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Mechanism of Inhibition of the Avian Myeloblastosis Virus Deoxyribonucleic Acid Polymerase by Adriamycin†

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ABSTRACT: The avian myeloblastosis virus (AMV) DNA polymerase utilizes endonuclease-nicked DNA as a substrate for processive DNA synthesis, adding ~25 nucleotides each time it binds this template. This number can be lowered by increasing the KCl concentration of the reaction but is unaffected by shifts in pH which significantly alter reaction rate. Inhibition of the polymerization reaction by adriamycin has been probed with respect to the basic steps of DNA synthesis: binding of enzyme to DNA, catalysis, and enzyme translocation. Adriamycin has essentially no effect on initial enzyme–DNA interaction, as measured by a nitrocellulose filter binding assay. This assay measures total binding, not necessarily specific binding at 3′ termini. Kinetic assays demonstrate that inhibition of the reaction by adriamycin is competitive with respect to DNA terminus concentration. This suggests that inhibition involves specific binding to 3′ termini by the DNA polymerase or dissociation from these termini during synthesis. Inhibition is essentially noncompetitive with respect to deoxynucleoside triphosphate concentration, confirming that catalytic steps are not perturbed by the durg. The extent of processive synthesis falls to 22% of the uninhibited value at a drug concentration causing 96% inhibition of the rate of DNA synthesis. This indicates that a major effect of the drug is to cause premature dissociation of the DNA polymerase during synthesis. The component of inhibition not accounted for by lower processivity must result from a lowering of specific binding to 3′ termini by the DNA polymerase. The above information is incorporated into a comprehensive model for the inhibitory action of adriamycin.

The avian myeloblastosis virus (AMV)[1] DNA polymerase has been purified and extensively studied with respect to its role in the life cycle of the RNA tumor virus and its catalytic activity (Verma, 1977). It is a reverse transcriptase, responsible for transcription of the RNA genome of the virus into DNA (Verna et al., 1976). The purified enzyme consists of two polypeptides of apparent molecular weight 65 000 (α) and 95 000 (β) (Kacian et al., 1971; Grandgenett et al., 1973; Gibson & Verma, 1974). It is active for DNA synthesis on both RNA and DNA primer–templates with a 3′-OH terminus (Temin & Baltimore, 1972). In addition to its DNA polymerizing activity, the enzyme possesses an RNase H activity which degrades the RNA strand of a DNA–RNA hybrid in both 5′ → 3′ and 3′ → 5′ directions (Leis et al., 1973). The AMV DNA polymerase does not, however, possess DNA nuclease activities (Verma, 1977; Seal & Loeb, 1976; Battula & Loeb, 1976).

This report addresses two aspects of the enzymology of this DNA polymerase: (a) the basic steps of the DNA polymerization reaction in vitro and (b) the molecular mechanism by which these steps are affected by the inhibitor adriamycin. An initial requirement is an understanding of the way that the DNA polymerase and DNA interact during the synthetic process. This interaction has been studied in vitro in other laboratories by using homopolymer primer–templates with seemingly contradictory results (Leis, 1976; Dube & Loeb, 1976). It has been reported that if synthesis is begun on poly(A)·poly(dT), the enzyme requires several minutes to distribute to and synthesize on newly added poly(I)·poly(dC) (Leis, 1976). This result suggests a processive mechanism of synthesis, that is, the addition of more than one nucleotide each time the DNA polymerase binds a primer terminus, accompanied by translocation of the bound DNA polymerase along the template strand.

However, it has also been reported that the enzyme will distribute from oligo(dT)·poly(A) to oligo(dG)·poly(C) very rapidly (Dube & Loeb, 1976), which suggests that the enzyme may dissociate from the primer–template after addition of each nucleotide. The problem with the template competition technique employed for these measurements is that it yields the rate of distribution of enzyme molecules among DNA templates, a parameter not necessarily related to the number of nucleotides added each time a polymerase molecule binds the DNA template. In addition, when oligonucleotide primers are used on homopolymer templates, the primer molecules may

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[1] Abbreviations used: AMV, avian myeloblastosis virus; dNTPs, deoxynucleoside triphosphates; drug/DNA phosphate or drug/DNA-P, molar ratio of adriamycin molecules to DNA nucleotides in a solution.

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also dissociate from the template, promoting dissociation of the enzyme.

We have now used kinetic methods developed in this laboratory (Bambara et al., 1978) to quantitate the extent of processive DNA synthesis by the AMV DNA polymerase, thereby resolving the issue of the translocation mechanism of this enzyme. Processivity is quantitated by measuring the rate of DNA synthesis in the presence of one, two, or three dNTPs and comparing it to the rate measured with all four dNTPs present in the reaction. The reaction with a limited complement of dNTPs indicates the number of termini on which binding and synthesis was attempted, and the reaction with all four dNTPs indicates the total number of nucleotides polymerized onto the templates in the solution. This information is then used to calculate the average number of nucleotides added per binding event. It is not always true that the polymerase molecules move from one template to the next just as often in both the limited dNTP and the four dNTP reactions. A correction factor is added to the analysis to account for this. A template analogue inhibitor is added to two additional reactions, one with a limited complement of dNTPs and one with all four dNTPs. The inhibitor binds the polymerase as it attempts to move from one primer–template to the next. A "cycling time" correction factor is obtained by comparing the extent of inhibition in the limited reaction to the inhibition of the reaction with all four dNTPs.

When this analysis was used, the $\alpha\beta$ form of the AMV DNA polymerase displayed a processive mode of DNA synthesis on the nicked template employed in these studies.

We have also examined the effect of changes in pH and ionic strength on the processivity of the AMV polymerase in order to determine whether changes in rate, as these conditions are varied, could be the result of altered translocation properties of the DNA polymerase.

