

Initiation of DNA Replication by a Third Parallel DNA Strand Bound in a Triple-Helix Manner Leads to Strand Invasion[†]

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ABSTRACT: According to current knowledge, DNA polymerases accommodate only two polynucleotide strands in their catalytic site: the template and the primer to be elongated. Here we show that in addition to these two polynucleotide strands, HIV-1 and AMV reverse transcriptases, human DNA polymerases β , γ , and λ , and the archaeobacterial Dpo4 can elongate 10-nucleotide primers bound in a triple-helix manner to hairpin duplex DNA tethered by a few thymidine residues. The elongation occurs when the primer is parallel to the homologous strand. This feature was confirmed by using complementary single-stranded DNA with restricted nucleotide composition which bound polypurine and polypyrimidine primers at an asymmetric site. The results unambiguously confirmed the previous experiments, showing binding of the primer strand parallel to the homologous sequence. The common feature of these DNA polymerases is that they all elongated dG-rich primers, whereas they behaved differently when other polynucleotide sequences were used. Interestingly, only five to seven dG residues at similar positions between the primer and its binding site can allow elongation, which may even be facilitated by a single C/C mismatch. We suggest that DNA polymerases displace the primer from Hoogsteen bonds to form Watson–Crick pairings, enabling subsequent priming of replication. These experiments indicate that DNA polymerases may bind three DNA strands, as RNA polymerases do, and provide a molecular basis for 3'-OH invasion at short similar sequences in the DNA double helix, yielding potential DNA rearrangements upon single-strand breakage.

The mechanism underlying the replication of genetic information involves several steps. Basically, upon separation of each polynucleotide strand of the double helix by DNA helicases, primases synthesize complementary oligonucleotides whose 3'-OH ends are elongated by DNA polymerases. They incorporate the complementary deoxyribonucleoside monophosphates through phosphodiester bonds, with the concomitant release of pyrophosphate (1). The free energy released from this reaction allows translocation and conformational changes of the enzyme, the catalytic activity of which involves an acidic amino acid triad and two metal ions (2). Hence, DNA polymerases have been considered to

bind only two DNA strands in their catalytic site: the template and the primer.

Studies on the origin of mitochondrial DNA rearrangements, first described in 1988 (3), showed that they mainly occur between two direct repeats (DR)¹ of 7–13 bp separated by several kilobases. We noticed that the neo-synthesized direct repeats display a skewed base composition (4) (purine-rich) as compared with the overall direct repeats of the mtDNA.

This observation suggested to us the possibility that a three-stranded structure could form between this neo-synthesized sequence and the double-stranded homologous sequence. This was first described for a two poly(U)-one poly(A) molecule in 1957 by Felsenfeld et al. (5) and then extended by a number of studies to other polynucleotides, including intramolecular (mirror) sequences (H-DNA) possibly involved in gene regulation (6). As a general rule, the third polypurine (G or A) or polypyrimidine (C, T, or U) strand binds in an antiparallel orientation (7) to the homologous strand in the major groove of the duplex DNA through Hoogsteen (pyrimidine strand) or reverse Hoogsteen (purine strand) bonds. Triple helices can induce inhibition of DNA replication (8), transcription (9), and recombination (10).

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¹ Abbreviations: THP, triple-helix primer; PBS, primer binding site; DR, direct repeat; Pol, DNA polymerase; mt, mitochondrial; ddG, dideoxyguanosine.

Protein binding to triplex DNA has been reported, and the access of triple-helix oligonucleotides during the cell cycle has been studied (11).

We thus proposed (12) a slippage mispairing mechanism in which the neo-synthesized (DR1-DNA polymerase) complex could dissociate from its template and reassociate with the homologous double-stranded DR2 site by the formation of a triple helix, thus bypassing the intervening sequence. Asymmetric synthesis of the two strands of mitochondrial DNA would lead to deletion of the intervening sequence. Previous experiments had shown that short oligonucleotides forming triple helices can prime DNA replication on double-stranded DNA (13) by phage T7 and bacterial DNA polymerases.

To evaluate the generality of these novel properties, we first tested several combinations of primers and double-stranded DNA templates, with various DNA polymerases of widely different origins and functions [HIV-1 and AMV reverse transcriptases, human mitochondrial DNA polymerase γ , human repair polymerases β and λ , and Dpo4 from the thermophilic Archaeobacteria *Sulfolobus solfataricus* (14)]. The unusual parallel orientation of the primer to the homologous strand of the template was then tested, and the product was sequenced using an RNA primer. Finally, longer primers presenting differences with the binding site revealed that microhomologies could allow primer elongation.

Taken together, these data highlight the common property of 3'-end invasion by deoxyguanosine-rich polynucleotides on homologous or partly heterologous double-stranded DNA by all tested DNA polymerases, suggesting a new mechanism involved in DNA metabolism.

EXPERIMENTAL PROCEDURES

HPLC-purified oligonucleotides were purchased from Eurogentec (Seraing, Belgium) or from Sequentia (Evry, France). DNA polymerase γ subunits were a gift of W. C. Copeland (National Institutes of Health, Bethesda, MD). Human DNA polymerase β was purchased from Trevigen (Gaithersburg, MD). HIV and AMV reverse transcriptases were from Amersham Biosciences (Saclay, France). Dpo4 was purified according to the method described in ref 14.

