Vaccinia DNA topoisomerase forms a covalent DNA-(3'-phosphotyrosyl)-enzyme intermediate at a specific target site 5'-C+C++C++T++TTPp \downarrow N^{-1} in duplex DNA. Here we study the effects of nonpolar pyrimidine isosteres difluorotoluene (F) and monofluorotoluene (D) and the nonpolar purine analog indole at individual positions of the scissile and noncscissile strands on the rate of single-turnover DNA transesterification and the cleavage-religation equilibrium. Comparison of the effects of nonpolar base substitution to the effects of abasic lesions reported previously allowed us to surmise the relative contributions of base-stacking and polar edge interactions to the DNA transesterification reactions. For example, the deleterious effects of eliminating the +2T base on the scissile strand were rectified by introducing the nonpolar F isostere, whereas the requirement for the +1T base was not elided by F substitution. We impute a role for +1T in recruiting the catalytic residue Lys-167 to the active site. Topoisomerase is especially sensitive to suppression of DNA cleavage upon elimination of the +4G and +3G bases of the noncissile strand. Indole provided little or no gain of function relative to abasic lesions. Inosine substitutions for +4G and +3G had no effect on transesterification rate, implying that the guanine exocyclic amine is not a critical determinant of DNA cleavage. Prior studies of 2-aminopurine and 7-deazaguanine effects had shown that the O6 and N7 of guanine were also not critical. These findings suggest that either the topoisomerase makes functionally redundant contacts with polar atoms (likely via Tyr-274, a residue important for pre-guanine were also not critical. These findings suggest that either the requirement for the 5'-CCCTT/3'-GGGAAT target sequence triggers conformational changes in the enzyme that recruit the full set of catalytic side chains into the active site, a process that entails protein contacts with several of the nucleotide bases and specific atoms of the phosphate backbone of DNA within and immediately flanking the CCCTT element (7–12).

Position-specific base modifications have been used to systematically address the features of the individual target site bases that affect the kinetics of DNA transesterification (13–17). The sensitivity of this method depends on the degree to which the particular modification alters the size, shape, stacking, and hydrogen-bonding potential of the base or base pair. Studies to date have entailed relatively small additions to, or subtractions from, the standard base structures as well as modification by more bulky adducts. The substituent addition approach has pinpointed many DNA structural elements that are not functionally relevant.

To survey the base moieties that are relevant to catalysis, Tian et al. (10) replaced each nucleoside of the 5'-CCCTT \downarrow A/3'-GGGAAT target site with a tetrahydrofuran abasic nucleoside. They found that the rate of DNA cleavage was reduced by factors of 350, 250, 60, and 10 when abasic sites replaced the −1A, +1T, +2T, and +4C bases of the scissile strand, but abasic lesions at +5C and +3C had little or no effect. Abasic lesions in the noncissile strand in lieu of +4G, +3G, +2A, and +1A reduced the rate of cleavage by factors of 130, 150, 10, and 5, whereas abasic lesions at +5G and −1T had no effect. The striking positional asymmetry of abasic interference on the scissile and noncissile strands highlighted the importance of individual bases (+4G, +3G, +2T, and +1T), not base pairs, in promoting DNA cleavage. The simple interpretation of the findings was that abasic interference is a consequence of the loss of base-specific contacts between the topoisomerase and the 5'-CCCTT/3'-GGGAAT element. Because the +4G, +3G, +2T, and +1T abasic lesions exclusively or selectively interfered with the cleavage reaction, but not the religation step, it was inferred that contacts to these bases are needed to promote active site assembly prior to cleavage but are not required to maintain the active site once the covalent intermediate is formed (10).
The missing base strategy does not parse the functionally relevant base atoms or the contributions of base stacking. To address these issues, we have conducted an analysis of the effects of nonpolar base isosteres and analogs at individual positions of the scissile and the nonscissile strands within the target sequence. The structures of the pyrimidine isosteres difluorotoluene (F₃, an analog of thymine) and monofluorotoluene (D, an analog of cytosine) are shown in Fig. 1. Both compounds lack the N1 and N3 atoms of the pyrimidine ring. The F base contains fluorine in lieu of the O2 and O4 atoms of thymine; the D base contains fluorine in place of O2 and a methyl instead of the 4-amino of cytosine. These pyrimidine isosteres are unable to hydrogen bond to the complementary purine base on the opposite strand or to functional groups on an interacting protein, but they mimic closely the shapes of the natural bases and are able to base-stack normally along the helical axis (18, 19). Indole is an undifferentiated nonpolar purine analog that lacks the N1, N3, and N7 atoms common to adenine and guanine (20). It also lacks the exocyclic substituents at C2 and C6 that distinguish adenine from guanine (Fig. 3). Nonetheless, indole has been shown to base stack proficiently in the DNA helix (20, 21).

