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Analysis of the Mechanism of ATP Stimulation of Calf Thymus DNA α-Polymerase†

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ABSTRACT: Biochemical kinetic analyses of the ATP stimulation of the A2 form of calf DNA α-polymerase show that when DNA or primer termini are the variable substrates, maximum reaction velocity is independent of ATP concentration. When dNTP concentration is the variable substrate, the apparent K_m is invariant with ATP. Such results indicate that the increase in the synthetic rate caused by ATP results from an improvement in synthesis initiation at primer termini. The effect of ATP on the DNA binding affinity of α-A2-polymerase was examined by using column chromatography. Passage of the polymerase through native DNA–cellulose at 70 mM ionic strength resulted in 40% binding of the enzyme. In the presence of 4 mM ATP, binding increased to 80%. In both cases, the bound polymerase could be eluted by a 370 mM ionic strength wash. An elution profile similar to that observed in the absence of ATP was obtained with 0.1 mM ATP, 4 mM GTP, or 4 mM each of the nonhydrolyzable ATP analogues adenyl-5′-yl imidodiphosphate or adenosine 5′-*O*-(3-thiotriphosphate). These results suggest that hydrolysis of the γ-phosphate occurs at millimolar levels of ATP and leads to a higher affinity of polymerase for DNA. To distinguish the effects of ATP on RNA priming from those on DNA synthesis, products synthesized processively by α-A2-polymerase were sized by gel filtration. Results indicate that essentially all products made on a gapped fd replicative form template in the presence of four dNTPs and 4 mM ATP result from the extension of preexisting DNA primers. When rNTPs other than or in addition to ATP are present, products primed de novo, approximately 30 nucleotides long, are observed. The length of processive synthesis of these products is unaffected by the presence of 4 mM ATP. At physiological concentrations (10 nM–10 μM), the ATP analogue P^1,P^4-di(adenosine-5′) tetraphosphate did not stimulate the synthetic rate of α-A2-polymerase. At millimolar concentrations, moderate stimulation was observed.

DNA α-polymerase has been implicated as the enzyme responsible for eukaryotic chromosomal replication (Weissbach, 1979; DePamphilis & Wasserman, 1980). One approach to identification of proteins and other factors involved in DNA replication has been to assess the ability of such factors to stimulate α-polymerase activity. Several protein factors that stimulate the catalytic activity of DNA α-polymerase have been identified from various sources (Herrick et al., 1976; Otto et al., 1977; Novak & Baril, 1978; Burke et al., 1980; Riva et al., 1980; Boxer & Korn, 1980; Lamothe et al., 1981).

Recently, it has been noted that ATP stimulates the activity of DNA α-polymerase. Smith & Berezney (1982) reported that nuclear matrix bound α-polymerase from regenerating rat liver is stimulated by ATP. An effect of ATP on the synthetic rate of α-polymerase obtained from Ehrlich ascites tumor cells has also been observed (Faust & Rankin, 1982). We previously reported that ATP stimulates both the synthetic rate and processivity (Wierowski et al., 1983) of the A and C enzyme forms of calf thymus DNA α-polymerase [as defined by Holmes et al. (1974, 1975)].

In this report, we investigate the mechanism of this stimulation of calf α-polymerase by ATP. Through biochemical kinetic analyses, we find that the primary effect of ATP is on the initiation phase of DNA synthesis. We have also demonstrated that ATP increases the affinity of the α-polymerase for DNA by carrying out DNA binding studies in the absence of DNA synthesis. This result further suggests an involvement of ATP in an initiation-related event.

Materials and Methods

Proteins. Escherichia coli DNA polymerase I was purchased from New England Biolabs. Pancreatic deoxyribonuclease I was purchased from Worthington Biochemicals. T4 DNA polymerase was purchased from Miles Laboratories. *E. coli* exonuclease III was a gift from Dr. L. Loeb (University of Washington). Calf thymus unwinding protein 1 (UP1) was isolated according to the method of Herrick & Alberts (1976).

Calf thymus DNA α-polymerase was isolated essentially as described by Holmes et al. (1974, 1975). The α-polymerase multiple species were eluted from DEAE-cellulose as described (Holmes et al., 1977) and rechromatographed over DEAE-

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cellulose to minimize cross-contamination. After isolation, the enzyme forms were made 50% in glycerol and stored at −20 °C. The DNA α-polymerase preparations were found to be essentially free of terminal deoxynucleotidyltransferase, β-polymerase, DNA exonuclease, and DNA single-stranded and double-stranded endonuclease activities as described previously (Hockensmith & Bambara, 1981; Wierowski et al., 1983). Enzyme units are as defined by Holmes et al. (1974).

Nucleotides and Polynucleotides. Ribonucleoside triphosphates were purchased from Sigma and P-L Biochemicals. Deoxynucleoside triphosphates were purchased from Sigma and ICN. [^{3}H]dTTP (78–80 Ci/mmol) was purchased from New England Nuclear. [γ-^{32}P]ATP (~3000 Ci/mmol) was purchased from Amersham. The ATP analogues adenyl-5′-yl imidodiphosphate (AMPPNP)[1] and ATP γ S were purchased from P-L Biochemicals and Boehringer Mannheim, respectively. Ap_4A was purchased from Sigma. Poly(dA) was purchased from Miles, and the oligonucleotides $p(dT)_{10}$, $p(dT)_{19-24}$, and $p(dT)_{40-60}$ were from P-L Biochemicals.

