Individual Nucleotide Bases, Not Base Pairs, are Critical For

Triggering Site-Specific DNA Cleavage by Vaccinia Topoisomerase

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ABSTRACT

Vaccinia DNA topoisomerase forms a covalent DNA-(3’-phosphotyrosyl)-enzyme intermediate at a specific target site 5’-C+C+C+T+T+1pN-1 in duplex DNA. Here we study the effects of abasic lesions at individual positions of the scissile and nonscissile strands on the rate of single-turnover DNA transesterification and the cleavage-religation equilibrium. The rate of DNA incision was reduced by factors of 350, 250, 60, and 10 when abasic sites replaced the –1N, +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 nonscissile 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 –1N had no effect. The striking positional asymmetry of abasic interference on the scissile and nonscissile strands highlights the importance of individual bases, not base pairs, in promoting DNA cleavage. The rate of single-turnover DNA religation by the covalent topoisomerase-DNA complex was insensitive to abasic sites within the CCCTT sequence of the scissile strand, but an abasic lesion at the 5’-OH nucleoside (–1N) of the attacking DNA strand slowed the rate of religation by a factor of 600. Nonscissile strand abasic lesions at +1A and –1N slowed the rate of religation by factors of ~140 and 20, respectively, and strongly skewed the cleavage-religation equilibrium toward the covalent complex. Thus, abasic lesions immediately flanking the cleavage site act as topoisomerase poisons.
INTRODUCTION

Poxvirus topoisomerases are exemplary type IB topoisomerase family members; they cleave and rejoin one strand of the DNA duplex through a transient DNA-(3'-phosphotyrosyl)-enzyme intermediate. Vaccinia topoisomerase cleaves duplex DNA at a pentapyrimidine target sequence, 5'-(T/C)CCTTp↓ (1). (The Tp↓ nucleotide is defined as the +1 nucleotide.) Topoisomerases encoded by other genera of poxviruses recognize the same DNA target sequence (2-6), despite the large variations in overall G/C contents of the genomes of the different poxvirus genera. Available structural and biochemical studies suggest that the assembly of a catalytically competent topoisomerase active site is triggered by recognition of the 5'-CCCTT/3'-GGGAA target sequence (7,8).

Early studies using nuclease footprinting, modification interference, modification protection, analog substitution, and UV crosslinking techniques suggested that vaccinia topoisomerase makes contact with several nucleotide bases and the sugar-phosphate backbone of DNA within and immediately flanking the CCCTT element (9-15). Recent studies have focused on delineating the features of the DNA interface that affect the kinetics of transesterification. For example, position-specific covalent polycyclic aromatic hydrocarbon diol epoxide–DNA adducts have been exploited to probe the minor groove interface (16) and the effects of intercalation at all of the dinucleotides steps spanning the target site (17,18). The aromatic hydrocarbon adduct studies delineated the margins of the functional DNA interface at atomic resolution, but did not reveal the nature of the DNA contacts within the essential zone of DNA.

Modifications of the nonbridging and 5'-bridging oxygens of the DNA phosphodiester backbone have been especially informative in that regard. Phosphorothioate and methylphosphonate modifications at the scissile phosphodiester have illuminated the chemical mechanism of topoisomerase IB, the roles of the individual amino acids in either transition-state stabilization or general acid catalysis, and the parameters affecting the stability of the covalent topoisomerase-DNA intermediate (19-23). Position-specific interference by methylphosphonate modifications at remote phosphates on the scissile and nonscissile strands has provided an
atomic-resolution map of the DNA backbone contacts required for active site assembly (8).
Whereas sterically subtle modifications of nonbridging phosphate oxygens flanking the cleavage site can have drastic effects on transesterification chemistry (8), phosphorothiolate substitutions for the 5' bridging oxygens of the scissile strand have no significant effect on the rate of DNA cleavage by vaccinia topoisomerase (6).

A major outstanding question is how the poxvirus topoisomerase reads the nucleotide sequence at its cleavage site. Available evidence suggests that most of the site-specificity is achieved at the level of transesterification chemistry rather than at the noncovalent DNA binding step (24). Whereas the affinity for the target site, the rate of cleavage, and the cleavage equilibrium constant are affected by the nucleotide sequence context surrounding the 5'- (C/T)CCTT target site (1,15) in ways that are not well understood in structural terms, the dominant factor triggering the DNA incision reaction is the pentamer 5'-CCCTT/3'-GGGAA. We have begun to systematically address the features of the individual bases that affect the kinetics of DNA cleavage, via position-specific base modifications entailing relatively small additions to, or subtractions from, the standard base structures (11,17) as well as modification by more bulky adducts (16,18). The addition of new substituents to the purine and pyrimidine rings provides a means of mapping functionally relevant sites of protein-DNA contact. The caveat to the new substituent approach is that a particular site of modification interference (presumably arising via steric hindrance) cannot be equated with a specific atomic interaction with the DNA. Rather, the power of the new substituent approach resides mainly in its ability to identify DNA structural elements that are not functionally relevant. Moreover, the sensitivity of the base modification method necessarily depends on the extent to which the particular modification alters the size, shape and hydrogen-bonding potential of the base or base-pair. In general, the most informative modification interference effects will be those elicited by the subtlest modifications.

