JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Subscriber access provided by American Chemical Society

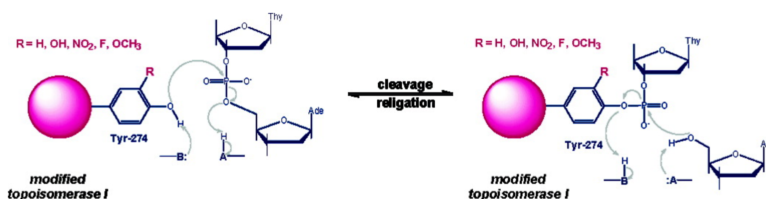
Article

Analogues of Vaccinia Virus DNA Topoisomerase I Modified at the Active Site Tyrosine

Rong Gao, Yi Zhang, Ambar K. Choudhury, Larisa M. Dedkova, and Sidney M. Hecht

J. Am. Chem. Soc., **2005**, 127 (10), 3321-3331• DOI: 10.1021/ja044182z • Publication Date (Web): 16 February 2005

Downloaded from http://pubs.acs.org on March 24, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 2 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S
ARTICLES
Published on Web 02/16/2005

Analogues of Vaccinia Virus DNA Topoisomerase I Modified at the Active Site Tyrosine

Rong Gao, Yi Zhang, Ambar K. Choudhury, Larisa M. Dedkova, and Sidney M. Hecht*

Contribution from the Departments of Chemistry and Biology, University of Virginia, Charlottesville, Virginia 22901

Received September 24, 2004; E-mail: sidhecht@virginia.edu

Abstract: The mechanism of type IB topoisomerase-mediated DNA relaxation was studied by modification of vaccinia topoisomerase I at the active site tyrosine (position 274) with several tyrosine analogues. These analogues had varied steric, electronic, and stereochemical features to permit assessment of those structural elements required to support topoisomerase function. Eleven tyrosine analogues were successfully incorporated into the active site of vaccinia topoisomerase I. It was found that only tyrosine analogues having the phenolic −OH group in the normal position relative to the protein backbone were active. Modifications that replaced the nucleophilic tyrosine OH ($pK_a \approx 10.0$) group with NH_2 (pK_a 4.6), SH ($pK_a \approx 7.0$), or I groups or that changed the orientation of the nucleophilic OH group essentially eliminated topoisomerase I function. For the active analogues, the electronic effects and H-bonding characteristics of substituents in the meta-position of the aromatic ring may be important in modulating topoisomerase I function. The pH profile for the functional analogues revealed a small shift toward lower pH when compared with wild-type topoisomerase I.

Introduction

DNA topoisomerases are ubiquitous nuclear enzymes that release the supercoiling and torsional tension of DNA to facilitate the DNA replication, transcription, recombination, and chromatin remodeling.[1−6] As a subfamily of topoisomerases, type IB topoisomerases modulate the topological state of DNA through the introduction of a single-strand break in the phosphodiester backbone. Vaccinia topoisomerase I is the smallest member of type IB topoisomerases family and constitutes an excellent model[7] for studying the interaction of type IB enzymes with duplex DNA because of its small size (314 amino acids) and target site specificity (5′(C/T)CCTTp$^{\downarrow}$ sites).[8]

The basic mechanism of the DNA relaxation by type IB topoisomerases involves a reversible transesterification reaction (Figure 1).[9,10] The active site tyrosine OH group of the enzyme mediates a nucleophilic attack on phosphodiester backbone to form a DNA-(3′-*O*-phosphotyrosyl)-enzyme intermediate, with the formation of a free DNA strand having a 5′-OH group. The free DNA strand can undergo passage around the unbroken strand, removing DNA supercoils; attack of the DNA 5′-OH group upon the covalent enzyme−DNA intermediate then regenerates the original strand and free topoisomerase IB.[11] DNA relaxation by topoisomerase I requires neither ATP nor added Mg^{2+}.[6]

Characterization of the transesterification reaction has been carried out previously using modified DNA substrates, such as those containing 2′,5′-phosphate ester linkages[12−14] or methylphosphonates[15−17] in proximity to high efficiency cleavage sites, as well as modified 5′-OH groups for the religation reaction.[18] A reasonable level of flexibility in the behavior of the vaccinia and human enzymes has been demonstrated, and the facility of the ligation reaction was responsive to physicochemical changes in the nature of the nucleophile responsible for initiating religation.

Recent efforts in our laboratory have focused on investigating the extent to which the transesterification reaction will tolerate the modification of the active site tyrosine of the type IB enzyme. Presently, vaccinia topoisomerases I have been modified by introducing tyrosine analogues with altered steric,

(1) Wang, J. C. *Harvey Lect.* **1985**, *81*, 93−110.
(2) Osheroff, N. *Pharmacol. Ther.* **1989**, *41*, 223−241.
(3) Chen, A. Y.; Liu, L. F. *Annu. Rev. Pharmacol. Toxicol.* **1994**, *34*, 191−218.
(4) Gupta, M.; Fujimori, A.; Pommier, Y. *Biochim. Biophys. Acta* **1995**, *1262*, 1−14.
(5) Wang, J. C. *Annu. Rev. Biochem.* **1996**, *65*, 635−692.
(6) Champoux, J. J. *Annu. Rev. Biochem.* **2001**, *70*, 369−413.
(7) Shuman, S. *Biochim. Biophys. Acta* **1998**, *1400*, 321−337.
(8) Shuman, S.; Prescott, J. *J. Biol. Chem.* **1990**, *265*, 17826−17836.
(9) Tse, Y. C.; Kirkegaard, K.; Wang, J. C. *J. Biol. Chem.* **1980**, *255*, 5560−5565.
(10) Champoux, J. J. *J. Biol. Chem.* **1981**, *256*, 4805−4809.
(11) Stivers, J. T.; Harris, T. K.; Mildvan, A. S. *Biochemistry* **1997**, *36*, 5212−5222.
(12) Arslan, T.; Abraham, A. T.; Hecht, S. M. *J. Biol. Chem.* **1998**, *273*, 12383−12390.
(13) Arslan, T.; Abraham, A. T.; Hecht, S. M. *Nucleosides Nucleotides* **1998**, *17*, 515−530.
(14) Krogh, B. O.; Claeboe, C. D.; Hecht, S. M.; Shuman, S. *J. Biol. Chem.* **2001**, *276*, 20907−20912.
(15) Gao, R.; Claeboe, C. D.; Eisenhauer, B. M.; Hecht, S. M. *Biochemistry* **2004**, *43*, 6167−6181.
(16) Tian, L.; Claeboe, C. D.; Hecht, S. M.; Shuman, S. *Mol. Cell.* **2003**, *12*, 199−208.
(17) Tian, L.; Claeboe, C. D.; Hecht, S. M.; Shuman, S. *Structure* **2004**, *12*, 31−40.
(18) Henningfeld, K. A.; Arslan, T.; Hecht, S. M. *J. Am. Chem. Soc.* **1996**, *118*, 11701−11714.

10.1021/ja044182z CCC: $30.25 © 2005 American Chemical Society

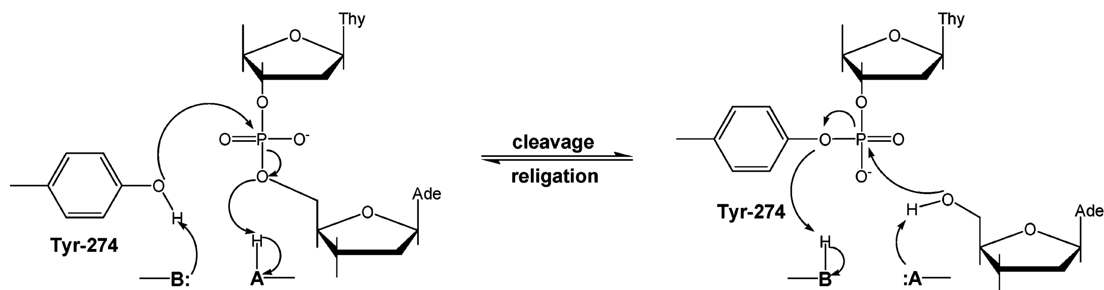


Figure 1. Mechanism of transesterification by vaccinia topoisomerase I.

