Inhibition of Klenow Fragment DNA Polymerase on Double-Helical Templates by Oligonucleotide-Directed Triple-Helix Formation†

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ABSTRACT: We have examined the capacity of oligonucleotide-directed triple helices to block the progress of primer extension by DNA polymerase. Occupancy of the major groove of a double-helical DNA substrate obstructed Klenow fragment progress at sites that map near the proximal boundary between duplex and triplex. Among a family of related third-strand oligonucleotides that all stably occupied the target duplex in the absence of polymerase, those forming longer triplexes were more effective polymerase inhibitors than shorter complexes. Kinetic analysis revealed that the triple-helical complex provided an effective blockade for times of at least 20 min. These observations provide the basis for considering and further dissecting repair DNA polymerase function and mechanism by using various defined local three-stranded DNA structures as probes.

Triple-helical DNA structures can be formed by the sequence-specific interaction of oligodeoxynucleotides with their cognate target sites in double-stranded DNA (Moser & Dervan, 1987; Le Doan et al., 1987). The third-strand oligonucleotide, which may be either purine-rich or pyrimidine-rich, interacts with purine residues in the major groove of the target duplex. Pyrimidine oligonucleotides bind in a parallel orientation relative to the purine-rich duplex strand through the formation of TAT and C^+GC triplets (Moser & Dervan, 1987; Rajagopal & Feigon, 1989; de los Santos et al., 1989). Purine oligonucleotides bind in an antiparallel orientation relative to the purine-rich duplex strand through the formation of GGC and AAT or TAT triplets (Cooney et al., 1988; Beal & Dervan, 1991; Radhakrishnan et al., 1991). Oligonucleotide-directed triple-helix formation has recently been used to study interactions between DNA and various DNA binding proteins, including restriction endonucleases (Maher et al., 1989, 1990; Francois et al., 1989; Hanvey et al., 1990), transcription factors (Maher et al., 1989, 1992), viral replication proteins (Huang et al., 1992), and RNA polymerases (Young et al., 1991; Maher et al., 1992; Maher, 1993). Effects of intermolecular triple-helix formation with double-helical templates on the function of DNA polymerases have not previously been reported, and these are the focus of this work.

The interaction of DNA polymerases with different forms of DNA is central for their DNA repair and replication functions. Recent studies have used structural, biochemical, genetic, and pharmacological approaches to focus on different aspects of this important protein–DNA interaction. X-ray cocrystal structures of *Escherichia coli* DNA polymerase I large fragment with DNA substrates have provided atomic-level resolution and led to detailed molecular models for the interaction of polymerase with primer and single-stranded template (Ollis et al., 1985; Joyce & Steitz, 1987; Derbyshire et al., 1988; Beese & Steitz, 1991; Beese et al., 1993), but similar structural data are not yet available for polymerase on a double-stranded DNA substrate. Others have probed the interactions of polymerase and double-stranded templates by studying effects that different site-specific noncoding template lesions have on the processivity and fidelity of *in vitro* DNA synthesis systems (Bhanot et al., 1991; Simha et al., 1991; Comess et al., 1992; Grevatt et al., 1991; Shibutani et al., 1993). In general, these lesions lead to very efficient blocks to elongation; however, they can be bypassed by the incorporation of bases, usually adenine, across from these modified sites (Strauss, 1991). Other studies have noted sequence-specific inhibition of DNA polymerase by small molecules that have high-affinity, noncovalent interactions with DNA (Wartell et al., 1974; Weiland & Dooley, 1991; Sun & Hurley, 1992) or by replication termination proteins such as the *E. coli ter*-binding protein (Lee & Kornberg, 1992). Furthermore, there has been recent interest in potentially inhibitory template structures and sequence composition effects such as homopurine tracts (Abbotts et al., 1988; Bedinger et al., 1989; Weisman-Shomer et al., 1989; Brinton et al., 1991). In some cases, blocks to extension occur at sites where intramolecular triple helices or local intermolecular triple helices, formed on single-stranded templates, can potentially be formed (Lapidot et al., 1989; Baran et al., 1991; Brinton et al., 1991; Dayn et al., 1992; Giovannangeli et al., 1993; Samadashwily et al., 1993).

In this study, we report efficient, sequence-specific inhibition of DNA synthesis on a double-stranded template through intermolecular pyrimidine oligonucleotide-directed triple-helix formation. Triple-helix-forming oligonucleotides of lengths ranging from 17 to 27 nucleotides (nt)[1] were used to map blocks to synthesis catalyzed by either *E. coli* Klenow fragment or a modified T7 DNA polymerase. This system has allowed us to study sequence-specific inhibition of primer extension without introducing chemical lesions.

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[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; nt, nucleotide(s); TE, Tris-HCl/EDTA; TNE, Tris-HCl/sodium chloride/EDTA; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; SSB(s), single-stranded binding protein(s).

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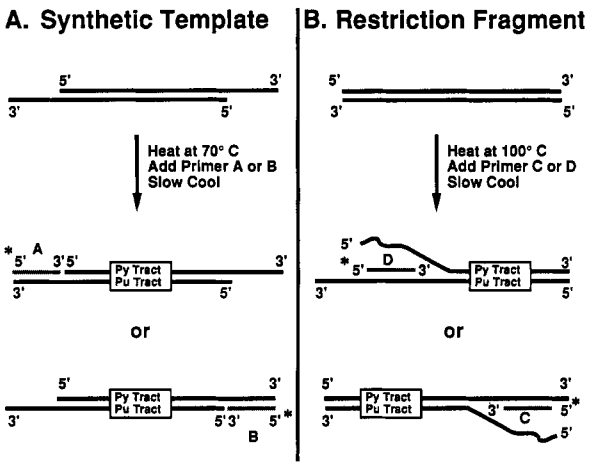

FIGURE 1: Representation of the two systems used in the primer extension inhibition assays. Oligonucleotides **S1** and **S3** are ligated together to form the pyrimidine-rich strand of the synthetic template while oligonucleotides **S2** and **S4** are ligated together to form the purine-rich strand of the synthetic template with **S2** and **S3** providing the 3′-overhangs complementary to primers **A** and **B**, respectively. Py Tract refers to the pyrimidine-rich strand of the target site while Pu Tract refers to the purine-rich strand.

