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DNA Contacts by Protein Domains of the Molluscum Contagiosum Virus Type-1B Topoisomerase

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All poxviruses studied encode a type 1B topoisomerase that introduces transient nicks into DNA and thereby relaxes DNA supercoils. Here we present a study of the protein domains of the topoisomerase of the poxvirus molluscum contagiosum (MCV), which allows us to specify DNA contacts made by different domains. Partial proteolysis of the enzyme revealed two stable domains separated by a protease-sensitive linker. A fragment encoding the linker and carboxyl-terminal domain (residues 82-323) was overexpressed in *Escherichia coli* and purified. MCV topoisomerase (MCV-TOP)(82-323) could relax supercoiled plasmids *in vitro,* albeit with a slower rate than the wild-type enzyme. MCV-TOP(82-323) was sensitive to sequences in the favored 5′-(T/C)CCTT-3′ recognition site and also flanking DNA, indicating that some of the sequence-specific contacts are made by residues 82-323. Assays of initial binding and covalent catalysis by MCV-TOP(82-323) identified the contacts flanking the 5′-CCCTT-3′ sequence at +10, +9, −2, and −3 to be important. Tests with substrates containing a 5-bridging phosphorothiolate that trap the cleaved complex revealed that correct contacts to the flanking sequences were important in the initial cleavage step. MCV-TOP(82-323) differed from the full-length protein in showing reduced sensitivity to mutations at a position within the 5′-(T/C)CCTT-3′ recognition site, consistent with a model in which the amino-terminal domain contacts this region. These findings provide insight into the division of labor within the MCV-TOP enzyme.

INTRODUCTION

Topoisomerases catalyze DNA cleavage and rejoining reactions important in relaxing supercoiled DNA. Type-1B topoisomerases act by binding to supercoiled DNA substrates and catalyzing formation of a covalent protein–DNA 3′ phosphotyrosine linkage (Fig. 1). After DNA relaxation, the reaction is completed by attack of the free 5′ hydroxyl group on the phosphotyrosine bond, thereby religating the cleaved strand. DNA relaxation by topoisomerases has been implicated as important for DNA replication, transcription, repair, and other functions (Wang, 1996).

All poxviruses studied to date encode a type-1B topoisomerase protein (Hwang *et al.,* 1998; Klemperer *et al.,* 1995; Massung *et al.,* 1993; Petersen *et al.,* 1997; Senkevich *et al.,* 1996; Shuman *et al.,* 1988). For a sequence alignment of these enzymes, see Petersen *et al.* (1997). Vaccinia topoisomerase, the most thoroughly studied model, has been inferred to be essential for viral replication because viruses deleted for the topoisomerase gene could not be isolated (Shuman *et al.,* 1989).

The poxvirus enzymes are attractive models for the study of topoisomerase function. The enzymes are small: molluscum contagiosum (MCV) topoisomerase (TOP) is 323 amino acids, whereas cellular type 1B topoisomerases range from 765 to 1019 amino acids. Poxvirus topoisomerases are also sequence specific, acting at the sequence 5′-(T/C)CCTT-3′, where the scissile phosphate is 3′ of the last T (position +1, numbers increasing leftward (Hwang *et al.,* 1999a, 1998; Shuman, 1991; Shuman and Prescott, 1990). This allows the enzyme to be placed at a defined position on DNA substrates for study. In previous work on MCV-TOP, correct flanking sequences at +10, +9, −2, and −3 were found to be important for covalent catalysis but not for initial DNA binding (Hwang *et al.,* 1999a). This permits precleavage or postcleavage complexes to be trapped experimentally for study *in vitro.*

Extensive studies of the structure, function, and kinetics of vaccinia topoisomerase have resulted in the proposal of a detailed model of enzyme function (Cheng *et al.,* 1998; Sekiguchi and Shuman, 1996; Sharma *et al.,* 1994). Structural studies of the two enzyme domains revealed that the carboxyl-terminal domain is related to the catalytic domains of human topoisomerase 1, Cre recombinase, lambda integrase, and other members of the lambda integrase family (Cheng *et al.,* 1998). The catalytic domain is thought to bind to the conserved pentamer near the scissile phosphate, whereas the amino-terminal domain is proposed to bind on the other side of the DNA helix (Cheng *et al.,* 1998; Sekiguchi and Shuman, 1996; Sharma *et al.,* 1994).

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FIG. 1. Diagram of DNA breaking and joining reactions carried out by a type-1 topoisomerase.

We have chosen to study the topoisomerase of the poxvirus MCV for both its interest as an additional model system and its potential importance as a drug target. MCV-TOP is 54% identical to vaccinia topoisomerase, clearly related but different enough to lend interest to a comparison of the two enzymes. MCV causes benign skin papules in healthy adults but disfiguring lesions in immunocompromised patients that are essentially untreatable (Gottlieb and Myskowski, 1994; Petersen and Gerstoft, 1992; Porter *et al.,* 1992; Schwartz and Myskowski, 1992). Understanding the structure and function of MCV-TOP may help guide the design of possible inhibitors for treating MCV infection.

Here we extend our study of catalysis by MCV-TOP by identifying and analyzing stable protein domains. Domain mapping by partial proteolysis indicated that MCV-TOP is composed of two domains separated by a protease-sensitive linker. We have isolated and analyzed an active catalytic domain fragment. Although this fragment is reduced in activity compared with the full-length protein, the fact that activity was detectable allowed the sequence discrimination by this domain to be tested. These results allowed DNA contacts made by each domain to be mapped and their contribution to specific reaction steps to be inferred.

RESULTS

Domain structure of MCV-TOP

As a first step in investigating DNA contacts made by MCV protein domains, stable protein fragments were mapped by partial proteolysis. MCV-TOP was partially digested with trypsin, and the resulting fragments were analyzed. Digestion with low amounts of trypsin yielded five predominant fragments of approximately 36 kDa (F1), 27 kDa (F2), 20 kDa (F3), 16 kDa (F4), and 9 kDa (F5) (Fig. 2A, lane 4). At high concentrations of trypsin, the 16- and 27-kDa fragments were reduced in abundance, and the fragments of 9 and 20 kDa were increased.

To determine the points of cleavage by trypsin, purified fragments were analyzed by amino-terminal amino acid sequencing. The amino-terminal sequences of the 36-kDa (F1), 16-kDa (F4), and 9-kDa (F5) fragments were found to be Gly-Ser-His-Met[1]-Lys-Arg-Phe-Phe-Phe. The first three amino acids are derived from the 20-amino acid hexahistidine tag used to purify the protein, indicative of cleavage within the tag by trypsin. The sequence continues with that of the MCV-TOP amino-terminus. The 20-kDa fragment (F3) contained an amino acid sequence beginning at 139, indicative of internal cleavage. Preparation and analysis of the 27-kDa fragment (F2) was challenging due to its lability in the presence of trypsin (data not shown). Partial amino acid sequence data were consistent with this fragment containing an amino-terminus at amino acid 82.

Analysis of the molecular weights of the observed fragments together with the amino-terminal sequences specified the identity of the fragments produced (Fig. 2A, right). Assuming that positions of trypsin cleavage indicate exposed or disordered regions, it is possible to infer that MCV-TOP is composed of two stable domains: residues 1-81 and 139-323. Between domains is a trypsin-sensitive linker (Fig. 2B). Fragments containing the linker region (82-138; F4 and F2) are efficiently degraded to shorter forms lacking the linker (F5 and F3) on prolonged digestion. The linker fragment itself (82-138) did not accumulate, potentially because it is disordered and rapidly digested. This domain structure parallels that found previously for vaccinia topoisomerase (Cheng and Shuman, 1998; Petersen *et al.,* 1997).

Expression and purification of the linker and catalytic domains.

