4922–4927 *Nucleic Acids Research, 2005, Vol. 33, No. 15*
doi:10.1093/nar/gki803

Efficient isothermal expansion of human telomeric and minisatellite repeats by *Thermococcus litoralis* DNA polymerase

Jörg S. Hartig and Eric T. Kool*

Department of Chemistry, Stanford University, Stanford, CA 94305-5080, USA

Received July 29, 2005; Revised and Accepted August 15, 2005

ABSTRACT

Repeating DNA sequences, such as telomeres, centromeres, and micro- and mini-satellites, comprise 50% of the genome and play important roles in regulatory and pathogenic mechanisms. In order to study structures and functions of such repeating sequences, it is important to have simple and efficient methods for making them *in vitro*. Here, we describe the efficient and convenient expansion of repetitive telomeric and minisatellite DNA sequences starting from small synthetic templates to final product lengths of several hundreds to thousands of nucleotides by the thermostable DNA polymerase from *Thermococcus litoralis* (Vent DNA polymerase). This enzyme was so far unknown to catalyze repeat expansion. Either single-stranded or double-stranded DNAs could be produced, depending on nucleotides present. Compared to earlier results obtained with other enzymes, the expansion reaction is highly efficient both in its yield and product length, and proceeds without thermal cycling. Moreover, the products are characterized by a narrow length distribution.

INTRODUCTION

Almost exactly half of the human genome comprises repetitive sequences (1). Some of these repeats are known to play important biological roles; e.g. telomeres, which are hexamer repeats of TTAGGG, act to 'cap' the chromosomes and are key components of regulatory processes such as the determination of cellular replicative life span (2–4). Other repeated motifs, such as micro- and mini-satellite sequences, have not yet been identified with a distinct function. These variable number of tandem repeats (VNTRs) show high polymorphism in length even in closely related individuals (5). This property predestines VNTRs as genetic markers in a wide variety of genetic analyses such as gene mapping, tumor diagnosis, and in forensic and parentage applications such as DNA fingerprinting. Beyond these examples, other repetitive stretches of the genome are very sensitive towards changes in length. For example, deletions and expansions of certain repeats are linked to a variety of diseases such as spinal and bulbular atrophy, spinocerebellar ataxia and schizophrenia (6). The most abundant class of unstable DNA, the trinucleotide repeat expansion, can also lead to myotonic dystrophy, fragile X syndrome, Huntington disease and other disorders (7).

Expansions and deletions, as well as the high polymorphism of repetitive sequences, mainly arise from slipped-strand mispairing, caused by a realignment of the primer/template complex during DNA replication (Figure 1) (8–10). This mechanism has been studied *in vitro* using a variety of polymerases (11–14). Importantly, most polymerases known to catalyze expansion of repetitive sequences are only able to add few repeats to the starting strands or otherwise need thermal cycling (12,14). For example, the expansion of telomeric sequences has been studied by Yoshida and co-workers (15), who demonstrated that a DNA polymerase α/primase complex can expand a template by a maximum of 40–90 nt. In a different study, longer products of a polyG:polyC duplex have been synthesized using the Klenow fragment of DNA Pol I (16).

Here we describe the observation of efficient synthesis of long repetitive DNA sequences based on repeat expansion using Vent DNA polymerase, extending short templates to a final length of >1000 nt. We have been developing approaches for the synthesis of long telomeric DNA in order to study its structure and function. Repetitive sequences are especially prone to adopt non-B-DNA-like structures such as quadruplexes (17) and i-motif (18). Telomeres are suspected to play an important role in regulatory pathways via alternate folding of the DNA sequence as well (19,20). Although telomeric structures have been studied in the context of small synthetic DNAs, higher-order structures and cooperative effects originating from interactions within long stretches of

*To whom correspondence should be addressed. Tel: +1 650 724 4741; Fax: +1 650 725 0259; Email: kool@stanford.edu

