Mechanisms for the Processing of a Frozen Topoisomerase-DNA Conjugate by Human Cell-free Extracts*

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The metabolic fate of covalently linked DNA-protein complexes (cross-links) is not clearly understood. Our aim was to investigate the processing of protein-DNA cross-links by cellular enzymes. As an example of a DNA-protein cross-link, we have constructed frozen topoisomerase-DNA conjugates and investigated their processing by human cell-free extracts. A suicide DNA substrate was constructed that upon reaction with vaccinia type I topoisomerase yielded a highly stable covalent DNA-protein cross-link. When this conjugate was treated with human nuclear or whole cell extracts, two sites of DNA breakpoints were detected: one set of double-stranded breaks occurred close to the 3’ side of the topoisomerase (topo) conjugation site, and there was another set of nicks about 30 nucleotides 3’ to the topo site. The double-stranded breaks were not made by extracts from xeroderma pigmentosum group A mutant cells, suggesting that the xeroderma pigmentosum group A damage recognition protein may be required for the occurrence of DNA breakage. In addition to these DNA breakage reactions, there was an activity that resulted in the delinking of the frozen topoisomerase (or proteolytic fragments thereof) from the DNA substrate, which was followed by a ligation step that restored the continuity of the broken DNA strand at the erstwhile topo attachment site. We suggest that frozen topoisomerase-DNA conjugates (and perhaps other types of covalent DNA-protein complexes) are processed by multiple pathways that may involve the cleavage of the DNA in the covalent protein-DNA complex and/or enzymatic delinking followed by ligation of the broken DNA ends. These processes may represent the “repair” of DNA-protein cross-links.

Clear-cut evidence for the occurrence or the repair of DNA-protein cross-links in normal living cells is lacking. Nevertheless, there are compelling reasons to believe that the protein-DNA cross-link is a distinct category in the repertoire of DNA damage in living cells (1). Protein-DNA cross-links can occur via a variety of processes. Extraneous agents such as UV (2) or therapeutic chemicals (e.g. cisplatin, furocoumarins, and anti-cancer topoisomerase poisons) (3–6) are known to induce DNA-protein cross-links. These processes may represent the “repair” of DNA-protein cross-links.

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*MATERIALS AND METHODS

DNA and Proteins—DNA oligos were purchased from Midland Reagent Co. (Midland, TX). The concentration of DNAs were determined from UV absorbance at 260 nm (ε260 = 10^5 M^-1 cm^-1). DNAs were either 5’-end labeled with T4 polynucleotide kinase and [γ-32P]ATP or 3’-end labeled with [α-32P]dideoxy-ATP and calf terminal deoxynucleotidyl transferase (14). The 32P-labeled and unlabeled DNAs were run on preparative 8 M urea-polyacrylamide gels, and the correct

1 The abbreviations used are: topo, topoisomerase; ds, double-stranded; ss, single-stranded; nt, nucleotide(s); TE, Tris-EDTA; TBE, Tris-borate-EDTA; XPA, xeroderma pigmentosum group A; DTT, dithiothreitol.
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...full-length DNAs were recovered by electroelution. Equimolar amounts (1 μmol) each of 58-mer, 94-mer, and 150-mer were mixed in kinase buffer (Tis-Mg buffer from New England Biolabs (Beverly, MA)), extracted with phenol-chloroform, and precipitated with EtOH (14). The DNAs were taken up in 10 μl Tris-HCl (pH 7.5), 1 mM MgCl₂/water, heated at 65 °C for 5 min, and cooled to room temperature overnight. The supernatant was collected, and the pellets were washed two times with 100 μl ethanol (70% ethanol). The pellets were then air-dried at room temperature overnight and redissolved in 12 μl of kinase buffer (14). The reactions were incubated at 30 °C for 10 min or heat-treated at 65 °C for 5 min, and then quenched by adding 1% SDS, which denatured the topoisomerase. The DNA samples were electrophoresed through 6% acrylamide-SDS gel and visualized by autoradiography with x-ray films and/or a phosphor screen. The DNA bands were visualized by autoradiography.

