Repair of Topoisomerase-Mediated DNA Damage in Bacteriophage T4

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ABSTRACT

Type II topoisomerase inhibitors are used to treat both tumors and bacterial infections. These inhibitors stabilize covalent DNA-topoisomerase cleavage complexes that ultimately cause lethal DNA damage. A functional recombinational repair apparatus decreases sensitivity to these drugs, suggesting that topoisomerase-mediated DNA damage is amenable to such repair. Using a bacteriophage T4 model system, we have developed a novel *in vivo* plasmid-based assay that allows physical analysis of the repair products from one particular topoisomerase cleavage site. We show that the antitumor agent 4′-(9-acridinylamino)-methanesulphon-*m*-anisidide (*m*-AMSA) stabilizes the T4 type II topoisomerase at the strong topoisomerase cleavage site on the plasmid, thereby stimulating recombinational repair. The resulting *m*-AMSA-dependent repair products do not form in the absence of functional topoisomerase and appear at lower drug concentrations with a drug-hypersensitive topoisomerase mutant. The appearance of repair products requires that the plasmid contain a T4 origin of replication. Finally, genetic analyses demonstrate that repair product formation is absolutely dependent on genes *32* and *46*, largely dependent on genes *uvsX* and *uvsY*, and only partly dependent on gene *49*. Very similar genetic requirements are observed for repair of endonuclease-generated double-strand breaks, suggesting mechanistic similarity between the two repair pathways.

THE type II topoisomerases play critical roles in many aspects of DNA metabolism, including replication, transcription, and chromosome segregation (for reviews, see Wang 1996; Berger 1998). These enzymes create a transient double-strand break (DSB) in one DNA molecule, through which a second intact double helix is passed. A number of important antitumor and antibacterial agents target the type II topoisomerases, stabilizing enzyme-DNA cleavage complexes that consist of topoisomerase covalently linked to the 5′ phosphates of the DSB (for reviews, see Burden and Osheroff 1998; Hooper 1998). Results in both prokaryotic and eukaryotic systems demonstrated that the cytotoxicity of the topoisomerase inhibitors results from the accumulation of these cleavage complexes, not from inhibition of the topoisomerase catalytic function (for reviews, see Chen and Liu 1994; Drlica and Zhao 1997). Thus, reduced levels of topoisomerase confer relative resistance to these drugs while overexpression of the enzyme confers hypersensitivity (Kreuzer and Cozzarelli 1979; Nitiss and Wang 1988; Neece *et al.* 1996).

Extensive research has been devoted to understanding how drug-stabilized cleavage complexes cause cell death, but the mechanism remains poorly understood. The cleavage complexes themselves are readily reversible upon drug removal and are necessary but not sufficient for cytotoxicity. These reversible cleavage complexes can apparently be converted into cytotoxic lesions, and the DNA replication machinery has been implicated in this conversion. First, cells in S phase are more sensitive to topoisomerase inhibitors than cells in G1 phase, and this increased sensitivity is abrogated by inhibiting DNA replication with aphidicolin (Wilson and Whitmore 1981; Nitiss and Wang 1988; Holm *et al.* 1989; D'Arpa *et al.* 1990). Second, a helicase can disrupt the drug-stabilized cleavage complex and convert it into an irreversible DNA break *in vitro* (Howard *et al.* 1994). Third, drug-stabilized cleavage complexes can block replication forks *in vitro* and *in vivo* (Hiasa *et al.* 1996; Hong and Kreuzer 2000). These blocked forks could potentially be processed into DNA breaks by an endonuclease (Michel *et al.* 1997).

During the conversion of reversible cleavage complexes into cytotoxic DNA lesions, the fate of the topoisomerase protein covalently linked to the 5′ DNA ends is unclear. A phosphodiesterase that specifically cleaves yeast type I topoisomerases from 3′ DNA ends has been found, and certain mutant cells lacking this enzyme are hypersensitive to the type I topoisomerase inhibitor camptothecin (Yang *et al.* 1996; Pouliot *et al.* 1999). An apparently similar human enzyme has also been described (Sastry and Ross 1998). However, no such enzyme has yet been found for processing type II topoisomerase cleavage complexes. Thus, the exact nature of the DNA ends following cleavage complex processing remains unclear.

Assuming that the drug-stabilized cleavage complex

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is ultimately converted into an irreversible DNA break, cell death will follow unless the lesion is repaired. Evidence has accumulated in both prokaryotic and eukaryotic systems that recombinational repair is involved in this process. Treating yeast and mammalian cells with type II topoisomerase inhibitors induces homologous recombination (Nitiss and Wang 1988; Pommier *et al.* 1988). In addition, recombinational repair mutants are hypersensitive to these agents. Mutations in the *recA* gene of *Escherichia coli*, the *RAD52* gene of *Saccharomyces cerevisiae*, or the *xrs* genes of Chinese hamster ovary cells all confer hypersensitivity to type II topoisomerase inhibitors (McDaniel *et al.* 1978; Nitiss and Wang 1988; Eng *et al.* 1989; Jeggo *et al.* 1989; Lewin *et al.* 1989; Caldecott *et al.* 1990; Urios *et al.* 1991). While these studies implicate recombinational repair in resolving topoisomerase-mediated DNA damage, the detailed mechanism of this repair is not understood.

Bacteriophage T4 provides a valuable model system in which to study recombinational repair mechanisms (Kreuzer and Drake 1994). The recombinational repair machinery of T4 has been extensively studied, and most of the proteins involved have been purified and characterized. Recombinational repair of a DSB is believed to begin with resection of the broken ends to generate 3′ single-stranded overhangs, a step for which RNase H 5′ → 3′ DNase activity and the gp46/47 (*g*ene *p*roducts 46 and 47) complex have been implicated (George and Kreuzer 1996; Mueller *et al.* 1996; Huang *et al.* 1999). Single-stranded ends can then invade homologous duplex DNA, directed by the strand-exchange protein UvsX (RecA homologue), along with its accessory protein UvsY and the single-stranded DNA-binding protein gp32 (Yonesaki and Minagawa 1985; Formosa and Alberts 1986; Hinton and Nossal 1986; Kodadek *et al.* 1989; Yonesaki and Minagawa 1989; Morrical and Alberts 1990). Recombinational repair is coupled to DNA replication by the assembly of a replication fork at the invading 3′ single-stranded end (for review, see Kreuzer 2000). The resulting recombination intermediate can presumably be resolved by the Holliday junction resolvase gp49 (endonuclease VII; Mizuuchi *et al.* 1982; George and Kreuzer 1996). Several detailed DSB repair models that include most or all of these basic steps have been proposed, including the Szostak *et al.* model (1983), the synthesis-dependent strand annealing (SDSA) model (Mueller *et al.* 1996; Paques and Haber 1999), and the extensive chromosome replication (ECR) model (George and Kreuzer 1996).