Base isosteres and other nonpolar analogs have been exploited to great effect to assess the contributions of Watson-Crick pairing and base stacking to a variety of DNA transactions, especially the basis for the fidelity of DNA polymerase (reviewed in Ref. 22). They have also been used to dissect sequence-specific interactions of repair enzymes with DNA (23). The rationale for using nonpolar bases to study site-specific DNA cleavage by topoisomerase is that a comparison of nonpolar analog effects with abasic interference effects might provide new insights to the relative contributions of base stacking versus base edge contacts to the enzyme in directing the assembly of the active site.

**EXPERIMENTAL PROCEDURES**

**DNA Substrates**—Oligodeoxyribonucleotides containing single pyrimidine isosteres or a single indole in lieu of adenine or guanine were synthesized by solid-phase methodology and purified by reverse-phase high performance liquid chromatography as described previously (23–25). The difluorotoluene phosphoramidite derivative (24) was purchased from Glen Research; the monofluorotoluene and indole phosphoramidite derivatives were prepared as described (20, 24). The CCCTT-containing scissile strands were 5'-32P-labeled by enzymatic phosphorylation in the presence of [γ-32P]ATP and T4 polynucleotide kinase. The labeled oligonucleotides were gel-purified and hybridized to standard or modified nonscissile strand oligonucleotides at a 1:4 molar ratio of scissile to nonscissile strand. Annealing reaction mixtures containing 0.2 μM NaCl and oligonucleotides were heated to 80 °C and then slow-cooled to 22 °C. The hybridized DNAs were stored at 4 °C. The structures of the annealed duplexes and the sequences of the component strands are depicted in the figures.

**Vaccinia Topoisomerase**—Recombinant vaccinia topoisomerase was produced in *Escherichia coli* (BL21) by infection with bacteriophage λCE6 (26) and then purified to apparent homogeneity from the soluble bacterial lysate by phosphocellulose and Source S-15 chromatography steps. Protein concentration was determined by using the dye-binding method (Bio-Rad) with bovine serum albumin as the standard.

**Effect of Pyrimidine Isosteres on Single-turnover DNA Cleavage**—Reaction mixtures containing (per 20 μl) 50 μM Tris-HCl (pH 7.5), 0.3 pmol of CCCTT-containing DNA, and 75 ng (2 pmol) of vaccinia topoisomerase were incubated at 37 °C. The reactions were initiated by adding topoisomerase to prewarmed reaction mixtures. Aliquots (20 μl) were withdrawn at various times and quenched immediately with SDS (1% final concentration). The products were analyzed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS. Free DNA migrated near the dye front. Covalent complex formation was revealed by transfer of radiolabeled DNA to the topoisomerase. The extent of covalent complex formation was quantified by scanning the dried gel using a FujiFilm BAS-2500 imager. A plot of the percentage of input DNA cleaved versus time established the end point values for cleavage. The data were then normalized to the end point values (defined as 100%), and the cleavage rate constants (kₜ) were calculated by fitting the normalized data to the equation 100 − % cleavage norm = 100e⁻kt.

