DNA repair is known as a defense mechanism against genotoxic insults. However, the most lethal type of DNA damages, double-strand DNA breaks (DSBs), can be produced by DNA repair. We have previously demonstrated that when long patch base excision repair attempts to repair a synthetic substrate containing two uracils, the repair produces DSBs (Vispe, S. and Satoh, M. S. (2000) J. Biol. Chem. 275, 27386–27392 and Vispe, S., Ho, E. L., Yung, T. M., and Satoh, M. S. (2003) J. Biol. Chem. 278, 35279–35285). In this synthetic substrate, the two uracils are located on the opposite DNA strands (separated by an intervening sequence stable at 37 °C) and represent a high risk site for DSB formation. It is not clear, however, whether similar high risk sites are also induced in genomic DNA by exposure to DNA damaging agents. Thus, to investigate the mechanisms of DSB formation, we have modified the DSB formation assay developed previously and demonstrated that high risk sites for DSB formation are indeed generated in genomic DNA by exposure of cells to alkylating agents. In fact, genomic DNA containing alkylated base damages, which could represent high risk sites, are converted into DSBs by enzymes present in extracts prepared from cells derived from clinically normal individuals. Furthermore, DSBs are also produced by extracts from cells derived from ataxia-telangiectasia patients who show cancer proneness due to an impaired response to DSBs. These results suggest the presence of a novel link between base damage formation and DSBs and between long patch base excision repair and human diseases that occur due to an impaired response to DSB.

It has been demonstrated that the most lethal type of DNA damages, double-strand DNA breaks (DSBs), are produced by exposure of DNA to γ- or x-rays (1, 2). These forms of ionizing radiations generate radical pairs by depositing their energy on water molecules (2). These pairs then lead to the formation of multiple damages, including oxidized bases and single-strand DNA breaks (SSBs), within a short stretch of DNA (2). These multiple damages or “closely spaced” damages compose DNA damage clusters (2). Within the clusters, if two “closely spaced” SSBs are produced on opposite DNA strands, DNA is spontaneously denatured, leading to DSB formation (1). Similarly, when two “closely spaced” oxidized base damages located on opposite DNA strands are removed by DNA glycosylases, SSBs are produced through the incision of the apurinic/apyrimidinic (AP) sites by AP-endonuclease (3). These SSBs can then be converted into a DSB (4–7). Although the Tm value of the intervening sequence between two SSBs or oxidized base damages is required to be below 37 °C to allow spontaneous denaturation of the sequence, “closely spaced” damage formation within a DNA damage cluster plays a critical role in the formation of radiation-induced DSBs.

Although radiation-induced DSBs are cytotoxic, the majority of cells have the ability to respond to DSBs by activating a pathway initiated by ataxia-telangiectasia mutated protein (ATM); this pathway eventually regulates DSB repair and cell cycle (8, 9). An abnormality in this pathway, thus, causes hypersensitivity of cells to γ- and x-rays (10). In fact, cells derived from homozygotes of a human autosomal recessive disorder, ataxia-telangiectasia (AT), and AT patients are extremely sensitive to ionizing radiations due to the impaired function of ATM (8–11). Such hypersensitivity is, however, not the only characteristic of AT patients, as they also exhibit various other clinical symptoms, including immune deficiency and progressive neuronal degeneration (11–13). In addition, AT homozygotes frequently develop lymphoid-type malignancies (14, 15). Furthermore, it has been suggested that AT heterozygotes also develop cancer, although in the form of breast cancer (13, 16–18). These symptoms appear to develop in the absence of obvious exposure to ionizing radiations, suggesting that DSBs are produced in cells under physiological conditions (19, 20). It has been suggested that these DSBs are created from other forms of DNA damages induced by reactive oxygen metabolites or other DNA damaging agents (19, 20), although the majority of endogenous metabolites or agents do not frequently induce “closely spaced” damages. Thus, it has been assumed that DSBs can be produced through alternative mechanisms that are yet to be understood.

If “closely spaced” damages are defined as damages pro-
duced on opposite DNA strands and separated by an intervening sequence that can be denatured at 37 °C, “spaced” damages can be defined as damages produced on opposite DNA strands and separated by an intervening sequence that has a $T_m$ value above 37 °C (Fig. 1A). Because “spaced” damages can be induced by various DNA damaging agents, we have previously developed a cell-free DSB formation assay using a synthetic substrate containing “spaced” uracils to investigate the mechanisms of DSB formation (Fig. 1B) (21). Uracil was selected to construct “spaced” base damages, as synthetic oligonucleotide acid-containing uracils is commercially available. In addition, extracts were prepared from DNA ligase I mutant cells (46BR) (22), which predominantly produce long repair patches at the sites of base damages (23), and from normal fibroblast, MRC5 cells (21). In this assay the “spaced” uracils, one of which is located on the 3’ side of the other on opposite DNA strands and both of which are separated by an 18- or 30-bp intervening sequence ($T_m$ values of intervening sequences are 62 and 75 °C, respectively), are converted into a DSB by extracts prepared from 46BR cells (21). This conversion is initiated through removal of uracils by uracil DNA glycosylase followed by incision at resulting AP sites by AP endonuclease (3) (Fig. 1B). Then flap endonuclease-1 (FEN-1), which is known to play a role in the repair of uracils sites (8, 9), extracts prepared from cells derived from AT heterozygotes and homozygotes were also used to determine whether “spaced” base damages could act as a risk factors for ATM gene mutation carriers.