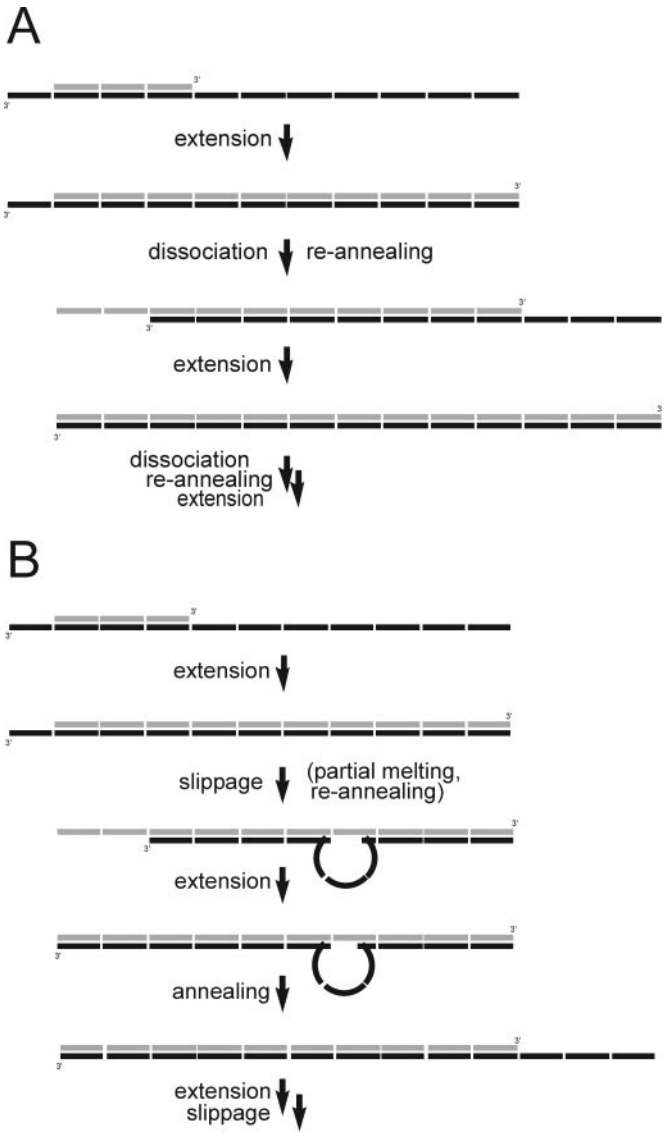

Figure 1. Possible mechanisms of repeat expansion by Vent DNA polymerase. The repetitive nature of primer and template allows for dissociation and reassociation of the elongated products, especially at elevated temperatures. If misalignment of the two strands occurs [by full dissociation as in (**A**) or partial melting as in (**B**)], additional extension can take place, resulting in products longer than the original template.

these sequences are likely to be missed (21–23). In addition, non-B-DNA structures in various repetitive sequences could be part of regulatory and pathogenic processes. For example, length dependence of such alternative structures has been found in sequences connected to Friedreich ataxia. Depending on the number of trinucleotide repeats related to the disease, different species of H-DNA (a structure composed of an intramolecular triplex) have been observed (24). Such findings demand more detailed structural investigations, particularly of long synthetic repeats, as a reference for possible structures formed *in vivo*. In order to do so, convenient methods for the synthesis of long stretches of repetitive sequences are needed.

We have described previously a new strategy for the synthesis of long repetitive DNA based on a rolling circle replication mechanism (25). Circular, single-stranded templates encoding the human telomeric sequence have been applied as effectively infinite templates in extension reactions to yield very long telomeric sequences, allowing for the synthesis of single-stranded G-rich and C-rich telomeric stretches (26,27). In control experiments for these former studies, we observed a surprising phenomenon when using *Thermococcus litoralis* (Vent) DNA polymerase. During characterization of the circle-dependent replication we performed controls with non-circular templates. As expected, no extension of the primers beyond the linear templates was observed for several enzymes; however, Vent DNA polymerase unexpectedly yielded long products (25,27). This intriguing observation gave rise to the present study.

MATERIALS AND METHODS

Oligonucleotides

All DNA oligonucleotides were synthesized on 1 μmol scale using standard phosphoramidite solid-phase chemistry using an ABI 394 synthesizer followed by purification by 15% PAGE.

Telomeric oligonucleotides

G-rich 21mer (TG21): $GGG(TTAGGG)_3$; C-rich 21mer (TC21): $CCC(TAACCC)_3$; G-rich 45mer (TG45): $GGG(TTAGGG)_7$; C-rich 45mer (TC45): $CCC(TAACCC)_7$; G-rich 69mer (TG69): $GGG(TTAGGG)_{11}$; C-rich 69mer (TC69): $CCC(TAACCC)_{11}$; G-rich 93mer (TG93): $GGG(TTAGGG)_{15}$; and C-rich 93mer (TC93): $CCC(TAACCC)_{15}$.

Scrambled sequences

Primer (SC-P): TCTACATCCTACCACCCC; template (SG-T): TGTGGTGGAGAGTTGTAGTGTTGTGGGGTGGTAGGATGT.

Mini-satellite sequences

MS-primer: TGGGAGAGGCTGGGAGAGGC; MS-template: GCCTCTCCCAGCCTCTCCCAGCCTCTCCCAGCCTCTCCC.

