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Systematic analysis of enzymatic DNA polymerization using oligo-DNA templates and triphosphate analogs involving 2′,4′-bridged nucleosides

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ABSTRACT

In order to systematically analyze the effects of nucleoside modification of sugar moieties in DNA polymerase reactions, we synthesized 16 modified templates containing 2′,4′-bridged nucleotides and three types of 2′,4′-bridged nucleoside-5′-triphospates with different bridging structures. Among the five types of thermostable DNA polymerases used, *Taq*, *Phusion HF*, *Vent(exo-)*, *KOD Dash* and *KOD(exo-)*, the *KOD Dash* and *KOD(exo-)* DNA polymerases could smoothly read through the modified templates containing 2′-*O*,4′-*C*-methylene-linked nucleotides at intervals of a few nucleotides, even at standard enzyme concentrations for 5 min. Although the *Vent(exo-)* DNA polymerase also read through these modified templates, kinetic study indicates that the *KOD(exo-)* DNA polymerase was found to be far superior to the *Vent(exo-)* DNA polymerase in accurate incorporation of nucleotides. When either of the DNA polymerase was used, the presence of 2′,4′-bridged nucleotides on a template strand substantially decreased the reaction rates of nucleotide incorporations. The modified templates containing sequences of seven successive 2′,4′-bridged nucleotides could not be completely transcribed by any of the DNA polymerases used; yields of longer elongated products decreased in the order of steric bulkiness of the modified sugars. Successive incorporation of 2′,4′-bridged nucleotides into extending strands using 2′,4′-bridged nucleoside-5′-triphospates was much more difficult. These data indicate that the sugar modification would have a greater effect on the polymerase reaction when it is adjacent to the elongation terminus than when it is on the template as well, as in base modification.

INTRODUCTION

Enzymatic DNA polymerizations using modified nucleotides have been used to study the mechanism of polymerase reactions (1–6), and to apply modified DNA to SELEX (systematic evolution of ligands by exponential enrichment) (7–13) or non-SELEX selections (14,15) that create modified DNAzymes and modified DNA aptamers (16–25). Several years ago, our group and Wengel and co-workers independently developed 2′-*O*,4′-*C*-methylene bridged/locked nucleic acid [2′,4′-BNA (26,27)/LNA (28)]. The 2′,4′-BNA/LNA and its analogs are one of the most promising candidates for antisense drugs, miRNA detecting probes, decoy oligonucleotides, etc. (29–31). These types of modification have been found to improve nuclease resistance to DNA (32,33), which is an important property for the biological use of DNAzymes, DNA aptamers and the aforementioned functional oligonucleotides. Therefore, in the current study, we synthesized oligo-DNA templates containing 2′,4′-bridged nucleotides [2′,4′-BNA/LNA (27,34), 2′,4′-BNA^{COC} (35) and 2′,4′-BNA^{NC} (36,37)] and their 5′-triphosphate derivatives to systematically analyze how the chemical structures of modified sugars affect the primer extension reaction. We also examined the effects of the type of DNA polymerase on polymerization using these types of modified nucleotides.

Regarding enzymatic polymerization using these modified nucleotides, we consider two reactions. One is the

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production of modified DNA from a natural DNA template using a modified triphosphate, and the other is the production of natural DNA from a modified DNA template using a natural triphosphate. Previously, we synthesized modified DNA primers and templates containing C5-modified thymidine, and demonstrated that modification to the extending strand decreased the catalytic efficiency of polymerase to a far greater extent than modification to the template strand did (38). Modification to the sugar backbone is also interesting to consider. Modification to this moiety has greater effects on the polymerase reaction than that to the base moiety in many cases, and the study of such effects may also be useful in clarifying the mechanism of the reaction.

MATERIALS AND METHODS

General

A TC-312 thermal cycler (Techne, Stone, Staffordshire, UK) was used for primer extension experiments and kinetic studies. Reaction products were resolved by denaturing PAGE using a vertical electrophoresis unit (Nihon Eido, Tokyo, Japan) at 48°C in an M-260F incubator (Taitec, Saitama, Japan). Bands were imaged using a Molecular Imager FX (Bio-Rad, Hercules, CA, USA) equipped with an external laser module and quantified with the software Quantity One (Bio-Rad).

