DNA Polymerase ϵ: Aphidicolin Inhibition and the Relationship between Polymerase and Exonuclease Activity†

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ABSTRACT: Calf thymus DNA polymerase ϵ readily uses short, synthetic oligonucleotides as substrates for both polymerase and exonuclease activity. These substrates were used to examine the mechanism of inhibition by aphidicolin. Aphidicolin competes with each of the four dNTPs for binding to a pol ϵ·DNA complex. Importantly, aphidicolin binds equally well regardless of the identity of the next template base to be replicated ($K_i \approx 0.6$ μM). Hydrolysis of synthetic templates of defined sequence by the 3′→5′ exonuclease was examined. pol ϵ preferred to hydrolyze single-stranded DNA 3-fold better than double-stranded DNA (V_{max}/K_M), while under V_{max} conditions single-stranded DNA was hydrolyzed 100-fold faster than double-stranded DNA. Aphidicolin did not inhibit exonuclease activity on single-stranded DNA; however, activity on double-stranded DNA was partially inhibited. Formation of an E·[template·primer]·aphidicolin ternary complex inhibits exonuclease activity. However, even under conditions where the polymerase site is completely blocked by a template·primer, the exonuclease retains significant activity.

DNA polymerase ϵ (pol ϵ)[1] is one of four known nuclear DNA polymerases. It contains a highly processive polymerase activity as well as 3′→5′ exonuclease activity (Crute et al., 1986; Focher et al., 1989; Syvaoja & Linn, 1989). Genetic studies in yeast suggest that pol ϵ is critical for DNA replication (Morrison et al., 1990). In addition to involvement in DNA replication, pol ϵ may also be involved in DNA repair (Bambara & Jessee, 1991). For both processes, however, the precise role of pol ϵ has not yet been defined.

The fungal metabolite aphidicolin is a potent inhibitor of eukaryotic DNA replication (Bucknall et al., 1973). It potently blocked mitotic division of sea urchin embryos while not affecting nondividing cells (Ikegami et al., 1978). Aphidicolin inhibits DNA pol α, pol δ, and pol ϵ, as well as the α-like polymerase of plant cells, and the herpes simplex and vaccinia virus encoded DNA polymerases (Crute et al., 1986; Pedrali-Noy & Spadari, 1980; Huberman, 1981; Spadari et al., 1984, 1985b; Fry & Loeb, 1986), while not affecting DNA methylation or RNA, protein, or nucleotide biosynthesis (Spadari et al., 1984, 1985). Given this high degree of specificity for DNA polymerase inhibition, aphidicolin has been widely used to study DNA repair and replication (Berger, 1979; Chang & Trosko, 1989; Decker et al., 1986). The accumulation of early replication intermediates in SV40 replication systems and mouse L1210 nuclei has been detected upon addition of aphidicolin (Decker et al., 1986; Nethanel et al., 1988; Nethanel & Kaufmann, 1990; D. Ilsley & R. Kuchta, unpublished data). Additionally, aphidicolin can be used to synchronize cells at the G1/S border (Pedrali-Noy et al., 1981; Dinter-Gottlieb & Kaufman, 1982; Spadari et al., 1984). Cells that have entered the S phase can no longer synthesize DNA, whereas cells in other phases continue the cell cycle and stop at the G1/S border.

Aphidicolin inhibits DNA polymerase α by binding to a pol α·DNA complex to form a pol α·DNA·aphidicolin ternary complex (Sheaff et al., 1991). Inhibition is competitive with respect to dNTPs. However, if the next template nucleotide to be replicated is deoxyguanosine, aphidicolin binds 10-fold tighter than if the next template base is deoxyadenosine, deoxycytosine, or deoxythymidine.

Aphidicolin also inhibits DNA pol ϵ, and inhibition of polymerase activity is described as being of similar potency to pol α inhibition (Bambara & Jessee, 1991). While the precise details of inhibition are unknown, Sabatino et al. (1990) demonstrated that inhibition proceeds via formation of a pol ϵ·DNA·aphidicolin ternary complex. Aphidicolin did not inhibit exonuclease activity on single-stranded DNA (Sabatino & Bambara, 1988). Using short, duplex DNAs that contained 4–10 single-stranded nucleotides at the primer terminus as substrates for the exonuclease, they found that addition of aphidicolin (30 μM) resulted in partial inhibition of exonuclease activity. Then, in experiments measuring exonuclease activity on single-stranded DNA in the presence of a duplex DNA containing single-stranded nucleotides at the primer terminus, addition of aphidicolin now gave partial inhibition of the exonuclease activity on the single-stranded DNA.

In this report we use synthetic oligonucleotides of defined sequence to elucidate the detailed mechanism of pol ϵ inhibition by aphidicolin and identify a potentially important difference between pol α and pol ϵ inhibition. We also show that the exonuclease of pol ϵ hydrolyzes single-stranded DNA more readily than double-stranded DNA (V_{max} and V_{max}/K_M).

MATERIALS AND METHODS

Materials. Unless noted, all materials and methods were as described previously (Kuchta & Willhelm, 1991; Sheaff et al., 1991). Calf thymus was obtained from Brown Packing Co. (New Holland, IL), and pol ϵ was purified as described by Focher et al. (1989) to a specific activity of 2000–5000 units mg^{-1}. One unit of DNA pol ϵ polymerizes 1 nmol of dTTP in 60 min at 37 °C in assays containing 5 mM $MgCl_2$, 50 mM Tris (pH 7.5), 20 μM poly(dA)·oligo(dT) (total nucleotide), and 10 μM dTTP (see below). Poly(dA) and

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid (sodium salt); pol α, DNA polymerase α; pol δ, DNA polymerase δ; pol ϵ, DNA polymerase ϵ; Tris, tris(hydroxymethyl)-aminomethane (HCl salt).

