Guanine-Rich Telomeric Sequences Stimulate DNA Polymerase Activity in Vitro†

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ABSTRACT: Guanine-rich oligonucleotides and short telomeric DNA sequences can self-associate into G-quartet stabilized complexes. We discovered that this self-association can occur in sequencing reactions and that higher-order structures stimulate DNA polymerase to synthesize extended DNA strands. Base analogues were used to identify Hoogsteen base pairings as stabilizing forces in these stimulatory DNA structures. Scanning force microscopy confirmed that quartet-DNA was formed from these oligomers and that these extended, four-stranded structures could be bound by DNA polymerase. Since guanine quartet-stabilized structures are proposed to exist in vivo, such structures may stimulate DNA polymerization in vivo.

Guanine-rich oligonucleotides can spontaneously self-assemble into four-stranded helices in vitro (*1, 2*). Four-stranded complexes can further associate into superstructures composed of 8, 12, or 16 oligomers (*3*). Some G-rich oligomers can also assemble in an offset, parallel alignment, forming a 'G-wire' (*4, 5*). These higher-order structures are stabilized by G-quartets, hydrogen-bonded structures that are formed when four planar guanines interact through a Hoogsteen base pairing scheme (Figure 3A). Three contiguous guanines within the oligomer are normally required for the formation of a four-stranded structure (*3*).

Four-stranded DNA structures stabilized by G-quartets may be biologically relevant. G-rich sequences occur at immunoglobulin switch regions and eukaryotic telomeres (*1*). These same sequences form G-quartet stabilized DNA in vitro (*1, 4*−*10*). It has been proposed that these alternative structures promote synapsis formation during meiosis, regulate telomere length, and inhibit HIV-1 integrase (*1, 10*−*12*). Additionally, although G-quartet stabilized structures have not been detected in vivo, the discovery of proteins that can act as molecular chaperones to promote structure formation (*14*−*16*) or dissociation (*13*) in vitro suggests that these DNA structures exist and function within living cells.

We discovered that self-association between G-rich oligonucleotides can occur in DNA sequencing reactions and that these higher-order structures can stimulate DNA polymerase to synthesize extended DNA strands. Here we describe our investigation of structures stabilized by G-quartets and the ability of these structures to stimulate DNA synthesis by *AmpliTaq*-FS, *AmpliTaq,* and *Pfu* thermostable DNA polymerases. Alternative DNA structures formed from human, *Tetrahymena*, *Oxytrichia,* and yeast telomeric sequences each stimulated DNA polymerase activity. These results suggest that an additional biological role for the alternative DNA structures formed from G-rich sequences may include the stimulation of DNA polymerase activity.

MATERIALS AND METHODS

Oligonucleotide Syntheses. DNA oligonucleotides were synthesized by the Gene Technologies Laboratory at Texas A&M University (Asub, 7-deaza-dG substituted Tet1.5 oligonucleotides, and all 2-AP substituted oligonucleotides), Genosys Biotechnologies, Inc. (Asub, ATsub, TAsub, Tsub, Zsub, Tet1.5, Oxy1.5, and both human and yeast telomeric oligonucleotides), and Eurogentec Bel S.A. (Asub). The 7-deaza-dG and 2-AP phosphoramidites were obtained from Glen Research.

Octamer-Primed DNA Sequencing. Automated octamer-primed DNA sequencing reactions were performed as described (*17*). Briefly, 10-μL reactions containing 25 pmol of octamer primer, 200 ng of plasmid template (when indicated) and 4 μL of ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit Premix were cycled for 99 cycles at 96 °C for 10 s, 40 °C for 1 min, and 60 °C for 4 min, precipitated, resuspended in loading buffer (5 parts deionized formamide: 1 part 25 mM EDTA (pH 8.0) containing 50 mg/mL blue dextran), heated to 96 °C for 2 min and loaded onto a 36 cm, 5% LongRanger sequencing gel. Data were collected in 4× mode by an ABI PRISM 377 DNA sequencer.

PCR Assays. Polymerase chain reactions were performed in 50-μL reactions containing 5 units of the indicated thermostable DNA polymerase, 25 pmol of primer, 200 μM