To explore the functions of the MCV-TOP domains, the fragment encoding the linker and carboxyl-terminal domains (amino acids 82-323) was purified and studied. The linker was included in this construction in the hopes of maximizing activity (Cheng and Shuman, 1998). For expression of MCV-TOP(82-323), the coding region was amplified and transferred to the T7 expression vector pET28B. This resulted in the fusion of a 20-amino acid linker containing a hexahistidine tag to the amino-terminus to facilitate purification. MCV-TOP(82-323) was purified from the soluble fraction of lysed *E. coli* cells by chromatography on Ni-chelating Sepharose and CM-Sepharose. SDS–PAGE analysis of the purified fraction used for subsequent studies is shown in Fig. 3, lane 2.

To probe the folding of the MCV-TOP(82-323) fragment, the purified protein was analyzed by trypsin digestion. Characterization of the reaction products by SDS–PAGE revealed that a stable form was produced that corresponded in size to the 139-323 fragment described above. This was consistent with the view that the MCV-TOP(82-323) fragment was folded as in the intact protein (data not shown).

Effects of NaCl and $MgCl_2$ on relaxation activity by MCV-TOP(82-323)

The DNA relaxation activity of MCV-TOP(82-323) was investigated next. Supercoiled DNA molecules were incubated with MCV-TOP(82-323), and relaxation products

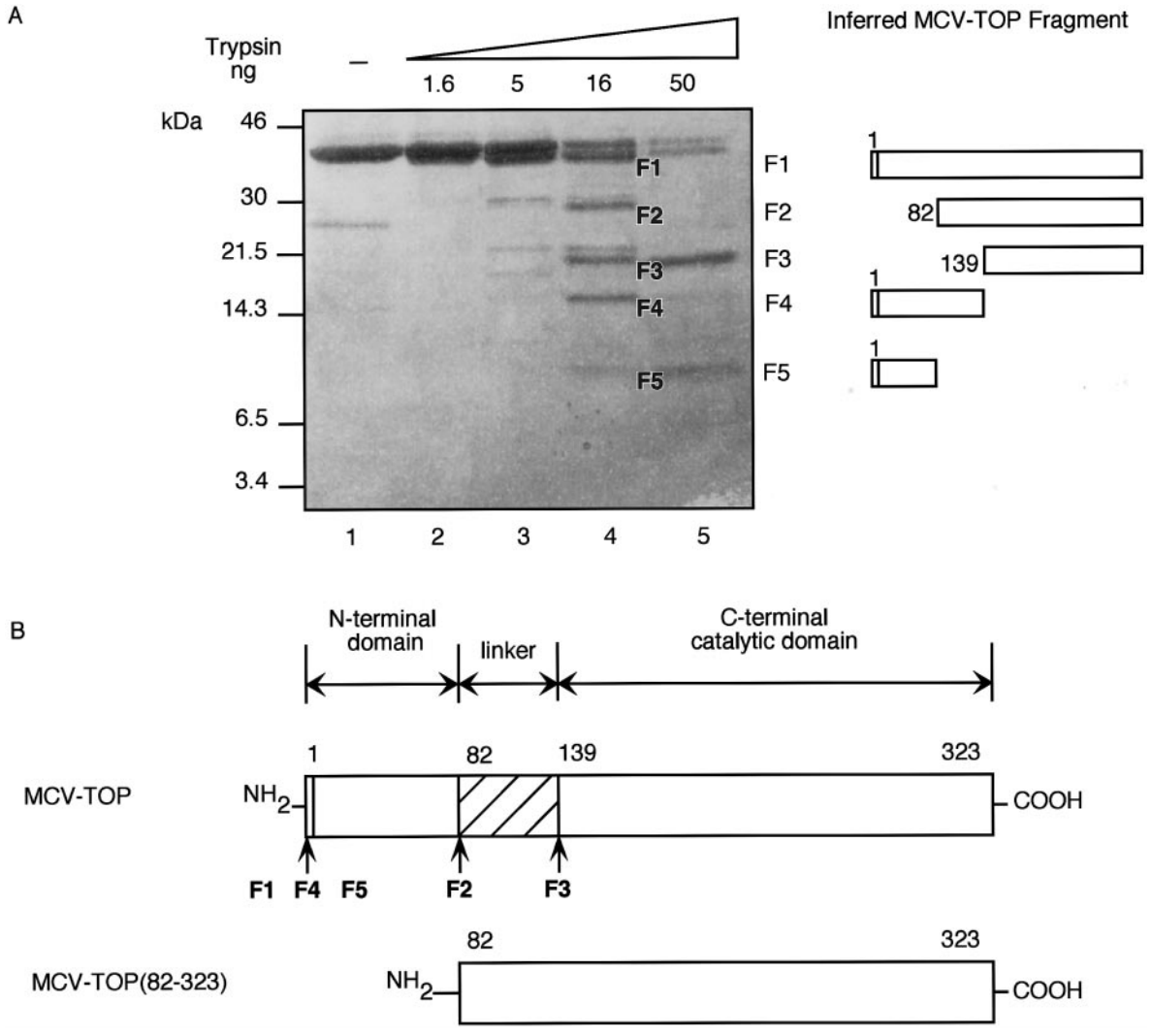

FIG. 2. Analysis of the domain structure of MCV-TOP by partial digestion with trypsin. (A) Digestion products analyzed by SDS–PAGE and staining with Coomassie blue. The positions and sizes of marker proteins are indicated on the left. The deduced structures of fragments F1 (36 kDa), F2 (27 kDa), F3 (20 kDa), F4 (16 kDa), and F5 (9 kDa) are shown on the right. (B) Domain structure of MCV-TOP. The trypsin-sensitive linker region is indicated by the hatched box. The trypsin cleavage sites deduced by amino acid sequencing are indicated by the arrows.

were visualized as slow-migrating bands on agarose gels (Fig. 4). MCV-TOP(82-323) was tested under conditions optimal for MCV-TOP(1-323), and little activity was found (Fig. 4, lanes 1-4). The addition of 200 mM potassium glutamate, conditions found to favor covalent complex formation for MCV-TOP, also had little effect (Fig. 4, lanes 5-8). The addition of 100 mM NaCl, however, increased the relaxation activity of MCV-TOP(82-323) (Fig. 4, lanes 9-12). The addition of $MgCl_2$ also stimulated slightly (Fig. 4, lanes 13-16). The addition of both was not obviously better than the addition of NaCl only (Fig. 4, lanes 17-20). Full-length MCV-TOP was not stimulated by Mg^{2+} and NaCl (Hwang *et al.,* 1998). Evidently the optimal conditions differ between the full-length enzyme and MCV-TOP(82-323). The highest rate of DNA relaxation by MCV-TOP(82-323) was about 0.1% that of wild type (data not shown).

DNA binding and covalent complex formation by MCV-TOP(82-323)

Plasmid pUC19 contains eight 5′-CCCTT-3′ pentamer sequences (Hwang *et al.,* 1998; Klemperer *et al.,* 1995; Shuman and Prescott, 1990). One site was cleaved by MCV-TOP with much higher efficiency than the others (Table 1, sub a; substrates henceforth are abbreviated "sub" and designated by a letter (Hwang *et al.,* 1998).

Binding of MCV-TOP(82-323) to a duplex oligonucleotide matching sub a was tested by native PAGE of protein–DNA complexes. The binding reactions contained

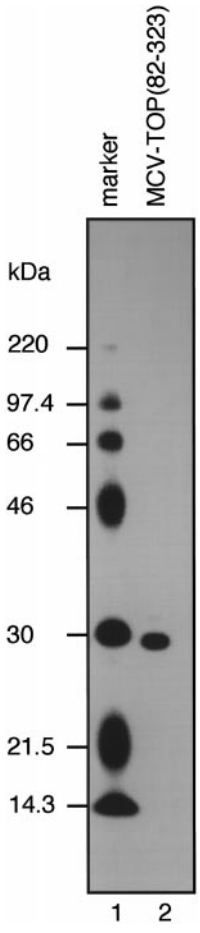

FIG. 3. Analysis of purified MCV-TOP(82-323) by SDS–PAGE. Lane 1 indicates size markers; lane 2, purified MCV-TOP(82-323).

