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Model of Elongation of Short DNA Sequence by Thermophilic DNA Polymerase under Isothermal Conditions

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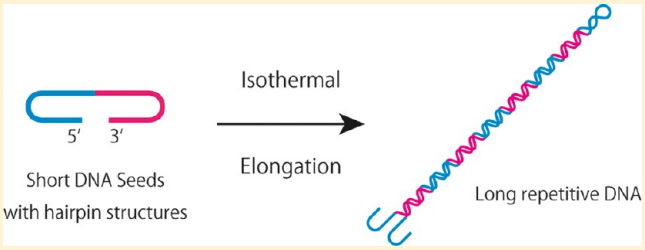
Supporting Information

ABSTRACT: Short DNA sequences, especially those that are repetitive or palindromic, can be used as the seeds for synthesis of long DNA by some DNA polymerases in an unusual manner. Although several elongation mechanisms have been proposed, there is no well-established model that explains highly efficient elongation under isothermal conditions. In the present study, we analyzed the elongation of nonrepetitive sequences with distinct hairpins at each end. These DNAs were elongated efficiently under isothermal conditions by thermophilic Vent (exo$^-$) DNA polymerase, and the products were longer than 10 kb within 10 min of the reaction. A 20-nucleotide DNA with only one hairpin was also elongated. Sequence analysis revealed that the long products are mainly tandem repeats of the short seed sequences. The thermal melting temperatures of the products were much higher than the reaction temperature, indicating that most DNAs form duplexes during the reaction. Accordingly, a terminal hairpin formation and self-priming extension model was proposed in detail, and the efficient elongation was explained. Formation of the hairpin at the 5′ end plays an important role during the elongation.



Repetitive DNA sequences are of interest because of their abundance, potential functions, contributions to molecular evolution, and relations with some genetic diseases.[1−5] In analyses that detect a special DNA sequence, unusual DNA amplification results in false positive detection.[6] In addition to PCR,[7,8] several isothermal DNA amplification methods have been developed (e.g., nucleic acid sequence-based amplification (NASBA),[9] rolling circle amplification (RCA),[10] strand displacement amplification (SDA),[11,12] loop-mediated isothermal amplification (LAMP),[13] and so on[14]). These methods are facile for practical applications because they do not require a thermal cycler. However, unexpected amplification occasionally occurs under isothermal conditions, especially if the region to be amplified contains repetitive sequences.[5,6] Thus, clarification of the mechanism for unusual DNA amplification is significant in both biology and biotechnology.

Several elongation mechanisms of repetitive sequences by DNA polymerase have been proposed, including slippage synthesis,[15−22] bulge migration,[21−24] hairpin−coil transition,[25,26] template switching,[27] and duplex elongation at melting equilibrium (DEME).[28,29] These models cannot explain the high efficiency of amplification of long repetitive sequences, however. For example, elongation efficiency should decrease after DNA becomes long for bulge migration and hairpin−coil transition models because only short lengths are extended for each elongation cycle. Strand switching and DEME models are energetically unfavorable and work only at high concentrations of a DNA substrate. Liang et al. proposed a hairpin elongation model to explain the efficient *de novo* DNA synthesis by the combination of a DNA polymerase and a restriction enzyme; however, no data were provided to prove it.[30] In the present study, we analyzed the highly efficient elongation of a single-stranded DNA with palindromic sequences at both ends. On the basis of our data, we propose and prove in detail the terminal hairpin formation and self-priming extension (THSP) model.

MATERIALS AND METHODS

Oligonucleotides and DNA Polymerases. All DNA oligonucleotides (ODNs) used in this study were purchased from Integrated DNA Technologies (sequences are shown in Figure 1 and Table S1 of the Supporting Information). ODNs were dissolved in sterile Milli-Q water to a concentration of 100 μM and frozen until use. Usually, the ODNs were diluted to 1.0 μM as the stock for elongation and to a final concentration of 100 nM. Vent (exo$^-$) DNA polymerase, Taq DNA polymerase, Deep Vent (exo$^-$) DNA polymerase, and 9°N$_m$ DNA polymerase were obtained from New England Biolabs. Phusion DNA polymerase was purchased from Finnzymes.

Elongation of ODNs. The standard reaction solution contained 100 nM ODN, dNTPs (0.5 mM each) (GE Healthcare), and 20 units/mL DNA polymerase in 100 μL of a 1× Thermopol buffer [20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, and 0.1% Triton X-100 (pH

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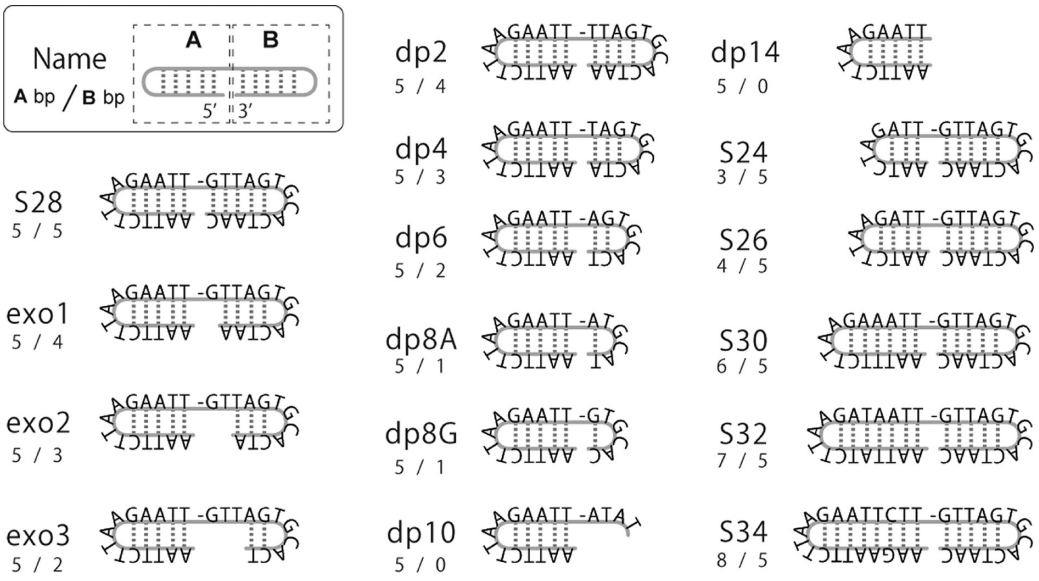

Figure 1. Design of DNA sequences used in this study. These ODNs have a hairpin at only the 5′ end or at both ends. The 5′ half of the ODN is termed part A and the 3′ half part B. The hairpins in **S28** have five base pairs. Forms of part A in **exo1**, **exo2**, and **exo3** and **dp2**, **dp4**, **dp6**, **dp8A**, **dp8G**, and **dp10** are identical to that of **S28** and are modified in part B. **dp14** is only the 5′ half of **S28**. **S24**, **S26**, **S30**, **S32**, and **S34** have the part B sequence of **S28** and vary in the 5′ region. The numbers of base pairs formed in each hairpin are indicated underneath the name of ODNs in the manner of part A/part B.

