Spontaneous Cleavage of Bleomycin-Induced Abasic Sites in Chromatin and Their Mutagenicity in Mammalian Shuttle Vectors†

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ABSTRACT: The stability of oxidized abasic sites induced by bleomycin and neocarzinostatin was examined in chromatin reconstituted from a supercoiled plasmid and core histones. Most of the drug-induced abasic sites were found to undergo spontaneous cleavage in chromatin, probably by reaction with histone amine groups. However, there was considerable heterogeneity in the rate of spontaneous cleavage, with some sites being cleaved almost immediately and some remaining intact even after 7 h. Bleomycin-induced abasic sites with closely opposed strand breaks were more unstable than lone abasic sites. Neocarzinostatin-induced abasic sites, which have a different chemical structure, were cleaved somewhat more slowly than those induced by bleomycin. To assess the mutagenic potential of bleomycin-induced abasic sites, bleomycin-treated shuttle vectors were transfected into mammalian cells, and mutations in progeny plasmids were sequenced. Bleomycin treatment resulted primarily in deletions of various sizes in the shuttle vectors, including a number of one-base deletions occurring at potential bleomycin damage sites. However, under certain conditions, substitutions occurring at expected sites of bleomycin attack were also observed. The results suggest that bleomycin-induced abasic sites have only a slight potential to produce base substitutions in mammalian cells and that a substantial fraction of the double-strand breaks induced by bleomycin and most of the double-strand breaks induced by neocarzinostatin are the result of spontaneous cleavage of abasic sites with closely opposed strand breaks. Inaccurate repair of these double-strand breaks may accout for the large deletions, and perhaps the one-base deletions, induced by bleomycin.

Abasic (AP)[1] sites have been proposed as intermediates in mutagenesis by a variety of genotoxic agents (Loeb, 1985; Strauss et al., 1982). Ionizing radiation and other agents which generate oxygen radicals produce AP sites by oxidation of deoxyribose sugars in DNA (Isildar et al., 1981; Povirk & Steighner, 1989). In general, oxygen radicals attack DNA indiscriminately, producing myriad base damages as well. However, bleomycin specifically attacks C-4′ of deoxyribose, producing AP sites in which the C-4′ position has been oxidized to a ketone (Rabow et al., 1986). Neocarzinostatin (NCS) induces AP sites which involve predominantly oxidation of C-1′ to a lactone (Kappen & Goldberg, 1989). Since these drugs induce AP sites with some sequence specificity and induce very little DNA base damage (Gajewski et al., 1991), they offer an opportunity to assess the role of oxidized AP sites in oxidative mutagenesis.

Some of the AP sites induced by bleomycin and NCS are accompanied by a closely opposed break in the complementary strand (Povirk & Houlgrave, 1988), which renders them resistant to cleavage by certain AP endonucleases and, at least in *Escherichia coli*, increases their mutagenic potential (Steighner & Povirk, 1990a; Povirk et al., 1988). However, the altered chemical structures of free radical-induced AP sites render them more susceptible than normal AP sites to cleavage by certain primary amines (Povirk & Houlgrave, 1988). Thus, it is possible that they would be too short-lived in chromatin, which is particularly rich in primary amines, to have significant mutagenic potential.

In order to address this question, the stability of bleomycin- and NCS-induced AP sites was examined in reconstituted chromatin. In addition, bleomycin-induced mutagenesis was examined in two SV40-based shuttle vectors, and the effect of methoxyamine treatment, which stabilizes AP sites against amine-catalyzed cleavage (Liuzzi & Talpaert-Borlé, 1985), was assessed.

MATERIALS AND METHODS

Plasmids. The shuttle vector pSVSU270 (Figure 1) was constructed from pSWC270 (Yarus et al., 1986), pSVod (Mellon et al. 1982), and pBR322. The 264-bp *Eco*RI fragment of pSWC270, which contains a promoterless *trpT180* suppressor tRNA allele, was cloned into the *Eco*RI site of pBR322, in which it was efficiently transcribed from the *tet* promoter. A partial *Eco*RI digest of this plasmid was treated with Klenow fragment plus dNTPs to produce blunt ends, and *Xho*I linkers were added. Following digestion with *Bam*HI and *Xho*I, a 646-bp fragment containing *trpT180* and part of *tet* was isolated. A derivative of pSVod, in which the *Hin*dIII site at the 5′-end of the *tet* gene was converted to an *Xho*I site, was generated by *Hin*dIII cleavage followed by ligation in the presence of an excess of AGCTCG linker. The *Xho*I/*Bam*HI fragment containing the 5′ half of *tet* was removed from this vector and replaced with the 646-bp *trpT180*-containing fragment to give pSVSU270. This vector lacks the poison sequences near the pBR322 replication origin and replicates efficiently in COS 1 cells.

pSVSU270 and pZ189 were grown in *recA E. coli* in the absence of chloramphenicol and isolated by the alkaline lysis method followed by banding in cesium chloride–ethidium bromide gradients. To obtain ^{32}P-labeled plasmid, the culture

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[1] AP, abasic (apurinic/apyrimidinic); dNTP, deoxynucleoside triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, disodium ethylenediaminetetraacetate; PMSF, (phenylmethyl)-sulfonyl fluoride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-pressure liquid chromatography; PCR, polymerase chain reaction; bp, base pair; kb, kilobase.

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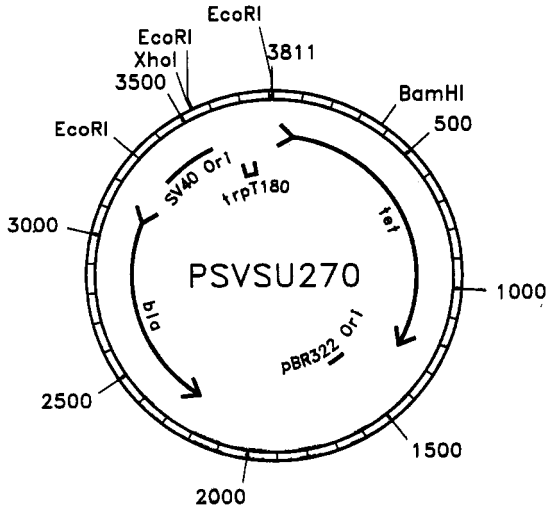

FIGURE 1: Shuttle vector pSVSU270 (3811 bp). The *trpT180* gene is from pSWC270, the 5′ portion of *tet* is from pBR322, and the remainder of the vector is from pSVod. *trpT180* is a mutant tryptophan tRNA gene which suppresses amber mutations and is the marker gene for bleomycin-induced mutagenesis. The vector also contains genes coding for resistance to tetracycline (*tet*) and ampicillin (*bla*), a pBR322 replication origin to support replication in *E. coli*, and an SV40 replication origin to support T-antigen-dependent replication in COS 1 cells.

broth was supplemented with 5 μCi/mL [^{32}P]orthophosphate (carrier-free, ICN). The specific activity of the DNA was approximately 15 000 dpm/μg.