**Effects of Indole Substitutions on Single-turnover DNA Cleavage**—Reaction mixtures containing (per 20 μl) 50 μM Tris-HCl (pH 7.5), 0.3 pmol of CCCTT-containing DNA, and 75 ng (2 pmol) of vaccinia topoisomerase were incubated at 37 °C. The reactions were initiated by adding topoisomerase to prewarmed reaction mixtures. Aliquots (20 μl) were withdrawn at various times and quenched immediately with SDS (1% final concentration). The samples were digested for 60 min at 37 °C with 10 μg of proteinase K, then mixed with an equal volume of 95% formamide/20 mM EDTA, heat-denatured, and analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 μM urea in Tris borate-EDTA. Transmethylation was evinced by the conversion of the input radiolabeled 18-mer scissile strand to a more rapidly migrating product consisting of labeled 12-mer DNA linked covalently to short peptides. The extent of covalent adduct formation was quantified by scanning the gel. A plot of the percentage of input DNA cleaved versus time established the end point values for cleavage. The data were then normalized to the end point values (defined as 100%), and the cleavage rate constants (kₜ) were calculated by fitting the normalized data to the equation 100 − % cleavage norm = 100e⁻kt.

**Single-turnover Religation**—Cleavage reaction mixtures containing (per 20 μl) 0.3 pmol of 32P-labeled 18-mer/30-mer DNA, and 75 ng of topoisomerase were incubated at 37 °C for 15 min (unmodified, −2F, +2F, +3D, +4D, +5D, and +1 indole substrates), 60 min (+1F substrate), or 120 min (+2, +3, and +4 indole substrates) to form the suicide intermediate. Religation was initiated by the simultaneous addition of NaCl to 0.5 M and a 5'-OH 18-mer acceptor strand d(ATTCCGATGACTACA) to a concentration of 15 pmol/22 μl (i.e., a 50-fold molar excess over the input DNA substrate). Aliquots (22 μl) were withdrawn at times specified in the figures and quenched immediately with 1% SDS. A time 0 sample was withdrawn prior to addition of the acceptor strand. The samples

The abbreviations used are: F, difluorotoluene; D, monofluorotoluene.
were digested for 60 min at 37 °C with 10 μg of proteinase K, then mixed with an equal volume of 95% formamide/20 mM EDTA, heat-denatured, and analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 M urea in Tris borate-EDTA. Religation of the covalently bound 12-mer strand to the 18-mer acceptor DNA yielded a 5′-32P-labeled 30-mer strand transfer product. The extent of religation (expressed as the percent of the covalent intermediate converted into 30-mer) was plotted as a function of reaction time. 

**Equilibrium Cleavage**—A 34-mer CCCTT-containing oligonucleotide (5′-32P-labeled, then gel-purified and annealed to an unlabeled complementary 30-mer strand to form a duplex containing 12-bp of DNA 5′ to the cleavage site and 18-bp 3′ to the cleavage site. Reaction mixtures containing (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 34-mer/30-mer DNA, and 9, 18, 37, 75, 150, or 300 ng of topoisomerase were incubated at 37 °C for 20 min. The reactions were initiated by the addition of topoisomerase to prewarmed reaction mixtures. The reaction 37 °C for 20 min. The reactions were initiated by the addition of topoisomerase to prewarmed reaction mixtures. The reaction was quenched by adding SDS to 1%. The samples were digested for 60 min at 37 °C with 10 μg of proteinase K, then mixed with an equal volume of formamide/EDTA, heat-denatured, and analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 M urea in Tris borate-EDTA. The cleavage product, a 32P-labeled 30-mer strand, was well resolved from the noncovalently bound DNA at the reaction end point under conditions of saturating enzyme and was calculated according to the equation $K_{cl} = \%$ cleaved/(100 − \% cleaved).

**RESULTS**

Effects of Nonpolar Pyrimidine Isosteres in the Scissile Strand—A series of oligodeoxynucleotide 18-mer scissile strands containing a single pyrimidine isostere within the 5′-C+5′C+C+4′T+2′T+1A−1T−2 sequence were 5′-32P-labeled and then annealed to an unlabeled 30-mer strand to form “suicide” cleavage substrates for vaccinia topoisomerase (Fig. 1). The cleavage transesterification reaction of vaccinia topoisomerases results in covalent attachment of the 3′P-labeled 12-mer 5′-pCGTGTCGCCCTTp to the enzyme via Tyr-274 (12, 27). The unlabeled 6-mer 5′-OH leaving strand ATTTCC dissociates spontaneously from the protein-DNA complex. Loss of the leaving strand drives the reaction toward the covalent state, so that the reaction can be treated kinetically as a first-order unidirectional process. The reaction of excess topoisomerase with the unmodified control substrate attained an end point at 20 s at which 96% of the DNA was converted to covalent topoisomerase-DNA complex. The extent of transesterification after 5 s was 93% of the end point value. From this datum, we calculated a single-turnover cleavage rate constant ($k_{cl}$) of 0.38 s⁻¹ (Fig. 1).