Preparation of Templates. Calf thymus DNA (Sigma) was activated with pancreatic deoxyribonuclease I according to Uyemura & Lehman (1976). The poly(dA)·$(dT)_{10}$ primer-templates were prepared as described by Fay et al. (1981) at the following dA:dT nucleotide molar ratios, 60:1, 30:1, 20:1, 15:1, and 7:1. All concentrations of DNA are given as total nucleotide.

Preparation of bacteriophage fd ss and RF DNA was previously described (Matson et al., 1980). Bacteriophage fd RF DNA was digested to generate gaps as described by Wierowski et al. (1983). The average gap size was 190 nucleotides, with approximately 7 3′-OH termini per fd RF molecule, as determined by methods previously described (Hockensmith & Bambara, 1981).

Measurement of Reaction Rates for Kinetic Analyses. Standard reaction conditions were 60 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 500 μg/mL bovine serum albumin. All reactions (50 μL) contained 0.124 unit of the A2 form of DNA α-polymerase. The synthetic primer-template poly(dA)·$(dT)_{10}$ was used in reactions at a final concentration of 200 μM, except where DNA concentrations were varied as described in the legend to Figure 1. The dA:dT nucleotide molar ratio of the primer-template was 20:1, except where the ratio was varied as described in the legend to Figure 1. For one analysis, described in the legend to Figure 1, [^{3}H]dTTP (1 Ci/mmol) was varied in concentration; otherwise, [^{3}H]dTTP (4 Ci/mmol) was present at 40 μM. Incubations were at 37 °C with aliquots (10 μL) removed at 0, 5, 10, and 20 min. Incorporation of radioactivity was determined as described earlier (Wu et al., 1974). The extent of incorporation was such that less than 5% of the DNA template was utilized for synthesis, so that all measurements were of initial rates.

Determination of the Effect of Ap_4A on Enzyme Activity. Reactions (50 μL) designed to determine the effect of Ap_4A on the rate of polymerization contained, in addition to the standard reaction mixture, 40 μM [^{3}H]dTTP (4 Ci/mmol), 100 μM poly(dA)·$(dT)_{10}$ (20:1), 0.209 unit of DNA α-A2-polymerase, and 10 nM–4 mM Ap_4A. Incubations were at 37 °C with 10-μL aliquots removed at 0, 5, 10, and 20 min and assayed for incorporation of radioactivity. Again, all measurements were of initial rates. Identical reaction conditions were used to determine the effect of Ap_4A on ATP stimulation of calf DNA α-polymerase, except that 4 mM ATP was present and the $MgCl_2$ concentration was increased to 10 mM.

To determine the effect of Ap_4A on the length of products synthesized, the processivity of DNA α-polymerase was measured by the method of Fay et al. (1981). The reactions (100 μL) contained the same components as described above, except for the following: 200 μM primer-template, 0.547 unit of the A2 form of α-polymerase, and Ap_4A at a concentration of 1 μM, 1 mM, or 4 mM. The reactions were processed as described previously (Wierowski et al., 1983). In all cases, less than 1 pmol of nucleotides was incorporated per pmol of 3′-OH termini, ensuring that the elongation of each reacted primer was the result of a single binding event of the polymerase.

Measurement of DNA Binding Affinity. DNA–cellulose chromatography was used to measure the binding affinity of calf DNA α-polymerase for DNA. Native DNA–cellulose was prepared by using herring sperm DNA and Whatman CF-11 cellulose according to the method of Alberts & Herrick (1971). DNA α-A2-polymerase (0.66 unit) was loaded onto a native DNA–cellulose column (0.5 × 3.0 cm) equilibrated with a low ionic strength buffer (60 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 500 μg/mL bovine serum albumin. Fractions (100 μL) were collected as the column was initially washed with the low ionic strength buffer and subsequently washed with a high ionic strength buffer. The high ionic strength buffer contained the same ionic components as the low ionic strength buffer plus 300 mM KCl. Additions to the buffers were as follows: 0.1 mM ATP, 4 mM ATP, 4 mM GTP, or 4 mM each of the nonhydrolyzable ATP analogues AMPPNP or ATPγS.

Since calf DNA α-polymerase activity is sensitive to high salt concentrations (Holmes et al., 1974), only a 5-μL aliquot of each fraction from the high ionic strength wash was assayed for DNA polymerase activity such that the final KCl concentration in each reaction was 15 mM. The entire volume (100 μL) of each fraction from the low ionic strength wash was assayed for polymerase activity. In order to keep the reaction conditions consistent, the fractions from the low ionic strength wash were supplemented to 15 mM KCl. Additionally, each synthetic reaction (120 μL) contained the standard reaction mixture, 200 μM activated calf thymus DNA, and dATP, dCTP, dGTP, and [^{3}H]dTTP each at 20 μM. The reaction also contained 4 mM ATP, 4 mM GTP, 4 mM AMPPNP, or 4 mM ATPγS, depending upon the particular buffer used. After a 30-min incubation at 37 °C, the reactions were assayed for incorporation of radioactivity.

