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Biochemistry

Mapping the active-site tyrosine of vaccinia virus DNA topoisomerase I

(covalent catalysis/tyrosyl phosphodiester linkage/yeast topoisomerase)

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ABSTRACT **Site-directed mutagenesis of the vaccinia virus gene encoding a type I DNA topoisomerase implicates Tyr-274 as the active-site residue that forms a covalent adduct with DNA during cycles of DNA-strand breakage and reunion. Replacement of Tyr-274 by phenylalanine results in loss of the ability of the enzyme to relax negatively supercoiled DNA as well as to form the covalent DNA–protein intermediate. Substitution of phenylalanine for tyrosine at nine other sites in the protein has no apparent effect on enzyme activity. Amino acid sequence alignment reveals Tyr-274 to be homologous to Tyr-727 and Tyr-771, respectively, of the type I topoisomerases from *Saccharomyces cerevisiae* and *Saccharomyces pombe*; Tyr-727 and Tyr-771 have been shown to represent the active-site tyrosines of those enzymes. Sequence comparison of the active-site regions defines a motif Ser-Lys-Xaa-Xaa-Tyr common to the viral and cellular type I topoisomerases, including the human enzyme.**

A mechanistic feature common to all known DNA topoisomerases is the formation of a covalent protein–DNA intermediate during each cycle of DNA-strand breakage and reunion (1). Type II topoisomerases, both prokaryotic and eukaryotic, bind covalently to the 5′-phosphoryl end of the cleaved DNA strand (2–4). The same is true of the prokaryotic type I DNA topoisomerases (2). In contrast, eukaryotic type I topoisomerases form a 3′-phosphoryl linkage to DNA (5). In each instance, DNA is bound to protein by means of a phosphodiester bond to a tyrosyl residue (2–6).

Interest in the structure of the enzyme–DNA intermediate has been fueled by the realization that numerous antimicrobial and antitumor agents of clinical import act by stabilizing or trapping the enzyme in its DNA-bound state (the so-called cleavable complex) (7). Initial studies have focused on the identification of the tyrosine residue(s) to which DNA is attached (8–11). This identification has been facilitated by the cloning, sequencing, and expression of the genes encoding topoisomerases I and II from prokaryotic and eukaryotic sources (12–26).

Our report addresses the identity of the active-site tyrosine of the vaccinia virus DNA topoisomerase, an M_r 32,000 type I enzyme that is encapsidated within the infectious poxvirus particle (17, 27, 28). Vaccinia topoisomerase, like eukaryotic cellular type I enzymes, forms a covalent bond to the 3′-phosphoryl group of a cleaved DNA strand (28, 29). The linkage of DNA to vaccinia topoisomerase is stable to acid, alkali, and hydroxylamine (S.S., unpublished observations), properties consistent with the formation of a phosphotyrosyl bond. The amino acid sequence of the vaccinia enzyme, deduced from the nucleotide sequence of the vaccinia gene that encodes topoisomerase, includes 14 tyrosine residues (out of 314 amino acids). To map the active-site tyrosine(s), we have taken advantage of the ability to express active vaccinia topoisomerase in a heterologous system, *Escherichia coli* (29). By studying the effects of a series of Tyr → Phe point mutations in the topoisomerase gene on the catalytic activity of the resulting mutant proteins, we deduced that Tyr-274 constitutes the active-site tyrosine of vaccinia topoisomerase. While our work was in progress, the laboratories of Wang and Sternglanz reported the mapping of the active-site tyrosines of *Saccharomyces cerevisiae* and *Saccharomyces pombe* type I topoisomerases to Tyr-727 and Tyr-771, respectively (10, 11). Sequence alignment shows that the active-site tyrosine residues are conserved in all known eukaryotic type I topoisomerases.

