

DNA Contacts Stimulate Catalysis by a Poxvirus Topoisomerase*

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Eukaryotic type 1B topoisomerases act by forming covalent enzyme-DNA intermediates that transiently nick DNA and thereby release DNA supercoils. Here we present a study of the topoisomerase encoded by the pathogenic poxvirus molluscum contagiosum. Our studies of DNA sites favored for catalysis reveal a larger recognition site than the 5'-(T/C)CCTT-3' sequence previously identified for poxvirus topoisomerases. Separate assays of initial DNA binding and covalent complex formation revealed that different DNA sequences were important for each reaction step. The location of the protein-DNA contacts was mapped by analyzing mutant sites and inosine-substituted DNAs. Some of the bases flanking the 5'-(T/C)CCTT-3' sequence were selectively important for covalent complex formation but not initial DNA binding. Interactions important for catalysis were probed with 5'-bridging phosphorothiolates at the site of strand cleavage, which permitted covalent complex formation but prevented subsequent religation. Kinetic studies revealed that the flanking sequences that promoted recovery of covalent complexes increased initial cleavage instead of inhibiting resealing of the nicked intermediate. These data 1) indicate that previously unidentified DNA contacts can accelerate a step between initial binding and covalent complex formation and 2) help specify models for conformational changes promoting catalysis.

Topoisomerases play important roles in DNA replication, recombination, and transcription (1). Type 1B topoisomerases act (Fig. 1) by first binding double-stranded DNA and then cleaving substrate DNA using a conserved enzyme tyrosine residue, forming a 3'-O-phosphotyrosine linkage. The DNA strands then isomerize, either by passing one strand through the break in the other or by rotation of the covalent complex, relaxing the DNA. The broken strand is then resealed by nucleophilic attack of the 5'-hydroxyl on the phosphotyrosine bond. Last, the topoisomerase dissociates from substrate.

All the poxviruses studied to date encode a type 1B topoisomerase (2–7) that is inferred to be required for viral replication (8). Poxvirus topoisomerases bind to the sequence 5'-(T/C)CCTT-3' and cleave just 3' of the last T (designated position +1), to form the covalent intermediate (7, 9, 10). Although many topoisomerases have been found to show some DNA sequence preferences (11–14), the high degree of specificity seen with the poxvirus topoisomerases is unique.

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In the case of vaccinia virus, the most thoroughly studied model, the topoisomerase enzyme consists of two domains linked through a trypsin-sensitive bridge (15, 16). The N-terminal domain (amino acids 1–80) contacts the 5'-(T/C)CCTT-3' element on the major groove side of the DNA (15–17). Chemical footprinting and interference studies indicate that the enzyme contacts several phosphates, including the scissile phosphate, on both strands and the minor groove side as well. Recently, the crystal structure of the C-terminal catalytic domain of vaccinia topoisomerase (amino acids 81–314) was determined and found to be a member of the λ -integrase family (18). The topoisomerase of MCV¹ is 54% identical to that of vaccinia and presumably resembles the vaccinia enzyme closely in structure and mechanism.

Here we present a study of the role of DNA sequence in the function of MCV topoisomerase. Poxvirus topoisomerases are experimentally tractable due to their small size (33 kDa) and function as monomers (19). The high degree of sequence specificity allows the enzyme to be placed precisely on test DNA substrates for analysis. In addition, MCV causes medically important opportunistic infections in immunocompromised patients (20–22), adding interest to understanding the mechanism in the hope of aiding the development of antiviral agents. We investigated DNA contacts important in different parts of the topoisomerase catalytic cycle. We found that previously unidentified DNA contacts increase the rate of a limiting step between initial binding and covalent bond formation.

EXPERIMENTAL PROCEDURES

Enzyme Purification—MCV topoisomerase was overexpressed in *Escherichia coli* and purified as described by Hwang *et al.* (7). The active fraction of MCV topoisomerase monomers was measured by titrating the enzyme with a suicide substrate derived from substrate A DNA (7). In this substrate (sequence 5'-TCCGTGTCGCCCTTATTCC-3' and 5'-GAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGG-3') the short duplex extension 3' of the 5'-CCCTT-3' sequence can dissociate upon covalent complex formation, trapping the covalent intermediate and allowing the active fraction to be determined.

Noncovalent Complex Formation—Oligonucleotide substrates were end-labeled at the 5'-end by treatment of one strand with [γ -³²P]ATP and T4 polynucleotide kinase. After labeling, the oligonucleotide was purified in a Sephadex G-25 spin column and then hybridized to the complementary oligonucleotide by heating and slow cooling. Reaction mixture contained 20 mM Tris-Cl (pH 8.0), 200 mM potassium glutamate, 1 mM dithiothreitol, 0.1% Triton X-100, 1 mM EDTA, and 50 nM of 5'-³²P-end-labeled DNA substrate. Reaction mixtures were prewarmed at 37 °C and then initiated by the addition of topoisomerase. Reaction mixtures were incubated for 5 min and cooled in ice water bath for 5 min. Reaction products were separated on 5% polyacrylamide gels, visualized by autoradiography, and the radioactivity was quantitated by PhosphorImager. All reactions were repeated and quantitated 2–4 times. Note that the difference between the amount of covalent complexes visualized on SDS-PAGE and complexes visualized on native gels allows the amount of noncovalent complexes to be determined by subtraction.

Covalent Complex Formation—Reaction mixtures contained 20 mM

¹ The abbreviation used is: MCV, molluscum contagiosum virus.

