Deletions at the Carboxyl Terminus of Vaccinia DNA Topoisomerase Affect DNA Binding and Enhance Distributivity in DNA Relaxation†

Li Kai Wang and Stewart Shuman*

Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021

Received November 5, 1996; Revised Manuscript Received January 27, 1997⊗

ABSTRACT: Vaccinia topoisomerase relaxes DNA through the formation of a covalent DNA−(3′-phosphotyrosyl)protein intermediate at sites containing the sequence 5′-(T/C)CCTT↓. The active site, Tyr-274, is situated near the carboxyl terminus of the 314 amino acid enzyme. Here, we report the effects of serial C-terminal deletions. Removal of five amino acids had no effect on topoisomerase activity. However, deletion of 10, 15, or 20 amino acids rendered the enzyme distributive in DNA relaxation, incrementally slowed the rate of single-turnover DNA cleavage, and progressively diminished DNA binding affinity, without altering the sequence specificity of DNA cleavage. These effects lead us to speculate that the region downstream of the active site, which is not well-conserved among the poxvirus-encoded topoisomerases, is a component of the proposed circumferential interface between the enzyme and duplex DNA.

DNA relaxation by the vaccinia virus type I topoisomerase entails a series of partial reactions common to all eukaryotic type I enzymes. These are (i) noncovalent binding of the protein to duplex DNA, (ii) cleavage of one DNA strand with formation of a covalent DNA−(3′-phosphotyrosyl)-protein intermediate, (iii) strand passage, and (iv) strand religation. A distinctive feature of the vaccinia topoisomerase is that it binds and cleaves duplex DNA at a specific target sequence, 5′-(T/C)CCTT↓ (Shuman & Prescott, 1990). The enzyme makes contact with the base pairs and with the sugar−phosphate backbone of DNA within the CCCTT recognition site (Shuman & Turner, 1993; Sekiguchi & Shuman, 1994b, 1996). Base-specific contacts are made in the major groove of the DNA, whereas contacts with specific phosphates, including the scissile phosphate, are made on both strands along the minor groove. The phosphate contacts are situated on the opposite face of the DNA helix from the base-specific contacts, which implies that vaccinia topoisomerase binds circumferentially to the DNA (Sekiguchi & Shuman, 1994b).

The 314 amino acid vaccinia topoisomerase consists of three structural domains demarcated by two protease-sensitive segments referred to as the bridge and hinge (Figure 1) (Sekiguchi & Shuman, 1995; Sharma et al., 1994). UV cross-linking experiments suggest that the 9-kDa N-terminal domain (from residues 1 to 80) contacts the target sequence in the major groove (Sekiguchi & Shuman, 1996). Transesterification reaction chemistry is carried out by the hinge and the adjacent C-terminal domain. The protease-resistant C-terminal domain includes the active-site nucleophile (Tyr-274) and three other residues (Lys-167, Arg-223, and His-265) that are essential for covalent catalysis (Shuman et al., 1989; Morham & Shuman, 1992; Klemperer & Traktman, 1993; Petersen et al., 1996; Petersen & Shuman, 1997). Two more essential residues (Arg-130 and Tyr-136) are located within the hinge (Wittschieben & Shuman, 1994). These

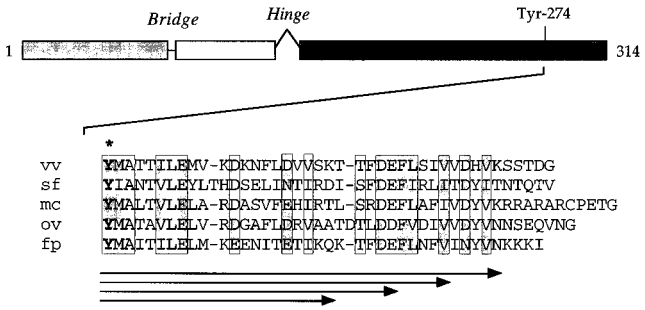

FIGURE 1: Domain structure and deletion mutations of vaccinia topoisomerase. The tripartite domain structure of the 314 amino acid vaccinia topoisomerase is illustrated. The protease-resistant structural domains are punctuated by protease-sensitive interdomain bridge and hinge segments (Sekiguchi & Shuman, 1995). The active-site Tyr-274 is situated within the C-terminal domain. The amino acid sequence of the C-terminus of vaccinia virus topoisomerase (vv) from residues 274 to 314 is aligned with the homologous segments of the topoisomerases encoded by other members of the poxvirus family: Shope fibroma virus (sf), *Molluscum contagiosum* virus (mc), Orf virus (ov), and fowlpox virus (fp). [For protein sequences, see Shuman and Moss (1987), Upton et al. (1990), Klemperer et al. (1995), Zantige et al. (1996), and Senkevitch et al. (1996).] The active site is indicated by an asterisk. Conserved residues are in shaded boxes. The distal boundaries of the four C-terminal deletion mutants are indicated below the sequence.

five functional groups (all of which are located upstream of the active-site tyrosine) are presumed to interact directly with the scissile phosphate during the strand cleavage step.

A 20-kDa carboxyl fragment of the topoisomerase (from residues 134 to 314) is inactive with respect to DNA cleavage but does bind noncovalently to duplex DNA, albeit without the specificity for the CCCTT target site characteristic of the full-sized protein (Sekiguchi & Shuman, 1995). The constituents of the C-terminal domain involved in sequence-independent DNA binding are as yet uncharted. To begin to understand the function of the C-terminal domain, we have analyzed the effects of serial 5-amino-acid deletions from the C-terminus on topoisomerase activity, single-turnover

† This work was supported by NIH Grant GM46330 and ACS Grant FRA-432 (S.S.). L.K.W. is supported by NIH training grant T32CA09512.
⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1997.

S0006-2960(96)02754-7 CCC: $14.00

DNA cleavage, and DNA binding. Deletion of five amino acids was without effect. However, removal of 10, 15, or 20 amino acids rendered the enzyme distributive in DNA relaxation, incrementally slowed the rate of single-turnover cleavage, and progressively diminished DNA binding affinity, without altering the sequence specificity of strand cleavage.

