We present a mutational analysis of vaccinia topoisomerase that highlights the contributions of five residues in the catalytic domain (Phe-88 and Phe-101 in helix α1, Ser-204 in α5, and Lys-220 and Asn-228 in α6) to DNA binding and transfection steps. When augmented by structural information from exemplary type IB topoisomerases and tyrosine recombinases in different functional states, the results suggest how closure of the protein clamp around duplex DNA and assembly of a functional active site might be orchestrated by internal conformational changes in the catalytic domain. Lys-220 is a constituent of the active site, and a positive charge at this position is required for optimal DNA cleavage. Ser-204 and Asn-228 appear not to be directly involved in reaction chemistry at the scissile phosphodiester. We propose that (i) Asn-228 recruits the Tyr-274 nucleophile to the active site by forming a hydrogen bond to the main chain of the tyrosine-containing α8 helix and that (ii) contacts between Ser-204 and the DNA backbone upstream of the cleavage site trigger a separate conformational change required for active site assembly. Mutations of Phe-88 and Phe-101 affect DNA binding, most likely at the clamp closure step, which we posit to entail a distortion of helix α1.

The type IB DNA topoisomerase family includes eukaryotic nuclear topoisomerase I and the topoisomerases encoded by vaccinia and other cytoplasmic poxviruses (1). The type IB enzymes relax supercoiled DNA via a multistep reaction pathway entailing noncovalent binding of the topoisomerase to duplex DNA, cleavage of one DNA strand with formation of a covalent DNA-(3'-phosphotyrosyl)-protein intermediate, strand passage, and strand religation. The poxvirus topoisomerases display stringent specificity for transfection at DNA sites containing the sequence 5′-C/CCTT-3′ immediately 5′ of the scissile bond. This specificity has facilitated a detailed kinetic analysis of the chemical steps of the topoisomerase IB reaction using defined DNA substrates containing a single CCTT cleavage site.

The 314-amino acid vaccinia virus topoisomerase consists of two structural modules (see Fig. 1). A 234-amino acid carboxyl segment, Topo-(81–314), comprises an autonomous catalytic domain that performs the same repertoire of reactions as the full-sized vaccinia topoisomerase: relaxation of supercoiled DNA, site-specific DNA transesterification, and DNA strand transfer (2). The crystal structure of the catalytic domain reveals an oblong-shaped globular protein consisting of 10 α-helices and a three-stranded antiparallel β-sheet packed against one surface (3). The smaller N-terminal domain forms a five-stranded antiparallel β-sheet with two short α-helices and connecting loops (4). The N- and C-terminal domains are connected via a trypsin-sensitive linker (4, 5). Vaccinia topoisomerase binds the DNA duplex circumferentially as a C-shaped protein clamp (3, 6).

Although the poxvirus topoisomerase has not been crystallized in the DNA-bound state, extensive biochemical data (6–9) plus the structure of the human topoisomerase I-DNA co-crystal (10) support a model of the vaccinia topoisomerase-DNA complex (3) whereby the N-terminal domain interacts with the major groove on the face of the helix opposite the cleavage site while the C-terminal catalytic domain interacts with the minor groove on the face of the helix that contains the scissile phosphodiester. Proteolytic footprinting of the vaccinia topoisomerase provides biochemical evidence of protein conformational changes at the interdomain linker triggered by DNA binding (5) that are presumed to reflect the clamp closure step (1). In addition, DNA binding elicits rearrangements within the catalytic domain that bring some of the catalytic side chains into the active site (3, 5). The protein constituents and molecular contacts that orchestrate these rearrangements are uncharted.

The tertiary structure of the catalytic domain of vaccinia topoisomerase is similar to the catalytic domains of nuclear topoisomerase IB and the tyrosine recombinases (3, 10–15). The latter are a family of site-specific DNA strand transferases that, like type IB topoisomerases, catalyze DNA breakage and rejoicing through covalent DNA-(3′-phosphotyrosyl)-enzyme intermediates. A constellation of four amino acid side chains of vaccinia topoisomerase (Arg-130, Lys-167, Arg-223, and His-265) catalyzes the attack of the tyrosine nucleophile (Tyr-274) on the scissile phosphodiester (16–19). Mutational and structural data suggest that the two arginines and the histidine interact directly with the scissile phosphodiester and enhance catalysis by stabilizing the developing negative charge on a pentacoordinate phosphorane transition state (3, 10, 16–18, 20). Lys-167, which sits atop the loop connecting β-strands 2 and 3 of the catalytic domain, serves as a general acid during the cleavage reaction, donating a proton to expel the 5′-OH leaving group (21).

