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Kinetics of Incorporation of O^6-Methyldeoxyguanosine Monophosphate during in Vitro DNA Synthesis†

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ABSTRACT: O^6-Methyldeoxyguanosine triphosphate (m^6dGTP), known to be produced in vivo by methylation of deoxyguanosine triphosphate with simple methylating mutagens, is utilized by prokaryotic DNA polymerases during in vitro replication of synthetic and natural DNA template-primers. A study of the kinetic behavior of m^6dGTP during DNA replication in vitro and of its effect on DNA replication indicates that m^6dGTP acts as an analogue of dATP with K_m^{app} of about 6 μM for *Escherichia coli* DNA polymerase I (Klenow fragment) compared to the K_m^{app} of about 0.8 μM for dATP. m^6dGTP is not incorporated in the complete absence of dATP (a competitive inhibitor). m^6dGTP also inhibits in vitro DNA synthesis. Different DNA polymerases behave differently in utilization and turnover of m^6dGTP. T4 DNA polymerase shows stronger discrimination against m^6dGMP incorporation than either T5 DNA polymerase or *E. coli* DNA polymerase I. The possibility that m^6dGTP is unlikely to contribute significantly to in vivo mutation is discussed.

O^6-Methylguanine is an important promutagenic lesion produced by direct methylation of DNA by simple methylating carcinogens (Loveless, 1969; Pegg, 1977; Lawley, 1979; Singer, 1979). Following the proposal of Loveless (1969) that O^6-alkylguanine is promutagenic, indirect in vitro and in vivo experiments (Abbott & Saffhill, 1979; Lawley & Martin, 1975; Coulondre & Miller, 1977) supported the idea that the pairing of the alkylated base with thymine during DNA replication causes mutation. More recently, Nagata et al. (1982) and Klopman & Ray (1982) proposed, on theoretical grounds, that the m^6dG·dT[1] pair is similar to the dA·dT pair in terms of energy of formation, bond angles, and bond distances. Our previous studies (Snow et al., 1984) indicate that m^6dG in the DNA template has a strong preference for dT during in vitro DNA synthesis, but at the same time, the m^6dG·dT pair is not recognized as a normal substitute for dA·dT by prokaryotic DNA polymerases. In the present studies, we have investigated the recognition of the m^6dG·dT pair by DNA polymerases during utilization of m^6dGTP as a substrate in DNA synthesis. This is important because it has been shown recently that m^6dGTP can also be produced in vivo by direct alkylation of

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[1] Abbreviations: m^6dGTP, O^6-methyldeoxyguanosine triphosphate; m^6dG, O^6-methyldeoxyguanosine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; HPLC, high-performance liquid chromatography.

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dGTP in the DNA precursor pool, and it was proposed that m^{6}dGTP can be promutagenic, acting as a substrate for DNA synthesis (Topal & Baker, 1982). Other base analogues such as 2-aminopurine and bromodeoxyuridine have previously been shown to be mutagenic when in the template during DNA replication (Hopkins & Goodman, 1980; Kaufman & Davidson, 1978) or after incorporation from the precursor pool (Watanabe & Goodman, 1981; Kaufman & Davidson, 1978). Recently, Dodson et al. (1982) have demonstrated that m^{6}dGTP present in high concentration in the precursor pool during T7 DNA replication can lead to increased mutagenesis.

The results in this paper show that while m^{6}dGTP is utilized as an analogue of dATP during in vitro DNA synthesis, different DNA polymerases behave differently in accepting the alkylated dNTP as a substrate.

