Response of Phage T4 Polynucleotide Kinase toward Dinucleotides Containing Apurinic Sites: Design of a ^{32}P-Postlabeling Assay for Apurinic Sites in DNA†

Michael Weinfeld,*,‡ Michel Liuzzi, and Malcolm C. Paterson

Laboratory of Molecular Genetics and Carcinogenesis, Department of Medicine, Cross Cancer Institute, Edmonton, Alberta T6G 1Z2, Canada

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ABSTRACT: We have examined the capacity of bacteriophage T4 polynucleotide kinase (EC 2.7.1.78) to phosphorylate the partially depurinated products of d-ApA, namely, d-SpA and d-ApS (where S represents an apurinic deoxyribose group). It was observed that the enzyme acted only on the latter isomer. Since molecules of this type (d-NpS) are the sole apurinic site containing products resulting from the combined digestion of lightly depurinated DNA by snake venom phosphodiesterase and calf alkaline phosphatase [Weinfeld, M., Liuzzi, M., & Paterson, M. C. (1989) *Nucleic Acids Res. 17*, 3735–3745], we were able to devise a postlabeling assay for these biologically important DNA lesions. The method offers several advantages, including (a) elimination of the need for prelabeled DNA, (b) high (femtomole range) sensitivity, and (c) nearest-neighbor analysis of bases 5′ to apurinic/apyrimidinic sites. Using this assay, we obtained a value for the rate of depurination of form I pRSVneo plasmid DNA, incubated at pH 5.2 at 70 °C, of approximately 3.3 apurinic sites per plasmid molecule per hour. This value compares favorably with previously published data of others, acquired by alternative approaches. The rate of depurination of poly(dA), treated in a similar fashion, was found to be ~1 base per 10^3 nucleotides per hour.

Loss of bases, primarily purines, from DNA occurs spontaneously in living cells (Lindahl & Nyberg, 1972). Extrapolating from results obtained at elevated temperatures, it has been postulated that approximately 10^4 apurinic/apyrimidinic (AP) sites arise in each mammalian cell per day (Lindahl & Nyberg, 1972). Additional AP sites can be induced chemically by DNA damaging agents, such as aliphatic and aromatic alkylating agents (Kochetkov & Budovskii, 1972; Osborne & Merrifield, 1985) and ionizing radiation (Ward, 1975), and also enzymatically by DNA glycosylases involved in DNA repair processes (Lindahl, 1982). AP sites are themselves repaired in vivo because of their potential as mutagenic lesions (Loeb & Preston, 1986).

In vitro experiments have shown variable responses of DNA acting enzymes toward these lesions. For example, several DNA polymerases, especially from eukaryotes, catalyze replication past AP sites with high efficiency, while prokaryotic polymerases experience great difficulty in copying past the damaged sites (Kunkel et al., 1983). In an earlier study (Weinfeld et al., 1989a), we examined how a number of exo- and endonucleases react with phosphodiester bonds adjacent to an abasic site. It was observed that the activity of each of these enzymes was dependent upon whether the AP site lay 5′ or 3′ to the phosphodiester linkage. Thus, nuclease P1 hydrolyzed d-ApS (a partially depurinated dinucleoside monophosphate containing an AP site 3′ to the internucleotide phosphate bond), but was unable to cleave the isomeric molecule (d-SpA). On the other hand, snake venom phosphodiesterase cleaved d-SpA but not d-ApS. Stuart and Chambers (1987) similarly found that complete digestion of a longer oligonucleotide by venom phosphodiesterase released the depurinated nucleotide in association with its 5′-neighboring nucleotide, i.e., as a molecule with the structure d-pNpS. Experiments such as these provide information concerning the nucleic acid components required by substrates for full enzymatic activity.

In this paper, we demonstrate that phage T4 polynucleotide kinase (EC 2.7.1.78) also displays a selective response toward the two AP site containing isomers mentioned above. The enzyme can catalyze phosphorylation at the 5′-hydroxyl group of d-ApS, but not of d-SpA, a result that suggests binding of the substrate to the kinase requires the presence of a base on the deoxyribose accepting the phosphate group. In addition, we describe how we were able to exploit our observations and those of Stuart and Chambers (1987) to set up a sensitive postlabeling assay for AP sites.

Materials and Methods

Chemicals. The four dinucleoside monophosphates, d-ApA, d-TpA, d-CpA, and d-GpA, as well as their respective 5′-phosphorylated analogues, oligo(dT)$_{16}$ and poly(dA)$_{600}$ were purchased from Pharmacia Canada (Dorval, PQ). Adenine, guanine, the four normal 5′-deoxyribonucleotides, ATP, and methoxyamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). pRSVneo plasmid DNA (Gorman et al., 1983) was kindly supplied by our colleague Dr. R. Aubin and shown to be ~80% form I double-stranded DNA and the remainder form II. [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol at a concentration of 3.3 μM) was purchased from both Amersham Canada (Oakville, ON) and NEN Canada (Montreal, PQ).

Enzymes. DNase I, calf alkaline phosphatase, and nuclease P1 were purchased from Boehringer Mannheim Canada (Dorval, PQ). Snake (*Crotalus adamanteus*) venom phosphodiesterase was obtained from Sigma and T4 polynucleotide

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‡ Present address: Radiobiology Research Program, Cross Cancer Institute, Edmonton, Alberta T6G 1Z2, Canada.

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kinase from Pharmacia. Definitions of the units for each of these enzymes were those given by the supplier.