The catalytic residues of vaccinia topoisomerase are conserved in nuclear topoisomerase IB and tyrosine recombinases, and they occupy similar positions in the tertiary structures of those proteins (10–15). Tyrosine recombinases have a two-domain structure, and they also form a C-shaped clamp around the DNA duplex with the N-terminal domain on the major
groove and the C-terminal catalytic domain on the minor groove face containing the scissile phosphate. Yet the structures of the N-terminal domains of tyrosine recombinases are not conserved from one recombinase to another and are completely different from those of type IB topoisomerases (4, 10–15).

To gain insights into the structural basis for DNA binding and transesterification by poxvirus topoisomerases, we have undertaken a comprehensive mutational analysis of the vaccinia virus protein. Testing the effects of mutations at 140 individual amino acids (16–19, 22–29) has thus far identified 10 residues that play important roles in transesterification (Arg-130, Gly-132, Tyr-135, Lys-167, Ser-204, Lys-220, Arg-223, Asn-228, His-265, and Tyr-274) and seven residues that contribute to DNA binding affinity (Arg-67, Tyr-70, Tyr-72, Arg-80, Arg-84, Phe-200, and Ser-204) (see Fig. 1). Here we extended the mutational analysis by performing an alanine scan of 15 new residues within the catalytic domain (see Fig. 1). Our findings illuminate roles for Phe-88 and Phe-101 in DNA binding, most likely at the clamp closure step. We also study the effects of conservative substitutions at residues Ser-204, Lys-220, and Asn-228, which had been previously defined via alanine scanning as important for DNA cleavage (18, 29). The results suggest that Lys-220 is a constituent of the active site, whereas Ser-204 and Asn-228 facilitate conformational rearrangements of the catalytic domain during assembly of the active site.

EXPERIMENTAL PROCEDURES

Topoisomerase Mutants—Mutations were introduced into the vaccinia virus topoisomerase gene by using the two-stage polymerase chain reaction overlap extension method (30). NdeI-BglII restriction fragment containing the mutated topoisomerase genes were cloned into the T7-based expression vector PET3c. All mutations were confirmed by dyeoxy sequencing. pET-Topo plasmids were transformed into Escherichia coli BL21. Topoisomerase expression was induced by infection with bacteriophage λCE6 (31). Wild-type and mutant topoisomerases were purified from soluble bacterial lysates by phosphocellulose chromatography (31). The protein concentrations of the phosphocellulose preparations were determined by using the dye-binding method (Bio-Rad) with bovine serum albumin as the standard.

Single-turnover Cleavage—An 18-mer CCCCTT-containing DNA oligonucleotide was 5'-end-labeled by enzymatic phosphorylation in the presence of [γ-32P]ATP and T4 polynucleotide kinase, then gel-purified, and hybridized to a complementary 30-mer strand. Cleavage reaction mixtures containing (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 18-mer 32P-labeled DNA, and topoisomerase as specified were incubated at 37 °C. The reactions were quenched by adding SDS to 1%. The samples were analyzed by SDS-polyacrylamide gel electrophoresis. Covalent complex formation was revealed by transfer of radiolabeled DNA to the topoisomerase polypeptide. The extent of covalent adduct formation (expressed as the percentage of the input 5'-32P-labeled oligonucleotide that was transferred to protein) was quantitated by scanning the dried gel using a FUJIX BAS2500 Bio-Imaging Analyzer. Cleavage rate constants (kcl) were determined by normalizing the data to the end point values (redefined as 100) and fitting to the equation 100 – %Cleaveage(norm) = 100e-kt.

Single-turnover Religation—Reaction mixtures containing (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 18-mer/30-mer DNA, and 75 ng of topoisomerase were incubated at 37 °C to form the covalent intermolecularly ligated DNA. Religation was initiated by the simultaneous addition of 10 μg/ml of wild-type or mutant topoisomerase to the reaction mixture containing 10 μg/ml of supercoiled plasmid DNA to a final concentration of the input DNA strand of 100 μg/ml. The reactions were quenched by adding SDS to 1% and analyzed by denaturing gel electrophoresis. The extent of religation (expressed as the percentage of the input labeled 18-mer strand recovered as 30-mer) was plotted as a function of reaction time. The data were normalized to the end point values, and krel was determined by fitting the data to the equation 100 – %Religation(norm) = 100e-kt.

