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DNA Substrate Structural Requirements for the Exonuclease and Polymerase Activities of Procaryotic and Phage DNA Polymerases†

Marlon Cowart, Katharine J. Gibson, Dwayne J. Allen, and Stephen J. Benkovic*
Department of Chemistry, The Pennsylvania State University, 152 Davey Laboratory, University Park, Pennsylvania 16802
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ABSTRACT: A DNA duplex covalently cross-linked between specific bases has been prepared. This and similar duplexes are substrates for the polymerase and exonuclease activities of the Klenow fragment of *Escherichia coli* DNA polymerase I and T4 and T7 DNA polymerases. The action of Klenow fragment on these duplexes indicates that the polymerase site does not require that the DNA duplex undergo strand separation for activity, whereas the exonuclease site requires that at least four base pairs of the primer strand must melt out for the exonucleolytic removal of nucleotides from the primer terminus. The exonucleolytic action of T4 and T7 DNA polymerases requires that only two and three bases respectively melt out for excision of nucleotides from the primer terminus. Klenow fragment and T4 DNA polymerase are able to polymerize onto duplexes incapable of strand separation, whereas T7 DNA polymerase seems to require that the primer terminus be at least three bases from the cross-linked base pair. A DNA duplex with a biotin covalently linked to a specific base has been prepared. In the presence of the biotin binding protein avidin, the exonucleolytic activity of Klenow fragment requires that the primer terminus be at least 15 base pairs downstream from the base with the biotin–avidin complex. On the other hand, the polymerase activity of Klenow fragment required that the primer terminus be at least six base pairs downstream from the base with the biotin–avidin complex. These results suggest that the polymerase and exonuclease sites of Klenow are physically separate in solution and exhibit different substrate structural requirements for activity.

Escherichia coli DNA polymerase I (Pol I)[1] is a 109-kDa protein required for repair and replication in vivo (Kornberg, 1980). In addition to a 3′→5′ polymerase activity, requiring a template to be copied and a primer strand to which nucleotides are added, the enzyme possesses a 3′→5′ exonuclease activity capable of removing nucleotides from the primer strand and a 5′→3′ exonuclease activity which removes nucleotides in front of the growing primer strand (Jovin et al., 1969). Limited proteolysis of Pol I yields a 68-kDa fragment (the Klenow fragment) which retains the polymerase and 3′→5′ exonuclease activity (Brutlag et al., 1969; Klenow & Henningsen, 1970). This fragment has been studied extensively by a variety of kinetic (McClure & Jovin, 1975; Bambara et al., 1976; Bryant et al., 1983; Mizrahi et al., 1985, 1986; Kuchta et al., 1987, 1988), stereochemical (Burgers & Eckstein, 1979; Brody & Frey, 1981; Gupta & Benkovic, 1984), genetic (Freemont et al., 1986), and structural (Joyce & Steitz, 1987; Ollis et al., 1985a) methods and is the chief object of this study.

X-ray crystallography has provided structural data of the Klenow fragment with substrates and inhibitors at 3.3-Å resolution (Steitz & Joyce, 1987; Ollis et al., 1985a). The location of the 3′→5′ exonuclease site was determined in crystals containing a nucleoside monophosphate (dTMP). Nucleoside monophosphates are known to be competitive inhibitors of 3′→5′ exonuclease activity. In the absence of metals, a single-stranded tetranucleotide DNA also was observed to bind to this site. A helical cleft proposed as a second binding site for double-stranded DNA was located in the enzyme, which from footprinting experiments would cover about eight base pairs of double-stranded DNA. By use of the X-ray structural data and guided by data from enzymes constructed with point mutations at the putative active sites that had been found to have altered polymerase or exonuclease activity (Freemont et al., 1986), B-DNA was model-built into the enzyme to approximate the location of the polymerase and exonuclease sites. One particularly intriguing observation gleaned from the model was that the putative polymerase and exonuclease sites were about 30 Å apart, prompting questions of how the two sites work together during DNA synthesis.

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* Author to whom correspondence should be addressed.

[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; kDa, kilodalton(s); TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DMT, 4,4′-dimethoxytrityl; CNE, 2-cyanoethyl; TEAB, triethylammonium bicarbonate; TEAA, triethylammonium acetate; DTT, dithiothreitol, Tris, tris(hydroxymethyl)aminomethane; dNMP, 2′-deoxynucleoside monophosphate; dNTP, 2′-deoxynucleoside triphosphate.

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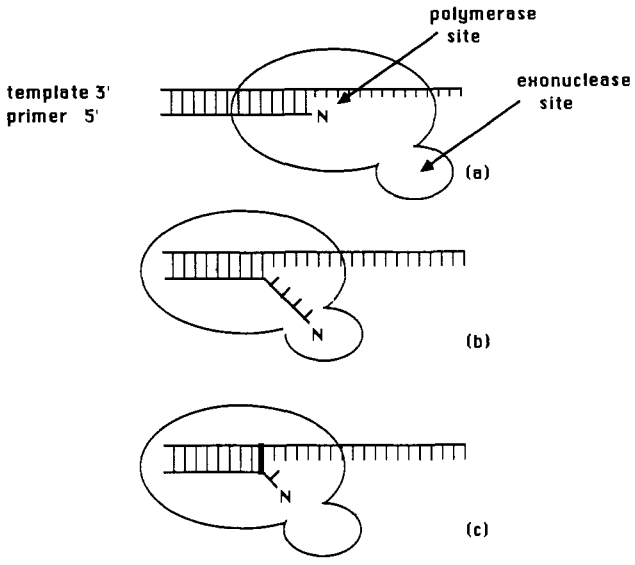
Chart I: Test for Requirement of Strand Separation by Klenow Fragment[a]

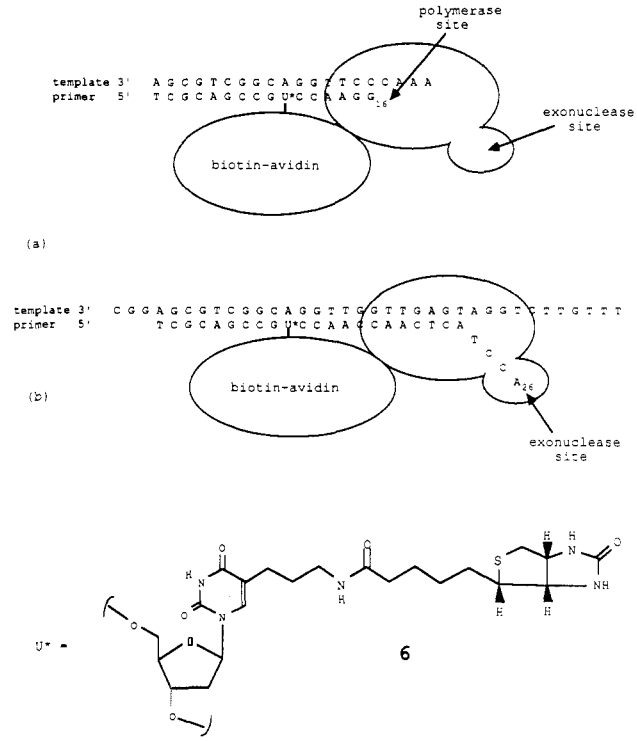


[a] (a) A primer terminal nucleotide N in the polymerase site; (b) the melting out of at least four or five base pairs of the primer terminus in order for the terminal nucleotide N to occupy the exonuclease site and be excised; (c) the presence of a covalently cross-linked base pair (thick line) preventing the primer from occupying the exonuclease site.