**MATERIALS AND METHODS**

*Cells and Cell Extract Preparation—*Lymphoblastoid cell lines, GM00621 (clinically normal), GM01953 (clinically normal), GM06351 (clinically normal), GM03334 (AT heterozygote), GM09588 (AT heterozygote), GM09585 (AT heterozygote), GM02782 (AT homozygote), and GM03189 (AT homozygote), were purchased from the NIGMS Human Genetic Cell Repository (Camden, NJ). Cell-free extracts were prepared as described previously (26, 27).

*Preparation of P2U18 and pBluscript KS II+ (pBS), Exposed to Either MNNG or γ-Rays—*P2U18 was constructed, and pBS was exposed to either 400 μM MNNG or 50 Gy of γ-rays (137Cs source, dose rate 1 Gy/min, Gamma Cell 40 Exactor, MDS Nordion) as described previously (26, 27). Covalently closed circular DNA was purified by CsCl-ethidium bromide (EtBr) centrifugation and stored in 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA (TE buffer). Formation of DNA damage by the treatment with MNNG or the exposure to γ-rays resulted in the conversion of about 40% of covalently closed circular pBS into the open circular form by endonuclease III (endo III) treatment.

*DSB Formation Assay—*DSB formation assays with 200 ng of P2U18, MNNG-treated pBS, or γ-ray-irradiated pBS were carried out as described (21) with 50 μg of extracts proteins in a 50-μl reaction mixture for 1 h at 30 °C. After termination of cell-free DSB formation reaction and DNA purification, plasmids were fractionated on a 1% agarose-EtBr gel. DNA was visualized by UV. To determine the amount of linearized DNA, images were created using Alphalager (Alpha Innotech Corp.), and DNA was quantified with Chemilumager 4000 (Alpha Innotech). Standard errors, which were determined from 6 independent experiments, were typically 25%.

*PCR and Sequencing of P2U18—*After fractionation of P2U18 by 1% agarose-EtBr gel electrophoresis, linearized P2U18 was extracted. Then linear P2U18 (5 μg/μl) was methylated by 0.6 units/μl of HaelIII methylase for 1 h at 37 °C. In some cases HaelIII-treated P2U18 (8 μg/μl) was incubated with 0.0005 units/μl of Klenow fragment for 15 min at 25 °C. The reaction was terminated by the addition of 20 mM EDTA. These linearized P2U18 (16 ng/μl) were mixed with 0.05 pmol/μl of BE fragment, which was prepared by annealing of Adf (5’-TGAAGCACATCGAGGACCTTATCCGG-3’) and Adr (5’-CCCGGATAAGTCCTCGACTGTGCCCTTCAAAATAA-3’), and incubated with 0.5 units/μl of T4 DNA ligase (Roche Applied Science) overnight at 16 °C. After heat inactivation of T4 DNA ligase, DNA was incubated with 0.5 units/μl of HaelII for 30 min at 37 °C to digest dimerized BE fragments. Then 80 ng of linearized P2U18 was used for PCR with 80 pmol of BE primer and either Pr-Reverse primer (5’-GTCGGTCCCCCACCTC-TGACTTGGCCT-3’) or Pr-Forward primer (5’-CAGCCCTCCGCGTAAACCCACCACCCCGGCGCGTTAAATGGCCCCCT-3’) in 100 μl of ThermoPol reaction buffer (New England Biolabs) containing 200 μM each of dNTPs, 20% of betaine, and 2 units of vent DNA polymerase. Amplification reaction (30 cycles) was performed with 1 min denaturation at 96 °C, 1 min annealing at 65 °C, and either a 20-s extension for Pr-Reverse primer or 40 s for Pr-Forward primer at 72 °C. Amplified products were fractionated by 1% agarose-EtBr gel electrophoresis and visualized by UV. Alternatively, amplified products were cloned into pCR® Blunt II TOPO using Zero Blunt TOPO PCR cloning kit (Invitrogen), and Escherichia coli Top 10 was transformed using this plasmid. The cloned plasmid was used for sequencing with either M13 reverse or T7 primers.
Sequence of Re-circularized P2U18—Linearized P2U18 (100 ng), extracted from 1% agarose-EtBr gel, was re-circularized using Quick Ligation kit (New England Biolabs). Then DNA was used for transformation of E. coli DH5α. Extracted P2U18 from cloned E. coli was used for DNA sequencing with E. coli DH5α.