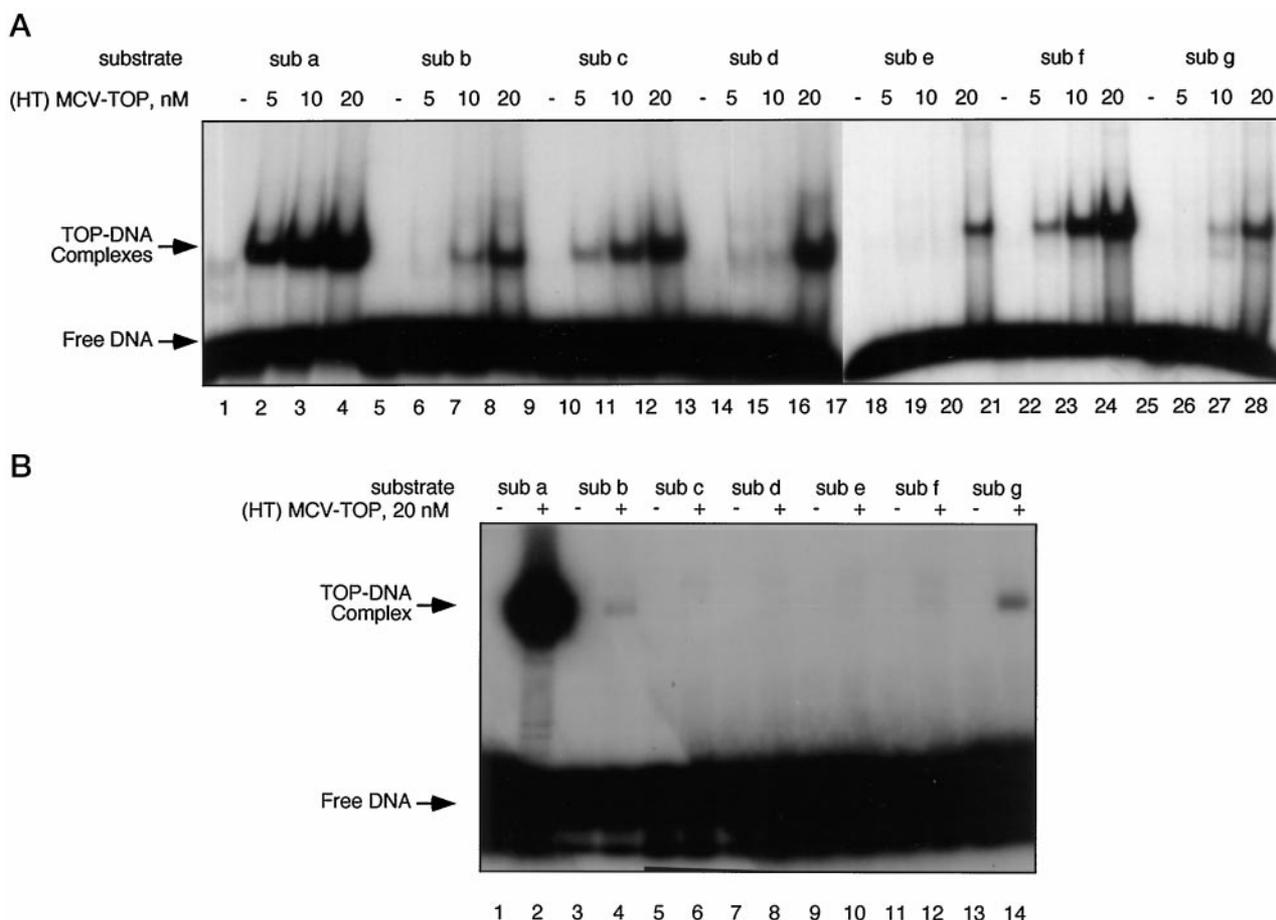


FIG. 2. DNA binding and covalent complex formation by MCV topoisomerase at various sequences from pUC19. The sequences of substrates (*sub*) tested are indicated in Table I. The input topoisomerase concentration is marked *above* the gel. Control reactions lacking topoisomerase are indicated by a *dash*. The positions of the free DNA substrates and topoisomerase-DNA complexes are indicated at the *left*. *A*, assay of DNA binding activity by native gel electrophoresis. *B*, assay of covalent complex formation by denaturing gel electrophoresis.

topoisomerase (9). Three additional favored sites had the sequence 5'-TCCTT-3' (Table I; substrates d, e, and f).

To study the function of MCV topoisomerase in more detail, sequences from pUC19 were synthesized as oligonucleotides and annealed to form duplexes. Their binding activities were then studied in gel retardation assays (summarized in Table I). The binding measured by the band shift assay represented contributions from both covalent and noncovalent complexes. The fraction of covalent complexes was determined as described below, allowing the fraction of noncovalent complexes to be deduced by subtraction. For example, for substrate a, 57% of DNA was bound in Fig. 2, *lane 4*, and of this 33% was in noncovalent and 24% in covalent complexes. The fraction bound in noncovalent complexes ranged from 33% (defined as 100% in Table I) for substrate a, to 3% for substrate k. Evidently, the sequence determinants for binding extended beyond the 5'-(T/C)CCTT-3' pentanucleotide found in all substrates.

DNA binding by the various substrates was tested under several solution conditions. Binding was assayed in the presence of 200 mM potassium glutamate, since this promotes accumulation of the covalent complex intermediate in the assays described below. Binding was also assayed in the presence of 100 mM NaCl, used previously for analyzing poxvirus topoisomerases (24). The efficiency of DNA binding was found to be similar for each substrate under both conditions (data not shown).