MATERIALS AND METHODS

Site-Directed Mutagenesis. A DNA fragment containing the vaccinia topoisomerase gene was excised from the plasmid pvtopS (29) by cleavage with enzymes *Bam*HI and *Hin*dIII and then subcloned into M13mp18 to generate M13topo. Uracil-substituted single-stranded phage DNA was isolated from M13topo that had been grown in *E. coli* CJ236 (dut$^-$, ung$^-$). Mutagenesis was performed by hybridizing to the uracil-substituted DNA various mutagenic oligonucleotides; these oligonucleotides, each 21 nucleotides long, contained at position 11 a single base change (relative to the wild-type topoisomerase gene) that converted a tyrosine codon (either TAT or TAC) to a phenylalanine codon (either TTT or TTC). The primed circles were then converted to replicative form by the action of the DNA polymerase III system and DNA ligase purified from *E. coli* (provided by K. Marians and R. Digate, Sloan–Kettering Institute). Phage plaques arising from transformation of the replicative form into *E. coli* JM105 were amplified. Single-strand DNA was isolated from phage supernatants, and the presence of the oligonucleotide-directed mutation was assayed by dideoxynucleotide sequencing. Typically, 50–90% of phage isolates contained the desired mutation. Replicative-form DNA isolated from phage-infected cells was cleaved with nucleases *Bam*HI and *Hin*dIII, and the resulting topo-containing fragment was subcloned into pUC19 to generate a series of mutant constructs designated pUCtopo-phe. This series of plasmids was digested with nucleases *Nde* I and *Bgl* II; the DNA fragments containing the topoisomerase gene were cloned into the T7-based expression plasmid pET3c (30) that had been cleaved with *Nde* I and *Bam*HI, thereby generating the series of mutant plasmids, pETtopo-phe. A plasmid containing the wild-type topoisomerase gene (designated pETtopo-wt) was constructed by cloning an *Nde* I/*Bgl* II fragment of pvtopS into pET3c. Each pETtopo plasmid was used to transform *E. coli* BL21.

Topoisomerase Expression. *E. coli* BL21 transformants carrying pETtopo plasmids were induced to express vaccinia

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topoisomerase by infection with bacteriophage λCE6 (containing the T7 RNA polymerase gene) as described (29). Bacteria (from 50 ml of culture) were harvested by centrifugation 4 hr postinfection, then stored frozen at −80°C. Soluble cell lysates were prepared as described (29) and stored at −80°C.

Assay of Vaccinia Topoisomerase. Soluble bacterial lysates were screened for EDTA-resistant DNA relaxing activity as described (29). Formation of a covalent complex (cleavable complex) of vaccinia topoisomerase with radiolabeled nick-translated λ DNA was assayed as described (28).

Materials. Sequenase and other DNA sequencing reagents were obtained from United States Biochemical. Reagents for nick translation were purchased from Bethesda Research Laboratories. Radionucleotides were from Amersham.

RESULTS

Expression of Mutated Vaccinia Topoisomerase Genes. A series of 10 mutant alleles of the vaccinia topoisomerase gene (each resulting in a specific Tyr → Phe change at the protein level) were generated by oligonucleotide-directed mutagenesis and then cloned into the T7-based expression vector pET3c (described in *Materials and Methods*). *E. coli* bearing either wild-type or mutant pETtopo plasmids were induced to express the cloned gene by infection of the bacteria with bacteriophage λCE6 (29, 31). Lysates of λCE6-infected cells were prepared, and soluble polypeptides were analyzed by SDS/PAGE. As shown in Fig. 1, lane WT, cells bearing pETtopo-wt accumulated high levels of a M_r 33,000 polypeptide shown previously to be vaccinia topoisomerase (29). A polypeptide with identical mobility was evident in cells bearing the pETtopo-phe plasmids (Fig. 1). The appearance of the topoisomerase polypeptide was absolutely dependent in each case on infection with λCE6 (data not shown). Wild-type and mutant proteins accumulated to similar levels in both the soluble fraction (Fig. 1) and in whole cell lysates (data not shown). No polypeptides of $M_r < 33,000$ were induced by bacteriophage λCE6 infection. Thus, the introduction of Tyr → Phe point mutations had no significant deleterious effect on the expression, solubility, or stability of the vaccinia topoisomerase in *E. coli*.