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Table I: Synthetic Oligonucleotides of Defined Sequence

DNA_G	TCC ATA TCA CAT(3′)
	AGG TAT AGT GTA GAT CTT ATC ATC T
DNA_C	TCC ATA TCA CAT(3′)
	AGG TAT AGT GTA CAT CTT ATC ATC T
DNA_T	TCC ATA TCA CAT GCG CCG(3′)
	AGG TAT AGT GTA CGC GGC TTT GTG TAA TAG TAA G
ss18	TCC ATA TCA CAT GCG CCG(3′)

oligo(dT)$_{12-18}$ were obtained from U.S. Biochemicals. Poly(dA)·oligo(dT) was annealed in a 20:1 ratio (total nucleotide). Synthetic oligonucleotides of defined sequence listed in Table I were purchased from Oligos ETC, Inc., and annealed as previously described (Sheaff et al., 1991). Unless otherwise noted, DNA concentrations are given in terms of the concentration of 3′-termini. Methyl bromoacetate was from Aldrich.

Methods. All assays were performed at 37 °C and were initiated by the addition of pol ϵ (0.05–0.2 unit).

Polymerization Assays. Assays (10–20 μL) contained 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, DNA, pol ϵ, dNTPs, and inhibitors as required. One dNTP was radiolabeled ([α-^{32}P]dATP or [α-^{32}P]dTTP) at a specific activity of ca. 3000–10000 cpm pmol^{-1}. Assays were quenched at single time points by the addition of 2.5 vol of 50 mM EDTA (pH 8.0). Assays were linear over the time points taken. Radioactivity incorporated into DNA was measured using a DE81 filter-binding assay followed by scintillation counting as described previously (Sheaff et al., 1991).

K_m and K_D Determinations. The K_m's for the DNAs were determined by Lineweaver–Burk plots using 0.05–1.0 μM DNA and 5 μM dNTPs. The K_D's of various DNAs were measured as a K_i. Assays contained one DNA [poly(dA)·oligo(dT)] that served as substrate and then varying concentrations of a second DNA (e.g., DNA_G) that was the inhibitor. Only [α-^{32}P]dTTP was present in the assay, so that no synthesis could occur on the inhibitor DNA. Data were analyzed via Dixon plots. The concentrations of poly(dA)·oligo(dT) were 33, 67, and 167 nM, while the inhibitor DNA concentration varied from 0 to 100 nM. The K_M of dTTP during polymerization onto poly(dA)·oligo(dT) was determined with 167 nM DNA. The K_M for dNTPs during polymerization onto DNA_C (2 μM) was determined by varying the concentration of all four dNTPs simultaneously.

Processivity Measurements. Assays (10 μL) contained pol ϵ, 1 μM DNA_G, 5 μM [α-^{32}P]dCTP, and 5 μM dATP, dGTP, and dTTP. Aliquots (4 μL) were quenched by the addition of 12 μL of gel loading buffer containing 90% formamide, and the products were analyzed by gel electrophoresis followed by phosphorimagery (Molecular Dynamics; Sheaff et al., 1991). The relative molar amount of each length product was calculated; hence, the fraction of each length product that was elongated (i.e., the processivity) could be determined. For these measurements to be valid, after dissociating from a product, pol ϵ must always rebind to DNA_G. This was confirmed in control experiments that showed that the size distribution of products did not change with increasing amounts of product synthesized, indicating that after dissociating from a DNA product, the pol ϵ always bound to unelongated DNA_G. Processivity measurements on DNA_T were performed similarly, except that [α-^{32}P]dATP was used as the radiolabeled dNTP.

K_D of Aphidicolin. The K_D for aphidicolin binding to pol ϵ·DNA was determined using equilibrium inhibition assays described previously (Sheaff et al., 1991). Assays contained 20 μM [α-^{32}P]dTTP and 330 nM poly(dA)·oligo(dT) as substrate DNA. Inhibitor DNA (DNA_C, DNA_G, or DNA_T) was present at 0–50 nM, and then aphidicolin was added to final concentrations of 0–2 μM. The presence of only [α-^{32}P]dTTP allows synthesis on poly(dA)·oligo(dT), but not on any of the inhibitor DNAs.

Exonuclease Assays. ss18 was gel purified by nondenaturing gel electrophoresis (18% acrylamide). The gel containing the ss18 was located by UV shadowing, and the portion of the gel containing ss18 was excised. ss18 was eluted from the gel by adding 1 mL of H_2O and then subjecting the gel to three freeze–thaw cycles. The gel was then discarded, and the ss18 was concentrated from the H_2O by removing the solvent in vacuo. After the ss18 was resuspended in 50 mM Tris (pH 7.4), the ss18 concentration was determined by UV spectroscopy. ss18 was 5′-^{32}P-labeled using polynucleotide kinase and [γ-^{32}P]ATP. It was either used as substrate directly or first hybridized to a template DNA to generate DNA_T. DNA_T was gel purified on a nondenaturing gel as described above, except that the gel was kept at 4 °C during electrophoresis. The concentration of DNA_T was determined by measuring the polymerization of [α-^{32}P]dNTPs into DNA by the Klenow fragment (Sheaff et al., 1991).