The specific activity of the [^{3}H]dTTP was 0.5 Ci/mmol for those reactions in which a high volume of the enzyme was used and 10 Ci/mmol for those reactions in which a low volume of the enzyme was used. Under these conditions, the 20-fold difference in specific activity of the [^{3}H]dTTP compensates for the 20-fold difference in enzyme volume. The accuracy of this compensation was verified in a separate experiment.

The relative binding affinity of the A2 and C forms of calf α-polymerase for DNA was determined by gradient elution of the enzyme activity from DNA–cellulose. Five units of α-A2- and α-C-polymerase were loaded onto separate native DNA–cellulose columns (1.2 × 2.0 cm) equilibrated with the previously described low ionic strength buffer which was supplemented with ATP at a final concentration of 4 mM. The columns were washed with 2 column volumes of low ionic

[1] Abbreviations: AMPPNP, adenyl-5′-yl imidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Ap_4A, P^1,P^4-di(adenosine-5′) tetraphosphate; ss, single stranded; RF, replicative form; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

strength buffer containing 4 mM ATP and then eluted with 10 volumes of a gradient of 0–200 mM KCl in low ionic strength buffer that contained 4 mM ATP. α-Polymerase activity was monitored by a synthetic assay described previously (Hockensmith & Bambara, 1981).

Analysis of the Primase Activity Associated with the DNA α-Polymerase. Four methods were used to analyze the primase activity associated with the A2 form of the calf DNA α-polymerase. In the first, the size distribution of products synthesized on the gapped fd RF template was analyzed as described previously (Wierowski et al., 1983). Three reactions (100 μL) were prepared, each containing the standard reaction mixture, 0.024 unit of α-A2-polymerase, 100 μM gapped fd RF DNA, 40 μM [^{3}H]dTTP (40 Ci/mmol), and the following: reaction 1, dATP (10 μM) and dGTP and dCTP (40 μM each); reaction 2, dATP (10 μM), dGTP and dCTP (40 μM each), and GTP, CTP, and UTP (100 μM each); reaction 3, dATP (10 μM), dGTP and dCTP (40 μM each), GTP, CTP, and UTP (100 μM each), and ATP (4 mM). Synthesis was allowed to proceed for 5 min at 37 °C and then terminated by addition of EDTA to 50 mM. Total incorporation was determined in a 5-μL aliquot of the reaction. Generally, less than 1 pmol of nucleotides was incorporated per pmol of 3′-OH termini. In cases where this value was exceeded, the average product size was sufficiently great that less than 3% of the available termini had sustained synthesis.

In the second method, the length of products synthesized by α-A2-polymerase was examined on an fd ss DNA template. Three reactions (50 μL) were prepared, each containing the standard reaction mixture, 0.365 unit of the A2 form of α-polymerase, 200 μM fd ss DNA, 40 μM [^{3}H]dTTP (10 Ci/mmol), and the following: additions to reactions 1, 2, and 3 as described above. After 10 min at 37 °C, each reaction was stopped and processed as previously described (Wierowski et al., 1983). Conditions identical with that of reaction 3 were used to determine the effect of UP1 on the length of products synthesized on fd ss DNA by α-A2-polymerase.

The third method was designed to demonstrate that the fd ss DNA is capable of supporting the synthesis of products that are several hundred nucleotides long. It involves the following two-step reaction: Initially, the reaction (50 μL) contained the standard reaction mixture, 0.365 unit of α-A2-polymerase, 200 μM fd ss DNA, 10 μM dATP, 40 μM each of dGTP, dCTP, and [^{3}H]dTTP (4 Ci/mmol), and ATP, GTP, CTP, and UTP at 100 μM each. After 12 min at 37 °C, DNA polymerase I was added in excess (36 units) to extend products that had been synthesized by the α-polymerase. This reaction was incubated for 60 min at 37 °C, terminated by the addition of EDTA, and processed as previously described (Wierowski et al., 1983).

Lastly, the ribonucleotide that is preferred by the α-A2-polymerase for priming activity was determined by using an fd ss DNA template. Reactions (50 μL) contained, in addition to the standard reaction mixture, 0.547 unit of α-A2-polymerase, 50 μM fd ss DNA, dATP, dCTP, dGTP and [^{3}H]dTTP (4 Ci/mmol) each at 40 μM, and combinations of ribonucleotides (100 μM each) as indicated. The reactions were incubated at 37 °C for 30 min and assayed for incorporation of radioactivity.

