Clustered DNA Damage, Influence on Damage Excision by XRS5 Nuclear Extracts and Escherichia coli Nth and Fpg Proteins*

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Marie-Hélène David-Cordonnier‡, Jacques Laval§, and Peter O’Neill

From the Medical Research Council, Radiation and Genome Stability Unit, Harwell, Didcot, Oxon, OX11 0RD, United Kingdom and UMR 8532 CNRS, Institut Gustave Roussy, 39 Rue Camille Desmoulins, 94805 Villejuif, France

Ionizing radiation and radiomimetic anticancer agents induce clustered DNA damage, which are thought to reflect the biological severity. *Escherichia coli* Nth and Fpg and nuclear extracts from XRS5, a Chinese hamster ovary Ku-deficient cell line, have been used to study the influence on their substrate recognition by the presence of a neighboring damage or an abasic site on the opposite strand, as models of clustered DNA damage. These proteins were tested for their efficiency to induce a single-strand break on a 32P-labeled oligonucleotide containing either an abasic (AP) site, dihydrothymine (DHT), 7,8-dihydro-8-oxo-2′-deoxyguanine, or 7,8-dihydro-8-oxo-2′-deoxyadenine at positions 1, 3, or 5 base pairs 5′ or 3′ to either an AP site or DHT on the labeled strand. DHT excision is much more affected than cleavage of an AP site by the presence of other damage. The effect on DHT excision is greatest with a neighboring AP site, with the effect being asymmetric with Nth and Fpg. Therefore, this large inhibition of the excision of DHT by the presence of an opposite AP site may minimize the formation of double-strand breaks in the processing of DNA clustered damages.

Radiation and radiomimetic anticancer agents cause DNA damage, and it is thought that clustered damage (in which at least two damages are produced within less than 10 base pairs) is implicated in the biological severity of radiation since it is less repairable (1, 2). The complexity of radiation-induced clustered DNA damage increases on increasing the ionizing density of the radiation (LET).1 From track structure simulations, ~20% of double-strand breaks are associated with other damages for low LET radiation but increased to >20% for double-strand breaks induced by high LET α-radiation (3). Indirect experimental evidence supporting the role of DNA damage complexity comes from the reduced repairability of double-strand breaks induced in cellular DNA by high LET radiation (4, 5) and the increased complexity of single-strand breaks on increasing radiation quality as revealed using cell extracts (6–9). If base damages within a cluster are on opposite strands and both excised, this gives rise to double-strand breaks. Therefore, it is of great significance to understand the way in which several damages in close proximity are recognized/processed by the cell.

Although the chemical nature of oxidative damage produced by oxidative stress and by ionizing radiation are similar, the unique feature of ionizing radiation and radiation mimetic agents is their ability to produce clustered damage. There are only few studies about the excision of a damage substrate in the vicinity of another damage. In particular, synthesized oligonucleotides containing damage at specific sites were used to focus on the efficiencies of endonucleases VIII (Nei) and III (Nth) to excise either thymine glycol or DHT when opposite a single-strand gap (10) as well as the efficiency of Fpg to excise 8-oxo-G or AP site opposite a gap (11) or 8-oxo-G near a formylamine on the same strand (12). Chaudhry and Weinfeld (13) focused on the efficiency of Nth to process either clustered abasic site or two DHTs on opposed strands within a plasmid.

These DNA glycosylases, e.g. bacterial Nth protein and formamidopyrimidine DNA glycosylase (Fpg), are involved in the first step of the base excision repair to remove specific modified bases from DNA (14–16) to create an abasic site (AP site, for apurinic/apurinic site) which is subsequently cleaved by their AP lyase activity giving a gap in the DNA strand. Nth protein excises mainly ring saturated pyrimidines (e.g. 5,6-dihydrothymine (DHT) and thymine glycol) (17–20), whereas Fpg excises mainly 2,6-diamino-4-hydroxy-5-N-methyl formamidopyrimidine (21) and 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxoG) (22, 23).

Since major base modifications produced by ionizing radiation are DHT (through reduction pathway), 8-oxoG, and 8-oxoA (through oxidative pathways) (24), specific DNA constructions have been synthesized with these lesions inserted at precisely known positions and varied the positions systematically relative to each other or to an AP site. This study represents the first investigation to address the way in which clustered DNA base damage is removed by nuclear extracts (from Ku-deficient CHO cells, XRS5; see Refs. 25–27) in comparison with damage removal by Nth- and Fpg-purified enzymes. The nuclear extracts from the Ku-deficient CHO-derived cell line XRS5 were used to gain insight into the role of a composite of enzyme present in the nucleus of mammalian cells.