100 mM NaCl, the optimal conditions for relaxation. MCV-TOP(82-323) formed complexes with reduced mobility and aggregated complexes that remain in the wells at the top of the gel (complexes 1 and 2, Fig. 5A). The maximum of complex 1 formation was reached at a 1:1 molar ratio of enzyme to sub a (Fig. 5A, lane 5). Increasing the ratio of MCV-TOP(82-323) to DNA to 2:1, 4:1 and 8:1 ratios resulted in increased formation of complex 2 and aggregated complexes in the wells (Fig. 5A, lanes 6–8). These forms likely represent binding of additional monomers of MCV-TOP(82-323) to sub a, with complex 2 likely formed by two monomers of MCV-TOP(82-323) bound to one DNA duplex.

To examine covalent complex formation by MCV-TOP(82-323), the enzyme was titrated into reactions containing 50 nM labeled sub a. Reactions were incubated for 3 h at 37°C, and production of the covalent complex was monitored by SDS–PAGE (Fig. 5B). The covalent complex formed by MCV-TOP(82-323) and DNA migrated with the mass ~34 kDa, the expected sum of the molecular masses. A plateau was reached at 100 nM MCV-TOP(82-323), at which point about 4% of the substrate was in the covalent form (Fig. 5B, lane 6).

The rate of covalent complex formation was then investigated. MCV-TOP(82-323) (100 nM) was incubated with 50 nM sub a at 37°C. Aliquots were withdrawn at different times, analyzed on SDS–PAGE, and quantified with a PhosphorImager (Fig. 5C). MCV-TOP(82-323) cleaved sub a slowly, with a half-time of approximately 2 h. The maximum amount of covalent complex (4%) was obtained at 12–24 h. In contrast, for MCV-TOP, 22% of the input DNA became covalently bound by 20 nM enzyme in less than 1 min at 37°C. The full-length enzyme catalyzed cleavage with an apparent rate of 0.55/min (Hwang *et al.,* 1998), whereas MCV-TOP(82-323) catalyzed cleavage with an apparent rate of 3.8×10^{-4}/min. These experiments indicated that deletion of the amino-terminal domain affects DNA binding relatively modestly but inhibits covalent complex formation profoundly.

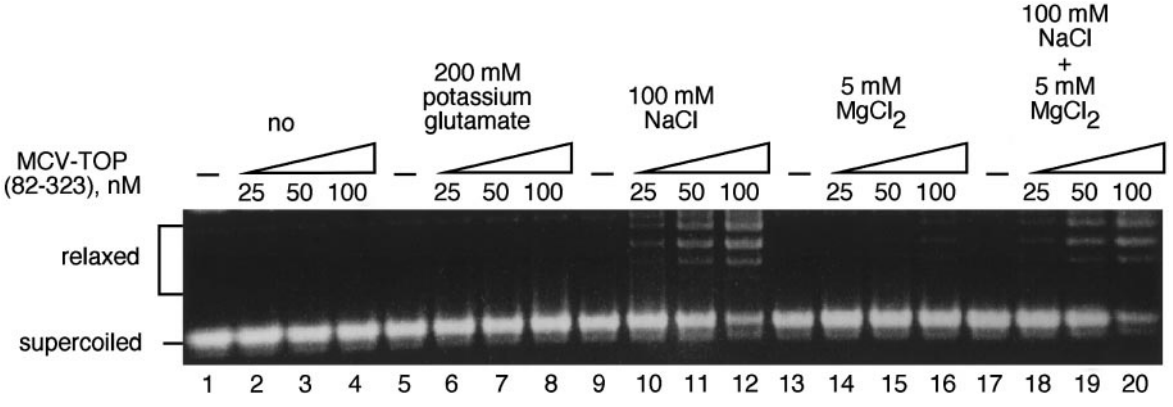

FIG. 4. Effects of NaCl and $MgCl_2$ on the DNA relaxation activity by MCV-TOP(82-323). Reaction mixtures contains 20 mM Tris–Cl (pH 8.0), 0.1% Triton X-100, 1 mM DTT, and 0.5 μg of pUC19 DNA. The reactions were started by adding the enzyme and incubated for 3 h at 37°C. The input MCV-TOP(82-323) is marked above the gel. Control reactions lacking enzyme are indicated by –. The salt concentrations added are indicated above the gel. Digestion of product DNAs with protease K and SDS did not alter mobility, indicating that the slower migrating forms are DNA circles with altered topology and not protein–DNA complexes (unpublished data).

TABLE 1

DNA Binding and Covalent Complex Formation by MCV-TOP on DNA Substrates Derived from pUC19

Substrate	Nucleotide sequence	Relative noncovalent binding	Relative covalent complex formation
	+10 +1 −10		
sub a	5′ TCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCT	100	100
(2457)	3′ GGCACAGCGGGAATAAGGGAAAAAACGCCGTAAAACGGAG		
sub b	5′ TACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCG	80	<0.2
(1514)	3′ TGGTTTTAGGGAATTGCACTCAAAAGCAAGGTGACTCGCG		
sub c	5′ TTCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACG	81	<0.2
(2677)	3′ AGTGCTCCGGGAAAGCGAGGCGCGCAAAGCCACTACTGCG		
sub d	5′ TCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCA	109	<0.2
(2492)	3′ GTATGAGAAGGAAAAAGTTATAATAACTTCGTAAATAGTG		
sub e	5′ TCGGTTAGCTCTTTCGGTCCTCCGATCGTTGTCAGAAGTA	101	<0.2
(2053)	3′ GCCAATCGAGGAAGCCAGGAGGCTAGCAACAGTCTTCATG		
sub f	5′ TCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCT	102	<0.2
(1461)	3′ GTTCTTCTAGGAAACTAGAAAAGATGCCCCAGACTGCGAG		
sub g	5′ TGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGG	98	<0.2
(1035)	3′ CGGAAAGAGGGAAGCCCTTCGCACCGCGAAAGAGTTACCG		
sub h	5′ CACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGC	104	<0.2
	3′ TGGCTAGCGGGAAGGGTTGTCAACGCGTCGGACTTACCGG		
sub i	5′ AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAG	110	<0.2
	3′ CGTGTAGGGGGAAAGCGGTCGACCGCATTATCGCTTCTCC		
sub j	5′ TGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAA	97	<0.2
	3′ CGCGAGCCGGGAAGGCCGACCGACCAAATAACGACTATTG		
sub k	5′ GCCCTTATTCCCTTTTTTGCGCATTTTGCCTTCCTGTTT	105	<0.2
	3′ GGGAATAAGGGAAAAAACGCCGTAAAACGGAAGGACAAAA		

Note. The coordinates of the cleavage sites in pUC19 are indicated in parentheses. The consensus sequence 5′-(T/C)CCTT-3′ is underlined. An arrow indicates location of the scissile phosphate. 100% noncovalent complex formation indicates 90% of substrate DNA converted to the noncovalently bound form. 100% covalent complex formation indicates 2% of conversion of substrate to covalent product.

DNA strand religation by MCV-TOP(82-323)

The religation activity was studied in isolation using covalent complexes trapped on "suicide substrates" (Fig. 6A). A cleavage substrate was used that contained only 5 bp of DNA 3′ of the 5′-CCCTT-3′ sequence. DNA cleavage accompanying covalent complex formation yielded a 5-bp hybrid, which melted under reaction conditions and was lost by diffusion. This trapped the covalent complex. Religation could then be analyzed in isolation by addition of high concentrations of a complementary 15-base strand. Religation yielded a 29-base labeled product.

MCV-TOP(82-323) was incubated with the suicide substrate, and the religation reaction was started by the addition of a 50-fold molar excess of the labeled 15 mers. Reaction products were analyzed by electrophoresis on a denaturing DNA sequencing type gel followed by autoradiography. Religation by MCV-TOP(82-323) was observed in 2 h and maximal after 4 h (Fig. 6B, lane 3). Initially 4% of the input substrate was converted to covalent complex. Of this, 50% (2% of the input substrate) participated in religation reactions within 4 h. For the case of full-length MCV-TOP, the conversion of covalent complex to religation product taking place within 25 min (data not shown). These data indicate that the active site itself is not greatly impaired in MCV-TOP(82-323) because the religation activity remains within 10-fold of wild type.

