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

Norio Ogata* and Takanori Miura

Taiko Pharmaceutical Company, Ltd., Suita, Osaka, Japan

Received June 9, 2000; Revised Manuscript Received September 7, 2000

ABSTRACT: DNA is replicated by DNA polymerase semiconservatively in many organisms. Accordingly, the replicated DNA does not become larger than the original DNA (template DNA), implying that replicative synthesis by DNA polymerase alone cannot explain the diversification of primordial simple DNA. We demonstrate that a single-stranded tandem repetitive oligodeoxyribonucleic acid (oligoDNA) composed of a palindromic or quasi-palindromic motif sequence and 25−50% GC content is elongated in vitro to more than 20 000 bases at 70−74 °C by the DNA polymerase of the hyperthermophilic archaeon *Thermococcus litoralis* without a bimolecular primer−template complex. The efficiency of elongation decreased when the palindromic structure of the oligoDNA was destroyed or when the GC content of the oligoDNA was outside the range of 25−50%. The thermal melting transition profile of the oligoDNA, as observed by ultraviolet spectroscopy, exhibited a biphasic curve, reflecting a duplex−hairpin transition at 31−40 °C and a hairpin−coil transition at 70−77 °C. The optimal reaction temperature for the elongation, for instance, of oligoDNA $(AGATATCT)_6$ (72 °C) was very close to its hairpin−coil transition melting temperature (70.4 °C), but was markedly higher than the temperature at which duplex oligoDNA can exist stably (<35.9 °C). These results suggest that a hairpin-based "intramolecular primer−template structure" is formed transiently in the oligoDNA, and it is elongated by the DNA polymerase to long DNA through repeated cycles of folding and melting of the hairpin structure. We discuss the implication of this phenomenon, "hairpin elongation", from the standpoint of potential amplification of simple DNA sequences during the evolution of the genome.

DNA polymerase (pol)[1] plays a central role in the replication of cellular DNA during cell division, base excision repair, and recombination and is therefore essential for maintaining the integrity of organisms (*1*). The enzyme catalyzes orderly addition of complementary nucleotide bases to the 3′-end of primer DNA along template DNA, forming a phosphodiester bond between a 3′-end OH moiety of the primer and an incoming α-phosphate moiety of the nucleotide. Finally, a complete copy of the original DNA sequence (template) is synthesized (*1*). According to this reaction, the DNA strand elongated from the primer DNA does not become larger than the template DNA. The genome of a present organism is composed of huge complex DNA that carries copious amounts of genetic information. It is reported that oligonucleotide can be synthesized nonenzymatically from modified monoribonucleotides (ribonucleoside 5′-phosphorimidazolides) in the reaction catalyzed by divalent metal ions (*2*, *3*) and is self-replicated using modified monoribonucleotides (*4*, *5*) or modified oligoribonucleotides (*6*, *7*). Although such reactions would be meaningful from the standpoint of early evolution of genetic material in that they do not require the presence of life or life-related structures, the synthesis of a large polymer of nucleotides has not been demonstrated in these reactions. In the primordial world, what was in short supply must have been the long template of a nucleotide chain to copy. How did long DNA appear on the early earth? Up to now, nothing has accounted for the ab initio synthesis of long DNA on the early earth.

In the genome of many organisms, there often exists a characteristic short stretch of DNA, e.g., microsatellite DNA, direct repeats, dA/dT tracts, and palindromes (self-complementary DNA or inverted repeats). It has been known that some of these DNAs can form unusual structures (*8*). For instance, palindromic DNA sequences especially have the potential to form an intramolecular hairpin structure, as confirmed by ultraviolet spectroscopy (*9*−*18*), differential scanning calorimetry (*10*, *11*), temperature-dependent circular dichroism spectroscopy (*11*, *19*), multiple fluorescence spectroscopy (*20*), and nuclear magnetic resonance spectroscopy (*13*, *21*, *22*). There are also cases where the palindromic sequences are repeated in tandem (*23*−*25*); such sequences exist frequently in genome and have been found near promoters (*25*, *26*), receptor binding sites (*27*, *28*), and

* To whom correspondence should be addressed: Division of Research and Development, Taiko Pharmaceutical Co., Ltd., 3-34-14 Uchihonmachi, Suita, Osaka 564-0032, Japan. Telephone: 81-6-6382-3100. Fax: 81-6-6382-1152. E-mail: ogata145@pearl.ocn.ne.jp.

[1] Abbreviations: pol, DNA polymerase; dNTP, deoxyribonucleoside 5′-triphosphate; dNMP, deoxyribonucleoside 5′-monophosphate; bp, base pair; kbp, kilobase pairs; *Tli*, *Thermococcus litoralis*; oligoDNA, oligodeoxyribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; β-gal, β-galactosidase.

10.1021/bi0013243 CCC: $19.00
Published on Web 10/13/2000

replication origins (*29*, *30*). Thus, it is likely that the palindromic sequences are or have been important and indispensable for the regulation of cellular metabolism. It is also likely that the repetitive palindromic sequences existed in the genome of primordial organisms and have played important roles, or they once existed as primordial genetic materials that were later used as sources for diversification of the genome of modern organisms.

We have recently discovered that pols of the hyperthermophilic archaeon *Thermococcus litoralis* (*Tli*) (*31*) and the hyperthermophilic eubacterium *Thermus thermophilus* (*32*) can synthesize large DNA of more than 20 kilobase pairs (kbp) without a set of primer and template DNAs, elements absolutely required for common replicative DNA synthesis by pol (*1*), under an isothermal condition of 74 °C (*33*, *34*), indicating that pols potentially have the ability to synthesize DNA without preexisting genetic information (ab initio DNA synthesis). The DNA synthesized in this fashion is composed primarily of tandem repetitive palindromic or quasi-palindromic sequences having four nucleotide bases (A, T, G, and C). For instance, long DNA having sequences of $(AGATATCT)_n$, $(TACATGTA)_n$, and $(TAGTTATAACTA)_n$ was synthesized ab initio (*33*−*35*). We also found that the GC content (mole percent of the sum of G and C in DNA) of the product DNA increased almost linearly when the reaction temperature increased (*35*). Interestingly, similar sequences are found in centromeric satellite DNA and noncoding regions of many natural genes (*33*−*35*).

On the basis of these findings, we thought that oligodeoxyribonucleic acid (oligoDNA), having the sequence described above or a similar sequence, might be elongated to long DNA when added as a "seed" to a mixture of a DNA synthesis reaction by *Tli* pol, despite the absence of a complex of primer and template DNAs. In this paper, we show a novel enzymatic reaction in which various tandem repetitive palindromic or quasi-palindromic oligoDNAs added to a reaction mixture for DNA synthesis are elongated by *Tli* pol to very long DNA at high temperatures. The important factors required in this reaction are high temperature, the DNA structure, and the GC content of the added oligoDNA. On the basis of our results, we propose a model "hairpin elongation" for the elongation of short DNA. We also discuss the implication of this novel reaction in terms of potential diversification of primordial genetic material.