Polymerase extension reactions

The reactions were carried out in 20 mM Tris–HCl buffer, pH 8.8, containing 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$ and 0.1% Triton X-100 (Thermopol buffer) in a final volume of 5 μl. The reactions contained a primer and a template at 1 μM concentration each, 1 mM dNTPs except dTTP at 0.05 mM in Figures 2 and 5 and Supplementary Figure S1, at 0.25 mM in Figure 3. The products were visualized by including 0.025 μCi of [α-^{32}P]dTTP in 5 μl each. Experiments using 5′-^{32}P-labeled primer (Supplementary Figure S1) were carried out at 1 mM dNTPs each. The reactions were initiated by adding 1 U Vent DNA polymerase (NEB) or 2.5 U of *Taq* DNA polymerase (NEB), followed by incubation at the given temperatures for 1 h. The reactions were quenched in 5 μl PAGE loading buffer and analyzed by 10% PAGE and autoradiography.

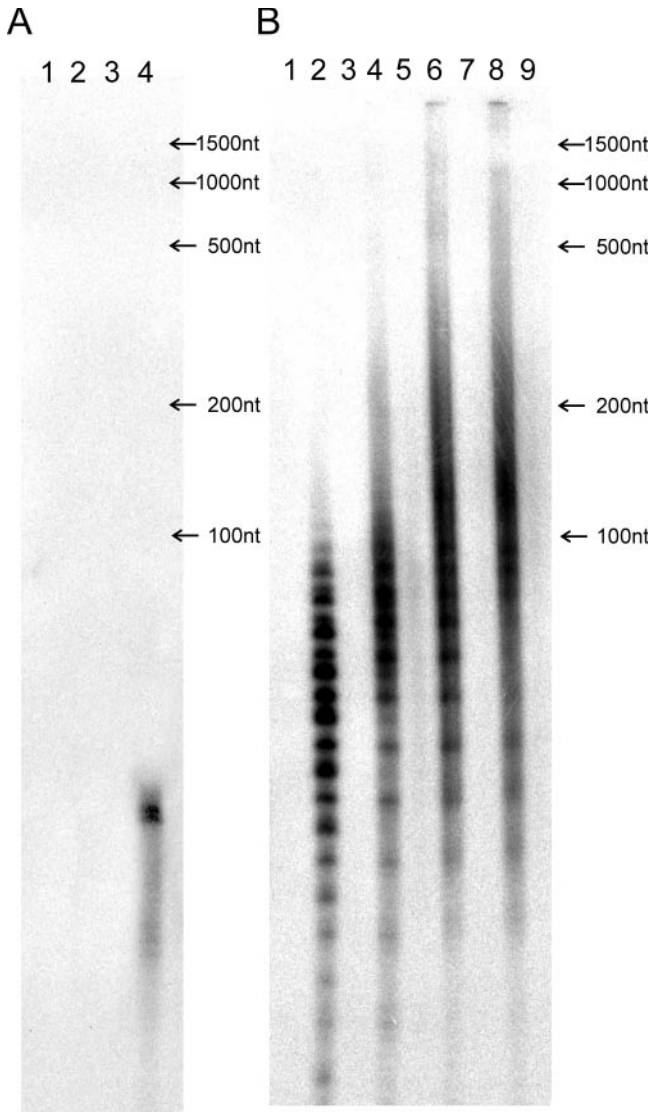

Figure 2. Effect of template length and sequence on expansion by Vent DNA polymerase. (**A**) A non-repetitive, scrambled sequence results in extension only to the template end. Lane 1: reaction lacking primer and template; lane 2: reaction containing primer SC-P but lacking template; lane 3: reaction containing template SG-T but lacking primer; and lane 4: reaction containing both SC-P and SC-T. (**B**) Varying template length. Lane 1: reaction containing only TC21; lane 2: reaction containing TC21 and TG21; lane 3: reaction containing only TG21; lane 4: TC21 and TG45; lane 5: TG45 only; lane 6: TC21 and TG69; lane 7: TG69 only; lane 8: TC21 and TG93; and lane 9: TG93 only.

Preparation of products for sequencing

A 50 µl Vent DNA polymerase reaction was carried out as described above except that all dNTPs were provided at 1 mM concentration and radioactivity was omitted. The reaction was extracted with phenol/$CHCl_3$ (1:1) and passed through a G25 Sephadex spin column (Amersham Biosciences). Sequencing was carried out (Stanford PAN facility) following the Sanger procedure [primer: $(CCCTAA)_3$].