Specificity of DNA binding and covalent complex formation by MCV-TOP(82-323)

Previous studies investigated the effects of DNA sequences on function of full-length MCV-TOP (Hwang *et al.,* 1999a, 1998), allowing a detailed comparison with MCV-TOP(82-323). The oligonucleotide matching the sub a site described above was found to be cleaved with much higher efficiency than the others (Table 1, sub a). Interestingly, there are eight 5′-CCCTT-3′ sequences in pUC19, but only four were cleaved detectably when tested as oligonucleotides (Table 1, sub a–c and g; sub h–k were not cleaved), implicating flanking sequences as also important. Three additional favored sites had the sequence 5′-TCCTT-3′ (Table 1, sub d, e, and f). The availability of active MCV-TOP(82-323) offered an opportunity to begin to delineate the domains of MCV TOP important for different contacts in the above DNA substrates.

The sequence specificity of DNA binding by MCV-TOP(82-323) was first assayed using these oligonucleotide substrates (Fig. 7A and Table 1). The efficiency of DNA-binding by MCV-TOP(82-323) varied slightly, but all

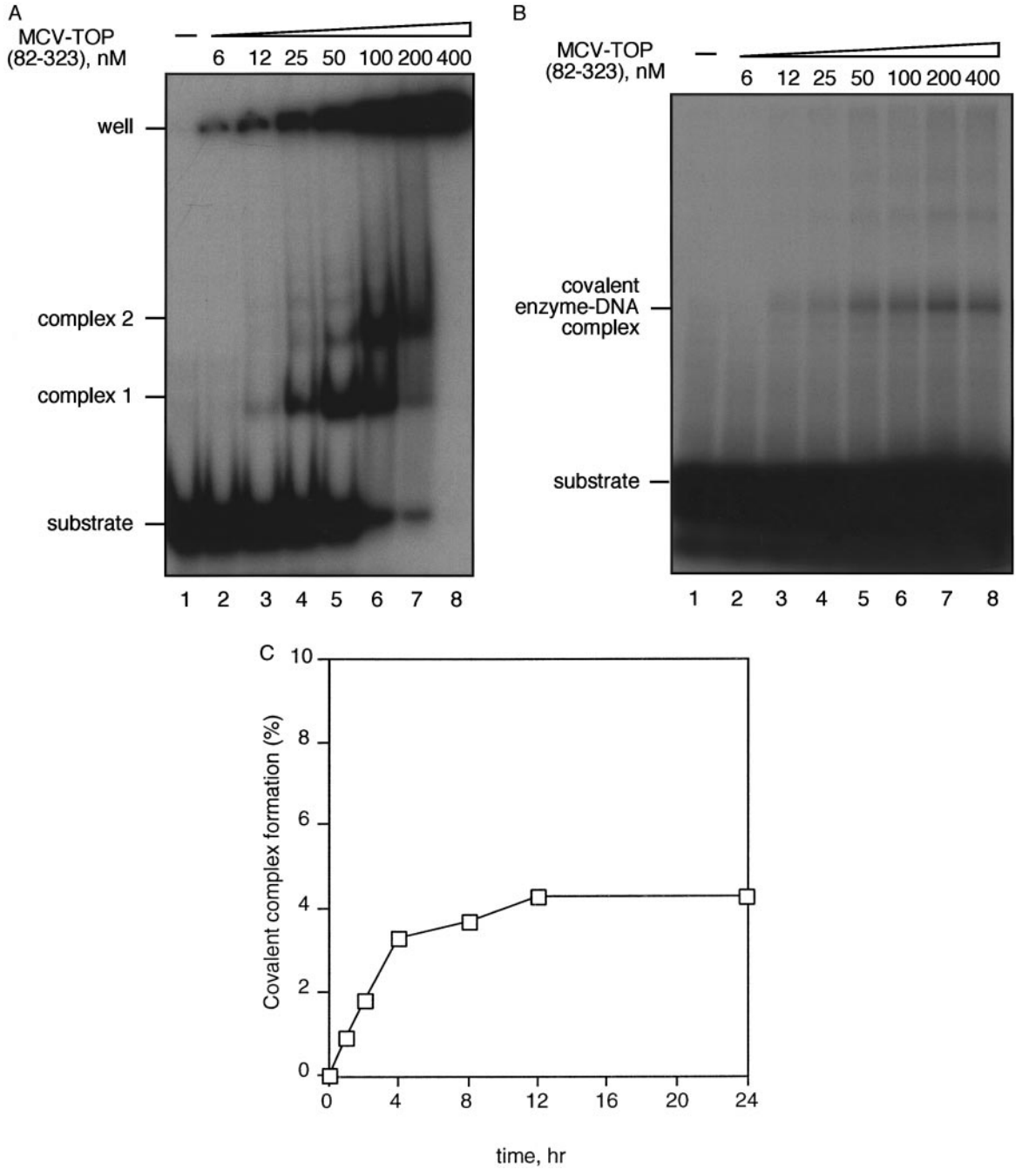

FIG. 5. DNA binding and covalent complex formation by MCV-TOP(82-323). Reaction mixture (per 10 μl) contained 20 mM Tris–Cl (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 1 mM DTT, and 500 fmol of radiolabeled sub a. The input MCV-TOP(82-323) is marked above the gel. Control reactions lacking enzyme are indicated by —. The positions of the free DNA substrates and topoisomerase–DNA complexes are indicated at the left. (A) DNA binding activity analyzed by native gel electrophoresis. The reactions were started by adding the enzyme and incubated for 5 min at 37°C. (B) Covalent complex formation activity by SDS–PAGE. The reactions were started by adding the enzyme and incubated for 3 h at 37°C. The products were analyzed on 12% SDS–PAGE. (C) Kinetics of covalent complex formation. Reaction mixture (per 100 μl) contained 20 mM Tris–Cl (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 1 mM DTT, and 5 pmol of radiolabeled sub a. The reactions were started by adding the enzyme and incubated at 37°C. Aliquots (10 μl) were withdrawn at various times, and the covalent complex trapped by adding SDS. The products were analyzed on 12% SDS–PAGE. The extent of covalent complex formation was visualized by autoradiography and quantitated by PhosphorImager.

substrates bound efficiently in the presence of 100 nM MCV-TOP(82-323).

Substrates sub a–k were next tested for covalent complex formation. Oligonucleotides were incubated with MCV-TOP(82-323), separated by SDS–PAGE, and quantified (Fig. 7B and Table 1). Covalent complex formation