The D or F isosteres spanning positions +5 to −2 on the scissile strand had no significant effect on the extents of DNA cleavage (86−92%), but they exerted disparate position-specific effects on the rate of the reaction. The $k_{cl}$ values for the +5D (0.37 s⁻¹) and −2F (0.32 s⁻¹) substrates were similar to that of unmodified DNA. The benigneffect of the +5D analog is consistent with the earlier finding that an abasic lesion at the +5 nucleoside had no significant effect on the kinetic of cleavage (Fig. 1) (10). The rate constants for cleavage of the +4D (0.075 s⁻¹) and +3D (0.1 s⁻¹) substrates were similar (within a factor of two) to the rates of cleavage of the +4 and +3 abasic substrates (Fig. 1). The situation was quite different at the +2T position, where the F isostere ($k_{cl} = 0.1$ s⁻¹) elicited a 14-fold gain-of-function compared with the overly deleterious +2 abasic lesion ($k_{cl} = 0.0073$ s⁻¹). Because the +2F isostere restores a near-normal cleavage rate, we surmise that base stacking or thymine shape recognition is the dominant contribution of the +2 thymine, with polar edge interactions playing a minor role. The +1T base is the most important of the scissile strand pyrimidines, insofar as a +1 abasic lesion slows cleavage by a factor of 200. Although the +1F isostere increased cleavage rate 6-fold compared with the abasic lesion, the fact that the nonpolar analog slowed $k_{cl}$ by nearly a factor of 40 (to 0.01 s⁻¹) relative to unmodified DNA underscores the importance of polar base edge atoms to the function of the +1 thymine base.

The effects of the pyrimidine isosteres on the religation reaction were studied under single-turnover conditions by assaying...
the ability of preformed suicide intermediate to transfer the covalently held 5'-32P-labeled 12-mer strand to a 5'-OH-terminated 18-mer strand to generate a 30-mer product (Fig. 2A). After forming the suicide intermediate on the unmodified 18-mer/30-mer DNA substrate or 18-mer/30-mer DNA containing an isostere at positions +5, +4, +3, +2, or +1 of the scissile strand, the religation reaction was initiated by adding a 50-fold molar excess of the 18-mer DNA acceptor strand complementary to the 5'-single-stranded segment of the nonscissile strand. The religation reaction yields a 30-mer product.

Religation by topoisomerase bound covalently on unmodified DNA was effectively complete within 5 s, the earliest time analyzed. The religation of covalent complexes containing +5D +4D +3D, +2F, or +1F nonpolar isosteres on the scissile strand was virtually indistinguishable from that of unmodified DNA, as gauged by the completeness of the reactions after 5 s (Fig. 2B). (Note that the religation rate constant of vaccinia topoisomerase (k_rel ~1.2 s⁻¹) is too fast to be measured manually, which means that the religation rate would have to be slowed at least severalfold to be detectable in our assay.) The religation results are consistent with the minimal or mild effects of the +5D, +4D, +3D, and +2F isosteres on the rate of the forward cleavage reaction. The +1F isostere, in contrast, exerted a selective slowing of cleavage. We conclude that polar edge contacts of the +1 thymine base play a role in the assembly of a catalytically competent active site during the forward cleavage reaction but are not critical during religation by the covalent topoisomerase-DNA complex, in which the active site is already established.

