Selective Inhibition of the 3′ to 5′ Exonuclease Activity Associated with DNA Polymerases: A Mechanism of Mutagenesis†

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ABSTRACT: The 3′ to 5′ exonuclease activity associated with both mammalian and bacterial DNA polymerases can be selectively inhibited by nucleoside 5′-monophosphates including 6-mercaptopurine ribonucleoside 5′-monophosphate, whereas the polymerase activity is not affected. Nucleosides, 3′ nucleotides, and cyclic nucleotides have no effect on exonuclease activity. With poly(dA-dT) as a template/primer both *Escherichia coli* DNA polymerase I and mammalian DNA polymerase δ catalyze the template-dependent conversion of deoxyribonucleoside triphosphate to monophosphate with the noncomplementary nucleotides dGTP and dCTP. This represents the incorporation of the noncomplementary nucleotide at the primer terminus by the polymerase activity and its subsequent hydrolysis by the proofreading exonuclease activity. Selective inhibition of the exonuclease activity results in a decrease in the amount of free dGMP or dCMP generated and an increase in the amount of dGMP or dCMP incorporated into poly(dA-dT), and, thus, to a higher error frequency. 6-Mercaptopurine is a known mutagen in *E. coli* and its derivative, Azathioprine, is a mutagen in *Salmonella typhimurium* and a carcinogen in man. Azathioprine is readily converted to 6-mercaptopurine in vivo. It is proposed that the mutagenicity of 6-mercaptopurine and Azathioprine in microorganisms and their carcinogenicity in man are due to the selective inhibition of the proofreading exonuclease associated with DNA polymerases by 6-mercaptopurine ribonucleoside 5′-monophosphate, the active metabolite of 6-mercaptopurine. This would result in a higher frequency of mispaired bases in DNA and consequent mutation.

The observed rate of spontaneous mutation in microorganisms (10^{-6} to 10^{-8}) suggests replication fidelity of a very high order. In vitro the error rate with *Escherichia coli* DNA polymerase I (Springgate and Loeb, 1973; Trautner et al., 1962) and bacteriophage T_4 DNA polymerase (Hall and Lehman, 1968) is one in 10^5 to 10^6 base pairs. However, the ΔG of discrimination between the correct and incorrect Watson-Crick base pairs is not sufficient to provide for a fidelity of replication of more than one error in 10^2 to 10^3 base pairs (Mildvan, 1974). The augmented ability of *E. coli* and T_4 DNA polymerases to copy DNA templates in vivo or in vitro with such high fidelity has been ascribed in part to the associated 3′ to 5′ exonuclease activity of these DNA polymerases (Brutlag and Kornberg, 1972). The 3′ to 5′ exonuclease activity has a proofreading function as it excises a mismatched nucleotide incorporated at the primer terminus prior to further chain extension, thereby helping to maintain replication fidelity. The importance of the exonuclease activity in maintaining fidelity of replication has been emphasized in studies with bacteriophage T_4. Mutations in the structural gene for T_4 DNA polymerase were found to result in either an increased rate (mutator) (Speyer, 1965) or a decreased rate (antimutator) (Drake et al., 1969) of spontaneous mutation. Examination of the ratios of exonuclease to polymerase activities of the partially purified DNA polymerases from these mutants has shown that mutator strains have a lower ratio of nuclease to polymerase activity than the wild type, whereas antimutator strains have a higher ratio (Muzcyzka et al., 1972).

Fidelity of DNA replication is also rigidly maintained in eukaryotes; however, 3′ to 5′ exonuclease activity is generally reported not to be associated with mammalian DNA polymerases (Bollum, 1975; Chang, 1973; Chang and Bollum, 1973; Loeb, 1974; Sedwick et al., 1975; Wang et al., 1974). We have recently purified a new species of mammalian DNA polymerase from erythroid hyperplastic bone marrow. This DNA polymerase (δ) in contrast to other mammalian DNA polymerases (α,β,γ) is associated with a 3′ to 5′ exonuclease activity. The data suggesting that the 3′ to 5′ exonuclease activity is an integral part of DNA polymerase δ are: (1) the DNA polymerase activity is not separable from the exonuclease activity by various chromatographic procedures; (2) the two activities are not separable by sedimentation in sucrose density gradients either at low or high ionic strength, which gives two molecular weight species of DNA polymerase activity; (3) both activities have identical rates of heat inactivation; and (4) both activities are coordinately inhibited by hemin and rifamycin AF/013 (Byrnes et al., 1976).

Inhibition of the 3′ to 5′ exonuclease activity associated with DNA polymerase while the DNA synthetic activity is not affected would be expected to lead to a higher incidence of mutation, since synthesis of DNA without this proofreading mechanism would lead to a higher frequency of misincorporation. In this report we will present evidence that the 3′ to 5′ exonuclease activity of both bacterial and mammalian DNA polymerases can be selectively inhibited by nucleoside 5′-monophosphates. We shall further show that the selective inhibition of the 3′ to 5′ exonuclease activity results in an increase in the incorporation of the noncomplementary nucleotides dGMP or dCMP into poly(dA-dT). One of these selective inhibitors of the 3′ to 5′ exonuclease activity, 6-mercaptopurine ribonucleoside 5′-monophosphate, is the active metabolite of a known mutagen in bacteria (Greer, 1958; Speck and

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Rosenkranz, 1976) and of a suspected carcinogen in man (Schein and Winokur, 1975; Penn and Starzl, 1972). We propose that the mutagenicity and carcinogenicity of 6-mercaptopurine are due to the selective inhibition of the 3′ to 5′ exonuclease activity associated with DNA polymerases.

