Site-specific Interaction of Vaccinia Virus Topoisomerase I with Base and Sugar Moieties in Duplex DNA*

(Received for publication, February 23, 1993, and in revised form, May 10, 1993)

Stewart Shuman† and Jennifer Turner
From the Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021

Vaccinia DNA topoisomerase specifically binds and forms a covalent adduct at DNA sites containing a conserved sequence element 5'CT(C/T)CCCTT1 in the scissile strand. The molecular interactions that contribute to recognition of the CCCTT motif in a synthetic DNA substrate have been examined using modification interference, modification protection, and analog substitution techniques. We report that topoisomerase makes contact with guanine nucleotide bases of the pentamer motif complementary strand (3'GGGAA) within the major groove of the DNA helix and that alteration of the binding surface by chemical modification is deleterious to the interaction. Additional contacts are made with guanine residues located outside the pentamer element. The enzyme is unable to form a covalent adduct with synthetic RNA substrates. Analysis of the cleavage of DNA duplexes containing 2'OMe sugars suggests that the inability of the vaccinia topoisomerase to cleave either an RNA duplex or an RNA:DNA hybrid can be accounted for by the interfering effects of a 2' sugar substituent at two or more sites within the pentamer. Interaction with the sugar at the +2T nucleotide appears to be the most critical, as judged by the effects of single sugar substitutions.

A requirement for covalent complex formation between vaccinia topoisomerase and DNA is that the CCCTT sequence be in duplex form (4). Single nucleotide substitutions within the CCCTT motif profoundly affect cleavage efficiency (5). A hierarchy of mutational effects is observed depending on the nature of the base alteration and the position within the CCCTT sequence. The +2 T:A base pair appears to be the most critical position, insofar as any base change (either on the scissile or the nonscissile strand) virtually abrogates the cleavage reaction. At other positions, pyrimidine-to-purine or purine-to-purine substitutions can be tolerated to varying degrees, whereas pyrimidine-to-purine alterations (and vice versa) are generally inimical to the cleavage reaction (5).

The vaccinia topoisomerase, upon binding to a duplex DNA containing a CCCTT motif, specifically protects the region around the site of covalent adduct formation from DNase I digestion (5). The DNase footprint spans both sides of the cleavage site, from +13 to −13 on the scissile strand (+1 being the site of cleavage) and from +13 to −9 on the noncleaved strand. The size of the footprint is greater than the 11 bp of duplex DNA that constitute the "minimal" substrate for covalent adduct formation (5). Also, the margins of the nuclease footprint extend beyond the minimal essential positions for strand cleavage (from +6 to −2) defined by DNA deletion and site phasing experiments (5).

Although the DNA mutational analyses and nuclease footprinting experiments confirm the sequence specificity of the topoisomerase-DNA interaction, they do not illuminate the pertinent principles of site recognition. This issue is addressed in the present study using a combination of modification interference, modification protection, and analog substitution techniques. The results demonstrate that vaccinia topoisomerase binds to the major groove of the DNA helix, making critical contacts with its recognition element at G residues on the noncleavable strand. Additional contacts are made with purine residues outside the pentamer element. Pentose analog substitution effects suggest that the enzyme also recognizes the sugar moiety of the polynucleotide chain and provide an explanation for the strict specificity of the vaccinia enzyme in its cleavage of DNA, but not RNA chains.

EXPERIMENTAL PROCEDURES

Enzyme Purification—Vaccinia DNA topoisomerase was expressed in Escherichia coli and purified as described (6). The heparin-agarose enzyme fraction used in the present study was the same preparation described previously (6).

Oligonucleotide Substrates—Synthesis of DNA and RNA oligonucleotides via Dimethoxytrityl-cyanoethyl phosphoramidite chemistry was performed by the Sloan-Kettering Microchemistry Laboratory using an Applied Biosystems model 380B or model 394 automated DNA synthesizer according to protocols specified by the manufac-

* This work was supported by National Institutes of Health Grant GM46330. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Recipient of a Pew scholarship in the biomedical sciences supported by the Pew Charitable Trusts. To whom correspondence should be addressed: Molecular Biology Program, Sloan-Kettering Institute, 1275 York Ave., New York, NY 10021. Tel.: 212-639-7143; Fax: 212-717-3623.

