5360–5365 Nucleic Acids Research, 1994, Vol. 22, No. 24

Requirements for noncovalent binding of vaccinia topoisomerase I to duplex DNA

JoAnn Sekiguchi and Stewart Shuman*
Molecular Biology Program, Sloan-Kettering Institute, New York, NY 10021, USA

Received August 12, 1994; Revised and Accepted November 3, 1994

ABSTRACT

Vaccinia DNA topoisomerase binds duplex DNA and forms a covalent adduct at sites containing a conserved sequence element 5′(C/T)CCTT↓ in the scissile strand. Distinctive aspects of noncovalent versus covalent interaction emerge from analysis of the binding properties of Topo(Phe-274), a mutated protein which is unable to cleave DNA, but which binds DNA noncovalently. Whereas DNA cleavage by wild type enzyme is most efficient with 'suicide' substrates containing fewer than 10 base pairs distal to the scissile bond, optimal noncovalent binding by Topo(Phe-274) requires at least 10-bp of DNA 3′ of the cleavage site. Thus, the region of DNA flanking the pentamer motif serves to stabilize the noncovalent topoisomerase–DNA complex. This result is consistent with the downstream dimensions of the DNA binding site deduced from nuclease footprinting. Topo(Phe-274) binds to duplex DNA lacking the consensus pentamer with 7–10-fold lower affinity than to CCCTT-containing DNA.

INTRODUCTION

Vaccinia DNA topoisomerase I binds duplex DNA and forms a covalent adduct at specific sites containing a pentapyrimidine sequence 5′-(C/T)CCTT↓ in the scissile strand (1). The T↓ residue (designated position +1) is linked via a 3′ phosphodiester bond to Tyr-274 of the enzyme (2). Synthetic model substrates have been used to examine the molecular interactions that contribute to specific cleavage at the CCCTT motif (3–9). 'Suicide' substrates that are optimal for detection of strand cleavage typically contain six or fewer base pairs 3′ of the scissile bond. For these DNAs, transesterification leads to spontaneous dissociation of the 3′ fragment of the cleaved strand from the protein–DNA complex. With no readily available acceptor for religation, the enzyme is covalently trapped on the DNA.

Nuclease footprinting, modification interference, modification protection, and analog substitution experiments suggest that the topoisomerase makes contact with specific base pairs and with the sugar–phosphate backbone within the CCCTT motif (4,6,9). However, the points of contact are not confined to the pentamer element (4,6). For example, the DNase I footprint (22–26 bp) spans both sides of the cleavage site, from +13 to −13 on the scissile strand and from +13 to −9 on the noncleaved strand. The margins of the footprint extend beyond the minimal essential positions for strand cleavage (from +6 to −2) defined by DNA deletion and site-phasing experiments (4). The footprinting experiments, performed using CCCTT-containing model duplexes 60–70 bp in length, are reflective of site occupancy under equilibrium conditions in which noncovalent topoisomerase binding predominates strongly over covalent adduct formation (1,7). It has therefore been inferred that the DNA functional groups defined by the interference or protection assays are important for pre-cleavage binding by topoisomerase to the CCCTT target (independent of, or in addition to, a requirement for these groups in strand cleavage).

In the present study, we examine the contributions of DNA length and of the CCCTT element to the noncovalent binding of vaccinia topoisomerase to duplex DNA. To exclude the contributions of covalent binding to site affinity, we focus on the DNA binding properties of Topo(Phe-274), an active site mutant protein which is unable to cleave DNA, but which binds DNA noncovalently (2,4). We find that Topo(Phe-274) does not form a stable complex with minimal substrates that are readily cleaved by the wild type enzyme. The DNA dimensions for effective noncovalent binding (extending from +10 to −10) agree with previous footprinting studies. Apparently, the flanking DNA regions, while not essential for transesterification, do stabilize the noncovalent topoisomerase–DNA complex. Binding of Topo(Phe-274) to synthetic DNAs lacking the CCCTT motif also occurs, albeit with lower affinity. These data suggest that topoisomerase may initially bind to duplex DNA nonspecifically (e.g., through sugar–phosphate backbone contacts) and subsequently locate its CCCTT recognition motif by a facilitated diffusion mechanism.