Materials

The following commercially available thermostable DNA polymerases were purchased: *Taq* (Takara Bio, Siga, Japan), *Phusion High-Fidelity* (Finzymes, Espoo, Finland), *Vent(exo-)* (New England Biolabs, Hitchin, Herts, UK) and *KOD Dash* (Toyobo, Osaka, Japan). *KOD(exo-)* DNA polymerase was supplied by Toyobo. *KOD(exo-)* is an enzyme genetically engineered to eliminate the 3′,5′ exonuclease activity from *KOD*, and *KOD Dash* is a mixture of *KOD* and *KOD(exo-)* (39,40). Natural 2′-deoxynucleoside-5′-triphosphates (dATP, dGTP, dCTP and TTP) were obtained from Roche Diagnostics, Basel, Switzerland. The chemical structures of 2′,4′-bridged nucleotides, i.e. **K**, **L**, **M** and $\mathbf{K_A}$, and thymidine 5′-triphosphate analogs, i.e. **KTP**, **LTP** and **MTP**, are shown in Figure 1. Four types of 2′,4′-bridged nucleosides, their amidite derivatives and the corresponding oligodeoxynucleotides were synthesized according to previously published procedures (34–37). The triphosphate analogs were synthesized according to the method of Kovács and Ötvös (41). Sequences of oligo-BNA templates (T2–T7, T9–T12) containing **K**, **L**, **M** and $\mathbf{K_A}$ are listed in Table 1. Primers P1 and P2 and templates T1, T8 and T13 were purchased from JBioS, Saitama, Japan. To detect and quantify extension products, the 5′-ends of the primers were labeled with 6-carboxyfluorescein (6-FAM). Synthetic procedures and spectroscopic data of the triphosphate analogs and oligo-DNA templates (T2–T13, T15–T18) are provided in the Supplementary Material.

Primer extension experiments using oligo-DNA templates containing 2′,4′-bridged nucleotides

Primer extension reactions were performed in a 20 μl reaction volume, containing 0.4 μM of a primer (P1), one of the templates (T1–T18) at 0.4 μM, an appropriate concentration of thermostable DNA polymerase, reaction buffer supplied with an enzyme (at 1× concentration) and 200 μM of 2′-deoxyadenosine-5′-triphosphate (dATP) when templates (T1–T13) were used, or thymidine-5′-triphosphate (TTP) when templates (T14–T18) were used. A reaction with a natural template (T1 or T14) was used as a positive control, and a reaction with water in place of T1 or T14 was used as a negative control. The assays were performed with one of the modified templates (T2–T13) containing the 2′,4′-bridged thymidine analogs (**K**, **L** or **M**) in place of T1. Also, one of the modified templates (T15–T18) contained the 2′,4′-bridged adenosine analog ($\mathbf{K_A}$) instead of T14. The final concentrations of the

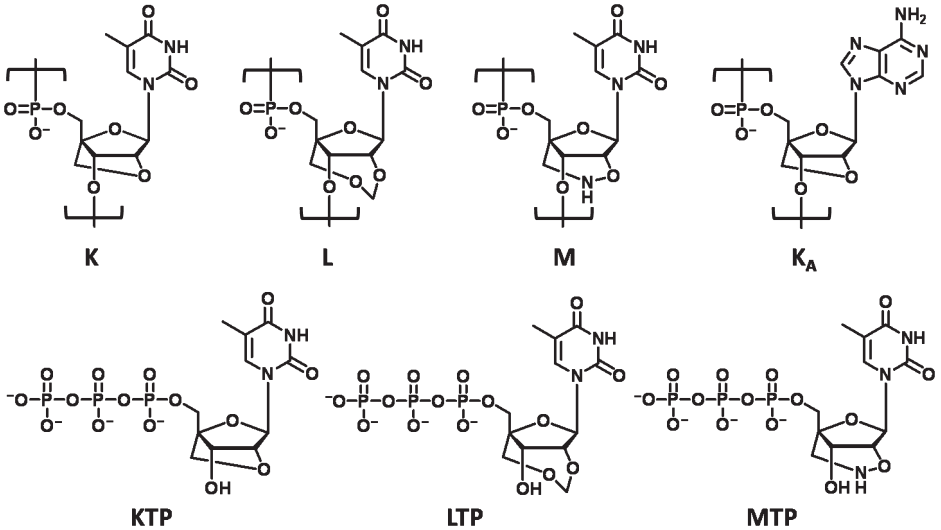

Figure 1. Chemical structures of 2′,4′-bridged nucleotides and triphosphate analogs used in this study; **K**, $\mathbf{K_A}$ and **KTP** (the type of 2′,4′-BNA/LNA), **L** and **LTP** (the type of 2′,4′-BNA^{COC}), and **M** and **MTP** (the type of 2′,4′-BNA^{NC}).