Bacteriophage T4 is also a valuable model system for studying the mechanism of type II topoisomerase inhibitors (for review, see Kreuzer 1998). The T4-encoded type II topoisomerase is inhibited by many of the antitumor drugs that inhibit the mammalian enzyme, and the mechanism of inhibition appears identical. DNA damage resulting from cleavage complex formation is amenable to recombinational repair, as mutations in genes *uvsX*, *uvsY*, and *46* all increased the sensitivity of phage T4 to the type II topoisomerase inhibitor 4′-(9-acridinylamino) methanesulphon-*m*-anisidide (*m*-AMSA). In addition, phage recombination was stimulated upon treatment with *m*-AMSA (Neece *et al.* 1996). Although this work implicated recombinational repair in the response to topoisomerase-mediated DNA damage, it did not allow direct monitoring of the repair reaction or a physical analysis of the repair products.

In this study, we present a novel plasmid-based assay to monitor recombinational repair from one particular *m*-AMSA-inducible topoisomerase cleavage site (topo site). The formation of drug-stabilized cleavage complexes at the topo site and the subsequent repair product formation are monitored by Southern blotting. Using this assay, we analyze the protein requirements for recombinational repair of damage at the topo site and ask how these requirements compare to those for repair of endonuclease-generated DSBs.

MATERIALS AND METHODS

m-AMSA was provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, and diluted in DMSO. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA), Nytran nylon transfer membranes from Schleicher and Schuell (Keene, NH), random-primed labeling kits from Roche Molecular Biochemicals (Indianapolis), and [α-^{32}P]dATP from New England Nuclear (Boston). Oligonucleotides were synthesized by the Duke University Cancer Center DNA Core Facility, and sequencing was performed by the Duke University Cancer Center DNA Analysis Facility. L-broth contained Bacto-Tryptone (10 g/liter), yeast extract (5 g/liter), and sodium chloride (10 g/liter). Ampicillin and tetracycline were obtained from Sigma (St. Louis) and used at concentrations of 25 μg/ml and 2 μg/ml, respectively, in plasmid-containing cultures.

Strains: *E. coli* strain JGD1 is a derivative of KL16 (λ$^-$, e14$^-$, *relA1*, *spoT1*, *thi-1*; Low 1968) with *acrA*::Tn*10-kan* and *recA*::Tn*9-200-cam*. The *acr* mutation increases *m*-AMSA sensitivity by eliminating a multidrug efflux pump (Nikaido 1996; J. W. George and K. N. Kreuzer, unpublished data). Phage strains were T4 K10 [*amB262* (gene *38*), *amS29* (gene *51*), *nd28* (*denA*), *rIIPT8* (*denB-rII* deletion)], K10-*uvsX* [as K10, with *am11* (gene *uvsX*)], K10-*uvsY* (as K10, with *uvsYΔ1*), K10-*46* [as K10, with *amB14* (gene *46*)], K10-*32* [as K10, with *amA453* (gene *32*)], K10-*49* [as K10, with *amE727* (gene *49*)], K10-*G269V* [as K10, with *G269V* (gene *52*)], and K10-*S79am* [as K10, with *amS79* (gene *52*)] (Kreuzer *et al.* 1988; Selick *et al.* 1988; Derr and Kreuzer 1990; Benson and Kreuzer 1992; George and Kreuzer 1996; Freudenreich *et al.* 1998; E. K. O'Reilly and K. N. Kreuzer, unpublished data).

Plasmids: Plasmid pBS1 was constructed as follows. The plasmid pJG2 (George and Kreuzer 1996) was first modified by replacing the *Xho*I-flanked I-*Tev*I cleavage site with an *Xho*I-flanked insert containing a strong *m*-AMSA-inducible topo site. The topo site was created by annealing the following oligonucleotides: 5′-TCGAGCTCAAGCTAAAGTTATATAACTTTATTCAAGGC-3′ and 5′-TCGAGCCTTGAATAAAGTTATATAACTTTAGCTTGAGC-3′. This insert was designed on the basis of previous work analyzing T4 topoisomerase sequence recognition (Freudenreich and Kreuzer 1993;

Hong and Kreuzer 2000). In addition, the 1644-bp *Bgl*II/*Nhe*I fragment of pJG2 was replaced with a PCR-generated 170-bp *Bgl*II/*Nhe*I fragment containing the T4 replication origin *ori(34)* (corresponding to T4 map coordinates 152,906–153,070 bp). This modified pJG2 plasmid (designated pJGH914-01) was digested with *Eco*RI and *Nhe*I and the fragment containing the topo site and T4 *ori(34)* was ligated to a 2259-bp PCR-generated fragment of pBR322 starting at the *Eco*RI site, including the ampicillin resistance gene and the pBR322 origin of replication, and ending with an *Nhe*I site engineered into the PCR primer.

Plasmids pBS2, pBS3, pBS4, and pBS5 are all derivatives of pBS1. Plasmid pBS2 lacks a topo site at the *Xho*I restriction site. Plasmid pBS3 contains a mutated topo site at the *Xho*I site created by annealing the following oligonucleotides (bases varying from the pBS1 topo site are underlined): 5′-TCG AGCTCAAGCTAAAGCTATATAGCTTTATTCAAGGC-3′ and 5′-TCGAGCCTTGAATAAAGCTATATAGCTTTAGCTTG AGC-3′. Plasmid pBS4 contains a I-*Tev*I cleavage site (described in George and Kreuzer 1996) in place of the topo site. Plasmid pBS5 was constructed by digesting pBS1 with *Bgl*II and *Nhe*I to excise the T4 *ori(34)*, filling in the overhanging ends with Klenow enzyme, and recircularizing the plasmid.