A plasmid encoding DNA polymerase λ was a gift from L. Blanco. Pol λ protein was purified by nickel chelation chromatography according to Novagen's protocol with the following modifications. Cells were lysed by being frozen (16 h at -70°C) and slowly thawed on ice in the presence of 20 mg/mL lysozyme and 1% Triton X-100. Proteins were concentrated and stored in buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 20% glycerol. Protein purity was estimated to be $>90\%$ by visual inspection of Coomassie Blue-stained 10% SDS-polyacrylamide gels.

Replication Assays. The 5' ^{32}P end-labeled primers (0.5–1 pmol) were mixed with 100 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 15 mM dithiothreitol, dNTPs (0.2 mM each), and 100 pmol of template in a volume of 10 μL , to give a template/primer (P) ratio of 100. Reactions were started by the addition of 4×10^{-8} M DNA polymerase γ ($\alpha\beta_2$), 7×10^{-8} M Dpo4, 1.5×10^{-8} M human DNA polymerase λ , or 2.9×10^{-8} M HIV-1 or AMV reverse transcriptase. After incubation for 30 min at 37°C (except human λ DNA

polymerase, 60 min), samples were heat-denatured and subjected to electrophoresis on a 20% polyacrylamide gel containing 8 M urea in 50 mM Tris-Borate (pH 7.5), 2 mM EDTA buffer and to autoradiography. For DNA polymerase β , reactions were conducted in buffer B containing 50 mM Tris-HCl (pH 8.8), 10 mM MgCl_2 , 10 mM KCl, 1 mM dithiothreitol, 0.2 mM dNTP, 1% glycerol, and 7×10^{-8} M human DNA polymerase β ($E/P = 7$).

Formation of Duplexes. Each single-stranded DNA (50 pmol) was heat-denatured at 95°C in buffer B lacking dNTPs and then slowly cooled to 37°C for 90 min.

DNA Sequencing. Hybridization mixtures of 20 pmol of each single-stranded DNA Pur 72 template prepared as described above were divided into two equal parts. Five picomoles of ^{32}P end-labeled DNA or RNA primers Pur 10 ($T/P = 2$) and 7×10^{-8} M human DNA polymerase β were added ($E/P = 1$). The sequencing reactions were conducted for 5 min by the dideoxy termination chain reaction (Amersham France). The mixes were as follows: A mix, dCTP, dGTP, and dTTP (280 μM each), 31.2 μM dATP, and 4.6 μM ddATP; G mix, dATP, dCTP, and dTTP (280 μM each), 31.2 μM dGTP, and 4.6 μM ddGTP; C mix, dATP, dGTP, and dTTP (280 μM each), 31.2 μM dCTP, and 5.6 μM ddCTP; T mix, dATP, dCTP, and dGTP (280 μM each), 31.2 μM dTTP, and 4.6 μM ddTTP. Reaction products were heat-denatured and separated by electrophoresis on a 20% polyacrylamide gel containing 8 M urea and then subjected to autoradiography.

Modeling of the Complex. Swiss PDB Viewer (Expasy) was used to generate the superposition of the finger domain of Klenow fragment [Protein Data Bank (PDB) entry 1knf] on the finger domain of Dpo4 (PDB entry 1jx4). Pymol (DeLano Scientific) was used to generate the image.

Oligonucleotide Sequences Not Presented in the Figures. For calculation of the T_m of PBS, we used the common formula $2(A+T) + 4(G+C)$, $T_m(\text{PBS DR}) = 72^\circ\text{C}$, $T_m(\text{PBS Pur}) = 72^\circ\text{C}$.

RESULTS

Comparison of Elongation of dG-Rich Primers on Hairpin Double-Stranded DNA Templates by Several DNA Polymerases. To maintain the DNA strand stoichiometry, we designed linked complementary strands tethered by four or two thymidine residues. The primer binding site of 10 bp displays a 5'–3' sequence similar to the primer on the lower strand and is followed by 20 bp. Elongation of the 5' ^{32}P end-labeled primers yields a 30-nucleotide product. Our previous experiments had been conducted with two enzymes belonging to the replicative family A (13). One is the human mitochondrial DNA polymerase γ , an ($\alpha\beta_2$) heterotrimeric complex whose 50 kDa β component is the processive factor and whose 130 kDa α component is the catalytic subunit (which shares homology with the phage T7 DNA polymerase of 80 kDa). Elongation of the dG-rich primer P1 (10 nucleotide) previously shown to form a triple helix (12) was studied with templates DR64 of 30 bp, DR64A, DR64C, and DR62C, having either three A/A mismatches or a single C/C mismatch and four or two thymidines as linkers. As shown in Figure 1A, comparable elongations of P1 to 30 nucleotides were observed with all these templates, and the presence of mismatches did not significantly increase the amount of P1

