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Disruption of a topoisomerase–DNA cleavage complex by a DNA helicase

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ABSTRACT **The type II DNA topoisomerases are targets for a variety of chemotherapeutic agents, including the antibacterial quinolones and several families of antitumor drugs. These agents stabilize an enzyme–DNA cleavage complex that consists of the topoisomerase covalently linked to the 5′ phosphates of a double-stranded DNA break. Although the drug-stabilized cleavage complex is readily reversible, it can result in cell death by a mechanism that remains uncertain. Here we demonstrate that the action of a DNA helicase can convert the cleavage complex into a nonreversible DNA break by displacing DNA strands from the complex. Formation of a nonreversible DNA break, induced by a DNA helicase, could explain the cytotoxicity of these topoisomerase poisons.**

Topoisomerases play a major role in cellular DNA metabolism by altering the topological state of DNA. Type II DNA topoisomerases effect this change by creating a transient double-stranded DNA (dsDNA) break through which a second DNA segment is passed (1–3). Several classes of chemotherapeutic drugs, including the acridines, anthracyclines, ellipticines, and epipodophyllotoxins, inhibit the type II topoisomerases (4, 5). These drugs stabilize a protein–DNA complex, called the cleavage complex, in which the enzyme is covalently linked to each 5′ phosphate of a staggered dsDNA break. Although both strands of the DNA helix are thought to be broken, the linear integrity of the DNA is maintained by a noncovalent topoisomerase bridge. The simplest model is that the inhibitors trap the cleavage complex by inhibiting the ligation step of the enzymatic cycle.

The type II topoisomerases are a primary cellular target for many of the chemotherapeutic drugs described above. Studies on drug-resistant mammalian cell lines revealed alterations in topoisomerase levels or activity, suggesting that the enzyme is an important component of the cytotoxic response (6, 7). Genetic studies in *Saccharomyces cerevisiae* have demonstrated that a temperature-sensitive mutation in the type II topoisomerase gene greatly decreased drug sensitivity at the semipermissive temperature, presumably due to a reduction in topoisomerase activity (8). Perhaps the clearest evidence for a type II topoisomerase being the primary drug target comes from the T4 bacteriophage system. The properties of the T4 topoisomerase closely resemble those of the mammalian enzyme (1, 9). Most importantly, both are sensitive to the aminoacridine *m*-AMSA [4′-(9-acridinylamino)methanesulfon-*m*-anisidide] (10, 11). A point mutation in one of the genes encoding the bacteriophage T4 topoisomerase allows drug-resistant phage growth, and topoisomerase purified from the mutant phage has drug-resistant topoisomerase activity (12, 13).

Topoisomerase inhibitors convert the enzymes that they target into poisons by inducing formation of the cleavage complex, a form of DNA damage. This view was first articulated when it was found that bacteriophage T7 could be inhibited by the quinolone nalidixic acid, even though phage growth was not significantly reduced by mutational inactivation of DNA gyrase (14). Importantly, mutational inactivation of DNA gyrase protected T7 from inhibition by the drug (14). More recent results imply that the cleavage complex is critical for the cytotoxicity of antitumor agents that inhibit topoisomerases. For example, defects in DNA repair have been shown to increase drug sensitivity (refs. 15–17; S.H.N., K. Carles-Kinch, and K.N.K., unpublished data), indicating that the topoisomerase inhibitors induce DNA damage. In spite of the importance of the cleavage complex in cytotoxicity, the complexes are reversible both *in vitro* and *in vivo*. To explain this apparent paradox, it has been proposed that an active mechanism, such as transcription or replication, converts reversible cleavage complexes into nonreversible cytotoxic DNA lesions (ref. 18; also see *Discussion*).

