Covalent DNA Binding by Vaccinia Topoisomerase Results in Unpairing of the Thymine Base 5’ of the Scissile Bond

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We have used potassium permanganate to probe contacts between vaccinia DNA topoisomerase and thymine residues in its 5’-CCCTT \( \rightarrow \) DNA target site. Two major conclusions emerge from the experiments presented: (i) permanganate oxidation of the +2T base of the scissile strand interferes with topoisomerase binding to DNA, and (ii) the +1T base of the scissile strand becomes unpaired upon formation of the covalent topoisomerase-DNA intermediate. Disruption of TA base pairing is confined to the +1-positions. Covalently bound DNAs that have experienced this structural distortion (such DNAs being marked by oxidation at +1T) are fully capable of being religated. We suggest that a protein-induced DNA conformational change is a component of the strand passage step of the topoisomerase reaction.

The eukaryotic family of type I DNA topoisomerases, which includes the nuclear enzymes and the poxvirus topoisomerases, comprises a group of proteins with structural similarity and a common catalytic mechanism. A single catalytic cycle for these proteins entails: (i) noncovalent binding of the enzyme to duplex DNA, (ii) scission of one DNA strand with concomitant formation of a covalent DNA-(3’-phosphotyrosyl)-protein intermediate, (iii) strand passage, and (iv) religation across the phosphodiester bond originally broken. The DNA binding, cleavage, and religation steps have been studied in detail using defined model substrates containing high affinity cleavage sites for the cellular or vaccinia virus type I enzymes (Stevnsen et al., 1989; Christiansen et al., 1993; Christiansen and Westergaard, 1994; Shuman, 1991a, 1991b, 1992a, 1992b; Sekiguchi and Shuman, 1994a, 1994b). Vaccinia topoisomerase displays the greatest degree of specificity in its interaction with DNA; it cleaves at sites containing the pentamer sequence 5’-(C/T)CCCTT \( \downarrow \) immediately 5’ of the scissile bond (Shuman and Prescott, 1990). The cellular topoisomerases exhibit a loose preference for a four-base motif, 5’-(A/T)(G/C)(A/T) \( \downarrow \) (Edwards et al., 1982; Been et al., 1984; J. Axel et al., 1991). A common structure at the topoisomerase catalytic center is likely, insofar as the active site tyrosine of every eukaryotic topoisomerase is located within a conserved motif, Ser-Lys-X-Tyr, situated near the COOH terminus of the protein (Lynn et al., 1989; Eng et al., 1989; Shuman et al., 1989), and because amino acid residues shown by mutational analysis to be essential for strand cleavage by the vaccinia virus topoisomerase are conserved in the cellular counterparts (Morham and Shuman, 1990, 1992; Klemperer and Traktman, 1993; Wittschieben and Shuman, 1994).

An outstanding question regarding the eukaryotic topoisomerase I family concerns the mechanics of “topoisomerization” (i.e. how the protein, once covalently bound to DNA, permits, executes, or controls the passage of the noncleaved strand of the DNA duplex through the nick in the incised strand). Two formal models have been discussed (Wang, 1985; Champsou, 1990). The free rotation model posits that the duplex DNA segment downstream of the cleavage site is unconstrained by the protein and therefore permitted free and unlimited rotation about the phosphodiester bond in the unbroken strand opposite the nick. According to the enzyme-bridging model, topoisomerase maintains noncovalent interactions with the downstream DNA and either actively assists in strand passage or, at the very least, controls the step increment of rotation.

Studies of binding, cleavage, and religation by vaccinia topoisomerase on CCCTT-containing linear DNAs, while not directly scoring changes in topology, do shed some light on the choice of models. For example, there is evidence that vaccinia topoisomerase makes contacts with the DNA downstream of the cleavage site. Increasing the length of the duplex region downstream of the scissile bond incrementally from 6 to 10 to 12 bp strongly enhances noncovalent binding (Sekiguchi and Shuman, 1994b). This suggests that the topoisomerase interacts with the DNA duplex at a distance from the CCCTT element of about one helical turn. This is consistent with the DNase footprint of the vaccinia protein, which reaches 9–13 bp downstream of the cleavage site (Shuman, 1993b). Interaction of topoisomerase with the DNA segment 3’ of the site of strand scission has also been suggested by studies of the cellular type I enzyme (Christiansen et al., 1993).