Joyce and Steitz have proposed that in order for the 3′ primer terminus to span the distance from the polymerase site to the exonuclease site the DNA would have to slide about eight base pairs through the enzyme while at the same time the terminal three to four base pairs of the primer terminus strand would have to melt out (Joyce & Steitz, 1987). Evidence for this hypothesis was derived from X-ray crystallographic data on a cocrystal of Klenow fragment with an eight base pair duplex with a three base single-stranded 5′ extension. Four bases of single-stranded DNA were found extending from the exonuclease site toward the duplex binding site. This suggests that the polymerase site binds double-stranded DNA, whereas the exonuclease site requires single-stranded DNA and could not excise nucleotides from the primer terminus if the DNA were forced to remain double stranded less than four bases away. DNA duplexes in which the two strands are covalently cross-linked have been prepared (Webb & Matteucci, 1986a,b). Such duplexes depicted in Chart I would seem to provide an ideal test of the structural requirements for the polymerase and exonuclease activities of Klenow fragment. In this paper, we describe the preparation of a cross-linked DNA duplex and report its suitability as a substrate for the polymerase and 3′→5′ exonuclease activities of Klenow fragment and for T4 and T7 DNA polymerases.

Another way to probe the separation of the polymerase and exonuclease sites in Klenow fragment is to attach a bulky group to the DNA at a specific nucleotide. Both activities of Klenow fragment are at the 3′ primer terminus of DNA duplexes; if the enzyme abuts the bulky group attached to the DNA, it might be blocked sterically from functioning. A variety of DNA and RNA oligomers with biotin covalently attached to a specific base have been prepared by chemical and chemical/enzymatic methods (Langer et al., 1981; Chu & Orgel, 1985; Cook et al., 1988) that can bind avidin, a large (66 kDa) protein which has a very high affinity for biotin ($K_d \leq 10^{-15}$ M). It was reasoned that such a large bulky group attached to the DNA would impart the necessary steric properties to the duplex as a substrate, since the sheer size of the avidin should prevent the Klenow fragment from reaching

Chart II: Biotinylated Oligomer Attached to Avidin[a]



[a] (a) Distance for active polymerization; a shorter primer is inactive. (b) Distance requirement for exonuclease activity; with a shorter primer bases cannot be excised.

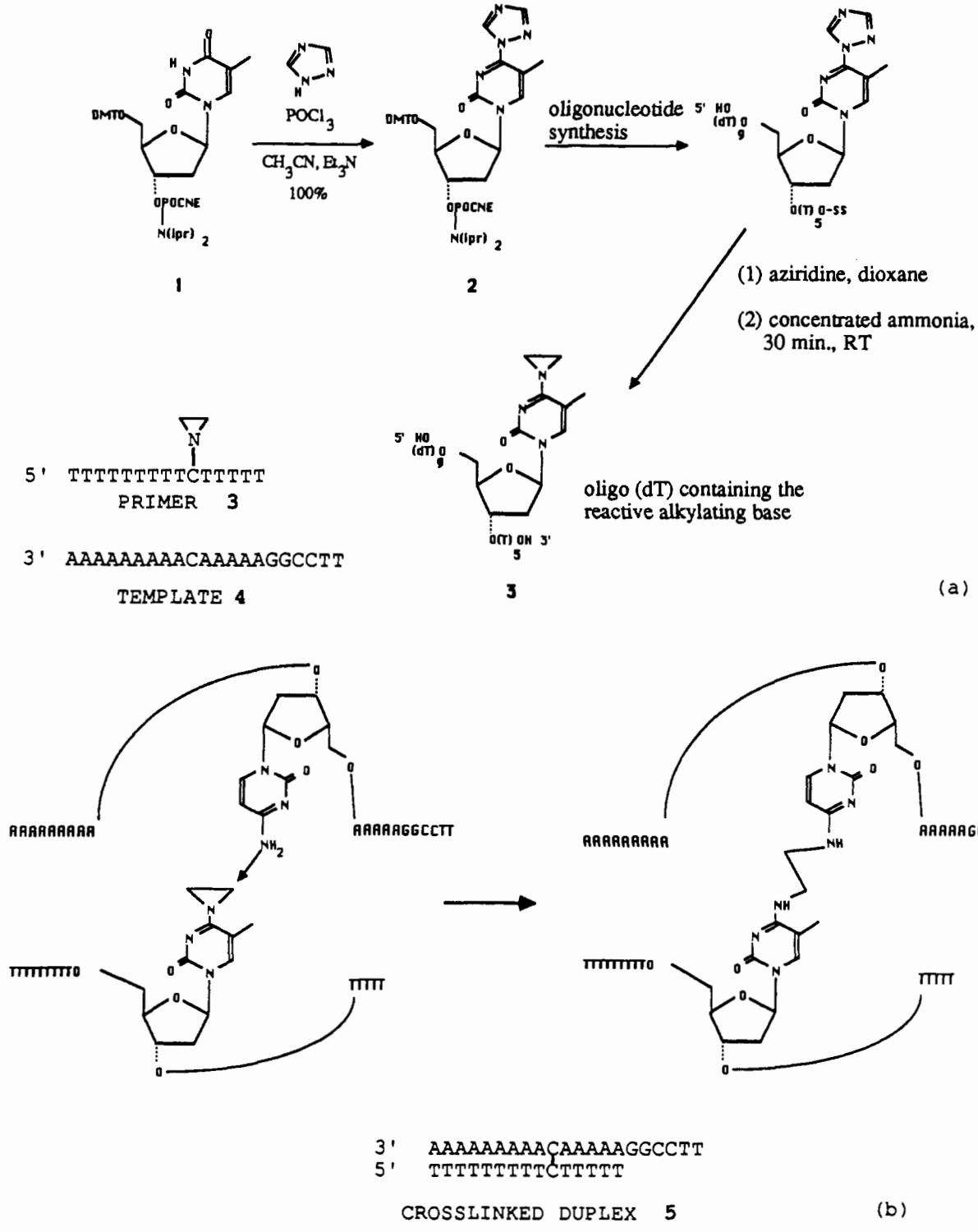
the primer terminus until the primer terminus is more distant from the base attached to the biotin–avidin complex. If the polymerase and exonuclease sites of the Klenow fragment are indeed physically separate, then they might have different distance requirements between the base attached to the biotin–avidin and the primer terminus (Chart II).