Equilibrium Cleavage—A 60-mer oligonucleotide containing a centrally placed CCCCTT element (17) was 5'-end-labeled, then gel-purified, and annealed to an unlabeled complementary 60-mer strand. Reaction mixtures containing (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of the 60-mer DNA duplex, and topoisomerase were incubated at 37 °C. The reaction was quenched by adding SDS to 1%. The samples were digested for 60 min at 37 °C with 10 μg/ml of proteinase K, then adjusted to 47% formamide, heat-denatured, and electrophoresed through a 17% polyacrylamide gel containing 7% urea in Tris borate-EDTA. Cleavage product, a 32P-labeled 60-bp duplex, was resolved from the input 60-mer substrate. The extent of strand cleavage was quantitated by scanning the gel with a PhosphorImager. The cleavage equilibrium constant (Kcl) is defined as the ratio of covalently bound DNA to noncovalently bound DNA at the reaction end point under conditions of saturating enzyme and was calculated according to the equation Kcl = % Cleavage(100 – % Cleavage).

DNA Binding—Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 0.3 pmol of the 5'-32P-labeled 60-bp duplex DNA, and vaccinia topoisomerase as specified were incubated at 37 °C for 15 min. The mixtures were adjusted to 5% glycerol and then analyzed by electrophoresis through a native 6% polyacrylamide gel containing 45 mM Tris borate, 1.2 mM EDTA. Free DNA and topoisomerase-DNA complexes were visualized by autoradiography of the dried gel.

RESULTS

Mutagenesis Strategy and Production of Mutant Proteins—New mutations were introduced in two regions of vaccinia topoisomerase: (i) the segment from positions 88 to 124 comprising the first two α-helices of the catalytic domain and (ii) a C-terminal segment from positions 296 to 299 comprising the proximal portion of the last α-helix of the catalytic domain (3). Twelve positions in α1 and α2 were substituted by alanine to yield the following mutants: F88A, R90A, R97A, N99A, F101A, R90A, R97A, N99A, F101A, N103A, K104A, K107A, and K108A. The residues mutated are denoted by dots in Fig. 1. Note that Phe-88, Phe-101, Glu-124, and Phe-101 are conserved in the topoisomerases of six other vertebrate poxviruses of widely different host range (Shope fibroma virus, myxoma virus, Yaba monkey tumor virus, molluscum contagiosum virus, orf virus, and fowlpox virus) and two insect poxviruses (Amsacta moorei entomopoxvirus and Melanoplus sanguinipes entomopoxvirus) (Fig. 1). The wild-type and mutant proteins were produced in E. coli and purified from soluble bacterial extracts. The topoisomerase polypeptide constituted the major species in the protein preparations as determined by SDS-polyacrylamide gel electrophoresis, and the extents of purification were essentially equivalent (Fig. 2A and data not shown).

Relaxation of Superoiled 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 the dissociation of topoisomerase from the relaxed plasmid product (32, 33). The rates of relaxation were determined 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 1 min (Fig. 2B, +NaCl). 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 DNA relaxation assays were also performed in the presence of 0.1 M NaCl plus 5 mM MgCl2. Magnesium enhances product off-rate without affecting the rate of DNA cleavage by the wild-type topoisomerase (32). Magnesium stimulated the
activity of the wild-type enzyme such that 2.7 ng of enzyme relaxed all supercoils in 15–30 s (Fig. 2B, +NaCl +MgCl₂).

We observed that the relaxation rates of 13 of the 15 alanine-substituted proteins were equivalent to that of the wild-type enzyme in the absence and presence of magnesium: these were R90A, R97A, N99A, N103A, K104A, K107A, K108A, Q116A, E124A, T296A, F297A, D298A, and E299A (data not shown). These 13 mutants also displayed wild-type activity in covalent adduct formation on a CCCTT-containing suicide cleavage substrate (not shown). On the basis of these findings, we concluded that the side chains of Arg-90, Arg-97, Asn-99, Asn-103, Lys-104, Lys-107, Lys-108, Gln-116, Gln-297, Asp-298, and Gln-299 are unimportant for topoisomerase function in vitro. These proteins were not analyzed further.

Effect of F88A and F101A Mutations on DNA Relaxation—Substitution of Phe-88 and Phe-101 by alanine had little effect on the kinetics of DNA relaxation in the presence of 0.1 M NaCl as gauged by the decay of the supercoiled substrate, but the mutations appeared to increase the abundance of partially relaxed intermediates compared with wild-type topoisomerase (Fig. 2B, +NaCl). The F88A and F101A mutants displayed an aberrant response to magnesium. Neither protein was stimulated by magnesium; indeed their rates of relaxation were actually lower by a factor of 4–5 in the presence of 5 mM magnesium than in its absence (Fig. 2B, +NaCl +MgCl₂). Therefore, the rates of relaxation by F88A and F101A in the presence of NaCl plus magnesium were reduced by a factor of 10–20 vis à vis wild-type topoisomerase. The paradoxical inhibition of F88A and F101A by magnesium (in contrast to the stimulatory effect on wild-type enzyme) suggested that these mutants had altered affinity for DNA.