HPLC. The instrumentation consisted of a computer-controlled Waters 840 system coupled to a Waters 490 multiwavelength detector (Waters Associates, Mississauga, ON). Peaks of UV-absorbing material were digitally integrated by the 840 system. Reverse-phase HPLC was performed on a Whatman Partisil 10 ODS-2 column (250 × 4.6 mm i.d.; Whatman Inc., Clifton, NJ). Elution conditions were as follows: 100% buffer A (50 mM NaH_2PO_4, pH 4.5) and 0% buffer B [100 mM NaH_2PO4, pH 4.5/methanol (1:1 v/v)] for 1 min followed by a linear gradient to 0% buffer A and 100% buffer B over 30 min. The flow rate was 1 mL/min.

Gel Electrophoresis. This was carried out on a Hoefer SE 620 system (Hoefer Scientific Instruments, San Francisco, CA), employing 18 × 32 cm plates and 1.5-mm spacers; 20% polyacrylamide/7 M urea gels were prepared according to published procedures (Maniatis et al., 1982) and run at 800 V until the bromophenol blue marker had migrated 11–12 cm (3–4 h). Kodak X-Omat K film was used for autoradiography.

Preparation of Substrates and Markers. The preparation of d-SpA and d-ApS by depurination of d-ApA in 0.1 M HCl at 37 °C and their characterization have been described previously (Weinfeld et al., 1989a). The other purine–purine dimers, d-GpG, d-pApA, and d-pGpG (2.5 A_{260} of each), were depurinated in a similar fashion; d-GpG was incubated for 30 min, and the terminally phosphorylated molecules were each incubated for 60 min. Their monodepurinated derivatives were isolated by HPLC using the gradient system detailed above. Molecules with a 5′-AP site (d-SpA, d-pSpA, d-SpG, and d-pSpG) were distinguished from their respective isomers with a 3′-AP site by cleavage of the internucleotide phosphodiester bond of the former in 0.2 M NaOH at 37 °C for 15 min. In the case of the pyrimidine–purine dimers, which show virtually no loss of the pyrimidine base, the acid depurination was carried out for 3–4 h to optimize the yield (~95%) of the depurinated derivatives.

After each compound was recovered from the HPLC column, it was taken to dryness and redissolved in 1 mL of distilled water. Half was desalted by passage through a Waters C_{18} SEP-PAK cartridge (Weinfeld et al., 1989a), evaporated, and dissolved in distilled water at a concentration of 0.2–0.5 A_{260} units/mL. The remaining 0.5 mL of recovered material was brought to pH 7.0 by addition of ~10 μL of 0.4 M NaOH, and to this solution was added 55 μL of 1 M methoxyamine (pH 7.0). The mixture was incubated for 1 h at 37 °C, and each methoxyamine addition product (d-NpM[1] and d-pNpM) was isolated by HPLC, desalted, and resuspended in distilled water as detailed above. The products were stored frozen at −20 °C. UV spectral characteristics and HPLC retention times for all the compounds produced are presented in Table I.

Treatment of d-ApA, d-SpA, d-ApS, d-MpA, and d-ApM with T4 Polynucleotide Kinase and Unlabeled ATP. Each reaction mixture (20 μL) contained kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, and 0.1 mM EDTA), 0.4 nmol of "dinucleoside" monophosphate, 2 nmol of ATP, and 10 units of kinase. The samples were incubated at 37 °C for 60 min and then analyzed by reverse-phase HPLC.

Table I: UV Spectral Properties[a] and HPLC Retention Times[b] of the Monodepurinated Compounds and Their Methoxyamine-Modified Derivatives

compound	λ_{max} (nm)	λ_{min} (nm)	A_{260}/A_{280}	retention time (min)
d-ApS	258	225	4.54	21.1
d-GpS	251	222	1.47	17.6
d-CpS	270	249	1.11	13.8
d-TpS	266	234	1.50	17.6
d-ApM	260	232	4.47	25.9
d-GpM	252	226	1.44	21.7
d-CpM	270	251	1.13	18.4
d-TpM	266	240	1.46	22.3
d-pApS	258	227	4.48	15.1
d-pSpA				15.8
d-pGpS	253	222	1.45	13.0
d-pCpS	273	246	0.85	9.8
d-pTpS	266	234	1.44	12.8
d-pApM	259	229	6.17	19.3
d-pGpM	252	225	1.47	16.7
d-pCpM	274	245	0.78	14.8
d-pTpM	266	235	1.44	16.7

[a] In distilled water. [b] Column and elution conditions described under Materials and Methods.

Preparation of Radiolabeled Markers. To generate labeled markers, an excess of each oligonucleotide (d-NpM) was incubated with ^{32}P-labeled ATP to ensure nearly complete uptake of radioactivity by the oligonucleotides. Each depurinated dinucleoside monophosphate (~50 nmol) was incubated in kinase buffer with 3.3 pmol of [γ-^{32}P]ATP and 10 units of polynucleotide kinase (total volume, 20 μL). The reaction was allowed to proceed at 37 °C for 1 h, after which time the samples were stored frozen at −20 °C.

Recovery of Labeled Markers from a Polyacrylamide Gel. Aliquots containing 6 × 10^5 cpm of each of the four labeled d-pNpM markers were mixed and applied to a single slot of a 20% polyacrylamide/7 M urea gel (see above for gel details). After the gel had been run and an autoradiogram taken, the area of the gel containing the radioactive contents was excised. The radiolabeled products were extracted from the gel by mashing up the gel slice and incubating it overnight at 37 °C in 1 mL of distilled water. (This treatment released about 2 × 10^6 cpm of the 2.4 × 10^6 cpm initially applied.) The gel material was removed by filtration through a glass wool pad and rinsed with another 2 mL of distilled water. Approximately 4 × 10^4 cpm of the filtrate was applied to the reverse-phase column, along with unlabeled markers of each d-pNpM. Eighty 0.3-mL fractions were collected and Cerenkov counted.

