^{32}P-Postlabeling Detection of Radiation-Induced DNA Damage: Identification and Estimation of Thymine Glycols and Phosphoglycolate Termini†

Michael Weinfeld* and Krista-June M. Soderlind

Radiobiology Program, Cross Cancer Institute, Edmonton, Alberta, Canada T6G 1Z2

Received June 25, 1990; Revised Manuscript Received September 28, 1990

ABSTRACT: A ^{32}P-postlabeling assay has been developed that permits detection of several radiogenic base and sugar lesions of DNA at the femtomole level. The technique is based on the inability of DNase I and snake venom phosphodiesterase to cleave the internucleotide phosphodiester bond immediately 5′ to the site of damage so that complete digestion of irradiated DNA with these nucleases and alkaline phosphatase yields lesion-bearing "dinucleoside" monophosphates. Because these fragments contain an unmodified nucleoside at the 5′-end of each molecule, they can be readily phosphorylated by T4 polynucleotide kinase and [γ-^{32}P]ATP and analyzed by polyacrylamide gel electrophoresis and reverse-phase HPLC. We observed a linear induction of total damage in DNA irradiated with 5–50 Gy. Virtually no damage was detected when the DNA was irradiated in solution containing 1 M DMSO, implicating hydroxyl radicals in the formation of these lesions. Evidence for the presence of thymine glycols and phosphoglycolate groups came from (i) a comparison of the radiation-induced products with those produced by OsO_4 and $KMnO_4$ and (ii) incubation of irradiated DNA with *Escherichia coli* endonuclease III and exonuclease III before analysis by the postlabeling procedure. This was confirmed by comigration of the radiogenic products with chemically synthesized markers. *G* values of 0.0022 and 0.0105 μmol J^{-1} were obtained for thymine glycol and phosphoglycolate production, respectively. The identity of the 5′-nucleotide of each isolated compound was obtained by nuclease P1 digestion. This analysis of nearest-neighbor bases to thymine glycols and phosphoglycolates indicated a nonrandom interaction between radiation-induced hydroxyl radicals and DNA.

The interaction of ionizing radiation with DNA produces a diverse array of modifications to base and sugar residues (Ward, 1975; von Sonntag, 1987). Approaches to their quantification include measurements of repair endonuclease-induced single-strand breaks by alkaline sucrose sedimentation (Paterson, 1978), immunochemical detection (Leadon, 1987; Hubbard et al., 1989; Fuciarelli et al., 1985), and chromatographic analysis of bases or nucleosides released chemically or enzymatically from irradiated DNA. After chromatographic separation, damaged bases or nucleosides have been detected by GC–MS (Dizdaroglu & Bergtold, 1986), electrochemically (Kasai et al., 1986), by fluorescence postlabeling (Sharma et al., 1990), or radiochemically, following incorporation of labeled nucleotides into the DNA prior to irradiation (Breimer & Lindahl, 1985; Frenkel et al. 1981).

The ^{32}P-postlabeling assay, originally devised by Randerath et al. (1981) to measure carcinogen–DNA adducts, is an alternative technique which has only recently been applied to the detection of oxidative DNA damage (Rosier & Van Peteghem, 1988; Hegi et al., 1989; Mouret et al., 1990). In the procedure, DNA is first digested by micrococcal nuclease and calf spleen phosphodiesterase to give nucleoside 3′-monophosphates (normal and modified) that are subsequently radiolabeled by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase. The radiolabeled compounds are then separated by two-dimensional TLC. The assay has two important advantages. First, there is no requirement for prelabeling of the DNA, which makes the assay particularly useful for the study of DNA lesions in tissues. Second, because γ-labeled ATP can be obtained with a high specific activity, the assay permits detection at the femtomole level. There are, however, a few drawbacks: (a) polynucleotide kinase must be able to act on the modified nucleoside 3′-monophosphate, and (b) the resulting labeled modified nucleoside diphosphate must be separated from the high background of normal nucleoside diphosphates. These problems are well illustrated in the report of efforts to detect thymine glycol in irradiated DNA (Hegi et al., 1989). The 3′-monophosphate of thymidine glycol (5,6-dihydroxy-5,6-dihydrothymidine) was shown to be a poor substrate for polynucleotide kinase under standard conditions and was phosphorylated with moderate efficiency only after the addition of beryllium ions. Then, in order to isolate the thymidine glycol diphosphate, an extra chromatography step (on a phenylboronate affinity column) was introduced before the TLC step. Nonetheless, the high sensitivity offered by the ^{32}P-postlabeling approach should permit measurement of base damage in the DNA of irradiated tissue.

In this paper, we provide an alternative approach that overcomes the problems noted above. The assay is based on previous observations that certain DNA lesions prevent the hydrolysis by snake venom phosphodiesterase and DNase I of adjacent 5′-internucleotide phosphodiester linkages (Liuzzi et al., 1989; Weinfeld et al., 1989a). Hence, digestion of damaged DNA with these enzymes and calf alkaline phosphatase generates lesion-containing oligonucleotides, which are good substrates for polynucleotide kinase, because the nucleoside at the 5′-end is unmodified (Figure 1). The remaining, unaffected bases are recovered as mononucleosides, which are not phosphorylated by this kinase. This procedure has been used to quantify cyclobutane pyrimidine dimers (Weinfeld et al., 1989b) and apurinic sites (Weinfeld et al., 1990). As a result of an observation by Dizdaroglu et al. (1978) that irradiated DNA could not be completely digested to mononucleotides by snake venom phosphodiesterase, we have now applied the technique to assay for radiogenic DNA

† These studies were supported by research grants from the Alberta Cancer Board and the National Cancer Institute of Canada.

