Mutational Analysis of 26 Residues of Vaccinia DNA Topoisomerase Identifies Ser-204 as Important for DNA Binding and Cleavage†

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ABSTRACT: Vaccinia DNA topoisomerase, a 314 amino acid type I enzyme, catalyzes the cleavage and rejoining of DNA strands through a DNA-(3′-phosphotyrosyl)-enzyme intermediate formed at a specific target sequence, 5′-(C/T)CCTT↓. To identify amino acids that participate in the DNA binding and transesterification steps, we introduced alanine substitutions at 18 positions within a centrally located 27 amino acid segment (181-RLYKPLLKLTDDSSPEEFLFNKLSERK-207) and at 8 positions near the N-terminus (1-MRALFYKDGK-10). All mutant proteins except two displayed wild-type activity in relaxing supercoiled DNA. F200A and S204A exhibited reduced rates of relaxation and were subjected to a kinetic analysis of the strand cleavage reaction under single-turnover and equilibrium conditions. The F200A and S204A mutations reduced the rate of single-turnover DNA cleavage by factors of 5 and 70, respectively. Both mutations shifted the cleavage−religation equilibrium in favor of the noncovalently bound state. The S204A mutation reduced the affinity of topoisomerase for CCCTT-containing DNA, but did not alter the site-specificity of DNA cleavage. Vaccinia residue Ser-204, which is conserved in all poxvirus topoisomerases, but not in the cellular homologues, may contribute to the unique cleavage site specificity of the poxvirus enzymes. Phe-200 is conserved in all members of the type IB topoisomerase family.

The type IB DNA topoisomerase family includes eukaryotic topoisomerase I, a ubiquitous nuclear enzyme, and the topoisomerases encoded by vaccinia and other cytoplasmic poxviruses (Wang, 1996). These proteins relax supercoiled DNA via a common reaction mechanism, which involves noncovalent binding of the topoisomerase to duplex DNA, cleavage of one DNA strand with concomitant formation of a covalent DNA-(3′-phosphotyrosyl)-protein intermediate, strand passage, and strand religation. A shared structural basis for transesterification is inferred from the considerable amino acid sequence conservation between the cellular and virus-encoded enzymes (Caron & Wang, 1994; Gupta et al., 1995). However, the cellular and viral proteins display different site-specificities for covalent adduct formation on DNA. Vaccinia topoisomerase cleaves at sites containing the pentamer sequence 5′-(C/T)CCTT↓ immediately 5′ of the scissile bond (Shuman & Prescott, 1990; Shuman, 1991, 1992). The cellular topoisomerases exhibit a loose preference for a four-base motif, 5′-(A/T)(G/C)(A/T)T↓ (Edwards et al., 1982; Been et al., 1984; Jaxel et al., 1991).

The 314 amino acid vaccinia virus topoisomerase is the smallest topoisomerase known and likely constitutes the minimal functional unit of a type IB enzyme (Shuman & Moss, 1987). (The cellular type IB enzymes vary in size from 765 to 1019 amino acids.) Our aim is to understand the structural requirements for site recognition and transesterification reaction chemistry via mutational analysis of the vaccinia topoisomerase. Four strategies have been employed: (i) random mutagenesis followed by *in vivo* genetic selection of mutations that adversely affect enzyme activity (Morham & Shuman, 1990, 1992); (ii) serial deletion of amino acids from the carboxyl terminus (Wang & Shuman, 1997); (iii) targeted mutagenesis of a specific class of amino acid side chains irrespective of location within the protein (Shuman et al., 1989; Petersen & Shuman, 1997); and (iv) comprehensive mutagenesis of specific regions of the enzyme (Wittschieben & Shuman, 1994; Petersen et al., 1996; Cheng et al., 1997).

In applying the regional mutagenesis strategy, we have already targeted 85 amino acids within 2 conserved protein segments—from residues 126−167 and residues 213−274. Six amino acids in addition to the active site Tyr-274 were defined as essential; i.e., substitution by alanine elicited at least a 10^{-2} effect on activity in DNA relaxation and the formation of the covalent DNA−protein intermediate. The six essential residues are Arg-130, Gly-132, Tyr-136, Lys-167, Arg-223, and His-265. Two other residues, Lys-220 and Asn-228, were defined as important for catalysis; i.e., alanine substitution slowed the rate of covalent adduct formation by at least 1 order of magnitude. Six of the essential and important amino acids (Arg-130, Gly-132, Lys-167, Lys-220, Arg-223, and His-265) are strictly conserved in every member of the eukaryotic type IB enzyme family. Tyr-136 and Asn-228 are conserved among the poxvirus-encoded topoisomerases. All other amino acids in the targeted regions of the vaccinia topoiosmerase, including many conserved residues, were found to be nonessential. We regard as nonessential those residues at which side-chain removal has less than an order of magnitude effect on catalysis.