A corollary of this proposition is that the most straightforward approach to assessing the relevance of a given base to topoisomerase catalysis is to remove the base rather than add new substituents to the base. Missing base analysis has been facilitated by the availability of
synthetic DNAs containing position-specific tetrahydrofuran (THF) abasic sites (Fig. 1). Other investigators have shown that abasic lesions flanking cleavage sites for mammalian DNA topoisomerase I or topoisomerase II can act as “topoisomerase poisons” that trap the normally transient covalent topoisomerase-DNA intermediate by causing a selective slowing of the religation transesterification reaction relative to the cleavage transesterification reaction (25,26). Such studies have broad physiological relevance insofar as: (i) abasic lesions arise in vivo with high frequency as a consequence of base-excision repair by DNA glycosidases and (ii) topoisomerases may reinforce the cytotoxicity of DNA lesions (27).

Here we conduct a systematic analysis of the effects of abasic lesions at six positions of the scissile strand and six positions of the nonscissile strand within the target sequence 5’-CCCTTA/3’-GGGAAT. The key instructive findings are that individual bases, not the base pairs, are critical determinants of the rate of DNA cleavage. We also find that abasic lesions at the +1 and −1 positions flanking the scissile phosphodiester slow the rate of religation and thereby poison the topoisomerase reaction equilibrium.
EXPERIMENTAL PROCEDURES

DNA substrates. Oligodeoxyribonucleotides containing single THF abasic sites were commercially synthesized and gel-purified by Oligos Etc. Inc (Wilsonville, OR) or synthesized by standard solid-phase methodology and purified by reverse-phase HPLC before and after removal of the 5'-dimethoxytrityl protecting group. 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 M 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 E. coli (BL21) by infection with bacteriophage λCE6 (28) 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 (Biorad) with bovine serum albumin as the standard.

Single-turnover DNA cleavage. Reaction mixtures containing (per 20 µl) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of CCCTT-containing DNA, and 75 or 150 or 300 ng (2 or 4 or 8 pmol) of vaccinia topoisomerase were incubated at 37 °C. 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 endpoint values for cleavage. The data were then normalized to the
endpoint values (defined as 100%), and the cleavage rate constants \((k_{\text{cl}})\) were calculated by fitting the normalized data to the equation \(100-\%\text{Cleavage}_{\text{norm}} = 100e^{-kt}\).

**Single-turnover religation.** Cleavage reaction mixtures containing (per 20 µl) 0.3 pmol of \(^{32}\text{P}\)-labeled 18-mer/30-mer DNA (unmodified or abasic) and 2, 4 or 8 pmol of topoisomerase were incubated at 37°C for 10 to 60 min 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 \(d(GTTCCGATAGTGACTACA)\) to a concentration of 15 pmol/22 µl (i.e., a 50-fold molar excess over the input DNA substrate). Aliquots were withdrawn at various times and quenched immediately with 1% SDS. A time zero sample was withdrawn prior to addition of the acceptor strand. 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 M urea in TBE. Religation of the covalently bound 12-mer strand to the 18-mer acceptor DNA yielded a 5'-\(^{32}\text{P}\)-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. The data were normalized to the endpoint values and \(k_{\text{rel}}\) was determined by fitting the data to the equation \(100-\%\text{Religated}_{\text{norm}} = 100e^{-kt}\).

**Equilibrium cleavage.** A 34-mer CCCTT-containing oligonucleotide was 5' \(^{32}\text{P}\)-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 10 min. The reactions were initiated by the addition of topoisomerase to prewarmed reaction mixtures. The reaction was quenched by adding SDS to 0.5%. The samples were digested for 60 min at 37°C with 10 µg of proteinase K, mixed with an equal volume of formamide/EDTA, and then analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 M urea in TBE. The cleavage product, a \(^{32}\text{P}\)-labeled 12-mer bound to a short peptide, was well resolved from the input 34-mer
substrate. The extent of strand cleavage was quantified by scanning the gel. The cleavage equilibrium constant ($K_{cl}$) is defined as the ratio of covalently bound DNA to noncovalently bound DNA at the reaction endpoint under conditions of saturating enzyme and was calculated according to the equation $K_{cl} = \%\text{Cleaved}/(100 - \%\text{Cleaved})$. 
RESULTS

Position-specific scissile strand abasic interference effects on DNA cleavage.