EXPERIMENTAL PROCEDURES

Substrate Oligonucleotides—The oligonucleotides were purchased from Genosys or Glen Research. The sequences of the 40-mer oligonucleotides are presented in Table I. Strand 2 contains either DHT, 8-oxoG, 8-oxoA, or a uracil (or the corresponding undamaged base as control) at a fixed position (position X). Strand 1 contains a uracil (that could be removed by uracil-DNA-glycosylase (Ung) to give an abasic site), a DHT, or thymine as a control at given but variable positions (position Y) opposite to the fixed damage on strand 2. The oligonucleotides were 32P 5′-end-labeled using 10 units of T4 polynucleotide kinase (Life Technologies, Inc.) with 50 μCi of [γ-32P]ATP (6,000 Ci/mmol, 10 mCi/ml, NEN Life Science Products) in 25 μl of the recommended buffer for 1 h at 37 °C. Following purification on a 12% denaturing polyacrylamide gel, the labeled oligonucleotide was hybridized with 1.5-fold
Excision of Clustered DNA Damage

**Fig. 1. Excision efficiency of several substrates by Nth, Fpg, and XRS5 nuclear extracts.** A, representation of the excision of a base damage (X) on the labeled strand (+) of a double-strand oligonucleotide by increasing amount of protein and its visualization after migration on a 12% denaturing polyacrylamide gel. B–D, comparison of the percentage of removal of several substrates by increasing amount of Nth, Fpg, and XRS5 nuclear extracts, respectively. The standard errors are represented, and the lanes are used to determine the quantity of protein that removes 50% of each base damage.

**Table I**

Oligonucleotide sequences

| -5 | 5′-ctcttagtca ggaatytgtN ttcatgcttg gacgaaaggc-3′ |
| -3 | 3′-gagaatcagt ctttatacaX agatacgacc ctggttccg-5′ |
| -1 | 5′-ctcttagtca ggaatytgtN ttcatgcttg gacgaaaggc-3′ |
| +1 | 3′-gagaatcagt ctttatacaX agatacgacc ctggttccg-5′ |
| +3 | 5′-ctcttagtca ggaatytgtN ttcatgcttg gacgaaaggc-3′ |
| +5 | 3′-gagaatcagt ctttatacaX agatacgacc ctggttccg-5′ |
| +8 | 5′-ctcttagtca ggaatytgtN ttcatgcttg gacgaaaggc-3′ |

X and Y represent AP, DHT, 8-oxoG, 8-oxoA or the normal corresponding base (T for AP and DHT, G for 8-oxoG or A for 8-oxoA). N represents normal base complementary to the X base (A opposite to AP or DHT, C opposite to 8-oxoG:C or T opposite to 8-oxoA). +1 up to +8, position of the X base 3′ from the Y base, on the complementary strand.

excess of the purified non-radiolabeled complementary strand. That the annealing was efficient was verified by migration on a native 10% polyacrylamide gel. To prepare the oligonucleotides containing an abasic site at given positions, the 32P-labeled double-stranded oligonucleotides containing a uracil were treated with 1 unit of uracil-DNA-glycosylase (Life Technologies, Inc.) for 30 min at 37 °C in 50 μl of buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA).

**Purified Proteins**—The purified Nth protein was a generous gift from Prof. Rick Wood (Imperial Cancer Research Fund) (28). The purified Fpg protein was extracted and purified as described by Boiteux et al. (29).