5' AAAAAAAAAA	64A10Para3A	TmPBS=40 °C
TTTTTTTTTTAAAGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
AAAAAAAAAAACATAGTAGAAGAGAATG3'		
5' AAAAAAAAAA	64A10Anti	
AAAAAAAAAAGCGGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
TTTTTTTTTTCGGCATAGTAGAAGAGAATG3'		
5' AAAAAAAAAA	64A10Anti3A	
AAAAAAAAAAGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
TTTTTTTTTTAAACATAGTAGAAGAGAATG3'		
5' CCCCCCCCCC	64C10Para3A	TmPBS=80 °C
GGGGGGGGGAAAGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
CCCCCCCCCAACATAGTAGAAGAGAATG3'		
5' CCCCCCCCCC	64C10Anti	
CCCCCCCCCGCGGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
GGGGGGGGGCGGCATAGTAGAAGAGAATG3'		
5' CCCCCCCCCC	64C10Anti3A	
CCCCCCCCCAAGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
GGGGGGGGGAAACATAGTAGAAGAGAATG3'		
5' TTTTTTTTTT	64T10Para3A	TmPBS=40 °C
AAAAAAAAAAGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
TTTTTTTTTTAAACATAGTAGAAGAGAATG3'		
5' TTTTTTTTTT	64T10Anti	
TTTTTTTTTTGCGGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
AAAAAAAAAAGCGCATAGTAGAAGAGAATG3'		
5' TTTTTTTTTT	64T10Anti3A	
TTTTTTTTTTAAAGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
AAAAAAAAAAGCATAGTAGAAGAGAATG3'		
5' GGATTACGAG	64RandomPara	TmPBS=60 °C
CCTAATGCTCGCGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
GGATTACGAGCGGCATAGTAGAAGAGAATG3'		
5' GGATTACGAG	64RandomPara3A	
CCTAATGCTCAAAGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
GGATTACGAGAAACATAGTAGAAGAGAATG3'		
5' GGATTACGAG	64RandomAnti	
GGATTACGAGCGCGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
CCTAATGCTCCGGCATAGTAGAAGAGAATG3'		
5' GGATTACGAG	64randomAnti3A	
GGATTACGAGAAAGTATCATCTTCTCTTAC5'		
T4 ::::::::::	::::::::::::::::	
CCTAATGCTCAAACATAGTAGAAGAGAATG3'		

elongation (compare lane 2 with lanes 3–5). However, the elongation rates were much slower than that observed for the control primer annealed with a single-strand template (lane 1), possibly due to dissociation of the processive subunits. We then examined the generality of these results

by testing the X family human DNA polymerase β (15) and λ (16) involved in DNA repair and Dpo4 (14) from the thermophilic Archaeobacteria *S. solfataricus* akin to the human DNA polymerase from family Y. Figure 1A shows that P1 was not significantly elongated by any of the DNA polymerases on DR64 presenting no mismatches (although a slight smear appeared with pol β and Dpo4, lane 2), while either three A/A mismatches or a single C/C mismatch allowed full elongation (lanes 3 and 4) comparable to that obtained with the single-stranded template (lane 1). The product of elongation with DNA polymerase λ was slightly shorter than 30 nucleotides, due to template misalignment and slippage (16). The number of thymidine residues in the linkers did not modify DNA elongation as exemplified by DNA polymerase γ (Figure 1A, lane 5).

As we previously showed that phage T7 DNA polymerase elongates P1 (13), and since *Escherichia coli* DNA polymerase I may behave as a reverse transcriptase, we tested other polymerases, i.e., the HIV-1 (17) and AMV (avian myeloblastoma virus) reverse transcriptases, which synthesize a double-stranded DNA from their retrotranscribed RNA template, for integration into the genome. The results displayed in Figure 1A show that they behave like DNA polymerases β and λ and Dpo4.

These experiments show that all DNA polymerases tested can elongate a primer bound to a double-stranded DNA presenting a similar sequence of parallel orientation and that a mismatch is generally required for their full elongation. An exception is DNA polymerase γ , which must replicate this mitochondrial DNA sequence. Nevertheless, the extent of elongation of P1 is 5–10-fold lower with DR64 (and DR64 templates containing a mismatch) than with the control single-strand 30 mer (lanes 1 and 2). To compare the different reaction rates between a single- and double-stranded DNA template, we studied the elongation by DNA polymerase β of P1 with either the 30 mer single-strand template or DR62C with a C/C mismatch. Figure 1B indicates only a 2–3-fold lower reaction rate with DR62C, as quantified by phosphorimaging. DNA polymerases behave differently with these G-rich primers and templates. Therefore, we tested other combinations of primers and templates.

Importance of the Primer Sequences on Their Elongation by Several DNA Polymerases. To test the importance of the sequence of the primer–template complex, the P1 sequence was modified to produce the symmetric Pur10, which, however, still contained eight dG residues [d(G₃AG₂AG₃)]. As shown in Figure 1C, full elongation by human DNA polymerase β was observed on Pur62, presenting no mismatch, as well as on template Pur62C, harboring one C/C mismatch (lanes 5 and 6). Thus, replacing 5'-d(TG₄AG₄) (P1) with d(G₃AG₂AG₃) (Pur10) and its homologous DNA binding site facilitated DNA elongation, indicating that productive elongation of primers depends on their sequence.