In the present study, we extended the regional mutational analysis to a 27 amino acid segment located between the 2 regions targeted previously. We also introduced mutations

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at the amino terminus of the topoisomerase. Twenty-six positions were substituted by alanine. Most of the mutations had little or no impact on DNA relaxation activity. Mutational effects at Ser-204 implicate this residue in target site recognition and strand cleavage.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Mutations were introduced into the segment of the vaccinia virus topoisomerase gene encoding residues 181−207 by using the two-stage PCR-based overlap extension method (Ho et al., 1989). Plasmid pA9topo (Shuman et al., 1988) was the template for the first-stage PCR reactions. Gene fragments with overlapping ends obtained from the first-stage reactions were paired and used as templates in the second-stage amplification. Mutations at the amino terminus of vaccinia topoisomerase were introduced by one-stage PCR. *Nde*I−*Bgl*II restriction fragments containing the entire topoisomerase gene were cloned into the T7-based expression vector pET3c. 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 with bacteriophage λCE6. Wild type and mutant topoisomerases were purified from soluble bacterial lysates by phosphocellulose column chromatography (Shuman et al., 1988). The protein concentrations of the phosphocellulose preparations were determined by using the dye-binding method (BioRad) with bovine serum albumin as the standard.

DNA Relaxation Assay. Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 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. Reaction products were analyzed by electrophoresis through a 1% horizontal agarose gel in TG buffer (50 mM Tris, 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 [γ-^{32}P]ATP and T4 polynucleotide kinase and then gel-purified and hybridized to a complementary 30-mer strand (present at 4-fold molar excess). Cleavage reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 0.5 pmol of 18-mer/30-mer DNA, and 90 ng of topoisomerase 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 at 1, 2, 5, 10, and 20 min, and the reaction was quenched immediately by adding SDS to 1%. The 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. A plot of the percent of input DNA cleaved versus time established end point values for cleavage. The data were normalized to the end point values, and k_{cl} was determined by fitting the data to the equation $(100 - \%Cl_{norm}) = 100e^{-kt}$.

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. Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 0.5 pmol of 60-mer DNA duplex, and topoisomerase were incubated at 37 °C for 20 min. Covalent complexes were trapped by adding 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, 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.

Site-Specificity of Cleavage of pUC19 DNA. 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 topoisomerase as specified, 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 5% polyacrylamide gel containing 7 M urea in TBE. 3′-End-labeled cleavage products were visualized by autoradiography of the dried gel.

Native Gel Mobility Shift Assay of DNA Binding. Reaction mixtures (20 μL) contained 50 mM Tris-HCl (pH 8.0), 170 fmol of 24 bp duplex DNA (5′-^{32}P-labeled on the scissile strand), and topoisomerase as specified. The nucleotide sequence of the scissile strand of the 24 bp DNA ligand was 5′-CGTGTCGCCCTTATTCCGATAGTG. The reaction mixtures 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, 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 extent of protein−DNA complex formation, expressed as [bound DNA/(bound DNA + free DNA)] × 100, was quantitated by scanning the gel with a Bio-Imaging Analyzer.

RESULTS

Mutagenesis Strategy and Production of Mutant Proteins. The 314 amino acid vaccinia topoisomerase consists of 3 protease-resistant structural domains demarcated by 2 protease-sensitive segments referred to as the bridge and hinge (Figure 1) (Sekiguchi & Shuman, 1995; Sharma et al., 1994). Residues that are essential or important for transesterification

