Very Efficient Template/Primer-Independent DNA Synthesis by Thermophilic DNA Polymerase in the Presence of a Thermophilic Restriction Endonuclease

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ABSTRACT: We have found that, in the presence of a thermophilic restriction endonuclease, thermophilic DNA polymerase efficiently synthesizes and amplifies DNA in the absence of any added template and primer nucleic acid under isothermal conditions. More than 10 μg of DNA can be synthesized by 1 unit of DNA polymerase in 1 h, and the reaction proceeds until available dNTPs are consumed. We used mostly the *Tsp*509I restriction endonuclease (recognition sequence: ↓AATT), the *Tsp*RI restriction endonuclease (recognition sequence: NNCA(G/C)TGNN↓), and Vent (exo$^-$) and Vent DNA polymerase. The synthesized double-stranded DNA has a highly repetitive palindromic sequence, e.g. $(AAAAATTTTT)_n$ and $(ATACACTGTATATACAGTGTAT)_n$. In every repeating unit, there are one or two recognition sites for the restriction enzyme. Our data show that the high efficiency of the restriction-endonuclease−DNA-polymerase (RE-pol) DNA synthesis results from an efficient exponential amplification involving digestion−elongation cycles: a longer DNA with numerous recognition sites for the restriction enzyme is digested to short fragments, and the short fragments are used as seeds for elongation to synthesize longer DNA. A possible role of RE-pol DNA synthesis in the evolutionary development of genetic materials is briefly discussed.

For all organisms, genetic information is carried by long DNA. Even the smallest genomic DNA found in mycoplasma genitalium is 580 kb in length (*1*). How is the long DNA synthesized in the primordial environment? This is an interesting but extremely difficult question because the first long DNA synthesis happened billions of years ago. Recently more and more tandem repetitive DNA sequences have been found in many organisms (*2*−*8*). Furthermore, many repetitive short DNA sequences can be elongated to more than 50-kilobase pairs in length by DNA polymerase (*9*−*15*). Thus, short repetitive DNA sequences are believed to be served as one of the origins of large genomic DNA in modern organisms. Ohno proposed that modern coding sequences of DNA evolved from primordial oligomeric repeats and these repeats were elongated progressively during the course of evolution (*16*−*19*).

Ogata and Miura found that 10−50 knt[1] DNA could be ab initio (i.e. in the absence of any initial nucleic acid) synthesized from dNTPs by thermophilic DNA polymerases (from archaea *Thermococcus litoralis* and archaea *Thermus thermophilus*, etc.) (*20*−*22*). The synthesized double-stranded DNA had a repetitive and palindromic sequence, like $(TACATGTA)_n$, and similar sequences were found in the genomes of many organisms (*20*, *21*). The authors claimed that the tandem repetitive structure in many genes might have been arisen by the ab initio DNA synthesis but not by gene duplication as commonly believed (*22*). They imagined that a primitive polypeptide having polymerase-like activity synthesized long stretches of DNAs with simple repetitive sequences, which are gradually "evolved" into degenerate sequences by accumulating mutations during the error-prone replication by primordial enzyme.

However, the ab initio synthesis of DNA observed by Ogata and Miura was very slow. The synthesized DNA could not be detected until the reaction proceeded for 40 min to 1 h (*20*, *21*). In this paper, we report that if a thermophilic restriction endonuclease is present, the speed of the template and primer independent DNA synthesis dramatically increases. We designate the novel restriction-endonuclease−DNA-polymerase isothermal DNA synthesis we discovered as RE-pol. The tandem repetitive sequences obtained from RE-pol DNA synthesis, which were restricted by the recognition sequences of restriction enzymes, were completely different from the ab initio synthesized DNA reported by Ogata and Miura. The high efficiency of RE-pol and digestion of the synthesized long DNA make the birth of genomic-like DNA (diversity of sequence) much more possible because different restriction enzymes can help build a library of DNA sequences and the combination of these sequences might produce a more information-rich long DNA sequence.

MATERIALS AND METHODS

Materials. Vent and Vent (exo$^-$) DNA polymerase (from archaea *T. litoralis*), restriction enzymes *Tsp*509I (recognition sequence: ↓AATT), *Tsp*RI (recognition sequence: NNCA(G/C)TGNN↓), *Tsp*45I (recognition sequence:

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[1] Abbreviations: RE-pol, restriction-endonuclease−DNA-polymerase; dNTP, deoxyribonucleotide 5′-triphosphate; bp, base pair; kbp, kilobase paris; nt, nucleotide; knt, kilonucleotides; ODNs, oligodeoxyribonucleotides; Tris, tris(hydroxymethyl)aminomethane; exo$^-$, no 3′→5′ exonuclease activity.

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↓GT(G/C)AC), *Tli* (recognition sequence: C↓TCGAG), *Bst*BI (recognition sequence: TT↓CGAA), *Tth*111I (recognition sequence: GACN↓NNGTC), *Bsa*BI (recognition sequence: GATNN↓NNATC), *Taq*$^{\alpha}$I (recognition sequence: T↓CGA), *Tfi* (recognition sequence: G↓A(A/T)TC), *Psp*GI (recognition sequence: ↓CC(A/T)GG), *Apo*I (recognition sequence: Pu↓AATTPy), *Hinc*II (recognition sequence: GTPy↓PuAC), and *Eco*RI (recognition sequence: G↓AATTC), plasmid pUC19, 10X ThermoPol reaction buffer, and 10X NEBuffer 2-4 were purchased from New England Biolabs. Deoxyribonucleoside triphosphates (dNTPs), restriction enzyme *Tas*I (*Tsp*EI, recognition sequence: ↓AATT), proteinase K, and RNase A were purchased from Fermentas (*23*). RNase-free DNase I was purchased from Promega, nuclease P1 from Roche Diagnostics, SYBR Green I from Molecular Probes, and DNase−RNase-free water from SIGMA Chemical. The nonenzymatic synthesized oligodeoxynucleotides (ODNs) were obtained from Integrated DNA Technologies.

