Kinetic Characteristics Which Distinguish Two Forms of Calf Thymus DNA Polymerase α†

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ABSTRACT: DNA polymerase α was isolated as previously described [Holmes, A. M., Hesslewood, I. P., & Johnston, I. R. (1974) *Eur. J. Biochem. 43*, 487]. This method yields five nuclease-free forms of α-polymerase, A1, A2, B, C, and D. Holmes and co-workers [Holmes, A. M., Hesslewood, I. P., Wickremasinghe, R. G., & Johnston, I. R. (1977) *Biochem. Soc. Symp. 42*, 17] have suggested that the C form is the core enzyme of α-polymerase and have demonstrated that removal of a protein subunit from the A1 form yields an enzyme with the physical properties of the C form. They did not investigate the function of the subunit because the A1 and C forms were not easily distinguished with biochemical kinetics. We have been able to demonstrate three kinetic differences between these forms: (1) the α-A1-polymerase adds more nucleotides per binding event to activated DNA (is more processive) than does α-C-polymerase. (2) The synthetic activity of the α-A1-polymerase is greater on a template with an average gap size of 65 nucleotides than it is on a template with an average gap size of 10 nucleotides whereas that of the α-C-polymerase is not. (3) The synthetic activity of the α-C-polymerase is inhibited by high concentrations of activated calf thymus DNA (>300 μM) whereas that of the α-A1-polymerase is not. The nature of the inhibitor was investigated and found to be a nuclear RNA component present in the DNA preparations. These kinetic differences may provide a means to assay for the protein subunit that converts α-C-polymerase to α-A1-polymerase, and provide a basis for isolation and characterization of other DNA replication–association proteins.

DNA polymerase α is widely accepted as the enzyme involved in DNA replication in eukaryotic cells (Bollum, 1975; Brun & Chapeville, 1977; Weissbach, 1979). However, the characterization of this enzyme has been complicated by its multiplicity of forms (Momparler et al., 1973; Holmes et al., 1974, 1977; Yoshida et al., 1974; Brakel & Blumenthal, 1977). These many forms have created problems both with purification of α-polymerase and with study of its mechanism of action (Holmes et al., 1974, 1975, 1976, 1977; Holmes & Johnston, 1975; McKune & Holmes, 1979a). Thus far, the forms have been shown to differ in such fundamental characteristics as chromatographic behavior, molecular weight, and *N*-ethylmaleimide sensitivity. It has been more difficult, however, to obtain biochemical kinetic information that is characteristic of each form.

Study of DNA polymerase α has revealed that there is a core enzyme with DNA polymerase activity and that some of the other forms are generated because additional protein subunits[1] bind to the core enzyme (Holmes & Johnston, 1975; Holmes et al., 1977; Mechali & DeRecondo, 1978). The character and role of the subunits are not clear, but such proteins could alter some functional parameter of the core enzyme and consequently could be involved in regulation of DNA replication.

McKune & Holmes (1979a) showed that calf thymus α-A1-polymerase can be converted by mild urea treatment to the α-C-polymerase (the core enzyme) plus a protein subunit. The α-A2-polymerase, when treated with urea, yields the core polymerase and a similar protein subunit. The conversion of these forms was monitored by chromatography on DEAE-cellulose. The A1 and A2 forms of the polymerase appear to be identical in all properties except the charge on their respective subunit proteins.

McKune & Holmes (1979a) found that the α-A2-polymerase differed from the α-C-polymerase in its rate of DNA synthesis on homopolymer vs. calf thymus DNA templates. These differences, however, are difficult to interpret in terms of enzyme function in vivo, and the role of the protein subunit(s) remains unclear.

In addition, McKune & Holmes (1979b) found that both the A and C forms of the polymerase were able to dissociate rapidly from a template of one nucleotide composition and begin synthesis on a template of a different composition, added during the course of a reaction. They concluded that both forms synthesize DNA by a mechanism which involves frequent dissociation of the DNA polymerase from the DNA template, but they did not demonstrate a kinetic difference between the forms of the enzyme with respect to dissociation during synthesis.

We have used a different method (Bambara et al., 1978) to measure the processivity (the average number of nucleotides added each time the DNA polymerase binds to the DNA template) of the A1 and C forms and find them to be processive to differing extents.

