Nucleic Acids Research, 1995, Vol. 23, No. 15 **2973–2979**

A novel suicide substrate for DNA topoisomerases and site-specific recombinases

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Received March 24, 1995; Revised and Accepted June 19, 1995

ABSTRACT

DNA topoisomerases and DNA site-specific recombinases are biologically important enzymes involved in a diverse set of cellular processes. We show that replacement of a phosphodiester linkage by a 5′-bridging phosphorothioate linkage creates an efficient suicide substrate for calf thymus topoisomerase I and lambda integrase protein (Int). Although the bridging phosphorothioate linkage is cleaved by these enzymes, the 5′-sulfhydryl which is generated is not competent for subsequent ligation reactions. We use the irreversibility of Int-promoted cleavage to explore conditions and factors that contribute to various steps of lambda integrative recombination. The phosphorothioate substrates offer advantages over conventional suicide substrates, may be potent tools for inhibition of the relevant cellular enzymes and represent a unique tool for the study of many other phosphoryl transfer reactions.

INTRODUCTION

DNA topoisomerases and site-specific DNA recombinases carry out cleavage and joining reactions involving phosphodiester bonds. Type I topoisomerases from eukaryotes (1) and members of the prokaryotic integrase family of recombinases (2) have similar biochemical properties, although the two proteins are phylogenetically disparate and share no obvious sequence similarity. In both cases strand cleavage is not the result of phosphodiester hydrolysis, but transesterification by an active site tyrosine, creating a 3′-DNA covalent intermediate and a 5′-terminus (5′-OH). Strand ligation occurs during a second transesterification event, when a 5′-OH displaces the enzyme (Fig. 1). In a typical topoisomerase reaction the 5′-OH created by cleavage displaces the protein subsequent to strand unwinding, restoring the duplex but permitting a change of DNA linking number. In the case of recombinases the reactive 5′-OH does not come from the original cleaved DNA strand, but from a similarly cleaved 'partner' strand, resulting in intermolecular strand ligation (4). These two pathways are not mutually exclusive, indeed, under some conditions, site-specific recombinases can display topoisomerase activity (5) and type I topoisomerases can catalyze intermolecular strand ligation (6–8).

Mechanistic studies of elementary topoisomerase reactions have been difficult because the 3′ covalent intermediate is very transient. It has been demonstrated for vaccinia topoisomerase I (9) and agreed to be generally true for topoisomerases and site-specific recombinases that the rate of cleavage (k_c) is slower than the rate of ligation (k_l). The enzyme-imposed 'equilibrium' ($K = k_c/k_l < 1$) therefore favors accumulation of the strand joining product, which has the same primary structure as the original substrate, making the analysis of these reactions cumbersome. Suicide substrates, which shift the equilibrium ($K >> 1$) and thereby trap the enzyme–DNA complex, have therefore been developed. These substrates contain nicks near the site of cleavage so that a small incised fragment is released following strand cleavage, thereby removing the critical 5′-OH necessary for strand ligation (10,11). Here we report that DNA containing a 5′-bridging phosphorothioate linkage (OPS) also acts as a suicide substrate. The modified DNA linkage (Fig. 1) is cleaved by calf thymus topoisomerase I (topo I) and lambda integrase (Int) proteins, but the 5′-sulfhydryl that is generated by cleavage is not competent for subsequent ligation reactions. Because this novel substrate contains no interruptions in the polynucleotide backbone prior to exposure to the enzyme under study, it offers several advantages (see Discussion) over nicked suicide substrates.

Although we demonstrate the generality of our approach by using phosphorothioate substrates with several enzymes that carry out cleavage/ligation reactions, our principal focus is the strand exchange activity of bacteriophage lambda Int. During lambda integrative recombination strand exchange (i.e. cleavage and ligation at two phosphodiesters) occurs at well-defined 'attachment' sites in the phage (*att*P) and bacterial (*att*B) chromosomes (12). Both *att*P and *att*B contain a 'core' region within which both strands of the duplex DNA are subject to a cleavage and a ligation event. In each core the two sites of strand cleavage/joining are separated by 7 bp (termed the overlap region) and are bounded by two inverted Int binding sites. *att*P also contains several distinct Int binding sites that lie outside the core and are not present in *att*B; these 'arm' sites are bound tightly

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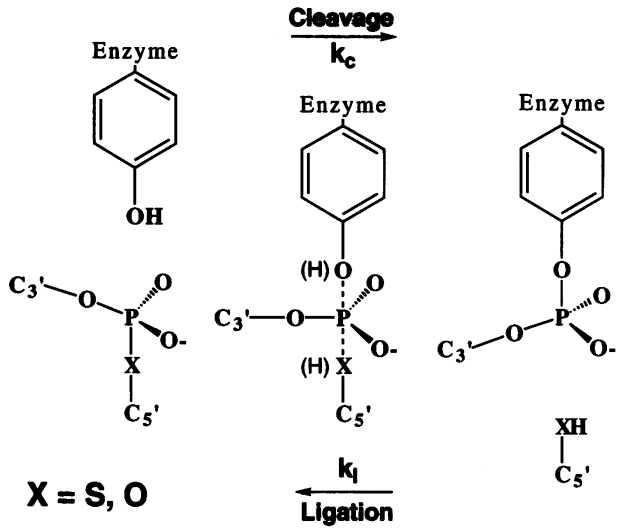