MATERIALS AND METHODS

Materials. The 5′-phosphorylated oligoDNAs and deoxyribonucleoside triphosphates (dNTPs) were obtained from Amersham Pharmacia Biotech. (DNA sequences are always shown from 5′ to 3′.) A 48-base fragment of an *Escherichia coli* β-galactosidase (β-gal) gene used as a control represents nucleotides 1705−1752 of its genomic sequence (GenBank accession number J01636). Recombinant *Tli* pol and Δ*Tli* pol, a mutant *Tli* pol in which the 3′−5′ exonuclease activity (proofreading activity) is lacking due to replacements of Asp^{141} and Glu^{143} with alanine (*36*), were obtained from New England Biolabs. T4 polynucleotide kinase and deoxyribonuclease I were obtained from Roche Diagnostics, and restriction endonucleases were from Toyobo.

DNA Synthesis Reaction. A mixture of a standard DNA synthesis reaction contained 0.8 unit of either *Tli* pol (38 ng) or Δ*Tli* pol (24 ng) and 4 nmol each of α-^{32}P-labeled (final specific activity of 110 μCi/μmol or 4.0 MBq/μmol) or nonlabeled dNTPs (dATP, dTTP, dGTP, and dCTP) in 20 μL of buffer A, which contained 20 mM Tris-HCl buffer (pH 8.8, at 25 °C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, and 6 mM $MgSO_4$. In many experiments, the mixture also contained 2 ng of oligoDNA, unless stated otherwise. The most frequently used oligoDNA, $(AGATATCT)_6$, is hereafter named "standard oligoDNA". Unless otherwise specified, the reaction mixture was incubated at 74 °C for 20 min, and the reaction was terminated by the addition of 1 μL of 500 mM ethylenediaminetetraacetic acid (pH 8.0). Its 5% trichloroacetic acid-insoluble material was next collected by filtration on a glass microfiber filter (Whatman), and the amount of radioactivity was measured using a liquid scintillation counter (*37*). The experimental error of this assay, in terms of the coefficient of variation, was 5%. In other experiments, the reaction mixture was electrophoresed on a 0.8% agarose gel (SeaKem GTG, FMC) as described previously (*37*). The gel was subsequently stained with 0.5 μg/mL ethidium bromide and photographed under ultraviolet illumination (*37*).

Demonstration of the Elongation of OligoDNA. The 5′-end phosphate moiety of oligoDNA was first labeled, by an exchange reaction, with [γ-^{32}P]ATP using T4 polynucleotide kinase according to the manufacturer's instructions. Next, the labeled oligoDNA (2 ng) was added to the standard reaction mixture containing 0.8 unit of *Tli* or Δ*Tli* pol and 4 nmol each of four nonlabeled dNTPs in 20 μL of buffer A. The mixture was incubated at 74 °C. Aliquots (2 μL) were next taken at the various indicated times and subjected to 6% polyacrylamide gel electrophoresis under denaturing conditions (in the presence of 8 M urea), followed by autoradiography (*37*).

Gel Electrophoresis of OligoDNA under Nondenaturing Conditions. Each oligoDNA was dissolved in buffer A containing 5% glycerol at a concentration of 2 μg/mL. The solution (25 μL) was heated at 74 °C for 10 min and then the reaction quenched in an ice/water mixture for 1 min. The solution was next electrophoresed on a 5% NuSieve 3:1 agarose gel (SeaKem GTG, FMC) in a solution containing 80 mM Tris-borate buffer (pH 8.9), 5 mM ethylenediaminetetraacetic acid, and 10 mM KCl at 25 °C. The gel was next stained with highly sensitive fluorescent dye GelStar (BioWhittaker Molecular Applications) and photographed under ultraviolet illumination using a yellow filter.

Molecular Cloning of the DNA Synthesis Product. The DNA synthesis product (elongation product) was prepared in the standard reaction mixture for 20 min as described above, except that the scale of the reaction was 5000-fold larger (100 mL). The product DNA was purified by phenol treatment, ethanol precipitation, and CsCl centrifugation (*37*). The yield determined by absorbance at 260 nm was 150 μg. A 10 μg aliquot of the product DNA was partially digested at 15 °C for 20 min in a 25 μL solution containing 50 mM Tris-HCl buffer (pH 7.6), 10 mM $MnCl_2$, 25 μg of bovine serum albumin, and 0.02 unit of deoxyribonuclease I. It was then purified with a MicroSpin S-400 HR column (Amersham Pharmacia Biotech). The partially digested DNA was next cloned into a pUC19 plasmid vector linearized by *Sma*I, and clones containing insert DNA were obtained after screening. The sequences of the insert DNA were determined by a dideoxy chain termination method on both strands (*38*).

Digestion of the DNA Synthesis Product with Restriction Endonucleases. Each DNA (0.8 μg) synthesized in the presence of either $(AGATATCT)_6$, $(ACTTAAGT)_6$, or $(AAAGCTTT)_6$ was digested with either 18 units of *Xba*I, 16 units of *Alu*I, 12 units of *Sca*I, or 18 units of *Hin*dIII at 37 °C for 2 h. The digested sample was electrophoresed on a 0.8% agarose gel, and the gel was stained and photographed as described above.

Measurement of the Thermal Melting Temperature of OligoDNA. The thermal melting temperature (T_m) of oligoDNA was determined in buffer A at a concentration of 1−10 μg/mL from an absorbance (260 nm) versus temperature curve. The temperature was increased from 20 to 100 °C, and then decreased from 100 to 20 °C at a rate of 0.25 °C/min using a diode array single-beam spectrophotometer (Ubest-50, Jasco) equipped with a thermal circulator (HD25, Julabo) and a program for temperature control (Labworld). The sample solution was overlaid with mineral oil to prevent evaporation during the measurement. The oligoDNA concentration was estimated from extinction coefficients corrected according to the nearest nucleotide values (*39*). Prior to the measurement of T_m, samples were pretreated by heating at 99 °C for 5 min. The heated sample was next cooled at a rate of 2.5 °C/min to 4 °C and was kept at 4 °C for at least 48 h until the measurement was taken. The T_m was determined from the peak of the computer-generated derivative of the absorbance versus temperature curve. The melting curves were further analyzed to obtain transition thermodynamic parameters as follows. The derivative (df/dT) was plotted against temperature, where f is a molar fraction of coil as calculated from the absorbance curve (*16*) and T is the absolute temperature. Next, the peak value, $(df/dT)_{max}$, was determined. The van't Hoff transition enthalpy (ΔH) was calculated from the equation $\Delta H = 4RT_m^2(df/dT)_{max}$, where R is the gas constant (8.31 J mol^{-1} K^{-1}). ΔS was calculated from the equation $\Delta S = \Delta H/T_m$. The experimental error (coefficient of variation) of the T_m measurement was 0.1%.