We investigated whether helicase action could convert a type II topoisomerase cleavage complex into a nonreversible DNA break by examining the DNA products generated when *Escherichia coli* helicase II unwinds a DNA substrate containing an *m*-AMSA-induced T4 topoisomerase cleavage complex. These two enzymes were selected for the following reasons: (*i*) The bacteriophage T4 topoisomerase closely resembles mammalian type II topoisomerases in reaction mechanism and sensitivity to inhibitors (see above). (*ii*) A strong *m*-AMSA-inducible T4 topoisomerase cleavage site has been cloned near the middle of a 703-bp DNA fragment (G. Hong and K.N.K., unpublished data), facilitating analysis of cleavage complexes occurring at a single site. The sequence of this cleavage site was based on mutational analyses of a T4 topoisomerase cleavage site and on experiments with *in vitro* substrates containing optimal sequences for DNA cleavage (ref. 19 and C. Freudenreich and K.N.K., unpublished data). (*iii*) Several topoisomerase inhibitors have been shown to block helicase activity *in vitro* (20–23); however, *E. coli* helicase II was found to be insensitive to *m*-AMSA (20). (*iv*) Unlike most other DNA helicases, *E. coli* helicase II does not require a single-stranded DNA (ssDNA) loading site to initiate DNA strand separation (24). This allows the use of fully duplex linear DNA substrates, which is important because the T4 topoisomerase interacts strongly with ssDNA and ssDNA–dsDNA junctions (25).

MATERIALS AND METHODS

DNA Substrate. The DNA substrate was prepared from a pBR322-derived plasmid, pGH2, containing a 28-bp insert between the unique *Bam*HI and *Sal* I restriction sites. The insert was designed based on the studies of Freudenreich and Kreuzer (19) to produce a very efficient *m*-AMSA-inducible

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Abbreviations: dsDNA, double-stranded DNA; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide); ssDNA, single-stranded DNA; ATP[γS], adenosine 5′-[γ-thio]triphosphate.

topoisomerase cleavage site (insert sequence is 5′-AAGCTAAAGTTATATAACTTTATTCAAG-3′). The 703-bp fragment was generated by cleaving pGH2 DNA with *Hin*dIII and *Nru* I, filling in the recessed 3′ end with the Klenow fragment of DNA polymerase I (Boehringer Mannheim), treating with calf intestinal phosphatase and proteinase K, and purifying by electrophoresis through a nondenaturing 6% polyacrylamide gel. The DNA fragment was electroeluted, ethanol-precipitated, and stored at −70°C. The substrate was labeled at the 5′ ends using T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham). Labeling was followed by a 5-min chase at 37°C with 50 μM (unlabeled) ATP to provide completely phosphorylated 5′ ends. Restriction enzymes, calf intestinal phosphatase, T4 polynucleotide kinase, and proteinase K were from New England Biolabs.

T4 Topoisomerase and Helicase II Reactions. Except where indicated, topoisomerase cleavage reaction mixtures (40 μl) contained 40 mM Tris·OAc (pH 7.8), 20 mM KOAc, 8 mM $Mg(OAc)_2$, 0.5 mM EDTA, 2.5 mM. ATP, 0.5 mM dithiothreitol, bovine serum albumin (35 μg/ml), 5% (vol/vol) glycerol, 12.5 μM *m*-AMSA (NSC 249992, provided by the National Cancer Institute), ≈1 fmol of 5′-^{32}P-end-labeled 703-bp DNA fragment (see below), and purified T4 type II topoisomerase (9, 13) at a molar ratio of ≈20 topoisomerase dimers per duplex DNA fragment. Controls lacking *m*-AMSA received 0.02% (final concentration) dimethyl sulfoxide (*m*-AMSA solvent) and those lacking enzyme received topoisomerase dilution buffer (9). Incubation was for 5 min at 32°C.

Helicase II reactions were initiated by adding 2.8 pmol (1 μl) of purified *E. coli* helicase II (26) to the cleavage complexes above; helicase storage buffer was added to samples that received no helicase. Incubation was then continued for 5 min at 32°C. After this incubation, either EDTA (50 mM, final concentration) or SDS (1%, final concentration) was added and incubation was continued for 20 min at 32°C. All samples were then adjusted to contain 50 mM EDTA and 1% SDS and were treated with proteinase K (380 μg/ml) for 20 min at 37°C.