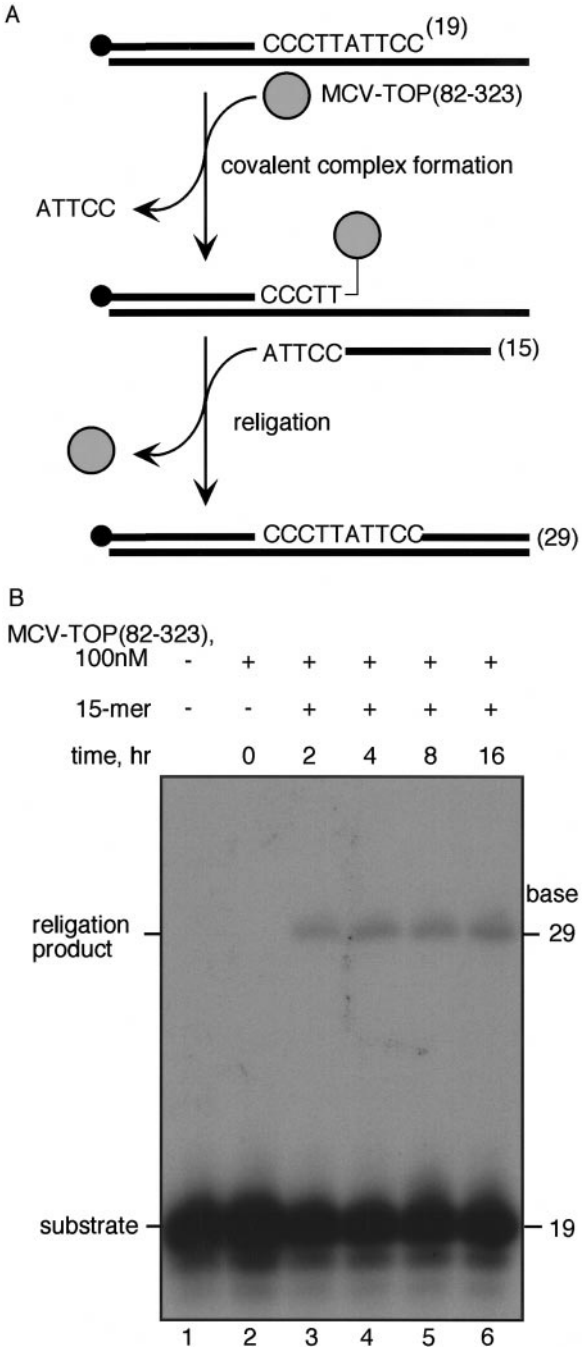

FIG. 6. DNA religation activity of MCV-TOP(82-323). (A) Diagram of the religation reaction. The suicide substrate labeled on the 5′ end of the top strand was incubated with MCV topoisomerase. Cleavage released the short duplex extension 3′ of the 5′-CCCTT-3′ sequence, trapping the covalent complex. This allowed the religation reaction to be carried out after the addition of a 15-mer donor DNA complementary to the single strand in the trapped covalent intermediate. Religation generates a product containing a radiolabeled 29-mer DNA strand. The 5′ end labeled position is indicated by the closed circle. MCV-TOP(82-323) protein is indicated by the shaded circle. The number of bases in single-stranded DNAs are indicated in parentheses. (B) DNA religation by MCV-TOP(82-323). Reaction mixtures (per 100 μl) contained 20 mM Tris–Cl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT, and 5 pmol of radiolabeled suicide substrate sub a. The reactions were started by adding the enzyme and incubated for 24 h at 37°C to generate covalent complex. The aliquots (10 μl) with covalent complex were transferred into individual tubes and incubated with 50-fold excess of the 15-mer donor DNA. The products were analyzed on DNA-sequencing type gel. The extent of religation product formation was visualized by autoradiography and quantified by PhosphorImager. Control religation reactions were performed without enzyme (lane 1) and with enzyme but without the 15-mer donor (lane 2). The sampling time to measure the religation products is indicated above the gel (lanes 3–6).

was efficient only with sub a, showing 2.0% conversion of substrate to product (defined as 100% in Table 1). Sub b–k were not detectably active. Overall, these data indicate that primary sequence outside the conserved pentamer can strongly influence covalent complex formation by MCV-TOP(82-323) and that the effects of sequence are exerted primarily after noncovalent DNA binding.

In a previous study of the full-length enzyme, important sequences outside the conserved pentamer were mapped (Hwang *et al.,* 1999a). By exchanging sequences between sub a and a disfavored site, it was found that bases at positions +10, +9, −2, and −3 were specifically important for covalent complex formation by MCV-TOP, but not for DNA binding (Hwang *et al.,* 1999a).

For the case of MCV-TOP(82-323), two substrates were compared. One was the favored sequence, sub a. The other, sub y, contained disfavored bases at +10, +9, −2, and −3 but was otherwise identical. DNA binding assayed by gel retardation revealed little difference between the two substrates (Fig. 8A). Assays of covalent complex formation revealed that sub a formed covalent complexes efficiently (Fig. 8B, lanes 2-6), whereas sub y was greatly reduced in activity (Fig. 8B, lanes 8-12). Quantification of the initial rates (Table 2) revealed that catalysis in the presence of sub a was eight times faster than for sub y. For the case of the full-length protein, the difference was 13-fold (Hwang *et al.,* 1999). Thus the substitutions at +10, +9, −2, and −3 in sub y specifically inhibited covalent complex formation without changing DNA binding, as in full-length MCV-TOP. This indicates that MCV-TOP(82-323) contains the determinants necessary for responding to the correct sequence at +10, +9, −2, and −3.

Mechanism by which flanking sequences stimulate covalent complex formation

The presence of the optimal flanking sequences could have stimulated accumulation of the covalent complex by either of two means. The optimal sequences could either have promoted the initial cleavage reaction or prevented the religation reaction, thereby driving an equilibrium toward the covalent form. In previous studies of the full-length enzyme, these alternatives were distinguished using sub-

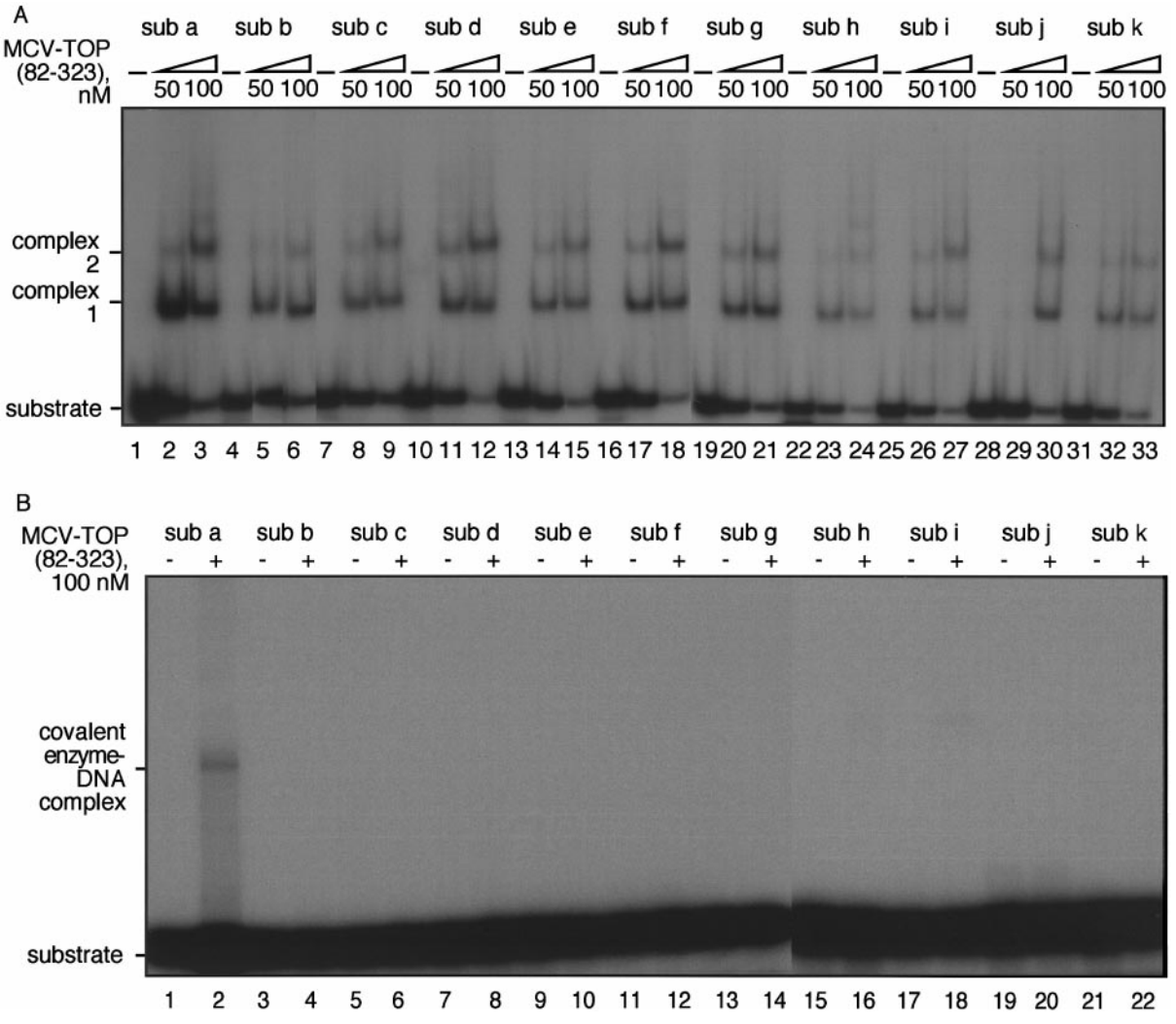

FIG. 7. DNA binding and covalent complex formation by MCV-TOP(82-323) at various sequences from pUC19. The sequences of substrates tested are indicated in Table 1. The input MCV-TOP(82-323) is marked above the gel. Control reactions lacking enzyme are indicated by −. The positions of the free DNA substrate 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 SDS–PAGE.