Substrates a–k were next tested for covalent complex formation. Oligonucleotides were incubated with MCV topoisomer-

ase, and then complexes were separated by SDS-polyacrylamide gel electrophoresis. Under these conditions, only covalently bound DNA strands migrated with the topoisomerase. An autoradiogram is shown in Fig. 2*B* and quantitated in Table I. The efficiency of covalent complex formation was drastically different for different DNAs, with substrate a showing 24% conversion of substrate to product (defined as 100% in Table I). Substrates b–k were much less active. Why the sequences in substrates h–k were not cleaved detectably in pUC19 is unclear, although cleavage at all sites except substrate a was inefficient, so cleavage at some of the weak sites may not have been detected. Overall, these data indicate that sequences outside the conserved pentamer can independently influence either DNA binding (for example, substrates e and k), or covalent complex formation (for example, substrates f and h).

To explore the importance of bases in the conserved pentanucleotide, mutants were constructed containing changes in the 5'-CCCTT-3' sequence (Table II; bases are numbered such that the T 5' of the scissile phosphate is +1). Altering base +5 (C to T) or +3 (C to T) had slight effects on binding, while altering base +1 (T to C) drastically reduced binding. For covalent complex formation, altering the base at +5 had only a modest effect, confirming that the consensus pentamer should include a C or T at this position. Altering base +3 reduced covalent complex formation 20-fold, while altering base +1 reduced covalent complex formation to less than 0.1% of the substrate a level. Evidently, the correct base at position +3 is most important for covalent complex formation after binding,

TABLE II

DNA binding and covalent complex formation on substrates containing mutations in the 5'-CCCTT-3' pentanucleotide

The mutated positions are underlined.

Substrate	Nucleotide sequence	Relative noncovalent binding	Relative covalent complex formation
a	+10 +1 -10 5' TCCGTGTCGCCCTTATTCCCTTTTTGCGGCATTTGCCT 3' GGCACAGCGGAATAAGGGAAAAACGCCGTAAACGGAG	100 ± 7	100 ± 8
a +5T	5' TCCGTGTCG <u>T</u> CCTTATTCCCTTTTTGCGGCATTTGCCT 3' GGCACAGCAGGAATAAGGGAAAAACGCCGTAAACGGAG	42 ± 4	30 ± 2
a +3T	5' TCCGTGTCGCC <u>T</u> TATTCCCTTTTTGCGGCATTTGCCT 3' GGCACAGCGGAAATAAGGGAAAAACGCCGTAAACGGAG	70 ± 6	4.8 ± 0.6
a +1C	5' TCCGTGTCGCCCTCATTCCCTTTTTGCGGCATTTGCCT 3' GGCACAGCGGGAGTAAGGGAAAAACGCCGTAAACGGAG	1.4 ± 0.5	0.2 ± 0.1

TABLE III

DNA binding and covalent complex formation on hybrid sites containing sequences from the highly active substrate a and the cleavage-impaired substrate h.

The hybrid sites containing sequences from the highly active substrate a are underlined. Shortened derivatives of substrates a and h were used in this experiment (substrate a 22-mer and substrate h 22-mer).

Substrate	Nucleotide sequence	Relative noncovalent binding	Relative covalent complex formation
a (22-mer)	+10 +1 -8 5' <u>TCCGTGTCGCCCTTATTCCCTT</u> 3' <u>GGCACAGCGGAATAAGGGAAA</u>	100 ± 11	100 ± 9
h (22-mer)	5' CACCGATCGCCCTTCCCAACAG 3' TGGCTAGCGGAAGGGTTGTCA	74 ± 6	2.5 ± 0.4
l	5' CACCGATCGCCCTTATTCCCTT 3' TGGCTAGCGGAATAAGGGAAA	82 ± 12	88 ± 8
m	5' TCCGTGTCGCCCTTCCCAACAG 3' GGCACAGCGGAAGGGTTGTCA	73 ± 7	60 ± 8
n	5' CACCGATCGCCCTTATTCCAG 3' TGGCTAGCGGAATAAGGGTCA	86 ± 4	57 ± 7
o	5' CACGTGTCGCCCTTCCCAACAG 3' TGCACAGCGGAAGGGTTGTCA	77 ± 9	54 ± 6
p	5' CACCGATCGCCCTTATTAACAG 3' TGGCTAGCGGAATAATTGTCA	66 ± 8	62 ± 8
q	5' CACCGATCGCCCTTACCAACAG 3' TGGCTAGCGGAATGGTTGTCA	76 ± 12	3.1 ± 0.8
r	5' TCCGGATCGCCCTTACCCCTT 3' GGCCTAGCGGAATGGGGAAA	76 ± 8	2.1 ± 0.5
x	5' TCCGTATCGCCCTTACCCCTT 3' GGCATAGCGGAATGGGGAAA	78 ± 9	47 ± 6

while position +1 is important for binding. These results parallel findings in the vaccinia system (24).

The ability to trap reaction intermediates by using different substrates provided a potential means for probing the reaction mechanism. As a first step, substrate sequences were mapped that were specifically important for covalent complex formation.

Sequence Determinants for Covalent Complex Formation—To identify the required sequences flanking the 5'-(C/T)CCTT-3' pentanucleotide, flanking sequences were systematically exchanged between substrate a, the most active substrate, and substrate h (Table III). Substrate h was chosen because it contains the 5'-CCCTT-3' sequence found in substrate a, and substrate h binds MCV topoisomerase almost as well as substrate a (72% of the substrate a level). However,

substrate h was impaired in forming the covalent intermediate (0.2% of substrate a). Note that substrate h differs from substrate a at only 12 out of 22 positions, facilitating the identification of sequences important for covalent complex formation. Independently replacing DNA to either side of the conserved pentanucleotide of substrate h with substrate a sequences restored high level covalent complex formation (Table III, substrates l and m). This indicates that both flanking regions must have disfavored nucleotides present for the substrate to display low cleavage activity. Substitution of sequences in substrate a with shorter flanking sequences of substrate h also yielded substrates active for cleavage (substrate n and substrate o, base pairs substituted extending from -1 to -5 and +9 to +11, respectively). Replacement of base pairs +7 to -3 in substrate h with sequences from substrate a yielded a highly active