RESULTS

DNA Synthesis Is Enhanced by OligoDNA Due to Its Elongation. Tli pol synthesized DNA from four [α-^{32}P]-dNTPs without a primer−template complex or oligoDNA, after a lag time of 40 min, at an initial rate of 6 pmol/min as determined by the amount of radioactivity incorporated into acid-insoluble material (Figure 1C, ▲), confirming our previous observation of ab initio DNA synthesis (*33*, *35*). When 0.1 μg/mL 48mer repetitive palindromic oligoDNA $(AGATATCT)_6$ (standard oligoDNA) was present in the reaction mixture, DNA synthesis by *Tli* pol started without a lag time at an initial rate of 94 pmol/min (Figure 1C, ●). We examined this oligoDNA first, because the sequence AGATATCT was most frequently found in the product of the ab initio DNA synthesis by *Tli* pol (*33*, *35*). The above result indicates that the oligoDNA enhances the DNA synthesis in the reaction mixture in which a bimolecular primer−template complex is absent.

The maximum amount of DNA synthesized in the reaction described above was 1100 pmol after 40 min, corresponding to 6.9% of the maximal DNA theoretically possible with all four dNTPs (16 nmol) in the reaction mixture. This amount

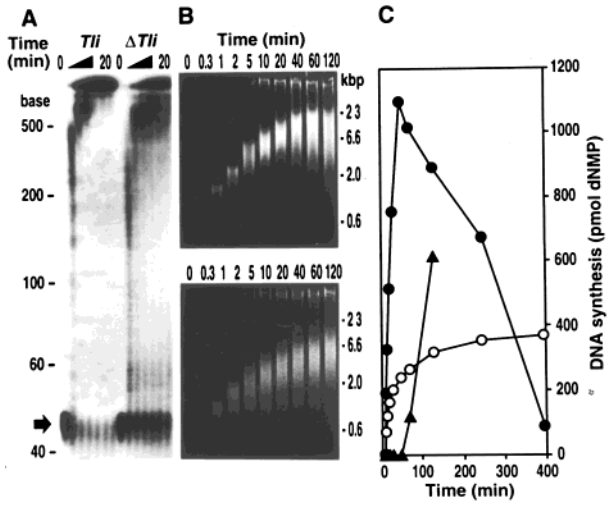

FIGURE 1: Time course of DNA synthesis by *Tli* and Δ*Tli* pols. (A) A standard reaction mixture containing 2 ng of 5′-^{32}P-labeled 48mer palindromic oligoDNA $(AGATATCT)_6$ (standard oligoDNA) and either *Tli* pol or Δ*Tli* pol was incubated at 74 °C, and 2 μL aliquots were taken at 0, 0.3, 1, 2, 5, 10, and 20 min. Samples were electrophoresed on a denaturing polyacrylamide gel and then subjected to autoradiography to measure the size of DNA as a single-stranded chain. Size markers are shown on the left. (B) The standard reaction mixture containing 2 ng of nonlabeled 48mer oligoDNA $(AGATATCT)_6$ was incubated at 74 °C with either *Tli* pol (upper panel) or Δ*Tli* pol (lower panel) for the indicated times, electrophoresed on an agarose gel under nondenaturing conditions, and stained with ethidium bromide. Size markers are shown on the right. (C) The standard reaction mixture containing 2 ng of nonlabeled 48mer oligoDNA $(AGATATCT)_6$ (circles) or no oligoDNA (triangles) was incubated with four [α-^{32}P]dNTPs at 74 °C for various times. The mixture also contained either *Tli* pol (black symbols) or Δ*Tli* pol (white symbols). The amount of DNA synthesized was then measured as the amount of radioactivity incorporated into acid-insoluble material.

of the newly synthesized DNA (1100 pmol, 360 ng) was 180 times larger than the amount of the added oligoDNA (2 ng). This means that the reaction is not a common replicative DNA synthesis in which the amount of newly synthesized DNA never exceeds that of template DNA (*1*). In other words, the reaction depicted in Figure 1C represents some DNA synthesis in which the added oligoDNA is markedly amplified either in its sequence or in its chain number (or both).

When the reaction with the oligoDNA and *Tli* pol was continued beyond 40 min, the amount of DNA that was synthesized decreased (Figure 1C, ●), presumably due to degradation of the synthesized DNA by the 3′−5′ exonuclease activity of *Tli* pol (*36*). This view was further supported by the finding that there was no decrease in the amount of DNA synthesized by Δ*Tli* pol, mutant *Tli* pol in which 3′−5′ exonuclease activity was lacking (*36*) (Figure 1C, ○). The appearance of a large amount of [^{32}P]dNMP monomers after reaction for 390 min with *Tli* pol also supports this view (data not shown). The maximum amount of [^{32}P]dNMP incorporated in DNA by *Tli* pol was greater than the amount incorporated in DNA by Δ*Tli* pol, suggesting that the 3′−5′ exonuclease activity plays an auxiliary, but not indispensable, role in this reaction. It is likely that a nucleotide inappropriately incorporated into the 3′-end of the growing chain is removed by the 3′−5′ exonuclease activity (activity present in *Tli* pol but not in Δ*Tli* pol) before the next nucleotide is incorporated.

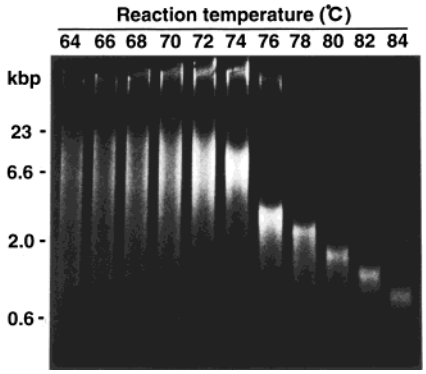

FIGURE 2: Effect of reaction temperature on DNA synthesis by *Tli* pol in the presence of oligoDNA. The standard reaction mixture for DNA synthesis was incubated with *Tli* pol and 2 ng of 48mer palindromic oligoDNA $(AGATATCT)_6$ (standard oligoDNA) at the various indicated temperatures for 20 min. The reaction mixture was next electrophoresed on an agarose gel under nondenaturing conditions. Size markers are shown on the left.

