scheme and 3 figures as described in the text. See DOI: 10.1039/x0xx00000x

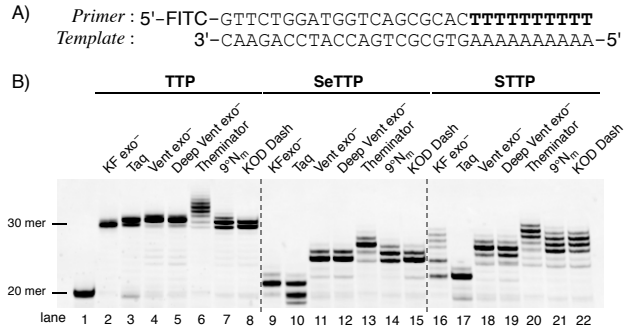

Fig. 2 Primer extension reactions in the presence of SeTTP, STTP or TTP. (A) Sequences of DNA templates and primer. Bold letters are elongated sequence. (B) Denaturing polyacrylamide gel image of the products. The duplex of 5′-FITC labeled primer (0.4 μM) and template (0.5 μM), corresponding triphosphate (50 μM), and each DNA polymerase (0.04–0.05 unit/μL) in a reaction buffer (20 μL) was incubated at 37 or 74 °C. Experimental details are described in the ESI.

random sequence was used as a template, the full-length products were observed (Fig. S1†). These enzymatic behaviors incorporating SeTTP were similar to those of 4'-thiothymidine triphosphate (STTP) (lanes 16–22).

To compare the enzymatic behaviour of SeTTP with STTP and TTP quantitatively, the kinetic parameters for single nucleotide insertion reactions, such as the Michaelis constant (K_m), the maximum rate of enzyme reaction (V_{max}) and the incorporation efficiency (V_{max}/K_m), were determined. As shown in Table 1, the V_{max}/K_m values for Klenow Fragment exo$^-$ nuclease minus (KF exo$^-$) followed the trend TTP > STTP >> SeTTP and were consistent with the results from the primer extension reaction shown in Fig. 2. Using KF exo$^-$, the V_{max} values of the triphosphates were similar. On the other hand, the K_m value for STTP was 23 times worse than that of TTP (K_m = 11.5 and 0.5 μM, respectively) and that for SeTTP (K_m = 187 μM) was 370 times worse than that of TTP. In general, the K_m value indicates the level of dNTP recognition by DNA polymerase. Atomic-level structure analysis of the complexes between KF and dNTPs in the replicative mode suggests that Phe667 forms a replicative platform[8] and that its side chain plays a critical role in distinguishing dNTPs from other sugar motifs as substrates.[9] The steric barriers for conformation changes in the sugar ring arising from the substitution of O4' with selenium or sulfur[5,10,11] could lead to a loss of the interaction between SeTTP or STTP and KF exo$^-$.

Next, PCR was studied in the presence of SeTTP using thermophilic DNA polymerases belonging to family B under

Table 1 Kinetic parameters for single nucleotide insertion using KF exo$^-$.[a]

Primer : 5'-FITC-GTTCTGGATGGTCAGCGCAC
Template : 3'-CAAGACCTACCAGTCGCGTG**A**TTACGGGTG-5'

entry	dNTP	K_m (μM)	V_{max} (min^{-1})	V_{max}/K_m (%·min^{-1}·M^{-1})
1	TTP	0.50 (0.013)[b]	31 (1.2)[b]	6.2×10^7
2	SeTTP	187 (8.3)	7.3 (0.78)	3.9×10^4
3	STTP	11.5 (0.48)	20 (0.78)	1.7×10^6

[a]Experimental details are described in the ESI. [b]Standard deviations are given in parentheses.

various dNTP conditions with an 87-mer template and two primers to give 104-bp amplified products.[3b] From the screening, only the use of KOD Dash DNA polymerase afforded small amounts of amplified products containing SeT units, whereas all of the enzymes used in this study gave amplicons containing thymidine and 4'-thiothymidine units under the condition used (Fig. 3). Bergen *et al.* have reported the crystal structures of KOD DNA polymerase in binary complexes with DNA in the replicative mode. There are fewer interactions (only two residues Asp540 and Arg612) with the ribose moiety of the primer in KOD.[12] Although there is no TP

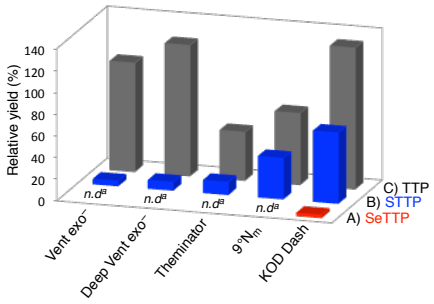

Fig. 3 Screening of DNA polymerases for PCR amplification. Relative yields of the PCR products using Vent exo$^-$, Deep Vent exo$^-$, Therminator, 9N°$_m$ and KOD Dash in the presence of (A) SeTTP with natural dATP, dGTP and dCTP, (B) STTP with natural dATP, dGTP and dCTP, and (C) all natural dNTPs were described. Y-axis indicates the replication yields relative to that Vent exo$^-$ in the presence of natural dNTPs. Reactions were performed in 20 μL of polymerase buffer, 0.5 μM primers, 200 μM corresponding each triphosphate mixture, and 0.1 pmol of the DNA template each with 0.100–0.125 unit/μL of the DNA polymerase. The extension times in each PCR cycle were 30 s. Experimental details are described in the ESI. [a]not detected.