RESULTS

The Topoisomerase-DNA Complex as an Example of Protein-DNA Conjugates—The monomeric (molecular mass, 36.6 kDa) type I topoisomerase of the vaccinia virus specifically binds and cleaves the conserved sequence 5'-CT/CT/CCTT ↓ in duplex DNA (18). During a single catalytic cycle, the enzyme cleaves only the top strand (the scissile strand) and forms a covalent enzyme-DNA intermediate (Fig. 1). Religation of the broken DNA bond occurs following strand passage/rotation. The covalent intermediate has a 3'-phosphodiester linkage with Tyr-274 (19, 20).

Our DNA substrate consisted of a 32P-58-mer, which ended in CCCTTAT-3′ (the cleaved strand) plus a cold 5′ phosphorylated 94-mer top strand and an unlabeled 150-mer bottom strand (Fig. 1). In this DNA substrate, there is only a single high-affinity consensus top site (CCCTT). Topo cleavage is highly specific at this site; even single base substitutions in the consensus sequence reduce DNA cleavage quite drastically (21). To demonstrate the formation of a stable covalent frozen intermediate, we titrated a fixed amount of 32P-58-mer DNA “suicide” substrate with different amounts of topo I. The reactions were then stopped by adding 1% SDS, which denatured the topo. The samples were electrophoresed through 6% acrylamide-SDS gels. In the experiment, we used two variations of the suicide substrate. In the first type of substrate, the 32P-58-mer cleavable strand was annealed to the unlabeled 150-mer bottom strand (i.e., the 94-mer was not included; Fig. 1B without the 94-mer). This resulted in a partially duplex structure with the topo cleavable site in the ds region. In the second case, all the three oligos (32P-58-, 94-, and 150-mer; Fig. 1A), were annealed...
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**FIG. 2. Covalent complexation of topo I with DNA suicide substrate.** Autoradiograms of 6% acrylamide-SDS gels. Vaccinia topoisomerase I was purified from overexpressing E. coli cells (35). Various amounts of Topo I were mixed with 32P-labeled 58-mer DNA suicide substrate (1.3 pmol) in 17 μl of ice-cold 50 mM Tris-HCl (pH 7.5) and incubated at 37 °C for 10 min. The reaction was brought to 1% SDS and loaded on 6% acrylamide-SDS gels. The gels were dried and the bands were visualized by autoradiography with an X-ray film. Panel A was with DNA substrate without the 94-mer. Lane 1 is without topo. Lanes 2–6 are with DNA plus topo at molar ratios of 1:1, 1:2, 1:4, 1:10, and 1:20, respectively. Panel B: lanes 1–7 are with a fully ds substrate. Lane 1 is without topo. Lanes 2–6 are with DNA plus topo at molar ratios of 1:1, 1:2, 1:4, 1:10, and 1:20, respectively. Lanes 7 and 8 are DNA-topo (molar ratio at 1:2) complexes plus 5 μl of whole cell extract using either a fully ds substrate (lane 7) or a partially ds substrate (lane 8). Panel C: lane 1 contained ds DNA substrate without topo. Lanes 2 and 3 are with DNA and with extract plus topo (DNA:topo at a molar ratio at 1:2) with either a partially ds (lane 2) or a fully ds (lane 3) substrate, wherein the extract was first added to DNA and then followed by the topo. Lane 4 is fully ds DNA plus 5 μl of whole cell extract.