0006-2960/91/0430-1091$02.50/0

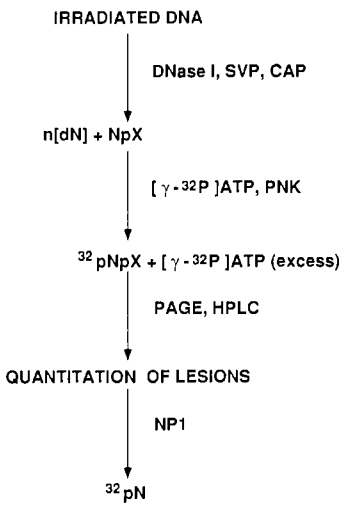

FIGURE 1: Strategy of postlabeling assay. X represents a damaged nucleoside.

damage. This paper describes the assay protocol and steps taken to identify some of the detectable products.

Materials and Methods

Enzymes. Snake venom phosphodiesterase (*Crotalus atrox*, type IV, 1 g/mL, 22 units/mL) and nuclease P1 (1 mg/mL, 340 units/mL) were purchased from Sigma Chemical Co. (St. Louis, MO); DNase I (10000 units/mL) and exonuclease III (65000 units/mL) was from BRL (Burlington, ON); calf alkaline phosphatase (1000 units/mL) was from Boehringer Mannheim Canada (Dorval, PQ); and T4 polynucleotide kinase was from Pharmacia Canada (Dorval, PQ). Unit definitions are as follows: snake venom phosphodiesterase, 1 unit hydrolyzes 1.0 μL of bis(*p*-nitrophenyl) phosphate per minute at pH 8.8 at 37 °C; nuclease P1, 1 unit hydrolyzes 1.0 μmol equiv of RNA phosphodiester linkages per minute at pH 5.3 at 37 °C; DNase I, 1 unit increases the absorbance of calf thymus DNA solution (50 μg/mL) at a rate of 0.001 A_{260} units min^{-1} mL^{-1} at pH 5.0 at 25 °C; exonuclease III, 1 unit produces 1 nmol of acid-soluble nucleotide from sonicated DNA in 30 min at pH 8.0 at 37 °C; calf alkaline phosphatase, 1 unit hydrolyzes 1 μmol of 4-nitrophenyl phosphate in 1 min at pH 9.8 at 37 °C; polynucleotide kinase, 1 unit catalyzes the transfer of 1 nmol of phosphate from ATP to polynucleotide in 30 min at pH 7.6 at 37 °C.

Escherichia coli endonuclease III (fraction IV) was purified from *E. coli* $\lambda N99_{cI857}$ carrying the pHIT1 plasmid (kindly provided by Dr. R. P. Cunningham of SUNY, Albany, NY) according to the procedure of Asahara et al. (1989).

Marker Compounds. Dinucleoside monophosphates containing thymine glycol were prepared by oxidation of 0.6 mM d-ApT or d-CpT with 1 mM $KMnO_4$ in 0.3 M NH_4Cl–NH_3 buffer (pH 8.6), at 0 °C for 2 h (Iida & Hayatsu, 1971). After sodium metabisulfite inactivation of the $KMnO_4$, the reaction mixture was resolved by reverse-phase HPLC with the system and gradient described below. To ascertain the peak containing the desired compound, an aliquot of each peak was incubated with nuclease P1 and then calf alkaline phosphatase and analyzed by HPLC for the release of thymidine glycol. An authentic sample of *cis*-thymidine glycol (predominantly the 5*R*,6*S* isomer) was kindly supplied by Dr. H. Ide of the University of Vermont, Burlington, VT.

The phosphoglycolate-bearing compounds, d-Apg, d-Cpg, and d-Tpg,[1] prepared by Dr. S. M. Hecht of the University of Virginia, Charlottesville, VA, were provided by Dr. W. D. Henner of Oregon Health Sciences University, Portland, OR.

The preparation of dinucleosides lacking a 3′-base has been previously described (Weinfeld et al., 1990).

HPLC. The instrumentation consisted of a Varian 5000 liquid chromatograph (Varian Canada Inc., Guelph, ON) coupled to a Waters μBondapak C_{18} RCM 8 × 10 RadialPak cartridge (Waters Associates, Mississauga, ON), a Tracor 970A UV detector (Tracor Instruments, Rexdale, ON), and a SP 4100 integrator (Spectra Physics, San Jose, CA). Gradient conditions were as follows: 100% buffer A (50 mM NaH_2PO_4, pH 4.5) and 0% buffer B [100 mM NaH_2PO_4, pH 4.5/methanol (1:1 v/v)] for 1 min followed by a linear gradient to 20% buffer A/80% buffer B over 30 min at a flow rate of 1 mL/min. Fractions were collected every 0.5 min.

Radiation and Chemical Treatment of DNA. (*A*) *Irradiation.* Calf thymus DNA (Sigma; 0.5 mg/mL) dissolved in air-saturated 10 mM sodium phosphate (pH 7.4) was irradiated at room temperature in a ^{60}Co Gammacell 220 (AECL, Ottawa, ON) at a dose rate of 10.8 Gy/min as determined by Fricke dosimetry. The DNA was then precipitated by addition of one-tenth volume of 2.5 M sodium acetate and 2 volumes of ethanol, collected by centrifugation, and redissolved in water to a concentration of 1 mg/mL.

(*B*) *Oxidation.* Calf thymus DNA was oxidized by potassium permanganate according to Frenkel et al. (1981) and osmium tetroxide by the procedure of Kow and Wallace (1985).

