Depurination-Induced Infidelity of Deoxyribonucleic Acid Synthesis with Purified Deoxyribonucleic Acid Replication Proteins in Vitro†

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ABSTRACT: Removal of purine bases from φX174 single-stranded DNA leads to increased reversion frequency of amber mutations when this DNA is copied in vitro with purified DNA polymerases. This depurination-induced mutagenesis is observed at three different genetic loci and with several different purified enzymes, including *Escherichia coli* DNA polymerases I and III, avian myeloblastosis virus DNA polymerase, and eukaryotic DNA polymerases α, β, and γ. The extent of mutagenesis correlates with the estimated frequency of bypass of the lesion and is greatest with inherently inaccurate DNA polymerases which lack proofreading capacity. With *E. coli* DNA polymerase I, conditions which diminish proofreading result in a 3–5-fold increase in depurination-induced mutagenesis, suggesting a role for proofreading in determining the frequency of bypass of apurinic sites. The addition of *E. coli* single-stranded DNA-binding protein to polymerase I catalyzed reactions with depurinated DNA had no effect on the extent of mutagenesis. Analysis of wild-type revertants produced during in vitro DNA synthesis by polymerase I or avian myeloblastosis virus DNA polymerase on depurinated φX174 *amber* 3 DNA indicates a preference for insertion of dAMP opposite the putative apurinic site at position 587. These results are discussed in relation both to the mutagenic potential of apurinic sites in higher organisms and to studies on error-prone DNA synthesis.

The loss of a base from DNA by hydrolysis of the N-glycosylic bond is one of the more frequent types of spontaneous and induced alterations in the cell genome. From the rate of depurination measured in vitro with purified DNA, it has been calculated that a mammalian cell containing 2×10^9 nucleotides spontaneously loses 10000 bases from its genome in a single 24-h period (Lindahl & Nyberg, 1972). The rate of base loss can be further increased by certain mutagenic DNA-damaging agents which modify bases with resultant destabilization of the glycosylic bond (Lawley & Brookes, 1963; Margison & O'Connor, 1973; Strauss et al., 1975; Singer, 1976). While cells have several mechanisms for repairing DNA damage, these repair processes may not be totally efficient, particularly in eukaryotic cells in which the DNA is tightly associated with chromosomal proteins. For this reason, we have been investigating the effects of unrepaired apurinic sites in DNA (Kunkel et al., 1981c; Schaaper & Loeb, 1981; Schaaper et al., 1982, 1983; Shearman & Loeb, 1979).

The mutagenic potential of unrepaired apurinic sites in DNA has been clearly demonstrated in vitro in a prokaryotic system. When φX174 DNA containing an amber mutation is depurinated and then transfected into spheroplasts, a large increase in reversion frequency, above a nondepurinated control, can be observed (Schaaper & Loeb, 1981). This increase is presumably due to error-prone DNA synthesis on the depurinated DNA, since the effect is only observed in spheroplasts made from bacteria previously exposed to UV light. Furthermore, the effect is *recA* dependent (Schaaper et al., 1982). This is in keeping with the SOS hypothesis (Witkin, 1976), which postulates that certain types of damage that are not easily copied by replicating DNA polymerases under normal conditions may be bypassed with greatly increased frequency upon induction of the SOS system. An analysis of the DNA sequence of depurination-induced revertants indicated a predominance of A for T substitutions opposite the putative apurinic site. In this paper, we have examined the mutagenic potential of apurinic sites by using purified DNA polymerases from prokaryotic and eukaryotic cells. The frequency of bypass of apurinic sites on DNA templates is quantitated both by the extent of incorporation and by the mutagenicity. The results focus on the relationship of the inherent accuracy of DNA polymerases to their ability to copy over apurinic sites.

Materials and Methods

Bacteria and Bacteriophage. Bacterial strains *Escherichia coli* HF4714 (*su-1*$^+$) and HF4704 (*su*$^-$) used for plating of φX174 phage and *E. coli* KT-1 for making spheroplasts have been described before (Kunkel & Loeb, 1979; Weymouth & Loeb, 1978). *E. coli* C520 (*su-1*$^+$) was obtained from Dr. I. Tessman, Purdue University. Bacteriophage φX174 *am*3, *am* to8, and *am*18 were obtained from Dr. Weisbeek, University of Utrecht. Phage *am*3 was grown on HF4704 (Weymouth & Loeb, 1978); *ambers* to8 and 18 were grown on C520 with addition of 0.2 M $MgSO_4$ at 5 min after infection to prevent lysis (Kunkel & Loeb, 1980). Revertant (wild-type) phage were grown on HF4704 as previously described (Kunkel & Loeb, 1980). Single-stranded (viral) DNA and replicative form (RFI) DNA were obtained as described previously (Kunkel & Loeb, 1979), as were restriction endonuclease fragments after treatment of RFI DNA with restriction endonucleases *Hae*III, *Hpa*I, or *Alu*I.