Plasmid pAC1000 was constructed from pJGH914-01 as follows. Plasmid pJGH914-01 contains inverted repeats of 1500 bp total length, one interrupted by the topo site and the other by a 284-bp *Nae*I fragment. Primers were constructed to amplify a 1271-bp region that included the *Nae*I fragment and ~500 bp of the inverted repeat on both sides of the *Nae*I fragment. One primer contained an *Afl*III site and the other a *Sal*I site. The PCR fragment and the parental pJGH914-01 were digested with these enzymes and ligated together, resulting in a pJGH914-01 derivative with inverted repeats of ~1000 bp total length (interrupted centrally by either the topo site or the 284-bp *Nae*I fragment). This plasmid was digested with *Eco*RI, and the resulting 1395-bp fragment containing the *Nae*I fragment and surrounding inverted repeat was inserted at the *Eco*RI site of pACYC184 to generate pAC1000.

Phage infections and DNA preparation: Aliquots of frozen log-phase JGD1 cells harboring pAC1000 and the appropriate pBS plasmid were diluted 1:200 into L-broth containing ampicillin and tetracycline and grown with shaking at 37° to an OD_{560} of 0.5 (roughly 4×10^8 cells/ml). Phage were added at three plaque-forming units per cell and incubated for 4 min at 37° without shaking to allow phage adsorption. Cultures were incubated an additional 2 min at 37° with vigorous shaking prior to the addition of *m*-AMSA (in DMSO) at 5 μg/ml (unless otherwise indicated; controls received an equivalent volume of DMSO). Cultures were incubated at 37° with vigorous shaking for an additional 18 min, and 1-ml aliquots were then removed. Cells from each aliquot were collected by centrifugation and frozen in an ethanol/dry ice bath. Cell pellets were resuspended in 300 μl lysis buffer (50 mm Tris-HCl pH 7.5, 10 mm EDTA, 100 mm NaCl, 0.2% SDS, 330 μg/ml proteinase K) and incubated for 2 hr at 55°. Total nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and dialyzed against TE buffer (10 mm Tris-HCl pH 8.0, 1 mm EDTA) overnight at 4°.

Gel electrophoresis and Southern blotting: Total nucleic acids (10 μl) were digested overnight at 37° with 40 units of *Ase*I (double digests contained 20 units of *Hae*III in addition). Digests were subjected to electrophoresis at 2 V/cm for 27 hr in a 1% agarose gel in 1× TBE (89 mm Tris, 89 mm boric acid, 2 mm EDTA) unless otherwise noted. Gels were treated for 20 min with 0.24 n HCl and then twice for 15 min with 0.4 m NaOH/0.6 m NaCl. Southern blotting was performed with Nytran membranes using procedures recommended by the manufacturer (Schleicher and Schuell). Probes were prepared from purified restriction fragments using the random-primed labeling kit (Roche Molecular Biochemicals; see Figure 1 for description of probes). Blots were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). When necessary, blots were stripped by boiling in 1% SDS three times for 15 min and checked for residual counts.

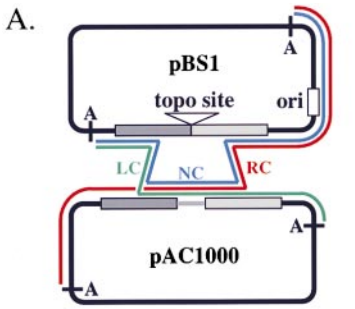
RESULTS

Assay to detect repair of topoisomerase-mediated DNA damage: A two-plasmid assay was developed to analyze repair from one particular topoisomerase cleavage site. Plasmids pBS1 and pAC1000 share ~1000 bp of homology (shaded boxes in Figure 1A), which allows damaged pBS1 to use pAC1000 as a template for homologous recombinational repair. The homologous region is interrupted centrally in both plasmids. Plasmid pBS1 has a 38-bp insert containing a strong *m*-AMSA-inducible topoisomerase cleavage site (topo site) while pAC1000 contains a 283-bp *Nae*I fragment at the corresponding location. Following T4 infection of *E. coli* harboring the two plasmids and subsequent addition of *m*-AMSA, topoisomerase cleavage complexes accumulated at the topo site (data not shown; also see below). In addition, plasmid pBS1 replicates following infection due to its cloned T4 origin of replication [*ori(34)*].

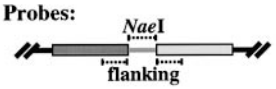
If the drug-stabilized cleavage complexes at the topo site cause DNA breaks requiring recombinational repair, the resulting repair products are expected to carry the 283-bp *Nae*I fragment in place of the topo site (when pAC1000 is used as repair template). Some repair events could be associated with a single crossover between the flanking regions of the two plasmids (the LC and RC products shown in Figure 1). Other repair events could lead to a reconstituted pBS1 plasmid with the 283-bp *Nae*I fragment replacing the topo site (the NC product in Figure 1; no crossover between flanking regions of the two plasmids). *Ase*I digestion of these different repair products and the original plasmids would yield fragments of varying size that can be resolved by Southern hybridization (Figure 1C).

The replication status of the various restriction fragments can be determined since phage-replicated DNA contains glucosylated hydroxymethylcytosine residues. While *Ase*I can cleave this modified DNA, other restriction enzymes such as *Hae*III cannot. Because *Hae*III cuts the plasmid DNA at many sites, it can be used to digest away any unmodified plasmid bands, leaving behind only modified, phage-replicated bands. In addition, the modified DNA migrates slightly more slowly than unmodified DNA during gel electrophoresis, giving it a characteristic shift.

Drug-stabilized cleavage complexes at the topo site stimulate recombinational repair: The two-plasmid assay was first used to determine if the topo site on plasmid pBS1 stimulates recombinational repair in the presence of *m*-AMSA. T4 strain K10 was used to infect *E. coli*



B. Probes:



C.