To create a fully ds substrate shown in Fig. 1B. In the first substrate, topo I cleaves off the 3’ overhanging terminal AT flank of the 32P-58-mer resulting in a frozen SDS-resistant 32P-56-mer-topo I conjugate (Fig. 2A). Because the 94-mer is absent, ligation is prevented and the complex remains frozen. In the second substrate, the frozen 32P-56-mer-topo I conjugate remains so in the presence of SDS because the 5’-end of the 94-mer was phosphorylated (Fig. 2B). The reason for testing both substrates is that the substrate without the 94-mer served as an “authentic control” suicide substrate because similar forms have often been used in topo binding assays (see, for example, Ref. 22). Thus, we can estimate the amount of frozen complex formed on the fully ds substrate relative to the authentic control. With the partially ds substrate a single complex representing the trapped topo-DNA conjugate was seen (Fig. 2A, lanes 2–6). Based on the relative phosphor counts in the free DNA versus cross-linked DNA, we estimated that on the average about 85–90% of the 32P-DNA was conjugated to the topo. A 2–4 fold molar excess of topo I was sufficient to produce the maximum amount of frozen conjugate. Similarly, when the fully ds substrate, which contained the cold phosphorylated 94-mer was used, up to 90% of the 32P-56-mer DNA was conjugated to the topo (Fig. 2B). The presence of excess topo did not change the amount of frozen complex formed. These experiments demonstrate that SDS-resistant stable topo-DNA complexes were consistently formed on the ds 150-mer suicide substrate. A small amount of free DNA was always seen whether the phosphorylated 94-mer was present or not. This may be simply because the conjugation reaction is never 100% efficient (just like almost all in vitro enzymatic reactions), or it may be because a small fraction of the DNA molecules were, for some reason, not “liked” by the topo I. We do not believe that the probable presence of a small fraction (10–15% or less) of free DNA in the reactions would affect the interpretation of our subsequent experimental results because a major fraction of the DNA was always covalently bound to the topo I. Any free topo is unlikely to have interacted with other sites on the DNA because we have not observed multiple complexes in SDS gels and the cleavage by topo is highly specific. Addition of Escherichia coli DNA polymerase Klonef fragment (plus all four cold dNTPs) to the frozen topo-DNA conjugates did not result in extension of the 5’ 32P-labeled 56-mer, indicating that the 3’-end of the 56-mer was inaccessible to Klonef because it is conjugated to the topo (not shown).

We have performed additional control experiments to assess the binding of topo I to a nonsuicide substrate (i.e., continuous top strand plus bottom strand or with a nonphosphorylated 94-mer) relative to the suicide substrate. In this nonsuicide substrate the topo I recognition site was the same as that shown in Fig. 1. Using our gel assay, we observed only a very faint band that represented an equilibrium complex on the nonsuicide DNA substrate. The amount of SDS-resistant topo-DNA complex that was formed on nonsuicide substrate was only about 10% compared with that formed on the suicide substrate.

When human cell-free extracts are added to topo-DNA conjugates, super-shifted bands were seen (Fig. 2B, lanes 7 and 8). These SDS-resistant complexes may represent higher-order complexes between topo-DNA conjugates and some factor(s) in the whole cell extracts. In the absence of the topo there were no SDS-resistant shifted bands with cell-free extracts alone (Fig. 2C, lane 4). Addition of extract, first, to the DNA and then the addition of topo dramatically reduced complex formation, suggesting that preformed topo-DNA complexes were required for the formation of higher-order complexes (Fig. 2C, lanes 2 and 3). These experiments demonstrated that human whole cell extracts contained factors that tightly bind frozen topo I-DNA conjugates.