Experimental Procedures

Materials. Klenow fragment was purified from *E. coli* CJ155 supplied by Catherine Joyce (Yale University) (Joyce & Grindley, 1983). T4 DNA polymerase was purified from a cloned overproducer supplied by William Konigsberg (Yale University School of Medicine). T4 polynucleotide kinase and Sequenase were from U.S. Biochemical Corp. T7 DNA polymerase was prepared by mixing T7 gene 5 protein and *E. coli* thioredoxin (both prepared from cloned overproducers supplied by Arne Holmgren of the Karolinska of Stockholm, Sweden) in a ratio of 1:7. (*N*-Hydroxysuccinimidyl)biotin and avidin were from Sigma. 5′-(Dimethoxytrityl)thymidine 3′-CED phosphoramidite was from American Bionetics. Nucleoside triphosphates were from Sigma. [α-^{32}P]dCTP and [γ-^{32}P]ATP (>3000 Ci/mmol) were from New England Nuclear. Scintiverse II was from Fisher. 1,2,4-Triazole from Aldrich was recrystallized before use. Acetonitrile and triethylamine from Aldrich were distilled from calcium hydride before use. All other reagents were of the highest quality commercially available.

Electrophoresis. DNA oligomers were analyzed by quenching reaction solutions into 90% formamide buffer (90 mM Tris–borate and 10 mM EDTA at pH 8.3), followed by electrophoresis on polyacrylamide denaturing gels. Bands corresponding to oligomers were visualized by autoradiography and quantified either by cutting out and counting the bands of the gel in Scintiverse II with a Beckman LS 6800 scintillation counter or by densitometry of the autoradiograph using an LKB Ultroscan XL densitometer.

Scheme I: (a) Synthesis of Modified Oligonucleotide **3** after the Method of Webb and Matteucci (1986a) and (b) Nature of the Cross-Link Formed on Mixing **3** and **4**



Synthesis of Cross-Linked DNA Duplex **5**. A modification of the method of Webb and Matteucci (1986b) was used to convert 5′-(dimethoxytrityl)thymidine 3′-CED phosphoramidite to its 4-triazole derivative in quantitative yield (Scheme I).

To a solution of 179 mg (0.25 mmol) of protected thymidine **1** and 388 mg (5.62 mmol) of 1,2,4-triazole in 0.8 mL of triethylamine and 2.5 mL of acetonitrile under argon was added 46 μL (0.49 mmol) of freshly distilled $POCl_3$. After stirring at room temperature for 7 h and monitoring the conversion (λ_{max} 267 nm → λ_{max} 328 nm), the mixture was poured into 50 mL of ethyl acetate and 10 mL of triethylamine and washed with 5% aqueous sodium carbonate (2 × 50 mL). The organic phase was separated, dried over Na_2SO_4, and concentrated in vacuo to a colorless syrup. The triazole derivative **2** was dried by coevaporation with toluene. Thin-layer chromatography and NMR indicated the absence of byproducts and starting material. Thin-layer chromatography of the product, **2**, revealed r_f = 0.66, 0.69 (diastereomeric mixture) on silica gel (45% dichloromethane, 45% ethyl acetate, 10% triethylamine). ^1H 300-MHz NMR ($CDCl_3$) characteristic resonances are at δ 9.22 (s, 1 H), 8.34 (d, 2 H, J = 14 Hz), 8.02 (s, 1 H), 7.1–7.4 (m, 9 H), 6.78 (m, 4 H), 6.29 (q, 1 H, J = 6 Hz), 2.58 (t, 1 H, J = 5 Hz), 2.39 (t, 1 H, J

= 5 Hz), 1.85 (d, 3 H, J = 3 Hz), and 1.12 (m, 12 Hz).

A 15-base oligonucleotide, **3**, containing the modified base 4-ethano-5-methylcytosine was synthesized by reaction of the corresponding 4-triazolo-5-methylcytosine-containing oligonucleotide (prepared on an Applied Biosystems 380A DNA synthesizer using phosphoramidite methodology) with aziridine. A complementary 21-base oligonucleotide template strand, **4**, was also synthesized. Both oligomers were judged to be >80% pure by C_{18} reversed-phase HPLC (linear gradient of 0% acetonitrile in 0.1 M aqueous triethylammonium acetate, pH 7.6, to 100% acetonitrile). An equimolar aqueous solution of the triethylammonium salts of the two oligomers (128 μM) was allowed to stand for 4 days at 22 °C to cross-link. After lyophilization, the mixture was purified by preparative gel electrophoresis on a 20% denaturing gel (Rickwood & Hames, 1983). After electrophoresis, bands corresponding to primer, template, and cross-linked duplex were located by UV shadowing and cut out and eluted from the gel with 0.1 M triethylammonium bicarbonate (pH 7.7). The eluate was purified by adsorption on 1 mL of DE-52 resin which had been prequilibrated with 0.1 mL of triethylammonium bicarbonate at pH 7.7; then, the resin was washed with 5 mL of 0.1 M triethylammonium bicarbonate and the product eluted with 1.0 M triethylammonium bicarbonate. Lyophilization gave pure primer **3**, template **4**, or cross-linked duplex **5**. Webb and Matteucci (1986b) sequenced a cross-linked duplex similar to **5** and determined that the two opposing cytosines were covalently attached to each other. Because the N4 of cytosine is quite nucleophilic, it is likely that the cross-link is formed as shown in Scheme I. Since polynucleotide kinase inefficiently labeled cross-linked duplex **5**, radiolabeled cross-linked duplex **5** was prepared by 5′-^{32}P labeling of purified template strand **4** with [γ-^{32}P]ATP by use of T4 polynucleotide kinase (Mizrahi et al., 1986). After hybridization and cross-linking with a 6-fold excess of *crude* (gel purification of the primer strand destroys its ability to form cross-linked duplex) primer strand as before, the cross-linked duplex was purified by gel electrophoresis on a 13% denaturing polyacrylamide sequencing gel and, following autoradiography, by DE-52 chromatography. This gave cross-linked duplex of high (>2 × 10^6 cpm/pmol) specific activity. The concentration of duplexes was determined by 3′ fill-in labeling with Klenow fragment using [α-^{32}P]dCTP and quantitation by the DE-81 filter binding assay (Bryant et al., 1983).

Interaction of the Cross-Linked Duplex with Polymerases. All reactions were performed at 22 °C. Unless otherwise noted, all reactions with the Klenow fragment of DNA polymerase I contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA. All reactions with T4 DNA polymerase contained 33 mM Tris–acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, and 0.1 mg/mL bovine serum albumin at pH 7.5. All T7 DNA polymerase reactions contained 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, and 0.1 mg/mL bovine serum albumin at pH 7.8.

The ability of Klenow fragment to remove exonucleolytically dTMP from cross-linked duplex **5** was examined by adding Klenow fragment to initiate a reaction containing 26 nM duplex **5** (2.7 × 10^5 cpm/pmol), 6.3 μM dATP (to protect the blunt end from excision), and 300 nM Klenow fragment. At appropriate intervals over 2 h, aliquots were withdrawn and analyzed by gel electrophoresis. Then dTTP was added to a concentration of 5.5 μM, and after 1 min an aliquot was withdrawn for analysis. Then dCTP was added to 2.5 μM, and after 1 min an aliquot was withdrawn for analysis. Because the K_d of Klenow fragment with DNA is quite low (Kuchta et al., 1988), most of the DNA is bound to enzyme, and the exonuclease reaction followed pseudo-first-order kinetics.