A series of oligodeoxynucleotide 18-mer scissile strands containing a single THF abasic site within the 5’-C^+5C^+4C^+3T^+2T^+1A-1 sequence were 5’ 32P-labeled and then annealed to an unlabeled 30-mer strand to form “suicide” cleavage substrates for vaccinia topoisomerase (Fig. 1B). The presence of the abasic lesions at the correct positions was assessed by limited digestion of the suicide substrates with *E. coli* exonuclease III, which possesses 3’ exonuclease and abasic endonuclease activities. Whereas exonuclease III digests the unmodified suicide substrate exclusively in the 3’ exonuclease mode to generate a ladder of partially shortened 5’ 32P-labeled products (21), the abasic suicide substrates were also cleaved endonucleolytically at the 5’-phosphodiester flanking the abasic nucleoside to yield a discrete 5’ 32P-labeled product (Fig. 2). Digestion in parallel of the 6 position-specific abasic substrates yielded a series of endonucleolytic cleavage products of the expected size and differing by 1-nucleotide spacing.

The cleavage transesterification reaction of vaccinia topoisomerase results in covalent attachment of the 32P-labeled 12-mer 5’-pCGTGTCGCCCTTp to the enzyme via Tyr274. The unlabeled 6-mer 5’-OH leaving strand ATTCCC 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 endpoint at which 95% of the DNA was converted to covalent topoisomerase-DNA complex; the reaction was complete within 20 s. The extent of transesterification after 5 s was 89% of the endpoint value. From this datum, we calculated a single-turnover cleavage rate constant (*k*~cl~) of 0.46 s⁻¹ (Fig. 1B).

Single abasic lesions spanning positions +5 to –1 on the scissile strand had no significant effect on the extents of DNA cleavage (90-98%), but they exerted disparate position-specific effects on the rate of the reaction. The *k*~cl~ values for the +5 abasic (0.25 s⁻¹) and +3 abasic substrates (0.18 s⁻¹) were within a factor of 2 or 3 of the value for unmodified control DNA. In contrast, abasic lesions at +2, +1 and –1 slowed *k*~cl~ by factors of 60 (to 0.0073 s⁻¹), 260 (0.0018 s⁻¹) and 70 (0.0014 s⁻¹), respectively.
and 350 (0.0013 s\(^{-1}\)), respectively. The +4 abasic lesion caused a 10-fold decrement in \(k_{cl}\) (0.043 s\(^{-1}\)). These results indicate that: (i) vaccinia topoisomerase does not rely on contacts to the +5C or +3C bases during the forward transesterification reaction and (ii) the most important contributions of the scissile strand bases are made by +2T, +1T and –1N.

**Nonscissile strand abasic effects on DNA cleavage.**

An unmodified 5' 32P-labeled 18-mer scissile strand was annealed to a series of 30-mer nonscissile strands containing a single THF abasic site within the 3'-G+5G+4G+3A+2A+1T-1 element (Fig. 3). Single abasic lesions spanning positions +5 to –1 on the nonscissile strand had no significant effect on the extents of DNA cleavage (80-97%), but they elicited position-specific effects on the rate of cleavage that, with the exception of the +5 abasic site, were drastically different than the effects exerted by the loss of the complementary base on the scissile strand.

The first instructive finding was that elimination of the +5G base at the “upstream” margin of the target site had no significant impact on \(k_{cl}\) (0.33 s\(^{-1}\)). Thus, neither component base of the +5 C:G base pair was functionally important for the forward cleavage reaction. A second notable finding was that loss of the –1T base at the “downstream” margin on the nonscissile strand also had no effect on \(k_{cl}\) (0.46 s\(^{-1}\)), but in this case, there was a huge disparity between the benign effect of an abasic lesion on the nonscissile strand compared with the 350-fold decrement in \(k_{cl}\) that occurred when the complementary –1A base was missing from the scissile strand.

The \(k_{cl}\) values for the +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 were slowed by factors of 5, 10, 150, and 130 relative to the \(k_{cl}\) for unmodified DNA. A gradient of increasing severity of abasic interference was evident as the nonscissile strand lesion was phased away from the cleavage site; this contrasts with a severity gradient of opposite directionality for cleavage interference by abasic lesions on the scissile strand. The strand-selectivity can be quantified as the ratio of the cleavage rate constants for the missing scissile strand and nonscissile strand bases of each base-pair. These SS/NS abasic ratios are as follows: 0.75 for +5C:G, 12 for +4C:G, 60 for +3C:G, 0.15 for +2T:A, 0.02 for +1T:A, and 0.003 for –1A:T.
**Effects of missing base pairs on DNA cleavage.**

5′[^32]P-labeled 18-mer scissile strands containing single abasic sites were annealed to abasic 30-mer nonscissile strands to form a series of suicide cleavage substrates lacking both complementary bases of each base-pair within the CCCTTA element (Fig. 4). The effects of missing base pairs ranged from modest (e.g., +5, +3 and −1) to severe (+4, +2 and +1). The missing base pair interference effects we classify as modest were those that had little effect on the extent ofcleavage (78-98%) and for which the rate decrement incurred by deleting both bases were either no worse than, or only modestly worse than, the interference caused by a single abasic lesion. We can quantify the missing base pair effect as the inverse ratio of the cleavage rate constant for the missing pair substrate to the slower of the two rate constants for a single abasic substrate lacking either the scissile or nonscissile strand base. For example, the missing base pair effect at position −1A:T was 1.1 (= 0.0013/0.0011), which means that complete elimination of the −1 base pair was no worse than deleting just the −1A base on the scissile strand. The missing base pair effect at +5C:G was 4 (= 0.25/0.059), but the notable finding was that the rate of cleavage of a DNA substrate lacking the +5C:G pair was slowed by only a factor of 8 compared to an unmodified DNA. The missing pair effect at +3C:G was 7 (= 0.003/0.00043).