Depurination of pRSVneo and Poly(dA). Fifteen microliters of pRSVneo (2.5 mg/mL) or 50 μL of poly(dA) (25 A_{260} units/mL, 970 μg/mL) was added to 1.5 mL of preheated (70 °C) depurination buffer (10 mM sodium citrate, 10 mM NaH_2PO_4, and 10 mM NaCl brought to pH 5.2 with citric acid) and held at 70 °C. At the time intervals indicated in Figure 4C, 250-μL aliquots were removed from these reaction mixtures, added to 0.25 mL of ice-cold methoxyamine solution (100 mM methoxyamine in 80 mM sodium phosphate, pH 7.5), and incubated for 30 min at 37 °C. The DNA was then dialyzed, for 3 h each time, at 4 °C against 2 × 1.5 L of distilled water, followed by 1.5 L of 20 mM Tris-HCl (pH 7.5) and 4 mM $MgCl_2$. Finally, the optical density of each solution was measured.

For the zero time point, 2.5 μL of the original pRSVneo solution was added to 0.25 mL of ice-cold depurination buffer

[1] Abbreviations: d-SpN, a dinucleoside monophosphate lacking a 5′ base; d-NpS, a dinucleoside monophosphate lacking a 3′-base; d-MpN, a methoxyamine-modified dinucleoside monophosphate lacking a 5′ base; d-NpM, a methoxyamine-modified dinucleoside monophosphate lacking a 3′ base; ODS, octadecasilyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TBE, Tris–borate–EDTA.

and mixed immediately with 0.25 mL of methoxyamine solution.

Assay for Apurinic Sites. To 100 μL of each sample of depurinated DNA or poly(dA) were added 200 units of DNase I, 0.005 unit of snake venom phosphodiesterase, and 8 units of calf alkaline phosphatase (total volume, 111 μL). After overnight incubation at 37 °C, the protein was removed from each sample by addition of 3 volumes of ice-cold ethanol and storage at −70 °C for 1 h, followed by centrifugation at 10000*g* for 15 min. The recovered supernatants were taken to dryness, and then each was resuspended in 100 μL of distilled water, heated at 100 °C for 5 min to inactivate any residual nuclease and phosphatase activity, and stored at −20 °C.

For enzymatic phosphorylation, 4 μL of this digested material was included in a reaction mixture (20 μL) containing kinase buffer, 3.3 pmol of [γ-^{32}P]ATP (3000 Ci/mmol), and 10 units of polynucleotide kinase and incubated at 37 °C for 60 min. Excess ATP was then consumed by incubation for a further 20 min with 1 μL of oligo(dT)$_{16}$ (5 A_{260} units/mL) and 5 units of the kinase. Half of each sample was then added to an equal volume of formamide loading buffer—90% formamide, 0.02% bromophenol blue, and 0.02% xylene cyanol in 1 × TBE (Maniatis et al., 1982)—and applied to a polyacrylamide gel. The radiolabeled products were visualized by autoradiography and excised from the gel, and their activity was counted.

The gel bands containing the d-pNpM species from each track were mashed up and soaked overnight at 37 °C in 0.4 mL of distilled water. Gel debris was removed by filtration through a glass wool pad and rinsed with 0.4 mL of water. The filtrate of each sample was counted, and 2–10 × 10^4 cpm of this material was added to UV-detectable quantities of the unlabeled markers and further resolved by HPLC. Fractions of 0.3 mL were collected from the chromatographed pRSVneo samples and 0.5 mL from the poly(dA) samples and counted.

Nearest-Neighbor Analysis. To complete the analysis of the nucleotides 5′ to the AP sites, it was necessary to quantitate separately d-pTpM and d-pGpM, which coelute on our HPLC system. The simplest way to do this was to convert these molecules to their respective 5′ mononucleotides, which were resolvable, by treatment with nuclease P1. Radiolabeled fractions containing d-pTpM and d-pGpM from the HPLC analysis of the 120-min depurination of pRSVneo were combined. The pH was raised to 7.0 by dropwise addition of 1 M NaOH, and then the solution was taken to dryness under vacuum. The residue was redissolved in 0.5 mL of distilled water and desalted by passage through a C_{18} SEP-PAK cartridge, evaporated, and redissolved in 50 μL of nuclease P1 buffer (10 mM sodium acetate, pH 5.3, 1 mM $ZnSO_4$). Nuclease P1 (0.3 unit) was added to the solution and incubated at 37 °C for 1 h before the mononucleotide products were separated by HPLC and 0.2-mL fractions were collected and counted. These results were combined with those obtained for d-pCpM and d-pApM from the HPLC analysis of the d-pNpM species.

Results

Action of Polynucleotide Kinase on Depurinated Oligonucleotides. The two isomers, resulting from partial depurination of d-ApA, were tested, individually and combined, for their substrate capacity toward the phosphorylating activity of T4 polynucleotide kinase. It can clearly be seen from Figure 1a,b that the loss of the base from the 5′ nucleoside (d-SpA) rendered the molecule totally refractory to the enzyme. In contrast, its isomer, d-ApS, was completely phosphorylated within 60 min to give d-pApS, which, being more hydrophilic than its precursor, had a shorter retention time on the reverse-phase column (15 vs 21 min). One possible explanation for the lack of phosphorylation of d-SpA could have been an inactivating interaction between the carbonyl group of the apurinic sugar and an amino group of the protein. This explanation was discounted after the enzyme reaction was repeated with d-MpA and d-ApM—the products of addition of methoxyamine to the carbonyl groups at the AP sites (Coombs & Livingston, 1969)—and the same outcome was obtained (Figure 1c,d).