8.8)]. Elongation by Phusion DNA polymerase was carried out in 1× Phusion HF buffer supplied by Finnzymes (the contents are not declared). Prior to the start of the elongation reaction, the reaction mixture lacking only dNTPs was prepared and incubated at the reaction temperature for several minutes, and then the reaction was started with the addition of dNTPs (in a 25 mM stock solution). Elongation was performed on a Thermal Cycler Dice (Takara Bio) or a PCR-320 (Astec). To quench the reaction, aliquots of the reaction solution were mixed with an equal volume of the 2× loading dye or a 60-fold excess volume of 0.5× TAE buffer. In cases of low concentrations, the products were ethanol precipitated and analyzed on an agarose gel. In some cases, products were analyzed on a denaturing agarose gel (30 mM NaOH, 1.0 mM EDTA).

Quantitative Analysis of DNA Products. Elongated DNA products were analyzed by fluorescence measurements after staining with SYBR Green I (Lonza). An aliquot of the reaction mixture was diluted 60-fold with a 2× SYBR Green I solution in a 0.5× TAE buffer. A 100 μL aliquot of the sample was transferred into a well of a 96-well clear-bottom microplate, and fluorescence measurements were performed on an FLA-3000 instrument (Fujifilm). When the DNA concentration was too high to measure, the samples were diluted until the fluorescence intensity was in the proper range.

Molecular Cloning of the Elongated S28. Elongated **S28** was purified by a PCI (a 25:24:1 phenol:chloroform:isoamyl alcohol ratio, Invitrogen) and CIA (a 24:1 chloroform:isoamyl alcohol ratio, Fluka Chemicals) treatment and precipitated with ethanol (Wako). Small molecules were removed with a sephadex G-25 spin column (GE Healthcare). The purified DNA was fragmented by 0.08 unit/mL DNase I (New England Biolabs) at 15 °C for 30 min in the presence of 10 mM $MnCl_2$. The fragments, which have blunt ends, were cloned into the *Sma*I site of a pUC19 plasmid (New England Biolabs) by ligation with T4 DNA ligase (Invitrogen) at 16 °C for 20 h. After harvesting and treatment with a GenElute plasmid extraction kit (Invitrogen), sequencing reactions were performed on a Bio-Rad DNA Engine Dyad PTC-220 Peltier Thermal Cycler by using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit. Single-pass sequencing was performed on an ABI 3730xl sequencer (Applied Biosystems), according to the protocols supplied by the manufacturer.

T_m Measurements. After purification by PCI and CIA treatment, ethanol precipitation, and spin-column treatment, as described in the previous section, the elongated DNA product was dissolved in a 1× Thermopol Detergent-Free (DF) Buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$ (pH 8.8)]. The midpoint of the thermal melting transition (T_m) was determined by monitoring the change of absorbance at 260 nm with temperature on a UV−vis spectrometer (UV-1800) equipped with a temperature-controlling device TMSPC-8 (Shimadzu). T_m's of short seed DNAs used as elongation substrates were also measured under the same conditions.

Elongation of RnA and/or RnB. Elongation of **RnA** (5′-AATTCTTAAGAATTAATTCTTAAGAATTAGATTGAAC-TCTCGAACTAT-3′) and **RnB** (5′-AATTCTTAAGAATTA-ATTCTTAAGAATTATAGTTCGAGAGTTCAATCT-3′) was performed at 70 °C under the following conditions: 100 nM **RnA** and/or **RnB**, dNTPs (0.5 mM each), 20 units/mL Vent (exo$^-$) DNA polymerase in 1× Thermopol buffer. Samples were electrophoresed on 1.0% agarose gels and stained by SYBR Green I. The elongation products were purified by treatment with PCI, CIA, ethanol precipitation, and a sephadex G-25 spin column before digestion by Taq$^\alpha$I.

Restriction Digestion of Elongated Products. After purification, elongated products were digested by the restriction enzyme Tsp509I (New England Biolabs). The reaction was carried out at 65 °C overnight with 10 units of Tsp509I in 50 μL of 1× NEBuffer 1 [10 mM bis-Tris/propane/HCl (pH 7.0), 10 mM $MgCl_2$, 1.0 mM dithiothreitol]. For the elongation products of **RnA** and **RnB**, restriction enzyme Taq$^\alpha$I (New England Biolabs) was used. After purification, the elongation

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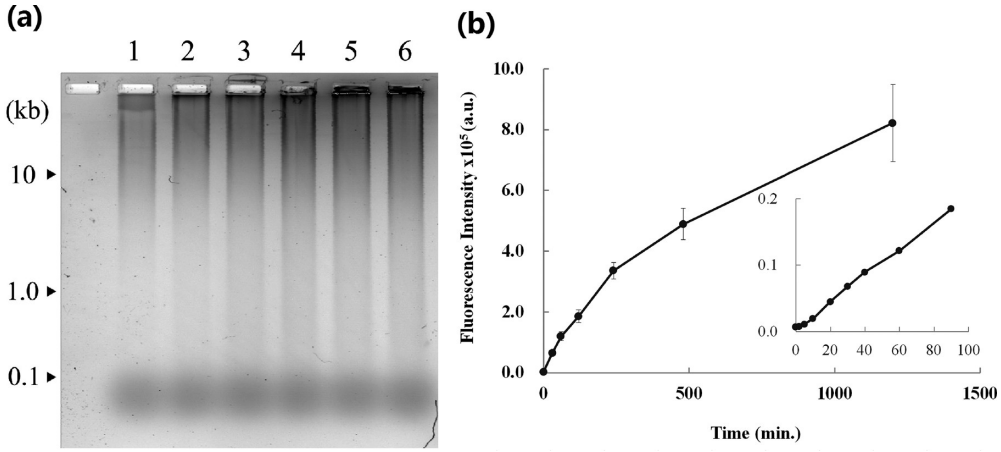

Figure 2. Time course of the elongation of **S28** by Vent (exo$^-$) DNA polymerase at 70 °C. (a) Image of the gel resolving elongation products after staining with SYBR Green I: lane 1, 10 min; lane 2, 30 min; lane 3, 60 min; lane 4, 90 min; lane 5, 120 min; lane 6, 150 min. (b) Quantitative analysis of the elongation of **S28**. Fluorescence intensity after staining with SYBR Green I was measured. The inset shows the result of the first 100 min.