Figure 1. Rationale for suicide substrate design. A phosphodiester linkage at the site of strand cleavage/ligation and an active site tyrosine are diagramed on the left; the cleaved DNA and the 3′-phosphotyrosyl enzyme covalent complex are diagramed on the right. In the unmodified phosphodiester, X represents oxygen; for the 5′-bridging phosphorothioate, X represents sulfur. Chiral phosphorothioate diesters, in which one of the non-bridging oxygens is replaced by sulfur (not diagramed) have been extensively used as mechanistic probes in enzymology (3) and should not be confused with the achiral 5′-bridging phosphorothioate diesters described here.

by a domain of Int that is separate from the domain that binds to the inverted core sites (13). The flanking arms of *att*P also contain multiple binding sites for an architectural protein, integration host factor (IHF). It is believed that supercoiled *att*P DNA, Int and IHF form a higher order structure, termed the 'intasome', which is the active species during integrative recombination, and *att*B enters the reaction without any bound protein (14,15). IHF is specifically required for DNA bending (16), presumably to assist the assembly of a functional intasome. We therefore view integrative recombination as a bimolecular, single turnover reaction with intasome acting as the enzyme and *att*B as the substrate.

MATERIALS AND METHODS

Preparation of 5′-(*S*-trityl)-mercapto-5′-deoxythymidine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylamino)phosphite

5′-Bridging phosphorothioates were synthesized by a modification of the method of Mag *et al.* (17). Thymidine (25 g, 100 mmol) (Aldrich) was dissolved in 80 ml dry pyridine (Aldrich) and then cooled to 0°C. Toluene sulfonyl chloride (23.75 g, 125 mmol) (Aldrich) in 20 ml dry pyridine was then added dropwise and stirred at 0°C for 16 h. Water (500 ml) was added and the solution was extracted three times with 200 ml chloroform. Chloroform layers were pooled, extracted sequentially with 500 ml 5% $NaHCO_3$ and 500 ml saturated NaCl and evaporated to an oil. 5′-*O*-Tosyl-thymidine was then recrystallized from 500 ml ethanol at –20°C overnight (36 g, 90% yield). The thio-anion of triphenylmethyl mercaptan was prepared by mixing 5.05 g KOH (90 mmol) in 200 ml ethanol and 27.6 g (100 mmol) triphenylmethyl mercaptan (Aldrich) in 200 ml toluene. The solution was immediately added to 19.8 g (50 mmol) 5′-tosyl-thymidine dissolved in 400 ml ethanol:toluene (1:1) at 75°C. The slurry was stirred for 4 h at 75°C, filtered and the solution was then extracted twice with 500 ml 10% $NaHCO_3$ and the remaining toluene was evaporated to an oil. The oily residue was dissolved in 200 ml ethyl acetate, extracted twice with 200 ml 5% $NaHCO_3$, twice with 200 ml saturated NaCl, dehydrated over sodium sulfate (Aldrich) and dried under vacuum to a yellow powder. 5′-*S*-Trityl-thymidine was purified (11 g, 45% yield) by flash chromatography (5% methanol in methylene chloride over 230–400 mesh 60A silica gel; Sigma) and its structure confirmed by chemical ionization mass spectroscopy. The 3′-phosphoramidite was prepared according to McBride and Caruthers (18).

Synthesis of 5′-bridging phosphorothioates

The synthesis of oligonucleotides was carried out on an ABI 380 or ABI 391EP DNA synthesizer. Addition of the 5′-*S*-trityl-phosphoramidite to the growing chain was performed following the standard procedure. The *S*-trityl group is not labile to mild acid, so to extend the oligonucleotide the 5′ protecting group was removed manually in freshly prepared 50 mM $AgNO_3$ (Aldrich) at room temperature for 10 min. The resin was then washed with 5 ml water, 2 ml 50 mM dithiothreitol (DTT), 2 ml water, 2 ml acetonitrile and 2 ml dry acetonitrile. Coupling time of the next phosphoramidite (i.e. addition to 5′-sulfhydryl) was increased to 5 min and all other steps were as recommended by ABI. The phosphorothioate oligonucleotides were typically obtained in 25–50% yield, as compared with unmodified oligonucleotides. We believe this is due to reaction of the 5′-SH with the solvent and/or resin. Oligonucleotides containing a 5′-sulfhydryl were prepared as described, except synthesis was stopped after removing the trityl protecting group.