Materials and Methods

Unlabeled deoxyribonucleoside triphosphates were obtained either from P-L Biochemicals or Calbiochem. ^{3}H-labeled deoxyribonucleoside triphosphates were obtained from New England Nuclear and their purity was checked by thin-layer chromatography on poly(ethylenimine)-cellulose (PEI-cellulose[1]). Nucleosides and nucleoside monophosphates were obtained from P-L Biochemicals. The purity of the nucleoside 5′-monophosphates was examined by PEI-cellulose thin-layer chromatography as described by Cashel et al. (1969), and no contamination by nucleoside diphosphates or triphosphates was detectable (<0.1%). However, because of the high concentrations of nucleoside 5′-monophosphates used in the experiments, they were further purified: dAMP was purified by chromatography on Dowex-1 (chloride) as described by Cohen (1966) and TMP by chromatography on Dowex-1 (formate) as described by Hurlbert and Furlong (1967). The barium salt of 6-mercaptopurine ribonucleoside monophosphate was obtained from P-L Biochemicals and converted to the sodium salt before use. PEI-cellulose thin-layer chromatography plates were purchased from Brinkman Instruments. *E. coli* B, ¼ log phase, was obtained from Grain Processing.

Preparation of Enzymes and Polynucleotides. DNA polymerase δ (step VI) was prepared from rabbit erythroid hyperplastic bone marrow as described by Byrnes et al. (1976). DNA polymerase I was prepared from *E. coli* through step VII (>95% pure) as described by Jovin et al. (1969). Poly(dA-dT) was synthesized according to the method of Schachman et al. (1960).

Preparation of 3′-Terminally Labeled Poly(dA-dT)·[^{3}H]TMP. Poly(dA-dT) labeled at the 3′ terminus with [^{3}H]TMP was prepared by incubating poly(dA-dT) with *E. coli* DNA polymerase I in the presence of a single substrate, [^{3}H]TTP. The reaction mixture contained in a final volume of 5.0 mL: 80 mM Tris-HCl, pH 7.8; 0.2 mM $MnCl_2$; 0.8–1.0 A_{260} unit of poly(dA-dT), chain length 250 nucleotides; 20 μCi of [^{3}H]TTP, 43–48 μCi/mmol, 0.8 mM 5′AMP; and 1.0 unit/mL DNA polymerase I. After a 15-min incubation at 37 °C, the reaction was stopped by the addition of 0.25 mL of 0.1 M EDTA, pH 7.0, and sodium dodecyl sulfate to 0.5%. The solution was deproteinized twice with chloroform–isoamyl alcohol (24:1) and poly(dA-dT)·[^{3}H]TMP was separated from unincorporated nucleotides on a Sephadex G-50 column (2.2 × 50 cm) equilibrated with 0.01 M Tris-HCl (pH 7.4)–0.06 M KCl. The excluded volume was collected, lyophilized, dissolved in 0.01 M Tris-HCl (pH 7.4)–0.06 M KCl, and dialyzed against the same buffer. The final specific activity of 3′-terminally labeled poly(dA-dT)·[^{3}H]TMP was 4–6 × 10^6 cpm/A_{260} unit.

DNA Polymerase Assays. For mammalian DNA polymerase δ, each reaction mixture contained: 80 mM Hepes buffer, pH 7.0; 60 mM KCl; 0.4 mM $MnCl_2$; 4.0 μM dATP; 0.2 μM [^{3}H]TTP, 41 Ci/mmol; 2 × 10^{-2} A_{260} unit of poly(dA-dT) and 2–4 units of DNA polymerase in a final volume of 0.25 mL. Unless otherwise indicated, the reaction was incubated for 15 min at 37 °C and stopped by the addition of 2 mL of cold 5% trichloroacetic acid. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976). One unit of DNA polymerase δ catalyzes the incorporation of 1 nmol of TMP/h at 37 °C.

For the *E. coli* DNA polymerase I assay, the reaction mixture contained in a final volume of 0.25 mL: 80 mM Tris-HCl, pH 7.8; 4 mM $(NH_4)_2SO_4$; 0.2 mM $MnCl_2$; 4.0 μM dATP; 0.12–0.24 μM [^{3}H]TTP, 41 Ci/mmol; 2 × 10^{-2} A_{260} unit of poly(dA-dT); 10 μg of bovine serum albumin; and 0.1–0.5 unit of DNA polymerase I. Unless otherwise indicated, the reaction mixture was incubated for 15 min at 37 °C and stopped by the addition of 2 mL of cold trichloroacetic acid containing 0.01 M sodium pyrophosphate. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1976). One unit of DNA polymerase I catalyzes the incorporation of 10 nmol of total nucleotide/30 min at 37 °C.

3′ to 5′ Exonuclease Assays. The 3′ to 5′ exonuclease activity of mammalian DNA polymerase δ was assayed by measuring the release of [^{3}H]TMP from poly(dA-dT)·[^{3}H]-TMP. Each reaction mixture contained: 80 mM Hepes buffer, pH 7.0; 1.0 mM $MnCl_2$; 120 mM KCl; 3 × 10^{-3} A_{260} unit of poly(dA-dT)·[^{3}H]TMP, 4–6 × 10^6 cpm/A_{260} unit; and 1–2 units of DNA polymerase δ in a final volume of 0.125 mL. The reaction was incubated at 37 °C for 15 min and stopped by the addition of 2 mL of cold trichloroacetic acid. The reaction was mixed and left on ice for 10 min, filtered on a Millipore HAWP filter disc, and washed with 30 mL of distilled water. The disc was dried and counted in a toluene–Omnifluor solution in a liquid scintillation spectrometer.