AseI product	Size (bp)
Nonrecombinant:	
pBS1 (►)	2892
pAC1000 (▻)	2761†
Recombinant:	
Left crossover (LC)	1608†
Right crossover (RC)	4282†
Noncrossover (NC)	3129†
Topo Cleavage:	
Small	769
Large (*)	2123

FIGURE 1.—Two-plasmid repair assay. (A) Schematic of plasmids pBS1 and pAC1000. The shaded boxes represent regions of homology shared between the two plasmids. The topo site and T4 origin of replication (ori) are described in the accompanying text and in MATERIALS AND METHODS. Bars labeled A indicate *Ase*I cleavage sites. Recombinational repair products are denoted by the colored lines labeled LC (left crossover), RC (right crossover), and NC (noncrossover). (B) Plasmid probes. The locations of the flanking and *Nae*I probes along the pAC1000 plasmid are indicated by dotted lines. Note that the flanking probe will detect the homologous regions of both plasmids, while the *Nae*I probe will hybridize only to pAC1000 and repair products that have picked up the 283-bp *Nae*I fragment of pAC1000 (shaded line). (C) Nonrecombinant and recombinant *Ase*I fragments. Only those *Ase*I fragments that will hybridize with probes are listed. All of the listed fragments will hybridize with the flanking probe, while only those marked with a dagger will hybridize with the *Nae*I probe. All recombinant repair products have lost the topo site and picked up the corresponding 283-bp *Nae*I fragment from pAC1000. The LC and RC products also have associated crossovers as shown in A, while the NC product does not. The small and large cleavage fragments are formed when cleavage occurs at the topo site, which splits the 2892-bp pBS1 band into 769-bp and 2123-bp fragments. The abbreviations and symbols used to mark the *Ase*I products on subsequent figures are shown in parentheses.

containing pAC1000 and either pBS1 or pBS2 (a control plasmid lacking the 38-bp topo site). At 6 min postinfection, each culture was split into two equal samples, one receiving *m*-AMSA (5 μg/ml). At 24 min, the infections were terminated and total DNA was isolated (control samples were isolated from uninfected cells, *i.e.*, time zero). Following *Ase*I digestion and Southern blotting, DNA samples were hybridized to the flanking probe, which detects the region of homology shared between pAC1000 and the pBS plasmids (Figure 1B).

Looking first at the nonrecombinant bands, the *Ase*I fragments of pBS1 and pBS2 appear as doublets at the 24-min time point since they contain a T4 replication origin (Figure 2A, lanes 2, 3, 5, and 6, solid arrowheads). The upper band in the doublet is the modified (phage-replicated) pBS DNA containing glucosylated hydroxymethylcytosine residues, while the lower band consists of unmodified pBS DNA present prior to phage infection (since replication from the bacterial plasmid origin shuts off following phage infection; MATTSON *et al.* 1983). Unmodified pAC1000 DNA (Figure 2A, open arrowhead) is visible just below the pBS bands. Drug-stabilized cleavage complexes at the topo site of pBS1 generate cleavage products of 769 bp and 2123 bp (see Figure 1C), the larger of which is visible in the drug-treated pBS1/pAC1000 sample (Figure 2A, lane 3, asterisk; the smaller cleavage product has run off the gel). As expected, the cleavage product appears only with *m*-AMSA treatment (compare lanes 2 and 3 of Figure 2A) and requires the topo site (compare lanes 3 and 6 of Figure 2A).

The recombinational repair products are best visualized using a probe for the 283-bp *Nae*I fragment of the pAC1000 plasmid (Figure 1B, *Nae*I probe). Figure 2B shows the same blot as in Figure 2A, but hybridized with the *Nae*I probe. In addition to detecting the nonrecombinant pAC1000 *Ase*I fragment, the *Nae*I probe detects all the expected crossover and noncrossover repair products (listed in Figure 1C). Without drug treatment, noncrossover (NC) repair product is undetectable whether or not the pBS plasmid contains the topo site (Figure 2B, lanes 2 and 5). Drug treatment induces the formation of this repair product, and the induction is much stronger when the pBS plasmid carries the topo site (compare lanes 3 and 6 of Figure 2B). Quantitation of multiple experiments indicates that approximately threefold more drug-induced noncrossover repair product forms when the topo site is present (Table 1). Thus, the majority of the noncrossover repair product observed with the pBS1 plasmid depends on the cloned topo site, presumably reflecting repair of DNA damage from the drug-stabilized cleavage complexes at this site. The small amount of drug-induced noncrossover repair product observed in the absence of the topo site is presumably due to low-level cleavage complex formation at other weak sites along the pBS2 plasmid.

Analysis of the crossover repair products (RC and LC) is complicated by significant drug-independent formation of these products (Figure 2B, lanes 2 and 5). This drug-independent signal requires the T4 origin of replication (see Figure 4) and is addressed in detail elsewhere (J. W. GEORGE, B. A. STOHR, D. J. TOMSO and K. N. KREUZER, unpublished results). Quantitation of multiple experiments nevertheless indicates that the topo site stimulates formation of crossover repair products just as it does noncrossover repair products (*i.e.*, approximately threefold more drug-induced crossover repair products with the topo site present; Table 1).