Furthermore, we have demonstrated that, if the two forms of DNA polymerase are normalized to equal synthetic activities on DNA with gaps of ten nucleotides, the α-A1-polymerase shows a relatively greater activity on DNA with larger gaps than does the α-C-polymerase. The α-A1-polymerase also has a greater specificity of interaction with the DNA template than does the α-C-polymerase.

Such differences in biochemical kinetics found among the enzyme forms are an important tool not only to distinguish between multiple enzyme forms but also to define the roles of subunit proteins when the DNA polymerase operates as a

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[1] The term subunit, as used here, is meant to imply a protein found associated with the DNA polymerase after purification. A function for such proteins in DNA replication is not assumed a priori.

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multisubunit complex in vivo.

Materials and Methods

Enzymes. *E. coli* DNA polymerase I was purchased from New England Biolabs. T4 DNA polymerase was purchased from Miles Laboratories. Pancreatic deoxyribonuclease I was purchased from Worthington Biochemicals. *E. coli* exonuclease III was a gift of Dr. L. Loeb. Pancreatic ribonuclease A was purchased from Sigma Chemical Co.

Calf thymus DNA polymerase α was isolated and assayed for synthetic activity essentially as described by Holmes et al. (1974, 1975). The multiple species of α-polymerase were eluted from DEAE-cellulose exactly as described (Holmes et al., 1977) with clear resolution of each species. Each polymerase species showed *N*-ethylmaleimide sensitivity, which is characteristic of DNA polymerase α. After isolation, the enzyme forms were made 50% in glycerol and 5 mM in β-mercaptoethanol and stored at −20 °C.

Terminal deoxynucleotidyl transferase and β-polymerase had been shown to separate from the α-polymerase during the isolation procedure (Holmes et al., 1974); this was confirmed in our preparation.

The α-polymerase preparations were also found to be essentially free of nuclease contamination. Incubation of 2.07 units (Holmes et al., 1974) of α-A1-polymerase or 2.17 units of α-C-polymerase with 9 nmol of nicked ^{3}H-labeled ColE1 DNA (9000 cpm/nmol) for 20 min at 37 °C under conditions similar to those for synthetic activity (with or without dNTPs)[2] resulted in conversion of less than 0.1% of the DNA to an acid-soluble form. In addition, an incubation of 0.45 unit of α-A1-polymerase or 0.17 unit of α-C-polymerase with 9 nmol of supercoiled ^{3}H-labeled ColE1 DNA was performed by using the conditions for the synthetic activity assay (but without dNTPs). The ColE1 DNA from this reaction was then layered onto a 5–20% alkaline sucrose gradient that contained 0.5 M NaCl, 10 mM EDTA, and 0.2 N NaOH and then centrifuged in a Beckman SW60 rotor at 50000 rpm for 135 min at 2 °C. This assay, in which closed circular and nicked circular DNA can be distinguished, showed that no detectable nicks were introduced (i.e., less than one nick per 150000 nucleotides). Consequently, nuclease contamination is far below any level which could alter the results of experiments described below.

The assay for ATPase activity was conducted by use of the synthetic activity assay conditions with the addition of 10 μM [^{3}H]ATP (1.0 Ci/mmol) and 0.05 unit of α-A1-polymerase or 0.16 unit of α-C-polymerase. After 14 min at 37 °C, the reaction mixture was brought to 0.6 mM unlabeled ATP and 5 mM EDTA and then spotted on a poly(ethylenimine)–cellulose plate (Brinkman Co.). The plate was developed in 0.9 M LiCl and 7 M urea and dried. The regions of the plate corresponding to AMP, ADP, and ATP were removed and soaked with 50 mM Tris, pH 7.5, a toluene–Triton X-100-based scintillation fluid then was added, and the samples were counted. Neither α-A1-polymerase nor α-C-polymerase showed any detectable ATPase activity.

Nucleotides and Polynucleotides. Deoxynucleoside triphosphates and monophosphates were purchased from Sigma Chemical Co. When highly purified deoxynucleoside triphosphates were needed in processivity assays (Bambara et al., 1978), the commercial compounds were further purified according to the procedure of Lehman et al. (1958). Purity was checked on poly(ethylenimine)–cellulose plates developed in 0.6 M LiCl and 7 M urea. [^{3}H]dTTP (40–60 Ci/mmol) was purchased from New England Nuclear Corp. [^{3}H]ATP (21 Ci/mmol) was purchased from Amersham Corp.