Assays contained pol ϵ, 5 mM $MgCl_2$, 50 mM Tris (pH 7.5), 1 mM DTT, 5′-^{32}P-labeled DNA, and inhibitors as required. After various amounts of time at 37 °C, aliquots were quenched with gel loading buffer. The products were analyzed by gel electrophoresis (18% acrylamide, 8 M urea) followed by phosphorimagery. Exonuclease activity results in a family of products—the DNA generated due to the removal of one dNMP is a substrate for the removal of another dNMP. To calculate the exonuclease rates, the amount of each product due to the removal of up to eight nucleotides from the starting DNA was quantified [i.e., products of length n (18-mer) to $n-8$ (10-mer)]. For each length product ($n-1$ to $n-8$ nucleotides long), the background was determined from an identical assay that lacked enzyme, and this value was then subtracted from the measured amount of product in the assay that contained enzyme. For the starting material (18-mer), the background was calculated from an equal area of the gel immediately above the 18-mer in the lane that lacked enzyme. The amount of each length product was converted to the percent of total DNA loaded onto the gel—this corrects for any error in the amount of sample loaded onto the gel. Finally, the percent of total DNA that each length product represents was multiplied by the number of exonuclease events required to give that product. This value, $\{(\%n)0 + (\%(n-1))1 + (\%(n-2))2 + (\%(n-3))3 + \ldots + (\%(n-8))8\} \times$ (amount of DNA in assay), then gives the exonuclease rate. K_M's and V_{max}'s were determined using Lineweaver–Burk plots.

Inactivation by Methyl Bromoacetate. pol ϵ is stored in the presence of DTT (0.5–1 mM). Since DTT can react with methyl bromoacetate, the DTT was first removed by gel filtration. Bovine serum albumin (1 mg mL^{-1}) was added to the pol ϵ (final volume ca. 200 μL), and this solution was subjected to gel filtration on a 1-mL Sephadex G-25 column equilibrated with 50 mM potassium phosphate (pH 7.5) and 10% glycerol. Fractions containing protein were detected using the Bradford reagent (Bradford, 1976). Enzyme was treated with methyl bromoacetate for various amounts of time, and then aliquots were withdrawn and the remaining polymerase and exonuclease activity was measured. Polymerase activity on poly(dA)·oligo(dT) and exonuclease activity on [5′-^{32}P]ss18 were measured as described above, except that the assays also contained 10 mM DTT. The DTT served to destroy any remaining methyl bromoacetate. In an alternative procedure

Table II: Kinetic Properties of Synthetic DNAs

DNA	K_M (DNA)[a] (nM)	V_{max}[b]	K_D(DNA) (nM)	K_m(dNTPs) (μM)
poly(dA)·oligo(dT)	300	1.0		9.6
DNA_C	140	0.45	20	5.5
DNA_G	190	0.39	28	
DNA_T			5.5	

[a] K_M's and K_D's are given in terms of the concentration of primer 3′-terminii. [b] V_{max}'s are given relative to poly(dA)·oligo(dT), which was normalized to 1.0. The V_{max} with poly(dA)·oligo(dT) was measured in assays containing 10 μM dTTP, and the V_{max} with DNA_G and DNA_C was determined in assays containing 5 μM dNTPs. [c] The oligo(dT) primer used is 12–18 nucleotides long. We assumed an average length of 15 nucleotides to obtain the concentration of 3′-terminii.

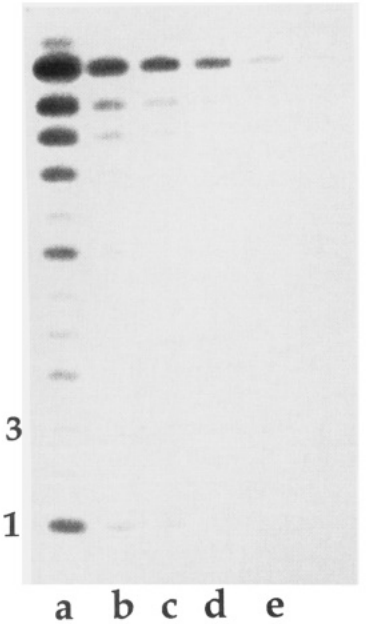


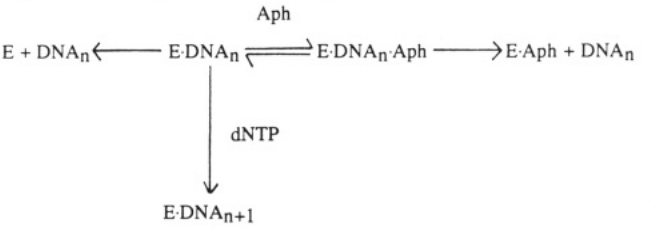
FIGURE 1: Effect of aphidicolin on pol ϵ activity. Assays were performed as described in the Materials and Methods section with DNA_G as the substrate. The first nucleotide polymerized onto the primer, dCTP, was α-^{32}P-labeled; hence, all products can be visualized. Lanes a–e show the polymerization products in the presence of 0, 0.5, 1, 2, and 3.5 μM aphidicolin, respectively. The products due to polymerization of 1 and 3 nucleotides onto the primer are noted.

for treating enzyme with methyl bromoacetate, the DTT was not removed from the enzyme, but rather sufficient methyl bromoacetate was added to react with the DTT present with the enzyme, and then the remaining methyl bromoacetate would give the desired concentration. Both methods gave similar rates of polymerase inactivation.