DNA Synthesis by DNA Polymerase in the Presence of Restriction Enzyme (RE-Pol Reaction). The standard reaction mixture (50 μL) for the restriction enzyme *Tsp*509I contained 1.0 unit of DNA polymerase, 0.5 unit of restriction enzyme, 0.4 mM dATP and dTTP in 1X ThermoPol reaction buffer (10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25 °C), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100). In the case of restriction enzyme *Tsp*RI, 0.4 mM dNTPs were used and the RE-pol DNA synthesis was carried out in a 1:1 (v/v) mixture of 1X ThermoPol reaction buffer and 1X NEB4 buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9 at 25 °C). The mixture was incubated at 70 °C for 45 min or various time intervals specified, and the reaction was terminated by adding 50 μL of 1X TBE buffer (pH 8.0 at 25 °C). Similar experiments were carried out in the cases of other restriction enzymes under various conditions. Then 7.5 μL of the products were electrophoresed on a 10% nondenaturing polyacrylamide gel (stained by ethidium bromide and photographed under ultraviolet illumination at 302 nm). Also, 10 μL of the above solution was added to 190 μL of SYBR Green I solution (2X final concentration) for fluorescence measurement. The samples were excited at 497 nm, and emission was measured at 530 nm (at 22 °C).

Enzymatic Treatment of DNA Polymerase and Restriction Enzyme. Twenty units of the restriction enzyme (or 10 units of DNA polymerase) was treated with 1 unit of DNase I or (and) 1 unit of RNase A in 50 μL of a solution containing 1X ThermoPol buffer and 0.1X DNase buffer (4 mM Tris-HCl, 1 mM $MgSO_4$, and 0.2 mM $CaCl_2$). DNase buffer was added because Ca^{2+} is essential to the DNase digestion. After treatment at 37 °C for 2 h, 50 °C for 10 min, and 70 °C for 30 min, 1 unit of treated DNA polymerase and 0.5 unit of the treated restriction enzyme were used for DNA synthesis under standard conditions described above. In some cases dNTPs were also treated with 5 units/mL of DNase I.

Similarly, 25 units of restriction enzyme was treated with 0.6 unit of proteinase K in 50 μL of 1X ThermoPol buffer at 65 °C for 1 h, or with 3 units of nuclease P1 in 50 μL of acetate buffer (30 mM, pH 5.3) at 70 °C for 1 h.

Molecular Cloning of Synthesized DNA and Characterization of DNA Product. The DNA product was prepared under the standard reaction conditions as described above, except that 1 U/mL of the *Tsp*509I restriction enzyme was used.

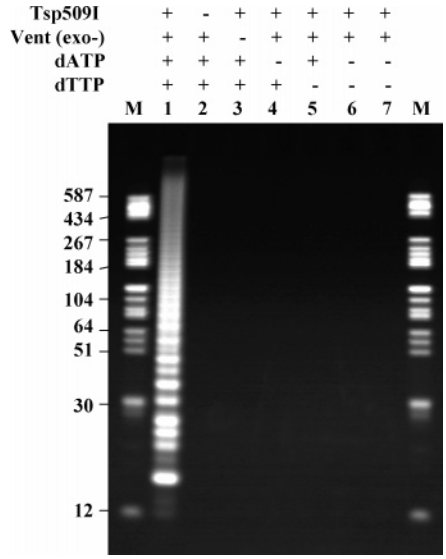


FIGURE 1: DNA synthesis by Vent (exo$^-$) DNA polymerase in the presence of *Tsp*509I restriction enzyme. Lane M, molecular marker in bp; lane 1, standard RE-pol reaction at 70 °C for 45 min; lanes 2−5, control experiments in the absence of *Tsp*509I, Vent (exo$^-$), dATP, and dTTP, respectively; lane 6, control experiment in the absence of both dATP and dTTP; lane 7, control experiment using dGTP and dCTP instead of dATP and dTTP. Ten percent nondenaturing polyacrylamide gel (stained with ethidium bromide) was used.

Then, the reaction solution was treated by phenol extraction, and the product was purified using ethanol precipitation. The obtained DNA was further digested by *Tsp*509I and cloned to a pUC19 plasmid at its unique *Eco*RI site (G↓AATTC). The insert-positive clones were sequenced by a dideoxy chain terminated method on one strand. In the case of *Tsp*RI, the RE-pol products were treated with nuclease mungbean at 30 °C for 30 min to remove the 9 nt 3′ overhangs, and then cloned to a pUC19 plasmid at its unique *Hinc*II blunt end site (GTPy↓PuAC).

Digestion-Elongation Cycles of RE-Pol Product in Separate Experiments. In the polymerization experiments, 1% of the digested DNA fragments (the RE-pol products were used in the first cycle) in the former digestion experiment were used as seeds for elongation by 1 unit of Vent (exo$^-$) DNA polymerase in the presence of 0.4 mM dATP and dTTP (or 0.4 mM dNTPs for *Tsp*RI). In the digestion experiments, the elongated DNA products in the former elongation experiment were digested by 0.5 unit of *Tsp*509I. All digestion and polymerization experiments were carried out in 50 μL of 1X ThermoPol buffer at 70 °C for 0.5 h (or a mixed buffer of ThermoPol and NEB4). Before every elongation or digestion experiment, the DNA was purified by phenol extraction and ethanol precipitation. Three digestion−elongation cycles were carried out. Similarly, the 26 nt long synthesized DNA (AATTTTTAAAAATTTTTAAAAATTTT) was also used as seeds and three digestion−elongation cycles were carried out.

RESULTS

Efficient RE-Pol DNA Synthesis in the Absence of Template or Primer Nucleic Acid. In the presence of the *Tsp*509I restriction endonuclease, which recognizes the ↓AATT sequence, a large amount of DNA was synthesized by Vent (exo$^-$) DNA polymerase at 70 °C for 45 min (Figure 1, lane

1). Here, no DNA was added as template or primer; only DNA polymerase, the restriction enzyme, dATP, and dTTP were present in the reaction buffer (10 mM KCl, 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.1% Triton X-100). Thus, DNA was efficiently synthesized from dATP and dTTP in the absence of DNA (or RNA) as initial material. The size of RE-pol product ranged from 10 nt to 1 knt after alkaline denaturation to single strands, as analyzed by denaturing polyacrylamide gel electrophoresis (data not shown). The synthesized DNA could be completely digested by DNase I but not by mung bean nuclease (a single-strand-specific nuclease), indicating that it consisted of (at least partially) double-stranded DNA (data not shown).