1 The abbreviation used is: bp, base pair(s).
DNA Cleavage by Topoisomerase I

The DNA was 5' radiolabeled on either the scissile or non-scissile strand. Control experiments established that topoisomerase-mediated cleavage was confined to the CCCTT-containing strand (data not shown). The 60-mer was methylated on purine residues by treatment with dimethyl sulfate. Methylated DNA was mixed with purified topoisomerase under standard binding reaction conditions described previously (4, 5, 7); the reactions were constituted such that about half of the input DNA was bound by the enzyme. Topoisomerase-DNA complexes were then separated from unbound 32P-labeled DNA by native gel electrophoresis (7). Bound and unbond DNA species were recovered from the gel, and the DNA was cleaved at methylated G residues via treatment with piperidine. Cleavage products were then analyzed by denaturing gel electrophoresis (Fig. 1).

N-7 methylation of any of the guanine bases within the 3'GGGAAA pentamer on the nonscissile strand strongly interfered with protein-DNA complex formation; this was evinced by the nearly complete exclusion, from the bound DNA fraction, of DNAs with piperidine-cleaveable sites corresponding to G positions +3, +4, and +5 (Fig. 1, left panel, lane B). DNAs methylated at these three sites were represented prominently in the population of unbound DNAs (lane F). Thus, integrity of each of the Gs within the pentamer motif was a key determinant of DNA binding. In contrast, methylation at the nearby +7G residue (situated outside the conserved motif) appeared not to interfere with complex formation (this was more obvious on longer autoradiographic exposure of the gel in Fig. 1). A partial effect of methylation was noted at the –4G position of the noncissile strand, as this cleavage site was underrepresented in the bound DNA population (Fig. 1, left panel, and other data not shown).

Methylation of two G residues of the scissile strand (+6G and +9G, located 5' of the pentamer element) interfered partially with protein-DNA complex formation (Fig. 1, right panel, and other experiments not shown). Note that a cleavage product of the radiolabeled scissile strand was detected in the protein-bound DNA fraction, but not in the free DNA population, which did not correspond to a G position in the scissile strand sequence (Fig. 1, right panel, denoted by the filled circle). Rather, this product was generated by strand scission at the topoisomerase +1 cleavage site. We suspect that the phosphotyrosyl DNA-protein intermediate was partially susceptible to hydrolysis during treatment with piperidine at 90°C.

**Methylation Protection**—Intimate contacts between vaccinia topoisomerase and guanine bases within the 60-bp DNA were identified on the basis of protection from base-specific chemical modification. Preformed protein-DNA complexes were reacted in solution with dimethyl sulfate, and the bound DNAs were resolved preparatively from unbound species by native gel electrophoresis. Control experiments established that dimethyl sulfate treatment did not affect the stability of the protein-DNA complex (data not shown). Comparison of piperidine cleavage products from the bound and free DNA populations indicated that the all three G residues (+3, +4, and +5) within the pentamer element on the noncissile strand were protected strongly from chemical modification when bound to the enzyme (Fig. 2, left panel). Partial protection was afforded at –4 and –5 G of the noncleavable strand. The –5G cleavage band migrated quite diffusely in lane B of

---

The DNA was 5' radiolabeled on either the scissile or non-scissile strand. Control experiments established that topoisomerase-mediated cleavage was confined to the CCCTT-containing strand (data not shown). The 60-mer was methylated on purine residues by treatment with dimethyl sulfate. Methylated DNA was mixed with purified topoisomerase under standard binding reaction conditions described previously (4, 5, 7); the reactions were constituted such that about half of the input DNA was bound by the enzyme. Topoisomerase-DNA complexes were then separated from unbound 32P-labeled DNA by native gel electrophoresis (7). Bound and unbound DNA species were recovered from the gel, and the DNA was cleaved at methylated G residues via treatment with piperidine. Cleavage products were then analyzed by denaturing gel electrophoresis (Fig. 1).