Experimental Procedures

Materials

Unlabeled m^{6}dGTP (Foote et al., 1980) and [8-^{3}H]m^{6}dGTP (5.8 Ci/mmol) (Foote et al., 1983) were prepared as previously described. After further purification on a Waters μBondapak C-18 column (Dodson et al., 1982) the fractions containing [^{3}H]m^{6}dGTP in 0.125 M ammonium phosphate (pH 7.5) were pooled and stored in liquid nitrogen until required. [α-^{32}P]-dNTPs and [^{3}H]dNTPs were obtained from New England Nuclear. Poly(dA-dT) was synthesized in a primed reaction with *Escherichia coli* DNA polymerase I (Radding et al., 1962). Activated calf thymus DNA (Mechali et al., 1980) was a gift of C. C. Morse of this laboratory. T5 phage DNA polymerase was purified according to Fujimura & Roop (1976). T4 phage DNA polymerase, prepared according to Morris et al. (1979), was a gift of C. R. Wobbe of this laboratory. The Klenow fragment of *E. coli* DNA polymerase I (Klenow & Hennigsen, 1970) was purchased from Boehringer-Mannheim. *E. coli* DNA polymerase I was a gift from L. A. Loeb (University of Washington, Seattle). All other materials were of reagent grade.

Methods

DNA Replication Using m^{6}dGTP as a Substrate. Incorporation of labeled precursors was measured by acid precipitation as described elsewhere (Snow et al., 1984). Poly(dA-dT), poly(dA)–oligo(dT), and activated calf thymus DNA were replicated with various DNA polymerases at 30 °C in a 50-μL reaction mixture containing 67 mM Tris-HCl (pH 8.6), 6.7 mM $MgCl_2$, 10 mM dithiothreitol, 25 μg/mL bovine serum albumin, and 50 μM each of the required dNTPs (unless otherwise indicated). The specific activities of labeled dNTPs used in DNA synthesis were 50–800 cpm/pmol for m^{6}dGTP and 20–400 cpm/pmol for normal dNTPs. Replication with *E. coli* DNA polymerase I or the Klenow fragment of DNA polymerase I was carried out in 67 mM potassium phosphate (pH 7.4) in place of Tris-HCl, and albumin and dithiothreitol were omitted.

Assay of Deoxynucleotide Turnover. Utilization and turnover were determined as previously described (Snow et al., 1984). In some experiments, the replicated DNA along with carrier DNA was precipitated with 3.5% perchloric acid for 10 min at 0 °C. An aliquot of the supernatant was removed after low-speed centrifugation and neutralized by shaking with an equal volume of 0.4 M Alamine 336 (tricaprylylamine) in Freon-TF (Du Pont) (Khym, 1975). The upper aqueous layer was removed and brought to pH 7.0 by dropwise addition of 1 M KOH. The amount of labeled dNMP and dNTP in the sample was measured by liquid scintillation spectrometry of the fractions recovered after separation of the nucleotides by anion-exchange chromatography on an Aminex A-28 column (Khym, 1975).

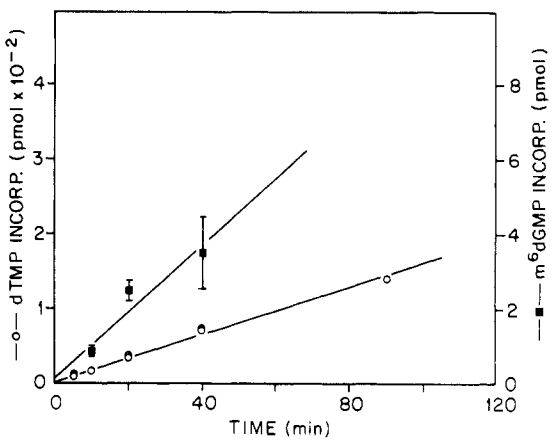

FIGURE 1: Kinetics of incorporation of dTMP and m^{6}dGMP. Poly(dA-dT) (0.8 nmol) (expressed as nucleotide equivalent) was replicated by the Klenow fragment of *E. coli* DNA polymerase I (0.03 μg) in the presence of 60 μM dTTP and 10 μM each of m^{6}dGTP and dATP. Error bars indicate the standard deviation for three or more independent determinations.

Nearest-Neighbor Analysis of Incorporated m^{6}dGMP. Poly(dA-dT) was replicated with the Klenow fragment of DNA polymerase I and [α-^{32}P]dTTP (60 μM), [^{3}H]m^{6}dGTP (60 μM), and dATP (10 μM). After 60 min at 30 °C, the reaction was stopped with 10 mM EDTA and 15 μg of calf thymus DNA. After phenol extraction the DNA was desalted on a Sephadex G-50 column, precipitated with ethanol, and dried under vacuum. The DNA was digested to 3′-dNMPs with micrococcal nuclease and spleen phosphodiesterase as described elsewhere (Snow et al., 1984) and separated by HPLC on a Waters μBondapak C-18 column (Wang et al., 1981). Fractions were collected and counted for ^{3}H and ^{32}P in Amersham ACS scintillation solvent.

