Double-strand DNA breaks are the most lethal type of DNA damage induced by ionizing radiations. Previously, we reported that double-strand DNA breaks can be enzymatically produced from two DNA damages located on opposite DNA strands 18 or 30 base pairs apart in a cell-free double-strand DNA break formation assay (Vispé, S., and Satoh, M. S. (2000) J. Biol. Chem. 275, 27386–27392). In the assay that we developed, these two DNA damages are converted into single-strand interruptions by enzymes involved in base excision repair. We showed that these single-strand interruptions are converted into double-strand DNA breaks; however, it was not due to spontaneous denaturation of DNA. Thus, we proposed a model in which DNA polymerase $\delta$,$\varepsilon$, by producing repair patches at single-strand interruptions, collide, resulting in double-strand DNA break formation. We tested the model and investigated whether other enzymes/factors are involved in double-strand DNA break formation. Here we report that, instead of DNA polymerase $\delta$,$\varepsilon$, flap endonuclease-1 (FEN-1), an enzyme involved in base excision repair, is responsible for the formation of double-strand DNA break in the assay. Furthermore, by transfecting a flap endonuclease-1 expression construct into cells, thus altering their flap endonuclease-1 content, we found an increased number of double-strand DNA breaks after $\gamma$-ray irradiation of these cells. These results suggest that flap endonuclease-1 acts as a double-strand DNA break formation factor. Because FEN-1 is an essential enzyme that plays its role in DNA repair and DNA replication, DSBs may be produced in cells as by-products of the activity of FEN-1.

Double-strand DNA breaks (DSBs) are the most lethal type of DNA damage induced by $\gamma$- and X-rays (1, 2). Energy deposition by these forms of ionizing radiations in water results in production of multiple radical pairs (3). When this energy deposition occurs in close proximity to DNA, multiple damages are produced, often within a short stretch of DNA, resulting in the formation of DNA damage clusters (2). Such energy deposition results in production of various types of damages, including oxidized DNA bases and single-strand DNA breaks (1, 4). If two single-strand DNA breaks are produced on opposite DNA strands within a few bases, DNA can be denatured spontaneously and thus converted into DSBs (1). Alternatively, if two oxidized DNA bases are produced on opposite DNA strands a few bases apart, these damaged bases are converted into single-strand interruptions (SSIs) by base excision repair (BER) enzymes (5, 6); DNA glycosylases remove the oxidized DNA bases, leading to the formation of apurinic/apyrimidinic (AP) sites (5, 6), and then such sites are incised by AP-endonucleases (6). Two closely spaced SSIs on opposite DNA strands are thus produced by this mechanism, resulting in spontaneous denaturation of DNA (7, 8). If the denaturation temperature of the sequence between the two DNA damages is over 37 °C (12 base pairs apart, assuming a GC content of 50%), the induction of SSIs at the sites of DNA damage, however, cannot produce DSBs due to spontaneous denaturation.

Using a model substrate DNA and the cell-free DSB formation assay we developed, we previously demonstrated that two DNA damages produced on opposite DNA strands can be converted into DSBs even when the denaturation temperature of the intervening sequence is over 37 °C (9). This conversion is associated with long repair patch formation, which is mediated by BER enzymes. At the sites of SSIs produced by BER enzymes, DNA $\alpha$-polymerase $\delta$ ($\alpha$-pol $\delta$) and DNA ligase I (lig I) can produce repair patches over 2 nucleotides in length (a long repair patch) (10, 11). During the synthesis of a long repair patch, the DNA strand is cleaved from the 5′ termini (11). A 5′–3′ exonuclease activity of flap endonuclease (FEN-1) is involved in this cleavage (12).

In the cell-free DSB formation assay, circular substrate DNA is expected to be linearized when DSBs are produced between two DNA damages. With extracts prepared from MRC5 cells, which are normal fibroblasts that mainly produce short repair patches, some DSB formation was observed. However, when 46BR cell extracts, which produce long repair patches due to an abnormality in lig I (13), were employed, more DSBs were observed. Thus, these results suggest that the amount of DSBs produced is correlated with the size of the repair patches. Based on these observations, we proposed that enzymes involved in long repair patch formation collide between two DNA damages, resulting in DSB formation (9). Thus, if DSBs are produced by this mechanism, two distantly formed DNA damages located on opposite DNA strands can be converted into DSBs, as repair patches of over 40 bases are found in vivo (2, 14, 15).