Drugs. Fe^{III}·bleomycin was prepared from clinical blenoxane (Bristol-Myers) as described previously (Povirk & Houlgrave, 1988) and stored at −20 °C at a concentration of 1 mM. NCS chromophore, at a concentration of 100 μM, was prepared by methanol extraction of clinical NCS (Povirk et al., 1981) and was diluted on ice in 20% methanol/20 mM sodium citrate, pH 4.

Other Materials. CM Sephadex C-25 (Pharmacia) minicolumns were prepared as described previously (Steighner & Povirk, 1990b). Sephadex G-50 columns were prepared similarly except G-50 Sephadex slurry (Pharmacia) was used. Micrococcal nuclease (Sigma) was dissolved in distilled water at a concentration of 12.5 units/mL, stored in 10-μL aliquots at −70 °C, and diluted in water immediately before use. COS 1 cells, an SV40-transformed African Green Monkey kidney fibroblast-like cell line (Gluzman, 1981) permissive for replication of pSVSU270, and human 293 cells were from American Type Culture Collection.

Chromatin Reconstitution. Human core histones were prepared from a leukopheresis pack from a patient with hairy cell leukemia. The cells were washed four times with phosphate-buffered saline by gravity sedimentation at 4 °C, with the red cell layers being discarded. A total of 4×10^{11} nucleated cells, predominantly lymphocytes, was lysed with 0.5% nonidet P40/10 mM Hepes–NaOH/1 mM EDTA/0.2 mM PMSF/0.2 mM DTNB, pH 7.4, and chromatin was pelleted by centrifugation. The pellet was extracted six times with the same buffer, containing 0.15 M NaCl and no detergent. The pellet was then suspended in deionized water containing 0.2 mM PMSF and 0.2 mM DTNB and homogenized with a Dounce homogenizer. The slurry was mixed with an equal volume of 0.4 M H_2SO_4, homogenized on ice for 10 min, and centrifuged at 2000*g* for 10 min. Following extraction of the pellet with 0.2 M H_2SO_4, the supernatants, containing histones, were combined and extensively dialyzed against 0.1 mM PMSF/0.1 mM DTNB. This preparation contained approximately equal amounts of histones H2a, H2b, H3, and H4 and a lesser amount of H1, as determined by polyacrylamide gel electrophoresis.

Chromatin was reconstituted by a modification of the procedure of Stein (1987). The reconstitution mix contained 22.5 μg of plasmid DNA and 90 μg of histones in 1.2 mL of 0.8 M NaCl/10 mM 2-mercaptoethanol/1 mM EGTA/1 mM EGTA/10 mM Tris-HCl, pH 8. After 2 h at 22 °C, the NaCl concentration was decreased by dilution to 0.6 M and after an additional 2 h to 0.4 M. After a final 2 h at 22 °C, the mixture was stored at 4 °C.

In order to concentrate and desalt the sample and remove excess histones, the reconstituted chromatin was pelleted in a sucrose gradient. The gradient was prepared by layering 2.3 mL of 10% sucrose/0.4 M NaCl over 2.3 mL of 20% sucrose, both in 50 mM Hepes–NaOH (pH 8)/0.1 mM EDTA (HE8), in a 5-mL Beckman Ultraclear tube. The tube was sealed with Parafilm and tilted at 90° for 1 h to allow some diffusion between the layers. The reconstitution mix (0.4 mL per tube) was layered atop the gradient, and it was centrifuged in a Beckman SW55 rotor for 6 h at 45 000 rpm at 20 °C. The supernatant was immediately poured off and a clear, wet pellet was evident. Following the addition of 0.2 mL of distilled water, the tube was shaken for 14 h at 22 °C, and the resuspended chromatin was removed and stored at 4 °C.

To examine nucleosome periodicity, chromatin samples with a DNA concentration of 10 μg/mL were digested with micrococcal nuclease in 20 mM Tris/2 mM $CaCl_2$, pH 8. After incubation at 37 °C for various times, 30-μL aliquots were removed, and the reaction was stopped by addition of EGTA to 5 mM and sodium acetate to 0.3 M, followed immediately by phenol/chloroform extraction. Each sample was concentrated 6-fold by ethanol precipitation and electrophoresed on a 2% agarose gel.

DNA Cleavage Assays. Bleomycin reaction mixtures contained 10 μg/mL DNA or an equivalent amount of reconstituted chromatin and 0.01–0.16 μM Fe^{III}·bleomycin in HE8. The drug was activated by addition of 2-mercaptoethanol to 25 mM followed by incubation for 1 h at 37 °C or by addition of H_2O_2 to 1 mM followed by incubation for 20 min at 22 °C. To stop the reaction and remove drug, the sample was diluted into 2 mL of 50 mM EDTA, pH 8, centrifuged in a Centricon 30 concentrator (Amicon) for 30 min at 8000*g*, then diluted in 2 mL of HE8, and again centrifuged for 30–40 min. Control experiments showed that this procedure rendered the sample devoid of any DNA-cleaving activity. The sample was adjusted back to its initial volume and then incubated for various times at 37 °C. Histones were then removed by addition of NaCl to 0.2 M followed by extraction with 1:1 phenol/chloroform. The sample was then split in two and incubated for 45 min at 37 °C in the presence or absence of 20 mM putrescine, a concentration which was sufficient to cleave all AP sites.

NCS reactions were performed as above except that activation was effected by glutathione (5 mM) at 22 °C for 20 min in the dark. Since the activation and inactivation of NCS chromophore are complete within a few minutes (Kappen et al., 1980), samples were then incubated immediately at 37 °C, without drug removal. However, after histone removal the samples were desalted by dilution in HE8 followed by Centricon 30 reconcentration because cleavage of NCS-induced AP sites by putrescine was found to be partially inhibited by 0.2 M NaCl. Complete cleavage was effected by treatment with 0.1 M putrescine for 1 h at 37 °C in HE8.