Several control experiments were done to clarify the RE-pol DNA synthesis. Vent (exo$^-$) DNA polymerase could not synthesize DNA in the absence of *Tsp*509I (Figure 1, lane 2), indicating that the restriction enzyme was essential to the DNA synthesis. As expected, *Tsp*509I could not synthesize DNA itself in the absence of Vent (exo$^-$) (Figure 1, lane 3). Also, DNA could not be synthesized in the presence of only one deoxyribonucleoside triphosphate (dATP or dTTP) (Figure 1, lane 4, 5). When dGTP and dCTP were used instead of dATP and dTTP, no DNA was detected either (Figure 1, lane 7). When dNTPs (consisting of dATP, dTTP, dGTP, and dCTP) were used, DNA could also be synthesized but the gel pattern of the synthesized DNA was different (Supplemental Figure 1, Supporting Information).

Naturally, one could think that the DNA synthesis was due to a contaminant. To address this problem, we pretreated Vent (exo$^-$) DNA polymerase and the *Tsp*509I restriction enzyme with some nucleases. If some contaminating nucleic acids were present, the nucleases would digest them. However, the RE-pol DNA synthesis proceeded after both Vent (exo$^-$) and *Tsp*509I were treated with DNase I and RNase A at 37 °C for 2 h (Figure 2, lane 2−4). Similarly, DNA was synthesized after *Tsp*509I and Vent (exo$^-$) were treated with nuclease P1 at 70 °C for 1 h (Figure 2, lane 7). Nuclease P1 was chosen because its optimal reaction temperature is 70 °C, the same temperature at which the RE-pol DNA reaction proceeded. It is a single-strand-specific enzyme, but it can also digest double-stranded DNA at a very slow rate (*7*). Again, DNA could not be synthesized in the presence of nuclease P1 but in the absence of *Tsp*509I (Figure 2, lane 5). Treatment of the RE-pol reaction buffer with DNase I, RNase A, and nuclease P1 did not slow the reaction either.

Another question is whether the digestion activity of *Tsp*509I is essential to the RE-pol DNA synthesis. After *Tsp*509I was pretreated at 65 °C for 1 h with proteinase K, and the proteinase K was heat-inactivated at 95 °C for 10 min, no DNA was detected using the treated restriction enzyme incubated with Vent (exo$^-$), dATP, and dTTP (Figure 2, lane 8). It indicates that proteinase K inactivated *Tsp*509I and the active *Tsp*509I protein is essential for the RE-pol reaction. One may argue that the incomplete inactivation of proteinase K could be responsible for the prevention of DNA synthesis, due to the inactivation of Vent (exo$^-$). However, DNA could be synthesized again when a new portion of the *Tsp*509I restriction enzyme was added (Figure 2, lane 9), indicating that Vent (exo$^-$) retained its polymerase activity.

RE-Pol DNA Synthesis under Various Conditions. To better understand the RE-pol DNA synthesis, the reaction

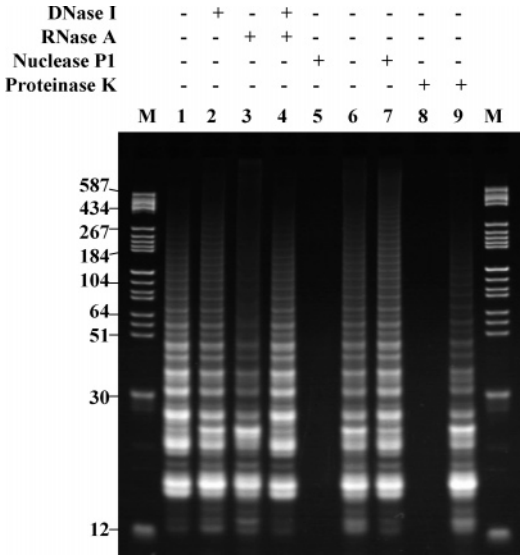


FIGURE 2: RE-pol DNA synthesis using the nuclease-treated *Tsp*509I restriction enzyme and Vent (exo$^-$) DNA polymerase. Both *Tsp*509I and Vent (exo$^-$) were treated according to the same protocol if not especially described. The products of RE-pol DNA synthesis (70 °C, 45 min) were analyzed by 10% nondenaturing gel electrophoresis. Lane M: molecular marker in bp. Lane 1: control of DNase and RNase treatment. Lane 2: DNase I treatment. Lane 3: RNase A treatment. Lane 4: treatment by both DNase I and RNase A. Lane 5: nuclease P1 treatment of Vent (exo$^-$), no *Tsp*509I was added. Lane 6: control of nuclease P1 treatment. Lane 7: nuclease P1 treatment. Lane 8: proteinase K treatment of *Tsp*509I. Lane 9: a new portion of nontreated *Tsp*509I was added to the reaction mixture of lane 8.

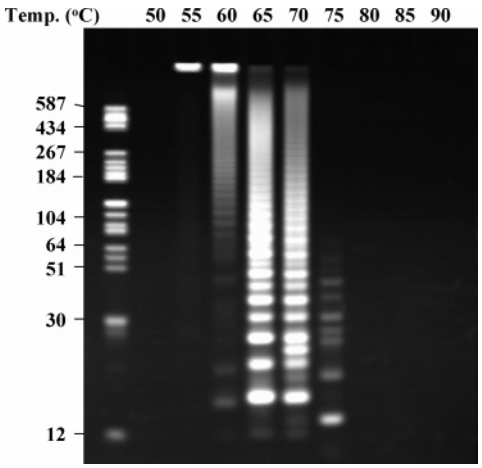


FIGURE 3: RE-pol DNA synthesis by Vent (exo$^-$) DNA polymerase in the presence of *Tsp*509I restriction enzyme at various temperatures. Except for temperature, the standard reaction conditions were used and the DNA products were analyzed by nondenaturing polyacrylamide gel (10%) electrophoresis.

was studied in detail under various conditions. Here, *Tsp*509I, Vent (exo$^-$), and dATP-dTTP were used. Figure 3 shows the results of the RE-pol DNA synthesis at various temperatures. DNA can be synthesized in the temperature range of 55−75 °C, with the optimal temperature range of 65−70 °C. At lower temperature (<60 °C), the reaction slowed considerably, and longer DNA molecules were synthesized (>1 knt); At higher temperature (>75 °C), a very small amount of DNA (or no DNA at all) was synthesized.