Postlabeling Assay. Samples (10 μg) of unirradiated, irradiated, or oxidized DNA were incubated overnight at 37 °C with 0.4 unit of DNase 1, 0.04 unit of snake venom phosphodiesterase, and 0.4 unit of calf alkaline phosphatase in 40 μL of digestion buffer (10 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$). After a further incubation period of 3 h with an additional 0.02 unit of venom phosphodiesterase and 0.2 unit of phosphatase, the enzymes were precipitated by addition of 3 volumes of ice-cold ethanol and removed by centrifugation (10000*g*, 15 min). The supernatants were evaporated and the resulting residues dissolved in 100 μL of distilled water, heated at 100 °C for 10 min to inactivate residual nuclease and phosphatase activity, and then stored at −20 °C.

Each phosphorylation 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), 5 μL of enzyme-digested DNA, 2.2 pmol of [γ-^{32}P]ATP (4500 Ci/mmol, ICN Canada, Montreal, PQ), and 5 units of polynucleotide kinase. The samples were incubated at 37 °C for 1 h, and then the bulk of the excess ATP was consumed by incubation for a further 30 min with 1 μL of oligo(dT)$_{12}$ (5 A_{260} units/mL, Pharmacia) and 2.5 units of the kinase. 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) was added to each sample, and half of the reaction mixture was loaded onto a 20% polyacrylamide/7 M urea gel. Gel electrophoresis equipment and conditions were the same as previously described (Weinfeld et al., 1990). Radiolabeled products were visualized by autoradiography, excised from the gel, and counted (without addition of scin-

[1] Abbreviations: T^g, thymidine glycol; d-Npg, 2′-deoxynucleoside 3′-(phospho-2″-*O*-glycolic acid); d-pNpg, 2′-deoxynucleoside 3′-(phospho-2″-*O*-glycolic acid) 5′-phosphate; d-pNpS, dinucleotide lacking a 3′-base; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TBE, Tris–borate–EDTA; SVP, snake venom phosphodiesterase; CAP, calf alkaline phosphatase; PNK, polynucleotide kinase; PAGE, polyacrylamide gel electrophoresis; NP1, nuclease P1.

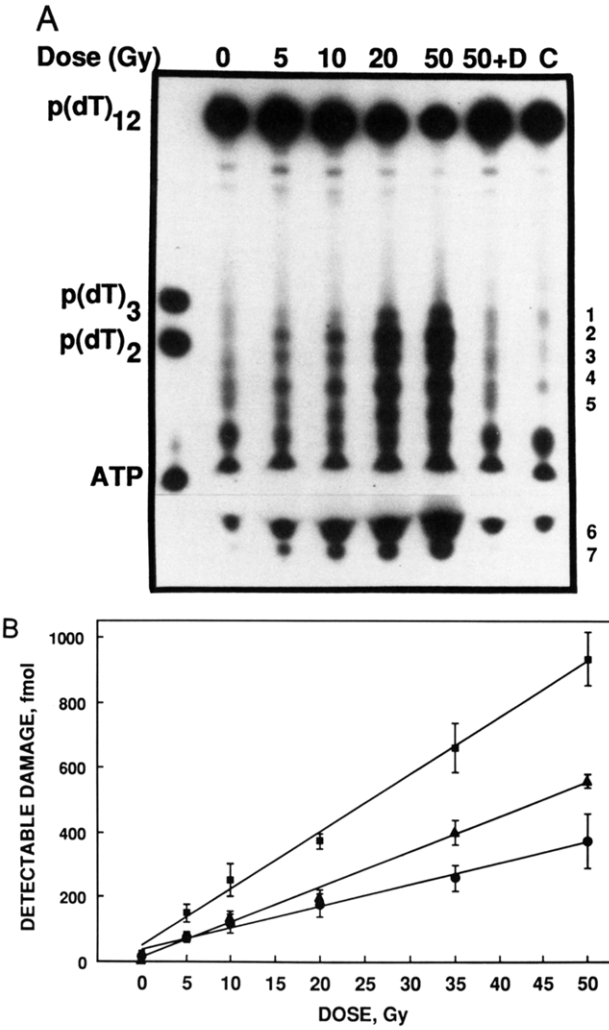

FIGURE 2: Dose–response for induction of radiogenic DNA damage. (A) Gel electrophoresis of the end-labeled compounds obtained by digestion and phosphorylation of irradiated DNA. Lane C shows the control. Lane 50+D shows the effect of irradiation in the presence of 1 M DMSO. Oligo(dT)$_{12}$ was used to consume the bulk of the excess ATP, thereby reducing the background radioactivity in the vicinity of the small products. (B) Plot of detectable damage from 0.5 μg of DNA vs dose: bands 1–5 (●), bands 6 and 7 (▲), and bands 1–7 (■) are ±SD from three independent determinations. The lines drawn through these points were generated by linear regression of the data points.

tillant).

The radioactive gel bands were then mashed up and soaked overnight at room temperature in 0.5 mL of distilled water. Gel debris was removed by filtration through a glass wool pad and rinsed with 0.5 mL of water. The combined filtrate was counted and ~20000 cpm from each band analyzed by HPLC as described above.

Peaks isolated by HPLC were taken to dryness and resuspended in P1 nuclease buffer (10 mM sodium acetate, pH 5.3, 10 mM $ZnCl_2$). To this was added 2 units of P1 nuclease and a UV-detectable quantity of d-TpA to check for enzyme activity. The reaction mixture was incubated for 3 h at 37 °C and then resolved by HPLC with UV markers for all four 5′-mononucleotides.