The oligonucleotides $p(dA)_3$, $p(dT)_{10}$, and $p(dA)_{19-24}$ were purchased from P-L Biochemicals.

Native ColE1 DNA was isolated according to Blair et al. (1972). Bacteriophage fd and native single-stranded fd DNA were prepared by the method of Sadowski & Hurwitz (1969a,b). Calf thymus DNA (lot no. 579-18) and the DNA duplex poly(dG)·poly(dC) were purchased from P-L Biochemicals.

Number of 3′-Hydroxyl Termini in DNA Samples. The concentration of 3′-hydroxyl termini produced in a DNA sample by partial endonucleolytic digestion was determined by measurement of the maximum incorporation of three dNTPs with 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 incorporation and the concentration of 3′ termini in the DNA solutions. The data of Fisher et al. (1979) indicate that 3′ → 5′ exonuclease activity of DNA polymerase I does not produce significant errors in such determinations.

Size of Gaps in DNA Samples. Gap size is defined as the amount of single-strand template (in nucleotides) per 3′-hydroxyl terminus. This value was determined by two different methods.

In the first method, the assumption is made that nucleotides hydrolyzed during procedures that produce gaps in a double-strand DNA are measurable as the acid-soluble nucleotide component of the resulting DNA mixture. Division of the number of acid-soluble nucleotides by the number of 3′-hydroxyl termini (measured as described above) gave the average number of nucleotides hydrolyzed per 3′-hydroxyl terminus (i.e., the average gap size).

The second method involves use of the "random nuclease" model of Fisher et al. (1979) for comparison of the extents of polymerization in the presence of a limited and a complete complement of dNTPs, and an excess of T4 DNA polymerase. Conditions for the T4 DNA polymerase assay were as described by Matson et al. (1980) with the following exceptions: The reactions (0.1 mL) contained 5 μM DNA and 330 ng of T4 DNA polymerase. The second method yields gap values 10–20 nucleotides larger than the first, possibly because T4 DNA polymerase can add several nucleotides to a nicked site before synthesis stops. Alternatively, some short oligonucleotides associated with gaps in the DNA may not easily solubilize in acid. The gap sizes presented in this paper are an average of the values provided by these two methods.

Preparation of Templates. Calf thymus DNA was activated with pancreatic endonuclease according to Uyemura & Lehman (1976). The typical preparations of activated DNA were from 1 to 2% acid soluble and contained one 3′-hydroxyl terminus every 521–658 nucleotides. Digestion under these conditions produces some gapped structure, with an average gap size of 29 nucleotides.

Preparation of exonuclease-gapped DNA was started by nicking calf thymus DNA essentially as described by Uyemura & Lehman (1976). Nicking, a milder procedure than activation, produces less than 0.1% acid solubility. The gapping reaction (1.0 mL) was a modification of that used by Uyemura & Lehman (1976). It contained 1.0 mg of nicked calf thymus DNA, 20 mM Tris-HCl, pH 8.5, 3 mM $MgCl_2$, 1 mM β-mercaptoethanol, and exonuclease III. The amount of exonuclease III used depended on the desired gap size and ranged from 0 to 140 units. Following incubation for 2 h at 37 °C, the mixture was made 3 mM in EDTA and 10 mM in *N*-

[2] Abbreviations used: dNTPs, deoxyribonucleoside triphosphates; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

ethylmaleimide and then incubated for 15 min at 70 °C.

Synthetic Activity Measurements during Kinetic Studies. Reactions (90 μL) contained 60 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, 500 μg/mL BSA, and dATP, dCTP, dGTP, and [^{3}H]dTTP (1.0–10.0 Ci/mmol) each at 50 μM. In addition, each reaction contained potassium phosphate and glycerol contributed by the enzyme stock solutions. The potassium phosphate concentration of each enzyme stock solution varied, depending on its position of elution from DEAE-cellulose, and was determined by using a conductivity bridge. The concentration of enzyme in the stock solutions varied among different preparations. When different enzyme forms were being compared, use of a specific number of enzyme units would result in different amounts of salt and glycerol being added to different reactions. However, the salt and glycerol contribution to the reaction by enzyme additions from each enzyme stock solution was calculated, and the reactions were supplemented as needed with potassium phosphate (pH 7.5) and glycerol such that the concentrations of these components were held constant at 7.5 mM and 3.8%, respectively. DNA concentrations were varied as described in the figures, and the DNA was always present in great excess relative to the enzyme. Each reaction contained from 0.03 to 0.45 unit of DNA polymerase. Incubations were at 37 °C, and aliquots (25 μL) were removed at the times specified in the figure legends. They were assayed for acid-soluble radioactivity as described earlier (Wu et al., 1974).