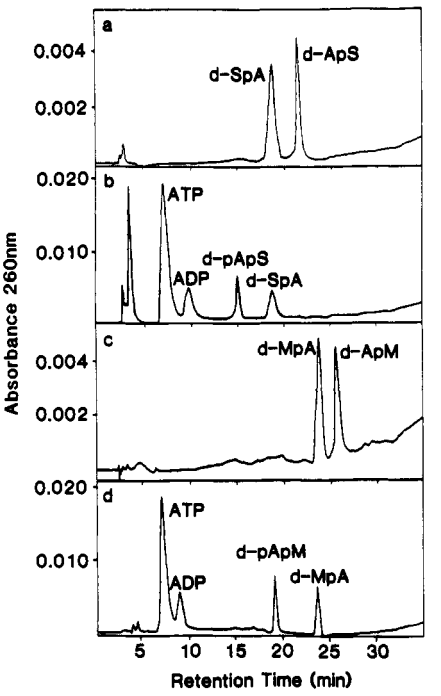

FIGURE 1: Response of d-SpA and d-ApS and their methoxyamine-modified derivatives toward the phosphorylating activity of polynucleotide kinase: (a) unreacted d-SpA and d-ApS; (b) 0.4 nmol of d-SpA and d-ApS incubated for 1 h at 37 °C with 2 nmol of ATP and 10 units of enzyme; (c) unreacted d-MpA and d-ApM; (d) 0.4 nmol of d-MpA and d-ApM incubated for 1 h at 37 °C with 2 nmol of ATP and 10 units of kinase. See Materials and Methods for elution conditions.

Chart I: Outline of the Steps Involved in the Postlabeling Assay for Apurinic Sites

1. Prepare DNA containing AP sites.
2. React with methoxyamine and dialyze; measure concentration of recovered DNA.
3. Digest DNA with DNase I, snake venom phosphodiesterase, and calf alkaline phosphatase (37 °C, overnight).
4. Remove proteins by ethanol precipitation; evaporate supernatant.
5. Resuspend digested DNA in H_2O; heat inactivate residual enzyme activity (100 °C, 5 min).
6. End label d-NpM species with polynucleotide kinase and [γ-^{32}P]ATP; add excess oligo(dT)$_{16}$ to mop up excess ATP.
7. Run samples on 20% polyacrylamide/7 M urea gel.
8. Autoradiograph; excise major bands and count radioactivity.
9. Elute material from d-pNpM band; run on HPLC and count fractions.

Preparation of Markers. The assay for apurinic sites, outlined in Chart I, required radiolabeled markers of the four d-pNpM compounds for the gel electrophoresis step and unlabeled UV markers for the HPLC analysis. In the case of the pyrimidine–adenine dimers, isolation of the apurinic products generated by acid treatment was straightforward and almost quantitative, because depyrimidination was negligible. The preparation of the partially depurinated molecules from the purine–purine dimers was more complicated, because (a) these products are intermediates in the complete depurination

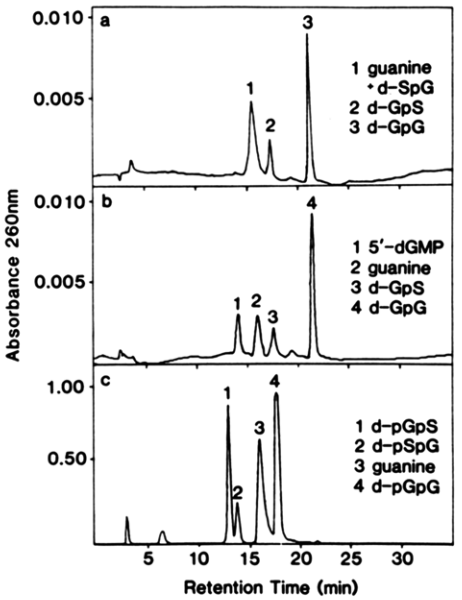

FIGURE 2: Chromatographic separation of the products arising from (a) the 30-min depurination of d-GpG in 0.1 M HCl at 37 °C, (b) the subsequent 15-min hydrolysis in 0.2 M NaOH at 37 °C, and (c) the 60-min depurination of d-pGpG in 0.1 M HCl.

of the starting material and (b) the correct product had to be chosen from the two monodepurinated isomers that were produced. The length of time of acid treatment needed to produce optimal yields of each partially depurinated compound (see Materials and Methods) was obtained from preliminary time course experiments (data not shown). To distinguish the 5′-depurinated isomers from their 3′-depurinated counterparts, a sample of the reaction mixture was incubated in 0.2 M NaOH at 37 °C. Under these conditions, the compounds with a depurinated 5′ nucleoside readily undergo phosphodiester cleavage mediated by a base-catalyzed β-elimination reaction (Kochetkov & Budovskii, 1972).

The results of depurination of d-GpG and d-pGpG are shown in Figure 2a,c. Figure 2b illustrates the use of the alkaline treatment to identify the d-SpG and d-GpS peaks in Figure 2a. An interesting difference between the depurination of d-GpG and d-pGpG emerges from the integration of the peaks in their HPLC elution profiles. Incubation of d-GpG at low pH gave rise to almost equal quantities of the two partially depurinated isomers (as judged by comparison of the peaks of 5′-dGMP and d-GpS in Figure 2b), but similar treatment of d-pGpG yielded 3 times as much d-pGpS as d-pSpG (Figure 2c). The same phenomenon was observed with d-ApA and d-pApA (data not shown).