Enzymes. The sources of all enzymes used here have been described in detail, including *E. coli* polymerase I (Pol I)[1] (Kunkel & Loeb, 1980) and Pol III (Kunkel et al., 1979), avian myeloblastosis virus DNA polymerase (Gopinathan et

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[1] Abbreviations: Pol I, *Escherichia coli* DNA polymerase I; Pol III, *Escherichia coli* DNA polymerase III; AMV, avian myeloblastosis virus; AP, apurinic; SSB, single-strand DNA-binding protein; dNTP, deoxynucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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al., 1979), calf thymus DNA polymerase α (Kunkel & Loeb, 1981), rat Novikoff hepatoma DNA polymerase β (Kunkel & Loeb, 1981), HeLa cell DNA polymerase γ (Kunkel & Loeb, 1981), HeLa cell apurinic endonuclease (Schaaper et al., 1983), and SSB (Kunkel et al., 1979).

ϕX174 Mutagenesis Assay. The reversion frequency of the amber mutation in the copied DNA was determined by transfection into *E. coli* spheroplasts and titering the resultant progeny phage on bacterial indicators either permissive or nonpermissive for the amber mutation. A detailed account of the methodology for this assay has been published (Kunkel & Loeb, 1979, 1980; Weymouth & Loeb, 1978). The reversion frequency values are the average of the number of determinations indicated for individual experiments, after subtracting the background reversion frequency of uncopied DNA in unincubated reactions [typically $(1.5–3.5) \times 10^{-6}$]. Day to day fluctuations in the progeny phage titering procedure can be as large as 2-fold. For error rate determinations, only reversion frequencies at least twice the background were considered significant.

The procedure for preparations of revertant DNA and sequencing by the chain terminator method (Sanger et al., 1977) has been described (Kunkel & Loeb, 1980). The ratio of dideoxy- to deoxyribonucleotides was 100:1. Depurination of DNA was achieved by heating the DNA in 10 mM sodium citrate–100 mM KCl buffer (pH 5.00) at 70 °C. These conditions produce approximately one apurinic site per single-stranded ϕX174 molecule for every 7 min of incubation.

Results

Mutagenesis at Different Loci with Purified DNA Polymerases. We have previously shown that when ϕX174 viral DNA containing the *amber* 3 mutation is depurinated and then copied in vitro with purified Pol I (Kunkel et al., 1981c) or AMV DNA polymerase (Schaaper et al., 1983), the phage which are produced upon transfection of the DNA into spheroplasts have a much higher reversion frequency compared to those resultant from nondepurinated DNA. Table I extends these observations to other amber mutations, demonstrating that depurination-induced mutagenesis is not limited to the *amber* 3 locus. Furthermore, comparing the results with AMV DNA polymerase in copying different amber mutations, it can be seen that both mutagenesis relative to nondepurinated DNA and mutagenesis per AP site vary over a severalfold range. Expressed per apurinic site, depurination contributes the amount of 265×10^{-6}, 268×10^{-6}, and 81.6×10^{-6} to the reversion frequency of *am*3, *am*18, and *am* to8, respectively.

In vitro DNA synthesis on depurinated DNA by all three classes of eukaryotic DNA polymerases is also highly mutagenic (Table I). The relative enhancement is largest with Pol-α (25-fold, 104×10^{-6} vs. 4.13×10^{-6}), the enzyme thought to be the replicative DNA polymerase in eukaryotes (Weissbach, 1977). Although the relative effect is lower, the absolute mutagenicity per AP site is even greater for the normally less accurate DNA polymerases β and γ.

The use of primed *amber* 18 DNA is advantageous for these studies; all three nucleotides of the amber locus are mutable, and a primer can be used to start synthesis only six nucleotides before the amber mutation (Zakour & Loeb, 1982). Thus, the mutagenic potential of depurination under conditions of limited synthesis can be examined. Depurination-induced mutagenesis can even be observed with the replicative DNA polymerase from *E. coli*, Pol III [in this case the core enzyme, containing only the α, ϵ, and θ subunits (McHenry & Crow, 1979)]. In spite of the limited ability of the core enzyme to add only approximately 50–200 nucleotides to the primed single-strand DNA (data not shown), this synthesis is sufficient to copy past the amber site. To date, this is the only situation in which we have observed mutagenicity during the copying of natural DNA with this highly accurate enzyme (Loeb et al., 1980). The depurination-induced mutagenicity observed with this enzyme is of interest, since the in vivo mutagenicity observed previously with ϕX174 DNA in SOS$^+$ spheroplasts (Schaaper & Loeb, 1981) is presumably also a result of errors by the replicative DNA polymerase III. It is interesting to note that synthesis on depurinated ϕX174 *amber* 18 by Pol

Table I: Mutagenicity of Depurination at Three Amber Loci with Purified DNA Polymerases[a]

DNA polymerase	reversion frequency ($\times 10^{-6}$) nondepurinated	reversion frequency ($\times 10^{-6}$) depurinated	mutation frequency per AP site ($\times 10^{-6}$)
Experiment 1: *amber* 3 (586–588)		2 AP sites	
E. coli Pol I	<1.50	9.47	4.74
AMV	34.0	564.0	265.0
calf thymus Pol-α	4.13	104.0	49.9
rat hepatoma Pol-β	29.6	181.0	75.7
HeLa cell Pol-γ	23.7	382.0	179.0
Experiment 2: *amber* 18 (23–25)		3 AP sites	
E. coli Pol III	<27.0	114.0	38.0
E. coli Pol I	<27.0	158.0	52.7
AMV	297.0	1100	268.0
calf thymus Pol-α	53.2	219.0	55.3
Experiment 3: *amber* to8 (5132–5134)		3 AP sites	
AMV	19.1	264.0	81.6