MATERIALS AND METHODS

Deletion Mutations. C-Terminal deletion variants of vaccinia topoisomerase were generated by PCR amplification with oligonucleotide primers designed to introduce stop codons at positions encoding amino acids 310, 305, 300, and 295. The mutagenic primers also introduced a *Bam*HI site distal to the stop codon. Plasmid pA9topo (Shuman et al., 1988) was used as the template for the PCR reaction. The PCR products were digested with *Nde*I and *Bgl*II and then inserted into pET3c (which had been digested with *Nde*I and *Bam*HI) to yield plasmids pET-Topo(1−309), pET-Topo(1−304), pET-Topo(1−299), and pET-Topo(1−294). All mutations were confirmed by dideoxy sequencing.

Topoisomerase Expression and Purification. pET-based plasmids were transformed into *Escherichia coli* BL21. Topoisomerase expression was induced by infection of 200-mL bacterial cultures with bacteriophage λCE6 (Shuman et al., 1988). Wild-type topoisomerase, Topo(1−309), and Topo(1−304) were purified from soluble bacterial lysates by phosphocellulose column chromatography as described (Shuman et al., 1988). The overexpressed Topo(1−299), and Topo(1−294) proteins were recovered exclusively in the insoluble pellet. However, these truncated topoisomerases could be partially solubilized by resuspending the pellet in buffer A [50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, and 10% glycerol] containing 1 M NaCl. The suspension was clarified by centrifugation and the supernatant was diluted with 2 volumes of buffer A. This material was applied to a 1-mL phosphocellulose column that had been equilibrated in buffer A containing 0.2 M NaCl. The column was eluted stepwise with buffer A containing 0.5 and 1.0 M NaCl. The elution profiles of the topoisomerases were monitored by SDS−PAGE. The truncated topoisomerase polypeptides (like the wild-type enzyme) were recovered in the 1.0 M NaCl eluate. The phosphocellulose preparations were further purified by sedimentation through 4.8-mL 15−30% glycerol gradients (Sekiguchi & Shuman, 1995). The gradients were centrifuged for 38 h at 50 000 rpm in a Beckman SW50 rotor. Fractions were collected from the bottom of the tubes. The sedimentation profiles were examined by SDS−PAGE. Each deletion mutant sedimented as a discrete peak consistent with a monomeric structure, as described previously for the wild-type enzyme.

DNA Relaxation Assay. Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 0.3 μg of pUC19 DNA, recombinant topoisomerase, and other components as specified were incubated at 37 °C. The reactions were initiated by the addition of enzyme. Aliquots (20 μL) were withdrawn at the times indicated and quenched immediately by adding a solution containing SDS (0.3% final concentration), glycerol, xylene cyanol, and bromophenol blue. "Time 0" samples were taken prior to addition of enzyme. Reaction products were analyzed by electrophoresis through a 1% horizontal agarose gel in TG buffer (50 mM Tris and 158 mM glycine). The gels were stained in a 0.5 μg/mL ethidium bromide solution, destained in water, and photographed under short-wave UV illumination.

Suicide Cleavage Assays. An 18-mer CCCTT-containing DNA oligonucleotide was 5′ end-labeled by enzymatic phosphorylation in the presence of [$\gamma-^{32}$P]ATP and T4 polynucleotide kinase, then gel-purified and hybridized to a complementary 30-mer strand (present at 4-fold molar excess). Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 0.25 pmol of 18-mer/30-mer DNA, and topoisomerase were incubated at 37 °C. Covalent complexes were denatured by addition of SDS to 1%. The denatured samples were electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. Free DNA migrated near the bromophenol blue dye front. Covalent complex formation was revealed by transfer of radiolabeled DNA to the topoisomerase polypeptide. The extent of covalent adduct formation (expressed as the percent of the input 5′ ^{32}P-labeled oligonucleotide that was transferred to protein) was quantitated by scanning the dried gel using a Fujix BAS1000 bio-imaging analyzer.

Native Gel Mobility Shift Assay of DNA Binding. Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 100 fmol of 24-bp duplex DNA (5′ ^{32}P-labeled on the scissile strand), and topoisomerase as specified were incubated at 37 °C for 5 min. Glycerol was added to 5% and the samples were electrophoresed through a 6% native polyacrylamide gel in 0.25× TBE (22.5 mM Tris-borate and 0.6 mM EDTA) at 100 V for 2.5 h. Topoisomerase−DNA complexes of retarded mobility were visualized by autoradiographic exposure of the dried gel. The nucleotide sequence of the scissile strand of the 24-bp DNA ligand was 5′-CGTGTCGCCCTTATTCCGATAGTG.

Equilibrium Cleavage Assays. A 60-mer oligonucleotide containing a centrally placed CCCTT element was 5′ end-labeled, then gel-purified, and annealed to an unlabeled complementary 60-mer strand (Shuman & Turner, 1993). Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 0.2 pmol of 60-mer DNA duplex, and topoisomerase were incubated at 37 °C for 20 min. Covalent complexes were trapped by addition of SDS to 1%. The samples were then digested for 60 min at 37 °C with 10 μg of proteinase K. The volume was adjusted to 100 μL and the digests were then extracted with an equal volume of phenol/chloroform. DNA was recovered from the aqueous phase by ethanol precipitation. The pelleted material was resuspended in formamide and the samples were electrophoresed through a 10% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris-borate and 2.5 mM EDTA). The cleavage product, a ^{32}P-labeled 30-mer bound to a short peptide, was well-resolved from the input 60-mer substrate. The extent of strand cleavage was quantitated by scanning the wet gel using a bio-imaging analyzer.