EXPERIMENTAL PROCEDURES

Materials. All DNA synthesis reagents were obtained from Applied Biosystems Inc. except for 5-methylcytidine phosphoramidite obtained from Cruachem Inc. and chemical phosphorylation reagent obtained from Glen Research. Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, and DNase I were purchased from Boehringer Mannheim. Sequenase version 2.0 DNA sequencing kit and *E. coli* DNA polymerase I large (Klenow) fragment were purchased from U. S. Biochemicals. Adenosine 5′-[γ-^{32}P]triphosphate and deoxyadenosine [α-^{32}P]triphosphate were obtained from New England Nuclear.

Synthesis of Oligonucleotides. Oligodeoxyribonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer using β-cyanoethyl phosphoramidite chemistry. Oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis, visualized by UV shadowing, and electroeluted from gel slices using an Elutrap apparatus (Schleicher & Schuell). They were ethanol-precipitated and resuspended in 0.5 × TE before being stored at −80 °C. The concentrations of oligonucleotides were determined by A_{260} measurements using the following molar extinction coefficients: 15 400 (A), 11 700 (G), 7300 (C), 8800 (T), and 5800 (MeC).

Plasmid Construction. The plasmid pTER I was obtained by cloning the oligonucleotides 5′-pTCGACTTTTTTTC-TTTTTCTTTCTTTTTTTTTTTTGGCGCATG-3′ and 5′-pCGCCAAAAA AAAAAAAGAAAGAAAAAGAAAA-AAAG-3′ into the large *Sal*I/*Sph*I restriction fragment of pUC19. Large-scale preparation of plasmid was performed using Qiagen purification kits. The sequence of the insert was confirmed by dideoxynucleotide sequencing.

Construction of Synthetic Templates for Inhibition of DNA Synthesis. The synthetic template shown in Figure 1 was made by mixing 2 nmol of oligonucleotides **S1** (5′-CGACG-GCCAGTGAATTCGAGCTCGGTACCCGGGGATCC-TCTAGAGTCGACTTTTTTTCTTTTTCTTTCTTTTTTT-3′), **S2** (5′-pAAAAAGTCGACTCTAGAGGATCCCCGG-GTACCGAG CTCGAATTCACTGGCCGTCGTTTTA-CAAGCTCGTGACTGG-3′), **S3** (5′-pTTTTTGGCGCA TGCAAGCTTGGCGTAATCATGGTCATAGCTGTTT-CCTGTGTGAAATTGTTATCCGCTCACAATT-3′), and **S4** (5′-TTCACACAGGAAACAGCTATGACCATGAT-TACGCCAAGCTTGCA TGCGCCAAAAAAAAAAAAA-GAAAGAAAAAGAA-3′) in a total volume of 50 μL of 1× TNE. The reaction was heated to 100 °C for 2 min and slowly cooled to room temperature. To the reaction were added 38 μL of water, 10 μL of 10× ligation buffer, 1 μL of 100 mM ATP, and 10 units of T4 DNA ligase. It was placed at 37 °C for 8 h, terminated by ethanol-precipitation, and resuspended in 40 μL of nondenaturing load dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol). The samples were heated to 70 °C for 2 min and chilled on ice prior to electrophoresis on a 10% nondenaturing polyacrylamide gel. The annealed full-length target duplex was visualized by UV-shadowing, excised, and extracted through electroelution. The duplex was ethanol-precipitated and resuspended in 1× TE buffer prior to storage at −80 °C.

DNase I Footprinting. The 438 bp *Afl*III–*Eco*RI restriction fragment of pTER I was 3′-end-labeled with deoxyadenosine [α-^{32}P]triphosphate, and chemical sequencing A+G-specific reactions were carried out by standard procedures (Sambrook et al., 1991). Labeled DNA (50 000 cpm) in 9 μL of 1.1× Sequenase reaction buffer (44 mM Tris-HCl, pH 7.5, 55 mM NaCl, and 22 mM $MgCl_2$) was either heated to 100 °C under mineral oil for 2 min prior to slow-cooling to room temperature or incubated for an equivalent amount of time at 37 °C, as indicated. Oligonucleotide (1 μL) was added, and the reaction was allowed to equilibrate for 2 h at 37 °C. Immediately prior to addition of 2 μL of DNase I (0.01 unit/mL in 1× TE), 1 μL of 100 mM $CaCl_2$ was added to the reaction. Digestion proceeded for 2 min at room temperature before termination by ethanol-precipitation and resuspension in Sequenase stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). The samples were analyzed by electrophoresis on 8% denaturing polyacrylamide gels.

Inhibition of DNA Synthesis Using Synthetic Templates. DNA template (0.1 pmol) was added to either 5′-end-labeled primer **A** (5′-CCAGTCACGAGCTTGTAAAA-3′) or 5′-end-labeled primer **B** (5′-AATTGTGAGCGGATAACAAT-3′) (20 000 cpm/reaction) in a volume of 9 μL of 1.1× Sequenase reaction buffer. The mixture was heated to 70 °C for 2 min and slow-cooled to 37 °C. Oligonucleotide was added in a volume of 1 μL and incubated at 37 °C for 2 h. A 15-μL volume of primer extension buffer (12.5 mM DTT, 50 μM in each dNTP, 15 mM NaCl, 2.5 mM Tris-HCl, pH 7.5, 12.5 μg/mL BSA, and 3.25 units of either Klenow fragment or Sequenase 2.0) was added to each reaction and allowed to incubate for the specified time at 37 °C. This amount of polymerase theoretically represents a 10-fold excess of enzyme to third-strand oligonucleotide (present at 50 nM concentration). Addition of 10-fold greater amounts of polymerase did not change experimental results (data not shown), implying that the primer extension reactions are done with saturating amounts of DNA polymerase. Reactions were stopped by the addition of 14 μL of Sequenase stop buffer. Molecular weight markers were generated by terminating primer extension reactions by ethanol precipitation and resuspension in 40 μL of nondenaturing load dye. The samples were heated to 70 °C for 2 min and chilled on ice prior to electrophoresis on a 10% nondenaturing gel. 5′-End-labeled full-length target duplex was visualized by autoradiography, excised, and electroeluted. Chemical sequencing A+G- and C+T-specific reactions were carried out as described (Sambrook et al., 1989). Samples were electrophoresed on 8%