[a] Primers for each amber mutation, prepared as described previously (Kunkel & Loeb, 1979), were *Hae*III fragment 5 (310 base paris, 81 nucleotides from amber site) for *amber* 3, *Hpa*I fragment 2 (1264 base pairs, 6 nucleotides from amber site) for *amber* 18, and *Alu*I fragment 5 (337 base pairs, 77 nucleotides from amber site) for *amber* to8. The reversion frequencies of these primed templates prior to DNA synthesis (and independent of the presence of AP sites when transfected into noninduced spheroplasts; Schaaper & Loeb, 1981) were 1.5×10^{-6}, 27×10^{-6}, and 0.7×10^{-6}, respectively. These values are subtracted from the reversion frequencies for copied DNA shown in the table. Hybridization of primer to template was as described (Kunkel & Loeb, 1979). Copying reactions (50 μL) contained 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 10 mM $MgCl_2$, 500 μM (or 25 μM for Pol I) each of dATP, dGTP, dCTP, and [α-^{32}P]dTTP (100–500 cpm/pmol), 0.2 μg of primed ^{3}H-labeled ϕX174 amber DNA (18 800–29 300 cpm/μg), and the following amounts of enzyme: Pol I, 2.8 units (10:1 molar ratio of enzyme to DNA); Pol III (core enzyme), 10 units; AMV DNA polymerase, 10 units; calf thymus Pol-α, 0.64 unit; rat hepatoma Pol-β, 0.76 unit; or HeLa cell Pol-γ, 0.40 unit. Reactions were scaled up (in exact proportions) 3–5-fold for synthesis on depurinated DNA in order to compensate for the decreased survival of depurinated DNA in the biological transfection assay. Incubation at 37 °C was for 60 min (Pol I) or 30 min (others), at which time the reactions were stopped by addition of EDTA to 15 mM. The extent of synthesis was determined as previously described (Kunkel & Loeb, 1979) by measuring the acid-insoluble radioactivity in 1-μL aliquots. For all conditions shown here, the number of nucleotides added per input template molecule, as a calculated average, was more than sufficient to copy past the amber mutation. The remaining copied DNA was used to determine the reversion frequency of progeny phage in the transfection assay, as described in detail (Kunkel & Loeb, 1979, 1980). "Less than" values indicate that the reversion frequency was less than 2 times the control reversion frequency of uncopied DNA. The positions of the amber mutations are shown in parentheses for *amber* 3, *amber* 18, and *amber* to8 (unpublished sequencing data of R.M.S.). The number of apurinic sites per genome is shown for each experiment and determined from survival curves as described (Schaaper & Loeb, 1981).

Table II: Reversal of Mutagenesis by Pretreatment of Depurinated DNA with Alkali or Apurinic Endonuclease[a]

template treatment	reversion frequency ($\times 10^{-6}$) nondepurinated	depurinated
Experiment 1: Avian Myeloblastosis Virus		
		1 AP site
none	49.5	399.0
alkali	54.0	55.1
Experiment 2: *E. coli* Pol I		
		2 AP sites
none	2.92	11.35
AP-endonuclease	2.64	2.01

[a] Pretreatment of both nondepurinated and depurinated ϕX174 *amber* 3 DNA with alkali was carried out in 0.1 M NaOH for 2.5 h at 20 °C. After neutralization with HCl, the DNA was copied with AMV DNA polymerase as indicated in the legend to Table I. Pretreatment with AP-endonuclease was carried out in a reaction volume of 50 μL containing 1.0 μg of ϕX174 DNA (either nondepurinated or depurinated), 25 mM Tris-HCl (pH 7.5), 0.005% Triton X-100, 0.1 mM EDTA, and 10 units of HeLa apurinic endonuclease (Schaaper et al., 1983). Incubation was for 30 min at 37 °C. The DNA was then copied and transfected as described in the legend to Table I. In all cases, synthesis as a calculated average was sufficient to copy past the amber mutation. The uncopied DNA reversion frequency, which is subtracted from the values shown, was 1.50×10^{-6} (experiment 1) and 3.60×10^{-6} (experiment 2).

III (core) polymerase in vitro results in a reversion frequency of 38×10^{-6} per AP site, comparable to a reversion frequency of $(30\text{–}35) \times 10^{-6}$ per AP site for DNA polymerase III in vivo in SOS$^+$-induced spheroplasts (Schaaper & Loeb, 1981). While these comparisons may be entirely circumstantial, it is interesting to speculate that SOS induction might result in the dissociation of the normal Pol III holoenzyme into the core enzyme, and it is the latter that might be responsible for the observed mutagenesis.

Reversal of Depurination-Induced Mutagenesis. We have shown that alkali pretreatment of depurinated DNA reverses the enhancement in mutagenicity observed when the DNA is copied by Pol I in vitro (Kunkel et al., 1981c). The experiments in Table II substantiate the conclusion that the AP site is the premutagenic lesion. Pretreatment with alkali also reverses the depurination-induced mutagenesis with AMV DNA polymerase. Moreover, hydrolysis of depurinated DNA with an apurinic endonuclease abolishes the enhancement in mutagenesis after copying with Pol I. These data make it unlikely that another, perhaps minor, lesion is responsible for mutagenesis with either Pol I or AMV DNA polymerase. These results complement the studies on mutagenicity of depurinated sites in vivo observed in SOS-induced spheroplasts in that similar treatments abolished mutagenicity (Schaaper & Loeb, 1981; Schaaper et al., 1983).