The 3′ to 5′ exonuclease activity of *E. coli* DNA polymerase I was also assayed by measuring the release of [^{3}H]TMP from the 3′-terminally labeled poly(dA-dT)·[^{3}H]TMP. Each reaction mixture contained in a final volume of 0.15 mL: 67 mM Tris-HCl, pH 7.8; 0.13 mM $MnCl_2$; 2.0 mM β-mercaptoethanol; 130 mM KCl; 3 × 10^{-3} A_{260} unit of poly(dA-dT)·[^{3}H]TMP, 4–6 × 10^6 cpm/A_{260} unit; 10 μg of bovine serum albumin and 0.2–0.5 unit of DNA polymerase I. The reaction was incubated for 15 min at 37 °C and stopped by the addition of 2 mL of cold 5% trichloroacetic acid. The precipitate was collected, washed, and counted as described above.

The exonuclease activity is expressed as follows: 100% activity is the amount of [^{3}H]TMP released in the control reaction which ranged from 4000 to 8000 cpm. This amount corresponds to the hydrolysis of 25 to 50% of the total poly(dA-dT)·[^{3}H]TMP added.

Results

Inhibition of 3′ to 5′ Exonuclease Activity by Nucleoside 5′-Monophosphates. The exonuclease activities of both the mammalian and bacterial DNA polymerases are inhibited by nucleoside 5′-monophosphates (Table I). The mammalian enzyme is inhibited by the purine ribonucleotides 5′AMP and 5′GMP about 50% at 0.2 mM, whereas at the same concentration the pyrimidine ribonucleotides and the deoxyribonucleotides are less effective. The exonuclease of the bacterial enzyme is equally sensitive to inhibition by either purine ribonucleotides or deoxyribonucleotides as well as the pyrimidine deoxyribonucleotides dTMP and dUMP at a concentration of 0.33 mM. Nucleosides, 3′-nucleotides, and cyclic nucleotides cause little or no inhibition of the exonuclease activities of both the mammalian and bacterial DNA polymerases.

Inhibition of the 3′ to 5′ Exonuclease Activity of DNA Polymerase by Nucleoside 5′-Monophosphates Is Selective. The effects of increasing concentrations of dAMP on the 3′ to 5′ exonuclease activity of *E. coli* DNA polymerase I, as well

[1] Abbreviations used: PEI-cellulose, poly(ethylenimine)-cellulose; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Effects of Nucleotides on DNA Polymerase Associated 3′ to 5′ Exonuclease Activity.[a]

Nucleoside or nucleotide added	Exonuclease act. (%)	
	Mammalian DNA polymerase δ	*E. coli* DNA polymerase I
5′AMP	57	29
5′GMP	42	28
5′CMP	83	75
5′UMP	83	55
5′dAMP	89	12
5′dGMP	73	35
5′dCMP	91	64
5′dTMP	87	18
5′dUMP	94	31
3′AMP	100	97
Cyclic 3′,5′AMP	100	91
Nucleosides	100	100

[a] Assays of 3′ to 5′ exonuclease activities were as described in Materials and Methods except for the addition of nucleosides or nucleotides as indicated. The concentrations of the nucleosides and nucleotides were 0.2 mM in the assay for the exonuclease of mammalian DNA polymerase δ and 0.33 mM in the assay for the exonuclease activity of *E. coli* DNA polymerase I.

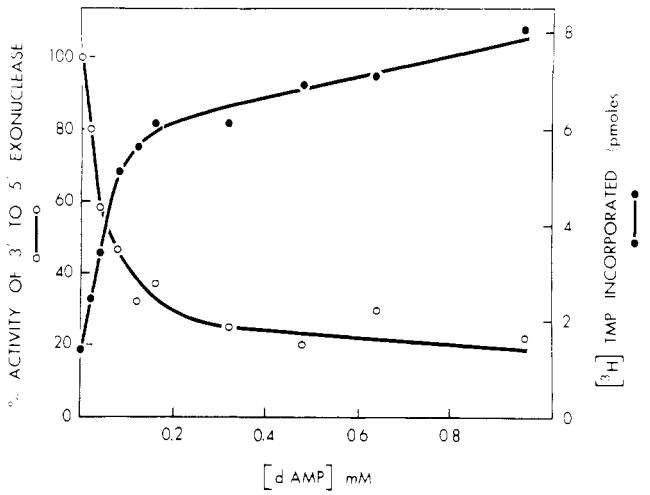

FIGURE 1: Effects of dAMP on 3′ to 5′ exonuclease activity of *E. coli* DNA polymerase I and on nucleotide incorporation. DNA polymerase and 3′ to 5′ exonuclease were assayed separately as described in Materials and Methods except for the addition of dAMP as indicated.

as the effects on nucleotide incorporation at low substrate concentrations, are shown in Figure 1. Increasing concentrations of 5′dAMP result in a progressive inhibition of exonuclease activity, as measured by the release of [^{3}H]TMP from the 3′ terminus of poly(dA-dT)·[^{3}H]TMP, and an increase in [^{3}H]TMP incorporation. At a concentration of 0.05 mM 5′-dAMP, the exonuclease activity is inhibited about 50% and [^{3}H]TMP incorporation is increased threefold. The degree of stimulation observed is dependent on the time of incubation, being greater at longer incubation times. Similar results were obtained with the other 5′ nucleotides listed in Table I, although different concentrations of nucleotide were required to give 50% inhibition of the exonuclease activity. Those nucleotides or nucleosides which have no effect on exonuclease activity also have no effect on nucleotide incorporation.