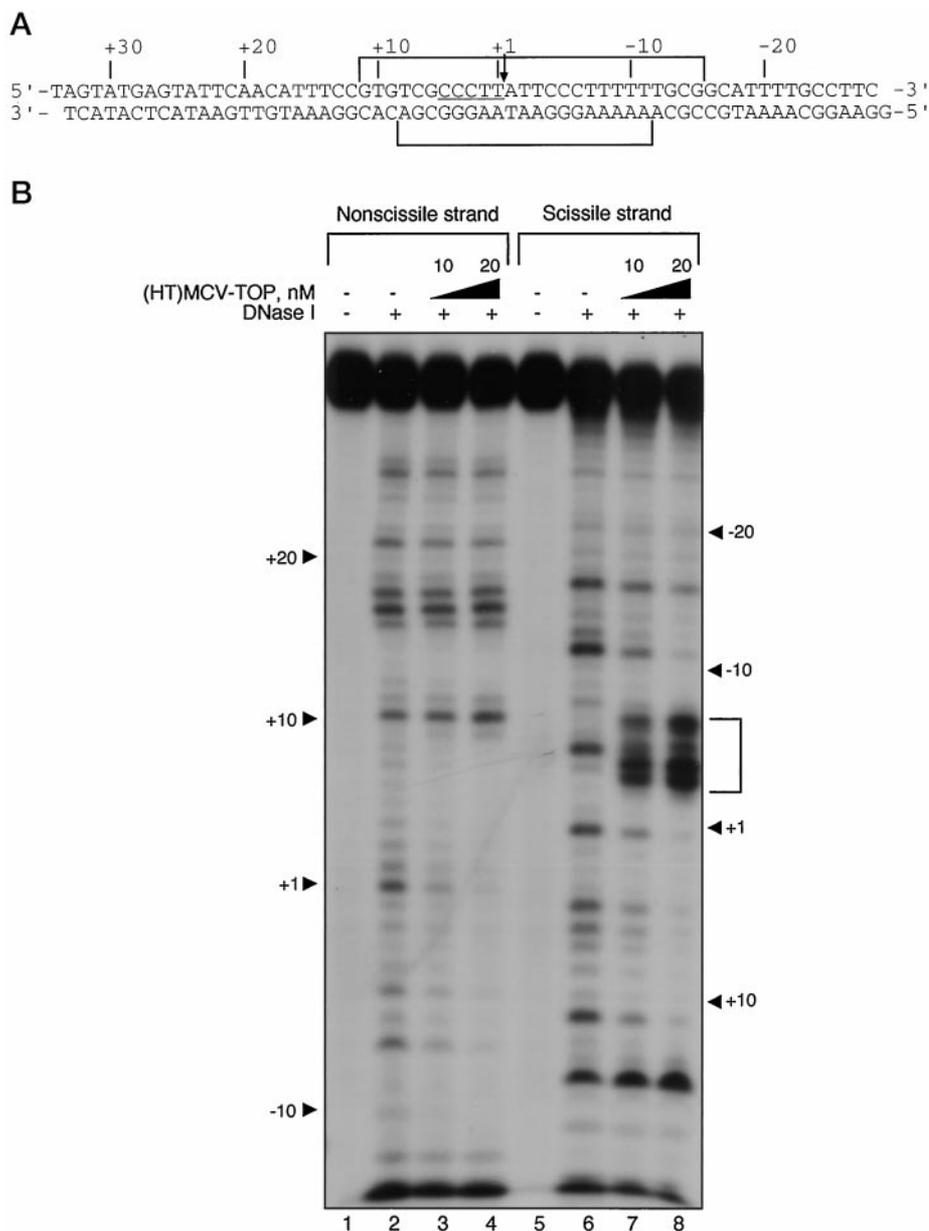


FIG. 3. DNase I footprint analysis of MCV topoisomerase binding to substrate a. *A*, DNA sequence of the 62-mer substrate used in the footprinting experiment. The consensus site, 5'-CCCTT-3' is *underlined*. The favored cleavage site is marked by the *arrow*. The *brackets* indicate the extent of the footprint. *B*, analysis of binding of MCV topoisomerase to DNA by DNase I protection. The 62-mer substrate a was 5'-end labeled on either the scissile strand or the nonscissile strand as indicated. The inclusion or omission of topoisomerase or DNase I in the reaction mixture is indicated as a *plus* or *minus* sign, respectively, above the *lanes*. The location of the topoisomerase cleavage product is marked by the *bracket* at the *right*. The relative positions of DNase I digestion sites were determined by coelectrophoresis with labeled DNA markers.

substrate (substrate p), indicating that at least one flanking sequence was favorable. However, replacing base pairs -2 and -3 in substrate p with sequences from substrate h yielded a substrate with low activity (substrate q). This implicated base pairs -2 and -3 on the right as important. On the left side, comparison of substrates m and q indicates that bases at +9 to +11 must be important, since disfavored nucleotides needed to be present on both sides of the conserved pentanucleotide to impair function. To confirm these findings, a derivative of substrate a containing base pairs from substrate h at -2, -3, +9, and +10 was tested (substrate r). This substrate displayed low activity for cleavage (3.1% of substrate a), indicating that the four bases changed are indeed important for cleavage activity. Changing only bases +9, -2, and -3 to match substrate h in a substrate otherwise matching substrate a yielded a substrate with high activity (substrate x), indicating that on the left side base +10 is particularly important.

Substrates l-q and substrate x all displayed noncovalent DNA binding activity within 66% of substrate a, indicating that the altered activities observed with mutant substrates were mainly due to changes at postbinding steps. Evidently, in the

context of substrate a, optimal bases at positions +10, -2, and -3 are required for efficient covalent complex formation but not for initial binding.