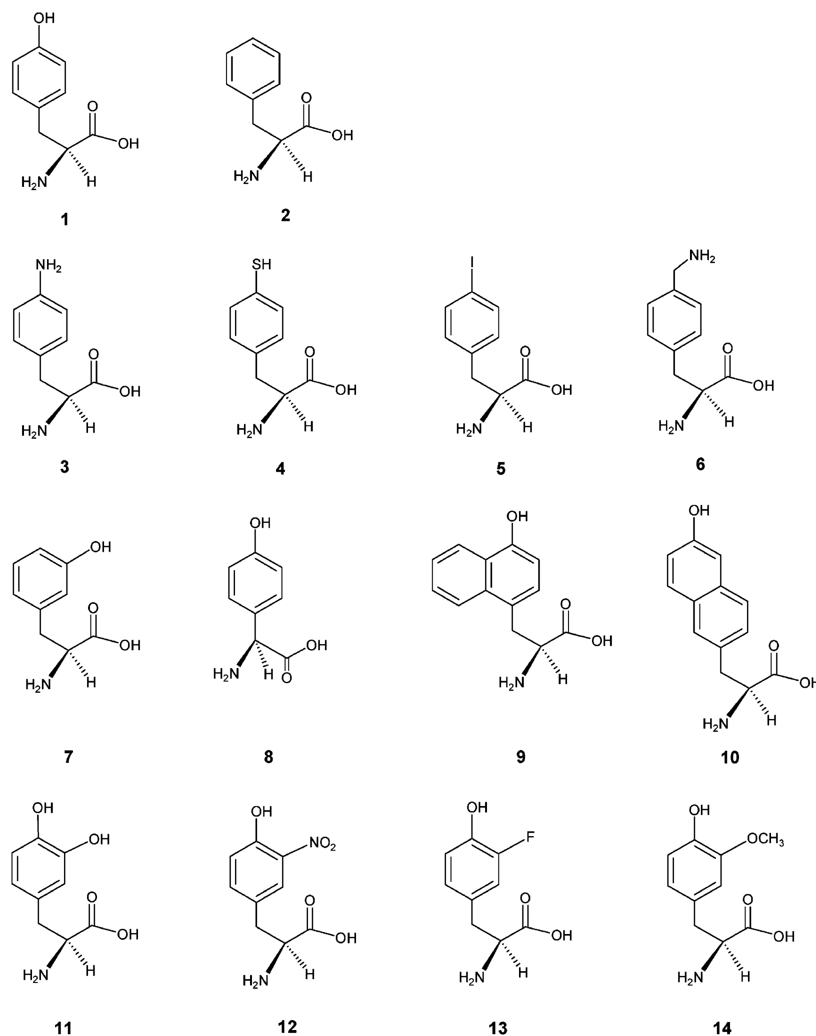


Figure 2. Tyrosine analogues incorporated into vaccinia topoisomerase I at position 274. Analogues **7**, **9**, **10**, and **13** were prepared in racemic form.

electronic, and regiochemical features (Figure 2) in lieu of the active site tyrosine at position 274. The results provide functional biochemical data that demonstrate the importance of the nature and position of the tyrosine OH group in supporting the type IB topoisomerase I-mediated transesterification reaction. In contrast to the flexibility of the enzyme in processing modified DNA substrates, there is little flexibility permitted at the level of modification of the active site tyrosine.

Results

Construction of Expression Plasmid. The DNA fragment containing the gene for vaccinia topoisomerase I was excised from its original plasmid pET3c-Top1(wt) (Figure 3) and inserted into plasmid vector pET28b(+). The newly constructed plasmid pET28b(+)-Top1(wt) included the DNA sequence corresponding to an N-terminal hexahistidine motif MGSSH-HHHHHSSGLVPRGSH immediately prior to the initiator codon. The inclusion of the hexahistidine moiety was intended to facilitate purification of the derived vaccinia topoisomerase I by Ni-NTA chromatography.[19] The vaccinia topoisomerase I containing the fusion peptide at the N-terminus was found to have the same activity in relaxing supercoiled DNA as wild type. In addition, the coding sequence of the vaccinia topoisomerase I was altered to include a nonsense (TAG) codon at position 274 using site-directed mutagenesis techniques[20,21] to construct plasmid pET28b(+)-Topo1(274).

(19) Janknecht, R.; de Martynoff, G.; Lou, J.; Hipskind, R. A.; Nordheim, A.; Stunnenberg, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8972−8976.

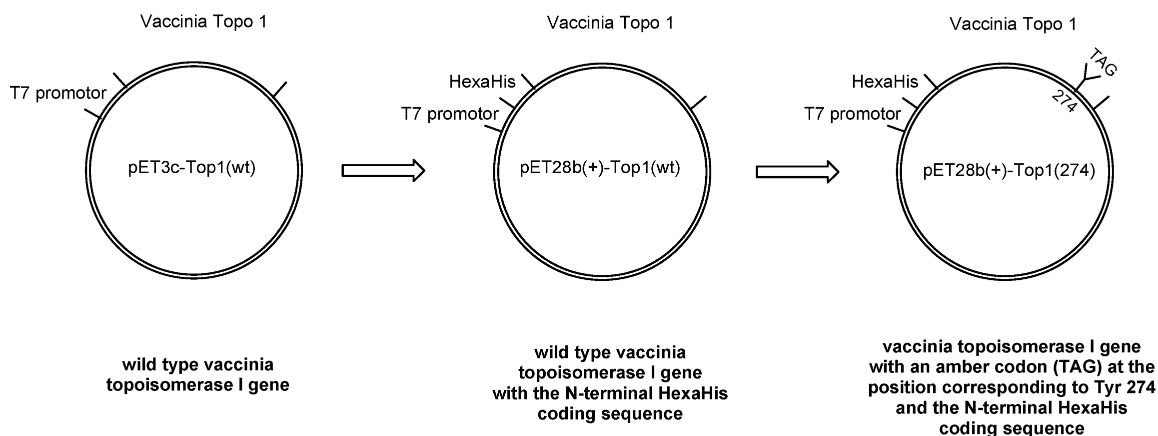
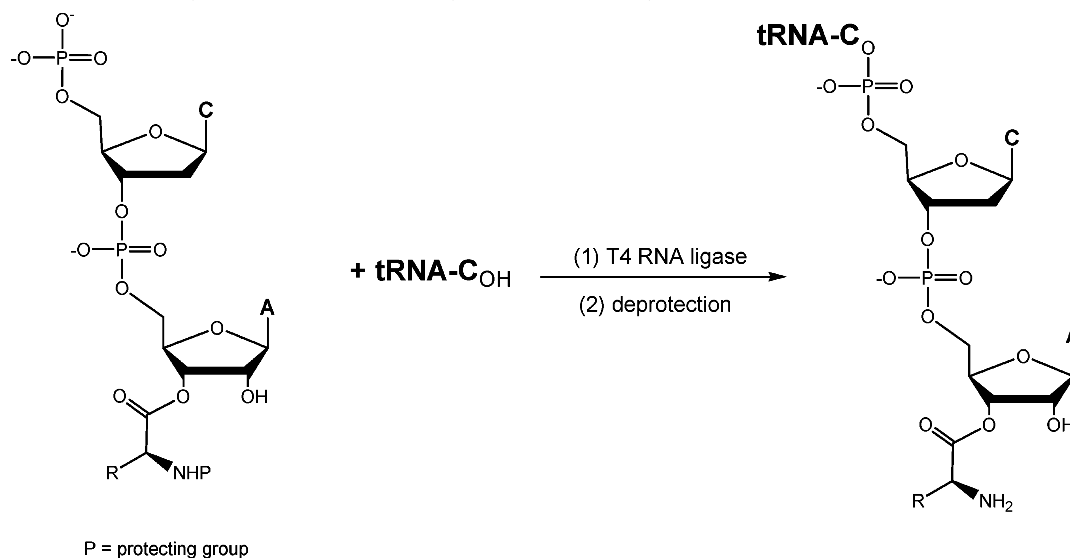


Figure 3. Plasmid constructs used for expression of the vaccinia topoisomerase I gene.

Scheme 1. Preparation of Misacylated Suppressor tRNAs by Chemical Aminoacylation



Preparation of Misacylated tRNA$_{CUA}$s. Suppressor tRNA$^{Phe}_{CUA}$ lacking the 3′-terminal cytidine and adenosine moieties was prepared by in vitro transcription using T7 RNA polymerase as described in the Experimental Section. The abbreviated tRNA transcript tRNA$_{CUA}$-C$_{OH}$ was coupled with N-protected 2′(3′)-*O*-aminoacyl-pdCpA derivatives via the agency of T4 RNA ligase (Scheme 1). The efficiency of ligation was estimated by polyacrylamide gel electrophoresis at acidic pH.[22]

In Vitro Elaboration of Vaccinia Topoisomerase I. A number of vaccinia topoisomerase I analogues modified at position 274 have been prepared using the general strategy shown in Scheme 2.[23−34] These were prepared by translation in a cell free system utilizing *Escherichia coli* S-30 extract. Figure 4 shows SDS-PAGE analysis of the elaborated vaccinia topoisomerases I incorporating 14 different tyrosine derivatives (**1**−**14**) into position 274. As a control, a full-length protein was synthesized by transcription and translation from a DNA template containing the wild-type vaccinia topoisomerase gene (Figure 4, lane 1). The synthesis of the modified full-length proteins by translation of the mRNA containing UAG at position 274 was dependent on the presence of an aminoacylated tRNA$_{CUA}$. In the absence of the misacylated suppressor tRNA$_{CUA}$, protein translation was terminated at the nonsense codon by release factors and gave the truncated protein (Figure 4, lane 2); in the presence of aminoacylated tRNA$_{CUA}$, full-length vaccinia topoisomerases I were obtained by read-through of the UAG codon by the individual tyrosyl-tRNA$_{CUA}$s (Figure 4, lanes 3−16). Quantification of the elaborated proteins was carried out by phosphorimager analysis and is summarized in Table 1. Although there was a substantial variation from one

