

# Mechanism of DNA transesterification by vaccinia topoisomerase: catalytic contributions of essential residues Arg-130, Gly-132, Tyr-136 and Lys-167

John Wittschieben and Stewart Shuman\*

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

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## ABSTRACT

**Vaccinia topoisomerase, a eukaryotic type IB enzyme, catalyzes relaxation of supercoiled DNA by cleaving and rejoining DNA strands through a DNA–(3′-phosphotyrosyl)–enzyme intermediate. We have performed a kinetic analysis of mutational effects at four essential amino acids: Arg-130, Gly-132, Tyr-136 and Lys-167. Arg-130, Gly-132 and Lys-167 are conserved in all members of the type IB topoisomerase family. Tyr-136 is conserved in all poxvirus topoisomerases. We show that Arg-130 and Lys-167 are required for transesterification chemistry. Arg-130 enhances the rates of both cleavage and religation by  $10^5$ . Lys-167 enhances the cleavage and religation reactions by  $10^3$  and  $10^4$ , respectively. An instructive distinction between these two essential residues is that Arg-130 cannot be replaced by lysine, whereas substituting Lys-167 by arginine resulted in partial restoration of function relative to the alanine mutant. We propose that both basic residues interact directly with the scissile phosphate at the topoisomerase active site. Mutations at positions Gly-132 and Tyr-136 reduced the rate of strand cleavage by more than two orders of magnitude, but elicited only mild effects on religation rate. Gly-132 and Tyr-136 are suggested to facilitate a pre-cleavage activation step. The results of comprehensive mutagenesis of the vaccinia topoisomerase illuminate mechanistic and structural similarities to site-specific recombinases.**

## INTRODUCTION

The type IB DNA topoisomerase family includes eukaryotic topoisomerase I, a ubiquitous nuclear enzyme, and the topoisomerases encoded by vaccinia and other cytoplasmic poxviruses (1). 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.

The 314 amino acid vaccinia virus topoisomerase is the smallest topoisomerase known and likely constitutes the minimal functional unit of a type IB enzyme (2). The cellular type IB enzymes vary in size from 765 to 1019 amino acids (3). Our aim is to understand the structural basis for transesterification reaction chemistry via mutational analysis of the vaccinia protein. Four strategies have been employed: (i) random mutagenesis followed by *in vivo* genetic selection of mutations that adversely affect enzyme activity (4,5); (ii) serial deletion of amino acids from the C-terminus (6); (iii) targeted mutagenesis of a specific class of amino acid side chains irrespective of location within the protein (7,8); and (iv) comprehensive mutagenesis of specific regions of the enzyme (9–12).

In applying the regional mutagenesis strategy, we previously targeted 103 amino acids within three conserved protein segments: from residues 126–167, 181–207 and 213–274, respectively. 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. Three other residues, Ser-204, 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 one 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, Ser-204 and Asn-228 are conserved among the poxvirus-encoded topoisomerases.

In the present study, we present a detailed kinetic analysis of the mutational effects at four essential positions: Arg-130, Gly-132, Tyr-136 and Lys-167. We analyzed the effects of alanine substitutions and conservative substitutions on the rates of DNA strand cleavage and religation under equilibrium and single-turnover conditions. We show that Arg-130 and Lys-167 are essential for both cleavage and religation chemistry and suggest a model for their action at the scissile phosphate. In contrast, Gly-132 and Tyr-136 are required for strand cleavage, but mutations at these positions elicit only mild effects on religation.

\*To whom correspondence should be addressed. Tel: +1 212 639 7145; Fax: +1 212 717 3623; Email: s-shuman@ski.mskcc.org

## MATERIALS AND METHODS

### Mutant proteins

Wild type topoisomerase and mutant proteins R130A, R130K, G132S, G132A, Y136S, Y136A, K167A and K167R were expressed in bacteria and purified from soluble bacterial lysates by phosphocellulose column chromatography as described (5,9,10). The topoisomerase polypeptide constituted the major species in each protein preparation, as determined by SDS-PAGE, and the extents of purification were essentially equivalent (5,9,10). The protein concentrations of the topoisomerase preparations were determined by using the dye-binding method (BioRad) with bovine serum albumin as the standard.

### Equilibrium cleavage assays

A 60mer oligonucleotide containing a centrally placed CCCTT element was 5'-end-labeled by enzymatic phosphorylation in the presence of [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase, then gel-purified, and annealed to an unlabeled complementary 60mer strand. Reaction mixtures containing (per 20  $\mu$ l) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 60mer DNA duplex and 38–76 ng of topoisomerase were incubated at 37°C. The reactions were initiated by the addition of topoisomerase to prewarmed reaction mixtures. Aliquots (20  $\mu$ l) were withdrawn at various times and the reaction was quenched immediately by adding SDS to 0.5%. The samples were then digested for 60 min at 45°C with 10  $\mu$ g of proteinase K. The volume was adjusted to 100  $\mu$ l 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 17% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris-borate, 2.5 mM EDTA). The cleavage product, a  $^{32}$ P-labeled 30mer bound to a short peptide, was well-resolved from the input 60mer substrate. The extent of strand cleavage was quantitated by scanning the wet gel using a FUJIX BAS1000 Bio-Imaging Analyzer. A plot of the percent of input DNA cleaved versus time established endpoint values for cleavage. The data were normalized to the endpoint values and  $k_{\text{obs}}$  for approach to equilibrium was determined by fitting the data to the equation  $(100 - \%Cl_{\text{norm}}) = 100e^{-kt}$ . The cleavage equilibrium constant ( $K_{\text{cl}}$ ) is defined as the ratio of covalently bound DNA to noncovalently bound DNA at the reaction endpoint under conditions of saturating enzyme and was calculated according to the equation  $K_{\text{cl}} = \%Cl/(100 - \%Cl)$ . Enzyme titration experiments established that the conditions used for measuring approach to equilibrium were saturating with respect to input topoisomerase. The experimental values for  $k_{\text{obs}}$  and  $K_{\text{cl}}$  were used to calculate the rate constants for the cleavage and religation steps ( $k_{\text{cl}}$  and  $k_{\text{rel}}$ ) according to equations  $K_{\text{cl}} = k_{\text{cl}}/k_{\text{rel}}$  and  $k_{\text{obs}} = k_{\text{cl}} + k_{\text{rel}}$ .