Synthesis of 3′-phosphotyrosine oligonucleotides

Oligonucleotides containing a 3′-phosphotyrosine were synthesized by a modification of the method of Pan *et al.* (19). The resin (TentaGel S OH, capacity 0.29 mmol/g; Rapp Polymere) was first derivatized by adding 0.69 g (1.5 mmol) F-moc-t-butyl-tyrosine (Applied Biosystems/Perkin Elmer) in 10 ml dry dichloromethane (Aldrich), 0.018 g (0.15 mmol) dimethylaminopyridine (Aldrich) in 1 ml dry dichloromethane and then 0.31 g (1.5 mmol) dicyclohexylcarbodiimide (Aldrich) in 1 ml dry dichloromethane to 0.52 g (0.015 mmol) resin. The slurry was stirred at room temperature under argon for 2 h, washed sequentially with 10 ml dichloromethane, 10 ml acetonitrile, 10 ml dimethylformamide and then dried *in vacuo*. The t-butyl protecting group was removed by adding 0.2 ml 50% trifluoroacetic acid (Applied Biosystems) in dimethylformamide (Aldrich) to the resin at room temperature for 10 min. The resin was washed sequentially with 5 ml dimethylformamide, 5 ml acetonitrile, 5 ml dichloromethane and then dried *in vacuo*. Synthesis (and deblocking) of the oligonucleotides was performed as recommended by Applied Biosystems, except that the coupling time of the first phosphoroamidite was increased to 2 min. The 3′-phosphotyrosine oligonucleotides were typically only obtained in 60–75% yield, as compared with unmodified oligonucleotides.

Oligonucleotide purification and sequences

All oligonucleotides were purified by polyacrylamide gel electrophoresis [12–18% polyacrylamide (19:1)–8 M urea, 0.5× TBE

(44 mM Tris, 44 mM boric acid, 1 mM Na_2-EDTA)], electro-eluted (Schleicher and Schuell Elutrap) in 0.5× TBE, concentrated by lyophilization and desalted over a Bio-Gel P6 (BioRad) spin column. The following oligonucleotides were used in this study (written 5′→3′, s designates position of 5′-bridging phosphorothioate, hs designates a 5′-sulfhydryl, Y designates tyrosine): oHN69S, TCCGTTGAAGCCTGCTTTsTTTATACTAACTTGAGC; oHN69, TCCGTTGAAGCCTGCTTTTTTATACTAACTTGAGC; oHN68, TCGCTCAAGTTAGTATAAAAAAGCAGGCTTCAACG; oHN204S, GAGGATCTAAAAGACTTsTGAAAAATTT; oHN204, GAGGATCTAAAAGACTTTGAAAAATTT; oHN205, AAATTTTTCAAAGTCTTTTAGATCCTC; oHN206, GAGGATCTAAAAGACTTTG; oHN66Y, TCCGTTGAAGCCTGCTTTY; oHN67, TTTATACTAACTTGAGC; oHN67SH, hsTTTATACTAACTTGAGC.

Oligonucleotide labeling reactions

The oligonucleotides were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Typically, a 50 µl reaction mixture containing 200 µCi [γ-^{32}P]ATP (800 Ci/mmol; NEN), 10 U T4 polynucleotide kinase (New Engand Biolabs), 0.5 µmol oligonucleotide, 50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine and 0.05% NP-40 was incubated at 37°C for 30 min, heated to 80°C for 5 min and passed over a Bio-Gel P-6 spin column (BioRad). The specific activity of the oligonucleotide was determined following polyethyleneimine thin layer (Sigma) chromatographic analysis (0.75 M KH_2PO_4, pH 3.5) and scintillation counting. The oligonucleotides were 3′-end-labeled using [α-^{32}P]dATP and terminal deoxynucleotide transferase. Typically, a 50 µl reaction mixture containing 200 µCi [α-^{32}P]dATP (800 Ci/mmol; NEN), 20 U terminal deoxynucleotide transferase (TdT; Pharmacia LKB), 0.5 µmol oligonucleotide, 50 mM sodium cacodylate, pH 7.0, 5 mM $MgCl_2$, 1 mM DTT was incubated at 37°C for 20 min. It should be noted that TdT is more active when $CoCl_2$ is the only divalent cation. However, $MgCl_2$ was substituted since $CoCl_2$ led to moderate hydrolysis of 5′-bridging phosphorothioate-containing oligonucleotides. Reaction products resulting from a single nucelotide addition were purified on a 16% acrylamide (19:1)–8 M urea, 0.5× TBE gel, electroeluted (Schleicher and Schuell EluTrap apparatus) in 0.5× TBE, concentrated by lyophilization and desalted over a Bio-Gel P6 spin column (BioRad). Duplex substrates were prepared by mixing the appropriate oligonucleotides (a 5-fold molar excess of unlabeled oligonuceotide) in 0.1 M KCl and 10 mM Tris–HCl, pH 8.0, at 37°C for 10 min. The concentration of duplex substrates was based on the specific activity of the limiting oligonucleotide.

Cleavage, ligation and recombination reactions

Topoisomerase cleavage reactions (20 µl) were incubated at room temperature and contained 50 mM Tris, pH 7.5, 1 mM Na_2-EDTA, 1 mM DTT, 10% glycerol, 50 mM NaCl, 200 µg/ml BSA, 20 nM substrate, 50 nM calf thymus topo I. Reactions were quenched by adding an equal volume of 10 M urea, 0.2% SDS and heating to 95°C for 2 min. Calf thymus topo I was a generous gift of Dr Leroy Liu.