The RE-pol DNA synthesis rate and the length of synthesized DNA changed with the concentration of the *Tsp*509I restriction enzyme (Figure 4). From the viewpoint

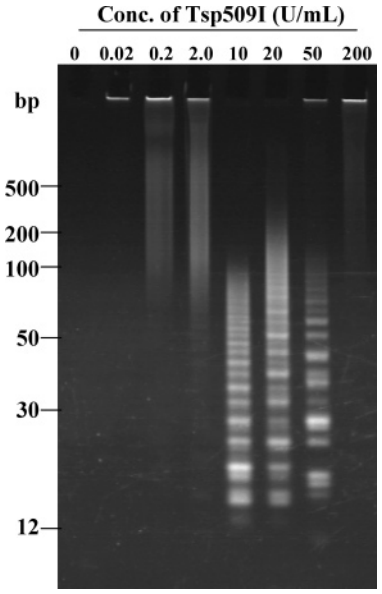


FIGURE 4: RE-pol DNA synthesis at various concentration of *Tsp*509I restriction enzyme. Except for the concentration of *Tsp*509I, the reaction was carried out under standard conditions and the DNA products were analyzed by nondenaturing polyacrylamide gel (10%) electrophoresis.

of synthesis efficiency, the optimal concentration was within the 10−20 units/mL range. At lower concentration of the restriction enzyme, longer DNA was synthesized (>1 knt) and the reaction slowed; at higher concentration, DNA became longer and the reaction slowed again (Figure 4).

Figure 5 shows the time course of the RE-pol DNA synthesis. Under the optimal conditions (10 units/mL of *Tsp*509I), the DNA synthesis was almost completed in 1 h with a short lag period of 4 min (Figure 5a,b). After 2 h, the synthesized DNA was digested by *Tsp*509I to very short fragments (<15 nt). By measuring fluorescence (stained by SYBR Green I) and comparing with standard DNA ($(AAAAATTTTT)_2$), the RE-pol reaction was quantitatively analyzed. The yield of synthesized DNA is as high as 90 ± 10% (theoretically possible amount of DNA product is 12.3 μg). Thus, DNA was synthesized with an average speed of 3 ng/s in the first hour. In a control experiment in which the restriction enzyme was absent, no DNA was detected even after incubation at 70 °C for 24 h. The speed of synthesizing DNA was increased at least 10^5 times by the added restriction enzyme. Our RE-pol reaction was even 100 times faster than the ab initio DNA synthesis Ogata and Miura reported (with an initial rate of 0.031 ng/s after a lag period of 40 min), in which Vent DNA polymerase was used instead of Vent (exo$^-$) and the restriction enzyme was absent (*20*−*22*). The efficiency of the template/primer-independent DNA synthesis was dramatically improved by combining the restriction enzyme with the DNA polymerase.

When a lower concentration of *Tsp*509I was used (0.02 unit/mL), the speed of the DNA synthesis slowed (0.125 ng/s). The synthesis was linear with time (Figure 5c,d). The length of the product was longer than 2 knt (Figure 5c, on agarose gel). Again, nearly all dATP and dTTP were consumed after 24 h and the yield was close to 100%.

Digestion−*Elongation Cycles of RE-Pol Products.* Since the RE-pol products changed from longer ODNs to very short ones as the reaction proceeded (Figure 5a), it was clear that

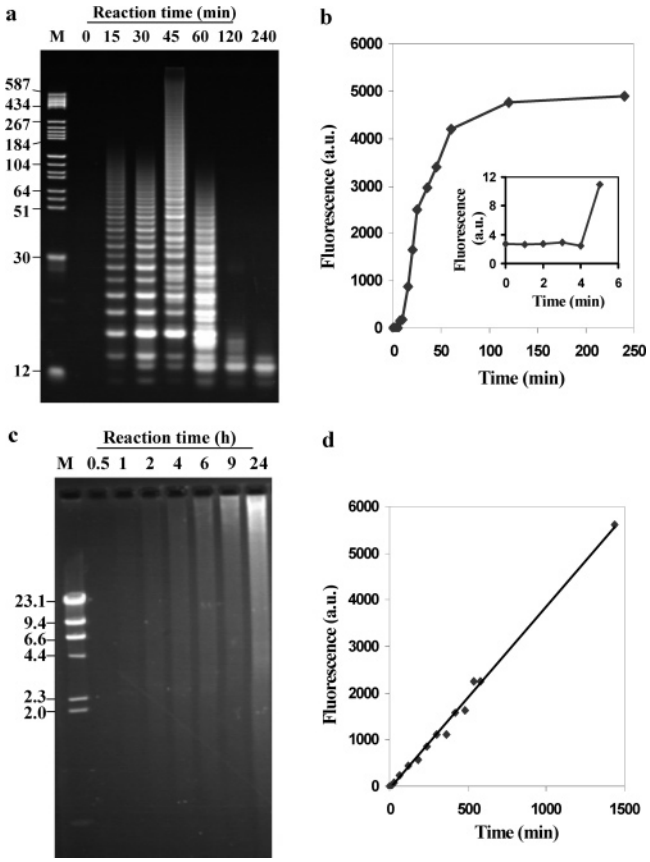


FIGURE 5: Time course for RE-pol DNA synthesis by Vent (exo$^-$) DNA polymerase in the presence of *Tsp*509I restriction enzyme. (a) Nondenaturing polyacrylamide gel (10%) electrophoresis in the case of 10 units/mL of *Tsp*509I. DNA size markers are shown on the left in bp. (b) Quantitative analysis by fluorescence measurement in the case of 10 units/mL of *Tsp*509I. The inset shows the results for the first 5 min. (c) One percent agarose gel electrophoresis in the case of 0.02 unit/mL of *Tsp*509I. DNA size markers are shown on the left in kb. (d) Quantitative analysis by fluorescence measurement in the case of 0.02 unit/mL of *Tsp*509I.