Because long repair patches are produced by pol $\delta$,$\varepsilon$, we previously proposed that collision of pol $\delta$,$\varepsilon$ between two DNA damages was responsible for DSB formation. However, here we report that FEN-1 is actually involved in the productions of DSBs. Our results suggest that FEN-1 initiates DNA strand cleavage from two SSIs produced on opposite DNA strands, thus lowering the
denaturation temperature of the intervening sequence. When the denaturation temperature falls below 37 °C, the DNA strands separate and a DSB is produced. In addition, cells with a higher FEN-1 content produced more DSBs after exposure to γ-rays. Thus, based on these results, we conclude that FEN-1 has an activity to produce DSBs both in vitro and in vivo. Because FEN-1 is an essential enzyme that plays its roles in DNA repair and DNA replication (16–20), DSBs may be produced in cells as by-products of the activity of FEN-1.

**MATERIALS AND METHODS**

**Preparation of Substrate DNA—**PIU and P2U18 DNA were prepared as described previously (9). γ-Irradiated DNA was prepared by exposure of pBluescript KS+ (3.4 mg/ml) in 10 mM Tris-HCl and 1 mM EDTA to 100 Gy of γ-rays (Catp, dose rate: 0.51 Gy/min, Gamma Cell 220, Atomic Energy of Canada). After irradiation, nicked circular DNA produced due to the formation of SSIs, and linearized DNA was removed by ethidium bromide (EtBr)-CsCl centrifugation (21), and the remaining closed circular DNA was used for cell-free DSB formation assay.

**Cell-free DSB Formation Assay—**Cell-free DSB formation assay was carried out as described previously (9). Briefly, either PIU, P2U18, or closed circular pBluescript KS+ irradiated with 100 Gy of γ-rays (160 ng) was incubated with cell-free extracts (50 μg) were extracted from the agarose gel using Qiagen gel extraction kit (Qiagen). The DNA was then digested from E. coli

**Analysis of DNA Fragments by Sequencing-Gel Electrophoresis—**Linearized DNA by DSB formation from the cell-free DSB formation assay with His6 cell extracts (50 μg) was extracted from the agarose gel using Qiagen gel extraction kit (Qiagen). The DNA was then incubated with [γ-32P]ATP together with T4 polynucleotide kinase to label the 5’ ends. After ethanol precipitation of the DNA fragment, labeled DNA was digested with either SacI or NolIII. Alternatively, linearized DNA was incubated with [α-32P]dATP with terminal transferase to label the 3’ ends. After precipitation of the DNA by ethanol, DNA was digested with either SacI or NolIII. These digested DNA fragments were denatured, and then separated by 8.5% polyacrylamide sequencing-gel electrophoresis. To prepare the size markers, sequencing reactions were carried out with a primer hybridized to the SacI site (5’-GACGCGCCGGGTCGCGGGG-3’). The PCR product was purified from the gel using Qiagem gel extraction kit (Qiagen). The PCR product was then purified by ethanol precipitation and redissolved in 10 mM Tris-HCl, 0.1 mM sodium chloride, and 0.1 mM phenylmethylsulfonyl fluoride. After elution of the proteins, the positive fractions were determined by SDS-polyacrylamide gel electrophoresis. Concentration of NolIII in the positive fractions was then brought up to 1 μl, and the fractions were applied to a Superdex-75 gel filtration column pre-equilibrated with 50 mM HEPES-KOH, pH 7.5, 10 mM MgCl2, 1 mM NaCl, 10 mM glycerol, and 0.1 mM phenylmethylsulfonfyl fluoride (buffer FE). After washing the column with 10 volumes of buffer A, proteins were eluted on a linear gradient from 0 to 1.6 mM NaCl over 5 column volumes. Positive fractions were determined by SDS-polyacrylamide gel electrophoresis. Concentration of NolIII in the positive fractions was then brought up to 1 μl, and the fractions were used for the assay.