strates containing 5′-bridging phosphorothiolates at the scissile phosphate ("thio" substrates, Fig. 9A) (Hwang *et al.,* 1999a). The thio DNA is an efficient cleavage substrate, but the 5′ sulfur atom produced by cleavage is a poor nucleophile at phosphorous, so the religation reaction is blocked. This traps the first cleavage event on each molecule and so allows the forward rate of cleavage to be measured in isolation.

DNA binding was tested first and found to be roughly similar for the sub a–thio and sub y–thio substrates (Fig. 9B and Table 2). Covalent complex formation, however, was prominent for sub a–thio but greatly diminished for sub y–thio (Fig. 9C and Table 2). The presence of the 5′-bridging phosphorothiolate did not result in increased accumulation of the covalent complex, suggesting that the religation reaction is not quantitatively significant under the conditions studied. This indicates that the effects of the flanking sequences that distinguish sub a and sub y are primarily on the initial cleavage step, a conclusion also reached for the full-length protein.

The reactions containing MCV-TOP(82-323) and sub a–thio also yielded additional covalent complex forms revealed as additional bands on the autoradiogram. Such extra bands were seen with variable frequency in reactions on other substrates as well (data not shown). These bands may be formed by additional monomers of MCV-TOP(82-323) binding to the substrate and forming covalent complexes, as seen, for example, in the band-shift assays (complex 2).

Effect of mutations within the conserved pentamer on binding and cleavage by MCV-TOP(82-323)

To explore the importance of bases in the conserved pentamer, mutant DNA substrates were constructed

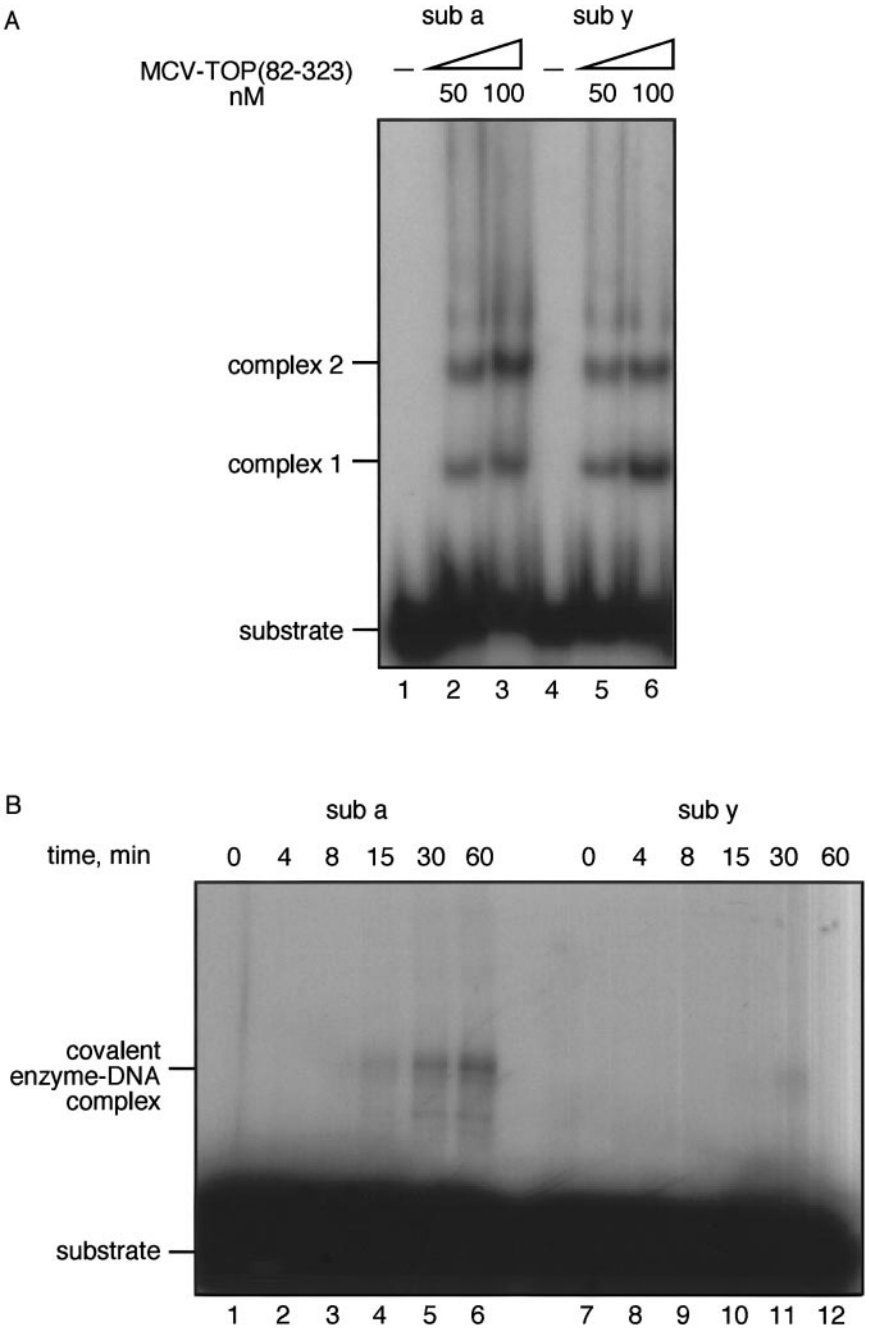

FIG. 8. Effect of mutations on sub a at positions +10, +9, −2, and −3 by MCV-TOP(82-323). (A) Assay of DNA binding by sub a and sub y. (B) Covalent complex formation by sub a and sub y.

containing changes in the 5′-(T/C)CCTT-3′ sequence and tested in the presence of MCV-TOP(82-323) (Table 3). Altering bases +5 (C to T) or +3 (C to T) had at most modest effects on binding, whereas altering base +1 (T to C) drastically reduced binding. This indicated that the DNA binding seen with MCV-TOP(82-323) was indeed sequence specific because binding was greatly impaired by a single base pair change.

For covalent complex formation, altering the base pairs at +5 and +3 had only modest effects, whereas altering the base pair at +1 reduced covalent complex formation to less than 0.1% of the sub a level.