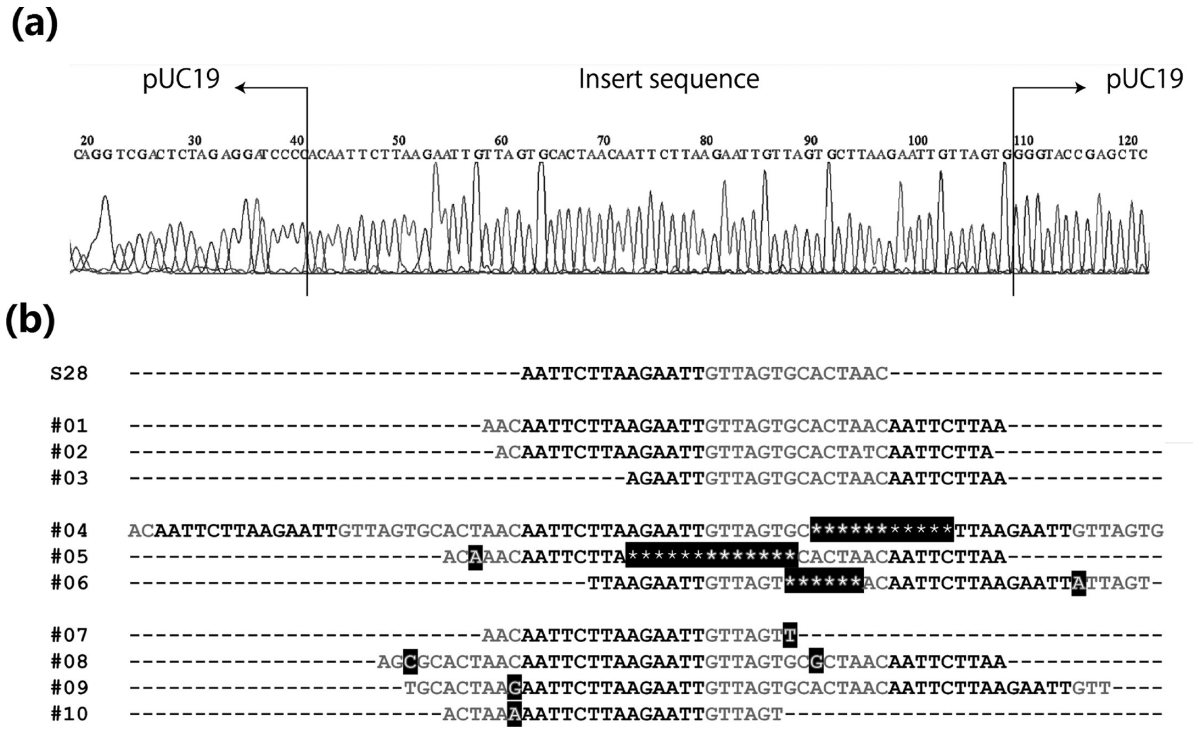

Figure 3. Results of the sequence analysis of elongated products from **S28**. (a) An example of the sequencing pattern (the result of clone 4 is shown). Elongated **S28** was purified and fragmented by DNase I in the presence of $MnCl_2$. The fragments were cloned into the *Sma*I site of the pUC19 plasmid. (b) Obtained sequences after elongation of **S28**. The asterisk indicates a deleted base, and highlights indicate mutation sites. Black letters show the sequence of the 5′ half, and the gray ones are that of the 3′ half of **S28**.

product was digested at 65 °C overnight by 20 units of Taq$^{\alpha}$I in 50 μL of 1× NEBuffer 4 [50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate (pH 7.9), 1.0 mM dithiothreitol].

RESULTS

Sequence Design of Short Seed Sequences for Elongation. Mispriming is often the cause of unusual elongation. Here, we concentrated on the self-priming of palindromic sequences due to hairpin formation. A palindromic sequence at the 3′ end can easily initiate the DNA synthesis by self-priming. For continuous DNA synthesis after the initiation, the 5′ end is also important. A palindromic sequence at the 5′ end can increase the probability of self-priming after the primer extension reaches the 5′ end because the newly synthesized 3′ end also contains the palindromic sequence. Accordingly, we designed a 28-nucleotide ODN (**S28**) with palindromic sequences at both the 5′ and 3′ ends for forming hairpin structures (Figure 1). At relatively high concentrations, the ODN can also form an intermolecular duplex (Figure S1 of the Supporting Information). To avoid unexpected mis-pairing during elongation, **S28** is AT rich (71% AT content). For the 14-nucleotide sequence at the 5′ end of **S28**, the AT content is 85.7%. To investigate in detail the effect of hairpin formation on elongation, a series of modified sequences based on **S28** were also used (Figure 1).

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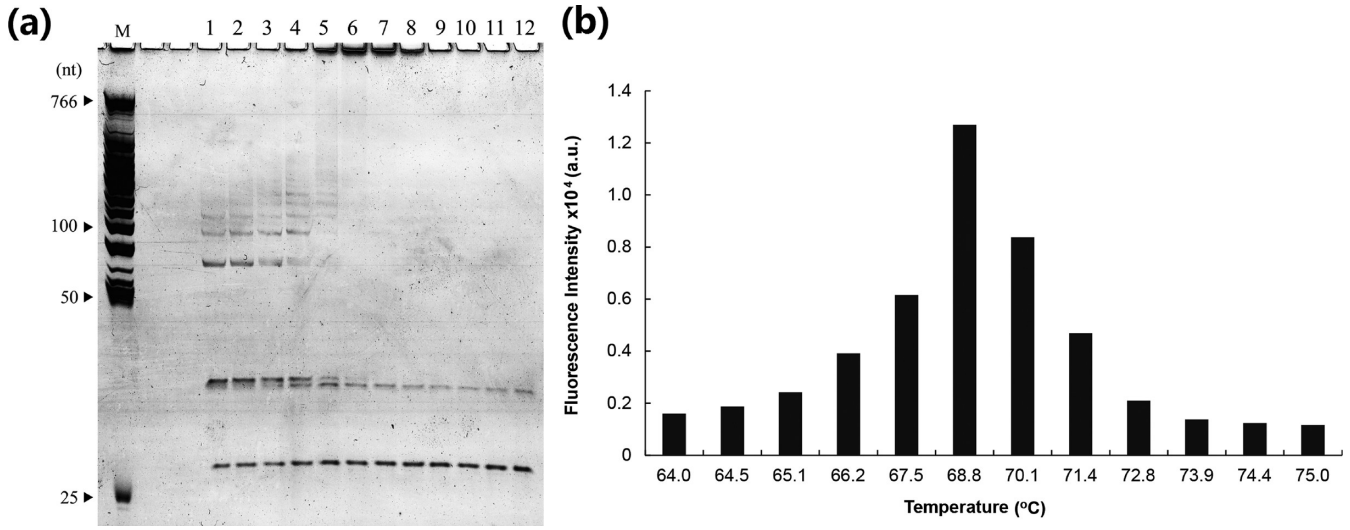