Determination of the Hydrolysis of [γ-^{32}P]ATP. Thin-layer chromatography was used to measure hydrolysis of [γ-^{32}P]-ATP by the A2 form of DNA α-polymerase. Reactions (50 μL) contained, in addition to the standard reaction mixture, 1.39 units of α-A2-polymerase, 200 μM poly(dA)·(dT)$_{10}$ (20:1), 40 μM dTTP, 2 mM ATP, and 15 nM [γ-^{32}P]ATP (~600 Ci/mmol). Incubations were carried out at 37 °C with 10-μL aliquots removed at 0, 20, and 60 min and spotted onto Polygram CEL 300 poly(ethylenimine) plates (Brinkmann Instruments). Samples were chromatographed in 0.6 M LiCl and 7 M urea, pH 6.8, for 4 h. The thin-layer plates were then covered with plastic wrap, overlaid with Kodak X-Omat RP film, and exposed at −20 °C for 3–5 h. The percent conversion of [γ-^{32}P]ATP to ADP + [^{32}P]P$_i$ at each time point was determined by comparing radioactivity profiles obtained by cutting each lane into 0.5-cm strips and measuring radioactivity by scintillation counting in toluene–Triton fluor.

Results and Discussion

Kinetic Analyses of ATP Stimulation. Biochemical kinetics were used to establish whether the stimulation of the α-A2-polymerase reaction rate by ATP on the poly(dA)·(dT)$_{10}$ primer-template occurs at the initiation or the elongation step of the polymerization reaction. Results are shown as double-reciprocal plots of initial reaction rates in the presence of various levels of ATP (Figure 1). In the first experiment (panel A), the primer-template (dA:dT = 20:1) was the variable substrate. Results show that when the concentration of DNA is extrapolated to infinity (at the *y*-axis intercept), no stimulation of the reaction rate by ATP can be observed. This result indicates that the effects of ATP are only manifested when the concentration of DNA is partially limiting the reaction rate. The implication is that the initial interaction of the polymerase with some site on the DNA, or the specific interaction of the polymerase with 3′-OH termini, is the step stimulated by ATP.

In order to distinguish between these possibilities, the DNA concentration was held constant, and the number of primers was varied (Figure 1, panel B). As in panel A, a similar intersection of lines occurred at the *y* axis. Such a result suggests that ATP stimulates specific interactions between the polymerase and the primer terminus.

Another characteristic of these plots is that ATP decreases the K_m (as determined from the *x*-axis intercept) for DNA and for 3′-OH termini. In these experiments, K_m values for DNA change from 2200 μM in the absence of ATP to 43 μM in the presence of 4 mM ATP. The K_m values for primer termini change from 11 μM in the absence of ATP to 0.57 μM with 4 mM ATP. The results indicate that ATP stimulates the rate of binding of 3′-OH termini by the DNA α-polymerase.

Different results were obtained when the elongation reaction was accelerated by the addition of increased concentrations of dTTP (Figure 1, panel C). Even at an extrapolated infinite concentration of dTTP, considerable stimulation by ATP could be observed. The K_m for the reaction with respect to dTTP was essentially unaffected by ATP. Such a result suggests that ATP has no direct effect on the rate of nucleotide addition reactions.

DNA Binding Affinity. Since the kinetic data indicated that the primary effect of ATP was on binding of α-polymerase to DNA, we investigated this effect of ATP by using column chromatography. Affinity measurements were made by passing the calf DNA α-polymerase over a native DNA–cellulose column and comparing the amount of activity that flowed through the column to that which remained bound on the column (see Materials and Methods). Although the DNA on the column is primarily double stranded, there are nicks and gaps in the DNA which presumably create sites to which the α-polymerase could bind. The effect of ATP on the affinity of α-A2-polymerase for DNA is shown in Figure 2. In the absence of ATP, approximately 60% of the total α-A2-

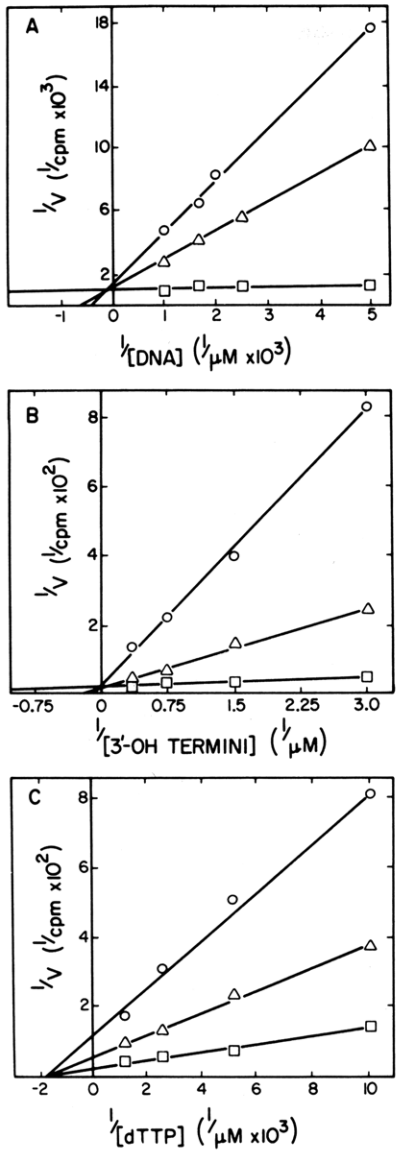

FIGURE 1: Kinetic analyses of ATP stimulation. The initial rate of dTTP incorporation by the A2 form of α-polymerase on a poly-(dA)·$(dT)_{10}$ template was determined at various levels of ATP concentration as described under Materials and Methods. Results are presented as double-reciprocal plots. In panel A, DNA (dA:dT = 20:1) was varied in concentration from 200 to 1000 μM. In panel B, the DNA concentration was held constant at 200 μM while the primer density (dA:dT) was varied from 60:1 to 7:1. In panel C, the DNA (dA:dT = 20:1) concentration was 200 μM, and the dTTP concentration was varied from 100 to 800 μM. (○), (△), and (□) represent 0, 1, and 4 mM ATP, respectively, except in panel A where (△) represents 0.5 mM ATP.