Gel Electrophoresis. Reaction products were resolved by electrophoresis through polyacrylamide gels and visualized by autoradiography. Denaturing gels contained 7 M urea and 6% polyacrylamide [acrylamide/*N*,*N*′-methylenebisacrylamide = 20:1 (wt/wt)], with 1× TBE (89 mM Tris/89 mM borate/2 mM EDTA) as running buffer; electrophoresis was at 21–23 V/cm for ≈4 hr. Samples for the denaturing gels consisted of 20-μl aliquots that were mixed with 15 μl of loading solution [85% (vol/vol) formamide/1× TBE/0.1% bromophenol blue/0.1% xylene cyanole] and heated for 5 min at 100°C immediately before loading. Nondenaturing gels contained 5% polyacrylamide, 0.1% SDS, and 25% glycerol, with 1× TBE containing 0.1% SDS as running buffer; electrophoresis was at 4.4 V/cm for ≈21 hr. Samples for the nondenaturing gels consisted of 20-μl aliquots that were mixed with 15 μl of loading solution (40% glycerol/50 mM EDTA/0.5% SDS/0.02% bromophenol blue/0.02% xylene cyanole). DNA markers (BioVentures, Murpheesboro, TN) were labeled by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP.

RESULTS

DNA Helicase II Prevents Reversal of *m*-AMSA-Induced T4 Topoisomerase Cleavage Complexes. Cleavage complexes were established by incubating the T4 type II topoisomerase with *m*-AMSA and the 703-bp 5′-end-labeled DNA substrate. The DNA substrate contains a strong topoisomerase II cleavage site located 337 bp from one end. The amount of T4 topoisomerase cleavage complex was determined by stopping the reactions with SDS, incubating with proteinase K, and analyzing the products on polyacrylamide gels. Labeling the DNA substrate at the 5′ ends confines analysis to the cleavage products that were not covalently linked to the topoisomerase. An autoradiograph of a denaturing polyacrylamide gel revealed the two major products of cleavage at the strong topoisomerase site along with bands generated from minor topoisomerase cleavage sites (Fig. 1*A*, lanes 1 and 2).

To determine the impact of helicase activity on an *m*-AMSA-induced cleavage complex, we used a cleavage reversal assay in which the samples were exposed to EDTA after the enzymatic reaction but prior to treatment with SDS and proteinase K. This treatment causes reversal of cleavage complexes formed by eukaryotic (27, 28), T4 (29), and *E. coli* (30) type II topoisomerases. The mechanism of reversal is unknown; however, the simplest explanation is that chelation of magnesium prevents topoisomerase from initiating a new round of cleavage but does not prevent DNA religation. When EDTA was added to the topoisomerase reactions without helicase, about 80% of the cleavage complexes at both the major and minor sites were reversed (Fig. 1*A*, lanes 2 and 3). However, EDTA reversal was almost completely blocked in reactions that contained helicase (Fig. 1*A*, lanes 4 and 5). The presence of the cleavage products was dependent on *m*-AMSA (Fig. 1*A*, lanes 7 and 8) and topoisomerase (Fig. 1*A*, lanes 9 and 10). Thus, reversible *m*-AMSA-induced type II topoisomerase cleavage complexes were converted to nonreversible DNA breaks by helicase II.

Aliquots of the same reactions were analyzed on a nondenaturing polyacrylamide gel to examine further the nature of the cleavage products and determine the extent of helicase unwinding. The addition of helicase to topoisomerase reactions resulted in the appearance of several new bands (Fig. 1*B*, lanes 2 and 4). These bands comigrated with bands produced when the helicase-free topoisomerase cleavage reactions were subjected to heat denaturation prior to electrophoresis (Fig. 1*B*, lane 6; note that one of the major ssDNA cleavage products comigrates with one of the major dsDNA cleavage products). Therefore, helicase II can unwind DNA cleavage complexes to yield ssDNA cleavage products. Furthermore, the ssDNA cleavage products generated by topoisomerase plus helicase are not reversible by EDTA treatment (Fig. 1*B*, lanes 4 and 5). Helicase II also unwound the topoisomerase-free substrate DNA to generate full-length ssDNA molecules that migrated slower than the full-length duplex DNA (Fig. 1*B*, lanes 4, 5, and 7–10). Again, the presence of cleavage products was strictly dependent on *m*-AMSA (Fig. 1*B*, lanes 7 and 8) and topoisomerase (Fig. 1*B*, lanes 9 and 10). We conclude from this analysis that, coincident with converting reversible cleavage complexes to nonreversible DNA breaks, helicase II unwinds DNA constrained within the cleavage complex to produce ssDNA cleavage products.