Next, we examined the time course of the change in size of the added standard oligoDNA $(AGATATCT)_6$, in which the 5′-end had been labeled with ^{32}P, in the *Tli* pol reaction using nonlabeled dNTPs as substrates. The size of the ^{32}P-labeled oligoDNA increased from 48 bases to about 500 bases in 20 s with *Tli* and Δ*Tli* pols, as demonstrated by denaturing polyacrylamide gel electrophoresis (Figure 1A). This finding indicates that the oligoDNA added to the reaction mixture is elongated and that its apparent DNA synthesis-enhancing activity is due to its elongation during the reaction. The size of the reaction product, namely, the elongated oligoDNA, reached well above 20 kbp after reaction for 40 min, as judged by agarose gel electrophoresis (Figure 1B). Some DNAs, as a smear, were much larger than 20 kbp, and they appeared near the top of the gel (Figure 1B). It is unclear why there are two types of DNAs (small DNAs and large DNAs) on the gel. All DNA synthesis reactions described hereafter were carried out for 20 min; DNA synthesis therefore solely represents the elongation of added oligoDNA, since ab initio DNA synthesis does not occur during this period as shown in Figure 1C. Although we chose 6 mM for a Mg^{2+} concentration (this was optimal) in most of the experiments, the elongation of standard oligoDNA also occurred in 0.5−50 mM Mg^{2+} (data not shown). When the reaction temperature was changed from 64 to 84 °C, the amount of DNA synthesized by *Tli* pol in the presence of the standard oligoDNA was maximal at 72 °C (Figure 2). The synthesized DNA became shorter and smaller in amount above 72 °C. A similar result was found in the reaction with Δ*Tli* pol (data not shown). The result indicates that the optimal temperature of the reaction is about 72 °C.

Various Repetitive Palindromic OligoDNAs with 25−50% GC Content Are Efficiently Elongated. To determine what kind of oligoDNA is preferentially elongated by *Tli* and Δ*Tli* pols, we investigated the effect of the length, GC content, and a base sequence of the repetitive palindromic oligoDNAs on DNA synthesis by these pols. The elongation of the added oligoDNA, as determined by the amount of radioactive DNA synthesized by *Tli* and Δ*Tli* pols, was observed with oligoDNAs which were more than 24 bases long, e.g., $(AGATATCT)_3$ (Figure 3). This indicates that more than three palindromic repeat units are required for efficient

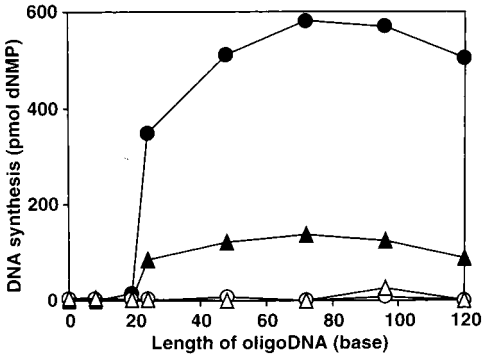

FIGURE 3: Effect of the length of the added oligoDNA on DNA synthesis by *Tli* and Δ*Tli* pols. A standard reaction mixture containing 134 pmol of palindromic oligoDNA of $(AGATATCT)_n$ series ($n = 0-15$) was incubated at 0 °C (white symbols) or at 74 °C (black symbols) for 20 min with either *Tli* pol (circles) or Δ*Tli* pol (triangles). The amount of acid-insoluble radioactivity was then determined. The motif sequence AGATATCT is the same as that of the standard oligoDNA $(AGATATCT)_6$.

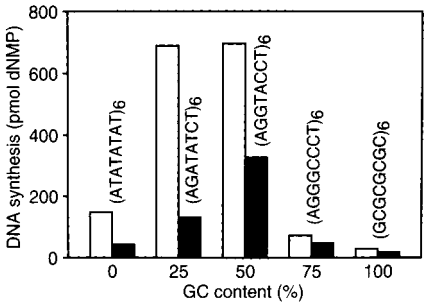

FIGURE 4: Effect of GC content of the added oligoDNA on DNA synthesis by *Tli* and Δ*Tli* pols. A standard reaction mixture containing 2 ng of 48mer repetitive palindromic oligoDNA with differing GC content as indicated and either *Tli* pol (white bars) or Δ*Tli* pol (black bars) was incubated at 74 °C for 20 min. The amount of acid-insoluble radioactivity was then determined.

elongation of the oligoDNA. We found that oligoDNAs with a shorter repetitive motif sequence, e.g., $(GAC)_3$, $(AGCT)_3$, $(AGACT)_3$, $(AGATCT)_3$, and $(AGATACT)_3$, were not elongated (data not shown). When the reaction was performed with oligoDNAs with either 25 or 50% GC content, the amount of dNMP incorporated in DNA was 650 or 640 pmol, respectively, by *Tli* pol (Figure 4, white bars). The level of DNA synthesis by *Tli* pol decreased dramatically when the GC content of added oligoDNA was outside the range of 25−50% (Figure 4, white bars). Essentially similar results were found for DNA synthesis by Δ*Tli* pol (Figure 4, black bars). This means that oligoDNAs with 25−50% GC content are preferentially elongated in the DNA synthesis reaction at 74 °C.

When various repetitive palindromic oligoDNAs with 25% GC content were added to the DNA synthesis reaction mixture, the amount of dNMP incorporated into DNA varied depending upon its base sequence (Figure 5). Although there are substantial differences in the amount of DNA synthesized, it could be concluded that repetitive 25% GC oligoDNAs are elongated efficiently regardless of their base sequences, if their repetitive motif sequences have a palindromic structure (Figure 5). It is noteworthy that while the presence of the palindromic structure in the repetitive motif sequence is important, oligoDNA having a quasi-palindromic sequence is also elongated, albeit not efficiently, provided that the

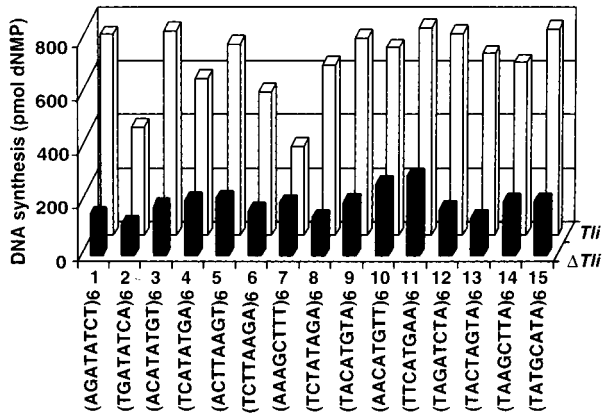

FIGURE 5: Effect of a base sequence of palindromic oligoDNA on DNA synthesis by *Tli* and Δ*Tli* pols. A standard reaction mixture containing 2 ng of 48mer repetitive palindromic oligoDNA with 25% GC content as indicated and either *Tli* pol (white bars) or Δ*Tli* pol (black bars) was incubated at 74 °C for 20 min. The amount of acid-insoluble radioactivity was then determined.

deviation of the motif sequence from the palindromic structure is small, as we demonstrate in the next paragraph.