Figure 4. Temperature dependence of the elongation of **S28**. (a) Image of a gel stained by SYBR Green II separating products of elongation of **S28** at various temperatures: lane 1, 64.0 °C; lane 2, 64.5 °C; lane 3, 65.1 °C; lane 4, 66.2 °C; lane 5, 67.5 °C; lane 6, 68.8 °C; lane 7, 70.1 °C; lane 8, 71.4 °C; lane 9, 72.8 °C; lane 10, 73.9 °C; lane 11, 74.4 °C; lane 12, 75.0 °C. The elongation reaction was stopped at 30 min and analyzed on a 10% denaturing PAGE containing 8.0 M urea in a 1× TBE buffer. The bands with the highest mobility are assigned to be **S28**. The bands between 25 and 50 nucleotides are the 42-nucleotide intermediate of elongation. (b) Quantitative analysis of elongated products under various temperatures using fluorescence measurement stained by SYBR Green I. The fluorescence background in the absence of short oligos was not subtracted. The elongation reaction was performed for 60 min.

Elongation of S28 by Vent (exo⁻) DNA Polymerase and Characterization of the Elongated Products. The elongation reaction by Vent (exo⁻) DNA polymerase was first performed at 70 °C using 100 nM **S28** and 0.5 mM dNTPs. A higher concentration of dNTPs was used here for producing more elongation products, especially for a long reaction time. The elongation products were analyzed by a 1.0% agarose gel (Figure 2a). Smeared bands were observed close to the well of the gel, indicating that very long DNA was synthesized. The short fragment observed at the bottom of the gel was nonelongated **S28**. **S28** was elongated very efficiently, and products of longer than 10 kb were observed within only 10 min (Figure 2a, lane 1). Similar results were also obtained when a denaturing agarose gel (30 mM NaOH) was used (data not shown).

As shown by the time course in Figure 2b, elongation continued after 20 h. As intermolecular duplex formation may result in extension, we evaluated the concentration dependence of the elongation (Figure S2 of the Supporting Information). When 100 pM **S28** was used, DNA products longer than 10 kb were observed in 20 min. Even with 10 pM, much lower than the K_m (90 pM) of Vent (exo⁻) DNA polymerase,[31] **S28** was elongated efficiently, and the elongated products were detected after 1.0 h.

For sequencing, elongated products were purified, digested by DNase I, and cloned into the *Sma*I site of the pUC19 vector. To obtain blunt-ended fragments, Mn^{2+} ion was added into the DNase I digestion buffer. As shown in Figure 3, the sequences of elongated products were tandem repeats of **S28**, although several point mutations were observed. In clones 4, 5, and 6, 6–13-nucleotide deletions occurred, probably as a result of the ligation of the digested fragments before insertion into the plasmid. Point mutations may arise due to misincorporation by Vent (exo⁻) DNA polymerase, which lacks proofreading activity.

To determine whether longer products were elongated from **S28**, the elongated DNA was digested by the restriction enzyme Tsp509I with a recognition site of 5′-↓AATT-3′ (Figure S3 of the Supporting Information). All DNA products were digested efficiently, and no DNA longer than 50 nucleotides remained, indicating that all elongated products contained the 5′-AATT-3′ sequence at short intervals. The electrophoresis pattern of Tsp509I digestion products indicated that the elongation products consisted of repeats of the sequence of **S28**.

Temperature Dependence of Elongation of S28. As shown in Figure 4a, long DNA was only obtained during elongation of **S28** within a narrow temperature range (66–74 °C), indicating that the elongation efficiency was very sensitive to the reaction temperature. The optimal reaction temperature was 68.8 °C, and the amount of elongation product decreased abruptly when the temperature was outside the optimal range by only 1–2 °C (Figure 4b). Interestingly, at temperatures lower than 66.2 °C, distinct bands were observed, and all the elongation products were shorter than 500 bp. At temperatures higher than 74 °C, however, almost no elongation was observed (Figure 4). In addition to the bands due to **S28**, another band of approximately 42 nucleotides was observed at all temperatures (Figure 4a). This band is the product with elongation stopped after replication of the 5′ part of **S28**.

T_m Analysis of Elongated Seeds. The highly sensitive temperature dependence of elongation efficiency promoted us to hypothesize that the structure of the DNA template (i.e., duplex or single strand) during elongation may be very important. To verify this consideration, we determined the melting temperatures (T_m) of both short seeds and the elongated products. For these experiments, a Thermopol DF buffer was used as Triton X-100, a nonionic surfactant, caused turbidity at temperatures higher than 65 °C. Figure 5 shows an example of T_m curves of **S28** and the purified products obtained from elongation by Vent (exo⁻) DNA polymerase at 70 °C for 4.0 h. T_m's of **S28** and the product were 53.1 and 79.4 °C, respectively. As the elongation of **S28** was carried out at a temperature below 73 °C, the elongated products should be mostly in duplex states, suggesting that the mechanism

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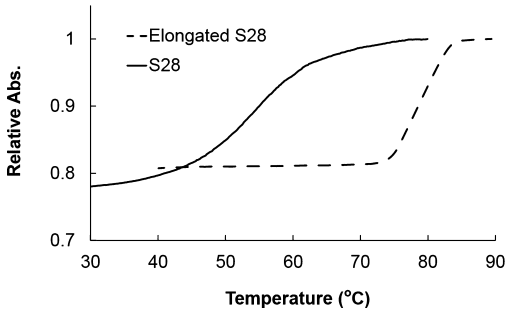

Figure 5. T_m curves of **S28** (solid line) and elongated products (dashed line). Samples for T_m measurements were prepared in a 1× Thermopol DF buffer. The T_m's for **S28** and the product were 53.1 and 79.4 °C, respectively.

proposed by Ogata does not explain the elongation we observe.[28] In the absence of DNA polymerases at the elongation reaction temperature (>64 °C), **S28** will not have a hairpin structure (shown in Figure 1) as the T_m is significantly lower than the reaction temperature. Thus, binding of DNA polymerase must promote and stabilize self-priming of **S28**.