Heat Denaturation Assay with MNNG-treated and γ-Ray-irradiated pBS—MNNG-treated or γ-ray-irradiated pBS (25 ng/µl) was incubated with 0.1 units/µl of endo III for 30 min at 37 °C. Then reaction mixtures were incubated at either 37, 50, 60, or 80 °C for 5 min followed by chilling on ice. Incubated DNA was fractionated on a 1% agarose gel and visualized by UV.

Preparation of Hybrid-pBS—Non-damaged pBS (1 µg/µl) was digested with 2 units/µl SmaI for 1 h at 25 °C. MNNG-treated and γ-ray-irradiated pBS (1 µg/µl) was cleaved by 2 units/µl EcoRV for 1 h at 37 °C. Then, cleaved non-damaged pBS and either MNNG-treated or γ-ray-irradiated pBS were mixed and incubated at 96 °C for 5 min. After gradual cooling of the reaction mixture to allow annealing of denatured DNA strands, the mixture containing 500 µg of DNA was incubated with 1250 units of T4 DNA ligase in a 500-ml reaction mixture containing 10 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 1 mM ATP, and 1 mM dithiothreitol for 12 h at 16 °C. By annealing of a non-damaged DNA strand with a damaged strand, single-strand tails were created at both ends (Fig. 5A), and this ligation reaction allowed the circularization of this hybrid-pBS through these tails. The ligation reaction mixture was then concentrated by centrifugation at 100,000 × g for 4 h in a SW55 rotor and covalently closed-circular hybrid-pBS was purified by CsCl-EtBr centrifugation. The resulting DNA was stored in TE buffer.

Exposure of Cells to Either MNNG or γ-Rays and Preparation of Circularized Genomic DNA—HeLa S3 cells were exposed to either 2 or 10 Gy of γ-rays (¹³⁷Cs source; dose rate, 1 Gy/min) on ice. Alternatively, HeLa S3 cells were treated with either 10 or 50 µM MNNG in serum-free Dulbecco’s modified Eagle’s medium at 37 °C for 15 min and placed on ice. After harvesting cells through scraping, cells were lysed using a buffer containing 1% SDS, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.4 mg/ml proteinase K for 30 min at 37 °C. DNA was purified by chloroform-phenol extractions, precipitated by ethanol, and treated with 0.03 mg/ml RNase A in a buffer containing 10 mM Tris-HCl, pH 8.0, and 10 mM EDTA for 1 h at 37 °C. Extracted genomic DNA (0.1 µg/µl) was digested with 0.3 units/µl of SpeI, 0.3 units/µl of NheI, and 0.6 units/µl of XbaI to create CTAG-5′ protruding ends for 2 h at 37 °C. Genomic DNA (100 µg) was circularized in a 100-ml ligation reaction and purified by CsCl-EtBr centrifugation as described under “Preparation of hybrid-pBS.” The resulting DNA was stored in TE buffer.

DSB Formation Assay with Circularized Genomic DNA—DSB formation reaction was carried out with 200 ng of circularized genomic DNA and 50 µg of protein extracts as described (21). After termination of the reaction and purification of DNA, 0.2 units/µl of calf intestinal alkaline phosphatase was incubated with the DNA (8 µg/µl) for 1 h at 37 °C. Then, after ethanol precipitation of DNA (200 ng), duplex oligonucleic acid prepared by annealing of AdF4 (5′-TAGAAGGGC-ACAGTCGAGGACTTATCACGATTTGGCCTACTAGGdideoxyC-3′) and AdR4 (5′-pCCGGTAGGCGACCTATCATGGCGTGATGCTTTCCCTTAAAdideoxy[³²P]A-3′) was ligated to DSB ends by 5 units of T4 DNA ligase in a 10-µl reaction mixture containing 33 µM Tris-HCl, pH 7.9, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP, and 15% of polyethylene glycol 8000 (28) at 20 °C for overnight. The ligation reaction was then fractionated on a 1% agarose-EtBr gel containing a 5% agarose layer as illustrated in Fig. 6E. DNA was visualized using UV. Alternatively, the gel was dried and then exposed to an x-ray film for autoradiography.
Endo III–Endo IV Treatment of Circularized Genomic DNA—Circularized genomic DNA (250 ng) or pBS (250 ng) exposed to either MNNG or γ-rays were treated with 2 units of endo III in a 20-μl reaction mixture containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin for 30 min at 37 °C. After DNA ethanol precipitation,