The assay for DNA synthesis in the template utilization measurements (90 μL) contained 65 mM Tris-HCl, pH 7.5, 6.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 500 μg/mL BSA, 7.5 mM potassium phosphate, pH 7.5, 3.8% glycerol, 200 μM DNA, and dATP, dCTP, dGTP, and [^{3}H]dTTP (1.0–10.0 Ci/mmol) each at 40 μM. Each reaction contained from 0.03 to 0.45 unit of DNA polymerase (salt and glycerol contributions were maintained as described above). Incubations were at 37 °C, with aliquots (20 μL) removed at 0, 5, 10, and 15 min and assayed for acid-soluble radioactivity.

Measurement of Processivity. Processivity was quantitated (Bambara et al., 1978) by measuring the rate of DNA synthesis in the presence of 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 containing all four dNTPs indicates the total number of nucleotides polymerized onto the templates. The average number of nucleotides added per binding event can then be calculated. The polymerase molecules may not move from one template to the next as often in the limited dNTP reaction as in the four dNTPs reaction. To account for this, a correction factor is added and is calculated from two additional reactions: one with three dNTPs and one with all four dNTPs, but both containing a template analogue inhibitor (described below). The inhibitor reversibly binds the polymerase as it tries to move from one primer template to the next. By comparison of the extent of inhibition in the limited reaction to the inhibition of the reaction with all four dNTPs, a "cycling-time" correction factor is obtained.

Processivity values were obtained in the presence of excess DNA template for α-A1-polymerase and α-C-polymerase. Processivity was measured on activated calf thymus DNA. The reactions (90 μL) contained the same components as described above for the template utilization studies, with the following exceptions. Reactions requiring a limited complement of dNTPs contained [^{3}H]dTTP (4.0–10.0 Ci/mmol), dCTP, and dGTP at 40 μM each. Reactions requiring a template analogue inhibitor contained 50 μM activated calf thymus DNA and 150 μM activated poly(dG)·poly(dC) (preparation and role described below). All assays were performed at 37 °C. Aliquots (20 μL) were removed at 0, 5, 10, and 15 min, and acid-insoluble radioactivity was determined.

Preparation of Inhibitor DNA. The typical preparation of activated poly(dG)·poly(dC) was performed in a 0.6-mL reaction. Each reaction mixture contained 90 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 448 μM poly(dG)·poly(dC), and 15 ng of pancreatic endonuclease. The mixture was incubated at 37 °C for 15 min followed by a 30-min incubation at 75 °C.

This activated poly(dG)·poly(dC) demonstrated ability to support synthesis when used as a template and displayed classical competitive inhibition with respect to incorporation of [^{3}H]dTTP into activated calf thymus DNA for both forms of DNA polymerase α. The activated poly(dG)·poly(dC) fulfills the requirements for inhibitor DNA for processivity measurement (Bambara et al., 1978) because it is an analogue of the calf thymus DNA template and does not incorporate the radiolabeled dTTP. Also, because of the presence of dGTP and dCTP in all reactions, the poly(dG)·poly(dC) should interact with the DNA polymerase to the same extent in all reactions.

Results

Processive DNA Synthesis. Quantitative values of processivity were determined for the two forms of DNA polymerase α by using activated calf thymus DNA as a primer template. On this substrate, α-C-polymerase has a processivity of 11.17 ± 0.77 (standard error, 37 determinations) nucleotides. The α-A1-polymerase has a greater processivity of 17.20 ± 0.89 (18 determinations). The "*t*" test statistical evaluation indicates that the probability of a real difference in the mean values is greater than 99.9%.