N-7 methylation of any of the guanine bases within the 3'GGGAAA pentamer on the noncissile strand strongly interfered with protein-DNA complex formation; this was evinced by the nearly complete exclusion, from the bound DNA fraction, of DNAs with piperidine-cleaveable sites corresponding to G positions +3, +4, and +5 (Fig. 1, left panel, lane B). DNAs methylated at these three sites were represented prominently in the population of unbound DNAs (lane F). Thus, integrity of each of the Gs within the pentamer motif was a key determinant of DNA binding. In contrast, methylation at the nearby +7G residue (situated outside the conserved motif) appeared not to interfere with complex formation (this was more obvious on longer autoradiographic exposure of the gel in Fig. 1). A partial effect of methylation was noted at the –4G position of the noncissile strand, as this cleavage site was underrepresented in the bound DNA population (Fig. 1, left panel, and other data not shown).

Methylation of two G residues of the scissile strand (+6G and +9G, located 5' of the pentamer element) interfered partially with protein-DNA complex formation (Fig. 1, right panel, and other experiments not shown). Note that a cleavage product of the radiolabeled scissile strand was detected in the protein-bound DNA fraction, but not in the free DNA population, which did not correspond to a G position in the scissile strand sequence (Fig. 1, right panel, denoted by the filled circle). Rather, this product was generated by strand scission at the topoisomerase +1 cleavage site. We suspect that the phosphotyrosyl DNA-protein intermediate was partially susceptible to hydrolysis during treatment with piperidine at 90°C.

**Methylation Protection**—Intimate contacts between vaccinia topoisomerase and guanine bases within the 60-bp DNA were identified on the basis of protection from base-specific chemical modification. Preformed protein-DNA complexes were reacted in solution with dimethyl sulfate, and the bound DNAs were resolved preparatively from unbound species by native gel electrophoresis. Control experiments established that dimethyl sulfate treatment did not affect the stability of the protein-DNA complex (data not shown). Comparison of piperidine cleavage products from the bound and free DNA populations indicated that the all three G residues (+3, +4, and +5) within the pentamer element on the noncissile strand were protected strongly from chemical modification when bound to the enzyme (Fig. 2, left panel). Partial protection was afforded at –4 and –5 G of the noncleavable strand. The –5G cleavage band migrated quite diffusely in lane B of

---

The DNA was 5' radiolabeled on either the scissile or non-scissile strand. Control experiments established that topoisomerase-mediated cleavage was confined to the CCCTT-containing strand (data not shown). The 60-mer was methylated on purine residues by treatment with dimethyl sulfate. Methylated DNA was mixed with purified topoisomerase under standard binding reaction conditions described previously (4, 5, 7); the reactions were constituted such that about half of the input DNA was bound by the enzyme. Topoisomerase-DNA complexes were then separated from unbound 32P-labeled DNA by native gel electrophoresis (7). Bound and unbound DNA species were recovered from the gel, and the DNA was cleaved at methylated G residues via treatment with piperidine. Cleavage products were then analyzed by denaturing gel electrophoresis (Fig. 1).

N-7 methylation of any of the guanine bases within the 3'GGGAAA pentamer on the noncissile strand strongly interfered with protein-DNA complex formation; this was evinced by the nearly complete exclusion, from the bound DNA fraction, of DNAs with piperidine-cleaveable sites corresponding to G positions +3, +4, and +5 (Fig. 1, left panel, lane B). DNAs methylated at these three sites were represented prominently in the population of unbound DNAs (lane F). Thus, integrity of each of the Gs within the pentamer motif was a key determinant of DNA binding. In contrast, methylation at the nearby +7G residue (situated outside the conserved motif) appeared not to interfere with complex formation (this was more obvious on longer autoradiographic exposure of the gel in Fig. 1). A partial effect of methylation was noted at the –4G position of the noncissile strand, as this cleavage site was underrepresented in the bound DNA population (Fig. 1, left panel, and other data not shown).