OligoDNA Having a Palindromic Structure Is Preferentially Elongated. We next examined the influence of the destruction of the palindromic structure in the oligoDNA on the DNA synthesis reaction to determine to what extent the palindromic structure in the oligoDNA molecule is required for the reaction. When repetitive quasi-palindromic oligoDNA (AGATATC**A**)$_6$ (a nucleotide different from the standard oligoDNA shown by the letter in heavy type) was added to the DNA synthesis reaction mixture with *Tli* or Δ*Tli* pols, the amount of [^{32}P]dNMP incorporated into DNA dramatically decreased (Figure 6A, bars 3). Likewise, the level of DNA synthesis decreased with other oligoDNAs having quasi-palindromic or nonpalindromic repetitive motif sequences (Figure 6A, bars 4−9). (The borderline between nonpalindromic and quasi-palindromic sequences is not strictly defined in this work.) The influence of the destruction of the palindromic structure was marked in (AGAT**TAGA**)$_6$ (Figure 6A, bars 9), in which the degree of destruction was most marked (1.2 vs 750 pmol of standard oligoDNA by *Tli* pol).

We next examined the effect of various repeats of the nonpalindromic motif sequence AGAT**TA**CT introduced between the repeats of the palindromic motif sequence AGATATCT to determine to what extent the repetitiveness of the palindromic motif sequence in the oligoDNA is required for the reaction. When the number of repeats of the nonpalindromic motif sequence AGAT**TA**CT was increased, the level of DNA synthesis gradually decreased (Figure 6B, bars 3−7). The effect of the position of the nonpalindromic motif sequence AGAT**TA**CT introduced in the oligoDNA (repeat of the palindromic motif sequence AGATATCT) on the DNA synthesis reaction by *Tli* pol was marked when AGAT**TA**CT was present at the 5′-end, whereas this directional specificity, namely, whether at the 3′-end or at the 5′-end, was unclear in the Δ*Tli* pol reaction (data not shown).

When the DNA synthesis reaction was performed in the presence of both nonpalindromic oligoDNA (AGATATC**A**)$_6$ and nonpalindromic, but complementary, oligoDNA (**T**GATATC**T**)$_6$, the amount of DNA that was synthesized

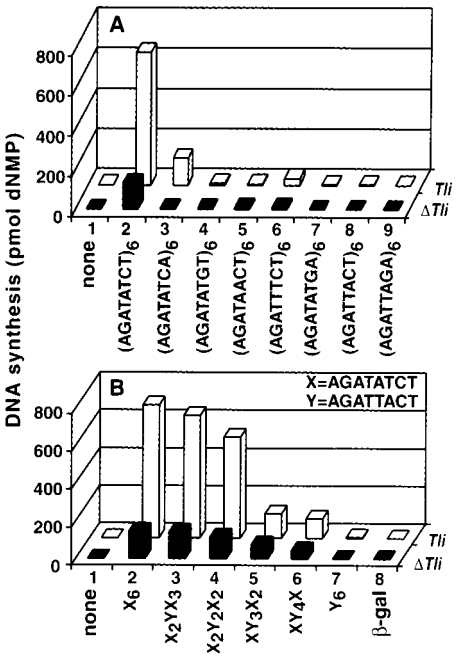

FIGURE 6: Effect of a palindromic structure of oligoDNA on DNA synthesis by *Tli* and Δ*Tli* pols. A standard reaction mixture containing 2 ng of fully repetitive but nonpalindromic 48mer oligoDNA (A) or partially repetitive palindromic 48mer oligoDNA (B) as indicated was incubated at 74 °C for 20 min with either *Tli* pol (white bars) or Δ*Tli* pol (black bars). The amount of acid-insoluble radioactivity was then determined. Note that in panel B, the sequence motif X = AGATATCT is palindromic, whereas the sequence motif Y = AGAT**TA**CT is nonpalindromic. Fully repetitive palindromic oligoDNA (AGATATCT)$_6$ (standard oligoDNA) and a 48mer fragment of *β*-galactosidase gene (*β*-gal) were used as controls.

was almost the same as the sum of the amounts of DNA synthesized when each oligoDNA was added individually. In a similar experiment, when two oligoDNAs (AGATAA**A**CT)$_6$ and (AG**T**TATCT)$_6$, which were complementary to each other, were incubated together, elongation was not found at all (data not shown). These results suggest that duplex (dimer) DNA, namely, a primer−template complex that could be formed between complementary strands (homoduplex in palindromic oligoDNA and heteroduplex in nonpalindromic oligoDNA), is not formed at 74 °C. Elongation may well occur if duplex is a necessary and sufficient structure, because the two oligoDNAs described above are completely complementary to each other and they can form a dimer, but not a hairpin, structure. The absence of DNA synthesis with these oligoDNAs strongly excludes the possibility that duplex is not formed under our reaction condition or duplex, if present, is not utilized for elongation. This interpretation was further supported by a thermal melting experiment as we show later.

DNA Elongated by Tli Pol Contains the Same Sequence as the Added OligoDNA. To determine the base sequences of the product DNA synthesized by *Tli* pol in the presence of oligoDNA, it was partially digested with deoxyribonuclease I, and was cloned into a plasmid vector. In the DNA synthesized in the presence of various repetitive palindromic oligoDNAs, the sequences of the cloned DNAs were the same as those of the added oligoDNAs (data not shown), indicating that the added oligoDNAs were faithfully elongated without changing their original base sequences. However, we cannot rule out rigorously the possibility that

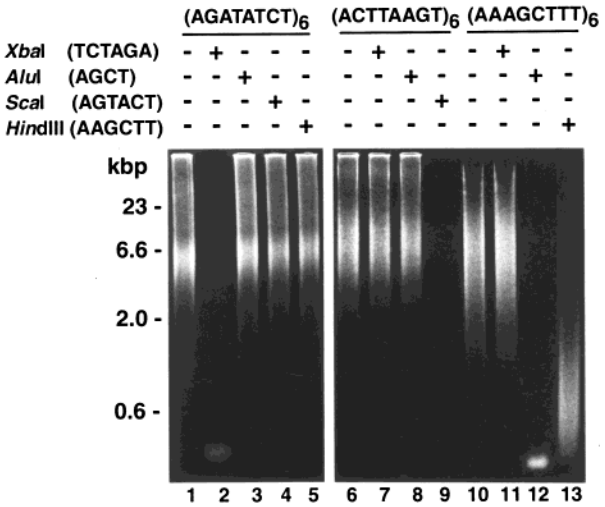

FIGURE 7: Restriction enzyme analysis of the DNA product synthesized by *Tli* pol in the presence of various 48mer oligoDNAs. Each DNA product synthesized by *Tli* pol in the presence of various repetitive palindromic oligoDNAs with 25% GC content, as indicated in the top line, was treated individually with or without the indicated restriction enzyme at 37 °C for 2 h, and the treated samples were then electrophoresed on an agarose gel under nondenaturing conditions. Size markers are shown on the left of the gel. The recognition sequence of each restriction enzyme that was used (*Xba*I, *Alu*I, *Sca*I, or *Hin*dIII) is shown on the right of each enzyme name.