The +4, +2 and +1 missing pair interference effects we classify as severe affected the cleavage endpoint (11-62%) and elicited strongly synergistic effects on \( k_{cl} \) compared to the single abasic lesions (Fig. 4). The missing base pair effects at +4C:G, +2T:A and +1T:A were 440, 1300, and 240, respectively. It is likely that these severe effects of deleting both bases of the pair are caused by secondary structural changes to the DNA target site, especially to the phosphodiester backbone with which the topoisomerase makes electrostatic contacts that are essential for the cleavage reaction (8).

**Position-specific abasic interference with DNA religation.**

The observed abasic interference effects on the rate of DNA cleavage could reflect a requirement for specific bases for either: (i) chemical catalysis of transesterification or (ii) DNA-
assisted assembly of a catalytically competent active site. The observed rate of covalent complex formation by vaccinia topoisomerase with unmodified DNA is believed to be limited by the chemical step itself rather than by requisite pre-cleavage conformational steps (29). The topoisomerase catalytic cycle entails two transesterification reactions, cleavage and religation. Religation occurs via the attack of the DNA 5’-OH on the covalent intermediate, leading to expulsion of the Tyr274 leaving group and restoration of the DNA phosphodiester backbone (30). Religation is believed to be the microscopic reversal of the cleavage reaction. Thus, changes in the structure of the topoisomerase or the DNA target site that inhibit the chemical step directly will likely slow both the forward cleavage reaction and the DNA religation reaction. However, changes that impede active site assembly to the point that it becomes limiting for cleavage will affect the cleavage reaction selectively; they would have less impact on the rate of religation by the pre-formed covalent topoisomerase-DNA intermediate, in which the active site is already assembled correctly.

Abasic effects on the religation reaction were studied under single-turnover conditions by assaying the ability of pre-formed 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. 5A). After forming the suicide intermediate on the unmodified 18-mer/30-mer DNA substrate or 18-mer/30-mer DNA containing an abasic lesion 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. The sequence of the added 18-mer is fully complementary to the 5’ single-stranded tail of the suicide intermediate. The ionic strength was adjusted simultaneously to 0.5 M NaCl to promote dissociation of the topoisomerase after strand ligation and prevent recleavage of the 30-mer strand transfer product. Aliquots were withdrawn immediately prior to the addition of 18-mer and NaCl (defined as time 0) and at various times afterward and the extent of religation at each time point was expressed as the fraction of the 32P-labeled DNA present as covalent adduct at time zero that was converted to 30-mer strand transfer product (Fig. 5B). Religation by topoisomerase bound covalently on unmodified DNA was effectively
complete within 5 s, the earliest time point analyzed. The religation of covalent complexes containing +5, +4, or +3 abasic sites on the scissile strand was virtually indistinguishable from that of unmodified DNA, as gauged by the completeness of the reactions after 5 s (Fig. 5B). The religation results are consistent with the minimal/mild effects of the +5, +4 and +3 abasic lesions on the rate of the forward cleavage reaction. Note that the religation rate constant of vaccinia topoisomerase ($k_{rel}$ ~ 1.0-1.2 s$^{-1}$) is too fast to measure manually, which means that the religation rate would have to be slowed at least several fold to be detectable in our assays.

The religation of covalent complexes containing +2 or +1 abasic lesions was effectively complete in 10 s; the reactions attained 56% and 52% of the endpoint values after 5 s, from which we estimated $k_{rel}$ values of 0.16 s$^{-1}$ and 0.15 s$^{-1}$, respectively. We surmise that the +2 and +1 T bases contribute no more a ~6-fold enhancement of the rate of religation, which contrasts with their 60-fold and 250-fold contributions to the rate of cleavage. We infer that the +2T and +1T bases play a role in the assembly of a catalytically competent active site.

The role of the –1 scissile strand base in DNA religation was assessed by forming the suicide intermediate on an unmodified DNA substrate and then initiating religation by adding a 5’-OH 18-mer strand containing a 5’-terminal THF abasic moiety in lieu of a 5’-terminal deoxyadenosine nucleoside (Fig. 6). Religation to the –1 abasic acceptor was efficient but slow; the reaction was complete after 30 min and the observed single-turnover religation rate constant of 0.0017 s$^{-1}$ was slower by a factor of 600 than religation to an unmodified DNA strand. Thus, the –1 base on the scissile strand is crucial for both the cleavage and religation transesterification steps.

