Analysis of Incision Sites Produced by Human Cell Extracts and Purified Proteins during Nucleotide Excision Repair of a 1,3-Intrastrand d(GpTpG)-Cisplatin Adduct*

(Received for publication, October 27, 1995)

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Nucleotide excision repair by mammalian enzymes removes DNA damage as part of ~30-mer oligonucleotides by incising phosphodiester bonds on either side of a lesion. We analyzed this dual incision reaction at a single 1,3-intrastrand d(GpTpG)-cisplatin cross-link in a closed circular duplex DNA substrate. Incisions were formed in the DNA with human cell extracts in which DNA repair synthesis was inhibited. The nicks were mapped by restriction fragment end labeling and primer extension analysis. Principal sites of cleavage were identified at the 9th phosphodiester bond 3' to the lesion and at the 16th phosphodiester bond 5' to the lesion. The predominant product was found to be a 26-mer platinated oligonucleotide by hybridization to a 32P-labeled complementary DNA probe. Oligonucleotides were formed at the same rate as the 3' cleavage, suggesting that both incisions are made in a near-synchronous manner. There was, however, a low frequency of 5' incisions in the absence of 3' cleavage. The dual incision reaction was reconstituted using the purified mammalian proteins XPA, RPA, XPC, TFIIH, XPG, and a fraction containing ERCC1-XPF and IF7. All of these components were required in order to observe any cleavage.

The dual incisions produced during nucleotide excision repair (NER) in vertebrates have been studied for several DNA lesions, including UV photoproducts, thymine-pyrimidines monoadducts, cisplatin cross-links, and acetylaminofluorene adducts (1–4). For these lesions, an asymmetric pattern of incisions has been found. Oligonucleotides 27–29 nucleotides long are released after cleavage at the 22nd to 24th phosphodiester bonds 5' and at the 5th phosphodiester bond 3' to a thymine dimer (1). A few other DNA lesions have been reported to be removed in this way (2–4), leading to the view that this is the general incision pattern during NER in eukaryotes.

The incision reaction requires XPA, RPA, XPG, XPC, TFIIH, ERCC1-XPF complex, and IF7 (5), although it is not clear that all of these components directly participate in the formation of both the 3' and 5' incisions. XPA preferentially binds damaged DNA (6), suggesting a role in damage recognition. The single-stranded DNA-binding protein RPA is also required for the incision stage (7–9) and acts synergistically with XPA to bind damaged DNA (10). Two “structure-specific” nuclease is involved that can recognize bubble or splayed arm structures, with opposite polarity. The XPG nuclease is responsible for incisions 3' to a DNA lesion (11), while the human ERCC1-XPF complex is believed to mediate the 5' incision, by analogy with the structure-specific nuclease activity of the homologous RAD1-RAD10 complex of Saccharomyces cerevisiae (12, 13).

The roles of IF7 and XPC are not yet known, although the latter is a single-stranded DNA-binding protein that may stabilize an incision reaction intermediate (14, 15). The TFIIH complex contains 3' to 5' and 5' to 3' ATP-dependent helicase activities, in the XPB and XPD subunits, respectively (16, 17). These may allow localized unwinding of DNA and assist opening of the DNA helix so that the structure-specific nucleases can act. It is not known whether the dual incisions are made sequentially or simultaneously, but it is likely that the nucleases achieve selectivity in cleavage of the damaged DNA strand through interactions with other repair proteins. For example, the XPA protein has been found to interact with ERCC1, RPA, and TFIIH (10, 18–22).

To investigate the roles of these proteins further we have examined the incision reaction using a closed circular DNA substrate containing a single 1,3-intrastrand d(GpTpG)-cisplatin cross-link at a specific site. Cisplatin is an important antitumor drug (23) that reacts with DNA to form intrastrand and interstrand cross-links (24) that are removed by nucleotide excision repair with varying efficiencies. Several new methods are reported here for characterization of the lesions made by human cell extracts during NER and for mapping the structure of the excised oligonucleotide. Although the oligonucleotides released during repair of the 1,3-intrastrand d(GpTpG)-cisplatin cross-link have sizes (4) and repair patches (25) consistent with those observed for several other DNA lesions, we find that the platinum lesion has a novel incision pattern that may be dictated by its unusual structure. This dual incision reaction has been reconstituted using purified repair proteins and this has provided information on the degree to which the incisions are coupled and on the proteins required for cleavage 3' and 5' to a DNA lesion.

EXPERIMENTAL PROCEDURES

Synthetic Oligonucleotides—Oligonucleotides were synthesized by the cyanoethyl phosphoramidite method using an Applied Biosystems model 380B DNA synthesizer. For primer extension analysis, oligonucleotides were purified on 20% denaturing polyacrylamide gels and recovered by soaking in 0.3 M NaAc (pH 5.2), 10 mM MgCl2 followed by precipitation with ethanol. The 5' termini were phosphorylated with ATP or [γ-32P]ATP using T4 polynucleotide kinase. 32P-Labeled oligonucleotides were purified by centrifugation through Sephadex G-25 (Pharmacia Biotech Inc.) columns.