EXPERIMENTAL PROCEDURES

Enzyme purification

Vaccinia DNA topoisomerase was expressed in *Escherichia coli* as described (10). Purification from soluble extracts was achieved by sequential phosphocellulose and SP5PW column

*To whom correspondence should be addressed

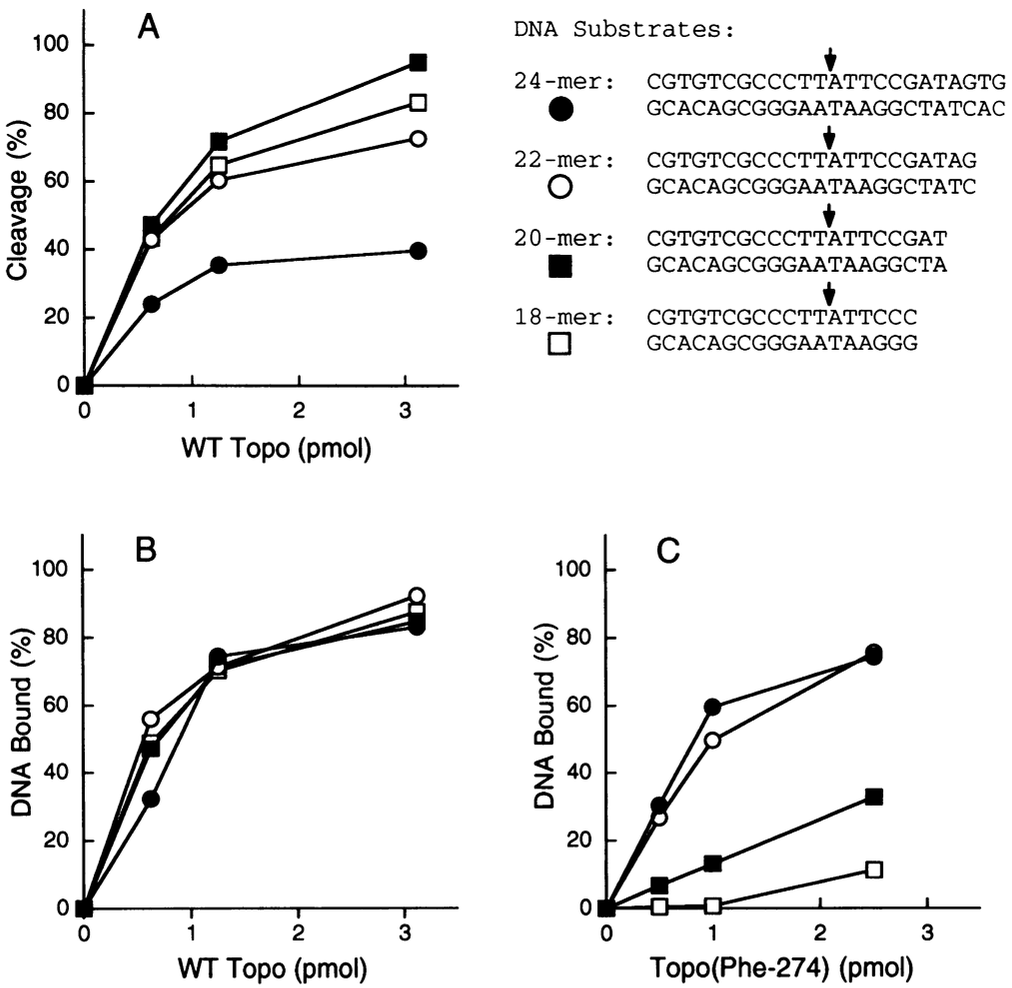

Figure 1. Effect of downstream DNA length on covalent and noncovalent DNA binding. The structures of the duplex DNA substrates used in this experiment are shown. Each substrate was 5′ radiolabeled in the scissile DNA strand. The site of strand cleavage at the CCCTT element is indicated by the arrow. The symbols for each substrate in graphs A–C are shown. (**A**) Covalent adduct formation by wild type topoisomerase (WT) was assayed as described under Experimental Procedures. The extent of DNA cleavage (as percent of the input substrate) is plotted as a function of input protein. (**B**) Total DNA binding was assayed by native gel mobility shift assay as described under Experimental Procedures and in the legend to Fig. 2. The data in Fig. 2A were quantitated by scintillation counting of gel slices containing the bound and free DNA species from each reaction. (**C**) The binding of each substrate to Topo(Phe-274) was determined from the native gel shift experiment shown in Fig. 2B.

chromatography steps (8). Expression and purification of the Phe-274 mutant protein was identical to that of the wild type protein. Both preparations appeared homogeneous with respect to the 33 kDa topoisomerase polypeptide, based on SDS–PAGE separation and polypeptide visualization by staining with Coomassie blue (not shown). Protein concentration was determined using the Biorad dye reagent, taking bovine serum albumin as the standard.