The effect of nucleoside 5′-monophosphate on nucleotide incorporation is dependent on the concentration of the substrates TTP and dATP (Table II). At very low substrate concentrations (10^{-8} M) [^{3}H]dAMP incorporation is increased

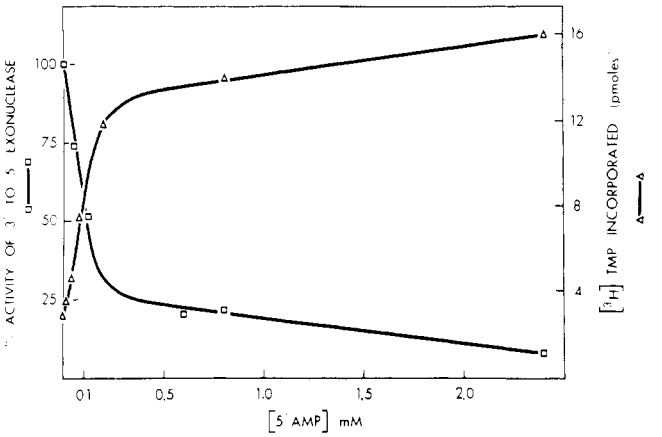

FIGURE 2: Effects of 5′ AMP on 3′ to 5′ exonuclease activity of mammalian DNA polymerase δ and on nucleotide incorporation. DNA polymerase and 3′ to 5′ exonuclease were assayed separately as described in Materials and Methods except for the addition of 5′ AMP as indicated.

over sixfold by 1.6 mM dAMP, whereas at high substrate concentrations (10^{-5} M) comparatively less effect on incorporation is observed, although the absolute amount of incorporation is increased.

The 3′ to 5′ exonuclease activity of mammalian DNA polymerase δ is also selectively inhibited by nucleoside 5′-monophosphates. The effects of increasing concentrations of 5′AMP on both 3′ to 5′ exonuclease activity and nucleotide incorporation are shown in Figure 2. As is the case with the bacterial enzyme, increasing concentrations of 5′AMP result in increasing inhibition of exonuclease activity and a corresponding increase in [^{3}H]TMP incorporation at low substrate concentrations. At 0.15 mM AMP the exonuclease activity is inhibited approximately 50% and DNA synthesis is increased threefold.

Inhibition of Exonuclease Activity Results in Increased Nucleotide Incorporation. To determine whether the enhancement of nucleotide incorporation at low substrate concentration is due to the stimulation of DNA polymerase activity by nucleoside 5′-monophosphates, or whether it is the result of the inhibition of the exonuclease activity, the radioactive products of the DNA polymerase reaction were analyzed by thin-layer chromatography on PEI-cellulose. By this procedure, it is possible to study both the polymerase activity and the exonuclease activity simultaneously since the products of the DNA polymerase reaction, which include both the [^{3}H]TMP incorporated into polynucleotide (the product of the polymerization reaction) and [^{3}H]TMP released as free mononucleotide (the product of the exonuclease reaction) can be separated (Hershfield and Nossal, 1972). As shown in Figure 3 increasing concentration of 5′AMP resulted in a decrease in the amount of free [^{3}H]TMP released and a corresponding increase in the amount of [^{3}H]TMP incorporated into polynucleotide. However, the total amount of [^{3}H]TMP formed, either as free [^{3}H]TMP or as residues in polynucleotide, remained constant at all concentrations of 5′AMP studied. This demonstrates that, under conditions where both polymerase and exonuclease are active, the increase in nucleotide incorporation in the presence of 5′AMP is due to the selective inhibition of exonuclease activity by this nucleotide, which prevents the hydrolysis of the newly incorporated [^{3}H]TMP. This further demonstrates that 5′AMP has no effect on DNA polymerase activity under these conditions.

Selective Inhibition of 3′ to 5′ Exonuclease Activity of Bacterial and Mammalian DNA Polymerases by 6-Mer-

TABLE II: Effect of 5′dAMP on Nucleotide Incorporation at Various Substrate Concentrations with *E. coli* DNA Polymerase I.[a]

Substrate concn (M)	[^{3}H]dAMP incorp (pmol)		Absolute increase (pmol)	Rel increase (fold)
	−5′dAMP	+5′dAMP		
1.2×10^{-8}	0.24	1.53	1.29	6.4
2.4×10^{-8}	0.60	2.77	2.17	4.6
3.6×10^{-8}	1.08	4.52	3.44	4.2
4.8×10^{-8}	1.46	5.57	4.11	3.8
4.5×10^{-7}	30.7	41.3	10.6	1.3
4.0×10^{-6}	266	280	14	1.1
4.0×10^{-5}	603	727	124	1.2

[a] Assays were carried out as described in Materials and Methods except: (1) the concentrations of the substrates TTP and [^{3}H]dATP were varied as indicated and (2) 5′dAMP (1.6 mM) was added as indicated. The time of incubation was 15 min.