Endonuclease III and Exonuclease III Treatment of Irradiated DNA. (A) Endonuclease III. Twenty micrograms of irradiated (50 Gy) DNA was incubated for 3 h at 30 °C in a total volume of 80 μL containing 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 1 mM EDTA, and 0.1 mM dithiothreitol with 6 ng of endonuclease III. The DNA was then drop dialyzed

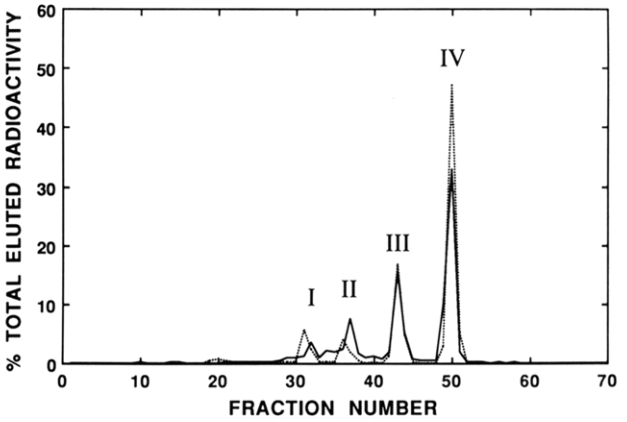

FIGURE 3: HPLC analysis of products in irradiated and OsO_4-treated DNA. Gel bands 2 from irradiated and OsO_4-treated DNA (see Figure 4) were excised, and the contents were eluted in water and then analyzed on reverse-phase HPLC. Irradiated DNA, solid line; OsO_4-treated DNA, dotted line.

against 500 mL of distilled water for 1 h. Half of this material was further incubated with exonuclease III, while the remainder was immediately digested by DNase I, venom phosphodiesterase, and alkaline phosphatase.

(B) Exonuclease III. Ten micrograms of irradiated (50 Gy) DNA (treated or untreated with endonuclease III) was incubated for 1 h at 37 °C with 130 units of exonuclease III in 100 μL of buffer containing 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, and 1 mM EDTA. The DNA was then completely digested as described above.

(C) Controls. Controls for all three samples were incubated in the absence of the endonuclease and exonuclease.

Results

Postlabeling Detection of Radiation Products. The first objective was to determine whether radiogenic DNA lesions could be detected autoradiographically with the same basic postlabeling protocol, outlined in Figure 1, that had been successfully applied to UV-induced DNA damage (Weinfeld et al., 1989b). Aqueous samples of calf thymus DNA were irradiated under air-saturated conditions by ^{60}Co γ-rays with doses of 5–50 Gy, and the DNA fragments in 0.5 μg of each sample were end labeled. Figure 2A shows the resulting autoradiogram of the polyacrylamide gel and demonstrates the appearance, with increasing dose, of several distinct bands (numbered 1–7), each representing one or more damage-containing short oligonucleotides. The control for this experiment (lane C) was an incubated mixture containing ~0.125 μg of each of the four mononucleosides together with [^{32}P]ATP and polynucleotide kinase. The nature of the low levels of radioactivity in both the 0 Gy and control lanes is unknown. Also included in Figure 2 is a track (50+D) that shows that the presence of 1 M DMSO in a solution of DNA during irradiation with 50 Gy significantly decreases formation of the detectable radiation-induced lesions. DMSO is known to be a good scavenger of hydroxyl radicals (Reuvers et al., 1973).

To quantify the total detectable damage, the radioactive areas of each lane were excised from the gel and counted. The counts in the areas containing DNA damage products were divided by the total number of counts in each lane and multiplied by 2200 fmol (the amount of ATP used in each phosphorylation reaction). This value was corrected by subtraction of the value obtained for the equivalent region in the control lane. Replicate samples of the irradiated DNA were analyzed, and the reproducibility is indicated by the standard

Table I: Summary of PAGE and HPLC Analysis of Irradiated (50 Gy) DNA

	retention time (min)	% ^{32}P in gel band	% total detectable damage	fmol in 0.5 μg of DNA	5′-base(s)	proposed structure
gel band 1			3.4			
HPLC peak I	16.5	8.1	0.27		G, T (4:1)	
HPLC peak II	19.0	9.6	0.32		G, T (2:1)	
HPLC peak III	21.5	14.8	0.5		A, T (4:1)	
HPLC peak IV	24.5	50.7	1.7	15.7	G	d-pGpT^g
gel band 2			10.2			
HPLC peak I	16.0	5.1	0.52		C, T, G (4:3:2)	
HPLC peak II	18.5	12.8	1.3		A, C, G, T (4:3:1:1)	
HPLC peak III	21.5	23.1	2.4	21.4	T	d-pTpT^g
HPLC peak IV	25.0	44.3	4.5	41.1	A	d-pApT^g
gel band 3			6.2			
HPLC peak I	16.0	5.8	0.36		C, G, T (6:2:1)	
HPLC peak II	17.0	5.9	0.37		C, T (7:1)	
HPLC peak III	19.0	14.9	0.92		G	
HPLC peak IV	21.0	52.4	3.2	29.6	C	d-pCpT^g
gel band 4			10.6			
HPLC peak I	18.5	31.2	3.3		T	
HPLC peak II	21.0	31.2	3.3		A	
HPLC peak III	23.0	15.2	1.6		A	
gel band 5			9.1			
HPLC peak I	14.5	55.0	5.0		C	
HPLC peak II	16.5	23.1	2.1		C	
gel band 6a			15.5			
HPLC peak I	5.0	90.8	14.1	128.1	G	d-pGpg
gel band 6b			37.0			
HPLC peak I	5.5	46.1	17.0	155.2	T	d-pTpg
HPLC peak II	7.5	51.4	19.0	173.2	A	d-pApg
gel band 7			8.0			
HPLC peak I	4.5	96.7	7.7	70.4	C	d-pCpg

deviations shown in Figure 2B.

Greater resolution of the radiation products could be achieved by eluting the radioactive contents from each band and analyzing them by reverse-phase HPLC. This procedure was carried out for DNA irradiated with 50 Gy. The elution profile for band 2 is given as an example of such an analysis in Figure 3, and a complete listing of retention times of all the HPLC peaks is provided in Table I.