T4 topoisomerase can cleave ssDNA and form a covalent protein–DNA complex that is resistant to reversal by EDTA treatment (25). Therefore, an alternative interpretation seemed possible: perhaps the nonreversible DNA breaks analyzed in this study were due to formation of cleavage complexes on the ssDNA product of the helicase II unwinding reaction. To test this possibility, topoisomerase-induced cleavage products were analyzed using the 703-bp DNA substrate that had been heat-denatured immediately prior to the topoisomerase reaction. Although cleavage sites were detected on the ssDNA, the products did not comigrate with the nonreversible topoisomerase cleavage products generated from dsDNA in the presence of helicase (Fig. 1*C*, lanes 5–7). In addition, the binding of helicase II to the heat-denatured substrate inhibited the ssDNA cleavage by the T4 topoisomerase (Fig. 1*C*, lanes 7 and 9). Therefore, the nonreversible cleavage complexes were not formed by the

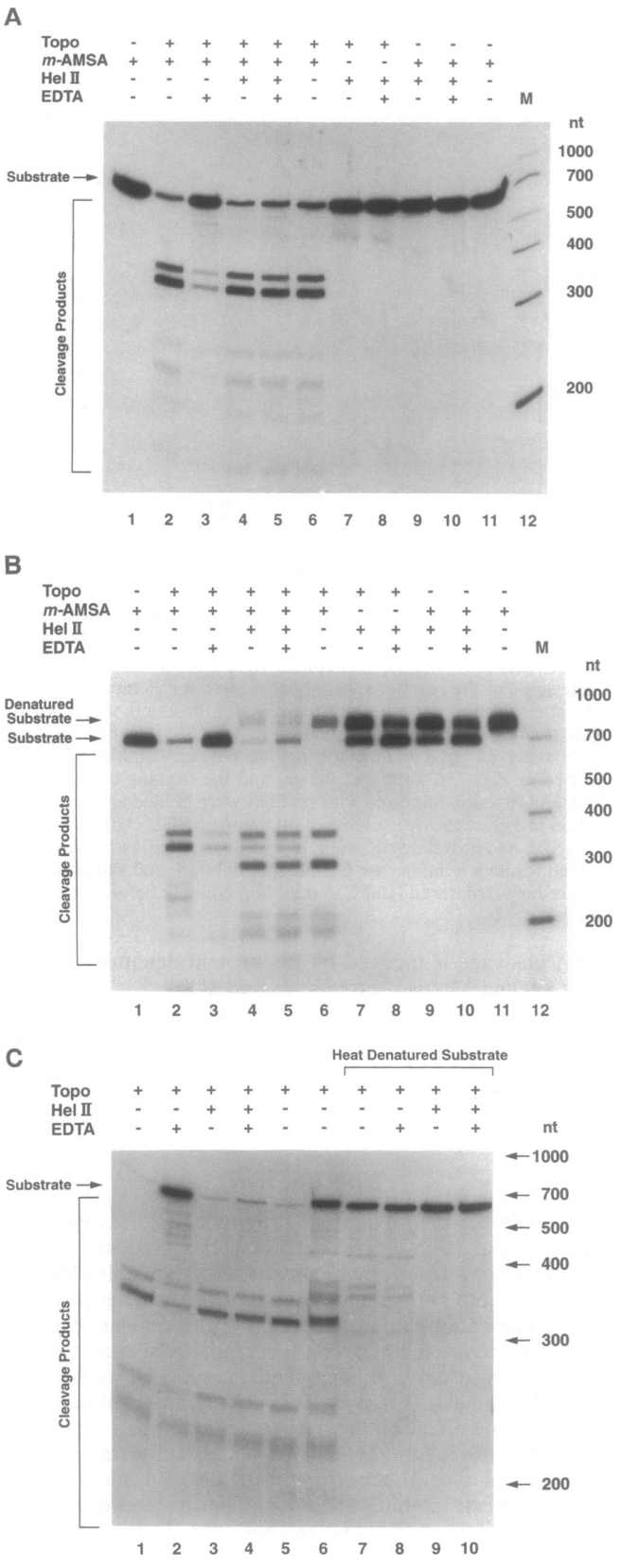

FIG. 1. Effect of *E. coli* helicase II on the T4 topoisomerase cleavage reaction. In the first stage of the reactions, the 5′-^{32}P-end-labeled DNA substrate was incubated for 5 min at 32°C with (+) or without (−) T4 type II topoisomerase (Topo) and *m*-AMSA. The 703-bp substrate contains a strong *m*-AMSA-inducible cleavage site 337 bp from one end. In the second stage of the reactions, *E. coli* helicase II (Hel II) was added to some reactions (+), but not others (−), and incubation was continued for 5 min at 32°C. In the third stage, either EDTA (+) or SDS (−) was added, and incubation was continued for 20 min at 32°C. After the third stage, all samples were adjusted to contain the same final concentrations of EDTA and SDS, respectively. Samples were then treated with proteinase K and subjected to electrophoresis on a denaturing 6% polyacrylamide/7 M urea gel (*A*) and on a nondenaturing 5% polyacrylamide gel (*B*). All samples in *A* and samples 6 and 11 in *B* were heat-denatured (100°C, 5 min) immediately before loading on the gel. In *C*, the DNA substrate in samples 7–10 was heat-denatured immediately prior to the topoisomerase reaction and samples were subjected to electrophoresis on a denaturing gel as in *A*. Sample 6 in *C* is composed of a mixture of samples identical to samples 5 and 7. Samples 9 and 10 in *C* had helicase added in the first stage of the reactions and topoisomerase added in the second stage. DNA markers (M), with sizes in nucleotides, are in lanes 12 of *A* and *B*; the sizes and positions of markers are indicated on the right in *C*.