### Single-turnover religation on the 60 bp DNA

Equilibrium cleavage reaction mixtures containing (per 18  $\mu$ l) 0.3 pmol of the 60 bp DNA and 38–76 ng of topoisomerase were incubated at 37°C for a period sufficient to attain equilibrium. At this time (time zero), an aliquot (18  $\mu$ l) was removed and quenched with SDS. The reaction mixtures were then adjusted to 0.5 M NaCl and aliquots (20  $\mu$ l) were taken at various times

thereafter. The samples were digested with proteinase K and processed for electrophoresis as described in the preceding section. The decrease in the abundance of the covalent complex (expressed as percent of input DNA) was plotted as a function of time. The data were normalized to the time zero and endpoint values and  $k_{\text{rel}}$  was determined by fitting the data to the equation  $\%Cl_{\text{norm}} = 100e^{-kt}$ .

### Suicide cleavage assays

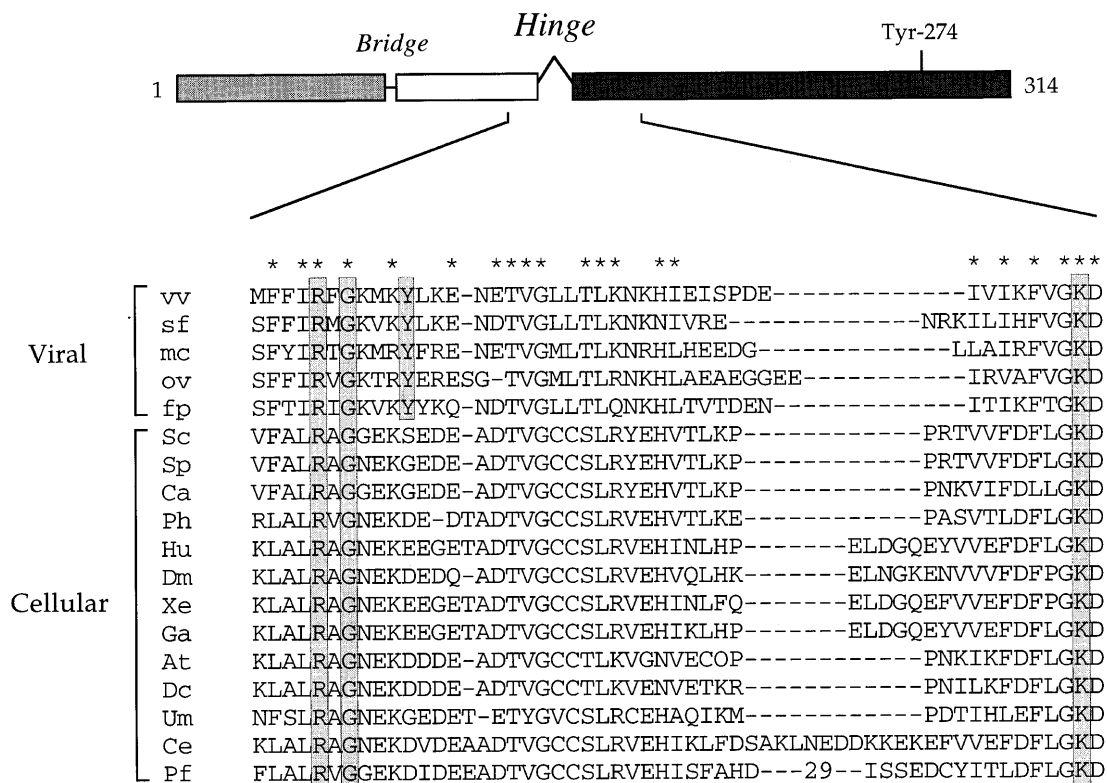
An 18mer CCCTT-containing DNA oligonucleotide was 5'- $^{32}$ P-labeled, then gel-purified and hybridized to a complementary 30mer strand. Cleavage reaction mixtures containing (per 20  $\mu$ l) 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 18mer/30mer DNA and 38–76 ng of topoisomerase were incubated at 37°C. The cleavage reactions were initiated by the addition of topoisomerase to prewarmed reaction mixtures. Aliquots (20  $\mu$ l) were withdrawn at various times 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. A plot of the percent of input DNA cleaved versus time established endpoint values for cleavage. The data were normalized to the endpoint values and  $k_{\text{cl}}$  was determined by fitting the data to the equation  $(100 - \%Cl_{\text{norm}}) = 100e^{-kt}$ .