Construction of Closed Circular DNA Molecules Containing a Site-
specific 1,3-intrastrand d(GpTpG)-cisplatin cross-link—The 24-mer 5'-TCTCTTCTGTCAGCTTTCTC-3' was allowed to react at a concentration of 1 mM with a 3-fold molar excess of cisplatin (3 mM) for 16 h at 37°C in containing 3 mM NaCl, 0.5 mM Na2HPO4, and 0.5 mM NaH2PO4. The identity of the 1,3-intrastrand d(GpTpG)-cisplatin cross-link, bridging bases 10 and 12 of the 24-mer, was established by enzymatic digestion of the platinated oligonucleotide to its constituent nucleosides and reverse phase high performance liquid chromatography analysis. In addition the oligonucleotide was observed on a denaturing 20% polyacrylamide gel as a single band (purity measured at 97%) that reverted to an unmodified 24-mer following treatment with cyanide (data not shown).

Covalently closed circular DNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin cross-link (Pt-GTG) was produced by priming 30 μg of plus strand M13mp18GTG (150 ng/μl) with a 5-fold molar excess of 5-phosphorylated platinated oligonucleotide in a 200-μl reaction mixture containing 10 mM Tris-Cl (pH 7.9), 50 mM NaCl, 10 mM MgCl2, 1 mM DTT, 600 μM each of dATP, dCTP, dGTP, and TTP, 40 units of T4 DNA polymerase gp43 subunit (HT Biotechnology Ltd.) and 36 Weiss units of T4 DNA ligase (New England Biolabs) at 60°C for 4 h prior to electrophoresis in nondenaturing 12% polyacrylamide gels. Counts collected with a PhosphorImager (Molecular Dynamics) were corrected to take account of the base composition of the damaged strand of each restriction fragment.

Formation of Incised DNA Intermediates—Mammalian cell extracts and/or purified repair proteins were incubated with DNA for the times indicated under the same conditions as repair synthesis reactions, except that [α-32P]dNTPs were omitted from the reaction buffer. Uvra and Uvrb were preincubated in repair reaction buffer (Fig. 5A) or a reaction buffer (Fig. 5B) containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 84 mM KCl, 2 mM DTT, and 2 mM ATP for 10 min at 37°C. DNA substrate (300 ng) and UvraC were then added and incubated for a further 30 min at 37°C.

Restriction Fragment End Labeling Analysis of Incised DNA Intermediates—For end labeling the damaged DNA strand 3' to the lesion, 300 ng of purified incised DNA intermediates were incubated with 6 units of XhoI (New England Biolabs) in a 20-μl reaction at 37°C for 90 min. The reactions were chilled on ice before adding dATP, dCTP, and dGTP to final concentrations of 50 μM each, 2 μCi of [α-32P]dTTP and 1.5 units of E. coli DNA polymerase I large (Klenow) fragment (New England Biolabs). After incubation for 10 min on ice, TTP was added to a final concentration of 50 μM, and reactions were incubated for a further 5 min on ice. For end labeling the damaged DNA strand 3' to the lesion, 300 ng of purified incised DNA intermediates were incubated with 6 units of HindIII (New England Biolabs) in a 10-μl reaction containing TA buffer (Epicentre Technologies Corp.) for 90 min at 37°C. The reaction was supplemented with 5 mM CaCl2, and 1 unit of HK phosophatase (Epicentre Technologies Corp.) and incubated for 90 min at 30°C. After heat inactivation for 15 min at 65°C, the reaction was supplemented with ATP to 1.2 μM, [γ-32P]ATP (300 Ci/mmol) to 0.2 μM, 5 units of T4 polynucleotide kinase (New England Biolabs) and incubated for 60 min at 37°C followed by heat inactivation for 15 min at 65°C.

Primer Extension Analysis of Incised DNA Intermediates—Purified incised DNA intermediates (300 ng) were added to a 10-20-fold molar excess of 32P-labeled 17-mer primer 5'-CAGGAAACAGCTATGAC-3' in a 15-μl reaction mixture containing 40 μl Tris-HCl (pH 7.5), 20 mM MgCl2, 50 mM NaCl, and 80 μM each of dATP, dCTP, dGTP, and dTTP. The reaction mixture was incubated at 95°C for 4 min, 48°C for 4 min, and then 37°C for 1 min before adding 3 units of T7 DNA polymerase (Sequenase v2.0) (U. S. Biochemical Corp.) and incubating for a further 30 min at 37°C.

**Fig. 1.** Closed circular duplex DNA containing a single 1,3-intrastrand d(GA1Gp)G-cisplatin cross-link. A, structure of Pt-GTG DNA, a modified M13mp18 molecule containing a specifically located 1,3-intrastrand d(GpGp)G-cisplatin cross-link in the (−)DNA strand. The expanded region illustrates the DNA sequence flanking the cisplatin cross-link. The eight BstNI restriction fragments and the target sites of other restriction enzymes are indicated. Non-damaged Con-GTG DNA contains a sequence identical to Pt-GTG DNA, without a Pt lesion. B, 0.8% agarose gel demonstrating the presence of the cisplatin cross-link. Lane 1, uncut Pt-GTG; lanes 2–7, digestion of Pt-GTG with Bsu36I (Bsu), HindIII (H), ApalI (A), EcoRI (E), XhoI (X), and BsaHI (Bsa). Lane 8, uncut Con-GTG. Lanes 9–14, digestion of Con-GTG with the same enzymes. The mobilities of covalently closed circular (cc), linear (lin), and nicked circular (nc) Pt-GTG and Con-GTG DNA are indicated alongside the gel.
Fig. 2. Inhibition of DNA repair synthesis at the site of the cisplatin cross-link. Autoradiographs of 12% polyacrylamide gels demonstrating the occurrence of DNA repair synthesis in the region of the cisplatin cross-link. DNA was incubated with cell extract, purified, and digested with BstNI before electrophoresis. Lanes 1–9, HeLa cell extract. Lanes 10–18, HeLa cell extract in the presence of aphidicolin. The size of the eight BstNI restriction fragments is shown alongside each autoradiograph. In order to make the 68-bp band visible, lanes 10–18 are shown at a darker exposure than lanes 1–9.