Since this property might be restricted to dG-rich primer–template forms, DNA polymerases were then tested with homopolynucleotide primers d(A)₁₀, d(T)₁₀, and d(C)₁₀, known to form triple helices (7, 18), along with their corresponding hairpin templates. We also tested with or without three A/A mismatches, and the homologous strand in parallel or antiparallel orientation to the THP (triple helix primer) (Experimental Procedures). No significant elongation was observed with any of the polymerases tested when the

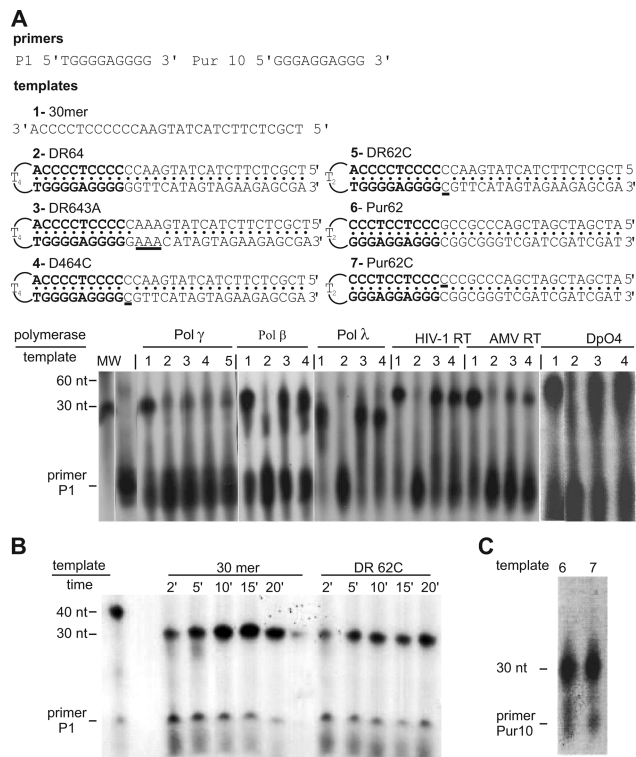


FIGURE 1: (A) Elongations of primer P1 on hairpin DNA templates by several DNA polymerases. Autoradiogram showing elongation products with DR templates primed by P1. Pol γ : human mitochondrial DNA polymerase γ . Pol β : human or rat DNA polymerase β . Pol λ : human DNA polymerase λ . HIV-1 RT: human immunodeficiency virus-1 reverse transcriptase. AMV RT: avian myeloblastosis virus reverse transcriptase. Dpo4: archaeobacterial DNA polymerase from *Thermophilus solfataricus*. (B) Kinetic experiment with human DNA polymerase β on single- and double-stranded templates. Elongation of P1 at different times on the 30 mer (left panel) and DR62C (right panel). (C) Comparison of primer elongation by human DNA polymerase β on hairpin templates with or without a mismatch. Lane 6 shows the elongation product on template Pur62 and lane 7 on template Pur62C primed by Pur10.

THPs were in antiparallel orientation to the homologous strand even when presenting three A/A mismatches (data not shown).

Elongation only occurred (Table 1) when the THPs were parallel to the homologous strand, and no mismatch was required. Since these variations differed from one DNA polymerase to another (Table 1), they ruled out the trivial hypothesis of an opening of the hairpin templates and elongation on the single-stranded template.

Interestingly, although human DNA polymerase β elongated all the primers that were tested, Dpo4 elongated specifically d(T)₁₀ and d(C)₁₀ while human DNA polymerases γ and λ , and the reverse transcriptases, did not show any elongation of d(A)₁₀, d(C)₁₀, and d(T)₁₀ even with a 3' end ribose. The use of random primer 5'-G₂AT₂ACGAG-3' and its corresponding primer binding site parallel or antiparallel with a three-A/A mismatch with DNA polymerase β did not lead to any elongation. Oligonucleotide primers known to form triplexes enable DNA elongation. However, their unusual orientations require further experiments.

Triple-Helix Primers Are Oriented Parallel to the Homologous Strand. Since the parallel binding of the triple helix primer to the double-strand DNA binding site is noncon-

ventional (7, 18) and may be due to an artefact of the thymidine linker, we investigated this result using an orthogonal method. DNA duplexes were designed by hybridizing equimolar amounts of two complementary single-stranded DNA fragments of 72 (or 73) nucleotides (one polypurine, one polypyrimidine). They were composed of 20 bp, followed by 10 bp (or 11, Pyr11) corresponding to the priming binding site flanked on each side by a C/C mismatch to favor elongation in both orientations, and ending with 40 bp (Figure 2).

The products from the 5' end ³²P-labeled THP in parallel or antiparallel orientation to the homologous strand could be distinguished by the size of the elongation product. Depending on which strand is replicated, a choice determined by the THP orientation and the dNTPs included in the reaction mixture, the length of the elongation product must be either 31 nucleotides in the presence of dATP and dGTP or 51 nucleotides in the presence of dGTP, dCTP, and dTTP. As shown in Figure 2, in lanes 1–4 corresponding to the elongation products using either d(A)₁₀ (lanes 1 and 2) or d(T)₁₀ (lanes 3 and 4) as primers and the two same aliquots of the hybridized DNA solution, no elongation of d(A)₁₀ could be detected using dATP and dGTP (lane 1), whereas an elongation product of approximately 50 nucleotides was observed with dGTP, dCTP, and dTTP (lane 2). These results demonstrate that the lower strand was replicated, thus indicating an orientation of d(A)₁₀ parallel to the homologous sequence of the upper strand. Conversely, using d(T)₁₀ as primer, elongation products of approximately 30 nucleotides were formed using dATP and dGTP (lane 3) while no elongation was detected with dGTP, dCTP, and dTTP (lane 4). By using primer P1 (TG₄AG₄) and the two dNTP compositions as well as a binding site ending in a C/C mismatch, a product of approximately 30 nucleotides was obtained (lanes 5 and 6), indicating that the lower strand is replicated and thus that the primer binds parallel to its homologous sequence. Similar results were obtained with other primers displaying symmetry: Pur10 G₃AG₂AG₃ (lanes 7 and 8) and Pyr11 CT₂CT₃CT₂C (lanes 9 and 10). Elongation products of approximately 30 nucleotides confirmed the parallel orientation of these THPs to the homologous strand, even for the polypyrimidine primer (Pyr11), despite its known antiparallel binding. Consequently, these data allow us to generalize the concept that triple helices can prime DNA replication on double-stranded DNA through a primer parallel to the homologous strand.