One potential implication of these findings is that the enzyme may control the rate of topoisomerization during relaxation of supercoiled DNA by binding and releasing the downstream segment of the DNA molecule in synchrony with cycles of cleavage and religation. Alternatively, the topoisomerase may actively separate the strands of the duplex immediately 3’ of the cleavage site as part of a gating step in strand passage. We set out in this study to test the latter hypothesis by chemical probing of the topoisomerase-DNA covalent intermediate using potassium permanganate, a reagent that selectively modifies unpaired thymine bases (Hayatsu and Ukita, 1967). Counter to our expectations, we observed no strand separation 3’ of the cleavage site. Rather, we found that the +1T base on the 5’ side of the cleaved bond became highly accessible to permanganate modification.

EXPERIMENTAL PROCEDURES

Enzyme Purification—Wild type vaccinia topoisomerase and the Topo(Phe-274) mutant were expressed in Escherichia coli BL21 by infection with bacteriophage λCE6 (Shuman et al., 1988). The proteins were purified from soluble bacterial lysates by sequential phosphocel-

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1 The abbreviations used are: C, cytosine; T, thymine; A, adenine; G, guanine; bp, base pair(s).

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lulose and SPSPW ion exchange chromatography steps (Morham and Shuman, 1992). The SPSPW protein preparations were homogeneous with respect to the topoisomerase polypeptide, as judged by SDS-polyacrylamide gel electrophoresis.

Oligonucleotide Substrates—Synthesis of DNA oligonucleotides via DMT-cyanoethyl phosphoramidite chemistry was performed using an Applied Biosystems model 394 automated DNA synthesizer. Oligonucleotides were 32P-5’-end-labeled using T4 polynucleotide kinase and [γ-32P]ATP and then gel-purified and annealed to a complementary DNA strand (present at 4-fold molar excess) as described (Shuman, 1991a).

KMnO4 Modification of DNA Strands—A mixture (0.2 ml) containing 100–200 pmol of 5’-end-labeled 60-mer oligonucleotide (either the scissile or nonscissile strand) and 5 mM KMnO4 (from a freshly made 100 mM stock solution) was incubated at 37 °C for 30 s. The reaction was quenched by the addition of 20 μl of β-mercaptoethanol. The DNA was recovered by ethanol precipitation and dissolved in 0.1 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The modified strands were then hybridized to the unlabeled complementary strand.

KMnO4 Modification Interference—Reaction mixtures containing 50 mM Tris-HCl, pH 8.0, 5 pmol of 60-mer duplex DNA (KMnO4-modified and 32P-5’-end-labeled either on the top or bottom strand), and 330 ng of purified topoisomerase were incubated at 37 °C for 5 min and then adjusted to 5% (v/v) glycerol. To separate topoisomerase-DNA complexes from unbound DNA, the samples were electrophoresed through a nondenaturing 6% polyacrylamide gel containing 0.25 × TBE (22.5 mM Tris borate, 0.6 mM EDTA) at 100 V for 3 h. The gel was autoradiographed wet. A discrete topoisomerase-32P-DNA complex was resolved from the free 60-mer DNA; about half of the input DNA was bound. Free and bound DNAs were electroeluted from excised gel slices using an IBI electroelution apparatus (model UEA). The labeled DNA was ethanol-purified and then resuspended in 80 μl of 1 mM β-mercaptoethanol. The DNA was recovered by ethanol precipitation and resuspended in 0.1 ml of water, followed by lyophilization and resuspension in 4 μl of formamide. The samples were incubated at 95 °C for 5 min and then electrophoresed through a 12% polyacrylamide sequencing gel containing 7 M urea in TBE (25 mM Tris borate, 0.6 mM EDTA) at 100 V for 3 h. The gel was autoradiographed wet. A discrete topoisomerase-32P-DNA complex was resolved from the free 60-mer DNA; about half of the input DNA was bound. Free and bound DNAs were electroeluted from excised gel slices using an IBI electroelution apparatus (model UEA). The labeled DNA was ethanol-purified and then resuspended in 80 μl of 1 mM β-mercaptoethanol. The DNA was recovered by ethanol precipitation and resuspended in 0.1 ml of water, followed by lyophilization and resuspension in 4 μl of formamide. The samples were heated at 95 °C for 1 min and then subjected to three cycles of lyophilization and resuspension in 50 μl of water, followed by lyophilization and resuspension in 4 μl of formamide. The samples were heated at 95 °C for 5 min and then electrophoresed through a 12% polyacrylamide sequencing gel containing 7 M urea in TBE (25 mM Tris borate, 0.6 mM EDTA) at 100 V for 3 h. The gel was autoradiographed wet. A discrete topoisomerase-32P-DNA complex was resolved from the free 60-mer DNA; about half of the input DNA was bound. Free and bound DNAs were electroeluted from excised gel slices using an IBI electroelution apparatus (model UEA). The labeled DNA was ethanol-purified and then resuspended in 80 μl of 1 mM β-mercaptoethanol. The DNA was recovered by ethanol precipitation and resuspended in 0.1 ml of water, followed by lyophilization and resuspension in 4 μl of formamide. The samples were heated at 95 °C for 5 min and then electrophoresed through a 12% polyacrylamide sequencing gel containing 7 M urea in TBE (25 mM Tris borate, 0.6 mM EDTA) at 100 V for 3 h.