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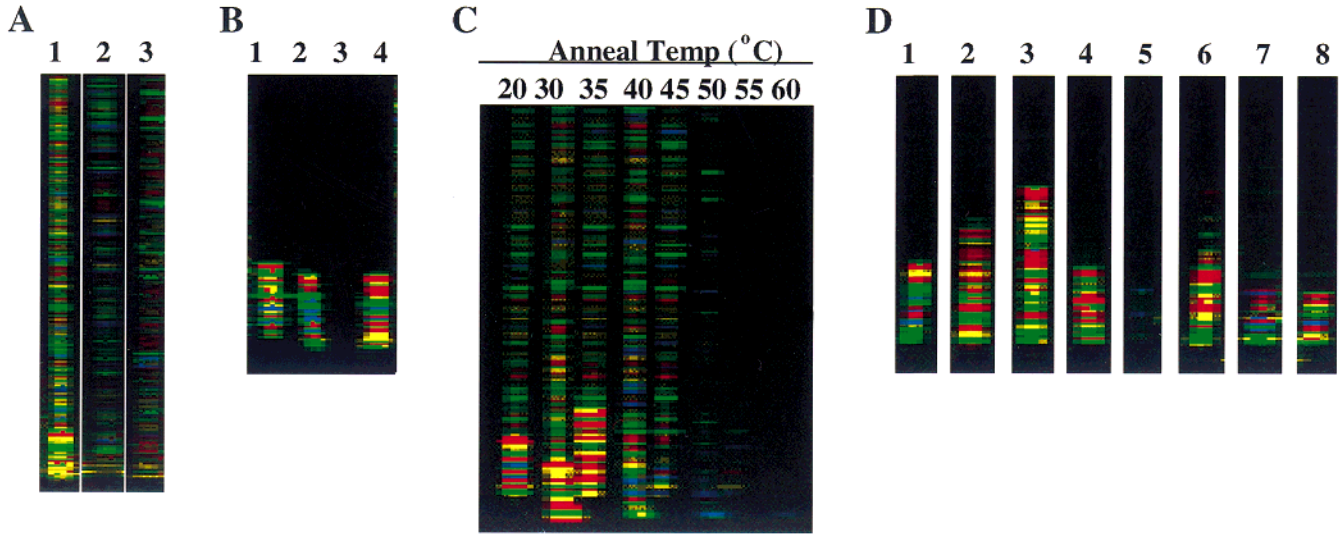

FIGURE 1: (A) Asub primer produces high-intensity data, HID, in sequencing reactions in the presence or absence of a conventional replication template. Lane 1, HID occurs at the beginning of the sequencing data when a conventional template is included in the octamer primed sequencing reaction. Lane 2, sequencing reaction with the pGEM plasmid template and the 18-base, −21 M13 primer. Lane 3, a representative sequencing reaction primed by a non-HID producing octamer. Data were collected on an ABI377 automated DNA sequencer and are presented in compressed gel file format. (B) Exonuclease 1 (Exo 1) treatment of reaction components. Octamer sequencing reactions were performed after the following single-strand nuclease treatments: Lane 1, control reaction of Asub primer with 10 units of heat inactivated Exo 1. Lane 2, heat-denatured sequencing reaction premix was quickly cooled on ice, 10 units of Exo 1 were added, and the reaction was incubated at 37 °C for 15 min. Subsequently, Exo 1 was heat inactivated (15 min at 85 °C), and Asub primer was added (no conventional template). Lane 3, sequencing reaction premix containing Asub primer was heat-denatured and quick-cooled on ice, 10 units of Exo 1 were added, the reaction was incubated at 37 °C for 15 min, and Exo 1 was heat inactivated. Lane 4, control sequencing reaction of Asub with no Exo 1 treatment. (C) Sequencing reactions performed using the Asub primer and a dsDNA template at the indicated annealing temperatures. (D) Sequencing reactions using individual Asub variants. Lane 1, Asub: 5′ GGAGGGAG 3′. Lane 2, ATsub: 5′ GGAGGGTG 3′. Lane 3, TAsub: 5′ GGTGGGAG 3′. Lane 4, Tsub: 5′ GGTGGGTG 3′. Lane 5, A3sub: 5′ GGAGAGAG 3′. Lane 6, P1: 5′ GAGGGAGG 3′. Lane 7, P2: 5′ AGGGAGGG 3′. Lane 8, P3: 5′ GGGAGGGA 3′.

of each dNTP, 1× PCR buffer [*Pfu* buffer contains 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 0.1 mg/mL nuclease-free BSA; *AmpliTaq* buffer contains 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, and 50 mM KCl] unless otherwise indicated. Reactions were first denatured at 95 °C for 2 min and then cycled 50 times at 95 °C for 45 s, at 40 °C for 1 min, and at 60 °C for 4 min with a final incubation at 60 °C for 10 min. The amplified products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized using a Kodak EDAS 120.

Gel Mobility Assays. Asub, Zsub, and Tet1.5 oligonucleotides were 5′ phosphorylated with T4 polynucleotide kinase (New England BioLabs, Inc.) using 25 pmol of [^{32}P]ATP (6000 Ci/mmol, New England Nuclear) following the New England Biolabs protocol. Approximately 10 000 counts of the radiolabeled oligonucleotide were mixed with 100 pmol of the unlabeled oligonucleotide in 10 μL of 1× DNA sequencing reaction buffer [80 mM Tris (pH 9), 2 mM $MgCl_2$]. The samples were heat denatured at 96 °C for 2 min and incubated at 40 °C overnight. These 10-μL samples were heated to 96 °C for 2 min in loading buffer (final concentrations 12.5% glycerol, 0.05% SDS, 0.25× TAE, 0.025% bromophenol blue, 0.025% xylene cyanol), and electrophoresed at either 550 V for 1.5 h through 20 cm 15% denaturing polyacrylamide gels (Tet1.5) or 350 V for 2.5 h through 15% nondenaturing polyacrylamide gels (Asub and Zsub).