RESULTS

Carboxyl-Terminal Deletions of Vaccinia Topoisomerase. The active-site tyrosine of every eukaryotic type I topoisomerase (cellular and poxviral) is located near the carboxyl-terminus of the protein (Caron & Wang, 1994). The vaccinia topoisomerase contains 40 amino acids downstream of its active site, Tyr-274. In order to gauge the function, if

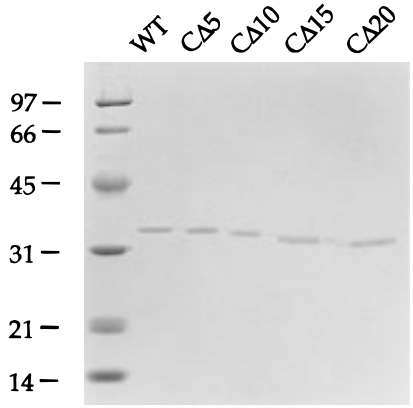

FIGURE 2: Topoisomerase purification. The glycerol gradient enzyme fractions were analyzed by SDS−PAGE. Protein (0.33 μg) was applied to each lane of a 10% polyacrylamide gel. Polypeptides were visualized by staining the gel with Coomassie Brilliant Blue dye. The positions and molecular mass (in kilodaltons) of coelectrophoresed protein standards are indicated on the left. The identity of the protein preparation is indicated above each lane.

any, of this C-terminal protein segment, we constructed a series of deletion mutants encoding polypeptides truncated from the C-terminus in 5-amino-acid increments. For convenience, we will refer to deletion mutants Topo(1−309), Topo(1−304), Topo(1−299), and Topo(1-294) as CΔ5, CΔ10, CΔ15, and CΔ20, respectively. The wild-type and four mutant alleles of vaccinia topoisomerase were expressed in *E. coli* BL21. The wild-type, CΔ5, and CΔ10 proteins were purified from soluble bacterial extracts by phosphocellulose column chromatography and glycerol gradient sedimentation. The more extensively truncated versions, CΔ15, and CΔ20, were insoluble when overexpressed but were rendered partially soluble by extraction of the insoluble pellet fractions with 1 M NaCl. The salt-extracted CΔ15 and CΔ20 proteins were then purified by phosphocellulose chromatography and glycerol gradient sedimentation. The topoisomerase polypeptide constituted the major species in the glycerol gradient protein preparations, as determined by SDS−PAGE (Figure 2). The extents of purification were essentially equivalent. Note that the electrophoretic mobilities of the recombinant proteins increased with serial deletion of C-terminal amino acids, as expected.

Mutational Effects on Relaxation of Supercoiled DNA. To assess the impact of these deletions, the proteins were tested for their ability to relax supercoiled plasmid DNA *in vitro*. Screening assays were performed in 0.1 M NaCl in the absence of magnesium. The rate-limiting step for wild-type topoisomerase under these conditions is the dissociation of enzyme from the relaxed plasmid product (Sekiguchi & Shuman, 1994a; Stivers et al., 1994). The rates of relaxation were determined at a fixed level of input protein. Wild-type topoisomerase (4 ng) relaxed 0.3 μg of supercoiled pUC19 DNA to completion within 2 min (Figure 3). The rate of relaxation by CΔ5 was similar to that of wild-type topoisomerase (Figure 3). This indicated that the last five amino acids were dispensable for catalysis. The rates of relaxation by CΔ10, CΔ15, and CΔ20 were approximately one-half, one-fifth, and one-tenth the wild-type rate, respectively (as gauged by the rate of disappearance of the fully supercoiled input DNA).

Relatively few reaction products of intermediate superhelicity were observed with the wild-type topoisomerase, suggesting that the enzyme relaxed individual DNA molecules to completion before dissociating and engaging a new DNA. The same distribution of products was observed for CΔ5. However, intermediate topoisomers were prominent during relaxation by CΔ10, CΔ15, and CΔ20 (Figure 3). This situation could arise if the rate of supercoil release on plasmid DNA were slowed or if the mutant enzyme became more distributive in its action, i.e., dissociating to a new substrate molecule before relaxing to completion.

The DNA relaxation assays were also performed in the presence of 5 mM magnesium. [Magnesium enhances product off rate without affecting the rate of DNA cleavage (Stivers et al., 1994).] Magnesium stimulated the activity of the wild-type enzyme such that 4 ng of enzyme relaxed nearly all supercoils within 15 s (Figure 3). CΔ5 was stimulated to a similar extent by magnesium. However, neither CΔ10, CΔ15, nor CΔ20 was stimulated by magnesium; indeed, the rates of relaxation by these mutants were actually *lowered* by about a factor of 10 in the presence of magnesium. These results argued that a step other than product release was rate-limiting for relaxation by CΔ10, CΔ15, and CΔ20 in the absence of magnesium. Paradoxical inhibition of CΔ10, CΔ15, and CΔ20 by magnesium (in contrast to the stimulatory effect on wild-type enzyme) suggested that the more extensively truncated mutants may have altered affinity for DNA.

To determine which component step(s) of the topoisomerase reaction were affected by the deletion mutations, we subjected the CΔ5, CΔ10, CΔ15, and CΔ20 proteins to a more detailed biochemical characterization as described below.

Mutational Effects on Single-Turnover DNA Cleavage. A "suicide" substrate containing a single CCCTT↓ cleavage site for vaccinia topoisomerase was used to examine the transesterification reaction under single-turnover conditions. The substrate consisted of an 18-mer scissile strand annealed to a 30-mer complementary strand (Figure 4). Upon formation of the covalent protein−DNA adduct, the distal cleavage product 5′-ATTCCC is released and the topoisomerase becomes covalently trapped on the DNA (Shuman, 1991). In the experiment shown in Figure 4A, we measured the rates of DNA cleavage at equivalent concentrations of each protein, i.e., 50 ng/20 μL. The wild-type and CΔ5 proteins displayed identical rates and end points of cleavage; both enzymes cleaved >90% of the input DNA. The initial rates and cleavage end points of the CΔ10, CΔ15, and CΔ20 proteins were reduced. The cleavage values in each reaction series were normalized to the end points (Figure 4B). Comparing the rates of approach to the cleavage end point, we estimate that the observed rate constants for cleavage by the CΔ10, CΔ15, and CΔ20 proteins were ∼45%, 12%, and 6%, respectively, of the wild-type value. These decrements in the rate of cleavage were consonant with the mutational effects on the rate of DNA relaxation in the absence of magnesium.