The ability of Klenow fragment to remove exonucleolytically dAMP from cross-linked duplex **5** was examined by adding Klenow fragment to initiate a reaction containing 39 nM duplex **5** (10^5 cpm/pmol), 2.9 μM dTTP, and 450 nM Klenow fragment. At appropriate intervals, aliquots were withdrawn and analyzed by gel electrophoresis. Then dATP was added to 4 μM and dTTP to 3.9 μM, and at various timepoints aliquots were removed for analysis on a 13% polyacrylamide denaturing gel.

A control reaction was conducted to demonstrate that Klenow fragment can completely degrade the *un-cross-linked* duplex formed by mixing an equimolar solution of template **4** and primer **3**. Primer **3** was 5′-^{32}P end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and this mixed with purified template **4**. Klenow fragment was added to initiate a reaction containing 1.0 μM un-cross-linked duplex, 5 μM dATP, and 2.3 μM Klenow fragment. At appropriate intervals, aliquots were withdrawn and analyzed by gel electrophoresis on a 16% polyacrylamide denaturing gel.

The ability of T4 DNA polymerase to remove exonucleolytically dTMP from cross-linked duplex **5** was examined by adding T4 DNA polymerase to initiate a reaction containing 1.0 μM duplex **5** (70 000 cpm/pmol), 27 μM dATP, and 31 nM T4 polymerase in T4 polymerase buffer. At appropriate intervals, aliquots were withdrawn for analysis by gel electrophoresis. Then dTTP was added to 39 μM, and after 1 min an aliquot was removed for analysis. Then dCTP was added to 14.6 μM, and after 1 min an aliquot was removed for analysis on a 13% polyacrylamide denaturing gel.

The ability of Klenow fragment to elongate duplexes exonucleolytically degraded by T4 DNA polymerase was demonstrated by adding T4 DNA polymerase to initiate a reaction containing 470 nM duplex **5** (6 × 10^3 cpm/pmol), 80 μM dATP, and 31 nM T4 polymerase in T4 polymerase buffer. After 1 min, EDTA was added to 10.6 mM and the sample heated at 90 °C for 15 min. The sample was cooled to 22 °C and brought to pH 7.6 with Tris-HCl; $MgCl_2$ was added to 33 μM and dTTP to 100 μM. After 5 min an aliquot was removed for analysis. Klenow fragment was added to 3.2 μM, and aliquots were withdrawn for analysis after 1 and 2 min on a 13% polyacrylamide denaturing gel.

The ability of T7 DNA polymerase to remove exonucleolytically dTMP from the primer strand of cross-linked duplex **5** was examined by adding T7 polymerase to initiate a reaction containing duplex **5** (390 nM, 600 cpm/pmol), 260 μM dATP, and T7 polymerase (24 nM) in buffer. At appropriate time points, aliquots were removed for electrophoresis on a 16% polyacrylamide denaturing gel.

The ability of a modified T7 polymerase (Sequenase; Tabor & Richardson, 1987) to elongate duplexes produced by the exonucleolytic action of T4 DNA polymerase on **5** was examined. T4 polymerase was added to initiate a reaction containing labeled duplex **5** (354 nM, 600 cpm/pmol), 236 μM dATP, and 14 nM T4 polymerase in T4 polymerase buffer. After 2.5 min at 22 °C, EDTA was added to 10 mM, and the reaction was heated at 70 °C for 5 min and then cooled to 22 °C. $MgCl_2$ was added to 17 mM, and dATP, dTTP, and dCTP were added to 73, 144, and 54 μM, respectively. Sequenase (12.5 units) was added, and at appropriate time points, aliquots were quenched into formamide buffer and analyzed by autoradiography following electrophoresis on a