Results

Incorporation of m^{6}dGMP into DNA by E. coli DNA Polymerase I. Replication of the alternating copolymer poly(dA-dT) with *E. coli* DNA polymerase I (Klenow fragment) in 10 μM [8-^{3}H]m^{6}dGTP, 60 μM dTTP, and 10 μM dATP resulted in increasing incorporation of m^{6}dGMP for at least 40 min (Figure 1). Under these conditions, the rate of m^{6}dGMP incorporation was about 6% of the rate of dTMP incorporation. As expected, dAMP and dTMP were incorporated in equimolar amounts in the absence of m^{6}dGTP (data not shown). Increasing the concentration of m^{6}dGTP (up to 250 μM) increased m^{6}dGMP incorporation, although the overall DNA synthesis, as measured by dTMP incorporation, was significantly decreased (Figure 2).

We analyzed the kinetics of m^{6}dGMP incorporation by the Lineweaver–Burk procedure. As shown in Figure 3, dATP is a competitive inhibitor of m^{6}dGMP incorporation during replication of poly(dA-dT). The inhibition constant (K_i^{app}) for dATP was 0.4 μM. The incorporation of m^{6}dGMP in the complete absence of dATP was too low to be measured, but the approximate K_m^{app} of m^{6}dGTP, determined by extrapolation, was 6 μM. The K_m^{app} of dATP in the absence of m^{6}dGTP was 0.8 μM. m^{6}dGTP inhibited dAMP incorporation into poly(dA-dT) in a somewhat nonlinear fashion (Figure 4). Low concentrations of m^{6}dGTP were not inhibitory. It appears possible that, at low concentrations of both m^{6}dGTP and dATP, the inhibitory effect of m^{6}dGTP is compensated by a stimulation of replication as a result of an increase in the total purine deoxynucleotide pool. Similar nonlinear inhibition of

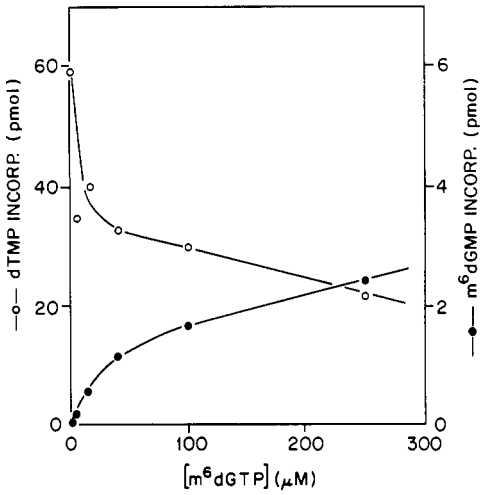

FIGURE 2: Incorporation of dTMP and m^{6}dGMP as a function of m^{6}dGTP concentration. The details of the enzyme and template are described in Figure 1. Incorporation of dTMP (○) and m^{6}dGMP (●) was measured during replication of poly(dA-dT) at 30 °C for 20 min by the Klenow fragment of *E. coli* DNA polymerase I in 60 μM dTTP, 10 μM dATP, and m^{6}dGTP as noted.