Effects of F88A and F101A Mutations on Single-turnover DNA Cleavage and Religation—To determine which component(s) of the topoisomerase reaction was affected by the F88A and F101A mutations, we subjected the mutant proteins to further biochemical characterization as described below. The wild-type topoisomerase was analyzed in parallel.

A suicide substrate containing a single CCCTT cleavage site for vaccinia topoisomerase was used to examine the trans-esterification reaction under single-turnover conditions in the absence of added salt and magnesium. The substrate consisted of an 18-mer scissile strand annealed to a 30-mer strand (Fig. 2C). 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. The extent of cleavage of 0.3 pmol of DNA substrate by the wild-type topoisomerase during a 5-min reaction was proportional to added enzyme and reached saturation at 37 ng of protein at
cleavage by wild-type topoisomerase, F88A, and F101A under conditions of enzyme excess. The apparent rate constant ($k_{cl}$) for the wild-type enzyme was 0.34 s$^{-1}$, whereas the rate constants for F88A and F101A were 0.21 and 0.17 s$^{-1}$, respectively (not shown).

The religation reaction was studied under single-turnover conditions by assaying the ability of the covalent intermediate to transfer the covalently held 5'-$^{32}$P-labeled 12-mer strand to a 5'-hydroxyl-terminated 18-mer strand to form a 30-mer product (34). The rates of single-turnover religation by covalently bound wild-type topoisomerase, F88A, and F101A were indistinguishable within the limits of detection of the manually performed kinetic assay ($k_{rel} \geq 0.3$ s$^{-1}$) (not shown).

Transesterification was also examined under equilibrium conditions by reacting wild-type topoisomerase, F88A, and F101A with a 60-bp DNA duplex containing a centrally placed CCCTT cleavage site (17). The extent of cleavage of 0.3 pmol of the 60-bp DNA substrate by the wild-type topoisomerase and F88A during a 5-min reaction was proportional to added enzyme and reached saturation at 75 ng of protein at which point 23% of the input DNA became covalently bound ($K_{cl} = 0.3$) (not shown). F101A cleaved 17% of the CCCTT DNA strand at saturation ($K_{cl} = 0.2$). The slightly lower cleavage equilibrium constant for F101A was consistent with the modest reduction in single-turnover rate constant for the forward cleavage step (note that $K_{cl} = k_{cl}/k_{rel}$).

**Effects of NaCl and Magnesium on DNA Cleavage by F88A and F101A**—Single-turnover DNA cleavage reactions are routinely performed at low ionic strength in the absence of a divalent cation. 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. It has been argued that salt and magnesium stimulate relaxation by enhancing product dissociation rather than by affecting the chemical steps of transes-terification (32, 33). The finding that DNA relaxation by the F88A and F101A mutant enzymes was either unstimulated or actually inhibited by magnesium plus salt suggested that pre-cleavage binding might be limiting under these conditions.

To address this issue, we examined the effects of salt and magnesium on single-turnover cleavage. The amounts of covalent adduct formed in 10 s in the presence of 50, 100, and 150 mM NaCl or 2, 4, 6, 8, and 10 mM MgCl$_2$ were measured and normalized to the extent of cleavage in unsupplemented control reactions (Fig. 3). We observed that the wild-type topoisomerase was unaffected by up to 100 mM NaCl but was inhibited by 42% at 150 mM NaCl. In contrast, covalent adduct formation by F88A and F101A was salt-sensitive. F88A was inhibited by 65% at 100 mM NaCl and by 96% at 150 mM NaCl (Fig. 3A). Transesterification by F101A was reduced by 87% at 100 mM NaCl and essentially eliminated at 150 mM NaCl (Fig. 3A).

Wild-type topoisomerase was unaffected by magnesium up to 10 mM, whereas F88A and F101A were inhibited progressively at 2–10 mM MgCl$_2$ (Fig. 3B). Susceptibility to salt and magnesium inhibition suggested that F88A and F101A bound less avidly to the CCCTT-containing DNA substrate under the solution conditions used to assay DNA relaxation.