DNA Sequencing of the Elongation Products. We then sequenced the elongation product of the ³²P Pur10 primer by DNA polymerase β on the double-stranded template Pur72 using the dideoxy termination method. The sequence revealed an unexpected incorporation of additional dideoxynucleotides, namely, ddA, ddT, and ddC (small letters in Figure 3). This suggested that slippage of the primer had occurred in the forward and reverse directions, as previously inferred from sequencing with Tth sequenase at the purine–pyrimidine–purine transitions of the double-stranded template (13), or that there had been double incorporation of dideoxynucleotides facing the nontemplate strand (Figure 3A).

However, under the same experimental conditions, but using an RNA primer of the same sequence, there was no additional incorporation of ddNTPs. This result may be

Table 1: Elongation by DNA Polymerases of Different DNA Primers with Their Corresponding Hairpin DNA Templates^a

DNA Polymerase	Pol	Pol	Pol	HIV-1 RT	Dpo4
Primers/Templates	beta	gamma	lambda	AMV RT	
5' TGGGGAGGGG P1 / DR64 ACCCCTCCCCCAAGTATCATCTTCTCGCT 5' T ₄ ::::::::::::::::::::::::::::::::::::::: TGGGGAGGGGGGTTTCATAGTAGAAGAGCGA 3'	-	+	-	-	-
5' TGGGGAGGGG P1 / DR64C ACCCCTCCCCCAAGTATCATCTTCTCGCT 5' T ₄ :::::::::::::: ::::::::::::::::::::::: TGGGGAGGGGCGTTTCATAGTAGAAGAGCGA 3'	+	+	+	+	+
5' GGGAGGAGGG Pur10 / Pur62 CCCTCCTCCCGCGCCAGCTAGCTAGCTA 5' T ₂ ::::::::::::::::::::::::::::::::::::::: GGGAGGAGGGCGGCGGGTCGATCGATCGAT 3'	+	+	+	+	+
5' AAAAAAAAAA d(A) ₁₀ / 64A ₁₀ para TTTTTTTTTTTGGCGTATCATCTTCTCTTAC 5' T ₄ ::::::::::::::::::::::::::::::::::::::: AAAAAAAAAACGGCATAGTAGAAGAGAATG 3'	+	-	-	-	-
5' TTTTTTTTTT d(T) ₁₀ / 64T ₁₀ para AAAAAAAAAAGCGGTATCATCTTCTCTTAC 5' T ₄ ::::::::::::::::::::::::::::::::::::::: TTTTTTTTTTTCGGCATAGTAGAAGAGAATG 3'	+	-	-	-	+
5' CCCCCCCCCC d(C) ₁₀ / 64C ₁₀ para GGGGGGGGGCGGTATCATCTTCTCTTAC 5' T ₄ ::::::::::::::::::::::::::::::::::::::: CCCCCCCCCGGCATAGTAGAAGAGAATG 3'	+	-	-	-	+

^a Reaction conditions were as described in Experimental Procedures. The primers of the double-stranded templates are of parallel orientation to their homologous sequence. The other tested template sequences are listed in Experimental Procedures.

interpreted by the higher stability of RNA–dsDNA versus DNA–dsDNA structures determined by biophysical methods (19). The lower stability of the DNA primer on the DNA duplex could enable sliding or stuttering of the DNA polymerase.

Biological Function. We then assessed the biological relevance of these new properties of DNA polymerases within the framework of our proposed mechanism. Specifically, we investigated the DNA invasion process in the absence of any double-strand break, as proposed in our model of DNA rearrangement. To this end, a longer 40 mer polynucleotide, Pur40, ending in the 3′ 10-nucleotide sequence of Pur10, was tested with hairpin templates Pur62 and Pur62C for elongation by human DNA polymerases γ and β , HIV-1 reverse transcriptase, and Dpo4. DR64 and DR62C served as controls.

Elongation products of 60 nucleotides were expected to result from the elongation of 40 mer primer Pur40 by 20 nucleotides. Figure 4 shows that elongation products of 60 nucleotides were obtained when using DNA polymerase γ and a homologous 3′ end primer and binding site (lanes 1 and 2), while no detectable elongation occurred in the heterologous system (lanes 3 and 4). Note the high 3′–5′ exonuclease activity (a property of the N-terminal domain of the enzyme) at the 3′ end of the primer, notably in the heterologous system (lanes 3 and 4).