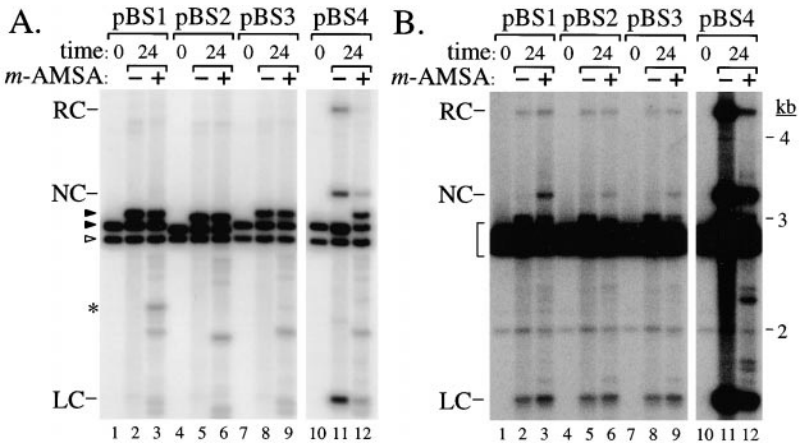


FIGURE 2.—Topo site stimulates plasmid recombination in the presence of *m*-AMSA. Plasmid pBS1 is described in Figure 1A. The other pBS plasmids contain the following inserts in place of the topo site: no insert (pBS2), mutated topo site (pBS3), and I-*Tev*I recognition site (pBS4). DNA sample collection times are in minutes postinfection. The zero time point samples were collected immediately preceding phage addition. Drug-treated samples received *m*-AMSA 6 min after infection as described in MATERIALS AND METHODS. The blot was hybridized with the flanking probe in A and the *Nae*I probe in B. The three repair products are labeled RC (right crossover), NC (noncrossover), and LC (left crossover); we do not know whether these three bands are transferred to the blot with the same efficiency. The large cleavage product (visible in A, lane 3 only) is marked with an asterisk, while the small cleavage product migrated off the gel. The open arrowhead in A indicates unmodified pAC1000, while the solid arrowheads indicate the unmodified (bottom arrowhead) and modified (phage-replicated; top arrowhead) pBS plasmid bands (modified DNA migrates slower due to glucosylation). The pBS2 bands migrate slightly faster than the other pBS bands since they lack a topo site or I-*Tev*I site. The bracket in B indicates the pAC1000 band. The molecular markers in this and subsequent figures were generated by measuring the migration of *Xba*I fragments of unmodified T4 DNA (unless otherwise noted). The majority of unmarked bands in drug-treated samples in this and subsequent figures is presumably due to low-level cleavage complex formation at other sites on the plasmids (HONG and KREUZER 2000).

Because the effect of the topo site on recombinational repair is most readily apparent with the noncrossover product, we focus on this repair band in the figures that follow.

Mutation of topo site reduces stimulatory effect on recombinational repair: Previous mutational analysis of an *m*-AMSA-inducible topoisomerase cleavage site showed that particular sequence changes close to the sites of DNA cleavage could reduce or eliminate *in vitro* drug-stabilized cleavage complex formation (FREUDENREICH and KREUZER 1993). On the basis of this *in vitro* work, we mutated the topo site, changing two critical positions symmetrically flanking the sites of DNA cleavage from A/T to G/C base pairs (see MATERIALS AND METHODS). A plasmid containing this altered topo site was constructed (pBS3) and compared to the above-described plasmids pBS1 and pBS2.

Drug-stabilized cleavage complex formation at the mutated topo site was dramatically reduced compared to the unaltered topo site (compare lanes 3 and 9 of Figure 2A, asterisk), but a weak cleavage product band was detected in an overexposure (data not shown). Drug-induced noncrossover repair product (NC) formation was much lower with the mutated topo site (pBS3) than with the unaltered topo site (pBS1; compare lanes 3 and 9 of Figure 2B) and was roughly equivalent to that with no topo site (pBS2; compare lanes 6 and 9 of Figure 2B). Quantitation of multiple experiments indicated that the mutated topo site had, at most, a weak stimulatory effect on drug-induced repair product

TABLE 1

Topo site stimulates *m*-AMSA-induced repair product formation

	Relative intensity of *m*-AMSA-induced repair product bands between the three plasmids		
Repair product	pBS1	pBS2	pBS3
Noncrossover (NC)	1.64 ± 0.25	0.49 ± 0.15	0.87 ± 0.22
Left crossover (LC)	1.64 ± 0.43	0.66 ± 0.33	0.69 ± 0.19
Right crossover (RC)	1.82 ± 0.67	0.41 ± 0.25	0.77 ± 0.50

Plasmid pBS1 and the repair products are described in Figure 1. Plasmid pBS2 lacks the topo site, and pBS3 carries a mutated topo site in place of the strong topo site. The intensity of each drug-induced repair product band was determined by measuring the intensity of the repair band in the plus-drug lane and then subtracting out the intensity of the equivalent repair band in the accompanying minus-drug lane. These values were normalized between the three different plasmids to allow comparison between experiments and between the different repair product types (*i.e.*, LC, NC, and RC). For example, the three NC values from one experiment (representing the drug-induced repair with pBS1, pBS2, and pBS3) were normalized by dividing each NC value by the sum of the three NC values. All three repair product types (*i.e.*, LC, NC, and RC) in all four experiments were normalized in this way. Values represent the mean ± standard deviation for four experiments.

formation (Table 1). This result provides additional evidence that drug-stabilized cleavage complex formation at the topo site stimulates recombinational repair with the pBS1 plasmid.

As an additional control, recombinational repair of endonuclease-generated double-strand breaks was analyzed using the two-plasmid repair assay (Figure 2, A and B, lanes 10–12); those experiments are described in detail below.

Topoisomerase mutants alter recombinational repair from topo site: If drug-stabilized cleavage complexes at the topo site are responsible for the stimulation of recombinational repair, certain mutations in the T4 type II topoisomerase should influence the level of drug-induced recombinational repair. One previously described T4 topoisomerase mutant is hypersensitive to a variety of topoisomerase inhibitors including *m*-AMSA (Freudenreich *et al.* 1998). The hypersensitivity results from a glycine-to-valine substitution at position 269 of the gp52 subunit of the enzyme. This mutant demonstrates the same cleavage site preference as the wild-type protein in the presence of *m*-AMSA (E. K. O'Reilly and K. N. Kreuzer, unpublished data). We compared a phage carrying the *G269V* mutation to wild-type K10 at various *m*-AMSA concentrations. As expected, drug-induced noncrossover repair product appeared at lower drug concentrations with the hypersensitive mutant than with the wild type (Figure 3A). We also analyzed a T4 strain carrying an amber mutation (*S79am*) in gene *52* (Freudenreich *et al.* 1998). Because the *E. coli* strain (JGD1) is nonsuppressing, no functional T4 topoisomerase should be made during infection with the *S79am* mutant. Indeed, no drug-induced stimulation of recombinational repair was apparent with this mutant (Figure 3B). The results with these two topoisomerase mutants provide further evidence that the drug-induced recombinational repair in the two-plasmid system depends upon the formation of T4 type II topoisomerase cleavage complexes.

Origin of replication is required for repair product formation: Evidence has accumulated that DNA replication plays a role in converting reversible cleavage complexes into irreversible DNA breaks (see Introduction). To address this issue, we asked whether the T4 origin of replication on the pBS1 plasmid is required to observe recombinational repair from the topo site. We constructed pBS5, which is identical to pBS1 except that it lacks the T4 origin of replication. While pBS1 and pBS5 both have a plasmid replication origin, that origin is shut down upon T4 infection (Mattson *et al.* 1983). As expected, plasmid pBS5 undergoes minimal replication following phage infection compared to pBS1. After *Hae*III digestion to remove unmodified plasmid bands, a strong modified (phage-replicated) pBS1 *Ase*I band remains (Figure 4A, lanes 5 and 6, top solid arrowhead) but very little modified pBS5 is evident (Figure 4A, lanes 7 and 8, top arrow).