Incorporation into Depurinated DNA and Product Analysis. The observation that mutagenicity in vivo depends on error-prone DNA repair suggests that apurinic sites slow down or block the movement of the DNA synthetic apparatus. This led us to examine in more detail the relationship between bypass of an apurinic site and mutagenicity. As shown previously (Schaaper et al., 1983) and extended in Figure 1, the presence of a large number of apurinic sites in the template inhibits both Pol I and AMV DNA polymerase. However, AMV polymerase, which is inherently error prone (Gopinathan et al., 1979) and lacks a proofreading 3′ → 5′ exonuclease (Battula & Loeb, 1976), copies over AP sites with a much higher frequency than does Pol I (75% vs. <10% bypass; Schaaper et al., 1983). This differential ability to bypass correlated with depurination-induced mutagenesis; i.e., AMV

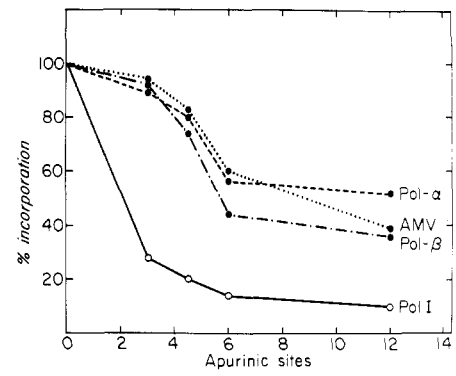

FIGURE 1: Effect of depurination on the extent of DNA synthesis by purified DNA polymerases. Reactions were performed as described in Table I, using Z8-primed ϕX174 *amber* 3 DNA containing increasing numbers of apurinic sites as indicated. The 100% incorporation values (calculated average number of nucleotides added per input template molecule) were as follows: Pol I, 3520 (○—○); AMV DNA polymerase, 3350 (●···●); calf thymus Pol-α, 867 (●---●); rat hepatoma Pol-β, 969 (●–·–●).

Table III: Restriction Endonuclease Fragment Analysis of Products of DNA Polymerase Reactions on Depurinated DNA[a]

DNA polymerase	no. of apurinic sites	cpm in Z7 fragment	% of control
Pol I	0	134	100
	6	64	48
	24	22	16
AMV	0	178	100
	6	194	109
	24	182	102
Pol-α	0	74	100
	12	56	76
Pol-β	0	171	100
	12	134	78

[a] Reactions were performed as described in the legend to Table I by using Z8-primed ϕX174 *am*3 DNA. The products of the reactions were phenol extracted, dialyzed, ethanol precipitated, resuspended, and digested with restriction endonuclease *Hae*III. The digest was subjected to electrophoresis in a 5% polyacrylamide gel as described previously (Schaaper et al., 1983). Fragments were identified by autoradiography, cut out, and counted for determination of ^{32}P radioactivity in the Z7 fragment. The order of fragments synthesized is Z8, Z5, and Z7. Z7 is the second fragment after the primer, representing synthesis of 10% of the ϕX174 cycle.

polymerase shows frequent bypass and high mutagenesis, while Pol I shows less frequent bypass and mutagenesis. The higher depurination-induced mutagenesis seen with the eukaryotic DNA polymerases when compared to Pol I suggests that these enzymes, like AMV DNA polymerase, might copy over AP sites more frequently (Table I). The data in Figure 1 and Table III support this idea. The inhibition of synthesis by apurinic sites is similar for Pol-α, Pol-β, and AMV DNA polymerase, and all of these are substantially less inhibited than Pol I. Since measurements of the average incorporation per added template may not be indicative of the average length of newly synthesized DNA, the products of these reactions were analyzed by gel electrophoresis after restriction by endonuclease *Hae*III. In the case of fragment Z7 (Table III), containing the *amber* 3 locus and representing synthesis of 10% of the circle (starting from a Z8 primer), the amount of fragment produced on depurinated relative to nondepurinated DNA is least for Pol I, greatest for AMV DNA polymerase, and intermediate for Pol-α and -β. Although not absolutely quantitative, these data correlate well with the level of mutagenesis shown in Table I and support the conclusion that bypass is mutagenic.

Table IV: Effect of Diminished Proofreading on Incorporation and Mutagenesis by Pol I on Depurinated ϕX174 DNA[a]

condition	incorporation[b] nondep	incorporation[b] depur	incorporation[b] % (depur/nondep)	reversion frequency ($\times 10^{-6}$) nondep	reversion frequency ($\times 10^{-6}$) depur
Experiment 1: Variable "Next Nucleotide"					
25 μM dNTP	1070	89	8.3	<1.50	9.47
500 μM dNTP	1310	153	11.7	4.37	45.5
Experiment 2: Monophosphate Inhibition					
0 mM dNMP	1070	89	8.3	<1.50	6.64
20 mM dGMP[c]	1070	99	9.2	4.19	25.5
20 mM dCMP[c]	1190	144	12.2	4.22	56.7

[a] *E. coli* DNA polymerase I copying reactions were performed as described in the legend to Table I but with the variations listed. Abbreviations: nondep, nondepurinated; depur, depurinated. [b] Incorporation is expressed as the average number of nucleotides added per input template molecule, assuming all molecules are utilized to the same extent. All reversion frequency measurements were performed on 0 vs. 2 apurinic sites per template. Incorporation data were obtained for 0 vs. 12 AP sites per template. [c] The monophosphate concentration for the incorporation data was 10 mM. The control reversion frequency of uncopied DNA in these experiments averaged 1.50×10^{-6} for six determinations and was subtracted from the values shown.