Table 1. Primers and templates used in this study

Primers			
P1	5′-FAM-	GGCGTTGAGTGAGTGAATGAGTGAGT	–3′
P2	5′-FAM-	GGCGTTGAGTGAGTGAATGAGTGAGTA	–3′
Templates			
T1	3′-	CCGCAACTCACTCACTTACTCACTCATTTTTTTTTTT	–5′
T2	3′-	CCGCAACTCACTCACTTACTCACTCATKTTTKTTTTT	–5′
T3	3′-	CCGCAACTCACTCACTTACTCACTCATKTTKTTKTTT	–5′
T4	3′-	CCGCAACTCACTCACTTACTCACTCATKTKTKTKTTT	–5′
T5	3′-	CCGCAACTCACTCACTTACTCACTCATKKKKKKKTTT	–5′
T6	3′-	CCGCAACTCACTCACTTACTCACTCATLTTTLTTTTT	–5′
T7	3′-	CCGCAACTCACTCACTTACTCACTCATLTTLTTLTTT	–5′
T8	3′-	CCGCAACTCACTCACTTACTCACTCATLTLTLTLTTT	–5′
T9	3′-	CCGCAACTCACTCACTTACTCACTCATLLLLLLLTTT	–5′
T10	3′-	CCGCAACTCACTCACTTACTCACTCATMTTTMTTTTT	–5′
T11	3′-	CCGCAACTCACTCACTTACTCACTCATMTTMTTMTTT	–5′
T12	3′-	CCGCAACTCACTCACTTACTCACTCATMTMTMTMTTT	–5′
T13	3′-	CCGCAACTCACTCACTTACTCACTCATMMMMMMMTTT	–5′
T14	3′-	CCGCAACTCACTCACTTACTCACTCAAAAAAAAAAAA	–5′
T15	3′-	CCGCAACTCACTCACTTACTCACTCAAK$_A$AAAK$_A$AAAAA	–5′
T16	3′-	CCGCAACTCACTCACTTACTCACTCAAK$_A$AAK$_A$AAK$_A$AAA	–5′
T17	3′-	CCGCAACTCACTCACTTACTCACTCAAK$_A$AK$_A$AK$_A$AK$_A$AAA	–5′
T18	3′-	CCGCAACTCACTCACTTACTCACTCAAK$_A$K$_A$K$_A$K$_A$K$_A$K$_A$K$_A$AAA	–5′
T19	3′-	CCGCAACTCACTCACTTACTCACTCAAAAAAAAA	–5′

thermostable DNA polymerase in each reaction mixture was 0.025 U/μl for *Taq*, 0.010 U/μl for *Phusion HF*, 0.020 U/μl for *Vent(exo-)*, 0.0025 U/μl for *KOD Dash* with templates (T1–T13), 0.0050 U/μl for *KOD Dash* with templates (T14–T18), 0.0025 U/μl for *KOD(exo-)* with templates (T1–T13) and 0.0050 U/μl for *KOD(exo-)* with templates (T14–T18) for the 'lower enzyme concentrations'. The concentrations were 0.25 U/μl for *Taq*, 0.10 U/μl for *Phusion HF*, 0.20 U/μl for *Vent(exo-)*, 0.025 U/μl for *KOD Dash* with templates (T1–T13), 0.050 U/μl for *KOD Dash* with templates (T8–T12), 0.025 U/μl for *KOD(exo-)* with templates (T1–T7) and 0.050 U/μl for *KOD(exo-)* with templates (T14–T18) for the 'higher enzyme concentrations'. The lower concentrations are the standard conditions recommended by manufacturers, except for *KOD Dash* and *KOD(exo-)* DNA polymerases; the recommended concentrations of these two polymerases are around 0.025–0.050 U/μl. The higher concentrations were set 10-fold higher than the lower concentrations. All reactions were performed by denaturation for 1.5 min at 94°C, annealing for 0.5 min at 52°C and extension for 5 min at 74°C (72°C only for *Phusion HF*), successively. The reaction products were resolved by denaturing PAGE, and gel images were recorded with excitation of the 5′-labeled fluorophore at 488 nm (Figure 2). The yields of elongated products were calculated from the intensity of each band on gel images visualized by the detection of the 5′-labeled fluorophore. The total amount of elongated products was set at 100% in each reaction mixture, and the calculated yields were the averages of three independent experiments.

Kinetic analysis of the nucleotide incorporation opposite 2′,4′-bridged thymidine

To study how 2′,4′-bridged nucleotides on the template, located on the opposite site of elongation terminus of the primer, affect the accuracy of nucleotide incorporation

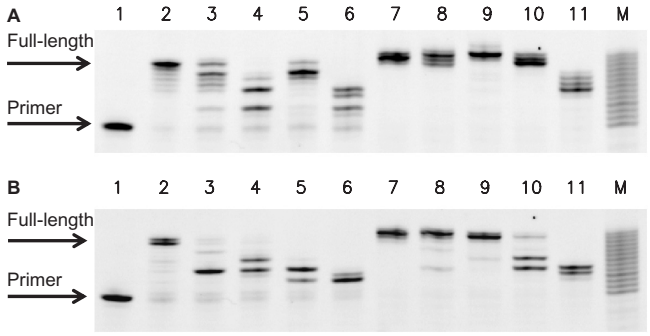

Figure 2. Representative gel images of the reactions using template BNA containing **K** with *KOD Dash* and *Taq* DNA polymerases. The reaction mixtures contained template T1 (lanes 2 and 7), T2 (lanes 3 and 8), T3 (lanes 4 and 9), T4 (lanes 5 and 10) or T5 (lanes 6 and 11). Extension was performed at lower (lanes 2–6) and higher concentrations (lanes 1, 7–11). The negative control does not contain the template strand (lane 1). The thermostable DNA polymerases used were *KOD Dash* (**A**) and *Taq* (**B**).