**Cell-free DSB Formation Assay—**Cell-free DSB formation assay was carried out following the method of Harrington and Lieber (12) with minor modifications. Briefly, a fresh substrate was prepared by annealing three oligonucleotides: 5’-CGTGTGAATCCTGGCGGCAATATCCCTTCCCGGGGTTT-3’ (base strand), 5’-GGAGGATTCGTTAGGCGGCCATGGTC-3’ (adjacent strand), and 5’-GGACCGCCGTTAAGATCC-3’ (intron strand). The oligonucleotides were denatured by using the same conditions as for the cell-free DSB formation assay with the addition of 1 μl of flap substrate and 2 μg of salmon sperm DNA. After termination of the reactions by addition of 1% SDS and 250 μg/ml proteinase K, DNA was purified, denatured, and fractionated on 10% polyacrylamide-8% urea gel. The dried gel was used for autoradiography to visualize 32P-labeled DNA.

**Constant-field Gel Electrophoresis—**Constant-field gel electrophoresis of FEN-1 was carried out with an anti-FEN-1 antibody (N-17) (100-fold dilution) purchased from Santa Cruz Biotechnology Inc.

**Separation of Cells Transfected with the Expression Construct of FEN-1—**FEN-1 mammalian expression construct (0.05 μg, pcDNA 3.1- GS/FEN-1, ResGene), pMACS K5.2 (2.2 μg, Miltenyi Biotec), containing a genetically modified H-2Kb DNA, and FEN-1 (4.25 μg) were mixed with 19.5 μl of Trix-20 (Promega) and 5.2 ml of serum-free DMEM. As a control, pBluescript KS+ (2.25 μg) was transfected. After 15 min of incubation at room temperature, the mixture was added to a 10-cm cell culture dish containing HeLa S3 or MOS9J (American Type Culture Collection) cells at 80% confluence. The cells were incubated for 1 h at 37 °C, then 25 μl of complete DMEM was added, and the cells were cultured for another 24 h. The cells were then harvested with trypsin-EDTA and resuspended in complete DMEM, and × 106 cells were centrifuged. The pellet was resuspended in 300 μl of complete DMEM and mixed with 40 μl of colloidal superparamagnetic microbeads coated with anti-H-2Kb antibody (MACSelect K6 MicroBeads (Miltenyi Biotec)). To the H-2Kb-expressing cells to the beads, the suspension was gently mixed for 15 min at room temperature prior to the addition of 1600 μl of phosphate-buffered saline containing 0.5% bovine serum albumin and 5 mM EDTA (PB). An MS column (Miltenyi Biotec), held by a magnet, was pre-equilibrated with 500 μl of PBE. Cells were then loaded onto the column and washed four times with 500 μl of PBE. Magnetically retained cells were eluted with 1 ml of PBE after removing the magnet.
RESULTS