KMnO4 Sensitivity of the Topoisomerase-DNA Complex—Reaction mixtures (20 μl) containing 50 mM Tris-HCl, pH 8.0, 1–2 pmol of 32P-5’-end-labeled DNA, and topoisomerase as specified were incubated at 37 °C for 5 min. The mixtures were adjusted to 1 mM KMnO4 (from a freshly made 100 mM stock solution) and incubated at 22 °C for 1 min. The modification reactions were quenched by the addition of 1 mM β-mercaptoethanol, and the DNA was recovered by ethanol precipitation. Alternatively, the topoisomerase-DNA complexes were first digested with proteinase K (10 μg) in 0.2% SDS for 1 h at 37 °C and then electrophoresed sequentially with phenol-chloroform and chloroform. The DNA was recovered by ethanol precipitation and resuspended in 18 μl of TE buffer. The deproteinized DNA samples were then treated with 1 mM KMnO4 for 1 min at 22 °C, and the reactions were quenched with β-mercaptoethanol. The modified DNAs were cleaved with piperidine and analyzed by polyacrylamide gel electrophoresis as described above for the KMnO4 interference assay.

RESULTS

Permanganate Interference—Potassium permanganate oxidizes the 5,6-double bond of the thymine base to produce a 5,6-glycol, thereby altering the DNA surface in the major groove. Initial experiments addressed whether permanganate modification of T residues had any impact on topoisomerase binding to DNA. A 60-mer duplex DNA containing a single CCCTT motif was employed for this analysis; the sequence of the DNA in the vicinity of the cleavage site is shown in Fig. 1. The individual strands were 32P-5’-end-labeled and treated with permanganate prior to hybridization to an unmodified complementary strand. The 60-mer duplex was mixed with purified topoisomerase, and protein-DNA complexes were separated from unbound 32P-labeled DNA by native gel electrophoresis. Bound and unbound DNA species were recovered from the gel, and the DNA was cleaved at oxidized T residues by treatment with piperidine. Cleavage products were then analyzed by denaturing gel electrophoresis. Modification of the +2T base on the scissile (top) strand interfered with protein-DNA complex formation, as evinced by the exclusion of DNA molecules with a piperidine-degradable site at +2T from the bound DNA fraction (Fig. 1, compare lanes 3 and 4). Modification of the +1T did not interfere with binding, since the +1T cleavage product was evenly distributed in the bound and free populations. DNAs modified at −2T and −3T positions of the top strand were recovered preferentially in the protein-bound DNA fraction (Fig. 1, lane 4), as was DNA containing an oxidized T at the −1-position of the noncleaved bottom strand (Fig. 1, lane 8). These findings suggest that topoisomerase makes an essential contact with the +2T base of the CCCTT element in the major groove. They also suggest that distortion
of the DNA immediately 3' of the cleavage site enhances DNA binding to the topoisomerase.