Results

Closed Circular DNA Containing a Single 1,3-Intrastrand (GpG)-Cisplatin Cross-link—A 24-mer oligonucleotide containing a single 1,3-intrastrand (GpG)-cisplatin cross-link was incorporated into closed circular duplex DNA using methods described previously (11, 28). This adduct is a good substrate for the vertebrate NER system (4, 11, 25), and cellular extracts described previously (11, 28). This adduct is a good substrate for the vertebrate NER system (4, 11, 25). The 10% 18-bp substrate, designated Pt-GTG, contains the platinated (GpG) sequence within the unique recognition sequence of the restriction enzyme ApaLI (Fig. 1A). Closed circular Pt-GTG DNA was resistant to cleavage by ApaLI (Fig. 1B, lanes 1 and 4), while non-platinated Con-GTG DNA was completely linearized (Fig. 1B, lanes 8 and 11). Restriction enzymes with unique sites in DNA sequences flanking the cisplatin cross-link were used for Southern hybridization to detect oligonucleotides formed by the dual incision reaction. The distance from the cisplatin cross-link to the 3' termini of oligonucleotides was determined by treatment with 3' to 5' exonuclease.

10 min at 37 °C. Where indicated, DNA was first linearized with PvuI (New England Biolabs) to enable more efficient primer extension than was possible with unnicked closed circular DNA. Primer extension or restriction fragment end labeling reactions were separated by electrophoresis in denaturing 10 or 12% polyacrylamide gels, dried, and exposed to x-ray film (Kodak X-Omat) or a PhosphorImager screen. To determine the size of incision fragments, dideoxy sequencing reactions were performed on single-stranded M13mp18GTGx DNA using a32P-labeled 16-mer primer 5'-GTAAAACGACGGCCAGT-3'.

A24-mer oligonucleotide containing a single 1,3-intrastrand (GpG)-cisplatin cross-link.

Strategies for analysis of incised DNA intermediates and oligonucleotides formed by dual incisions. A, extension from a primer annealed 3' to the lesion on the damaged DNA strand locates the cisplatin cross-link and incisions 3' to the lesion. Linearization with PvuI enables detection of translesion DNA synthesis and also of completed repair events. B, end labeling the damaged DNA strand at HinII reveals 3' incisions and uncoupled 5' incisions. C, end labeling the damaged DNA strand at HinIII allows analysis of 5' incisions. D, an oligonucleotide complementary to the DNA spanning the site of the cisplatin cross-link was used for Southern hybridization to detect oligonucleotides formed by the dual incision reaction. The distance from the cisplatin cross-link to the 3' termini of oligonucleotides was determined by treatment with 3' to 5' exonuclease. E, the 5' termini of oligonucleotides was determined by primer extension from an oligonucleotide complementary to the DNA spanning the site of the cisplatin cross-link.

2 D. E. Szymkowski and R. D. Wood, unpublished data.
Trapping Incision Intermediates in Damaged DNA by Inhibition of DNA Repair Synthesis—In order to trap incised DNA intermediates during nucleotide excision repair, we used aphidicolin to inhibit DNA repair synthesis by human cell extracts. Aphidicolin inhibits both of the DNA polymerases (α and ε) that are implicated in nucleotide excision repair (29, 30) and has been shown to lead to accumulation of incised DNA intermediates in closed circular DNA substrates (31). To facilitate analysis of the DNA repair patch, the cisplatin lesion is located near the center of the 33-bp BstNI fragment of Pt-GTG, and the 68-bp fragment is located on the 5′ side (Fig. 1A). Pt-GTG was incubated with HeLa cell extract in the presence of [α-32P]dNTPs, and the DNA was then purified and cleaved with BstNI. Most of the damage-dependent DNA repair synthesis by HeLa cell extract was in the 33- and 68-bp fragments (Fig. 2, lanes 2 to 7). A maximum level was reached in the 33-bp fragment by 30 min (Fig. 2, lane 5). A background level of nonspecific DNA synthesis was detected in other fragments of Pt-GTG and all fragments of Con-GTG, in proportion to the length of the fragment. In the presence of aphidicolin, DNA repair synthesis was inhibited by >90%. This abolished synthesis in the 33-bp fragment (compare lanes 4 and 13 in Fig. 2) and reduced synthesis by 70% in the 68-bp fragment (where the repair patch begins).

Several strategies were used to detect incisions produced during repair. These are summarized schematically in Fig. 3 and discussed in turn below.