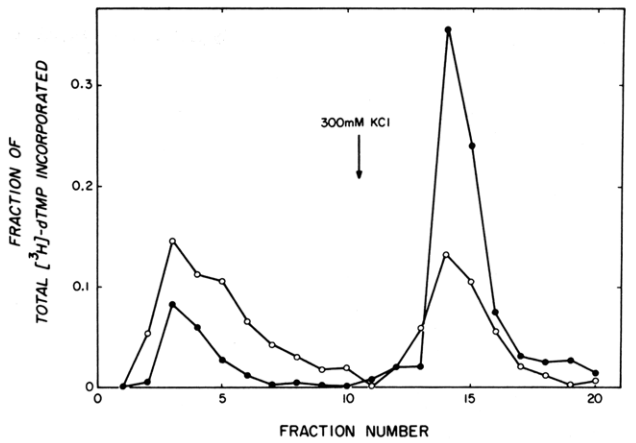

FIGURE 2: Effect of ATP on DNA binding affinity. The affinity of α-A2-polymerase for DNA was measured by DNA–cellulose chromatography. As described under Materials and Methods, the A2 form of α-polymerase was applied to a native DNA–cellulose column, and the column was washed with low (70 mM) ionic strength buffer (fractions 1–10) and high (370 mM) ionic strength buffer (fractions 11–20). Results are presented as a profile of α-polymerase activity vs. elution position in the absence (open circles) or presence (closed circles) of 4 mM ATP.

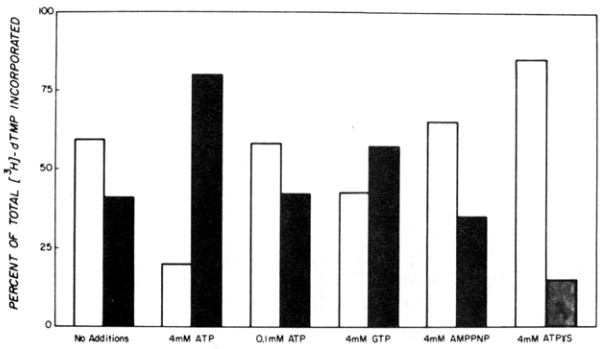

FIGURE 3: Binding to DNA–cellulose under various conditions. The fraction of α-A2-polymerase which binds to DNA–cellulose under several different conditions described under Materials and Methods was determined. Results are presented as a bar graph in which the opened and stippled bars represent the amount of α-polymerase activity recovered in the low (70 mM) and high (370 mM) ionic strength elutions, respectively.

polymerase activity recovered was found in fractions 1–10, i.e., in the low ionic strength wash. The remaining 40% was eluted with the high ionic strength wash (fractions 11–20). In the presence of 4 mM ATP, the amount of α-A2-polymerase activity which binds to the column (recovered by the high ionic strength wash) increased to approximately 80%. Thus, ATP increases the affinity of the A2 form of calf α-polymerase for DNA.

Figure 3 summarizes the DNA binding affinity of α-A2-polymerase under a variety of conditions. The presence of 0.1 mM ATP resulted in a pattern of binding affinity identical with that obtained in the absence of ATP, indicating that millimolar levels of ATP are necessary to observe the increase in DNA binding affinity. We had previously shown that GTP at 4 mM had no effect on the synthetic activity of the A2 form of calf α-polymerase (Wierowski et al., 1983). The affinity of α-A2-polymerase for DNA was slightly increased in the presence of 4 mM GTP. However, the effect was much smaller than that observed with 4 mM ATP.

When present at a concentration of 4 mM, the nonhydrolyzable ATP analogues AMPPNP and ATPγS decreased the affinity of α-A2-polymerase for DNA (Figure 3). Perhaps this lower binding affinity is part of the mechanism by which these compounds inhibit the synthetic activity of calf DNA α-polymerase, as previously observed (Wierowski et al., 1983). The results obtained with the two nonhydrolyzable ATP analogues suggest that even in the absence of DNA synthesis, hydrolysis of ATP may be required to increase the affinity of α-polymerase for DNA. Furthermore, this requirement for hydrolysis of the γ-phosphate implies that the increased DNA binding affinity is not the result of ATP-dependent priming on the DNA–cellulose column by the α-A2-polymerase.