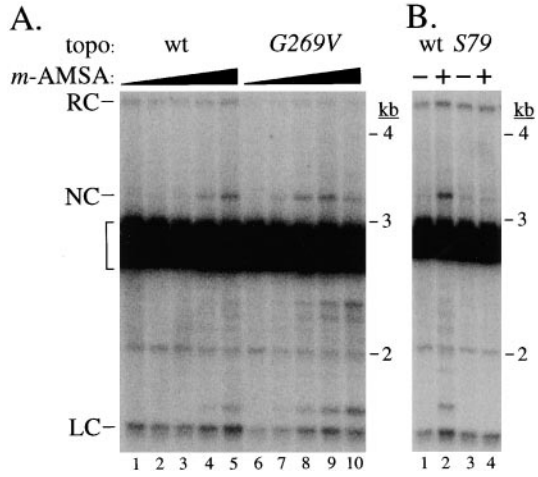


Figure 3.—Topoisomerase mutations alter *m*-AMSA-induced plasmid recombination. (A) Cells harboring the pBS1 and pAC1000 plasmids were infected with K10 (wt) or K10-*G269V* phage strains. After 6 min of infection, *m*-AMSA was added at increasing concentrations: 0, 0.625, 1.25, 2.5, and 5 μg/ml. DNA samples were collected at 24 min after infection. The blot was hybridized with the *Nae*I probe. Nonrecombinant and repair bands are labeled as in Figure 2B. (B) Infections with K10 (wt) and K10-*S79am* phage strains and sample collection were done as for A, except that *m*-AMSA was added at either 0 (-) or 5 (+) μg/ml. Note that the samples in B were run using slightly different electrophoresis conditions, so the band separations vary slightly from A.

As in the above experiments, drug-induced noncrossover repair product is readily observed with pBS1 (Figure 4B, lane 2, NC), but no such product is detected with pBS5 (Figure 4B, lane 4, NC′). Note that the pBS5 noncrossover repair band, if present, would run faster than the corresponding pBS1 repair band since it would lack the 170-bp T4 origin. The bracket labeled NC′ in Figure 4B notes the expected position of noncrossover repair product in the pBS5 lanes. The absence of noncrossover repair products from pBS5 is consistent with DNA replication playing a role in the processing of topoisomerase-mediated DNA damage. However, this interpretation is weakened by the fact that more drug-stabilized cleavage complexes form on the modified pBS1 (Figure 4A, lane 2, asterisk) than on the unmodified pBS5 (Figure 4A, lane 4, double asterisk). This difference, which likely results from a preference for cleavage complex formation on modified DNA, could contribute to the lack of recombinational repair product formation in the absence of a T4 replication origin.

Repair of topoisomerase-mediated damage and endonuclease-generated DSBs requires the same recombinational repair proteins: The protein requirements for repair of endonuclease-generated DSBs have been studied previously in bacteriophage T4 using a one-plasmid repair assay (George and Kreuzer 1996; also see Mueller *et al.* 1996). This assay analyzed recombinational repair of a DSB generated by the phage-encoded endonuclease I-*Tev*I. The products of genes *uvsX*, *uvsY*,

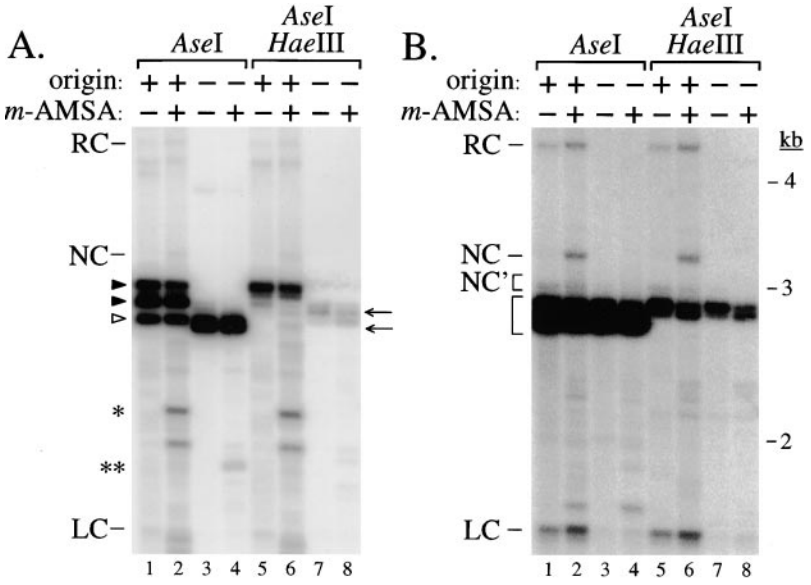


FIGURE 4.—T4 origin of replication is required for *m*-AMSA-induced repair product detection. Cells containing pAC1000 and pBS1 [lanes 1–2 and 5–6; with T4 origin *ori(34)*] or pAC1000 and pBS5 (lanes 3–4 and 7–8; without origin) were infected with T4 strain K10, and *m*-AMSA was added where indicated 6 min after infection. Samples were collected at 24 min postinfection, and purified total DNA was digested with *Ase*I alone (lanes 1–4) and *Ase*I plus *Hae*III (lanes 5–8). The blot was hybridized with the flanking (A) and the *Nae*I (B) probes. Repair bands (RC, NC, and LC) are most easily seen in B. The large cleavage product (visible in A, lanes 2, 4, and 6) is marked with an asterisk for *ori(34)*$^+$ lanes and a double asterisk for *ori(34)*$^-$ lanes. The small cleavage band migrated off the gel. In A, unmodified pAC1000 is indicated by the open arrowhead, unmodified and modified pBS1 fragments by the bottom and top solid arrowheads, respectively, and unmodified and modified pBS5 fragments by the bottom and top arrows, respectively. Note that the unmodified pBS5 band partially overlaps the unmodified pAC1000 band (A, lanes 3 and 4). In B, the bracket labeled NC′ indicates the expected position of NC repair product in the *ori(34)*$^-$ samples. The NC (and RC) repair products would migrate faster in the *ori(34)*$^-$ samples due to the absence of the 170-bp T4 *ori(34)*. The unlabeled bracket indicates the nonrecombinant plasmid bands. The molecular markers in this figure were generated by measuring the migration of *Ase*I-digested T4 DNA fragments.