Int-dependent cleavage reactions (20 µl) were incubated at room temperature and contained 50 mM Tris–HCl, pH 8.0, 1 mM Na_2-EDTA, 70 mM KCl, 10% glycerol, 200 µg/ml BSA, 100 nM substrate, 50 nM lambda Int. Int and IntY342F were prepared as previously described (21). Intasome-dependent cleavage and recombination reactions (20 µl) were incubated at room temperature and contained 50 mM Tris–HCl, pH 8.0, 1 mM Na_2-EDTA, 70 mM KCl, 10% glycerol, 200 µg/ml BSA, 100 nM *att*B substrate, 20 nM supercoiled *att*P plasmids (pHN894; described in 20), 300 nM Int, 100 nM IHF. IHF was prepared as previously described (22). Reactions were quenched by adding an equal volume of 10 M urea, 0.05% SDS and heating to 95°C for 2 min.

Int-dependent ligation reactions (50 µl) contained 100 nM substrate, 100 nM IntY342F, 50 mM Tris–HCl, pH 9.0, 20 µg/ml BSA, 60 mM KCl. Reactions were incubated at room temperature and aliquots were removed, quenched by adding an equal volume of 10 M urea, 0.05% SDS, 5 mM DTT and heated to 95°C for 2 min.

RESULTS

The effect of a phosphorothioate substitution on the cleavage/ligation equilibrium of an enzyme can best be examined when the modified phosphodiester is placed at a preferred point of attack. Sequence preference is a defining characteristic for the site-specific recombinases. Moreover, although eukaryotic topo I can act on any DNA, a preferred DNA binding and cleavage site has been defined (23). When a phosphorothioate linkage is placed at a preferred site of cleavage of either calf thymus topo I or bacteriophage lambda Int, cleaved DNA accumulates. To demonstrate this, 3′-end-labeled DNA containing a single 5′-bridging phosphorothioate linkage was incubated with or without enzyme and the products resolved on a denaturing gel (Fig. 2). Incubation of the OPS substrates with heavy metals (Hg^{2+}) produces fragments which co-migrate with the enzyme-dependent cleaved products (data not shown), confirming that cleavage is at the site of the modification. When 5′-end-labeled substrates are analyzed by SDS–PAGE, a radioactive band accumulates that is of the size expected for the protein covalently joined to DNA (see Figs 4 and 5 below). For the Int reaction, if the DNA contains the OPS linkage but lacks an Int binding site or if a mutant Int lacking an active tyrosine (Int Y342F) is used, no cleaved product is observed (data not shown). Thus cleavage reflects an effect of Int at its normal site of action. In addition, pre-incubation of Int with an excess of OPS-containing DNA destroys its capacity to recombine standard substrates and the presence of OPS-containing *att*B DNA decreases the rate and extent of recombinant product formed with standard *att*B in an integrative recombination reaction. These inhibitory effects provide evidence that the modified substrate binds to its enzyme irreversibly.

In contrast to the substantial amount of cleaved product seen with phosphorothioate substrates, little or no cleaved DNA is visible when unmodified DNA is incubated with topo I or Int (Fig. 2). This low level cleavage presumably represents the steady-state concentration of cleaved intermediate that is established during the reaction with unmodified DNA and has been reported before (1,5). It should also be noted that when the Cre recombinase (a gift from R. Hoess), another member of the integrase family of recombinases (24), is incubated with the appropriate OPS-containing substrate, cleaved DNA also accumulates (data not shown).

Elementary theory suggests that the primary effect of the OPS modification is to suppress the ligation reaction (see Discussion). To test the competence of the 5′-sulfhydryl that is generated by

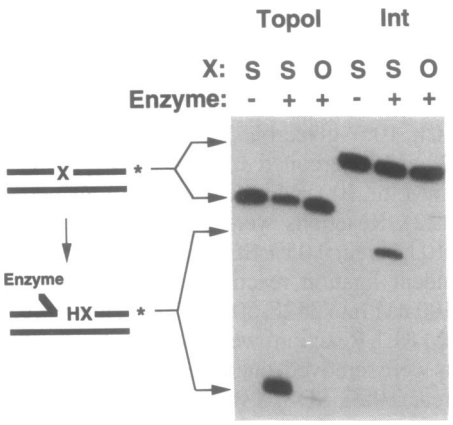

Figure 2. Phosphorothioates trap strand cleavage products. The topo I substrates (lanes 1–3) were prepared by hybridizing 3′-end-labeled oHN204 (O) or oHN204S (S) with oHN205. The Int substrates (lanes 4–6) were prepared by hybridizing 3′-end-labeled oHN69 (O) or oHN69S (S) with oHN68. After incubation (20 min) with the appropriate enzyme, samples were boiled and immediately loaded onto an 18% acrylamide (19:1)–8 M urea, 0.5× TBE gel. The position of topo I and Int substrates (23 and 35 nt, respectively) and the resulting cleavage products (10 and 17 nt) are indicated to the left of the autoradiogram.