**Breakage of DNA in the Protein-DNA Complex—**Initially, to assay the fate of frozen topo-DNA cross-links, we attempted to work with purified topo-DNA conjugates (i.e., without any free DNA), which were obtained either by excising the DNA bands representing the conjugates in native gels and subsequently electroeluting the complexes, or by purifying topo-DNA reactions through a high performance liquid chromatography gel-filtration column. With the former procedure, we could recover only about 5–10% of the topo-DNA conjugates even after prolonged (24 h) electroelution. Hence, this procedure was abandoned because it was extremely inefficient. The high performance liquid chromatography gel filtration procedure was somewhat more efficient for separating free DNA from topo-DNA conjugates (data not shown). However, with both procedures, the quality of the recovered topo-DNA conjugates, as assessed from the UV absorption spectra, was uncertain. The isolated topo-DNA conjugates did not show the expected dominant broad transition at λ_{max} of 260 nm, which is a signature of nondenatured native DNA-protein conjugates because the stronger nucleic acid base transitions (centered at 260 nm) dominate those from aromatic (e.g., Trp and Tyr) residues of the protein. We concluded that the isolation and purification of native topo-DNA conjugates was fraught with difficulties and that the isolated conjugates may not represent native states. Hence, we decided to carry out the following experiments with topo-DNA conjugates without further purification.

We incubated topo-DNA conjugates that were formed on the suicide substrate with human nuclear or whole cell-free extracts. The extracts were prepared by published procedures (see “Materials and Methods”). The biochemical competence of these extracts was ascertained by performing a transcription assay driven by the adenovirus major late promoter (assay was carried out in Prof. Robert Roeder’s laboratory at Rockefeller University). When increasing amounts of human nuclear extract were incubated with preformed topo I-DNA conjugates followed by digestion with proteinase K, a new band(s) migrating at ~88 nt was seen (Fig. 3B, lanes 3–8, arrow). This band suggested breakage of the topo-conjugated DNA substrate (see below for more details). In the presence of topo I without the extract (following treatment with proteinase K) or in the ab-
Treatment of topo I-DNA conjugates with nuclear extract. Autoradiogram of a 15% acrylamide-urea gel. In panels A and B, only the 58-mer in the substrate was $^{32}$P-5'-end labeled (asterisk). Panel A: lane 1, topo complexes were treated with proteinase K; lane 2, DNA substrate was treated with proteinase K; lanes 3–6, DNA substrate without topo treated with 0.5, 1, 2, 2.5, 3, and 5 $\mu$l of nuclear extract, respectively. Panel B: lane 1, topo-DNA complexes were treated with proteinase K; lane 2 contained DNA-topo complexes that were treated with 0.5 $\mu$l of a 10× dilution of extract; lanes 3–8 were DNA-topo complexes that were treated with 0.5, 1, 2, 2.5, 3, and 5 $\mu$l of nuclear extract, respectively. In panels C and D, the DNA substrate was 5'-end labeled on the 150-mer bottom strand. Panel C: lane 1, DNA treated with proteinase K; lane 2, topo-DNA complexes were treated with proteinase K; lanes 3–6, DNA substrate without topo that was treated with 0.5, 2, 3, and 5 $\mu$l of nuclear extract, respectively. Panel D: lane 1, DNA treated with proteinase K; lane 2, DNA-topo complexes were treated with proteinase K; lanes 3–6, DNA-topo complexes were treated with 0.5, 2, 3, and 5 $\mu$l of nuclear extract, respectively. The other lanes contained ss DNA markers.

Mapping the DNA breakpoints—To define the sites of the DNA breakage, we $^{32}$P-labeled the ds suicide substrate on either the 94-mer or the 150-mer (Fig. 1). Our attempts to assay for cleavage of the 5'-end labeled 150-mer bottom strand were frustrated because the nuclear and/or the whole cell extract contained a strong phosphatase that for unknown reasons specifically dephosphorylated the 5'-32P-end of the bottom strand (Fig. 3, C and D in Fig. 3). To circumvent this problem, we $^{32}$P-labeled the 3'-end of the 150-mer bottom strand using [$\alpha$-32P]dideoxy-ATP and terminal deoxynucleotidyl transferase. As shown in Fig. 5A, the cleavage of the bottom strand generated a cluster of bands that were predominantly centered at ~62 nt (Figs. 5A, 6H 150B-C, and 5D). This indicated that the bottom strand, which is complementary to the topo-conjugated strand (i.e. the scissile top strand), is also cleaved (Fig. 5D). (Note that the topo I itself does not cleave the bottom strand.) When the 94-mer was $^{32}$P-labeled, fragments measuring ~65 nt from the 3'-end (Figs. 5B and 6, 94-C) or ~32 nt from the 5'-end were seen (Fig. 5C). The ~65 nt and the ~32 nt fragments represented cleavage of the suicide substrate 3' to the topo site (Fig. 5D). When cell-free extract was added to a mixture of non-suicide DNA plus topo I, we did not observe specific cuts in the DNA (not shown). This experiment indicated that the presence of frozen topo-DNA conjugates was necessary for DNA cleavage.