FIGURE 2. DSB end mapping. A, linearized P2U18 was extracted from agarose gel. Then oligonucleic acid (BE fragment) was ligated to DSB ends. PCR was carried out with BE primer together with either Pr-Forward or Pr-Reverse primers, which produced about 380 and 560 bp in length of amplified products, respectively, when DSBs were produced between the two uracil residues. These fragments were cloned into a vector for sequencing. B, amplified DNA fragments produced by PCR with linearized P2U18 were fractionated on a 1% agarose–EtBr gel and visualized using UV. Linearized pBS produced by GM09585 extracts was also used for PCR amplification. C, cloned fragments were used for sequencing with T7 and M13 reverse primers to map DSB ends.
DNA was treated with 2 units of endo IV in a 10-μl reaction mixture containing 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, and 1 mM dithiothreitol for 30 min at 37 °C. Then, 40 μl of 50 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, and 1 mM dithiothreitol was added and incubated at 80 °C for 5 min. After the addition of 50 μl of preheated 2X Thermolip reaction buffer containing 200 μM each of dNTPs at 80 °C, 1 unit of vent DNA polymerase was added. The temperature was then shifted to 72 °C and incubated for 1 min. After purification of DNA by Nucleospin Extract II kit, DNA was treated with alkaline phosphatase, labeled with 32P, and fractionated on agarose gel as described under “DSB formation assay with circularized genomic DNA.”

RESULTS

DSB Formation Assay with P2U18—We have previously developed a cell-free DSB formation assay using model substrates, circular DNAs containing two uracils separated by intervening sequences (Fig. 1B) (21). One of the model substrates, P2U18, contains the intervening sequence with a Tₘ value of 65 °C. Thus, SSBs produced at the uracils sites do not lead to denaturation of DNA at 37 °C (21). The Tₘ value of the intervening sequence is, however, reduced by a FEN-1-mediated 5’ to 3’ DNA strand cleavage that initiates from these SSBs, leading to DNA strand denaturation and DSB formation (26) (Fig. 1B). This cleavage can be efficiently counteracted by DNA ligase I (21). Therefore, to experimentally detect DSB formation, we previously used extracts prepared from DNA ligase I mutant cells, 46BR (22), or added an excess of purified FEN-1 to the DSB formation assays (21, 26). In the present work we performed our study without the addition of FEN-1. Thus, we first characterized DSBs produced by extracts prepared from cells derived from clinically normal individuals, AT heterozygotes, and homozygotes using our cell-free DSB formation assay with P2U18. As shown in Fig. 1C, about 6–16% of P2U18 were converted into linear P2U18, whereas 0–8% of non-damaged pBS were linearized, suggesting that DSBs are more frequently produced in P2U18 when compared with pBS by extracts prepared from various cells.

Formation of a DSB between Two Uracil Residues—To examine whether DSBs are produced at uracils sites, we performed PCR analysis. After extraction of linearized P2U18 from agarose-EtBr gels, duplex oligonucleotides (BE primer), which contained a primer binding site for PCR, was ligated to DSB ends (Fig. 2A). PCR was then performed with a primer for BE (BE primer) and either with Pr-Forward or Pr-Reverse primers. These primers, in the case of DSBs generated between two uracil residues, were expected to produce PCR products of about 380 (U1 side of DSB) and 560 (U2 side of DSB) bp in length (Fig. 2A). In fact, we detected DNA fragments of the expected lengths when linearized P2U18 produced by extracts prepared from cells derived from clinically normal (GM06315 and GM00621), AT heterozygotes (GM09588 and GM09585) and AT homozygote (GM03189) were used (Fig. 2B). As a control, pBS linearized by GM09588 extract (see Fig. 1C) was also used for PCR analysis. After PCR amplification, however, no discrete bands were produced (Fig. 2B, pBS). Thus, results suggest that amplified DNA fragments found in assays with linearized P2U18 were generated due to the formation of DSB within a region of P2U18 containing two uracils.

Mapping of DSB Ends—To further characterize DSBs produced by extracts prepared from various cells, DSB ends were mapped (Fig. 2C). Amplified DNA fragments (Fig. 2B) were then cloned into a pCR® Blunt II vector, and recombinant plasmids extracted from cloned E. coli were used for sequencing. As summarized in Fig. 2C, the U1 side of DSB ends was mapped between two uracils residues. Mapping showed that ATAT sequences located within an intervening sequence were a preferential DSB formation site. Furthermore, the U2 side of DSB ends was also mapped between the two uracils residues. Although some DSB ends were found outside of the two uracils, possibly due to the processing of DNA ends by nuclease, these results reveal that DSB ends were predominantly produced at uracils sites. As an alternative approach to characterize DSBs, linearized P2U18 produced by GM00621 (normal) and GM09585 (AT heterozygote) extracts were re-circularized (Fig. 3A) and used for sequencing analysis. Through this analysis, we found 1–3 base deletions which were likely generated upon DSB formation, in the intervening sequences and at the uracils sites (Fig. 3B). Of note, to create a one-base deletion at the uracils site, DNA polymerization and DNA cleavage are required to occur in a coordinated manner either during the denaturation process of the intervening sequence or through the processing of DSB ends. Thus, in addition to FEN-1, other factors may be involved in the process of DSB formation. Nevertheless, these results suggest that DSBs are predominantly produced in the region of P2U18 containing the two uracils by cell-free extracts prepared from either normal, AT heterozygote, or homozygote cells even without any addition of purified FEN-1.