Effects of NaCl and Mg on DNA Cleavage. Suicide cleavage reactions are routinely performed at low ionic strength in the absence of a divalent cation. Prior studies showed that the rate of single-turnover cleavage by the wild-type vaccinia topoisomerase is unaffected by the levels of salt and magnesium that strongly stimulate DNA relaxation under steady-state conditions (Stivers et al, 1994; Sekiguchi and Shuman, unpublished results). It has been argued that

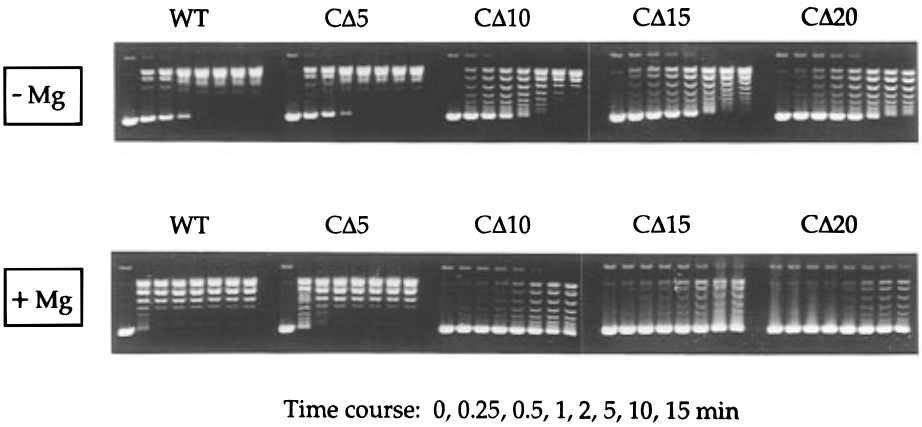

FIGURE 3: Kinetics of DNA relaxation by mutant topoisomerases. Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, either 2.5 mM EDTA (−Mg) or 5 mM $MgCl_2$ (+Mg), 0.3 μg of pUC19 DNA, and 4 ng of wild-type topoisomerase (WT) or the indicated mutant proteins were incubated at 37 °C. Aliquots were withdrawn at the times indicated and quenched immediately in SDS. The reaction products were analyzed by agarose gel electrophoresis. The predominant DNA species in the time zero lanes is supercoiled pUC19 monomer (2.7 kbp); the higher molecular weight species corresponds to supercoiled pUC19 dimer (5.4 kbp).

salt and magnesium stimulate relaxation by enhancing product dissociation rather than by affecting the chemical steps of transesterification (Stivers et al., 1994; Sekiguchi & Shuman, 1994a). The apparently distributive character of the relaxation reactions catalyzed by the CΔ10, CΔ15, and CΔ20 proteins suggested that C-terminal deletions might reduce the affinity of topoisomerase for DNA, resulting in dissociation of product prior to complete relaxation. The findings that DNA relaxation by these deletion mutants is inhibited by magnesium suggested that precleavage binding might be partially rate-limiting under these conditions.

To address this issue, we examined the effects of salt and magnesium on suicide cleavage. The amounts of covalent adduct formed in the presence of 50, 100, and 150 mM NaCl or 1, 2.5, 5, and 7.5 mM $MgCl_2$ were measured and normalized to the extent of cleavage in unsupplemented control reactions. The salt effects are shown in Figure 5A; the magnesium effects, in Figure 5B. We observed that the wild-type and CΔ5 proteins were unaffected by either salt or magnesium. In contrast, the extents of covalent adduct formation by CΔ10, CΔ15, and CΔ20 were reduced progressively by inclusion of 100−150 mM NaCl and by 1−7.5 mM $MgCl_2$. Susceptibility to salt and magnesium inhibition suggested weaker binding of the truncated topoisomerases to the DNA substrate.

DNA Relaxation in the Absence of NaCl. DNA relaxation by vaccinia topoisomerase under steady-state conditions is stimulated markedly by 100−150 mM NaCl (Shuman et al., 1988; Sekiguchi & Shuman, 1994a). This is because NaCl accelerates the rate-limiting step of the reaction, i.e., dissociation of the enzyme from the relaxed DNA product. Relaxation by CΔ10, CΔ15, and CΔ20 appeared distributive when reactions were performed in 0.1 M NaCl (Figure 3). In light of the inhibitory effects of 0.1−0.15 M NaCl on single-turnover cleavage by these mutants (presumably reflective of weaker DNA binding), we reevaluated the mutational effects on DNA relaxation under nonstringent conditions, i.e., in the absence of added salt or divalent cation. Under these circumstances, 10 ng of wild-type topoisomerase or CΔ5 relaxed 0.3 μg of supercoiled pUC19 DNA to completion within 10−15 min and no topoisomers of intermediate superhelicity were detected (Figure 6). CΔ10, CΔ15, and CΔ20 relaxed the DNA as fast or faster (in the case of CΔ15) than the wild-type enzyme. This was in contrast to the mutational effects noted in the presence of salt, in which case the truncated enzymes relaxed more slowly than the wild-type (compare Figures 3 and 6). Given that product dissociation is more profoundly rate-limiting in the absence of salt (relative to the cleavage step, for example) than it is in the presence of salt, we suggest that the modest mutational effects on cleavage rate are unlikely to render the cleavage step rate-determining in the absence of salt and that cleavage rate effects may be obscured or overshadowed by a mutational enhancement of the product dissociation rate. Intermediate topoisomers were detected during relaxation by CΔ15 and CΔ20 in the absence of salt (Figure 6) but were less prominent than when salt, or salt and magnesium, was present.

Mutational Effects on DNA Binding. A native gel mobility shift assay (Morham & Shuman, 1992; Sekiguchi & Shuman, 1994c) was used to analyze the DNA binding properties of the mutant proteins. The ^{32}P-labeled DNA ligand was a 24-bp CCCTT-containing duplex. Binding of wild-type topoisomerase to the 24-bp DNA resulted in the formation of two discrete protein−DNA complexes of retarded electrophoretic mobility (Figure 7). The more rapidly migrating species contained topoisomerase bound to the 24-mer duplex, whereas the upper complex contained topoisomerase bound covalently to a 5′-tailed DNA cleavage product (Morham & Shuman, 1992). The upper complex arises via dissociation of the 3′ segment of the scissile strand.