A native gel mobility shift assay was used to directly analyze DNA binding by the F88A and F101A proteins. The ligand was a 60-bp DNA duplex containing a centrally placed CCCTT site (Fig. 3C). Binding of wild-type topoisomerase to the 60-bp DNA resulted in the formation of a discrete protein-DNA complex of retarded electrophoretic mobility, the yield of which was proportional to the amount of input topoisomerase (Fig. 3C). Nearly all of the input DNA was bound by 100 ng of the wild-type enzyme. The F88A and F101A proteins bound less...
Conservative Mutations of Amino Acids Important for DNA Cleavage—In previous studies, we classified mutational effects on cleavage rate as follows. Residues at which side chain removal by alanine substitution results in a \( \geq 10^{-2} \) effect on cleavage rate are defined as essential. Residues at which side chain removal elicits a \( \geq 10^{-1} \) but \( \leq 10^{-2} \) effect on reaction rate are deemed important. Residues at which alanine substitution causes less than an order of magnitude rate effect are regarded as nonessential. These definitions are reasonable when one considers that the wild-type topoisomerase accelerates the rate of transesterification by an estimated factor of \( 10^{9} \)–\( 10^{12} \) (32).

Amino acids identified by alanine scanning as important for transesterification are Ser-204, Lys-220, and Asn-228. To gauge which structural features of the amino acid side chains are functionally relevant, we introduced alternative functional groups in lieu of Ser-204, Lys-220, and Asn-228. The findings are described below.

Serine 204—Ser-204 is located at the beginning of helix \( \alpha \)5 of the catalytic domain and is conserved in every poxvirus topoisomerase except that of \( \text{A. moorei} \) entomopoxvirus in which the serine is replaced by asparagine (Fig. 1). The residue analogous to Ser-204 is an asparagine in mammalian topoisomerase I, an aspartic acid in \( \text{Saccharomyces cerevisiae} \) topoisomerase I, and a serine in \( \text{Schizosaccharomyces pombe} \) topoisomerase I (35).

Replacement of Ser-204 by alanine reduced the rate of single-turnover DNA cleavage by a factor of 70 and shifted the cleavage-religation equilibrium in favor of the noncovalently bound state (39). The S204A mutation also reduced the affinity of vaccinia topoisomerase for CCCCTT-containing DNA but did not alter the site specificity of DNA cleavage (29). Consistent with the DNA binding and cleavage defects, the S204A protein relaxed supercoiled DNA slowly and was strongly inhibited in the presence of salt plus magnesium.

Here we studied the effects of replacing Ser-204 with asparagine. The S204N protein (Fig. 4A) relaxed supercoiled DNA with wild-type kinetics in the presence of 0.1 M NaCl and was stimulated to the same extent as wild-type topoisomerase in the presence of 0.1 M NaCl plus 5 mM MgCl\(_2\) (not shown). The extent of cleavage of 0.3 pmol of the 18-mer/30-mer DNA substrate by the S204N protein during a 5-min reaction was proportional to added enzyme and reached saturation at 37 ng of protein at which point 94% of the input DNA became covalently bound. The concentration dependence of covalent adduct formation by S204N was the same as that of the wild-type enzyme (not shown). The single-turnover cleavage rate constant for S204N under conditions of enzyme excess was 0.3 s\(^{-1}\). The rate of single-turnover religation by covalently bound S204N was indistinguishable from wild type within the limits of detection of the assay. S204N cleaved 34% of the 60-bp equilibrium DNA strand at saturation (39). Thus, by all criteria tested, the
Asparagine 228—Asn-228 is a catalytically important residue that flanks the essential side chain Arg-223. Arg-223 and Asn-228 reside within helix a6, which comprises part of the conserved active site of topoisomerase IB and the tyrosine recombinases (3). Asn-228 is strictly conserved as an asparagine in all poxvirus topoisomerases (Fig. 1). The equivalent position of cellular type IB topoisomerase is always occupied by a serine (35). Tyrosine recombinases generally have a serine or threonine at this position (36). Cheng et al. (18) reported that replacement of Asn-228 by alanine reduced the rates of supercoil relaxation and single-turnover DNA cleavage and shifted the cleavage-religation equilibrium in favor of the noncovalently bound state with little effect on religation rate. Here we replaced Asn-228 with aspartic acid, glutamine, and serine and then compared the relaxation and transesterification activities of the purified recombinant N228D, N228Q, and N228S proteins (Fig. 4) to those of N228A and wild-type topoisomerase.