Conversion of Alkylated Base Damages into DSBs—Next, we have investigated whether “spaced” damages, which can represent potential sites for DSB formation, were produced by exposure of DNA to an alkylating agent. pBS was, thus, exposed to MNNG to produce alkylated base damages, and the resulting MNNG-treated pBS was incubated with endo III, which induces DNA nicks at the site of modified thymidines (29). As shown in Fig. 4A, lanes 7 versus 8, MNNG-treated pBS (super-
coiled) was converted to the open-circular form after nick induction, whereas no detectable amount of linear DNA was produced after incubation at 37 °C. Thus, if it is assumed that “spaced” alkylated base damages are produced by MNNG treatment, the intervening sequences that separate the alkylated base damages is stable at 37 °C. Incubation of the nicked MNNG-treated pBS at 80 °C for 5 min (Fig. 4B, lane 6) when the plasmid was not treated with endo III. Thus, these results suggest that a fraction of nicked MNNG-treated pBS is linearized by a 5-min incubation at 80 °C and that multiple “spaced” alkylated base damages separated by intervening sequences, stable at 37 °C but denatured at 80 °C, are produced after exposure of pBS to MNNG. Judging from the temperature required for denaturation, these alkylated base damages should be separated by intervening sequences between 20 and 40 bp in length if GC contents are assumed to be 100 and 0%, respectively.

As a control we used /H9253-ray-irradiated pBS, which was subjected to CsCl-EtBr centrifugation to remove pBS containing SSBs induced by radicals (27). The resulting /H9253-ray-irradiated pBS was converted into a nicked circular form by endo III treatment, although small amounts of linear DNA could also have been produced. This suggests that DNA damage clusters, which contain “closely spaced” oxidized base damages, are produced in pBS by /H9253-ray irradiations (Fig. 4A, lanes 13 versus 14). However, because increased amounts of linear DNA were found when /γ-ray-irradiated pBS, which was nicked by endo III, were incubated at 80 °C (Fig. 4A), “spaced” oxidized base damages were also produced by the /γ-ray irradiations.

MNNG-treated and /γ-ray-irradiated pBS were then used for cell-free DSB formation assays. Previously, we observed the conversion of /γ-ray-irradiated pBS into the linear form due to the formation of DSBs in the assay with extracts prepared from normal human fibroblasts (26). Consistent with this observation, /γ-ray-irradiated pBS was also converted into linear form by extracts prepared from cells derived from clinically normal individuals, AT heterozygotes, and homozygotes (Fig. 4C). Similarly, the linear form of pBS was produced from MNNG-treated pBS by these extracts, suggesting that DSBs are
produced in MNNG-treated pBS (Fig. 4C). Furthermore, the addition of FEN-1 to the assay with MNNG-treated pBS promoted the formation of DSBs (Fig. 4D). Thus, these results suggest that pBS containing “spaced” alkylated base damages is converted into linear form due to DSB formation by a mechanism mediated by FEN-1.