### Single-turnover religation by the suicide intermediate

Cleavage reaction mixtures containing (per 14  $\mu$ l) 0.3 pmol of the 18mer/30mer DNA and 38 ng of topoisomerase were incubated at 37°C to form the suicide intermediate. Religation was initiated by the simultaneous addition of NaCl to 0.5 M and a 5' hydroxyl-terminated 18mer acceptor strand (5'-ATTCCGA-TAGTGACTACA) to a concentration of 30 pmol/16  $\mu$ l (i.e., a 100-fold molar excess over the input DNA substrate). Aliquots (16  $\mu$ l) were withdrawn at the times indicated and quenched immediately in an equal volume of buffer containing 2% SDS, 76% formamide and 20 mM EDTA. A time zero sample was withdrawn prior to addition of the acceptor strand. The samples were heat-denatured and then electrophoresed through a 17% polyacrylamide containing 7 M urea in TBE. Religation of the covalently bound 12mer strand to the 18mer acceptor DNA will yield a 5'- $^{32}$ P-labeled 30mer strand transfer product. The extent of religation (expressed as the percent of the input labeled 18mer strand recovered as 30mer) was plotted as a function of reaction time. The data were normalized to the endpoint values and  $k_{\text{rel}}$  was determined by fitting the data to the equation  $(100 - \%Re_{\text{norm}}) = 100e^{-kt}$ .

### Native gel mobility shift assay of DNA binding

Reaction mixtures (20  $\mu$ l) contained 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 60 bp duplex DNA (5'- $^{32}$ P-labeled on the scissile strand) and topoisomerase as specified. The reaction mixtures were incubated at 37°C for 5 min. Glycerol was added to 10% and the samples were electrophoresed through a 6% native polyacrylamide gel in 0.25 $\times$  TBE (22.5 mM Tris-borate, 0.6 mM EDTA)



**Figure 1.** Domain structure and essential residues 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 126 to 168 is aligned with the homologous segments of viral topoisomerases encoded by Shope fibroma virus (sf), molluscum contagiosum virus (mc), orf virus (ov) and fowlpox virus (fp), and with the cellular type I topoisomerases of *Schizosaccharomyces pombe* (Sp), *Saccharomyces cerevisiae* (Sc), *Candida albicans* (Ca), *Physarum polycephalum* (Ph), *Homo sapiens* (Hu), *Drosophila melanogaster* (Dm), *Xenopus laevis* (Xe), *Gallus gallus* (Ga), *Arabidopsis thaliana* (At), *Daucus carota* (Dc), *Ustilago maydis* (Um), *Caenorhabditis elegans* (Ce) and *Plasmodium falciparum* (Pf). Sequence gaps are indicated by dashes (-). A non-conserved 29 amino acid insert in the sequence of the *P.falciparum* protein was omitted from the alignment and is simply abbreviated as (-29-). Residues conserved in all of the polypeptides are indicated by an asterisk above the vaccinia sequence. The four residues of the vaccinia protein that were mutated in this study are demarcated by the shaded boxes. Where the mutated residues are conserved in other topoisomerases, they are also shaded.

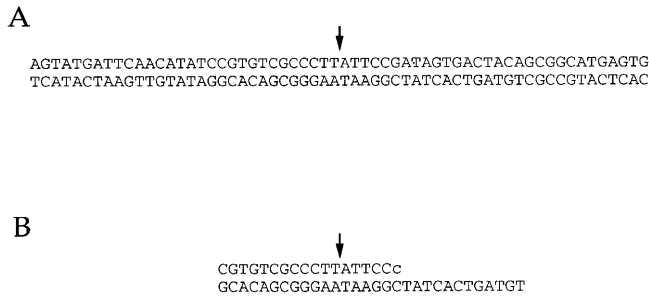
at 100 V for 2 h. Topoisomerase–DNA complexes of retarded mobility were visualized by autoradiographic exposure of the dried gel. The extent of protein–DNA complex formation, expressed as  $[\text{Bound DNA}/(\text{Bound DNA} + \text{Free DNA})] \times 100$ , was quantitated by scanning the gel with a Bio-Imaging Analyzer.

## RESULTS

The 314-amino acid vaccinia topoisomerase consists of three protease-resistant structural domains demarcated by protease-sensitive interdomain segments referred to as the bridge and hinge (Fig. 1) (13,14). The amino acid sequence in the vicinity of the hinge region (vaccinia residues 126–168) is conserved at 21/43 positions in other cellular and viral type IB topoisomerases (Fig. 1). Four residues that are essential for DNA relaxation are located within this segment of the vaccinia enzyme: Arg-130, Gly-132, Tyr-136 and Lys-167. The essential residues are shown in shaded boxes in Figure 1. Our initial studies indicated that replacement of Arg-130 with alanine or lysine abolished activity in DNA relaxation (9). Substitution of Gly-132 by Ser caused a 100-fold decrement in relaxation activity, as did replacement of Tyr-136 with either Asp or Ala (5,9). Replacement of Lys-167 by either Ala or Glu reduced topoisomerase activity by more than two

orders of magnitude, but substitution by arginine had less severe effects (10). Preliminary studies suggested that all these mutants proteins were defective in formation of the covalent topoisomerase–DNA intermediate, as determined by the yield of covalent adduct formed by the mutant protein on a short DNA duplex containing a single CCCTT↓ cleavage site for the vaccinia topoisomerase. We have since developed substrates and assays suitable for the determination of the kinetic and equilibrium parameters of the transesterification reactions under single-turnover and equilibrium conditions (8,11,15,16). This has permitted us to quantitate the catalytic contribution of several functional groups on the enzyme to the estimated  $10^9$ – $10^{12}$ -fold enhancement of the rate of transesterification by topoisomerase (8,11,15). We have now applied these methods to assess the roles played by vaccinia residues Arg-130, Gly-132, Tyr-136 and Lys-167.