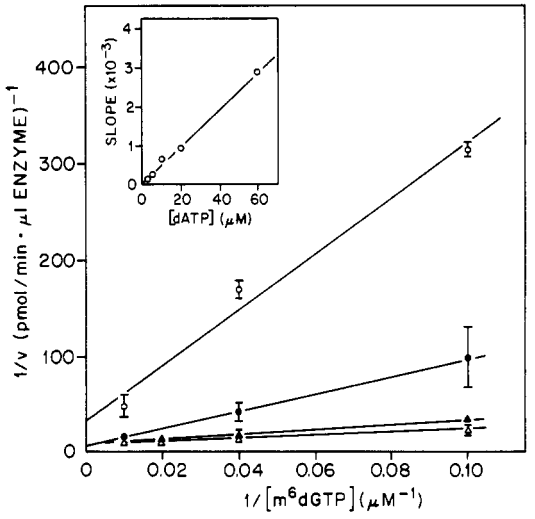

FIGURE 3: Lineweaver–Burk plot of m^{6}dGMP incorporation during replication of poly(dA-dT). Details of enzyme and template are as in Figure 1. The incorporation of m^{6}dGMP was measured in the presence of 2 (△), 5 (▲), 20 (●), and 60 (○) μM dATP for 20 min at 30 °C. The insert shows a replot of the slope vs. dATP concentration.

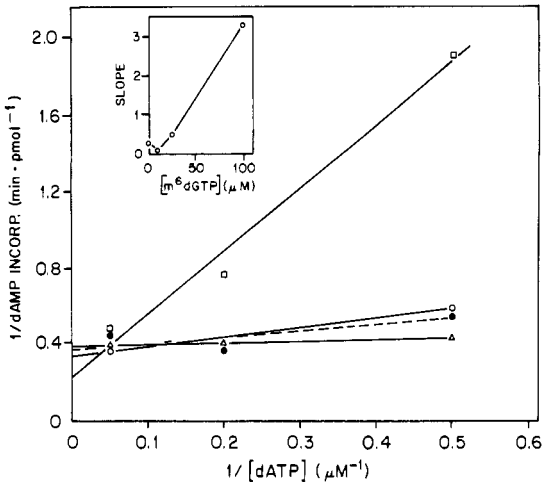

FIGURE 4: Lineweaver–Burk plot of dAMP incorporation during replication of poly(dA-dT). The incorporation of dAMP was measured in the presence of 0 (●), 10 (△), 25 (○), or 100 (□) μM m^{6}dGTP under the same conditions as in Figure 3. The insert shows a replot of the slope vs. m^{6}dGTP concentration.

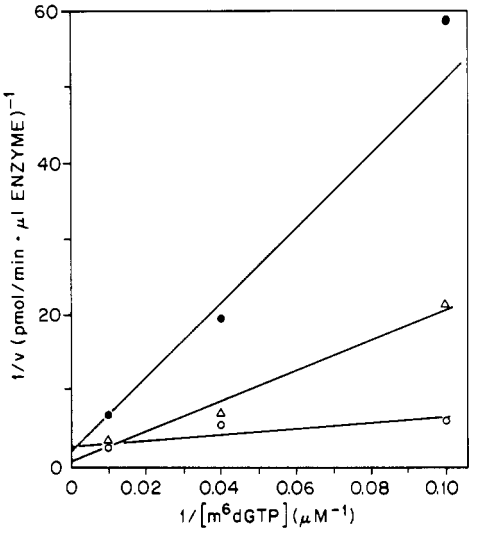

FIGURE 5: Effect of dTTP on m^{6}dGMP incorporation. Poly(dA-dT) (0.8 nmol) was replicated for 30 min at 30 °C with *E. coli* DNA polymerase I (0.09 μg). Incorporation of m^{6}dGMP was measured in the presence of 5 μM dATP, 2 (●), 5 (△), or 20 (○) μM dTTP, and m^{6}dGTP as given.

dAMP incorporation by m^{6}dGTP was also seen during replication of poly(dA-dT) by T5 DNA polymerase (data not shown). If the slope of dAMP incorporation is determined for concentrations of dATP above 10 μM, a least-squares fit of a plot of the slope vs. m^{6}dGTP concentration gives a K_i^{app} of about 6 μM for m^{6}dGTP. The fact that the K_i^{app} of m^{6}dGTP is comparable to its K_m^{app} is indicative of a direct competition between dATP and m^{6}dGTP during DNA synthesis.

The likelihood that m^{6}dGTP substitutes for dATP during replication of poly(dA-dT) was confirmed by nearest-neighbor analysis of the product DNA. [8-^{3}H]m^{6}dGMP was incorporated during replication of poly(dA-dT), in the presence of 10 μM dATP and 60 μM [α-^{32}P]dTTP. Following digestion of the replicated DNA to 3′-deoxynucleotides, 92% of the tritium label in m^{6}dGMP was associated with ^{32}P, indicating that the modified base had been incorporated adjacent to the thymine, thus behaving as an analogue of adenine.