To map the fragmentation pattern in a better way and to rule out the possibility that very short peptides (end-products of proteinase K treatment) may be still attached to the DNAs (thus complicating the assignment of the cleavage sites), we isolated the cleaved products from the denaturing gels, such as those in Figs. 4 and 5. The isolated cleaved DNA fragments
were extracted with phenol:chloroform, precipitated with EtOH, and then subjected to Maxam-Gilbert G-reaction with dimethyl sulfate followed by hot piperidine or with hot piperidine alone (23). We reasoned that this harsh procedure (hot alkali, etc.) will hydrolyze any peptide-DNA bonds but will leave most unmodified naked DNA molecules intact. Fig. 6 shows a collection of isolated fragments after such a treatment. Surprisingly, the 32P-88-mer (shown by the arrow in Fig. 4), after gel isolation and Maxam-Gilbert G-reaction, migrated at 62 nt in a high resolution denaturing gel (Fig. 6I, lane 2). There is indeed a faint 88-mer band in Fig. 6I, lane 2 representing the residual nonhydrolyzed DNA-peptide conjugate. All reactions were finally treated with proteinase K. Panel D, schematic illustration of approximate positions of the cleavage sites. The 5’ and 3’ 32P-labeled ends are denoted by the asterisk.

This 62-nt fragment perfectly matched the sequence of the full-length 150-mer top strand (which was purified from gels such as that represented by the triangle in Fig. 4) starting with the 62nd-nt breakpoint and counting from the 5’-end (compare Fig. 6I, lanes 1 and 2). The alternative explanation that peptide fragments from topo that were still covalently conjugated to the 32P-56-mer resulted in its anomalous migration to 62-mer (in Fig. 6I, lane 2) is unlikely because cleavage of the tyr-phosphodiester bond in hot alkali will release a 56-mer but not a 62-mer (see Ref. 22 for a discussion of the stability of the tyr-phosphodiester). These results indicated that at least in a fraction of the topo-DNA conjugated suicide substrate molecules, there was a cleavage of the DNA 5’ to the topo attachment site. Because such a cleavage resulted in ~62 nt with sequences from the 94-mer, there might have been a ligation event with the 94-mer (see below for more details). Our extracts did possess ligase activity (and the reactions contained added ATP) because the top strand 32P-150-mer was also produced in the absence of the topo (Fig. 3, A and B and Fig. 6I, lane 1). To find out whether ligase itself was sufficient to generate the...
150-mer top strand in the topo-DNA complexes, we incubated topo-DNA conjugates that were formed on the suicide DNA substrate with T4 DNA ligase alone (plus ATP) and/or supplemented with extracts (not shown). In the presence of T4 DNA ligase alone, we did not observe the formation of the 150-mer top strand. However, when the reaction was supplemented with whole cell extracts, we observed the 150-mer and DNA breakage. This suggested that ligase by itself was insufficient for the formation of the 150-mer top strand from the topo-DNA conjugates and that, at least in some topo-DNA conjugates, the extract was needed to first remove the topo I before ligation occurred (see below for more evidence).