Table V: Effect of *E. coli* Single-Strand DNA-Binding Protein on Depurination-Induced Mutagenesis with Pol I[a]

SSB[b]	incorporation nondep	incorporation depur	incorporation % (depur/nondep)	reversion frequency ($\times 10^{-6}$) nondep	reversion frequency ($\times 10^{-6}$) depur
Experiment 1: 25 μM dNTP, 0 vs. 2 AP Sites					
0.0	2510	790	31.5	2.71	19.8
0.1				2.23	9.62
0.5	2020	632	31.3	<1.50	6.52
1.0	1830	590	32.2	<1.50	8.16
Experiment 2: 500 μM dNTP, 0 vs. 2 AP Sites					
0.0	3200	1470	45.9	4.96	42.8
0.5	2060	833	40.4	2.70	9.07
1.0	1930	752	39.0	<1.50	14.8
Experiment 3: 25 μM dNTP, 0 vs. 12 AP Sites					
0.0	1070	89	8.3		
0.5	1660	191	11.5		
1.0	1290	179	13.9		

[a] Reactions were performed as described in the legend to Table I. The DNA was phenol extracted before transfection. [b] The SSB concentration is expressed as the amount needed to saturate 10, 50, or 100% of the single-stranded ϕX174 DNA template, calculated as described previously (Kunkel et al., 1979).

Effect of Proofreading on Depurination-Induced Mutagenesis. It has been suggested that the inability of a DNA polymerase to copy over blocking lesions, in particular pyrimidine dimers, is caused by the (3′ → 5′) proofreading exonuclease activity associated with the enzyme which effectively causes it to "idle" at the lesion (Villani et al., 1978). Since the limited ability of Pol I to copy over apurinic sites is in accord with this argument, we examined its proofreading in relation to the mutagenic bypass of apurinic sites. The approach was to measure both incorporation and mutagenesis by Pol I on normal vs. depurinated DNA by two different methods which diminish proofreading activity. The first method (Table IV, experiment 1) utilizes low (25 μM) vs. high (500 μM) dNTP substrates. With nondepurinated DNA, proofreading is diminished at high dNTP concentrations presumably due to the rapid rate of incorporation of the next nucleotide immediately after a misincorporation event, resulting in less time to excise the error (Kunkel et al., 1981b). With depurinated DNA, increasing the dNTP concentration from 25 to 500 μM had little effect on incorporation with depurinated vs. nondepurinated DNA (8.3% compared to 11.7%). However, measurements of incorporation are less sensitive than measurements of reversion frequency in the biological assay. The reversion frequencies showed a 4.5-fold increase in depurination-induced misincorporation (45.5×10^{-6} vs. 9.47×10^{-6}) at high dNTP concentrations. These data suggest a small but significant role for proofreading in determining the degree of mutagenesis with depurinated DNA.

The second approach to diminish proofreading was to add deoxynucleoside monophosphates to the in vitro DNA synthesis reactions. Any of the four dNMPs are capable of reducing proofreading (Kunkel et al., 1981b), presumably by binding to the active site of the 3′ → 5′ exonuclease and diminishing the preferential hydrolysis of misincorporated terminal nucleotides. The results (Table IV, experiment 2) are in accord with the first approach, i.e., little effect on the total incorporation but a severalfold increase in mutagenesis. These results suggest that the 3′ → 5′ exonuclease actively hydrolyzes during polymerization terminally incorporated nucleotides opposite depurinated sites at a greater rate than at correctly base-paired termini.

Effect of Single-Stranded DNA-Binding Protein on Mutagenesis. E. coli single-stranded DNA-binding protein (SSB) has been shown to be essential for DNA replication (Meyer et al., 1979). Furthermore, SSB has also been shown to increase the accuracy of DNA polymerases in vitro (Kunkel et al., 1979). We examined the effect of SSB on incorporation and mutagenesis by using depurinated templates. In two separate experiments, the first at low dNTP concentration (25 μM) to allow proofreading (Table V, experiment I) and the second at 500 μM dNTP to decrease proofreading and give higher depurination-induced mutagenesis (Table V, experiment 2), the addition of SSB to in vitro DNA synthesis reactions catalyzed by Pol I had little effect on synthesis but resulted in a 2–5-fold decrease in mutagenesis on depurinated templates (e.g., cf. 19.0×10^{-6} vs. 9.62×10^{-6}, or 42.8×10^{-6} vs. 9.07×10^{-6}). When incorporation into DNA containing a large number of apurinic sites was determined to allow a more sensitive measure of potential changes in incorporation caused by SSB, no significant increase was observed (Table V, experiment 3).