Table 1: Sequences of Oligonucleotides and 31 bp Target Site

Homopurine Target Site

5' A A A A A A A A A A A A G A A A G A A A A A G A A A A A A A A G 3'
3' T T T T T T T T T T T T C T T T C T T T T T C T T T T T T T C 5'

Oligonucleotides

Oligo			Length
y1	5'	T T T T T T T T T C T T T C T T T T T C T T T T T T T	3' 27 nt
y2	5'	T T T T T T T T T T T C T T T C T T T T T C T T T T	3' 27 nt
y3	5'	T T T T T T T T T C T T T C T T T T T	3' 19 nt
y4	5'	T T T C T T T T T C T T T T T T T	3' 17 nt
y5	5'	T T T T T T T C T T T T T C T T T C T T T T T T T T T	3' 27 nt
r1	5'	A A A A A A A A A G A A A G A A A A A G A A A A A A A	3' 27 nt
r2	5'	A A A A A A A G A A A A A G A A A G A A A A A A A A A	3' 27 nt

denaturing polyacrylamide gels that were dried and placed on film upon completion. Afterward, gels were exposed to a storage phosphor screen (Kodak storage screen S0230, obtained from Molecular Dynamics), and data were analyzed on a Molecular Dynamics 400S PhosphorImager using ImageQuant v. 3.22 software.

Inhibition of DNA Synthesis Using Restriction Fragments. Molecular weight markers consisted of dideoxy sequencing reactions performed on alkaline-denatured pTER I supercoiled template using 5'-end-labeled primers. The 349 bp *Pvu*II restriction fragment of pTER I was isolated by electrophoresis on a 1% agarose gel followed by electroelution. Restriction fragment (10 ng) was added to either 5'-end-labeled primer **C** (5'-TGCTTCCGGCTCGTATGTTGTGTGG-3') or 5'-end-labeled primer **D** (5'-ACGCCAGGGTTTTCCCAGT-CACGAC-3') (200 000 cpm/9-μL final reaction) in a volume of 9 μL of 1.1× Sequenase reaction buffer per final reaction. The mixture was heated to 100 °C under mineral oil for 2 min and slow-cooled to 37 °C. Reaction aliquots (9 μL) were placed in separate tubes, and oligonucleotide was added in a volume of 1 μL. The triple-helix-annealing reaction was allowed to equilibrate for 2 h at 37 °C. Primer extension buffer (15 μL) was added to each reaction and incubated for the specified time at 37 °C. Reactions were stopped by the addition of 14 μL of Sequenase stop buffer and heated at 100 °C for 2 min. Samples were electrophoresed on 8% denaturing polyacrylamide gels that were dried and placed on film and a storage phosphor screen for analysis as stated above.

RESULTS

Design of a Duplex DNA Target Site and Corresponding Third-Strand Oligonucleotides. To examine whether and how intermolecular triplex structures affect DNA synthesis reactions, a 31 bp homopurine tract was designed to act as the target site. The large size of this purine sequence allows for the formation of several different triple-helical complexes nested within the target site. Potential third-strand oligonucleotides used in all triplex formation and primer extension assays are shown in Table 1. Reagents **y1–y4** are all expected to occupy the target in the orientation shown. However, their individual lengths and frame of occupancy on the target are anticipated to be different. Together, these probes provide the means to map termination patterns positioned across the target site in a predictable manner and to compare how well triple-helical complexes of varying stability inhibit polymerase. Reagents **y5**, **r1**, and **r2**, which are not expected to bind to the target site under the conditions used in this study, serve to measure polymerase termination that is unrelated to triple-helix formation but might depend on template composition or the mere presence of oligonucleotides. Reagent **y5** has the same sequence composition as y1 but is antisense to the purine-rich target strand, so it can be used to test for possible Watson–Crick interactions and their effects on the polymerase reactions. Reagents **r1** and **r2** are purine-rich oligonucleotides, with **r1** being antisense to the homopyrimidine target strand.

Occupancy of Triple-Helix Target Sequence. DNase I footprinting assays were performed to show that the target site is occupied by oligonucleotides **y1–y4**, as expected from their design, and that negative control oligonucleotides did not form triplexes. The target duplex is embedded in a restriction fragment (*Eco*RI–*Afl*III) from the plasmid pTER I. It consists of a 31 bp homopurine run flanked by sequences common to the small *Pvu*II fragment from pTER I and the synthetic oligonucleotide duplex depicted in Figure 1. Studies using the native fragment shown in Figure 2, panel A, demonstrate complete occupancy of the target site by oligonucleotides **y1**, **y2**, **y3**, and **y4** under the same conditions that are used for DNA polymerase assays. The size and location of the protected regions correlate with the formation of the expected oligonucleotide-directed triple helices. Moreover there is a distinctive hypersensitive cleavage site present at the 3'-end of the triple-helical complexes in panel A, lanes 3, 5, and 6. Oligonucleotides **y5**, **r1**, and **r2** show no protection of the triple-helix target site, as expected since they have no triple-helical homology to the target site. Studies performed on the renatured target fragment shown in panel B further demonstrate complete occupancy of the target site by the triple-helix-forming oligonucleotides. The control oligonucleotides **y5**, **r1**, and **r2** do not occupy the target site. The similarity of DNase I patterns in panels A and B indicates that, within the limits of detection, the vast majority of the heat-denatured template has reannealed to completion. The anticipated orientation of the third strand was confirmed for oligonucleotide **y1** by standard affinity cleavage analysis (Dreyer & Dervan, 1985; Moser & Dervan, 1987) (data not shown).