during DNA polymerase reaction, we performed nucleotide incorporation reactions using the template T1 or T5 and 5′-(6-FAM)-labeled primer (P2) together with dNTP. The reaction was performed at 40°C because it was difficult to monitor the reaction at the optimal temperature of the enzymes (~75°C). The templates T1 and T5 have the same sequence, but T1 consists of four natural nucleotides and T5 has seven consecutive 2′,4′-bridged thymidines (**K**), as shown in Table 1. Two types of DNA polymerases, *Vent(exo-) and KOD(exo-)*, which lack 3′,5′ exonuclease activity, were used as the enzyme to compare the fidelity of DNA polymerases. Reaction mixtures (18 μl) containing the primer (P2) at 0.4 μM, the template T1 or T5 at 0.4 μM, one of the 2′-deoxynucleoside-5′-triphosphates (dATP, dGTP, dCTP or TTP) at 800 μM and the reaction buffer supplied with the enzyme (at 1× concentration) were denatured at 95°C for 1.5 min with the thermal

cycler and then annealed at room temperature for 30 min. The mixtures were then set aside on an ice bath for 10 min. Subsequently, enzyme solutions [0.4 U for *Vent(exo-)*, 0.0125 U for *KOD(exo-)*] were added to the mixture, and the reaction tube was quickly placed in a thermoregulated bath and incubated at 40°C during the reaction. After the reactions were started, the reaction tubes were removed from the bath sequentially, and immediately quenched by freezing in liquid nitrogen. The frozen reaction mixtures were then mixed with 4 μl of 40 mM EDTA containing 0.1% bromophenol blue and 24 μl of 7 M urea containing 3 mM EDTA, and then were melted into a homogeneous solution by vortexing. The sample solutions were resolved by denaturing PAGE, and gel images were recorded on the imager. The amount of reactant and products was measured from the intensity of each band with excitation at 488 nm to visualize the 5′-labeled fluorophore. The decrease of the primer ratio (%) was obtained from band intensities of the primer and its elongated products (Figure S3). The data of the time-dependent decrease were fit to hyperbolic saturation curves by the least squares method using OriginPro ver.8.

Primer extension experiments using 2′,4′-bridged nucleoside triphosphate analogs

To investigate enzymatic incorporation of 2′,4′-bridged nucleotides into a DNA strand, primer extension reactions were performed in a 20 μl reaction volume containing 0.4 μM of a primer (P1), 0.4 μM of a template (T19), an appropriate concentration of a thermostable DNA polymerase, a reaction buffer supplied with the enzyme (at 1× concentration) and a nucleoside triphosphate at 200 μM. A reaction mixture with natural TTP was used as a positive control. The assays were performed with one of the 2′,4′-bridged nucleoside triphosphate analogs (**KTP**, **LTP** or **MTP**) in place of TTP; a reaction with water in place of TTP was used as a negative control. The final concentrations of the thermostable DNA polymerase in each reaction mixture were 0.10 U/μl for *Phusion HF* and 0.050 U/μl for *KOD Dash*. At these enzyme concentrations, the high polymerase activity of *Phusion HF* and *KOD Dash* polymerases caused an overreaction and decreased the yield of the product for the positive controls; therefore, we reduced their concentrations to 0.010 and 0.0050 U/μl, respectively, to obtain an optimal yield of the product. All reactions were performed by denaturation for 1.5 min at 94°C, annealing for 0.5 min at 52°C and extension for 5 min at 72°C for *Phusion HF* and 74°C for *KOD Dash*, successively. The reaction products were resolved by denaturing PAGE, and gel images were obtained, as mentioned earlier (Figure 2).

RESULTS AND DISCUSSION

Primer extension experiments using a variety of template BNA

We systematically investigated the effects of insertion intervals of bridged nucleotides, chemical structures of bridged moieties, the base type of bridged nucleotides and the types of DNA polymerases on DNA polymerization (Figures 2 and 3). Template BNA T2, T6, T10 and T15 contain **K**, **L**, **M** and $\mathbf{K_A}$ at intervals of three nucleotides; T3, T7, T11 and T16 contain **K**, **L**, **M** and $\mathbf{K_A}$ at intervals of two nucleotides; T4, T8, T12 and T17 contain **K**, **L**, **M** and $\mathbf{K_A}$ at intervals of one nucleotide, respectively (Table 1). Templates T5, T9, T13 and T18 contain sequences of the seven successive **Ks**, **Ls**, **Ms** and $\mathbf{K_A}$**s**, respectively.