The extent of protein−DNA complex formation was proportional to the amount of wild-type topoisomerase added. The protein dependence of DNA binding by CΔ5 was similar to that of wild type enzyme. The CΔ10 mutation reduced DNA binding affinity by a factor of 2−4. The effects of the CΔ15 and CΔ20 mutations on DNA binding were more severe; most of the DNA remained unshifted and discrete topoisomerase−DNA complexes were barely detectable (Figure 7).

Mutational Effects on Equilibrium DNA Cleavage. We used a 60-bp DNA duplex containing a centrally placed cleavage site with 30 bp upstream and 30 bp downstream of the scissile bond to study topoisomerase cleavage under equilibrium conditions. In this assay, the topoisomerase−DNA adduct is trapped by addition SDS and then digested with proteinase K to yield a 5′-labeled 30-mer oligonucleotide linked to a small peptide. This product migrated during

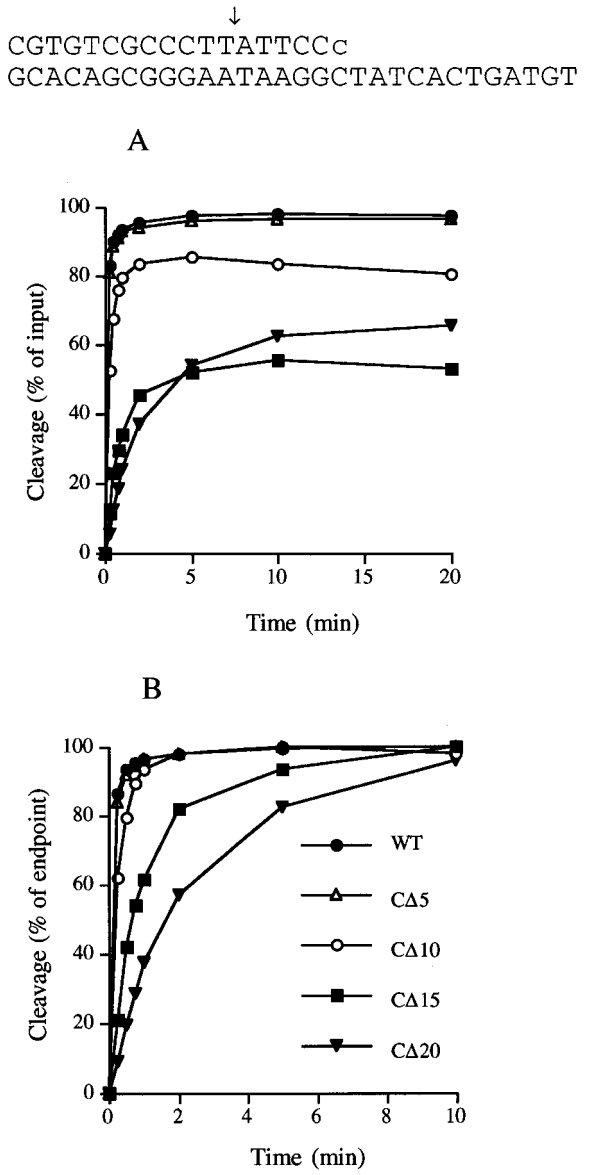

FIGURE 4: Mutational effects on suicide DNA cleavage. Suicide cleavage was assayed as described under Materials and Methods. Reaction mixtures containing (per 20 μL) 0.25 pmol of the 5′ ^{32}P-labeled suicide substrate (depicted at the top of the figure) and 50 ng of WT or mutant proteins were incubated at 37 °C. The cleavage reactions were initiated by the addition of topoisomerase to prewarmed reaction mixtures. Aliquots (20 μL) were withdrawn at 15, 30, and 45 s and 1, 2, 5, 10, and 20 min and quenched immediately by adding SDS. Covalent complex formation is plotted as a function of time in panel A. The data were normalized to the end-point values and replotted in panel B.

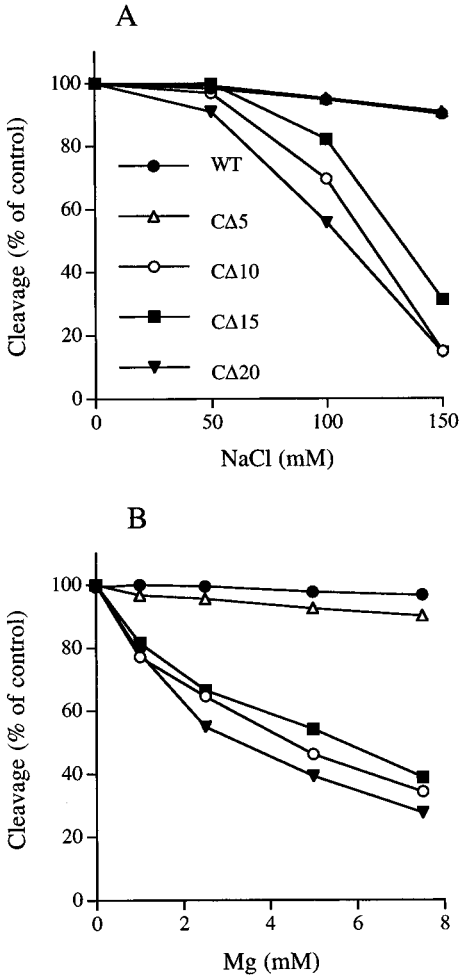

FIGURE 5: Effects of salt and magnesium on suicide cleavage. Reaction mixtures containing 0.25 pmol of DNA substrate and 50 ng of protein were supplemented with NaCl (panel A) or $MgCl_2$ (panel B) as indicated. Reactions were initiated by adding protein and terminated after incubation at 37 °C for either 1 min (WT, CΔ5, and CΔ10) or 5 min (CΔ15 and CΔ20). The extents of covalent complex formation were normalized to that of the unsupplemented control reaction (defined as 100%). The normalized cleavage data are plotted as a function of the concentration of added salt (panel A) or magnesium (panel B).