Adriamycin is an anthracycline antibiotic with a strong affinity for double-stranded DNA (Zunino et al., 1977). It has been shown to be a particularly effective inhibitor of RNA tumor virus DNA polymerases (Chandra & Zunino, 1972; Zunino et al., 1975), but a detailed explanation of the action of the drug in disruption of the process of DNA synthesis has been lacking. We have dissected the polymerization process into four steps: (1) AMV DNA polymerase binding to DNA; (2) interaction with 3′ termini for initiation of synthesis; (3) deoxynucleoside triphosphate (dNTP) condensation reactions at the primer terminus; (4) translocation of the DNA polymerase along the DNA template to the next site of dNTP reaction. The effect of adriamycin on each of these steps is considered experimentally.

Materials and Methods

Enzymes. Purified AMV DNA polymerase was obtained through the Office of Program Resources and Logistics, Viral Cancer Program, National Cancer Institute, from Dr. Joseph Beard. Purification and assays of this enzyme have been described (Houts et al., 1979). The enzyme is greater than 90% pure as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Incubation of 0.5 μg of enzyme with 1 nmol of nicked ^{3}H-labeled ColE1 DNA (12 000 cpm/nmol) for 60 min under conditions described below (–dNTPs) resulted in the release of less than 1% acid-soluble radioactivity. A similar incubation with 2 nmol of supercoiled ^{3}H-labeled ColE1 DNA produced less than 1 nick/37 000 base pairs. The number of nicks was determined from alkaline sucrose centrifugal separation of whole and nicked DNA. These results indicate that nuclease contamination is far below any level which could alter the results of the experiments described below.

Pancreatic DNase was purchased from Worthington Biochemicals. Micrococcal nuclease was obtained from Boehringer Mannheim. *Escherichia coli* DNA polymerase I [fraction VII prepared by the procedure of Jovin et al. (1969)] was kindly provided by Dr. A. Kornberg (Stanford University). DNA polymerase from T4-infected *E. coli* B and *E. coli* RNA polymerase were purchased from Miles Laboratories, Inc.

Nucleotides and Polynucleotides. Unlabeled deoxynucleoside triphosphates (dNTPs) used in limited reactions in processivity assays were purified by the procedure of Hall & Lehman (1968). Purity was checked on poly(ethylenimine) (PEI) plates (Brinkman Co.) developed in 1.2 and 1.5 M LiCl solvents. Unlabeled dNTPs used in unlimited reactions were purchased from P-L Biochemicals. [^{3}H]dTTP (40–60 Ci/mmol) was purchased from New England Nuclear Corp. or Amersham Corp.

Micrococcal nuclease treated DNA used in processivity studies was prepared according to Bambara et al. (1978). DNA with 3′-PO_4 termini is an effective inhibitor of the AMV DNA polymerase (data not shown). ColE1 DNA was isolated according to Blair et al. (1972). Nicking of substrate DNA was performed as described by Uyemura & Lehman (1976). Nicked DNA used to compare the activities of three DNA polymerases was prepared by using 25% of the level of pancreatic DNase recommended by Uyemura & Lehman. This level was used to assure the absence of gapped DNA structures.

The concentration of 3′ termini produced in a DNA sample by partial endonucleolytic digestion was determined by measurement of the maximum incorporation of three dNTPs using excess *E. coli* DNA polymerase I. Equation 20, with D_n = 1 (Bambara et al., 1978), was used to calculate the relationship between the amount of nucleotide incorporated and the concentration of 3′ termini in the DNA solutions. The data of Fisher & Korn (1979) indicate that the 3′→5′-endonuclease activity in the DNA polymerase I does not produce significant errors in such determinations.

Bacteriophage fd and fd DNA were prepared by the method of Sadowski & Hurwitz (1969a,b). Preparation of replicative form I (RFI) fd employed the method of Model & Zinder (1974) with the following alterations: cell lysis was carried out by the method of Reuben et al. (1974), and preparation of the DNA-containing supernatant was carried out by the method of Blair et al. (1972). Primer DNA fragments were prepared by digesting fd RFI (200 nmol/mL) with pancreatic DNase (500 ng/mL) for 15 min at 37 °C. This method yields fragments ~100 nucleotides long after boiling to separate DNA strands, as judged by thin-layer chromatography on PEI plates developed in 1.2 M LiCl and 7 M urea. Primed fd DNA was prepared by annealing these fragments to fd phage DNA and purifying the product, employing the method of Sherman & Gefter (1976).

Adriamycin. Adriamycin was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

Measurement of Processivity. Processivity was measured in the presence of excess DNA template for the AMV polymerase by using the kinetic techniques of Bambara et al. (1978). For processivity determinations, reaction volumes were 65 μL. Uninhibited reactions contained 53 mM Tris-HCl (pH 8), 5.6 mM sodium glycine, 6 mM potassium phosphate, 0.28 mM $CaCl_2$, 5 mM $MgCl_2$, 11.5 mM KCl, 5 mM dithiothreitol, 40 μg/mL BSA, 1.5 mM NH_4Cl, 36.4 μM nicked ColE1 DNA, 55.3 μM untreatred ColE1 DNA, 22.4 ng of AMV

DNA polymerase, and 10 μM each of [^{3}H]dTTP (13 Ci/mmol), dATP, dCTP, and dGTP. Several of the buffer components above are present because they remain from the preparation of the DNA substrate. The procedure requires that some reactions contain less then the normal complement of dNTPs. These contained [^{3}H]dTTP alone or in conjunction with one or two of the remaining three dNTPs at 10 μM each. Also required are inhibited reactions in which mixtures were altered to contain 22.4 μM nicked ColE1 DNA and 67.3 μM micrococcal nuclease treated ColE1 DNA. All assays were conducted at 37 °C. Aliquots (25 μL) were removed at 20 and 40 min, and acid-insoluble radioactivity was determined (Wu et al., 1974). Changes in buffer components where ionic strength or pH is varied are shown in the figure legends.