Oligonucleotide substrates

Synthesis of DNA oligonucleotides via DMT-cyanoethyl phosphoramidite chemistry was performed by the Sloan-Kettering Microchemistry Laboratory using an Applied Biosystems model 394 automated DNA synthesizer according to protocols specified by the manufacturer. Standard deoxynucleoside phosphoramidites were purchased from Applied Biosystems Inc. Synthetic DNA oligonucleotides were labeled at the 5′ end via enzymatic phosphorylation in the presence of [γ^{32}P]ATP and T4 polynucleotide kinase. The labeled oligonucleotide was gel-purified and hybridized to complementary strand (present at 4-fold molar excess) as described (3,4).

Assay of covalent complex formation

Reaction mixtures (20 μl) containing 50 mM Tris HCl, pH 7.5, 5′ ^{32}P-labeled DNA substrate (0.5 pmol), and vaccinia topoisomerase were incubated at 37°C for 5 min. Covalent complexes were trapped by addition of SDS to 1%. Samples were heated for 2 min at 95°C and then electrophoresed though a 10% polyacrylamide gel containing 0.1% SDS. Free DNA migrated with the bromophenol blue dye front. Covalent complex formation was revealed by transfer of radiolabeled DNA to the topoisomerase polypeptide as detected by autoradiographic exposure of the dried gel. The extent of adduct formation was quantitated by scintillation counting of an excised gel slice containing the labeled protein and was expressed as the percent of the input 5′ ^{32}P-labeled oligonucleotide that was covalently transferred to protein.