Using thin-layer chromatography, we have shown that α-A2-polymerase is able to hydrolyze [γ-^{32}P]ATP to ADP + [^{32}P]P_i (not shown). Measurements indicate that 1.39 units of α-A2-polymerase were able to hydrolyze approximately 2.5 nmol of ATP per h with the reaction conditions described

under Materials and Methods. Although the hydrolysis does not appear to be dependent upon the presence of either template DNA or deoxynucleoside triphosphate, a stimulation of hydrolysis was observed when DNA was present in the reaction (not shown). Because the α-A2-polymerase is not homogeneous, we cannot conclude that the enzyme itself is responsible for the observed ATP hydrolysis. This issue is currently under investigation with a more purified form of the polymerase.

The affinity of the C form of calf α-polymerase for DNA–cellulose was also increased from 40% bound in the absence of ATP to 80% bound in the presence of 4 mM ATP (data not shown). When the A2 and C forms of calf α-polymerase were eluted from the DNA–cellulose with a KCl gradient, the peak of α-A2-polymerase activity eluted at 25 mM KCl, and α-C-polymerase activity eluted at 60 mM KCl. These results indicate that essentially all polymerase activity was eluted from the DNA–cellulose columns by the high ionic strength buffer.

Effect of Ap_4A on Enzyme Activity. Since the ATP analogue Ap_4A has been shown to bind to calf DNA α-polymerase (Grummt et al., 1979), we were concerned that a possible trace contaminant of Ap_4A in our stock solution of ATP could contribute to the observed stimulation effects. At concentrations expected for a trace contaminant (10 nM–10 μM), which also represents the physiological concentration range of Ap_4A (Rapaport & Zamecnik, 1976), there was no detectable stimulation in the rate of α-A2-polymerase synthesis on the poly(dA)·$(dT)_{10}$ primer-template (data not shown). At much higher than physiological Ap_4A concentrations, 100 μM–4 mM, a modest stimulation was observed. This stimulation reached a maximum of 3.2-fold at 1 mM Ap_4A. Stimulation by Ap_4A decreased to 1.7-fold at 4 mM, the concentration at which maximal ATP stimulation was observed (Wierowski et al., 1983). Under these conditions of stimulation, Ap_4A may be acting as a hydrolyzable analogue of ATP since we have observed that high levels of Ap_4A (1–4 mM) compete with ATP (4 mM) in the stimulation of α-A2-polymerase synthetic rate on poly(dA)·$(dT)_{10}$ (data not shown).

We have previously shown that 4 mM ATP causes an increase in the length of processive synthesis by the A2 form of calf DNA α-polymerase from approximately 15 to 50 nucleotides added (Wierowski et al., 1983). There was no effect on the processivity of the A2 enzyme form by Ap_4A at either 1 μM or 4 mM (data not shown). At 1 mM Ap_4A, the concentration at which maximum synthetic rate stimulation was observed, the processive synthesis by α-A2-polymerase was increased from approximately 15 to 30 nucleotides added (data not shown).

DNA Polymerase Associated Primase Activity. Hübscher (1983) has reported that calf thymus DNA α-polymerase has a tightly associated primase activity. We previously developed a method to distinguish the effects of ATP on the DNA synthesis reaction from its effects as a substrate for DNA primase (Wierowski et al., 1983). This method involves the use of an excess of gapped fd RF DNA substrate. Extension of the 3′ end of the double-stranded DNA adjacent to the gaps by the polymerase results in very long products, with the length derived primarily from the preexisting primers. These long products are detected in the excluded volume after alkaline gel filtration on Bio-Gel A-5m resin. Products synthesized de novo by the primase–polymerase coupled reaction are expected to be shorter and elute in fractions within the included volume of the resin. Using this method, we previously demonstrated that under reaction conditions that gave ATP stimulation (four dNTPs plus 4 mM ATP), α-A2-polymerase showed no detectable primase activity (Wierowski et al., 1983). However, when the four dNTPs are supplemented with rNTPs other than or in addition to ATP, a DNA polymerase associated priming activity is detectable on the gapped fd RF template. These results indicate that α-A2-polymerase can initiate priming de novo in the presence of existing 3′-OH termini.