electrophoresis as a cluster of bands with an apparent chain length of 32−34 nucleotides. The heterogeneity of the cleavage product is attributable to the covalent attachment of one or more amino acids to the 3′ end of the cleaved fragment (Shuman, 1991). The extent of cleavage of the 60-mer duplex by the wild-type topoisomerase increased with input enzyme and plateaued at ~20 ng (Figure 8). At saturation, 21% of the substrate was cleaved. The cleavage equilibrium constant (K_{cl} = covalent complex/noncovalent complex) was 0.26. Covalent complex formation by CΔ5 was 20% at saturation, indicating that removal of the last five amino acids did not affect the cleavage equilibrium. In contrast, the extents of covalent adduct formation by CΔ10, CΔ15, and CΔ20 were 6%, 3%, and 2%, respectively (Figure 8). Hence, their cleavage−religation equilibrium constants (0.06 for CΔ10, 0.03 for CΔ15, and 0.02 for CΔ20) were lower than that of the wild-type enzyme. Kinetic analysis of 60-mer cleavage by 50 ng of each protein confirmed that all reactions had achieved equilibrium (data not shown). Note that the 5′-labeled cleavage products generated by CΔ10, CΔ15, and CΔ20 were identical to those of the wild-type topoisomerase (not shown), suggesting that the truncated enzymes cleaved the 60-mer specifically at the CCCTT site.

Cleavage Site Specificity. We considered the possibility that carboxyl-terminal deletion mutations of vaccinia topoisomerase might affect cleavage site specificity. This was tested by incubating wild-type, CΔ5, CΔ10, CΔ15, and CΔ20 topoisomerases with a 2464-bp *Ava*II fragment of pUC19 that had been 3′ end-labeled with [α-^{32}P]dGMP on both DNA strands. Addition of SDS to the mixture traps the covalently bound protein on the unlabeled portion of the DNA strand, permitting localization of the sites of strand cleavage by size analysis of the cleavage products under denaturing conditions (Figure 9). The sizes of the radiolabeled cleavage products reflect the distance of the cleavage

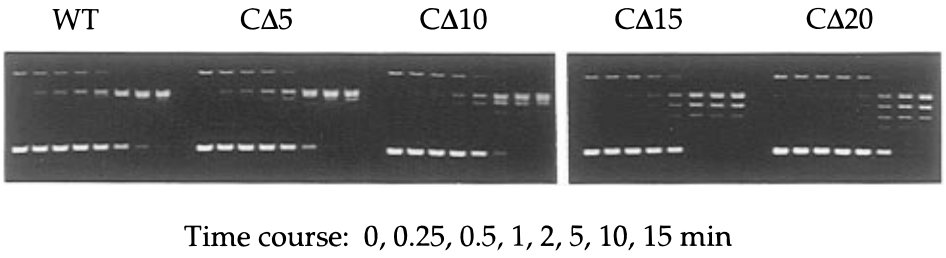

FIGURE 6: DNA relaxation in the absence of NaCl. Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 0.3 μg of pUC19 DNA, and 10 ng of wild-type topoisomerase (WT) or the indicated mutant proteins were incubated at 37 °C. Aliquots (20 μL) were withdrawn at the times indicated and quenched immediately in SDS. The reaction products were analyzed by agarose gel electrophoresis.

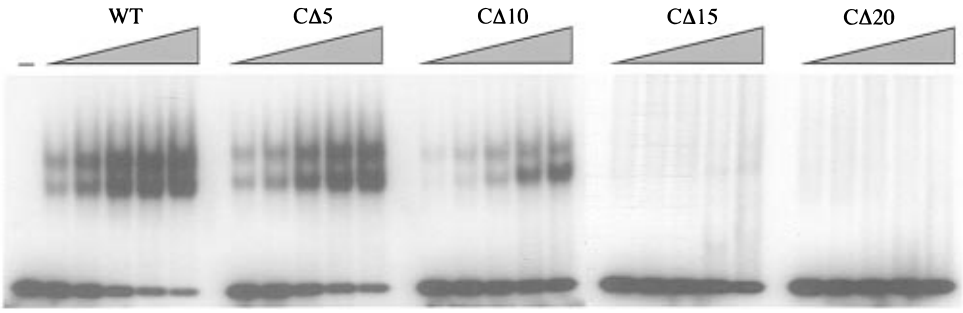

FIGURE 7: C-Terminal deletions affect DNA binding. Reaction mixtures (20 μL) contained 50 mM Tris HCl, pH 8.0, 100 fmol of 5′ ^{32}P-labeled 24-bp DNA, and increasing amounts of wild-type or mutant topoisomerase (0.9, 1.85, 3.7, 7.4, and 14.8 ng, proceeding from left to right within each titration series). The samples were analyzed by native gel electrophoresis. An autoradiograph of the gel is shown. A control reaction containing no topoisomerase is included in the lane at the extreme left.

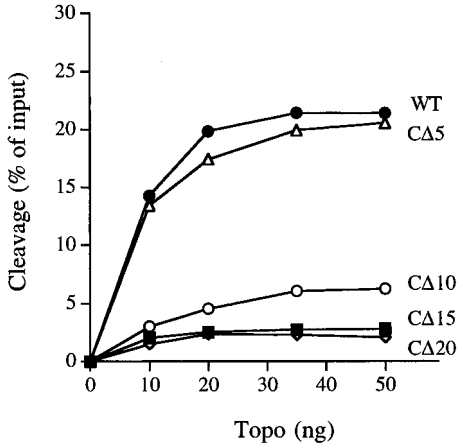

FIGURE 8: Mutational effects on equilibrium DNA cleavage. Covalent complex formation on a 60-bp duplex substrate was assayed as described under Materials and Methods. The extent of covalent complex formation is plotted as a function of input protein.