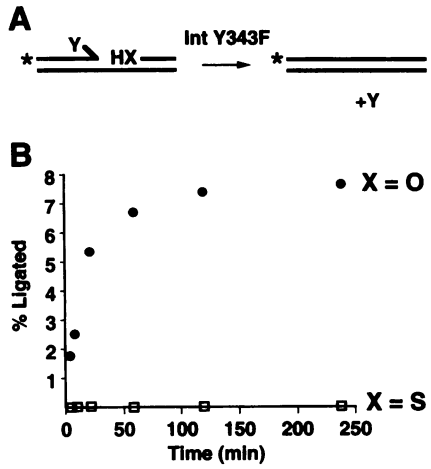

Figure 3. 5′-Sulfhydryl is incompetent for strand ligation. (**A**) Lambda integrase substrates containing a pre-activated 3′-phosphotyrosine and a 5′-hydroxyl or 5′-sulfhydryl were incubated with IntY342F so that the ligation reaction could be monitored in the absence of competing cleavage reactions. The duplex corresponds to an *att*B substrate containing a nick at the site of top strand exchange and was formed by hybridizing three oligonucleotides (oHN66Y, oHN68 and either oHN67 or oHN67SH). (**B**) The oligonucleotide containing the 3′-phosphotyrosine (oHN66Y, 17 nt) was 5′-end-labeled so that strand ligation results in a unique size fragment (35 nt) in a denaturing gel [16% acrylamide (19:1)–8M urea, 0.5× TBE]. The fraction of the substrate converted to this product was quantitated with a phosphorimager and subsequent densitometry analysis.

cleavage for ligation, we exploited a strategy that specifically isolates the ligation step (k_l) in the topoisomerase cycle. Pan *et al.* (19) have shown that Int can ligate a DNA strand bearing a 5′-OH to another strand that is activated by the presence of a 3′-phosphotyrosine. If a mutant recombinase that is defective in cleavage (IntY342F) is used, the ligation reaction can be studied in the absence of the competing cleavage reaction. Accordingly, duplex DNA substrates with nicks containing a 3′-phosphotyrosine and a 5′-hydroxyl or 5′-sulfhydryl were incubated with IntY342F (Fig. 3A). The fraction of the DNA ligated as a function of time is shown in Figure 3B. No ligation product is observed when a 5′-sulfhydryl is present at the joining site. Addition of divalent cations (Mg^{2+}, Mn^{2+}, Co^{2+} or Zn^{2+}) or a reducing agent (DTT) or changing the pH (from 7 to 11) did not activate the 5′-sulfhydryl (data not shown).

To compare the effectiveness of the chemical modification described in this work with more traditional approaches, we have measured the rate of topo I-induced cleavage of two substrates, one containing a phosphorothioate and the other a half-site designed to block re-ligation by release of a short (2 nt) fragment. Each substrate (Fig. 4) was 5′-end-labeled and incubated with calf-thymus topo I, aliquots were removed after various times, quenched with SDS and resolved on an SDS–polyacrylamide gel. In both cases a band (shown in the insets) accumulates that migrates at the position expected for the enzyme–DNA covalent complex (~60 kDa) and is both protease and nuclease sensitive (data not shown). As shown in Figure 4B, the OPS substrate is >100 times more efficient (based on comparison of initial rates) at trapping the enzyme–DNA complex than the topo I half-site. Half-site substrates that generate a 6 nt fragment were also tested and were even less efficient at trapping the enzyme–DNA complex than the substrate used in Figure 4 (data not shown). As a control, unmodified full sites were incubated with topo I; only very low levels of topo I–DNA complexes accumulated (such complexes are not visible with similar autoradiogram exposure times and are not shown in the Fig. 4 insets). The amount of these complexes did not increase with time, a result that is expected if this product represents the steady-state concentration of enzyme–DNA covalent intermediate during multiple cleavage/ligation cycles.

Although Int protein alone is clearly able to attack a phosphorothioate-modified *att*B (Fig. 2), we believe this capacity is only indirectly germane to the recombination reaction. This is because the affinity of Int for the core sites of *att*B is so low that, under recombination conditions, virtually all of the Int is bound specifically and/or non-specifically to the supercoiled partner DNA (15). Indeed, when supercoiled pBR322 DNA is added to reaction mixtures, Int-promoted cleavage of an *att*B phosphorothioate suicide substrate drops to low levels (data not shown). The same result is seen when the partner DNA contains an *att*P (Fig. 5, lanes 3 versus 5). However, in this case (but not with pBR322) addition of IHF protein restores the capacity of Int to covalently alter *att*B (Fig. 5, lane 2). As diagramed in Figure 5, two kinds of products are generated. One is a duplex bearing a covalently attached Int; this is the same product that is made in the Int-only reaction (Fig. 5, lane 5). The other is a recombinant product in which the 5′-OH of a broken DNA strand from the *att*P partner has displaced the Int covalently attached to *att*B and thereby joined the two DNAs. It is important to note that both kinds of product depend upon IHF (Fig. 5, lanes 2 versus 3). Since IHF has no effect on the Int-only reaction (Fig. 5, lanes 4 versus 5), we conclude that IHF does not directly influence Int or *att*B, but serves to activate *att*P so that it can attack the *att*B partner, i.e. IHF is needed to make an active intasome. The experimental distinction between Int-dependent cleavage and intasome-dependent cleavage can be made even simpler by labeling the 3′-end of the top strand of an *att*B phosphorothioate suicide