Location of the 3′ Incision by Primer Extension Analysis—The positions of phosphodiester bond cleavage in the damaged DNA strand were located by primer extension analysis (Fig. 3A). Incised DNA intermediates were obtained by incubating Pt-GTG DNA with HeLa cell extract in the presence of aphidicolin as in Fig. 2, except that [α-32P]dNTPs were omitted from the reaction mixture. A 17-mer primer complementary to the damaged strand was used, with its 3′ end 25 nucleotides away from the cisplatin adduct. The primer was annealed to heat-denatured duplex DNA and extended with modified T7 DNA polymerase (Sequenase v2.0). The cisplatin cross-link efficiently blocked extension after a residue was incorporated opposite the C immediately 3′ to the adduct (Fig. 4A, lane 1). About 5% of extension products apparently bypassed the 3′-stratifying damage-dependent incisions 3′ to the lesion and blocking of Sequenase by the cisplatin cross-link. DNA was incubated with HeLa whole cell extract (wce) for 30 min as described. Purified DNA was digested with PvuI, a 32P-labeled 17-mer primer was annealed to denatured DNA and extended by Sequenase prior to electrophoresis. Lanes 1 and 2, no incubation of DNA prior to primer extension analysis (N/A, not applicable); Lanes 3 and 4, incubation with buffer only. Lanes 5 and 6, HeLa cell extract. Lanes 7 and 8, HeLa cell extract in the presence of aphidicolin. The lanes G, A, T, and C show dideoxy sequencing reactions of Con-GTG using the same 32P-labeled 17-mer primer. The DNA sequence of the complementary strand surrounding the cisplatin cross-link is written at the side. The positions of blocks to extension at the damaged cross-link, the PvuI restriction site and those resulting from cleavage of the damaged DNA strand are indicated at the left side. An arrow indicates a predominant 3′ incision at the 9th phosphodiester bond from the lesion. B, restriction fragment end labeling analysis. Autoradiograph of a denaturing 12% polyacrylamide gel demonstrating damage-specific incisions around the cisplatin cross-link. DNA was incubated for the times incubated (min) with HeLa cell extract, purified, digested with XhoI, and labeled with [α-32P]dNTPs using DNA polymerase I (Klenow fragment) prior to electrophoresis. Lanes 1–10, +aphidicolin; lanes 11–16, –aphidicolin. The expanded region illustrates the DNA sequence surrounding the cisplatin cross-link. An arrow indicates a predominant 3′ incision at the 9-th phosphodiester bond from the lesion. The positions of strand cleavage were determined by comparison with dideoxy sequencing ladders and by comparison with the positions of primer extension reaction products. Phosphodiester bonds are indicated by bars at the left side; the bold bars show the bonds within the cross-link.

**Fig. 4. Analysis of incised DNA intermediates.** A, primer extension. Autoradiograph of a denaturing 12% polyacrylamide gel demonstrating damage-dependent incisions 3′ to the lesion and blocking of Sequenase by the cisplatin cross-link. DNA was incubated with HeLa whole cell extract (wce) for 30 min as described. Purified DNA was digested with PvuI, a 32P-labeled 17-mer primer was annealed to denatured DNA and extended by Sequenase prior to electrophoresis. Lanes 1 and 2, no incubation of DNA prior to primer extension analysis (N/A, not applicable); Lanes 3 and 4, incubation with buffer only. Lanes 5 and 6, HeLa cell extract. Lanes 7 and 8, HeLa cell extract in the presence of aphidicolin. The lanes G, A, T, and C show dideoxy sequencing reactions of Con-GTG using the same 32P-labeled 17-mer primer. The DNA sequence of the complementary strand surrounding the cisplatin cross-link is written at the side. The positions of blocks to extension at the damaged cross-link, the PvuI restriction site and those resulting from cleavage of the damaged DNA strand are indicated at the left side. An arrow indicates a predominant 3′ incision at the 9-th phosphodiester bond from the lesion. B, restriction fragment end labeling analysis. Autoradiograph of a denaturing 12% polyacrylamide gel demonstrating damage-specific incisions around the cisplatin cross-link. DNA was incubated for the times incubated (min) with HeLa cell extract, purified, digested with XhoI, and labeled with [α-32P]dNTPs using DNA polymerase I (Klenow fragment) prior to electrophoresis. Lanes 1–10, +aphidicolin; lanes 11–16, –aphidicolin. The expanded region illustrates the DNA sequence surrounding the cisplatin cross-link. An arrow indicates a predominant 3′ incision at the 9-th phosphodiester bond from the lesion. The positions of strand cleavage were determined by comparison with dideoxy sequencing ladders and by comparison with the positions of primer extension reaction products. Phosphodiester bonds are indicated by bars at the left side; the bold bars show the bonds within the cross-link.
added G residue and proceeded to a block opposite the T between the platinated G residues. Approximately 7% of extension products proceeded to the PvuII restriction site, indicating some translesion DNA synthesis. Bypass of 1,3-intrastrand d(GpNpG)-cisplatin cross-links by Sequenase has been observed previously (32).