there are some sequences that have changed during the DNA synthesis reaction, due to the limited number of clones that were analyzed. The base sequence of the product DNA was also confirmed by a restriction enzyme. As shown in Figure 7, the product DNA was completely digested by a restriction enzyme when its recognition sequence was also found in the sequence of the oligoDNA added to the DNA synthesis reaction mixture. For instance, when $(AGATATCT)_6$, which could be written as $(TCTAGATA)_n$, was added to the DNA synthesis reaction mixture, the product DNA (Figure 7, lane 1) was completely digested by *Xba*I (Figure 7, lane 2), whose recognition sequence is TCTAGA. This means that the sequence TCTAGA is ubiquitous in the product DNA. [Note that, judging from the cutting frequency of the 6-base cutter *Xba*I, the average fragment length of random-sequence DNA digested by *Xba*I is 4^6 (4096) bases.] On the other hand, the same product DNA was not digested at all by other restriction enzymes (*Alu*I, *Sca*I, and *Hin*dIII) whose recognition sequences were not found in $(AGATATCT)_6$ (Figure 7, lanes 3−5).

The Reaction Temperature for the DNA Synthesis Is Close to the Hairpin Melting Temperature of the Added OligoDNA. Repetitive palindromic oligoDNAs were thermally melted, and the change in the absorbance at 260 nm was measured as a function of temperature by ultraviolet spectroscopy (Figure 8). When the temperature of a solution containing the standard oligoDNA $(AGATATCT)_6$ (0.1 μg/mL) was increased from 20 to 100 °C, an absorbance versus temperature curve exhibited a biphasic pattern of melting transitions, i.e., "transition 1" (duplex−hairpin transition) at 35.9 °C (Figure 8, white arrowhead) and "transition 2" (hairpin−coil transition) at 70.4 °C (Figure 8, black arrowhead), as demonstrated by Xodo and co-workers regarding various palindromic oligoDNAs (*12*, *16*−*18*). ("Coil" is defined as unstructured single-stranded DNA in which no specific

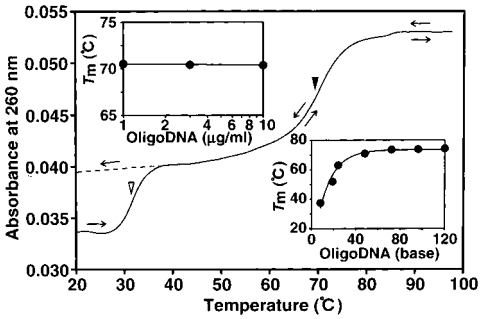

FIGURE 8: Thermal melting profile of repetitive palindromic 48mer oligoDNA $(AGATATCT)_6$. The absorbance of oligoDNA, at a concentration of 1 μg/mL in buffer A, was measured at 260 nm as a function of increasing (—) or decreasing (− − −) temperature. The point of the T_m of transition 1 (duplex−hairpin transition) is shown by a white arrowhead, and the point of the T_m of transition 2 (hairpin−coil transition) is indicated by a black arrowhead. The left inset shows the T_m's of transition 2 as a function of oligoDNA concentration, each point being a mean of three or four experiments. The right inset shows the T_m's of $(AGATATCT)_n$ series, where $n = 1-15$, as a function of their nucleotide lengths.

secondary structure is present.) The melting temperature (T_m) of transition 2 was always independent of the concentration of oligoDNA (Figure 8, left inset), as expected from the fact that this transition is an intramolecular event. On the other hand, when the temperature of the oligoDNA solution was gradually decreased from 100 to 20 °C, a typical absorbance versus temperature profile exhibited a monophasic pattern due to the disappearance of transition 1 (Figure 8). The absence of transition 1 in the decreasing-temperature experiment would be due to the fact that duplex formation, which is responsible for the absorbance change of transition 1, is a very slow process; the duplex formation would hardly be observed at the rate at which the temperature is decreasing in this experiment (0.25 °C/min) as pointed out by Xodo and co-workers (*16*). The T_m of transition 2 was again independent of the oligoDNA concentration in the decreasing-temperature experiment. Essentially the same biphasic melting profiles have been reported by many workers who assessed the thermal melting of a series of palindromic oligoDNAs, including repetitive palindromic $(dA\text{-}dT)_n$ (*9*, *11*, *12*, *16*−*18*).

The findings of the above investigators clearly demonstrated that transition 1 was due to interstrand homoduplex formation and that transition 2 was due to intrastrand hairpin formation (*9*, *11*, *12*, *16*−*18*). In addition, when the repetitive palindromic 48mer oligoDNAs $(AGATATCT)_6$, $(\mathbf{T}GATATC\mathbf{A})_6$, $(A\mathbf{ACATGT}T)_6$, and $(AG\mathbf{G}TA\mathbf{C}CT)_6$ were incubated at 74 °C and electrophoresed immediately on a nondenaturing polyacrylamide gel at 25 °C, their electrophoretic mobilities were the same as those of 24mer duplex oligoDNA used as a marker (Figure 9, white arrow), implying that the oligoDNA had a hairpin structure under the experimental conditions. [Note that the electrophoretic mobility of single-stranded DNA becomes almost the same as that of half-length double-stranded DNA, when it takes a hairpin structure (*16*, *18*).] When the T_m of transition 2 (hairpin−coil transition) of the oligoDNA of the $(AGATATCT)_n$ series was measured as a function of its length, it was 63.0 °C in 24mer DNA and 70.4 °C in 48mer DNA; a further increase in length did not increase T_m substantially (Figure 8, right inset). It is noteworthy that the T_m (70.4 °C) of transition 2 of the