RESULTS

Initially, we examined the ability of the polymerase activity of pol ϵ to use short, synthetic DNAs of defined sequence as substrates (Table I). Steady-state polymerization was compared on several DNAs (Table II and Figure 1). Both DNA_C and DNA_G showed normal Michaelis–Menten kinetics, and the K_M and V_{max} were similar for poly(dA)·oligo(dT), DNA_C, and DNA_G. Additionally, both DNA_C and poly(dA)·oligo(dT) gave similar values for the K_M of dNTPs. The K_M values for poly(dA)·oligo(dT) and dTTP are similar to previously published values (Focher et al., 1989). Using poly(dA)·oligo(dT) as substrate, pol ϵ is highly processive (Focher et al., 1989). Consistent with this high processivity, pol ϵ efficiently elongated DNA_G (Figure 1) and bound these short DNAs very tightly (Table II). DNA_C and DNA_G are identical except for the identity of the first template base to be replicated, and

Scheme I: Kinetic Scheme Depicting the Effect of Aphidicolin on pol ϵ Processivity



both had similar K_D's. DNA_T, which is somewhat longer than DNA_C or DNA_G, bound 4-fold tighter. Together, these data show that pol ϵ will readily use short, synthetic oligonucleotides. Binding of pol ϵ to single-stranded DNA was also measured (ss18, Table I). In assays containing 190 nM DNA_G and 1 μM dNTPs, the IC_{50} for ss18 was 5 μM, indicating that pol ϵ weakly binds short, single-stranded DNA.

Aphidicolin Inhibition. Aphidicolin potently inhibited DNA synthesis on poly(dA)·oligo(dT), DNA_C, and DNA_G, with K_i's of 1.5, 0.45, and 0.55 μM, respectively (Dixon plots, data not shown). With each DNA substrate, inhibition was competitive with respect to dNTPs (on DNA_G and DNA_C, all four dNTPs were varied simultaneously). Aphidicolin inhibition of pol ϵ on all three DNA substrates was of similar potency, regardless of whether or not the template contained a dG, thus suggesting that the identity of the template base being replicated has a minimal effect on aphidicolin inhibition of pol ϵ.

As will be described later, aphidicolin binds primarily to the pol ϵ·DNA complex. To directly obtain the K_D for aphidicolin binding to various pol ϵ·DNA complexes, we used the equilibrium inhibition assay (Sheaff et al., 1991). Whereas K_i's measured via direct competition with dNTPs reflect aphidicolin binding to multiple pol ϵ·DNA species due to dNTP polymerization onto the substrate DNA, equilibrium inhibition assays measure aphidicolin binding to a single pol ϵ·DNA species. We measured aphidicolin binding to E·DNA_G, E·DNA_C, and E·DNA_T complexes, conditions where the next nucleotide to be replicated is dG, dC, and dT, respectively. K_D's of 0.64, 0.90, and 0.50 μM, respectively, were obtained, indicating that aphidicolin binds equally well regardless of the identity of the next nucleotide to be replicated.

Aphidicolin Primarily Binds to the pol ϵ·DNA Complex. Using DNA_C as template, aphidicolin inhibition was uncompetitive with respect to the DNA (parallel lines in a plot of $(\text{rate})^{-1}$ versus [aphidicolin] at 33, 133, and 670 nM poly(dA)·oligo(dT), data not shown). This suggests that aphidicolin binds to the pol ϵ·DNA complex but not to free pol ϵ. If binding to free enzyme was very weak, however, this method may not have been sensitive enough to detect such binding. To examine further the possibility that aphidicolin weakly bound to free pol ϵ, we examined the effect of aphidicolin on the processivity of pol ϵ.

Processivity is a competition between dissociation and addition of the next correct nucleotide (Bryant et al., 1983). For aphidicolin to decrease processivity, it must increase the rate of DNA dissociation relative to polymerization. The data described above show that aphidicolin converts a pol ϵ·DNA binary complex to a pol ϵ·DNA·aphidicolin ternary complex that cannot polymerize dNTPs. Two possible fates exist for this complex: aphidicolin could dissociate, leaving a pol ϵ·DNA complex capable of further elongation, or DNA could dissociate, leaving a pol ϵ·aphidicolin complex (Scheme I). The latter event would increase the rate of DNA dissociation relative to polymerization and could be measured

as a decrease in processivity, thereby suggesting that aphidicolin can bind free enzyme.

In the absence of aphidicolin, pol ϵ processively elongated DNA_G (Figure 1). For each DNA species generated during polymerization onto DNA_G, >95% of that species was further elongated until pol ϵ came within four nucleotides of the end of the template. At this point, pol ϵ became much less proficient at polymerizing the next dNTP. Addition of sufficient aphidicolin to inhibit polymerization by 94% resulted in either no change in processivity or very small decreases in processivity (positions 1 and 3, Figure 1). The fraction of these DNAs elongated decreased by 8% and 5%, respectively. Similar data were also obtained when processivity was examined on DNA_T (not shown). These small decreases in processivity indicate that DNA can occasionally dissociate from a pol ϵ·DNA·aphidicolin complex and, therefore, that aphidicolin can bind very weakly to free pol ϵ.[2]