A possible error in the magnitude of processivity changes which are measured in the presence of adriamycin may occur if the adriamycin distributes unevenly between DNA species necessary for the reactions, i.e., nicked and micrococcal nuclease treated DNAs. The exact distribution of the drug, however, is not readily measurable.

The total distance over which synthesis occurs with excess enzyme at each 3′ terminus was determined according to Bambara et al. (1978). The reaction (75 μL) contained 53 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 30 mM KCl, 5 mM dithiothreitol, 40 μg/mL BSA, 8 mM potassium phosphate (pH 7.2), 1.7 μM nicked ColE1 DNA, 2.4 μM untreated ColE1 DNA, and 450 ng of AMV DNA polymerase. Limited reactions contained either [^{3}H]dTTP (13 Ci/mmol) alone or in conjunction with dCTP and dATP at 10 μM each. Unlimited reactions contained all four dNTPs, with [^{3}H]dTTP as the labeled precursor, at 10 μM each. Reactions were incubated until synthesis no longer increased (3 to 4 h). Final values obtained were essentially independent of the number of dNTPs in the limited reaction (data not shown). Consequently, the value presented is the average of all of the experimental data.

Kinetic Measurements. Measurements for Figures 2 and 3 were made in reaction mixtures identical with those used for uninhibited processivity reactions with all four dNTPs, with the following exceptions: nicked ColE1 DNA was 44 μM and no untreated ColE1 DNA was added. The [^{3}H]dTTP used was 10 Ci/mmol. Different terminus concentrations were the result of different levels of endonuclease used in producing the nicked DNA. The concentration of termini in each DNA sample was determined by using *E. coli* DNA polymerase I as described above. Reactions were started by addition of 22.4 ng of AMV DNA polymerase. Terminus concentration and dNTP concentration were varied as indicated by the figures.

Protein–DNA Binding Assays. Filter binding assays were performed essentially as described by Hinkle & Chamberlin (1972). AMV polymerase was added to 50 μL of binding buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 5 mM DTT, 40 μg/mL BSA) and 1 nmol of ^{3}H-labeled ColE1 DNA previously nicked by pancreatic DNase. The mixture was incubated for 10 min at 37 °C, diluted to 3.0 mL with binding buffer, and passed through a Millipore type HA filter at a flow rate of 4 mL/min. The filters were dried and counted in a toluene-based scintillant. The effects of adriamycin on the binding of AMV DNA polymerase to DNA were measured at a DNA polymerase concentration where binding increases in an approximately linear fashion with addition of DNA polymerase. The percent decrease of DNA retained on the filter caused by the presence of adriamycin is then a close approximation of the percent decrease in DNA polymerase molecules bound to the DNA.

Template Utilization Reactions. Volumes for the *E. coli* DNA polymerase I reactions were 75 μL and they contained 15 mM Tris-HCl (pH 8), 5 mM sodium glycine, 5 mM potassium phosphate, 10 mM KCl, 9 μg of BSA, 1.9 mM NH_4Cl, 4 mM $MgCl_2$, 20 μM [^{3}H]dTTP (13 Ci/mmol), 40 μM each of dATP, dCTP, and dGTP, 22 ng of *E. coli* DNA polymerase I, and 44 μM nicked ColE1 DNA or 18 μM primed single-strand fd DNA. The nicked ColE1 DNA has ~1 nick/4000 nucleotides, and the primed fd DNA has ~7 3′-OH termini/single-strand circle. Aliquots (25 μL) were removed at 15 and 30 min and assayed for acid-insoluble radioactivity (Wu et al., 1974). Data represent the average of 15- and 30-min measurements.

Reaction mixtures when AMV polymerase was used contained 54 mM Tris-HCl (pH 8), 5 mM sodium glycine, 5 mM potassium phosphate, 11.5 mM KCl, 5 mM dithiothreitol, 40 μg/mL BSA, 1.5 mM NH_4Cl, 5 mM $MgCl_2$, 20 μM [^{3}H]-dTTP (13 Ci/mmol), 40 μM each of dATP, dCTP, and dGTP, 22 ng of AMV DNA polymerase, and 44 μM ColE1 DNA or 18 μM primed single-strand fd DNA. Reaction volumes and methods were as described above.

Reaction mixtures when bacteriophage T4 DNA polymerase was used contained 15 mM Tris-HCl (pH 8), 5 mM sodium glycine, 5 mM potassium phosphate, 10 mM KCl, 9 μg of BSA, 1.9 mM NH_4Cl, 9 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20 μM [^{3}H]dTTP (13 Ci/mmol), 40 μM each of dATP, dCTP, and dGTP, 22 ng of T4 DNA polymerase, and 44 μM nicked ColE1 DNA or 18 μM primed single-strand fd DNA. Reaction volumes and methods were as described above.

Results

Synthetic Activity and Processive Synthesis on Nicked ColE1 DNA. Nicked ColE1 DNA is a double-strand DNA polymer with phosphodiester bond breakages but essentially no single-strand regions (Uyemura & Lehman, 1976). DNA with this structure was used in all the experiments described here because (a) as demonstrated below it is an effective substrate for the AMV DNA polymerase and (b) it is essentially 100% double-strand DNA, the DNA structure most thoroughly studied with respect to its interactions with adriamycin (Zunino et al., 1977).

Since the AMV DNA polymerase possesses no associated nuclease activities and since the enzyme preparation used does not contain significant levels of contaminating nuclease (as described under Materials and Methods), it was first necessary to demonstrate that nicked DNA is effectively used by the DNA polymerase as a template for DNA synthesis. Its synthetic ability on nicked DNA (~1 nick/4000 nucleotides) was compared to that on a primed single-strand DNA template with approximately the same concentration of 3′-OH termini. The enzyme utilized both templates for synthesis. By use of the same enzyme concentration in each reaction, the synthetic rate on nicked DNA was 55% of that on primed DNA. In order to demonstrate that the two DNA preparations showed expected template properties, we tested them with two other well characterized enzymes. DNA polymerase I, from *E. coli*, which is known to utilize nicked or primed single-strand DNA as a template (Goulian et al., 1968; Inman et al., 1965), did so, with a synthetic rate on nicked DNA 202% of that on primed DNA. Bacteriophage T4 DNA polymerase, which does not synthesize on nicked DNA, showed negligible activity on the nicked substrate, synthesizing at a rate that was 3% of that observed on the primed substrate.