Inhibition of pol δε—We have previously developed a DSB formation assay based on a cell-free assay (9) used for the study of BER (25). Our assay uses whole cell-free extracts and a defined substrate: a closed circular DNA construct containing two uracils on opposite DNA strands (Fig. 1A). The whole cell-free extracts contain uracil DNA glycosylase, which removes the uracils, leaving AP sites. These sites can then be cleaved by AP-endonuclease, producing two SSIs (Fig. 1A). For P2U18, which contains two uracils on opposite DNA strand 18 base pairs apart (the uracils are designated as U1 and U2), the denaturation temperature of the intervening sequence is 62 °C, and the formation of two SSIs at the uracil sites does not cause linearization of P2U18 by spontaneous denaturation (9). Thus, incised P2U18 can only be linearized by enzymatic formation of DSBs (Fig. 1A) (9). Using this assay, we previously observed that 46BR cell extracts, which produce long repair patches (over two nucleotides in length) due to an abnormality in lig I (13), generated more linearized P2U18 than extracts prepared from MRC5 cells (normal fibroblasts), which mainly produce short repair patches prepared from MRC5 cells (normal fibroblasts), which mainly produce short repair patches (Ref. 9 and see Fig. 1B, lane 5 versus lane 7). However, such DSB formation was not observed when P1U, which contains one uracil residue, was used (Ref. 9 and see Fig. 1B, lane 3 versus lane 7), suggesting that two DNA damages are required for the DSB formation. We proposed that the collision of enzymes involved in long repair patch synthesis initiated at those two SSIs converts these two damages into a DSB (Fig. 1A). pol δε were potential candidates for the enzymatic production of DSBs, as they both mediate long patch BER. Thus, to investigate the molecular mechanism of DSB formation, we first tested the effect of aphidicolin, an inhibitor of pol δε, on DSB formation. As shown in Fig. 1B (autoradiography), aphidicolin inhibited incorporation of [α-32P]dAMP into DNA, suggesting that DNA strand synthesis by pol δε was inhibited by aphidicolin. However, linearization of P2U18 by 46BR cell extracts due to DSB formation was not inhibited (Fig. 1B, lane 7 versus lane 8 and C). Instead, we found an increased amount of DSBs in DSB formation assay with MRC5 cell extracts and aphidicolin (Fig. 1B, lane 5 versus lane 6 and C). Thus, these results suggest that DSBs are not produced by collision of pol δε, but, on the contrary, that pol δε have an inhibitory effect on DSB formation.

DSB End Structure—To identify other potential enzymes involved in DSB formation, the DSB end structures were analyzed. Linearized P2U18 produced by 46BR cell extracts was 32P-labeled at either the 3′ or 5′ end (Fig. 2A) and was digested with either SacI or NlaIII. The resulting fragments were denatured, and their lengths were determined by sequencing-gel electrophoresis. The expected length of Fragment 1 from the SacI site to the SSI at the first uracil (U1) is 84 bases. If DNA polymerization occurs from the SSIs, the length of this fragment is expected to increase. However, as shown in Fig. 2B, the majority of Fragment 1 was 84 bases in length (85 bases on the gel due to ddAMP labeling), suggesting that no DNA synthesis occurred on this fragment. A one-base extension occurred on remaining Fragment 1 (85 bases in length (86 bases on the gel)), although no base extension is unlikely to be sufficient to reduce the denaturation temperature of the intervening sequence below 37 °C to produce DSBs. We also did not find any increase in the length of Fragment 3; its expected length from NlaIII to the second uracil (U2) is 55 bases (56 bases on the gel) (Fig. 2C). Thus, the lack of DNA elongation on the majority of Fragments 1 and 3 suggests that DNA polymerization has no major role in DSB formation, consistent with the results obtained from the assay with aphidicolin (Fig. 1, B and C).

On the other hand, the majority of the 5′ labeled Fragment 2 (expected length of 107 bases) and Fragment 4 (expected length of 78 bases) were shorter than expected, suggesting that the
DNA strands are cleaved in the 5’ to 3’ direction from the SSIs. Taken together, these results suggest that DSBs are produced by cleavage of DNA strands from the 5’ ends, as summarized in the legend to Fig. 2D.

Effect of FEN-1 on DSB Formation—In long patch BER, FEN-1 is known to cleave DNA strands from the 5’ to 3’ direction by releasing fragments of 2 to 3 nucleotides (20, 26). Thus, we decided to investigate the possible involvement of FEN-1 in DSB formation. In the DSB formation assay, we added up to three times the amount of FEN-1 (corresponding to 3U) normally present in 50 μg of extracts. As shown in Fig. 3, A and B, adding 3U of FEN-1 using MRC5 cell extracts produced a 4-fold increase in the amount of DSBs formed. When P1U1, which contains one uracil, was used, no DSB formation was found (Fig. 3, A and B). Thus, increased DSB formation is not due to nonspecific nicking of DNA by FEN-1, suggesting that FEN-1 can generate DSBs from two DNA damages located on opposite DNA strands. Addition of PCNA, which promotes the activity of FEN-1 (27, 28), to the DSB formation assay did not increase DSB formation (data not shown), possibly due to the fact that the extracts already contain sufficient amount of PCNA. In the same experimental conditions, but using 46BR cell extracts, FEN-1 did not promote DSB formation (Fig. 3, A and B). We also did not observe the formation of more than 28–30 ng of linearized DNA, even after addition of an increased amount of FEN-1 in the assay using MRC5 cell extracts (data not shown). As illustrated in Fig. 1A, two uracil residues are required to be simultaneously converted into SSIs for DSB formation. Thus, if such simultaneous SSI formation occurs only in 10% (30 ng) of a given substrate DNA (P2U18), over 30 ng of DSB cannot be produced in the cell-free DSB formation assay, even after addition of increased amounts of FEN-1.