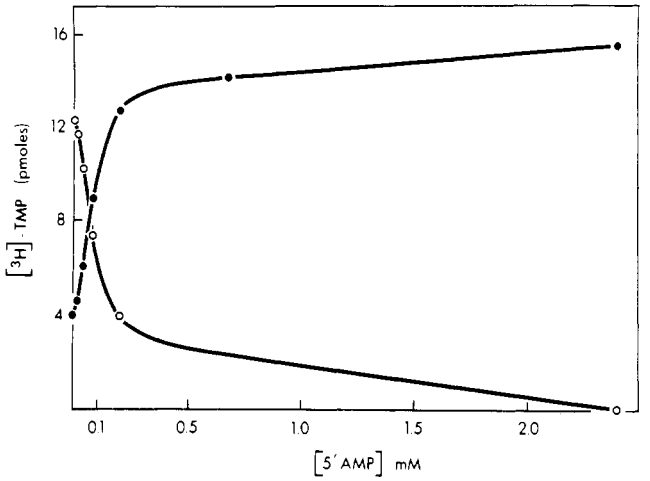

FIGURE 3: Effect of 5′ AMP on the conversion of [^{3}H]TTP to either [^{3}H]TMP as polynucleotide or free [^{3}H]TMP by DNA polymerase δ. The reaction mixture was as described in Materials and Methods for the DNA polymerase δ assay except for the addition of 5′ AMP as indicated. After 15 min incubation at 37 °C, the reaction was terminated by the addition of EDTA to a final concentration of 2.5 mM. [^{3}H]TMP as polynucleotide and free [^{3}H]TMP were separated by thin-layer chromatography on PEI-cellulose and quantitated as previously described (Byrnes et al., 1976).

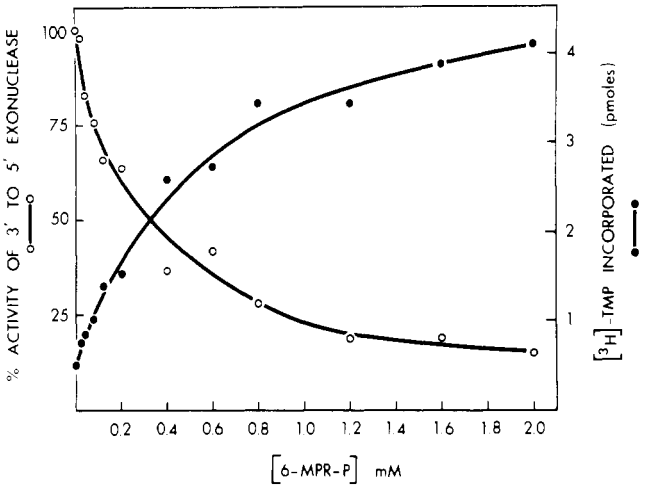

FIGURE 4: Effect of 6-MPR-P on the 3′ to 5′ exonuclease activity of *E. coli* DNA polymerase I and on nucleotide incorporation. Assay conditions were as described in Materials and Methods except for the addition of 6-MPR-P as indicated.

captopurine Ribonucleoside 5′Monophosphate. In addition to the nucleotides listed in Table I, nucleotide analogues inhibit the exonuclease activities of both bacterial and mammalian DNA polymerases. Figure 4 shows the effects of increasing concentrations of the active metabolite of 6-mercaptopurine (6-MP), 6-mercaptopurine ribonucleoside 5′-monophosphate (6-MPR-P), on 3′ to 5′ exonuclease activity, and on [^{3}H]TMP incorporation with DNA polymerase I. Similar to the effect of 5′dAMP, 6-MPR-P selectively inhibits the exonuclease activity while DNA polymerase activity is not affected. At 0.3 mM 6-MPR-P, the exonuclease activity is inhibited about 50% while net DNA synthesis is stimulated about fourfold. Mammalian DNA polymerase δ is similarly affected by increasing concentrations of 6-MPR-P (data not shown).

Selective Inhibition of Exonuclease Activity Results in Misincorporation. The effect of the inhibition of the 3′ to 5′ exonuclease of *E. coli* DNA polymerase I on the incorporation of the noncomplementary nucleotide [^{3}H]dGMP into poly(dA-dT) is shown in Table III. In the absence of 5′AMP, this enzyme incorporated very little or no [^{3}H]dGMP into polynucleotide, 0.1 pmol being the limit of detection under the assay conditions. Under identical conditions 86 pmol of [^{3}H]dAMP are incorporated into polynucleotide resulting in an error frequency of less than 1/860. However, in the presence of 5′AMP, 0.7 pmol of [^{3}H]dGMP is incorporated into polynucleotide, resulting in an error frequency of 1/123. At the concentration of dATP and TTP used in this experiment, i.e., 1.3 μM, no apparent stimulation of poly(dA-dT) synthesis is observed at short incubation times.

TABLE III: Misincorporation of dGMP into Poly(dA-dT) with *E. coli* DNA Polymerase I.[a]

	Minus 5′AMP	Plus 5′AMP
[^{3}H]dGMP incorp (pmol)	<0.1	0.70
[^{3}H]dAMP incorp (pmol)	86	86
Error frequency	<1	1
	860	123

[a] The reaction mixtures contained in a final volume of 0.25 mL: 80 mM Tris-HCl, pH 7.8; 0.2 mM $MnCl_2$; 80 mM KCl; 1×10^{-2} A_{260} unit of poly(dA-dT); 8 μM TTP; 0.5 unit of *E. coli* DNA polymerase I; and 1.3 μM either [^{3}H]dATP, 11.97 Ci/mmol, or [^{3}H]dGTP, 13 Ci/mmol. The concentration of 5′AMP, when present, was 1.6 mM. After incubation at 37 °C for 5 min, the reaction was stopped by the addition of 2 mL of cold 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The precipitate was collected, washed, and counted as described in Materials and Methods.