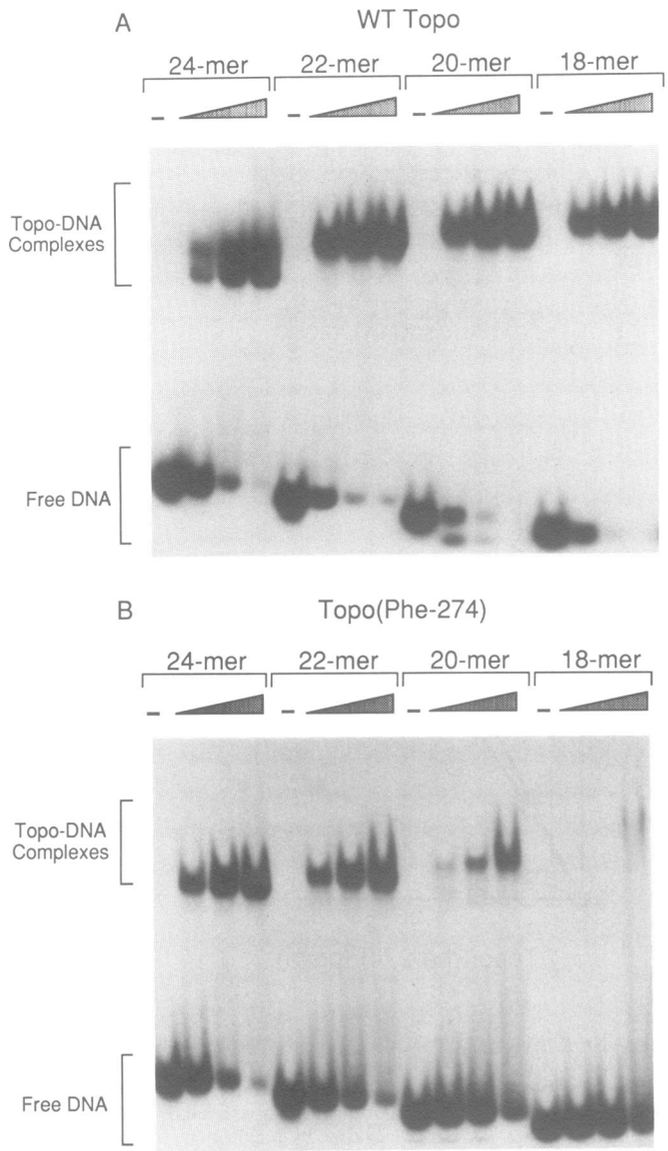

Figure 2. Assay of DNA binding by native gel electrophoresis. The binding of wild type topoisomerase (Panel **A**) and Topo(Phe-274) (Panel **B**) to DNA substrates differing in the length of their 3′ segments was assayed by native gel electrophoresis as described under Experimental Procedures. Autoradiographs of the gels are shown. The substrates used in each topoisomerase titration series (demarcated by brackets above the lanes) are indicated according to DNA size. The structures of the substrates are shown in Fig. 1. (Panel A) The reactions mixtures in each series included, proceeding from left to right, 0.6, 1.2, and 3.1 pmol of wild type topoisomerase. (Panel B) reaction mixtures in each series included, proceeding from left to right, 0.5, 1, and 2 pmol of Topo(Phe-274). Control reactions lacking topoisomerase are indicated by '−'. The positions of the free DNA substrates and topoisomerase−DNA complexes are indicated at the left.

Assay of DNA binding by native gel electrophoresis

Binding reaction mixtures (20 μl) containing 50 mM Tris HCl, pH 7.5, 0.5 pmol of 5′ ^{32}P-labeled DNA, and topoisomerase were incubated for 5 min at 37°C. Reactions were adjusted to 10% glycerol and then electrophoresed through a native 6% polyacrylamide gel containing 0.25× TBE (22.5 mM Tris borate, 0.6 mM EDTA) at 100 V for 3 h. Topoisomerase−DNA complexes of retarded electrophoretic mobility were visualized by autoradiographic exposure of the dried gel. The extent of protein−DNA complex formation was quantitated by scintillation counting of excised gel slices containing bound and free DNA from each reaction.

RESULTS

Requirements for noncovalent binding of topoisomerase to DNA

A role for the DNA region 3′ of the scissile bond in stabilizing the noncovalent topoisomerase−DNA complex was suggested by comparison of the binding properties of the wild type and Topo(Phe-274) proteins to duplex DNAs containing the same 12-bp sequence, 5′-CGTGTCGCCCTT, upstream of the scissile bond, but having 3′ segments of varying length (6, 8, 10, or 12-bp) (Fig. 1). The extent of strand cleavage by wild type topoisomerase increased as a function of enzyme concentration for all substrates. However the yield of covalent complex at saturation depended acutely on the length of the distal segment (Fig. 1A). The 24-mer substrate containing 12 downstream base pairs was cleaved to the extent of 38% of the input DNA. A nearly two-fold increase in the yield of covalent adduct occurred upon 3′ deletion of two base pairs (Fig. 1A, 22-mer). The yield of cleaved DNA increased further with more extensive 3′ deletions (Fig. 1A, 20-mer and 18-mer). The yield of covalent adduct in the case of the 18-mer was 95% of the input DNA.

Total binding of topoisomerase to DNA by the wild type protein (covalent plus noncovalent complexes, assessed by native gel electrophoresis) displayed no such variation as a function of 3′ segment length (Fig. 1B and Fig. 2A). All substrates were bound by topoisomerase with nearly the same affinity and to the same extent at saturation (Fig. 1B). The behavior of the topoisomerase−DNA complexes during native gel electrophoresis confirmed that the equilibrium of the cleavage reaction was influenced profoundly by 3′ segment length. As shown previously (8), two discrete species were formed upon topoisomerase binding to a 24-bp duplex (Fig. 2A). The predominant and more rapidly migrating species contained topoisomerase bound to the 24-mer duplex, whereas the upper complex contained enzyme bound covalently to a tailed DNA cleavage product. The upper complex arises via spontaneous dissociation of the 3′ segment of the scissile strand (8). The 3′ deletion of 2 base pairs to generate the 22-mer duplex reduced the binding reaction product to a single species, a trend that persisted for the 20-mer and the 18-mer substrates (Fig. 2A). Thus, as 3′ segment length was shortened to 10-bp or less, the 3′ strand dissociated more readily and drove the reaction equilibrium toward covalent adduct formation. Note that the mobility of the topoisomerase−DNA complex in the native gel actually decreased as the amount of DNA in the complex was reduced (in contrast to the free DNAs, which migrated more rapidly when length was decreased) (Fig. 2A). This suggested that net negative charge, rather than mass, is a dominant factor in the electrophoretic behavior of the protein−DNA complex.