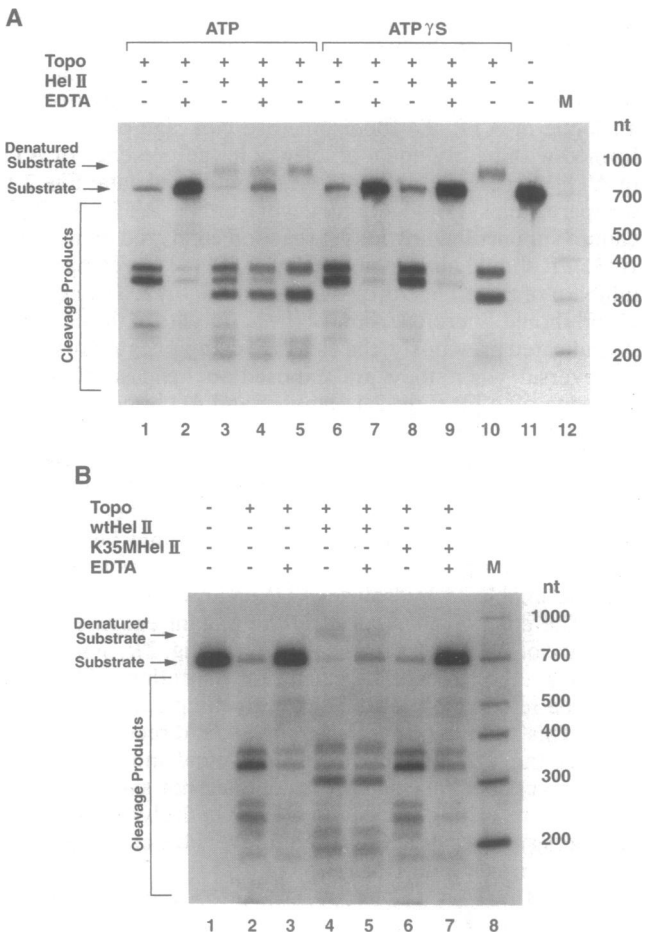

FIG. 2. Requirement for ATP hydrolysis and DNA unwinding by helicase II. (*A*) The three stages of the reactions were the same as those in Fig. 1 except that reaction mixtures 6–10 contained ATP[γS] instead of ATP. All reaction mixtures contained *m*-AMSA. Reaction products were analyzed on a nondenaturing 5% polyacrylamide/0.1% SDS gel as described in Fig. 1. Samples 5 and 10 were heat-denatured (100°C, 5 min) immediately before loading on the gel. (*B*) The three stages of the reactions were the same as those in Fig. 1 except that some samples were incubated with wild-type helicase II (wtHel II; lanes 4 and 5) and others with the K35M mutant helicase II (K35MHel II; lanes 6 and 7; same concentration as the wtHel II). All reactions contained *m*-AMSA. Reaction products were analyzed on a nondenaturing 5% polyacrylamide/0.1% SDS gel as described in Fig. 1.

action of topoisomerase on the ssDNA products of helicase II unwinding.

The Effect of DNA Helicase II on *m*-AMSA-Induced Cleavage Complexes Is Dependent on ATP Hydrolysis. We next investigated whether the generation of a nonreversible DNA

break by helicase II was dependent on the unwinding activity of the enzyme. In the first approach, topoisomerase cleavage reactions were performed in the presence of ATP or adenosine 5′-[γ-thio]triphosphate (ATP[γS]), a poorly hydrolyzed analogue of ATP. T4 topoisomerase cleavage products are formed with nearly equal efficiency in the presence of ATP or ATP[γS] (S.H.N. and K.N.K., unpublished data; Fig. 2*A*, compare lanes 1 and 6); however, unlike ATP, ATP[γS] cannot support the unwinding reaction catalyzed by helicase II (31). It should also be noted that ATP[γS] is a competitive inhibitor of the ATP hydrolysis reaction catalyzed by helicase II and should, therefore, bind at the active site of the enzyme. As indicated previously, the cleavage complexes are resistant to reversal when they are exposed to helicase II in the presence of ATP (Fig. 2*A*, lanes 3 and 4). In contrast, the reactions that were exposed to helicase II in the presence of ATP[γS] were readily reversible (Fig. 2*A*, lanes 8 and 9), indicating that the impact of helicase II on the cleavage complex is dependent on ATP hydrolysis by helicase II. In a second approach, we tested the K35M mutant helicase II, which contains a single amino acid substitution rendering the enzyme unable to hydrolyze ATP or unwind duplex DNA (32). The complexes exposed to the mutant enzyme, in the presence of ATP, were readily reversed (Fig. 2*B*, lanes 6 and 7). Finally, the effect of increasing concentrations of helicase II on the reversibility of the cleavage complexes was examined. This analysis revealed roughly a direct correlation between the total amount of DNA unwound and the quantity of nonreversible cleavage products (data not shown). Thus, these results strongly argue that ATP hydrolysis and DNA unwinding are necessary to convert reversible cleavage products to nonreversible DNA breaks.