The average size of the single-strand region next to the 3′-hydroxyl termini in the substrate is 29 nucleotides, suggesting that a greater processivity would have been possible if either enzyme had the capability. The fact that the average gap size is larger than either value of processivity, however, does not completely preclude an influence of the template structure on processivity. The synthesizing enzyme could be encouraged to dissociate as it nears the 5′ terminus of a strand of DNA hydrogen bonded to the template ahead of it. Such behavior has been suggested for the α DNA polymerase from human KB cells (Wang & Korn, 1980).

Template Utilization. Initial rates of DNA synthesis with each DNA polymerase were measured on calf thymus DNA that had been gapped to produce single-stranded regions adjacent to the 3′-hydroxyl termini. The average size of these regions was varied for different DNA substrates, as indicated in Figure 1. Results are plotted as a ratio of the activity on each gapped template divided by the activity on the template with the shortest gaps. Figure 1 clearly shows that both α-A1-polymerase and α-C-polymerase utilize gaps of 54 nucleotides better than shorter or longer gaps.

It is also apparent that the synthetic activity of α-A1-polymerase increases with respect to that of α-C-polymerase as the gap size is increased. For example, α-A1-polymerase is substantially more active on a template with 65 nucleotide gaps than on one with 10 nucleotide gaps while α-C-polymerase is not. The activity of both enzymes is depressed on DNA with 110 nucleotide long gaps, relative to DNA with 10 nucleotide gaps, but the depression is greater for the α-C-polymerase.

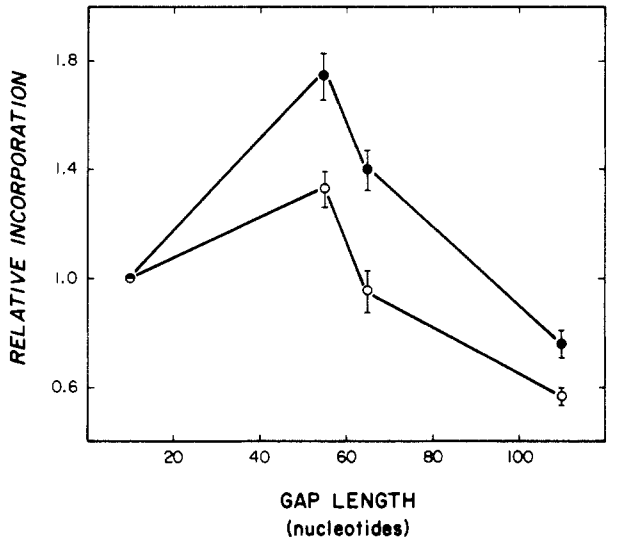

FIGURE 1: Template utilization vs. size of gap. Reaction mixtures for template utilization measurements are described under Materials and Methods. The DNA substrates were prepared by nicking and then gapping calf thymus DNA as described under Materials and Methods. All exonuclease gapped substrates were obtained from a single preparation of nicked calf thymus DNA and consequently have the same number of 3′-hydroxyl termini (one per 674 nucleotides). The data are expressed as the ratio of the synthetic activity on each gapped template to the synthetic activity on the template with the shortest gaps (10 nucleotides). Each data point represents the average of at least 18 determinations, with the deviation expressed as the standard error of the mean. Open and closed circles represent α-C-polymerase and α-A1-polymerase, respectively.

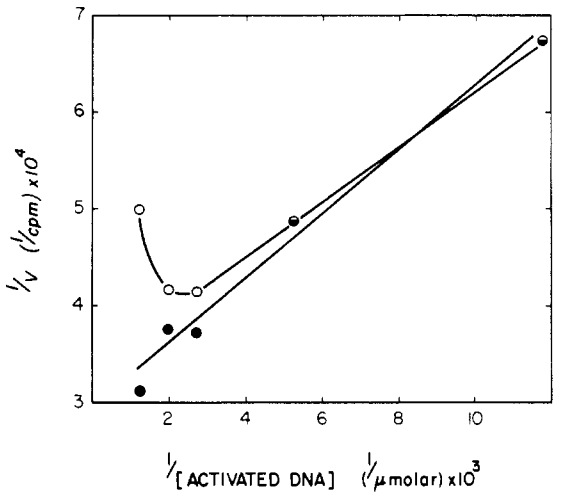

FIGURE 2: Inhibition at high DNA concentrations. Reaction mixtures are described under Materials and Methods. The activated DNA had one 3′-hydroxyl terminus per 658 nucleotides and was 1.46% acid soluble. The data are expressed as the average of values obtained after 7 and 14 min of incubation. At least eight determinations were made for each point. Open and closed circles represent α-C-polymerase and α-A1-polymerase, respectively.