Chart III: Structures (a) of the Biotinylated 11-mer Used as Primer, (b) of the 20-mer and 36-mer Used as Templates, and (c) of the Products Formed by Treatment with Klenow Fragment and Appropriate Triphosphates

```
5'  TCGCAGCCGU'C          5'   TCGCAGCCGU*C    11-mer

    U'= 5-aminopropyl-2'-deoxyuridine
    U*= 5-biotinylated-2'-deoxyuridine 6                    (a)

3'  AGCGTCGGCAGGTTCCCAAA                       20-mer template

3'  CGGAGCGTCGGCAGGTTGAGTAGGTCTTGTTT           36-mer template (b)

3'  AGCGTCGGCA-GGTTCCCAAA
5'  TCGCAGCCGU*C                               11/20 mer

3'  CGGAGCGTCGGCA-GGTTGAGTAGGTCTTGTTT
5'     TCGCAGCCGU*C                            11/36 mer

3'  AGCGTCGGCA-GGTTCCCAAA
5'  TCGCAGCCGU*CC                              12/20 mer

3'  AGCGTCGGCA-GGTTCCCAAA
5'  TCGCAGCCGU*CCAA                            14/20 mer

3'  AGCGTCGGCA-GGTTCCCAAA
5'  TCGCAGCCGU*CCAAGGG                         17/20 mer

3'  CGGAGCGTCGGCA-GGTTGAGTAGGTCTTGTTT
5'     TCGCAGCCGU*CCAACTCATCCAG                26/36 mer

3'  CGGAGCGTCGGCA-GGTTGAGTAGGTCTTGTTT
5'     TCGCAGCCGU*CCAACTCATCCAGAACAAA          33/36 mer       (c)
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16% polyacrylamide denaturing gel.

Preparation of Biotinylated DNA. An 11-mer (**6**), shown in Chart III, containing 5-(3-aminopropyl)-2′-deoxyuridine was synthesized by methods described previously (Gibson & Benkovic, 1987). To 0.5 mg of (*N*-hydroxysuccinimidyl)biotin in 100 μL of 0.2 M TEAB (pH 9.5) was added 25 μL of a 0.5 mM solution of the modified 11-mer **6**. After 3 h at 22 °C, the products were purified by reversed-phase chromatography on a C_{18} column using a linear gradient of 10% CH_3CN in 0.1 M TEAA (pH 7.0) to 100% CH_3CN for elution. Two products were isolated: unbiotinylated 11-mer and biotinylated 11-mer, eluting at 47.7% CH_3CN/0.1 M TEAA and 48.3% CH_3CN/0.1 M TEAA, respectively.

Interaction of Biotinylated DNA with Klenow Fragment. The ability of Klenow fragment to polymerize duplexes with attached avidin was examined. Biotinylated 11-mer was 5′-^{32}P end labeled by the method of Mizrahi et al. (1985). The 11-mer was annealed to a 20-mer as a template and elongated to various lengths by the reaction of 50 nM 11/20-mer, 10 nM Klenow fragment, and 5 μM nucleoside triphosphates in 50 mM Tris-HCl and 5 mM $MgCl_2$ at pH 7.6 for 15 min at 22 °C. Addition of dCTP gave 12/20-mer; addition of dCTP and dATP gave 14/20-mer; addition of dCTP, dATP, and dGTP gave mostly 17/20-mer. In each case, in addition to the product named, there were formed smaller amounts of shorter products, thus generating a "ladder" of oligonucleotides. After addition of avidin to the reaction mixtures, the susceptibility of the products to elongation with Klenow fragment was examined. Reactions were made 27 nM in oligonucleotide, 33 μM each in dATP, dCTP, dGTP, and dTTP, and 267 nM in avidin in 50 mM Tris-HCl, pH 7.6, and 5 mM $MgCl_2$. After 5 min Klenow fragment was added to 200 nM, and aliquots were quenched into formamide buffer after 0, 10, and 30 min for analysis by 12% polyacrylamide denaturing gel electrophoresis followed by autoradiography (samples were heated at 100 °C for 5 min prior to electrophoresis). Densitometry of bands on the autoradiograph allowed determination of which products could be elongated by Klenow fragment.

The ability of Klenow fragment to remove exonucleolytically bases from a duplex with attached avidin was examined. The biotinylated 11-mer was 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Mizrahi et al. (1985). The biotinylated 11-mer was then elongated to 26-mer in a reaction containing 100 nM 11-mer, 200 nM 36-mer as template, 10 μM each of dCTP, dATP, and dTTP, and 20 nM Klenow fragment in 50 mM Tris-HCl and 5 mM $MgCl_2$ at pH 7.6. After 15 min at 22 °C, the reaction was heated at 100 °C to destroy the polymerase and cooled to 22 °C, and glucose was added to 5 mM. The coupled action of nucleoside diphosphate kinase and hexokinase (0.1 unit each) was used to convert the nucleoside triphosphates to diphosphates by incubation of the above reaction mixture at 37 °C for 40 min. At the end of the incubation, all enzymes were destroyed by heating at 100 °C for 2 min. The labeled biotinylated 26/36-mer (30 nM) was treated with avidin at 400 nM for 5 min and then with Klenow fragment (200 nM) in 50 mM Tris-HCl and 5 mM $MgCl_2$, pH 7.6, at 37 °C to examine the action of exonuclease activity of Klenow on the DNA. Control reactions contained no avidin. Aliquots were withdrawn after 0.5, 1, 2, 3, 4, and 5 h and analyzed by gel electrophoresis on a 12% polyacrylamide denaturing gel, followed by autoradiography.

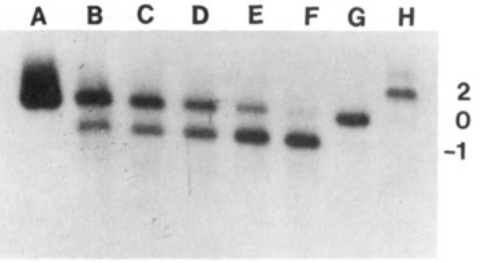


FIGURE 1: Interaction of Klenow fragment with cross-linked duplex **5** in the presence of dATP. DNA and excess enzyme after 0, 1, 2, 4, 8, and 16 min (A–F) demonstrate exonuclease activity; addition of dTTP allows polymerization back to the original duplex (G), while addition of dCTP extends the duplex (H) (13% denaturing gel). Numbers indicate the number of nucleotides removed from, or added to, the primer terminus.

By the method used to elongate the 11/36-mer to 26/36-mer, 11/36-mer was elongated to 33/36-mer, except that all four deoxynucleoside triphosphates were in the polymerization reaction. The elongated duplex was treated with excess Klenow fragment as previously described, and aliquots of the reaction were quenched after 30 s and 1 and 2 h and analyzed by 12% denaturing polyacrylamide gel electrophoresis, followed by autoradiography.

Results

Two types of chemically modified DNA duplexes have been used to test the proposal that the polymerase and exonuclease sites of Klenow fragment are physically separate in solution with bound substrates: covalently cross-linked duplexes and duplexes containing a base with an attached biotin–avidin complex.

Cross-Linked Duplexes. The cross-linked duplex **5** was found to be a competent substrate for the exonuclease and polymerase activities of Klenow. Klenow fragment removes one base from the primer strand of the duplex but is incapable of removing bases three or less from the cross-linked base pair (Figure 1).[2] Gel electrophoretic analysis of the products