the *Tsp*509I restriction enzyme can digest the RE-pol products. To clarify the mechanism of the RE-pol reaction, the RE-pol DNA products were further analyzed by independent treatment with Vent (exo$^-$) and *Tsp*509I (Figure 6). We expected DNA synthesized via the RE-pol reaction to be amplified by Vent (exo$^-$) in the absence of *Tsp*509I, and amplified DNA to be digested by *Tsp*509I in the absence of Vent (exo$^-$). Indeed, short DNA molecules obtained from the RE-pol reaction (Figure 6, lane 1) were extended to much longer molecules (>2 knt) after the DNA polymerase and restriction enzyme were inactivated by phenol extraction and only Vent (exo$^-$) DNA polymerase, dATP, and dTTP were added (Figure 6, lane 2). After Vent (exo$^-$) was removed by phenol extraction, the long DNA molecules were digested by *Tsp*509I (Figure 6, lane 3). Then the digestion products were elongated again by Vent (exo$^-$) in the absence of *Tsp*509I (Figure 6, lane 4). Such digestion−elongation cycles were repeated more than 3 times. During every elongation reaction, only 1% of the former DNA product was added. DNA was elongated from 10−50 nt to longer than 2 knt, and the amount of DNA increased more than 100-fold. This result shows that DNA can be exponentially amplified with extremely high efficiency under isothermal conditions.

RE-Pol DNA Synthesis Using Other Restriction Enzymes. To check whether other restriction enzymes can carry out

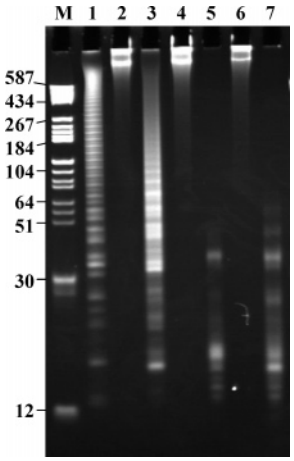


FIGURE 6: Independent digestion and elongation cycles of RE-pol products. Lane M: molecular marker in bp. Lane 1: DNA products under standard RE-pol reaction conditions. Lanes 2, 4, 6: products of elongation reaction by Vent (exo$^-$) DNA polymerase in the absence of restriction enzyme. Lanes 3, 5, 7, products of *Tsp*509I restriction enzyme digestion in the absence of DNA polymerase. In every elongation experiment, 1% of the products of the former digestion experiment were used as seeds.

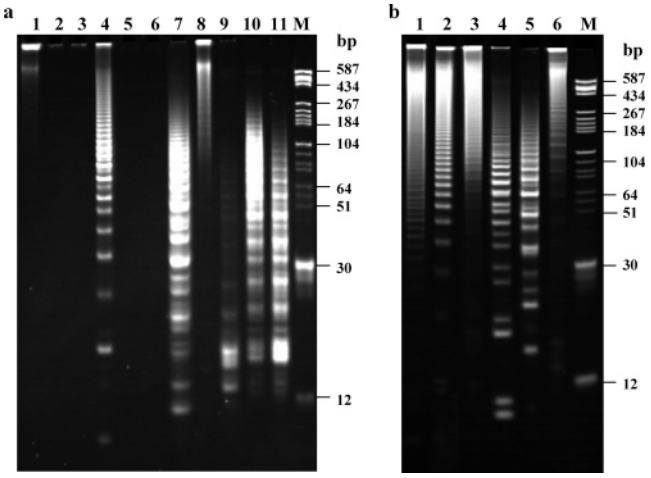


FIGURE 7: RE-pol DNA synthesis by other thermophilic restriction enzymes: (a) 1 unit Vent (exo$^-$) DNA polymerase, 0.5 unit restriction enzymes (5 units for *Taq*$^\alpha$I), 0.4 mM dNTPs, 70 °C (60 °C for *Apo*I), 1 h in 50 μL of 1X ThermoPol buffer; lane 1, *Psp*GI; lane 2, *Bsa*BI; lane 3, *Tli*I, lane 4, *Tth*111I; lane 5, *Tsp*45I; lane 6, *Tfi*I; lane 7, *Tsp*RI; lane 8, *Taq*$^\alpha$I; lane 9, *Apo*I; lane 10, *Tas*I; lane 11, *Tsp*509I; lane M, molecular marker in bp; (b) the reaction conditions were optimizing (only changed conditions are shown); lane 1, *Tli*I, 2 units, 75 °C, 2 h; lane 2, *Tfi*I, 6 h; lane 3, *Psp*GI, 85 °C, 2 h; lane 4, *Bsa*BI, 5 units, 60 °C, 2 h; lane 5, *Tsp*45I, 5 units, mixed buffer containing 0.5X NEB4 and 0.5X ThermoPol buffer; lane 6, *Taq*$^\alpha$I, 5 units, 65 °C, 4 h; lane M, molecular marker in bp.

RE-pol DNA synthesis, a dozen restriction enzymes were also used. Figure 7a shows the gel pattern of synthesized DNA using restriction enzyme *Tsp*RI, *Tsp*45I, *Tli*, *Bas*BI, *Tth*111I, *Taq*$^\alpha$I, *Apo*I, *Tfi*, *Psp*GI, *Tas*I, and *Tsp*509I. The RE-pol reaction proceeded under the following conditions: 1 unit of Vent (exo$^-$) DNA polymerase, 0.5 unit of restriction enzymes (5 units for *Taq*$^\alpha$I), 0.4 mM dNTPs, 70 °C (60 °C for *Apo*I), 1 h in 50 μL of 1X ThermoPol buffer. Here all four dNTPs are necessary because the recognition sites of some restriction enzymes consist of A, T, G, and C. For *Psp*GI (Figure 7a, lane 1), *Tth*111I (Figure 7a, lane 4), *Tsp*RI (Figure 7a, lane 7), *Taq*$^\alpha$I, (Figure 7a, lane 8), *Apo*I (Figure 7a, lane 9), *Tas*I (Figure 7a, lane 10), and *Tsp*509I (Figure 7a, lane 11), DNA was synthesized. After optimizing the reaction conditions, all the other restriction enzymes could also carry out RE-pol DNA synthesis (Figure 7b). For *Tli*I, DNA was synthesized at 75 °C for 2 h when 2 units of *Tli*I was used (Figure 7b, lane 1). For *Tfi*, DNA bands appeared after 6 h (Figure 7b, lane 2). For *Psp*GI, higher temperature (>80 °C) was favorable (Figure 7b, lane 3). For *Bsa*BI, DNA was synthesized at lower temperature (60 °C, Figure 7b, lane 4). For *Tsp*45I, DNA was obtained using the mixed buffer containing 0.5X NEB4 and 0.5X ThermoPol buffer (Figure 7b, lane 5). *Bst*BI restriction enzyme also showed RE-pol activity when the mixed buffer containing 0.5X NEB4 and 0.5X ThermoPol buffer was used (data not shown). Thus, all these 12 restriction enzymes could carry out RE-pol reaction using Vent (exo$^-$) DNA polymerase under the respective optimal reaction conditions. For *Tsp*RI, the RE-pol reaction under various conditions was also studied in detail and results similar to those of *Tsp*509I were obtained (Supplemental Figures 2−4, Supporting Information). The differences were that, for *Tsp*RI, DNA could also been synthesized at higher temperature (>85 °C); and the efficiency of RE-pol DNA synthesis kept higher at high concentration of restriction enzyme.