The two types of substrates used for this study are shown in Figure 2. A 60 bp duplex containing a centrally placed CCCTT cleavage site is employed to assay transesterification under equilibrium conditions (Fig. 2A). We determined by enzyme titration that wild type vaccinia topoisomerase cleaved 19% of the input substrate at saturation. The cleavage–religation equilibrium constant ( $K_{cl} = \text{covalent complex}/\text{noncovalent complex} = k_{cl}/k_{rel}$ ) was thus 0.23. The individual rate constants for DNA cleavage



**Figure 2.** CCCTT-containing DNA substrates for vaccinia topoisomerase. **(A)** Equilibrium cleavage. The structure of the 60 bp duplex is shown. Topoisomerase bound at the CCCTT site establishes a cleavage–religation equilibrium. **(B)** Suicide cleavage. The structure of the 18mer/30mer suicide substrate is shown. Transesterification to the scissile phosphate results in dissociation of the 6mer leaving group, which effectively traps the topoisomerase in the covalently bound state.

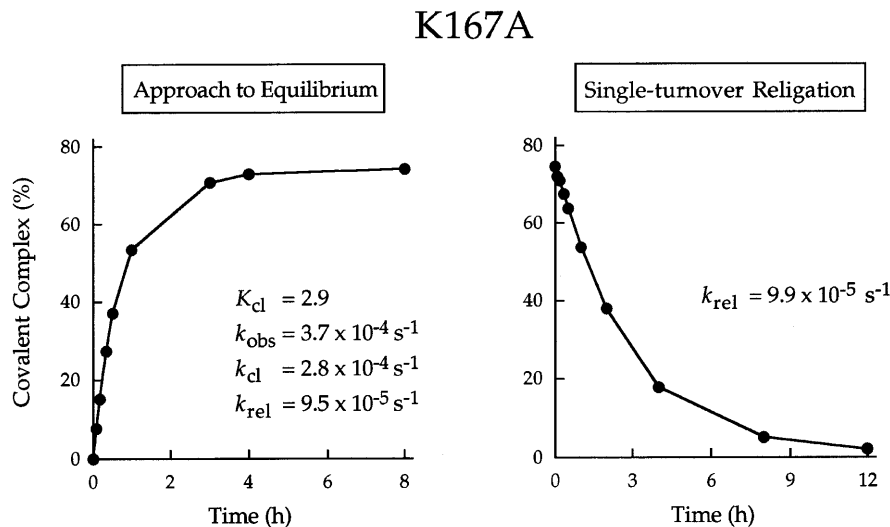
( $k_{cl}$ ) and religation ( $k_{rel}$ ) on the 60mer can be determined from the rate constant ( $k_{obs}$ ) of the approach to the equilibrium cleavage value. In practice, it is not feasible to manually measure  $k_{obs}$  for the wild type topoisomerase on the 60mer duplex because the rates of the component steps are too fast ( $k_{cl} = 0.28 \text{ s}^{-1}$  and  $k_{rel} \sim 1.2 \text{ s}^{-1}$ ). However, approach to equilibrium is an assay well-suited to the analysis of mutant proteins for which the rate is much slower than that of the wild type enzyme (8,11).

A suicide cleavage substrate consisting of an 18mer CCCTT-containing strand annealed to a 30mer strand is used to examine transesterification under single turnover conditions (Fig. 2B). Upon formation of the covalent topoisomerase–DNA adduct, the distal cleavage product 5'-ATTCCC is released and the enzyme becomes covalently trapped on the DNA; we refer to this complex as the suicide intermediate. The cleavage rate constant ( $k_{cl}$ ) of the wild type enzyme on this suicide substrate is  $0.28 \text{ s}^{-1}$  (8).

### Alanine substitutions at Lys-167 and Arg-130 profoundly decrease the rates of the cleavage and religation reactions

The K167A mutant displayed a slow approach to equilibrium over 4 h (Fig. 3, left panel). Remarkably, 74% of the input 60mer was covalently bound at equilibrium. Hence, the observed equilibrium constant ( $K_{cl} = 2.9$ ) was more than 10 times higher than that of the wild type enzyme. The rate constant  $k_{obs}$  for approach to equilibrium by K167A was  $3.7 \times 10^{-4} \text{ s}^{-1}$ . Knowing that  $K_{cl} = 2.9$  and that  $k_{obs} = k_{cl} + k_{rel}$ , we calculated that  $k_{cl} = 2.8 \times 10^{-4} \text{ s}^{-1}$  and  $k_{rel} = 9.5 \times 10^{-5} \text{ s}^{-1}$ . Because neither the extent of cleavage at equilibrium nor the observed rate constant for approach to equilibrium was affected significantly by a 2-fold variation of the amount of input protein (data not shown), we surmise that the mutation did not affect pre-cleavage binding. (This was confirmed by additional experiments described below.)