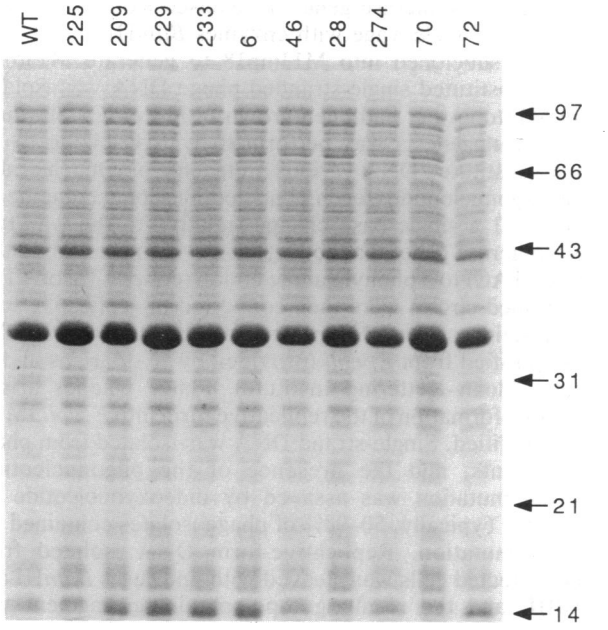

FIG. 1. Expression of wild-type and mutant topoisomerases in *E. coli* BL21. The polypeptide composition of soluble cell lysates from λCE6-infected *E. coli* BL21(pETtopo) was examined by electrophoresis through a 15% polyacrylamide gel containing 0.1% SDS. Protein bands were visualized by staining with Coomassie brilliant blue dye. Each lane contained 25 μl of soluble lysate; the identity of the pETtopo plasmid carried by the phage-infected bacteria is indicated above the lane. Numbers of plasmids correspond to the amino acid position at which tyrosine was replaced by phenylalanine. Protein concentrations of the soluble lysates, determined by dye binding (32), were as follows: WT, 4.0 mg/ml; Phe-225, 3.8 mg/ml; Phe-209, 3.5 mg/ml; Phe-229, 4.4 mg/ml; Phe-233, 3.7 mg/ml; Phe-6, 3.3 mg/ml; Phe-46, 4.3 mg/ml; Phe-28, 3.9 mg/ml; Phe-274, 3.8 mg/ml; Phe-70, 4.6 mg/ml; Phe-72, 3.1 mg/ml. Positions and molecular masses (in kDa) of coelectrophoresed protein standards are indicated by arrows.

Effect of Tyr → Phe Mutations on Topoisomerase Activity. Topoisomerase activity in *E. coli* lysates was assayed by the relaxation of negatively supercoiled plasmid DNA in the presence of EDTA—i.e., under conditions that limit DNA relaxation to that catalyzed by the vaccinia enzyme. As shown in Fig. 2, the soluble protein fraction from cells expressing wild-type topoisomerase relaxed the supercoiled DNA to completion. Extracts of cells expressing topoisomerases that contained phenylalanine in lieu of tyrosine at positions 6, 28, 46, 70, 72, 209, 225, 229, or 233 also relaxed the DNA fully. In contrast, an equivalent amount of extract from cells expressing topoisomerase mutated to phenylalanine at position 274 showed no appreciable topoisomerase activity. A more thorough analysis of topoisomerase activity is shown in Fig. 3. Wild-type extract catalyzed extensive DNA relaxation, even when diluted 1000-fold, whereas the Phe-274 mutant extract failed to relax the plasmid DNA at any level of dilution. Thus, within the limitations of this assay, it is concluded that the effect of the amino acid substitution at position 274 is to reduce the specific activity by 1000-fold or more, if not to wholly inactivate the enzyme. Similar analyses of the remaining nine mutant topoisomerase extracts revealed little difference in activity of the mutants compared to wild-type extract (data not shown).

Wild-type and Phe-274 topoisomerases were partially purified from the soluble bacterial extracts by phosphocellulose column chromatography, as described (29). The chromatographic properties of wild-type and mutant proteins (as determined by SDS/PAGE analysis of column fractions) were indistinguishable: both eluted from the column between 0.5 M and 1.0 M NaCl. Quantitative assay of DNA relaxation by partially purified enzymes confirmed the 1000-fold or greater disparity in specific activity between the wild-type and Phe-274 mutant (data not shown). A mixing experiment showed that the relaxing activity of wild-type enzyme was unaffected by prior addition of Phe-274 mutant protein, even at concentrations representing up to a 700-fold excess of mutant topoisomerase over wild-type enzyme (data not

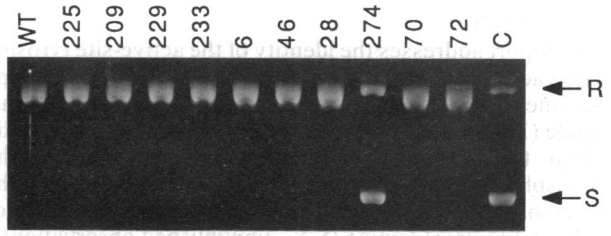

FIG. 2. DNA relaxation by wild-type and mutant topoisomerases. Topoisomerase reaction mixtures contained 0.3 μg of supercoiled plasmid DNA and 1 μl of soluble bacterial lysate that had been diluted 10-fold with lysis buffer (29). Reaction products were electrophoresed through 1.2% agarose gels, and DNA was visualized by subsequent staining with ethidium bromide. A photograph of the stained gel is shown. The source of the soluble lysate is indicated above each lane. The lane labeled C shows the product of a reaction from which enzyme was omitted. Positions of relaxed (R) and supercoiled (S) forms of the plasmid DNA are indicated by arrows.