In the case of full-length MCV-TOP, the correct base at position +3 is important for covalent complex formation. Altering this base (C to T as above) reduces complex formation to 21-fold, whereas for MCV-TOP(82-323), the reduction was only 5-fold (with the value uncorrected for the 2-fold reduction in initial binding). This reduced discrimination by MCV-TOP(82-323) at this base pair sug-

TABLE 2

DNA Binding and Covalent Complex Formation on sub a and sub y

Substrate	Nucleotide sequence	Relative noncovalent binding	Relative covalent complex formation (initial rate, fmol/min)
	+10 +1 −10		
sub a	5′ TCCGTGTCGCCCTToATTCCCTTTTTTGCGGCATTTTGCCT	100	100 (1.8×10^{-2})
	3′ GGCACAGCGGGAAoTAAGGGAAAAAACGCCGTAAAACGGAG		
sub y	5′ TCCGGATCGCCCTToACCCCCTTTTTTGCGGCATTTTGCCT	98	13 (2.3×10^{-3})
	3′ GGCCTAGCGGGAAoTGGGGGAAAAAACGCCGTAAAACGGAG		
sub a–thio	5′ TCCGTGTCGCCCTTsATTCCCTTTTTTGCGGCATTTTGCCT	96	46 (8.3×10^{-3})
	3′ GGCACAGCGGGAAoTAAGGGAAAAAACGCCGTAAAACGGAG		
sub y–thio	5′ TCCGGATCGCCCTTsACCCCCTTTTTTGCGGCATTTTGCCT	109	6 (1.1×10^{-3})
	3′ GGCCTAGCGGGAAoTGGGGGAAAAAACGCCGTAAAACGGAG		

Note. The nucleotides that differ between sub a and sub y are underlined. The apparent initial rate of covalent complex formation in fmol/min is indicated in parenthesis. "o" and "s" indicate the normal oxo- or phosphorothiolate 5′-linkages, respectively.

gests that the contact may normally be made in the full-length enzyme in part by the amino-terminal domain.

DISCUSSION

We present a study of the domain structure of MCV-TOP. Partial proteolysis identified three regions in the protein: an amino-terminal domain from residues 1-81, a linker from residues 82-138, and a carboxyl-terminal domain from residues 139-323. The linker is inferred to be flexible because it was readily removed from either domain by proteolysis and did not accumulate as a stable form. The carboxyl domain contains the conserved Tyr residue that in poxviruses topoisomerase was inferred to form the protein–DNA phosphotyrosine linkage (Shuman *et al.,* 1989). The relaxation and DNA cleavage activities were greatly reduced in MCV-TOP(82-323) compared with the full-length protein. However, the religation activity of MCV-TOP(82-323) was within 10-fold of the full length, indicating that the enzyme active site is largely intact and contained within residues 82-323. The general domain organization is similar to that described for vaccinia topoisomerase (Cheng *et al.,* 1998), indicating that the domain structure is conserved in the poxvirus topoisomerase family (Cheng and Shuman, 1998; Petersen *et al.,* 1997).

The fact that MCV-TOP(82-323) could carry out detectable DNA cleavage allowed the response to specific DNA sequences to be tested. In previous studies, we found that contacts to DNA sequences at +10, +9, −2, and −3 strongly influenced activity of the full-length enzyme. Studies reported here indicate that the contacts to the flanking sequences at +10, +9, −2, and −3 can now be attributed to MCV-TOP(82-323). Models for binding of the topoisomerase to DNA could be constructed based on structural studies of vaccinia (Cheng *et al.,* 1998; Sharma *et al.,* 1994) and biochemical studies on the MCV and vaccinia systems (Cheng and Shuman, 1998; Hwang *et al.,* 1999a; Sekiguchi and Shuman, 1996). For the MCV case, studies with inosine-modified substrates indicated that +10, +9, −2, and −3 are contacted on the face of the DNA likely apposed to the catalytic domain (Hwang *et al.,* 1999a). Analysis with substrates containing 5′-bridging phosphorothiolates reported here revealed that correct contacts to +10, +9, −2, and −3 act to promote initial cleavage by MCV-TOP(82-323), consistent with these DNA contacts being made by the catalytic domain.

These findings also constrain models for function of the amino-terminal domain. Binding of MCV-TOP(82-323) was not greatly reduced compared with MCV-TOP, suggesting that the amino-terminal domain contributes relatively little to DNA affinity. Binding by MCV-TOP(82-323) was still specific, as indicated by the finding that altering the base at +1 in the conserved pentamer abrogated complex formation. Thus the amino-terminal domain is not strictly required for specific binding. A specific amino-terminal domain–DNA contact is suggested by the finding of a DNA site (+3) at which discrimination is diminished with MCV-TOP(82-323) compared with the full-length protein. In support of such a view, for the case of vaccinia topoisomerase, residues Tyr^{70} and Tyr^{72} in the amino-terminal domain can be cross-linked to the +3 and +4 bases of the recognition site (Sekiguchi and Shuman, 1996).

Evidently, optimal catalysis requires correct contacts by both the amino-terminal domain and MCV-TOP(82-323) to position and activate the enzyme. One specific model posits that correct contacts serve to position the active site tyrosine for cleavage. This step appears to be necessary because the active site tyrosine is not posi-

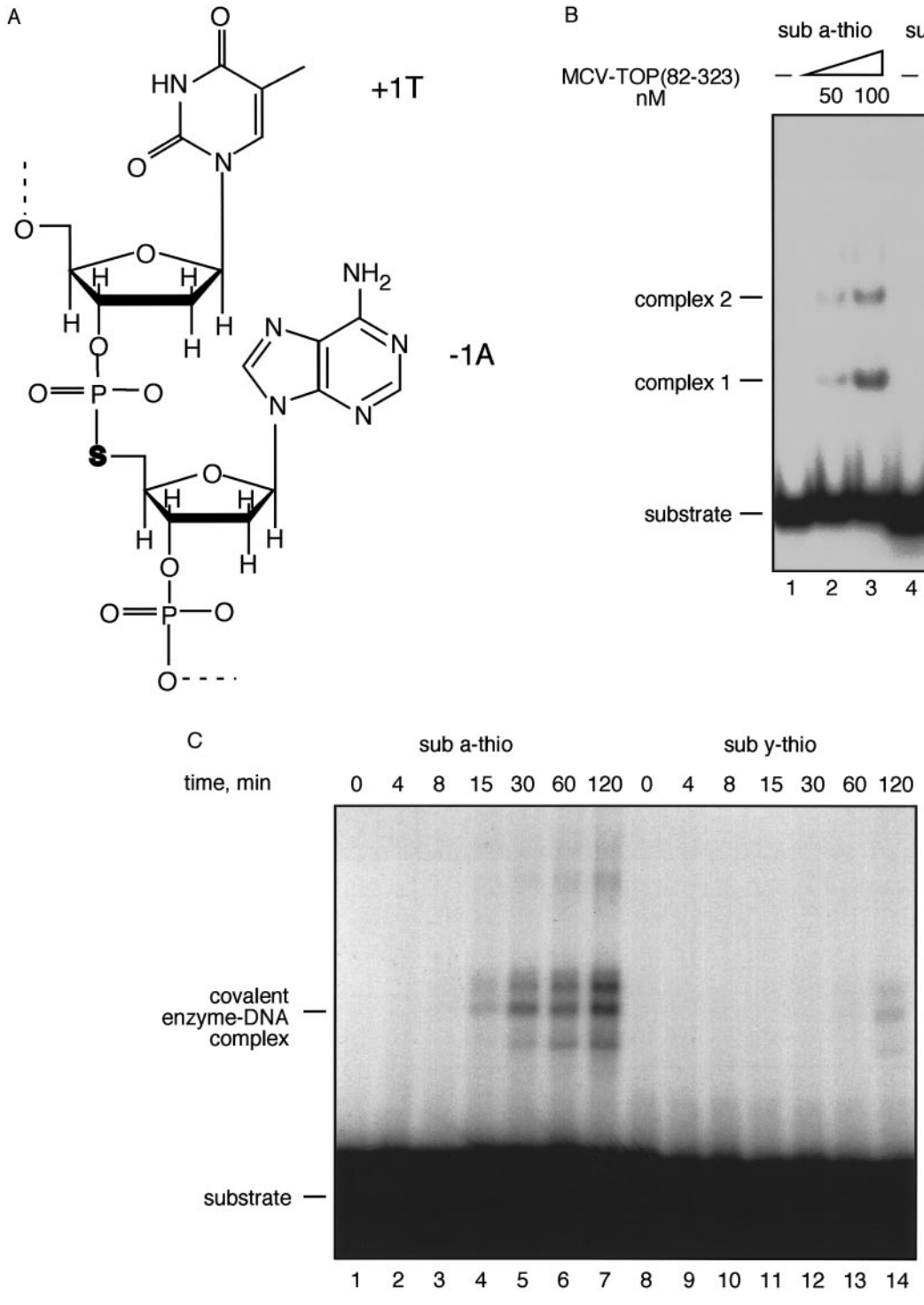

FIG. 9. Use of 5′-bridging phosphorothiolates to study the forward rate of cleavage of sub a and sub y. (A) Diagram of the 5′-bridging phosphorothiolate structure. The sulfur atom at the 5′ position is shown in bold. (B) DNA binding by sub a–thio and sub y–thio assayed by native gel electrophoresis. (C) Covalent complex formation by sub a–thio and sub y–thio assayed by PAGE.