We measured single-turnover religation on the 60mer substrate by allowing the cleavage reaction to reach equilibrium and then adjusting the reaction mixtures to 0.5 M NaCl (8,11). This concentration of salt blocks both equilibrium cleavage and single turnover-cleavage by interfering with DNA binding. Topoisomerase pre-bound to an equilibrium cleavage substrate at low ionic strength is dissociated when the salt concentration is raised to 0.5 M. Hence, topoisomerase molecules that have catalyzed strand closure on the 60mer DNA will be dissociated from the DNA by salt and will be unable to rebind and re-cleave. The decrease in covalent complex as a function of time after addition of NaCl is plotted in Figure 3 (right panel). K167A covalent complex declined slowly over 8 h ( $k_{rel} = 9.9 \times 10^{-5} \text{ s}^{-1}$ ). Note that the observed rate constant for K167A in single-turnover religation on the 60mer agreed with the value calculated from the rate of approach to equilibrium on the 60mer ( $9.5 \times 10^{-5} \text{ s}^{-1}$ ). By comparing these values to those for wild type topoisomerase, we surmise that elimination of the basic side chain at position 167



**Figure 3.** Approach to equilibrium and single-turnover religation by K167A on the 60mer DNA. **(Left panel)** Approach to equilibrium. Reaction mixtures contained (per 20  $\mu\text{l}$ ) 0.3 pmol of labeled 60 bp DNA and 38 ng of K167A protein. Covalent complex formation is plotted as a function of time. The data were used to determine the cleavage equilibrium constant ( $K_{cl}$ ) and the first-order rate constant ( $k_{obs}$ ) for approach to equilibrium; these values were used to calculate the rate constants for the cleavage and religation steps. **(Right panel)** Single-turnover religation. Cleavage reaction mixtures containing (per 18  $\mu\text{l}$ ) 0.3 pmol of 60mer duplex DNA and 38 ng of K167A protein were incubated for 4 h at 37°C. At this time (time 0), an aliquot (18  $\mu\text{l}$ ) was removed and quenched with SDS. The reaction mixtures were then adjusted to 0.5 M NaCl and aliquots (20  $\mu\text{l}$ ) were taken at the times indicated. The decrease in the abundance of the covalent complex (expressed as percent of input DNA) is plotted as a function of time. The data were used to determine the first-order rate constant for religation.

	Approach to Equilibrium				Single-turnover Religation (60mer)
	$K_{cl}$	$k_{obs}$ ( $s^{-1}$ )	$k_{cl}$ ( $s^{-1}$ )	$k_{rel}$ ( $s^{-1}$ )	$k_{rel}$ ( $s^{-1}$ )
WT	0.23		(0.28)	(1.2)	
K167A	2.9	$3.7 \times 10^{-4}$	$2.8 \times 10^{-4}$	$9.5 \times 10^{-5}$	$9.9 \times 10^{-5}$
K167R	0.53	0.019	0.0067	0.012	0.022
R130A	0.22	$1.3 \times 10^{-5}$	$2.3 \times 10^{-6}$	$1.1 \times 10^{-5}$	$6.3 \times 10^{-6}$
R130K	0.42	$8.0 \times 10^{-5}$	$2.4 \times 10^{-5}$	$5.6 \times 10^{-5}$	$4.2 \times 10^{-5}$

**Figure 4.** Effects of R130 and K167 mutations on equilibrium DNA cleavage. Equilibrium cleavage and single-turnover religation on the 60mer DNA were assayed as described in Methods and Materials. For each mutant, we determined the cleavage equilibrium constant ( $K_{cl}$ ), the rate constant ( $k_{obs}$ ) for approach to equilibrium (these values were used to calculate the rate constants for the cleavage and religation steps) and the rate constant for single-turnover religation.

resulted in a  $10^{-3}$  decrement in the rate of cleavage and a  $10^{-4.1}$  effect on religation.

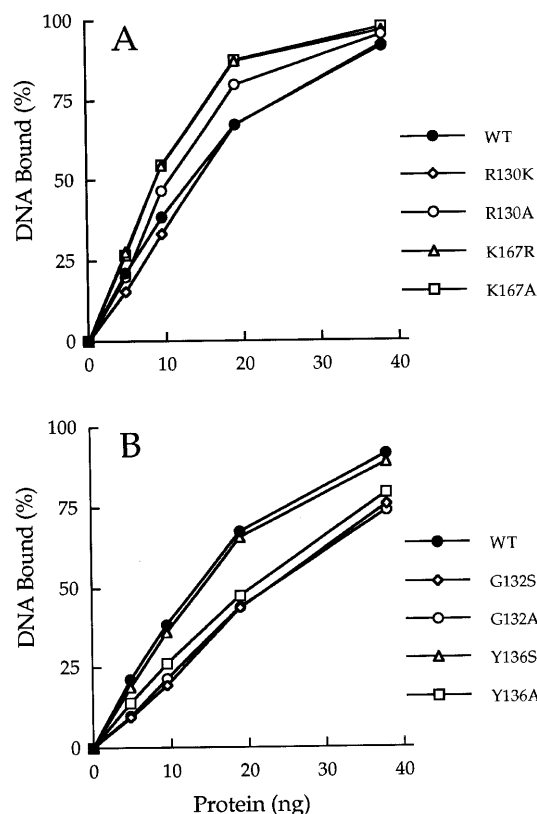
Alanine substitution at position Arg-130 was even more deleterious to reaction chemistry. The rate constants for R130A determined from approach to equilibrium were  $k_{cl} = 2.3 \times 10^{-6} s^{-1}$  and  $k_{rel} = 1.1 \times 10^{-5} s^{-1}$  (Fig. 4). Hence, removal of this basic side chain caused a  $10^{-5.1}$  decrement in cleavage rate and a  $10^{-5}$  effect on religation relative to wild type enzyme. The balanced mutational effects of R130A on the forward and reverse transesterification reactions meant that the equilibrium constant (0.22) was not different from the wild type value (Fig. 4). The rate of single-turnover religation by R130A on the 60mer ( $6.6 \times 10^{-6} s^{-1}$ ) agreed well with the value determined from approach to equilibrium.