Recovery of Radiolabeled Markers from a Polyacrylamide Gel. The radiolabeled markers were used in a control study to check that the four d-pNpM compounds could be recovered without selective loss from a polyacrylamide gel. Equal quantities of the radioactive compounds were combined and loaded into a single well of a 20% polyacrylamide/7 M urea gel (Figure 3A, d-pNpM track). The gel was run at 800 V for 4 h, and its radioactive contents were located by autoradiography. The radioactive bands from this track were excised from the gel as a single slice, mashed up, and incubated overnight in 1 mL of distilled water at 37 °C. After filtration, an estimated 80% of the radioactivity was recovered in the aqueous fraction. A portion of this material was analyzed by HPLC (Figure 3B) and found to contain the markers d-pCpM, d-pTpM + d-pGpM, and d-pApM, in the ratio 1.0:1.99:1.1, indicating little, if any, preferential recovery of the four species.

Assay for Apurinic Sites. Since d-ApS and d-ApM are

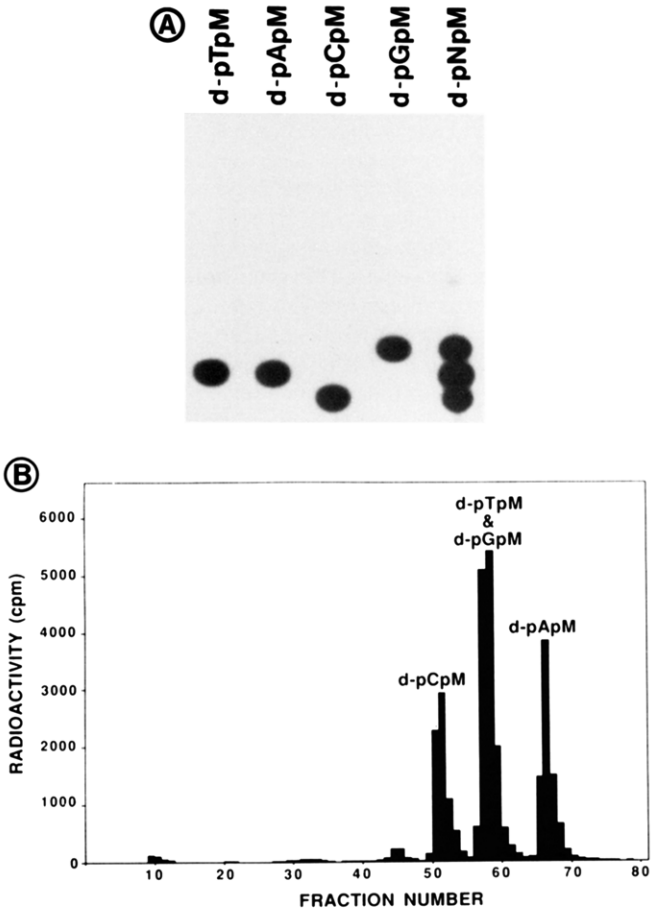

FIGURE 3: Preparation of radiolabeled markers and their recovery from a polyacrylamide gel. (A) An excess of each d-NpM compound was incubated with 3.3 pmol of [γ-^{32}P]ATP and 10 units of polynucleotide kinase. Aliquots of each reaction were analyzed individually (tracks 1–4) and in combination (track 5) on a 20% polyacrylamide/7 M urea gel. (B) The band of radiolabeled material in track 5 was excised, and the products were eluted from the gel as described under Materials and Methods. They were separated in 0.3-mL fractions by HPLC and counted.

substrates for polynucleotide kinase, we were able to exploit our previous observations (Weinfeld et al., 1989a) that these molecules are totally resistant to hydrolysis by snake venom phosphodiesterase and that d-ApS is the sole AP site containing species generated by complete venom phosphodiesterase/alkaline phosphatase digestion of lightly depurinated poly(dA). The protocol outlined in Chart I is a modification of an assay we have successfully applied to the measurement of cyclobutane pyrimidine dimers in UV-irradiated DNA (Weinfeld et al., 1989b). Key features of the assay and our experimental approach to test it are detailed further.

(*i*) *Source of DNA and Its Depurination.* Two groups have described assays in which AP sites, generated under slightly acidic conditions, in double-stranded closed circular DNA molecules (pAT153 plasmid and the SV40 genome) were measured on the basis of conversion, by alkali or AP endonuclease, of the DNA to its nicked open circular form (Ciomei et al., 1984; Gentil et al., 1984). To make a useful comparison, we employed form I DNA of comparable molecular weight (pRSVneo plasmid) and carried out the depurination reaction under the conditions described by Ciomei et al. (1984), i.e., incubation at 70 °C in a citrate buffer (pH 5.2) for various times up to 2 h. A single-stranded linear molecule, poly(dA), was also treated under these conditions for up to 1 h.

(*ii*) *Methoxyamine Treatment.* As can be appreciated from the outline of the protocol, the depurinated DNA undergoes

prolonged digestion at 37 °C, followed by a brief incubation at 100 °C to inactivate any residual nuclease or phosphatase activity. These steps can, of themselves, generate apurinic sites, leading to an increase in the background that would almost certainly affect the sensitivity of the assay when small quantities of AP sites are measured. Reaction with methoxyamine provided a means to circumvent this problem by allowing us to separate AP sites present at the start of the assay from those produced in the course of the procedure. In other words, those AP sites that we intended to quantitate were isolated as d-pNpM molecules, while the others existed as d-pNpS species. Methoxyamine addition also increased the retention time of the AP site molecules on the reverse-phase column, thereby improving their resolution.