(20) Smith, M. *Annu. Rev. Genet.* **1985**, *19*, 423−462.
(21) Kunkel, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 488−492.
(22) Varshney, U.; Lee, C. P.; RajBhandary, U. L. *J. Biol. Chem.* **1991**, *266*, 24712−24718.
(23) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182−188.
(24) Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. *J. Biol. Chem.* **1978**, *253*, 4517−4520.
(25) Pezzuto, J. M.; Hecht, S. M. *J. Biol. Chem.* **1980**, *255*, 865−869.
(26) Heckler, T. G.; Zama, Y.; Naka, T.; Hecht, S. M. *J. Biol. Chem.* **1983**, *258*, 4492−4495.
(27) Heckler, T. G.; Chang, L. H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468−1473.
(28) Heckler, T. G.; Chang, L. H.; Zama, Y.; Naka, T.; Hecht, S. M. *Tetrahedron* **1984**, *40*, 87−94.
(29) Roesser, J. R.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1986**, *25*, 6361−6365.
(30) Payne, R. C.; Nichols, B. P.; Hecht, S. M. *Biochemistry* **1987**, *26*, 3197−3205.
(31) Heckler, T. G.; Roesser, J. R.; Xu, C.; Chang, P. I.; Hecht, S. M. *Biochemistry* **1988**, *27*, 7254−7262.
(32) Roesser, J. R.; Xu, C.; Payne, R. C.; Surratt, C. K.; Hecht, S. M. *Biochemistry* **1989**, *28*, 5185−5195.
(33) Lodder, M.; Golovine, S.; Hecht, S. M. *J. Org. Chem.* **1997**, *62*, 778−779.
(34) Lodder, M.; Golovine, S.; Laikhter, A. L.; Karginov, V. A.; Hecht, S. M. *J. Org. Chem.* **1998**, *63*, 794−803.

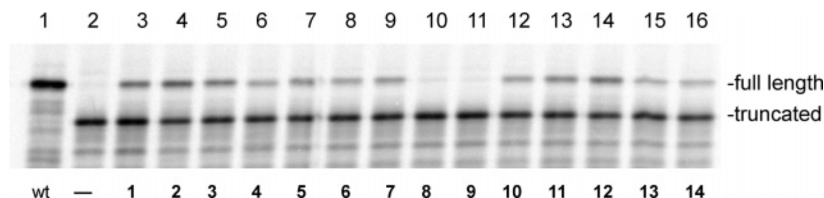


Figure 4. Analysis of in vitro synthesis of vaccinia topoisomerase I analogues modified at position 274. Lane 1, wild-type topoisomerase I; lane 2, truncated topoisomerase I-274 (no suppressor tRNA); lanes 3−16, modified topoisomerases I containing amino acids **1**−**14** at position 274, respectively.

Scheme 2. General Strategy Employed for Elaboration of Vaccinia Topoisomerases I Modified at Position 274 by Incorporation of Unnatural Amino Acids

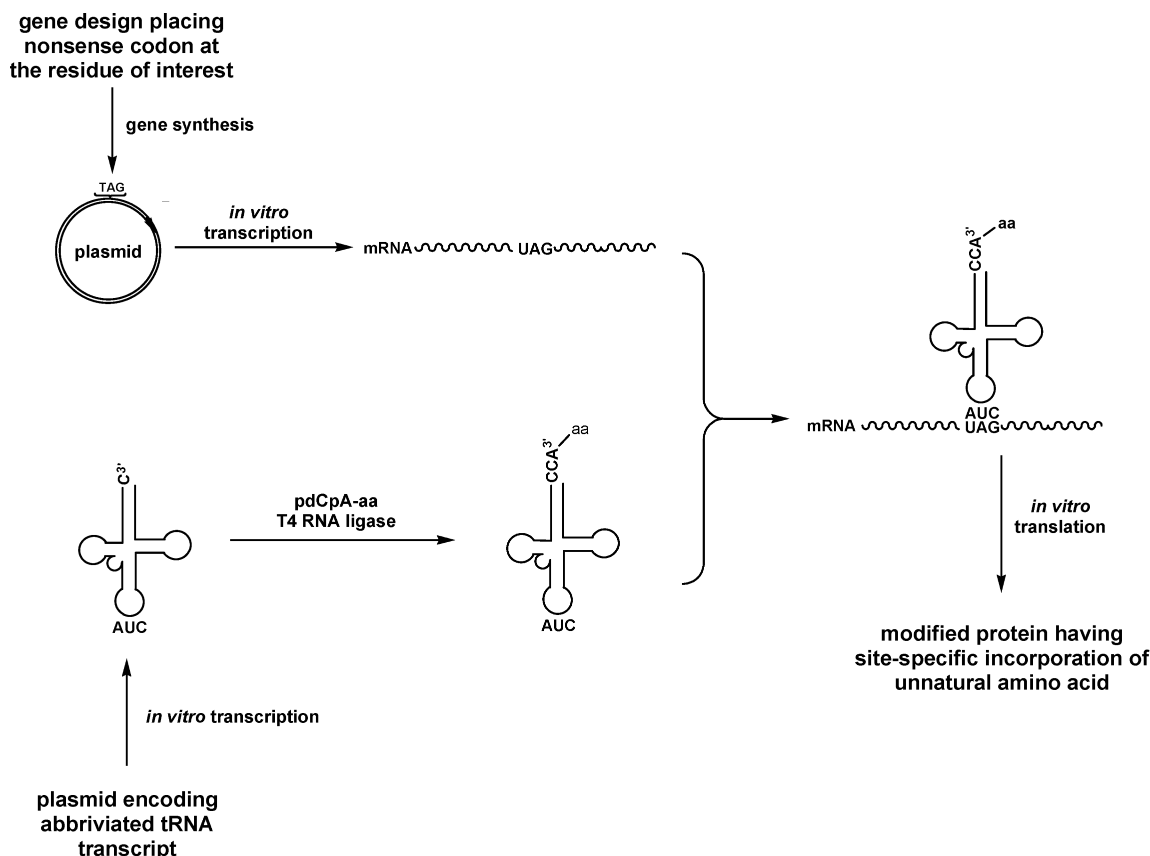


Table 1. In Vitro Synthesis of Vaccinia Topoisomerases I Having Tyrosine Analogues at Position 274

amino acid	suppression efficiency (%)[a]	topoisomerase activity
1	19	+
2	34	−
3	21	−
4	11	−
5	17	−
6	11	−
7	13	−
8	0.5	N.D.[b]
9	0.4	N.D.[b]
10	12	−
11	18	+
12	28	+
13	9	+
14	9	+

[a] Yield of full-length topoisomerase I as a percentage of all protein synthesized. [b] Not determined.

tyrosyl-tRNA analogue to another in efficiency of read-through at position 274, all of the analogues of vaccinia topoisomerases I were expressed at a useable level except the $tRNA_{CUA}$s bearing tyrosine analogues **8** and **9**.

Assay for Topoisomerase I Function. The 12 modified vaccinia topoisomerases I were assayed for their ability to relax supercoiled DNA plasmid. Relaxation activity was determined by incubation of 0.5 μL of the crude translation mixture with 250 ng of supercoiled DNA plasmid in a 10 μL reaction mixture at 37 °C for 30 min. The reactions were analyzed by running a 1% agarose gel sequentially in the absence and then in the presence of ethidium bromide to separate the relaxed DNA plasmid (form IV) from the supercoiled DNA plasmid (form I) and (adventitious) nicked DNA plasmid (form II). The results are shown in Figure 5, and those topoisomerases exhibiting DNA relaxation activity are summarized in Table 1. As anticipated, introduction of the tyrosine "analogue" phenylalanine (**2**) resulted in loss of topoisomerase activity. The topoisomerase I analogues having an altered nucleophile such as NH_2 (**3**), SH (**4**), or I (**5**) in place of the active site tyrosine OH group were also essentially inactive (Figure 5, lanes 6−8). In addition, any modification that changed the position of the nucleophilic OH group on the active site tyrosine (**6** and **7**) or which added a phenyl ring (**10**) also resulted in enzyme inactivation (Figure 5, lanes 9−11). Only those modifications

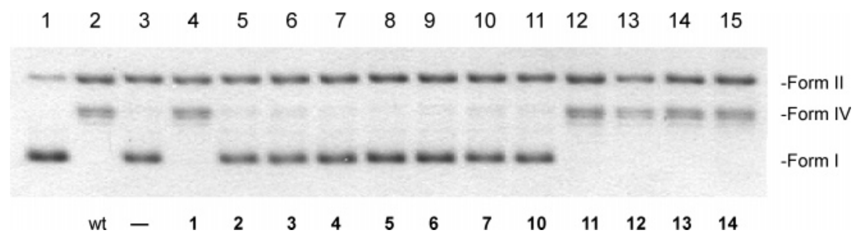


Figure 5. Relaxation assay of vaccinia topoisomerases I modified at position 274. Lane 1, plasmid pSP64 alone; lane 2, DNA + wild-type topoisomerase I; lane 3, DNA + truncated topoisomerase I; lanes 4−15, DNA + modified topoisomerases I.