The samples (40–50 μL) were electrophoresed on 14 × 10 × 0.7 cm 1.8% agarose gels containing 12 mM sodium acetate/12 mM Hepes–NaOH/0.6 mM EDTA, pH 7.5, at 11.5 V/cm

for 3.5 h with buffer recirculation. The gels were vacuum-dried on filter paper, and radioactivity in the supercoiled, relaxed, and linear molecules was determined on a Betagen 630 blot reader. Single- and double-strand breaks were calculated as described previously (Povirk & Houlgrave, 1988). Single-strand breakage in samples not exposed to drug, usually ~0.15 breaks per plasmid, was substracted. There was no detectable double-strand breakage in the untreated samples.

Thymine Propenal Release. In order to measure bleomycin-induced release of thymine propenal and free thymine base, reactions were performed with H_2O_2 activation as above, except that the bleomycin concentration was increased to 3–6 μM and the plasmid used was [^{3}H]thymidine-labeled colE1, prepared as described previously (Povirk & Houlgrave, 1988). The undiluted reaction mix (0.32 mL) was then subjected to Centricon 30 ultrafiltration for 70 min at 8000*g*. The filtrate, containing low molecular weight released species, was evaporated to 200 μL and subjected to HPLC on a 4.6 × 250 mm Spherisorb 5-μm ODS2 column eluted for 10 min at 1 mL/min with aqueous 5 mM ammonium acetate/5 mM acetic acid, followed by a 20-min gradient of 0–50% acetonitrile containing the same buffer. Thymine eluted at 14 min, thymidine at 19 min, and thymine propenal at 22 min. Fractions of 1 mL were collected, and released compounds were quantified by liquid scintillation counting.

Bleomycin Treatment of Shuttle Vectors. pSVSU270 or pZ189 (40 μg) was reacted with 3.0 or 6.0 μM bleomycin in 50 mM Hepes–NaOH (pH 7.5)/0.1 mM EDTA (HE7.5) and 25 mM 2-mercaptoethanol in a total volume of 400 μL. The reaction mixture was prepared at 22 °C and then incubated for 30 min at 37 °C. Following termination of the reaction by incubation for 10 min in the presence of 50 mM EDTA, bleomycin was removed by retention in CM Sephadex C-25 columns spun at 3200*g* for 10 min or by dilution to 2 mL in HE7.5 and concentration in a Centricon 30 microconcentrator spun at 8000*g* for 30 min. To one-half of each sample was added methoxyamine to a final concentration of 10 mM followed by another 30-min incubation at 37 °C. Control experiments showed that this concentration of methoxyamine protected greater than 90% of bleomycin-induced AP sites from cleavage by putrescine, but did not itself induce any detectable mutagenesis. Methoxyamine was removed from samples by passage through a Sephadex G50 column (3200*g*, 10 min) or by dilution in HE7.5 to 2 mL and concentration in a Centricon 30 concentrator.

Transfection. Trypsinized COS 1 cells were harvested in exponential growth from six 100 × 20 mm tissue culture dishes and resuspended in 6 mL of 137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM glucose/20 mM Hepes–NaOH, pH 7.05, resulting in a concentration of (2.0–3.5) × 10^6 cells/mL. Ten micrograms of DNA (100 μL) plus 11 μL of 10× phosphate-buffered saline were added to each 1 mL of cell suspension. The COS 1 cells were transfected with DNA by electroporation at 200 V using a gene pulser (Bio-Rad) with a 0.4-cm gapped cuvette and a 500-μF capacitance, yielding a time constant between 8 and 10 ms. Electroporated cells remained on ice until their transfer to Dulbecco's modified Eagle medium containing penicillin and streptomycin (Gibco) and 10% defined, supplemented calf serum (HyClone). Cells were cultured in 100 × 20 mm tissue culture dishes and incubated for 48 h in 5% CO_2 and 95% humidified air to allow plasmid replication. Human 293 cells were transfected by the calcium phosphate procedure as described previously (Wang et al., 1990) and likewise incubated for 48 h.

Mutation Analysis. Progeny plasmid was isolated from COS 1 or 293 cells, treated with *Dpn*I to digest any unreplicated

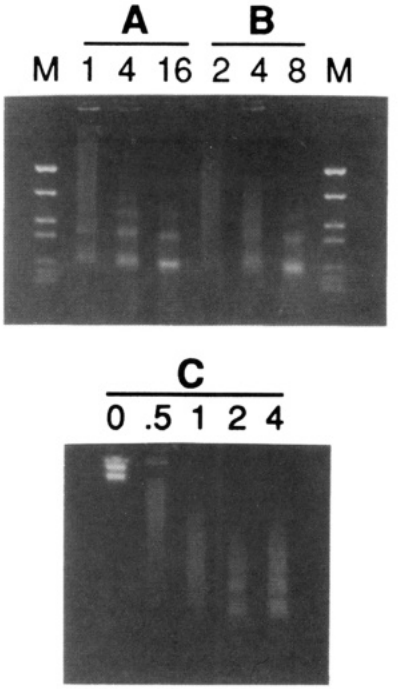

FIGURE 2: Internucleosomal cleavage by micrococcal nuclease (A), bleomycin (B), or NCS (C). Chromatin reconstituted from plasmid pZ189 and core histones, with a final DNA concentration of 10 μg/mL, was treated with 0.006 unit/mL micrococcal nuclease for 1, 4, or 16 min, treated with 2, 4, or 8 μM bleomycin in the presence of 2-mercaptoethanol, or treated with 0, 0.5, 1, 2, or 4 μM NCS and then treated with putrescine to cleave AP sites. The lane marked M contains molecular weight markers of (top to bottom) 955, 585, 341, 258, 141, 105, and 75 bp, obtained by digestion of pUC19 with *Dpn*I.

molecules, and transfected by electroporation into *lacZ*(Am) indicator cells (Wang et al., 1990). For pZ189, MBM7070 cells (Hauser et al., 1986) were used; for pSVSU270, a tetracycline-sensitive derivative, MBM7070S, was isolated as described by Bochner et al. (1980). Transformants were plated on Luria-Bertani plates with tetracycline (12.5 μg/mL), ampicillin (50 μg/mL), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (147 μM), and isopropyl β-D-thiogalactopyranoside (30 μM). White or light blue colonies were stabbed and streaked on a new plate and then grown in 6 mL of LB medium with antibiotics for plasmid DNA preparation.