(*iii*) *DNA Digestion.* The DNA was digested by DNase I, snake venom phosphodiesterase, and calf alkaline phosphatase. The first of these enzymes is an endonuclease, used here at a limiting concentration to cleave the large DNA molecules into smaller fragments, which can be more rapidly digested by the snake venom exonuclease. HPLC analysis of an aliquot of each pRSVneo reaction mixture confirmed that the overnight digestion by these enzymes went to completion. Four peaks were observed, corresponding to the four mononucleosides. The presence of any normal dinucleoside monophosphates, resulting from incomplete DNA hydrolysis, was not detected.

(*iv*) *Addition of Oligo*$(dT)_{16}$. On the polyacrylamide gel ATP migrates just ahead of the d-pNpM species and, when in large excess, overshadows them. Therefore, after phosphorylation of the d-NpM molecules, the bulk of the excess ATP was consumed by reaction with a long oligonucleotide whose migration through the gel was significantly slower than that of the AP site species. This also meant that considerably less radioactivity had to be applied to the HPLC column in the final step. An autoradiogram of a gel used in the quantitation of AP sites in pRSVneo is shown in Figure 4A.

(*v*) *HPLC Analysis.* In the assay for UV dimers there is no necessity for HPLC because the radiolabeled dimer-containing species are trinucleotides, which are efficiently isolated by gel electrophoresis alone. Moreover, the diversity of these modified trinucleotides (potentially 16 compounds) makes quantitation by reverse-phase HPLC an unattractive proposition. In the case of AP sites, on the other hand, there are only four d-pNpM compounds to be taken into consideration. Since we still found residual ATP (and possibly ADP) contamination of the molecules after gel electrophoresis, despite the use of oligo$(dT)_{16}$, and a small level of d-pNpS molecules, HPLC was used to improve the isolation and quantitation of the depurinated dinucleotides. In addition, HPLC provided a partial nearest-neighbor analysis of the AP sites (see below). The HPLC analysis of AP sites introduced in the plasmid DNA after 120 min is shown in Figure 4B.

(*vi*) *Calculation of AP Sites.* The following factors were taken into account in calculating the number of AP sites introduced at each time point: (a) 1 A_{260} unit is equivalent to 50 μg of plasmid DNA (Maniatis et al., 1982), the pRSVneo molecule has 5736 bp (Gorman et al., 1983), and the average molecular weight for each nucleotide residue is ~309; (b) 1 A_{260} unit of poly(dA) is equivalent to 38.8 μg, and the molecular weight of each nucleotide residue is 313. It is a relatively simple matter to calculate the frequency of AP sites per 10^6 purines and hence, in the case of pRSVneo, the number of AP sites per plasmid molecule for each time point. An example of such a determination is detailed in Table II. The kinetics of depurination of pRSVneo and poly(dA) are shown in Figure 4C. Linear regression of the values obtained for each time point reveals that an average of 575 AP sites per 10^6 purines were introduced per hour, or 3.3 AP sites per plasmid molecule. This provides a rate constant for depurination of the double-stranded molecule at pH 5.2 and 70