Inhibition at High DNA Concentration. The initial rate of DNA synthesis by the two polymerases was determined at various concentrations of activated calf thymus DNA. Results are given in a Lineweaver–Burk plot (Figure 2). The α-C-polymerase demonstrated an inhibition at DNA concentrations greater than 300 μM that is similar in appearance to classical substrate inhibition. When compared at an equal level of synthetic activity, the α-A1-polymerase did not show such an inhibition. A control experiment with *E. coli* DNA polymerase I also did not show any inhibition at DNA concentrations up to 858 μM when assayed on this template (data not shown). Data presented in earlier sections were obtained at a DNA concentration of 200 μM and are therefore not subject to this inhibitory effect.

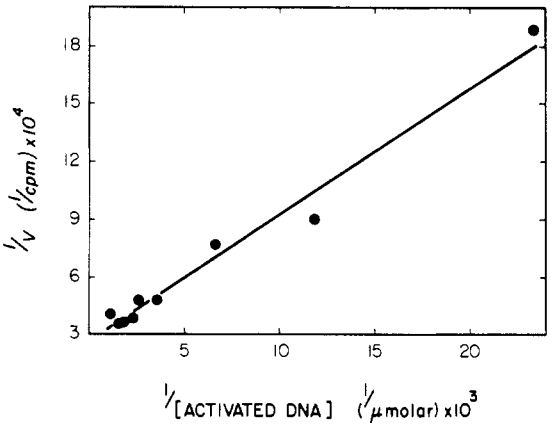

FIGURE 3: Effect of salt on inhibition at high DNA concentrations. Reaction mixtures are described under Materials and Methods. The activated DNA had one 3′-hydroxyl terminus per 658 nucleotides and was 1.46% acid soluble. Each reaction mixture had an additional 30 mM potassium phosphate (pH 7.5) added. This extra salt reduced the activity of the enzyme by 73%. The data are expressed as the average of values obtained with α-C-polymerase after 7 and 14 min of incubation. Each point represents the average of two determinations.

The insensitivity of α-A1-polymerase, compared to α-C-polymerase, to this inhibition at concentrations of activated DNA up to 858 μM is one of the most apparent differences between the forms. This difference indicates that the α-A1-polymerase has a greater specificity for binding to DNA than that of the α-C-polymerase.

The high specificity of α-A1-polymerase can be mimicked by the addition of high concentrations of salt. In Figure 3, 30 mM potassium phosphate, pH 7.5, was included in addition to the salts normally present in the reaction mixture for α-C-polymerase. This extra salt reduced the activity of the polymerase by 73% and eliminated the inhibition seen at DNA concentrations greater than 300 μM. Higher salt concentration shields and weakens charge interactions. This result implies that the inhibitory process involves such interactions.

Nature of the Inhibitor. Experiments were performed to determine whether calf thymus DNA itself or some other component of the DNA preparation was responsible for the inhibition seen at high DNA concentrations. Phenol extraction and EDTA addition to the activated DNA had no effect on the inhibition (data not shown). Variation of the concentration of deoxynucleoside triphosphates or addition of deoxynucleoside monophosphates, single-stranded closed circular fd phage DNA, double-stranded native ColE1 plasmid DNA, or synthetic oligonucleotides $p(dA)_3$, $p(dT)_{10}$, or $p(dA)_{19-24}$ were all without effect (data not shown).

Treatment of the activated DNA with excess ribonuclease A, however, eliminated the inhibition seen at high DNA concentrations (Figure 4). Digestion of a calf thymus DNA sample with excess ribonuclease A released approximately 1% of the polynucleotide as acid-soluble RNA, quantitating the level of RNA present in DNA samples used for these experiments. This is nuclear RNA which remains from the purification of the calf thymus DNA. Molecular sizing of an activated DNA sample by agarose gel filtration indicated that the inhibitor could be removed. The RNA segments responsible for inhibition were found to be approximately 100 nucleotides in length or smaller (data not shown).