Identification of the 5′-Base in the End-Labeled Products. The labeled 5′-nucleotides of the compounds in each peak were ascertained by P1 nuclease hydrolysis (see Figure 1) of the HPLC-purified material, followed by cochromatography of the P1 digest with UV markers for the four 5′-mononucleotides. The results of such an analysis are included in Table I.

Identification of the Radiogenic Lesions in the End-Labeled Products. Ionizing radiation produces such a wide diversity of DNA lesions that it was clearly impractical to prepare dinucleotide markers of all the potential compounds as a means of identifying the lesions detected by this assay. Clues as to the nature of the nucleoside damage in the 3′-position of some of the labeled compounds came from a comparison with oxidative damage caused by other agents and from the use of repair enzymes.

(*A*) *Comparison with Chemically Oxidized DNA.* DNA damage caused by potassium permanganate and osmium tetroxide partially mimics oxidative damage induced by irradiation in the presence of oxygen, in particular the formation of thymine glycols (5,6-dihydroxy-5,6-dihydrothymine) (Kochetkov & Budovskii, 1972). Calf thymus DNA oxidized by these agents was therefore subjected to the postlabeling procedure and compared to irradiated DNA. It appeared from the autoradiogram of the gel (Figure 4) that all three agents produced three bands in common (bands 1–3). When further analyzed by HPLC, these bands were shown to have very similar constituents. This is shown in Figure 3, where the HPLC profile for band 2 from OsO_4-treated DNA is compared

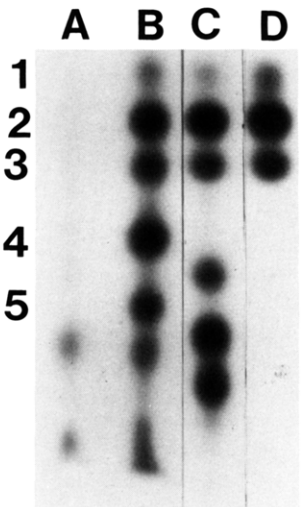

FIGURE 4: Comparison of irradiated DNA with chemically oxidized DNA. (Lane A) Unirradiated DNA; (lane B) irradiated (50 Gy) DNA; (lane C) $KMnO_4$-treated DNA; (lane D) OsO_4-treated DNA. Bands 1–5 are equivalent to bands 1–5 in Figure 2.

with that from γ-irradiated DNA. This provided strong suggestive evidence that bands 1–3 contained dinucleotides bearing a thymine glycol as the 3′-base. Figure 4 also shows that $KMnO_4$ gives rise to other lesions, probably thymine fragmentation products, capable of being detected by the postlabeling assay.

(*B*) *Influence of Damage-Recognizing Enzymes.* Both *E. coli* endonuclease III and *E. coli* exonuclease III act on irradiated duplex DNA. The former has a DNA-glycosylase activity which cleaves the N-glycosylic bond of several pyrimidine ring saturation products, such as thymine glycol and dihydrothymine, and ring contraction and fragmentation products, such as 5-hydroxy-5-methylhydantoin and methyltartronylurea (Wallace, 1988). The enzyme subsequently cleaves the phosphodiester 3′ to the AP site leaving a 2′,3′-

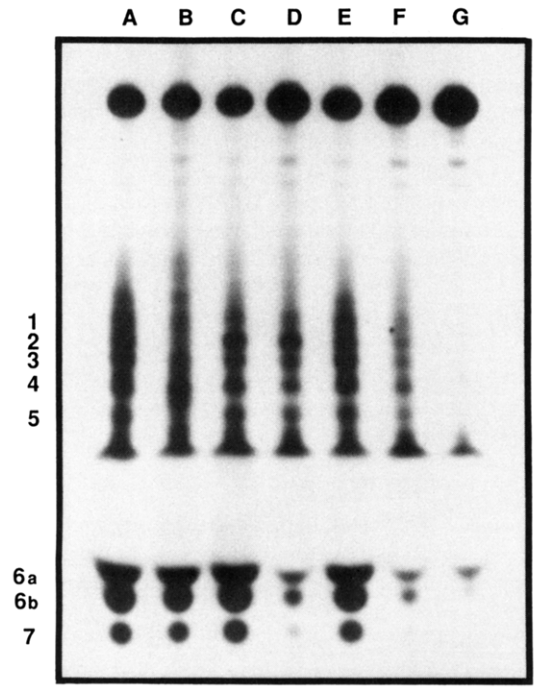

FIGURE 5: Influence of repair enzymes. Irradiated (50 Gy) DNA was incubated with *E. coli* endonuclease III (track B), exonuclease III (track D), or both (track F) prior to complete digestion and end labeling. Tracks A, C, and E represent incubated controls, and lane G shows incubated unirradiated DNA.

didehydro-2′,3′-dideoxyribose group at the strand terminus (Bailly & Verly, 1987). Exonuclease III, in addition to its exonuclease activity on double-stranded DNA and class II AP endonuclease activity, can process phosphoglycolate residues found at many strand break termini (Henner et al., 1983) and the 2′,3′-didehydro-2′,3′-dideoxyribose groups described above (Kim & Linn, 1988). It does not incise DNA at base-modified sites such as thymine glycols, formamidopyrimidines, or β-ureidoisobutylic acid groups (Kow, 1989).