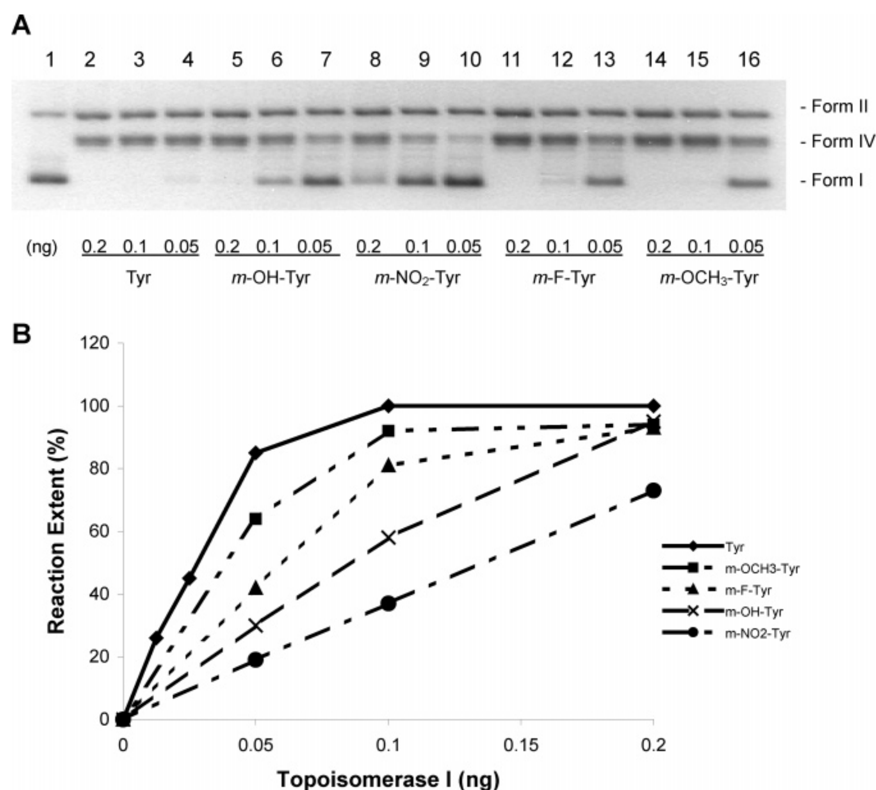


Figure 6. (A) Electrophoretic analysis of DNA relaxation mediated by (modified) topoisomerases I at three concentrations. The relaxation assay was carried out by incubation with 0.2, 0.1, or 0.05 ng of each topoisomerase I with 250 ng of supercoiled plasmid pSP64 at 37 °C for 30 min. Lane 1, plasmid pSP64 alone; lanes 2−4, wild-type topoisomerase I; lanes 5−7, vaccinia topoisomerase I analogue containing **11**; lanes 8−10, vaccinia topoisomerase I analogue containing **12**; lanes 11−13, vaccinia topoisomerase I analogue containing **13**; lanes 14−16, vaccinia topoisomerase I analogue containing **14**. (B) Concentration dependence of DNA relaxation by wild-type topoisomerase I and four analogues modified at position 274.

containing tyrosine analogues (**11**−**14**) having an −OH group in the "normal" position relative to peptide backbone exhibited relaxation activity comparable to wild-type vaccinia topoisomerase I (Figure 5, cf. lanes 2 and 4 vs lanes 12−15).

For those vaccinia topoisomerase I analogues having DNA relaxation activity, purification was carried out by Ni-NTA chromatography,[19] and the protein concentrations were quantified as described in the Experimental Section. To test the concentration dependence of the relaxation assay, wild-type topoisomerase I and topoisomerase analogues containing **11**−**14** were diluted twice from the same concentrations. The relaxation reactions were carried out at 37 °C for 30 min by incubation of each topoisomerase analogue at each of three concentrations with 250 ng of supercoiled DNA plasmid. A 1% agarose gel was run to separate the relaxed DNA plasmid (form IV) from the supercoiled DNA plasmid (form I) and (adventitious) nicked DNA plasmid (form II). Visualization of the relaxed bands was performed under UV light and quantified using ImageQuant software. The dose dependence of DNA relaxation by wild-type topoisomerase I and the four analogues (**11**−**14**) were compared as shown in Figure 6A by electrophoretic analysis and summarized graphically in Figure 6B. Relaxation activity was found to be greatest for wild type (i.e., having tyrosine at position 274). For the topoisomerase analogues, activity decreased in the order **14** > **13** > **11** > **12** when these tyrosine derivatives were substituted at position 274 of the enzyme. This finding seems consistent with altered nucleophilicity of the *p*-tyrosine OH group in the active site. Thus, while each of the meta-substituted analogues was less active than the wild-type enzyme, the election-donating OCH_3 substituent adjacent to the OH group resulted in a more active topoisomerase I analogue than the electron-withdrawing NO_2 substituent. It also seems possible that the substituent ortho to the tyrosine OH group may alter enzyme function via H-bonding, a factor that could contribute to the activity of those enzyme analogues containing the OCH_3 (**14**), F (**13**), and OH (**11**) substituents.

In addition to measuring the concentration dependence of relaxation, the rate constants (k_{obs}) of DNA relaxation were also determined for each of the vaccinia topoisomerase I analogues.

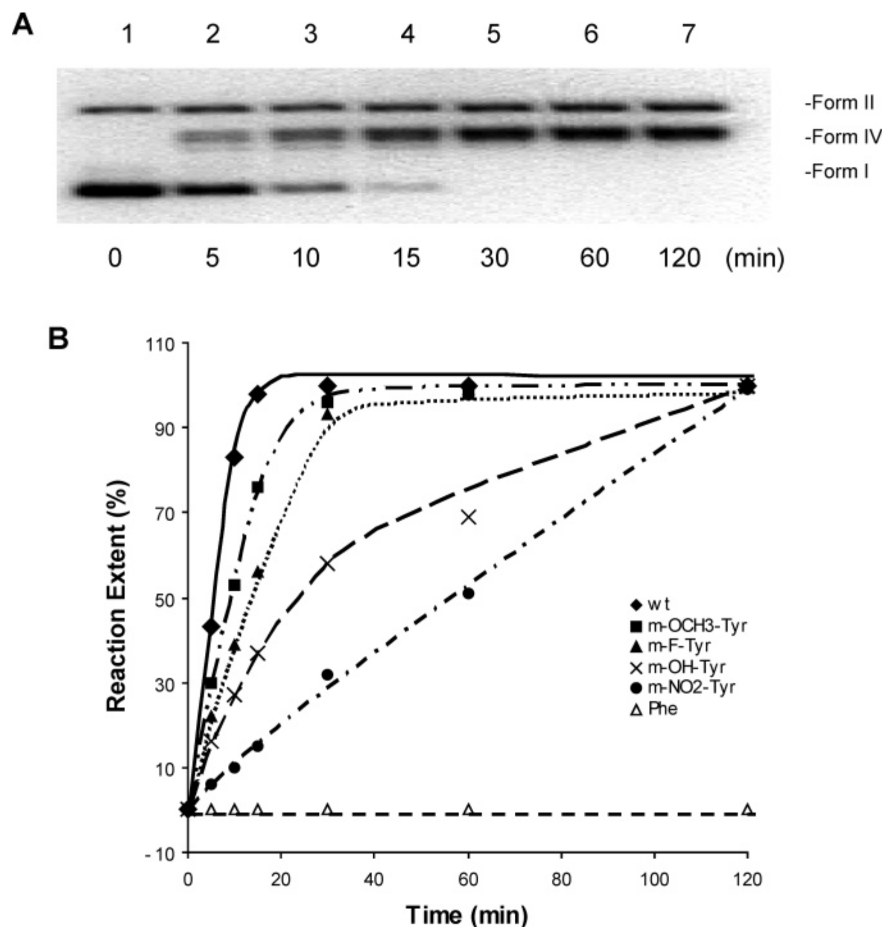


Figure 7. (A) Electrophoretic analysis of the time course of relaxation mediated by vaccinia topoisomerase I analogue containing **14**. The reaction was carried out as described in the Experimental Section. Aliquots were removed at the times indicated, and the reaction was quenched by adding 1% sodium dodecyl sulfate. (B) Time course of vaccinia topoisomerase I-mediated DNA relaxation comparing the activity of wild-type vaccinia topoisomerase I and four analogues modified at position 274.

Table 2. Kinetic Parameters for DNA Relaxation Mediated by Analogues of Vaccinia Topoisomerases I Modified at Position 274[a]

amino acid at position 274	k_{obs} ($\times 10^4$ s^{-1})
Tyr (wild type) (**1**)	43 ± 3
m-OCH_3-Tyr (**14**)	16 ± 2
m-F-Tyr (**13**)	9 ± 1
m-OH-Tyr (**11**)	5 ± 1
m-NO_2-Tyr (**12**)	2 ± 1

[a] Rate constants were obtained under pseudo-first-order reaction conditions using a large excess of DNA substrate.

An analysis was also performed using wild-type topoisomerase I for comparison. In each case, 0.1 ng of the enzyme was used. Each reaction was quenched by treatment with sodium dodecyl sulfate, followed by agarose gel electophoretic analysis to separate the relaxed DNA. Figure 7A shows a representative agarose gel electrophoresis performed for the topoisomerase I analogue containing tyrosine derivative **14** at position 274. The time course of relaxation is shown in Figure 7B, and the rate constants are summarized in Table 2. All of the mutant topoisomerases I were less efficient than wild type; the observed rates were less than half that of the wild-type topoisomerase I. The rank order of the rate constants was the same as that observed in the concentration dependence experiment (Figure 6B).