Plasmids were analyzed by agarose gel electrophoresis to assay for gross deletions and insertions. pSVSU270 mutants were also screened for loss or alteration of the 264-bp *Eco*RI fragment containing *trpT180*. Mutants with no apparent large deletions were processed for dideoxy sequencing through *trpT180* or *supF* using avian myeloblastosis virus reverse transcriptase (Promega) and a pBR322 *Hin*dIII counter-clockwise primer for pSVSU270 or a pBR322 *Eco*RI clockwise primer for pZ189 (New England Biolabs or Promega) (Wang et al., 1990).

RESULTS

Characterization of Reconstituted Chromatin. Partial digestion of the reconstituted chromatin with micrococcal nuclease produced a nucleosomal ladder of up to six bands with a periodicity of 145 bp (Figure 2A). This interval is smaller than that of native chromatin (~200 bp) and is typical of the close nucleosome spacing resulting from reconstitution with core histones (Stein, 1987). Polyacrylamide gel electrophoresis of histones extracted from the chromatin revealed equal amounts of histones H2a, H2b, H3, and H4; a small amount of H1 present in the original histone preparation was not present in histones extracted from the reconstituted chromatin (data not shown).

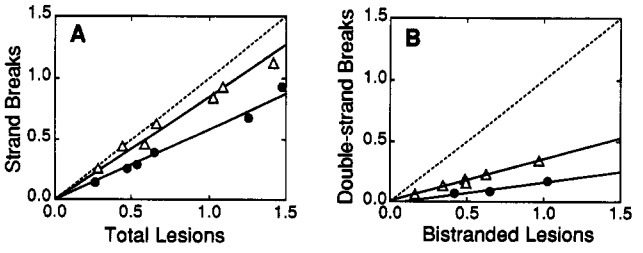

FIGURE 3: Comparison of direct strand breaks and AP sites induced by bleomycin (A) or NCS chromophore (B) in chromatin (△) or in naked DNA (●). The ordinate shows the total number of lesions, i.e., the number of breaks following cleavage of AP sites with putrescine, while the abscissa shows the number of direct breaks determined without putrescine treatment. Thus, the deviation of each line from a slope of 1 (dashed line) is a measure of the fraction of lesions which were uncleaved AP sites before putrescine treatment. Drug concentrations were 0.005–0.04 μM bleomycin and 0.005–0.03 μM NCS chromophore.

Treatment of the reconstituted chromatin with either bleomycin (Figure 2B) or NCS (Figure 2C) also produced a nucleosomal ladder, but in each case the bands were less well-defined then those generated by nuclease digestion. Thus, these drugs show some selectivity for internucleosomal DNA, but it is not as strong as that of micrococcal nuclease.

Spontaneous Cleavage of AP Sites in Reconstituted Chromatin. Both bleomycin and NCS induce AP sites by oxidation of DNA sugars. Since these AP sites are cleaved by putrescine, they can be assayed as the increase in strand breakage resulting from exposure to putrescine following drug treatment, where strand breakage is calculated from the conversion of supercoiled molecules into nicked circular and linear molecules (Povirk & Houlgrave, 1988).

When naked DNA was treated with various concentrations of bleomycin in the presence of 2-mercaptoethanol, about 40% of total strand breaks was putrescine-dependent (i.e., AP sites), and 60% was direct breaks (Figure 3A). For bleomycin-treated chromatin, the proportion of putrescine-dependent breaks was only about 15%, suggesting that most of the AP sites had spontaneously broken down in chromatin. In an attempt to reduce AP cleavage during drug treatment, bleomycin was activated with 1 mM H_2O_2, so that the reaction time and temperature could be decreased. Under these conditions, 28% of breaks in chromatin was putrescine-dependent immediately following drug removal, as opposed to 45% in naked DNA (Figure 4A). Spontaneous cleavage of bleomycin-induced AP sites in chromatin continued for several hours, so that by 7 h, only 8% of the breaks remained putrescine-dependent. Bleomycin-induced AP sites were much more stable in naked DNA than in chromatin. Bistranded lesions induced by bleomycin (i.e., AP sites with closely opposed strand breaks, assayed as putrescine-dependent double-strand breaks) were even more rapidly broken down in chromatin than single-strand lesions (Figure 4B).

In all of the above experiments, histones were not removed from bleomycin-treated chromatin until 1 h after treatment, due to the need to first remove drug by ultrafiltration. In an attempt to reduce this interval, activated bleomycin was generated separately by reacting metal-free bleomycin with 1 mM $Fe(NH_4)_2(SO_4)_2$ for 10 s at 6 °C (Burger et al., 1981). The activated bleomycin was then rapidly diluted and added to the chromatin. After 10 min at 22 °C, which should be sufficient for inactivation of all of the activated bleomycin, the histones were removed, and strand cleavage assays were preformed as above. Under these conditions (data not shown), the fraction of putrescine-dependent breaks was still only 30 ± 6% (SD, n = 7), the same as with H_2O_2 activation.

These results suggest that either a portion of the AP sites was very rapidly cleaved in the reconstituted chromatin (within 10 min at 22 °C) or that the chemistry of DNA damage was altered in chromatin such that the initial ratio of direct strand breaks to AP sites was greater in chromatin than in naked DNA. To address this question, release of thymine propenal (which is associated with direct strand cleavage (Giloni et al., 1981)) and free thymine base was measured in [^{3}H]thymine labeled DNA and reconstituted chromatin. Bleomycin concentrations of 3–6 μM resulted in release of 2–4% of the total ^{3}H label (data not shown). In DNA, the ^{3}H release was 50% thymine and 50% thymine propenal (±2% SD, n = 3), while in chromatin it was 52% thymine and 48% thymine propenal (±2% SD, n = 3). Thus, packaging into chromatin did not alter the ratio of direct strand breakage to base release, and the paucity of putrescine-dependent breaks in bleomycin-treated chromatin must be attributed to rapid spontaneous cleavage of AP sites.