**Effects of Indole Substitutions at Purine Bases of the Nonscissile Strand**—An unmodified 5'-32P-labeled 18-mer scissile strand was annealed to a series of 30-mer nonscissile strands containing a single nonpolar indole in lieu of either guanine or adenine within the 3'-G-G+3A-A+1T-1 element (Fig. 3). The effects of a +5 indole substitution were not tested because previous studies showed that a +5 abasic lesion had no apparent effect on single-turnover DNA cleavage or religation. The k_rel values for the +1 indole (0.18 s⁻¹), +2 indole (0.03 s⁻¹), +3 indole (0.0013 s⁻¹), and +4 indole (0.01 s⁻¹) substrates were slowed by factors of 2, 13, 300, and 40 relative to the k_rel for unmodified DNA (Fig. 3). These values were similar to the cleavage rate constants reported previously for +1 abasic (0.085 s⁻¹), +2 abasic (0.046 s⁻¹), +3 abasic (0.003 s⁻¹), and +4 abasic (0.0035 s⁻¹) substrates (Fig. 3) (10). The instructive point was that indole failed to substantially reverse the severe cleavage defects accompanying loss of the essential +4G and +3G bases. Indole also conferred no benefit at +2A compared with the abasic lesion. These results suggest that the topoisomerase makes polar contacts to the +4G, +3G, and +2A bases during the forward cleavage reaction.

We assayed religation by preformed topoisomerase-DNA complexes containing indole substitutions on the nonscissile strand. The +1, +2, and +4 indoles had little impact on the rate or extent of religation compared with the unmodified control; these reactions achieved an end point in less than 60 s with 80–90% of the covalent complex present at time 0 being converted to the religated 30-mer product (Fig. 4A). In contrast, the +3 indole-substituted topoisomerase-DNA complex (which had been formed during a 2 h cleavage reaction prior to adding the 18-mer acceptor strand for religation) attained only 40% religation during the same time frame (Fig. 3A). Tracking the +3 indole religation reaction for longer times revealed a biphasic accumulation of 30-mer product, in which a relatively rapid burst of ~40% rejoining (within 30 s) was followed by a slow phase from 1 to 60 min during which the religation product accumulated to ~90% (Fig. 4B). This kinetic profile suggested the existence of two functionally distinct forms of the covalent topoisomerase-DNA complex on the +3 indole-containing DNA (see “Discussion”).
Indole Effects on the Cleavage-Religation Equilibrium—A synthetic 5'-32P-labeled CCCTT-containing duplex containing 12-bp of DNA upstream of the cleavage site and 18-bp of DNA downstream of the cleavage site was employed to assay trans-esterification under equilibrium conditions (Fig. 3). This DNA is an equilibrium substrate because the 5'-OH leaving strand generated upon cleavage at CCCTT remains stably associated with the topoisomerase-DNA complex via base-pairing to the nonscissile strand. We determined by enzyme titration that vaccinia topoisomerase cleaved 23% of the unmodified substrate at saturation. The cleavage-religation equilibrium constant \(K_{cl}\) of the unmodified DNA was 0.29. The cleavage-religation equilibrium constant for the +1 indole-substituted DNA, despite the 8-fold difference in their rates of forward cleavage, underscores that the +1 indole modification also slows the rate of re-ligation under equilibrium conditions. An estimate of the cleavage rates from the observed single-turnover cleavage rate constants and the equilibrium constants (via \(k_{rel} = k_{cl}/K_{cl}\)) gives values of 1.4 and 0.13 s\(^{-1}\) for unmodified DNA and +3 indole DNA, respectively. The +2 indole modification elicited a modest reduction in \(K_{eq}\) to 0.08, which mimicked the effect of the +2 abasic lesion reported previously. These results, which highlight the concordance of indole and abasic effects in suppressing the cleavage phase of equilibrium transesterification, indicate that the contributions of the +4 guanine, +3 guanine, and +2 adenine to the reaction equilibrium are derived mostly from polar interactions of the protein with these bases rather than base stacking.

Base Stacking at the +1A Position Rectifies the Poisoning Effect of an Abasic Lesion—An abasic lesion of at the +1A position of the nonscissile strand acts as a potent topoisomerase poison, reflected in a measured \(K_{cl}\) value of 11.5 (Fig. 3). Measurements of the component rate constants underscored that the +1 abasic lesion had only a modest impact on \(k_{cl}\) but strongly suppressed \(k_{eq}\) (10). Here we found that replacing the +1 adenine with indole reinstated a more balanced cleavage-religation equilibrium (\(K_{eq} = 0.89\)) (Fig. 3). This salutary effect of +1 indole versus +1 abasic modifications necessarily stems from an increase in the rate of re-ligation. These results show that the loss of base stacking in the complementary strand opposes the scissile phosphate largely accounts for the poisoning effect of the abasic lesion.