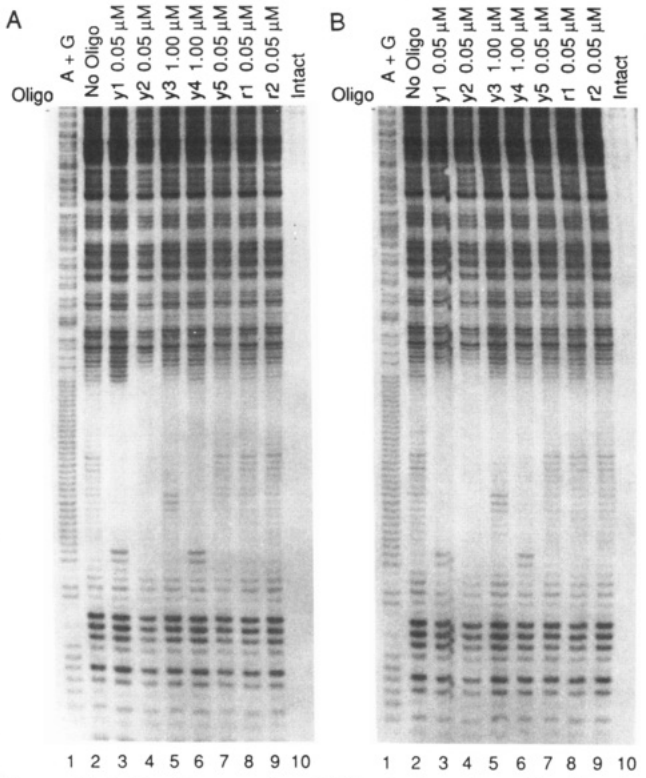

FIGURE 2: Autoradiogram of an 8% denaturing polyacrylamide gel showing DNase I footprinting patterns of the oligonucleotides listed in Table 1. Panel A represents footprinting reactions performed on the native *Afl*II–*Eco*RI restriction fragment of pTER I while panel B represents the same reactions performed on the denatured and reannealed fragment. Lanes 1A and 1B, A+G-specific chemical sequencing lanes; lanes 2A and 2B, DNase I control lanes; lanes 3A and 3B, 0.05 μM **y1**; lanes 4A and 4B, 0.05 μM **y2**; lanes 5A and 5B, 1 μM **y3**; lanes 6A and 6B, 1 μM **y4**; lanes 7A and 7B, 0.05 μM **y5**; lanes 8A and 8B, 0.05 μM **r6**; lanes 9A and 9B, 0.05 μM **r7**; lanes 10A and 10B, untreated DNA control lanes.

Synthetic Gapped Template System of DNA Synthesis Inhibition. Two templates carrying the same target sequence were used in this study (Figure 1). These substrates differ in the manner in which they provide an entry and initiation point for DNA polymerase. The first was designed to provide a nick in an otherwise perfect duplex. The nick is positioned upstream of the triplex target site and is expected to serve as a specific entry site and primer for the DNA polymerase. To create a homogeneous population of this substrate, its components were each synthesized chemically, followed by annealing reactions and subsequent gel purification (Experimental Procedures). Preliminary experiments verified that the nick acts as a primer for DNA synthesis using Klenow fragment. Triple-helix-forming oligonucleotides were added to the annealed template to test for inhibition of DNA synthesis. The sequence of the template's double-stranded region is identical to that of the pTER I restriction fragment used in the footprinting and restriction fragment termination assays. This should permit direct comparison of the synthetic gapped template system and the restriction fragment system described below.

Restriction Fragment System of DNA Synthesis Inhibition. In this alternative system, the small *Pvu*II fragment of pTER I is heat-denatured in the presence of excess primer, as depicted schematically in Figure 1. After annealing, primer bound to the fragment template can be extended with either the Klenow fragment of DNA polymerase I or Sequenase 2.0 (data not shown), as would be expected from prior studies showing the ability of these enzymes to execute strand displacement synthesis (Tabor & Richardson, 1989; Kornberg & Baker, 1992). For reasons detailed below, this class of substrates was used only to provide initial observations which were then verified on the better defined synthetic template described above. However, this substrate should have the advantage of most nearly approximating the region upstream of DNA polymerase performing strand displacement synthesis, and all major conclusions proved the same in both systems. For this set of templates, there is clearly greater uncertainty concerning substrate homogeneity and conformation because the majority of primer is expected to be branch-migrated off the template strand during the reannealing stage. This means that priming and DNA synthesis will come from a minority of the DNA in the reaction. For this reason, it is not possible to definitively and directly characterize the conformation of DNA (and its triple-stranded status where appropriate oligonucleotides are present) at positions downstream of the primer termini. It is easy to imagine that competing side reactions on higher order structures could become significant in this system. Nevertheless, as shown below, both substrate types gave remarkably similar results when triple-helix-forming oligonucleotides are added to the reaction followed by challenge with polymerase. Effects of the same set of oligonucleotides tested on the fully defined, nicked substrate were determined for the restriction fragment substrates.

Inhibition of DNA Polymerase Priming from the Pyrimidine-Rich Strand. In the synthetic template system, primer extension terminates efficiently and quite abruptly a few bases internal to the 5′-end of the triple helix as shown in Figures 3A and 4. This stoppage is distinct in position and efficiency from the intrinsic polymerase pausing (Figure 4, lane 11) characteristic of homopurine tracts (Weisman-Shomar et al., 1989). Both oligonucleotide probes **y1** and **y2** cause greater than 95% inhibition of primer extension at the homopurine target site (Figure 4, lanes 2 and 3). The shorter oligonucleotides **y3** and **y4** show termination efficiencies of 85% and 40%, respectively, when present at concentrations sufficient to saturate the target site by the criterion of in parallel DNase I footprinting (Figure 4, lanes 4 and 5). At oligonucleotide concentrations no longer sufficient for full occupancy of the target site, there is a further decrease in termination efficiency (Figure 4, lanes 6 and 7). Oligonucleotides with identical 5′-ends (**y1** and **y3**) show similar patterns of inhibition. This appears to be due to the position of the triple-helical region itself, rather than an effect due to a particular sequence in the context of the helix. Thus, oligonucleotide **y2**, which has a different 5′-end than **y1** and **y3**, yields a different termination pattern in the same homopurine run. That termination is similarly positioned a few bases downstream from the 5′-end of the triplex. Of the oligonucleotides that do not bind the target site significantly, only **y5** displays slight (less than 5%) inhibition of DNA synthesis localized near the 3′-end of the homopurine run (Figure 4, lane 8).