**Preparation of Nuclear Extracts**—The nuclear extracts were prepared from a Ku-deficient CHO-derived cell line, XRS5 (25–27), to avoid possible interference by Ku binding to linear DNA (30). The cells were grown in exponential phase in a-complemented minimum Eagle’s medium (ICN Biomedicals Inc.) supplemented with 10% fetal calf serum (Glasform). The cells were harvested by centrifugation at 1,000 × g for 10 min at 4 °C, and the pellet was washed twice in 30 ml of phosphate-buffered saline volume of phosphate-buffered saline. The pellet of cells (1 volume) was then resuspended in an equal volume of buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT) and incubated on ice for 15 min. The cytoplasmic membranes were broken by drawing 10 times into a 0.5-μm diameter needle. After a brief centrifugation at 12,000 × g, the supernatant was removed, and the nuclear pellet was resuspended in 2/3 volume of high salt buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM MgCl2, 0.5 mM DTT, 0.5 mM PMSF) for 30 min with agitation on ice. After a 10-min centrifugation at 12,000 × g, the supernatant was dialyzed 3 times over a period of 2 h against 1 liter of incubating buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol containing 0.5 mM DTT, 0.5 mM PMSF). The protein concentration was estimated using the Bradford colorimetric technique, and the aliquots of nuclear extracts were stored at −80 °C.

**Cleavage Assays for Single-strand Break Analysis**—The double-stranded oligonucleotides (10,000 cpm, 200 fmol) were incubated with increasing amounts of Nth, Fpg proteins, or XRS5 nuclear extracts, as specified in the legends of the figures, in 5 μl of the incubation buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol) for 30 min at 37 °C. Subsequently, 5 μl of the denaturing stop solution was added (98% formamide, 0.025% bromphenol blue, 0.025% xylene cyanol, 2 mM EDTA, pH 8.0) to the samples that were then subjected to electrophoresis on a 12% denaturing polyacrylamide gel containing 8 M urea in 1 × TBE (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 90 min at 85 watts. The dried gel was then exposed to a Bio-Rad PhosphorImager screen to visualize cleaved and full-length DNA fragments using phosphorimaging (Bio-Rad, Molecular Imager® FX) and quantified using Quantity One software (Bio-Rad) to determine the excision efficiency of each enzyme for each of the DNA sequences used.
The number of nicks reflects the number of modified bases excised. The activity of the cleaved strand is expressed as a percentage of the total activity of the cleaved and intact strand. The efficiencies for multiple damages are compared with that for single damage to assess the effect of the second damage, present on the unlabeled strand, on the excision of the damage on the labeled strand by the various proteins.

**Measure of Double-strand Breaks Induced by the Proteins**—The experiments were done as described above for the analysis of single-strand breaks, except the reactions were stopped by adding 5 μl of a non-denaturing solution (40% sucrose, 0.025% bromphenol blue, 0.025% xylene cyanol, 5 mM EDTA, pH 8.0). The samples were run on a 10% native polyacrylamide gel in 1× TBE for 3 h at 300 V, dried, and quantified using the Bio-Rad PhosphorImager as described above.

**RESULTS**

**Excision Efficiency of Different Damages by Nth, Fpg, and XRS5 Nuclear Extracts**—The efficiency of the various proteins to remove specific single substrates from DNA was first compared with that for single damage to assess the effect of the second damage, present on the unlabeled strand, on the excision of the damage on the labeled strand by the various proteins.

**Effect of the presence of another opposite damage on the AP site excision efficiency by Nth.** The oligonucleotide containing an AP site is labeled and hybridized to a complementary strand containing or not (control) another damage opposite the positions between 5 bases 5′ (−5) and 8 bases 3′ (+8) from the AP site (see Table I). Double-stranded oligonucleotides containing an AP site (at the variable Y position (A), 8-oxoG (B), 8-oxoA (C), a DHT (D), or a corresponding normal base opposite to the AP site on the labeled strand are incubated with increasing amounts of Nth (1 pg up to 5 ng). The diagrams reflected the fold inhibition/activation by comparison to the control containing no other damage on the strand opposite to the AP site. The error bars represent the standard deviation from 3 to 5 different experiments.

**Effect of the presence of another opposite damage on the AP site excision efficiency by Fpg.** The same oligonucleotides as in Fig. 2 are incubated with an increasing amounts of Fpg (1 pg up to 5 ng). −5 up to +8 represent the different positions of the opposite damage from 5 bases 5′ up to 8 bases 3′ from the AP site as described in Table I. The diagrams reflected the fold inhibition/activation by comparison to the control containing no other damage on the strand opposite to the AP site from 3 to 5 different experiments. The error bars represented the standard deviation of the values presented on the right of the diagrams.
give 50% cleavage of the DNA. As shown in Fig. 1C with Fpg, the AP site is also the most efficiently removed damage, whereas 8-oxoG and DHT are excised less efficiently (7.5- and 75-fold, respectively) than an AP site. It is worth noting that Fpg does not excise 8-oxoA from DNA even when using a high concentration (200 ng) of protein.