Inosine Substitutions at +4G and +3G—the large cleavage rate decrements elicited by either removing the +3 and +4 guanines of the nonscissile strand, or replacing them with indole, imply a need for polar contacts to the base edges at these positions. To probe the possible contributions of the exocyclic N2-amine of guanine, we measured the rate of single-turnover cleavage on 18-mer/30-mer suicide substrates...
containing deoxyinosine in lieu of +4G or +3G. Inosine lacks the C2-amine but is otherwise identical to guanosine. The rates and extents of cleavage of the inosine substrates were identical to that of unmodified control DNA tested in parallel (data not shown). We conclude that interactions of topoisomerase with the guanine N2 in the DNA minor groove are not critical for assembly of the active site for the forward cleavage reaction.

DISCUSSION

By probing the effects of nonpolar pyrimidine isosteres and the nonpolar purine analog indole on site-specific DNA trans-esterification by vaccinia virus DNA topoisomerase, we have gained new insights to the relative contributions of base stacking and base edge contacts to the precleavage assembly of the active site and the set point of the cleavage-religation equilibrium. We discuss our findings in light of the crystal structure of the covalent intermediate of poxvirus topoisomerase on a CCCTT-containing DNA, reported recently by Van Duyne and colleagues (12), which provides an invaluable framework for understanding the cornucopia of functional data on the protein-DNA interface (4–10, 13–17, 28–33) and, by comparison to the apoenzyme structure (8), reveals the conformational transitions that accompany DNA-triggered active site assembly.

Fig. 5 shows a stereo view of the topoisomerase-DNA complex (12) that highlights the polar main chain and side chain contacts to the phosphates and bases of the 5'-CCCTT/3'-GGGAA target site. Tyr-274 is attached covalently to the scissile phosphate, which is coordinated by the catalytic residues Arg-130, Arg-223, and His-265. The fourth catalytic residue, Lys167, is located nearby in the minor groove, where it donates a hydrogen bond to the O2 atom of the 1T base. All other polar base contacts are located in the major groove and are made by Gln-69, Tyr-136, and Lys-133, respectively. Tyr-136 makes a bifurcated contact to the N7 atoms of +3G and +4G on the nonscissile strand, while Lys-133 makes a bifurcated hydrogen bond to the O6 and N7 atoms of +5G. Tyr-136 and Lys-133 are located within a putative “specificity helix” extending from residues 132–140 that makes additional interactions with the phosphates of the nonscissile strand that are critical for active site assembly (9). The interactions between the specificity helix and the DNA serve to recruit Arg-130 to the active site. The polypeptide segment that includes Arg-130 and the specificity helix is disordered in the apoenzyme structure (8). This region of the topoisomerase is sensitive to trypsin, chymotrypsin, and V8 proteases but becomes protease-resistant upon DNA binding (7). The crystal structure of the protein-DNA complex reveals how the sites of protease accessibility in the apoenzyme (Lys-135, Tyr-136, and Glu-139) become masked when the helix inserts into the DNA major groove. In the same vein, footprinting with a lysine-modifying agent showed that DNA binding protects at least two of the three lysines in the specificity helix: Lys-133, Lys-135, and/or Lys-139 (34). Gln-69 is the only side chain in the N-terminal domain that make a base-specific DNA contact, a bidentate hydrogen bond from Gln-69 N and O atoms to the 2 adenine N7 and the exocyclic N6 atoms, respectively (Fig. 5).
**Vaccinia Virus DNA Topoisomerase**

![Image](50x57 to 407x734)

**FIGURE 5. Interactions of covalent bound topoisomerase with the 5'CCCTT/3'GGGAA site.** The stereo image was prepared in PyMol (39) using coordinates of the structure of the covalent topoisomerase-DNA complex (12) (Protein Data Bank code 2H7F). Protein contacts to the phosphodiester backbone and the bases are denoted by dashed lines.

cally poised position in the minor groove (Fig. 5). Whereas the recruitment of Lys–167 to the active site might be rate-limiting for cleavage of the +1F substrate, the polar groups on the thymine are apparently not as critical for religation chemistry once the active site has been assembled in the +1F covalent intermediate.