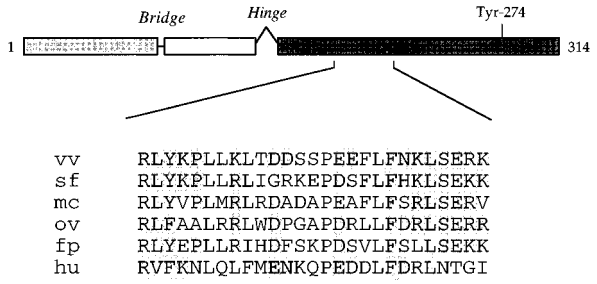

FIGURE 1: Domain structure and regional mutagenesis of vaccinia topoisomerase. The tripartite domain structure of the 314 amino acid vaccinia topoisomerase is illustrated. The protease-resistant structural domains are punctuated by protease-sensitive interdomain bridge and hinge segments. The active site Tyr-274 is situated within the C-terminal domain. The amino acid sequence of vaccinia virus topoisomerase (vv) from residues 181−207 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). The sequence of the human topoiosmerase I (hu) is shown below that of the poxvirus enzymes. The residues of the vaccinia protein that were targeted for mutagenesis in this study are demarcated by the shaded boxes. Where the targeted residues are conserved in other topoisomerases, they are also shaded.

reaction chemistry are located within the hinge and the adjacent C-terminal domain. In the present study, we performed a mutational analysis of a segment of the C-terminal domain from positions 181 to 207. This region is conserved in the topoisomerases encoded by five different genera of poxviruses: vaccinia (genus *Orthopoxvirus*), Shope fibroma virus (genus *Leporipoxvirus*), Orf virus (genus *Parapoxvirus*), fowlpox virus (genus *Avipoxvirus*), and molluscum contagiosum virus (genus *Molluscivirus*) (Upton et al., 1990; Klemperer et al., 1995; Zantige et al., 1996; Senkevich et al., 1996). Of 27 residues, 14 are conserved in all 5 poxvirus enzymes (Figure 1). The vaccinia and human topoisomerases are conserved at 15/27 positions within this region (Figure 1).

We assessed the role of individual amino acid side chains by alanine-scanning mutagenesis. Fifteen positions were substituted singly by alanine to yield the following mutants: R181A, L182A, Y183A, K184A, P185A, K188A, E196A, F200A, N201A, K202A, L203A, S204A, E205A, R206A, and K207A. In addition, we constructed two double-mutants, D191A−D192A and E196A−E197A, in which adjacent acidic side chains were both replaced by alanine. The mutant alleles of vaccinia topoisomerase were expressed in *E. coli*. The wild-type and mutant proteins were purified from soluble bacterial extracts by phosphocellulose column chromatography. The topoisomerase polypeptide constituted the major species in the protein preparations, as determined by SDS−PAGE, and the extents of purification were essentially equivalent [data not shown; see Wittschieben and Shuman (1994), Petersen et al. (1996), Petersen and Shuman (1997), and Cheng et al. (1997) for comparable purifications of mutant topoisomerases].

Mutational Effects on Relaxation of Supercoiled DNA. To assess the impact of these mutations, all proteins were tested for their ability to relax supercoiled plasmid DNA. Screening assays were performed in 0.1 M NaCl in the absence of magnesium. The rate-limiting step under these conditions is believed to be the dissociation of topoisomerase from the relaxed plasmid product (Stivers et al., 1994; Sekiguchi & Shuman, 1994a). The rates of relaxation were determined

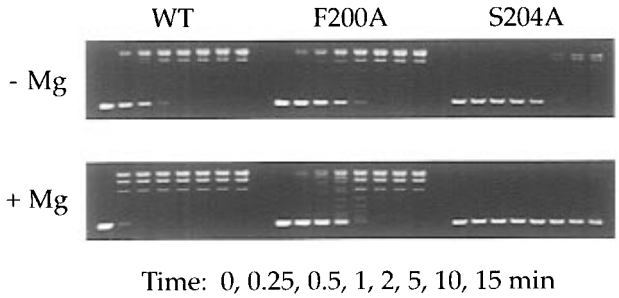

FIGURE 2: Kinetics of DNA relaxation by mutant topoisomerases. Reaction mixtures containing (per 20 μL) 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, either 2.5 mM EDTA (−Mg) or 5 mM $MgCl_2$ (+Mg), 0.3 μg of pUC19 DNA, and 2.7 ng of wild-type topoisomerase (WT) or the indicated mutant protein were incubated at 37 °C. The reactions were initiated by the addition of enzyme. Aliquots (20 μL) were withdrawn at the times indicated and quenched immediately. The "time 0" sample was taken prior to addition of enzyme. Reaction products were analyzed by agarose gel electrophoresis. A photograph of the ethidium bromide-stained gel is shown.

at a fixed level of input protein; 2.7 ng of wild-type topoisomerase relaxed 0.3 μg of supercoiled pUC19 DNA to completion within 2 min (Figure 2, −Mg). We observed that the relaxation rate of 16 of the mutant proteins was equivalent or nearly equivalent (within a factor of 2) to that of the wild-type enzyme (data not shown). The exception was S204A, which relaxed supercoiled DNA at about one-tenth the rate of the wild-type topoisomerase (Figure 2).