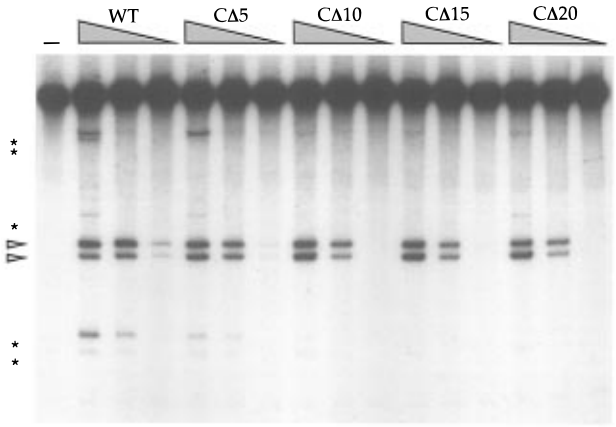

FIGURE 9: Cleavage site specificity. A 2464-bp *Ava*II restriction fragment of pUC19 was gel-purified and 3′ end-labeled on both strands in the presence of [α-^{32}P]dGTP and Klenow DNA polymerase. The labeled DNA was purifed by phenol-chloroform extraction and gel filtration through Sephadex G50. Topoisomerase cleavage reaction mixtures (20 μL) containing 50 mM Tris HCl, pH 8.0, 0.74 ng of 3′ end-labeled *Ava*II DNA fragment, and 1, 0.2, or 0.04 ng of the indicated topoisomerase (from left to right within each titration series), were incubated at 37 °C for 20 min. Reactions were halted by addition of SDS to 0.5% final concentration. The samples were adjusted to 60% formamide and then heated at 95 °C for 5 min. Cleavage products were analyzed by electrophoresis through a 4% polyacrylamide gel containing 7 M urea in TBE. An autoradiograph of the gel is shown. A control reaction (lane −) contained no topoisomerase.

sites from the 3′ end of the cleaved strand. As reported previously (Shuman & Prescott, 1990; Sekiguchi & Shuman, 1996), the wild-type enzyme cleaved at two "high-affinity" sites at low enzyme concentration (these are denoted by arrowheads in Figure 9). The cleavage products generated at 0.04 ng of input enzyme migrated at ∼375 bp and ∼395 bp during denaturing PAGE. (The sizes were estimated in a separate experiment in which the topoisomerase cleavage products were electrophoresed with marker fragments generated by restriction endonuclease digestion of the labeled DNA.) Increasing the amount of enzyme to 0.2 and 1.0 ng resulted in cleavage at multiple additional sites (denoted by asterisks in Figure 9). The spectrum of cleavage sites and the protein concentration dependence of site occupancy by CΔ5 were essentially identical to those of the wild-type enzyme. In the case of CΔ10, CΔ15, and CΔ20, cleavage at sites that yield the 375-bp and 395-bp fragments required higher levels of input protein (Figure 9). The CΔ10, CΔ15, and CΔ20 deletions either reduced or eliminated strand scission at most other target sites. It is noteworthy that none of the mutant enzymes cleaved at sites not normally cleaved by the wild-type enzyme. Hence, loss of the carboxyl terminus did not loosen the stringent site specificity of the vaccinia topoisomerase.

DISCUSSION

Multiple points of contact between vaccinia topoisomerase and DNA are likely to contribute to site specificity and site affinity. Few of these are documented. Specific residues in the N-terminal domain make major groove contacts with the +4C and +3C bases of the CCCTT element, suggesting that site specificity is determined, at least in part, by this domain (Sekiguchi & Shuman, 1996). The capacity of the 20-kDa C-terminal segment for nonspecific low-affinity DNA binding suggests that this domain may interact with the phosphodiester backbone. The proposed model for circumferential binding of the enzyme at the target site entails protein−phosphate interactions along the minor groove on the side of the helix that includes the scissile bond (Sekiguchi & Shuman, 1994b). The amino acid residues that make these contacts have not been identified.

The effects of incremental C-terminal deletions of vaccinia topoisomerase suggest that the region downstream of the active-site Tyr-274 contributes to site affinity, potentially as a constituent of the proposed circumferential structure. We found that deletion of the last five amino acids (310−314) had no effect on the enzyme. These functionally unimportant terminal residues are conspicuously not conserved among the poxvirus-encoded topoisomerases. However, deletions encompassing positions 294−309 did affect the mode of DNA relaxation, the rate of strand cleavage, and the stability of the topoisomerase−DNA interaction. These mutational effects are considered below.

Incremental deletion of residues 305−309 (Val-Asp-His-Val-Lys) resulted in a transition from a processive to a distributive mode of action, as judged by the presence of intermediate topoisomers during relaxation of plasmid DNA by the CΔ10 mutant. Single-turnover DNA cleavage was minimally affected by this deletion when activity was assayed under nonstringent binding conditions, i.e., in the absence of salt or magnesium. We conclude that these five residues are not essential for reaction chemistry. [We regard as nonessential those residues at which side-chain removal, e.g., by alanine substitution or by deletion, slows covalent catalysis by an order of magnitude or less. An essential residue is one at which side-chain removal causes a drastic (i.e., ≥100-fold) decrement in strand cleavage. These definitions are reasonable when one considers that the wild-type topoisomerase accelerates the rate of transesterification by an estimated factor of 10^9 (Stivers et al., 1994).]

The binding affinity of CΔ10 to a 24-bp CCCTT-containing DNA under nonstringent conditions (no added salt or magnesium) was about one-fourth that of the wild-type enzyme, as determined by a native gel mobility shift assay. The inhibitory effects of 100−150 mM salt on suicide cleavage by CΔ10 suggested that the distributive pattern of relaxation was a consequence of dissociation of the enzyme from partially relaxed DNAs. (Note that the standard topoisomerase reaction mixtures contain 100 mM NaCl.) The observed inhibition of single-turnover cleavage by magnesium would account for the paradoxical magnesium inhibition of DNA relaxation by CΔ10. The distributivity and magnesium inhibition may both be attributable to lowered affinity of the CΔ10 protein for DNA (relative to that of the wild-type enzyme) under the reaction conditions employed. This is supported by the observation that intermediate topoisomers were not detected during DNA relaxation by CΔ10 under nonstringent reaction conditions (no added salt or magnesium).