Since the AMV DNA polymerase cannot degrade DNA ahead of the growing 3′ termini, the observed synthesis on

nicked DNA with this enzyme is likely to be accompanied by strand displacement.

The processivity of polymerization of the AMV polymerase was then determined on nicked ColE1 DNA (~1 nick/2000 nucleotides). On this template the AMV polymerase adds 26 ± 4.8 nucleotides (14 determinations) each time it binds to and extends a primer terminus.[2] The value of processivity is essentially independent of the level of nuclease digestion of the DNA when DNA with approximately 1–10 nicks/12 000 nucleotides is used in the reaction (data not shown). All further experiments, except those shown in Figure 2, are done with DNA containing ~1 nick/2000 nucleotides.

When excess DNA polymerase is used, the total distance over which synthesis occurs at each 3′ terminus can be determined. This value is 47 ± 6.9 nucleotides (five determinations). Since *E. coli* DNA polymerase I can add several hundred nucleotides to each 3′ terminus on this DNA template (Bambara et al., 1978), the value of 47 is not determined by the physical length of the template. Instead, some sequence in the template or structure produced during this reaction prevents extensive synthesis at each 3′ terminus.

Insensitivity of Processive DNA Synthesis to pH Changes. The processivity of the AMV DNA polymerase on nicked ColE1 DNA was determined at pH 7.0, 7.4, 7.8, 8.3, 8.7, and 9.1 and compared to the processivity at pH 8.1 (Figure 1A). It was found that although the initial rate of the polymerization reaction changes as the pH is varied throughout this range, the processivity of polymerization remains essentially constant throughout the region tested.[3] This result suggests that some step in the polymerization process other than translocation is sensitive to changes in pH.

Effect of Ionic Strength on the Processivity of AMV Polymerase. As the KCl concentration of the reaction mixture is increased, increasing the ionic strength, the synthetic activity of the AMV polymerase remains level and then decreases (Figure 1B). The processivity, however, continuously decreases, presumably because increasing the ionic strength increases shielding of charge interactions between DNA polymerase and DNA. Rate and processivity values do not change identically, presumably because the polymerization rate is determined by several factors, including processivity.

Effect of Adriamycin on the Binding of the AMV DNA Polymerase to the DNA Template. Since adriamycin strongly interacts with DNA (Zunino et al., 1977), it was possible that the inhibitory effect is caused simply because adriamycin

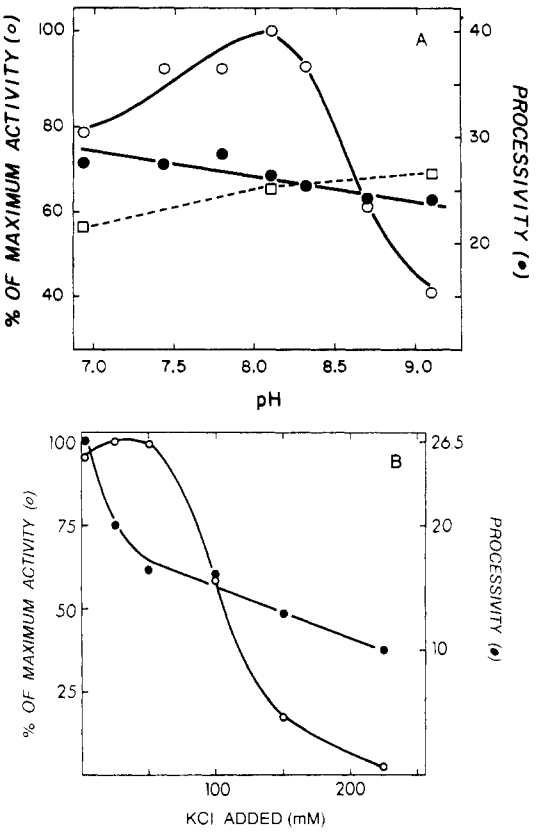

FIGURE 1: Reaction mixtures to measure processivity contained excess DNA template and other components as described under Materials and Methods, with the exception of additional procedures given below to adjust pH and ionic strength. Synthetic activity is measured in a reaction identical with the uninhibited processivity reaction containing all four dNTPs. At least four determinations were made for each processivity graph point, which includes determinations done with different combinations of three dNTPs in the limited reaction. Synthetic activity graph points are the average of three determinations, each of which was calculated from the average of 20- and 40-min measurements. Standard deviations of rates and processivity values on this and subsequent figures are <20% of average values. In panel A, Tris-HCl buffer was used to give the appropriate pH for each reaction. In addition, for the pH 7.0 and 7.4 reactions, the untreated ColE1 DNA and micrococcal nuclease treated ColE1 DNA were dialyzed for 12 h against 1000 volumes of the buffers (adjusted to pH 7.0 and 7.4, respectively) used in the preparation of micrococcal nuclease treated DNA in order to attain the correct reaction pH. The actual pH of each reaction was determined by directly measuring the pH of an aliquot (2 mL) of the reaction mixture, without the DNA and enzyme, by a pH meter at 25 °C. Control experiments were done to determine the effect of pH-dependent increases and decreases in the ionic strength of the reaction mixture on the processivity of polymerization. These experiments were done at pH 8 with the Tris-HCl buffer at 10 and 90 mM to simulate the ionic strength conditions of 50 mM Tris-HCl at pH 9.2 and 7.2, respectively. The data from these control experiments are represented by the dashed line and open squares. Open circles represent synthetic activity, and closed circles represent processivity. The line described by the closed circles is plotted by linear regression analysis of the data. In panel B, both processivity and synthetic activity reactions were supplemented with KCl as shown. [^{3}H]dTTP at 27 Ci/mmol was used when the synthetic rate fell below 25% of the maximum rate.