We also assayed religation by preformed topoisomerase-DNA complexes containing abasic lesions on the nonscissile strand (Fig. 5C). The +5 abasic lesion, which had no significant effect on cleavage, also had no apparent impact on religation, which was complete in 5 s. The +4 abasic lesion had no apparent effect on religation, but this was in contrast to its 130-fold reduction in the rate of cleavage. We infer that the +4G base is critical for assembly of the topoisomerase active site. Loss of the +2A base did not slow religation appreciably, whereas it
caused a 10-fold reduction in the rate of cleavage. Religation by the +3 abasic topoisomerase-
DNA complex was not complete until 60 s; we derived a single-turnover religation rate constant
of 0.09 s\(^{-1}\). The ~10-fold slowing effect of the +3 abasic site on \(k_{\text{rel}}\) was less severe than its 150-
fold decrement in \(k_{\text{cl}}\).

Religation was severely and selectively affected by abasic lesions at nonscissile strand
positions +1 and −1 (Fig. 5C). The religation rate constant of the +1 abasic topoisomerase-DNA
complex (0.0072 s\(^{-1}\)) was slowed by a factor of ~140 (Fig. 5C). This finding contrasts with the 5-
fold slowing effect of the +1 abasic site on the rate of the forward cleavage reaction. The rate of
religation by the −1 abasic complex (0.044 s\(^{-1}\)) was about 4% of the normal religation rate; the
same −1 abasic lesion had no effect on the forward cleavage rate.

**Abasic 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
transesterification under equilibrium conditions (Fig. 7). 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 25% of the unmodified
substrate at saturation. The cleavage-religation equilibrium constant (\(K_{\text{cl}} = \text{covalent}
complex/noncovalent complex} = k_{\text{cl}}/k_{\text{rel}}\)) was thus 0.33 for the unmodified DNA.

The yield of covalent intermediate as a function of input topoisomerase was determined for
a series of modified equilibrium substrates containing single abasic lesions in the nonscissile
strand. The +5 abasic substrate was cleaved to an extent of 40% of the input DNA. The
observed \(K_{\text{cl}}\) of 0.66 indicated that the +5 abasic site exerted a 2-fold greater effect on ligation
than cleavage. The +4, +3 and +2 abasic lesions reduced the yield of covalent adduct at
equilibrium to 1%, 5% and 9% of input DNA, respectively. The measured \(K_{\text{cl}}\) value of 0.01 for
the +4 abasic substrate was in good agreement with the ratio of the separately determined
single-turnover cleavage and religation rate constants (\(k_{\text{cl}}/k_{\text{rel}} = 0.016\)). Similarly the measured
$K_{cl}$ values of 0.05 and 0.1 for the +3 and +2 abasic DNAs were in good agreement with the calculated $k_{cl}/k_{rel}$ values of 0.03 and 0.16, respectively (Fig. 7). These results underscore the strongly biased effects of the +4 and +3 abasic lesions on the forward cleavage step compared to the religation step.

Entirely different effects on the transesterification equilibrium were elicited by removal of the +1A or −1T bases of the nonscissile strand, whereby the extents of cleavage of the +1 abasic and -1 abasic substrates were increased to 92% and 94%, respectively. The measured $K_{cl}$ values of 11.5 and 15.7 for cleavage of the +1 and -1 abasic DNAs were in good agreement with the calculated $k_{cl}/k_{rel}$ values of 11.8 and 10.5, respectively. Thus, these two abasic lesions acted as potent topoisomerase poisons.
DISCUSSION

Abasic effects on site-specific DNA cleavage

We systematically probed the contributions of individual bases and base-pairs to site-specific DNA transesterification by vaccinia virus DNA topoisomerase, by replacing each nucleoside of the 5'-CCCT\underline{T}A/3'-GGGAAT target site with an abasic THF nucleoside. 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 nonscissile strand in lieu of +4G, +3G, +2T, and +1T reduced the rate of cleavage by factors of 130, 150, 10, and 5, whereas abasic lesions at +5G and −1T had no effect. The reduced rates of cleavage of abasic DNAs were not caused by interference with noncovalent binding, insofar as the observed rate constants did not increase when the concentration of topoisomerase was doubled or quadrupled (data not shown). The simplest interpretation of these findings is that abasic interference is a consequence of the loss of base-specific contacts between the topoisomerase and the 5'-CCTT/3'-GGAA element.

The striking positional asymmetry of abasic interference on the scissile and nonscissile strands highlights the importance of individual bases, not base pairs, in promoting DNA cleavage. This result surprised us, as classical models of protein binding to specific sequences in double-stranded DNA highlight hydrogen bonding interactions between amino acid side chains and the DNA base-pairs as determinants of sequence specificity (32,33). Fig. 8 depicts a space-filling view of a B-form 17-bp duplex DNA containing the topoisomerase target site (5'-CGTGCCTp↓ATTCC, reading from left to right in the model) with the scissile phosphodiester colored red and four essential bases of the 5'-CCTT/3'-GGAA element colored green. Our operational definition of an essential base is one at which a THF substitution elicited at least a 20-fold decrement in $k_{cl}$. The view looking into the major groove in the top panel of Fig. 8 shows that the essential +2 and +1 T bases on the scissile strand and the +4 and +3G bases on the nonscissile strand present a continuous interaction surface for the vaccinia
topoisomerase. The lower panel of Fig. 8 highlights the limited surface accessibility of the minor
groove face of these four bases.