Parallel analysis of the binding of Topo(Phe274) (which was exclusively noncovalent) showed that the formation of a stable complex, i.e. one that could be resolved by native gel electrophoresis, was very much dependent on the length of the 3′ region. Topo(Phe-274) bound with similar affinity to the 24-bp and 22-bp duplexes, yielding a single species after native gel electrophoresis (Fig. 2B). A clear decrement in binding (2.5-fold) was noted for the 20-mer substrate (Figs. 1C and 2B). Almost no binding of Topo(Phe-274) to an 18-mer duplex was observed

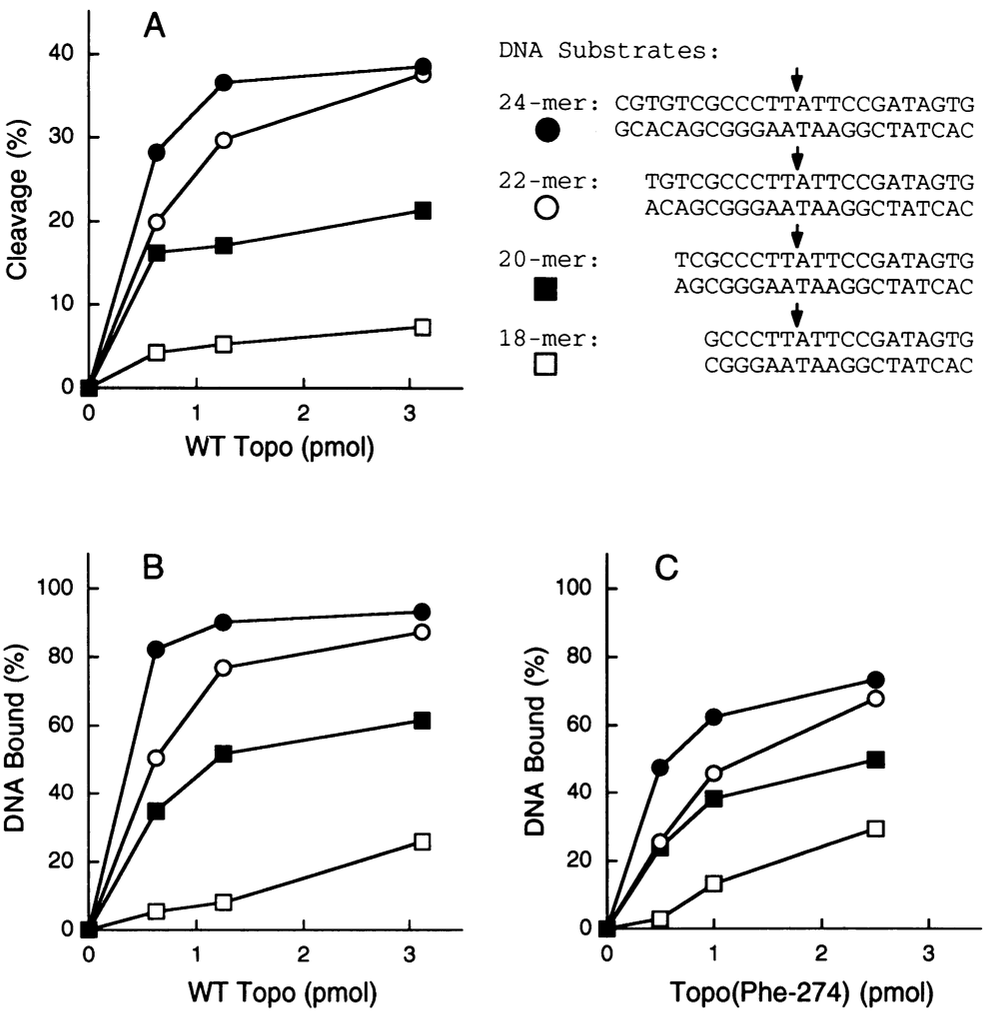

Figure 3. Effect of upstream DNA length on covalent and noncovalent DNA binding. The structures of the duplex DNA substrates used in this experiment are shown. Each substrate was 5′ radiolabeled in the scissile DNA strand. The site of strand cleavage at the CCCTT element is indicated by the arrow. The symbols for each substrate in graphs A–C are indicated. (**A**) The extent of DNA cleavage (as percent of the input substrate) by wild type topoisomerase is plotted as a function of protein added. Total DNA binding by wild type protein (**B**) and Topo(Phe-274) (**C**) was determined by native gel mobility shift assay and is plotted as a function of added protein.

at levels of input protein sufficient to bind half of the 22-mer. Only at much higher concentrations of protein could binding to the 18-mer be detected (Figs. 1C and 2B). Noncovalent binding of vaccinia topoisomerase was clearly stabilized by the region of duplex DNA downstream of the cleavage site, with the optimal substrate containing at least 10 bp of 3′ DNA.