The DNA relaxation assays were also performed in the presence of 5 mM magnesium. Magnesium enhances the product off-rate, without affecting the rate of DNA cleavage by the wild-type topoisomerase (Stivers et al., 1994). Magnesium stimulated the activity of the wild-type enzyme such that 2.7 ng of enzyme relaxed nearly all supercoils within 15 s (Figure 2, +Mg). Fifteen of the mutant enzymes were stimulated by magnesium (data not shown). Two of the alanine mutants displayed aberrant responses to magnesium. F200A, which relaxed at about half the wild-type rate in the absence of magnesium, was unaffected by inclusion of 5 mM magnesium. In effect, the rate of relaxation by F200A with metal present was about one-tenth the wild-type rate under the same conditions (Figure 2). This suggested that a step other than product release might be rate-limiting. S204A was also not stimulated by magnesium; indeed, its rate of relaxation was actually *lower* by about a factor of 5 in the presence of 5 mM magnesium than in its absence (Figure 2). Paradoxical inhibition of S204A by magnesium (in contrast to the stimulatory effect on wild-type enzyme) suggested that this mutant may have altered affinity for DNA. To determine which component steps of the topoisomerase reaction were affected by the F200A and S204A mutations, we subjected these two mutant proteins to a detailed biochemical characterization as described below. The wild-type topoisomerase was analyzed in parallel.

Mutational Effects on 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 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

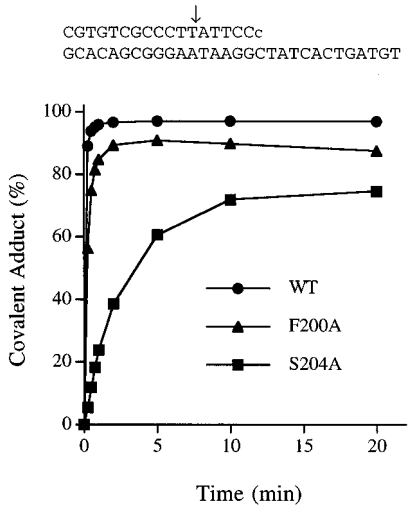

FIGURE 3: Mutational effects on suicide DNA cleavage. Suicide cleavage was assayed as described under Materials and Methods. The structure of the 5′^{32}P-labeled suicide substrate is depicted at the top of the figure. Covalent complex formation is plotted as a function of time.

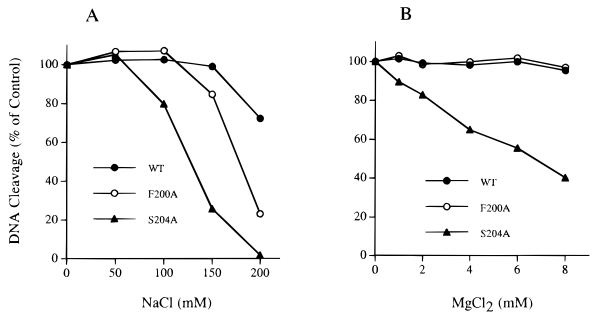

FIGURE 4: Effects of salt and magnesium on suicide cleavage. Reaction mixtures (20 μL) containing 0.5 pmol of DNA substrate and 90 ng of topoisomerase were supplemented with NaCl (panel A) or $MgCl_2$ (panel B) as indicated. Reactions were initiated by adding protein and terminated after incubation at 37 °C for either 10 s (WT), 1 min (F200A), or 10 min (S204A). 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).

on the DNA. In the experiment shown in Figure 3, we measured the rates of DNA cleavage at equivalent concentrations of each protein. The wild-type topoisomerase cleaved >90% of the input DNA; the reaction was essentially complete at the earliest time point analyzed (15 s). We reported previously that the rate constant for single-turnover cleavage (k_{cl}) by the wild-type enzyme on this suicide substrate was 0.28 s^{-1} (Petersen & Shuman, 1997). F200A attained a similar end point, but the initial rate of cleavage was slower than wild type; from the cleavage values at 15 and 30 s, we calculated that k_{cl} was 0.05 s^{-1}. S204A cleaved 75% of the input DNA; however, the rate of approach to the cleavage end point was slowed significantly ($k_{cl} = 0.004$ s^{-1}). Hence, simple elimination of the hydroxyl moiety of Ser-204 reduced the rate of covalent adduct formation by a factor of 70.