The relaxation activity of each of the topoisomerase I analogues was also investigated over a range of pH values and compared with wild-type topoisomerase I. In each case, the supercoiled DNA plasmid (form I) was treated with one of the topoisomerases I and incubated at 37 °C for 30 min at each of several pH values (pH 4.0−11.0). The reactions were analyzed by agarose gel electophoresis. Figure 8A shows a representative agarose gel electrophoresis performed for the wild-type topoisomerase I.

The pH dependence of relaxation assay gave a bell-shaped curve for each of the enzymes (Figure 8B). Within experimental error, each analogue had essentially the same pH dependence. The pH maximum for each was shifted toward lower pH by about 0.3 pH unit when compared to wild-type topoisomerase I.

Discussion

Since the first report by Bauer et al. in 1977,[35] vaccinia topoisomerase I has been studied as a model type IB enzyme because of its small size and specificity for a pentapyrimidine sequence.[8] A very stringent requirement for tyrosine at the active site has also been demonstrated. Peterson and Shuman found that replacement of the active site tyrosine at position 274 with serine or threonine afforded enzymes having activity 10^{-7} that of wild type.[36] Loss of DNA relaxation activity has also been

(35) Bauer, W. R.; Ressner, E. C.; Kates, J.; Patzke, J. V. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 1841−1845.
(36) Petersen, B. O.; Shuman, S. *Nucleic Acids Res.* **1997**, *25*, 2091−2097.

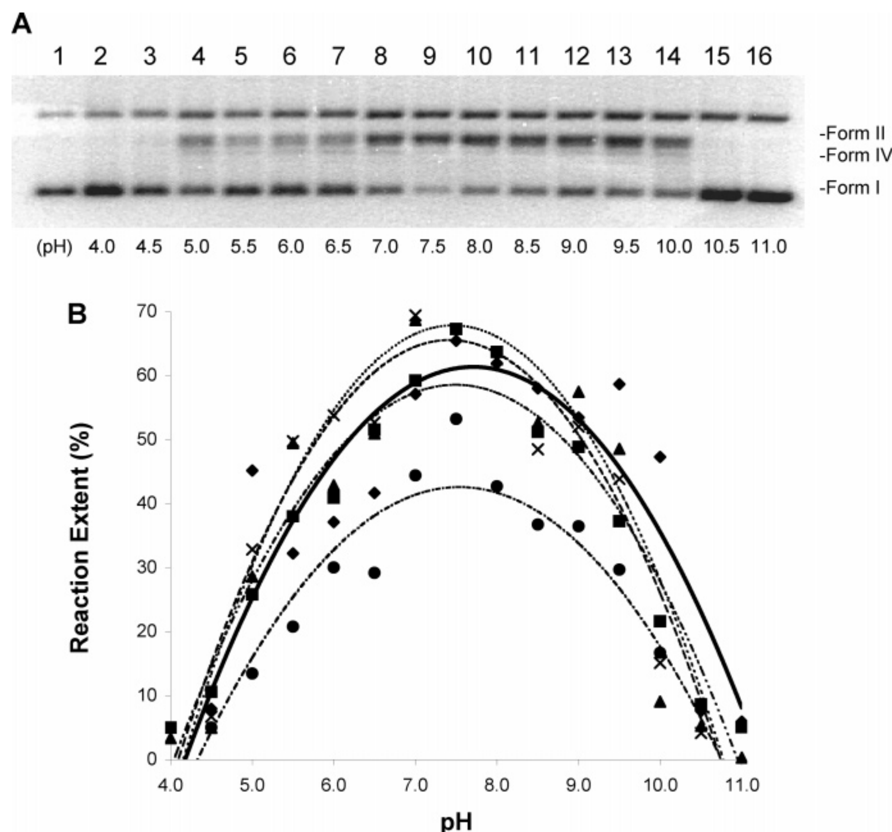


Figure 8. (A) Electrophoretic analysis of the pH dependence of DNA relaxation mediated by wild-type vaccinia topoisomerase I. The reaction was carried out as described in the Experimental Section. Lane 1, plasmid pSP64 alone; lanes 2−16, relaxation assay at different pH values, as indicated. (B) pH profile of vaccinia topoisomerase I-mediated DNA relaxation comparing the activity of wild-type vaccinia topoisomerase I (◆) and four analogues modified at position 274 (with **11** (×), **12** (●), **13** (▲), and **14** (■)).

reported as a consequence of serine substitution for the active site tyrosine of *Saccharomyces cerevisiae* topoisomerase I.[37] By utilizing "chemically" misacylated suppressor tRNAs to effect the read-through of a nonsense codon[23−34] at position 274, we have been able to incorporate unnatural amino acids to study the effect of enzyme function with greater chemical precision. Accordingly, 11 tyrosine analogues were incorporated into the active site of vaccinia topoisomerase I, permitting the study of the structure−function relationships for the derived topoisomerases I in some detail.[38]

Topoisomerase I-mediated DNA relaxation is a multistep process,[5−7] which includes (i) noncovalent binding of the enzyme to the DNA duplex substrate, (ii) sequence-selective cleavage of a single DNA strand involving formation of a covalent DNA-(3′-phosphotyrosyl)-enzyme intermediate, (iii) strand passage of the cleaved DNA strand around the intact strand, (iv) religation of the original cleaved strand in a process that is superficially the reverse of the cleavage reaction, and (v) dissociation of the enzyme from the DNA substrate. Alteration of any of these steps can result in a change in efficiency of the overall process. It may be noted that the intermediate topoisomerase I−DNA covalent binary complex is ordinarily formed transiently in an exceptionally facile process and exists as a minor species in equilibrium with much greater amounts of free enzyme and DNA. The potential liability of a covalent enzyme−DNA complex is underscored by the cytotoxicity that results when agents such as camptothecin stabilize the covalent binary complex formed from mammalian topoisomerases I.[39,40] Thus, it seemed reasonable to anticipate that active site function may have been carefully optimized for tyrosine and that any alteration could result in significant diminution of enzyme function.

The modifications made at the active site of vaccinia topoisomerase I can be classified into three groups based on the properties of the tyrosine analogues. In the first group, the tyrosine OH group, which acts as a nucleophile group in the topoisomerase I-mediated cleavage reaction (Figure 1), was modified by replacement with H (**2**), NH_2 (**3**), SH (**4**), I (**5**), or CH_2NH_2 (**6**) to change the nucleophilicity and acidity of the active site residue involved in the cleavage reaction. We have previously studied the religation reaction for human topoisomerase I using a modified DNA substrate having the 5′-OH group (Figure 1) replaced by 5′-thio, amino, and hydroxymethylene groups and demonstrated a surprising flexibility in the ability of the enzyme to process the modified DNA substrates.[18] However, the present results indicate that replacement of the nucleophilic OH group in the active site tyrosine resulted in loss of DNA relaxation ability (cf. Figure 5, lanes 5−9). Even when assayed over a large pH range, topoisomerase

(37) Lynn, R. M.; Bjornsti, M. A.; Caron, P. R.; Wang, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3559−3563.
(38) Four of the analogues, namely **7**, **9**, **10**, and **13**, were employed in racemic form, but the normal ribosomal bias against *R*-amino acids undoubtedly assured that only the *S*-isomers were incorporated into protein. See: Dedkova, L. M.; Fahmi, N. E.; Golovine, S. Y.; Hecht, S. M. *J. Am. Chem. Soc.* **2003**, *125*, 6616−6617 and references therein.
(39) Hsiang, Y.-H.; Hertzberg, R.; Hecht, S.; Liu, L. F. *J. Biol. Chem.* **1985**, *260*, 14873−14878.
(40) Hsiang, Y.-H.; Lihou, M. G.; Liu, L. F. *Cancer Res.* **1989**, *49*, 5077−5082.

activity was not recovered (data not shown). Loss of DNA relaxation activity presumably resulted from a change in equilibrium between the cleavage and religation reactions because of the altered nucleophilicity of the active site tyrosine residue.

A second group of modifications focused on alteration of the placement of the phenolic OH group of the active site tyrosine by either changing the position of the nucleophilic OH group relative to the protein backbone (analogues **7** and **8**) or introducing an additional phenyl ring (analogues **9** and **10**). Only two of these four modified topoisomerases could be produced in useable amounts (Figure 4). The loss of activity resulting from modifications of this type (Figure 5, lanes 10 and 11) again demonstrated a stringent requirement for the placement of the OH group in the catalytic domain of the enzyme.