Inhibition of FEN-1-mediated DSB Formation by Lig I—We have previously reported that adding lig I to 46BR cell extracts reduces the formation of DSBs (9). In addition, when FEN-1 activity in 46BR cell extracts was neutralized by an anti-FEN-1 antibody (data not shown), the DSB formation activity of 46BR cell extracts was also reduced by the neutralization (Fig. 3, C and D). Furthermore, as shown in Fig. 3, E and F, addition of 10 units of lig I to this assay inhibited the DSB formation promoted by FEN-1 with MRC5 cell extracts. Among the DNA ligases that we tested, lig I showed the most significant inhibitory effect on DSB formation. Therefore, the results suggest that FEN-1 can act as a factor converting two DNA damages produced on opposite DNA strands into DSBs, while lig I can counteract this activity.

Conversion of γ-Ray-induced DNA Damage into DSBs by FEN-1—To further investigate the involvement of FEN-1 in DSB formation, we used γ-irradiated plasmid DNA instead of the model substrate, P2U18. pBluescript K/S + 32P-labeled was exposed to γ-rays, and plasmid DNA containing SSIs and linearized DNA (products of direct action of free-radicals) were removed by EtsBr-CsCl centrifugation (21). The remaining closed circular DNA was used for the DSB formation assay. With this substrate, FEN-1 was able to promote the linearization of the irradiated plasmid DNA (Fig. 4), consistent with results obtained from assays using P2U18. These results suggest that γ-ray-induced DNA damages can be converted into DSBs, and FEN-1 promotes this conversion.

FEN-1-mediated DSB Formation in Vivo—We then tested the effect of FEN-1 on DSB formation in vivo using HeLa S3 cells, in which the FEN-1 content was increased by transfection of a mammalian FEN-1 expression construct (FEN-1 content was increased about 2-fold, as estimated by Western blotting using anti-FEN-1 antibody (data not shown)). These cells and control cells were then exposed to γ-rays on ice and incubated at 37 °C to allow enzymes to act on DNA damage. In control cells, as expected, the initial level of DSBs non-enzymatically induced by oxygen free radicals declined during the post-exposure period due to DSB repair (Fig. 5, Control). Compared with control cells, the amount of DSBs found in cells transfected with the FEN-1 expression construct was higher (Fig. 5, Transfected). These results can be explained by enzymatic production of DSBs by FEN-1 during the post-exposure period.

To confirm this interpretation, MO59J cells, a DNA-dependent protein kinase (DNA-PKcs) mutant, which have reduced DSB repair activity due to an abnormality in non-homologous end joining (29), were tested. In MO59J cells, DSB repair...
during the post-exposure period was less significant when compared with HeLa S3 cells (data not shown), consistent with a previous report by Allalunis-Turner et al. (29). In MO59J cells transfected with the FEN-1 expression construct, the amount of DSBs increased above the initial levels during the post-exposure period (data not shown), suggesting that DSBs are enzymatically produced by FEN-1 in these cells. Interestingly, following this increase, the DSBs were repaired, as the DSB levels began to decrease after 60 min of incubation despite the impairment of non-homologous end joining activity. As 3′-protruding ends could have been produced by FEN-1 at the DSB ends (Fig. 2D), this repair was perhaps mediated by another DSB repair pathway, such as homologous recombination, which catalyzes DNA strand invasion using 3′ protruding ends (10, 30). Taken together, these observations indicate that FEN-1 is involved in DSB formation in vivo.