Scanning Force Microscopy. Tet1.5 primers were mixed with 1× DNA sequencing reaction buffer [80 mM Tris (pH 9), 2 mM $MgCl_2$] at the concentration of 15 pmol/μL, denatured at 96 °C for 2 min, and incubated at 40 °C for 20 h. Reaction buffers were adsorbed to a freshly cleaved mica surface, washed with ddH_2O, dried with nitrogen, and imaged using fullerene nanotube probe tips in tapping mode on a Digital Instruments Nanoscope III. *AmpliTaq*-FS DNA polymerase (1:10 dilution) was added immediately prior to adsorbing the Tet1.5 sample to the freshly cleaved mica and imaged as above.

RESULTS

Guanine-Rich Oligonucleotides Stimulate DNA Polymerization. While developing a DNA sequencing strategy using octamer oligonucleotides to prime fluorescent dye-labeled terminator cycle sequencing reactions with *AmpliTaq* DNA polymerase-FS (*17*−*19*), four of 97 octamers produced an unusual sequencing profile. Specifically, intense fluorescent signals, termed high-intensity data (HID), obscured the first 70−140 bases of sequence data, after which normal intensity sequence data was observed (Figure 1A, lane 1). Typically, signal intensity profiles for sequencing reactions primed either by other octamers or by 18 base primers show relatively uniform signal intensities from the beginning to the end of data collection (Figure 1A, lanes 2 and 3). Since each of these four oligomers acted similarly, we characterized the oligomer with minimal sequence complexity in more detail ('Asub', 5′ GGAGGGAG 3′).

Three lines of evidence show that the HID was not templated by contaminating DNA from any of the reaction components. First, the HID-producing octamer Asub was synthesized by three companies, and each synthesis product generated HID (four independent syntheses, one of which

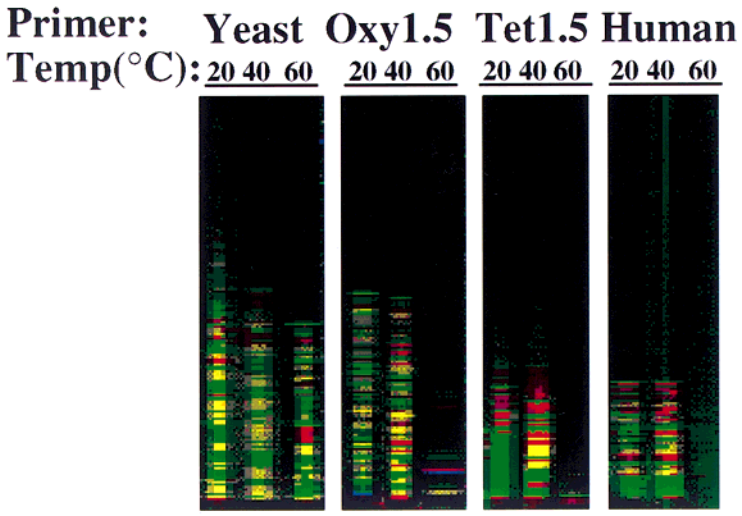

FIGURE 2: Telomeric DNA oligonucleotides produce HID. Octamer primed sequencing conditions were used in reactions of telomeric sequences from yeast (5′ GGTGTGTGGGTGT 3′), *Oxytricha* (Oxy1.5, 5′ $G_4T_4G_4$ 3′), *Tetrahymena* (Tet1.5, 5′ $G_4T_2G_4$ 3′), and human (5′ GGGTTAGGG 3′) lacking conventional sequencing templates. Reactions were cycled using the indicated annealing temperature.

was purified by reverse-phase chromatography). Second, sequencing reaction premix that was heat-denatured, cooled, and treated with exonuclease I prior to the addition of octamer primer still produced HID (Figure 1B, lane 2). However, if the primer was added prior to exonuclease treatment, production of the HID was eliminated (Figure 1B, lane 3). Finally, if the annealing temperature of the reaction was increased, HID production was eliminated, but conventionally primed sequence data was unaffected (Figure 1C). The preferential elimination of HID suggested that the structure formed by Asub was different than a Watson−Crick duplex.

To gain insight into HID production, sequencing reactions were performed using Asub oligomer *without* the addition of a conventional DNA template (Figure 1D, lane 1). Surprisingly, the primer itself was sufficient to produce the high-intensity sequence products. Although the oligomer was responsible for HID, it should be noted that Watson−Crick DNA base pairing could not occur between individual octamer primers. The polymerase was essential for HID production since similar sequencing reactions performed without the addition of the DNA polymerase did not produce HID (data not shown). Temperature cycling was also essential for HID production, since reactions held at the temperature steps used in thermocycling (40, 60, or 96 °C) for the time equivalent to that required to complete the thermocycling regime (11.5 h) did not produce HID (data not shown).

Guanine Richness and Three Consecutive Guanines Are Important for HID Production. To further understand this phenomenon, variants of this primer were assayed to determine whether its sequence per se or the guanines it contained were important for HID production. Independent sequencing reactions were assembled using octamers Asub, ATsub, TAsub, or Tsub without the addition of a conventional sequencing template (Figure 1D, lanes 1−4). The reactions were cycled using conditions in which only a perfectly matched octamer primes a conventional sequencing template (*11*) and analyzed for the production of HID. Each of these substituted octamers produced HID, demonstrating that the adenines were not required for HID production (Figure 1D). There was a critical requirement for three consecutive guanines in the octamer since elimination of the G track from Asub abolished HID even at reduced annealing temperatures (Figure 1D, lane 5). However, the position of the triple G track in the Asub sequence was not critical, since each permutation of Asub produced HID (Figure 1D, lanes 6−8).