Permanganate Probing for Unpaired Bases in the Topoisomerase-DNA Complex—Acquired permanganate sensitivity in duplex DNA provides a sensitive measure of unpaired thymine bases within protein-DNA complexes. All thymine bases in the top and bottom strands of the topoisomerase substrate were susceptible to permanganate oxidation and piperidine cleavage when the 60-mer oligonucleotides were in single-stranded form (Fig. 2, lanes 1 and 8). Modification was suppressed when the strands were annealed to form a fully base-paired duplex (Fig. 2, lanes 2 and 9), but no topoisomerase, were treated with permanganate and piperidine. The DNA samples were analyzed by denaturing gel electrophoresis. The positions of the T-specific cleavage products are indicated at the left of each autoradiogram. The +1T residue on the top strand that was rendered KMnO₄-sensitive in the presence of the topoisomerase is circled. The peptide-DNA products of proteinase K digestion of covalent complexes formed by topoisomerase binding to the radiolabeled top strand are denoted by a vertical bar.

**Fig. 2.** Topoisomerase binding results in sensitivity of the +1 thymine base to permanganate oxidation. Permanganate modification of preformed topoisomerase-DNA complexes was performed as described under “Experimental Procedures.” The DNA binding reaction mixtures contained 1 pmol of 60-mer DNA (5'-labeled on the top strand (lanes 1–7) or the bottom strand (lanes 8–14)) and 165 ng of topoisomerase (where indicated by Topo + above the lanes). The protein-DNA complexes in lanes 4–7 and 11–14 were depurinated by digestion with proteinase K in SDS prior to treatment with permanganate and/or piperidine as indicated in the legend. Control reactions containing radiolabeled 60-mer duplex DNA (lanes 2 and 9) or labeled 60-mer single strands (lanes 1 and 8), but no topoisomerase, were treated with permanganate and piperidine. The DNA samples were analyzed by denaturing gel electrophoresis. The positions of the T-specific cleavage products are indicated at the left of each autoradiogram. The +1T residue on the top strand that was rendered KMnO₄-sensitive in the presence of the topoisomerase is circled. The peptide-DNA products of proteinase K digestion of covalent complexes formed by topoisomerase binding to the radiolabeled top strand are denoted by a vertical bar.

**Fig. 3.** KMnO₄ sensitivity as a function of topoisomerase concentration. Reaction mixtures (20 μl) containing 2 pmol of 60-bp DNA substrate (5'-end-labeled either on the top (left panel) or bottom (right panel) strand) and 60, 120, 240, or 480 ng of topoisomerase (proceeding from left to right) were incubated at 37 °C for 5 min. The mixtures were treated with KMnO₄ and then subjected to cleavage with piperidine. Control reactions containing radiolabeled 60-mer duplex DNA, but no topoisomerase (lane −) or labeled 60-mer single strands (lane T), were processed in parallel. The samples were analyzed by electrophoresis through a denaturing polyacrylamide gel. The nucleotide coordinates of the T-specific cleavage products are indicated at the left of each autoradiogram. The nucleotide sequence of the DNA duplex in the vicinity of the cleavage site is shown at the bottom. The +1T residue rendered KMnO₄-sensitive upon topoisomerase binding is circled.

Base-flipping by DNA Topoisomerase I?
strand was rendered permanganate-sensitive. If sensitivity were simply a function of strand cleavage and "fraying" of ends at the nick, then we would have expected to see modification of the –1T on the bottom strand. Our inability to detect an unpaired –1 thymine on the bottom strand 3' of the scissile bond is subject to two interpretations: (i) strand separation does not occur; or (ii) the strands are separated, but the bases are inaccessible to the chemical agent because they are bound by protein.

We reasoned that if protein contacts prevented permanganate oxidation at –1T on the bottom strand, then elimination of the protein at the nick should render this base permanganate-sensitive. Similarly, if the observed hypersensitivity of the +1T on the top strand was simply a consequence of nicking, then this modification should be unaffected by removal of the protein. On the other hand, if the +1T sensitivity reflected a true disruption of base pairing induced by the topoisomerase, then elimination of the protein should also eliminate the sensitivity to oxidation. We addressed these points by subjecting the topoisomerase-DNA complexes to digestion with proteinase K prior to treatment with permanganate and piperidine (Fig. 2). In the absence of proteinase K treatment, the 5'-labeled 30-mer product of topoisomerase cleavage remained covalently bound to the protein and did not enter the polyacrylamide gel.