RESULTS

In early studies we observed that 18mer C-rich telomeric repeat primers in the presence of longer 42–60 nt

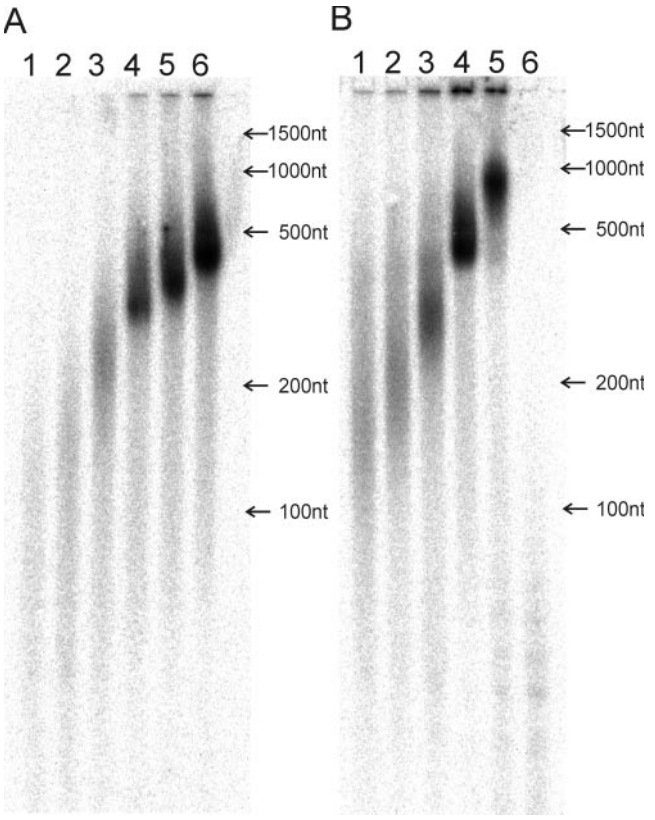

Figure 3. Effects of time and temperature on expansion of telomeric sequences by Vent DNA Polymerase using TC21/TG69 as primer/template. (**A**) Time-course of expansion reaction at 70.9°C. Lane 1: 1 min; lane 2: 2 min; lane 3: 5 min; lane 4: 15 min; lane 5: 30 min; and lane 6: 60 min. (**B**) Expansion reactions at varied temperatures (after 60 min). Lane 1: 60.0°C; lane 2: 61.6°C; lane 3: 65.6°C; lane 4: 70.9°C; lane 5: 76.0°C; and lane 6: 79.6°C.

complementary templates could be extended with Vent DNA polymerase to lengths exceeding the templates. With this polymerase there have been reports describing primer- and template-free *de novo* synthesis of DNA oligomers (28,29). To exclude the possibility of a primer and/or template-independent polymerization reaction in the current studies, we performed three control reactions (Figure 2A, lane 1: lacking primer; lane 2: lacking template; lane 3: lacking primer and template). Under these conditions, none of the reactions displayed any detectable product formation. A different source of uncontrolled polymerization might arise from the ability of the telomeric sequences to fold into higher-order structures such as G-quadruplexes and i-motifs which in principle could facilitate DNA synthesis by a self-priming mechanism. Such a primerless polymerization has been described for other repetitive sequences using Vent DNA polymerase (30). For each primer/template-containing reaction we therefore performed a control experiment that lacked the primer. There was no polymerization visible within these controls (Figure 2B, uneven lanes). Even with long templates (up to 93 nt; Figure 2B, lane 9), no self-priming was observed.

Vent DNA polymerase is frequently used as a thermostable enzyme in DNA preparation and PCR amplification techniques for non-repetitive sequences, without the occurrence of extension beyond the template. We, therefore, tested whether the repetitive nature of our sequence was a

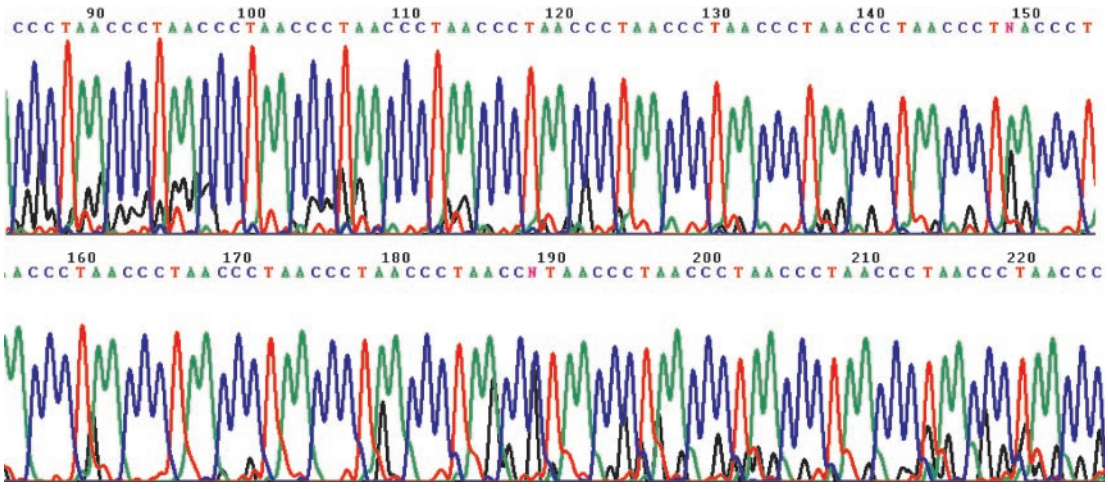

Figure 4. Electropherogram of sequenced expansion products. A Vent DNA polymerase expansion reaction using TC21/TG69 as primer/template was purified and Sanger sequenced using the primer $(CCCTAA)_3$.