In Figure 3, the *y*-axis of these graphs indicates the yield of the product, and the *x*-axis indicates the number of residues (dAs or Ts) incorporated into the extending strand. The gray and black bars indicate the yield of the reaction at lower and higher enzyme concentrations, respectively. The primer is elongated by 11 nt to give the full-length product. However, incorporation of over 11 nt was observed even in the positive controls, except for graph 6A for *Phusion HF*. This may have occurred because of DNA template slippage (42,43), or nontemplated nucleotide addition at the 3′-end by the action of the polymerases used (44). In the cases of T2–T4, when *KOD Dash* was used at higher enzyme concentrations, the sum of the yields of products that included an 11 or more nucleotide elongation was quantitative, as shown in graphs 1B–D. Similarly, when *Vent(exo-)* and *KOD(exo-)* were used at higher enzyme concentrations, the full-length and longer elongated products were given in quantitative yields (graphs 7B–D and 8B–C) or ∼90% yield (graph 8D). On the other hand, when *Taq* was used at higher enzyme concentrations, the yield was ∼90% in the case of T2 and T3 (graphs 5B and C), but only 13% in the case of T4 (graph 5D). Also, when *Phusion HF* was used at higher enzyme concentrations, the yield was only 8, <1 and 3% in the case of T2, T3 and T4, respectively (graphs 6B–D). In addition, degradation of the primer was observed due to its strong 3′,5′ exonuclease activity. These results indicate that *KOD Dash*, *Vent(exo-)* and *KOD(exo-)* are superior to *Taq* and *Phusion HF* in the production of natural DNA from the template BNA. Among these DNA polymerases used, only *Taq* DNA polymerase, which belongs to family A, is derived from bacteria, and the rest were obtained from archaea. Previously, it was demonstrated that family B polymerases are suitable for polymerase reactions involving base-modified nucleotides, compared with polymerases belonging to families A and D (45,58,59). The results of the present study indicate that family B polymerases with non or moderate 3′,5′ exonuclease activity, such as *Vent(exo-)* and *KOD Dash*, are also useful for polymerase reactions involving sugar-modified nucleotides, although ribonucleotides are generally poor substrates for most DNA polymerases.

The effects of insertion intervals of 2′,4′-bridged nucleotides in the templates would be well reflected, when the polymerases were used at lower enzyme concentrations. Extensions stopped discontinuously or at the particular sites, for example, where the third, fifth or sixth residue was incorporated (see gray bars in Figure 3). There may be some sites at which distortion of the ternary complex between extending-primer/template duplex and polymerase would reach a local maximum, so that the polymerase would likely dissociate from the duplex at these sites. This would be supported by the result that extension did not