To investigate the molecular basis for the increased incorporation of the noncomplementary nucleotide [^{3}H]dGMP into poly(dA-dT) in the presence of 5′AMP, the products of the reaction with the noncomplementary nucleotide were analyzed by chromatography on PEI-cellulose. As shown in Table IV,

TABLE IV: Analysis of dGMP Incorporation with Poly(dA-dT) and *E. coli* DNA Polymerase I.[a]

	Minus 5′AMP	Plus 5′AMP
[^{3}H]dGMP incorp into polynucleotide (pmol)	<0.1	4.0
[^{3}H]dGMP released as free monophosphate (pmol)	9.2	5.8

[a] The reaction mixture contained in a final volume of 0.25 mL: 80 mM Tris-HCl, pH 7.8; 0.2 mM $MnCl_2$; 8 μM TTP; 1.7 μM [^{3}H]-dGTP, 9.4 Ci/mmol; 2×10^{-2} A_{260} units of poly(dA-dT); 1.6 mM 5′AMP, when present; and 0.5 unit of *E. coli* DNA polymerase I. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of EDTA to a final concentration of 5 mM. The amount of free dGMP generated was quantitated by spotting an aliquot of each reaction mixture (0.1 mL) in 1.5-cm lanes on a prewashed PEI-cellulose plate with dAMP, dGMP, dCMP, and TMP markers. The plate was prerun with methanol and developed with 1 M acetic acid to 1 cm followed by 0.5 M LiCl. Markers were located by UV absorption. Radioactivity was located by cutting the lanes into 0.75-cm sections and counting in a liquid scintillation counter with toluene–Omnifluor scintillant. The counting efficiency for tritium was 7%. No detectable [^{3}H]dGMP was generated in the absence of either poly(dA-dT) or DNA polymerase. The amount of dGMP incorporated into polynucleotide was quantitated by precipitation of 0.1 mL of each reaction mixture with 2 mL of 5% trichloroacetic acid containing 0.01 M sodium pyrophosphate. The precipitate was collected, washed, and counted as described in Materials and Methods.

TABLE V: Misincorporation of dCMP into Poly(dA-dT) with Mammalian DNA Polymerase δ.[a]

	Minus 5′GMP	Plus 5′GMP
[^{3}H]dCMP incorp (pmol)	<0.05	0.5
[^{3}H]dTMP incorp (pmol)	27.3	77.9
Error frequency	<1	1
	540	156

[a] Reaction mixtures contained in a final volume of 0.25 mL: 80 mM Hepes buffer, pH 7.0; 0.4 mM $MnCl_2$; 80 mM KCl; 4 μM dATP; and 0.65 μM [^{3}H]dCTP, 23 Ci/mmol, or 0.65 μM [^{3}H]dTTP, 48 Ci/mmol; 2×10^{-2} A_{260} unit of poly(dA-dT); 1.6 mM 5′GMP when present; and 2 units of DNA polymerase δ. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 2 mL of cold 5% trichloroacetic acid containing 1 μmol of dCTP. The precipitate was collected, washed, and counted as previously described (Byrnes et al., 1975).

in the absence of 5′AMP, almost all [^{3}H]dGMP that has been incorporated into poly(dA-dT) is rapidly hydrolyzed and released as free [^{3}H]dGMP, resulting in little or no net incorporation of [^{3}H]dGMP into poly(dA-dT). However, in the presence of 5′AMP, 4 pmol of [^{3}H]dGMP was incorporated into poly(dA-dT) and the amount of [^{3}H]dGMP generated was reduced from 9.2 pmol to 5.8 pmol. This clearly shows that the incorporation of the noncomplementary nucleotide, [^{3}H]dGMP, into poly(dA-dT) is due to the selective inhibition of the 3′ to 5′ exonuclease activity.

Since the 3′ to 5′ exonuclease activity associated with mammalian DNA polymerase δ is similarly inhibited by nucleoside 5′-monophosphate, the effect of 5′GMP on the incorporation of the noncomplementary nucleotide, [^{3}H]dCMP, into poly(dA-dT) with mammalian DNA polymerase δ was also investigated (Table V). In the absence of 5′GMP, little or no incorporation (less than 0.05 pmol) of [^{3}H]dCMP into poly(dA-dT) is observed. Under identical conditions, 27.3 pmol of [^{3}H]TMP is incorporated into polynucleotide, resulting in an error frequency of less than 1/540. However, in the presence of 5′GMP, 0.5 pmol of [^{3}H]dCMP and 77.9 pmol of [^{3}H]TMP are incorporated and this gives an error frequency of 1/156. Analysis of the products of the reaction (Table VI) shows that, similar to the results obtained with *E. coli* DNA polymerase I, selective inhibition of the 3′ to 5′ exonuclease activity of DNA polymerase δ results in a reduction in the amount of free [^{3}H]dCMP generated from 2 pmol to 0.3 pmol and incorporation of 1.4 pmol of [^{3}H]dCMP into poly(dA-dT).