DNase I Footprinting of MCV Topoisomerase—How much of the DNA surrounding the 5'-(T/C)CCTT-3' sequence does MCV topoisomerase cover when bound? Are the +10, -2, and -3 sequences likely to be contacted by bound enzyme? In previously reported DNase I footprinting studies, vaccinia topoisomerase protected base pairs +13 to -13 on the cleaved strand and from +13 to -9 on the noncleaved strand (9). To determine where MCV topoisomerase binds on substrate DNAs, a footprinting experiment was carried out on a 61-mer duplex DNA fragment derived from substrate a (Fig. 3A). MCV topoisomerase was incubated with oligonucleotide radiolabeled uniquely at one 5'-end, and DNase I was added to cleave exposed DNA. Reaction products were treated with proteinase K and SDS, denatured, separated on DNA sequencing-type gels, and visualized by autoradiography.

Treatment of the labeled substrate with DNase I produced a partial digestion pattern (Fig. 3B, lanes 2 and 6). Digestions were repeated in the presence of MCV topoisomerase. DNase I

TABLE IV
DNA binding and covalent complex formation on sites containing inosine substitutions

The hybrid sites containing sequences from the highly active substrate a are underlined. The inosine-substituted positions are indicated as i.

Substrate	Nucleotide sequence	Relative noncovalent binding	Relative covalent complex formation
a (22-mer)	+10 +1 -8 ↓ 5' <u>TCCGTGTCGCCCTTATTCCCTT</u> 3' <u>GGCACAGCGGAATAAGGGAAA</u>	100 ± 9	100 ± 10
r	5' <u>TCCGGATCGCCCTTACCCCTT</u> 3' <u>GGCCTAGCGGAATGGGGAAA</u>	76 ± 11	2.1 ± 0.6
s	5' <u>TCCGiATCGCCCTTACCCCTT</u> 3' <u>GGCCTAGCGGAATGGGGAAA</u>	68 ± 7	2.3 ± 0.4
t	5' <u>TCCGGATCGCCCTTACCCCTT</u> 3' <u>GGCCTAGCGGAATiGGGAAA</u>	80 ± 4	3.1 ± 0.3
u	5' <u>TCCGiATCGCCCTTACCCCTT</u> 3' <u>GGCCTAGCGGAATiGGGAAA</u>	92 ± 8	0.7 ± 0.3
v	5' <u>TCCGTGTCGCCCTTATTCCCTT</u> 3' <u>GGCACAGCGGiAATAAGGGAAA</u>	88 ± 9	21 ± 2.2
w	5' <u>TCCGTGTCGCCCTTATTCCCTT</u> 3' <u>GGCACAGCGGAATAAGGGAAA</u>	75 ± 8	4 ± 0.6

cleavage products were mapped by comparison with labeled DNA size markers. MCV topoisomerase protected bases +11 to -15 on the cleaved strand (Fig. 3B, lanes 7 and 8) and bases +8 to -11 on the noncleaved strand (Fig. 3B, lanes 3 and 4). Bases +9 to +11 on the noncleaved strand were slightly enhanced for DNase I cleavage. Exact boundaries of the protected region are somewhat ambiguous due to the weak DNase I cleavage at some sequences. In addition, bands were seen on the cleaved strand that did not depend on the addition of DNase I, corresponding to DNA cleavage by topoisomerase-mediated covalent complex formation (data not shown). The heterogeneity of this product (Fig. 3B, bracket) probably results from incomplete removal of protein fragments by proteinase K. No topoisomerase-dependent cleavage product was observed when the nonscissile strand was labeled, as expected (7, 9).

Probing the Location of Topoisomerase-DNA Interactions Using Inosine Substitutions—To investigate protein-DNA contacts at +10, -2, and -3, oligonucleotides containing inosine substitutions were synthesized and tested. Changing T:A to I:C base pairs leaves the minor groove side of the DNA matching that of T:A, while the major groove side matches G:C (25). Thus, if major groove contacts at a T:A pair are important, then substitution of an I:C pair should be harmful to the interaction. Conversely, if the essential contacts are in the minor groove, then the I:C substitution should be tolerated.

DNA substrates were synthesized that contained inosine substitutions in the context of substrate r, which matches the highly active substrate a except at positions +10, +9, -2, and -3 and so displays weak activity. Since substrates that contain one favorable and one unfavorable flank are active (Table III), it is expected that conversion of a disfavored flanking sequence to a favored flanking sequence will restore activity. MCV topoisomerase bound to all seven DNA substrates efficiently (Table IV), allowing the relative activities in covalent complex formation to be compared. All I:C replacements at +10, -2, and -3 failed to restore covalent complex formation (Table IV, substrates s-u). These data are consistent with a model in which important contacts at the positions +10, -2, -3 are made at least in part in the major groove.

As a control, the effect of an inosine substitution were tested within the conserved pentamer (substrate a 3T, Table II). This substrate, containing a T substituted for C at position +3 in the

5'-CCCTT-3' pentamer, was reduced 25-fold relative to the wild type substrate for covalent complex formation (Table II). Substituting a C:I pair at +3 boosted activity 5-fold (substrates v and w, Table IV). This finding suggested that the recognition of position +3 is at least in part in the major groove, although activity of this substrate was still down 5-fold from substrate a, indicating a possible component of minor groove recognition also. This result was consistent with the previous finding that positions +2 and +1 are contacted in the major groove by vaccinia virus topoisomerase (24) but that the enzyme wraps around the helix in this region (18).