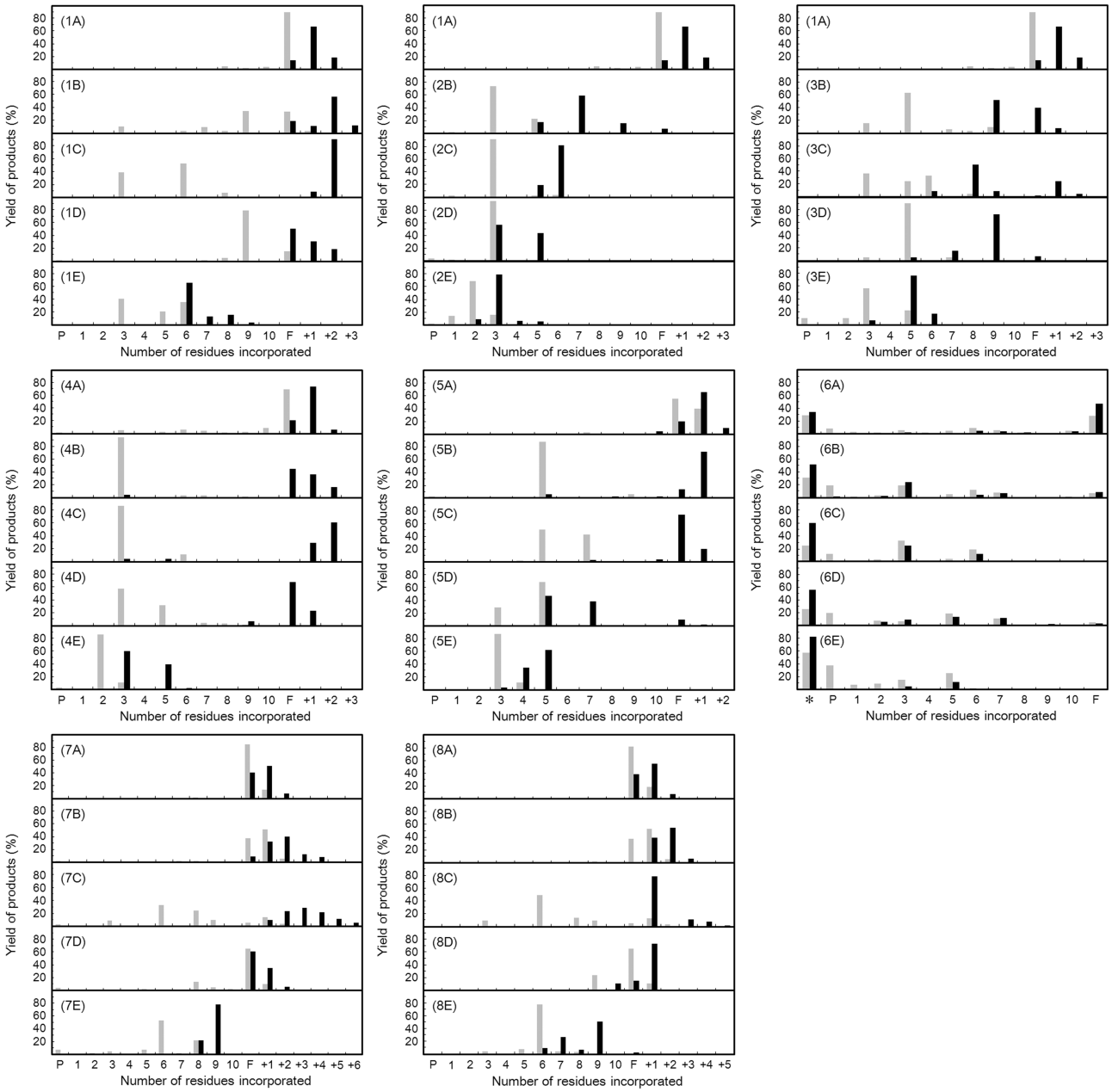

Figure 3. Yield of the natural DNA generated by primer extension reactions involving natural DNA templates or various BNA templates together with (1A–E, 2B–E, 3B–E and 4A–E) *KOD Dash* DNA polymerase, (5A–E) *Taq* DNA polymerase, (6A–E) *Phusion HF* DNA polymerase, (7A–E) *Vent(exo-)* DNA polymerase and (8A–E) *KOD(exo-)* DNA polymerase. The reaction mixture contained templates (1A and 5A–8A) T1, (1B and 5B–8B) T2, (1C and 5C–8C) T3, (1D and 5D–8D) T4, (1E and 5E–8E) T5, (2B) T6, (2C) T7, (2D) T8, (2E) T9, (3B) T10, (3C) T11, (3D) T12, (3E) T13, (4A) T14, (4B) T15, (4C) T16, (4D) T17 and (4E) T18. The *x*-axis indicates the number of residues incorporated, and the *y*-axis indicates the yield of the products. The asterisk (*), P and F on the *x*-axis represent degradation products, the primer and the full-length product, respectively. The gray and black bars indicate the yields of the reaction at lower and higher enzyme concentrations, respectively. The relative SDs were less than ±5% for all reactions.

stop there and further proceeded in most cases when the enzyme concentration was raised 10-fold (see black bars in Figure 3). Interestingly, in the reaction with *KOD Dash*, *Vent(exo-)* and *KOD(exo-)* at lower enzyme concentrations, the use of template T4 provided much better yields of longer elongated products compared with T3, although T4 contains **K** at shorter intervals than T3 (compare graph 1C with 1D, 7C with 7D, and 8C with 8D). On the contrary, at higher enzyme concentrations, the products in which over 11 nt were incorporated were given in higher yield when T3 was used than when T4 was used; the elongations stopped more promptly when T4 was used rather than when T3 was used. These are presumably because the conformation of the duplex with T3 does not fit well within the DNA-binding site of the polymerases. In the cases of T5, T9, T13 and T18 containing sequences of seven successive **Ks**, **Ls**, **Ms** and $\mathbf{K_A}$**s**, all the polymerases used except for *KOD(exo-)* could not accomplish

extensions to give full-length products (graphs 1E–7E); a full-length product was barely given in 3% yield using *KOD(exo-)* at higher enzyme concentrations (graph 8E). In the reaction with the template T5 using *Vent(exo-)* and *KOD(exo-)* at higher concentrations, ~80 and 50% of strands stopped elongation after the ninth residue was incorporated, although the nucleotides located on the opposite side of the ninth to eleventh residues are not **Ks** but natural Ts (graphs 7E and 8E). The polymerases seem to run off the template BNA depending on the conformational strain around the 3′-end of extending strand.

The effects of chemical structures of bridged moieties were found to depend on their ring size (graphs 1B–E, 2B–E and 3B–E). The bridged nucleotides **K**, **L** and **M** involve five-, seven- and six-membered rings, respectively (Figure 1). Use of templates containing bridged nucleotides with a larger ring yielded shorter elongated products. For example, extension mainly stopped after the sixth, third and fifth nucleotide was incorporated when the reaction was performed at higher enzyme concentrations using template T5 containing **Ks**, T9 containing **Ls** and T13 containing **Ms**, respectively (see black bars in graphs 1E–3E). This is consistent with the steric bulkiness of the bridged ring in the template strand.

The nucleobase type of the bridged nucleotide in the template significantly affected the extension reaction, at lower enzyme concentrations (see gray bars in graphs 1A–E and 4A–E). This might be due to the different thermodynamic stabilities of the base-stacking interaction between the 3′-end of extending strand and the incoming dATP or TTP. Also, it might be because the base orientation toward the helical axis, which is constrained by cross-bridging, resulted in unfavorable conformational distortion adjacent to the active site of the polymerase, so that the size difference between thymine and adenine of the bridged nucleotide in the template may sensitively be reflected in the distortion; the larger the base, the greater the steric distortion. However, at higher enzyme concentrations, extensions hardly stopped on the way, and the full-length and longer elongated products were provided in high yields, except for templates T5 and T18 (see black bars in graphs 1B–D and 4B–D). The concentrations of *KOD Dash* DNA polymerase (0.025 and 0.05 U/μl), set as higher enzyme concentrations in this experiment, are within the range of values that the manufacturer recommends for polymerase reactions under standard conditions. Thus, *KOD Dash* polymerase could transcribe the sequence information of the template BNA to natural DNA strands under standard reaction conditions not involving excessively high concentrations of enzyme and substrate triphosphates, extremely long reaction times (e.g. over an hour), etc.