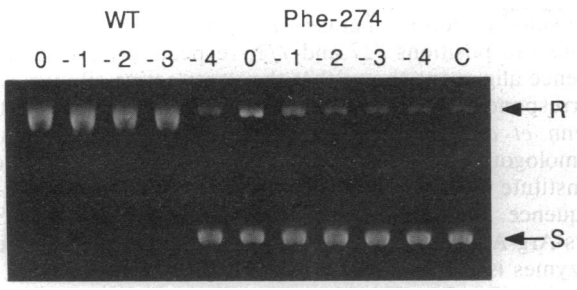

FIG. 3. Titration of wild-type and Phe-274 mutant topoisomerases. Lysates of phage-infected *E. coli* BL21 bearing pETtopo-WT or pETtopo-phe-274 were diluted serially by a factor of ten in lysis buffer and assayed for DNA relaxation activity. Each reaction contained 1 μl of the diluted enzyme. A photograph of the stained gel is shown. The logarithm of the dilution factor is indicated above each lane. The lane labeled C shows the product of a reaction from which enzyme was omitted. Positions of relaxed (R) and supercoiled (S) forms of the plasmid DNA are indicated by arrows.

shown). Thus, the inactivity of the Phe-274 protein in DNA relaxation is inherent to the mutant enzyme and not attributable to the presence of an inhibitor in the enzyme preparation.

Covalent Complex Formation. Partially purified wild-type topoisomerase was incubated with ^{32}P-labeled nick-translated λ DNA. DNA was then digested with DNase I, and the resulting products were analyzed by SDS/PAGE. As shown in Fig. 4, lane WT, this procedure resulted in the formation of a labeled polypeptide of M_r 37,000 as well as minor labeled polypeptides of M_r 34–36,000. These species correspond to vaccinia topoisomerase linked covalently to ^{32}P-labeled oligodeoxynucleotides (28). The size heterogeneity of the labeled protein derived presumably from linkage to oligonucleotides of various length. No labeled polypeptides were seen in control reactions from which topoisomerase had been omitted (Fig. 4, lane C). Partially purified Phe-274 mutant topoisomerase did not form the covalent adduct with DNA (Fig. 4, lane 274), in concordance with the inability of the mutant enzyme to relax supercoiled DNA.

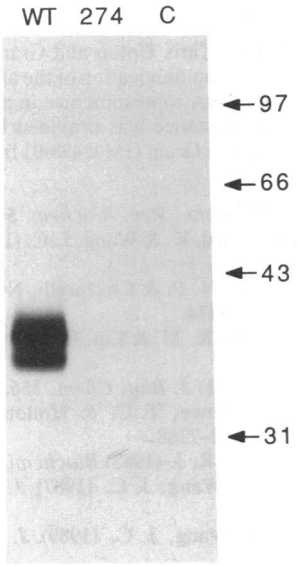

FIG. 4. Formation of a covalent topoisomerase–DNA complex. Covalent complex formation was assayed by label transfer from DNA to protein (28). Each reaction contained (in 20 μl) 50 mM Tris·HCl (pH 7.5), 10 ng of nick-translated λ DNA, and 5.9 ng of partially purified topoisomerase (1 M NaCl phosphocellulose fraction). DNase-resistant material was analyzed by electrophoresis through a 12.5% polyacrylamide gel containing 0.1% SDS. The gel was stained with Coomassie brilliant blue and then dried. Radiolabeled protein was visualized by autoradiography. A photograph of the autoradiogram is shown. The source of the partially purified enzyme is indicated above each lane. The lane labeled C shows the product of a reaction from which topoisomerase enzyme was omitted. Positions and molecular masses (in kDa) of coelectrophoresed protein standards are indicated by arrows.