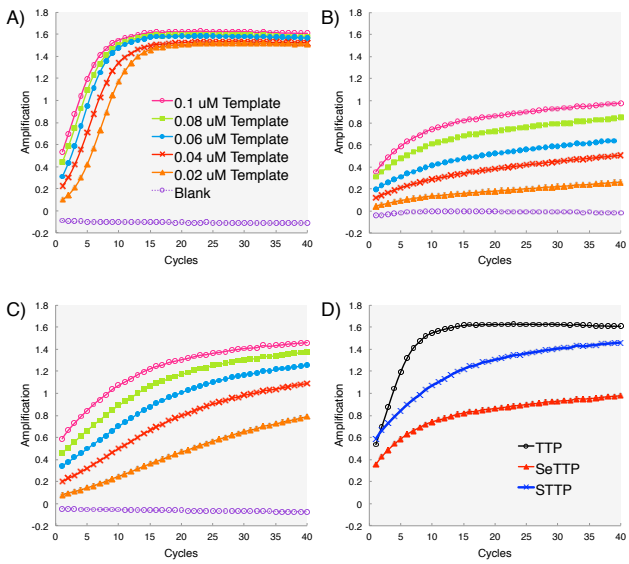

Fig. 4 Real-time quantitative PCR amplification plots under the conditions containing (A) TTP, (B) SeTTP, and (C) STTP, and (D) a comparison of each plot when 0.1 μmol template was used. Reaction mixtures were include 10 nM primers, 100,000-fold diluted SYBR Green I, 50-fold diluted ROX as a reference dye, 2 μM triphosphate mixture and 0.1 pmol of DNA template and 0.1 unit/μL of DNA polymerase in 20 μL of KOD Dash buffer. The extension time in PCR was 60 s. Experimental details are described in the ESI.

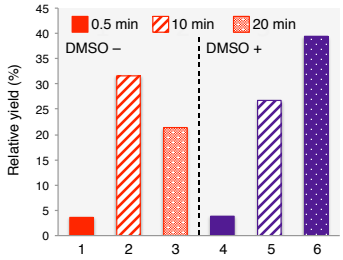

Fig. 5 Optimization of the extension times in each PCR cycle using SeTTP. Y-axis indicates the replication yields relative to that Vent exo$^-$ in the presence of natural dNTPs (see Fig. 3). Reactions were performed in 20 μL of polymerase buffer, 0.5 μM primers, 200 μM corresponding each triphosphate mixture, 0.1 pmol of DNA template and each 0.1 unit/μL of KOD Dash DNA polymerase. In case of reactions for lanes 4–6, the mixture included 5% DMSO. The extension times in each PCR cycle were 30 s (lanes 1 and 4), 10 min (lanes 2 and 5) and 20 min (lanes 3 and 6). Experimental details are described in the Electronic Supplementary Information (ESI).

incorporated in the X-ray structure of the KOD complex, KOD DNA polymerase is tolerant to chemical modification at the 4'-position of the sugar moiety during replication. To improve the effectiveness of the amplification incorporating SeTTP, we performed real-time PCR using KOD Dash DNA polymerase. Amplification curves for SeTTP, STTP, and TTP are shown in Fig. 4. The amplification curves using TTP was logarithmic in a dose-dependent manner (Fig. 4A). Although the amplifications incorporating STTP and SeTTP also increased dose-dependently, their slopes were not as steep as that of TTP (Fig. 4B and 4C, respectively). This indicates that the amplification efficiency of PCR is lower when the atomic number of the element at the 4'-position in the sugar moiety is larger. Using the same template concentration, the slope of the amplification curve in PCR incorporating STTP was even more moderate than that of TTP (Fig. 4D). After 40 cycles, however, both afforded similar amounts of products. In contrast, the amplification yield of PCR incorporating SeTTP was much lower than those of TTP and STTP even after 40 cycles. Thus, we thought that, if the number of PCR cycles and/or the extension times in each PCR cycle were increased, the amount of PCR product containing SeT units would increase. Therefore, we performed PCR with SeTTP using longer extension times (Fig. 5). The yields of the amplified products containing SeT units were dramatically increased with an increase in the extension times from 0.5 to 10 min (lanes 1 and 2, respectively). However, the extension time longer than 10 min was not effective, as shown in lane 3. On the other hand, the amounts of the PCR products increased with an increase in the extension time up to 20 min, when 5% DMSO was added to the reaction mixtures (lanes 4–6).[13] In other words, PCR incorporating SeTTP effectively gave 4'-selenoDNA. In addition, the accuracy of the sequence in the amplicons including SeT units was verified using sequencing analysis (Fig. S3†). These results indicate that substituting at O4' in dNTPs with homologous elements is an appropriate strategy for the development of a chemically modified ODN which is replicable using DNA polymerases.

In this communication, we not only performed primer extension but also amplification of 4'-selenoDNA using KOD Dash DNA polymerase. This is first synthesis of 4'-selenoDNA, which will make it possible to elucidate the structure of DNAs using X-ray crystallography with a MAD phase correlation strategy.[4i] Our results represent a new methodology for designing chemically modified nucleos(t)ide analogues, which are replicable and can be used in biotechnological applications.

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11 The conformation changes in the sugar ring upon substituting O4' with selenium or sulfur are summarized in Fig. S2†.

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