bound to the DNA template prevents the binding of the DNA polymerase. To test this hypothesis, it was necessary to measure the binding interaction between the DNA polymerase and the DNA in the absence of dNTPs, so that no synthetic processes were taking place. The work of Moelling et al. (1979) suggests that the DNA polymerase can bind to DNA with sufficient strength to retain DNA on nitrocellulose filters. We have confirmed this result by using labeled nicked ColE1 DNA. The binding activity cochromatographs with the DNA

[2] In a processivity assay four reactions are run simultaneously, producing reaction rates for the variable given in parentheses: a reaction with one, two, or three dNTPs (P_x), a reaction with one, two, or three dNTPs and a template analogue inhibitor ($P_{x,i}$), a reaction with four dNTPs (P_4), and a reaction with four dNTPs and a template analogue inhibitor ($P_{4,i}$). The data from a representative assay are P_x = 1352.3 cpm, $P_{x,i}$ = 371.2 cpm, P_4 = 23 292.0 cpm, $P_{4,i}$ = 2817.0 cpm, and a blank sample with no enzyme = 35.0 cpm. All samples are counted for 10 min. The blank is subtracted from each of the above rates, and these values are substituted into eq 14 and 26 of Bambara et al. (1978). In this reaction there is a 74% inhibition of the dNTP-limited reaction and there is a 88% inhibition of the unlimited reaction. The processivity is calculated to be 28.0. The average of this and other processivity values, and the standard deviations for these values, are presented in the text. The standard deviations on all processivity data presented in this report are <20% of the mean value.

[3] As pH is changed in the buffer system used, there are unavoidable changes in ionic strength. Figure 1A includes an approximation of the effect of this ionic strength change on the value of processivity (dashed line). The slope in the curve of processivity measurements is in the opposite direction to the expected effect of ionic strength, and therefore this slope may have been somewhat steeper if ionic strength could have been held constant.

Table I: Effect of Adriamycin on DNA Polymerase-DNA Binding Activity[a]

AMV DNA polymerase[c] (ng)	polymerase/DNA[b] (molar ratio)	adriamycin drug DNA-P	adriamycin μM	DNA retained (%)
320	23.4			97
128	9.4			90
32	2.4			44
16	1.2			27
16	1.2	0.025	0.5	31
16	1.2	0.050	1.0	36
16	1.2	0.125	2.5	28
16	1.2	0.300	6.0	21
16	1.2	0.600	12.0	21

[a] All binding assays contain 1 nmol of ^{3}H-labeled nicked ColE1 DNA (10 200 cpm/nmol) and buffer components as described under Materials and Methods. [b] The polymerase/DNA molar ratio is expressed as moles of DNA polymerase molecules per mole of full-size DNA molecules. [c] As a positive control, *E. coli* RNA polymerase was used in binding reactions identical with those described for AMV polymerase. At an RNA polymerase/DNA molar ratio of 4.2, 46% of the ^{3}H-labeled ColE1 DNA was retained, and at an RNA polymerase/DNA molar ratio of 8.4, 69% of the ^{3}H-labeled ColE1 DNA was retained.

polymerase on Sephadex G-100, confirming that minor contaminations of viral proteins with DNA binding properties are not responsible for the observed binding activity (data not shown).

The upper part of Table I shows that binding of the DNA polymerase can retain essentially 100% of the labeled DNA in the filter. Under the conditions of the experiment, ~3 polymerase molecules/DNA molecule are required to retain 50% of the DNA.

Measurement of the effects of adriamycin was performed with ~1 DNA polymerase molecule/DNA molecule, a ratio which caused retention of 27% of the DNA. Perturbation of binding should be quite evident at this binding level, where DNA retention is linearly related to the concentration of DNA polymerase molecules. Binding was then measured at successively higher adriamycin concentration. Results given in the lower part of Table I demonstrate that levels of adriamycin, which are shown in later experiments to be sufficient to halt DNA synthesis (0.15–0.2 drug/DNA phosphate), have only a small effect on the binding activity. In fact, a slight enhancement of DNA retention is observed at adriamycin levels less than 0.125 drug/DNA phosphate, followed by a small decrease in retention at 0.3–0.6 drug/DNA phosphate.

An important characteristic of the strong DNA binding property of the AMV DNA polymerase is that it is not dependent on the presence of 3′ termini (data not shown). Therefore, it is still possible that although total binding of polymerase to DNA is not weakened by the drug, specific interaction at 3′ termini during initiation of synthesis and primer elongation may be disrupted.

Effect of 3′-Terminus Concentration on the Inhibition of DNA Synthesis by Adriamycin. Inhibition kinetics were examined in a series of reactions in which 3′ termini are the variable substrate and DNA and adriamycin concentrations are held constant in order to test whether adriamycin interferes with specific interactions of the AMV DNA polymerase with 3′ termini. Results are shown on a Lineweaver–Burk plot (Figure 2). On the abscissa, the group of reactions that are represented contain identical concentrations of DNA but the DNA utilized for each reaction has been digested to a different extent with pancreatic endonuclease. The concentration of 3′ termini has been determined by using *E. coli* DNA polymerase I as described under Materials and Methods, and