**DISCUSSION**

The results reported here demonstrate that DSBs can be produced by a FEN-1-mediated mechanism from two DNA damages produced on opposite DNA strands. In Fig. 6A, we present a model for this FEN-1-mediated DSB formation. Two
DNA damages located on opposite DNA strands are converted into SSIs by BER enzymes. FEN-1 then initiates DNA strand cleavage from the 5’ termini of SSIs. With P2U18, which contains two uracils 18 base pairs apart, the denaturation temperature of the intervening sequence is 65 °C (9). Thus, if 4 nucleotides are cleaved from each SSI or 8 nucleotides are removed from either SSIs, a DSB can be produced as the denaturation temperature of the remaining DNA duplex between the damages falls below 37 °C. When DNA damage is present on one DNA strand (Fig. 6B), FEN-1 cleavage activity can result in long repair patch formation, as DNA gaps produced by FEN-1 are filled in by pol δε, and the resulting DNA nicks are sealed by lig I. In a previous report, we used P1U, which contains one uracil, to analyze the length of repair patches, and we found that 46BR cell extracts produce long repair patches (9). As these extracts produced larger amounts of DSBs than MRC5 cell extracts, which mainly produce short repair patches, we first proposed that in DSB formation assays, as DNA gaps produced by FEN-1 are filled in by pol δε, and the resulting DNA nicks are sealed by lig I. In a previous report, we used P1U, which contains one uracil, to analyze the length of repair patches, and we found that 46BR cell extracts produce long repair patches (9). As these extracts produced larger amounts of DSBs than MRC5 cell extracts, which mainly produce short repair patches, we first proposed that in DSB formation assays, collision of pol δε between two DNA damages is responsible for DSB formation. However, as illustrated in Fig. 6A, FEN-1, rather than pol δε, has been identified as an enzyme directly involved in DSB formation both in vivo and in vitro.

Any activity counteracting the cleavage of DNA ends by FEN-1 can potentially reduce the risks of DSB formation. In fact, in Fig. 3B, we show that lig I counteracts FEN-1-promoted DSB formation, lig I seals DNA nicks by forming covalent bonds between 5’-phosphate and 3’-hydroxyl groups, whereas if these nicks are not rejoined, the 5’ ends are likely susceptible to cleavage by FEN-1. In fact, the 5’ to 3’ cleavage activity of FEN-1 is found in an assay with substrates containing 5’-recessed ends (12). The effect of lig I in counteracting DSB formation by FEN-1 can therefore be explained by its nick sealing activity prior to the cleavage of 5’ ends by FEN-1, and thus, lig I can be considered as an enzyme that reduces the risks of DSB formation. In addition to lig I, pol δε can also be considered as such an enzyme. pol δε fills the DNA gaps produced by the cleavage activity of FEN-1. As pol δε produces nicks that can be sealed by lig I, efficient gap filling by pol δε apparently reduces the risk of gap expansion by FEN-1. In this context, inhibition of pol δε could increase the risk of DSB formation. In fact, we found the promotion of DSB formation by inhibition of pol δε activity by aphidicolin (Fig. 1, B and C). The cleavage activity of FEN-1 is, however, essential for repair of 5’ oxidized deoxyribose termini at SSIs (11). On the other hand, expansion of DNA gaps by FEN-1 increases the risk of DSB formation as we proposed here. Thus, cleavage of DNA ends by FEN-1, DNA polymerization, and DNA ligation apparently must be carried out in a coordinated fashion. One factor that has been proposed to coordinate these processes is PCNA (32), which is a DNA sliding clamp protein that interacts with FEN-1, pol δε, and lig I (27, 28, 33–35). Through such interactions, pol δε and lig I are proposed to be recruited to the sites of DNA cleavage by FEN-1 (32). This recruitment may allow the removal of oxidized deoxyribose from 5’ termini by cleavage of DNA ends and, at the same time, termination of repair without further expansion of DNA gaps. The repair pathway mediated by these enzymes is referred to as long patch BER. However, because pol δε and lig I counteract FEN-1-mediated DSB formation, the actual function of pol δε and lig I may be considered to be to complete repair by formation of the shortest possible patches to reduce the risks of DSB formation.