Relaxation of supercoiled DNA by N228D in the presence of NaCl and no divalent cation was slower by a factor of 40 than relaxation by wild-type topoisomerase (Fig. 4B). N228Q was slowed by a factor of 5, N228A by a factor of 8, and N228S by a factor of 2. Intermediate topoisomers were prominent during relaxation by N228A and N228S. N228A, N228Q, and N228S were not stimulated by including magnesium with NaCl. N228D was paradoxically inhibited by magnesium (Fig. 4A). The rates of relaxation in the presence of NaCl plus magnesium were reduced vis à vis wild type by >100-fold for N228D, >40-fold for N228Q and N228A, and 20-fold for N228S.

The kinetics of cleavage of the 18-mer/30-mer DNA by N228D, N228Q, N228S, and N228A are shown in Fig. 5A. The \( k_{\text{eq}} \) values were as follows: N228D = 0.0015 s\(^{-1}\), N228Q = 0.014 s\(^{-1}\), N228S = 0.04 s\(^{-1}\), and N228A = 0.01 s\(^{-1}\). Thus, the Asn-228 side chain enhanced the rate of covalent adduct formation by a factor of 30 as gauged by a comparison of the wild-type and N228A proteins. Introduction of a serine at position 228 resulted in a 4-fold increase in activity over the alanine mutant, whereas a glutamine failed to restore activity. An aspartic acid at position 228 was extremely detrimental; cleavage by the N228D enzyme was slower than wild type by a factor of 200. The N228D, N228Q, N228S, and N228A mutants were sensitized to cleavage inhibition by NaCl (Fig. 5B) and \( \text{MgCl}_2 \) (Fig. 5C). The hierarchy of solute effects paralleled the mutational effects on cleavage rate, i.e. N228D was most sensitive to salt and magnesium inhibition, whereas N228S was least sensitive.

The rates of single-turnover religation by covalently bound N228D, N228Q, N228S, and N228A were indistinguishable from wild type within the limits of detection of the assay (not shown). The equilibrium constants (\( K_{\text{eq}} \)) for transesterification on the 60-bp CCCTT-containing DNA at saturating enzyme were as follows: N228D = 0.006, N228Q = 0.05, N228S = 0.05, and N228A = 0.02 (not shown).

We conclude that the hydrogen bonding potential of the side chain at position 228 is important for the DNA cleavage activity of topoisomerase IB and that this function can be fulfilled in part by serine. Failure of glutamine to restore activity suggests that steric constraints imposed by the longer glutamine side chain may negate the anticipated salutary effects of restoring the amide functional group. Introduction of negative charge at position 228 is evidently detrimental in its own right.

Lysine 220—Lys-220 is strictly conserved in all viral and cellular type IB topoisomerases, but the tyrosine recombinases characteristically have a histidine at the equivalent position (35, 36). Replacement of Lys-220 by alanine reduced the rate of supercoil relaxation and single-turnover DNA cleavage and shifted the cleavage-religation equilibrium in favor of the noncovalently bound state with little effect on religation rate (18). Here we replaced Lys-220 with arginine, glutamine, and histidine and compared the activities of the purified recombinant K220R, K220Q, and K220H proteins (Fig. 4) to those of K220A and wild-type topoisomerase.

Supercoil relaxation by K220A, K220H, and K220Q in the presence of NaCl and no divalent cation was slower by a factor of 5 than relaxation by wild-type topoisomerase (Fig. 4C). K220R was slowed by a factor of 2. Intermediate topoisomers were conspicuous during relaxation by K220H. The K220A, K220H, and K220Q mutants were inhibited by including magnesium with NaCl, whereas K220R was neither stimulated nor inhibited by magnesium (Fig. 4C). The rates of relaxation in the presence of NaCl plus magnesium were reduced vis à vis wild type by ≥40-fold for K220H and K220Q, 20-fold for
either 0, 50, 100, or 150 M NaCl (7.5), 0.3 pmol of 18-mer/30-mer DNA, 75 ng of topoisomerase, and K220R, K220H, K220Q, and K220A were indistinguishable as a function of NaCl (B) or MgCl2 (C) concentration. The extent of covalent complex formation were normalized to that of a control (K220A, K220H, and K220Q) and then quenched with SDS. The extents of covalent complex formation are plotted as a function of time.