Assessing the Kinetic Steps Influenced by Different Sequences Using 5'-Bridging Phosphorothiolates—The activating sequences at +10, -2, and -3 could have increased recovery of the covalent complex by either of two means. The forward rate of initial cleavage might have been increased, or the rate of resealing the covalent intermediate might have been decreased. To distinguish between these possibilities, reactions were studied using suicide substrates containing 5'-bridging phosphorothiolate linkages at the site of strand cleavage and religation (Fig. 4A). The 5'-bridging sulfur atoms in such substrates are good leaving groups, so covalent intermediates form efficiently. However, sulfur is a poor nucleophile at phosphorus, so attack of the 5'-sulfur atom on the phosphotyrosine linkage is inhibited. Thus, the religation step is blocked, allowing the forward rate of initial cleavage to be studied in isolation (23, 26, 27).

The optimal sequence (substrate a) was compared with substrate y, which is identical to substrate a but contains disfavored bases at +10, +9, -2, and -3. Derivatives of substrate a and substrate y containing 5'-bridging phosphorothiolates were synthesized and compared with the normal forms containing bridging oxygens (Table V). The incorporation of the sulfur atom was confirmed by demonstrating that the phosphorothiolate bond was cleaved by incubation with heavy metal (Fig. 4B, lanes 3-6), while the normal oxygen-containing substrate was insensitive as expected (Fig. 4B, lanes 1 and 2).

Substrate a and substrate y were then assayed in the presence and absence of the 5'-bridging phosphorothiolate at the scissile bond. DNA binding assayed by gel retardation revealed little difference between substrate a (Fig. 4C, lanes 2-4), substrate y (lanes 6-8), and the phosphorothiolate derivatives

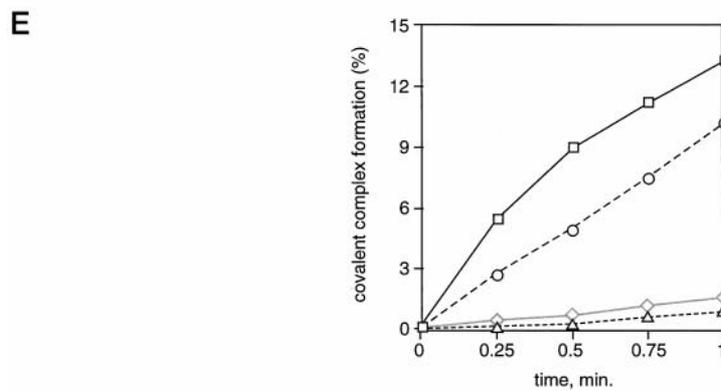
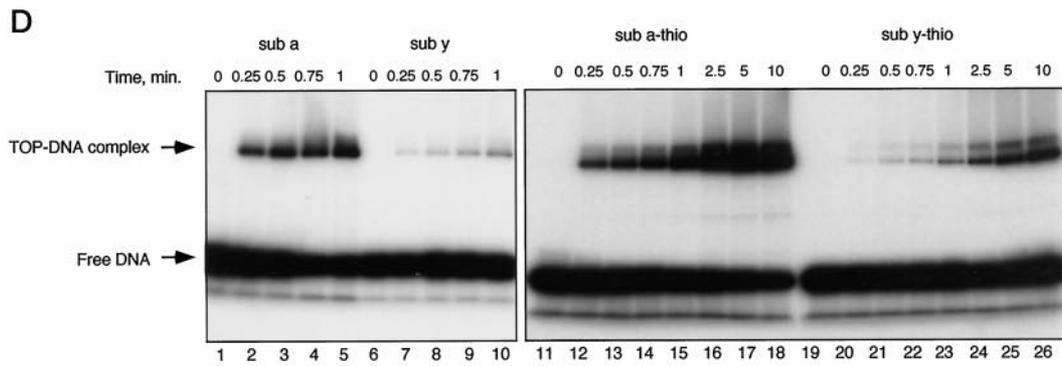
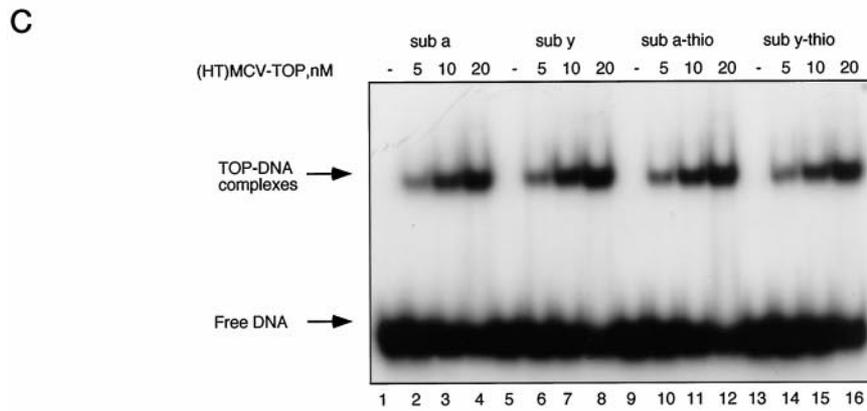
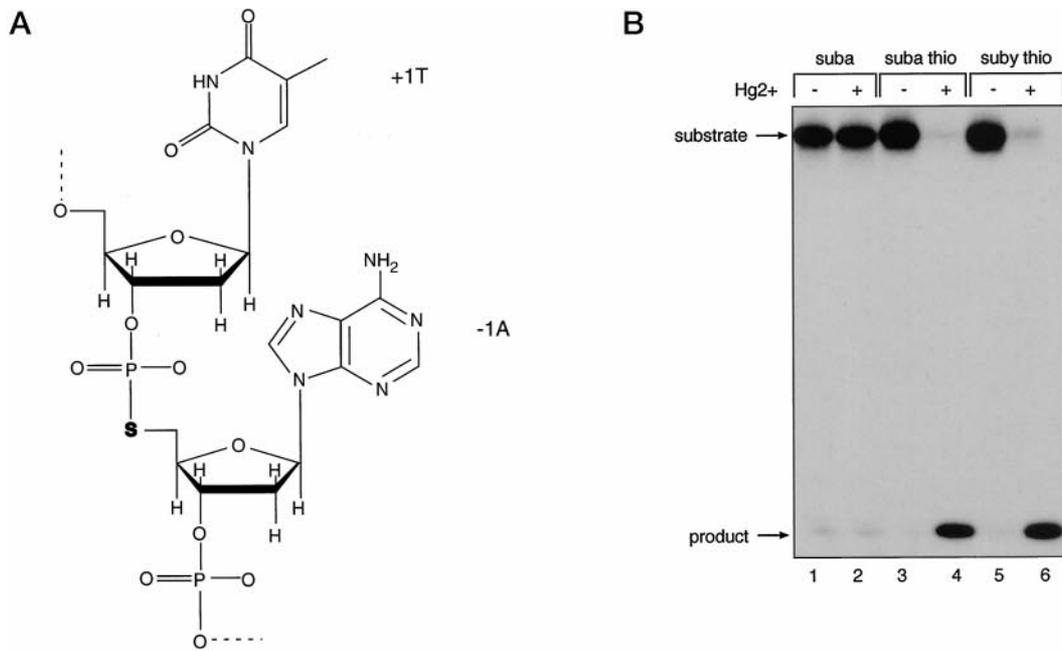