The 94-mer and 150-mer-derived cleaved fragments (Figs. 5, A–C and 6I) appeared as multiple bands indicating a clustered cleavage pattern (Fig. 5D). The cleavages at ~32 nt from the 5′-end of the 94-mer (downstream to the topo-conjugated site; Fig. 5, C and D) appear to be single-stranded nicks because we did not observe breakage at the corresponding place on the 150-mer bottom strand. We isolated and performed G-reactions on the cleaved 3′-end labeled 94-mer and the bottom 150-mer-derived fragments (Fig. 6, III and IV). The sequence and the positions of the breakpoints are consistent with the fact that cleaved fragments originated from 3′-end labeled top strand 94-mer and the bottom strand 150-mer (Fig. 6, III and IV).

The composite DNA breakpoints are shown in Fig. 5D. Note that the approximate sizes of the fragments are given, consistent with the clustered appearance of the cleaved bands and the decreased resolution toward the top of the gels (± 3–5 nt). In summary, it appears that the major breakage in the topo-DNA conjugate was a cluster of ds breakpoints immediately 3′ to the topo-conjugation site. In addition, ss nicks downstream of the ds breakpoints were seen on the 3′ side of the topo-conjugated site. These breakpoints are not due to footprinting by unbound topo of some other proteins from the extracts that may be bound to the DNA substrate because the topo is very specific to the unique consensus sequence at the end of the 56-mer topo strand.

A DNA Ligation Event Is Carried Out by the Extracts—Earlier, we suggested that the 150-mer top strand was formed by ligation of the 56-mer to the 94-mer in the presence of the extract and topo (Figs. 3B and 4B). To prove that this is indeed the case, we examined isolated complexes from native gels (Fig. 7). In a reaction containing topo-DNA conjugates plus extract, without the addition of SDS, the complexes did not migrate very far into the native gel (Fig. 7, I and II). When SDS was added to the reaction mixture before deposition into the gel wells, the complexes migrated into the gel (Fig. 7, III and IV). Prolonged incubation of the topo-DNA complexes with extract resulted in appearance of smearable bands (which were retarded compared with the free 150-base pair substrate) representing various species of DNA-protein complexes (Fig. 7IV, lanes 1–4). To test the possibility that these protein-bound DNA molecules could contain ligation products, we isolated the DNA from the complexes (such as those indicated by brackets A–C in Fig. 7IV,
Fig. 7. Panels I–IV, native gel electrophoresis of topo-DNA-extract complexes. The figure shows autoradiograms of 5% acrylamide nondenaturing gels that were made and run with TBE buffer. Panel I, the suicide DNA substrate was $^{32}$P labeled at the 5'-end of the 58-mer. Lane 1, suicide DNA alone; lane 2, suicide DNA + topo; lane 3, suicide DNA + topo + 3 μl of whole cell extract; lane 4, suicide DNA + 3 μl of whole cell extract. All reactions were incubated at 30 °C for 2 h. Panel II, the suicide DNA substrate was $^{32}$P labeled at the 5'-end of the 94-mer. Lane 1, suicide DNA alone; lane 2, suicide DNA + topo; lane 3, suicide DNA + topo + 3 μl of whole cell extract; lane 4, suicide DNA + 3 μl of whole cell extract. All reactions were incubated at 30 °C for 2 h. In panels I and II, the samples did not receive SDS after the incubation time. Panel III, the suicide DNA substrate was $^{32}$P labeled at the 5'-end of the 94-mer. Lane 1, suicide DNA alone; lane 2, suicide DNA + topo; lane 3, suicide DNA + topo + 3 μl of whole cell extract; lane 4, suicide DNA + 3 μl of whole cell extract. All reactions were incubated at 30 °C for 15 min. Panel IV: lane 1, suicide DNA $^{32}$P labeled at the 5'-end of the 58-mer + topo + 3 μl of whole cell extract were incubated for 2 h; lane 2, suicide DNA $^{32}$P labeled at the 5'-end of the 94-mer + topo + 3 μl of whole cell extract were incubated for 4 h; lane 3, suicide DNA $^{32}$P labeled at the 5'-end of the 94-mer + topo + 3 μl of whole cell extract were incubated for 2 h; lane 4, suicide DNA $^{32}$P labeled at the 5'-end of the 94-mer + 3 μl of whole cell extract were incubated for 2 h. All incubations were done at 30 °C. Brackets A–C indicate examples of regions in the gels that were excised to extract the DNAs after in situ proteinase digestion (see “Materials and Methods”). Panels V and VI, autoradiograms of 15% acrylamide-8 M urea gel showing the DNA that was isolated from complexes that were previously run on native gels (see “Materials and Methods”). Panel V: lanes 1–3 contained suicide DNA substrate that was $^{32}$P labeled on the 5'-end of the 58-mer. Lane 1, DNA that was extracted from complexes from reactions containing topo + whole cell extract (as in A, panel IV); lane 2, DNA that was extracted from complexes from the reactions containing whole cell extract; lane 3, DNA that was extracted from complexes in the reactions containing topo only. Panel VI: lanes 1–3 contained suicide DNA substrate that was $^{32}$P labeled on the 5'-end of the 94-mer. Lane 1, DNA that was extracted from complexes in the reactions containing topo + whole cell extract (as in B, panel IV); lane 2, DNA that was extracted from complexes in the reactions containing whole cell extract (as in C, panel IV); lane 3, DNA that was extracted from complexes in the reactions containing topo only.