DNA polymerase β also elongated Pur40 in the homologous system (lanes 1 and 2) with templates Pur62 and Pur62C, as in Figure 1C. Surprisingly, elongation also occurred with the “control” heterologous template DR62C (lane 4). However, no elongation was observed with DR64 presenting no mismatch (lane 3). Similar results were observed with HIV-1 reverse transcriptase and Dpo4. These experiments indicate that invasion of the duplex DNA by a heterologous primer enables elongation by human DNA polymerase β , HIV-1 reverse transcriptase, and Dpo4 but is not for DNA polymerase γ . These results probably reflect the particular structure of DR64, which can be primed by P1 without mismatch using DNA polymerase γ (as shown in Figure 1A, lane 2) but not accommodate and elongate Pur40. This may be due to the structural peculiarity of the DR resulting from the successive G₄ sequences in 5′-d(CCCCTCCCCA-TTTT-TGGGGAGGGG)-3′ which can self-associate into highly stable tetramers forming G-tetrads (20). Alternatively, the other polymerases may be unable to fit to the 3′ end G₃AG₂AG₃ of Pur40 annealed with DR64, unless a C/C mismatch is introduced into this template. These data indicate that the C/C mismatch decreases the extent of neighbor base pairing and stacking and so enables elongation on the DR templates. This was previously noticed with T7 DNA polymerase, even when the mismatch is 5 bp ahead of the theoretical 3′-OH end of the primer (13), and may be

induced, in vivo, by DNA supercoiling. Our data show that mismatches between the primer and its binding site can be productive with as few as five to seven dG residues bound at similar positions of the double helix and can be used to aid priming of further DNA elongation.

DISCUSSION

[illegible]

These data point first to the fact that DNA polymerases display a common enzymatic property through binding and unwinding double-stranded DNA to synthesize a templated polynucleotide from a primer-forming triple helix. This novel observation leads us to conclude that, as with RNA polymerases, these enzymes are able to bind three DNA strands. Previous structural studies had shown that RNA polymerases' catalytic center displays common features (21), but no information concerning possible strand displacement was available for DNA polymerases. On the other hand, to the best of our knowledge, DNA replication always occurs on single-stranded templates mostly provided by helicase activity, in contrast to transcription in which RNA polymerases themselves open the DNA double helix. The DNA strand separation reported here is supported by the recent observation of similar strand displacement activity exhibited by T7 RNA polymerase and *E. coli* DNA polymerase I (22), notably in the form of the Klenow fragment which has been

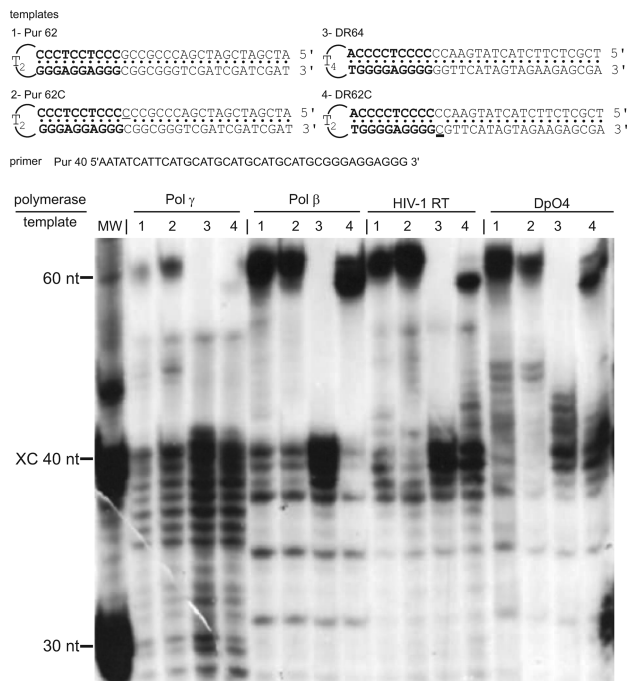


FIGURE 4: Experiments of elongation by several DNA polymerases with primer Pur40 and different double-stranded DNA templates. Autoradiogram showing the elongation of ^{32}P end-labeled primer Pur40 tested with templates Pur62 (lane 1) Pur62C (lane 2), DR64 (lane 3), and DR62C (lane 4) in the replication assay described in Experimental Procedures.

shown (13) to behave like the DNA polymerases tested here. The strand separation is due to the interaction between a conserved phenylalanine located in the three-helix bundle in the finger domain that unzip the DNA duplex (771 in DNA polymerase I and 644 in T7 RNA polymerase). Phenylalanine 53 of Dpo4 also present in the finger domain containing the three-helix bundle (Figure 5) could also explain the strand displacement activity of Dpo4 (F. Boudsocq and R. Woodgate, unpublished observation).

Structural similarities between helical hairpin helical domains (HhH) between DNA polymerase β and RuvA have been pointed out. The formation of a RuvA-ATP tetramer provides the structure selectivity for Holliday junction binding (residues 98–117 and 73–92, respectively) (23). These motifs closely contact the sugar-phosphate backbone of DNA.

The repair human DNA polymerase β elongates all the primers tested, except the random one. Its overexpression in ovarian tumor cells and in chronic myelogenous leukemia has been reported to enhance genomic instability (24) and increase the frequency of the mutator phenotype. Its overexpression has been shown to increase the level of DNA strand breakage and homologous recombination, leading to higher genetic instability (25).

In *E. coli*, several repair pathways have been described: double-stranded break repair, recombination-dependent replication, and replication-dependent recombination (26). These steps involve transient three-stranded structures mediated by RecA. However recombination between homologous sequences in *recA* strains (27) has also been described, suggesting other mechanisms.