The extent of inhibition of DNA synthesis by m^{6}dGTP as measured by dTMP incorporation with poly(dA-dT) template, in the presence of less than 25 μM dATP, was variable and depended on the enzyme preparation and reaction conditions. For example, the inhibition of dTMP incorporation in the presence of 5 μM dATP showed uncompetitive kinetics for the Klenow fragment of DNA polymerase I but competitive kinetics for DNA polymerase I. The kinetics of inhibition were complex for T4 and T5 DNA polymerases as well (data not shown). In general, however, increasing dTTP concentration caused an increase in m^{6}dGMP incorporation at low dATP concentrations (Figure 5). Qualitatively similar results were obtained for incorporation of m^{6}dGMP and inhibition by m^{6}dGTP with calf thymus DNA template-primer (data not shown).

Utilization and Turnover of m^{6}dGTP by Different DNA Polymerases. The above results show that *E. coli* DNA polymerase I discriminated against m^{6}dGTP as a substrate. It was, therefore, of interest to see if other DNA polymerases behaved in the same way as the *E. coli* enzyme in accepting m^{6}dGTP as a substrate substituting for dATP. We have, thus, measured the relative efficiency of *E. coli* DNA polymerase I and T4 and T5 phage DNA polymerases in the utilization

Table I: Incorporation of dAMP and m^{6}dGMP (Picomoles) with Poly(dT) as Template[a]

	DNA polymerase		
	T5	T4	Pol I
(a) dAMP	859	744	1924
(b) m^{6}dGMP	2.9	0.02	1.7

[a] Poly(dT) (1.1 μg) with an oligo(dA) hairpin primer was replicated for 60 min at 30 °C with either (a) 60 μM [^{3}H]dATP (specific activity 200 cpm/pmol) and no m^{6}dGTP or (b) 2 μM dATP and 60 μM [^{3}H]m^{6}dGTP (specific activity 800 cpm/pmol).

and turnover of m^{6}dGTP. Table I shows that T4 DNA polymerase behaved significantly differently from the other two polymerases in the incorporation of m^{6}dGMP with the synthetic DNA template. The relative incorporation of m^{6}dGMP was also measured with calf thymus DNA template-primer. Under comparable reaction conditions (50 μM each of [α-^{32}P]dTTP, dCTP, and dGTP, along with 10 μM dATP and 25 μM [^{3}H]m^{6}dGTP), DNA polymerase I and T5 DNA polymerase utilized m^{6}dGMP much more efficiently (0.4% relative to dTMP incorporation) than T4 polymerase (0.05% relative to dTMP incorporation).

Because the utilization of dNTPs during DNA synthesis by prokaryotic DNA polymerases depends on the relative polymerase and 3′-exonuclease activities of these enzymes (Kornberg, 1980), we determined the relative turnover of m^{6}dGTP by the three DNA polymerases with both synthetic and natural DNA templates. In all cases, synthesis was low compared to the amount of template added and less than 25% of the input dNTPs were utilized. As shown in Figure 6, incorporation of m^{6}dGMP was very low compared to dAMP incorporation and turnover of m^{6}dGMP was higher than dAMP. Turnover of both nucleotides is higher with poly(dA-dT) than with calf thymus DNA, which may be due to the weaker hydrogen-bond strength of dA·dT compared to the average of all the pairs in natural DNA (Patten, 1982). The relative turnover of m^{6}dGMP compared to that of dAMP was greater for T4 DNA polymerase than the other polymerases assayed, indicating greater discrimination against m^{6}dGMP by T4 DNA polymerase, as was demonstrated earlier.