requirement for the expansion phenomenon. We performed a reaction with a non-repetitive primer–template pair that was derived by scrambling the telomeric repeat sequence. As expected, the extension reaction generated a product that corresponded to the length of the template used (Figure 2A, lane 4).

In contrast, if the repetitive telomeric sequence was used, products much longer than the present template were observed. Four reactions were carried out with increasing template lengths (21, 45, 69 and 93 nt; lanes 2, 4, 6 and 8 in Figure 2B). With increasing template sizes, longer products were generated. We next raised dTTP concentration since it seemed limiting in the expansion reactions (dTTP was present at low concentrations for assisting the visualization of products by incorporating [α-^{32}P]dTTP). Significantly, by raising the dTTP concentration from 0.05 to 0.25 mM, a more narrow distribution of length was observed (when all four dNTPs were present at 1 mM concentration, a very sharp length distribution of products was generated, see below). At a temperature of 70.9°C, an increase in product quantity as well as of length was observed over time. After 1 h, the majority of products had grown to a length of 500 nt, starting from a 69 nt template and a 21 nt primer (Figure 3A). We next characterized the temperature dependence of the isothermal reaction. When testing a range of temperatures from 60 to 79.6°C, an increase in product quantity and length was observed, with lengths reaching 1 kb at 76°C. Interestingly, at higher temperatures no significant product formation was observed. This is most probably owing to inefficient primer–template annealing, as the calculated melting point for the primer/template interaction using nearest neighbor algorithms is 58.4°C.

To check whether the sequence expansion proceeds from a small or large fraction of primers, we carried out an expansion reaction as before but instead of visualizing products by incorporating [α-^{32}P]dTTP, we used a 5′-^{32}P-end-labeled primer. The results show that >95% of the primer was elongated to very long products (Supplementary Figure S1). The dNTP concentration was 1 mM each in this case, which resulted in a very narrow length distribution of the expanded products. Comparison with markers suggested that >90% of the products fell into the 550–650 bp range. This finding suggests that the 0.25 mM dTTP concentration used in the Figure 3 experiments was limiting the reaction.

Next we evaluated whether the DNA strands synthesized are polymerized in a template-specific manner, yielding repeats of the telomeric hexamer sequence. First we sequenced the obtained products using an 18 nt primer $(CCCTAA)_3$, which confirmed that the hexamer repeat of the telomeric sequence was indeed obtained (Figure 4). In addition, we performed expansion reactions lacking specific dNTPs. Owing to the nucleotide sequence of the hexamer repeat, product formation should not take place in reactions lacking dATP and dTTP. Since we used [α-^{32}P]dTTP to monitor the reaction, we only performed the reaction lacking dATP. No product was observed for C-rich primer/G-rich template reaction (lane 2, Figure 5) as well as G-rich primer/C-rich template reaction (lane 6, Figure 5) in accordance with a sequence-specific polymerization mechanism. Since the telomeric sequence contains no guanines in one strand and hence no cytosines in the complementary strand, one would expect that only one strand can be synthesized in reactions lacking either dGTP or dCTP.

When using a G-rich template strand and a C-rich primer, product formation was in fact observed for the reaction lacking dGTP (lane 3, Figure 5), suggesting the production of a long single strand of the C-rich sequence. The same was observed with reactions containing the C-rich template strand and the G-rich primer lacking dCTP (lane 8, Figure 5), suggesting synthesis of single-stranded G-rich sequence. In these products, the length distribution was considerably broader than that observed with all 4 nt. These experiments suggested that the 69 nt template is able to slip along the extending primer as it increases in length.