DNA Sequence Analysis of Depurination-Induced Revertants. When depurination-induced *amber* 3 revertants obtained in vivo from experiments using SOS-induced spheroplasts were analyzed by DNA sequence analysis, in 12 of 13 instances an A → T base-substitution mutation was observed (Schaaper et al., 1983). These presumably result from the incorporation of an A opposite a putative apurinic site at position 587, the middle position of the TAG amber codon. This unexpected preference for insertion of A was observed in vivo at two additional loci (Schaaper et al., 1983). In order to understand this preference, we examined the DNA sequence of revertants from in vitro DNA synthesis reactions catalyzed by Pol I or AMV DNA polymerase on normal vs. depurinated ϕX174 *amber* 3 DNA. As expected from previous studies (Kunkel et al., 1981b), revertants from Pol I reactions on nondepurinated DNA are predominantly produced by misincorporation of dCMP opposite the A at position 587 (9 of 11, Table VI, experiment 1), an event which produces the true wild-type DNA sequence. In contrast, with depurinated DNA, the revertants produced during in vitro DNA synthesis are predominantly caused by misincorporation of dAMP opposite

Table VI: DNA Sequence Analysis of Depurination-Induced Misincorporation at the *amber* 3 Locus by Pol I and AMV DNA Polymerase[a]

no. of apurinic sites	reversion frequency ($\times 10^{-6}$)	substitution at 587		
		C	A	G
Experiment 1: *E. coli* DNA Polymerase I, 500 μM dNTP				
0	4.83[b]	9	2	0
2	45.6	2	5	0
Experiment 2: AMV DNA Polymerase, 500 μM dNTP				
0	62.4	1	8	1
2	910.0	0	10	0

[a] DNA sequencing of independent revertants was performed as described (Kunkel & Loeb, 1980). [b] The value shown is the reversion frequency obtained in the experiment, representing only a 2-fold increase above the background reversion frequency (2.62×10^{-6}). For this reason, the actual revertants sequenced shown for nondepurinated DNA were obtained from copying reactions performed with a 50-fold excess of incorrect substrates (dCTP, dATP, dGTP) over correct substrate (dTTP). In this case, the reversion frequency was 177.0×10^{-6}, well above background. The revertants sequenced are therefore due to errors by Pol I under conditions in which the enzyme had an equal opportunity to misincorporate any one of three incorrect substrates. In all other reactions, the revertants sequenced were those indicated by the change in reversion frequency shown.

the putative apurinic site at position 587. The much less accurate AMV DNA polymerase shows a distinctly different substitution pattern from Pol I on nondepurinated DNA. In 8 of 10 instances, revertants were the result of misincorporation of dAMP, with one each of the two remaining possible incorrect substitutions (dCMP and dGMP). Depurination results in a 15-fold increase in mutagenesis, and thus the majority of substitutions sequenced result from depurination. Misincorporation by AMV DNA polymerase at position 587 on depurinated template was exclusively dAMP (10 of 10, Table VI, experiment 2). Therefore, the specificity observed in vivo repeats itself in vitro with two different DNA polymerases and might reflect a template–polymerase interaction common to all polymerases.

Discussion

The data in this paper clearly demonstrate the mutagenic potential of unrepaired apurinic sites in DNA. These in vitro studies with defined proteins were intended to address two basic issues concerning depurination-induced mutagenicity. The first was to determine the extent of the potential challenge presented by unrepaired apurinic sites. The in vitro data indicate qualitatively that misincorporation resultant from depurination is restricted neither to specific sites nor to DNA polymerase; mutagenesis was observed at three different loci and with all DNA polymerases tested (Table I). Several implications of these observations can be deduced. (1) Apurinic sites are a potential source for mutations throughout the genome, although quantitative differences in mutagenic potential are noted (vide infra). (2) All DNA polymerases examined, including the highly accurate *E. coli* DNA polymerases, exhibited enhanced misincorporations. This includes DNA polymerases considered to function in different aspects of DNA metabolism, including nuclear replication, repair, recombination, and mitochondrial replication. Encountering an apurinic site may thus be mutagenic in any of these processes, although the frequency of substitutions will presumably be modulated by other proteins. (3) Depurination-induced mutagenicity is even more frequent with eukaryotic DNA polymerases, suggesting that apurinic sites may represent a major mutagenic challenge in higher organisms containing 100–1000-fold more DNA than prokaryotes.

The realization that DNA polymerases do not work independently of other proteins in vivo led us to examine the effect of addition of a second protein known to be important for in vivo DNA synthesis, single-stranded binding protein, on mutagenesis. This protein has been shown to increase the accuracy of Pol I when copying nondepurinated ϕX174 DNA in vitro (Kunkel et al., 1979). Similarly, the depurination-induced mutagenicity by Pol I is decreased in the presence of SSB by 2–5-fold (Table V). However, even at saturating levels of SSB, substantial mutagenesis is still observed with depurinated DNA. Thus, the mutagenic potential of unrepaired apurinic sites is not eliminated in this multicomponent in vitro system and is consistent with the mutagenicity observed in vivo (Schaaper & Loeb, 1981).