AP sites induced by NCS chromophore showed qualitatively similar behavior. Since nearly all NCS-induced AP sites are accompanied by a closely opposed break in the complementary strand (Povirk et al., 1988), these sites were assayed as putrescine-dependent double-strand breaks. When naked DNA was treated with various concentrations of NCS, only 16% of bistranded lesions was direct double-strand breaks while 84% was putrescine-dependent, i.e., AP sites with closely opposed breaks (Figure 3B). When reconstituted chromatin was treated, 35% of the total bistranded lesions was expressed as double-strand breaks, suggesting that some of the AP sites had been rapidly cleaved. The ratio of bistranded lesions to total lesions (i.e., the ratio of double-strand breaks to total breaks following putrescine treatment) was the same, approximately 0.33, in chromatin as in DNA (data not shown).

When NCS-treated chromatin was incubated following drug treatment, the number of double-strand breaks gradually increased, reaching about 72% of total bistranded lesions by 7 h (Figure 4C). Thus, slow spontaneous cleavage of AP sites in chromatin continued for several hours, until the majority of sites were cleaved; nevertheless, bistranded lesions induced by NCS were somewhat more stable than those induced by bleomycin (compare Figure 4B,C).

Mutagenesis in Shuttle Vectors. To investigate the potential of bleomycin-induced AP sites to produce mutations in a mammalian system, mutagenesis was examined in bleomycin-treated shuttle vectors which were replicated in either simian COS 1 or human 293 cells and then rescued in indicator bacteria. Mutations in a suppressor gene (*supF* or *trpT180*) were revealed by a blue/white colony screen (Wang et al., 1990); in both of the vectors used, the suppressor gene is flanked by essential plasmid sequences, thus providing a selection against deletions larger than about 250 bp. To address the possible significance of spontaneous AP site cleavage, methoxyamine was used to stabilize bleomycin-induced AP sites. Methoxyamine adds nucleophilically to the aldehydic carbonyl to create the methyl oxime derivative; this modification prevents the amine-catalyzed β-elimination reaction leading to strand cleavage (Liuzzi & Talpaert-Borlé, 1985).

Mutagenesis was first examined in pSVSU270 replicated in COS 1 cells. The *trpT180* target gene of pSVSU270 contains two occurrences of the sequence C-G-C-C, which was the sequence most frequently mutated by bleomycin in λ phage (Povirk, 1987). Because COS 1 cells constitutively express T-antigen, initiation of plasmid replication should occur rapidly upon transfection, without the need for complete repair of lesions in the plasmid. With transfection by electroporation the background *trpT180* mutation frequency was about 0.1%

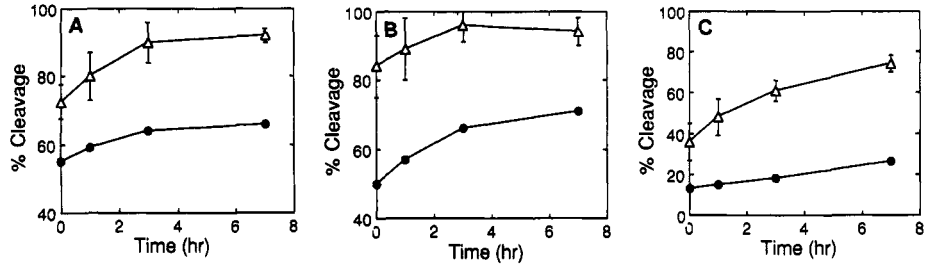

FIGURE 4: Time course of spontaneous cleavage of AP sites induced by bleomycin (A) or cleavage of AP sites with closely opposed breaks induced by bleomycin (B) or NCS (C) in chromatin (△) or in naked DNA (●). The percent cleavage was determined by taking the ratio of the number of strand breaks measured before and after putrescine-induced cleavage of AP sites. Error bars show the standard deviation of three separate experiments. For naked DNA, results are from a single experiment. Drug concentrations were 0.015–0.03 μM NCS and 0.03–0.06 μM bleomycin.

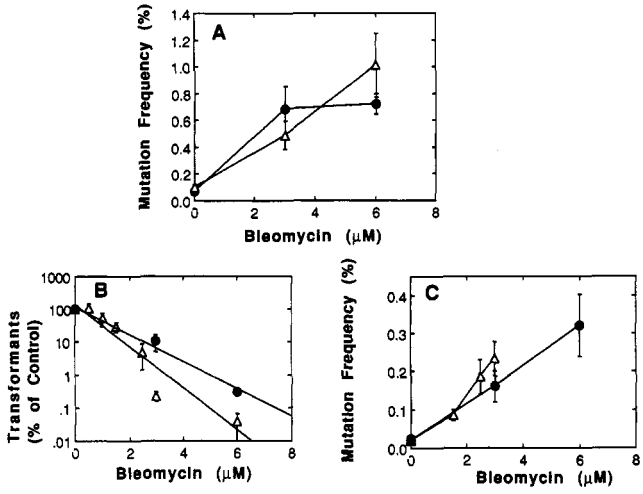

FIGURE 5: Mutagenesis by bleomycin in shuttle vectors. (A) Mean mutation frequency in bleomycin-treated pSVSU270 either with (●) or without (△) subsequent methoxyamine treatment, as determined from the fraction of white colonies recovered from Hirt lysates of COS 1 cells. Error bars indicate the standard error of the mean ($n \geq 4$). (B) Relative transformation frequency for bleomycin-treated pZ189 as determined from the number of bacterial colonies recovered from Hirt lysates of human 293 cells. (C) Mean mutation frequency for bleomycin-treated pZ189 determined as in A ($n = 2$ or 3). Symbols in B and C are the same as in A.