Is the distributive DNA relaxation pattern of CΔ10 caused by elimination of a specific functional group? To answer this question, we introduced single alanine substitution mutations at three of the five positions: Asp-306, His-307, and Lys-309. The activities of the purified recombinant proteins D306A, H307A, and K309A in DNA relaxation were identical to that of the wild-type topoisomerase and these proteins appeared to act processively (L. K. Wang, B. Ø. Petersen, and S. Shuman, unpublished results). This suggests that loss of the protein main chain, rather than a specific side chain, may elicit the observed mutational effect.

DNA relaxation by CΔ15 and CΔ20 remained distributive, as noted for CΔ10; however, relaxation by these two mutants was slower than that of CΔ10. This was in keeping with their slower rates of single-turnover cleavage. The cleavage rates were about one-tenth the wild-type rate. Hence the 10 amino acids from positions 294−305 are not essential for covalent catalysis by the criteria cited above. We did not evaluate the effects of individual substitutions within this segment. The reduced rates of cleavage by the CΔ10, CΔ15, and CΔ20 mutants was reflected in their lower cleavage−religation equilibrium constants compared to the wild-type topoisomerase. K_{cl} is equivalent to the ratio of the rate constants for cleavage and religation (k_{cl}/k_{rel}). That the effects of the CΔ10, CΔ15, and CΔ20 mutations on K_{cl} were quantitatively similar to effects on k_{cl} suggests that these mutations had relatively little impact on the strand religation step of the catalytic cycle.

CΔ15 and CΔ20 bound poorly to the CCCTT-containing 24-mer in the mobility shift assay. This cannot be attributed to mutational effects on the cleavage equilibrium, insofar as prior studies established that complete loss of the capacity for transesterification (i.e., by replacement of the active-site Tyr with Phe) had little impact on the noncovalent binding of vaccinia topoisomerase to its CCCTT target site (Shuman, 1991; Sekiguchi & Shuman, 1994c). Detection of a discrete topoisomerase-DNA complex in the mobility shift assay requires that the protein be bound stably to the DNA ligand. Formation of very low levels of shifted complex by CΔ15, and essentially no shifted complex by CΔ20, presumably reflects reduced stability of the mutant protein-DNA complex during the electrophoresis. Thus, the deletion of the 10 amino acids from positions 294−305 exacerbated the binding defect that was seen first in CΔ10.

Although the structural basis for the loss of binding affinity is not clear at present, we would speculate that the C-terminal segment of vaccinia topoisomerase contributes to the proposed circumferential interface with DNA. We imagine that this protein segment may reside at one the margins of the opening in a putative C-shaped protein ring within which resides the protein-bound DNA. In this view, loss of the deleted segments would reduce the "topological" contribution to DNA binding, i.e., by widening the opening in the ring. This would occur without altering binding specificity. Indeed, the deleted forms of the topoisomerase all cleaved linear pUC19 DNA with the same stringent site specificity as the wild-type enzyme. We cannot exclude the alternative view that residues within the segment from 295 to 309 make direct contacts with the DNA. However, if this were the case, we might expect the C-terminal region to be more highly conserved among the poxvirus-encoded topoisomeras-

es. Also, the segment downstream of the active site conspicuously lacks conserved basic amino acids that might be predicted to interact with the phosphodiester backbone of DNA.

REFERENCES

Caron, P. R., & Wang, J. C. (1994) *Adv. Pharmacol. 29B*, 271−297.

Klemperer, N., & Traktman, P. (1993) *J. Biol. Chem. 268*, 15887−15899.

Klemperer, N., Lyttle, D. J., Tauzin, D., Traktman, P., & Robinson, A. J. (1995) *Virology 206*, 203−215.

Morham, S. G., & Shuman, S. (1992) *J. Biol. Chem. 267*, 15984−15992.

Petersen, B. Ø., & Shuman, S. (1997) *J. Biol. Chem.* (in press).

Petersen, B. Ø., Wittschieben, J., & Shuman, S. (1996) *J. Mol. Biol. 263*, 181−195.

Sekiguchi, J., & Shuman, S. (1994a) *J. Biol. Chem. 269*, 29760−29764.

Sekiguchi, J., & Shuman, S. (1994b) *J. Biol. Chem. 269*, 31731−31734.

Sekiguchi, J., & Shuman, S. (1994c) *Nucleic Acids Res. 22*, 5360−5365.

Sekiguchi, J., & Shuman, S. (1995) *J. Biol. Chem. 270*, 11636−11645.

Sekiguchi, J., & Shuman, S. (1996) *EMBO J. 15*, 3448−3457.

Senkevich, T. G., Bugert, J. J., Sisler, J. R., Koonin, E. V., Darai, G., & Moss, B. (1996) *Science 273*, 813−816.

Sharma, A., Hanai, R., & Mondragon, A. (1994) *Structure 2*, 767−777.

Shuman, S. (1991) *J. Biol. Chem. 266*, 11372−11279.

Shuman, S., & Moss, B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7478−7482.

Shuman, S., & Prescott, J. (1990) *J. Biol. Chem. 265*, 17826−17836.

Shuman, S., & Turner, J. (1993) *J. Biol. Chem. 268*, 18943−18950.

Shuman, S., Golder, M., & Moss, B. (1988) *J. Biol. Chem. 263*, 16401−16407.

Shuman, S., Kane, E. M., & Morham, S. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9793−9797.

Stivers, J. T., Shuman, S., & Mildvan, A. S. (1994) *Biochemistry 33*, 327−339.

Upton, C., Opgenorth, A., Traktman, P., & McFadden, G. (1990) *Virology 176*, 439−447.

Wittschieben, J., & Shuman, S. (1994) *J. Biol. Chem. 269*, 29978−29983.

Zantige, J. L., Krell, P. J., Derbyshire, J. B., & Nagy, E. (1996) *J. Gen. Virol. 77*, 603−614.

BI962754P