**Hybrid-pBS**—To further confirm that “spaced” base damages were indeed required for DSB formation, we prepared an hybrid-pBS containing base damages on only one DNA strand, thus devoid of “spaced” base damages. To prepare hybrid-pBS, non-damaged and damaged pBSs were digested with SmaI and EcoRV, respectively (Fig. 5A). Then, digested pBSs were mixed and denatured at 96 °C. Annealing of denatured DNA strands created either original pBSs or hybrid-pBSs, which were composed of one damaged and one non-damaged DNA strand. Only hybrid-pBSs can be circularized through the ligation of complementary single-stranded DNA tails. After the circularization reaction, hybrid-pBS was purified by CsCl-EtBr centrifugation. Although hybrid-pBSs prepared from MNNG-treated pBSs was unstable (converted into the nicked circular form during preparation; data not shown), covalently closed circular hybrid-pBS was obtained from γ-irradiated pBSs. By incubating hybrid-pBS prepared from γ-irradiated pBSs with endo III, covalently closed circular hybrid-pBS was converted into nicked circular form (Fig. 5B, lanes 7 versus 8), suggesting that the hybrid-pBS contains oxidized base damages. However, no detectable amounts of linear hybrid-pBSs were produced, even after a 80 °C incubation, implicating the absence of “spaced” oxidized base damages in the hybrid-pBS. During DSB formation assays with hybrid-pBSs, only negligible amounts of linear hybrid-pBSs were produced (Fig. 5C), indicating that, in the absence of “spaced” base damages, DSBs are not produced (GDS fragment), designed to ligate to DSB ends, was prepared. To prevent ligation between GDS fragments, one end of the GDS fragment was modified to ddCMP, and an AAA tail was attached to the other end (Fig. 6C). Similarly, genomic DNA was treated with alkaline phosphatase to prevent ligation of linearized genomic DNAs. Alkaline phosphatase treated genomic DNA and GDS fragment were then ligated in the presence of polyethylene glycol 8000, which stimulates the DNA ligation reaction (28). Detection limit of this DSB formation assay was about 5 ng of pBS, linearized by SmaI (Fig. 6D). Then, to detect DSBs produced in circularized genomic DNA, 1% agarose gel containing a 5% agarose gel layer, was used (Fig. 6E). Although GDS fragment migrated through the layer, genomic DNA was retained in the 5% agarose layer (Fig. 6E, EtBr). Allowing concentration of genomic DNA and separation of unligated GDS fragments from genomic DNA. Because genomic DNA labeled with 32P was indeed concentrated in the 5% agarose layer (Fig. 6E, autoradiography (Auto.)), this modified DSB formation assay was used to study the formation of “spaced” base damages produced in genomic DNA.

**Formation of “Spaced” Base Damage in Genomic DNA**—To induce alkylated base damages in genomic DNA, cells were exposed to 10 or 50 μM MNNG. As controls, cells exposed to 2 or 10 Gy of γ-rays were also used. After preparation of circularized genomic DNA, endo III treatment was carried out with the genomic DNA (Fig. 7A). Because endo III produces α,β-unsaturated-aldehyde ends at the 3’ terminus (29), this end group was removed by an endo IV treatment (27). Then, circularized genomic DNA treated with endo III and endo IV was incubated at 80 °C followed by incubation with vent DNA polymerase to create blunt ends that can be ligated with a 32P-labeled GDS fragment. Because this assay involved three steps of enzymatic

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**FIGURE 5. DSB formation assay with hybrid-pBS.** A, to prepare hybrid-pBS containing DNA base damages on only one DNA strand, non-damaged pBS and damaged pBS were digested by SmaI and EcoRV, respectively. After DNA denaturation, original pBS and hybrid-pBS were produced by annealing of non-damaged and damaged strands. In the self-circularization reaction with T4 DNA ligase, hybrid-pBS was selectively circularized. Then, the circularized hybrid-pBS was purified by CsCl-EtBr centrifugation. B, hybrid-pBS, prepared from γ-ray-irradiated pBS, was treated with endo III followed by incubation at 80 °C. After 1% agarose gel electrophoresis, DNA was visualized using UV. C, DSB formation assays were carried out with the hybrid-pBS. Extracts prepared from GM00621 were used. Standard errors are shown. OC, closed circular form; OC, open circular form.
treatments, results may represent an under-estimated value. However, as shown in Fig. 7B, linearized MNNG-treated or γ-ray-irradiated pBS, both labeled with [32P]-labeled GDS fragment, were detected through this method, suggesting that blunt ends are indeed produced after three steps of enzymatic treatments.

Circularized genomic DNA, which was prepared from DNA extracted from cells exposed to MNNG or γ-rays, was then used, and as shown in Fig. 7C, [32P]-labeled genomic DNA was in fact found in the 5% agarose layer. To evaluate the effect of the degradation of heat labile damages induced by MNNG or γ-rays on DSB formation, circular genomic DNAs were incubated at 80 °C for 5 min without treatment with endo III, and DNA break ends were labeled with [32P]ddAMP using terminal transferase. However, no significant amounts of [32P] were transferred to the incubated genomic DNA (data not shown), suggesting that no significant degradation of heat labile damages occurs during the incubation at 80 °C for 5 min. Thus, results shown in Fig. 7C indicate that “spaced” alkylated or oxidized base damages with an intervening sequence that is denatured at 80 °C are produced in cells after exposure to MNNG or γ-rays.

**DSB Formation in Circularized Genomic DNA**—Circularized genomic DNA, prepared from DNA extracted from cells exposed to MNNG or γ-rays, was used for DSB formation assay. Without the addition of cell-free extracts, only negligible amounts of [32P] activity were retained in the 5% agarose layer, whereas as a result of DSB formation, larger amounts of [32P]-labeled genomic DNA were found when GM00621 and GM03189 extracts were used (Fig. 8A). This suggests the occurrence of conversion of “spaced” damages into DSBs.