Effects of Topoisomerase Expression on *E. coli*. The physiologic consequences to *E. coli* of vaccinia topoisomerase expression have been examined in *E. coli* BL21(DE3)pLysE, a λ lysogen that contains an inducible T7 RNA polymerase gene (under the control of a lacUV5 promoter) inserted into the λ *int* gene (33). It was shown that induction of topoisomerase expression resulted in *recA*-dependent lysogenic induction and cell lysis (33). In the course of those studies, it was noted that the lysogen strain BL21(DE3) could not be transformed with the vaccinia topo-bearing plasmid pA9topo to generate ampicillin-resistant colonies (S.S., unpublished data). This situation would arise if the low level of T7 RNA polymerase in the uninduced bacterium prevented establishment of plasmids because the target gene carried on the plasmid is toxic. The tolerance of BL21(DE3) for toxic target plasmids can be increased by the presence of the plasmid pLysE that carries the T7 lysozyme gene. T7 lysozyme decreases the level of active T7 polymerase in the uninduced state by specifically binding to and inhibiting the polymerase (34). The requirement for pLysE to establish the topo-bearing plasmid suggested that even uninduced levels of vaccinia topoisomerase expression were toxic to the bacteria. Bjornsti and Wang (35) have reported that overexpression of yeast topoisomerase I was also toxic to *E. coli*. The basis for the toxicity of vaccinia topoisomerase was addressed by studying the effects of the Phe-274 point mutation on the ability of pETtopo plasmids to transform BL21(DE3). Because BL21 strains made competent by the calcium chloride method were poorly transformed by any plasmid, we used electroporation to introduce DNA into the cells. As shown in Table 1, a single point mutation in the topoisomerase gene resulted in a 1000-fold increase in the transformation efficiency of the mutant pETtopo-phe-274 plasmid relative to that of pETtopo-wt. In contrast, another phenylalanine substitution mutant, pETtopo-phe-225, transformed BL21(DE3) with the same low efficiency as the wild-type gene. The transformation efficiency of pETtopo-phe-274 in BL21(DE3) was comparable to that obtained with the vector plasmid pET3c (data not shown). The pETtopo-wt and pETtopo-phe plasmids transformed BL21 with essentially equal efficiency (data not shown). These results indicate that the intolerance of *E. coli* BL21(DE3) for topoisomerase-bearing plasmids is abrogated selectively by mutations that render the topoisomerase inactive in catalysis. Thus topoisomerase activity, rather than some unrelated feature of the expressed vaccinia protein, is responsible for the apparent toxicity to *E. coli*.

Table 1. Efficiency of transformation of *E. coli* BL21(DE3) by pETtopo plasmids

Plasmid	Transformation efficiency, colonies/μg of DNA
pETtopo-wt	5.0×10^4
pETtopo-phe-274	4.9×10^7
pETtopo-phe-225	5.3×10^4

E. coli BL21(DE3) was electrotransformed with plasmid DNA using a Bio-Rad Gene Pulser electroporation apparatus. Preparation of bacterial cells and electrotransformation were done as prescribed by the manufacturer. Serial dilutions of each DNA sample (ranging from 0.5 pg to 50 ng of plasmid DNA per transformation) were used to determine the transformation efficiencies, expressed as the number of ampicillin-resistant colonies per μg of plasmid DNA.

DISCUSSION

The nature of the topoisomerase I catalytic intermediate, involving a tyrosyl phosphodiester bond between eukaryotic type I enzymes and DNA, predicts that the phenolic hydroxyl group of the active site tyrosine should be essential for enzyme activity. Substitution of phenylalanine for tyrosine, a conservative change in terms of overall protein structure, eliminates the potential for phosphodiester bond formation. The conservative nature of such amino acid substitutions on vaccinia topoisomerase is underscored by the effects of a series of Tyr → Phe mutations on enzyme activity. In 9 of 10 instances, substitution of phenylalanine for tyrosine had no discernable effect on enzyme accumulation, stability, or activity when mutant topoisomerases were expressed in *E. coli*. Only one mutation, resulting in replacement of tyrosine by phenylalanine at position 274, had an adverse effect on enzyme activity. This mutant topoisomerase, when expressed in *E. coli*, was catalytically inert in DNA relaxation. Moreover, the Phe-274 mutant was unable to form the covalent DNA–protein intermediate. It is construed from these data that Tyr-274 constitutes the active-site tyrosine of vaccinia virus DNA topoisomerase.