Telomeric Sequences Stimulate DNA Synthesis. The experiments described above demonstrated that the guanines but not the adenines in the Asub oligonucleotide were critical for HID production. Since the properties of the Asub sequence were similar to sequences found at telomeres (guanine richness and presence of guanine triplets), we assayed short telomeric oligonucleotides from yeast, *Oxytricha*, *Tetrahymena*, and human and discovered that each stimulated HID production (Figure 2). The lengths of the HID products were ∼120−170 bases for the 13 base yeast oligo, ∼130 bases for the 12 base Oxy1.5 oligo, ∼120 bases for the 10 base Tet1.5 oligo, and ∼110 bases for the 9 base human telomeric sequence oligo, lengths approximately equivalent to 9−13 primer molecules. Thus, there was a correlation between the length of the input oligonucleotide and the resulting HID length.

Base Analogues Identify Positions within Guanine That Stabilize the Structure. Since short telomeric repeats can associate into higher-order structures stabilized by G-quartets in vitro (*1*, *4*−*10*), analysis of our results suggested that G-quartets contributed to HID production. To test this hypothesis, we modified a *Tetrahymena* telomeric repeat oligonucleotide (Tet1.5; 5′ GGGGTTGGGG 3′) by incorporating base analogues predicted to weaken the strength of the G-quartet via eliminating hydrogen-bonding interactions between Hoogsteen paired bases. The base analogues used in these experiments were 7-deaza-deoxyguanine (7-deaza-dG) and 2-aminopurine (2-AP). 7-Deaza-dG was chosen since guanine N7 is replaced with a carbon atom (changing this position from a hydrogen acceptor to a neutral C−H site) that is predicted to disrupt Hoogsteen base pairing (Figure 3A). 2-AP was chosen since it eliminates guanine O6 and H1, positions critical for G−G recognition in Hoogsteen base pairing (Figure 3A).