After the delinking and dephosphorylation of the 3'-topo linkage, consistent with previous results (Fig. 4) some amount of the 150-mer top strand was formed in the absence of topo with the extract (Fig. 7, VI, and V, lane 2).

In discussing Fig. 5D, we implied that ds cuts occurred in the vicinity of the topo-conjugated site. Double-stranded fragments equivalent to 60–65 base pairs representing these cuts (migrating, as expected, with bromphenol blue dye) were seen in native gels (Fig. 7IV, lanes 1–3). Fragments from reactions involving topo or with the cell extract alone were of a different length. These results indicated that 60–65-base pair ds cuts are made in reactions containing DNA-topo conjugates treated with cell extracts. The larger DNA molecules running just above the 60–65-base pair fragments (Fig. 7IV, lanes 1–3) may represent fragments with bound proteins and/or other cleaved fragments.

XPA Protein Is Required for the Major DNA Breakage 3' to the topo Site—The XPA mutant cells are drastically reduced in the repair of UV-induced bulky adducts (24). The XPA protein is essential for the nucleotide excision repair pathway (and perhaps for other repair pathways as well) (24). XPA is a damage recognition protein, and interacts with several other proteins in the nucleotide excision repair pathway (24–26). We wanted to test whether XPA protein played a role in DNA...


Fig. 8. Topo-DNA complexes were treated with whole cell XPA extracts. Note that only the 58-mer was 32P labeled in this assay (asterisk). Panel A: lane 1 is DNA that was treated with proteinase K; lanes 2–6 are DNA substrates without topo that were treated with 1, 2, 3, 5, and 10 μl of extract, respectively. Panel B: lane 1 is DNA-topo complexes that were treated with proteinase K; lanes 2–6 are DNA-topo reactions that were treated with 1, 2, 3, 5, and 10 μl of extract, respectively. The other lanes contained ss DNA markers. Panel C: lane 1, contained DNA-topo complexes that were treated with 1 μl of XPA whole cell extract, whereas lane 2 contained 0.5 μl of XPA whole cell extract that was mixed with 0.5 μl of wild type whole cell extract.

CLEAVAGE ACTIVITIES REPORTED HERE. Fig. 8B shows that XPA mutant whole cell extracts cannot produce the 32P-88-mer band (which is actually 62 nt, as illustrated by isolation and sequencing; Fig. 6f, lane 2). Mixing XPA whole cell extract with wild type whole cell extract restored the 32P-88-mer band (Fig. 8C, lane 2, arrow), indicating that at least for the top strand breakage step near the topo, the XPA damage recognition protein may be required. However, the top strand 150-mer (Fig. 8, triangle) is produced with XPA extracts because, as shown above, it was most probably generated by a ligation step.