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 was unaffected by the levels of salt and magnesium that strongly stimulate DNA relaxation under steady-state conditions (Stivers et al., 1994). It has been argued that 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 above findings, that DNA relaxation by F200A and S204A in 0.1 M NaCl was slower than wild type and the activities of the mutant enzymes were either unaffected or 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, 150, and 200 mM NaCl, or 1, 2, 4, 6, and 8 mM $MgCl_2$, were measured and normalized to the extent of cleavage in unsupplemented control reactions. The reaction times differed for the wild-type and mutant enzymes: wild-type cleavage reactions were quenched after 10 s. F200A reactions were terminated after 1 min, and S204A reactions were halted after 10 min. The reaction times were chosen to attain comparable sensitivity for the effects of solution parameters on the cleavage reaction. The salt effects are shown in Figure 4A; magnesium effects are shown in Figure 4B. We observed that the wild-type topoisomerase was unaffected by up to 150 mM NaCl, but was inhibited by 28% at 200 mM NaCl. In contrast, covalent adduct formation by S204A was salt-sensitive; S204A was inhibited by 20, 74, and 98%, respectively, by 100, 150, and 200 mM NaCl. Wild-type enzyme was unaffected by magnesium up to 8 mM, whereas S204A was inhibited progressively by 1−8 mM $MgCl_2$. Susceptibility to salt and magnesium inhibition suggested that the S204A protein bound less avidly to the CCCTT-containing DNA substrate. F200A was only slightly more sensitive than wild-type topoisomerase to NaCl and was unaffected by magnesium.

DNA Binding by F200A and S204A. A gel mobility shift assay (Sekiguchi & Shuman, 1994b) was used to analyze the DNA binding properties of the F200A and S204A proteins. The ^{32}P-labeled DNA ligand was a 24 bp CCCTT-containing duplex (Figure 5). The binding reaction mixtures contained no added salt or magnesium. The topoisomerase−DNA complexes were resolved from free DNA by electrophoresis through a native polyacrylamide gel. The extent of DNA binding increased with the amount of input topoisomerase (Figure 5). The apparent affinity of F200A for the 24-mer was about half that of the wild-type topoisomerase, whereas S204A bound about one-sixth as well as the wild-type topoisomerase.

F200A and S204A Mutations Shift the DNA Cleavage−Religation Equilibrium. 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, which was performed in the absence of added salt or divalent cation, the topoisomerase−DNA adduct was denatured by adding SDS, and then digested with proteinase K to yield a 5′-labeled 30-mer oligonucleotide linked to a small peptide. The extent of cleavage of the 60-mer duplex by the wild-type topoisomerase increased with input enzyme and plateaued at >70 ng (Figure 6). At saturation, 21% of the substrate was cleaved. The cleavage equilibrium constant (K_{cl} = covalent complex/noncovalent complex) was 0.26.

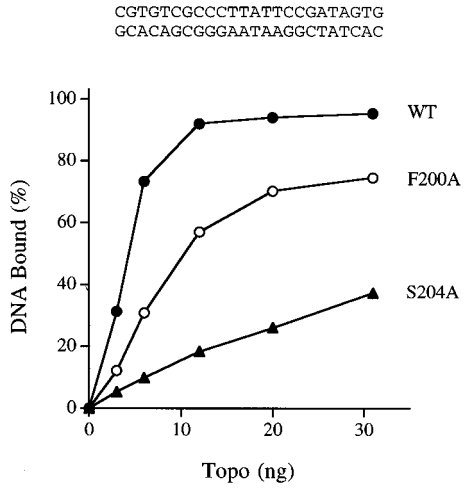

FIGURE 5: Mutational effects on DNA binding. Native gel-shift assays of topoisomerase−DNA complex formation were performed as described under Materials and Methods. The structure of the 24 bp ligand is shown. DNA binding (expressed as the percent of input DNA associated with the shifted topoisomerase−DNA complex) is plotted as a function of input protein.