In mammalian cells, the production of SSIs during repair of DNA damage induced by alkylating agents by BER gives rise to normal 5’ deoxyribose termini (6). The majority of these SSIs are repaired by short patch BER mediated by DNA polymerase β and either lig I or DNA ligase III (6). These SSIs can also be substrates of FEN-1, as long repair patches are found in cells exposed to alkylating agents (14, 15). Furthermore, in MRC5 cell extracts, which mainly produce short repair patches, DSBs are also produced (Ref. 9 and Fig. 1, B and C), although the amount was significantly less compared with that produced by 46BR cell extracts (Ref. 9 and Fig. 1, B and C). Thus, in the context of DSB formation, short patch repair mediated by DNA polymerase β and either lig I or DNA ligase III can be considered as a prime defense mechanism against DSB formation.

Although the majority of SSIs produced by BER is repaired

![Fig. 6. Model for FEN-1-mediated DSB formation.](image-url)
by short patch repair, a small portion of SSIs can be subjected to cleavage by FEN-1 as described. Thus, even in the presence of various mechanisms preventing DSB formation from closely spaced DNA damages on opposing DNA strands, there is still a risk of DSB-induced cytotoxicity, as only a small number of DSBs is sufficient to kill a cell (36). Such DNA damages can be found within DNA damage clusters induced by ionizing radiations (2). Typically, multiple DNA damages can be produced in clusters within an 80-base pair range (2). In vivo, repair patches of 40 base pairs are observed (2, 14, 15), and thus, two DNA damages produced on opposite DNA strands even 90 base pairs apart can be converted into a DSB (assuming an intervening sequence with a 50% GC content and spontaneous deamination occurring when the length of duplex DNA reaches 10 base pairs or less). Thus, DNA damage clusters induced by ionizing radiations can be susceptible sites for DSB formation by this FEN-1-mediated mechanism. In the case of alkylating agents and reactive oxygen free radicals, they do not produce DNA damage clusters. In cells, however, DNA damage produced by these agents is suggested to be concentrated in certain DNA regions (DNA damage hot spots). Such hot spots are found in the p53 gene (37, 38). Legault et al. (39) also found hot spots at DNA attachment sites of the nuclear scaffold. Furthermore, hot spots are found in DNA break clustering regions of AF-4 and MLL genes (40, 41). Interestingly, chromosome fragments are frequently translocated between the break clustering regions of MLL and AF-4 genes (42:4111 translocations) (40, 41), leading to acute child B-cell lymphomas (42–44). It has been proposed that multiple DNA damage formation by endogenous or exogenous DNA damaging agents in the break clustering regions triggers this translocation (40). The distance between two DNA damages within hot spots is typically from 10 to 100 base pairs. Thus, if multiple DNA damages are produced within hot spots by alkylating agents or reactive oxygen free radicals, these hot spots could be potential sites for DSB formation.

The presence of enzymatic pathways responding to DSB formation (45, 46) suggests that DSBs can be frequently produced in cells. These pathways, collectively called “the DNA damage response,” regulate various responses such as cell-cycle arrest and cell death by apoptosis (45, 46), and one factor, ataxia telangiectasia mutated protein, is activated by DSBs (47). Abnormalities in ataxia telangiectasia mutated protein lead to a human inherited disease, ataxia telangiectasia, and ataxia telangiectasia patients show an increased sensitivity to ionizing radiations and cancer proneness (31), due to an impaired response to DSBs (45). In addition, AT patients develop the disease even without exposure to ionizing radiations, and thus, it has been suggested that DSBs can be produced under normal living conditions (45). It has been assumed that DNA damages induced by endogenous reactive oxygen metabolites are enzymatically converted into DSBs, although the link between oxidative DNA damage and DSB formation is not known. Multiple DNA damages can be produced within DNA damage hot spots and FEN-1 can convert two DNA damages produced on opposite DNA strands into DSBs, DSBs may be produced under normal living conditions by the mechanism that we reported here.

In conclusion, FEN-1 produces DSB from two DNA damages located on opposite DNA strands. Since FEN-1 is an essential enzyme for DNA replication and repair, DSBs are perhaps produced by this essential enzyme as a by-product of its actions.

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