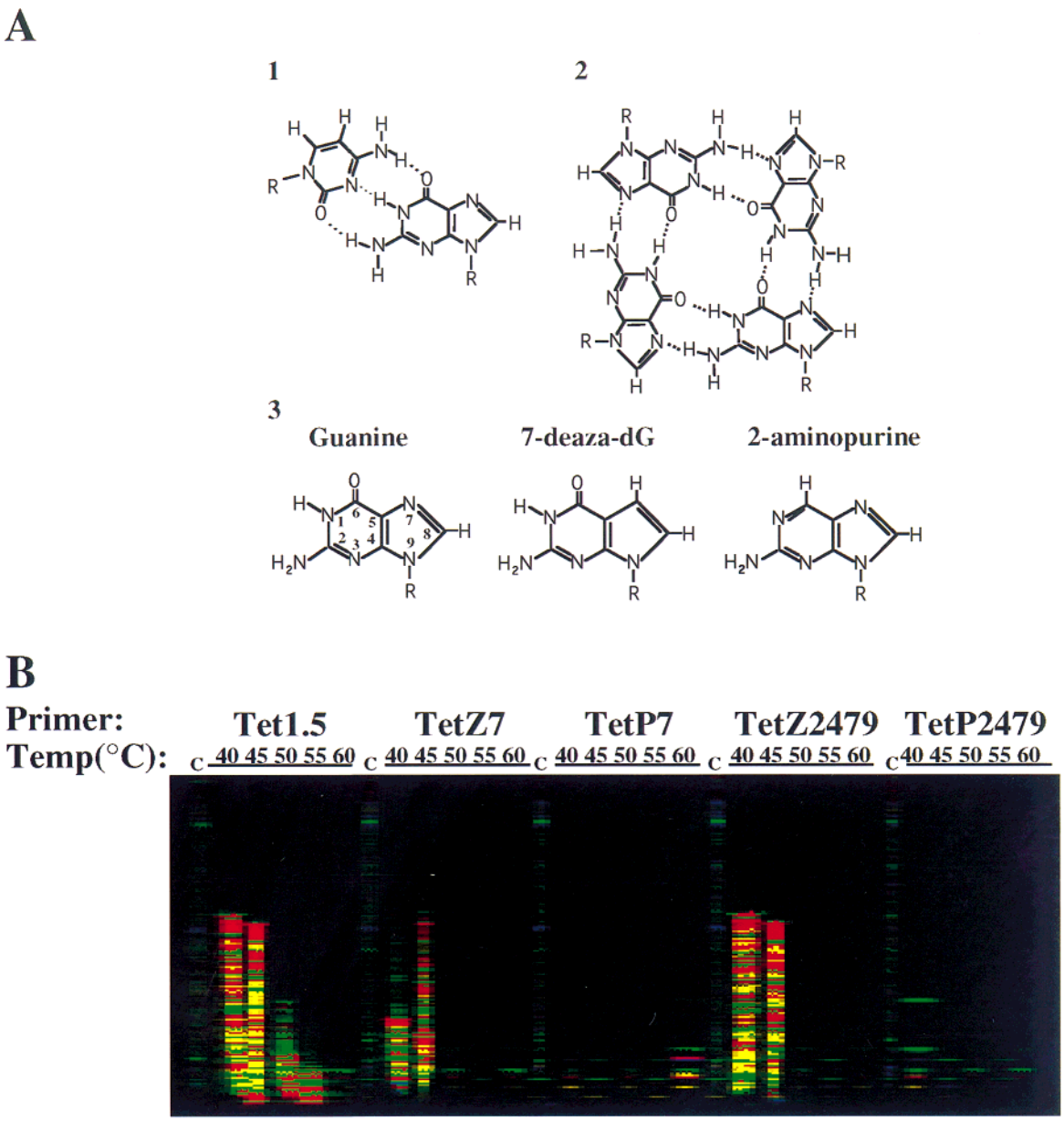

FIGURE 3: (A) 1, chemical structure of a Watson−Crick G−C base pair. 2, structure of a G-quartet formed through Hoogsteen base pairings. 3, structures of guanine and its analogues, 7-deaza-dG (Z) and 2-aminopurine (P). (B) HID thermostability profiles of Tet1.5, the 7-deaza-dG substitutions, TetZ7 (5′ ZZZZTTZZZG 3′) and TetZ2479 (5′ GZGZTTZGZG 3′), and the 2-AP substitutions, TetP7 (5′ PPPPTTPPPG 3′) and TetP2479 (5′ GPGPTTPGPG 3′). Reactions were cycled using the indicated annealing temperature. 'C' indicates lanes containing sequencing reactions of pGEM plasmid template and the 18-base, −21 M13 primer.

In one series of Tet1.5 oligonucleotides, all except the 3′ guanine were substituted with either 7-deaza-dG (TetZ7) or 2-AP (TetP7). In another series of Tet1.5 oligonucleotides, guanines at the second, fourth, seventh, and ninth positions in the sequence were substituted with either 7-deaza-dG (TetZ2479) or 2-AP (TetP2479). The 7-deaza-dG-substituted Tet1.5 oligomers, TetZ7 and TetZ2479, reduced HID thermostability (Figure 3B). Therefore, N7 is a position that stabilizes the structure responsible for HID production. 2-AP modified base eliminated HID production in both substituted oligonucleotides (Figure 3B), identifying O6 and H1 as positions essential for stabilization of the stimulatory structure. Since both 7-deaza-dG and 2-AP substitutions were predicted to decrease the stability of Hoogsteen base pairing by altering one of the two hydrogen bonds comprising a Hoogsteen base pair, these data suggest that Hoogsteen base pairings function as stabilizing forces in the structure recognized by the DNA polymerase.

Base analogues were also used in the context of the Asub oligonucleotide. Since the central base in the guanine triplet was essential for the HID production (GGAG***G***GAG), this position was substituted with either 7-deaza-dG (Zsub) or 2-AP (Psub). Consistent with the data obtained using the substituted Tet1.5 primers, reduced thermostability was observed when 7-deaza-dG was substituted for guanine, while no HID was observed when 2-AP was similarly substituted (Figure 4). However, the ability of these substituted oligonucleotides to prime conventional sequence extension products was not affected, demonstrating that the reduction or elimination of HID was not due to a low-quality primer or to the inability of the polymerase to extend a primer containing either of these substitutions (Figure 4). These

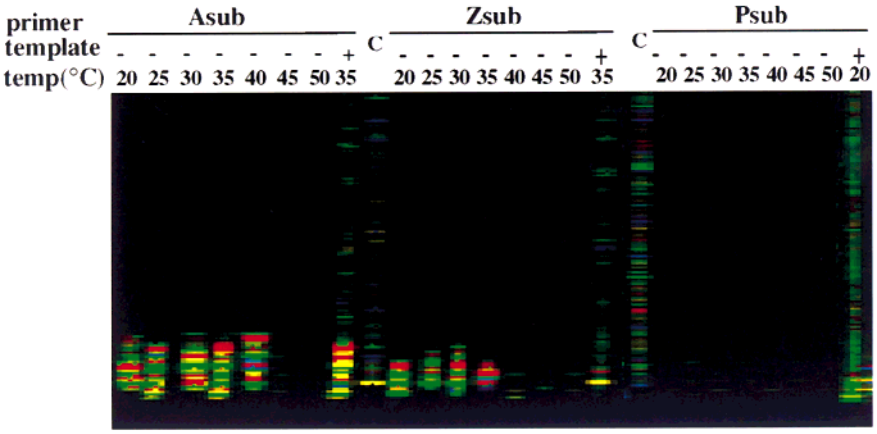

FIGURE 4: Atomic mutagenesis of Asub octamer. The fourth guanine in Asub was substituted with either 7-deaza-dG (Zsub, GGAG***Z***GAG) or 2-AP (Psub, GGAG***P***GAG). Reactions using these oligonucleotides were cycled at the indicated annealing temperature. With a conventional dsDNA template present (+), both Asub and Zsub oligos prime the reaction from the sequence 5′ GGAGGGAG 3′, while Psub primes from the sequence 5′ GGAGAGAG 3′. 'C' indicates lanes containing sequencing reactions of pGEM plasmid template and the 18-base, −21 M13 primer.

experiments confirmed that guanine H1, O6, and N7 were important for HID production. Each of these positions is also integral to G-quartet formation.