A third type of modification was carried out by adding a meta-substituent in the phenyl ring without changing the position of the nucleophilic OH group. These substituents, which are ortho to the nucleophilic OH groups, can affect the nucleophilicity and basicity of the OH group by electronic and H-bonding effects. These four topoisomerase I analogues were all found to relax supercoiled plasmid DNA with reasonable efficiency (Figure 5, lanes 12−15). The order of the analogue activity was found to be **1** > **14** > **13** > **11** > **12**, where the numbers refer to the tyrosine at position 274 in each topoisomerase I (Figures 6 and 7). The analogue containing an electron-rich OCH_3 group was the most efficient of the analogues, whereas the analogue containing the electron-withdrawing NO_2 group was the least efficient. The potential for H-bonding between the nucleophilic OH group and adjacent substituent was clearly also important, as judged by the reasonably efficient relaxation ability of the topoisomerase I analogue containing the (electron-withdrawing) F substituent. H-bonding of the OH group of the topoisomerase I analogue containing **14** to the ortho OCH_3 group may also have facilitated the function of this species. The topoisomerase I analogue containing an ortho OH group (**11**) was less efficient at DNA relaxation than those containing OCH_3 or F substituents. This may plausibly reflect the ability of the additional OH group to participate in both *acceptor* and *donor* H-bonds with the nucleophilic OH group of the enzyme. It is interesting that the differences in rates of the individual analogues containing tyrosine derivatives **11**−**14** are much smaller than the differences in pK_a's of the active site tyrosine OH groups, indicating that facility of H-abstraction from this group (Figure 1) does not determine enzyme efficiency.

The observed pH dependence of the DNA relaxation activity (Figure 8) for wild-type topoisomerase I and its active analogues is consistent with the general acid and base catalysis mechanism proposed for the enzyme.[41] As shown in Figure 1, a general base in the enzyme (−B:) accepts a proton from the attacking nucleophile concomitant with the formation of a covalent enzyme−DNA complex, whereas a general acid (−AH) donates a proton to facilitate expulsion of the 5′-OH leaving group. Our results indicated that the optimum pH values for the analogues were quite similar to wild type, being shifted by no more than 0.3 pH unit toward lower pH compared with wild-type topoisomerase I. These observations could plausibly result from altered acidity of the phenolic OH group because of the additional substituents in the ortho position, but are perhaps more likely related to a possible pH-sensitive precleavage conformational step that has been noted previously.[7]

Experimental Section

General Methods. ^{1}H NMR spectra were recorded on a Varian 500 (500 MHz) spectrometer. Racemic 2-amino-3-(4-hydroxy-1-naphthyl)-propanoic acid and racemic 2-amino-3-(6-hydroxy-2-naphthyl)propanoic acid were synthesized according to a literature procedure.[42] 6-Nitroveratryl chloroformate, 3-nitro-*S*-tyrosine, 4-iodo-*S*-phenylalanine, *S*-phenylalanine, Et_3N, *N*,*N*-diisopropylethylamine, chloroacetonitrile, CH_3CN, sodium hydrogen sulfate, *S*-tyrosine, 3-fluoro-*R*,*S*-tyrosine, 4-amino-*S*-phenylalanine, *S*-phenylglycine, anhydrous DMF, and *R*,*S*-*m*-tyrosine were purchased from Sigma-Aldrich Chemicals. 3-Methoxy-*S*-tyrosine was purchased from ICN Chemicals. Chemicals were used without further purification. Flash chromatography was performed using 230−400 mesh silica gel, purchased from Silicycle.

Plasmid pET3c-Topo1(wt) containing the vaccinia topoisomerase I gene was obtained from Dr. Stewart Shuman. Plasmid pET28b(+) and *E. coli* competent cells BL21 and BL21 (DE3) were obtained from Novagen (Madison, WI). *E. coli* competent cell JM109, recombinant RNasin ribonuclease inhibitor, amino acid mixture (complete), and Wizard plus DNA purification system were purchased from Promega Corporation (Madison, WI). DNA primer for sequence or mutagenesis was ordered from Invitrogen Life Technologies (Grand Island, NY). [^{35}S]-methionine (1000 Ci/mmol, 10 μCi/μL) was purchased from Amersham Corporation (Piscataway, NJ). Protease inhibitor (complete, EDTA-free) was obtained from Boehringer Mannheim Corp. (Indianapolis, IN).

Endonucleases *Fok*I, *Hin*dIII, *Nde*I, and *Dpn*I, as well as calf intestinal alkaline phosphatase, *Taq* DNA ligase, T4 DNA ligase, T4 polynucleotide kinase, and T4 RNA ligase were purchased from New England Biolabs (Beverly, MA). *Pfu*I DNA polymerase was purchased from Stratagene Cloning Systems (La Jolla, CA). GELase and AmpliScribe transcription kits were obtained from Epicentre Technologies (Madison, WI).

Ni-NTA agarose was obtained from Qiagen (Valencia, CA). Ultrapure agarose was from Bethesda Research Laboratories (Bethesda, MD). Agarose (low melting point) was obtained from Sigma Chemicals (St. Louis, MO). Triton X-100 was from Shelton Scientific Inc. (Shelton, CT).

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. UV spectral measurements were made using a Perkin-Elmer Lambda 20 UV/vis spectrometer.

General Procedure for the Synthesis of pdCpA Derivatives of Tyrosine Analogues. To a conical vial containing 5 μmol of protected amino acid cyanomethyl ester[33,34] was added a solution of 1 μmol of the tris(tetrabutylammonium) salt of pdCpA in 50 μL of anhydrous DMF, followed by 10 μL of Et_3N. The reaction was kept in the dark and stirred at room temperature for 36 h and was monitored by HPLC. Five μL of the reaction mixture was diluted with 45 μL of 1:1 CH_3CN−50 mM NH_4OAc buffer, pH 4.5. Ten μL of the diluted aliquot was analyzed by HPLC on a C_{18} reversed-phase column (250 × 10 mm). The column was washed with a linear gradient of 1% → 63% CH_3CN in 50 mM NH_4OAc buffer, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). After 3 days, the reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN−50 mM NH_4OAc buffer, pH 4.5, and purified on a semipreparative C_{18} reversed-phase HPLC column. After lyophilization of the appropriate fractions, the product was obtained as a solid.

***N*-(6-Nitroveratryl)oxycarbonyl-*S*-tyrosyl-pdCpA (1).** Isolated as a colorless solid: yield 2.1 mg (65%); HPLC retention times 26.6 and

(41) Stivers, J. T.; Shuman, S.; Mildvan, A. S. *Biochemistry* **1994**, *33*, 15449−15458.

(42) Vela, M. A.; Fronczek, F. R.; Horn, G. W.; McLaughlin, M. L. *J. Org. Chem.* **1990**, *55*, 2913−2918.

27.2 min; mass spectrum (ESI), *m/z* 1039.1 $(M + H)^+$, theoretical *m/z* 1039.2 $(M + H)^+$.

***N*-(4-Pentenoyl)-*S*-phenylalanyl-pdCpA (2).** Isolated as a colorless solid: yield 1.2 mg (52%); HPLC retention times 24.9 and 25.2 min; mass spectrum (ESI), *m/z* 866.5 $(M + H)^+$, theoretical *m/z* 866.2 $(M + H)^+$.[34]

Di-*N*-(6-Nitroveratryl)oxycarbonyl-4-amino-*S*-phenylalanyl-pdCpA (3). Isolated as a colorless solid: yield 1.0 mg (30%); HPLC retention time 32.8 min; mass spectrum (ESI), *m/z* 1277.3 $(M + H)^+$, theoretical *m/z* 1277.3 $(M + H)^+$.

***N*-(6-Nitroveratryl)oxycarbonyl-4-(*S*)-thiophenylalanyl-pdCpA Butyl Disulfide (4).** Isolated as a colorless solid: yield 1.2 mg (45%); HPLC retention time 34.4 min; mass spectrum (ESI), *m/z* 1143.5 $(M + H)^+$, theoretical *m/z* 1143.2 $(M + H)^+$; HRMS (FAB), *m/z* 1141.2210 $(M - H)^-$ ($C_{42}H_{51}N_{10}O_{20}P_2S_2$ requires 1141.2198).

***N*-(4-Pentenoyl)-4-iodo-*S*-phenylalanyl-pdCpA (5).** Isolated as a colorless solid: yield 1.3 mg (56%); HPLC retention time: 27.2 min; mass spectrum (ESI), *m/z* 992.4 $(M + H)^+$, theoretical *m/z* 992.1 $(M + H)^+$; HRMS (FAB), *m/z* 990.1077 $(M - H)^-$ ($C_{33}H_{39}IN_9O_{15}P_2$ requires 990.1086).

Di-*N*-(4-Pentenoyl)-4-aminomethyl-*S*-phenylalanyl-pdCpA (6). Isolated as a colorless solid: yield 1.0 mg (45%); HPLC retention time 24.0 min; mass spectrum (ESI), *m/z* 977.6 $(M + H)^+$, theoretical *m/z* 977.3 $(M + H)^+$; HRMS (FAB), *m/z* 975.2792 $(M - H)^-$ ($C_{39}H_{49}N_{10}O_{16}P_2$ requires 975.2803).

***N*-(6-Nitroveratryl)oxycarbonyl-*m*-*R*,*S*-tyrosyl-pdCpA (7).** Isolated as a colorless solid: yield 1.1 mg (49%); HPLC retention times 25.8 and 26.2 min; mass spectrum (ESI), *m/z* 1039.4 $(M + H)^+$, theoretical *m/z* 1039.2 $(M + H)^+$.

***N*-(6-Nitroveratryl)oxycarbonyl-*S*-phenylglycyl-pdCpA (8).** Isolated as a colorless solid: yield 0.9 mg (41%); HPLC retention times 25.1 and 25.9 min; mass spectrum (ESI), *m/z* 1025.6 $(M + H)^+$, theoretical *m/z* 1025.2 $(M + H)^+$.