Discussion

We have described three kinetic methods which distinguish between calf thymus DNA α-C-polymerase and α-A1-

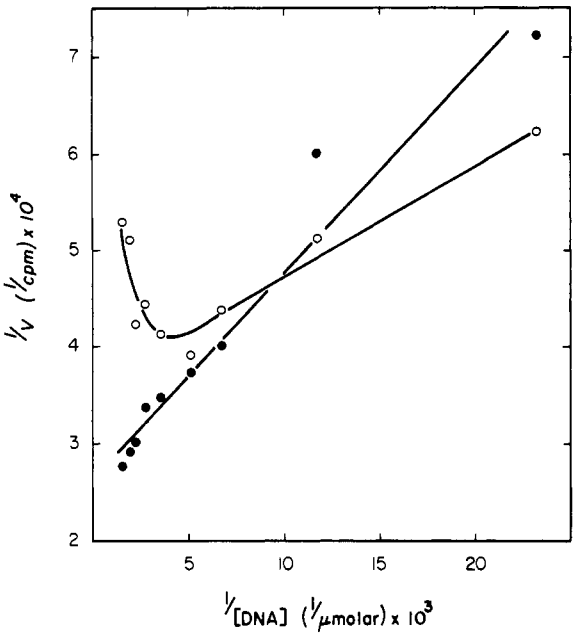

FIGURE 4: Effect of ribonuclease on inhibition at high DNA concentrations. Reaction mixtures are described under Materials and Methods. The activated DNA had one 3′-hydroxyl terminus per 521 nucleotides and was 1.26% acid soluble. The data are expressed as the average of values obtained with α-C-polymerase after 5 and 10 min of incubation. Each point represents the average of four determinations. Open circles represent synthetic activity on activated DNA. Closed circles represent synthetic activity on activated DNA which had been treated with ribonuclease A. Ribonuclease treatment was performed in a 1.0-mL reaction containing 25 mM Tris-HCl, pH 7.5, 1.25 mg of activated DNA, and 2 mg of ribonuclease A. The mixture was incubated at 37 °C for 1 h and then 70 °C for 15 min.

polymerase. By the criteria of these measurements, the two forms of the enzyme are functionally different. α-A1-Polymerase shows a greater processivity than does α-C-polymerase. If the enzymes are normalized to equal activity on DNA with gaps of 10 nucleotides, α-A1-polymerase shows a relatively greater activity than α-C-polymerase on DNA with gaps of 54–110 nucleotides. α-A1-Polymerase also demonstrates a greater specificity for binding of polynucleotide substrates than does α-C-polymerase, as can be observed by using preparations of activated DNA containing small amounts of RNA.

Calf thymus α DNA polymerase appears to have multiple forms with a common catalytic core (Holmes & Johnston, 1975). Evidence has been provided (Holmes et al., 1977; McKune & Holmes, 1979a) that this catalytic core is α-C-polymerase and that the core can be derived from other forms of α-polymerase. The α-A1-polymerase is separated into α-C-polymerase plus a protein subunit by mild urea treatment (McKune & Holmes, 1979a). These two forms were distinguished readily by column chromatography, but it had previously been difficult to demonstrate significant differences in their kinetic behavior.

While the forms of α-polymerase used in these studies are prepared by methods that do not yield homogeneous proteins, great care was taken to see that all possible contaminating DNA metabolizing activities (i.e., endonucleases, exonucleases, terminal transferase, ATPases, and other DNA polymerases) were absent from the preparations. The purification scheme also precludes proteins in the size range of eukaryotic single-strand DNA binding proteins (Herrick & Alberts, 1976). Therefore, it is most likely that the kinetic differences between the enzyme forms which we have observed result from the presence of the protein subunit associated with α-A1-DNA polymerase. Because of the presence of other proteins in each enzyme sample, it is not possible to completely discount effects that these proteins may have on the properties of the DNA polymerases.