46, and *32* were found to be essential for repair, and a mutation in gene *49* inhibited repair significantly.

We asked whether recombinational repair of topoisomerase-mediated damage exhibits the same protein requirements as repair from I-*Tev*I-generated DSBs. For this purpose, we constructed plasmid pBS4, which is identical to pBS1 except that it contains the I-*Tev*I endonuclease recognition sequence in place of the topo site. Following T4 infection, I-*Tev*I is expressed and cleaves pBS4 at the recognition sequence. The DSBs stimulate massive amounts of recombinational repair of pBS4 with pAC1000 serving as repair template, and the resulting crossover and noncrossover repair products are the same size as those seen with the topo site (Figure 2, A and B; compare lanes 11 and 12 to lanes 2 and 3). As expected, the accumulation of repair products occurs with or without *m*-AMSA. However, the level of repair product formation is somewhat lower in the presence of the drug for unknown reasons (Figure 2, A and B; compare lanes 11 and 12).

Repair product formation with both the topo site (pBS1) and the I-*Tev*I site (pBS4) was dramatically reduced by mutations in *uvsX* or *uvsY* (Figure 5B, lanes 1–3 and 7–9; note that *m*-AMSA was added to all infections in this experiment). With the topo site, noncrossover repair product formation dropped ~10-fold in the absence of either protein, compared to an ~100-fold drop with the I-*Tev*I site. Thus, a large majority of repair events in both cases required UvsX and UvsY, although repair independent of these two proteins was more prominent with the topo site. One concern with this type of genetic analysis is that the mutations in *uvs*X and *uvs*Y could decrease recombinational repair through an indirect effect, for example, by decreasing topoisomerase expression. This seems unlikely, however, because formation of drug-stabilized cleavage complexes appears to be unaffected by the *uvs*X or *uvs*Y mutations (Figure 5A, lanes 1–3, asterisks).

Absence of gp46 completely abolished repair from both the topo site (pBS1) and the I-*Tev*I site (pBS4; Figure 5B, lanes 4 and 10). The absence of gp46 also led to a dramatic increase of I-*Tev*I cleavage product (Figure 5A, lane 10, asterisks). This increase has been previously documented and is believed to result from stabilization of the broken ends due to the absence of gp46 exonuclease activity (ALBRIGHT and GEIDUSCHEK 1983; KREUZER *et al.* 1995; GEORGE and KREUZER 1996). Interestingly, there was no increase in topo site cleavage products in the absence of gp46 (Figure 5A, lane 4, asterisks). This finding is consistent with the ends of the DSB being shielded from exonuclease activity *in vivo* by the covalently linked topoisomerase protein within the drug-stabilized cleavage complex. In all of our experiments, DNA samples are treated with proteinase K, thereby freeing any DNA ends that are protein linked *in vivo*. These results suggest that the *in vivo* conversion of drug-stabilized cleavage complexes to protein-free DSBs is a relatively infrequent event.

Mutation of gene *32* also abolished recombinational repair from both the topo site (pBS1) and the I-*Tev*I site (pBS4; Figure 5B, lanes 5 and 11). The loss of repair from the topo site occurred despite significant formation of drug-stabilized cleavage complexes on the unmodified pBS1 plasmid (Figure 5A, lane 5, bottom aster-

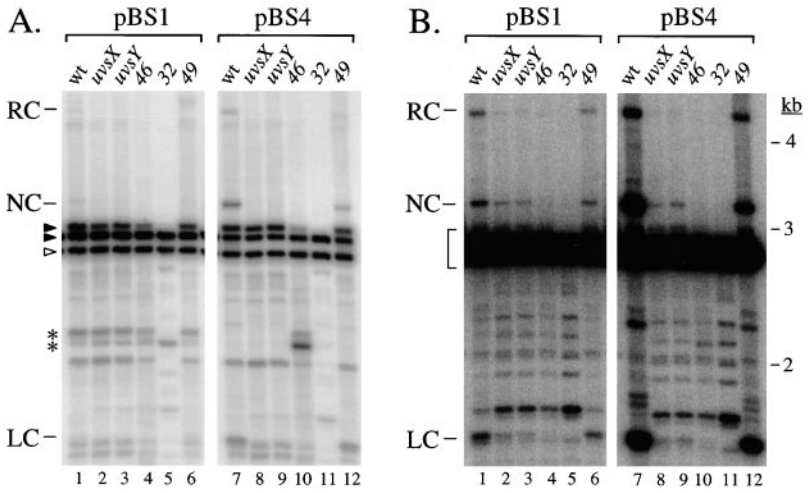


FIGURE 5.—Repair of both topoisomerase-mediated damage and I-*Tev*I breaks share similar protein requirements. Cells harboring pAC1000 and either pBS1 (topo site; lanes 1–6) or pBS4 (I-*Tev*I site; lanes 7–12) were infected with K10 (wt) or the indicated K10 mutant (see MATERIALS AND METHODS). All samples received *m*-AMSA at 6 min postinfection, and samples were collected at 24 min postinfection. Purified total DNA was digested with *Ase*I. The blot was hybridized with the flanking (A) and *Nae*I (B) probes. Repair bands (RC, NC, and LC) are most visible in B. In A, unmodified pAC1000 is indicated by the open arrowhead, while the unmodified and modified pBS bands are indicated by the bottom and top solid arrowheads, respectively. The unmodified and modified large cleavage bands are indicated by the bottom and top asterisks, respectively. The small cleavage bands have migrated off the gel. In B, the bracket indicates the pAC1000 band. The experiments presented in this figure were repeated multiple times with comparable results.

isk). The absence of repair induced by these cleavage complexes might result from gp32 playing a direct role in recombinational repair, but could also be due in part to the critical role of gp32 in DNA replication. As expected, no replication of either plasmid was observed in the infections lacking gp32 (Figure 5A, lane 5, top solid arrowhead).