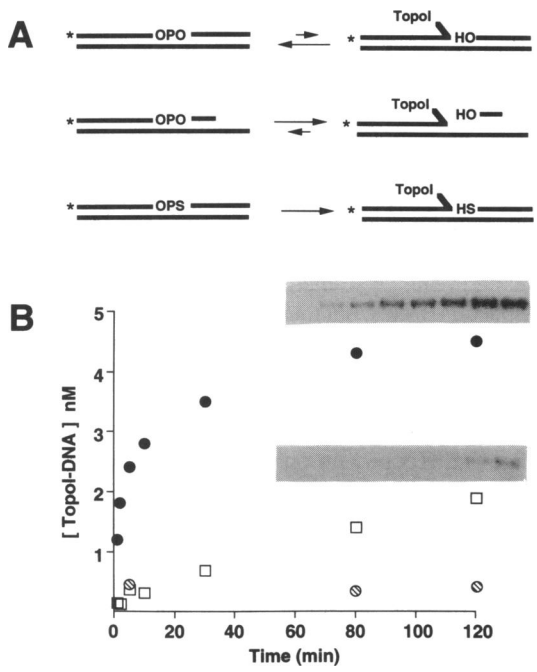

Figure 4. Comparison of phosphorothioate and half-site suicide substrates. (**A**) The top panel diagrams a topoisomerase reaction on a conventional duplex (oHN204, oHN205). The middle panel diagrams a half-site reaction (oHN206, oHN205); cleavage by topo I generates a 2 nt fragment that is subject to loss by diffusion. The bottom panel diagrams a phosphorothioate (OPS) cleavage reaction (oHN204S, oHN205); cleavage by topo I generates a 5′-sulfhydryl that is chemically inert in the ligation reaction. An asterisk marks the position of the radiolabel (5′-end of cleaved strand). The full duplex substrates contain the entire topo I binding site, as defined by Christiansen *et al.* (25), except for a single A→T transversion immediately 3′ to the cleavage site, introduced to permit use of the modified thymidine phosphoramidite. (**B**) Reaction aliquots (5 µl) were removed at 2, 5, 10, 15, 30, 80 and 120 min, quenched and then resolved on a 15% acrylamide (33:1)–SDS–tricine gel. The amount of this product was quantitated by densitometric analysis of product and substrate bands and is graphed as a function of time. Data for the phosphorothioate, half-site and conventional duplex substrates are shown as filled circles, empty squares and hatched circles, respectively.

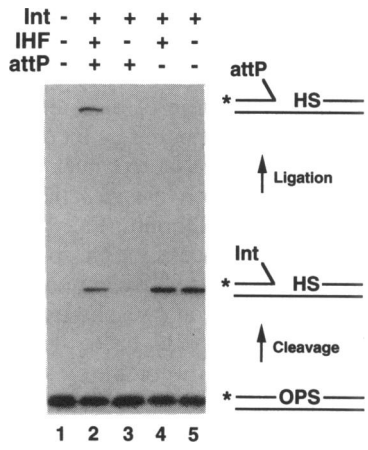

Figure 5. Int versus intasome cleavage reactions. Phosphorothioate-containing (oHN69S, oHN68) *att*B substrates, radiolabeled at the 5′-end of the modified strand, were incubated (30 min) in the presence (+) or absence (–) of Int, IHF and a supercoiled *att*P plasmid. Products were resolved on a 3.5% acrylamide (33:1)/15% acrylamide (33:1)–SDS–tricine gel. The gel was fixed (10% acetic acid, 10% methanol), dried and the resulting autoradiogram is shown. Cleavage of the OPS-containing *att*B substrate results in covalent attachment of Int to the DNA. If the Int–DNA phosphodiester bond (3′-phosphotyrosine linkage) is attacked by a 5′-OH from *att*P (generated when another protomer of Int cleaves *att*P), the ligated product will migrate near the top of the gel. The structure of the substrate (23 kDa) and products of the cleavage (60 kDa) and ligation (2950 kDa) reactions are diagramed to the right of the autoradiogram (the asterisk marks the position of the radiolabel). Because of the inability of the 5′-sulfhydryl end of the broken strand of *att*B to participate in any ligation reaction, the *att*P component of the 'recombinant' product (i.e. the *att*P–*att*B complex) is expected to contain a broken DNA strand.

substrate and analyzing the products on a denaturing gel. Here the only product from both the Int and intasome pathways is the released fragment of the top strand. Nevertheless, the same pattern of IHF dependence is seen; IHF is required for cleavage when an *att*P partner is present and does not enhance the level of Int-only cleavage (data not shown).