Effect of the Hairpin Structure and Stability on Elongation. To investigate in detail the effect of the hairpin structure of the short seed template, 16 ODNs were used as elongation templates (see detailed structure in Figure 1). Sequences of these short seeds were designed based on **S28**. Since the self-priming at the 3′ end should be the key step in initiation of elongation, sequences able to form hairpins of various stabilities at the 3′ end were used. For example, **exo1**, **exo2**, and **exo3** lack 1−3 bases relative to the 3′ end of **S28**; **dp2**, **dp4**, **dp6**, **dp8A**, **dp8G**, **dp10**, and **dp14** are sequences lacking 1−6 base pairs (Figure 1). **dp8A** has the potential to form only a single A-T pair, and **dp8G** can form one G-C pair at the 3′ end. For **S24**, **S26**, **S30**, **S32**, and **S34**, the length of the 5′ hairpin was varied. As shown in Figure 6a, all short seeds except for **dp8A**, **dp10**, and **dp14** were elongated at 70 °C within 1.0 h, and long smeared bands were observed. The T_m's of **S24**, **S26**, **S30**, **S32**, and **S34** and their elongated products were measured (Table 1). As expected for all of these sequences, T_m's of short seeds were lower than and T_m's of the elongated products were higher than 70 °C (the reaction temperature).

As shown in Figure 6b, the elongation efficiency depended on the number of base pairs in the hairpin at the 3′ end of the short seed template. For **exo1**, **exo2**, and **exo3**, elongation efficiency decreased with the number of deleted bases. Similarly, elimination of base pairs caused a significant decline of elongation efficiency for the series **dp2**, **dp4**, **dp6**, **dp8A**, and **dp8G**. For **dp10** and **dp14**, no elongation was observed, even after 20 h (data not shown), demonstrating that self-priming at the 3′ end is necessary for initiating the elongation. Interestingly, even for **dp8G**, in which only a 6-nucleotide sequence (5′-GTGCAC-3′) was left at the 3′ end, a very long DNA product was obtained in 1.0 h (lane 12, Figure 6a). Even at a low concentration of 1.0 nM, relatively efficient elongation of **dp8G** was also observed (data not shown). More interestingly, for **dp8A** which also has a 6-nucleotide sequence (5′-ATGCAT-3′) at the 3′ end but lower GC content, elongation was also observed, although the efficiency was very low (lane 11, Figure 6a). Hairpin length in the 5′ half region also affected the elongation efficiency. Reactions from the longer sequences such as **S30**, **S32**, and **S34** yielded

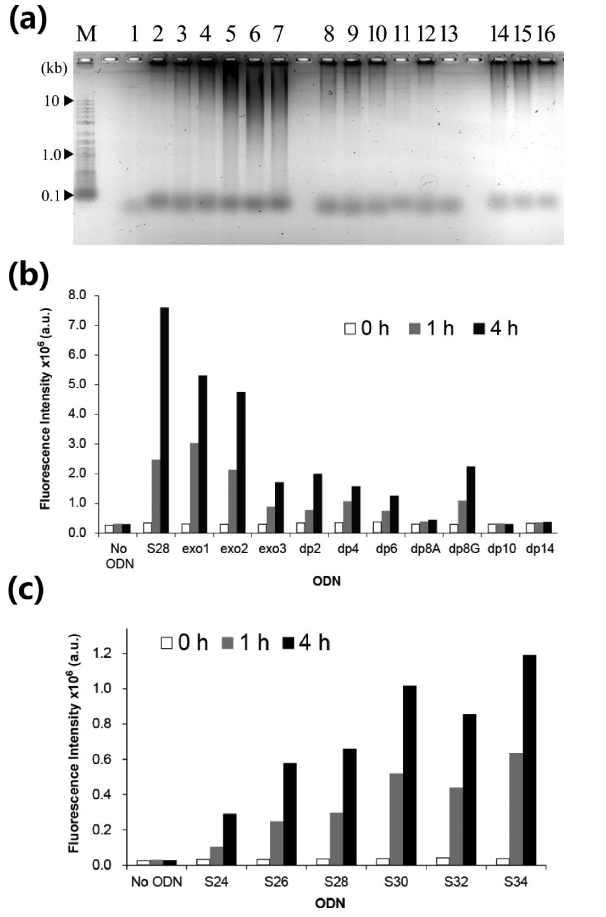

Figure 6. Elongation of short seeds with various sequences. (a) Separation of elongation products on a 1.0% agarose gel stained by SYBR Green I. The elongation was carried out at 70 °C for 1.0 h: lane 1, **dp14**; lane 2, **S24**; lane 3, **S26**; lane 4, **S28**; lane 5, **S30**; lane 6, **S32**; lane 7, **S34**; lane 8, **dp2**; lane 9, **dp4**; lane 10, **dp6**; lane 11, **dp8A**; lane 12, **dp8G**; lane 13, **dp10**; lane 14, **exo1**; lane 15, **exo2**; lane 16, **exo3**. (b) Quantitative analysis of products elongated from **S28** derivatives of the **exo** and **dp** series (stained with SYBR Green I). (c) Quantitative analysis of products elongated from **S28** derivatives of the **S** series of short seeds.