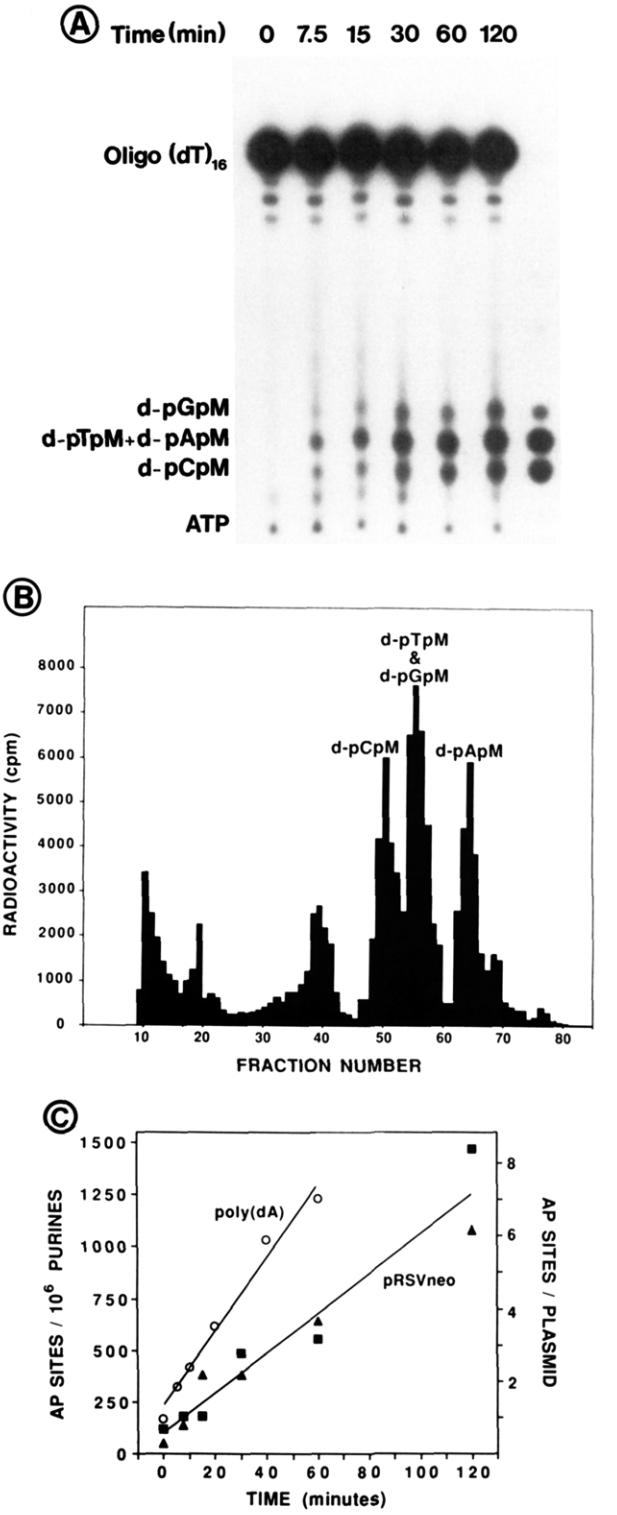

FIGURE 4: Postlabeling assay for AP sites. (A) Gel electrophoresis of the end-labeled compounds following depurination and enzymatic digestion of pRSVneo and phosphorylation of the products. Oligo-$(dT)_{16}$ was used to remove the bulk of the excess ATP from the vicinity of the d-pNpM species. (B) Chromatographic separation of the radiolabeled d-pNpM compounds arising from 120-min depurination of pRSVneo and isolation from a gel. (C) Result obtained by the postlabeling assay for the depurination of pRSVneo (■ and ▲, two separate determinations) and poly(dA) (○). The lines drawn through these points were generated by linear regression of the data points.

Table II: Calculation of the Number of AP Sites in pRSVneo as a Function of Treatment Time

time (min)	[DNA] A_{260}[a] (A)	% cpm in [^{32}P]d-pNpM in gel[b] (B)	% cpm in [^{32}P]d-pNpM on HPLC (C)	combined percentage (BC) (D)	fmol of AP sites[c] (E)	pmol of AP sites/A_{260}[d] (F)	AP sites/10^6 purines[e] (G)
0	0.255	0.61	19.6	0.12	3.9	3.9	48
7.5	0.230	2.20	14.4	0.32	10.4	11.3	140
15	0.225	1.62	51.6	0.84	27.6	30.7	378
30	0.192	1.47	48.5	0.71	23.5	30.6	378
60	0.195	2.06	59.8	1.23	40.7	52.1	643
120	0.208	3.54	62.3	2.20	72.7	87.4	1079

[a] Concentration of DNA per milliliter in solution following methoxyamine treatment and dialysis. [b] Contains ATP and other impurities. [c] $D \times 3.3$ pmol (the amount of ATP added in the kinase reaction). [d] $E \div A \div 0.004$ (4 μL of DNA solution used in the kinase reaction). [e] $F \div 0.081$ (micromoles of purines per A_{260} of DNA).

°C of $k = 1.6 \times 10^{-7}$ s^{-1}. Similarly, an average of 1074 adenines were released from 10^6 nucleotides of poly(dA) per hour, giving a rate constant of $k = 3.0 \times 10^{-7}$ s^{-1}.

(*vii*) *Analysis of Nucleotides 5′ to AP Sites.* The reverse-phase chromatography failed to separate d-pGpM from d-pTpM, but it was capable of resolving 5′-dGMP from 5′-dTMP. Nuclease P1, which has been shown to cleave d-ApS and d-ApM to release deoxyadenosine (Weinfeld et al., 1989a), was therefore used to complete the analysis of the nucleotides 5′ to AP sites introduced by 120-min depurination of pRSVneo. After the HPLC fractions containing d-pGpM and d-pTpM were pooled, evaporated, and desalted (to remove methanol and phosphate present in the elution buffer), the material was redissolved in a zinc-containing buffer and incubated with sufficient nuclease for full hydrolysis of the apurinic species. The two radiolabeled mononucleotides were then isolated by HPLC, and their radioactivity was counted. Combining the results from the HPLC separation of the d-pNpM compounds with those from the nuclease P1 hydrolysate, we obtained a ratio for the 5′- neighboring nucleotides of 1.0:0.70:0.86:0.94 (dCMP:dGMP:dTMP:dAMP).

Discussion

Phage T4 polynucleotide kinase is a 136-kDa protein capable of catalyzing the phosphorylation of DNA, RNA, oligonucleotides, and nucleoside 3′-monophosphates (Lillehaug, 1977; Richardson, 1965). Richardson showed, at the time of his original description of the enzyme, that a 3′-phosphate is necessary for kinase activity, since neither mononucleosides nor nucleoside 2′-monophosphates are substrates (Richardson, 1965). Recently, we have used a variety of modified oligonucleotides to further define the structural requirements that allow a molecule to be a substrate for this enzyme. First, it was observed (Weinfeld & Livingston, 1986) that neutralization of the charge on the internucleotide phosphate of d-TpT, by ethyl esterification, abolishes the substrate capacity of the dinucleoside monophosphate. We have also found that the introduction, by UV irradiation, of a cyclobutane pyrimidine dimer or 6,4-photoproduct renders d-TpT refractory to the kinase (Weinfeld et al., 1989b). The results reported here show that a deoxyribose group, bound at its C-3 position to a phosphate group but lacking a base, as in d-SpA, cannot be phosphorylated by this enzyme. The refractory nature of d-SpA could not be attributed to an interfering reaction between the protein and the carbonyl group of the ring-opened sugar, because the methoxyamine-capped molecule, d-MpA, responded in the same manner as d-SpA. Furthermore, unlike the UV-damaged compounds that have bridging bonds between the adjoining bases, the 5′-depurinated molecule presents no apparent steric hindrance to the enzyme if part of its substrate binding includes the internucleotide phosphate. Thus, the most plausible explanation for the failure of the kinase to act on d-SpA is that the enzyme must bind to the base of the phosphate-accepting nucleotide in the course of carrying out its activity. In agreement with this, Hegi et al. (1989) have shown that *cis*-5,6-dihydroxy-5,6-dihydrothymidine 3′-phosphate, which has a nonaromatic and therefore nonplanar base, is a poor substrate for polynucleotide kinase. However, the fact that the addition of bulky substituents to the base does not significantly impede phosphorylation (van de Sande & Bilsker, 1973; Reddy & Randerath, 1986), providing the base retains its aromatic character, suggests that it is unlikely that the enzyme fully encloses the base.