We have used the ability to define both Int- and intasome-dependent *att*B cleavage reactions to better understand the requirements of the recombination reaction (Table 1). For example, it has been previously shown that spermidine is absolutely required for recombination *in vitro* (26). We found that spermidine is required for intasome-dependent cleavage, however, it does not affect the rate of Int-dependent cleavage. Similarly, supercoiling of intasome DNA is required for both intasome-dependent cleavage and recombination. In the integrative recombination reaction the intasome forms on *att*P; in a different reaction (termed excisive recombination) the products of integrative recombination, *att*L and *att*R, recombine with each other. Although both *att*L and *att*R contain high affnity Int arm-type binding sites, *att*L and *att*R are not capable of recombining with *att*B. We tested the ability of *att*L or *att*R to function as an intasome in the cleavage of *att*B; neither *att*L nor *att*R could replace *att*P. Presumably they do not generate the proper architecture. Finally, the excisive recombination reaction requires an additional protein co-factor, Xis, which inhibits integrative recombination. We found that Xis also inhibits intasome-dependent cleavage, but has no effect on the rate of Int-dependent cleavage.

Table 1. Summary of requirements for Int- and intasome-dependent reactions with *att*B

	OPS Cleavage		Recombination
	Int	Intasome	Intasome
IHF	No effect	Required	Required
Spermidine	No effect	Required	Required
Xis	No effect	Inhibitory	Inhibitory
*att*P supercoiling	NA	Required	Required
*att*L or *att*R (no *att*P)	NA	Unreactive	Unreactive

*att*B duplexes, containing either a phosphorothioate or phosphodiester linkage at the scissile position of the top strand were assayed for cleavage or recombination, respectively, as described in Materials and Methods.
NA indicates not applicable.

DISCUSSION

We have shown that, for DNA containing 5′-bridging phosphorothioate (OPS) linkages, the equilibrium associated with elemen-

tary topoisomerase reactions is shifted in favor of the enzyme–DNA complex. Unmodified DNA can be cleaved by Int or topo I, but the covalent enzyme–DNA complex does not accumulate, presumably because of more efficient re-ligation. OPS-containing DNA is also cleaved by Int or topo I, but in this case the covalent enzyme–DNA complex accumulates. We conclude that the modification changes the direction of the enzyme cleavage/ligation equilibrium (Fig. 1) from $K^O << 1$ to $K^S >> 1$. How does the presence of the phosphorothioate perturb the cleavage–ligation equilibrium? We reasoned that since sulfur is usually a poor nucleophile at phosphorus (27), the primary effect of the modification would be to depress the ligation reaction. We tested this directly for Int by synthesizing oligonucleotides containing a 5′-sulfhydryl (5′-SH) and comparing it with a conventional (5′-OH) oligonucleotide in an assay that uncouples topoisomerase catalyzed ligation (k_l) from cleavage (k_c). Using this assay we have not been able to detect ligation product when a 5′-SH is present at the ligation site (Fig. 3). Although this experiment does not allow the direct measurement of k_l, it does set boundaries on the relative rates of k_l^O and k_l^S; we estimate $k_l^O > 100 \times k_l^S$. Future experiments will analyze the kinetics of binding, cleavage and ligation. However, it can be initially concluded that the observed equilibrium shift is due, at least in large part, to a dramatic decrease in the rate of re-ligation (k_l). The ability of the phosphorothioate linkage to shift the enzyme-imposed equilibrium may also benefit from an increase in k_c. We have not measured k_c, but one would expect $k_c^S > k_c^O$, since a thio-anion is a better leaving group than an oxy-anion (28). We doubt that this difference is responsible for the higher efficiency of OPS suicide substrates than nicked suicide substrates; the bulk of this difference is probably due to the higher binding efficiency of the full site (see Discussion below).

The ability of the phosphorothioate linkage to efficiently trap the enzyme–DNA intermediate contrasts with the results of Picirilli *et al.* (29), who have shown that in a transesterification reaction catalyzed by the *Tetrahymena* ribozyme a 3′-bridging phosphorothioate is cleaved 1000 times more slowly than an unmodified phosphodiester. The ribozyme requires Mg^{2+} ions for cleavage in the unmodified reaction. In the phosphorothioate cleavage reaction addition of Mn^{2+} or Zn^{2+} relieves the negative effect. Since sulfur binds Mn^{2+} and Zn^{2+} much better than Mg^{2+}, these results argue that a metal ion contributes directly to catalysis by stabilizing negative charge on the leaving group. Unlike the *Tetrahymena* ribozyme, both Int and topo I have no divalent cation requirement. Indeed, all experiments described here were performed in the presence of a chelating agent (EDTA). Kinetic studies have suggested that although divalent cations may stimulate some type I topoisomerases, they do not participate directly in catalysis (9). Taken together, these results argue that bridging phosphorothioates can help distinguish two fundamentally different classes of phosphoryl transfer reactions, those that do and do not use magnesium to stabilize leaving groups. Our results also argue that 3′-bridging phosphorothioates (30) will be useful in the analysis of enzymes that form transient 5′-DNA protein linkages, for example members of the resolvase family of recombinases (31).