Table 1. T_m Values and GC% of Short Seeds and Their Elongated Products

sequence	GC content (%)	T_m of short seeds (°C)	T_m of elongated product (°C)
S24	33.3	53.1	80.4
S26	30.8	52.4	80.1
S28	28.6	53.1	79.4
S30	26.7	53.1	79.2
S32	25.0	54.8	77.8
S34	31.3	57.9	80.0

amounts of elongated products (Figure 6c) greater than that of **S28**. By contrast, **S26** and **S24**, which have fewer base pairs than **S28** at the 5′ end, were elongated less efficiently. Reaction with **S24**, which has a 10-nucleotide palindromic sequence, provided significantly less product than that with **S28**. As a control, the sequence without the 5′ half region (**S14**: 5′-GTTAGTGCACTAAC-3′) was also tested; no elongated product was observed within 1.0 h (Figure S4 of the Supporting Information). Furthermore, the sequence with 14 thymines at

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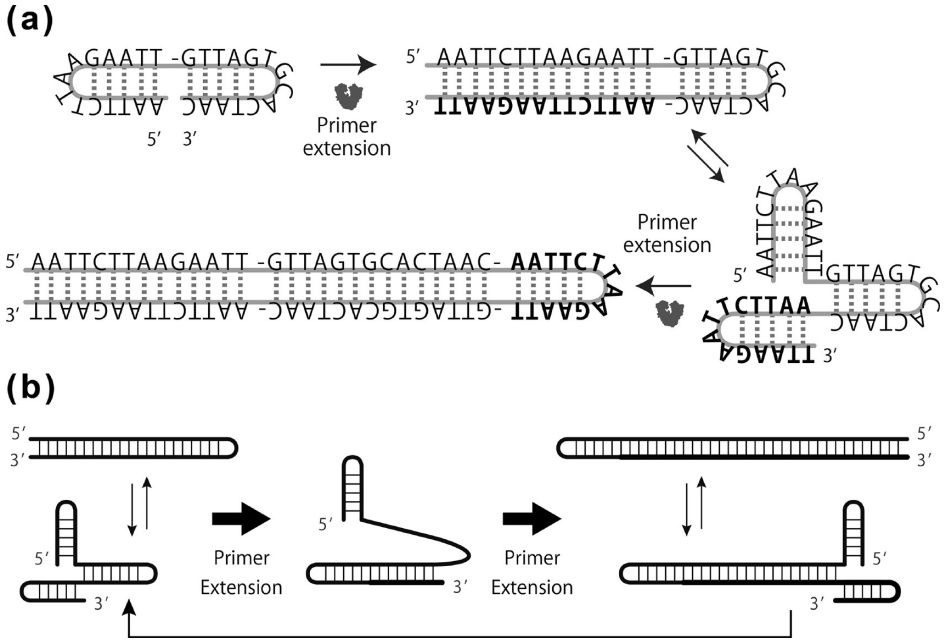

Figure 7. Proposed THSP model for efficient elongation of short seeds. (a) Self-priming and elongation of **S28** at the initiation step. Once elongation starts, conserved palindromic sequences at the 5′ end enable efficient elongation. (b) Successive elongation according to the THSP model. A hairpin structure forms at the 3′ terminus, and primer extension occurs. The duplex stem of the hairpin opens during primer extension due to the strand displacement activity of the DNA polymerase. When the primer extension reaches the 5′ end, a longer hairpin structure is formed, and a new elongation cycle starts.

the 5′ end that cannot form a hairpin (**T-S14** in Figure S4 of the Supporting Information) was not elongated.

■ DISCUSSION

Short 20−30 nucleotide DNA sequences that can form hairpins at either end due to the palindromic nature of the sequence were efficiently elongated by DNA polymerases. In some cases, elongation products longer than 10 kb were observed within only 10 min. The elongation efficiency depended on the stability of the hairpin at both ends of the short seed template. During elongation, DNA is mostly in a duplex state, as shown by our analysis of melting temperatures of short seeds and products. This suggests that the elongation is not due to a simple back-primed primer extension caused by slippage. Other previously reported elongation mechanisms do not explain well why long DNA products are obtained so quickly. Here, we used a new model, which we call terminal hairpin formation and self-priming extension (THSP), to explain this unusual elongation (Figure 7).

As shown in Figure 7a, once the hairpin at the 3′ end forms, primer extension to the 5′ end of the short seed template will first give a 42-nucleotide product that can form a longer hairpin structure. This 42-nucleotide product is clearly observed in Figure 4a. Self-priming from this product occurs when hairpins form at each end; each of these hairpins has the same sequence as the 5′ half of **S28** (Figure 7a). The hairpin at the 3′ end is similar to a usual template/primer structure that can be elongated by a primer extension. After extension to a 70-nucleotide sequence, a larger hairpin can form. Obviously, this self-priming and elongation can be carried out continuously with the length of ODN almost doubled after each cycle (Figure 7b). The above process, including dissociation of some base pairs, self-priming, and primer extension, enables successive efficient elongation under isothermal conditions. It is noteworthy that the DNA forms the duplex, and only the termini melt transiently in this model. According to the THSP model, the hairpin formation at the 3′ end plays the role of the initiator of elongation, and the hairpin at the 5′ end allows successive elongation. Obviously, even mispairing at the 3′ end can initiate elongation. Once the initiation starts, the sequence at the 5′ end will dominate the elongation.

Strand displacement activity of DNA polymerase is likely required to dissociate the hybridized nontemplate regions prior to elongation (Figure 7). To confirm this, we examined the elongation by other thermophilic DNA polymerases, Deep Vent (exo$^-$), 9°N$_m$, Taq, and Phusion. Like Vent (exo$^-$), Deep Vent (exo$^-$) and 9°N$_m$ have strand displacement activity, but the other polymerases do not. As expected, DNA polymerases with strand displacement activity efficiently elongated **S28**, but no elongation was observed in the presence of DNA polymerases Taq and Phusion, which do not have strand displacement activity (Figure S5 of the Supporting Information). The requirement for strand displacement activity strongly supports the THSP model.

Our other results can also be explained by the THSP model. The intramolecular self-priming and primer extension enable elongation at a low DNA concentration (Figure S2 of the Supporting Information). The elongated products can be digested completely by Tsp509I to give very clear bands of 28 and 18 nucleotides (Figure S3 of the Supporting Information), indicating that the elongated products consist of the repeated sequence of **S28** (Figure 3). Efficient elongation occurred in the temperature range of 66−77 °C; **S28** is in a single-stranded state in this range, whereas the elongated products are in a duplex state. As the T_m of **S28** is 53.1 °C, the hairpin with the sequence of 5′-AATTCTTAAGAATT-3′ does not form at the reaction temperature in the buffer. Binding of the DNA polymerase likely promotes hairpin formation and allows self-priming. At lower temperatures, the duplex is stable, and we observed that elongation did not occur after the initial primer

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extension. The end of a long DNA duplex can partly dissociate by breathing even at a temperature lower than its T_m.[32] Smolina et al. reported that a 13-nucleotide PNA could even invade at 37 °C to the end of a DNA duplex longer than 100 bp.[33] At higher temperatures, the hairpin does not form, even in the presence of polymerase, and no primer extension was detected.