The second basic issue concerns the frequency and specificity of the depurination-induced mutagenicity. Quantitative differences in the frequency of mutagenicity per apurinic site are observed at different loci and with different polymerases (Table I). Several explanations can be invoked. It is conceivable that depurination is not random, and the rate of depurination of an A or G may vary considerably at different sites. Certain misincorporation events at amber codons may produce nonfunctional changes in the amino acid composition of the protein, causing site-specific differences in the apparent mutagenicity. Also, damage-dependent mutagenesis might not always be targeted at the site of the lesion but could occur at nucleotides at some distance away from the lesion. Site-specific differences as observed here then might also reflect variations in untargeted [or semitargeted (Schaaper & Glickman, 1982)] mutagenesis.

Enzyme-mediated differences in the degree of mutagenicity associated with apurinic sites at a particular locus can be quite large (e.g., 60-fold for Pol I vs. AMV) (Table I). One reasonable explanation for those differences is that mutagenesis results from misincorporation opposite an apurinic site, the degree being a reflection of how frequently the enzyme copies over such a site. This explanation is supported qualitatively by the incorporation and mutagenicity data in vitro. The explanation, while attractive, must be carefully qualified, since enzymes with the same apparent bypass frequency (Figure 1) can have a 5-fold difference in depurination-induced mutagenicity (cf. Pol-α, 104×10^{-6}, to AMV polymerase, 564×10^{-6}). Experimentally, both determinations are subject to considerable error. Nevertheless, it is clear that those enzymes which are least inhibited by apurinic sites are also the ones that produce more revertants per apurinic site. This is consistent with the concept inherent in the SOS hypothesis that mutagenicity results from the bypass of otherwise blocking lesions.

In the consideration of what determines the bypass frequency, the encounter of a DNA polymerase with an apurinic site can be envisioned as follows: (1) The enzyme may or may not insert a nucleotide opposite the apurinic site, the probability of insertion being a reflection of the inherent (insertion) accuracy of the enzyme. (2) If a nucleotide has been inserted, the enzyme may excise the new residue with a certain frequency. This possibility applies only to enzymes that contain a $3' \rightarrow 5'$ exonuclease. In more extreme cases, the enzyme may idle, as has been described in some detail in the case of pyrimidine dimers (Villani et al., 1978). (3) Once stably inserted, the enzyme may or may not extend from the new and somewhat unusual primer terminus, depending on the properties of the enzyme. While by definition DNA polymerases are capable of extending from mismatched bases (since they

all make base substitution errors), enzymes clearly differ in this ability. Only in the case of a successful extension can a mutation result. Finally, all three of these possibilities are expected to be position dependent, since site-specific differences have been found for undamaged templates (Fersht, 1979; Fersht & Knill-Jones, 1981; Hibner & Alberts, 1980; Sinha & Haimes, 1981).

In light of the above-mentioned complexity, a simple experimental answer to the question of the mechanism of mutagenesis at apurinic sites cannot yet be formulated. Nevertheless, several valuable conclusions may be drawn. Consider the results with AMV DNA polymerase and calf thymus DNA polymerase α (Table I). Both enzymes are highly purified and lack detectable proofreading activity. On nondepurinated ϕX174 DNA, AMV DNA polymerase is less accurate than Pol-α by a factor of 5–8-fold (Table I). On depurinated DNA, induced mutagenicity under identical experimental conditions is 5-fold less for Pol-α (Table I). Thus, the inherently less accurate enzyme with nondepurinated DNA is also less accurate with depurinated DNA. In the absence of proofreading, this may reflect a difference in bypass due to discrimination at the insertion and/or the extension step. Note that the 5-fold difference between the enzymes is not readily observed in the direct incorporation assay. The limited sensitivity of this assay, particularly in the range observed, however, may obscure small differences. Alternatively, AMV DNA polymerase may be particularly prone to untargeted mutagenesis.

The second enzyme comparison, the one which first led us to examine incorporation data quantitatively, was of Pol I to AMV DNA polymerase. With nondepurinated DNA, Pol I is more accurate at the insertion step (Kunkel et al., 1981a,b) and in addition can excise mismatches by proofreading (Kunkel et al., 1981a,b). Thus, the large difference between Pol I and AMV DNA polymerase in incorporation and mutagenicity on depurinated DNA could reflect either or both mechanisms of reducing errors. The attempts to diminish proofreading (Table IV) show a 2–5-fold effect on the reversion frequency, suggesting that proofreading is involved in error discrimination at apurinic sites. This is not unexpected, since proofreading exonucleases are most active on mismatched bases and frayed ends (Kornberg, 1980), and in one sense, a base incorporated opposite an apurinic site is mismatched or frayed. However, proofreading may not be solely responsible for the differential discrimination between Pol I and Pol-α against incorporation at an apurinic site, since Pol I is approximately 2–3-fold more accurate at the level of insertion than is Pol-α (Kunkel & Loeb, 1981; Kunkel et al., 1981a,b). On depurinated DNA, under conditions of diminished proofreading where misinsertion frequencies play a major role in fidelity, Pol I is also 2-fold more accurate than Pol-α, e.g., Pol I, 45.5×10^{-6} (Table IV, experiment 1), vs. Pol-α, 104×10^{-6} (Table I, experiment 1). It thus appears in two instances (Pol-α vs. AMV polymerase and Pol-α vs. Pol I in the presumed absence of proofreading) that relative fidelities on normal templates are maintained on apurinic templates. This may be fortuitous but could also have a more fundamental significance as to the mechanism(s) which determine(s) the accuracy of DNA replication.