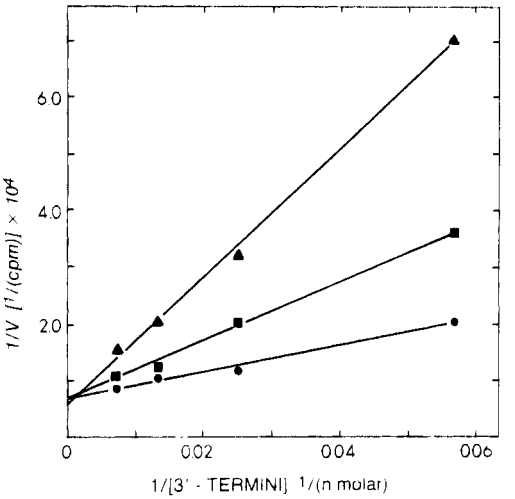

FIGURE 2: Reaction mixtures are described under Materials and Methods. Data are expressed as the average of at least three determinations of the reaction rate after 12 min of incubation. Graph points show the following values for adriamycin: 0.1 drug/DNA phosphate, 4.4 μM drug (▲); 0.07 drug/DNA phosphate, 3.1 μM drug (■); no drug addition (●). All lines are plotted by using linear regression analysis of the data.

these concentrations are the sole difference among the DNA substrates in the reactions.[4] The extent of nicking used in this experiment is kept low enough so that the double-stranded character of the DNA is maintained (data not shown).

The plot indicates that an increase in the terminus concentration can overcome the inhibitory effect of adriamycin. This is strong evidence that the drug interferes with the specific binding of the DNA polymerase to 3′ termini during DNA synthesis. It may weaken binding to the 3′ termini before the synthetic reaction begins, cause premature dissociation during synthesis, or have both of these activities. Since the effect of the drug can be overcome at high 3′-terminus concentration, it does not appear that nucleotide addition reactions are affected.

Effect of Adriamycin on Deoxynucleoside Triphosphate Reactions. Reaction rates were measured with respect to changes in dNTP concentration in order to detect effects of adriamycin on DNA polymerization steps which involve dNTPs. These rates were then measured at different levels of adriamycin. The results are depicted as a Hofstee plot (Figure 3). When the adriamycin concentration is raised to 0.025 drug/DNA phosphate, both the V_{max} and K_m of the reaction are affected by the drug. The effect on K_m suggests that a reaction involving binding or condensation of the dNTPs is being perturbed. However, at higher adriamycin concentrations, successive nearly parallel lines are produced, indicative of a decreasing V_{max} but no change in K_m. This latter behavior is the distinctive indicator of noncompetitive inhibition and suggests that at higher adriamycin concentrations further

[4] This type of experiment is fundamentally different from those in which DNA concentration is the variable substrate (Zunino et al., 1975). If DNA concentration is varied and adriamycin concentration is held constant, eventually a DNA concentration will be reached in which drug molecules are bound so infrequently to DNA that no inhibition of DNA synthesis will be observed. The Lineweaver–Burk plot of such data shows the convergence of lines on the ordinate characteristic for pure competitive inhibition, but the result cannot be interpreted in terms of the mechanism of inhibition. Instead, it indicates that the inhibitory properties of the drug are related to the relative concentrations of drug and DNA and not to the absolute drug concentration. However, if DNA concentration is held constant, as in the experiment shown here, the kinetic effect of varying only the 3′-terminus concentration can be measured.

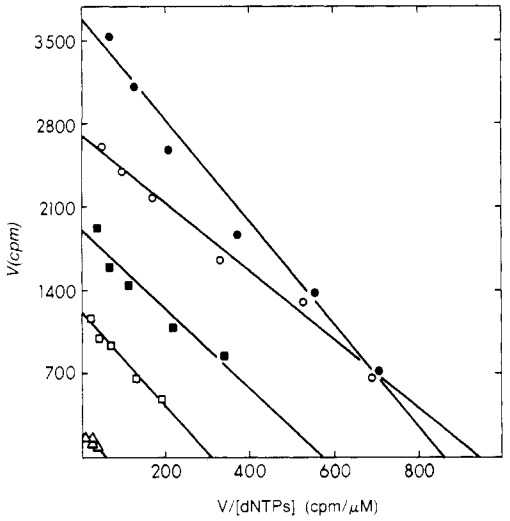

FIGURE 3: Reaction mixtures are described under Materials and Methods. The data are expressed as the average of at least three determinations of the reaction rate after 14 min of incubation. Graph points show the rate of reaction obtained with varying levels of dNTPs at the following adriamycin levels: 0.025 drug/DNA phosphate, 1.1 μM drug (○); 0.050 drug/DNA phosphate, 2.2 μM drug (■); 0.100 drug/DNA phosphate, 4.4 μM drug (□); 0.140 drug/DNA phosphate, 6.2 μM drug (△); no drug addition (●). All lines are plotted by using linear regression analysis of the data.

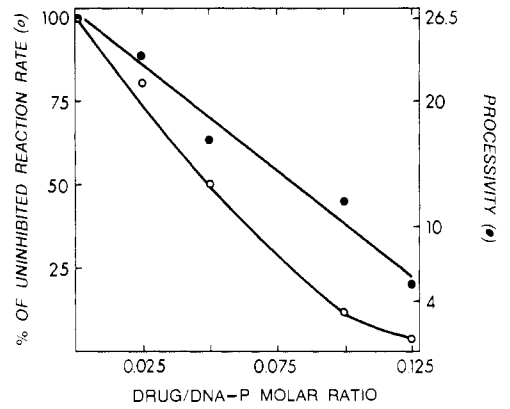

FIGURE 4: Reaction mixtures are described under Materials and Methods. The data are expressed as the average of values obtained after 20 and 40 min of incubation. At least four determinations were made for each point. [^{3}H]dTTP at 27 Ci/mmol was used when the synthetic rate fell below 25% of the maximum rate. At a drug/DNA phosphate ratio of 0.100, the adriamycin concentration is 9.2 μM.

additions of the drug affect polymerization at steps other than those involving dNTPs.