Comparison of the initial rate of natural nucleotide incorporation on modified templates

The initial rates (v_0) of nucleotide incorporation opposite **K** (2′,4′-bridged thymidine) and T (thymidine) were determined from a time-dependent decrease of the primer ratio (%) to an extent of 0–15% or less (Table 2). The v_0-values were confirmed to reach almost plateau of the apparent maximum rates (V_{max}) at 800 μM of dNTP concentrations in the experimental condition (data not shown). Therefore, we used this dNTP concentration to obtain values of v_0 that are close to V_{max}. The ratio of the initial rates (f') indicates the upper limit of a misincorporation ratio and was calculated according to the equation

$$f' = \frac{(v_0)_{wrong}}{(v_0)_{correct}} \approx \frac{(V_{max})_{wrong}}{(V_{max})_{correct}}; f' > f = \frac{(V_{max}/K_m)_{wrong}}{(V_{max}/K_m)_{correct}},$$

because the apparent Michaelis constants (K_m) are normally $(K_m)_{wrong} > (K_m)_{correct}$ in single-nucleotide incorporation reactions like this (46,47). Here, f is the misincorporation ratio (48,49). The effect of the bridged group on nucleotide incorporation (e) was calculated according to the equation $e = (v_0)_{bridged}/(v_0)_{natural}$. The v_0 value itself has no quantitative meaning, because it depends on the enzyme concentration. However, ratios of v_0-values (f' and e) in use of the same enzyme at the same concentration reflect fidelities of DNA polymerases used and effects of the bridged group in sugar moieties on nucleotide incorporation. In the nucleotide incorporation reactions opposite T using *Vent(exo-)* DNA polymerase, the v_0-value of the misincorporation was ~18- to 36-fold lower than that of the correct incorporation, and similarly, ~9- to 29-fold lower in the incorporation reactions opposite **K**. These results indicate that the presence of the bridged nucleotide **K** on the template, located opposite the 3′-end of the primer, did not significantly affect the incorporation accuracy. Also, *KOD(exo-)* DNA polymerase showed the same tendency as above. Under this experimental condition, the v_0 value of the correct incorporation reactions opposite T using *Vent(exo-)* and *KOD(exo-)* were 130%/min and 140%/min, respectively. Although these values are at almost the same levels, the v_0-value of the misincorporation was found to be ~240- to 2300-fold lower than that of the accurate incorporation when *KOD(exo-)* was used. It is noteworthy that *KOD(exo-)* DNA polymerase could clearly distinguish a correct substrate from an incorrect one like this. The e-values were 0.42–0.86 for *Vent(exo-)* and 0.11–0.45 for *KOD(exo-)*. In either of the enzymes, the rate of incorporation opposite **K** was found to virtually decrease compared to that of the reaction opposite T.

Enzymatic incorporation of 2′,4′-bridged nucleotides

Finally, we investigated substrate properties of 2′,4′-bridged nucleoside triphosphate analogs for thermostable polymerases during a primer extension reaction. Figure 4 shows that the elongated products, in which two and three incorporated consecutive **K** nucleotides, were mainly observed when *KOD Dash* was used. It was observed that *KOD Dash* could incorporate up to two **L** nucleotides, but only a single **M** nucleotide. The use of *Phusion HF* allowed incorporation of two and three consecutive **K** nucleotides, but the longer one was a minor product. The results are consistent with recent reports by Veedu *et al.* (50,51). Under these conditions, the primer was degraded by strong 3′,5′ exonuclease activity of *Phusion HF* DNA polymerase when **LTP** or **MTP** was used as well as the negative control. Optimization of

Table 2. Natural nucleotide incorporation opposite modified/natural template using *Vent(exo-)* and *KOD(exo-)* DNA polymerase[a]

dNTP

5′-FAM-· · · · · · · · · TGAGTGAGTA

3′-· · · · · · · · · ACTCACTCAT**X**· · · · · · · · ·-5′

Primer **P2**
Template **T1**, **T5**

DNA polymerase	X	dNTP	v_0 (% min^{-1})	The ratio of the initial rate (f')[b]	Effect of bridged group (e)[d]	DNA polymerase	X	dNTP	v_0 (% min^{-1})	The ratio of the initial rate (f')[b]	Effect of the bridged group (e)[d]
Vent(exo-)	**K**	A	57	1[c]	0.42[d]	*KOD(exo-)*	**K**	A	55	1[c]	0.39[d]
	K	G	2.6	0.046[b]	0.45[d]		**K**	G	0.12	0.0021[b]	0.38[d]
	K	C	2.0	0.035[b]	0.53[d]		**K**	C	0.024	0.00044[b]	0.11[d]
	K	T	6.4	0.11[b]	0.86[d]		**K**	T	0.23	0.0042[b]	0.45[d]
	T	A	130	1[c]	–		**T**	A	140	1[c]	–
	T	G	5.8	0.045[b]	–		**T**	G	0.31	0.0022[b]	–
	T	C	3.7	0.028[b]	–		**T**	C	0.22	0.0016[b]	–
	T	T	7.3	0.057[b]	–		**T**	T	0.51	0.0036[b]	–