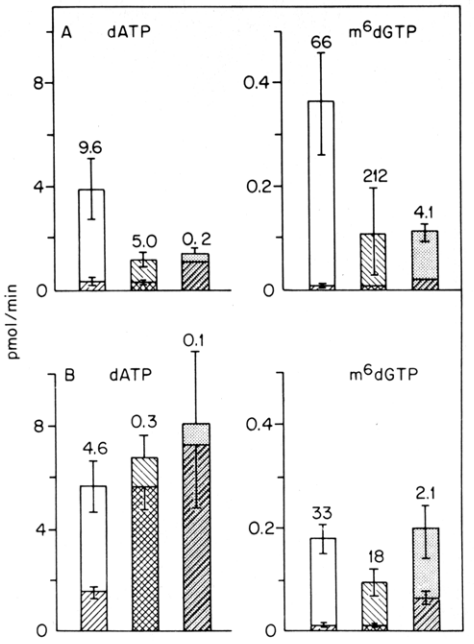

FIGURE 6: Utilization and turnover of dNTPs. Poly(dA-dT) (0.8 nmol) (A) and calf thymus DNA (70 nmol) (B) were replicated for 30 and 90 min, respectively, with 0.03 μg of T5 DNA polymerase (left, nonhatched upper portion), 0.3 unit of T4 DNA polymerase (center, hatched upper portion), and 0.09 μg of *E. coli* DNA polymerase I (right, stippled upper portion). Varying concentrations of dNTPs were used (10–50 μM [α-^{32}P]dATP, 100 μM each of dTTP, dCTP, and dGTP as required, and less than 50 μM [8-^{3}H]m^{6}dGTP) under conditions as described under Experimental Procedures. Total utilization (total height of bar) of dATP and m^{6}dGTP was determined from the sum of the acid-insoluble incorporation (lower portion) plus the released dNMP (upper portion). Turnover ratios (dNMP released/dNMP incorporated) for each polymerase are given above the bars. For low incorporations, the bars are not drawn to scale.

Discussion

While it is generally believed that O^6-methylation of guanine in DNA is a major mutagenic event, Topal & Baker (1982) showed recently that, on a molar basis, O^6-methylation of dGTP in the nucleotide precursor pool in vivo is greater than the corresponding alkylation of guanine in DNA; m^{6}dGTP could therefore be a mutagenic precursor, acting as a substrate for DNA polymerase in DNA synthesis. The result of m^{6}dGMP incorporation opposite dT in the DNA template and subsequent dealkylation of the alkylated base (Foote et al., 1980; Olsson & Lindahl, 1980) would lead to a dA·dT → dG·dC transition (Dodson et al., 1982). In these studies, we have investigated this possibility with several purified prokaryotic DNA polymerases during in vitro DNA synthesis. In view of the recent theoretical prediction that the m^{6}dG·dT pair is quite similar to dA·dT in terms of H bonding, bond angle, and bond distances (Nagata et al., 1982; Klopman & Ray, 1982), we also investigated whether the DNA polymerases can utilize m^{6}dGTP as well as they do dATP.

Our studies clearly indicate that while m^{6}dGTP does act as an analogue of dATP, and while these deoxynucleotides are competitive inhibitors of each other for the DNA polymerases, m^{6}dGTP also acts as an inhibitor for the DNA polymerases. Because m^{6}dGTP cannot completely replace dATP for DNA synthesis in vitro, we conclude that m^{6}dG·dT is not accepted as a normal pair by the DNA polymerases. While this paper was in preparation, Hall & Saffhill (1983) also showed that m^{6}dGTP is a substrate for *E. coli* DNA polymerase I and DNA polymerase α and that it also inhibits DNA polymerase I.

The fidelity of in vitro DNA replication by prokaryotic DNA polymerases depends on their relative polymerase and 3′-exonuclease (editing) activities as well as on other factors such as dNTP concentration, type of template-primer, and concentration of cofactors like Mg^{2+} (Kornberg, 1980; Fujimura & Das, 1980). Clayton et al. (1979) have shown that, with T4 DNA polymerase, the fidelity of DNA synthesis improves when the rate of DNA synthesis is low and the 3′-exonuclease activity of the polymerase is high in the presence of low concentrations of dNTPs. Our observation that the amount of m^{6}dGMP incorporation during replication of a poly(dA-dT) template with DNA polymerase I (Klenow fragment) depended not only on the concentration of dATP but also on that of dTTP is consistent with this observation. This result supports the notion that the polymerase recognizes m^{6}dG opposite dT in the template as an error in DNA replication.