Using the restriction fragment system, similar results were obtained (Figure 3B). Again the extension process is halted several bases internal to the 5′-terminus of the triple helix. Oligonucleotides **y1** and **y2** cause greater than 95% inhibition of primer extension past the homopurine target site. The shorter triple-helix-forming oligonucleotides **y3** and **y4** show significant termination efficiencies of 75% and 60%, respectively. An unanticipated result, novel to this substrate, was that oligonucleotide **y5** caused significant polymerase stoppage, even though it does not form a stable triplex complex as measured by footprinting. This may be due to interactions of the oligonucleotide with the displaced strand during the

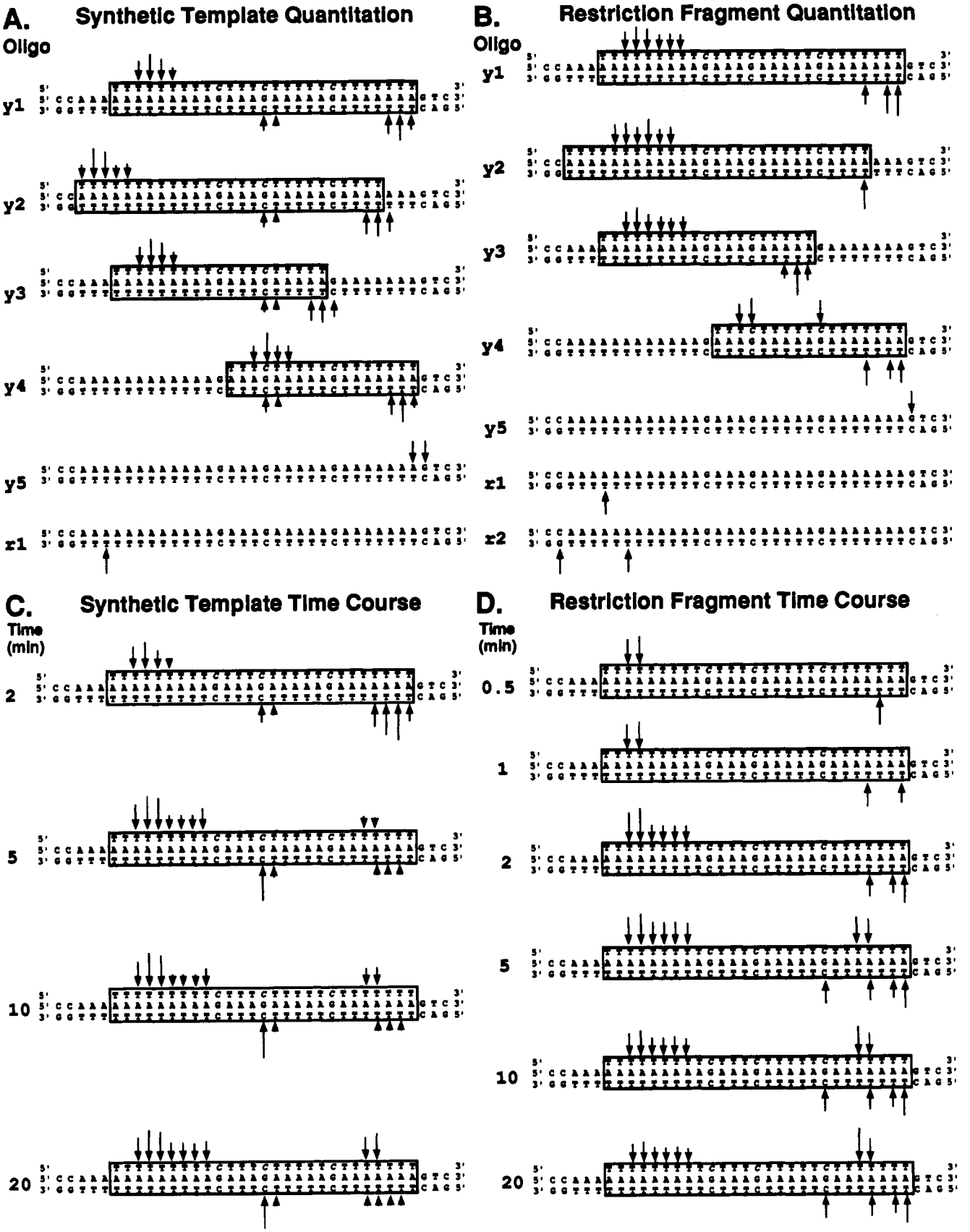

FIGURE 3: Histograms representing oligonucleotide-mediated termination patterns. Arrows indicate the location and relative intensities of polymerase stoppage. The upper arrows indicate pauses observed with primers **B** and **C** in the synthetic template and restriction fragment systems, respectively. The lower arrows indicate pauses observed with primers **A** and **D** in the synthetic template and restriction fragment systems, respectively. Pause sites that appear in the control and are independent of the oligonucleotide have been subtracted as background. (Panel A) Synthetic template system using oligonucleotides **y1**, **y2**, **y5**, **r1**, and **r2** at 0.05 μM concentration and oligonucleotides **y3** and **y4** at 1 μM concentration. Reactions were allowed to proceed for 2 min. (Panel B) Same conditions as panel A using the restriction fragment template. (Panel C) Oligonucleotide-mediated termination kinetics using the synthetic template system. Oligonucleotide **y1** is present at 0.05 μM concentration in all cases with the reaction time given. (Panel D) Same conditions as panel C using the restriction fragment template.