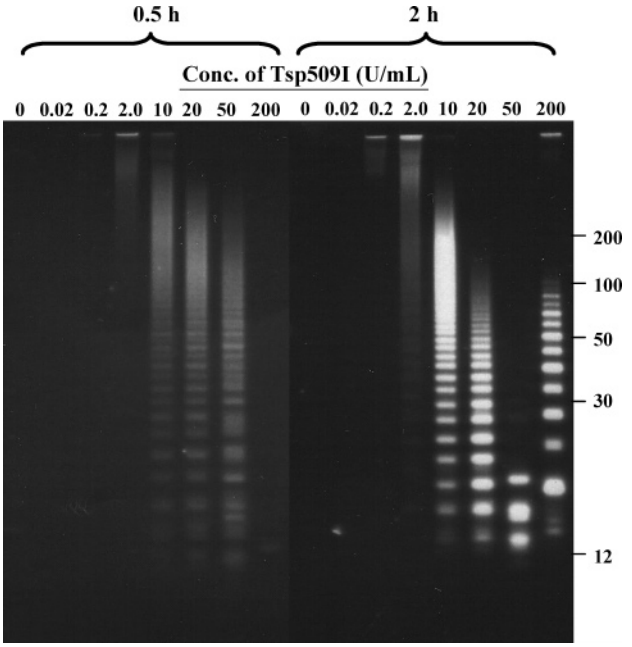


FIGURE 8: RE-pol DNA synthesis by Vent DNA polymerase in the presence of restriction enzyme *Tsp*509I. Except for the concentration of *Tsp*509I, the reaction was carried out under standard conditions (dATP and dTTP were used). Size markers are shown on the right.

RE-Pol DNA Synthesis by Other DNA Polymerases. In Vent (exo$^-$), the 3′→5′ proofreading exonuclease activity associated with Vent DNA polymerase is eliminated via genetic engineering, without altering kinetic parameters for polymerization on a primed single-stranded template. Vent (exo$^-$) has a stronger strand displacement activity than Vent (*24*). We checked whether the natural Vent DNA polymerase can carry out the RE-pol DNA synthesis. Figure 8 shows the products of RE-pol DNA synthesis by Vent at various concentrations of *Tsp*509I for different reaction time. The results are similar to those for Vent (exo$^-$): DNA was efficiently synthesized; the DNA products could be digested to very short ODNs (<15 nt); and the length of DNA

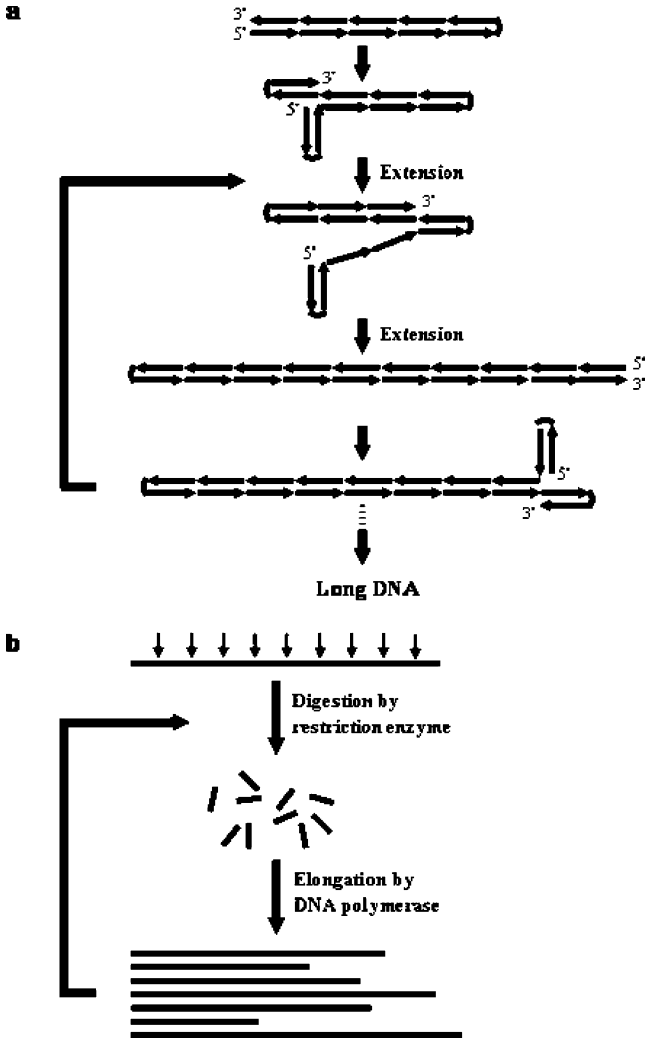
Sequence	Number of clones	length /nt
AATTTT(AAAATTTT)$_2$AAAATT	3	28
AATTTTTAAAAATTTTTAAAAATT	7	24
AATTTTT(AAAAATTTTT)$_2$AAAAATT	7	34
AATTTTT(AAAAATTTTT)$_3$AAAAATT	3	44
AATTTTT(AAAAATTTTT)$_4$AAAAATT	3	54
AATTTTT(AAAAATTTTT)$_6$AAAAATT	1	74
AATTTTT(AAAAATTTTT)$_7$AAAAATT	1	84
AATTATT(AAAAATTTTT)$_2$AAAAATT	1	34
AA (TTTTTAAAAA)$_2$TTTTTAATAATT	1	34
AATTTTA(AAAAATTTTT)$_2$AAAAATT	1	34
AATTTATT(AAAAATTTTT)$_2$AAAAATT	1	35
AATTTTT(AAAAATTTTT)$_2$AAAATTTTTT--AAAATTTTTAAAAATTTTTAAAAATT	1	63
AA(TTCTTAAGAA)$_2$TT	3	24
AA(TTCTTAAGAA)$_3$TT	5	34
AATTGTTAAATATTTAACAATT	7	22
AA(TTGTTAAATATTTAACAA)$_2$TT	1	40