The sequences we used here are different from previously reported sequences that result in unusual products; those sequences are usually simple repetitive sequences.[15−21] Obviously, the mechanisms previously proposed cannot explain the unusual elongation of short single-stranded ODNs we used here. For the in vitro elongation of the short duplex with the repetitive sequence, slippage may be the main model.[15−22] In our case, however, slippage cannot happen because the strands would have to move 28 nucleotides to find another point for primer extension, and the activation energy is too high. Similarly, bulge migration[21−24] is unlikely to occur through the long repeats of **S28**. The previously proposed hairpin-coil transition model[25,26] is close to our model; however, hairpin formation occurs only at the initiation of priming in that model. The template-switching model[27] requires high concentrations of DNA. The DEME model[28] can explain the elongation only at a temperature close to the T_m of the elongated product. In contrast, our proposed mechanism explains the elongation of single-stranded simple repetitive sequence to some extent.

According to the THSP mechanism, palindromic sequences at the 5′ and 3′ ends of the template are important for successive elongation. To verify this, we used two sequences, **RnA** and **RnB**, with a palindromic sequence only at the 5′ end (Table 1). At their 3′ ends, **RnA** and **RnB** have two 20-nucleotide random (nonpalindromic) sequences that are complementary to each other. For sequence analysis, the recognition site of Taq$^{\alpha}$I (5′-T↓GCA-3′) was also present. During elongation, the 3′ ends of **RnA** and **RnB** hybridize and allow primer extension. To simulate the 70-nucleotide intermediate as shown in Figure 4a, two repeats of the 5′ half of **S28** were designed. When both **RnA** and **RnB** were added, efficient elongation was observed in 30 min (lanes 7−9, Figure 8a). If only **RnA** was added, elongation was barely detected. A slight amount of elongation of **RnB** was also observed in the absence of **RnA**, probably due to the mispriming from the 3′ end of **RnB** (lanes 4−6, Figure 8a). To analyze the sequence of the elongation product from **RnA**/**RnB**, digestion by the restriction enzyme, Taq$^{\alpha}$I (5′-T↓GCA-3′), was carried out. As shown in Figure 8b, the elongated product was digested efficiently, indicating that it has the repetitive sequence of **RnA**/**RnB**. The fragment length deduced from bands on the gel also coincided well with the possible fragments when the elongation was carried out according to our THSP model.

CONCLUSIONS

Short single-stranded DNA with sequences able to form hairpin structures at each end were elongated efficiently by a thermophilic DNA polymerase under isothermal conditions. Even an ODN with a palindromic sequence only at the 5′ end could be elongated once priming from the 3′ end occurred. Our model, THSP, can explain well the results presented here. The high efficiency under isothermal conditions is due to hairpin formation that allows self-priming and primer extension. THSP may also explain in vitro elongation of a simple repetitive sequence, as long as a short hairpin structure can form at the 5′ end of the DNA strand after the initiation step. This model may clarify the molecular evolution of nucleic acids and explain the observation of nonspecific amplification observed during PCRs. Development of a novel isothermal DNA-amplification method based on this model is underway.

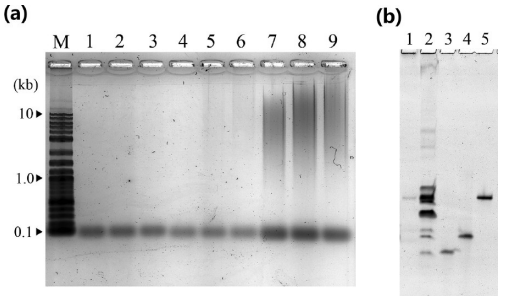

Figure 8. Elongation of DNA sequences (**RnA** and **RnB**) with palindromic sequences at the 5′ ends and mixed sequences at the 3′ ends. (a) Image of gel separation of elongation products. Samples were electrophoresed on 1.0% agarose gel and stained by SYBR Green I: lane 1, **RnA** only (0.5 h); lane 2, **RnA** only (1.0 h); lane 3, **RnA** only (2.0 h); lane 4, **RnB** only (0.5 h); lane 5, **RnB** only (1.0 h); lane 6, **RnB** only (2.0 h); lane 7, **RnA** and **RnB** (0.5 h); lane 8, **RnA** and **RnB** (1.0 h); lane 9, **RnA** and **RnB** (2.0 h). The reaction was performed at 67.0 °C. (b) Digestion of elongated product from **RnA**/**RnB** by restriction enzyme Taq$^{\alpha}$I. Samples were analyzed on a 10% denatured PAGE gel containing 8.0 M urea in a 0.5× TBE buffer. The gel was stained by SYBR Green II: lane 1, after elongation (purified); lane 2, after digestion; lane 3, 28-nucleotide marker; lane 4, 34-nucleotide marker; lane 5, 50-nucleotide marker.

ASSOCIATED CONTENT

Supporting Information

A list of the sequences used in this study is shown in Table S1. Supporting experimental results are shown in Figures S1−S5: structure of self-dimer (Figure S1), elongation of S28 at a lower concentration (Figure S2), assignment of the fragments of elongated products (Figure S3), controls for short seed experiments (Figure S4), elongation of **S28** by other thermophilic DNA polymerases (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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T.K. performed all experiments and wrote the manuscript.

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H.A. discussed the results, gave suggestions, and reviewed the manuscript.

The manuscript was written via the contributions of all authors. All authors have given approval for the final version of this manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

DEME, duplex elongation at melting equilibrium; ODN, oligodeoxyribonucleotide; THSP, terminal hairpin formation and self-priming extension; T_m, melting temperature.

■ REFERENCES

(1) Hastings, P. J., Lupski, J. R., Rosenberg, S. M., and Ira, G. (2009) Mechanisms of change in gene copy number. *Nat. Rev. Genet. 10*, 551−564.

(2) Treangen, T. J., and Salzberg, S. L. (2012) Repetitive DNA and next-generation sequencing: Computational challenges and solutions. *Nat. Rev. Genet. 13*, 36−46.

(3) Jurka, J., Kapitonov, V. V., Kohany, O., and Jurka, M. V. (2007) Repetitive sequences in complex genomes: Structure and evolution. *Annu. Rev. Genomics Hum. Genet. 8*, 241−259.

(4) Lovett, S. T. (2004) Encoded errors: Mutations and rearrangements mediated by misalignment at repetitive DNA sequences. *Mol. Microbiol. 52*, 1243−1253.