Fig. 6. Effect of Lys-220 mutations on DNA cleavage. A, kinetics. Reaction mixtures contained (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 18-mer/30-mer DNA, and 75 ng of topoisomerase (wild type (WT), K220A, K220H, K220R, or K220Q). The extent of covalent complex formation is plotted as a function of time. B, effect of NaCl. C, effect of MgCl2. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 18-mer/30-mer DNA, 75 ng of topoisomerase, and either 0, 50, 100, or 150 mM NaCl (B) or 0, 2, 4, 6, 8, or 10 mM MgCl2 (C) were incubated at 37 °C for 10 s (wild type and K220R) or 5 min (K220A, K220H, and K220Q) and then quenched with SDS. The extents of covalent complex formation were normalized to that of a control reaction without added salt or magnesium (defined as 100%) and plotted as a function of NaCl (B) or MgCl2 (C) concentration.

K220A, and 8-fold for K220R.

The kinetics of single-turnover DNA cleavage by K220R, K220H, K220Q, and K220A are shown in Fig. 6A. The cleavage rate constants (kcle) were as follows: K220R = 0.2 s−1, K220H = 0.009 s−1, K220Q = 0.004 s−1, and K220A = 0.014 s−1. Thus, the Lys-220 side chain enhanced the rate of covalent adduct formation by a factor of 20 compared with the K220A protein. Introduction of an Arg at position 220 restored activity to near wild-type level, whereas introduction of histidine and glutamine failed to restore activity and even elicited slightly more deleterious effects than did the alanine mutation. The K220A, K220H, K220Q, and K220R mutants were sensitized to cleavage inhibition by NaCl (Fig. 6B) and MgCl2 (Fig. 6C).

The rates of single-turnover religation by covalently bound K220R, K220H, K220Q, and K220A were indistinguishable from wild type within the limits of detection of the assay (not shown). The apparent equilibrium constants (Kcle) for transes-
tional data for vaccinia topoisomerase, this glutamine side chain makes no contact with DNA in the human topoisomerase-DNA cocrystal. Nonessential basic residues Arg-90 and Arg-97 of vaccinia topoisomerase are also conserved in the tertiary structure of human topoisomerase I (as Arg-449 and Lys-456, respectively) where they are located on the protein surface and far away from the DNA.

 Function of Lys-220, Asn-228, and Ser-204—Catalytically important residues Lys-220 and Asn-228 are located within helix α6, which is the most highly conserved segment of the type IB topoisomerase protein family. Mutation of Lys-220 to Ala slows the rate of DNA cleavage by a factor 20. The 3-fold effect of the K220A mutation on the cleavage equilibrium constant suggests that loss of the lysine also slows religation. In addition, the K220A mutant was sensitive to salt and magnesium, indicating a role for this side chain in DNA binding. Remarkably the introduction of an arginine at position 220 restored the rate of transfection to near wild type yet did not rectify the DNA binding defect. Indeed the K220R mutant was more sensitive than K220A to inhibition by magnesium. These findings highlight the requirement for a positive charge at position 220 for optimal DNA cleavage. Other polar residues such as glutamine and histidine, which are capable in principle of hydrogen bonding to phosphate, failed to correct the cleavage defect and were actually more deleterious than alanine.

Lys-220 is invariant in all type IB topoisomerases; the equivalent position is a histidine in the tyrosine recombinases. Based on the structure of the vaccinia topoisomerase catalytic domain with bound sulfate ions, it was proposed that Lys-220 interacts directly with the scissile phosphodiester (3). However, in the human topoisomerase-DNA cocrystal, the equivalent lysine side chain (Lys-587) engages in a hydrogen bond with the phosphate immediately 5’ of the cleavage site (10). The corresponding histidine of Flp recombinase contacts the scissile phosphate in the Flp-DNA cocrystal (15). In the crystal structure of Cre recombinase bound to DNA, the histidine contacts the scissile phosphate in the covalent complex but moves away from the scissile phosphate in the noncovalent complex (11). These crystallographic snapshots suggest that the interactions of the Lys or His side chain with DNA are flexible and may be synchronized with the steps of the catalytic cycle. In the case of topoisomerase IB, the Lys-220 residue may interact transiently with the scissile phosphodiester during the cleavage step, thereby accounting for the mutational effects on cleavage. There is clear evidence for a shift in the position of the tyrosine-containing helix during transfection by Cre recombinase (11). In this light it is noteworthy that Arg-297 of Cre (the counterpart of Asn-228 of vaccinia topoisomerase) also engages in a hydrogen bond to the main chain carboxyl of the tyrosine nucleophile (Tyr-324) in the Cre-DNA cocrystal. The interaction of Arg-297 with the backbone of the tyrosine-containing helix is remodeled after the covalent intermediate is formed such that Arg-297 donates a hydrogen bond to the carboxyl of residue 327 instead of Tyr-324.