Specificity of Mutation in Vitro. In our in vivo studies on specificity, it was observed that the enhancement of mutagenesis by depurination primarily represented the substitution of dAMP opposite the putative apurinic site. Transfection of uncopied *am*3 ϕX DNA in SOS-induced spheroplasts resulted in a 20-fold increase in the reversion frequency, and in 12 of 13 revertants, dAMP was inserted at position 587 (Schaaper et al., 1983). Explanations for this specificity in vivo include the following: (1) a substitution preference for dATP or possibly rATP by the SOS-altered replication complex; (2) alteration in nucleotide pools during SOS induction; (3) repetitive recognition of the next nucleotide (T) at position 586 resulting from looping out of the apurinic site; (4) nontemplate-directed preferential binding of dATP by the polymerase; and (5) apurinic site instructed incorporation of deoxyadenosine. The fact that a similar preference for misincorporation of dAMP is exhibited by purified *E. coli* DNA polymerase I and AMV DNA polymerase in vitro (15 of 17 revertants contained dAMP at position 587) suggests that in vivo specificity is not the result of pool alterations or SOS induction. Recently, Strauss et al. (1982) observed preferential insertion of dAMP opposite pyrimidine dimers during synthesis with Pol I. Based on the tight binding of Pol I to dATP and dGTP in the absence of template, they suggested that the specificity of misincorporation opposite noncoding lesions mimics the inherent affinity of the isolated enzyme for dNTP substrates. This concept is consistent with our results, since apurinic sites clearly can be classified as noncoding lesions.

Our studies on apurinic sites suggest that, if unrepaired, such lesions could result in significant mutagenesis. This is especially true in eukaryotes with large genomes and with purified DNA polymerases that copy past apurinic sites with high frequency. Furthermore, the mutagenic potential of apurinic sites might be underestimated, since the ϕX system is limited to base-substitution events and does not score for frame shifts and deletions. In vivo experiments in mammalian cells analogous to the *E. coli* experiments have not yet been carried out. In light of our observations with the eukaryotic polymerases, a highly mutagenic response is predicted.

Registry No. Pol I, 9012-90-2; Pol III, 37217-33-7.

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Relationship between Histone H1 Poly(adenosine diphosphate ribosylation) and Histone H1 Phosphorylation Using Anti-Poly(adenosine diphosphate ribose) Antibody†

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ABSTRACT: The chromatin-associated enzyme poly(ADP-Rib) polymerase catalyzes the posttranslational modification of histones. Antibody to poly(ADP-Rib) has been coupled to Sepharose, and the resultant immunoadsorbent was used to fractionate, specifically, histone H1 subpopulations undergoing this nuclear protein modification. When this method of separation was used, it was additionally observed that poly(ADP-ribosylated) H1 species were highly accessible to in vitro phosphorylation by nuclear protein kinase. Phosphorylated H1 molecules were retained by the anti-poly(ADP-Rib)–Sepharose column due to the presence of endogenous poly(ADP-Rib) components. Degradation of the latter moieties on phosphorylated H1 reversed their adsorption to the column.

Histone H1 has been demonstrated to play a major role in the higher ordered structure of chromatin (Worcel, 1978; Thoma & Koller, 1977). Multiple forms of histone H1, in tissues and cells, differ in their primary structure. In addition, postsynthetic modifications of histone H1 such as poly(ADP-ribosylation) and phosphorylation are thought to modulate the regulation of various biological processes. Our laboratory has been particularly interested in the poly(ADP-ribosylation) of histone H1 (Nolan et al., 1980; Butt et al., 1980; Wong et al., 1982, 1983). With the recent development of a highly specific antibody directed against the modified moiety, poly(ADP-Rib),[1] we thought it timely to test whether an immunological affinity method could be employed to bind selectively those very limited subpopulations of histone H1 within chromatin which undergo ADP-ribosylation. Second, it was of importance to ascertain whether or not phosphorylation and poly(ADP-ribosylation) of histone H1 are coordinated within the cell.

Poly(ADP-Rib) polymerase, a chromatin-associated enzyme, catalyzes the successive transfer of the ADP-Rib moiety of NAD to various nuclear proteins. One novel product of this reaction is an H1 complex, consisting of two molecules of H1 cross-linked by 15–16 units of poly(ADP-Rib) (Stone et al., 1977). We have speculated that this poly(ADP-Rib) complex of H1 may help stabilize nucleosomes during various physiological events of the cell cycle (Wong et al., 1983). From reconstitution studies, poly(ADP-ribosylated) H1 complex has been indirectly shown to be involved in the condensation of those limited domains of chromatin undergoing this modification (Butt et al., 1980; Wong et al., 1982).

Kidwell & Mage (1976) have shown that the maximal accumulation of cellular poly(ADP-Rib) occurs during the G2 phase of the eukaryotic cell cycle; recent immunological data have also suggested very high activity for the enzymatic synthetic reaction during metaphase of the cell cycle (Tanuma

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[1] Abbreviations: ADP-Rib, adenosine diphosphate ribose; NAD, nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; KSCN, potassium thiocyanate; Cl_3CCOOH, trichloroacetic acid; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; PCA, perchloric acid; DTT, dithiothreitol.

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