Our results on the relative turnover of m^{6}dGTP and the normal dNTPs (Figure 6) show the importance of the proofreading 3′-exonuclease activity of the DNA polymerases. The exonuclease activity determining the turnover of deoxynucleotides depends on the template-primer, and the higher turnover observed with poly(dA-dT) than with calf thymus DNA may reflect the weaker bond strength of the dA·dT pair (Patten, 1982). Wild-type T4 DNA polymerase has 10–100-fold more 3′-exonuclease than the *E. coli* DNA polym-

erase I (Kornberg, 1980). Therefore, it is expected that the T4 enzyme caused a much higher turnover of both dATP and m^6dGTP than the *E. coli* polymerase. The higher relative turnover of m^6dGTP than that of dATP and the inhibition of DNA synthesis by m^6dGTP suggest that m^6dGMP incorporation causes premature chain termination or inhibition of the rate of chain elongation. Although T5 DNA polymerase had a somewhat higher turnover than the T4 enzyme, it is surprising that the turnover of m^6dGTP was 6–20 times that of dATP for T5 DNA polymerase and *E. coli* DNA polymerase I and 40–60 times for the T4 enzyme. Thus, it appears that the T4 enzyme is more discriminatory in accepting the m^6dG·dT pair at the growing fork than either the T5 or the *E. coli* enzyme. This is supported by the observation that, with poly(dT) template and a low concentration of dATP, only the T4 DNA polymerase allowed no measurable incorporation of m^6dGMP (Table I). Furthermore, with calf thymus DNA template and 50 μM each of dATP and m^6dGTP, the ratio of dAMP and m^6dGMP incorporation was about 100 for T5 DNA polymerase and 600–700 for the T4 enzyme (data not shown). Because it is known that relative 3′-exonuclease activity and the fidelity of DNA synthesis are significantly different in mutator and antimutator T4 DNA polymerases (Clayton et al., 1979; Muzyczka et al., 1972; Nossal & Hershfield, 1973), it will be interesting to compare the relative turnover of m^6dGTP for these mutant enzymes.

We can calculate (Segel, 1975) the maximum incorporation of m^6dGMP relative to dAMP on the basis of the kinetic constants of *E. coli* DNA polymerase I for m^6dGTP and dATP. Assuming an intercellular concentration of m^6dGTP equal to 10^{-3} times that of dATP (50 μM) (Topal & Baker, 1982), the level of m^6dG incorporation into newly synthesized DNA in vivo could be as high as that produced in template DNA by direct methylation of dG (Topal & Baker, 1982; Smith et al., 1980; Newbold et al., 1980). However, the true rate of incorporation of m^6dGMP during in vivo DNA replication in *E. coli* must be significantly less, since dA·dT → dG·dC transitions, as predicted by incorporation of m^6dGMP into DNA opposite dT and its subsequent dealkylation, are relatively rare in *E. coli* treated with simple alkylating mutagens (Coulondre & Miller, 1977). The lack of significant m^6dGMP incorporation in vivo may also be due to degradation of the methylated deoxynucleoside (Pegg & Swann, 1979; Stankowski, et al., 1983; Ball et al., 1983), "channeling" of the deoxynucleotide precursors during replication (Reddy & Mathews, 1978; Wovcha et al., 1976), and the apparent inability of *E. coli* kinases to phosphorylate m^6dGTP precursors (R. S. Foote and S. Mitra, unpublished results). The only successful experiment, so far, to show that m^6dGTP is a mutagenic precursor (Dodson et al., 1982), in an assay much more sensitive than the measurement of incorporation of deoxynucleotides, required concentrations of m^6dGTP about 1000 times that calculated by Topal & Baker (1982) to be produced in vivo by methylating agents.

Acknowledgments

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Registry No. dATP, 56-65-5; dAMP, 653-63-4; dTMP, 365-07-1; dTTP, 365-08-2; m^6dGTP, 63642-16-0; DNA polymerase, 9012-90-2.

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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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