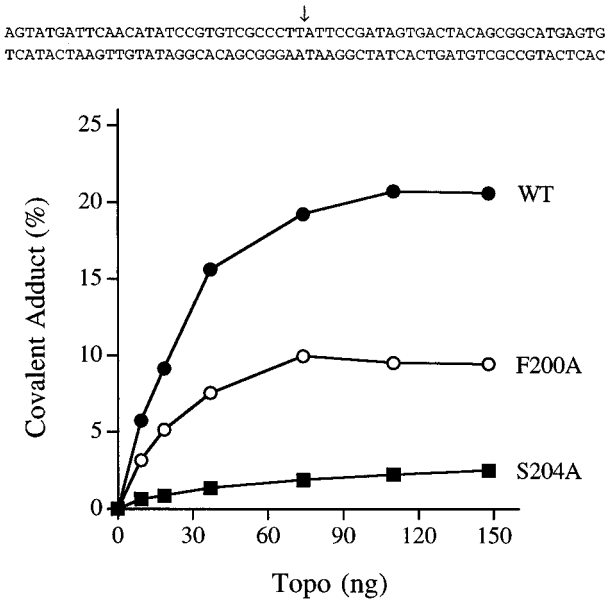

FIGURE 6: Mutational effects on equilibrium DNA cleavage. Covalent complex formation on a 60 bp duplex substrate was assayed as described under Materials and Methods. The structure of the substrate is shown. The extent of covalent complex formation is plotted as a function of input protein.

Covalent complex formation by F200A was 10% at saturation, whereas only 3% was bound covalently by S204A (Figure 6). The cleavage−religation equilibrium constants were 0.11 for F200A and 0.03 for S204A. Hence, both mutations shifted the cleavage religation equilibrium toward the noncovalently bound state. Because $K_{eq} = k_{cl}/k_{rel}$ (i.e., the ratio of the rate constants of the cleavage and religation reactions), we surmise that the F200A and S204A mutations must have a greater impact on cleavage than religation. From the K_{eq} and k_{cl} values measured in this study, and the previously reported k_{cl} of 0.28 for wild-type topoisomerase, we calculated k_{rel} values for the wild-type, F200A, and S204A proteins of 1.1, 0.45, and 0.13 s^{-1}, respectively.

Cleavage Site Specificity. The wild-type and mutant topoisomerases were incubated with a 2464 bp *Ava*II fragment of pUC19 that had been 3′-end-labeled with [α-^{32}P]-

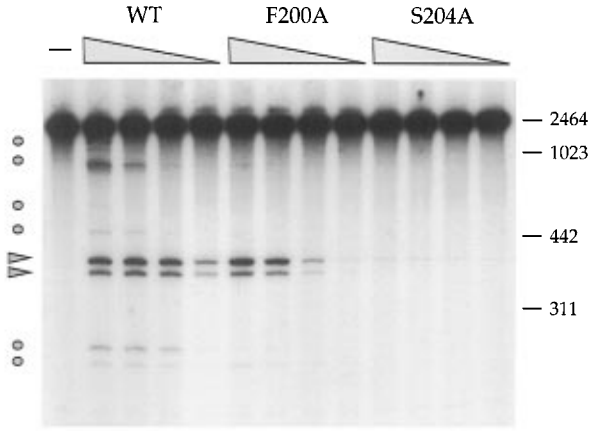

FIGURE 7: Cleavage site-specificity. Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 0.74 ng of 3′-end-labeled *Ava*II DNA fragment of pUC19 DNA, and either 1000, 330, 110, or 37 pg of topoisomerase (from left to right within each titration series) were incubated at 37 °C for 20 min. Cleavage products were analyzed by electrophoresis through a 5% polyacrylamide gel containing 7 M urea in TBE. An autoradiograph of the gel is shown. A control reaction (lane −) contained no topoisomerase. The positions and sizes (in nucleotides) of coelectrophoresed restriction endonuclease fragments of the 3′-labeled substrate are indicated at the right.

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 7). The sizes of the radiolabeled cleavage products reflect the distance of the cleavage 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 7). The cleavage products generated at 0.37 pg of input enzyme migrated at ∼375 bp and ∼395 bp during denaturing gel electrophoresis. Increasing the amount of enzyme to 0.33 and 1.0 ng resulted in cleavage at multiple additional sites (denoted by circles in Figure 7). The spectrum of cleavage sites and the protein concentration dependence of site occupancy by F200A were essentially identical to the wild-type enzyme. In the case of S204A, cleavage to yield the 395 bp fragment required higher levels of input protein (Figure 7). The S204A mutation either reduced or eliminated strand scission at most other target sites. It is noteworthy that neither F200A nor S204A cleaved at sites other than those cleaved by the wild-type enzyme. Hence, neither mutation loosened the stringent site-specificity of the vaccinia topoisomerase.