#### Effects of conservative substitutions at Lys-167 and Arg-130

Changing Arg-130 to lysine resulted in a catalytic defect that was only marginally less severe than that caused by side chain removal (Fig. 4). The R130K mutation caused a  $10^{-4.1}$  rate decrement in cleavage rate and a  $10^{-4.3}$  effect on religation rate relative to the wild type enzyme. We conclude that Arg-130 is specifically required for cleavage and religation chemistry. At position Lys-167, the conservative arginine substitution resulted in a more substantial restoration of function relative to the alanine mutant. The religation rate constant of K167R (0.012–0.022) was  $\geq 120$ -fold higher than that of K167A; the cleavage rate constant of K167R ( $0.0067 s^{-1}$ ) was 24-fold higher than that of K167A (Fig. 4). Still, the rates of cleavage and ligation by K167R were lower than the wild type rates by factors of 40 and 100, respectively. We surmise that a basic side chain is essential for catalysis, but that lysine is required for optimal activity.

#### DNA binding by catalytically defective R130 and K167 mutants

A native gel mobility shift assay was used to analyze the binding of the R130 and K167 mutant proteins to the 60mer equilibrium



**Figure 5.** Native gel-shift assay of DNA binding by wild type and mutant topoisomerases. Assays of topoisomerase–DNA complex formation on the 60mer DNA were performed as described in Materials and Methods. DNA binding (expressed as the percent of input 60mer DNA associated with the shifted topoisomerase–DNA complex) is plotted as a function of input protein.

cleavage substrate. Binding resulted in the formation of a discrete protein–DNA complex of retarded electrophoretic mobility (17; data not shown). The extent of DNA binding was proportional to the amount of input topoisomerase (Fig. 5A). The affinities of R130A, R130K, K167A and K167R for the 60mer were similar to that of the wild type protein (Fig. 5A). Hence, these mutant enzymes are not compromised in DNA binding; rather they are defective for transesterification chemistry.

#### Mutations at Gly-132 selectively impair the DNA cleavage reaction

Mutant G132S was originally identified through a genetic selection for catalytically defective topoisomerase alleles (4). In the present study, we introduced a less bulky alanine substitution for Gly-132 and subjected the G132S and G132A proteins to kinetic analysis of strand cleavage and religation. Both mutants formed very low levels of the covalent intermediate on the 60mer equilibrium cleavage substrate; G132S and G132A cleaved <1% of the input DNA at saturating enzyme ( $K_{cl} = 0.0063$  for G132S;  $K_{cl} = 0.0077$  for G132A) (Fig. 5). The drastic decrement in the equilibrium constant (to  $\sim 1/30$  of the wild type  $K_{cl}$ ) implied that these mutations significantly decreased the rate of cleavage, but had a relatively modest effect on the rate of religation. A native gel mobility shift assay of the binding of G132S and G132A to

	Approach to Equilibrium	Single-turnover Cleavage and Religation (18/30mer)		
	$K_{cl}$	$k_{cl}$ (s <sup>-1</sup> )	$k_{rel}$ (s <sup>-1</sup> )	$K_{cl}$ ( $k_{cl}/k_{rel}$ )
WT	0.23	0.28	>0.5 (1.2)	
G132A	0.0077	0.0011	0.094	0.012
G132S	0.0063	$8.9 \times 10^{-4}$	0.13	0.0068
Y136A	0.0084	$4.9 \times 10^{-4}$	0.051	0.0096
Y136S	0.11	0.073	0.26	0.28

**Figure 6.** Effects of Gly-132 and Tyr-136 mutations on equilibrium DNA cleavage, single turnover cleavage and single-turnover religation. Equilibrium cleavage on the 60mer DNA was assayed as described in Materials and Methods. The observed cleavage equilibrium constants ( $K_{cl}$ ) are shown. Suicide cleavage and single-turnover religation by the suicide intermediate were assayed as described in Materials and Methods. The rate constants for the cleavage and religation steps are indicated, as is the equilibrium constant calculated from the ratios of the experimentally determined single-turnover rate constants.

the 60mer DNA showed that both mutant proteins displayed nearly wild type affinity for the substrate (Fig. 5B).

Kinetic parameters for the G132S and G132A mutants were determined by assaying single-turnover cleavage on the suicide substrate. The cleavage rate constants for G132S and G132A were  $8.9 \times 10^{-4} \text{ s}^{-1}$  and  $0.0011 \text{ s}^{-1}$ , respectively (Fig. 6). Hence, these mutations caused an  $\sim 10^{-2.4}$  reduction in the rate of cleavage.

The religation reaction was studied under single-turnover conditions by assaying the ability of pre-formed suicide intermediate to transfer the covalently held 5'-<sup>32</sup>P-labeled 12mer strand to a 5' hydroxyl-terminated 18mer strand to form a 30mer product (8,11). Religation by wild type topoisomerase was extremely fast; >90% of the endpoint value was attained in 5 s. This result indicated that  $k_{rel}$  was at least  $0.5 \text{ s}^{-1}$ . (For gauging mutational effects, we derived a value for  $k_{rel}$  of the wild type topoisomerase of  $1.2 \text{ s}^{-1}$  based on the experimental values for  $K_{cl}$  on the 60mer and  $k_{cl}$  on the suicide substrate.) The observed religation rate constants for G132S and G132A were 0.13 and  $0.094 \text{ s}^{-1}$ , respectively, i.e.,  $\sim 1/10$  of the wild type value (Fig. 6). Note that the equilibrium constants calculated for G132S and G132A from the ratios of the single-turnover rate constants on the suicide substrate agreed well with the experimental values for  $K_{cl}$  determined on the 60mer (Fig. 6).