In contrast, no synthesis was observed when dCTP was omitted in reactions containing C-rich template and G-rich primer, as well as when dGTP was omitted when a G-rich template and C-rich primer was used (lanes 4 and 7 for G-rich template and C-rich template, respectively) This finding established that the template cannot be extended when the complementary primer is not extended as well. In other words, the longer oligonucleotide always acts as a template; no 'priming' of the shorter oligonucleotide (primer) by the longer one occurred. These experiments showed that the formation of

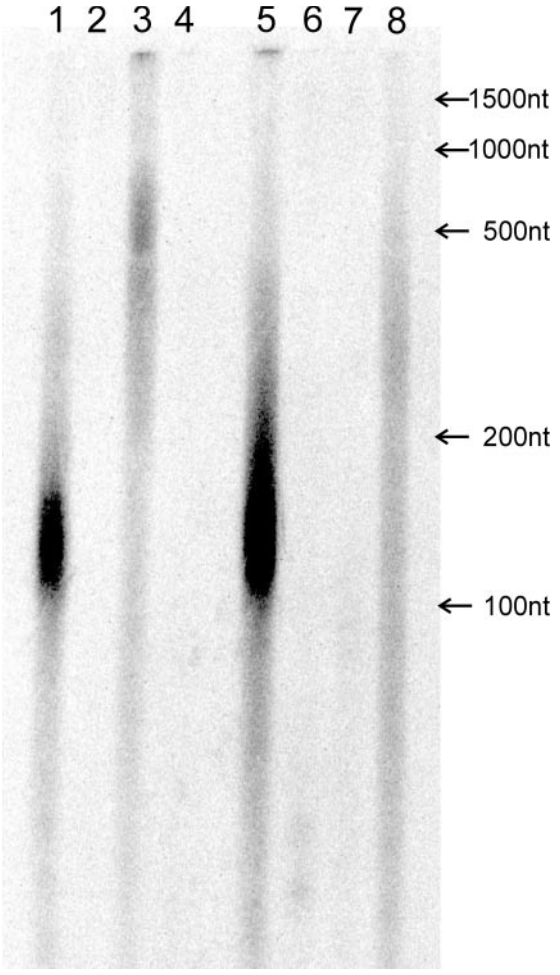

Figure 5. Vent expansion lacking distinct dNTPs. Lanes 1–4: TC21/TG69 as primer/template; lanes 5–8: TG21/TC69 as primer/template, all reactions contained dTTP at a concentration of 0.05 and 1 mM of the indicated dNTPs. Lane 1/5: all four dNTPs present; lane 2/6: lacking dATP; lane 3/7: lacking dGTP; and lane 4/8: lacking dCTP.

long single-stranded G-rich as well as C-rich telomeric repeats is possible, although with reduced efficiency compared with double-strand synthesis. The most efficient product formation was obtained in reactions containing all four dNTPs (lanes 1 and 5, containing 0.05 mM dTTP). Taken together, the experiments complement the sequencing data by demonstrating that mis-insertion is not observed and, thus, the telomeric sequence is generated in a template-specific manner.

Next we studied the expansion of a DNA sequence different from the telomeric repeats. We tested a mini-satellite sequence composed of a repetitive unit 10 nt in length (31). In these experiments the satellite DNA was also efficiently expanded by Vent DNA polymerase, but with an optimum temperature higher than the telomeric sequence (Supplementary Figure S2). A possible explanation lies in the increased length of the decamer repetitive unit compared with the telomeric hexamer repeat. For expansion by a slipped strand mispairing mechanism, a larger stretch of double-stranded DNA (dsDNA) would need to be melted, requiring higher temperatures.

To check whether the expansion activity of Vent DNA polymerase is a more general feature of thermostable polymerases when acting on repetitive sequences, we next tested the DNA polymerase of *Thermus aquaticus* in our studies. *Taq* DNA polymerase has been reported to moderately facilitate the expansion of repeats (11,12,14,15). No product formation was observed under our conditions (data not shown). This discrepancy could be explained by the fact that no thermal cycling was carried out in our experiments, in contrast to the previous studies with this enzyme.

DISCUSSION

We have shown that the Vent DNA polymerase isolated from the archaeon *T.litoralis* can be used to generate long repetitive sequences with high efficiency. The mechanism of repeat expansion is most probably based on strand slippage resulting in primer–template misalignment. The mechanism depicted in Figure 1A represents primer–template slippage after full dissociation of the two strands; however, partial melting of the ends of dsDNA followed by mis-annealing to leave a loop would be sufficient to give an extendable structure (Figure 1B).

A possible explanation for the efficient expansion activity of Vent DNA polymerase when replicating repetitive sequences might be represented by its combination of low processivity (7 nt are incorporated at each enzyme binding event) and a relatively high extension rate (4000 nt/min) (32). This combination results in high turnover of polymerase-bound and free primer–template complex compared with other thermostable polymerases such as *Taq* (33). Such rapid turnover would facilitate primer/template mis-annealing which can probably only occur if the enzyme is not bound (34). This hypothesis would also explain the activity of *Taq* and other enzymes in repeat expansion when thermal cycling is carried out, since the elevated temperature results in dissociation of the enzyme as well as the primer–template complex.