Exonuclease Activity. Initially, we developed an assay for measuring exonuclease rates on single-stranded DNA of defined length and sequence (ss18). The potential difficulty with measuring exonuclease rates is that the product of one exonuclease event is a substrate for another exonuclease event, and this must be accounted for. Exonuclease activity on a substrate of length n produces a family of products of lengths $n-1$, $n-2$, etc. The total number of exonuclease events required to produce a given product distribution is $(n-1)1 + (n-2)2 + (n-3)3 + \ldots$, and thus can be calculated. Importantly, since the exonuclease is nonprocessive (Sabatino & Bambara, 1988; Figure 2), a plot of exonuclease events versus time will be linear, and one can thus calculate an exonuclease rate, *provided that* all of the DNAs generated during exonuclease activity (i.e., length $n-1, n-2$, etc.) are equivalent as exonuclease substrates. As shown in Figure 2, a plot of exonuclease events versus time is linear for ca. 15 min.[3] At this point, only 17% of the starting DNA remains. These data indicate that pol ϵ removes each dNMP from ss18 with similar efficiency (V_{max}/K_M). Exonuclease activity on a synthetic {5′-^{32}P-labeled primer}·template (DNA_T) was measured similarly. Prior to use, DNA_T was purified on a nondenaturing polyacrylamide gel to ensure that no single-stranded DNA was present. The exonuclease nonprocessively hydrolyzed the primer (Figure 3), and a plot of exonuclease events versus time was again linear until less than 40% of the starting DNA remained (data not shown). Just as with single-stranded DNA, pol ϵ removed each dNMP from a duplex template·primer with equal efficiency.

Using the assay described above, we measured the kinetic parameters for exonuclease activity on ss18 and DNA_T. With ss18 as substrate, K_M was 800 nM and V_{max} was 1.2 nmol dNMP h^{-1} (unit pol ϵ)$^{-1}$ (plot of $[DNA]^{-1}$ versus (exonuclease rate)$^{-1}$; for comparison, 1 unit of pol ϵ polymerizes 1 nmol of dTTP h^{-1} in the presence of 10 μM dTTP and 20 μM poly(dA)·oligo(dT)). With DNA_T as substrate, K_M was 22 nM and V_{max} was 0.011 nmol dNMP h^{-1} (unit pol ϵ)$^{-1}$. While pol

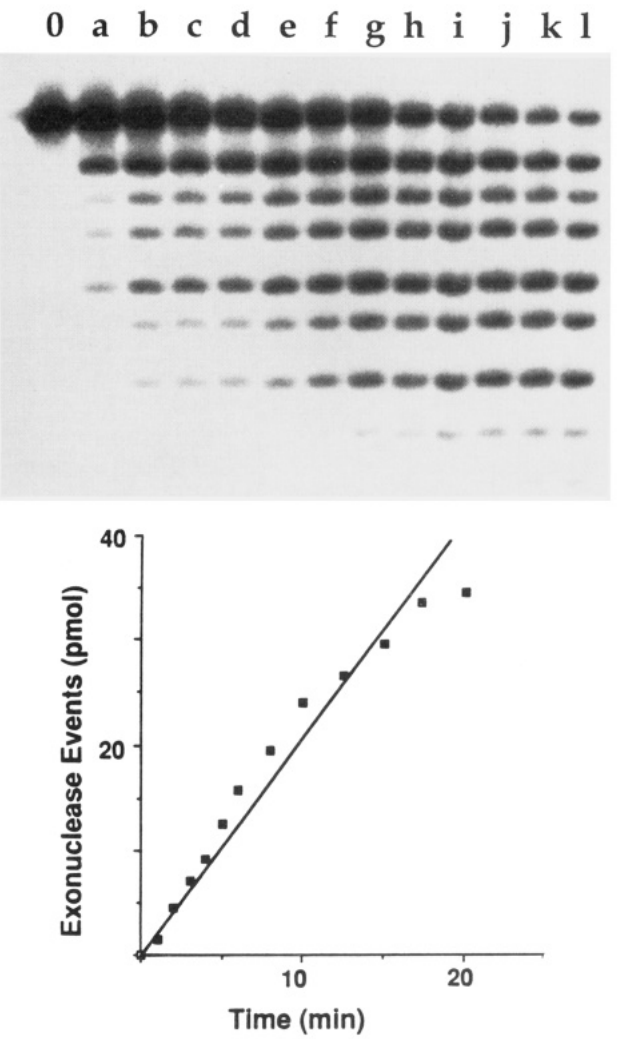


FIGURE 2: Exonuclease activity on ss18. The reaction (20 μL) contained 10 pmol of [5′-^{32}P]ss18. At various times, aliquots were quenched with gel loading buffer and analyzed by gel electrophoresis followed by phosphorimagery as described in Materials and Methods. (a, top) Lane 0 shows ss18 prior to addition of enzyme, while lanes a–l show the products after incubation with enzyme for 1, 2, 3, 4, 5, 6, 8, 10, 12.5, 15, 17.5, and 20 min, respectively. (b, bottom) Products were quantified as described in Materials and Methods, and the picomoles of exonuclease events are plotted as a function of time.

ϵ hydrolyzes single-stranded DNA 100-fold more rapidly than double-stranded DNA under V_{max} conditions, comparison of V_{max}/K_M for single-stranded versus duplex DNA shows that pol ϵ prefers to hydrolyze single-stranded DNA by only a factor of 3. Importantly, the sequences of the DNAs being hydrolyzed are identical; thus the differences in rates are not due to differences in the sequences.

Exonuclease Inhibition by Aphidicolin. Similar to previous data (Sabatino & Bambara, 1988), up to 100 μM aphidicolin inhibited the exonuclease rate on ss18 by less than 5% and had no effect on the size distribution of products (data not shown). Aphidicolin partially inhibited exonuclease activity on DNA_T. In assays containing 10 nM DNA_T, 0–200 μM aphidicolin was added to the assays. With 50 μM aphidicolin, exonuclease activity was inhibited by 21%, and further increases in the aphidicolin concentration did not increase the amount of inhibition (data not shown). Additionally, there was no change in the size distribution of the products (i.e., the exonuclease was still nonprocessive).

The effect of a DNA bound in the polymerase site on exonuclease activity on a second DNA molecule was analyzed.