The recombination steps require invasion of a 3'-OH primer end to form a three-stranded structure, transformation

to a Holliday junction, and migration of the junction mediated by RuvA, RuvB, and RuvC (26). Our data agree with these observations as *E. coli* DNA polymerase I, Dpo4, and other bacterial polymerases (13) may bind a three-strand structure. Hypothetically, the primer strand resulting from arrest of nucleolytic activity at *E. coli chi* sequences (GCTGGTGG) (26) could initiate DNA replication (27). Interestingly, this *chi* sequence displays high dG content and is similar to the human minisatellite VTR1 (28) which is highly recombinogenic, notably with the immunoglobulin light chain, as well as with the *c-myc* oncogene (29). Deletions and fusions in the hamster *aprt* locus involve short homologous sequences (30) and dyad symmetries, implying nonhomologous end joining upon double-strand breakage (31) with the Ku and DNA ligase complex. These palindromes expose one strand to cleavage which may then invade a homologous sequence. This may be the case for translocation t(11;22), where the breakpoint regions are AT-rich (32) and may adopt a self-complementary structure with a loop prone to nuclease cleavage.

A second emerging general structural feature encountered in translocation is a non-B-DNA structure (33, 34) possibly sensitive to nuclease attack, in particular at G-rich sequences. These generally occur at similar sequences or direct repeats between the donor and acceptor sequences (35). Some imperfect repeats and the addition of nucleotides, sometimes templated (36), agree with our finding that microhomologies of as few as five to seven nucleotides may prime replication.

In vivo, triplex-forming primers may result from the dissociation of the replication complex at peculiar template structures such as palindromes (37), polypurine/polypyrimidine sequences, G-quartets (38), and intramolecular H-DNA-forming (39) triplex structures, all highly prone to mutations through DNA strand breaks. Stalling of the replication fork at sites of DNA damage resulting from oxidative stress or adducts associated with the processivity factors of DNA polymerases may increase the rate of single-strand breakage.

Extended to the reverse transcriptases HIV-1 and AMV, these results may be related to the first step of the primer strand template switch between RNA and donor DNA to acceptor RNA (40). The recombination rate of these retroviruses which display the unusual property of having two parallel RNA molecules bound by a stem and autocomplementary loops critical for replication is high (ref 41 for a review). This feature may be relevant to our observations of apparent sliding of polymerization during primer elongation on each strand and contribute to high mutation rates. Alternatively, these repeats and associated mechanisms may be used by other viral RNAs (42).

Our experiments demonstrate that the triple-helix-forming primers are in parallel orientation to the homologous strand, and that their priming property contrasts sharply with all previous reports. Using computer modeling of the complex, we previously inferred that the primer lies in the major groove of the double helix with its 3'-OH end near the catalytic acidic amino acid triad of the phage T7 DNA polymerase (12). A computer model of Dpo4 (43) with a triple DNA helix and the incoming dNTP (shown in Figure 5) allows us to speculate that the 3'-OH end of the primer that binds via Hoogsteen bonds could be elongated as shown in Praskash's laboratory with Pol ι (44). The 3'-OH end of