[2] Examination of lanes B–F in Figure 1 reveals that besides starting duplex **5** only one product is produced by exonuclease activity, but one cannot be certain from this evidence alone that only one and not more bases were removed. However, comparison of the products from Klenow exonuclease action on **5** with the "ladder" of fragments formed by the successive removal of bases from **5** by T4 DNA polymerase confirms that there are no intermediate "rungs" on the ladder. Additionally, exonuclease action on DNA should be nonprocessive because the rate of dissociation of DNA from Klenow fragment (0.1 s^{-1}) has been found to be much faster than the rate of exonuclease activity (about 5×10^{-3} s^{-1}).

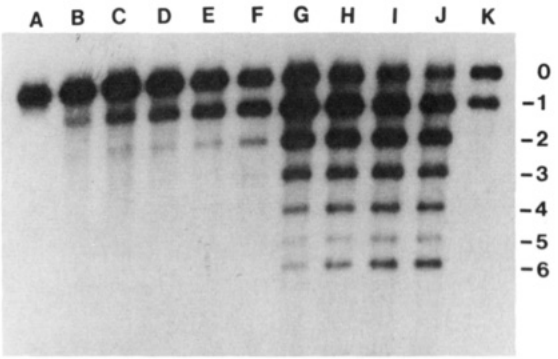


FIGURE 2: Interaction of Klenow fragment with cross-linked duplex **5** in the presence of dTTP. DNA and excess enzyme after 0, 1, 2, 4, 8, 16, 30, 45, 60, and 75 min (A–J) demonstrate that enzyme can remove only six nucleotides; addition of dATP allows polymerization back to the original duplex after 1 min (I) (13% denaturing gel) (see footnote 3). Numbers indicate the number of nucleotides removed from, or added to, the primer terminus.

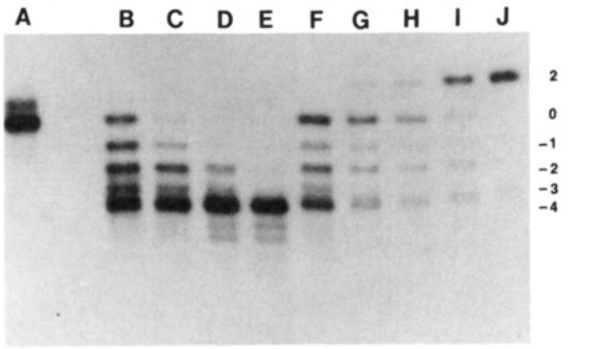


FIGURE 3: Interaction of T4 DNA polymerase with cross-linked duplex **5** in the presence of dATP. DNA (1 μM) and T4 polymerase (31 nM) after 0, 1, 2, 5, and 10 min (A–E) demonstrate that the enzyme exonucleolytically removes four bases. Addition of dTTP (F) allows the enzyme to polymerize the DNA back to the original duplex, while addition of dCTP allows further polymerization after 1, 2, 5, and 15 min (G–J). Numbers indicate the number of nucleotides removed from, or added to, the primer terminus.

formed by the action of excess Klenow on the duplex revealed that with excess enzyme the reaction was pseudo first order and that the rate of exonuclease activity was 4.6×10^{-3} s^{-1}. This is comparable to the rates of exonuclease activity of Klenow found with other *un-cross-linked* duplexes of 10^{-3} to 7×10^{-3} s^{-1} (Kuchta et al., 1988). The enzyme was able to remove one additional base from the primer strand, but this action is exceedingly slow, at a rate less than 5×10^{-5} s^{-1}. These DNA duplexes also were found to be competent substrates for polymerization by Klenow fragment (Figure 1, lanes G and H).

The ability of Klenow fragment to remove dAMP from the blunt end of cross-linked duplex **5** was examined in the presence of dTTP to fill in any dTMP removed at the other end. Gel electrophoretic analysis of the ladder of products formed clearly reveals that the enzyme can remove six nucleotides of the duplex as seen in Figure 2,[3] but cannot remove bases three or less from the cross-linked base pair. These duplexes were found to be competent substrates for polymerization.

A control reaction with Klenow fragment and *un-cross-linked* duplex **5** revealed that the exonucleolytic activity can completely degrade the primer strand within 15 min to the dinucleotide (data not shown). This amounts to a very rapid rate of exonuclease activity on this substrate (about 5-fold faster when compared to un-cross-linked duplexes containing all four bases) and may reflect the weak A-T base pairing in this duplex, as well as the presence of the C-C mismatch, which would allow DNA melting to occur readily. This would allow the primer terminus to more easily occupy (and be excised by) the exonuclease site.

T4 DNA polymerase (Kornberg, 1980) utilized cross-linked duplex **5** as a substrate for exonuclease activity as seen in Figure 3. This enzyme has an exceedingly active 3′→5′ exonuclease activity, about 400 times faster than that of Klenow fragment (Capson, unpublished results), and accordingly displays quantitatively as well as qualitatively different activity with cross-linked duplex **5**. Comparison with Klenow fragment reveals that a lower concentration of T4 polymerase removes bases from the primer strand of **5** faster and that the enzyme is capable of removing bases up to one base away from the cross-link. All these duplexes were efficiently elongated by the T4 polymerase. When cross-linked duplex **5** was subjected to the exonuclease activity of T4 DNA polymerase for 1 min, a "ladder" of products was formed, representing duplexes shorter than **5** by one or more bases. After destruction of the T4 polymerase, and addition of dTTP, Klenow elongated the ladder of products to the original duplex (data not shown).

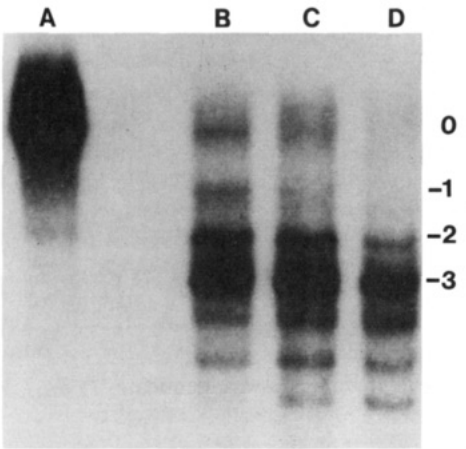


FIGURE 4: Exonucleolytic degradation of cross-linked duplex **5** by T7 DNA polymerase. Cross-linked duplex (390 nM) in the presence of dATP (260 μM) and T7 DNA polymerase (24 nM) at time = 0, 1, 2, and 4 min. It apparent that the enzyme quickly removes three bases from the 3′ end of the primer strand. The lower bands seen at longer time points are due to the removal of bases from the 3′ end at the other (blunt) end of the cross-linked duplex. Numbers indicate the number of nucleotides removed from, or added to, the primer terminus.

Bacteriophate T7 DNA polymerase was able to utilize cross-linked duplex **5** as a substrate for exonuclease activity as seen in Figure 4. The enzyme is capable of removing three nucleotides from the 3′ primer strand to leave duplexes with two nucleotides downstream from the cross-linked base pair. The ability of T7 polymerase to elongate various cross-linked duplexes produced by brief treatment of **5** with T4 polymerase was examined. Following partial degradation of **5** by T4 polymerase, and addition of T7 polymerase and nucleotide triphosphates, reactions were analyzed by densitometry fol-

[3] It should be noted that the exonuclease activity of Klenow is not fast enough to completely remove the nucleotides from the blunt end in the time allotted for exonuclease action, but it is apparent from the ladder of products formed that only six bases can be removed from this end. As seen in lane H, the enzyme does not fill in primers to blunt ends as efficiently as recessed 3′ ends. This fact has been previously observed in this laboratory. It has also been reported that the K_d of Klenow fragment for blunt-ended DNA is 2 orders of magnitude greater than that for DNA with a 5′ extension of even four bases (Joyce et al., 1986).

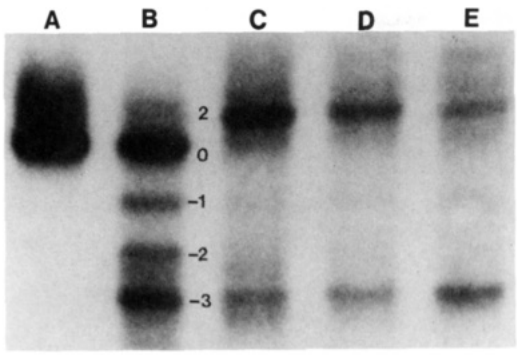