Our finding that the F isostere of +2 thymine restores nearly normal cleavage rate compared with the inhibitory +2 abasic lesion argues that base stacking suffices absent any polar base edge atoms. Indeed, the crystal structure shows no polar contacts between the protein and the +2T (Fig. 5). It is conceivable that the +2 abasic lesion affects cleavage indirectly via changes in the backbone conformation of the scissile strand flanking the abasic site. The crystal structure and methylphosphonate interference experiments (9) highlight the importance of protein contacts to the phosphates that flank the +2T nucleoside (Fig. 5).

The modest effects of the D isosteres in lieu of the +3, +4, and +5 cytosines are similar to those noted previously for the corresponding abasic lesions. These results are in keeping with the absence of any polar contacts to the scissile strand cytosines in the topoisomerase-DNA crystal. Rather, the +3C and +4C deoxycytidine analogs make van der Waals interactions in the major groove with Tyr-72 and Tyr-70. The Tyr-72 and Tyr-70 hydroxyls also coordinate the phosphate backbone of the scissile strand. Yet, mutational analyses and methylphosphonate experiments indicate that the aromatic quality of the Tyr-70 and Tyr-72 side chains (not their polar contacts to phosphates) enables their contributions to supercoil relaxation, DNA binding, and DNA cleavage (9, 35). If, as this suggests, the van der Waals contacts to the +3 and +4 deoxycytidine nucleosides are most relevant, then we might expect them to be unperturbed by substitutions with the D isostere. Thus, the 4–5-fold cleavage rate decrements seen with the +3D and +4D substrates could be indirect consequences of subtle effects of the isostere (or abasic lesions) on the opposing +3 and +4 guanine bases of the nonscissile strand, which clearly are critical for activity.

**Contributions of the Bases of the Nonscissile Strand—**The strong cleavage suppressive effects of abasic lesions at +3G and +4G are not rectified by indole, implying that polar edge interactions to one or more of the guanine N1, N2, N3, O6, or N7 atoms are critical. Recent studies using other guanine analogs shed some light on which contacts might be relevant. It is unlikely that the topoisomerase makes essential contacts to the O6 atoms of the +4G or +3G bases, insofar as replacing these guanines with 2-aminopurine (which lacks O6) had no effect on $k_{cl}$ (14, 17). Here we see that indole at +3 or +4 did not affect $k_{cl}$, signifying that the enzyme makes no essential interaction with the guanine 2-amino groups in the minor groove. The benign 2-aminopurine and indole effects are consistent with the absence of contacts to the O6 or N2 atoms of +3G or +4G in the topoisomerase-DNA complex (Fig. 5). Nonetheless, an interaction of topoisomerase with the +3G base in the major groove was proposed in light of the finding that 8-oxo modification of +3G resulted in a 35-fold decrement in $k_{cl}$ (14). Reference to the crystal structure suggests an explanation whereby the additional O8 atom on +3G might create a steric clash with the Tyr-136 side chain that projects into the major groove from the specificity helix (Fig. 5). Tyr-136 is critical for the forward cleavage reaction; its mutation to alanine reduces $k_{cl}$ to 0.0005 s$^{-1}$ (a ~600-fold decrement) (36). Tyr-136 coordinates the N7 atoms of both +3G and +4G. Thus, the cleavage suppressing effects of abasic and indole nucleosides at +3G and +4G might reflect weakening of the DNA contacts to Tyr-136. Yet, the recent report by Nagarajan and Stivers (17) that single 7-deazaguanine substitutions at +3G and +4G have no effect on $k_{cl}$ compared with unmodified DNA implies that the simple loss of one N7 atom in major groove does not account for the cleavage-suppressing indole/abasic effects. It is possible that the +3 and +4 guanine-N7 contacts of Tyr-136 are functionally redundant during the forward cleavage reaction.