To demonstate the utility of these substrates, we have applied them to the analysis of lambda integrative recombination. In this reaction the active enzyme is not Int by itself, but a higher order structure termed the intasome, composed of Int, IHF and supercoiled *att*P. This analysis, however, is complicated by the fact that although only the intasome is capable of carrying out the entire recombination reaction, both Int and the intasome are capable of cleaving *att*B (5,32). Suicide substrates have therefore often relied upon the ability to isolate subsequent strand joining products in order to confirm the involvement of the intasome. Using the phosphorothioate-containing *att*B substrate we show that it is possible to assay *att*B cleavage directly and to define alternative conditions where the observed cleavage is either due to Int (Int-dependent) or to the intasome (intasome-dependent). Earlier experiments examined intasome-dependent cleavage of heteroduplex *att*B sites, but these substrates contained distortions that might have perturbed their function. Our current study confirms the earlier conclusion that capture of *att*B by the intasome requires that the latter be supercoiled and have both P and P′ arms. In addition, we have examined the influence of spermidine and Xis protein on the capture process. It has been previously demonstrated that spermidine is required to obtain recombinant product (26). However, since intermediates during lambda integration are not easily visualized, it was not known what stage in the reaction depends on a polyvalent cation. Because spermidine is not required for (in fact, inhibits) topoisomerase activity and resolution of Holliday structures (33), it is unlikely that this agent is needed in the catalysis of the overall reaction. Consistent with this view, we find that spermidine is not required for Int-dependent cleavage of *att*B. However, since this polyamine is needed for intasome-dependent cleavage, spermidine must be required for an early step, such as formation of an active intasome or synapsis with *att*B. Of course, spermidine may have multiple roles and we cannot rule out the possibility that spermidine is also involved in later steps in the recombination pathway. In addition, it had been previously demonstrated that the protein Xis, which is required as a co-factor in the excision reaction, inhibits integrative recombination (34,35). We have found that Xis inhibits intasome-dependent cleavage, but has no effect on Int-dependent cleavage of *att*B. These results argue that Xis does not interact with Int directly, modifying its activity, but interacts through the intasome. This conclusion supports the idea that Xis modifies the structure of the intasome so that it is incapable of binding or cleaving *att*B (20,36).

DNA substrates containing bridging phosphorothioate linkages offer several advantages over nicked suicide substrates (half-sites) in the analysis of elementary topoisomerase reactions. First, the chemical modification minimizes pleiotropic effects on the reactions that follow from structural perturbations introduced by the nick. This is probably why a DNA duplex containing a phosphorothioate linkage is a much better substrate than a half-site in the topoisomerase cleavage reaction (Fig. 4). Christiansen *et al.* (25) have demonstrated that topo I recognizes sequences 3′ to the cleavage site; such sequences are not stably duplex in the half-site substrates. Similar considerations probably explain the superior performance of phosphorothioate substrates compared with half-site substrates in intasome-dependent cleavage reactions (unpublished observations). Another structural distinction between phosphorothioate and nicked suicide substrates is only apparent after cleavage: because no oligonucleotides are lost, the OPS DNA generates a product that much more closely mimics that generated under standard conditions. This is of particular concern in site-specific recombination, since the overlap region, a portion of which is entirely removed by cleavage of a nicked suicide substrate, plays a significant role in later steps in the reaction. We have used OPS substrates to

investigate the role of the overlap region in maintaining the fidelity of the reaction Burgin and Nash, submitted. Secondly, the modification can be placed within a supercoiled DNA duplex. This is important, since the natural substate of eukaryotic topo I is superoiled DNA and the phage attachment site (*att*P) must be supercoiled to obtain recombinant product. In addition, it is not possible to place multiple suicide nicks within a single molecule using nicked suicide substrates. This is important, since two strand exchange reactions, which are only separated by 7 bp, occur during lambda recombination. We have placed the OPS modification at both sites of strand exchange in *att*B and have identified factors dictating *att*B polarity that had been previously unrecognized (Burgin, Robertson and Nash, in preparation). Finally, the efficiency of nicked suicide substrates depends upon the rate of diffusion of the released incised fragment relative to the rate of strand ligation, a ratio that cannot be easily controlled or measured. In addition, it has been demonstrated that the small incised fragment that is released by nicked suicide substrates can re-attack the enzyme–DNA complex (8). The use of OPS-containing substrates avoids these limitations.

In conclusion, because bridging phosphorothioate substrates are very efficient at trapping the covalent enzyme–DNA complex, these analogs are potential topo I inhibitors *in vivo*. For example, duplex oligonucleotides containing the modification could be delivered to cells leading to depletion of functional topo I. Moreover, phosphoryl transfer reactions, even those without enzyme-bound intermediates, are potentially subject to reversal; our results indicate that bridging phosphorothioates can be used to disrupt the equilibria associated with many different enzyme-catalyzed phosphoryl transfer reactions.

ACKNOWLEDGEMENTS

We thank Serge Beaucage for assistance with oligonucleotide synthesis and Todd Capson for advice on phosphoramidite synthesis. AB was supported in part by the Damon Runyon–Walter Winchell Cancer Research Fund. BH was supported by the Howard Hughes Medical Insititute. We thank Drs Fritz Eckstein and James Wang for reading early drafts of the manuscript.

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