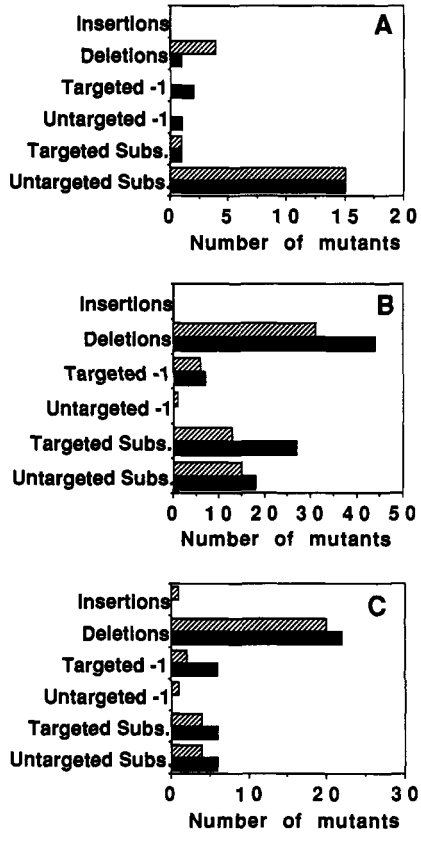

FIGURE 6: Distributions of mutations in sequenced mutant plasmids recovered from Hirt lysates. One-base-pair deletions (–1) and substitutions are divided into "targeted" (i.e., occurring at a primary or secondary site of bleomycin attack) and "untargeted" classes. Solid bars, methoxyamine treatment; striped bars, no methoxyamine. (A) Spontaneous mutations in pSVSU270 (*trpT180*) replicated in COS 1 cells. (B) Bleomycin-induced mutations in the same system. (C) Bleomycin-induced mutations in pZ189 (*supF*) replicated in human 293 cells.

and was not increased by treatment with methoxyamine alone. Bleomycin treatment resulted in an increase in mutation frequency of up to 10-fold (Figure 5A). Agarose gel electrophoresis of the treated plasmid prior to transfection (not shown) revealed that at a dose of 6 μM there was no remaining supercoiled plasmid and slightly more linear than nicked circular plasmid. This distribution indicates that there was, on average, approximately 1 double-strand break per plasmid, and from the known relative frequencies of other lesions (Povirk & Houlgrave, 1988), it can be inferred that there were about 10 single-strand breaks, 10 AP sites, and 0.5–1.0 AP sites with closely opposed breaks per plasmid. Within experimental error, bleomycin treatment did not result in any decrease in the number of replicated plasmids recovered in Hirt extracts. Since there was essentially no undamaged plasmid remaining in the bleomycin-treated samples, this result suggests that bleomycin-induced lesions were either efficiently repaired or efficiently bypassed in COS 1 cells. In fact, even isolated linear plasmids resulting from bleomycin-induced double-strand cleavage were efficiently repaired and replicated when transfected into COS 1 cells (data not shown).

Mutant plasmids from treated samples giving at least a 4-fold increase in mutation frequency, as well as from untreated samples, were sized on agarose gels, and those with the same mobility as pSVSU270 were sequenced. Point mutations were classified according to whether they occurred at potential sites of bleomycin damage (Figure 7). Primary sites for bleomycin damage are *GY* sequences (Y = C or T), indicated by bold capitals in each spectrum; secondary sites (light capitals) in the opposite strand are seldom *GY* sequences and are either directly opposite the primary site or opposite the base one position to the 3′-side (e.g., the A opposite T in GCT) (Povirk et al., 1989). Damage at secondary sites occurs only in the context of concomitant damage at the corresponding primary site (Steighner & Povirk, 1990a). In *trpT180*, 33% of the base pairs is potential primary or secondary bleomycin targets.

Of the mutant plasmids recovered from untreated samples or samples treated with methoxyamine only, all had normal electrophoretic mobility and nearly all were base substitutions (Figure 6A). For the samples treated with bleomycin, 91% (without methoxyamine) or 96% (with methoxyamine) had