[2] Two additional explanations should be considered. pol ϵ could dissociate, thus resulting in a DNA·aphidicolin complex. However, previous studies suggest that aphidicolin does not interact with free DNA (Dicioccio et al., 1980; Sheaff et al., 1991; Spadari et al., 1984). Alternatively, the decreased processivity could reflect simultaneous dissociation of DNA and aphidicolin. This requires, however, that ternary binding also occur, a very unlikely event.

[3] We only measure the removal of the first 6–8 nucleotides from the DNA substrate. Hence, the loss of linearity at long times may simply reflect the fact that we are not measuring some products. While the amount of these products may be small, they each represent at least 7–9 exonuclease events.

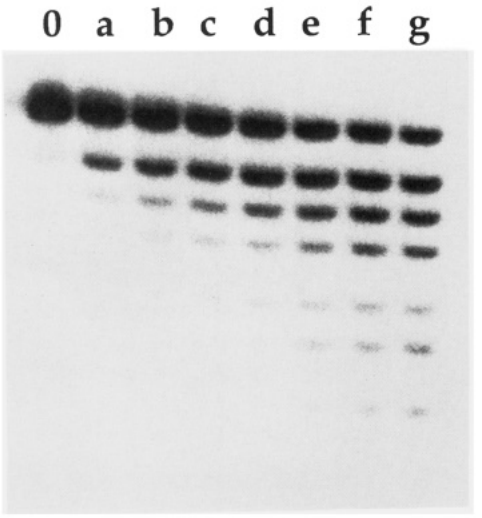


FIGURE 3: Exonuclease activity on DNA_T. The primer strand of DNA_T was 5′-^{32}P-labeled, and exonuclease activity was measured as described in Materials and Methods. Lane 0 shows DNA_T prior to the addition of enzyme, while lanes a–g show the products after incubation with enzyme for 5, 10, 15, 20, 30, 40, and 50 min, respectively.

Table III: Inhibition of Exonuclease Activity on ss18

DNA_G (nM)	[aphidicolin] (μM)	exonuclease rate[a]
0	0	1.0
200	0	0.50
200	2	0.37
200	10	0.28
200	40	0.25
200	70	0.24
200	100	0.24

[a] Exonuclease rates were measured on 100 nM 5′-^{32}P-labeled ss18 as described in Materials and Methods, and aphidicolin and DNA_G were added as noted. Rates are given relative to the rate in the absence of DNA_G and aphidicolin, which was normalized to 1.

Exonuclease activity was measured on 100 nM 5′-^{32}P-labeled ss18, and 200 nM DNA_G was added (Table III). This resulted in 50% inhibition of the exonuclease. Then, increasing amounts of aphidicolin were added to determine the maximum amount of inhibition that could be obtained. Surprisingly, in the presence of 100 μM aphidicolin, 24% of the exonuclease activity remained uninhibited. For the polymerase site, the K_D of DNA_G was 28 nM, and the K_D of aphidicolin for E·DNA_G was 0.64 μM; hence, under these conditions, >99.9% of the enzyme should contain DNA_G bound in the polymerase site. Thus, simple occupancy of the polymerase site by a template·primer cannot completely inhibit exonuclease activity. Similarly, effects of 5 nM DNA_G and increasing aphidicolin concentrations on exonuclease activity were also measured (100 nM [5′-^{32}P]ss18 as exonuclease substrate, data not shown). The 5 nM DNA_G alone inhibited the exonuclease by less than 5%, and subsequent addition of high concentrations of aphidicolin (>70 μM) increased inhibition to 60%.

The fact that formation of an E·DNA_G·aphidicolin complex where the DNA_G is likely bound in the polymerase site did not completely inhibit the exonuclease activity on a second DNA suggested that the polymerase and exonuclease activities are at least partially independent of each other. Consistent with this idea, inhibition of exonuclease activity on [5′-^{32}P]-ss18 by DNA_G was found to be of mixed type (Figure 4). If binding of DNA_G to the polymerase site completely prevented binding of ss18, inhibition should have been competitive.

Differential Inactivation of Polymerase and Exonuclease Activities by Methyl Bromoacetate. We then examined

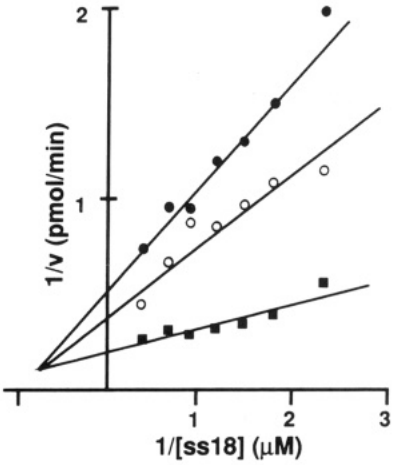


FIGURE 4: Inhibition of exonuclease activity on ss18 by DNA_G. Exonuclease activity on [5′-^{32}P]ss18 was measured as described in Materials and Methods. Assays contained various concentrations of [5′-^{32}P]ss18 and (■) 0 nM DNA_G, (○) 160 nM DNA_G, or (●) 320 nM DNA_G.