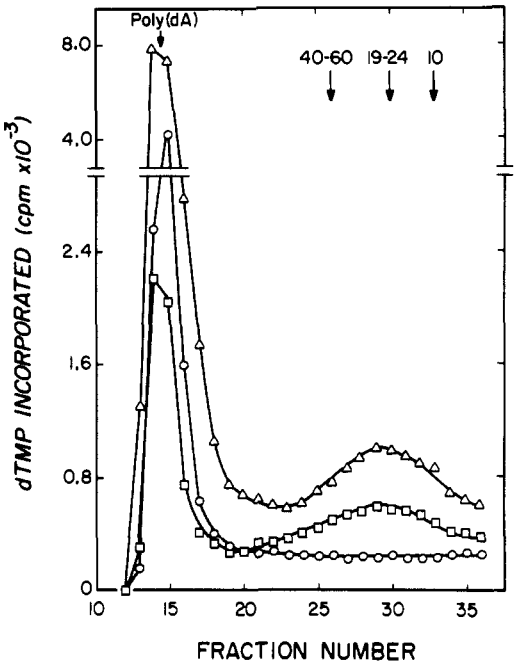

FIGURE 4: Analysis of products synthesized on gapped fd RF DNA. Synthesis by α-A2-polymerase on gapped fd RF DNA was performed as described under Materials and Methods. Results are presented as a profile of radioactivity incorporated vs. position of elution from an alkaline Bio-Gel A-5m column. In the presence of four dNTPs (open circles), 0.66 pmol of dTMP was incorporated (0.21 pmol of dTMP/pmol of 3′-OH termini). In the reaction containing four dNTPs plus GTP, CTP, and UTP (open squares), 0.64 pmol of dTMP was incorporated (0.21 pmol of dTMP/pmol of 3′-OH termini). Finally, in the reaction containing four dNTPs plus GTP, CTP, UTP, and 4 mM ATP (open triangles), 1.65 pmol of dTMP was incorporated (0.54 pmol of dTMP/pmol of 3′-OH termini). The number of picomoles of 3′-OH termini was calculated on the basis of the 3′-OH termini originally present on the gapped fd RF DNA (see Materials and Methods). Arrows indicate the elution positions of (from left to right) poly(dA), $(dT)_{40-60}$, $(dT)_{19-24}$, and $(dT)_{10}$.

Figure 4 illustrates the size distribution of products synthesized on gapped fd RF DNA under various conditions. As discussed above, elongation of existing primers in the presence of four dNTPs results in products that are excluded from the gel filtration media. When synthesis was allowed to take place in the presence of four dNTPs and three rNTPs (GTP, CTP, and UTP), short products were also observed. The length of these products is consistent with that expected for primers generated de novo (10–20 nucleotides) [from Conaway & Lehman (1982b) using *Drosophila melanogaster* α-polymerase] plus one length of processive DNA synthesis by α-A2-polymerase (10–20 nucleotides) (Hockensmith & Bambara, 1981). When 4 mM ATP was included in the reaction, a stimulation was observed in the synthesis of excluded products resulting from a direct effect of ATP on the DNA synthesis reaction. A stimulation was also observed in the synthesis of short, included products. This stimulation presumably results from a combination of the effects of ATP on the priming reaction and its effects on DNA synthesis.

We have previously shown that 4 mM ATP increases the processivity of α-A2-polymerase on the synthetic primer-

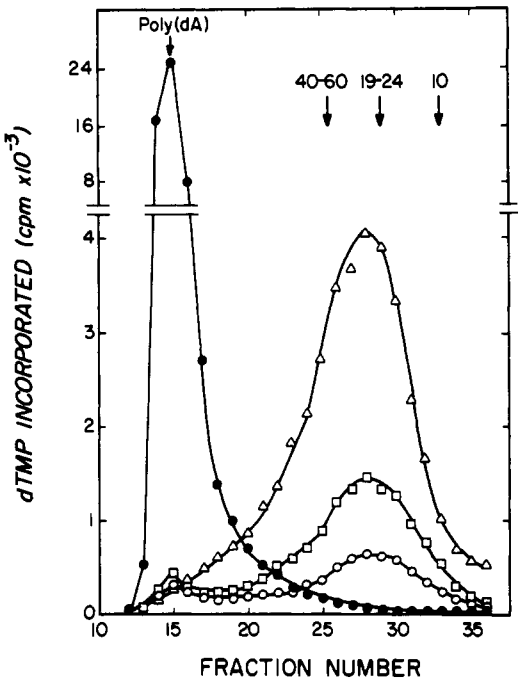

FIGURE 5: Analysis of products synthesized on fd ss DNA. Synthesis by α-A2-polymerase on fd ss DNA was performed as described under Materials and Methods. Results are presented as in Figure 4. In the presence of four dNTPs (open circles), 1.16 pmol of dTMP was incorporated. In the reaction containing four dNTPs plus GTP, CTP, and UTP (open squares), 2.37 pmol of dTMP was incorporated. In the reaction containing four dNTPs plus GTP, CTP, UTP, and 4 mM ATP (open triangles), 5.95 pmol of dTMP was incorporated. Finally, in the DNA polymerase I reaction (closed circles), 280 pmol of dTMP was incorporated.

template poly(dA)·$(dT)_{10}$ (Wierowski et al., 1983). Using the gapped fd RF template, we can now determine whether ATP affects the size distribution of the short products synthesized by the primase–polymerase coupled reaction. In the presence of 4 mM ATP, there was no apparent shift in the size profile of the short products (Figure 4, compare open squares and open triangles in the product size range 0–60 nucleotides). These results suggest that 4 mM ATP does not increase the length of processive synthesis by α-A2-polymerase during the primase–polymerase coupled reaction nor does it cause the polymerase to interact more than once with each RNA primer since several additions of the normal processive number of nucleotides would significantly lengthen the products.