Mutational Analysis of the Amino Terminus. We introduced alanine substitutions at 8 of the first 10 amino acids at the N-terminus of vaccinia topoisomerase (MRALFYKDGK). The mutant proteins (R2A, L4A, F5A, Y6A, K7A, D8A, G9A, and K10A) were expressed in *E. coli* and purified from soluble extracts by phosphocellulose column chromatography. The topoisomerase polypeptide constituted the major species in the protein preparations, and the extents of purification were essentially equivalent (not shown). All eight proteins were as active as wild-type topoisomerase in relaxing supercoiled plasmid DNA in the absence of magnesium (not shown), and all eight exhibited a stimulation of relaxation rate by 5 mM magnesium (not shown). We

conclude that none of the amino acid side chains in this region is important for catalysis.

DISCUSSION

In continuing our mutational analysis of the vaccinia DNA topoisomerase, we focused on a conserved segment of the 20 kDa C-terminal structural domain. We found that 17 of the 18 amino acids side chains mutated were nonessential for catalysis. Nonessential residues are those at which side chain removal by alanine substitution either has no effect on transesterification or else slows covalent catalysis by less than an order of magnitude. This definition is 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). Of the 17 nonessential residues, 4 are conserved in all viral and cellular type IB topoisomerases. These are the following: Phe-200 and Leu-203, which are strictly conserved; Leu-182, which is either Leu or Val; and Tyr-183, which is either Tyr or Phe (Caron & Wang, 1994; Gupta et al., 1995). Alanine substitution at invariant residue Phe-200 had only modest effects on transesterification rate, cleavage equilibrium, and DNA binding.

Essential residues are those at which side chain removal results in a $\geq 10^{-2}$ effect on catalysis; residues at which side chain removal elicits a $\geq 10^{-1}$ but $\leq 10^{-2}$ effect on reaction rate are deemed important. By these criteria, Ser-204 was found to be important for topoisomerase activity. The S204A enzyme relaxed supercoiled DNA at a reduced rate and was paradoxically inhibited by magnesium. Replacement of Ser-204 by alanine reduced the affinity of vaccinia topoisomerase for a CCCTT-containing 24 bp DNA by about a factor of 6. In addition, S204A slowed the rate of suicide cleavage by a factor of 70 under nonstringent conditions (i.e., in the absence of NaCl and magnesium); the effect on cleavage was exacerbated in the presence of salt or divalent cation. The hydroxyl group at position 204 is evidently important for DNA binding and cleavage. Ser-204 is strictly conserved in every poxvirus-encoded topoisomerase. The analogous residue of the cellular type IB enzymes is occupied by asparagine (human, mouse, *Drosophila*), aspartic acid (*Arabidopsis*, *S. cerevisiae*), or serine (*Sc. pombe*).

It is remarkable that after performing site-directed mutagenesis of more than 100 amino acids of the vaccinia topoisomerase, we have identified only 3 residues at which alanine substitution decreased noncovalent DNA binding affinity: Ser-204, Tyr-70, and Tyr-72. The two neighboring tyrosines are the sites of UV cross-linking between topoisomerase and the +4 and +3 cytosine bases, respectively, of the CCCTT element (Sekiguchi & Shuman, 1996). We have found that Y70A and Y72A mutations of vaccinia topoiosmerase have effects similar to those of the S204A mutation described in the present study. DNA relaxation by Y70A and Y72A is slowed instead of stimulated by magnesium. Their rates of single-turnover cleavage in the absence of salt or magnesium are reduced 2-fold and 7-fold, respectively (k_{cl} for Y70A = 0.12 s^{-1}; k_{cl} for Y72A = 0.04 s^{-1}), and suicide cleavage by both proteins is sensitive to inhibition by salt or magnesium (Sekiguchi and Shuman, unpublished results). Gel shift experiments showed that the Y70A and Y72A mutations reduced noncovalent DNA binding by factors of 4 and >20, respectively (Sekiguchi & Shuman, 1996).