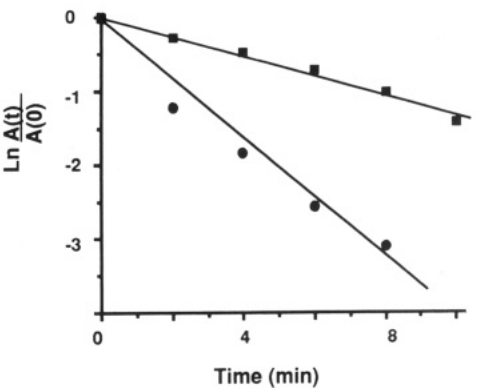


FIGURE 5: Inactivation of polymerase and exonuclease activities by methyl bromoacetate. Enzyme was incubated with 2 mM methyl bromoacetate at 37 °C for various amounts of time, and the amount of remaining polymerase (●) and exonuclease (■) activity was measured. The assay mixtures contained 10 mM DTT to destroy any residual methyl bromoacetate (the enzyme/methyl bromoacetate was diluted 1:5 into assay mixture; hence, the maximum remaining methyl bromoacetate concentration would be 0.4 mM). $A(t)$ is the amount of activity remaining after treating the enzyme with methyl bromoacetate for t minutes, and $A(0)$ is the amount of activity after 0 min of treatment. The pseudo-first-order rate of polymerase inactivation was 0.39 min^{-1}, and the rate of exonuclease inactivation was 0.13 min^{-1}.

inactivation of the exonuclease and polymerase activities by methyl bromoacetate. The bromine can be easily displaced by nucleophiles such as sulfur and thereby covalently modify an enzyme. Incubation of pol ϵ with methyl bromoacetate resulted in a time-dependent loss of polymerase activity (measured using poly(dA)·oligo(dT) and [α-^{32}P]dTTP, Figure 5). Activity was not restored by incubating methyl bromoacetate treated enzyme with 10 mM DTT for 1 h at 0 °C. However, if 10 mM DTT was included with the enzyme when methyl bromoacetate (2 mM) was added, there was no detectable inactivation of polymerase activity. Presumably, the DTT displaces the reactive bromine of the methyl bromoacetate, thereby protecting the enzyme. We also compared the rate of inactivation of polymerase and exonuclease activities (measured on [5′-^{32}P]ss18, Figure 5). Polymerase activity decreased 3-fold faster than the exonuclease activity, indicating that different amino acid residues must be modified to lose each activity and that the polymerase and exonuclease activities must be at least partially independent.

DISCUSSION

pol ϵ polymerizes dNTPs onto short, synthetic template·primers of defined sequence with efficiency similar to that observed with poly(dA)·oligo(dT). pol ϵ also binds these DNAs very tightly, consistent with the high processivity of pol ϵ (Bambara & Jessee, 1991). Since processivity is a competition between dissociation of the E·DNA complex versus polymerization of a dNTP (Bryant et al., 1983), tight DNA binding would enhance processivity. Binding of these synthetic DNAs by pol ϵ can be compared to binding by pol α, an enzyme that exhibits low processivity (Lehman & Kaguni, 1989). With pol α, the K_D for DNA_G and DNA_C is ca. 500 nM (Sheaff et al., 1991), while for pol ϵ, K_D's of 20 and 28 nM were measured, consistent with the higher processivity of pol ϵ. These short DNAs may understate how tightly pol ϵ can bind DNA. DNA_T, which is slightly larger than DNA_C or DNA_G, binds 3–4-fold tighter than either DNA_C or DNA_G. This suggests that pol ϵ interacts with a substantial amount of DNA and raises the possibility that DNAs larger than DNA_T bind with even greater affinity. An alternative possibility that we cannot exclude, however, is that the tighter binding of DNA_T reflects the different DNA sequences rather than length differences.

During polymerization on these synthetic DNAs, pol ϵ efficiently incorporates dNTPs into the growing strand until it gets within ca. 3–4 nucleotides of the template terminus. At this point the processivity decreases dramatically, either due to an increased rate of E·DNA dissociation or a decreased rate of dNTP polymerization. Additionally, these data indicate that pol ϵ interacts in a kinetically significant manner with at least 3–4 nucleotides of the single-stranded template.

The exonuclease activity of pol ϵ also readily uses short, synthetic DNAs as substrates. With each of the substrates examined, the efficiency of the exonuclease remained relatively constant as the length of the DNA decreased from 18 to 11 nucleotides and as the identity of the base excised changed. Interestingly, the rate of hydrolysis of single-stranded DNA by the exonuclease is similar to the rate of dNTP polymerization by the polymerase. Previous data indicated that pol ϵ "prefers" single-stranded DNA as a substrate (Sabatino & Bambara, 1988). Comparison of single- and double-stranded substrates of identical sequence revealed that, in terms of V_{max}, the exonuclease preferred single-stranded DNA by a factor of 100. However, if V_{max}/K_M (selectivity) is considered, pol ϵ preferred single-stranded DNA by only a factor of 3 due to the much higher K_M for single-stranded DNA. The much greater V_{max} for single-stranded DNA suggests that pol ϵ must unwind several basepairs of duplex DNA in order for the duplex DNA to be an appropriate substrate for the exonuclease. If unwinding base-paired DNA is slow compared to phosphodiester bond hydrolysis, perhaps due to the energetically unfavorable nature of disrupting base pairs, V_{max} for base-paired DNA will be lower than V_{max} for single-stranded DNA. Consistent with this idea, duplex primer·templates containing 1–6 single-stranded nucleotides appended to the primer 3′-terminus appear to be better substrates for the exonuclease than otherwise identical molecules containing greater than 6 or no single-stranded nucleotides on the primer 3′-terminus (Sabatino & Bambara, 1988).