Effect of Adriamycin on DNA Polymerase Translocation (Processivity) during DNA Synthesis. Figure 4 shows a simultaneous comparison of reaction rate and extent of processive synthesis as the adriamycin concentration is increased. Processivity decreases continuously with adriamycin concentration to ~22% of the uninhibited value, when the adriamycin level reaches 0.125 drug/DNA phosphate. This result indicates that steps in the polymerization involving translocation of the bound DNA polymerase along the DNA template are sensitive to adriamycin. Premature dissociation of the DNA polymerase from the primer terminus during synthesis is a primary action of the drug.

Discussion

In this report we have examined specific steps of the polymerization reaction of the avian myeloblastosis virus DNA polymerase and the perturbation of these steps by the inhibitor adriamycin. The AMV DNA polymerase was found to utilize endonuclease-nicked DNA for processive DNA synthesis. The action of adriamycin on the polymerization reaction was examined with respect to binding of DNA polymerase to DNA, interaction of the DNA polymerase with the primer terminus to initiate synthesis, utilization of dNTPs for addition of nucleotides to the DNA primer terminus, and translocation (processivity) of the DNA polymerase during synthesis. Although some effect of adriamycin was detected on all of these steps, by far the major effects of the drug are to cause premature dissociation of the DNA polymerase during synthesis and to interfere with reinitiation of synthesis at new 3′ termini.

Before the actions of adriamycin were examined, experiments were designed to elucidate the basic properties of the uninhibited reaction. Double-strand, endonuclease-nicked DNA was chosen as a substrate because, as discussed shortly, its interaction with adriamycin is well understood. Synthesis on this substrate presumably occurs by a strand displacement mechanism, since the polymerase has no nuclease activities. The ability of the enzyme to displace DNA strands is consistent with its reported DNA unwinding activity (Collett et al., 1978).

The polymerization was shown to occur via a processive mechanism. The processive reaction was examined with respect to changes in pH and ionic strength, both of which have a considerable effect on reaction rate. The lower processivity observed at higher ionic strengths is expected because of the shielding effect that higher salt could have on enzyme–DNA interactions. The lack of response of processive synthesis to changes in pH, which have substantial effects on rate, is more unusual. It suggests that some steps in the reaction, which are affected by the changes in pH, are not involved in the process of translocation of the DNA polymerase during DNA synthesis. This, and evidence described later, also shows that translocation steps are experimentally separable from initial binding and nucleotide condensation steps.

The tumor virus DNA polymerases are a class of enzymes sensitive to inhibition by adriamycin (Zunino et al., 1972). In order to examine the effect of this drug on a process as complex as DNA synthesis, it was necessary to dissect the synthetic reaction and examine the effect of the drug at each step.

Initial binding of the DNA polymerase to DNA was measured, using a nitrocellulose filter binding assay. A similar approach used earlier (Mizuno et al., 1975) had shown that binding of *E. coli* RNA polymerase to DNA can be essentially eliminated by daunomycin, which is a structural analogue of adriamycin. The binding of the AMV DNA polymerase to DNA, however, is quite insensitive to adriamycin.

In fact, the high adriamycin levels necessary to demonstrate inhibition of binding suggest that this inhibition occurs by a different process than the drug-induced decrease in DNA synthesis. This conclusion is supported by data which show that adriamycin and derivatives interact with double-strand DNA via two modes of binding (Zunino et al., 1972; Goodman et al., 1977; Pigram et al., 1972; Blake & Peacocke, 1968; Waring, 1970, 1971). The primary (stronger) interaction is an intercalation between bases of double-strand DNA; the secondary (weaker) interaction is electrostatic and involves the positively charged amine group on the adriamycin molecule and the negatively charged phosphate groups on the DNA backbone. The shift from intercalative binding to electrostatic binding occurs at an adriamycin level of 0.15–0.25 drug/DNA phosphate (Zunino et al., 1972, 1977) after intercalation sites are filled. Loss of synthetic activity correlates well with the filling of intercalation sites by adriamycin. The majority of

inhibition of DNA polymerase–DNA binding, however, occurs above 0.2 drug/DNA phosphate. It is reasonable to conclude that DNA polymerase–DNA binding is unaffected as intercalation sites are being filled but begins to be weakened as nonspecific electrostatic interactions of adriamycin and DNA are occurring. A change in the inhibitory properties of the drug when electrostatic binding begins had in fact been observed earlier by Goodman et al. (1977). They saw changes in the kinetics of inhibition of bacteriophage T4 DNA polymerase by adriamycin when drug levels were raised to the point where the shift in binding modes occurred.

Since interaction of adriamycin with single-strand DNA is less clearly understood, our experiments were specifically designed to avoid using single-strand-containing substrates.

The DNA polymerase presumably equilibrates from initial binding sites, which are not necessarily at or near 3′ termini, to the 3′ termini where DNA synthesis can take place. An informative experiment on this subject involved measurement of adriamycin inhibition of synthesis in a series of reactions containing DNA with progressively increasing amounts of 3′ termini. High 3′-terminus concentrations were found to completely overcome the inhibitory effect of the drug. This experiment indicates that the primary effect of the drug is to interfere with interaction of the DNA polymerase and 3′ termini, either during initiation of synthesis or during the translocation steps of processive synthesis.

It is obvious that a decrease in binding affinity during initiation of synthesis could be overcome by more 3′ termini. However, if the average number of nucleotides added per binding event is lowered, this effect on rate can also be overcome by very high terminus concentrations. The reason why is that very high terminus concentrations allow prematurely dissociated DNA polymerase molecules to immediately associate with new 3′ termini so that synthesis proceeds with no loss of overall rate.

The mechanisms just proposed require that the nucleotide addition reaction be essentially unaffected by the drug, a fact demonstrated in Figure 3. The premature dissociation model also required a direct effect of the drug on processivity, and this is seen in Figure 4. Under the conditions of the processivity measurements, the reduced reaction rate cannot result solely from a reduction in processive DNA synthesis. However, the remaining component of inhibition not caused by lower processivity is also overcome by raising the 3′-terminus concentration. We propose that the drug also lowers the affinity of 3′ termini for DNA polymerase molecules which are attempting to initiate synthesis. Therefore, both premature dissociation and less efficient reinitiation of synthesis account for the lowered reaction rate.