Methylation of two G residues of the scissile strand (+6G and +9G, located 5' of the pentamer element) interfered partially with protein-DNA complex formation (Fig. 1, right panel, and other experiments not shown). Note that a cleavage product of the radiolabeled scissile strand was detected in the protein-bound DNA fraction, but not in the free DNA population, which did not correspond to a G position in the scissile strand sequence (Fig. 1, right panel, denoted by the filled circle). Rather, this product was generated by strand scission at the topoisomerase +1 cleavage site. We suspect that the phosphotyrosyl DNA-protein intermediate was partially susceptible to hydrolysis during treatment with piperidine at 90°C.

**Methylation Protection**—Intimate contacts between vaccinia topoisomerase and guanine bases within the 60-bp DNA were identified on the basis of protection from base-specific chemical modification. Preformed protein-DNA complexes were reacted in solution with dimethyl sulfate, and the bound DNAs were resolved preparatively from unbound species by native gel electrophoresis. Control experiments established that dimethyl sulfate treatment did not affect the stability of the protein-DNA complex (data not shown). Comparison of piperidine cleavage products from the bound and free DNA populations indicated that the all three G residues (+3, +4, and +5) within the pentamer element on the noncissile strand were protected strongly from chemical modification when bound to the enzyme (Fig. 2, left panel). Partial protection was afforded at –4 and –5 G of the noncleavable strand. The –5G cleavage band migrated quite diffusely in lane B of

---
The G bases shown to be protected from chemical modification when bound to the topoisomerase were the very same residues whose prior methylation interfered with DNA binding. The strongest and most critical interactions were with the three G bases of the pentamer element in DNA cleavage was examined (5' TCAGCCCTTATTC). Covalent complex formation was assayed by transfer of 5' end-labeled scissile strand from the duplex substrate to the enzyme to yield an SDS-resistant adduct detectable by SDS-polyacrylamide gel electrophoresis. The wild-type 12-mer substrate readily formed the covalent adduct (Table I). Complex formation was unaffected by I substitution for G at either position +5 or +4. Thus, the loss of the C-2 exocyclic amino group of the purine residue at +5 or +4 (situated within the minor groove) had no significant impact on cleavage efficiency. A 3-fold reduction in cleavage on both sides of the site of strand scission.

Inosine Substitution Effects—The role of individual purine bases of the pentamer element in DNA cleavage was examined by substitution of inosine at positions +5 to +1 on the nonscissile DNA strand. Synthetic nonscissile strand oligonucleotides (12-mer) containing a single inosine substitution were annealed to a 5' radiolabeled 12-mer scissile strand (5'TCGCCCTTATTC). Covalent complex formation was assayed by transfer of 5' end-labeled scissile strand from the duplex substrate to the enzyme to yield an SDS-resistant adduct detectable by SDS-polyacrylamide gel electrophoresis. The wild-type 12-mer substrate readily formed the covalent adduct (Table I). Complex formation was unaffected by I substitution for G at either position +5 or +4. Thus, the loss of the C-2 exocyclic amino group of the purine residue at +5 or +4 (situated within the minor groove) had no significant impact on cleavage efficiency. A 3-fold reduction in cleavage efficiency was observed in the presence of inosine at position +5 or +4. Therefore, inosine substitution at these positions has a significant impact on cleavage activity.

The DNA cleavage products were analyzed by denaturing gel electrophoresis. The positions of the G-specific cleavage products relative to the consensus element are indicated at the left of each autoradiogram. The nucleotide sequences of the DNA strands are displayed at the right of the cleavage ladders; the pentamer motif is demarcated by a line. Sites of methylation interference are denoted by arrowheads. A non-G cleavage product of the scissile strand that was unique to the bound DNA population (lane B, right panel) is indicated by the solid dot.