2-(*R*,*S*)-(6-Nitroveratryl)oxycarbonylamino-3-(4-hydroxy-1-naphthyl)propionyl-pdCpA (9). Isolated as a yellow solid: yield 2.1 mg (62%); HPLC retention times 29.1 and 29.6 min; mass spectrum (ESI), *m/z* 1089.5 $(M + H)^+$, theoretical *m/z* 1089.2 $(M + H)^+$; HRMS (FAB), *m/z* 1087.2248 $(M - H)^-$ ($C_{42}H_{45}N_{10}O_{21}P_2$ requires 1087.2236).

2-(*R*,*S*)-(6-Nitroveratryl)oxycarbonylamino-3-(6-hydroxy-2-naphthyl)propionyl-pdCpA (10). Isolated as a yellow solid: yield 2.4 mg (69%); HPLC retention time 28.6 min; mass spectrum (ESI), *m/z* 1089.2 $(M + H)^+$, theoretical *m/z* 1089.2 $(M + H)^+$; HRMS (FAB), *m/z* 1087.2247 $(M - H)^-$ ($C_{42}H_{45}N_{10}O_{21}P_2$ requires 1087.2236).

***N*-(6-Nitroveratryl)oxycarbonyl-3-hydroxy-*S*-tyrosyl-pdCpA (11).** Isolated as a colorless solid: yield 1.4 mg (48%); HPLC retention times 24.5 and 25.3 min; mass spectrum (ESI), *m/z* 1055.2 $(M + H)^+$, theoretical *m/z* 1055.2 $(M + H)^+$.

***N*-(4-Pentenoyl)-3-nitro-*S*-tyrosyl-pdCpA (12).** Isolated as a yellow solid: yield 1.2 mg (57%); HPLC retention time 24.2 min; mass spectrum (ESI), *m/z* 927.3 $(M + H)^+$, theoretical *m/z* 927.2 $(M + H)^+$; HRMS (FAB), *m/z* 927.2063 $(M + H)^+$ ($C_{33}H_{41}N_{10}O_{18}P_2$ requires 927.2076).

***N*-(6-Nitroveratryl)oxycarbonyl-3-fluoro-*R*,*S*-tyrosyl-pdCpA (13).** Isolated as a colorless solid: yield 1.8 mg (55%); HPLC retention time 26.5 min; mass spectrum (ESI), *m/z* 1057.4 $(M + H)^+$, theoretical *m/z* 1057.2 $(M + H)^+$; HRMS (FAB), *m/z* 1055.1995 $(M - H)^-$ ($C_{38}H_{42}FN_{10}O_{21}P_2$ requires 1055.1985).

***N*-(6-Nitroveratryl)oxycarbonyl-3-methoxy-*S*-tyrosyl-pdCpA (14).** Isolated as a colorless solid: yield 1.2 mg (49%); HPLC retention times 27.9 and 28.1 min; mass spectrum (ESI), *m/z* 1069.2 $(M + H)^+$, theoretical, *m/z* 1069.2 $(M + H)^+$; HRMS (FAB), *m/z* 1067.2195 $(M - H)^-$ ($C_{39}H_{45}N_{10}O_{22}P_2$ requires 1067.2185).

Gene Construction for Recombinant Vaccinia Topoisomerase I Containing a Hexahistidine Fusion Peptide. To constuct a plasmid containing a hexahistidine fusion peptide, the DNA fragment containing the gene for vaccinia topoisomerase I was excised from 6 μg of plasmid pET3c-Topo1(wt) with restriction endonucleases *Hin*dIII (80 U) and *Nde*I (80 U) at 37 °C overnight and purified on a 1% low melting agarose gel (50 V at 4 °C, 3 h). The band of interest was excised and digested with GELase (1.5 U) for 1 h. The nucleic acid was concentrated by centrifugation at 14000*g* (YM-50 Microcon centrifugal filter, Millipore).

The vector plasmid (12 μL, conc 0.17 μg/μL), which included the same digestion sites and also contained an N-terminal hexahistidine motif (MGSSHHHHHHSSGLVPRGSH), was digested overnight using endonucleases *Hin*dIII (60 U) and *Nde*I (60 U) at 37 °C. The 5′-end of the linearized vector was dephosphorylated using calf intestinal alkaline phosphatase (10 U) for 1 h at 37 °C, followed by phenol extraction before religation.

The concentrations of vector and insert DNAs were estimated by running a 1% agarose gel against known concentration standards. The vector and insert DNAs (1:6 molar ratio) were ligated with T4 DNA ligase (400 U) at 22 °C for 1 h. After transformation of the ligation product into competent cells of an appropriate host strain (JM109 or BL21), colonies were screened by restriction analysis and protein expression in *E. coli*.

Site-Directed Mutagenesis of Position 274 of Vaccinia Topoisomerase I.[43] The primer for modification of Tyr-274 was CA TCA ATT TCA AAA AGA GCT TAG ATG GCA ACG ACT ATT TTA GAA. This primer (200 pmol) was phosphorylated at the 5′-end with T4 polynucleotide kinase (30 U).

A polymerase chain reaction was carried out in a 50-μL (total volume) reaction mixture containing 50 ng of template plasmid DNA, 14 pmol of primer, 10 nmol of dNTPs, 2.5 U of cloned *Pfu* DNA polymerase, and 20 U of *Taq* DNA ligase in 20 mM Tris-HCl, pH 8.2, 5 mM KCl, 5 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 12.5 mM KOAc, 5 mM $Mg(OAc)_2$, 5 mM DTT, 0.5 mM NAD, 0.1% Triton X-100, and 0.05 mg/mL BSA. The thermal cycle was programmed as follows: preincubation at 65 °C for 5 min, allowing the ligase to repair any nicks in the template; initial denaturation at 95 °C for 2 min; 18 cycles at 95 °C for 30 s, 51 °C for 30 s, and 65 °C for 13 min; postincubation at 75 °C for 7 min. The restriction enzyme *Dpn*I (20 U) was added to the sample, which was incubated at 37 °C for 1 h to eliminate the methylated and hemimethylated wild-type DNA template. Then the sample was denatured at 95 °C for 30 s, followed by two cycles at 95 °C for 30 s, 51 °C for 1 min, and 70 °C for 7 min. Five μL of the final sample was transformed into 50 μL of *E. coli* competent cell line JM109.

Preparation of S-30 Extract from *E. coli* BL21(DE3). A single colony of *E. coli* BL21(DE3) was inoculated in two flasks of 150 mL of LB medium and shaken at 37 °C until the optical density at 600 nm was 0.5. The cells were then diluted with 2.4 L of LB medium containing 0.5 mM IPTG to OD_{600} 0.05 and shaken at 37 °C until the OD_{600} was 1.2. The cells were collected by centrifugation at 6000*g* (5 min, 4 °C). The cell pellet was washed with 420 mL of ice cold 10 mM Tris-OAc, pH 8.2, containing 14 mM $Mg(OAc)_2$, 60 mM KOAc, 1 mM DTT, and 0.5 mL/L β-mercaptoethanol and then centrifuged at 6000*g* (4 °C for 5 min). The washing step was repeated two more times. The cell pellet was then washed once with 60 mL of ice-cold 10 mM Tris-OAc, pH 8.2, containing 14 mM $Mg(OAc)_2$, 60 mM KOAc, 1 mM DTT, and 0.05 mL/L β-mercaptoethanol. The cell pellet (7.3 g) was resuspended in 9.3 mL of 10 mM Tris-OAc, pH 8.2, containing 14 mM $Mg(OAc)_2$, 60 mM KOAc, and 1 mM DTT. S-30 premix (4.9 mL) containing 320 mM Tris-OAc, pH 8.2, 9.4 mM $Mg(OAc)_2$, 13.4 mM ATP, 13.4 mM GTP, 84 mM phosphoenol pyruvate, 4 mM DTT, 0.05 mM of a mixture of 20 amino acids, and 430 U/mL of pyruvate kinase were added. After being mixed gently, 18 μL of pyruvate kinase (319 U), 43 μL of 50 mg/mL lysozyme, 43 μL of 1.0 M $Mg(OAc)_2$, and 532 μL of 0.1 M EGTA, pH 7.5, were added. The cells were lysed by incubation of the mixture at 37 °C for 30 min, then frozen at −80

(43) Sawano, A.; Miyawaki, A. *Nucleic Acids Res.* **2000**, *28*, E78.

°C and thawed at room temperature twice. Finally, 532 μL of 0.1 M $CaCl_2$ was added, and the mixture was stirred gently. After freezing at −80 °C for 30 min, the sample was centrifuged at 14000*g* (30 min, 4 °C). The supernatant was stored at −80 °C as S-30 extract.

In Vitro Transcription of Abbreviated Suppressor tRNA-C_{OH}.[44] Plasmid pYRNA8, encoding the yeast suppressor $tRNA^{Phe}_{CUA}$,[45] was linearized with *Fok*I and then transcribed using an AmpliScribe T7 RNA polymerase transcription kit. A reaction mixture (300 μL total volume) containing 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, and 120 μg of plasmid DNA was digested with 120 units of *Fok*I. The reaction mixture was incubated at 37 °C for 4 h, extracted with phenol-chloroform, and precipitated by treatment with 3 volumes of ethanol.