After incubation of Pt-GTG with HeLa cell extract in the presence of aphidicolin, primer extension revealed a pattern of products corresponding to distances from 2 to 22 phosphodiester bonds 3' to the lesion. The principal band at the 9th phosphodiester bond 3' to the lesion appeared to represent the most frequent primary incision product (Fig. 4A, lane 7). In the absence of aphidicolin, little 3' nicking was detected (Fig. 4A, lane 5), consistent with completion of most repair events by repair synthesis and ligation. In such reactions, there was a concomitant increase in extension to the PvuII restriction site, representing restoration of an undamaged DNA strand. Quantification of the diagnostic bands in Fig. 4A indicates that —10—20% of Pt-GTG DNA molecules are incised.

Analysis of Incision Positions by Restriction Fragment End Labeling—The primer extension assay suggested that the major 3' incision was located 9 phosphodiester bonds away from the lesion. A different approach led to a similar conclusion. Incised DNA intermediates were produced as above and purified. XhoI was then used to cleave the DNA 43 nucleotides from the adduct, and the restriction products were 3' end-labeled with E. coli DNA polymerase I (Klenow fragment) as in Fig. 3B. A pattern of incision fragments was produced corresponding to cleavage of the damaged DNA strand on the 3' side of the lesion, and this was similar to that seen during primer extension analysis. After 5 min, the predominant 3' incision was 9 phosphodiester bonds away from the lesion (Fig. 4B, lane 2).

In addition, a weaker new pattern of bands appeared that corresponded to cleavage at the 16th to 20th phosphodiester bonds 5' to the lesion (Fig. 4B, lanes 2-7). In order to be detectable by 3' end labeling, the fragments representing incision 5' to the lesion must have occurred in the absence of a 3' incision and are henceforth referred to as "uncoupled 5' incisions." These incisions 5' to the lesion would not have been detected by the primer extension assay above, because the polymerase was efficiently blocked by the cisplatin cross-link. Omitting aphidicolin from the reaction buffer led to the loss of the uncoupled 5' incisions and >80% reduction in the level of 3' incisions at the 9th phosphodiester bond from the lesion (compare lanes 4 and 12 in Fig. 4B), consistent with completion of the majority of repair events.

Incisions Around the 1,3-Intrastrand d(GpTpG)-Cisplatin Cross-link Made by E. coli UvrABC Endonuclease—A location of the major 3' incision 9 phosphodiester bonds from the cisplatin adduct was unexpected, because previous studies have shown that the human NER system incises pyrimidine dimer-containing DNA primarily at the 5th phosphodiester bond on the 3' side of the lesion (1, 2). We used our methods to determine the positions of incisions made around the cisplatin cross-link by purified UvrABC endonuclease from E. coli. The 5' incision was located by end labeling incised DNA at a unique HindIII restriction site (Fig. 3C), and the position of the 3' incision was determined by primer extension (Fig. 3A). The major damage-dependent incisions were found at the 8th phosphodiester bond 5' to the lesion (Fig. 5A, lane 1) and at the 4th phosphodiester bond 3' to the lesion (Fig. 5B, lane 3). These incision positions correspond exactly with those previously found for UvrABC endonuclease cleavage of a 1,3-intrastrand d(GpCpG)-cisplatin cross-link in a 96-bp DNA fragment (33).

Incisions Formed by PCNA-depleted Cell Extracts—An alternative strategy to trap incised DNA intermediates was used to be sure that the positions of incisions observed in whole cell extracts were not influenced by the drug aphidicolin. PCNA-depleted (CFII) cell extracts supplemented with purified RPA protein can form stable incised DNA intermediates in the absence of DNA repair synthesis (8). Primer extension analysis (Fig. 6A) and restriction fragment end labeling (Fig. 6B) were used to locate these incisions. HeLa CFII extract produced the same pattern of incisions around the cisplatin cross-link as did HeLa whole cell extract in the presence of aphidicolin (compare lane 1 in Fig. 6A with lane 7 in Fig. 4A). When a CFII extract from an XPG-defective cell line was used, no incisions were detected (Fig. 6, A and B, lane 3), in agreement with our previous findings (11). Complementation of a CFII extract from XP-G cells with purified XPG protein restored a pattern of strong 3' and weaker uncoupled 5' incisions that was the same as produced by HeLa cell extracts (Fig. 6B, lane 2).

Reconstitution of the 3' Incision Reaction with Purified Repair Proteins—The nucleotide excision repair reaction has recently been reconstituted in vitro using purified proteins (5). It
is already established that XPA (8, 34) and RPA (8, 9) are needed for any incisions and that XPG is required for the 3'-incision (11) (lanes 2 and 3 of Fig. 6). To determine the minimum protein requirements for the 3'-incision reaction, we tested these proteins in combination with the other factors involved in the early steps of nucleotide excision repair. Reactions were carried out with purified XPA, XPG, RPA, XPC, and TFIIH proteins, as well as purified ERCC1-XPF complex (CM-Sepharose, step 5), which also contains IF7 activity (5). These proteins were sufficient to reconstitute the 3' incision reaction, giving a predominant band at the 9th phosphodiester bond 3' to the lesion as observed by both end labeling and primer extension analysis (Fig. 6, A and B, lane 6). In reactions that omitted TFIIH, XPC, or the fraction containing ERCC1-XPF complex and IF7, no incisions were detected (Fig. 6, A and B, lanes 4, 5, and 7, respectively). Separate experiments (data not shown) confirmed that XPA, XPG, and RPA were also required for the 3' incision reaction, as expected.