#### A hydroxyl moiety at position 136 is essential for DNA cleavage, but not for religation

We noted previously that replacement of Tyr-136 with alanine reduced DNA relaxation activity to 1% of the wild type level, whereas substitution by serine had little effect (9). Kinetic analysis of transesterification by the Y136A protein revealed properties similar to those of the Gly-130 mutants. Y136A displayed a much reduced level of cleavage of the 60mer at saturating enzyme ( $K_{cl} = 0.0084$ ). This was attributable to a  $10^{-2.8}$  decrement in the rate

of single-turnover cleavage ( $k_{cl} = 4.9 \times 10^{-4} \text{ s}^{-1}$ ) that was far greater than the  $\sim 24$ -fold rate effect on single-turnover religation ( $k_{rel} = 0.051$ ) (Fig. 6). In contrast, the catalytically active Y136S protein displayed only a mild ( $\sim 4$ -fold) reduction in cleavage and religation rates (Fig. 5). A native gel mobility shift assay showed that Y136S and Y136A displayed nearly wild type affinities for the substrate (Fig. 5B).

## DISCUSSION

The results presented in this study enhance our understanding of the vaccinia topoisomerase in three respects: (i) they provide a quantitative assessment of the contribution of four essential amino acids located within and immediately flanking the hinge region; (ii) they illuminate a clear distinction between functional groups required for transesterification chemistry in general and those required specifically for the cleavage reaction; and (iii) they suggest a model for how Arg-130, Lys-167 and other essential residues act at the active site to promote catalysis.

### Transesterification chemistry

Mutational rate effects on the religation reaction can be viewed as the simplest measure of the contribution of a given amino acid residue to transesterification reaction chemistry. This is because single-turnover religation entails no site-recognition step, i.e., the topoisomerase is already bound covalently to the DNA. Based on the effects of the R130A and R130K mutations on single-turnover strand closure by the covalent intermediate, we conclude that Arg-130 enhances the rate of transesterification by a factor of  $10^5$ . This is quantitatively similar to the  $10^5$  contribution of another basic residue, Arg-223, to transesterification chemistry (11). The magnitude of the mutational effects at Arg-130 and Arg-223 argues that both arginine residues interact directly with the scissile phosphate. A key distinction between the two essential arginines is that Arg-130 cannot be replaced by lysine (this study), whereas Arg-223 can be changed to lysine with relatively little catalytic cost (11).

The religation reaction entails an attack by the 5' hydroxyl moiety of the DNA strand on the DNA-(3'-phosphotyrosyl)-protein intermediate. This is likely to proceed through a pentacoordinate phosphorane transition state in which the attacking group (the 5' OH) and the leaving group (Tyr-274) are positioned apically. We speculate that Arg-130 makes bivalent hydrogen bond interactions with the phosphate oxygens in the transition state. The same bidentate contacts would stabilize the transition state during the forward cleavage reaction, which involves attack by Tyr-274 and expulsion of a 5' hydroxyl-terminated DNA. Lysine at position 130 would be less effective in transition state stabilization in this scheme because it would contact only one of the scissile phosphate oxygens. The side chain of Arg-223 might make an essential contact with one phosphate oxygen in the transition state. This monovalent interaction could potentially be sustained by lysine, which may account for the mild effects of the R223K mutation on catalysis. Lys-167 enhances the rates of transesterification by a factor of  $10^3$ – $10^4$  and an arginine at this position can only partially restore function. Lys-167 may interact with the bridging 5' phosphate oxygen of the leaving DNA strand and serve as a general acid to promote its expulsion during the cleavage reaction (16).

Topoisomerase	vac	IKQTAEVVGHTPSISKRAY
	sfv	IKQTADIVGHTPSISKRAY
	mcv	IRQTAEAIHGSPHIAEQAY
	orf	VRQTAEIVGHTPSISRSAY
	fpv	IKQTADTIGHTPNISKQAY
Recombinase	epv	VKRTANIIGHTPTISKNSY
	hp1	LLVLKEILLGHSSTEMTRY
	int	DKFAQHLLGHKSDTMASQY
	xer	LRVVQMLLGHSDLSTTSIY

**Figure 7.** Essential residues His-265 and Tyr-274 of vaccinia topoisomerase are conserved in site-specific recombinases. The amino acid sequence of the vaccinia virus topoisomerase (vac) from residue 256 to the active site Tyr-274 is aligned with the homologous segments of the topoisomerases encoded by other poxviruses, Shope fibroma virus (sfv), molluscum contagiosum virus (mcv), Orf virus (orf), fowlpox virus (fpv) and *Amsacta moorei* entomopox virus (epv), and with the homologous segment immediately preceding the active site tyrosines of recombinase family members HP1 integrase (hp1), lambda integrase (int) and XerD recombinase (xer). Positions conserved with respect to the vaccinia sequence are shaded.

Vaccinia residues Arg-130, Lys-167 and Arg-223 are conserved in other type IB topoisomerases. Hence, we suspect that these side chains play the same role during catalysis by the cellular and viral enzymes. Although the cellular topoisomerases have not been mutagenized as extensively as the vaccinia enzyme, the limited data available are consistent with a common structural basis for transesterification. For example, Jensen and Svejstrup (18) mutated the residues corresponding to Arg-130 and Lys-167 and concluded that they are essential for DNA relaxation. Yet, the deleterious changes they introduced into the human enzyme (Arg→Gln and Lys→Glu) did not reveal the underlying structure–function relationships (e.g., Lys→Glu entails charge inversion). Levin *et al.* (19) reported that a yeast Top1 mutant containing an Arg→Lys mutation at the position corresponding to vaccinia Arg-130 was defective in relaxing supercoiled DNA.