An amber mutation in gene *49* decreased recombinational repair about fourfold with either the topo site or the I-*Tev*I site, suggesting a role for this protein in the repair mechanism. The residual repair in the gene *49* mutant might reflect repair events that do not require the Holliday junction resolvase. Alternatively, some other protein(s) might resolve Holliday junctions in the absence of gp49, or a small amount of gp49 might be expressed despite the amber mutation.

The major conclusion is that repair of both topoisomerase-mediated damage and I-*Tev*I breaks requires the same proteins, which argues for very similar repair mechanisms.

DISCUSSION

Previous genetic studies in both prokaryotic and eukaryotic organisms have suggested that recombinational repair acts upon topoisomerase-mediated DNA damage, thereby limiting sensitivity to the type II topoisomerase inhibitors (see Introduction). Most of these studies have relied on cell or phage sensitivity as an experimental endpoint, thus limiting detailed analysis of the repair reaction. We present here a novel plasmid-based assay that allows physical analysis of the recombinational repair products from one particular topo site. The site specificity of the assay is demonstrated by the approximately threefold drop in drug-induced repair product formation when the topo site is absent or mutated (Figure 2). The involvement of topoisomerase is confirmed by the loss of signal in a topoisomerase null mutant and the appearance of signal at lower drug concentrations with an *m*-AMSA-hypersensitive topoisomerase mutant (Figure 3). To our knowledge, this is the first assay that detects repair from a particular topoisomerase recognition site.

Our results indicate that repair from the topo site requires the same recombinational repair proteins as repair of endonuclease-generated DSBs. Both types of repair are largely dependent on the UvsX and UvsY proteins and absolutely require gp46 and gp32. In addition, both types of repair are reduced severalfold by an amber mutation in gene *49*. Taken together, these results strongly suggest that the drug-stabilized cleavage complexes at the topo site are processed into protein-free DSBs that are subsequently acted upon by the phage recombinational repair apparatus. Nonetheless, two results indicate that only a small fraction of detectable cleavage complexes is converted into DSBs. First, much less repair product appears to be formed from cleavage complexes than from endonuclease-generated DSBs (see Figures 2 and 5). Second, we were unable to detect an increase in DSBs at the topo site in a gp46-deficient infection, where protein-free breaks are stabilized (Figure 5).

Assuming that drug-stabilized cleavage complexes are processed into protein-free DNA ends, the mechanism of this processing remains mysterious. The appearance of repair products in our system requires an active origin of replication on the plasmid with the topo site, consistent with the replication apparatus playing a role in cleavage complex processing. One possibility is that collision of a replication fork disrupts the drug-stabilized cleavage complex, generating at least one protein-free double-strand end (HOWARD *et al.* 1994). Alternatively

or additionally, drug-stabilized cleavage complexes might be processed by an enzyme that cleaves the covalently linked topoisomerase protein from the ends of the DNA, thereby generating a protein-free double-strand break. Such phosphodiesterases have been described in eukaryotic systems for type I topoisomerase cleavage complexes, but it is not clear whether comparable enzymes act upon type II topoisomerase cleavage complexes, or whether they are present in prokaryotic systems (Yang *et al.* 1996; Sastry and Ross 1998; Pouliot *et al.* 1999).

Recent work has shown that drug-stabilized cleavage complexes can stall T4 replication forks *in vivo* (Hong and Kreuzer 2000) and *E. coli* replication forks *in vitro* (Hiasa *et al.* 1996). In the case of the T4 system, these stalled forks might be cleaved by gp49 (endonuclease VII) to generate double-strand DNA ends capable of undergoing recombinational repair (G. Hong and K. N. Kreuzer, unpublished data cited in Kreuzer 2000). If gp49-mediated processing of stalled replication forks was the primary mechanism by which drug-stabilized cleavage complexes caused DSBs, inactivation of gp49 should decrease repair from the topo site more than repair from the I-*Tev*I site. Our results demonstrate, however, that a mutation in gene *49* impacts both types of repair equally. Therefore, unless the *49* amber mutant has residual endonuclease VII activity, topoisomerase-mediated DSBs can be generated through a gp49-independent mechanism.

However the DSBs are generated from drug-stabilized cleavage complexes, it is important to consider the mechanism by which these breaks are repaired. The requirement for the UvsX, UvsY, and gp32 proteins strongly suggests that single-stranded DNA (ssDNA) is generated during the repair process, because these proteins all play a role in the DNA strand exchange reaction. The gp46/47 protein complex is also required for repair and may be responsible for the generation of the ssDNA ends. This protein complex contains both helicase and nuclease motifs and is homologous to the eukaryotic proteins Rad50 and Mre11 (Sharples and Leach 1995). The partial requirement for gp49 indicates that branched DNA structures are formed during repair, perhaps following UvsX-catalyzed strand invasion.

Numerous DSB repair models have been proposed, including the Szostak *et al.* (1983) model, SDSA model (for review, see Paques and Haber 1999), and the ECR model (George and Kreuzer 1996). Modified versions of each of these models could potentially explain the findings presented in this article. Thus, while our results suggest a common mechanism for repair of topoisomerase-mediated damage and repair of endonuclease-generated damage, additional work will be required to determine which repair model most accurately reflects this shared mechanism.

It is very likely that homologous recombination pathways also repair topoisomerase-mediated DNA lesions in mammalian systems. Recent studies have shown that DSBs in the mammalian genome are frequently repaired by homology-directed pathways, through both single-strand annealing and gene conversion mechanisms (Liang *et al.* 1998). The latter pathway likely utilizes human proteins with functional and/or sequence homology to the T4 proteins described in this article (Sharples and Leach 1995; Thacker 1999; Flores-Rozas and Kolodner 2000; Kreuzer 2000). One potential consequence of such repair is loss of heterozygosity, which could lead to carcinogenesis if it occurs at a tumor suppressor locus. Another possible consequence is that recombination between closely related sequences (*e.g.*, *Alu* repeats) on nonhomologous chromosomes might generate chromosomal translocations, which could also lead to carcinogenesis. Thus, homologous recombinational repair of topoisomerase-mediated DSBs might contribute to the development of therapy-induced tumors, a well-recognized consequence of chemotherapy with the type II topoisomerase inhibitors (Felix 1998). Development of specific inhibitors to this repair pathway might increase the clinical efficacy of these inhibitors while also limiting the risk of therapy-induced tumors.

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