Irradiated DNA samples were treated with each of these enzymes individually and in combination, endonuclease III followed by exonuclease III, and then subjected to the postlabeling process. Controls were incubated in the appropriate buffers without enzyme. Several observations can be made from the autoradiogram of the polyacrylamide gel shown in Figure 5. First, from a comparison of the endonuclease III treated DNA (track B) with its control (track A), it is clear that the enzyme acted so as to reduce the counts in bands 1–3 and increase the radioactivity in the region between bands 4 and 5. This is consistent with a glycosylase-mediated removal of damaged bases, giving rise in the postlabeling assay to products—"dinucleotides" with a 2′,3′-didehydro-2′,3′-dideoxyribose group at the 3′-end—that migrate faster through the gel, because of their lower molecular weight. Second, incubation of endonuclease III treated DNA with exonuclease III (track F), led, as would be expected, to a sharp reduction in the radioactivity associated with the abasic site containing dimers. Third, the exonuclease III treatment (tracks D and F) drastically reduced (~90%) the radioactivity in bands 6 and 7. The sensitivity of these products to exonuclease III, coupled with their high charge to mass ratio (greater than that of ATP), provided strong evidence that bands 6 and 7 contained the four 2′-deoxynucleoside 3′-(phospho-2″-*O*-glycolic acid) 5′-phosphates.

(*C*) *Comparison with Markers.* The presence, in the postlabeled mixture derived from irradiated DNA, of some of the structures predicted from the previous experiments was

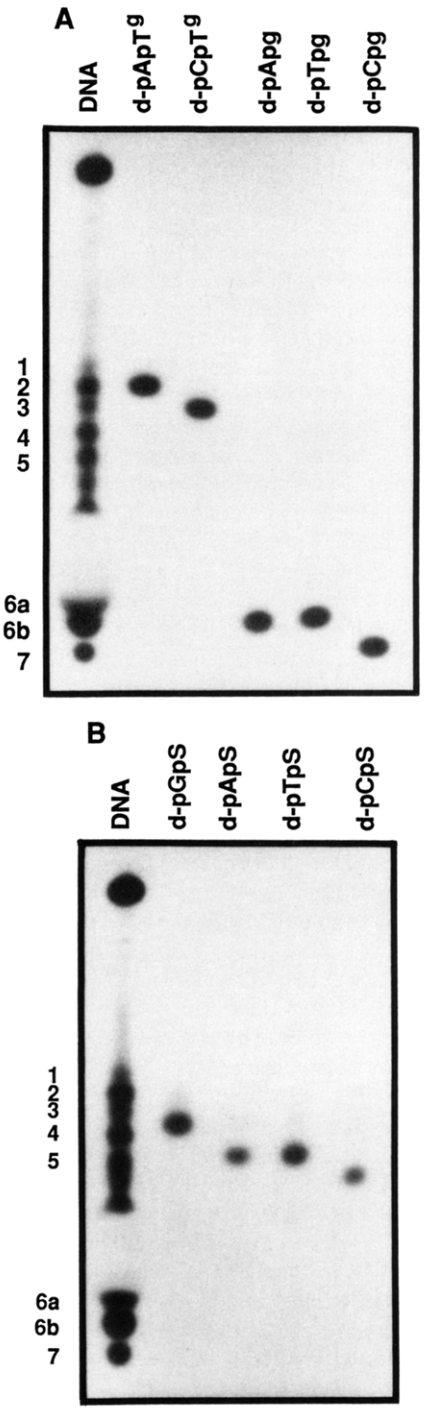

FIGURE 6: Comparison of radiation products with synthetic markers. (A) Comparison with thymine glycol and phosphoglycolate-containing "dinucleotides". (B) Comparison with dinucleotides lacking a 3′-base.

confirmed by comparison with the electrophoretic mobilities (Figure 6A) and HPLC retention times of chemically synthesized compounds. We were thus able to fully establish that the fourth HPLC peaks from gel bands 2 and 3 are the *cis*-thymine glycol containing dinucleotides with adenine and cytosine, respectively, in the 5′-position. Similarly, gel bands 6b and 7 were shown conclusively to contain the 3′-phosphoglycolate esters of the 5′-monophosphates of thymidine, deoxyadenosine, and deoxycytidine. We were unable, however, to confirm the presence of significant quantities of 2-deoxy-D-*erythro*-pentose groups (the abasic sites resulting from acid-catalyzed hydrolysis of N-glycosylic bonds) in DNA irradiated to 50 Gy. Figure 6B shows the comparison of irradiated DNA with synthetic markers derived by acid depuri-

nation of dinucleotides (Weinfeld et al., 1990). It can be seen that d-pGpS does not migrate with any of the major bands in the first track and that, while d-pApS and d-pTpS have similar mobilities to the compounds in band 5, the nuclease P1 digestion of the labeled products in this band indicated the presence of only deoxycytidine monophosphate (Table I).

Discussion

In this study we have shown that a variety of radiogenic DNA lesions can be simultaneously detected by a ^{32}P-post-labeling procedure, which is based on the enzymatic phosphorylation of oligonucleotide fragments generated by snake venom phosphodiesterase and calf alkaline phosphatase digestion of irradiated DNA. As stated in the introduction, an important advantage of the approach is that polynucleotide kinase acts on the normal nucleoside found at the 5′-end of each fragment. This permits detection of base and sugar damage that in the form of monomeric 3′-monophosphate units (generated in the Randerath method) would not serve as substrates for the kinase. Among the lesions in the radiolabeled products isolated by electrophoresis and HPLC, we have identified thymine glycols and phosphoglycolates. [Implicit in their detection by this procedure is the fact thymine glycols and phosphoglycolates render the adjacent 5′-phosphodiester linkage refractory to snake venom phosphodiesterase. Studies with model compounds bear this out (Weinfeld and Soderlind, unpublished data; Henner et al., 1983).] An inspection of Table I, in particular gel bands 4 and 5, suggests that two other, as yet unidentified, major lesions will be quantifiable by this approach. Comparison with markers indicated that neither of these are abasic sites of the type produced by simple hydrolysis of a damaged base from its sugar. This is at variance with previously published observations, in which the products of irradiation were examined by GC–MS (Isildar et al., 1981). These authors themselves suggest, however, that the abasic sites may have arisen in the course of their procedure.