DNA Helicase II Can Disrupt an *m*-AMSA-Induced Cleavage Complex in the Absence of Protein Denaturation. While DNA within the cleavage complex is thought to be cleaved, the continuity of the DNA duplex is maintained by a noncovalent topoisomerase bridge. The DNA break is generally observed only when the cleavage complex is treated with a protein denaturant (e.g., SDS) that disrupts the topoisomerase bridge (2, 3). In all of the above experiments, the products were treated with SDS and proteinase K. One could argue that unwinding the DNA around the cleavage complex alters the complex without disrupting it, so that it cannot be reversed. In this model, cleaved DNA products are not released until treatment with SDS and proteinase K. However, if helicase II actually disrupts the cleavage complex, then DNA cleavage products should be detectable without protein denaturation. To distinguish between these alternatives, we analyzed reaction products directly on a nondenaturing gel without SDS or proteinase K treatment (Fig. 3, lanes 1–6). Indeed, cleavage products were observed in a reaction that was strictly dependent on helicase II (Fig. 3, lane 3). These cleavage products were further analyzed by including a mild heat treatment (65°C) prior to electrophoresis, which releases helicase II from the ssDNA (M.T.H. and S.W.M., unpublished data). Because the DNA substrate is labeled at the 5′ ends, the cleavage products that are covalently linked to T4 topoisomerase are not detected in this analysis. After being released from helicase II, the cleavage products observed in the absence of SDS and proteinase K (Fig. 3, lane 4) comigrated with topoisomerase cleavage products that had been treated with SDS and proteinase K and then heat-denatured at 100°C (Fig. 3, lane 7). We conclude that helicase II can displace ssDNA from the cleavage complex in a reaction that is independent of protein denaturation or heat treatment. These results also provide compelling evidence for the prevalent view that the DNA within the topoisomerase cleavage complex is cleaved, even without protein denaturation, and contradict an alternative model in which

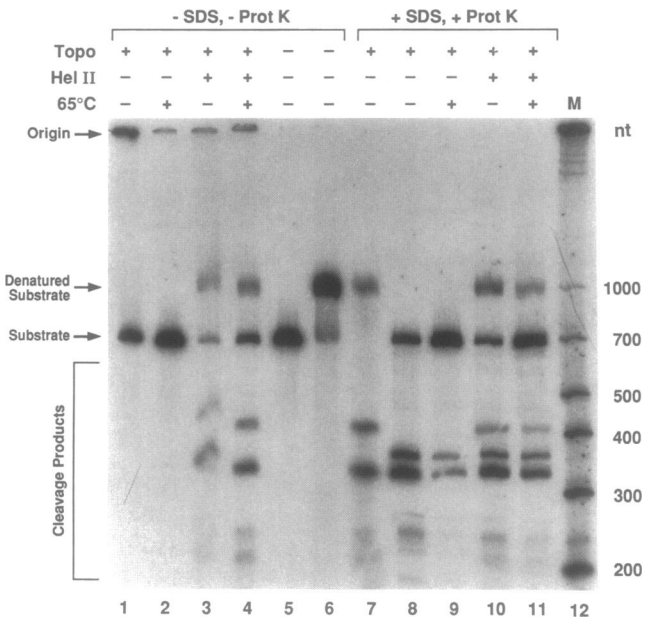

FIG. 3. Release of ssDNA fragments from topoisomerase cleavage complexes. The first two stages of the reactions were the same as those in Fig. 1. All reactions contained *m*-AMSA. After the second stage some of the reactions were heat-treated for 20 min at 65°C (+) and others were not (−). Only the reaction products in lanes 7–11 were treated with SDS and proteinase K (Prot K). Reaction products were analyzed on a nondenaturing 5% polyacrylamide gel as described in Fig. 1, except that the gel and the running buffer lacked SDS and reaction mixtures with no SDS were chilled and loaded on the gel in a glycerol/dye solution containing no SDS. After treatment with SDS and proteinase K, sample 7 was extracted with phenol and passed through a Sepharose CL-6B spin column, and samples 6 and 7 were heat-denatured (100°C, 5 min) immediately before loading on the gel.

DNA cleavage is induced by the protein denaturation step (refs. 30 and 33; also see refs. 28 and 34).

The results in Fig. 3 also provide an independent confirmation of the nonreversibility of DNA breaks induced by helicase II. The 65°C treatment resulted in reversal of topoisomerase cleavage complexes in the absence, but not in the presence, of helicase II (Fig. 3, lanes 8–11).