Gel Mobility Shift Analyses Demonstrate That Higher-Order DNA Structures Are Formed by HID-Producing Oligonucleotides. Formation of G-quartet structures by Tet1.5 is facilitated and stabilized by K^+ or Na^+ (*4*, *5*). However, the conditions used to generate HID did not require addition of either K^+ or Na^+. To determine whether G-quartet structures were formed in our sequencing reaction conditions, Tet1.5 oligonucleotides were incubated at 40 °C in sequencing reaction buffer and analyzed in gel mobility shift assays. A ladder of higher-order DNA structures, representing different extents of oligonucleotide association, was observed. These structures were relatively resistant to denaturation, since higher-order structures remained after heating to 96 °C and size separation under denaturing conditions (Figure 5A; *4*). A parallel set of experiments was carried out with Asub and Zsub. As with Tet1.5, Asub formed higher-order structures when incubated in sequencing reaction buffer (Figure 5B, lane 1). In contrast, Zsub failed to form higher-order structures under identical conditions (Figure 5B, lane 2). These results demonstrate that higher-order structures were assembled from these HID-producing oligonucleotides and that their formation involved guanine N7.

Tet1.5 Forms G-Wire Structures That Interact with DNA Polymerase. Scanning force microscopy (SFM) was used to define the physical characteristics of structures formed from wild-type Tet1.5 oligonucleotide and to detect interactions between these structures and the DNA polymerase. Samples were prepared on mica substrates according to standard protocols (*20*) and imaged using multiwall nanotube probe tips (*21*). Higher-order DNA structures, similar to the G-wires observed and characterized by Marsh et al. (*5*) were observed in the Tet1.5 oligonucleotide samples (Figure 6A). In our assay conditions, the average height measurement for the Tet1.5 structures was 1.85 ± 0.14 nm (n = 86), which is similar to the previous SFM studies of Tet1.5 (*5*). These measurements were larger than those we determined for double-stranded plasmid DNA under identical conditions (0.55 ± 0.12 nm). The lengths of the Tet1.5 G-wires ranged from 10 to 170 nm, with an average length of 37 ± 17 nm

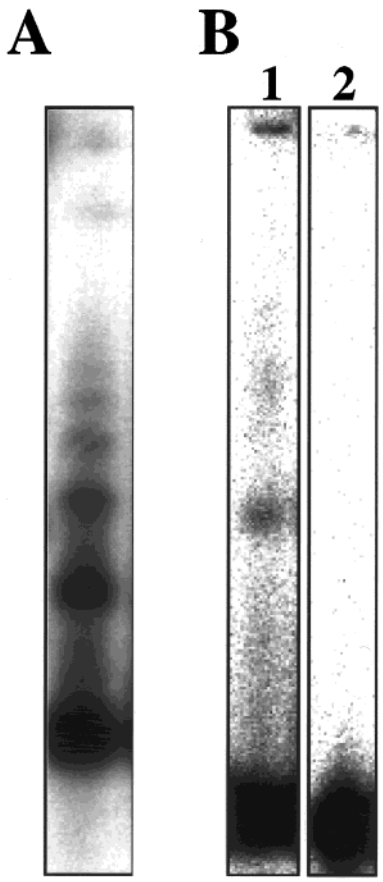

FIGURE 5: Gel mobility assays of oligonucleotides. (A) G-wires assembled from Tet1.5 in 1 X DNA sequencing reaction buffer. (B) Asub in 1 X DNA sequencing reaction buffer (lane 1). Zsub in 1 X DNA sequencing reaction buffer (lane 2).

(n = 41). This average corresponds to 109 base length ± 49 bases (assuming 0.34 nm between bases) and is consistent with observed HID lengths.

Since Tet1.5 can be extended by *AmpliTaq*-FS DNA polymerase to produce HID, we tested whether the polymerase could interact with Tet1.5 G-wires. DNA polymerase was added to a Tet1.5 sample just prior to being adsorbed onto the mica surface and imaged through SFM. The results demonstrate that the DNA polymerase does interact with

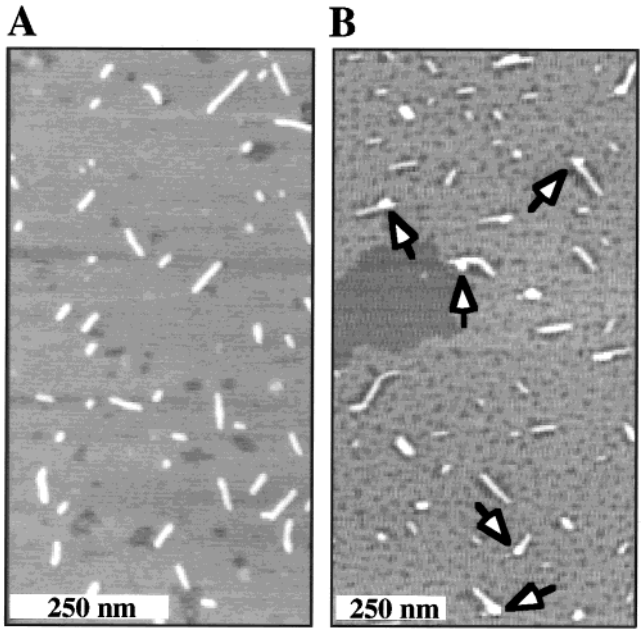

FIGURE 6: SFM imaging of Tet1.5 oligonucleotide. (A) G-wires formed from Tet1.5 (5′ GGGGTTGGGG 3′). (B) Binding of *AmpliTaq*-FS, DNA polymerase to the G-wires. Arrows indicate representative polymerase locations.