Using the values provided in Table I and taking into account the concentration of DNA in the irradiated solution, we obtain a radiation-chemical yield (G value) for total thymidine glycol production of $\sim$0.0022 μmol J^{-1} [or 0.021 molecule (100 eV)$^{-1}$]. This is in good agreement with a figure of 0.018 molecule (100 eV)$^{-1}$ determined by radioimmunoassay (West et al., 1982). The calculated G value for total phosphoglycolate yield is 0.0105 μmol J^{-1} [0.101 molecule (100 eV)$^{-1}$]. Since this is the first time a G value has been obtained for this product, it cannot be directly compared to a literature value. A G value of $\simeq$0.023 μmol J^{-1} has been determined for malondialdehyde production from γ-irradiated *E. coli* DNA (von Sonntag, 1987). Malondialdehyde is produced in the same reaction that gives rise to phosphoglycolate groups. However, the thiobarbituric acid based assay for malondialdehyde, though sensitive, is not particularly specific, and it is possible that other products may have contributed to the G value given for malondialdehyde (Langfinger & von Sonntag, 1985).

The principal drawback of the postlabeling procedure described here is that for every type of lesion generated four radiolabeled products can potentially be isolated. On the other hand, advantage can be taken of this situation through the use of nuclease P1 to obtain a nearest-neighbor analysis of the bases 5′ to the damaged nucleosides. The outcome of this type of analysis for both thymine glycols and phosphoglycolates (Table I) indicates that the reaction of radiation-induced hydroxyl radicals with thymine bases and deoxyribose groups within double-stranded DNA is nonrandom. For example, the nearly 3-fold difference in yield between d-pGpT^s and d-pApT^s does not reflect the nearest-neighbor distribution of thymidine within calf thymus DNA (Setlow, 1975). At present, however, we cannot offer a theoretical interpretation of these results.

The radioprotection afforded by DMSO (Figure 2) provided strong evidence that the radiogenic lesions under examination in this study were produced by hydroxyl radicals. Many other DNA-damaging agents are believed to act through oxygen radical mediated mechanisms, including hydrogen peroxide (Imlay & Linn, 1988; Blakely et al., 1990), bleomycin (Stubbe & Kozarich, 1987), mitomycin C (Lown & Chen, 1981; Dusre et al., 1990), and iron(II)–EDTA (Tullius, 1987). The postlabeling assay described above should find application to both in vitro and in vivo studies of these agents, as well as ionizing radiation.

Acknowledgments

We thank Drs. J. D. Chapman, M. Liuzzi, and M. C. Paterson for useful discussions. We are very grateful to Dr. R. P. Cunningham for supplying us with the endonuclease III overproducing strain of *E. coli*, Drs. W. D. Henner and S. M. Hecht for providing us with phosphoglycolate markers, and Dr. H. Ide for providing us with a sample of thymidine glycol. The skillful assistance of Gina Kennedy in preparing the manuscript is appreciated.

Registry No. d-pGpT^s, 130614-82-3; d-pTpT^s, 130614-83-4; d-pApT^s, 130641-40-6; d-pCpT^s, 130641-41-7; d-pGpg, 84871-00-1; d-pTpg, 97920-30-4; d-pApg, 97920-32-6; d-pCpg, 97920-31-5; thymine glycol, 2943-56-8; phosphoglycolate, 13147-57-4; hydroxy, 3352-57-6.

References

Asahara, H., Wistort, P. M., Bank, J. F., Bakerian, R. H., & Cunningham, R. P. (1989) *Biochemistry 28*, 4444–4449.
Bailly, V., & Verly, W. G. (1987) *J. Biochem. 242*, 565–572.
Blakely, W. F., Fuciarelli, A. F., Wegher, B. J., & Dizdaroglu, M. (1990) *Radiat. Res. 121*, 338–343.
Breimer, L. H., & Lindahl, T. (1985) *Biochemistry 24*, 4018–4022.
Dizdaroglu, M., & Bergtold, D. S. (1986) *Anal. Biochem. 156*, 182–188.
Dizdaroglu, M., Hermes, W., Schulte-Frohlinde, D., & von Sonntag, C. (1978) *Int. J. Radiat. Biol. 33*, 563–569.
Dusre, L., Rajagopolan, S., Eliot, H. M., Covey, J. M., & Sinha, B. K. (1990) *Cancer Res. 50*, 648–652.
Frenkel, K., Goldstein, M. S., Duker, N. J., & Teebor, G. W. (1981) *Biochemistry 20*, 750–754.
Hegi, M. E., Sagelsdorff, P., & Lutz, W. K. (1989) *Carcinogenesis 10*, 43–47.
Henner, W. D., Rodriguez, L. O., Hecht, S. M., & Haseltine, W. A. (1983) *J. Biol. Chem. 258*, 711–713.
Hubbard, K., Huang, H., Laspia, M. F., Ide, H., Erlanger, B. F., & Wallace, S. S. (1989) *Radiat. Res. 118*, 257–268.
Iida, S., & Hayatsu, H. (1971) *Biochim. Biophys. Acta 240*, 370–375.
Imlay, J. A., & Linn, S. (1988) *Science 240*, 1302–1309.
Isildar, M., Schuchmann, M. N., Schulte-Frohlinde, D., & von Sonntag, C. (1981) *Int. J. Radiat. Biol. 40*, 347–354.
Kasai, H., Crain, P. F., Kuchino, Y., Nishimura, S., Ootsuyama, A., & Tanooka, H. (1986) *Carcinogenesis 7*, 1849–1851.
Kim, J., & Linn, S. (1988) *Nucleic Acids Res. 16*, 1135–1141.
Kochetkov, N. K., & Budovskii, E. I. (1972) *Organic Chemistry of Nucleic Acids*, Part B, pp 287–288, Plenum Press, London.
Kow, Y. W. (1989) *Biochemistry 28*, 3280–3287.