A similar approach was taken to define the role of the proximal DNA region in covalent and noncovalent binding. In this case, the substrates contained the same 12-bp element downstream of the cleavage site, but differed with respect to the length of the 5′ portion of the duplex (either 12, 10, 8, or 6 bp). All DNAs included the CCCTT recognition motif (Fig. 3A). The extent of strand cleavage at saturating levels of wild type topoisomerase depended on the length of the proximal region. A two-fold decrease accompanied shortening of the 5′ segment from 10-bp to 8-bp, with another 3-fold decrement upon further deletion to 6 proximal base pairs (Fig. 3A). The effect of duplex length on cleavage mirrored the effect on total DNA binding (Fig. 3B). Two species were resolved by native gel electrophoresis for all substrates in this series; the relative amount of the tailed covalent complex *vs.* the more rapidly migrating species was essentially the same in each case (not shown). Calculations of the cleavage equilibrium constants for the 5′ deletion series (by the equation Kcl = Covalent Binding/[Total Binding − Covalent Binding]) showed Kcl to be relatively independent of the proximal segment length. Thus, the effect of 5′ deletions on the wild type protein was likely at the level of noncovalent protein–DNA interaction.

This was confirmed by examining the binding of these substrates by Topo(Phe-274) (Fig. 3C). Noncovalent binding was reduced in a step-wise fashion for each 2-bp deletion. Thus, the region upstream of the CCCTT recognition motif, though not required absolutely, does serve to enhance the noncovalent interaction of topoisomerase with DNA.

Specific vs. nonspecific DNA binding by topo(Phe-274)

Previous studies showed that the binding of wild type topoisomerase to the labeled 24-mer duplex used above was competed much more effectively by an unlabeled CCC-

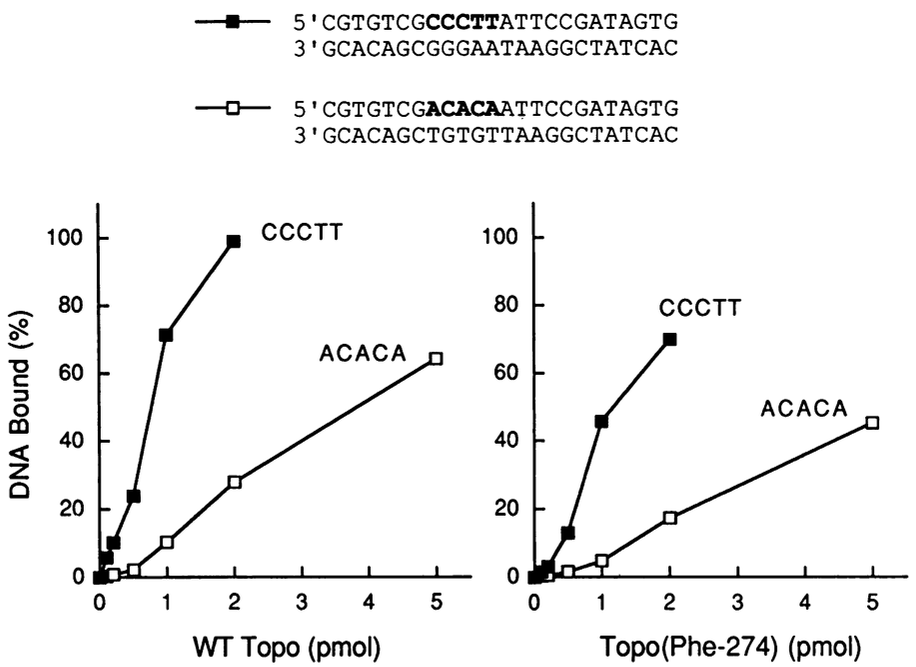

Figure 4. Specific vs. nonspecific DNA binding. The structures of the specific (CCCTT-containing) and non-specific (ACACA-containing) 24-bp substrates are shown. Binding reaction mixtures contained 1 pmol of DNA (5′ radiolabeled in the CCCTT-containing or ACACA-containing strands) and the indicated amounts of wild type or Phe-274 mutant proteins. DNA binding was assayed by native gel mobility shift.