In an approach to mimic DNA damage processing in cells, XRS5 nuclear extracts have been used to determine its efficiency to excise AP site, uracil, 8-oxoG or DHT from DNA. Fig. 1D shows that an AP site is cleaved most efficiently by nuclear extracts, whereas uracil and 8-oxoG are excised less efficiently (70- and 40-fold, respectively) by nuclear extract than an AP site. DHT is the least efficiently excised damage, requiring 180-fold greater quantity of nuclear extract to cleave 20% of the DNA compared with that for excision of the AP site (Fig. 1D).

The 3' end of the AP site containing oligonucleotides obtained after cleavage of the AP site Nth, Fpg proteins, or XRS5 nuclear extracts migrates at a different position in gel (data not shown). These bands correspond to a 3'phosphoaldehyde obtained by a beta elimination using Nth, to a 3'phosphate processed via a beta-d elimination by Fpg (31–33) and 3'OH termini using nuclear extracts in which exonucleases or 3'phosphatases clean up the 3' ends to give the 3'OH termini required by DNA polymerases to repair the strand break.

**Effect of Neighboring Damage on the Protein Recognition of an AP Site**—The efficiency of Nth to excise an AP site was determined when further damage is positioned on the nonlabeled strand at defined positions opposite to the AP site of interest (position X) on the labeled strand of DNA (see Table I). The relative positions are from 5 bases 5' to 5 bases 3' from the fixed AP site (Fig. 2). The diagrams reflected the fold inhibition/activation by comparison to the control containing no other damage on the strand opposite the AP site. The white bars, gray bars, and black bars represent the opposite damage on the nonlabeled strand at position 1, 3 or 5 nucleotides, respectively, in 5' or 3' from the AP site on the labeled strand. Triplicate reactions were performed, and the standard deviations are presented.

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Fig. 6. Effect of the presence of a neighboring damage on the efficiency of DHT excision by Nth. The oligonucleotide containing a DHT is labeled and hybridized to its complementary strand containing or not another damage at positions 5' (−5) up to 8 bases 3' (+8) from the DHT. Double-stranded oligonucleotides containing an AP site (A), uracil (B), DHT (C), 8-oxoG (D), 8-oxoA (E), or the corresponding normal base opposite to the DHT on the labeled strand are incubated with increasing amounts of Nth (250 pg up to 50 ng). The diagrams reflected the fold inhibition/activation by comparison to the control containing no other damage on the strand opposite to the AP site. The error bars represented the standard deviation from 3 to 5 different experiments.

Effect of Neighboring Damage on the Excision of DHT by Nth—We have tested the ability of Nth, Fpg, and XRS5 nuclear extracts to excise DHT from DNA when another damage is positioned on the other nonlabeled strand. The largest inhibitory effect of DHT excision by Nth is seen in the presence of an AP site opposite to the DHT, and the effect is asymmetrical (Fig. 6). The inhibition occurs when the AP site is between 2 and 8 bases 5' from the AP site on the labeled strand.

Similar results were obtained using XRS5 nuclear extracts where 8-oxoG, 8-oxoA, or DHT do not significantly influence the efficiency of cleavage of the AP site excision (Fig. 4, B and C and data not shown, respectively). However, the presence of another AP site results in an enhancement of the excision efficiency of the AP site when the other AP site is positioned between 1 and 5 bases 5' from the AP site of interest on the complementary strand (Fig. 5A), with maximum activation seen when the other AP site is 3 bases away in the 5' direction (position +3, Fig. 4A). This enhancement contrasts with the inhibition seen for excision by Fpg and Nth. No effect was observed if the other AP site is 3' or more than 8 bases 5' from the AP site on the labeled strand.