As a preparative method, the use of Vent DNA polymerase to generate long, repetitive stretches of DNA has some distinct advantages over other approaches. Compared with other enzymes used previously (12,14,16), Vent DNA polymerase is more efficient both in terms of product quantity (as judged by fraction of primer extension) and by length of the products. In addition, the products are characterized by a narrow length distribution. Importantly, the observed expansion occurs without thermal cycling, which greatly simplifies the reaction and obviates the need for costly thermal cyclers. Moreover, the polymerase displays high replication fidelity (32,35), and the length of the generated product can be controlled by adjusting incubation time and temperature.

A rolling circle strategy has been reported to produce long telomeric repeats isothermally as well (25–27). The new Vent-catalyzed expansion may be complementary to that approach, since the current strategy most efficiently generates double-stranded products, while the rolling circle strategy produces single strands. For single-stranded expansion, the current approach generates shorter products than the rolling circle approach, but is simpler since it does not require the preparation of a circular template.

Finally, the current isothermal mechanism of DNA polymerase-mediated expansion of repetitive sequences

could have biological significance. Although most human tumors express telomerase to maintain their telomeres, a fraction (7–10%) were demonstrated to use an alternative form of telomere maintenance (36). Alternative lengthening of telomeres is believed to occur via a recombination mechanism (possibly involving inter-telomeric copying, t-loops, as well as linear and/or circular extrachromosomal telomeric DNA), although the precise mechanism remains unknown (37). Therefore, it cannot yet be ruled out that DNA polymerase-mediated strand slippage expansions of telomeric sequences contribute to alternative mechanisms of telomere elongation. Notably, it has been proposed that the polymerase α/primase complex could be involved in telomere maintenance (15).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

We thank the US National Institutes of Health (GM069763) for support. J.S.H. was supported by a Fellowship of the Deutsche Forschungsgemeinschaft (DFG). Funding to pay the Open Access publication charges for this article was provided by the U.S. National Institutes of Health.

Conflict of interest statement. None declared.