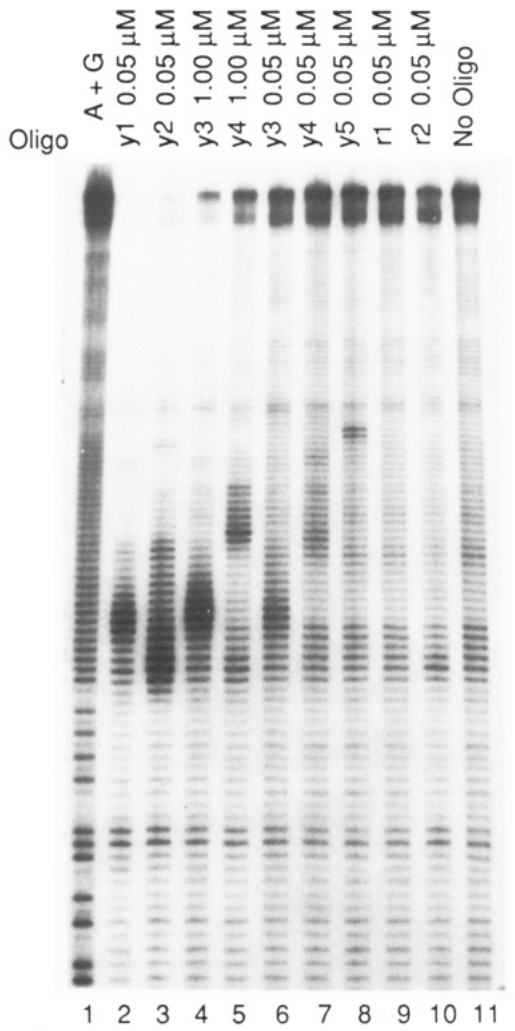

FIGURE 4: Autoradiogram of an 8% denaturing polyacrylamide gel showing the effect of oligonucleotides on primer extension reactions performed on a synthetic template. Klenow fragment was used to extend primer **B** off the strand containing the pyrimidine-rich tract in this 2-min reaction. Lane 1, A+G-specific chemical sequencing lane performed on the extension product of 5′-end-labeled primer **B**; lane 2, 0.05 μM **y1**; lane 3, 0.05 μM **y2**; lane 4, 1 μM **y3**; lane 5, 1 μM **y4**; lane 6, 0.05 μM **y3**; lane 7, 0.05 μM **y4**; lane 8, 0.05 μM **y5**; lane 9, 0.05 μM **r1**; lane 10, 0.05 μM **r2**; lane 11, no oligonucleotide control.

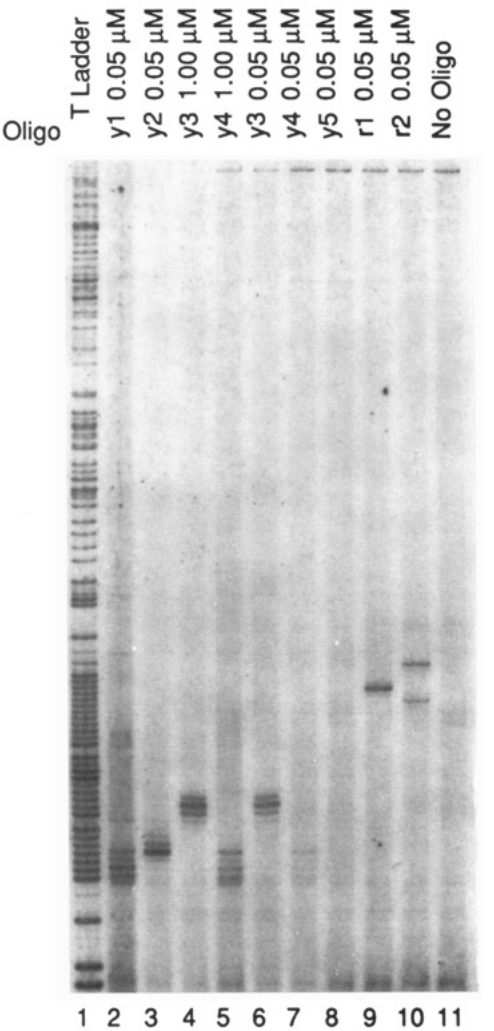

FIGURE 5: Autoradiogram of an 8% denaturing polyacrylamide gel showing the effect of oligonucleotides on primer extension reactions performed on a restriction fragment. Klenow fragment was used to extend 5′-end-labeled primer **D** off the strand containing the purine-rich tract in this 2-min reaction. Lane 1, T dideoxynucleotide sequencing reaction performed on plasmid pTER I; lane 2, 0.05 μM y1; lane 3, 0.05 μM y2; lane 4, 1 μM y3; lane 5, 1 μM y4; lane 6, 0.05 μM y3; lane 7, 0.05 μM y4; lane 8, 0.05 μM y5; lane 9, 0.05 μM r1; lane 10, 0.05 μM r2; lane 11, no oligonucleotide control.

primer extension reaction or to incomplete reannealing of the template, yielding oligonucleotide-stabilized higher order structures.

Inhibition of DNA Polymerase Priming from the Purine-Rich Strand. Using the synthetic template system, primer extension terminates directly internal to the 3′-end of the triple helix (Figure 3A). Both oligonucleotide probes **y1** and **y2** cause greater than 95% inhibition of primer extension past the target site. The shorter oligonucleotides **y3** and **y4** show a decreased amount of inhibition with termination efficiencies of 75% and 70%, respectively, when present at concentrations sufficient for full binding site occupancy. At lower concentrations, there is a further decrease in termination efficiency. Oligonucleotides with identical 3′-ends (**y1** and **y4**) show similar patterns of inhibition. Of the oligonucleotides that do not bind to the target site, only **r1** displays slight (less than 5%) inhibition of primer extension near the 5′-end of the homopurine run.