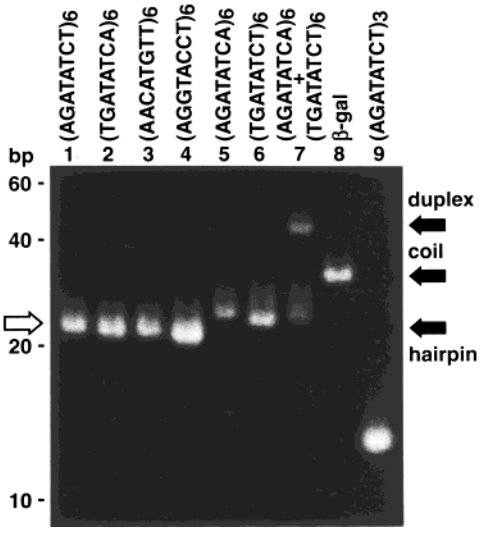

FIGURE 9: Nondenaturing agarose gel electrophoresis of various repetitive oligoDNAs. Each oligoDNA was dissolved in buffer A containing 5% glycerol and heated at 74 °C for 10 min and then the reaction quenched in an ice/water mixture. The solution was next electrophoresed on a nondenaturing 5% NuSieve 3:1 agarose gel and stained with the fluorescent dye GelStar. A 48-base fragment of the β-galactoside gene (β-gal) was used as a control for a 48-base coil. Positions of 48-base duplex, coil, and hairpin are shown by black arrows. The position of 24-base duplex DNA is shown by a white arrow. Size markers, as double-stranded DNA, are shown in base pairs (bp) on the left.

Table 1: Thermodynamic Parameters of the Hairpin−Coil Transition of Various Repetitive Palindromic 48mer OligoDNAs with 25% GC Content

oligoDNA[a]	T_m (°C)	ΔH (kJ/mol^{-1})	ΔS (J mol^{-1} K^{-1})
$(AGATATCT)_6$	70.4	327	952
$(TGATATCA)_6$	71.5	299	863
$(ACATATGT)_6$	72.1	302	725
$(TCATATGA)_6$	72.3	303	727
$(ACTTAAGT)_6$	72.9	305	881
$(TCTTAAGA)_6$	72.5	303	877
$(AAAGCTTT)_6$	76.8	321	917
$(TCTATAGA)_6$	70.8	296	860
$(TACATGTA)_6$	70.9	297	863
$(AACATGTT)_6$	73.4	307	886
$(TTCATGAA)_6$	73.0	306	884
$(TAGATCTA)_6$	71.8	301	873
$(TACTAGTA)_6$	71.0	297	863
$(TAAGCTTA)_6$	74.6	312	897
$(TATGCATA)_6$	73.4	307	886
mean ± standard deviation	72.5 ± 1.7	306 ± 9	864 ± 61

[a] The sequences are shown from 5′ to 3′.

standard oligoDNA $(AGATATCT)_6$ was very close to the optimal temperature (72 °C) of the DNA synthesis reactions (Figure 2).

The T_m's of transition 2 of various repetitive palindromic oligoDNAs with 25% GC content were between 70.4 and 76.8 °C (Table 1). These temperatures were within ±3.6 °C of the reaction temperature of 74 °C (Table 1). On the other hand, the T_m's of transition 1 were between 31 and 40 °C. Therefore, it is strongly suggested that the 48mer 25% GC repetitive palindromic oligoDNAs (Table 1 and Figure 5) are elongated at a hairpin−coil transitional state when incubated at 74 °C, and that a duplex structure is not involved in this reaction. When the T_m of transition 2 of 0% GC oligoDNA $(ATATATAT)_6$ was measured, it was 60.5 °C.

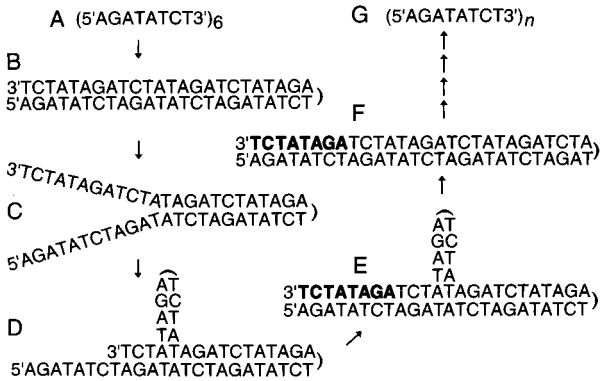

FIGURE 10: Model of hairpin elongation of repetitive palindromic oligoDNA $(AGATATCT)_6$ by *Tli* pol. OligoDNA (A) forms a hairpin structure by folding back (B), and then its ends melt partially due to the high temperature near the hairpin−coil transition (C). Next, the 3′-end arm of the hairpin locally forms a small hairpin (D), and *Tli* pol elongates the oligoDNA from the 3′-end by making use of the intramolecular primer−template structure thus formed (E). The newly synthesized stretch of DNA is shown in heavy type. The small hairpin is next incorporated into the large hairpin by slippage (F) because of the high temperature at which base pairing of the nucleotides is unstable. These cycles of hairpin formation, elongation, and slippage are repeated until large DNA (G) is made.

Since the amount of dNMP incorporated into DNA by *Tli* pol at 74 °C in the reaction mixture containing $(ATATATAT)_6$ was only 110 pmol as compared to 750 pmol of standard oligoDNA $(AGATATCT)_6$, the deviation of the added oligoDNAs from the hairpin−coil transitional state may be critical for its efficient elongation.

The values of transition enthalpy (ΔH) and transition entropy (ΔS) were next calculated to examine whether there was a correlation between these values and the amount of dNMP incorporated into DNA in the presence of various repetitive palindromic oligoDNAs with 25% GC content. These thermodynamic parameters were not correlated with the amount of dNMP incorporated into DNA (Table 1). This means that the efficiency of the elongation of the added repetitive palindromic oligoDNA with 25% GC content in the *Tli* pol reaction is not directly related to its ΔH or ΔS. In summary, tandem repetitive palindromic or quasi-palindromic oligoDNA markedly enhances the DNA synthesis by *Tli* and Δ*Tli* pols. This is because the added oligoDNA works as a "primer" and is elongated in the reaction at high temperature, in which it exists in a hairpin−coil transitional state.

DISCUSSION

On the basis of the result that the reaction temperature, at which added oligoDNA is efficiently elongated, is close to its hairpin melting temperature, we propose a model "hairpin elongation" for DNA elongation by *Tli* and Δ*Tli* pols in the presence of repetitive palindromic or quasi-palindromic oligoDNA (Figure 10). According to this model, the added oligoDNA (Figure 10A) first forms a hairpin structure by folding back at or near its center at a high reaction temperature (Figure 10B). The hairpin next melts partially and transiently (Figure 10C). The partially melted 3′-end arm of the hairpin folds back locally to form a small hairpin, eventually resulting in an "intramolecular primer−template structure" whose 3′-end is recessed (Figure 10D). The 3′-end is next elongated by pol (Figure 10E). Finally, the small

hairpin will be integrated into a single large hairpin by slippage (Figure 10F), because hairpin structures are unstable at high temperatures (around 72 °C). We speculate that such a round of hairpin formation and intrahairpin slippage is repeated, and finally, the oligoDNA added to the reaction mixture is elongated to form huge DNA (Figure 10G). While the model in Figure 10C shows partial melting of the hairpin, it would also be possible that the hairpin melts completely and then folds back again to form a 3′-recessed structure. It is possible that, in the intermediate of Figure 10D, the 5′-end arm also folds back and takes a hairpin structure, and the structure may temporarily hinder the elongation from the 3′-end arm. However, we think that there will be instants in which a hairpin is formed only in the 3′-end arm of the DNA molecule. In the same instants, *Tli* pol will be able to elongate DNA from the 3′-end.