DISCUSSION

This work provides evidence for mechanisms that process topo-DNA cross-links. Some of the results may have relevance to the processing of other types of protein-DNA cross-links as well. We report that the DNA in a frozen topo-DNA conjugate was broken at specific sites by nuclease activity in human cell extracts, and there appears to be a mechanism that delinks the topo-DNA cross-link, perhaps by first removing the frozen topo (or proteolyzed parts thereof) and then restoring the continuity of the broken DNA strand by ligation. Our observations are admittedly at the phenomenological level ("proof-of-principle"), and for that reason, we cannot yet propose a repair pathway. Nonetheless, because the ds breakpoints are located immediately 3' to topo-DNA conjugation site, there is an intriguing possibility that these ds breakpoints may represent steps in the processing of these conjugates. The dependence of the DNA breakage on XPA, which is a damage recognition protein (26), suggests that a frozen topo-DNA conjugate (or parts thereof) may be perceived by the cell as a potential DNA damage problem. We cannot yet mechanistically or temporally relate the ds break points and the topo delinking-ligation activity. The production of the 150-mer top strand occurred via a pathway that may first involve the delinking of the DNA conjugated topo. While the early versions of this work were being written-up for publication, a report appeared describing a DNA phosphodiesterase that is specific for phospho-tyrosyl bonds (27). These workers suggested that the phosphodiesterase would remove a frozen DNA-topo. In our case, this event may be followed by a phosphatase that converted the erstwhile 3'-end to an OH, which is then ligated to the phosphorylated 94-mer. The temporal relationship, if any, between the ligation event that generated the 150-mer and the cleavages 3' to the topo is unknown. The breakage of the DNA in the frozen DNA-topo conjugate may be an alternate pathway for the processing of frozen topo conjugates, in addition to the topo-delinking-ligation pathway. It is possible that the ds breaks may signify a breakage and rejoicing step that may be akin to recombination repair.

We chose the topo-DNA complex as a model substrate for our assay because it is a very well-characterized example of a protein-DNA cross-link and because the chemical nature of the covalent linkage is well established (see "Introduction"). Moreover, it is a naturally occurring cross-link. Frozen topo-DNA conjugates may be formed in patients undergoing chemotherapy with anticancer topoisomerase poisons, such as camptothecins (6). The topo-I DNA covalent bond is a 3'-phosphodiester linkage. Topo II DNA complexes are 5' phosphoryl-linked (6). UV and ionizing radiations also generate protein-DNA cross-links (26). Either bonds or C-C bonds involving Tyr and the ring carbons of thymine or cytosine are induced by far and near UV or ionizing radiation or by photosensitzers, such as psoralen plus near UV (2, 28–30). Other covalent linkages, such as Schiff's bases, may also occur. If protein-DNA cross-links occur frequently, as we believe they do, enzymatic pathways for their removal must be ubiquitous. Accepting the principle of cellular parsimony, it is hard to imagine that there is a distinct enzyme that can catalyze the reversal of each type of covalent bond (akin to the tyrosylphosphodiesterase for topo I (27)) in the wide variety of protein-DNA cross-links. Therefore, we believe that the breakage of DNA that we have demonstrated here may suggest a pathway that is capable of handling a wide spectrum of protein-DNA cross-links. In this context, it is worth noting that nucleotide excision repair can repair a wide spectrum of DNA bulky adducts (24, 26). Some enzymes from the nucleotide excision repair, base excision or mismatch, or recombinational repair pathways may also participate in DNA-protein cross-link repair.

Lastly, previous reports regarding protein-DNA cross-link repair are difficult to relate to our work because those assays were carried out with undefined substrates (31, 32). In conclusion, this work represents experimental evidence toward an understanding of the mechanisms that process topo-DNA cross-links and perhaps other DNA-protein cross-links.

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