The restriction fragment system gave similar termination patterns with the extension process again being halted nearby the 3′-terminus of the triple helix as shown in Figure 3B and Figure 5. Both oligonucleotide probes **y1** and **y2** cause greater than 95% inhibition of primer extension past the homopurine target site (Figure 5, lanes 2 and 3). The shorter triple-helix-forming oligonucleotides **y3** and **y4** still show significant termination efficiencies of 95% and 85%, respectively (Figure 5, lanes 4 and 5). Oligonucleotides with identical 3′-ends show similar patterns of extension inhibition. Although having no affinity for the duplex target site, the homopyrimidine antisense oligonucleotides **r1** and **r2** cause significant stoppage. Again, it may reflect interactions of the oligonucleotide with the displaced strand during primer extension or more complicated higher order structures.

Kinetics of DNA Synthesis Inhibition Priming from the Pyrimidine-Rich Strand. Using the synthetic template system, triple-helix formation with oligonucleotide **y1** blocks primer extension at greater than 95% efficiency for at least 20 min as shown in Figures 3C and 6. During this 20-min time span (37 °C), the minor stop sites toward the 3′-end of the triple helix present at the 2-min time point become more prominent (Figure 6, lanes 2–5). Similar results are found using the restriction fragment based assay as shown in Figure 3D.

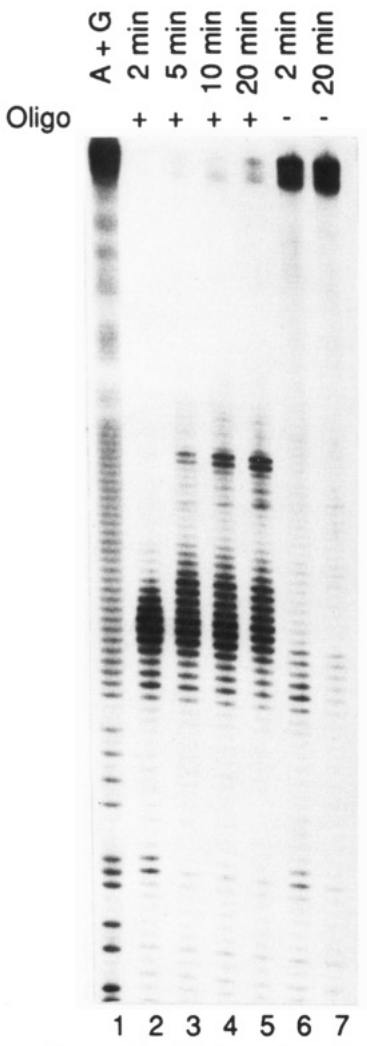

FIGURE 6: Autoradiogram of an 8% denaturing polyacrylamide gel showing the kinetics of a Klenow fragment primer extension reaction priming off the strand containing the pyrimidine-rich tract in the synthetic template system. The time of the reaction is given, and when indicated, reactions were done in the presence of 0.05 μM **y1**. Lane 1, A+G-specific chemical sequencing lane performed on the extension product of 5′-end-labeled primer **B**; lane 2, 2 min with **y1**; lane 3, 5 min with **y1**; lane 4, 10 min with **y1**; lane 5, 20 min with **y1**; lane 6, 2 min; lane 7, 20 min.

Kinetics of DNA Synthesis Inhibition Priming from the Purine-Rich Strand. The synthetic template system used in mapping termination events demonstrates that the triple helix formed with **y1** can block primer extension at greater than 95% efficiency for at least 20 min (Figure 3C). Once again, the minor stop sites toward the 5′-end of the triple helix present at the earlier 2-min time point become more prominent during the course of this time span. The restriction fragment system yields similar results as shown in Figure 3D.

DISCUSSION

We have found that triple-helical complexes of lengths ranging from 17 to 27 nt and of differing sequence composition inhibit primer extension by Klenow or modified T7 DNA polymerases. DNase I footprinting showed that only those oligonucleotides that stably occupied the target sequence were able to inhibit polymerase extension. Primer extension that copied from either strand of the duplex target in the synthetic template system was efficiently stalled adjacent to the first junction between triplex and duplex in the substrate. This block was stable for at least 20-min in the cases studied, although at longer time points there was an increase in larger termination products implying entry into the triplex or possible polymerase stuttering at the triplex junction. Interestingly, the efficiency of the polymerase blocks depended not only on triple-helix occupancy but also on the length of the triplex. When priming from either strand, shorter complexes were less efficient than longer ones. This length dependence is not a simple reflection of steady-state occupancy since DNase I footprinting assays showed complete occupancy of the target site by these oligonucleotides under polymerase assay conditions. However, it may indicate that polymerase remains on the blocked complex and can complete read-through upon oligonucleotide dissociation, thereby revealing more rapid dissociation rates for shorter oligonucleotides. An alternative or additional mechanism is that partial unwinding of the triple helices by polymerase is an active process which ultimately destabilizes the block, an effect that would be more efficiently disruptive to shorter complexes than longer ones.

A possible precedent for similar partial read-through by Klenow fragment has been observed in the blockage of an *in vitro* DNA replication fork by the *E. coli ter*-binding protein (Lee & Kornberg, 1992). In this case, the *ter*-binding protein (TBP) was shown to occupy a 22 base pair stretch of DNA, but allowed the T7 DNA polymerase to read 3 or 4 bp into the protected region in either direction. Interestingly, the same *ter* complex was effective in preventing any read-through into the 22 bp protected site by an *in vitro* DNA replication system containing DNA polymerase III holoenzyme, helicases, and single-strand binding proteins. This observation was attributed to TBP-mediated helicase inhibition in conjunction with the relative lack of strand-displacing activity of the DNA polymerase III holoenzyme. The inherent T7 or Klenow fragment strand-displacing activities, whose mechanism is not yet understood, may carry a local DNA melting activity that secondarily disrupts the interaction of either oligonucleotide or protein in its path. Alternatively, the ability to make partial progress into such obstacles might be revealing their capacity to physically disrupt diverse noncovalent interactions.