The deleterious effect of the loss of a base is limited to the 5′-nucleoside, since d-ApS and d-ApM were readily phosphorylated. This made it possible to radioactively label the AP site containing species in the snake venom phosphodiesterase and alkaline phosphatase digest of depurinated DNA. The double-stranded DNA molecule employed to test the assay and the conditions of depurination were chosen so as to permit comparison with results obtained by others using an alternative approach. Both Ciomei et al. (1984) and Gentil et al. (1984) measured the induction of AP sites by either alkali or AP endonuclease mediated conversion of form I to form II DNA. The former group depurinated the plasmid pAT153 (3657 bp) under conditions identical with ours and obtained a rate of 2.0 AP sites per plasmid per hour. The latter workers incubated SV40 DNA (5243 bp) at 70 °C under slightly more acidic conditions (pH 4.8 vs 5.2) and observed a depurination rate of 4.0 AP sites per DNA molecule per hour. Our result with pRSVneo (5735 bp) of 3.3 AP sites per plasmid per hour is clearly in good agreement with these previously published data. In addition, the value of $k = 1.6 \times 10^{-7}$ s^{-1} for the rate constant for depurination of the plasmid DNA incubated at 70 °C in a buffer at pH 5.2 is reasonably close to the value of 3.8×10^{-7} s^{-1} found by Lindahl and Nyberg (1972) for depurination of double-stranded DNA at pH 5.0 and 70 °C. The latter was obtained by measuring radioactively labeled ethanol-soluble material released from [^{14}C]purine-labeled *Bacillus subtilis* DNA. Also in accord with these authors, we observed that the rate of depurination of a single-stranded polymer, in our case poly(dA), was greater than that of double-stranded DNA.

Various other AP site assays have been devised. These include (i) the measurement of alkali or AP endonuclease induced strand breaks in radiolabeled DNA either by sedimentation in a sucrose gradient (Brent et al., 1978) or by alkali elution from polycarbonate filters (Kohn et al., 1981), (ii) the incorporation of [^{14}C]methoxyamine (Talpaert-Borlé & Liuzzi, 1983), and (iii) enhanced fluorescence after addition of 9-aminoellipticene (Bertrand et al., 1987). Several features of our approach may make it the method of choice in certain circumstances. First, by definition, it does not require labeled DNA, nor is it dependent upon the nature of the DNA, working equally well with double- or single-stranded, linear

or covalently closed circular molecules. This makes postlabeling assays particularly suitable for quantitation of lesions in cellular DNA, especially in tissues, where prelabeling of the DNA is not possible. It would permit, for example, the study of cellular repair of AP sites as a function of age and tissue type in a manner similar to that recently reported by Randerath and co-workers (Randerath et al., 1986) for covalent DNA modifications. Second, although we have not fully optimized our procedure, it should enable quantitation of AP sites at the femtomole level. (By use of ATP with a specific activity of 3000 Ci/mmol, 1 fmol is ~6000 dpm.) This degree of sensitivity cannot be achieved by either the fluorescence assay or the other postlabeling technique employing [^{14}C]-methoxyamine. Third, as demonstrated, it is possible to obtain the ratio among the four nucleotides for the nucleotides immediately 5′ to the AP sites. An important concern raised when DNA damage is examined is the possibility of "hotspots" governed, in part, by base sequence. The nearest-neighbor analysis in this paper was presented more as an example of how this type of analysis can be carried out for AP sites than for its possible biological significance. [The DNAs of living organisms rarely encounter the combined conditions of low pH and elevated temperature used here to depurinate poly(dA) and pRSVneo.] However, AP sites created by other agents, especially those where intercalation may play a role such as aromatic amines like *N*-acetoxy-2-(acetylamino)fluorene (Tarpley et al., 1982), may be produced more selectively.

Because of efficient repair processes, AP sites have a short half-life in vivo (Moran & Ebisuzaki, 1987; Den Engelse et al., 1980). Techniques used so far to monitor AP sites, generated by chemicals or by DNA glycosylases in cells, measure the small number that exist at any given time. A paper by Reznick and Shapiro (1975) suggests that methoxyamine is capable of entering mammalian cells. Providing that methoxyamine-modified AP sites are themselves not rapidly repaired, it may prove possible to induce the accumulation of AP sites over a period of time with the use of this chemical. [For example, the calf thymus AP endonuclease recently purified by Henner and co-workers is unable to cleave alkoxyamine-modified sites (Sanderson et al., 1989).] This would greatly facilitate their quantitation and may lead to the identification of new repair pathways involving DNA glycosylases and AP endonucleases.

One further potential application of the postlabeling assay is in distinguishing type I from type II AP endonucleases (Linn, 1982). The former cleave the phosphodiester bond 3′ to an AP site. Subsequent digestion of the DNA by snake venom phosphodiesterase and phosphatase should therefore give rise to AP site containing, enzymatically phosphorylatable "oligonucleotides". A similar digestion of DNA treated with a 5′-acting (type II) AP endonuclease, on the other hand, will release AP site as deoxyribose, which is not a substrate for polynucleotide kinase.

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