We suggest that protein contacts to the +1 and +2T bases of the scissile strand and the +3
and +4G bases of the nonscissile strand trigger assembly of a catalytically competent active site
subsequent to initial noncovalent binding of topoisomerase to the CCCTT target site. Whereas
the proposed precleavage conformational step required for active site assembly is not normally
rate-limiting when topoisomerase cleaves unmodified DNA, we suggest that it becomes rate-
limiting when topoisomerase cleaves these four abasic DNAs. Because the +4G, +3G +2T and
+1T abasic lesions exclusively or selectively interfere with the cleavage reaction, but not the
religation step, we infer that the putative base contacts needed to promote active site assembly
prior to cleavage are not required to maintain the active site once the covalent intermediate is
formed.

The abasic effects tell us that neither the +5C:G base pair, nor the individual +2A, +1A, +3C
or +4C bases are essential for the DNA cleavage step. Thus, the essential contributions of +4G,
+3G, +2T and +1T are not contingent on hydrogen bonding to the base on the opposing strand.
Extensive additional interference studies, entailing subtle modifications of the individual atoms
of the pyrimidine and purine bases, will be required to delineate which atomic contacts to the
bases are functionally relevant. However, we can speculate a bit in light of available structural
and functional studies. For example, it is likely that some interaction of vaccinia topoisomerase
with the +3G base of the nonscissile strand takes place in the major groove, because 8-oxo
modification of +3G resulted in a 35-fold decrement in $k_{cl}$ (17). On the other hand, it is unlikely
that the topoisomerase makes essential contacts in the major groove to the O6 atom of the +4G
or +3G bases, insofar as replacing these guanines with 2-aminopurine had no effect on $k_{cl}$ (17).
Contacts to the +2T and +1T are also likely to occur in the major groove, because early studies
showed that replacing the +2 T:A or +1 T:A pair with a cytosine:inosine base pair strongly
suppressed the yield of the covalent topo-DNA intermediate (10). Changing T:A to C:I alters the
surface of the major groove, but the minor groove is identical in both cases.
Although the minor groove affords a narrower ingress for protein functional groups to contact the DNA bases, it is evident from multiple DNA co-crystal structures of topoisomerase IB and tyrosine recombinase enzymes that there is a conserved contact between an invariant lysine side chain of the topoisomerase/recombinase (corresponding to Lys167 in vaccinia topoisomerase) and the minor groove face of the +1 base immediately 5' of the scissile phosphodiester (34-38). This lysine, which is an essential general acid catalyst of the transesterification reaction (19), is located atop a conformationally mobile beta-hairpin loop. The contact between the Lys Nζ and the O2 of the +1 thymine is the only base-specific contact seen in the human topoisomerase IB-DNA co-crystal structure (38). In light of our present demonstration that the +1T base is important for cleavage, but not religation, we posit that an equivalent contact of Lys167 with the +1T base helps recruit the beta-hairpin loop and Lys167 from its ground-state position outside the circumference of the DNA double helix (7) to a catalytically competent position within the minor groove adjacent to the scissile phosphodiester. Whereas the recruitment of Lys167 to the active site might be rate-limiting for cleavage of the +1 abasic substrate, the +1 thymine is apparently not critical for transesterification chemistry once an active site has been assembled in the covalent intermediate.

Role of the –1 base in DNA cleavage

Vaccinia topoisomerase displays no preference for a particular base pair immediately 3' of the scissile phosphodiester (1,18). Here we found that an abasic lesion at –1 on the scissile strand slowed \( k_{cl} \) by a factor of 350, whereas a –1 abasic lesion on the nonscissile strand had no effect at all. How can we rationalize the apparent requirement for a –1 base, when neither the base pair, not the identity of the –1 base, is important? We propose that the severe abasic interference at –1 reflects a requirement for continuous base stacking in the scissile strand to ensure optimal orientation of the scissile phosphodiester for coordination by the catalytic residues (Arg130, Lys157, Arg220 and His265) or nucleophilic attack by Tyr274 or proper orientation of the 5'-O leaving group of the –1 nucleoside sugar.
Abasic effects on religation

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 nonscissile strand. This is especially critical in the two-nucleotide segment immediately 3’ of the scissile phosphate (5'-CCCTTN-1N-2), where the potential to form –1 and –2 base pairs accelerated $k_{rel}$ 350-fold compared to an otherwise identical covalent intermediate in which the 5'-OH strand could not pair at these two positions (31). Here we find that elimination of the –1 base on the attacking 5-OH strand slowed religation by a factor of 600, whereas loss of the –1 base on the nonscissile strand slowed religation by a factor of 20. The nonscissile strand abasic effect provides an upper bound estimate of the contribution of base pairing at the –1 position to catalysis of religation (i.e., 20-fold). The ~30-fold greater interference by the abasic 5'-OH nucleoside on the attacking strand suggests that continuous stacking of the scissile strand bases on both side of the cleavage site is particularly important. It is also conceivable that the 5'-THF sugar perturbs the positions of the 5'-O at the end of the attacking strand. A previous study showed that an abasic lesion immediately 3’ of the scissile phosphodiester increased the extent of equilibrium cleavage by calf thymus topoisomerase IB and impeded salt-induced religation by the topoisomerase-DNA intermediate (25).