It is known that a long polymer of simple repetitive DNAs can be synthesized in the presence of oligoDNA $(AT)_6$ by *E. coli* pol I at 37 °C (*40*). Kornberg and co-workers suggested that a mechanism of the elongation of $(AT)_6$ is probably intermolecular slippage-replication cycles of two molecules of the oligoDNA that form a primer−template complex (*40*). Schlotterer and Tautz reported (*41*) that bimolecular (heteroduplex) repetitive oligoDNAs, e.g., $(TCC)_5$/$(GGA)_3$, were elongated by *E. coli* pol I at 37 °C. It suggests that the same intermolecular slippage mechanism proposed for $(AT)_6$ elongation (*40*) occurs. In the case of $(AT)_6$ and $(TCC)_5$/$(GAA)_3$ elongations, hairpin formation was not pointed out (*40*, *41*). The $(TCC)_5$/$(GGA)_3$ example (*41*) strongly indicates that the intermolecular slippage synthesis occurs in the DNA synthesis by *E. coli* pol I in the presence of oligoDNAs, because these oligoDNAs are theoretically unable to form a hairpin structure. Contrary to the examples described above, it is noteworthy that the elongation of oligoDNA occurs (Figure 1A), as a unimolecular reaction, with a single species of oligoDNA that forms a hairpin structure (Figures 8 and 9). Therefore, our finding of the DNA elongation and our hairpin elongation model must be discriminated from the intermolecular slippage elongation reported previously (*40*, *41*).

All eukaryotic genomes contain a number of various simple repetitive DNA sequences, e.g., microsatellite DNAs, which are composed of di-, tri-, tetra-, and pentanucleotide motifs. They are widely dispersed in the genome, and exhibit frequent instability that is caused by deletion and expansion events during replication. In the human genome, there are also many simple tandem DNA repeats. For instance, trinucleotide repeat sequences (triplet repeats) are familiar as a cause of genetic diseases, such as Huntington's disease, fragile X syndrome, myotonic dystrophy, and Friedreich's ataxia (*23*, *24*, *42*, *43*), which are collectively called "triplet repeat diseases". Many triplet repeat diseases are caused by the instability of CNG repeats, where N is either A, T, G, or C. It has recently been postulated that the triplet repeats of a CNG type (*44*, *45*) can form a kind of partial hairpin structure and can be expanded by an intermolecular slippage event following hairpin formation during DNA replication in a lagging strand (*44*, *46*, *47*). If this reaction mechanism really exists, our finding of the DNA elongation reaction partly resembles the triplet repeat expansion. Further investigation into the elongation mechanism of repetitive palindromic oligoDNA may provide useful information for elucidating the mechanism of triplet repeat expansion and many genetic diseases.

Smith proposed that DNA whose sequence is not maintained by natural selection will exhibit tandem repetitive patterns as a result of unequal crossing over, if a strand exchange in the recombination process depends on a sequence similarity (*48*). Replicative slippage has also been proposed as a mechanism of gene amplification by other authors (*49*). Our example of DNA elongation might be another example of gene amplification, if the gene to be amplified satisfies two conditions, namely, a sequence close to a palindromic structure and GC content appropriate for the temperature where the organism lives, since the melting temperature of DNA is strongly influenced by its GC content; the higher the GC content, the higher the melting temperature (*16*−*18*). For instance, if the organism is living at 70−74 °C, 25−50% GC content would be appropriate. We also found that a similar phenomenon, namely, elongation of repetitive palindromic oligoDNA with about 25% GC content at 74 °C, occurred with DNA polymerases from *Thermus aquaticus* (*Taq*), *Thermus brockianus*, and *Pyrococcus* sp. (N. Ogata, unpublished data). This suggests that expansion of the genome by hairpin elongation might have been a ubiquitous phenomenon in organisms living in high-temperature environments on the early earth. The mechanism of gene amplification by means of hairpin elongation would occur frequently on the chromosomal DNA, in which 3′-ends are copious, such as on a newly synthesized lagging strand. The hairpin elongation model requires a free 3′-end, but not a free 5′-end. This means that if such a phenomenon is really taking place in the chromosomal DNA, elongation would occur frequently in the newly synthesized lagging strand.

Ohno proposed that the first set of coding sequences that arose in the prebiotic world were repeats of oligomer, the number of bases in these oligomeric units not being multiples of three (*50*, *51*). His idea for nucleotide elongation is that nonpalindromic pentanucleotides polymerize from mononucleotides in the presence of Zn^{2+} and imidazole by a nonenzymatic event. They are then ligated to decamers, each consisting of these two repeat units. These decamers, as the template, are replicated by the same event, and finally, decamer duplexes are elongated to long duplexes through a melting−reannealing process, just like a polymerase chain reaction. However, a practical elongation synthesis, which is compatible with these models for yielding a long polynucleotide, has never been reported in a nonenzymatic event.

The minimal genome size required for life has recently been estimated to be 315−318 kbp by several investigators using completely different methods (*52*−*54*). It is not at all clear how such huge polynucleotides were synthesized by pol. By means of the elongation mechanism demonstrated by us in this investigation, long DNA of more than 20 kbp is easily synthesized when short (at least 24-base long) DNA having a palindromic or quasi-palindromic repetitive structure exists as a starting material (Figure 3). If homologous recombination and gene duplication (*55*) additionally occur, then gigantic DNA might easily be synthesized. Shiba and co-workers recently reported that a set of 42mer primer pairs, forming 8−10 complementary base pairs in the 3′-region and having double-mismatch pairs at their 3′-ends, was elongated up to 12 kbp by thermophilic pols (*56*). Thus, it is plausible

that a primitive oligopeptide with pol-like activity elongated short repetitive palindromic DNA to long DNA in a high-temperature environment, and that the long DNA then gradually evolved into a variety of nonrepetitive and non-palindromic DNAs by accumulating numerous mutations during replications catalyzed by the primordial enzyme which fortuitously would have had a low replication fidelity. The crucial point of our hairpin elongation model is that huge DNA can be created from short DNA without complementary template DNA in a simple in vitro reaction system.

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BI0013243