FIGURE 9: Sequences of DNA products obtained from RE-pol reaction by *Tsp*509I restriction enzyme and Vent (exo$^-$) DNA polymerase. In the last four sequences, G and C were also incorporated when dNTPs were used instead of dATP and dTTP.

products as well as the reaction speed changed with the concentration of *Tsp*509I. A difference in behavior of these two DNA polymerases with respect to the RE-pol reaction was that detectable DNA could be synthesized by Vent from dATP and dTTP in the absence of *Tsp*509I after 1−2 h, whereas for Vent (exo$^-$) no DNA was detected even after 24 h at 70 °C (data not shown). Several other thermophilic DNA polymerases such as *Bst* (large fragment) and 9°N$_m$ DNA polymerase could also carry out RE-pol DNA synthesis(Supplemental Figure 5, Supporting Information).

Sequencing and Characterization of RE-Pol DNA Products. Synthesized DNA was sequenced with standard cloning methods. In the case of RE-pol using restriction enzyme *Tsp*509I, since synthesized DNA can be elongated and digested again by *Tsp*509I, it was expected to have the recognition sequence of AATT. Accordingly, the DNA product was cloned into the plasmid pUC19 vector at its unique *Eco*RI site (G↓AATTC). More than 30 insert-positive clones were obtained when dATP, dTTP, and Vent (exo$^-$) DNA polymerase were used, and the lengths of the insert DNA were 24−84 bp (Figure 9). Most of the obtained sequences were $(A_4T_4)_n$ and $(A_5T_5)_n$ (Supplemental Figure 6, Supporting Information). They were highly palindromic and repetitive, and the number of repeats varied from 2 to 8. Several sequences had deletions (from AAAAA to AAAA), insertions (from TTTTT to TTTATT), or replacement (from TTTTT to TTATT) of a single base. The similar sequences (20 clones) were obtained when Vent instead of Vent (exo$^-$) DNA polymerase was used. When dNTPs were used instead of dATP and dTTP (Vent (exo$^-$) DNA polymerase), the repetitive sequences of $(TTCTTAAGAA)_n$ (8 clones) and $(TTGTTAAATATTTAACAA)_n$ (8 clones) were obtained. The RE-pol products of *Tsp*RI were cloned into pUC19 at the *Hin*cII site (GTPy↓PuAC). Six insert-positive clones were obtained, and the lengths of the insert DNA were 30−55 bp. The sequences are also palindromic and repetitive: $(ATACACTGTATATACAGTGTAT)_n$. Sequences similar to those of RE-pol DNA products can be found in many genomic DNAs (Supplemental Figure 7, Supporting Information).

Scheme 1: The Proposed Mechanism of the RE-Pol DNA Synthesis[a]



[a] (a) Hairpin-elongation model for the expansion of a tandem repetitive sequence. The free 3′-end forms a hairpin structure as a primer−template complex, and then it extends to the 5′-end through strand displacement by DNA polymerase. The length of DNA is doubled after every cycle, and a very long hairpin-structured DNA is synthesized by repeating the strand displacement extension. (b) Digestion−elongation model for efficient amplification of DNA. The synthesized long double-stranded DNA with numerous recognition sites is digested by the restriction enzyme to short fragments; then the short fragments are elongated by DNA polymerase to give long DNA. The cycles are repeated again and again so the synthesized DNA is amplified with extremely high efficiency. All reactions proceed isothermally.

DISCUSSION

From the presented data, the digestion−elongation mechanism for RE-pol DNA synthesis can be proposed (Scheme 1), although it remains unclear how the DNA synthesis is initiated. Assuming that a short DNA with a palindromic repetitive sequence is somehow synthesized, it can be elongated to longer double-stranded DNA by DNA polymerase as reported by other research groups (*9*−*15*). Scheme 1a shows a hairpin-elongation model we assume. At the open end of a long DNA hairpin, two short hairpin structures are formed (*25*). The free 3′-end of one short hairpin initiates the primer extension reaction of DNA polymerase, which proceeds through strand displacement to the end of the original molecule. The length of DNA is almost doubled in

every cycle, and a very long double-stranded DNA is eventually synthesized via repeating cycles of the strand displacement-extension reaction. Then the long DNA is digested by the restriction enzyme to short fragments to be used as new seeds for elongation. Thus, DNA will be exponentially amplified through the repeating digestion−elongation cycles under isothermal conditions (Scheme 1b).

Suppose that DNA is elongated with a speed of n bp/min, and DNA can be immediately digested to short ODN with an average length of l after the elongation; the number of DNA molecules (N) can be expressed as

$$dN = (Nn\, dt)/l \quad (1)$$

$$N = N_0 \exp(nt/l) \quad (2)$$

Here, t is the reaction time (min), N_0 is the number of initial molecules. If $n = 1000$ bp/min (*24*), $l = 50$ bp, so $N = N_0 \exp(20t) \approx N_0 \times 10^{8.7t}$.