Analysis of Platinated Oligonucleotides Produced by Dual Incision—The experiments described above indicated that the principal 3' incision was at the 9th phosphodiester bond from the 3'-platinated guanine and suggested that 5'-incisions were made as near as the 16th phosphodiester bond on the 5' side of the lesion. To investigate whether these were primary sites of cleavage, rather than products formed by exonucleolytic digestion after cleavage, it was necessary to examine the length and structure of the oligonucleotides produced by dual incision. A Southern hybridization procedure was devised for this purpose (Fig. 3D). DNA from NER reactions was separated by electrophoresis and then transferred to a nylon membrane. A 27-mer oligonucleotide complementary to the sequence around the adduct was 5'-labeled with 32P and used as a probe to detect excised fragments.

Oligonucleotides 24–32 nucleotides in length were formed during incubation with HeLa cell extract (Fig. 7A, lanes 2–8). A 26-mer platinated oligonucleotide predominates after 20-min incubation with the next most abundant 29- and 30-mer oligonucleotides reaching only 50% of the level of the 26-mer. XPG-defective cell extract formed no oligonucleotides (Fig. 7A, lane 11), but complementation with purified XPG protein fully restored dual incision activity (Fig. 7A, lanes 12 and 13). An identical (but weaker) pattern of platinated oligonucleotides was also observed in reactions that contained purified XPA, XPG, RPA, XPC, TFIIH, ERCC1-XPF complex, and IF7 (not shown).

Correspondence of the Structure of Excised Platinated Oligonucleotides with the Positions of the Dual Incisions—To measure the distance from the 3' end of the excised oligonucleotides

![Fig. 6. Analysis of the incisions made by PCNA-depleted (CFII) cell extracts and purified mammalian repair proteins. Autoradiographs of denaturing 12% polyacrylamide gels demonstrating damage-specific incisions made around the cisplatin cross-link by primer extension (A) and restriction fragment end labeling analysis (B) as described in the legends to Figs. 3 and 4, except that Pvu digestion prior to primer extension was omitted. The samples in A and B are aliquots from the same reactions after purification of incised DNA intermediates. Lane 1, HeLa CFII extract and 100 ng RPA. Lane 2, XPG415A CFII extract, 100 ng of RPA and 30 ng of purified XPG protein (47). Lane 3, XPG415A CFII extract and 100 ng of RPA. Lanes 4-9, combinations of purified repair proteins as indicated below each lane. The following amounts of purified repair proteins were used: 30 ng of XPA, 150 ng of RPA, 30 ng of XPC, 30 ng of XPG, 12 ng of ERCC1-XPF complex (step 5 CM-Sepharose fraction containing IF7 activity (5)), and 150 ng of TFIIH. The expanded region in B illustrates the DNA sequence surrounding the cisplatin cross-link. Brackets delineate the size range of extension products (A) or incision fragments (B) resulting from cleavage of the damaged DNA strand. An arrow indicates a predominant 3' incision at the 9th phosphodiester bond from the lesion. The positions of strand cleavage in B were determined by comparison with dideoxy sequencing ladders and with the mobility of primer extension reaction products.](http://www.jbc.org/)
to the platinum adduct, the oligonucleotides were digested with the 3' to 5' exonuclease activity of T4 DNA polymerase prior to electrophoresis (Fig. 3D). The strong 3' → 5' single-stranded DNA exonuclease activity exhibited by this enzyme in the absence of dNTPs is known to be inhibited 1–3 nucleotides away from cisplatin cross-links (35, 36). The major products after treatment with T4 DNA polymerase were 19-, 22-, and 23-mer oligonucleotides (compare lanes 1 and 2, Fig. 7B), consistent with removal of seven nucleotides from the 3' end of 26-, 29-, and 30-mer oligonucleotides. Treatment of the platinated oligonucleotides with calf intestinal phosphatase shifted their positions by approximately 1 nucleotide, consistent with the presence of 5' phosphate groups (data not shown). When aphidicolin was present during the repair reaction, there was no significant effect on the formation of platinated oligonucleotides (compare lanes 12 and 13 in Fig. 7A), supporting the observation that aphidicolin specifically inhibits DNA repair synthesis and not the dual incision reaction.

The position of the major 3' incision at the 9th phosphodiester bond from the lesion and the predominant formation of a 26-mer platinated oligonucleotide suggested that the major 5' incision was made 16 phosphodiester bonds 5' to the lesion. This prediction was tested directly by primer extension analysis using a primer that was complementary to the DNA spanning the lesion. The 3' terminus of this oligonucleotide was opposite the 6th phosphodiester bond 5' to the lesion in the damaged DNA strand. The bracket alongside the autoradiograph delineates the range of primer extension blocks, and arrows indicate the predominant blocks 16, 19, and 20 phosphodiester bonds 5' to the lesion.