FIGURE 5: Cross-linked duplex **5** (354 nM) (A) was exonucleolytically degraded by the action of T4 polymerase (14 nM) for 2.5 min before addition of EDTA and heat to destroy the enzyme. dATP, dTTP, and dCTP were added (to 73 μM, 144 μM, and 54 μM, respectively), along with $MgCl_2$ (B). Sequenase (12.5 units) was then added and polymerization allowed for 5, 10, and 30 min (C–E). Densitometry revealed that duplexes that had three dTMPs excised from the primer terminus could not be elongated, although duplexes with longer primer terminii could be extended. Numbers indicate the number of nucleotides removed from, or added to, the primer terminus.

lowing denaturing gel electrophoresis. It was found that the enzyme could elongate only those duplexes where the primer terminus was three or more bases downstream from the cross-linked base pair. Because it was possible that the T7 polymerase might be too sluggish in initiating polymerization on these shortened duplexes (in comparison to the exonuclease activity which would remove bases), we examined the action of a modified T7 polymerase (Sequenase; Tabor & Richardson, 1987) on the shortened duplexes (Figure 5). Sequenase is a chemically modified T7 polymerase with reduced exonuclease activity. The action of the modified T7 polymerase on the shortened duplexes was identical with that of T7 DNA polymerase, although the reduced exonuclease activity of the modified enzyme made the reaction easier to follow.

Biotinylated Oligonucleotides. A biotinylated oligonucleotide of defined sequence was prepared by treatment of a 5-(3-aminopropyl)-2′-deoxyuridine-containing 11-mer with (*N*-hydroxysuccinimidyl)biotin. The derivatized and underivatized oligonucleotides were separated and purified by reversed phase HPLC. The biotinylated 11-mer was then used as a primer after annealing to either a 20-mer or a 36-mer as a template.

A set of DNA duplexes with different distances between the biotinylated base and the primer terminus was generated by partial extension of the duplexes (Chart III). In the absence of avidin, Klenow fragment was used to elongate 11/20-mer to various lengths. In the presence of dCTP, 12/20-mer was formed; in the presence of dCTP and dATP, 14/20-mer was formed; and addition of dCTP, dATP, and dGTP gave mostly 17/20-mer. Conditions were such that the enzymatic extensions were not 100% efficient, so in every case, a ladder of products resulting from incompletely elongated duplexes was seen by denaturing gel electrophoresis. The ability of these oligomers to serve as substrates for polymerization by Klenow fragment was examined by densitometry of autoradiographs following denaturing gel electrophoresis of aliquots of polymerization reactions containing biotinylated oligomers in the presence of avidin, a large (66 kDa) biotin binding protein. The results revealed that in the presence of avidin 17/20-mer could be extended by Klenow fragment, whereas 16/20-mer was not extended by the enzyme. It was thus found that polymerization by Klenow fragment was possible only when the primer was six or more bases away from the base with the biotin–avidin complex.

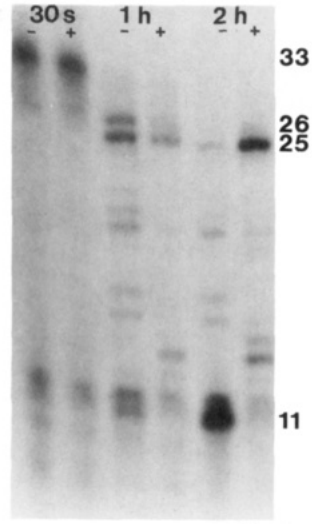


FIGURE 6: Biotinylated 33/36-mer (30 nM) in the presence of avidin (400 nM) and Klenow fragment (200 nM) after various times. Minus lanes are control reactions of biotinylated 33/36-mer (5′-^{32}P labeled primer 33-mer strand) with Klenow fragment (but no avidin) after 30 s and 1 and 2 h. It is apparent that in the absence of avidin the 33/36-mer is degraded to the 11/36-mer after 2 h. In contrast, biotinylated 33/36-mer in the *presence* of avidin (plus lanes) is only degraded to the 25/36-mer after 2 h (see footnote 4). Numbers indicate the length (in bases) of the primer strand.

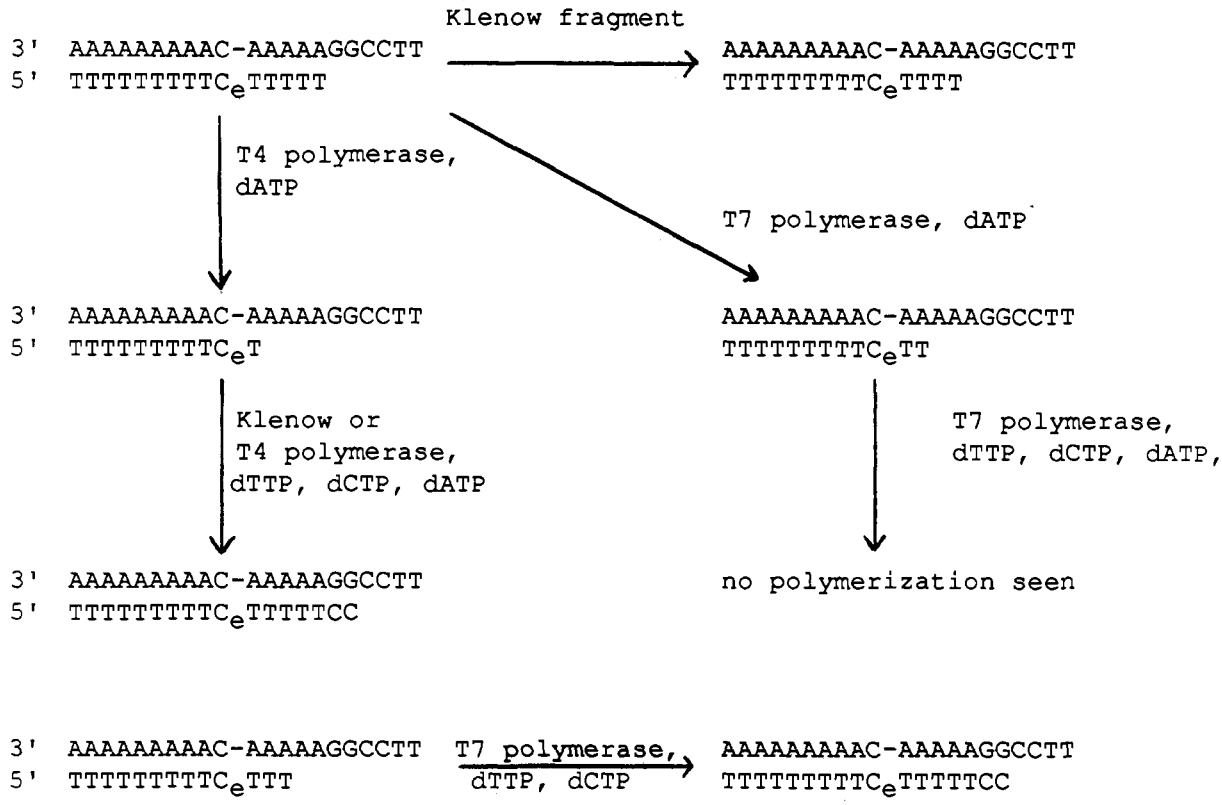
The ability of Klenow fragment to excise nucleotides from DNA duplexes with avidin attached was examined (Figure 6). Biotinylated 11/36-mer was elongated to 26/36-mer and 33/36-mer with Klenow fragment and the appropriate triphosphates. Excess triphosphates were destroyed by the combined action of nucleosidediphosphate kinase and hexokinase in the presence of glucose as the phosphate acceptor, and avidin was added. On addition of Klenow fragment, exonuclease action removed nucleotides from the primer strand of the 26/36-mer and 33/36-mer until 25/36-mer was produced.[4] It was thus found that the exonuclease site of Klenow can act only on duplexes where the primer terminus is 15 or more bases away from the base with the attached biotin–avidin complex.

Discussion

The results described clearly demonstrate the utility of covalently cross-linked DNA duplexes as probes of structural and mechanistic aspects of DNA utilizing enzymes. The action of polymerases on cross-linked duplex **5** is depicted in Scheme II. These results provide support for the hypothesis that the polymerase and exonuclease sites of Klenow fragment are separate and have different structural requirements for the DNA to act as a substrate. The results demonstrate that the polymerase site of Klenow fragment does not require that the DNA strands be separated to act as a substrate and are consistent with the observations of Kuchta et al. (1988) that a correctly base-paired primer terminus is a much better substrate for polymerization than an incorrectly base-paired primer terminus. If the strands are not separated during polymerization, then a long-distance relay of information in the selection of the complementary dNTP (relative to the template strand) would be avoided. In contrast to the polymerase site, the exonuclease site requires that the primer terminus be at least four bases from the cross-linked base pair

[4] Actually, at much longer time points (>3 h), the enzyme is seen to degrade some of the 25/36-mer to shorter oligonucleotides, but it is quite apparent that the 25/36-mer is quite resistant to the exonuclease action of Klenow fragment when in the presence of avidin.

Scheme II: Representation of the Action of Enzymes on Cross-Linked Duplex **5**



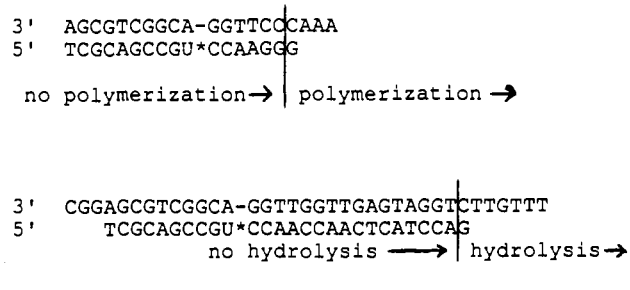
for activity and implies that a strand separation of at least four base pairs of the primer strand may be required for activity. The higher rate of exonucleolytic hydrolysis of single-strand DNA (relative to double-stranded DNA) is in accord with this view (Kuchta et al., unpublished observations).