To further confirm the presence of “spaced” base damages in genomic DNA, which represents a high risk site for DSB formation, we employed aphidicolin. Previously, we have demonstrated that conversion of “spaced” uracils into DSBs was promoted by aphidicolin, an inhibitor of DNA polymerase δ/ε (26). Indeed, formation of DSB in the MNNG-treated plasmid was also promoted by the addition of aphidicolin in the DSB formation assay (Fig. 8B). Furthermore, increased amounts of DSBs were produced when the assay was carried out with circular genomic DNA, which was prepared from DNA extracted from cells exposed to MNNG (Fig. 8C), thus suggesting that “spaced” damages in circular genomic DNA are converted into DSBs.

Therefore, we concluded that “spaced” base damages, which represent a potential site for DSB formation, are produced in cells not only by γ-rays but also by MNNG, an alkylating agent.

**DISCUSSION**

In this work we have demonstrated that “spaced” alkylated base damages induced by MNNG represent potential sites for DSB formation. Because such potential sites were generated in genomic DNA by exposure of cells to MNNG, DSBs could be produced through this mechanism in cells.

**Formation of “Spaced” Base Damages in Cells**—To generate “spaced” damages, two damages are required to be produced on
Base Damage and Double-strand DNA Break Formation

opposite DNA strands, within a distance that can be converted into a DSB by the FEN-1-mediated mechanism. If DNA damaging agents induce damages throughout genomic DNA in a random manner, the chance that such “spaced” base damages will be produced seems to be relatively small unless base damages are concentrated in particular regions of genomic DNA. Several lines of evidences in fact suggest that DNA damages could preferentially be produced in certain regions of genomic DNA known as mutational hot spots. For example, hot spots are frequently found in codons 157, 245, 248, and 273 of the p53 gene in lung cancer (30). Although frequent identification of mutations in these codons of the p53 gene in lung cancer can be explained by the selection of clones carrying these mutations during tumorigenesis, it has been demonstrated that aromatic hydrocarbons, including benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE), produce adducts with guanines in these codons (31, 32). Thus, frequently mutated codons found in the p53 gene in lung cancer are in fact frequently modified codons by BPDE. It has been demonstrated that BPDE produces adducts through formation of covalent bounds with N2 guanine and that the adducts are repaired by nucleotide excision repair in biochemical settings (33, 34). BPDE-N2-deoxyguanine is, however, labile at neutral pH, and adducts often undergo depurination in cells, resulting in the formation of AP sites (35). As illustrated in Fig. 1B, AP sites can be converted into SSBs, which could lead to DSB formation if two SSBs are produced on opposite DNA strands. Thus, if depurination of guanines occurs in mutational hot spots, it could create potential sites for DSB formation. Because mutational hot spots are found in many other genes and because we also found “spaced” base damages after exposure of cells to MNNG, formation of “spaced” damages, which represent potential sites for DSB formation, are likely to be frequently produced in genomic DNA.

SSBs and DSB Formation—Various types of DNA damaging agents, endogenous reactive oxygen metabolites, and reactive nitrogen species produce diverse classes of base damages, which can be converted into SSBs during the process of repair by BER (3, 36). We have been suggesting that such SSBs play a critical role in DSB formation. Furthermore, an alternative model for DSB formation also suggests a critical role for SSBs in DSB formation; this model predicts that DSBs are produced by replication of DNA containing SSBs (37). Kuzminov (38) in fact experimentally tested this model by employing nicked M13 and found that replication of nicked M13 transfected into E. coli induces DSB. Furthermore, a recent report suggests that DSBs are produced in the E. coli chromosome by the replication of the chromosome, containing SSBs that are derived from uracils (39). Thus, SSBs appear to be involved in DSB formation in E. coli; although it has not been known whether DSBs are produced by the replication of SSBs containing DNA in mammalian cells. Thus, to investigate the role of SSBs in DSB formation in mammalian cells, Saleh-Gohari et al. (40) have employed model SSBs created by a topoisomerase I inhibitor. During the process of supercoiled DNA relaxation, topoisomerase I transiently induces DNA strand scissions through covalent complex formation with DNA 3′ ends (41, 42). Stabilization of topoisomerase I-DNA complexes by a topoisomerase I inhibitor, thus, retains SSBs in genomic DNA. Through replication of genomic DNA containing these SSBs, Saleh-Gohari et al. (40) demonstrated that DSBs can indeed be produced by replication. Although it is often difficult to experimentally detect DSBs produced by the replication of DNA containing these SSBs,
DSBs are likely to frequently be produced during DNA replication. Indeed, homologous recombination mutants cells, which show impaired response to DSB formation, are sensitive to an alkylating agent, methyl methanesulfonate, even when no detectable amounts of DSBs were found in cells using conventional methods (43). The currently proposed models for DSB formation, therefore, consistently suggest that SSBs play a critical role in DSB formation.