REFERENCES

1. Makalowski,W. (2001) The human genome structure and organization. *Acta Biochim. Pol*., **48**, 587–598.
2. Chan,S.R. and Blackburn,E.H. (2004) Telomeres and telomerase. *Philos. Trans. R Soc. Lond. B Biol. Sci*., **359**, 109–121.
3. Cech,T.R. (2000) Life at the end of the chromosome: telomeres and telomerase. *Angew. Chem. Int. Ed. Engl*., **39**, 34–43.
4. Shay,J.W. and Wright,W.E. (2000) Hayflick, his limit, and cellular ageing. *Nature Rev. Mol. Cell. Biol*., **1**, 72–76.
5. Armour,J.A. and Jeffreys,A.J. (1992) Biology and applications of human minisatellite loci. *Curr. Opin. Genet. Dev*., **2**, 850–856.
6. Mohmood,S., Sherwani,A., Khan,F., Khan,R.H. and Azfer,A. (2003) DNA trinucleotide repeat expansion in neuropsychiatric patients. *Med. Sci. Monit*., **9**, RA237–RA245.
7. Vincent,J.B., Paterson,A.D., Strong,E., Petronis,A. and Kennedy,J.L. (2000) The unstable trinucleotide repeat story of major psychosis. *Am. J. Med. Genet*., **97**, 77–97.
8. Levinson,G. and Gutman,G.A. (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Mol. Biol. Evol*., **4**, 203–221.
9. Steinberg,E.K., Lindner,K.R., Gallea,J., Maxwell,A., Meng,J. and Allendorf,F.W. (2002) Rates and patterns of microsatellite mutations in pink salmon. *Mol. Biol. Evol*., **19**, 1198–1202.
10. Kunkel,T.A. (1992) DNA replication fidelity. *J. Biol. Chem*., **267**, 18251–18254.
11. Ji,J., Clegg,N.J., Peterson,K.R., Jackson,A.L., Laird,C.D. and Loeb,L.A. (1996) *In vitro* expansion of GGC:GCC repeats: identification of the preferred strand of expansion. *Nucleic Acids Res*., **24**, 2835–2840.
12. Wu,M.J., Chow,L.W. and Hsieh,M. (1998) Amplification of GAA/TTC triplet repeat *in vitro*: preferential expansion of $(TTC)_n$ strand. *Biochim. Biophys. Acta*, **1407**, 155–162.
13. Ohshima,K. and Wells,R.D. (1997) Hairpin formation during DNA synthesis primer realignment *in vitro* in triplet repeat sequences from human hereditary disease genes. *J. Biol. Chem*., **272**, 16798–16806.
14. Takahashi,N., Sasagawa,N., Suzuki,K. and Ishiura,S. (1999) Synthesis of long trinucleotide repeats *in vitro*. *Neurosci. Lett*., **262**, 45–48.
15. Nozawa,K., Suzuki,M., Takemura,M. and Yoshida,S. (2000) *In vitro* expansion of mammalian telomere repeats by DNA polymerase alpha-primase. *Nucleic Acids Res*., **28**, 3117–3124.
16. 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.
17. Simonsson,T. (2001) G-quadruplex DNA structures—variations on a theme. *Biol. Chem*., **382**, 621–628.
18. Ahmed,S., Kintanar,A. and Henderson,E. (1994) Human telomeric C-strand tetraplexes. *Nature Struct. Biol*., **1**, 83–88.
19. Blackburn,E.H. (2001) Switching and signaling at the telomere. *Cell*, **106**, 661–673.
20. Stansel,R.M., de Lange,T. and Griffith,J.D. (2001) T-loop assembly *in vitro* involves binding of TRF2 near the 3′ telomeric overhang. *EMBO J*., **20**, 5532–5540.
21. Parkinson,G.N., Lee,M.P. and Neidle,S. (2002) Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature*, **417**, 876–880.
22. Kanaori,K., Shibayama,N., Gohda,K., Tajima,K. and Makino,K. (2001) Multiple four-stranded conformations of human telomere sequence d(CCCTAA) in solution. *Nucleic Acids Res*., **29**, 831–840.
23. Phan,A.T. and Mergny,J.L. (2002) Human telomeric DNA: G-quadruplex, i-motif and Watson–Crick double helix. *Nucleic Acids Res*., **30**, 4618–4625.
24. Potaman,V.N., Oussatcheva,E.A., Lyubchenko,Y.L., Shlyakhtenko,L.S., Bidichandani,S.I., Ashizawa,T. and Sinden,R.R. (2004) Length-dependent structure formation in Friedreich ataxia (GAA)n*$(TTC)_n$ repeats at neutral pH. *Nucleic Acids Res*., **32**, 1224–1231.
25. Lindstrom,U.M., Chandrasekaran,R.A., Orbai,L., Helquist,S.A., Miller,G.P., Oroudjev,E., Hansma,H.G. and Kool,E.T. (2002) Artificial human telomeres from DNA nanocircle templates. *Proc. Natl Acad. Sci. USA*, **99**, 15953–15958.
26. Hartig,J.S. and Kool,E.T. (2004) Small circular DNAs for synthesis of the human telomere repeat: varied sizes, structures and telomere-encoding activities. *Nucleic Acids Res*., **32**, e152.
27. Hartig,J.S., Fernandez-Lopez,S. and Kool,E.T. (2005) Guanine-rich DNA nanocircles for the synthesis and characterization of long cytosine-rich telomeric DNAs. *Chembiochem*, **6**, 1458–1462.
28. Ogata,N. and Miura,T. (1997) Genetic information 'created' by archaebacterial DNA polymerase. *Biochem. J*., **324**, 667–671.
29. 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.
30. 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.
31. Boan,F., Gonzalez,A.I., Rodriguez,J.M. and Gomez-Marquez,J. (1997) Molecular characterization of a new human minisatellite that is able to form single-stranded loops *in vitro* and is recognized by nuclear proteins. *FEBS Lett*., **418**, 251–257.
32. Kong,H., Kucera,R.B. and Jack,W.E. (1993) Characterization of a DNA polymerase from the hyperthermophile archaea *Thermococcus litoralis*. Vent DNA polymerase, steady state kinetics, thermal stability, processivity, strand displacement, and exonuclease activities. *J. Biol. Chem*., **268**, 1965–1975.
33. Pavlov,A.R., Pavlova,N.V., Kozyavkin,S.A. and Slesarev,A.I. (2004) Recent developments in the optimization of thermostable DNA polymerases for efficient applications. *Trends Biotechnol*., **22**, 253–260.
34. Viguera,E., Canceill,D. and Ehrlich,S.D. (2001) Replication slippage involves DNA polymerase pausing and dissociation. *EMBO J*., **20**, 2587–2595.
35. Mattila,P., Korpela,J., Tenkanen,T. and Pitkanen,K. (1991) Fidelity of DNA synthesis by the *Thermococcus litoralis* DNA polymerase—an extremely heat stable enzyme with proofreading activity. *Nucleic Acids Res*., **19**, 4967–4973.
36. Stewart,S.A. (2005) Telomere maintenance and tumorigenesis: an 'ALT'ernative road. *Curr. Mol. Med*., **5**, 253–257.
37. Henson,J.D., Neumann,A.A., Yeager,T.R. and Reddel,R.R. (2002) Alternative lengthening of telomeres in mammalian cells. *Oncogene*, **21**, 598–610.