Elimination of the Asn-228 side chain of vaccinia topoisomerase reduces $k_\text{cat}$ by a factor of 30 and $K_\text{d}$ by a factor of 15, indicating that Asn-228 is specifically required for the cleavage step. This result is consistent with the hypothesis that Asn-228 helps tether Tyr-274 at the active site prior to covalent adduct formation because once cleavage has occurred the tyrosine is held in place by its covalent linkage to the DNA and is therefore already poised for displacement by the 5’-OH DNA strand during the religation step. N228A is less processive than wild-type topoisomerase in relaxing supercoiled DNA, which suggests that the proposed rearrangement facilitated by Asn-228 is also a component of the clamp-closure step.

Ser-204 of vaccinia topoisomerase enhances the rate of DNA cleavage by a factor 70 and contributes to noncovalent DNA binding affinity (29). Ser-204 is located on the surface of the vaccinia protein at the proximal end of the α5 helix far away from the active site residues. Thus, a direct role for Ser-204 in reaction chemistry at the scissile phosphodiester is virtually inconceivable. A serine is present at the equivalent position in the tertiary structure of Cre recombinase (Ser-257) at the amino end of a helix that interacts with the major groove of the DNA at a point 10–11 bp 5’ of the scissile phosphate. The serine of Cre engages in a hydrogen bond to the +10 phosphate of the scissile DNA strand, i.e., the 10th phosphate on the covalently held strand of the Cre-DNA intermediate (11). The human topoisomerase I equivalent of Ser-204 is Asn-569, which is also located at the amino end of a helix facing the major groove (10). N6 of Asn-569 is pointed toward the phosphodiester backbone of the scissile strand at a distance of 4.6 Å from the +10 phosphate. A similar scissile strand phosphate contact for Ser-204 of vaccinia topoisomerase is quite plausible given that (i) the footprint of the vaccinia protein on DNA extends 13 bp 5’ of the cleavage site (9, 41), (ii) both noncovalent and covalent binding of vaccinia topoisomerase to CCGTT-containing DNA are reduced significantly when there are fewer than 10 bp on the 5’-side of the cleavage site (42), and (iii) the rate

Asn-228 of vaccinia topoisomerase is invariant among the poxvirus topoisomerases. Although the Asn-228 side chain makes no intramolecular contacts in the free vaccinia enzyme, the corresponding residue of human topoisomerase I (Ser-595) engages in a bidentate hydrogen bond to the main chain of the helix that contains the Tyr-723 nucleophile. The serine donates a hydrogen bond to the carbonyl of Tyr-723 and receives a hydrogen bond from the amide of residue 725. The Tyr-724 nucleophile in the free vaccinia topoisomerase is out of place to participate in catalysis, and the formation of a catalytically competent active site depends perforce on a conformational rearrangement of the α8 helix following DNA binding (3). We propose that Asn-228 participates in this rearrangement by tethering the α8 helix containing Tyr-724 to the α6 helix. The structure-activity relationships at Asn-228 are consistent with a requirement for hydrogen bonding of the type seen in the human topoisomerase-DNA complex; to wit (i) introduction of a serine, the residue found in the human topoisomerase, results in a gain of function compared with the N228A mutant, and (ii) introduction of aspartate significantly inhibited enzyme activity.
of DNA cleavage is affected by alterations of the +9 and +10 base pairs in the major groove (43).

The catalytic and binding defects of the S204A mutant were reversed completely by the introduction of an asparagine, consistent with the requirement for a hydrogen bond to a DNA phosphate. A contact between Ser-204 and a DNA phosphate would account for the effects of the S204A mutation on noncovalent DNA binding. Why the mutation has such a strong effect on cleavage is less clear and is most plausibly explained by positing that the contact of Ser-204 with DNA is important for inducing a conformational change required for assembly of the active site. Indeed reference to the crystal structures of the vaccinia and human topoisomerases suggests that movement of the α5 helix toward the major groove might bring the disordered apex of the β-sheet into the minor groove near the scissile phosphate and thereby deliver the essential general acid catalyst Lys-167 or else contribute to the organization of the disordered segment that includes the essential Arg-130 side chain that interacts directly with the scissile phosphate.

In summary, the mutational analysis highlights the contributions of five residues (Phe-88, Phe-101, Ser-204, Lys-220, and Asn-228) to DNA binding and cleavage by vaccinia topoisomerase. When integrated with structural information for type IB topoisomerases and tyrosine recombinases, the results suggest how internal conformational changes in the vaccinia topoisomerase might orchestrate closure of the protein clamp around duplex DNA and assembly of a functional active site.

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