Kow, Y. W., & Wallace, S. S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8354–8358.
Langfinger, D., & von Sonntag, C. (1985) *Z. Naturforsch. 40C*, 446–448.
Leadon, S. A. (1987) *Br. J. Cancer 55* (Suppl. VIII), 113–117.
Liuzzi, M., Weinfeld, M., & Paterson, M. C. (1989) *J. Biol. Chem. 264*, 6355–6363.
Lown, J. W., & Chen, H. H. (1981) *Can. J. Chem. 59*, 390–395.
Maniatis, T., Fritsch, R. R., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mouret, J. F., Odin, F., Polverelli, M., & Cadet, J. (1990) *Chem. Res. Toxicol. 3*, 102–111.
Paterson, M. C. (1978) *Adv. Radiat. Res. 7*, 1–53.
Randerath, K., Reddy, M. V., & Gupta, R. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6126–6129.
Reuvers, A. P., Greenstock, C. L., Borsa, J., & Chapman, J. D. (1973) *Int. J. Radiat. Biol. 24*, 533–536.
Rosier, J., & Van Peteghem, C. (1988) *J. Chromatogr. 434*, 222–227.
Setlow, P. (1975) Nucleic Acids, in *Handbook of Biochemistry and Molecular Biology*, 3rd ed. (Fasman, G. D., Ed.) Vol. II, p 317, CRC Press, Cleveland, OH.
Sharma, M., Box, H. B., & Paul, C. R. (1990) *Biochem. Biophys. Res. Commun. 167*, 419–424.
Stubbe, J., & Kozarich, J. W. (1987) *Chem. Rev. 87*, 1107–1136.
Tullius, T. D. (1987) *Trends Biochem. Sci. 12*, 297–300.
von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor & Francis, London.
Wallace, S. S. (1988) *Environ. Mol. Mutagen. 12*, 431–477.
Ward, J. F. (1975) *Adv. Radiat. Biol. 5*, 182–239.
Weinfeld, M., Liuzzi, M., & Paterson, M. C. (1989a) *Nucleic Acids Res. 17*, 3735–3745.
Weinfeld, M., Liuzzi, M., & Paterson, M. C. (1989b) *J. Biol. Chem. 264*, 6364–6370.
Weinfeld, M., Liuzzi, M., & Paterson, M. C. (1990) *Biochemistry 29*, 1737–1743.
West, G. J., West, I. W.-L., & Ward, J. F. (1982) *Radiat. Res. 90*, 595–608.

A Thermodynamic Analysis of RNA Transcript Elongation and Termination in *Escherichia coli*†

Thomas D. Yager and Peter H. von Hippel*
Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403
Received August 14, 1989; Revised Manuscript Received September 26, 1990

ABSTRACT: In the first part of this paper we present a thermodynamic analysis of the elongation phase of transcription in *Escherichia coli*. The stability of the elongation complex is described by a "free energy of formation" function ($\Delta G_f°$) that is a sum of terms for forming (i) a locally denatured 17-base-pair DNA "bubble"; (ii) a constant-length hybrid between the 3′-terminal 12-nucleotide residues of the RNA transcript and the corresponding region of the DNA template strand; and (iii) a set of binding interactions between the polymerase and certain DNA and RNA residues within and near the "transcription bubble". The transcriptional elongation complex is very stable at most positions along a natural DNA template and moves in a highly processive fashion. At these positions, the $\Delta G_f°$ function provides a *quantitative* measure of the stability of the elongation complex. Besides allowing for the polymerization of the RNA transcript, the elongation complex also serves to define the context within which transcript termination occurs. In the second part of the paper the thermodynamic analysis is extended to discriminate between template positions at which the elongation complex is stable and positions at which it is rendered relatively unstable by the presence of a string of rU residues at the 3′-terminus of the RNA together with the formation of a specific RNA hairpin just upstream of this point. Most factor-independent (intrinsic) termination events are thermodynamically disallowed at the former positions and are thermodynamically allowed at the latter positions. The extended form of the analysis closely predicts the exact sites of termination at a number of intrinsic terminators (and attenuators) in the *E. coli* genome. It also correctly predicts bidirectional function for a number of bidirectional terminators. In some cases it may identify terminators that are similar to the intrinsic type but that require additional protein factors, unusual polymerase–nucleic acid interactions, or rate-limiting conformational changes in order to function. Finally, it successfully locates intrinsic terminators within a number of *E. coli* operons and discriminates between these terminators and the surrounding DNA sequence.

Transcription of RNA in prokaryotes occurs in six mechanistically distinct phases, which can be described as follows.

(*i*) *Promoter Location.* The RNA polymerase holoenzyme locates a promoter, perhaps in part by facilitated diffusion, and correctly "articulates" with this structure [see, e.g., von Hippel et al. (1984) and McClure (1985)]. This process occurs spontaneously, implying a (favorable) free energy decrease.

(*ii*) *Melting In.* The polymerase holoenzyme melts into (partially open) promoter DNA, which enables the template

†This work was supported in part by USPHS Grants GM-15792 and GM-29158 (to P.H.v.H.) and by USPHS Individual Postdoctoral Fellowship GM-10227 (to T.D.Y.). P.H.v.H. is an American Cancer Society Research Professor of Chemistry.

0006-2960/91/0430-1097$02.50/0