Oligonucleotides that could not stably occupy the double-helical DNA target, defined by DNase I footprinting criteria, had virtually no effect on primer extension in the synthetic fragment system. This included **y5** and **r1**, which are antisense complements to the two strands of the triple-helix target site, and **r2**, which shares partial Watson–Crick homology to the homopyrimidine strand. However, these reagents did display significant differences on the two substrate types studied: in the denaturation and annealing paradigm, the presence of an oligonucleotide that is the direct complement of the displaced strand target region gave a significant block to DNA synthesis. We believe that competing Watson–Crick pairing of the oligonucleotide generates alternative structures which then compete with the desired template product in the substrate preparation.

Possible Mechanisms of the Triplex-Mediated DNA Polymerase Block. There are several potential models for oligonucleotide-directed triple-helix inhibition of primer extension on duplex templates. In the systems we have studied, DNA synthesis occurs through a strand displacement mechanism. In order for the polymerase to continue primer extension, it must disrupt the downstream template duplex region. Although Klenow fragment lacks intrinsic helicase activity, it is able to perform strand displacement synthesis (Kornberg & Baker, 1992). One explanation for this phenomenon is that Klenow fragment may be able to exploit breathing of the template duplex beginning at a nick or gap and extending into

the fraying branch. Such end-melting effects are not fully understood, but it seems possible that the presence of a third strand together with the hydrogen-bonding and stacking interactions that accompany triplex formation might reduce this melting or fraying effect. In this model, the polymerase block would be secondary to enhanced duplex stability. One would predict from this model that if short triple helices could be generated using oligonucleotides with enhanced binding affinities due to improved third-strand stacking interactions, the efficiency of termination would be correspondingly improved.

An alternative model for the triplex-mediated polymerase blockade would be a significant distortion of the duplex template structure near the duplex/triplex junction. Previous chemical footprinting studies have shown that the double-helical regions near the triple-helical termini are structurally distorted from B-form geometry (Francois et al., 1988; Collier et al., 1991). Such distortion is also suggested by the DNase I footprinting experiment in this work; it revealed pronounced hypersensitivities at the 3′-junction of triple helices formed with oligonucleotides **y1**, **y2**, and **y4**. DNA polymerases may encounter such alterations in template topology and terminate the polymerization reaction. Other reports have suggested that Z-DNA-forming sequences adjacent to intramolecular triple-helical structures can cause stoppage that may be due to changes in template topology (Brinton et al., 1991). Nevertheless, the fact that shorter triple-helical complexes that share identical 5′- or 3′-termini with longer complexes gave the same pattern of DNA synthesis blockage, but have lesser termination efficiency, indicates that the junction by itself cannot be solely responsible for efficient termination.

There is no reported high-resolution structure of a paused DNA replication fork. In the most current crystal structure of the Klenow fragment, polymerase is bound to an 11 bp duplex with a single-stranded three-base 3′-overhang (Beese et al., 1993). Although this provides no information on the structure downstream of the 3′-end of an active extending primer, the dimensions of cleft 1 (containing the polymerase active site) and cleft 2 (formed upon binding duplex DNA) suggest that two DNA strands may be a tight fit, and this leads to speculation that three strands might be sterically disallowed. Indirect evidence concerning polymerase–DNA interactions at the displacement junction has come from DNase I footprinting on a nicked duplex template (Joyce et al., 1986). In this context, Klenow fragment protects a 4 base pair noncontact zone on the template strand. These investigators suggest that this is a region where the DNA strand curves away from the polymerase. If correct and important for function, it seems likely that such curvature would be altered by the presence of a third strand agent that can stiffen or distort the helix. This may account for the observed termination by the triple helix since the complex may prevent the polymerase from accepting the template into the polymerase active site. Moreover, while the current model for the Klenow fragment structure from crystallographic data does not provide guidance for strand displacement, it does suggest that double-helical DNA must bend 80° to enter the cleft containing the polymerase active site. This again presents a bending event that may be inhibited by the presence of a preformed triplex.

Inhibition of DNA Synthesis by Intramolecular Triple-Helix Formation. Several prior studies have reported that templates can form intramolecular, rather than intermolecular, triplexes and that these can inhibit DNA polymerase elongation (Lapidot et al., 1989; Baran et al., 1991; Brinton et al., 1991; Dayn et al., 1992). In all cases, these observations support the central conclusions from this study, although the intramolecular substrates undergo topological changes under triplex-favoring conditions, and the contributions due to such ancillary changes are difficult to clearly separate from direct triplex effects.

Effect of Triple-Helical Complexes on RNA Polymerases. A number of studies have focused on the ability of triple-helical complexes to alter transcription by preventing the binding of transcription factors, and this invites comparison with effects on DNA polymerase activities reported here. Several have shown that intermolecular triple helices positioned sufficiently far downstream so that they are presumed to be testing for effects on elongation showed either no blocking effect or minimal ones (Young et al., 1991; Maher, 1992; Duval-Valentin et al., 1992). Effects on transcriptional initiation are much more robust, and have been generated using triplex sites positioned either upstream (Maher et al., 1990; Maher, 1992) or just downstream (Duval-Valentin et al., 1993) of initiation sites. Not surprisingly, some of the inhibitory effects could be enhanced by covalently linking the third-strand reagent to the duplex target (Duval-Valentin et al., 1993). Thus, triple helices by themselves do not appear to be very effective in blocking elongation by either prokaryotic or eukaryotic RNA polymerases, and this result might reflect the fact that these polymerases are multiprotein holoenzyme complexes replete with DNA melting and helicase activities (Lewin, 1990). In contrast, triple helices substantially blocked elongation by the DNA polymerases studied in this work. It will now be interesting to extend this to additional DNA polymerase activities in the presence and absence of supporting activities provided by SSBs and helicases. Similarly, it will be interesting to test the effects of specifically modified third-strand oligonucleotides to discern how they block polymerase progress.

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