Therefore, the major effect on excision on an AP site is due to the presence of another AP site at various positions opposite the AP site at the fixed X position on the labeled strand presented above. To assess whether the inhibition of the cutting efficiency for both Nth and Fpg and the stimulation of AP excision by XRS5 nuclear extracts is strand- or sequence-specific, we performed similar experiments but using oligonucleotides that were 5'-labeled on the strand containing an AP site at the variable Y positions. The results are presented in Fig. 5. The inhibitory effect on excision of an AP site at the variable positions Y by Nth (Fig. 5A) is comparable with that at the fixed position X (Fig. 2A) and similarly shows an inhibitory effect of about 2 for an AP site 1 base 5' to the AP site of interest. The inhibitory pattern of an opposite AP site on the excision of an AP site by Fpg is similar when the AP site is in position Y (Fig. 5B) or X (Fig. 3A) of the labeled strand. The inhibitory effect is up to 3.9 and 3.3 when the AP site is positioned 1 base 5' opposite to the AP site at position Y (Fig. 5B) or X (Fig. 3A), respectively. Similar concordance of results is obtained for XRS5 nuclear extracts using oligonucleotide labeled on the strand that contains the AP site at position X (Fig. 4A) or Y (Fig. 5C). Some minor variations in the level of activation are seen and may reflect differences in the sequence context of the AP site, but activation of the excision of the AP site by the nuclear extracts is clearly correlated to the relative positions of the two opposite AP sites. The inhibitory/activation effects by an AP site on the excision of an opposite AP site does not depend significantly on sequence context.

Effect of Neighboring Damage on the Excision of DHT by Nth—We have tested the ability of Nth, Fpg, and XRS5 nuclear extract to excise DHT from DNA when another damage is positioned on the other nonlabeled strand. The largest inhibitory effect of DHT excision by Nth is seen in the presence of an AP site opposite to the DHT, and the effect is asymmetrical (Fig. 6). The inhibition occurs when the AP site is between −3...
and +8 bases from the DHT site, with a maximum of 52-fold inhibition when the AP site is positioned 1 base 5′ from the DHT (Fig. 6A). Uracil-containing oligonucleotides show a minor effect on the efficiency of excision of DHT by Nth (Fig. 6B). A minor effect was also observed with DHT opposite to DHT at the various positions shown in Fig. 6C. In contrast, 8-oxoG and 8-oxoA positioned 1 base 5′ from the DHT (positions +1, Fig. 6, D and E) have an inhibitory effect on the excision of DHT by Nth. Therefore, the inhibitory effect of the second damage is always asymmetrical in double-stranded DNA.

Since the presence of an AP site greatly inhibits the excision of DHT by Nth, we examined whether this inhibition of single-strand break formation correlated with the formation of a double-strand break. Therefore, the same experiment was performed as in Fig. 6A, but the samples were divided as follows: one-half ran on a 12% denaturing polyacrylamide gel, and the other half ran on a 10% native polyacrylamide gel. The percentage of single- or double-strand breaks induced by Nth in the DNA-specific oligonucleotides is represented in Fig. 7, A and B, respectively. A high yield of single-strand breaks but no double-strand breaks are produced in the control that contains only one damage. Fig. 7, A and B, shows similar dependence on the influence of the other damage for excision of the damages to give single- and double-strand breaks. Only oligonucleotide −5 has fewer double-strand breaks in comparison with the level of single-strand break. This is probably due to the fact that the 5 base pairs, between the two single-strands breaks, are not properly dehybridized in the sample and during the migration.

**Effect of a Neighboring Damage on the Excision of DHT by Fpg**—As shown in Fig. 8A, the largest inhibitory effect for Fpg excision of DHT occurs when an AP site is positioned opposite to the DHT of interest. This inhibition is asymmetrical and is greatest when the AP site is positioned 3 or 1 bases 3′ to the DHT (positions −3 and −1) with 50- and 43-fold inhibition, respectively. In contrast to the results with Nth, the removal of DHT by Fpg is also inhibited 8.4–12.2-fold by the presence of uracil located between 5 bases 3′ to 5 bases 5′ of the DHT on the opposite strand, as presented in Fig. 8B. The presence of an 8-oxoG opposite to the DHT also has an inhibitory effect and is maximum when 8-oxoG in positioned at 3 bases 3′ or 5′ of the DHT (positions −3 and +3 in Fig. 8D, respectively). In contrast, the presence of another DHT or an 8-oxoA, 1 base 5′ (position +1, Fig. 8C) and 1 base 3′ to the DHT (position −1, Fig. 8E), respectively, on the labeled strand gives 2.1- and 3.6-fold activation. No effect was seen when DHT or 8-oxoA is present at any other site opposite to the DHT of interest.