With the assumption that the protein subunit, which is dissociable from the α-A1-DNA polymerase, is responsible for the properties of that enzyme form, the data presented here support the concept that forms of DNA polymerase α that are more complex than the catalytic core should have properties more like those expected for the DNA polymerase in vivo. Investigation of other replicative DNA polymerases has provided the basis for such a concept, since some function as multimeric protein complexes during DNA replication. For example, the *E. coli* DNA polymerase III core enzyme cannot substitute for the DNA polymerase III holoenzyme in DNA replication model systems (Kornberg, 1980; Geider & Kornberg, 1974). Similarly, the T4 DNA polymerase core enzyme has been shown to have an increased processivity after the addition of specific replication-associated proteins (Liu et al., 1978; Alberts et al., 1980; Barry & Alberts, 1980; Huang et al., 1980). In the case of DNA polymerase α, the information we have obtained suggests that the presence of a subunit added to the core enzyme improves its ability to synthesize DNA in several respects.

Since DNA polymerase α is the major replication enzyme of eukaryotic cells, we might expect that its processivity would approach the length of Okazaki fragments. Okazaki fragments primed in positions where they can span the full distance between separated nucleosomes during DNA replication are about 200–300 nucleotides long (DePamphilis et al., 1980). The processivity value which we report for the calf thymus α-C-polymerase (11.17 ± 0.77) is similar to that reported by Fisher et al. (1979) for the DNA polymerase α from human KB cells, which may be a core component of a larger enzyme. Additionally, we have demonstrated that the α-A1-polymerase is more processive than the α-C-polymerase. The greater processivity of α-A1-polymerase presumably results from association of the protein subunit with the α-C-core polymerase. The processivity of α-A1-polymerase (17.20 ± 0.89) is still not as great as might be expected for the synthesis of an Okazaki fragment. This suggests that there may be other replication-associated proteins which act in conjunction with the α-C-polymerase during synthesis, further increasing its ability to translocate along the DNA template without dissociation.

Both DNA polymerases show more synthetic activity on DNA that has an average gap size of 54 nucleotides than on DNA with shorter or longer gaps. Also, the activity of the α-A1-polymerase increases with respect to that of the α-A1-polymerase as the gap size increases. This relative preference of the α-A1-polymerase for longer gaps must again be attributed to the presence of the protein subunit. Such a preference could be significant during DNA replication in vivo.

The two forms of DNA polymerase α can be readily distinguished by using appropriate gapped DNA templates. When the activities of the two forms are normalized to the same value by using DNA with 10 nucleotide long gaps, a comparison of the two enzymes by using DNA with 65 nucleotide long gaps reveals a considerably greater activity of α-A1-polymerase with respect to that of α-C-polymerase (Figure 1). Addition of the protein subunit to the α-C-polymerase and subsequent conversion to α-A1-polymerase could possibly be monitored by this type of template utilization experiment.

The presence of small quantities of nuclear RNA in calf thymus DNA was useful in differentiating the A1 and C forms of DNA polymerase α. This may be RNA involved in gene expression, RNA associated with DNA replication, or RNA having a structural role such as with that RNA found associated with the chromosome of *E. coli* (Kornberg, 1980). Only the core enzyme (α-C-polymerase) was inhibited by this RNA. The resistance of α-A1-polymerase to this inhibition most likely is caused by the protein subunit of the α-A1-polymerase. This protein subunit could increase the specificity of the active site of the DNA polymerase such that more DNA, and not RNA, is bound by the polymerase. Alternatively, the RNA may bind to the enzyme at a separate site from DNA, but blocking the active site, or causing a conformational change in the protein which inhibits DNA synthesis. The extra protein subunit of α-A1-polymerase may inhibit this second-site binding of RNA.

The higher specificity of the α-A1-DNA polymerase can be mimicked by high salt concentrations which confer resistance to this inhibition on the α-C-polymerase. Presumably, the higher ionic strength shields interactions between enzyme and inhibitor. The protein subunit may have a similar effect, or it may increase interactions between enzyme and template.

In summary, the results of our studies demonstrate that the multiple forms of DNA polymerase α can be readily differentiated by methods other than physical properties. We have developed three different assay methods by which we can distinguish between two forms of DNA polymerase α. These methods suggest that the protein subunit bound to the polymerizing subunit of α-A1-polymerase increases processivity, affects ability to use gapped DNA, and increases the specificity for binding to DNA termini. All three methods may be useful to assay the purification of, and assist in the characterization of, the protein subunit which converts α-C-polymerase to α-A1-polymerase. The processivity and template utilization assays should also prove to be useful in the search for other proteins involved in DNA replication.

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