TABLE V

DNA binding and covalent complex formation on substrates *a* and *y* containing 5'-bridging phosphorothiolates or unmodified controlsThe changed nucleotides from sub *a* are underlined. The initial rate of covalent complex formation in fmole/min is indicated in parenthesis (average of three time course studies). o and s indicate the normal oxo- or phosphorothiolate 5' linkages, respectively.

Substrate	Nucleotide sequence	Relative noncovalent binding	Relative covalent complex formation (initial rate, fmols/min)
<i>a</i>	5' TCCGTGTGCGCCCTT <u>o</u> ATTCCCTTTTTTGCGGCATTTCCT 3' GGCACAGCGGAAoTAAGGGAAAAACGCCGTAAACGGAG	100 ± 11	100 ± 2 (108)
<i>y</i>	5' TCCG <u>G</u> A <u>T</u> CGCCCTT <u>o</u> ACCCCTTTTTTGCGGCATTTCCT 3' GGCCTAGCGGAAoTGGGGAAAAACGCCGTAAACGGAG	96 ± 13	7 ± 0.6 (8)
<i>a</i> -thio	5' TCCGTGTGCGCCCTTsATTCCCTTTTTTGCGGCATTTCCT 3' GGCACAGCGGAAoTAAGGGAAAAACGCCGTAAACGGAG	92 ± 4	50 ± 5.4 (54)
<i>y</i> -thio	5' TCCG <u>G</u> A <u>T</u> CGCCCTTsACCCCTTTTTTGCGGCATTTCCT 3' GGCCTAGCGGAAoTGGGGAAAAACGCCGTAAACGGAG	87 ± 2	3 ± 0.4 (3.4)

(lanes 10–12 and 14–16). Assays of covalent complex formation revealed that substrate *a* and substrate *a*-thio formed covalent complexes efficiently (Fig. 4D, lanes 2–5 and 12–18), while substrate *y* and substrate *y*-thio were similarly reduced in activity (lanes 7–10 and 20–26). Quantitation (Fig. 4E; Table V) revealed that the initial rate for substrate *a* was 14 times faster than that for substrate *y*. Similarly, the initial rate for substrate *a*-thio was 16 times faster than that for substrate *y*-thio. Thus, the substitution of a 5'-bridging phosphorothiolate in substrate *y* did not lead to an accumulation of covalent complex, indicating that the base changes in substrate *y* specifically inhibit initial covalent complex formation without stimulating religation.

DISCUSSION

Our studies indicated that MCV topoisomerase recognizes a larger site than the previously described 5'-(C/T)CCTT-3' motif. Sequence contacts to the major groove outside the conserved pentamer were found to be selectively important for catalysis in the noncovalent protein-DNA complex. These data emphasize the importance of a reaction step between initial binding and covalent complex formation, a point also raised in structural studies of type 1B topoisomerases and related enzymes (26–30).

DNA Binding by Poxvirus Topoisomerases—DNase I footprinting studies of MCV topoisomerase and vaccinia topoisomerase (9) revealed that the enzyme bound to a recognition site spanning roughly 20 base pairs centered on the 5'-(C/T)CCTT-3' motif. The additional base pairs identified as important here, at +10, –2, and –3, are within the region bound on the cleaved strand, but the +10-position may not be contacted on the uncleaved strand. This differed slightly from results with vaccinia, in which the footprint was larger on the uncleaved strand for the same substrate, indicating possible subtle differences in the complexes. Previously, vaccinia topoisomerase substrates were found to have differential impairments in catalysis and binding, but the locations of base pairs important for each were not clarified. Data presented here for MCV represent the first elucidation of important sequences outside of the 5'-(C/T)CCTT-3' motif and their role in postbinding activation of catalysis.