By using double labeling probes containing an AP site opposite to DHT, the same level of DHT or AP site was excised by Nth or Fpg as that observed with the singly labeling probes described above (Figs. 2D and 6A and Figs. 3D and 8A). In the same way, identical results were obtained using 5′-32P-labeled probes on the DHT-containing strand that was hybridized to the complementary oligonucleotides containing an AP site with either a 5′-OH or a 5′-P termini. These results suggest that the presence of 5′ OH or 5′-P termini does not change the inhibitory effect of the presence of an AP site on the excision of DHT by Nth or Fpg.

**Influence of Another Damage on the Excision of DHT by XRS5 Nuclear Extract**—As with the purified Nth and Fpg proteins, the presence of an AP site opposite to a DHT causes a large decrease of the efficiency of the excision of DHT by the XRS5 nuclear extract (Fig. 9A). In contrast to the findings with Nth and Fpg (Figs. 6A and 8A, respectively), the effect is symmetric. The largest inhibition is seen when the AP site is 5 bases 3′ or 5′ of the DHT (positions −5 and +5 in Fig. 9A), and the inhibition is less when the AP site is at positions −1 and +1 to DHT (Fig. 9A). The effect of uracil on DHT excision (Fig. 9B) gives the same inhibitory profile as that of an AP site, but the level of inhibition is significantly less. The presence of either another DHT or 8-oxoG does not have any significant effect on the action of the nuclear extracts (Fig. 9C and D). However, it is worth noting that, as for the incubation with Fpg (Fig. 8D), the profile of the effect in the presence of 8-oxoG is the same but less efficient when incubated with nuclear extract (Fig. 9D).
The only significant effect of 8-oxoA is when positioned 1 base 3' (position -1, Fig. 9E) opposite to the DHT, giving a 2.1-fold inhibition.

**DISCUSSION**

The purpose of the present study was to determine the effect of neighboring DNA damage on the excision of a specific DNA damage to gain an insight into the effects of clustered DNA damage on DNA damage repair. Purified proteins involved in the DNA damage recognition and excision and nuclear extracts, as a composite of the cellular proteins, were used to assess how such clustered damage may compromise their efficiency to be repaired in cells even though the individual damages are readily processed (Fig. 1). This study is the first to assess the recognition and processing of complex damage by cell extracts, as a more complex model of repair, and compare the effects with base excision repair enzymes, used as benchmarks. For instance, the AP lyase activity of Nth and Fpg is about 1 order of magnitude greater than their glycosylase activities when comparing the cleavage of an AP site with that for excision of a base damage. The cleavage of an AP site by Nth is only affected by an immediately neighboring AP site. The cleavage of an AP site by Nth, Fpg, and extract is not greatly affected by the presence of a vicinal base damage, DHT, 8-oxoG, and 8-oxoA. For nuclear extracts, the efficiency of cleavage of an AP site is enhanced when another AP site is positioned opposite at 1, 3, and 5 and is not significantly dependent on the sequence context as demonstrated in Fig. 5.

The biggest effect on the efficiency of cleavage of an AP site, reflecting the AP lyase activity, by Nth, Fpg, and XRS5 nuclear extracts is the presence of a neighboring AP site on the complementary strand. The large inhibition (>4-fold) of the AP lyase activity occurs with Fpg when another AP site is situated from -5 to +8 of the AP site monitored (Figs. 3A and 5A). The cleavage of an AP site by Nth is only affected by an immediately neighboring AP site. The cleavage of an AP site by Nth, Fpg, and extract is not greatly affected by the presence of a vicinal base damage, DHT, 8-oxoG, and 8-oxoA. For nuclear extracts, the efficiency of cleavage of an AP site is enhanced when another AP site is positioned opposite at +1, +3, and +5 and is not significantly dependent on the sequence context as demonstrated in Fig. 5.