After proteinase K digestion, the 5'-labeled top strand cleavage product entered the gel and migrated as a cluster of bands with an apparent chain length of 32–34 nucleotides (Fig. 2, lane 4). The heterogeneity of the cleavage product and the slightly retarded mobility of these species relative to the size expected of a +1 topoisomerase cleavage product (30 nucleotides) were attributable to the covalent attachment of one or more amino acids to the 3' end of the cleaved fragment (Shuman, 1991a, 1991b). About 15% of the 32P-5'-end-labeled 60-bp DNA was covalently bound to the topoisomerase at saturating enzyme; this reflects the cleavage equilibrium at this particular CCCTT site.

Removal of the covalently bound topoisomerase by proteinase K prior to treatment with permanganate and piperidine completely eliminated the sensitivity of the +1T base on the scissile strand to permanganate oxidation (Fig. 2, lane 7). Similar experiments were performed using the 60-bp DNA substrate 5'-labeled on the bottom strand. In this case, prior digestion with proteinase K did not result in the new appearance of a permanganate/piperidine product at –1T or at any other thymine on the bottom strand (Fig. 2, lanes 10–14). We infer from these results that the sensitivity of +1T on the scissile strand to permanganate oxidation is not accounted for by fraying at a nick. Rather, we surmise that the +1T base is specifically unpaired by the topoisomerase in the context of the covalent protein-DNA complex.

Permanganate Hypersensitivity and Dissociation of the Leaving Group during Suicide Cleavage—Covalent adduct formation by vacuina topoisomerase at a single CCCTT site can be enhanced by truncating the length of the DNA duplex 3' of the scissile bond (Shuman, 1991a; Sekiguchi and Shuman, 1994b). "Suicide" substrates with less than 10 downstream base pairs have been used extensively to study the specificity and kinetics of the cleavage reaction (Stivers et al., 1994a, 1994b) and, more recently, to probe the structure of the covalent reaction intermediate (Sekiguchi and Shuman, 1995). An example of such a substrate, in which an 18-mer scissile strand is annealed to a 30-nucleotide bottom strand, is shown in Fig. 4. Without extensive base pairing to tether the 3' cleavage product to the bottom strand, the 6-mer leaving group AGACAG can be expected to dissociate from the covalent protein-DNA complex, leaving an 18-nucleotide single-stranded tail on the noncleaved strand. With no readily available acceptor for religation, the topoisomerase will become trapped on the DNA. This is reflected in a higher yield of covalent adduct, e.g., 82% of this DNA substrate becomes covalently bound to protein compared with 15% of the 60-bp DNA used above.

The +1 and +2 thymines in the top strand of the suicide substrate were susceptible to permanganate oxidation and piperidine cleavage when the 18-mer was in single-stranded form, but modification was suppressed when the 18-mer was annealed to the 30-mer bottom strand (Fig. 4, compare lanes 2 and 3). Treatment of the topoisomerase-DNA complex with potassium permanganate resulted in a profound enhancement of oxidation at +1T on the scissile strand, as demonstrated by the appearance of a prominent T-specific cleavage product (Fig. 4, lane 4). The formation of this species depended on piperidine treatment of the permanganate-modified protein-DNA adduct (Fig. 4, lane 5). Modification of +1T was not observed when

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2 Given that transesterification is required for permanganate sensitivity, we could not expect to detect unpairing of the –2T or –3T bases on the top strand, because only the 5' segment of the incised strand retains the 32P label.
Topo(Phe-274) was substituted for the wild type topoisomerase (Fig. 4, lane 6). This demonstrated that the acquisition of permanganate hypersensitivity was dependent on covalent adduct formation.