TABLE VI: Analysis of dCMP Incorporation with Poly(dA-dT) and Mammalian DNA Polymerase δ.[a]

	Minus 5′GMP	Plus 5′GMP
[^{3}H]dCMP incorp into polynucleotide (pmol)	<0.05	1.4
[^{3}H]dCMP released as free monophosphate (pmol)	2.0	0.3

[a] Duplicate reaction mixtures contained in a final volume of 0.25 mL: 80 mM Hepes buffer, pH 7.0; 0.4 mM $MnCl_2$; 80 mM KCl; 4 μM dATP; 0.87 μM [^{3}H]dCTP, 23 Ci/mmol; 2×10^{-2} A_{260} unit of poly(dA-dT); 1.6 mM 5′GMP when present; and 2 units of DNA polymerase δ. After incubation at 37 °C for 60 min, the reaction was stopped by the addition of EDTA to a final concentration of 5 mM or by the addition of 2 mL of cold 5% trichloroacetic acid containing 1 μmol dCTP. The free dCMP produced was quantitated by chromatography of the EDTA-stopped reaction on a prewashed PEI-cellulose plate as described in Table IV. No detectable [^{3}H]dGMP was generated in the absence of either poly(dA-dT) or DNA polymerase. The dCMP incorporated into polynucleotide was determined as described previously (Byrnes et al., 1975).

Discussion

The present studies demonstrate that the 3′ to 5′ exonuclease activities of both mammalian and bacterial DNA polymerases can be selectively inhibited by nucleoside 5′-monophosphates. Neither the mammalian nor the bacterial enzyme is inhibited by nucleosides, 3′-nucleotides, or cyclic 3′,5′-nucleotides.

At low substrate concentrations nucleotide incorporation is greatly enhanced by nucleoside 5′-monophosphates. The increase in nucleotide incorporation is the result of inhibition of exonuclease activity, for the total amount of TTP converted to TMP, either as acid-precipitable TMP, the product of the polymerase reaction, or as free TMP, the product of the polymerase and subsequent exonuclease reactions, is constant in the presence or absence of nucleoside 5′-monophosphates.

Since the overall rate of incorporation of labeled nucleotides into DNA is determined by both the rate of nucleotide polymerization and the rate of hydrolysis of newly synthesized DNA, it is not unexpected that selective inhibition of the 3′ to 5′ exonuclease activity results in an increase in net DNA synthesis at all substrate concentrations studied (Table II). However, at low substrate concentrations, there is a greater apparent stimulation of nucleotide incorporation relative to that at high substrate concentrations. This is probably because at limiting substrate concentrations the rate of polymerization is low and comparable to the rate of degradation, resulting in the hydrolysis of most of the newly incorporated nucleotides and very little net DNA synthesis. Thus, when the exonuclease activity is selectively inhibited by 5′-mononucleotides, there is a large increase in the rate of net nucleotide incorporation. In contrast, at high substrate concentrations, the rate of polymerization is much higher than the rate of degradation, and selective inhibition of the exonuclease activity has less effect on the overall rate of nucleotide polymerization. With *E. coli*

DNA polymerase I, it has been observed that the DNA polymerase activity is 15-fold greater than 3′ to 5′ exonuclease activity when each activity is measured under optimal conditions (Setlow, 1974).

The effects of nucleoside 5′-monophosphates on both the 3′ to 5′ exonuclease activity and DNA polymerase activity of *E. coli* DNA polymerase I, as well as the binding of nucleoside 5′-monophosphates to the polymerase, were previously investigated by Huberman and Kornberg (1970). These investigators found that there was a single binding site for nucleoside 5′-monophosphates, distinct from the nucleoside triphosphate binding site. The dissociation constants for the dNMPs ranged from 0.01 to 0.1 mM. They observed that nucleoside 5′-monophosphates had no effect on either DNA polymerase activity or exonuclease activity, except at concentrations considerably higher than their K_{diss}. Inhibition of the 3′ to 5′ exonuclease activity by dGMP, as measured by the hydrolysis of the dinucleotide, pTpT, was observed only at a dGMP concentration 50-fold higher than its K_{diss}. In the present study inhibition of exonuclease activity is observed at nucleotide concentrations of the same order of magnitude as the K_{diss} reported by Huberman and Kornberg (1970).

The difference between the concentration of 5′-nucleotide required to inhibit the 3′ to 5′ exonuclease of *E. coli* DNA polymerase I in the present study and that reported previously may be due to differences in assay conditions. In the present study poly(dA-dT)·[^{3}H]TMP was used as a substrate for the 3′ to 5′ exonuclease whereas Huberman and Kornberg used the dinucleotide [^{32}P]pTpT. Lehman and Richardson (1964) have shown that the K_m for pTpT is 3×10^{-4} M, while the K_m for poly(dA-dT) is 4×10^{-10} M. Thus, it would appear that poly(dA-dT) is a preferred substrate for the exonuclease. Furthermore, hydrolysis of each mole of pTpT results in the formation of 2 mol of 5′TMP, which can inhibit exonuclease activity.

The selective inhibition of the 3′ to 5′ exonuclease activity of DNA polymerases by 5′ nucleotides suggests that these compounds may be mutagenic, since continued DNA synthesis with the proofreading exonuclease inhibited would lead to a higher frequency of misincorporation, and, thus, to a higher incidence of mutation. This is analogous to the decreased ratio of exonuclease to polymerase activity seen in mutator strains of phage T_4 which have an increased rate of spontaneous mutation compared with wild type (Drake et al., 1969; Muzcyzka et al., 1972). With both *E. coli* DNA polymerase I and mammalian DNA polymerase δ, we have observed that, although the noncomplementary nucleotides [^{3}H]dCTP and [^{3}H]dGTP are incorporated into poly(dA-dT), they are rapidly hydrolyzed and released as free [^{3}H]dCMP and [^{3}H]dGMP, resulting in little or no net incorporation of [^{3}H]dCMP or [^{3}H]dGMP into poly(dA-dT). However, in the presence of nucleoside 5′-monophosphates, considerable incorporation of [^{3}H]dCMP or [^{3}H]dGMP into poly(dA-dT) is observed and the amount of free [^{3}H]dCMP or [^{3}H]dGMP generated is markedly reduced, demonstrating that selective inhibition of the exonuclease activity does result in increased misincorporation.