The major inhibition (>50-fold), however, is seen on conver-
sion of DHT into a single-strand break by Nth or Fpg (see below for DHT excision by Fpg) when an AP site is on the opposite strand and the influence extends over several base pairs of separation (Figs. 6A and 8A). Since the inhibitory effect of a neighboring AP site on excision of DHT is greater than on cleavage of an AP site, it is suggested that the AP site on the nonlabeled strand is rapidly converted into a single-strand break since both Nth and Fpg are more efficient at cleavage of an AP site than excision of DHT (Fig. 1, B and C). From comparison of the yields of single-strand and double-strand breaks (Fig. 7) following Nth excision of DHT (Fig. 1, B and C). From comparison of the yields of single-strand and double-strand breaks (Figs. 6A and 8A) following Nth excision of DHT, it is apparent that inhibition of double-strand break formation is of the same magnitude and trend as that for formation of a single-strand break. Therefore, it is suggested that a single-strand break on the non-labeled complementary strand at the AP site position is already formed prior to removal of the DHT. This finding is comparable with that for the removal of thymine glycol opposite a gap by Nth (10). However, the inhibitory effect of an AP site on the excision of thymine glycol is symmetrical in contrast to the asymmetry seen for excision of DHT opposite to an AP site by Nth. In contrast to the large inhibition by an AP site on the opposite strand, consistent with previous studies (10), is when 8-oxoG is more than 1 base 5’ or at every position 3’ to the DHT on the complementary strand (Fig. 6D). The asymmetrical effects observed may be correlated to the asymmetrical binding of Nth to the strand opposite to the recognized damage. Indeed, DNase I footprinting experiments (36) show that Nth protects an AP site from 4 bases 3’ to 4 bases 5’ of the AP site but only one base opposite the AP site on the complementary strand (38, 39). The influence of other damages is much more variable than that obtained with Nth. In particular, the presence of a uracil on the opposite strand has a 8.4–12.2-fold inhibitory effect on DHT excision by

**FIG. 9. Effect of the presence of a neighboring damage on the DHT excision by XRS5 nuclear extracts.** Double-stranded oligonucleotides as described previously (Fig. 6) are incubated with increasing amounts of XRS5 nuclear extracts (0.5 to 30 μg). The diagrams reflected the fold inhibition/activation by comparison to the control containing no other damage on the strand opposite to the DHT. The white bars, gray bars, and black bars represent the opposite damage on the nonlabeled strand at positions 1, 3, or 5 nucleotides, respectively, in 5’ or 3’ from the DHT on the labeled strand. Triplicates reactions were performed, and the standard deviation are presented.
Fpg (Fig. 8B) but no effect on DHT excision by Nth (Fig. 6B). This inhibition could allow the action of the Ung protein to form an abasic site that will be quickly converted into a single-strand break. After repair of this break, the Fpg protein can then act more efficiently on the DHT opposite a normal strand.

With XRS5 nuclear extracts, the major effect on excision of a DHT is the presence of an AP site (Fig. 9A) or uracil (Fig. 9B), in contrast to the small effects seen with either a neighboring DHT, 8-oxoG, or 8-oxoA. In contrast to the purified enzymes, the largest inhibitory effects of a vicinal damage are seen on base damage excision and not on cleavage of an AP site. The effect of uracil on the excision of DHT by XRS5 nuclear extracts gives a similar pattern to that for the influence of an AP site on DHT recognition by all the proteins tested.

It is suggested that the big inhibitory effect of an opposite AP site on DHT recognition by all the proteins tested may minimize the probability of formation of a double-strand break so that the repair enzymes have time to rejoin the gap, generated by the repair enzymes and not on cleavage of an AP site. The effect of uracil on the excision of DHT by XRS5 nuclear extracts might be due to the combined effects of an Fpg-like protein and Nth to give the lower inhibition. It is of interest that certain clustered damages could give an increased efficiency of excision of a base damage within the cluster. This is particularly apparent for a DHT 1 base 5’ or an 8-oxoA 1 base 3’ to DHT on the complementary strand with Fpg and an AP site 1 base up to 5 bases from another AP site with XRS5 nuclear extracts.

It is suggested that the big inhibitory effect of an opposite AP site on DHT recognition by all the proteins tested may minimize the probability of formation of a double-strand break so that the repair enzymes have time to rejoin the gap, generated at the AP site, prior to excision of DHT from the opposite strand.