**FIG. 7.** Analysis of oligonucleotides formed by the dual incision reaction. A, autoradiographs of Southern blots. Oligonucleotides formed during repair were transferred onto a nylon membrane and hybridized with a 32P-labeled complementary probe. The sizes (in nucleotides) of oligonucleotides are indicated alongside the autoradiograph and were determined by comparison with the mobility of the platinated 24-mer oligonucleotide used to make the substrate (see part B). A: Lanes 1–10, HeLa cell extract; lanes 11–13, XPG415A cell extract in the absence or presence of 30 ng of XPG protein or aphidicolin as indicated. Reaction times are in minutes. B, autoradiograph of a Southern blot. Lanes 1 and 2, HeLa cell extract. Lanes 3 and 4, HeLa cell extract in the presence of aphidicolin. Lane M, 250 pg of platinated 24-mer oligonucleotide 5'-TCTTCTTCTGACTTCTCTCTCTTT-3'. The samples in lanes 2 and 4 were incubated with T4 DNA polymerase in the absence of dNTPs at 37 °C for 30 min prior to electrophoresis. C, autoradiograph of 12% denaturing polyacrylamide gel demonstrating blocks to primer extension at the 5' termini of oligonucleotides. A 32P-labeled 16-mer primer was annealed to denatured DNA and extended by Sequenase prior to electrophoresis. Reaction mixtures contained HeLa cell extract ± aphidicolin as indicated. Reaction times (minutes) are shown. Lanes 1–7, 11–13, 8–10, and 14–16 are aliquots from the reactions shown in Fig. 4B, lanes 1–7, 8–10, 11–13, and 14–16, respectively. The 3' terminus of the 16-mer primer aligns with the 6th phosphodiester bond 5' to the lesion in the damaged DNA strand. The bracket alongside the autoradiograph delineates the range of primer extension blocks, and arrows indicate the predominant blocks 16, 19, and 20 phosphodiester bonds 5' to the lesion.
lesion, with lower levels of extension products blocked at the 19th and 20th phosphodiester bonds (Fig. 7C). Minor blocks at a few other sites were also detected between the 13th and 20th phosphodiester bonds 5′ to the lesion. No significant difference was seen in reactions with or without aphidicolin (compare lanes 4 and 7 with 9 and 10, Fig. 7C).

**DISCUSSION**

**Location of NER Incisions at a Lesion in Closed Circular DNA**—In this study, the incisions created by NER during repair of a single 1,3-intrastrand d(GpTpG)-cisplatin cross-link have been analyzed. The positions of 3′ and 5′ cleavage were determined for reaction intermediates formed under conditions where repair synthesis was prohibited and by detailed mapping of the structure of the excised oligonucleotide. Incised DNA intermediates could be trapped by inhibiting DNA repair synthesis with the drug aphidicolin or alternatively by use of PCNA-depleted human cell extracts that perform the incision steps of NER without repair synthesis (8).

The principal incisions made during repair of the 1,3-intrastrand d(GpTpG)-cisplatin cross-link are at the 9th phosphodiester bond 3′ and at the 16th phosphodiester bond 5′ to the lesion, forming a 26-mer platinated oligonucleotide. Secondary 5′ cleavage sites for this lesion were also found at the 19th and 20th phosphodiester bonds, resulting in 29- and 30-mer platinated oligonucleotides, respectively. Identical incision patterns were found when incised DNA intermediates were formed either by cell extracts in the presence of aphidicolin or with PCNA-depleted cell extracts. Consistent with this, aphidicolin had no effect on the size or levels of oligonucleotides formed during the reaction. The E. coli UvrABC endonuclease was found to cleave the strand containing the platinum adduct at the 4th phosphodiester bond 3′ and the 8th bond 5′ to the lesion, as expected (Fig. 8).

It has been proposed that after an initial 5′ or 3′ incision, a 5′-3′ exonuclease activity might remove several nucleotides in a controlled manner, before release of the damaged oligonucleotide (37). However, the major sites of incision that we observed in the duplex DNA by primer extension or end labeling analysis (Fig. 4) were in precise agreement with the structure determined for the excised oligonucleotide (Fig. 7). The same patterns of incision and oligonucleotide formation were observed throughout the time course. The data thus suggest that the principal cleavage sites observed are the “primary” ones and have not been significantly altered by an exonuclease. On the other hand, after incubation with cell extracts in which DNA repair synthesis was inhibited, some fragments were observed corresponding to nicks at the 13th, 16th, and 22nd phosphodiester bonds 3′ to the lesion (Figs. 4 and 6). No excised oligonucleotides were found that would correspond to such distant 3′ incisions, and therefore it is likely that these particular fragments result from limited exonucleolytic digestion of the plasmid by the cell extract, after the 3′ incision has been made. Reactions with purified proteins produced only the major 3′ incision products at the 8th and 9th phosphodiester bonds (Fig. 6).