[a]Experimental conditions are described in Materials and methods section.
[b]f', the ratio of the initial rate; $f' = (v_0)_{wrong}/(v_0)_{correct}$, i.e. $(v_0)_{K,dGTP}/(v_0)_{K,dATP}$, $(v_0)_{K,dCTP}/(v_0)_{K,dATP}$, $(v_0)_{K,TTP}/(v_0)_{K,dATP}$, $(v_0)_{T,dGTP}/(v_0)_{T,dATP}$, $(v_0)_{T,dCTP}/(v_0)_{T,dATP}$ and $(v_0)_{T,TTP}/(v_0)_{T,dATP}$.
[c]These are correct incorporations; $(v_0)_{K,dATP}/(v_0)_{K,dATP}$ and $(v_0)_{T,dATP}/(v_0)_{T,dATP}$.
[d]e, effect of the bridged group on nucleotide incorporation; $e = (v_0)_{bridged}/(v_0)_{natural}$, i.e. $(v_0)_{K,dATP}/(v_0)_{T,dATP}$, $(v_0)_{K,dGTP}/(v_0)_{T,dGTP}$, $(v_0)_{K,dCTP}/(v_0)_{T,dCTP}$ and $(v_0)_{K,TTP}/(v_0)_{T,TTP}$.

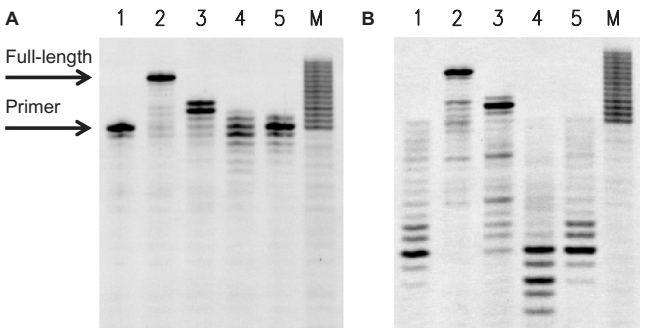

Figure 4. Successive incorporation of 2′,4′-bridged nucleotides using triphosphate analogs **KTP** (lane 3), **LTP** (lane 4) and **MTP** (lane 5). Except for the positive control (lane 2), the reaction mixtures did not contain natural TTP. The negative control does not contain any substrate triphosphates (lane 1). The thermostable DNA polymerases used were *KOD Dash* (**A**) and *Phusion HF* (**B**).

reaction conditions involving concentrations of enzyme or the triphosphate analogs, reaction times, addition of manganese chloride and use of a betaine enhancer solution were attempted, but obvious improvement was not observed.

CONCLUSION

Although the production of modified DNAs is limited by the substrate specificity of the DNA polymerases, there are many examples of the enzymatic preparation of modified DNAs by primer extension or polymerase chain reaction (PCR) using base-modified triphosphate analogs (52–68). Previously, we first showed that *KOD Dash* DNA polymerase is suitable for enzymatic production of modified DNA containing base-modified nucleotides (56). Using this DNA polymerase, we prepared a modified DNA library involving C5-modified thymidine and successfully screened modified DNA aptamers bound to sialyllactose, R-isomer of thalidomide derivative, and so on by SELEX (23–25). Recently, Inoue *et al.* (69) reported that double-stranded 4′-thioDNAs were directly amplified by PCR using *KOD Dash* and triphosphates of 4′-thionucleoside. Thus, *KOD Dash* DNA polymerase could accept a broad range of nucleotide modifications and might be best suited for enzymatic preparation of functional modified DNA.

The BNA templates containing sequences of seven successive 2′,4′-bridged nucleotides **Ks**, **Ls** and **Ms** could not be completely transcribed by any DNA polymerases used; yields of longer elongated products decreased in the order of steric bulkiness of the modified sugars. Successive incorporation of bridged nucleotides into extending strands using triphosphates **KTP**, **LTP** and **MTP** were much more difficult. These data indicate that the sugar modification would have a greater effect on the polymerase reaction, when it is adjacent to the elongation terminus than when it is on the template as well, as in base modification.

Polymerase reactions under extreme conditions and addition of manganese chloride would sometimes raise frequencies of misincorporation (70,71). Therefore, it is noteworthy that *KOD Dash* and *KOD(exo-)* DNA polymerases could smoothly read through the BNA templates containing **Ks** or $\mathbf{K_A s}$ at intervals of three nucleotides, two nucleotides and one nucleotide, respectively, and produce the corresponding complimentary natural DNA strand even under standard enzyme concentrations. Similarly, *Vent(exo-)* DNA polymerase also read through these BNA templates; however, kinetic study indicates that *KOD(exo-)* was found to be far superior to *Vent(exo-)* in accurate incorporation of nucleotides.

Although further research into other types of DNA polymerases, RNA polymerases and reverse transcriptases will be conducted, our current results suggest that applying BNA to the SELEX method may difficult but

not impossible; however, accurate transcription of natural DNA from templates containing 2′,4′-locked/bridged nucleotides at intervals of a few nucleotides by using *KOD Dash* and *KOD(exo-)* DNA polymerases would enable construction of non-SELEX selection systems to create aptamers with BNA/LNA.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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