#### Mutations in the hinge that selectively affect cleavage

The biased effects of the Gly-132 and Tyr-136 mutations on cleavage suggest that a post-binding, pre-chemical step may apply during the cleavage reaction, which is not pertinent (or at least not rate-limiting) during religation. Our results sound a note of caution in drawing conclusions about the structural requirements for topoisomerase reaction chemistry on the basis of mutational effects in a strand cleavage assay. The mutational effects at Gly-132 and Tyr-136 on  $k_{rel}$  are too modest to meet the criteria we have set for essentiality in transesterification.

Although the precise nature of the pre-cleavage steps remains unclear, the location of the Gly-132 and Tyr-136 residues within the interdomain hinge suggests a post-binding alteration of the protein–DNA interface. The hinge is highly susceptible to proteolysis when topoisomerase is free in solution; in fact, Tyr-136 is the principal site of cleavage by chymotrypsin. The hinge becomes protected from proteolysis when vaccinia topoisomerase is bound to DNA (13). Protection of the hinge does not require covalent adduct formation, indicating that it occurs prior to transesterification. The simple interpretation is that the hinge makes contact with the DNA ligand and that bound DNA excludes access by proteases. However, additional residues adjacent to the hinge become more accessible to proteolysis in the

DNA-bound state, which raises the possibility of a conformational change upon DNA binding (13). With this in mind, we speculate that hinge dynamics are involved in activating the noncovalent protein–DNA complex for cleavage, e.g., by properly orienting the catalytically essential residues flanking the hinge (Arg-130 and Lys-167) with respect to the scissile phosphate.

The mutational effects at Tyr-136 suggest an important hydrogen bonding interaction. The aromatic moiety is clearly not critical, because activity is preserved when Tyr-136 is replaced by serine. In addition to the requirement for a hydroxyl at this position, it is apparently critical that the functional group be constrained by the polypeptide backbone. We showed previously that a single interruption of the polypeptide backbone by chymotrypsin cleavage between Tyr-136 and Leu-137 elicited effects similar to those described here for the Y136A mutation (13). The amino and carboxyl fragments of the singly nicked wild type topoisomerase remained physically associated; the nicked protein bound to CCCTT-containing DNA, but was incapable of catalyzing strand cleavage.

The importance of the conformation of the hinge during strand cleavage is also suggested by the effects of Gly-132 mutations. Introduction of the methyl group in place of the glycine hydrogen may elicit a local conformational distortion (perhaps affecting nearby essential residue Arg-130) that in turn affects the pre-cleavage activation step. We consider it unlikely that Gly-132 mutations cause a global disruption of protein conformation, given that noncovalent DNA binding is preserved and religation chemistry is affected only modestly by the G132A and G132S changes. Vaccinia residue Gly-132 is invariant in every other type IB topoisomerase.

#### Mechanistic and structural similarities between vaccinia topoisomerase and site specific recombinases

Integrases and other recombinase family members catalyze the same transesterification reaction as the type IB topoisomerases, whereby attack by an active site tyrosine on one DNA strand results in the formation of a DNA–(3′-phosphotyrosyl)–protein intermediate. Functional similarities between vaccinia topoisomerase and the recombinases are probable, in so far as vaccinia topoisomerase promotes sequence-specific recombination *in vivo* and catalyzes site-specific resolution of Holliday junctions *in vitro* (22–24). Although recombinases and type IB topoisomerases have no apparent overall sequence similarity, they may well have converged with respect to the functional groups at their active sites. The recent reports of the crystal structures of the catalytic domains of bacteriophage lambda and HP1 integrases reveal two essential arginines and an essential histidine clustered at the active sites of both proteins (20,21).

We speculate that the Arg-130, Lys-167, Arg-223 triad of essential residues of the vaccinia topoisomerase is functionally analogous to the conserved Arg–His–Arg triad that defines the recombinase family. Further, we suggest that the catalytically essential His-265 of vaccinia topoisomerase (8), which is situated nine amino acids upstream of the active site tyrosine in all six known poxvirus topoisomerases, is the functional equivalent of a conserved histidine moiety present at the identical position relative to the active site tyrosines of lambda integrase, HP1 integrase and XerD recombinase (Fig. 7). Indeed, the segment proximal to the active site is remarkably well conserved between the poxvirus topoisomerases and the recombinases; particularly

the (V,I,L)-(V,I,L)-G-H-(T,S) motif, which includes vaccinia His-265 (Fig. 7). In the crystal structures of lambda and HP1 integrases, the histidine residue corresponding to vaccinia His-265 is situated at the active site pocket (21,22).

In summary, in this and other recent reports (8,11), we have defined the catalytic contributions of essential amino acid side chains of vaccinia topoisomerase, most of which are conserved in other type IB enzymes. We propose that Arg-130, Lys-167, Arg-223 and His-265 act directly on the scissile phosphate during catalysis. We speculate that vaccinia topoisomerase is related mechanistically and structurally to the catalytic domains of site-specific recombinases. Confirmation and further clarification of the topoisomerase I reaction mechanism will hinge on crystallization of topoisomerase in the DNA-bound state.

## ACKNOWLEDGEMENT

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