It is obvious that DNA can be exponentially amplified with extremely high efficiency. Separate digestion (by restriction enzyme) and elongation (by DNA polymerase) experiments strongly support this mechanism (Figure 6). DNA was exponentially amplified by digestion−elongation cycles with high efficiency ($N > 100^m$, m is the number of cycles). If we assume that 1000 copies of 50 bp long double-stranded DNA molecules were present after the first minute, the DNA would have to be amplified by more than 10^8 times in the following 4 min (Figure 5b, inset). Thus the amplification at the initial stage must be exponential. However, the RE-pol reaction changed to a linear process after a certain amount of DNA was synthesized because the amount of restriction enzyme or DNA polymerase was not enough to keep the exponential amplification, which needs a relatively high ratio of enzymes to DNA. In addition, after enough DNA was synthesized, the short DNA fragments could hybridize to longer DNA and worked as primers (*14*). As a result, we could observe only the linear kinetic data rather than exponential amplification (Figure 5b,d). When the DNA polymerase was in excess of the restriction enzyme, the rate of DNA synthesis was determined by the amount of restriction enzyme and longer DNA was obtained (Figure 4). When the restriction enzyme was in excess over DNA polymerase, the rate was determined by the amount of DNA polymerase, which resulted in shorter products. After the dNTPs were exhausted, the elongation stopped and the DNA products were gradually digested to very short fragments by the restriction enzyme (Figure 5a). When a very high concentration of the restriction enzyme (>100 units/mL) was used, however, longer DNA was synthesized (Figure 4). Unlike the DNA synthesized at lower concentrations of *Tsp*509I, it was difficult to further digest the long DNA by *Tsp*509I at low concentrations (<20 units/mL, Supplemental Figure 8, Supporting Information). Therefore, the long DNA product had a different sequence not containing AATT. The different sequence may come from a star activity of the *Tsp*509I restriction enzyme because of the extreme conditions such as a high enzyme to DNA ratio (>100 units/μg), low ionic strength (<25 mM), and high pH (>8.0). It was difficult to synthesize the DNA containing the AATT sequence because it would be digested into very short fragments which are poor substrates for elongation.

At lower temperatures (<55 °C), the restriction enzyme has a low digestion activity and it is more difficult for the transition between the regular duplex and a hairpin structure, as well as the strand displacement, to occur. At higher temperatures (>75 °C) the hairpin structure becomes unstable because the synthesized DNA has 100% AT content when only dATP and dTTP are used. As a result, an optimal temperature range for the RE-pol DNA synthesis of *Tsp*509I is 65−70 °C (Figure 3). In the case of *Tsp*RI, the DNA can also be synthesized even at 90 °C because of the higher GC content in the sequence of synthesized DNA (Supplemental Figure 2).

Let us now turn to the question of the origin of the seed DNA, which initiated the digestion−elongation cycles discussed above. The fact that the DNA synthesis was not affected by the treatment of the restriction enzyme with DNase I, RNase A, and nuclease P1 (Figure 2) indicated that the seed DNA was ab initio synthesized and was not present in the restriction enzyme as a contaminant. One possibility was that Vent (exo$^-$) DNA polymerase performed ab initio synthesis of DNA with random sequence with a very low efficiency. Among various sequences the tandem repetitive sequence with the recognition site (AATT) for the *Tsp*509I restriction enzyme accidentally appeared. Then the seed DNA was amplified via digestion−elongation cycles. We obtained a detectable amount of DNA after incubating Vent (exo$^-$), dATP, and dTTP in the absence of restriction enzyme at 70 °C for 3 days, although no DNA was detectable after incubation of 24 h (*23*). Ogata and Miura reported that several thermophilic DNA polymerases could ab initio synthesize DNA in the absence of primers or templates (*20*−*22*). Furthermore, Ramadan et al. found that human DNA polymerase λ, DNA polymerase μ, and terminal deoxyribonucleotidyl transferase could also ab initio synthesize DNA even with only one deoxyribonucleoside triphosphate such as dTTP (*26*). However, it is very difficult to be absolutely confident that the RE-pol DNA synthesis we describe did not result from contamination, because it was extremely efficient and DNA could be amplified even with several copies of DNA as seeds (*27*). For example, another possibility was that some DNA interacted tightly with the denatured restriction enzyme so DNase I could not digest it completely. This question must be addressed in further studies.

A reiteration and slippage mechanism was proposed by Kornberg et al. as early as in 1964 (*27*, *28*). According to this mechanism, one poly d(AT) strand slips on the other poly d(AT) strand by an AT dinucleotide pair during every slippage-replication cycle. This mechanism, however, cannot explain the elongation in the RE-pol reaction, because it requires slippage by 8 nt (for $(AAAATTTT)_n$) to 18 nt (for $(TTGTTAAATATTTAACAA)_n$) to form a new primer−template complex (Figure 9). Since DNA polymerase binds and stabilizes the template−primer structure, the hairpin-enlongation model works well when short hairpins form only transiently at the end of the duplex (Scheme 1). Even if the slippage happens, its impact should be much less compared to our mechanism since the hairpin forms easily at the end of duplex and the length of DNA is almost doubled after every cycle.

The RE-pol DNA synthesis seems a common characteristic of many thermophilic restriction enzymes and DNA polymerases. It was confirmed that several DNA polymerases

(such as *Bst* and 9°N_m DNA polymerase) and a dozen thermophilic restriction enzymes could carry out the RE-pol. In addition to a highly efficient amplification of ab initio synthesized DNA, a restriction enzyme may somehow help DNA polymerase to select the sequence to be synthesized. Only the recognition sequence of the restriction enzyme can be amplified via the RE-pol reaction mechanism we proposed. The palindromic and repetitive sequences of the RE-pol synthesized DNA also agreed well with the hairpin-elongation model (Scheme 1a), in which a hairpin structure has to form. It can be expected that when several restriction enzymes are mixed, the DNA involving more genetic information might be obtained from RE-pol DNA synthesis (under study). Our findings shed a new light on the template/primer-independent synthesis of nucleic acids as a necessary step of prebiological evolution (*29*). The findings suggest that the digestion of nucleic acids may play an important role in the evolution of genetic material for procreating the diversification of genetic information on the early earth. Using a mechanism similar to RE-pol, a reaction consisting of polymerization (growth) and digestion (generation of new seeds) might be responsible for the foundations of life. The polymerization and digestion activity could be carried out by either protein, RNA, or other functional molecule.

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SUPPORTING INFORMATION AVAILABLE

One figure showing the difference in gel pattern of RE-pol products between using dNTPs and using only dATP and dTTP, three figures showing gel pattern of RE-pol DNA products using *Tsp*RI restriction enzyme under various conditions, one figure showing gel pattern of RE DNA product using *Bst* and 9°N_m DNA polymerase, one figure showing the sequencing pattern of RE-pol product, one figure showing the similar sequences (as RE-pol products) found in natural genome, one figure showing the digestion pattern of RE-pol DNA products obtained at high concentration of *Tsp*509I, and one figure showing the length of RE-pol products on agrose gel. This material is available free of charge via the Internet at http://pubs.acs.org.

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