**Sensitivity of AT Cells to DNA Damaging Agents**—In this report we have demonstrated that “spaced” alkylated base damages can be converted into DSBs by extracts prepared from cells derived from AT homozygotes. Thus, if DSBs are produced in AT cells after exposure to alkylating agents, AT cells are expected to show increased sensitivity to alkylating agents. In fact, using a cell survival assay, it has been demonstrated that AT fibroblasts are more sensitive to MNNG (44). However, other reports suggest that AT cells show normal sensitivity to MNNG but have increased sensitivity to another alkylating agent, methyl methanesulfonate (45, 46). Because SSBs produced from alkylated base damages by BER enzymes could induce cytotoxicity through the activation of an abundant nuclear enzyme, poly(ADP-ribose) polymerase-1 (47, 48), which leads to cell death induction by depletion of NAD+ after binding to SSBs (49) or by triggering translocation of apoptosis induction factor from mitochondria to nuclei (50), the measurement of the cytotoxic effect of alkylating agents on AT cells may be significantly influenced by SSBs. Because the amount of SSBs produced in cells is likely to be affected by experimental conditions or cell types, AT cells may not show clear responses to MNNG in the case where SSBs generated from alkylated base damages induce a significant level of cytotoxicity in AT cells (45, 46). Nevertheless, these reports suggest that AT cells show an increased sensitivity to MMNG and/or methyl methanesulfonate (44–46) and that this sensitivity could be explained by the formation of DSBs, which are induced after exposure of AT cells to alkylating agents.

**BER, DSB Formation, and Human Diseases**—As illustrated in Fig. 9, “spaced” base damages, which can represent potential sites for DSB formation, could possibly be produced by alkylating agents and other DNA damaging agents. In mammalian cells SSBs produced from “spaced” damages can be repaired by two BER pathways, short-patch and long-patch BERs (51, 52). Short-patch BER mediated by DNA polymerase β and DNA ligase III is able to complete repair by creating one-base short-repair patches (51, 52). Thus, repair of “spaced” base damages by short-patch BER is likely associated with lower risks of DSB formation. On the other hand, we have suggested that, although long-patch BER is able to repair “spaced” base damages without DSB formation, repair of “spaced” base damages by BER is indeed associated with risks of DSB formation. Long-patch BER is, however, an essential pathway to repair SSBs with frayed 5’ termini, as long-patch BER is able to remove this terminus by cleaving the DNA strand in the 5’ to 3’ direction using FEN-1 (53, 54). Thus, if such spaced base damages are frequently produced in mammalian cells after exposure to DNA damaging agents, it is plausible that mammalian cells have acquired short-patch BER, which is lacking in yeast, as a primary defense mechanism against DSB formation to limit the involvement of long-patch BER only to SSBs that cannot be repaired by short-patch BER.

Despite the presence of such a defense mechanism, DSBs are still produced (21, 26). Thus, this most lethal type of DNA damage is required to be repaired either by the error prone or the error-free DSB repair pathway, non-homologous end joining, or homologous recombination (55–57). DSBs also activate the ATM protein, which regulates downstream effectors, including p53, to respond to DSB formation (8, 9). Thus, various clinical abnormalities and cancer found in AT patients are caused by an impaired function of ATM or cellular responses to DSBs, it appears that abnormalities found in AT patients are primarily related to DSB formation (11–15). These DSBs can be produced by attempts to repair “spaced” base damages by long-patch BER or replication of SSBs containing DNA, which are likely produced by BER (21, 26, 40). Therefore, although AT is not a disease that occurs because of a DNA repair abnormality, AT could be a disease related to BER. Furthermore, if DSBs are produced from base damages by BER, base damages induced by environmental carcinogens, endogenous reactive oxygen, or nitrogen species can be factors involved in the development of clinical symptoms of AT patients. Thus, administration of antioxidants, which could scavenge reactive oxygen species, to AT patients would be a relevant approach to improve their symptoms (8, 58).

It has been suggested that AT heterozygotes, estimated to be 1% of the total population (59), are also cancer-prone; particularly, they show a higher risk of breast cancer (13, 16–18). In the current work we have demonstrated that “spaced” base damages can be converted into DSBs by extracts prepared from cells derived from AT heterozygotes. Thus, the frequency of base damage formation may positively relate to the risk of developing breast cancer in AT heterozygotes. In addition, because products of breast cancer susceptibility gene-1 (BRCA-1) and -2 (BRCA-2) are also involved in the cellular response to DSBs or DSB repair, (60–62), the risk of breast cancer development in BRCA-1 or BRCA-2 heterozygotes may also relate to the frequency of “spaced” base damage formation and DSB formation.

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