Formation of a pol ϵ·{template·primer}·aphidicolin complex did not completely inhibit the exonuclease, suggesting that pol ϵ can simultaneously bind a single-stranded DNA and a template·primer and that the polymerase and exonuclease activities are at least partially independent. Consistent with these ideas, inhibition of exonuclease activity on single-stranded DNA by a template·primer was of mixed type, and methyl bromoacetate treatment inactivated the polymerase 3-fold faster than the exonuclease. A similar situation exists with DNA polymerase I (Klenow fragment) from *Escherichia coli* (Ollis et al., 1985), where the polymerase and exonuclease active sites are physically separate from each other. Additionally, formation of a Klenow fragment·[template·primer] complex, where the template·primer is bound in the polymerase active site, at most only partially inhibited exonuclease activity on a second template·{5′-^{32}P-labeled primer} (Catalano & Benkovic, 1989), analogous to the results obtained with pol ϵ. An alternative possibility that is also consistent with the data, however, is that some of the purified pol ϵ contains active exonuclease, but inactive polymerase. A template·primer may be unable to bind to this enzyme; thus, the exonuclease activity of this enzyme would not be inhibited by the addition of duplex DNA and aphidicolin.

Aphidicolin Inhibition of Polymerase and Exonuclease Activities. Use of synthetic oligonucleotides of defined sequence has provided a detailed description of pol ϵ inhibition by aphidicolin. Aphidicolin very weakly binds free pol ϵ, although it binds very tightly to a pol ϵ·DNA complex. Inhibition is competitive with respect to dNTPs, even though aphidicolin bears no obvious resemblance to a dNTP. Competitive inhibition with respect to dNTPs also suggests that the pol ϵ·DNA·aphidicolin complex is likely formed in the polymerase active site. Similar to previous data (Sabatino & Bambara, 1988), aphidicolin did not inhibit exonuclease activity on single-stranded DNA, while exonuclease activity on a template·primer was partially inhibited. Inhibition of exonuclease activity on a template·primer by aphidicolin could be due to either (i) "locking" the template·primer into the polymerase site such that this DNA cannot move into the exonuclease site or (ii) locking a DNA molecule into the polymerase active site such that it interferes with exonuclease activity on a second DNA molecule.

Qualitatively, inhibition of the DNA polymerase activity of pol α and pol ϵ is remarkably similar. In each case, inhibition proceeds via formation of a tightly bound E·DNA·aphidicolin ternary complex. Additionally, the K_D's for aphidicolin binding to pol α·DNA and pol ϵ·DNA binary complexes are similar [for pol α, K_D = 0.15–2.0 μM (Sheaff et al., 1991)]. These similarities suggest that the shape of each active site is similar. However, inhibition of the two enzymes is very different as regards the role of template guanosines. With pol α, when the next template base to be replicated is dG, aphidicolin binds 10-fold tighter than when the next template base is dA, dC, or dT. In contrast, pol ϵ binds aphidicolin equally well regardless of the identity of the next template base. While the cause of this difference is unknown, one possibility is that in the pol α·DNA·aphidicolin ternary complex, aphidicolin interacts with the next template base to be replicated; hence, binding is affected by the identity of this base. With pol ϵ, this interaction may not occur or the interaction is constant regardless of the identity of the template base. Alternatively, the conformation of the pol α active site may vary as the identity of the next template base varies, but an analogous conformational change does not occur in pol ϵ. Perhaps more importantly, this difference raises the possibility that aphidicolin can be used as an analytical tool to distinguish between pol α and pol ϵ catalyzed DNA synthesis. With appropriate dNTP and aphidicolin concentrations, pol α inhibition can largely be overcome by just increasing the dCTP concentration, whereas overcoming pol ϵ inhibition will require increasing the concentration of all four dNTPs.

Is aphidicolin a more potent inhibitor of pol ϵ than of pol α? The average K_i values with pol α and pol ϵ are similar, and the K_M values for dNTPs during polymerization onto preexistent DNA primers are similar for each enzyme. Since the K_i/K_M ratios are approximately equal, aphidicolin inhibits pol α and pol ϵ equally well under these conditions. In vivo, however, pol α largely elongates DNA primase synthesized primers (i.e., primase-coupled pol α activity). In vitro measurements of primase-coupled pol α activity using pure enzyme gave K_M(dNTPs) = 75 nM (Sheaff et al., 1991). The low K_M is due to rate-limiting primer synthesis. If one now considers the K_i/K_M values for pol α and pol ϵ, aphidicolin inhibits pol ϵ ca. 75-fold more potently than pol α.

In vivo, three polymerases may be involved in nuclear DNA replication, pol α, δ, and ϵ. The precise roles of each polymerase are unknown. In SV40 replication, synthesis of Okazaki fragments may require two polymerases (Nethanel et al., 1988; Nethanel & Kaufmann, 1990). Addition of aphidicolin to cell extracts containing replicating SV40 results in the accumulation of 30–60 nucleotide long products ("DNA primers") that appear to be precursors to full length Okazaki fragments. Presumably, these reflect primase-catalyzed primer synthesis followed by polymerization of 20–50 dNTPs by pol α. More recently, we have observed the accumulation of DNA primers in HeLa and L1210 cells upon the addition of low levels of aphidicolin (D. Ilsley & R. Kuchta, unpublished data). While the in vivo K_M(dNTP) values for each polymerase are unknown, the data with purified pol α and ϵ raise the possibility that aphidicolin inhibits pol ϵ more potently than pol α during DNA replication. If pol ϵ normally elongated the pol α·primase catalyzed synthesis of DNA primers and was more potently inhibited by aphidicolin, this could account for the accumulation of DNA primers in the presence of aphidicolin. (A similar argument might be made for pol δ, if $K_{i(aphidicolin)}/K_M$(dNTP) for pol δ is similar to that for pol ϵ.)

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