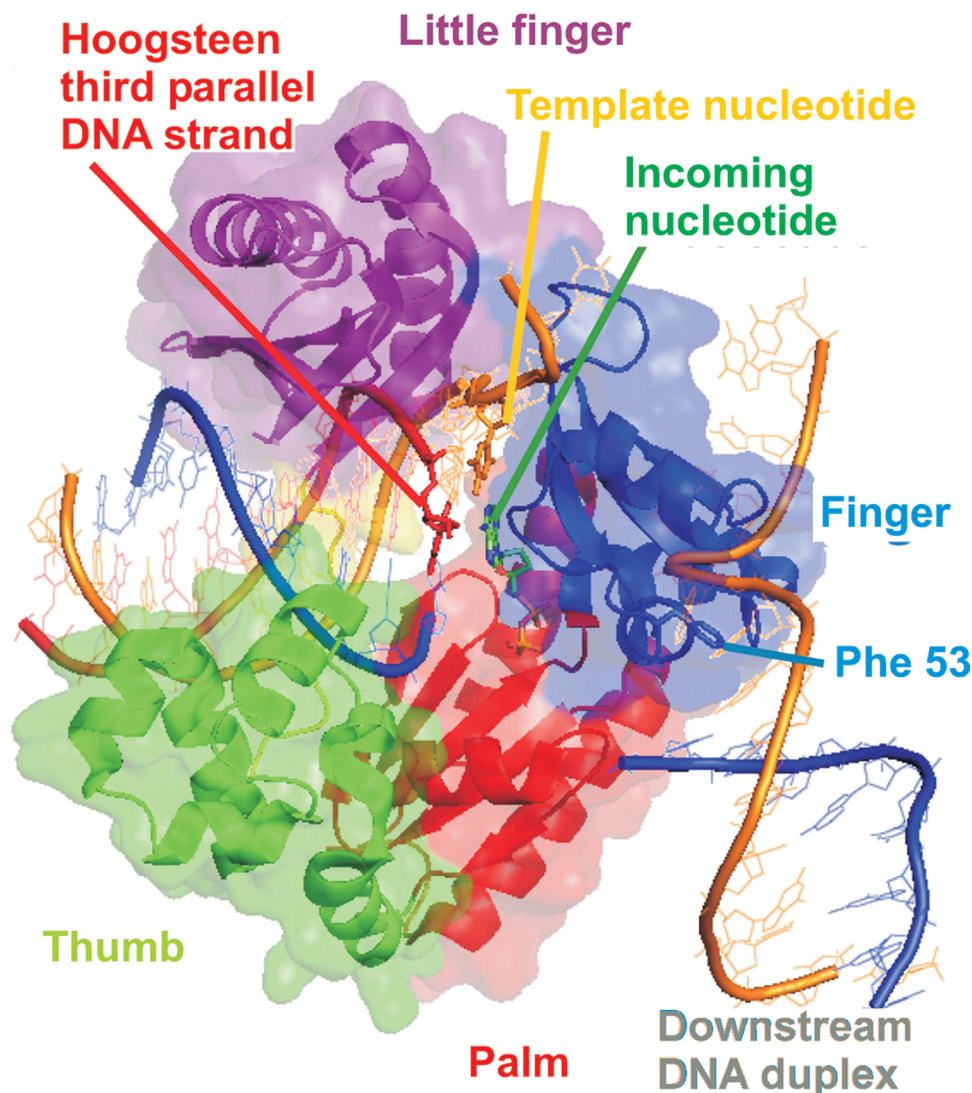


FIGURE 5: Modeling of a third parallel strand complexed in Hoogsteen with a double strand inside the catalytic site of DNA polymerase Dpo4 from *S. solfataricus*. The incoming nucleotide (green), the template nucleotide (yellow), and the 3'-OH nucleotide of the third invasive strand (red) (54) are represented as sticks and highlight the possibility of pairing between the incoming nucleotide and the third parallel strand. The ribbon representation of DNA polymerase Dpo4 is colored following the domain definition (43). The duplex DNA downstream could be separated by the finger domain (blue) as observed in other DNA polymerases (22).

the THP primer within the catalytic site in the presence of dNTP could induce the displacement of the homologous strand and allow subsequent DNA elongation. A symbolic representation of the transition between Hoogsteen or reverse Hoogsteen bonds in the major groove of DNA to the new Watson–Crick pairings that yields primers upon displacement of the homologous strand is shown in Figure 6.

Since dG-rich primers are elongated by all the tested polymerases, these results highlight their potential physiological importance. This is inferred from the reported cell cycle-regulated synthesis of dG-rich single-stranded DNA.

dG tracts show a skewed representation in oncogenes and tumor suppressors genes (45), suggesting that they may play a role in cell metabolism. Furthermore, endonuclease G, located within the mitochondrial intermembrane space (46), cleaves G-rich sequences, yielding a 3'-OH dG which could serve as a primer. With the number of diseases associated with such unusual structures as triple helices (47) or mirror sequences that form either triplex or mutagenic tetraplex such as those in the *c-myc* promoter (48), our results may provide

insight into how these rearrangements occur, as well as the incorporation of linear fragments into double-strand breaks.

The reported inhibition of replication by Mirkin (49) and by Mikhailov and Bogenhagen (50) was observed using single-stranded templates; in these cases, the replicated sequence interacts with the upstream one in an antiparallel orientation. However, the reported inhibition of transcription by T7 and SP6 RNA polymerases was carried out using double-stranded DNA and the homologous strand in an antiparallel orientation (7).

Taken together, these experiments reveal three novel basic properties of DNA polymerases relevant to their interaction with polynucleotides, a subject that has been studied for five decades. They indicate first a new role for intermolecular triple helices. Second is at least a new mechanism for DNA rearrangements, without any initial double-strand DNA strand break, by 3'-hydroxyl end DNA invasion from a single-stranded DNA. That was shown in *Drosophila* lacking the helicase, before induction of deletion (51) or rearrangements upon break-induced replication (52). Third, triple helices can be formed with a bound parallel homologous strand. De-

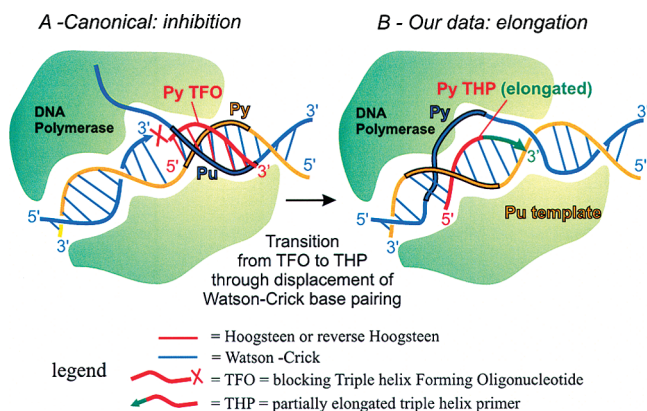


FIGURE 6: Comparison between the previously documented inhibition role of triples helices and interpretation of our enzymatic experiments. The left panel symbolizes the described inhibition of replication by a triple helix. Their orientation is antiparallel as the homologous sequence, preventing DNA replication. Our experiments show their priming possibility when oriented in parallel as the homologous strand, thus forming base pairs with the template strand. The transition from Hoogsteen pairings with the homologous strand to the Watson-Crick pairings would occur in the DNA polymerases catalytic center, by the displacement of the previously base paired complementary sequence, due to a conformational change of the catalytic center, from the 3'-OH end of the THP, localized within the double helix near the acidic amino acid catalytic triad, enabling DNA strand separation and further DNA elongation.

pending on the affinity of the invading strand-polymerase complex for the duplex and the stability of the resulting complex, our observations may be extended to various short triplexes active with other enzymes (53) involved in DNA transactions.

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