### REFERENCES

1. Goodhead, D. T., Thacker, J., and Cox, R. (1993) *Int. J. Radiat. Res.* **63**, 543–556
2. Ward, J. F. (1988) *Prog. Nucleic Acids Res.* **35**, 95–125
3. Nikjoo, H., O’Neill, P., Terrisol, M., and Goodhead, D. T. (1999) *Radiat. Environ. Biophys.* **38**, 31–38
4. Bücher, D. (1988) *Int. J. Radiat. Biol.* **54**, 761–771
5. Jenner, T. J., de Lara, C. M., O’Neill, P., and Stevens, D. L. (1993) *Int. J. Radiat. Biol.* **64**, 264–273
6. Satoh, M. S., Poirier, G. A., and Lindahl, T. (1993) *J. Biol. Chem.* **268**, 5480–5487
7. Hodgkins, P. S., Fairman, M. P., and O’Neill, P. (1996) *Radiat. Res.* **145**, 24–30
8. Hodgkins, P. S., O’Neill, P., Stevens, D., and Fairman, M. P. (1996b) *Radiat. Res.* **145**, 660–667
9. Cunniffe, S., and O’Neill, P. (1999) *Radiat. Res.* **152**, 421–427
10. Harrison, L., Hatahet, Z., Poirier, G. A., and Wallace, S. S. (1998) *Nucleic Acids Res.* **26**, 992–941
11. Harrison, L., Hatahet, Z., and Wallace, S. S. (1999) *J. Mol. Biol.* **290**, 667–684
12. Bourdat, A.-G., Gasparutto, D., and Cadet, J. (1999) *Nucleic Acids Res.* **27**, 1015–1024
13. Chaudhry, M. A., and Weinfield, M. (1997) *J. Biol. Chem.* **272**, 15650–15655
14. Laval, J., Jurado, J., Saparbaev, M., and Sodorkina, O. (1998) *Mutat. Res.* **402**, 93–102
15. Boiteux, S. (1993) *J. Photochem. Photobiol. B Biol.* **19**, 87–96
16. Dizdaroglu, M., Laval, J., and Boiteux, S. (1993) *Biochemistry* **32**, 12015–12111
17. Hatahet, Z., Kow, Y. W., Cunningham, R. P., and Wallace, S. S. (1994) *J. Biol. Chem.* **269**, 18814–18820
18. De Grado, W. F., and Laval, J. (1999) *Biochemistry* **38**, 2528–2536
19. Giraud, P.-M., Guibourt, N., and Boiteux, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4690–4694
20. Fuciarelli, A. F., Wegher, B. J., Blakery, W. F., and Dizdaroglu, M. (1990) *J. Biol. Chem.* **265**, 18814–18820
21. Calsou, P., Frit, P., and Salles, B. (1996) *Biochem. J.* **312**, 581–589
22. Boiteux, S., E. Davie, J. B., Blakery, W. F., and Dizdaroglu, M. (1990) *Int. J. Radiat. Biol.* **58**, 397–415
23. Chaudhary, M. A., and Weinfeld, M. (1997) *J. Biol. Chem.* **272**, 5159–5167
24. Girard, P.-M., Guibourt, N., and Boiteux, S. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4690–4694
25. Boitoux, S., O'Connor, T. R., and Laval, J. (1987) *J. Biol. Chem.* **262**, 2528–2536
26. Tchou, J., Kasai, H., Shibutani, S., Chung, M.-H., Laval, J., Grollman, A. P., and Nishimura, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 581–589
27. Boitoux, S., O’Connor, T. R., and Laval, J. (1989) *EMBO J.* **8**, 359–363
28. Tchou, J., Kasai, H., Shibutani, S., Chung, M.-H., Laval, J., Grollman, A. P., and Nishimura, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 581–589
29. Boitoux, S., O’Connor, T. R., and Laval, J. (1989) *EMBO J.* **8**, 359–363
30. Calsou, P., Frit, P., and Salles, B. (1996) *Biochem. J.* **312**, 581–589
31. Bailly, V., Verly, W. G., O’Connor, T., and Laval, J. (1989) *Biochem. J.* **262**, 581–589
32. O’Connor, T. R., and Laval, J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5222–5226
33. Bhagwat, M., and Gerlt, J. A. (1996) *Biochemistry* **35**, 659–665
34. Girard, P.-M., Guibourt, N., and Boitoux, S. (1997) *Nucleic Acids Res.* **25**, 3204–3211
35. Bailly, V., and Verly, W. G. (1987) *Biochem. J.* **242**, 27601–27607
36. O’Connor, T. R., and Laval, J. (1989) *Biochem. J.* **262**, 581–589
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Marie-Hélène David-Cordonnier, Jacques Laval and Peter O’Neill

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