(5) Dere, R., Napierala, M., Ranum, L. P. W., and Wells, R. D. (2004) Hairpin structure-forming propensity of the (CCTG·CAGG) tetranucleotide repeats contributes to the genetic instability associated with myotonic dystrophy type 2. *J. Biol. Chem. 279*, 41715−41726.

(6) Borst, A., Box, A. T. A., and Fluit, A. C. (2004) False-positive results and contamination in nucleic acid amplification assays: Suggestions for a prevent and destroy strategy. *Eur. J. Clin. Microbiol. Infect. Dis. 23*, 289−299.

(7) Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science 230*, 1350−1354.

(8) Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science 239*, 487−491.

(9) Compton, J. (1991) Nucleic acid sequence-based amplification. *Nature 350*, 91−92.

(10) Fire, A., and Xu, S. Q. (1995) Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci. U.S.A. 92*, 4641−4645.

(11) Walker, G. T., Fraiser, M. S., Schram, J. L., Little, M. C., Nadeau, J. G., and Malinowski, D. P. (1992) Strand displacement amplification: An isothermal, in vitro DNA amplification technique. *Nucleic Acids Res. 20*, 1691−1696.

(12) Walker, G. T., Little, M. C., Nadeau, J. G., and Shank, D. D. (1992) Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proc. Natl. Acad. Sci. U.S.A. 89*, 392−396.

(13) Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res. 28*, e63.

(14) Van Ness, J., Van Ness, L. K., and Galas, D. J. (2003) Isothermal reactions for the amplification of oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A. 100*, 4504−4509.

(15) Kornberg, A., Bertsch, L. R. L., Jackson, J. F., and Khorana, H. G. (1964) Enzymatic synthesis of deoxyribonucleic acid, XVI. Oligonucleotides as templates and the mechanism of their replication. *Proc. Natl. Acad. Sci. U.S.A. 51*, 315−323.

(16) Wells, R. D., Ohtsuka, E., and Khorana, H. G. (1965) Studies on polynucleotides L. synthetic deoxyribopolynucleotides as templates for the DNA polymerase of *Escherichia coli*: A new double-stranded DNA-like polymer containing repeating dinucleotide sequences. *J. Mol. Biol. 14*, 221−237.

(17) Wells, R. D., Büchi, H., Kössel, H., Ohtsuka, E., and Khorana, H. G. (1967) Studies on polynucleotides LXX. Synthetic deoxyribopolynucleotides as templates for the DNA polymerase of *Escherichia coli*: DNA-like polymers containing repeating tetranucleotide sequences. *J. Mol. Biol. 27*, 265−272.

(18) Wells, R. D., Jacob, T. M., Narang, S. A., and Khorana, H. G. (1967) Studies on polynucleotides LXIX. Synthetic deoxyribopolynucleotides as templates for the DNA polymerase of *Escherichia coli*: DNA-like polymers containing repeating trinucleotide sequences. *J. Mol. Biol. 27*, 237−263.

(19) Kotlyar, A. B., Borovok, N., Molotsky, T., Fadeev, L., and Gozin, M. (2005) In vitro synthesis of uniform poly(dG)−poly(dC) by Klenow exo$^-$ fragment of polymerase I. *Nucleic Acids Res. 33*, 525−535.

(20) Hartig, J. S., and Kool, E. T. (2005) Efficient isothermal expansion of human telomeric and minisatellite repeats by *Thermococcus litoralis* DNA polymerase. *Nucleic Acids Res. 33*, 4922−4927.

(21) Kurihara, H., and Nagamune, T. (2004) DNA polymerase-catalyzed elongation of repetitive hexanucleotide sequences: Application to creation of repetitive DNA libraries. *Biotechnol. Prog. 20*, 1855−1860.

(22) Lyons-Darden, T., and Topal, M. D. (1999) Effects of temperature, Mg^{2+} concentration and mismatches on triplet-repeat expansion during DNA replication in vitro. *Nucleic Acids Res. 27*, 2235−2240.

(23) Ogata, N., and Miura, T. (2000) Elongation of tandem repetitive DNA by the DNA polymerase of the hyperthermophilic archaeon *Thermococcus litoralis* at a hairpin-coil transitional state: A model of amplification of a primordial simple DNA sequence. *Biochemistry 39*, 13993−14001.

(24) Heidenfelder, B. L., Makhov, A. M., and Topal, M. D. (2003) Hairpin formation in Friedreich's ataxia triplet repeat expansion. *J. Biol. Chem. 278*, 2425−2431.

(25) Ogata, N., and Miura, T. (1998) Creation of genetic information by DNA polymerase of the archaeon *Thermococcus litoralis*: Influences of temperature and ionic strength. *Nucleic Acids Res. 26*, 4652−4656.

(26) Ogata, N., and Morino, H. (2000) Elongation of repetitive DNA by DNA polymerase from a hyperthemophilic bacterium *Thermus thermophilus*. *Nucleic Acids Res. 28*, 3999−4004.

(27) Tuntiwechapikul, W., and Salazar, M. (2002) Mechanism of in vitro expansion of long DNA repeats: Effect of temperature, repeat length, repeat sequence, and DNA polymerases. *Biochemistry 41*, 854−860.

(28) Ogata, N. (2007) Elongation of palindromic repetitive DNA by DNA polymerase from hyperthermophilic archaea: A mechanism of DNA elongation and diversification. *Biochimie 89*, 702−712.

(29) Miura, T. (2003) Origin of genomic DNA: Discussion from reverse-transcription and expression of repetitive oligonucleotides. *Viva Origino 31*, 46−61.

(30) Liang, X. G., Jensen, K., and Frank-Kamenetskii, M. D. (2004) Very efficient template/primer-independent DNA synthesis by thermophilic DNA polymerase in the presence of a thermophilic restriction endonuclease. *Biochemistry 43*, 13459−13466.

(31) Kong, H., Kucera, R. B., and Jack, W. E. (1993) Characterization of a DNA polymerase from the hyperthermophile archaea *Thermococcus litoralis*. *J. Biol. Chem. 268*, 1965−1975.

(32) Frank-Kamenetskii, M. D. (1987) DNA chemistry: How the double helix breathes. *Nature 328*, 17−18.

(33) Smolina, I. V., Demidov, V. V., Soldatenkov, V. A., Chasovskikh, S. G., and Frank-Kamenetskii, M. D. (2005) End invasion of peptide nucleic acids (PNAs) with mixed-base composition into linear DNA duplexes. *Nucleic Acids Res. 33*, e146.