Elimination of the +1A base of the nonscissile strand strongly impedes religation by vaccinia topoisomerase, but largely spares the forward cleavage reaction. This abasic lesion slowed $k_{rel}$ by more than two orders of magnitude and increased $K_{eq}$ by a factor of 35. In contrast, elimination of the opposing +1T base on the scissile strand slowed the cleavage reaction by a factor of 250 and had relatively little impact on religation. These results imply that the interaction of vaccinia topoisomerase with the +1T:A base pair is functionally important, but significantly different in atomic detail, during the cleavage and religation reactions.
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FIGURE LEGENDS

Fig. 1. Effects of scissile strand abasic lesions on single-turnover DNA cleavage by vaccinia topoisomerase. (A) The structures of a standard deoxynucleoside sugar and a THF abasic nucleoside sugar are shown. (B) The 18-mer/34-mer suicide substrate is shown at the bottom of the figure with the site of cleavage indicated by a vertical arrow. The 5’ 32P-label on the scissile strand is denoted by an asterisk. The unmodified control substrate is depicted in greater detail with the phosphodiester backbone drawn as horizontal bars and the base pairs as vertical bars. The numerical coordinates of the nucleotides are indicated above the sequence. The abasic sites are denoted by the absence of the appropriate vertical bar. Cleavage rate constants and reaction endpoints for transesterification by vaccinia topoisomerase are indicated to the right of each structure.

Fig. 2. Endonucleolytic cleavage of the phased abasic substrates by exonuclease III. Reaction mixtures (60 µl) containing 66 mM Tris-HCl (pH 8.0), 0.66 mM MgCl₂, 1.8 pmol of 5’ 32P-labeled 18-mer/34-mer DNA, and 1.0 unit of E. coli exonuclease III (New England Biolabs) were incubated at 22 °C for 1 min. The reactions were quenched by adding EDTA to 30 mM final concentration. The samples were adjusted to 47% formamide, heat-denatured, and analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 M urea in TBE. The reaction products were visualized by autoradiographic exposure of the gel. The sequence of the radiolabeled 18-mer strand is shown at bottom with the topoisomerase cleavage site indicated by the arrowhead. The positions of the abasic lesions relative to the topoisomerase cleavage site are specified above the lanes.

Fig. 3. Effects of nonscissile strand abasic lesions on single-turnover DNA cleavage. The substrates are depicted with the phosphodiester backbone drawn as horizontal bars and the base pairs as vertical bars. The abasic sites are denoted by the absence of the appropriate vertical bar. Cleavage rate constants and reaction endpoints for transesterification by vaccinia topoisomerase are indicated to the right of each structure.
Fig. 4. **Effects of missing base pairs on single-turnover DNA cleavage.** The substrates are depicted with the phosphodiester backbone drawn as horizontal bars and the base pairs as vertical bars. The missing base pairs are denoted by the absence of vertical bars. Cleavage rate constants and reaction endpoints for transesterification by vaccinia topoisomerase are indicated to the right of each structure.

Fig. 5. **Effects of abasic lesions on single-turnover DNA religation by the covalent topoisomerase-DNA intermediate.** (A) Single-turnover religation by the covalent topoisomerase–(12-mer)/30-mer intermediate was initiated by simultaneous addition of a 50-fold molar excess of an 18-mer acceptor strand complementary to the 5’ single-stranded segment of the nonscissile strand. The religation reaction yields a 30-mer product. (B) The kinetics of religation by the unmodified covalent intermediate and covalent intermediates containing abasic lesions at the indicated position of the scissile strand are shown. (C) The kinetics of religation by the unmodified covalent intermediate and covalent intermediates containing abasic lesions at the indicated position of the nonscissile strand are shown.

Fig. 6. **Single-turnover religation to a 5’-OH strand containing a 5’-terminal abasic lesion.** Single-turnover religation by the covalent topoisomerase–(12-mer)/30-mer intermediate was initiated by simultaneous addition of a 50-fold molar excess of an 18-mer acceptor strand complementary to the 5’ single-stranded segment of the nonscissile strand and containing a 5’-THF nucleoside. The kinetics of religation are shown.

Fig. 7. **Nonscissile strand abasic effects on the cleavage-religation equilibrium.** The 34-mer/30-mer substrate is shown at the bottom of the figure with the site of cleavage indicated by a vertical arrow. The unmodified and abasic substrates are depicted in greater detail with the phosphodiester backbone drawn as horizontal bars and the base pairs as vertical bars. The abasic sites are denoted by the absence of the appropriate vertical bar on the nonscissile strand. Equilibrium cleavage was assayed as described under Experimental Procedures. The observed reaction endpoints at saturating levels of topoisomerase and the experimental $K_{cl}$
values are indicated to the right of each structure. The ratios of the single-turnover cleavage and relocation rate constants are indicated in the last column on the right. (nd, not determined).