Vaccinia topoisomerase is predicted to contain 14 tyrosines, 10 of which were mutated in the present study. The possibility that 1 of the 4 remaining tyrosines might be the active-site residue, while not formally excluded, can be regarded as unlikely on the basis of sequence comparisons. The topoisomerase gene of Shope fibroma virus (a member of the leporipox genus of the poxvirus family) encodes a 314-amino acid polypeptide that is 60% identical at the amino acid level to the vaccinia virus topoisomerase (C. Upton and G. McFadden, personal communication). Twelve of the 14 tyrosines found in the vaccinia protein are conserved in the Shope fibroma virus protein; these include all 10 of the tyrosines studied herein. The nonconserved tyrosines, at positions 92 and 115 in the vaccinia enzyme, are replaced in the Shope fibroma virus enzyme by histidine and phenylalanine, respectively. We infer from such natural evolutionary variation in related viruses that tyrosines 92 and 115 are dispensible for poxvirus topoisomerase function. The remaining two tyrosines, at positions 136 and 183 of the vaccinia enzyme, lie within a region of significant amino acid similarity between the vaccinia topoisomerase and the type I topoisomerase of *S. cerevisiae* (12, 17), yet these tyrosines are not themselves conserved in the *S. cerevisiae* enzyme, or in topoisomerases I from *S. pombe* (13) or human (15), implying as before that these residues are not critical for catalysis. Indeed, Tyr-183 of vaccinia topoisomerase is replaced at an analogous position by phenylalanine in the type I enzyme from all three aforementioned cellular sources; Tyr-136 is replaced by arginine in each of the cellular enzymes.

The mapping of the vaccinia active site is predicated on a mutational analysis rather than on direct peptide sequencing of DNA-bound enzyme fragments, an approach that has been used by Wang and coworkers (8–10) to locate the active sites of several cellular DNA topoisomerases. In those cases, the yield of DNA-bound protein could be enhanced considerably by the formation of the cleavable complex in the presence of drugs that stabilize the covalent intermediate—i.e., oxolinic acid, tenoposide, or (in the case of eukaryotic topoisomerase I) camptothecin (8–10). This approach seemed less feasible for vaccinia DNA topoisomerase I because the enzyme is resistant to camptothecin (29). The mutational strategy was made more appealing by the relatively small size of the vaccinia protein and the limited number of tyrosine residues to be analyzed.

Our assignment of the vaccinia active site to Tyr-274 is sustained by the recently reported mapping of the active-site tyrosines of topoisomerases I from *S. cerevisiae* and *S. pombe* to positions 727 and 771, respectively (10, 11). Sequence alignment shows that the yeast active-site tyrosines correspond to Tyr-274 of vaccinia topoisomerase, as noted by Lynn *et al.* (10). The human topoisomerase contains a homologous tyrosine at position 773 that can be predicted to constitute the active site of that enzyme (15). The amino acid sequence immediately preceding vaccinia Tyr-274 is Ser-Lys-Arg-Ala-Tyr. The corresponding sequence in both yeast enzymes is Ser-Lys-Ile-Asn-Tyr and that of the human enzyme is Ser-Lys-Leu-Asn-Tyr. Thus, a motif Ser-Lys-Xaa-Xaa-Tyr is common to the active sites of viral and cellular topoisomerases. Species-specific variations at the Xaa positions of this motif appear of little significance insofar as mutation of these two amino acids in the yeast sequence to those amino acids present in the vaccinia enzyme had no effect on DNA relaxation activity of the variant yeast enzyme (10). Whether the conserved Ser-Lys portion of the active-site motif is functionally important remains unclear, but such a function is readily testable by site-directed mutagenesis.

Apparent structural homology in the active-site region, as well as in other portions of the enzyme molecule, tends to underscore the biochemical similarities between the eukaryotic viral and cellular type I topoisomerases. A distinctive property of the cellular enzymes—sensitivity to camptothecin—is not shared by the vaccinia topoisomerase. Comparative structural and mutational analyses of the vaccinia and cellular enzymes should provide insights into the mechanism of action of this antineoplastic agent.

Finally, the observation that mutations in the topoisomerase gene that inactivate the enzyme effect a 1000-fold increase in the transformation of BL21(DE3) can be exploited as a potentially powerful phenotypic screen for the isolation of additional (i.e., nontargeted) mutants that affect topoisomerase function.

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