In summary, the results of our studies with AMV DNA polymerase support the following model for the inhibitory mechanism of adriamycin. The AMV polymerase binds strongly to DNA, a process essentially unaffected by adriamycin. Enyzme molecules must then equilibrate with 3′ termini at which DNA synthesis can occur. The presence of the drug molecules bound to the DNA weakens the affinity of the enzyme for 3′ termini, slowing initiation of synthesis. Processive synthesis begins, and nucleotides are added to the primer terminus. The reaction of dNTPs and 3′-OH groups is essentially unaffected by the drug. As the DNA polymerase translocates along the template with the growing primer strand, the presence of drug molecules bound to the DNA leads to a premature dissociation event. The overall result of this slower initiation and premature dissociation is a decreased rate of reaction.

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Purification of Deoxyribonucleic Acid Polymerase δ from Calf Thymus: Partial Characterization of Physical Properties†

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ABSTRACT: Deoxyribonucleic acid (DNA) polymerase δ has been purified 7800-fold from calf thymus, to a specific activity of 28 000 units/mg of protein. Similar to DNA polymerase δ from bone marrow [Byrnes, J. J., Downey, K. M., Black, V. L., & So, A. G. (1976) *Biochemistry 15*, 2817], the calf thymus enzyme is associated with 3′- to 5′-exonuclease activity. Both DNA polymerase and 3′- to 5′-exonuclease activities copurify on hydroxylapatite, DNA–cellulose, and molecular sieve chromatography. The ratio of exonuclease activity to polymerase activity is approximately 1:12. When the most highly purified fraction is subjected to polyacrylamide gel electrophoresis under nondenaturing conditions, both DNA polymerase and exonuclease activities have the same mobility at several acrylamide gel concentrations. Isoelectric focusing experiments have shown that both activities have the same p*I*. These data suggest that 3′- to 5′-exonuclease activity is an intrinsic property of DNA polymerase δ. The molecular weight of the enzyme, as estimated from measurements of Stokes radius and sedimentation coefficient, is 152 000.

There are two mechanisms by which procaryotic DNA polymerases maintain the high fidelity of DNA synthesis: (1) the selection of a complementary deoxynucleoside triphosphate for incorporation at the primer terminus by the polymerase activity and (2) the excision of a mismatched nucleotide incorporated at the primer terminus by the 3′- to 5′-exonuclease activity (Brutlag & Kornberg, 1972; Kornberg, 1974; Topal & Fresco, 1976; Alberts & Sternglanz, 1977; Que et al., 1979). Although fidelity of DNA synthesis is also rigidly maintained in eucaryotes, it has generally been believed that eucaryotic DNA polymerases lack an error-correcting 3′- to 5′-exonuclease activity (Bollum, 1975; Chang & Bollum, 1973; Sedwick et al., 1975; Loeb, 1974; Wang et al., 1974; Sarngadharan et al., 1978). However, there have been several recent reports of the association of 3′- to 5′-exonuclease activity with high molecular weight DNA polymerases from lower eucaryotes: *Euglena gracilis* (McLennan & Keir, 1975), yeast (Wintersberger, 1974; Helfman, 1973; Chang, 1977), *Ustilago maydis* (Banks et al., 1976; Yarranton & Banks, 1977), *Cylindrotheca fusiformis* (Okita & Volcani, 1977), and *Chlamydomonas reinhardii* (Ross & Harris, 1978).

We have previously reported the purification of a high molecular weight DNA polymerase with 3′- to 5′-exonuclease activity from rabbit bone marrow, DNA polymerase δ (Byrnes et al., 1976, 1977). The bone marrow enzyme is the only mammalian DNA polymerase thus far reported to be associated with a 3′- to 5′-exonuclease activity (Byrnes et al., 1976; Byrnes & Black, 1978). In this report we present data demonstrating that DNA polymerase δ is also found in calf thymus tissue and is not unique to bone marrow and that the 3′- to 5′-exonuclease activity is an intrinsic property of calf thymus DNA polymerase δ. Some of the physical properties of this enzyme are also presented.

Materials and Methods

Deoxynucleoside triphosphates were obtained from P-L Biochemicals, and [^{3}H]dTTP (50 Ci/mmol) was obtained from Amersham/Searle. Poly(dA–dT) was purchased from Boehringer Corp.; other synthetic polydeoxyribonucleotide and oligodeoxyribonucleotides were from either P-L Biochemicals or Collaborative Research. Fetal calf thymus glands were obtained from Texas Biological Supply Specialists, Inc. Calf thymus DNA was purchased from Worthington Biochemical Corp. Sephacryl S-200 and DEAE-Sephadex A-25 were obtained from Pharmacia Fine Chemicals, phosphocellulose (P11) was from Whatman, and hydroxylapatite (Bio-Gel HT) was from Bio-Rad Laboratories. Acrylamide, methylenebis(acrylamide), Temed (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine), and Coomassie blue were purchased from Bio-Rad Laboratories. Ampholytes were purchased from LKB.

Denatured DNA–cellulose was prepared as described by Litman (1968). [^{3}H]Poly(dT)$_{50}$ was synthesized with terminal deoxynucleotidyltransferase using oligo(dT)$_4$ as primer and [^{3}H]dTTP (200 cpm/pmol) as previously described (Que et al., 1978). Poly(dA–dT) labeled at the 3′ terminus with [^{3}H]dTMP was prepared as previously described (Byrnes et al., 1977). Activated calf thymus DNA was prepared according to Fansler & Loeb (1974).

DNA Polymerase Assays. When poly(dA–dT) was used as template/primer, the reaction mixture contained in a final

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