DISCUSSION

To explain the cytotoxicity of drug-induced cleavage complexes, it has been argued that a cellular process, such as replication or transcription, converts the reversible cleavage complexes into permanent cytotoxic DNA lesions (18). The role of replication and transcription in the cellular response to topoisomerase inhibitors has been previously investigated by measuring drug cytotoxicity under conditions where DNA or RNA synthesis is inhibited (35–37). Transcription inhibitors had little effect on the cytotoxicity of type I topoisomerase inhibitors, but partially protected cells treated with inhibitors of type II topoisomerases (37). In addition, the DNA synthesis inhibitor aphidocolin abrogated the cytotoxicity of inhibitors targeting type I and type II topoisomerases (36, 37). Recent studies in a cell-free simian virus 40 replication system provided evidence that camptothecin-induced type I topoisomerase cleavage complexes arrest replication forks and lead to dsDNA breaks (38).

Helicase-driven separation of the double helix occurs during DNA replication and also during other DNA metabolic activities such as repair and recombination (39, 40). Therefore, we sought to determine whether helicase action on DNA containing a type II topoisomerase cleavage complex

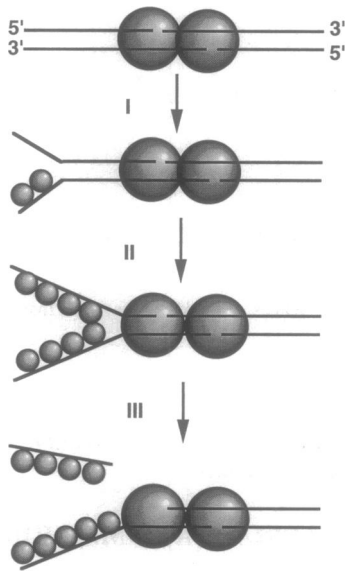

FIG. 4. Model for disruption of a topoisomerase cleavage complex by DNA helicase II. Steps: I, initiation of unwinding by helicase II on the DNA of an *m*-AMSA-induced topoisomerase–DNA cleavage complex; II, encounter of helicase II with the topoisomerase cleavage complex; III, release of a ssDNA cleavage product from the complex, with one of the possible remaining products shown.

could create a DNA lesion by displacing broken DNA from the complex. The generation of such a DNA lesion would be a plausible step in the pathway that generates a cytotoxic response to topoisomerase inhibitors.

The data presented in this paper provide biochemical evidence that DNA helicases can indeed convert drug-induced type II topoisomerase cleavage complexes to irreversible DNA lesions. We have shown that *E. coli* DNA helicase II converts a T4 topoisomerase cleavage complex into a DNA break that cannot be reversed by either EDTA or heat treatment. Furthermore, helicase II disruption of topoisomerase–DNA cleavage complexes releases cleaved ssDNA fragments in a reaction that requires ATP hydrolysis (Fig. 4).

Although *E. coli* helicase II disrupts cleavage complexes *in vitro*, we believe that a T4-encoded helicase is more likely to play a physiological role in disrupting T4 topoisomerase cleavage complexes during a phage infection *in vivo*. Nonetheless, the results presented here confirm the general premise that DNA helicases can disrupt topoisomerase cleavage complexes.

DNA helicases may also play a role in various recombination and/or mutation events induced by DNA topoisomerases. Topoisomerase inhibitors that induce the cleavage complex have been shown to stimulate homologous recombination, sister chromatid exchange, gross genetic rearrangements, and frameshift mutations at sites of cleavage complex formation (4, 18, 41). For each of these genetic events, the helicase-mediated generation of discrete DNA breaks from a cleavage complex would be a reasonable first step.

DNA helicases have recently been found to be sensitive to certain topoisomerase inhibitors, and each helicase appears to have its own unique spectrum of sensitivity (20–23). If a DNA helicase converts the topoisomerase cleavage complex into a cytotoxic lesion *in vivo*, then topoisomerase inhibitors that do not inhibit the helicase should be more cytotoxic than those that do.

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