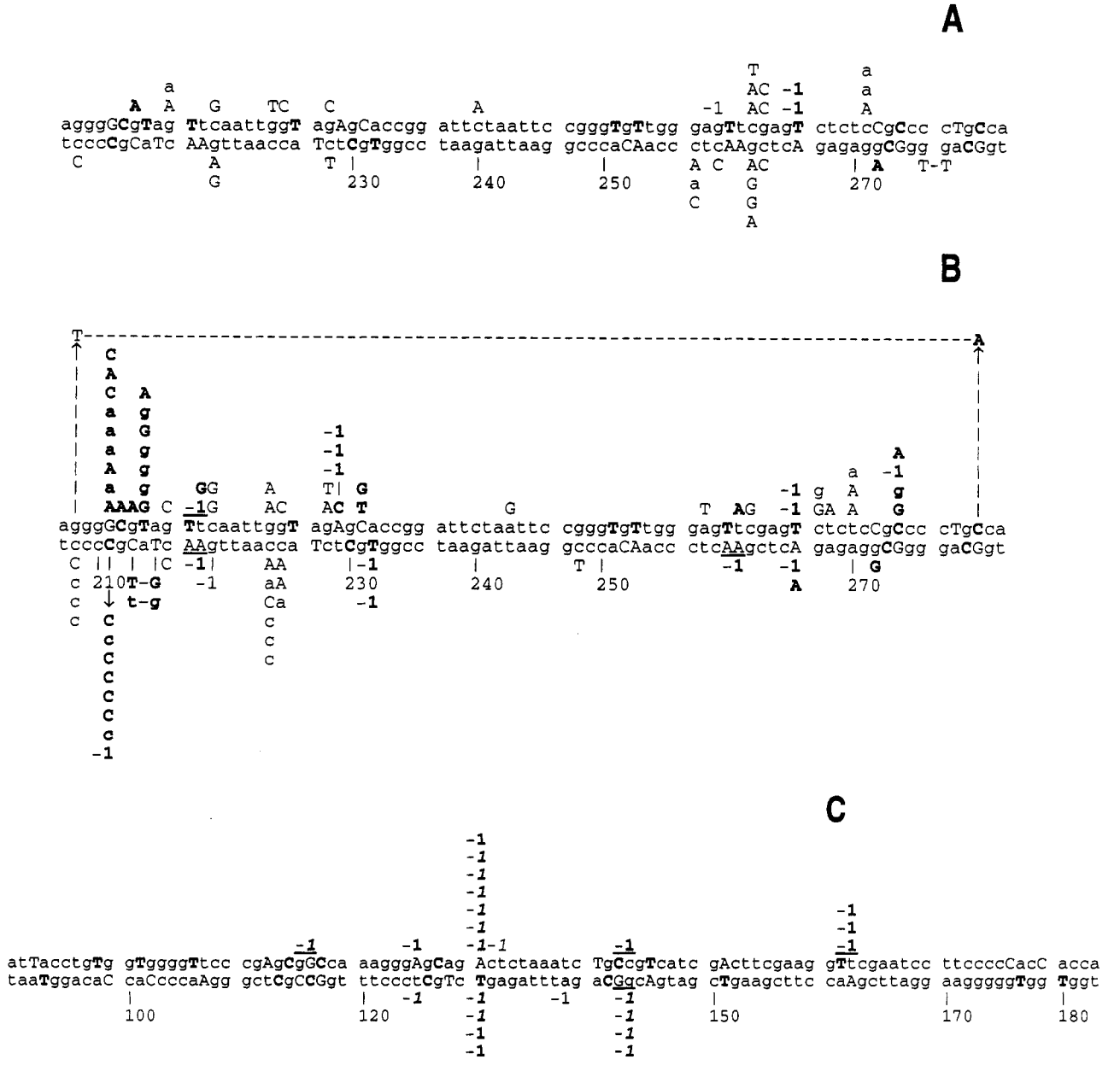

FIGURE 7: Spectra of spontaneous (A) or bleomycin-induced (B) one-base-pair deletions and base substitutions in *trpT180* and spectrum of bleomycin-induced one-base-pair deletions in *supF* (C). Bold capital letters in the gene are primary sites for bleomycin attack, and light capitals are secondary sites; lower case letters are not targets for bleomycin attack. Mutations occurring in samples which received methoxyamine treatment are above the gene sequence, while those occurring in samples which did not are below. Base substitutions reflect changes in the top strand sequence. Lower case letters above and below the sequence of the gene indicate duplicate mutants recovered more than once from a single transfection. Targeted mutations are in boldface type. In C, –1 refers to mutants recovered from Hirt lysates of 293 cells and *–1* from mutants recovered from Hirt lysates of COS 1 cells.

normal electrophoretic mobility, and in both cases about one-half of the mutations were deletions, ranging in size from 2 bp to >100 bp, while most of the remainder were base substitutions (Figure 6B). Of the base substitutions in the progeny of plasmids treated with bleomycin alone, 46% occurred at potential sites of bleomycin attack; methoxyamine increased this fraction to 60%, a statistically insignificant change. As in λ phage, base substitutions occurred frequently at the two C-G-*C*-C sequences in *trpT180*, particularly at base 211 (Figure 7B). In contrast, only 2 of 32 spontaneous base substitutions occurred at potential sites of bleomycin damage, and none of them were at C-G-*C*-C sequences (Figure 7A). A few one-base deletions were recovered from the drug-treated samples, and nearly all of them were at potential sites of bleomycin damage (Figures 6B and 7B). There were no obvious differences in the distributions of mutations seen at 3 and 6 μM bleomycin (data not shown).

Although these data suggested that bleomycin can induce targeted base substitutions, interpretation of the results is complicated by the relatively high spontaneous mutation frequency and by the large number of untargeted substitutions and deletions in the spectra. Therefore, bleomycin-induced mutagenesis was also examined in the shuttle vector pZ189 grown in human 293 cells, a system known to have a particularly low background mutation frequency (Yang et al., 1987). In contrast to pSVSU270 in COS 1 cells, recovery of replicated pZ189 from 293 cells was severely decreased by bleomycin treatment of the plasmid (Figure 5B). In fact, from comparisons of plasmid recovery with the levels of drug-induced damage in the plasmid, it appeared that even a single lesion (strand break or AP site) in the plasmid was sufficient to decrease the efficiency of transfection and/or replication in 293 cells dramatically. The frequencies of both spontaneous and induced mutations were somewhat lower than those of

pSVSU270 in COS 1 cells (Figure 5C). However, pZ189 was even more prone to deletions than pSVSU270; either with or without methoxyamine, about 40% of bleomycin-induced mutants (taken from samples with mutation frequencies of at least 4 × background) showed altered mobility on agarose, and of the remainder, more than one-half were deletions. Deletions in pSVSU270 and in pZ189 were similar; nearly all were <50 bp, about one-third were <10 bp, and most had short (1–3 bp) repeats at the termini, one copy of which was retained in the mutant (see the supplementary material for complete data). Only a few substitutions were recovered: about one-half of them were at potential damage sites (Figure 6C). Surprisingly, replication of bleomycin-damaged pZ189 in COS 1 cells, which gave spontaneous and induced mutation frequencies comparable to those of pSVSU270, gave a spectrum similar to that of pZ189 replicated in 293 cells, with a predominance of deletions and few base substitutions (see supplementary material). The infrequency of induced substitutions in pZ189 may be attributable to the lack of C-G-*C*-C sequences in *supF* and to the fact that there is 2 kb of DNA between the SV40 replication origin and *supF* in pZ189, as compared with 80 bp between the origin and *trpT180* in pSVSU270; this difference could allow more time for removal of AP sites prior to replication, particularly if replication is delayed at lesions between the origin and *supF*.

The mutation spectrum of bleomycin-induced mutations in pZ189, replicated in either 293 or COS 1 cells, also included a minor fraction of one-base deletions. These were seen both with and without methoxyamine, and nearly all of them occurred at potential sites of bleomycin damage (Figure 7C).

DISCUSSION

In λ phage, bleomycin, like NCS, produces a mutational spectrum which is dominated by base substitutions and is consistent with a model involving replicative bypass of sequence-specific AP sites (Povirk, 1987; Povirk & Goldberg, 1986). At certain sequences, such as C-G-*C*-C, bleomycin induces AP sites with directly opposed strand breaks (Steighner & Povirk, 1990a). The intrinsic difficulty of repairing these bistranded lesions and their resistance to AP endonucleases (Povirk & Houlgrave, 1988) probably account for the high frequency of bleomycin-induced base substitutions at C-G-*C*-C sites (Povirk, 1987). The present study was designed to examine the fate of bleomycin-induced AP sites in mammalian cells, particularly with regard to their potential to induce base substitutions.

Direct measurements of strand breakage in bleomycin-treated reconstituted chromatin indicated that, even though bleomycin selectivity attacks internucleosomal regions, most bleomycin-induced AP sites were subject to rapid spontaneous cleavage, presumably by reaction with amine groups of core histones (Figure 4A). It is particularly notable that bleomycin-induced AP sites with closely opposed breaks, which may be relatively resistant to enzymatic cleavage (Povirk & Houlgrave, 1988), were significantly more susceptible to spontaneous cleavage than lone AP sites, perhaps due to greater accessibility (Figure 4B). In view of these data, it appears likely that all bleomycin-induced AP sites would indeed have quite a short lifetime in the mammalian nucleus, being subject to cleavage by histone amines, as well as endogenous polyamines (Povirk & Houlgrave, 1988; McCormick, 1977) and AP endonucleases (Schyns et al., 1978). This instability is expected to markedly decrease the potential of these lesions to produce base substitutions by replicative bypass.