these nonconventional G-wire structures (Figure 6B). We observed that DNA polymerase formed complexes at the ends of G-wires more frequently than would be anticipated for random association. Out of 103 G-wire:polymerase complexes sampled, 68 had end-complexes. Making a conservative estimate that the G-wires were 6 times as long as the polymerase, a model in which the polymerase could complex at any of six sites was made and predicted 34 end-complexes, half the number observed. Thus, the DNA polymerase interacted with these nonconventional structures in a nonrandom manner.

The Tet1.5 oligonucleotide variants containing either 7-deaza-dG or 2-AP substitutions were also imaged using SFM (data not shown). The average height of TetZ2479 was 2.35 ± 0.40 nm ($n = 250$), and the average height of TetZ7 was 1.12 ± 0.62 nm ($n = 24$). However, neither of these oligonucleotides formed extended G-wires in SFM assays. This result is consistent with our hypothesis that extended, quartet-stabilized structures were less likely to form or remain stable if guanine N7 was removed. The non-HID producing oligonucleotides containing 2-AP showed neither extended structures nor height measurements greater than $\sim 0.7 \pm 0.40$ nm (TetP7, $n = 248$; TetP2479, $n = 881$). Thus, there is a correlation between the ability of an oligonucleotide to template HID and the ability of that oligonucleotide to form G-wires.

Clues to the Mechanism of HID Formation. Two pieces of evidence suggested that HID was produced by PCR amplification of a double-stranded DNA template containing the primer sequence at each 5′ end (and the Watson−Crick complement at each 3′ end). First, HID-producing reactions did not generate interpretable sequence data between the primer sites at each end. The presence of the primer complement explained why these reactions resembled doubly primed sequencing reactions. Second, HID signal intensity, but not HID length, increased with extended cycling (data not shown).

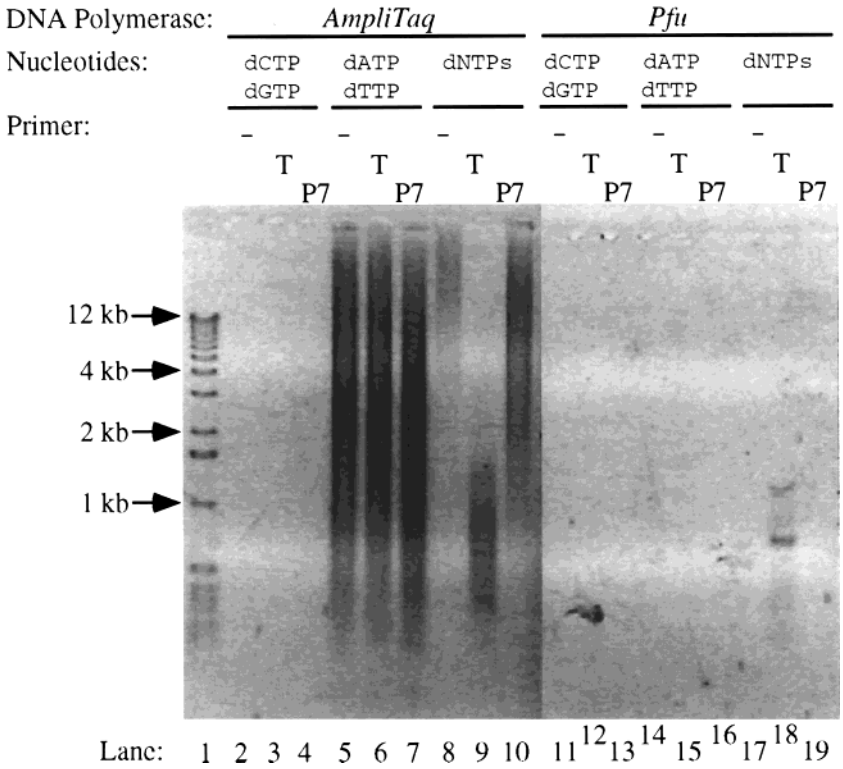

FIGURE 7: PCR assays for G-rich oligonucleotide stimulated polymerization activity. Lane 1, the 1 KB DNA ladder (Gibco/BRL). Reactions using *AmpliTaq* DNA polymerase (lanes 2−10). Reactions using *Pfu* DNA polymerase (lanes 11−19). Reactions containing dCTP and dGTP nucleotides (lanes 2−4 and 11−13). Reactions containing dATP and dTTP nucleotides (lanes 5−7 and 14−16). Reactions containing all four dNTPs (lanes 8−10 and 17−19). The primer added to each reaction is indicated: A minus sign (−) indicates that no primer was added (lanes 2, 5, 8, 11, 14, and 17); 'T' indicates that the Tet1.5 primer was added (Lanes 3, 6, 9, 12, 15, and 18); 'P7' indicates that the TetP7 primer was added (lanes 4, 7, 10, 13, 16, and19). Reactions were cycled as described in Materials and Methods.