It is remarkable that the +3G indole/abasic substitutions also slow the rate of religation (to 0.09 s$^{-1}$ for an abasic lesion under single turnover conditions and to ~0.13 s$^{-1}$ for indole under equilibrium conditions), albeit to a much lesser degree than they slow the forward cleavage step. This mimicks the Y136A mutation, which slows $k_{cl}$ to 0.05 s$^{-1}$ (36). These results implicate the Y136A/+3G interaction in maintaining the assembled active site in the context of the covalent topoisomerase-DNA intermediate. Our observation of biphasic kinetics of single-turnover religation by +3 indole-substituted suicide intermediate after a 2-h cleavage reaction suggests the existence of two functional states: one with a catalytically competent active site that religates relatively quickly to the incoming 5’-OH strand (comprising the ~40% burst in Fig. 4) and a second state in which the active site has decayed to an incompetent conformation (or less competent one) by virtue of the loss of contacts to +3G. We suspect that this transition entails loosening of the major groove contacts of the specificity helix,
resulting in displacement of the catalytic Arg-130 residue. This functional decay might be promoted by the addition of 0.5 M NaCl at the onset of the single-turnover cleavage reaction, a maneuver that could weaken the several interactions of the specificity helix with the phosphate backbone (Fig. 5). Thus, the slow phase of religation seen in Fig. 4B (comprising \(\sim 50\%\) of the religation events) could reflect either the rate of sealing by the covalent adduct in the decayed state or the rate at which the covalent adduct shifts between the decayed and reassembled active sites.

The crystal structure highlights a bifurcated hydrogen bond from Lys-133 to the N7 and O6 of the +5 guanine, yet an abasic lesion at +5 G has no effect on the rate of single-turnover DNA cleavage (10). Similarly, there is no effect on cleavage when the +5 G is substituted by either 8-oxoguanine, 2-aminopurine, or 7-deazaguanine (14, 17). These results indicate the contacts from Lys-133 to the +5 G are not critical per se for assembly of the active site.

The present study underscores the relatively modest contributions of the adenine-specific bidentate contact of Gln-69 to the N7 and N6 atoms of the +2 A base to assembly of the pre-cleavage active site. The cleavage rates for the +2 indole/abasic substituted suicide substrates (0.03/0.046 s\(^{-1}\)) are similar to the rate of cleavage of unmodified DNA by the Q69A mutant (0.09 s\(^{-1}\)) (37). Single-atom modifications of +2 A to 7-deazaadenine or purine have no effect on the cleavage rate (17), suggesting that a single Gln69 contact to either N7 or N6 suffices for full activity.

The +1 A base of the nonscissile strand is revealed here to be a key determinant of the cleavage-religation equilibrium, thereby reducing the base stacking interactions rather than base edge contacts, i.e. the poisoning effect of a +1 abasic lesion is largely rectified by +1 indole. This interpretation is consistent with the absence of protein contacts to the +1 A base in the topoisomerase-DNA cocrystal (Fig. 5). The +1 A base is not critical for the forward cleavage reaction, insofar as +1 A substitutions with indole or an abasic lesion elicit only 2–4-fold decrements in \(k_{eq}\). More subtle modifications of +1 A such as 8-oxoadenine, 7-deazaadenine, and purine have no effect on the cleavage rate (14, 17). The +1 abasic lesion slowed \(k_{eq}\) by a factor of \(\sim 140\), resulting in a net increase of \(K_{eq}\) by a factor of 35 (10). The religation reaction requires proper positioning of the 5′-OH DNA terminus for attack on the DNA-(3′-phosphotyrosyl)-enzyme intermediate. This positioning is normally attained by pairing of the bases of the 5′-OH DNA strand to the complementary bases on the noncislcle strand. The restoration of a near normal equilibrium by +1 indole suggests that continuous stacking of the noncislcle strand bases on both sides of the cleavage site is also particularly important. Such base stacking might influence the rotational freedom of the DNA around the phosphodiester opposite the topoisomerase-induced nick. Religation is possible only within a narrow slice of the rotational landscape (38).

In conclusion, we demonstrate the utility of nonpolar base isosteres and analogs to dissect the contributions of base stacking versus polar base edge interactions of a sequence-specific DNA cleaving enzyme. This approach can be extended to other enzymes that act at specific DNA sites.

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