Most of the mutations arising from replication of bleomycin-treated shuttle vectors in mammalian cells were deletions, presumably resulting from cellular processing of free DNA ends at sites of bleomycin-induced single- or double-strand breaks. However, when bleomycin-damaged pSVSU270 was transfected into COS 1 cells, where it should undergo rapid T-antigen-dependent replication, substitutions were also induced, and many of these were apparently targeted at C-G-*C*-C sites, just as in λ phage. While these substitutions probably arose by replicative bypass of AP sites, it is clear that only a small fraction of the AP sites were subject to such bypass. For example, at 6 μM bleomycin there were on average ~0.2 AP sites in the *trpT180* gene (10 AP sites/plasmid × 74 bp/3811 bp), but the frequency of putative targeted substitutions was only ~0.2%. Thus, assuming that all of the sequence positions in *trpT180* are mutable, only about one in 100 AP sites resulted in a targeted substitution. The rapid spontaneous breakdown of AP sites could account for this result. However, the failure of methoxyamine to increase the frequency of targeted substitutions in surprising and suggests that even stabilized AP sites are subject to rapid removal in COS 1 cells.

The observed mutation frequency of ~1% per bleomycin-induced AP site is similar to that reported by Gentil et al. (1992) for a similar SV40-based shuttle vector containing a single, site-specific AP site, also replicated in COS cells. Our results with bleomycin-treated pSVSU270 are also qualitatively similar to those of Klinedinst and Drinkwater (1992), who transfected a heat-depurinated Epstein–Barr virus-based shuttle vector into human cells and analyzed mutations in the HSV-*tk* gene. The majority of the mutations induced by heat depurination were deletions of various sizes, which may have resulted from processing of free DNA ends produced by AP site cleavage. Base substitutions were also induced, of which G·C → C·G transversions were most frequent. Assuming that the substitutions were targeted, this specificity suggests preferential incorporation of guanine opposite AP sites. G·C → C·G transversions were also the most frequent substitutions induced in bleomycin-treated pSVSU270 (Figure 7B); however, since the putative bleomycin-induced AP site could have occurred in either strand (Povirk & Steighner, 1990), it is uncertain whether G or C was incorporated opposite the putative lesion.

An intriguing feature common to all of the mutation spectra for bleomycin-treated shuttle vectors is the presence of one-base-pair deletions (Figure 7). Although these one-base deletions accounted for only 10–20% of bleomycin-induced mutations, virtually all of them (32/35) occurred at primary or secondary sites of bleomycin attack, suggesting that they were a result of bleomycin damage rather than a shuttle vector artifact. One-base deletions may be explained in several ways. First, they could be the result of an AP site bypass in which the AP site was folded out of the helix, allowing replication to continue at the next base in the template, thus giving a one-base deletion in the replicated strand at the position of the AP site. However, given the demonstrated tendency of AP site bypass to produce base substitutions (Gentil et al., 1990), this mechanism seems unlikely. Second, the one-base deletions may be the consequence of repair of an AP site opposite a strand break. For such a lesion, neither strand can provide an intact template for repair synthesis. Instead, removal of phosphoglycolate from the 3′-end of the strand break and folding of the AP site outside the helix could permit ligation of the strand break, with deletion of the nucleotide initially attacked in the formation of the break. The remaining bulge of one sugar residue (i.e., the AP site) in the backbone of the opposite strand would presumably be recognized and removed, and sealing of the nick by DNA ligase would give

an intact double helix with deletion of the base pair which was initially damaged. Finally, one-base deletions could result from double-strand break repair by a mechanism involving removal of phosphoglycolate blockages from both 3′-ends, followed by blunt-end ligation. This mechanism would likewise produce an intact double helix, with deletion of the base pair originally attacked in formation of the double-strand break.

An important feature of the latter two models is that they do not require DNA replication for fixation of the mutations. Further evidence in support of such a possibility comes from studies showing that, at the *aprt* locus of CHO-D422 cells, bleomycin is more mutagenic during the stationary phase than during exponential growth (Bennett & Povirk, 1992). Furthermore, preliminary sequencing data indicate that, among these mutants, large deletions are rare and that targeted one-base deletions, similar to those generated in the shuttle vectors, are the most frequent bleomycin-induced mutations. Although *aprt* is unusual in that it selects strongly against large deletions, a recent multiplex PCR analysis of bleomycin-induced *hprt* mutants in V79 cells (Köberle et al., 1991) indicated that a majority (20/35) of them contained only point mutations (i.e., yielded normal PCR products), even though *hprt* provides a much larger target for deletions than *aprt*.

Very large deletions induced by bleomycin (Doerr et al., 1989; Povirk & Austin, 1991) probably also result from incorrect repair of double-strand breaks, a process which may be strongly influenced by the sugar fragments blocking the DNA termini (Chang & Wilson, 1987). Direct double-strand breaks induced by bleomycin will have phosphoglycolates at both 3′-termini, while those resulting from AP site cleavage will presumably have an AP site at one 3′-terminus. Since the major mammalian AP endonuclease apparently cannot remove cleaved AP sites from 3′-ends (Bailly & Verly, 1988), this blockage may present a significant barrier to rejoining and may trigger nonhomologous recombination processes leading to large deletions.

With regard to the general question of oxidative mutagenesis, it appears that the oxidized AP sites induced by bleomycin do have some potential to produce base substitutions in mammalian cells, but only in a vector which is heavily damaged and rapidly replicated upon transfection. The finding that few substitutions were observed in pZ189 replicated in human cells suggests that in most cases the AP sites are too short-lived to produce significant base substitution mutagenesis. Hence, AP sites of similar structure, produced by other oxidative mutagens as a component of nonspecific free radical damage (Isildar et al., 1981), probably do not make a major contribution to base substitution mutagenesis by those agents. On the other hand, AP sites oxidized at the C-1′ position, such as those induced by NCS, are somewhat more stable (Figure 4C). Attempts to evaluate the mutagenic potential of these lesions are in progress, but these studies appear to be subject to the same complications (deletions and untargeted substitutions) encountered with bleomycin.

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SUPPLEMENTARY MATERIAL AVAILABLE

A complete listing of all mutants not shown in the manuscript, including deletion mutants (3 pages). Ordering information is given on any current masthead page. The listing will also be sent to individuals requesting reprints of the article.

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