The transcription reaction was carried out using an Ampliscribe T7 transcription kit in a buffered reaction mixture (800 μL) containing 7.5 mM each of ATP, CTP, and UTP, 5 mM GTP, 20 mM GMP, 10 mM dithiothreitol, 50 μg of linearized DNA template, and 80 μL of the T7 RNA polymerase preparation for 12 h at 42 °C. The elaborated tRNA-C_{OH} transcript was diluted 3-fold using 0.1 M NaOAc (pH 5.2) and applied to a 400-μL column of DEAE-Sepharose CL-6B. The column was washed with 600 μL of 0.1 M NaOAc (pH 5.2) and eluted successively with 600-μL portions of 0.1 M NaOAc, pH 5.2, containing 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, and then 0.9 M NaCl. Each of the 600-μL eluates was precipitated with 900 μL of 2-propanol for 20 min at 0 °C, washed with 70% ethanol, redissolved in 50 μL of RNase-free water, and analyzed by 8% denaturing polyacrylamide gel electrophoresis (50 V, 3 h). The fractions (0.6−0.8 M NaCl) containing $tRNA_{CUA}$-C_{OH} were pooled and stored at −80 °C.

Chemical Misacylation of Suppressor tRNA-C_{OH}. Suppressor tRNA aminoacylation was carried out in 100 μL (total volume) of 100 mM Na HEPES, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 100 μg of suppressor tRNA-C_{OH}, 2.0 A_{260} unit of protected aminoacyl-pdCpA (5−10-fold molar excess), 15% DMSO, and 200 units of T4 RNA ligase. After incubation at 37 °C for 1 h, the reaction was quenched by the addition of 0.1 volume of 3 M NaOAc, pH 5.3, and the aminoacylated tRNA was precipitated with 3 volumes of cold EtOH. The efficiency of ligation was estimated by gel electrophoresis (pH 5.0).[22]

Deprotection of NVOC-protected aminoacyl-tRNAs was carried out at a tRNA concentration of 3 μg/μL. The aminoacyl-tRNAs were cooled to 2 °C and irradiated with a 500 W mercury−xenon lamp using both Pyrex and water filters. Typically, NVOC-protected aminoacyl-tRNAs were deblocked for 5 min. After irradiation, deblocked aminoacylated suppressor tRNAs were used in in vitro suppression experiments.

Pentenoyl-protected aminoacyl-tRNAs were deprotected by treatment with 5 mM aqueous I_2 (30 min, 25 °C).[33] Following deprotection, the solution was centrifuged, and the cleared supernatant was adjusted to 0.3 M NaOAc and treated with 4 volumes of cold EtOH to precipitate the aminoacylated tRNA. The tRNA pellet was washed with 70% aq EtOH and then dissolved in aqueous solution for use in in vitro suppression experiments.

In Vitro Synthesis of Vaccinia Topoisomerase I Analogues.[46] Syntheses of vaccinia topoisomerase I and its analogues were performed using a bacterial S-30 extract from *E. coli* strain BL21(DE3). In a typical experiment, proteins were synthesized in a reaction mixture (100 μL total volume) that contained 7 μg of plasmid DNA, 40 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 11 mM magnesium acetate, 20 mM phospho(enol)pyruvate, 0.8 mg/mL of *E. coli* tRNA, 0.8 mM isopropyl β-D-thiogalactopyranoside, 20 mM ATP and GTP, 5 mM CTP and UTP, and 4 mM cAMP),[47] 100 μM of each of the 20 amino acids, 30 μCi of [^{35}S]-*S*-methionine, 10 μg/μL rifampicin, and 25 μL of S-30 extract. Suppression reaction mixtures contained 25 μg of deprotected misacylated $tRNA_{CUA}$ and were incubated at 37 °C for 60 min. As a control, in vitro translation was also carried out without addition of misacylated $tRNA_{CUA}$. Aliquots (1−2 μL) were taken for analysis by 10% SDS-PAGE.[48] Autoradiography of the gels was carried out to determine the location of ^{35}S-labeled protein; quantification of the bands was carried out using a phosphorimager.

Protein Purification by Ni-NTA Chromatography.[19] The in vitro translation reaction mixture (100 μL) was diluted with 200 μL of 50 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl and mixed gently with 50 μL of a 50% slurry of Ni-NTA resin for 1 h at 4 °C. After centrifugation for 10 s at 14000*g*, the resin pellet was washed twice with 100 μL of 50 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl, and once with 100 μL of 50 mM Tris-HCl, pH 7.5, containing 10% glycerol. The protein was eluted three times with 25 μL of 50 mM Tris-HCl, pH 7.5, containing 150 mM imidazole, 10% glycerol, and 100 μg/mL bovine serum albumin. After elution, 1 mM EDTA, adjusted to pH 8.0, containing 2 mM β-mercaptoethanol and 0.1% Triton X-100 was added to each eluate. Aliquots of each fraction were analyzed by 10% SDS-PAGE.

Quantification of Purified Protein. SDS-PAGE electrophoresis was used for determination of protein concentration. At first, a standard curve was determined using known concentrations of vaccinia topoisomerase I (Coomassie blue stain). The concentration of the in vitro synthesized wild-type topoisomerase I was then determined from the standard curve by comparison of band density. This wild-type in vitro-translated topoisomerase I was then used as a standard to determine the concentrations of the modified vaccinia topoisomerases I synthesized under the same conditions by comparison of the radioactivity of each band in a protein gel by the use of a phosphorimager.

Plasmid Relaxation Assay. A reaction mixture (10 μL total volume) containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM EDTA, 250 ng of supercoiled pSP64 DNA, and topoisomerase I was incubated at 37 °C for 30 min and quenched by addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, 0.125% bromophenol blue). The reaction mixture was analyzed by 1% agarose gel electrophoresis. After electrophoresis in 40 mM Tris-acetate, pH 8.0, containing 2 mM EDTA, the gel was stained with ethidium bromide and then rerun briefly to separate the relaxed DNA (form IV) and nicked DNA (form II).[49] Gels were visualized using UV light, and the reaction extent (expressed as the percentage of DNA plasmid relaxed by vaccinia topoisomerase I) was quantified by utilizing ImageQuant version 5.2 software.

DNA Relaxation Kinetics by Vaccinia Topoisomerase I. Supercoiled DNA plasmid (250 ng) was treated with 0.1 ng of vaccinia topoisomerase I in a 10-μL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 2.5 mM EDTA. The reaction mixture was incubated at 37 °C and quenched at predetermined times by the addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, and 0.125% bromophenol blue). The reaction mixture was analyzed by 1% agarose gel electrophoresis. After electrophoresis in 40 mM Tris-acetate, pH 8.0, containing 2 mM EDTA, the gel was stained with ethidium bromide, and then rerun briefly to separate the relaxed DNA (form IV) and nicked DNA (form II).[49] Gels were visualized using UV light, and the reaction extent (expressed as the percentage of DNA plasmid relaxed by vaccinia topoisomerase I) was quantified by utilizing ImageQuant version 5.2 software. The observed rate constants (k_{obs}) were determined by fitting the equation [100-% reaction extent] = 100 exp($-kt$).

Assay for pH Dependence of DNA Relaxation. Relaxation assays were performed at different pH values using the following buffers:

(44) Nazarenko, I. A.; Harrington, K. M.; Uhlenbeck, O. C. *Embo J.* **1994**, *13*, 2464−2471.
(45) Robertson, S. A.; Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Nucleic Acids Res.* **1989**, *17*, 9649−9660.
(46) Lesley, S. A.; Brow, M. A.; Burgess, R. R. *J. Biol. Chem.* **1991**, *266*, 2632−2638.
(47) Pratt, J. M. *Transcription and Translation: A Practical Approach*; IRL Press: Oxford, 1984; pp 179−209.
(48) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.
(49) Interthal, H.; Quigley, P. M.; Hol, W. G.; Champoux, J. J. *J. Biol. Chem.* **2004**, *279*, 2984−2992.

sodium acetate, pH 4.0−5.0; NaMES, pH 5.5−6.5; Tris-HCl, pH 7.0−9.0; NaCAPS, pH 9.5−11.0. A reaction mixture (10 μL total volume) containing 50 mM buffer, 100 mM NaCl, 2.5 mM EDTA, 250 ng of supercoiled pSP64 DNA, and topoisomerase I (0.045−0.18 ng) was incubated at 37 °C for 30 min and quenched by addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, and 0.125% bromophenol blue). The reaction mixture was analyzed by 1% agarose gel electrophoresis. After electrophoresis in 40 mM Tris-acetate, pH 8.0, containing 2 mM EDTA, the gel was stained with ethidium bromide, then rerun briefly to separate the relaxed DNA (form IV) and nicked DNA (form II).[49] Gels were visualized under UV light, and the reaction extent (expressed as the percentage of DNA plasmid relaxed by vaccinia topoisomerase I) was quantified by utilizing ImageQuant version 5.2 software.

Acknowledgment. We thank Dr. Stewart Shuman for the vaccinia topoisomerase I gene used for this study. We thank Dr. Chunhong Li and Dr. Mikell Paige for helpful discussions during the course of this work. This study was supported by NIH Research Grant CA78415, awarded by the National Cancer Institute.

JA044182Z