tioned to be able to attack the DNA backbone in the structure of the vaccinia catalytic domain solved in the absence of DNA (Cheng *et al.,* 1998; Cheng and Shuman, 1998). Similar mispositioning of the active site tyrosine residue is seen in structures of other members of the lambda integrase family in the absence of DNA (Cheng *et al.,* 1998; Kwon *et al.,* 1997; Redinbo *et al.,* 1998; Stewart *et al.,* 1998). Possibly, this represents a regulatory mechanism to prevent damage of DNA by cleavage at inappropriate sites. The findings here that the catalytic

TABLE 3

DNA Binding and Covalent Complex Formation on Substrates Containing Mutations in the 5′-CCCTT-3′ Pentanucleotide

Substrate	Nucleotide sequence	Relative noncovalent binding	Relative covalent complex formation
	+10 +1 −10		
sub a	5′ TCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCT	100	100
	3′ GGCACAGCGGGAATAAGGGAAAAAACGCCGTAAAACGGAG		
sub a +5T	5′ TCCGTGTCGTCCTTATTCCCTTTTTTGCGGCATTTTGCCT	53	28
	3′ GGCACAGCAGGAATAAGGGAAAAAACGCCGTAAAACGGAG		
sub a +3T	5′ TCCGTGTCGCCTTTATTCCCTTTTTTGCGGCATTTTGCCT	51	20
	3′ GGCACAGCGGAAATAAGGGAAAAAACGCCGTAAAACGGAG		
sub a +1C	5′ TCCGTGTCGCCCTCATTCCCTTTTTTGCGGCATTTTGCCT	<0.2	<0.2
	3′ GGCACAGCGGGAGTAAGGGAAAAAACGCCGTAAAACGGAG		

Note. The positions altered by mutation are underlined.

domain responds to the flanking sequences at +10, +9, −2, and −3 and that the step affected is cleavage after DNA binding suggest that correct contacts to the flanking bases by the catalytic domain might help to position and activate the active site tyrosine.

The data on domain structure of the MCV-TOP are useful for several reasons. The ability to study the activity of the catalytic domain in isolation has allowed the site of action of a MCV-TOP inhibitor, sansalvamide A, to be mapped to 82-323 (Hwang *et al.,* 1999b). The availability of individual domains may also be useful for structural studies of the enzyme, as has been demonstrated previously for vaccinia virus topoisomerase (Cheng *et al.,* 1998; Sharma *et al.,* 1994). Both types of studies may help in the eventual development of clinically useful inhibitors of MCV replication.

MATERIALS AND METHODS

Purification of MCV-TOP(82-323)

DNA encoding amino acids 82-323 was amplified by PCR from DNA encoding the full-length gene (Hwang *et al.,* 1998) using primers HW329 (5′-CAGCATATGCACGAGCGCCGCGAC-3′) and HW330 (5′-CGCGGATCCTCACCCCGTTTCGGG-3′). These primers added restriction sites for *Nde*I to the amino-terminal coding region and *Bam*HI to the carboxyl-terminal coding region. This DNA fragment was cloned into pCRII (InVitrogen) to generate pHW101 and sequenced. The *Nde*I–*Bam*HI insert was released by cleavage with these enzymes. The purified DNA fragment was then ligated with T7 expression vector pET28B (Novagen) that had been cleaved with *Bam*HI and *Nde*I, yielding pHW102. This manipulation results in the fusion of a sequence supplied by the vector encoding a hexahistidine tag to the amino-terminal coding region. The plasmid structure was confirmed by DNA sequencing. Plasmids were introduced into the *Escherichia coli* strain BL21/DE3, which supplies T7 RNA polymerase under control of the *lacZ* promoter. Transformed cells were grown to mid-log at 30°C in Terrific Broth (Sambrook *et al.,* 1989) and then induced to express MCV-TOP(82-323) by the addition of 0.1 mM IPTG to the culture medium.

MCV-TOP(82-323) was purified using Ni-chelating Sepharose as described (Hwang *et al.,* 1998) followed by chromatography on CM-Sepharose. Full-length wild-type MCV-TOP was purified using Ni-chelating Sepharose as described (Hwang *et al.,* 1998).

MCV-TOP activity assays

The standard conditions for assaying relaxation, DNA binding, covalent complex formation, and religation activities were 100 mM NaCl, 20 mM Tris–Cl (pH 8.0), 1 mM dithiothreitol, and 0.1% Triton X-100.

For the DNA binding assays, oligonucleotide substrate a (sub a) was 5′-end labeled with ^{32}P, added to MCV-TOP enzyme, and incubated for 5 min at 37°C. The reaction mixtures were separated on 5% polyacrylamide gels and visualized by autoradiography, and the radioactivity was quantified by PhosphorImager. Sub a was a duplex DNA of 5′-TCCGTGTCGCCCTTATTCCCTTTTTGCGGCATTTTGCCT-3′ and 5′-GAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGG-3′. The covalent complex formation assay was carried out as described (Hwang *et al.,* 1998).

For religation assays, a suicide substrate derived from sub a DNA was used. In this substrate (sequence 5′-TCCGTGTCGCCCTTATTCC-3′ and 5′-GAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGG-3′), the short duplex extension 3′ of the 5′-CCCTT-3′ sequence can dissociate on covalent complex formation, trapping the covalent complex but permitting religation to an added DNA strand. Covalent complex reaction mixtures were transferred to individual tubes, and the religation reaction was started by the addition of a 50-fold molar excess of a 15-mer

single-stranded DNA (5′-ATTCCCTTTTTGCGG-3′) to generate a product containing a 29-mer DNA. After incubation at 37°C for various times, formamide was added to 50% (v/v), the samples were denatured for 5 min at 95°C, and then the aliquots of samples were analyzed on DNA sequencing type gels. The extent of religation was visualized by autoradiography and quantified by PhosphorImager.

Synthesis of the 5′-bridging phosphorothiolate will be described elsewhere (A. Burgin, unpublished data).

Defining domain structure

Trypsin (Boehringer Mannheim) was used for partial protease digestion. Reaction mixtures (15 μl) containing 50 mM Tris–Cl (pH 8.0), 5 μg of MCV-TOP, and various concentrations of trypsin were incubated for 30 min at room temperature and terminated by the addition of SDS to a final concentration of 0.5%. The final products were separated either by SDS–PAGE or by HPLC. For SDS–PAGE, digestion products were transferred to PVDF membranes (BioRad), and visualized by staining with Amido-Black 10B (BioRad) as described (Fischer *et al.,* 1991). The bands of stained protein fragments were excised, and the amino-terminal sequences were determined by Edman degradation in an automated protein sequencer (model Procise 474; Perkin-Elmer ABI). The larger fragments F1 and F2 (Fig. 2A) transferred poorly under the conditions tested. For this reason, the trypsin digest was resolved by reversed-phase HPLC on a Vydac C-18 column (Separations Group). The resulting fractions were analyzed by SDS–PAGE and subjected to Edman degradation. Analysis of the amino-terminus of F2 was complicated by the presence of F1 and F3 in the fraction tested, but subtraction of the overlapping sequences yielded a unique placement of the amino-terminus.

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