Manganese ions have been shown to be mutagenic in vivo (Orgel and Orgel, 1965) and to increase misincorporation in in vitro systems (Hall and Lehman, 1968; Mitzutani and Temin, 1976). It is likely, therefore, that the use of the $MnCl_2$ as divalent cation in the present studies has contributed to the observed error frequency. However, under our assay conditions, in the absence of a selective inhibitor of 3′ to 5′ exonuclease activity, there is little or no detectable misincorporation.

It is difficult to say whether the in vivo concentrations of 5′AMP and other naturally occurring nucleoside 5′-monophosphates are ever high enough to inhibit the exonuclease activity of DNA polymerase. However, certain purine analogues, such as 6-MP or 6-thioguanine (6-TG), are substrates for adenylic pyrophosphorylase or inosinic–guanylic pyrophosphorylase and are converted to the ribonucleoside 5′-monophosphates (Elion, 1967; Timmis and Williams, 1967). Since 6-MPR-P and 6-TGR-P are only poorly converted to the diphosphate or triphosphate, the concentration of the nucleoside 5′-monophosphate derivatives of these purine analogues can reach very high levels in vivo, i.e., greater than 0.1 mM (Paterson, 1959; Moore and LePage, 1958). At these concentrations 6-MPR-P inhibits the exonuclease activities of both the bacterial and mammalian DNA polymerases.

6-MP is known to be mutagenic in *E. coli* (Greer, 1958) and recently a derivative of 6-MP, Azathioprine, was also shown to be mutagenic in *Salmonella typhimurium* (Speck and Rosenkranz, 1976). Azathioprine is converted to 6-MP in vivo. The mutagenicity of 6-MP has generally been attributed to its interference with purine nucleotide biosynthesis (Drake, 1970), although the exact mechanism is not understood. The ability of the nucleotide derivative of 6-MP to inhibit the proofreading exonuclease of DNA polymerase at in vivo concentrations provides a molecular mechanism for the mutagenicity of this purine analogue.

6-MP and Azathioprine are used clinically as chemotherapeutic and immunosuppressive agents. It is becoming apparent that Azathioprine is a carcinogen. It has been estimated that the relative risk of de novo cancer in patients receiving Azathioprine for prolonged periods is 80–100 times greater than that of the general population (Schein and Winokur, 1975; Penn and Starzl, 1972). This increased incidence of cancer has been attributed to the immunosuppressive effect of these agents. However, as we have suggested for the mutagenicity of 6-MP in *E. coli*, the selective inhibition of the exonuclease activity of the mammalian DNA polymerase by 6-MPR-P provides an alternate explanation for the carcinogenicity of 6-MP and Azathioprine. The correlation between carcinogenicity and mutagenicity of chemical compounds is well established (Ames et al., 1973; Miller and Miller, 1971). 6-MP may be another example of a mutagen being a carcinogen, but by a previously undescribed mechanism.

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Characterization of Chromatin Modified with Ethyl Acetimidate†

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ABSTRACT: Thymus chromatin was extensively modified with ethyl acetimidate, substituting up to 90% of the lysyl residues of the histones while retaining the positive charge of the basic amino acid. Physicochemical and immunochemical characterization of this derivative chromatin indicates a high degree of retention of the native structure of the nucleoprotein even after extensive modification. The alterations which are detected are most simply interpreted as resulting from a weakening of the interactions of histone H1 with DNA in the modified chromatin. The near-native character of amidinated chromatin contrasts with the more extensive structural alterations observed in acetylated chromatin. Our data demonstrate the suitability of this reagent for mapping available lysyl residues in this and other nucleoproteins and suggest that the related bisimido esters may be reagents of choice for cross-linking of chromatin histones.

Selective chemical modification of proteins has been one of the most useful approaches for identification of functional amino acid residues in enzyme active sites, delineation of surface topography, and determination of residues involved in subunit interactions. More recently, this experimental approach has been utilized for study of the topography of nucleic acid-protein complexes, with its most notable success in terms of chromatin structure thus far being the detection of histone octamers in nucleosome core particles and the definition of specific histone-histone interactions using cross-linking reagents (Hyde and Walker, 1975; Chalkley, 1975; Chalkley and Hunter, 1975; Thomas and Kornberg, 1975a,b; Martinson and McCarthy, 1975; Bonner and Pollard, 1975; Van Lente et al., 1975; Martinson et al., 1976). In both cross-linking studies and in studies of the relative reactivities of lysyl residues of histones when in the chromatin complex, the structural integrity of the modified species is of primary importance. Alterations in protein-protein or protein-nucleic acid interactions consequent to chemical modification may alter the reactivity of portions of the protein affected by the conformational changes. This is of particular concern, since many of the cross-linking reactions modify lysyl residues, which are expected to be a stabilizing force in histone-DNA interactions. Many of the chemical probes used in the past to study the structure of histones alone or in chromatin have resulted in the size, polarity, and/or charge of the modified residue being drastically altered, making it likely that extensive modification resulted in altered interactions among the constituents of chromatin. In the one case where structural characterization of a modified chromatin was performed, acetylation of chromatin lysyl residues led to definite changes in melting profile, consistent with destabili-

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