Bacteriophage T4 gene 43 codes for a 104-kDa protein, T4 DNA polymerase (Kornberg, 1980). This enzyme, like Klenow fragment and T7 polymerase, has a 3′→5′ polymerase activity requiring a template and primer DNA and a vigorous 3′→5′ exonuclease activity. The primary structure of T4 polymerase has been determined, and surprisingly, the enzyme is more similar to animal virus polymerases and human polymerase α than to *E. coli* Pol I or T7 polymerase (Spicer et al., 1988). The results of the interaction of cross-linked duplexes with T4 polymerase indicate that the exonuclease site of T4 DNA polymerase, in contrast to that of Klenow fragment, requires that the duplex melt out no more than two nucleotides for activity. One might speculate that this difference is related to the vigorous exonuclease activity of this enzyme. That is, since T4 polymerase requires a strand separation of two rather than the four or five bases required by Klenow fragment, the activation energy for exonuclease activity (which reflects the process of melting plus the hydrolysis step) may be lower in T4 polymerase. Because neither the polymerase nor the exonuclease site requires much strand separation, the two sites may be physically close together.

T7 DNA polymerase is comprised of the T7 gene 5 protein (80 kDa) and the host-encoded *E. coli* thioredoxin protein (12 kDa) in a 1:1 ratio. It has been found that several peptide regions of T7 gene 5 protein show strong amino acid sequence homology to Klenow fragment (Ollis et al., 1985). On the basis of the reported crystal structure of Klenow fragment, the homologous region in Klenow fragment is the DNA binding region, suggesting that the two enzymes evolved from a common precursor. It might be expected that if Pol I and T7 gene 5 protein are closely related, they might display similar DNA structural requirements.

The action of T7 DNA polymerase on cross-linked duplex **5** indicated that the enzyme cannot remove bases two or less from the cross-linked base pair. This enzyme has an extremely active 3′→5′ exonuclease activity (at least 1000 times that of Klenow fragment), which may again reflect the lesser degree of DNA strand melting required for activity. As previously mentioned, Klenow fragment and T4 polymerase are able to incorporate dNMP into cross-linked duplexes wherein the primer terminus is only one base from the cross-linked base pair, and thus require no strand separation for activity. When the ability of T7 DNA polymerase to catalyze addition of nucleotides to such duplexes was tested, it was found that those duplexes with a primer terminus only one or two bases from the cross-linked bases did not function as substrates for polymerization, although duplexes with primer termini more distant from the cross-linked bases are active substrates for polymerization. We can only speculate on the reason that T7 polymerase cannot add nucleotides to cross-linked duplexes unless the primer terminus is at least three bases away from the cross-linked base pair. It is possible (though we believe unlikely) that the primer terminus needs to be separated from the template strand during the polymerization event, despite the inefficiency relaying the information for the selection of the triphosphate complementary to the base on the template strand. Second, the two cross-linked cytosines may induce a minor change in the DNA structure near the primer which is recognized by the T7 DNA polymerase and which prevents primer extension by the enzyme, even though the other polymerases, Klenow fragment and T4 DNA polymerase, efficiently catalyze primer elongation at the closest possible distance to the cross-linked base pair. Finally, some portion of the catalytic cycle of T7 polymerase, not necessarily the actual chemical step of the polymerization event, may require partial strand separation of the DNA duplex. Whatever the explanation, it is apparent that although Klenow fragment and T7 polymerase share regions of similar peptide structure, the exonuclease and polymerase sites of the enzymes display

Scheme III[a]



[a] After addition of excess avidin, the 17/20-mer formed by partial extension of 11/20-mer is a substrate for polymerization by Klenow fragment; shorter duplexes are not extended in the presence of avidin. After addition of avidin, the 33/36-mer and 26/36-mer (formed by extension of 11/36-mer with Klenow fragment) are exonucleolytically degraded only to the 25/36-mer. The presence of avidin prevents further exonuclease action.

different substrate structural requirements.

A separation of the exonuclease and polymerase sites in Klenow was also implicated by the behavior of the enzyme toward a series of biotinylated DNA duplexes (Scheme III). The addition of avidin to biotinylated DNA provided duplexes having a very large group attached to a specific base. This bulky group should be able to impede the activity of Klenow fragment by blocking its access to the primer terminus. It was found that the enzyme could extend duplexes where the primer terminus was six or more bases away from the base containing the biotin–avidin adduct, whereas the exonuclease site of the enzyme could only remove nucleotides from the primer terminus if the primer terminus was 15 or more bases from the base with the biotin–avidin adduct. Thus, compared with the polymerase site, the exonuclease site requires nine more base pairs between the primer terminus and the base with the biotin–avidin complex.

The cumulative findings support the proposal that the polymerase and exonuclease sites of the Klenow fragment are physically separate and have distinctly different structural requirements for activity. It must be emphasized, however, that the data for the action of the Klenow fragment on the biotinylated DNA do not necessarily mean that the polymerase and exonuclease sites are separated by a distance equivalent to nine base pairs of DNA unless the disposition of the avidin to the enzyme–DNA complex during polymerase or exonuclease activity is identical. Nevertheless, the nine base pair difference between the polymerase and exonuclease sites represents about 30 Å, consistent with the observation of Joyce and Steitz (1987), who upon examination of the X-ray crystallographic data of Klenow fragment concluded that the polymerase and exonuclease sites are about 30 Å apart. Certainly the separation between the two sites observed in the crystallized enzyme is preserved in the functional enzyme in solution.

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