The −9T and −7T bases on the bottom strand of the suicide substrate were susceptible to permanganate oxidation, whereas the −5T, −3T, and −1T bases were shielded by virtue of base pairing to the scissile strand (Fig. 4, compare lanes 9 and 8). In the presence of wild type topoisomerase, the −5, −3, and −1 thymines became sensitive to permanganate oxidation (Fig. 4, lane 10). T-specific strand scission depended on piperidine treatment (lane 11). No enhancement of permanganate sensitivity was seen at the −5T, −3T, and −1T positions when Topo(Phe-274) was substituted for the wild type enzyme. We conclude that the 6-mer leaving group truly did dissociate spontaneously upon cleavage, thereby rendering the unpaired thymines of the bottom strand 5′ tail accessible to permanganate modification. We surmise from this result that the 3′ thymines were not protected from chemical modification by the covalently bound topoisomerase.

Religation of Covalently Bound DNA after Permanganate Oxidation of +1T—Does permanganate oxidation of the +1T base on the covalent intermediate affect the strand religation step of the topoisomerase catalytic cycle? This was addressed by assaying the ability of a preformed topoisomerase-DNA complex to transfer the covalently held 32P-5′-end-labeled strand to an exogenous acceptor (Shuman, 1992a, 1992b). In the experiment shown in Fig. 5, the substrate for covalent adduct formation consisted of a 32P-5′-end-labeled 18-mer scissile strand annealed to a 24-mer bottom strand. The topoisomerase-DNA intermediate was exposed to 1 mM KMnO4 for 1 min, at which point the oxidation reaction was terminated by the addition of β-mercaptoethanol. Piperidine treatment at this stage resulted in the appearance of an abundant +1T cleavage product, indicative of unpairing of the +1T base (Fig. 5, lane 3).

When the order of addition was reversed, such that the β-mercaptoethanol was added prior to KMnO4, the appearance of the +1T piperidine cleavage product was suppressed completely (Fig. 5, lane 4). This proved that all reactive KMnO4 was quenched upon addition of the reducing agent.

Religation by the covalent intermediate was initiated by the addition of a molar excess of an 18-mer acceptor strand (5′-ATTTCCGATAGTACTTCT) that was complementary to the 5′ tail of the covalent donor complex. Transfer of the 12-mer donor strand to an exogenous acceptor (Shuman, 1992a, 1992b). In contrast to the effects of

permanganate, which alters the major groove surface of the

substrate were susceptible to permanganate oxidation, whereas the −5, −3, and −1 thymines became sensitive to permanganate oxidation (Fig. 4, lane 10). T-specific strand scission depended on piperidine treatment (lane 11). No enhancement of permanganate sensitivity was seen at the −5T, −3T, and −1T positions when Topo(Phe-274) was substituted for the wild type enzyme. We conclude that the 6-mer leaving group truly did dissociate spontaneously upon cleavage, thereby rendering the unpaired thymines of the bottom strand 5′ tail accessible to permanganate modification. We surmise from this result that the 3′ thymines were not protected from chemical modification by the covalently bound topoisomerase.

Religation of Covalently Bound DNA after Permanganate Oxidation of +1T—Does permanganate oxidation of the +1T base on the covalent intermediate affect the strand religation step of the topoisomerase catalytic cycle? This was addressed by assaying the ability of a preformed topoisomerase-DNA complex to transfer the covalently held 32P-5′-end-labeled strand to an exogenous acceptor (Shuman, 1992a, 1992b). In the experiment shown in Fig. 5, the substrate for covalent adduct formation consisted of a 32P-5′-end-labeled 18-mer scissile strand annealed to a 24-mer bottom strand. The topoisomerase-DNA intermediate was exposed to 1 mM KMnO4 for 1 min, at which point the oxidation reaction was terminated by the addition of β-mercaptoethanol. Piperidine treatment at this stage resulted in the appearance of an abundant +1T cleavage product, indicative of unpairing of the +1T base (Fig. 5, lane 3).