Fig. 8. The functional interface of vaccinia topoisomerase with DNA bases of the 5′-CCCTT/3′GGGAA target site as revealed by abasic interference. An atomic model of a 17-bp B-form duplex DNA of sequence 5′-CGTGTGCCCTATTCC (reading from left to right in the model) is shown with the nucleotide bases color light gray and the sugar phosphate backbone colored yellow. The +4G, +3G, +2T, and +1T bases at which THF substitution elicited at least a 20-fold decrement in $k_{cl}$ are colored green. The scissile phosphodiester Tp↓A is colored red. The top panel shows the major groove interface; the bottom panel shows the minor groove interface.
**A**

![Deoxynucleoside and THF Abasic Site](image)

**B**

| Abasic Position | Sequence | $k_{cl}$ ($s^{-1}$) | End Point (% Cleaved) |
|-----------------|----------|----------------------|-----------------------|
| Unmodified      | G C C C T T A T T | 0.46                 | 95                    |
| +5 Abasic       | G G G A A T T | 0.25                 | 98                    |
| +4 Abasic       | G G G T A T | 0.043                | 93                    |
| +3 Abasic       | G C C T A T | 0.18                 | 93                    |
| +2 Abasic       | G G T T T A | 0.0073               | 90                    |
| +1 Abasic       | G C C T T A | 0.0018               | 92                    |
| -1 Abasic       | G C C C T T A | 0.0013               | 92                    |

**Sequence:**

```
*CGTGTCGCCCTTTATTCCC
GCAACGGGGAATAAGGCTATCAGCTGATG
```
|       | 5' | 3'       |     | 5' | 3'       |     |
|-------|----|----------|-----|----|----------|-----|
| unmodified | G C C | C T | T↓A | T |  | 0.46 | 95 |
| +5 abasic |  |  |  |  |  | 0.33 | 89 |
| +4 abasic |  |  |  |  |  | 0.0035 | 92 |
| +3 abasic |  |  |  |  |  | 0.003 | 80 |
| +2 abasic |  |  |  |  |  | 0.046 | 85 |
| +1 abasic |  |  |  |  |  | 0.085 | 89 |
| -1 abasic |  |  |  |  |  | 0.46 | 97 |

*CGTGCAGCCTATTTCTCC*
GCACAGCGGAATAGGCTATCGATGT
| Position | Sequence | $k_{eq} \, (s^{-1})$ | End Point (% Cleaved) |
|----------|----------|----------------------|-----------------------|
| unmodified | G C C C T T A T | 0.46 | 95 |
| +5 abasic | G G G A A T A | 0.059 | 78 |
| +4 abasic | G G G A A T A | 0.0000079 | 40 |
| +3 abasic | G G G A A T A | 0.00043 | 93 |
| +2 abasic | G G G A A T A | 0.0000055 | 11 |
| +1 abasic | G G G A A T A | 0.0000073 | 62 |
| -1 abasic | G G G A A T A | 0.0011 | 98 |

*CGTTGCTGCCCCCTATTCCCC
GCACAGCGGGAATAAGGCTATCCTAGTGT
A

Topo

\[ \text{CGTGTCGCCCTT} \]
\[ \text{GCACAGCGGAATAAGGCTATCACTGATGT} \]

\[ \text{CGTGTCGCCCTTATTCCGATAGTGACTACA} \]
\[ \text{GCACAGCGGAATAAGGCTATCACTGATGT} \]

B

\[ \text{Religation (%)} \]
\[ \text{Time (s)} \]

C

\[ \text{Religation (%)} \]
\[ \text{Time (s)} \]
Topo
CGTGTGCCCTT
GCACAGGGGAATAAGGCTATCAGGTAT

-1 abasic lesion on scissile strand

Religation (%) vs. Time (min)

0 30 60 90 120

0 20 40 60 80
| Abasic Position | End Point (% cleaved) | Equilibrium Constant ($K_{cl}$) | $k_{cl}$/$k_{rel}$ |
|-----------------|-----------------------|-------------------------------|-----------------|
| +6              | 25                    | 0.33                          | nd              |
| +5              | 40                    | 0.66                          | nd              |
| +4              | 1                     | 0.01                          | 0.016           |
| +3              | 5                     | 0.05                          | 0.03            |
| +2              | 9                     | 0.1                           | 0.16            |
| +1              | 92                    | 11.5                          | 11.8            |
| -1              | 94                    | 15.7                          | 10.5            |

*CGTGTGCCCCTTATCCGATAGTGACTACAGCGG
GCACAGCGGGAATAAGGCTATCAGTGATG*
Individual nucleotide bases, not base pairs, are critical for triggering site-specific DNA cleavage by vaccinia topoisomerase
Ligeng Tian, Jane M. Sayer, Donald M. Jerina and Stewart Shuman

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