If HID is produced via PCR amplification, then template-independent amplification should be observed with HID-producing oligonucleotides in traditional PCR reactions (i.e., not sequencing reactions). However, other template-independent polymerization activities have been described, but these activities are also reported to be primer-independent (*22*−*26*). *AmpliTaq* primer/template-independent polymerization is tightly correlated with the 5′ to 3′ exonuclease activity domain of the polymerase, while *Pfu* DNA polymerase contains neither of these activities (*22*, *23*). Thus, these enzymes were used to distinguish among these activities in traditional PCR. The ability to stimulate synthesis was tested in the absence of input primer and in the presence of either Tet1.5 or TetP7 oligonucleotides. Additionally, to distinguish *AmpliTaq* primer/template-independent polymerization (in which poly(AT) is preferentially synthesized; *22*, *23*) from polymerization stimulated from G-wire structures, reactions were assembled with dCTP and dGTP, dATP and dTTP, or all four dNTPs.

These assays demonstrated that *AmpliTaq* formed extended products in the presence of either dATP and dTTP or all four dNTPs, either with or without an input oligonucleotide (Figure 7). It is most likely that these products represent the primer/template-independent polymerization previously described (*22*, *23*, *25*, *26*). If the G-rich sequences were able to stimulate this polymerase, their amplification products were masked by the primer/template-independent activity. On the other hand, *Pfu* required the addition of G-wire-forming oligonucleotides and all four dNTPs for the production of extended DNA (Figure 7). This enzyme did not produce polymerization products in the presence of either dATP and dTTP alone or dCTP and dGTP alone. Extended PCR products were produced from the Tet1.5 oligonucleotide but not from the TetP7 oligonucleotide. Thus, data obtained using the high-fidelity *Pfu* DNA polymerase support a model of enzymatic DNA polymerization stimulated by oligonucleotides capable of forming G-quartet-stabilized structures. However, the mechanism that produces the extended strand that nucleates the PCR is not known.

DISCUSSION

DNA Synthesis Is Dependent upon the Formation of G-Quartet-Stabilized Structures. Since the properties of the HID-producing primers were similar to those of oligomers known to form four-stranded DNA (i.e., guanine-richness and presence of at least three consecutive guanines), we proposed that similar DNA quadruplexes were formed from these guanine-rich oligonucleotides and that these structures were stabilized by Hoogsteen base pairings. Base analogue studies support this hypothesis by revealing the importance of N7 guanine, a position that is not involved in Watson−Crick base pairing but is important for stabilization of the stimulatory structure.

Hoogsteen interactions stabilize parallel-stranded duplexes, triplexes, and G-quartets (*27*). However, triplex formation would require the presence of an additional pyrimidine-rich third strand, which is lacking from our assay. Stability under denaturing conditions and structure length are consistent with the formation of guanine-quartet-stabilized structures (see Figures 5 and 6). Furthermore, the differential effects of 7-deaza-dG and 2-AP demonstrate that elimination of the central H-bonded ring within a G-quartet by 2-AP substitution is more destabilizing to the structure than elimination of the peripheral H-bonded ring by 7-deaza-dG substitution (see Figure 3A). Perhaps this reflects the relative importance of cation stabilization by the central H-bonded ring on the G-quartet structure. Although no replicative polymerase is reported to synthesize extended strands from a structure other than a primer−template junction (specifically from an antiparallel DNA duplex), our data suggest that DNA polymerase can be stimulated by a G-quartet-stabilized structure.

The parallel quartet structure formed by the sequence TGGGGT is solved to 1.2 Å resolution and reveals that each side of the quartet has dimensions similar to a DNA duplex minor groove (*28*). These dimensions may be important for the activity detected in our assay systems since a *Taq* DNA polymerase:DNA cocrystal is solved and demonstrates that the enzyme binds to the minor groove of a DNA duplex (*29*). Thus, although the overall DNA structure is nonconventional, the polymerase may recognize its conventional binding structure and begin DNA synthesis.

Biological Relevance. The ability to use DNA structures stabilized by G-quartets as polymerization templates may be biologically relevant. As an example, these types of structures are proposed (but not proven) to exist at the telomere (*10*, *11*). Telomerase is essential for the maintenance of chromosome ends and, in particular, for the synthesis of telomeric DNA. However, some human tumor cell lines maintain telomeric DNA without detectable telomerase activity, suggesting that additional mechanisms exist to maintain chromosome ends in vivo (*30*). We have not assayed G-rich (HID-producing) oligonucleotides for their ability to stimulate a eukaryotic DNA polymerase. However, this proposed activity may explain how immortalized cell lines lacking detectable telomerase activity survive and maintain telomere length. If this alternative telomere maintenance mechanism occurs, it is not known whether it would result in the maintenance of the natural telomeric sequence.

It must also be noted that the short oligonucleotide primers were rapidly extended by the polymerases into several hundred base molecules. Perhaps a mechanism involving alternative DNA base pairings to stabilize templates is active in promoting expansion of a genome comprised of linear chromosomes, and it may have provided a mechanistic scaffold for primitive genome expansion.

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