The vaccinia virus topoisomerase catalytic domain (residues

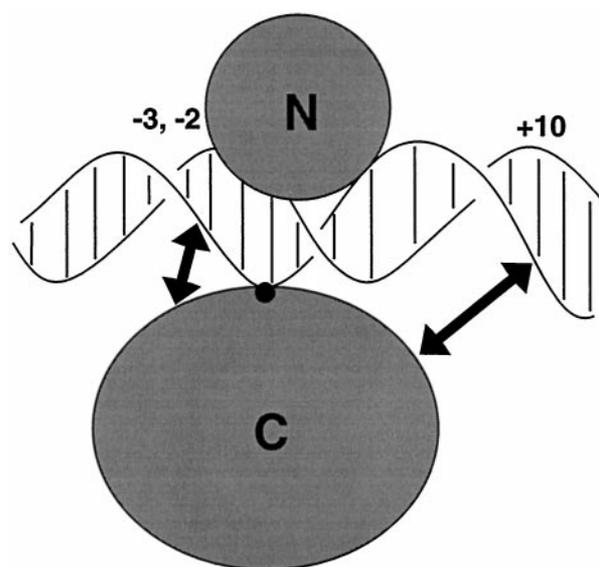


FIG. 5. Model of DNA contacts inferred to be important for covalent complex formation following initial binding. The model is adapted from Ref. 18. *N* and *C* indicate the amino- and carboxyl-terminal domains. The arrows indicate the inferred contacts between the topoisomerase catalytic domain and the substrate major groove important for covalent complex formation.

81–314) has been visualized by x-ray crystallography, and a model of the DNA complex has been built that satisfies several biochemical and structural constraints (18). In this model, the catalytic domain spans positions +10 to –9 with respect to the scissile phosphate, with varying degrees of engagement of the DNA in this region. The amino-terminal domain binds the conserved 5'-(C/T)CCTT-3' motif in the major groove. The catalytic domain lies on the other side of the DNA near the scissile phosphate (Fig. 5). Although the structure of the MCV topoisomerase has not been studied by x-ray crystallography, the high degree of sequence identity between the enzymes (54%) and functional similarity described here suggests that the vaccinia model probably describes MCV in some detail.

Postbinding Steps Potentiating Catalysis by Type 1B Topoisomerases and Related Enzymes—Vaccinia virus topoisomer-

FIG. 4. Effect of mutations in substrate *a* at positions +10, +9, –2, and –3 analyzed using 5'-bridging phosphorothiolate suicide substrates. *A*, diagram of the 5'-bridging phosphorothiolate structure. *B*, cleavage of 5'-bridging phosphorothiolate substrates with heavy metals. The substrates used (*sub*) and the presence or absence of Hg^{2+} are indicated above the gel. *C*, assay of DNA binding by substrates *a*, *y*, *a*-thio, and *y*-thio. *D*, covalent complex formation by substrates *a*, *y*, *a*-thio, and *y*-thio. *E*, kinetics of covalent complex formation by substrates *a* (□), *y* (◇), *a*-thio (○), and *y*-thio (△).

ase and several related proteins have been proposed to require a protein conformational change to potentiate covalent complex formation. In the structure of the catalytic domain of vaccinia virus topoisomerase, solved in the absence of DNA substrate, the tyrosine residue that acts as the nucleophile in forming the covalent intermediate was not correctly positioned to attack the scissile phosphate. A conformational change is thus necessary to activate the enzyme for covalent complex formation (18). Mutational studies have also supported a model in which a postbinding isomerization takes place prior to covalent complex formation (31).

Structural studies and sequence alignments indicate that the eukaryotic type 1B topoisomerases are members of the larger family of λ -integrase-related proteins. Several λ -integrase family protein structures, solved in the absence of DNA, also contain active site tyrosines in what appear to be inactive conformations. In XerD, the active site residues are apparently buried in the protein interior (28), while in the λ -integrase catalytic domain, the active site tyrosine is part of a disordered loop (30). For human type 1B topoisomerase, comparison of structures of catalytic domain fragments in both covalent and noncovalent complexes reveal clear although modest rearrangements of active site residues (26, 27).

In the case of the phage P1 Cre protein, a site-specific recombinase of the λ -integrase family, an x-ray structure is available of a recombination intermediate in which two of four Cre monomers have formed covalent complexes (29). In these structures, the positions of the active site tyrosines differ substantially, with the tyrosine in the two noncovalently bound complexes moved away from the scissile phosphate by a conformational change. Evidently, conformational transitions following the first pair of strand exchanges bring the second pair of active site tyrosines into alignment at the proper time for catalysis. These findings taken together emphasize the ubiquity of postbinding conformational changes and the potential of this step as a regulatory input.

DNA Binding and Catalysis by Poxvirus Topoisomerases—In our study of catalysis by MCV topoisomerase, an intermediate step between DNA binding and covalent complex formation appears to be rate-limiting, at least for some substrates. Correct DNA contacts may increase the rate of this intermediate step, potentially involving changes that bring the active site tyrosine into proper position. We hypothesize that the noncovalent complexes formed by MCV topoisomerase on poor substrates such as substrate γ mimic the normal noncovalent intermediate. According to this view, the suboptimal base pairs at +10, -2, and -3 obstruct the postbinding conformational change. Conversely, correct engagement of the key flanking sequences may help the catalytic domain to adopt the active conformation. In support of this model, we demonstrated using 5'-bridging phosphorothiolate substrates that the step activated by optimal flanking sequences was initial cleavage after DNA binding.

The activating contacts are probably made at least in part by the catalytic domain. Studies of inosine substitutions specify

the side of the helix toward the catalytic domain as the probable region contacted (Fig. 5). Furthermore, a catalytic domain fragment of MCV topoisomerase is sensitive to base sequence +10, -2, and -3, supporting this aspect of the model.³ In the model of the vaccinia catalytic domain structure docked on straight B-DNA (18), these flanking sequences may not be extensively engaged, indicating that the protein or DNA may need to bend to make these contacts (Fig. 5). A previous study of the vaccinia topoisomerase emphasized the role of the amino-terminal and linker domains in activating catalysis (18). However, in any model, conformational changes must ultimately position the active site tyrosine for cleavage, implying that changes must propagate to the catalytic domain as suggested here.

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