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Religation by the covalent intermediate was initiated by the addition of a molar excess of an 18-mer acceptor strand (5′-ATTTCCGATAGTACTTCT) that was complementary to the 5′ tail of the covalent donor complex. Transfer of the 12-mer donor strand to the 18-mer acceptor yielded a 32P-5′-end-labeled 30-mer (Fig. 5, lane 5). Covalent complexes that were treated with permanganate and quenched with mercaptoethanol prior to addition of acceptor DNA catalyzed the same extent of strand transfer as did untreated control complexes (Fig. 5, lanes 6 and 7, and data not shown). Hence, permanganate treatment did not inactivate the enzyme. The 30-mer religation products were gel-purified and then treated with piperidine. The 30-mer formed by covalent topoisomerase-DNA complexes that had been exposed to permanganate was cleaved by piperidine at +1T (Fig. 5, lane 7), whereas the 30-mer produced in control reactions was unaffected by piperidine (Fig. 5, lane 6). This proved that covalent complexes containing an oxidized +1T base were able to religate the modified strand.

**DISCUSSION**

We have used potassium permanganate to probe contacts between vaccinia topoisomerase and thymine residues in its CCCTT DNA target site. Two major conclusions emerge from the experiments presented: (i) permanganate oxidation of the +2T base of the scissile strand interferes with topoisomerase binding to DNA, and (ii) the +1T base of the scissile strand becomes unpaired upon formation of the covalent topoisomerase-DNA intermediate.

Permanganate Interference—The present results extend our understanding of the DNA component of the protein-DNA interface. Dimethylsulfate methylation interference and methylation protection experiments had shown that vaccinia topoisomerase makes contact in the major groove with the +3G, +4G, and +5G guanine bases on the noncleaved strand (Shuman and Turner, 1993). UV cross-linking experiments demonstrated protein contacts in the major groove with the +4C and +3C bases on the scissile strand (Segikuchi and Shuman, 1994a, 1996). We now find that oxidation of the +2T base by permanganate, which alters the major groove surface of the 2-position base pair, interferes with topoisomerase binding. It had been suggested earlier that the +2T-A base pair is the most critical position of the topoisomerase binding site, insofar as any base change at +2-position (either on the scissile or the nonscissile strand) abrogates the cleavage reaction (Shuman, 1991b). In contrast to the effects of +2T modification, we find that permanganate oxidation of the +1T base does not interfere with protein binding, which suggests that potential con-
tacks between the protein and the +1 base would not require integrity of the 5,6-double bond of the pyrimidine ring. Earlier base substitution experiments had shown that the requirement for a T:A base pair at the +1-position is less stringent than at the +2-position of the CCCCTT motif (Shuman, 1991b). Oxidation of the −2T and −3T bases on the scissile strand and the −1T base on the noncleaved strand enhanced the binding of vaccinia topoisomerase to DNA. Preferential recovery of these T-specific cleavage products in the protein-bound DNA fraction was observed consistently in multiple binding experiments. The enhancement of binding by the −2T and −3T modifications on the scissile strand cannot be accounted for by hypothetical effects of permanganate oxidation on the cleavage/relinkage equilibrium (i.e. because the scissile strand is 5′ end-labeled in these experiments, DNA molecules that are base covalently to the enzyme cannot yield T-specific cleavage products at sites 3′ of the scissile bond).

Unpairing of the +1T Base—Preformed topoisomerase-DNA complexes were probed with permanganate in an effort to determine whether the DNA strands became unwound during binding and/or catalysis. We expected to detect chemical modification of T residues in the region just 3′ of the cleavage site but found instead that the +1T of the CCCCTT element was specifically unpaired upon protein binding. The observation that permanganate sensitivity was conferred by the binding of wild type topoisomerase, but not Top1(Phc-274), engenders the conclusion that unpairing of the +1T base requires transesterification. The simplest explanation for this would be that the thymine base is unpaired either concomitantly with or subsequent to the strand cleavage step. A series of control experiments indicated that acquisition of permanganate sensitivity at the +1T base is not simply a consequence of strand nicking: base unpairing requires that an intact topoisomerase molecule be bound to the CCCCTT site. The finding of robust permanganate sensitivity at +1T in the suicide cleavage complex argues that strand religation is not germane to the unpairing step.

The present data correlate nicely with recent kinetic studies of the vaccinia topoisomerase, which reveal a conformational step occurring between the strand cleavage and strand religation reactions (Stivers et al., 1994b). The kinetic argument for a conformational step is based on the differing responses of the cleavage and religation rate constants to pH changes plus the disparate effects of phosphorothioate substitution at the scissile bond on cleavage and religation kinetics (Stivers et al., 1994a, 1994b). Whereas covalent chemistry is rate-limiting during the single-turnover cleavage reaction, it is the conformational step, rather than reaction chemistry, that appears to be rate-limiting for strand religation. The conformational change is suggested to be part of the topoisomerization or strand passage step of DNA relaxation (Stivers et al., 1994b).