TT-containing duplex (containing only 6-bp 3′ of the scissile bond) than by an otherwise identical duplex containing a mutated CCATT element (8). In such experiments, the CCCTT competitor functions as a suicide cleavage substrate and irreversibly traps any topoisomerase to which it binds. Because the CCATT duplex was not an effective cleavage substrate (3), it necessarily was less effective as a competitor in this assay. Now, knowing that noncovalent binding is stabilized by flanking sequences, we addressed the contribution of the CCCTT motif to the protein–DNA interaction by directly comparing topoisomerase binding to a 24-bp CCCTT-containing substrate to that of a 24-mer containing the sequence ACACA in lieu of CCCTT. The two substrates had identical flanking sequences (Fig. 4). Control experiments confirmed that the ACACA-containing duplex DNA was incapable of forming a covalent complex with wild type topoisomerase (not shown). Binding of either wild type protein or Topo(Phe-274) to the CCCTT-containing 24-mer (assayed by native gel electrophoresis) was 7 to 10-fold more efficient than to the substrate lacking a pentamer cleavage motif (Fig. 4). The magnitude of discrimination in overall binding between sites containing CCCTT *vs.* those lacking CCCTT was clearly less than that observed for strand cleavage, where specificity for CCCTT on duplex DNA, based on the mapping of cleavage sites, was akin to that of a restriction endonuclease (1). These results suggest that vaccinia topoisomerase may bind duplex DNA without strict sequence specificity and then locate the pentamer cleavage sequence by linear diffusion.

DISCUSSION

Among the several eukaryotic type I topoisomerases that have been studied in detail, the vaccinia enzyme displays the highest degree of sequence specificity in strand cleavage. The ability to express and purify substantial amounts of wild type and mutated versions of the topoisomerase, and to study individual partial reactions using defined DNA substrates, has made the vaccinia protein an attractive model for structural and functional analysis of the eukaryotic enzyme family. The present study of DNA binding by Topo(Phe-274) expands our understanding of the noncovalent binding interaction between the topoisomerase and and its DNA target.

Comparisons of covalent and noncovalent binding to 5′ and 3′ deleted duplex DNAs containing the CCCTT motif indicate that noncovalent binding is stabilized by flanking sequences on both sides of the pentamer motif. This is in keeping with DNase I and DMS footprinting studies demonstrating contacts between topoisomerase and the flanking regions (4,6). Flanking sequence interactions may explain the finding that pentapyrimidine consensus sites within plasmid DNA are cleaved by vaccinia topoisomerase with widely different affinities (up to 10-fold) (1). Because there is no apparent sequence conservation or nucleotide bias at positions outside the pentamer at these sites in plasmid DNA, we infer that flanking sequence context can exert a significant influence on overall site strength. The rules governing flanking sequence effects remain obscure.

The strong incremental effects of 3′ segment length in enhancing noncovalent binding suggest that the topoisomerase makes contact with the DNA duplex at a distance from the pentamer element of about one helical turn (i.e. at 10–12 bp 3′ of the scissile bond). Weak contacts with nucleotide bases of the nonscissile strand have been demonstrated in the intervening region at positions −1 and −4 (6,9). It is unlikely that the enzyme makes strong contacts with the scissile strand in the region immediately 3′ of the cleavage site, given the ready dissociation of the distal 'leaving group' oligonucleotide from the minimal

cleavage substrates containing 10 or fewer downstream base pairs. One implication of these findings is that the enzyme may control the rate of topoisomerization during relaxation of supercoiled DNA by alternately binding and releasing the downstream segment of the bound DNA molecule in synchrony with cycles of cleavage and religation.

Interaction of topoisomerase with the DNA segment downstream of the site of strand cleavage has also been suggested by studies of the cellular type I enzyme (11,12). However, the relative contributions of this interaction to noncovalent vs. covalent binding would appear to differ for the cellular and viral enzymes. Initial conclusions regarding the 'minimal' DNA duplex requirements for suicide cleavage by cellular topoisomerase I (13) have been revised, with the end result now being that covalent adduct formation requires a duplex segment of DNA extending 11-bp downstream of the site of strand scission (11). The downstream sequence requirement for DNA cleavage by human topoisomerase I reported by Westergaard's group is at variance with the earlier report by Champoux *et al.* (14) that 6-bp of 3′ duplex DNA was sufficent for cleavage of a fully duplex 22-bp substrate by wheat germ topoisomerase. Whether the minimal size requirement differs for cellular topoisomerases purified from different species, or may vary or according to the DNA site used to assay cleavage, remains an open question. Nevertheless, the downstream requirement for 11-bp for cleavage by human topoisomerase is not shared by the vaccinia topoisomerase, which readily cleaves fully duplex substrates with as few as 2-bp 3′ of the cleavage site (4). Rather, as shown herein, the influence of the downstream region on the vaccinia enzyme is on pre-cleavage binding. Assessment of the influence of DNA duplex length on noncovalent binding by the cellular topoisomerase has not (to our knowledge) been reported.