Tyr-70 and Tyr-72 are situated in the N-terminal domain just upstream of the interdomain bridge (Figure 1). A model of the DNA−protein interface based on the crystal structure of a 9 kDa N-terminal tryptic fragment fits the CCCTT major groove into a solvent-exposed face of the N-terminal domain (Sekiguchi & Shuman, 1996; Sharma et al., 1994). We suggested that the hinge and adjacent C-terminal domain (which contain all the amino acid residues identified as essential for transesterification chemistry) contact the target site along the minor groove on the opposite face of the helix. This surface of the DNA contains the seven phosphate residues shown by ethylation interference to be important for DNA binding (Sekiguchi & Shuman, 1994c, 1996). These phosphates, which include the scissile phosphate, are arrayed across the minor groove. Ser-204 is the first residue in the C-terminal domain implicated in noncovalent DNA binding. We speculate that Ser-204 hydrogen bonds with one of the six backbone phosphates (other than the scissile phosphate) that are important for DNA binding.

We found that the S204A mutation strongly skewed the cleavage−religation equilibrium of vaccinia topoisomerase toward the noncovalently bound state. This reflected the relatively greater impact of S204A on the cleavage reaction versus the religation reaction. We estimated from the experimental values for k_{cl} and K_{cl} that S204A had about an 8-fold effect on k_{rel}. Assays of single-turnover religation by the wild-type and S204A suicide intermediates [performed as described in Petersen and Shuman (1997)] did not reveal a significant difference in the extents of religation at 10 s, the earliest time point analyzed. Wild-type topoisomerase religated to >90% of the end point in 10 s, compared to 80% for S204A. The rate of the religation by wild-type topoisomerase is too fast to measure accurately when assays are performed manually; hence, it is difficult to discern less than 10-fold mutational effects on religation under single-turnover conditions. Note that religation entails no site-recognition step, because the protein is already bound covalently to the DNA. Therefore, we regard mutational rate effects on the religation reaction as the simplest measure of the contribution of a given amino acid residue to transesterification reaction chemistry. By this criterion, Ser-204 provides no more than a 10-fold rate enhancement of the chemical step. This is far less than the contribution of residues Arg-130 and Arg-223, which enhance the rate of single-turnover religation by a factor of 10^5 (Cheng et al., 1997; Wittschieben and Shuman, unpublished results). We presume that Arg-130, Arg-223, and Tyr-274 (the active site nucleophile) make direct contact with the scissile phosphate during transesterification. Remarkably, alanine substitution mutations at these three essential positions have no apparent impact on noncovalent DNA binding (Sekiguchi & Shuman, 1994b; Cheng et al., 1997; Wittschieben and Shuman, unpublished results).

Stivers et al. (1994) noted that the apparent second-order rate constant for suicide cleavage by vaccinia topoisomerase was far below the diffusion-controlled limit and suggested the existence of a precleavage step involving a conformational change in the enzyme or substrate. Given that the S204A mutation had a greater impact on cleavage than on religation, we suggest that Ser-204 may be involved in such a conformational step.

As noted above, the 9 kDa N-terminal domain of vaccinia topoisomerase includes two amino acid residues near the

interdomain bridge that make direct contact with the nucleotide bases of the CCCTT cleavage site. In the present study, we targeted mutations to the N-terminal peptide MRALFYKDGK. Our interest in this peptide arose after two independent mutations were identified in the analogous segment of the human topoisomerase I (LFRGRGA) that conferred resistance to camptothecin or 9-nitrocamptothecin (Benedetti et al., 1993; Rubin et al., 1994). In one drug-resistant mutant, human residue Gly-363 (which is a Lys in the vaccinia enzyme) was mutated to Cys (Benedetti et al., 1993); in the second drug-resistant mutant, human residue Phe-361, which is conserved in the vaccinia protein, was changed to Ser (Rubin et al., 1994). Our finding that alanine substitutions at Arg-2, Leu-4, Phe-5, Tyr-6, Lys-7, Asp-8, Gly-9, and Lys-10 had no effect on topoisomerase activity indicates that this segment of the vaccinia protein is not directly involved in catalysis. After we had performed the mutational analysis of this segment, reports of the sequences of other poxvirus-encoded topoisomerases made clear that this segment is not conserved among the viral enzymes (Klemperer et al., 1995; Zantige et al., 1996; Senkevich et al., 1996). In addition, the crystal structures of catalytically inactive fragments of the vaccinia and yeast topoisomerases revealed that these segments of the viral and cellular proteins adopt quite different structures (Sharma et al., 1994; Lue et al., 1995). Although none of the individual residues within the N-terminal decapeptide were essential for vaccinia topoisomerase activity, it is noteworthy that a deletion of the N-terminal eight residues caused the truncated recombinant protein to be completely insoluble when expressed in bacteria (J. Wittschieben, unpublished results). The N-terminal peptide may facilitate proper folding of the vaccinia enzyme.

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