This phenomenon was further investigated by examining the length of products synthesized by α-A2-polymerase on the natural template fd ss DNA. On this template, and with the reaction conditions used, only products resulting from a primase–polymerase coupled reaction should be observed. Figure 5 shows that in the presence of four dNTPs and three rNTPs (GTP, CTP, and UTP), the size distribution of products synthesized was similar to that of the small products obtained on the gapped fd RF template (Figure 4). The predominant effect of including 4 mM ATP in the reaction was a stimulation in synthesis with no significant increase in the average length of the products synthesized (Figure 5). This result further implies that the effect of ATP is primarily on initiation of DNA synthesis.

In order to test the possibility that some structural constraint on the fd ss DNA prevented the synthesis of long products, we used DNA polymerase I to further elongate the products synthesized on the template by α-A2-polymerase. The results obtained indicate that the fd ss DNA used was capable of supporting the synthesis of products several hundred nucleotides long (Figure 5). In all probability, the synthesis of relatively short products on the fd ss DNA by α-A2-polymerase is a characteristic of the coupled primase–polymerase reaction under the conditions used.

Table I: Determination of the Preferred rNTP for Priming Activity[a]

rNTPs	pmol of dTMP incorporated	rNTPs	pmol of dTMP incorporated
ATP	4.76	ATP, GTP	8.30
GTP	2.26	CTP, UTP	1.68
CTP	0.77	GTP, CTP, UTP	6.95
UTP	0.41	ATP, GTP, CTP, UTP	15.15

[a] The total number of picomoles of dTMP incorporated by α-A2-polymerase on the fd ss DNA template in the presence of each of the rNTPs listed was determined as described under Materials and Methods. A background of residual dTMP incorporation (1.16 pmol) that occurs in the absence of rNTPs was subtracted from the values shown.

Natural DNA templates differ significantly from homopolymer templates in their ability to assume secondary structures (hairpins). Apparently, α-A2-polymerase synthesized only short products on fd ss DNA because it was unable to traverse these secondary structures, irrespective of the presence of ATP. Hübscher et al. (1982) have shown that pausing sites (Kaguni & Clayton, 1982) are more easily overcome by a calf thymus DNA α-polymerase "holoenzyme" complex when the homologous ss DNA-binding protein (UP1) is included in the reaction. We find that UP1, in the presence or absence of ATP, has no effect on the average length of the products synthesized on fd ss DNA by α-A2-polymerase (data not shown). This suggests that UP1 was not successful in allowing the polymerase to synthesize through secondary structure. The implication is that an additional protein factor(s) may be required by the α-A2-polymerase for an increase in the length of products synthesized on a natural DNA template. Furthermore, in contrast to the findings of Herrick et al. (1976), who observed stimulation of partially purified calf DNA α-polymerase by UP1 on exonuclease-gapped bacteriophage λ DNA, we did not observe such stimulation with α-A2-polymerase on the fd ss template (data not shown).

Further Characterization of the Priming Reaction. Experiments were carried out to determine the rNTP requirements for priming by the A2 form of calf DNA α-polymerase on the natural template fd ss DNA. Of the four individual rNTPs, only addition of ATP or GTP resulted in significant levels of primase activity (Table I). These results are in agreement with the findings of Conaway & Lehman (1982a), who, while investigating the DNA α-polymerase from *Drosophila melanogaster*, identified an activity that catalyzed the purine rNTP dependent synthesis of DNA with a ss M13 template.

Although GTP is a substrate for the priming reaction, ATP appears to be the preferred rNTP for priming by α-A2-polymerase on the fd ss template (Table I). ATP clearly is much more effective in combination with the other rNTPs but has significant priming activity alone. This result can be contrasted with a previous result in which α-A2-polymerase did not demonstrate primase activity on gapped fd RF DNA when only ATP was present (see above). It is likely that the coupled primase–polymerase activities are in a balance that can be shifted depending upon the reaction conditions. We have shown that ATP increases the affinity of the α-polymerase for DNA primer 3′-OH termini and its rate of binding these termini. In a reaction where ATP is the sole rNTP, and a significant number of DNA 3′-OH termini are present, the role of ATP in improving binding to these termini may predominate over its role in priming. Only when rNTPs other

than or in addition to ATP are present would the coupled primase–polymerase reaction be evident. The apparent major role that ATP serves in the priming reaction further emphasizes the need to distinguish its effects on priming vs. those on DNA synthesis.

In summary, results show that the reason for the increase in the rate of DNA synthesis by α-polymerase on the poly(dA)·$(dT)_{10}$ primer-template is that initiation of synthesis at 3′-OH termini is promoted. Binding to DNA in the absence of synthesis is also improved specifically by ATP in a reaction that appears to require ATP hydrolysis. ATP is also a primary substrate of the primase that is associated with the calf α-polymerase. The length of processive synthesis by the primase–polymerase coupled reaction on natural DNA templates is not increased by ATP. This suggests that natural templates may require an additional DNA replication factor(s) for primase–polymerase coupled synthesis to be highly processive.

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Registry No. AMPPNP, 25612-73-1; ATP, 56-65-5; ATPγS, 35094-46-3; Ap_4A, 5542-28-9; GTP, 86-01-1; dTTP, 365-08-2; poly(dA)·$(dT)_{10}$, 60593-73-9; DNA primase, 64885-96-7; DNA α-polymerase, 9012-90-2.

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