The finding that vaccinia Topo(Phe-274) can bind to DNA lacking a pentamer element, albeit with lower affinity than to a pentamer-containing DNA, raises the question of how the enzyme accesses its target site for strand scission. Two possibilities are: (i) topoisomerase binds directly to the CCCTT motif (or related congeners), or (ii) topoisomerase binds initially to duplex DNA without sequence specificity (e.g., through interaction with the DNA backbone) and then diffuses linearly until it encounters a consensus pentamer. The importance of nonspecific DNA protein interactions and facilitated diffusion during site-specific strand scission has been described in a series of elegant studies of the *Eco*RI endonuclease by Modrich and colleagues (15–17). Given that the vaccinia topoisomerase can indeed bind nonspecifically, and because potential nonspecific binding sites in natural DNAs (including vaccinia genomic DNA) vastly outnumber specific pentapyrimidine motifs, we suspect that the facilitated diffusion mechanism is the major route by which the enzyme finds its target. It is worth noting that at low ionic strength, DNA relaxation by eukaryotic type I topoisomerases is processive because enzyme does not dissociate readily from the DNA molecule to which it is initially bound (18). Product release has been determined to be the rate-limited step during catalytic cleavage/religation of linear substrates by the vaccinia topoisomerase (7). Thus, under processive reaction conditions, an enzyme molecule could either remain at the same target site and catalyze multiple cycles of transesterification or diffuse from one cleavage site to another on the same DNA molecule without actually dissociating into solution.

ACKNOWLEDGEMENT

This work was supported by Grant GM46330 from the National Institutes of Health.

REFERENCES

1. Shuman, S. and Prescott, J. (1990) *J. Biol. Chem.* **265**, 17826–17836.
2. Shuman, S., Kane, E.M. and Morham, S.G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9793–9797.
3. Shuman, S. (1991) *J. Biol. Chem.* **266**, 1796–1803.
4. Shuman, S. (1991) *J. Biol. Chem.* **266**, 11372–11379.
5. Shuman, S. (1992) *J. Biol. Chem.* **267**, 8620–8627.
6. Shuman, S. and Turner, J. (1993) *J. Biol. Chem.* **268**, 18943–18950.
7. Stivers, J.T., Shuman, S. and Mildvan, A.S. (1994) *Biochemistry* **33**, 327–339.
8. Morham, S.G. and Shuman, S. (1992) *J. Biol. Chem.* **267**, 15984–15992.
9. Sekiguchi, J., and Shuman, S. (1995) *J. Biol. Chem.* (in press).
10. Shuman, S., Golder, M. and Moss, B. (1988) *J. Biol. Chem.* **263**, 16401–16407.
11. Christiansen, K., Svejstrup, A.B.D., Andersen, A.H. and Westergaard, O. (1993) *J. Biol. Chem.* **268**, 9690–9701.
12. Christiansen, K., and Westergaard, O. (1994) *J. Biol. Chem.* **269**, 721–729.
13. Svejstrup, J.Q., Christiansen, K., Andersen, A.H., Lund, K., and Westergaard, O. (1990) J. *Biol. Chem.* **265**, 12529–12535.
14. Champoux, J.J., McCoubrey, W.K., and Been, M.D. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49**, 435–442.
15. Jack, W.E., Terry, B.J. and Modrich, P. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4010–4014.
16. Terry, B.J., Jack, W.E., Rubin, R.A. and Modrich, P. (1983) *J. Biol. Chem.* **258**, 9820–9825.
17. Terry, B.J., Jack, W.E., and Modrich, P. (1985) *J. Biol. Chem.* **260**, 13130–13137.
18. McConaughy, B.L., Young, L.S. and Champoux, J.J. (1981) *Biochim. Biophys. Acta* **655**, 1–8.