**Structural Features of Particular Lesions That Determine Incision Sites**—The locations of primary incisions for the 1,3-intrastrand d(GpTpG)-cisplatin adduct (9 phosphodiester bonds on the 3′ side and 16, 19, or 20 phosphodiester bonds on the 5′ side) were initially surprising, because previous studies had clearly shown that UV-induced pyrimidine dimers are incised at the 4th or 5th bond on the 3′ side and the 22nd to 24th bond on the 5′ side of the dimer (1, 2), and a less detailed study had suggested a similar pattern for the 1,3-intrastrand d(GpTpG)-cisplatin adduct (4). The sizes of the principal excision fragments are similar in the two cases, however. The major excision product for the 1,3-intrastrand d(GpTpG)-cisplatin adduct was 26 nucleotides long, and for the TT cyclobutane dimer, 27–29 nucleotides long. Recently, a single cholesterol molecule incorporated into a phosphodiester backbone via a propanedioic linkage was also shown to be removed by NER in vitro. The principal sites of incision are at the 3rd phosphodiester bond on the 3′ side and the 25th bond on the 5′ side of the cholesterol, predominantly releasing a 27-mer (38). Thus, different lesions are released in almost identically sized oligonucleotides, but the incision positions change, depending on the adduct (Table I). We do not yet understand what controls the exact incision position, but these results suggest that a repair complex assembles in a way that opens a structure of similar size for each lesion, before catalysis of cleavage by the nucleases that act on the 3′ side (XPG) and the 5′ side (ERCC1-XPF complex).
One factor that may determine the incision position is the nature or degree of helical distortion at a particular lesion. The solution structure of a 1.3-intrastrand (GpTpG)-cisplatin cross-link in a 13-bp DNA duplex has been studied using high-resolution NMR (39). The modeled structure showed a helical distortion of the 5'-platinated G and central T residue, with 19° local duplex unwinding and a 20° kink at the platination site. Other studies have found similar distortions caused by 1.3-intrastrand cisplatin cross-links in different DNA sequence contexts (40, 41). While some aspects of these helical distortions are common to a variety of DNA lesions, including UV photoproducts (42, 43), the 1.3-intrastrand (GpTpG)-cisplatin cross-link produces novel base-stacking interactions in the damaged DNA strand (44). Base-stacking interactions are potentially important in damage recognition (45) and could influence protein-DNA interactions during damage recognition by XPA, RPA, and probably additional repair proteins. Altered protein-DNA contacts may influence subsequent protein-protein interactions, and thus the position on the damaged DNA strand to which structure-specific endonucleases are recruited, leading to variable positions of the dual incisions. It is interesting that although the human repair proteins appear to be susceptible to the unusual structural features of a 1,3-intrastrand (GpTpG)-cisplatin cross-link, the E. coli UvrABC endonuclease made dual incisions at positions consistent with those observed around other lesions (Ref. 45 and Fig. 5).

Proteins Required for Formation of Incisions—Reconstitution of the incision reaction using a combination of purified XPA, RPA, XPΔ, XPC, TFIIH, ERCC1-XPF, and IF7 (Fig. 6) demonstrates that the positions of cleavage observed with human cell extracts represent the activities of known nucleotide excision repair proteins. The absence of XPC, TFIIH, or ERCC1-XPF and IF7 led to loss of the 3' incision activity, indicating that these factors are needed in order for the 3' cut to be made, even though the XPG nuclease mediates the actual cleavage. Similarly, both 5' and 3' nicking activities are lacking in XPG cell extracts (Figs. 6B and 7A and Ref. 11), demonstrating that a defect in a protein involved in making the 3' incision can lead to loss of 5' incision activity as well. This dependence of both incisions on the presence of all components could reflect either assembly of the NER proteins into a large “repairosome” complex (46) or a sequential mechanism that depends on ordered interactions of each of the repair factors with one another. In a previous study we found that extracts from the ERCC1-defective mutant CHO 43–3B produced a weak band consistent with 3' cleavage (11). This is a more complex situation than with purified proteins, because mutant ERCC1 might still be assembled into a complex that is competent to make one or more incisions. The exact nature of the ERCC1 mutation in CHO 43–3B cells is not yet known.

Are the two NER incisions normally made in a particular order? The similar time courses of the 3' incision (Fig. 4) and oligonucleotide formation (Fig. 7) suggest that the two incisions are nearly synchronous. However, in this study we observed some uncoupled 5' incisions in the absence of any 3' cleavage, and Matsunaga et al. (38) recently presented evidence for some uncoupled 3' incisions during NER. These data suggest that either incision may occasionally occur without the other. This is compatible with a “bubble” model, whereby an opened structure is created during NER, for subsequent processing by structure-specific nucleases such as XPG and the presumed ERCC1-XPF nuclease (11–13). Tight coupling between the formation of incisions and subsequent reaction steps would be a preferable strategy, in order to avoid the exposure of a single-stranded gap and DNA ends to degradation by cellular proteins. The kinetics of incision and repair synthesis are nearly the same (Figs. 2, 4B, and 7A), consistent with such tight coupling.

General Applicability of the Methods—In addition to the insights on the mechanism of NER, our study is discussed above, the methods described here should be of more general utility. The primer extension and end labeling techniques can be used to observe the incisions produced in any suitable closed circular or linear DNA molecule containing a defined lesion. It is also worth drawing attention to the hybridization approach for detection of excised oligonucleotides. This allows the use of unlabelled damage-containing substrate which can be prepared in advance and stored until required. In these experiments we have used a detection scheme with a radioactive probe, but the method should be amenable to nonradioactive detection with appropriately designed complementary probes containing bio- tin or digoxigenin.
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*J. Biol. Chem.* 1996, 271:7177-7186.
doi: 10.1074/jbc.271.12.7177

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