We suggest that unpairing of the +1 thymine base by the topoisomerase constitutes a component of this conformational step. According to this view, the +1T base would be distorted prior to strand passage and returned to the paired state prior to strand religation. Our demonstration that covalently bound DNAs that have experienced disruption of the +1T-A pair (such DNAs being marked by oxidation at +1T) are fully capable of being religated is at least consistent with the idea that the permanganate-oxidized unpaired state is a reaction intermediate rather than a dead-end product of a side reaction. The extent of oxidation at +1T in the suicide cleavage complexes during a 1-min reaction with permanganate is remarkably high. This raises the possibility that the +1T base is held by the enzyme in the unpaired state in the absence of an acceptor strand.

What purpose might disruption of the +1T:A base pair serve during the topoisomerase reaction? We speculate that this structural distortion permits access of key functional groups on the topoisomerase to the 5′-OH end of the cleaved strand, the 3′-phosphotyrosyl intermediate, or the noncleaved strand opposite the nick and that these interactions may be necessary to control the strand passage step. In other words, unpairing the +1T base would open a conformational gate. A clearer picture of the disposition of the vaccinia protein on the DNA will be needed before these ideas can be tested. Conformational changes during topoisomerization need not be confined to the DNA substrate; indeed, the occurrence of protein conformational changes is likely in a reaction as complicated as DNA relaxation (Lima et al., 1994; Sekiguchi and Shuman, 1994a, 1995).

We suspect that disruption of base-pairing interactions upon covalent binding of topoisomerases to DNA are not be unique to the vaccinia protein. A similar mechanism may apply to the eukaryotic cellular type I topoisomerases, which are closely related structurally and functionally to the vaccinia enzyme, and perhaps to the recombinases, which, although structurally distinct, employ a broadly similar transsterification reaction mechanism involving a covalent 3′-phosphotyrosine-DNA intermediate. The vaccinia topoisomerase functionally resembles the recombinases with respect to its ability to promote sequence-specific recombination in vivo and to resolve recombination intermediates in vitro (Shuman, 1991c; Sekiguchi et al., 1996). When potassium permanganate was used to probe the complex of FLP recombinase bound to its DNA target site, no evidence was obtained for unpairing of thymines located 3′ of the scissile bond (Panigrahi et al., 1992); this is consistent with our findings for the vaccinia topoisomerase-DNA complex. However, because the nucleotide immediately 5′ of the scissile bond in the FLP target site is a cytosine, permanganate probing would not have revealed the type of specific unpairing that we encountered with the vaccinia protein.

In summary, our data show that covalent binding of vaccinia topoisomerase to DNA elicits a structural distortion 5′ of the scissile bond, manifest by accessibility of +1T to permanganate oxidation. Loss of T:A base pairing is confined to the +1-position; no evidence for disruption of the adjacent +2T:A or −1A:T base pairs was obtained. Note that this highly localized effect differs from the more extensive structural changes detected by permanganate probing of the open complex of RNA polymerase bound at a promoter or the complex of SV40 T antigen bound at the replication origin (Sasse-Dwight and Gralla, 1989; Borowiec and Hurwitz, 1988). Thus, hyperreactivity to permanganate at +1T in the topoisomerase-DNA complex is more likely to reflect protein-induced kinking of the DNA at or near the site of strand scission rather than duplex DNA melting, as invoked for RNA polymerase. Our results can also be accommodated by the DNA base-flipping paradigm described for Hha DNA cytosine-5-methyltransferase. In the crystal structure of Hha methyltransferase chemically trapped as the covalent enzyme-DNA reaction intermediate, the target cytosine base is flipped completely out of the helix (Klimasauskas et al., 1994). Of course, without a crystal structure for the vaccinia topoisomerase bound covalently to DNA, we cannot tell if the topoisomerase flips the +1T out of the helix or whether the structural distortion at the +1-position is more subtle.

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