FIG. 2. Methylation protection. Methylation of preformed topoisomerase-DNA complexes was performed as described under "Experimental Procedures." Protein-bound (lanes B) and unbound (free; lanes F) DNA populations from a single reaction mixture containing 60-mer DNA (5' labeled on the nonscissile strand (left panel) or scissile strand (right panel)) and topoisomerase were separated by native gel electrophoresis then subjected to piperidine cleavage. Control reactions containing radiolabeled DNA without added enzyme were processed in an identical fashion (lane G). Cleavage products were analyzed by denaturing gel electrophoresis. The positions of the G-specific cleavage products relative to the consensus element are indicated at the left of each autoradiogram. The nucleotide sequences of the DNA strands are displayed at the right of the cleavage ladders; the pentamer motif is demarcated by a line. Sites of methylation protection are denoted by arrowheads. A non-G cleavage product of the scissile strand that was unique to the bound DNA population (lane B, right panel) is indicated by the solid dot.
was noted when the +3 C:G base pair was changed to C:I (Table I); it was shown earlier that heteroduplex substitution of A for G at +3 on the noncissile strand had a similar mild effect (2-fold) on scission of the 12-mer (5). It appears then that the purine 2-amino group at +3 of the consensus motif (although not essential per se) may contribute to the topoisomerase-DNA interaction. Inosine substitution for A at +2 abrogated DNA cleavage; this was consistent with the stringent requirement for a T:A base pair at this position (5). A sharp reduction in cleavage was also observed when inosine was substituted for A at position +1 (Table I). These substitution effects could not be attributed to a failure of the radiolabeled scissile strand to hybridize to its I-substituted complement. Analysis of the annealed strands by native polyacrylamide gel electrophoresis revealed that the mobility of the hybridized scissile strand had been altered (i.e. retarded) relative to that of DNA that had not been subjected to annealing (data not shown). As in earlier studies (5), we interpreted this mobility shift as indicative of effective hybridization.

The deleterious effects of inosine substitution for A at +2 and +1 might have been caused by the single base mismatch per se. Thus, a second series of substrates was prepared in which the I-substituted noncleaved strand was annealed with a labeled scissile oligonucleotide containing a compensatory T → C mutation that would restore base pairing. Heteroduplex mutant substrates containing only the T → C mutations in the scissile strand were included as controls. As shown in Table II, the suppressive effects of the +2 C:A mispair on covalent adduct formation could not be overcome by replacement with the paired C:I moiety. Similarly, the cleavage of the +1 C:A heteroduplex substrate (which was reduced 20-fold relative to the standard substrate) was enhanced only slightly (2.5-fold) when the compensatory +1 C:I pair was introduced. It has been pointed out that changing T:A to C:I base pairs alters the surface of the major groove while preserving the potential binding surface of the minor groove (8). This has prompted a strategy for assessment of the relative contributions of major versus minor groove base substituents to DNA protein interactions (8), i.e. if major groove contacts at a T:A pair are critical, then substitution of an I:C pair should be detrimental to the interaction, whereas if the critical contacts are exclusively in the minor groove, then the I:C substitution should be well tolerated. That I:C substitution so strongly depresses the cleavage reaction (by 90-fold at +2 and by 10-fold at +1) suggests that the relevant contacts at positions +2 and +1 are in the major groove.

Uracil Substitution Effects—Individual thymines within the CCCTT motif of the scissile strand were replaced with deoxyuracil. U substitution for T at +2 or +1 had no influence on strand scission (Table III). Thus, the 5-methyl group of thymine was not essential for interaction of the enzyme with its recognition sequence. Vaccinia Topoisomerase Does Not Cleave RNA—The presumption that topoisomerases act exclusively on DNA substrates has been called into question by the recent findings of DiGate and Marians (9) that at least one DNA topoisomerase, *E. coli* topoisomerase III (a type I enzyme), is capable of cleaving RNA strands. RNA scission by topoisomerase III entailed covalent adduct formation between the enzyme and the 5' phosphate group of the incised bond. Furthermore, the nucleotide sequences of topoisomerase III RNA and DNA cleavage sites were found to be identical (9). A key question is whether other DNA topoisomerases have similar abilities to break and rejoin RNA strands. The sequence specificity of the vaccinia topoisomerase allows us to approach this issue in a straightforward fashion using synthetic RNA oligonucleotides identical in base sequence to the scissile and noncissile strands of standard DNA cleavage substrates.
As shown in Fig. 3, there was no detectable label transfer from 5' end-labeled RNA to the topoisomerase when the enzyme was incubated with an 18-bp RNA hybrid labeled either on the CCCU-containing strand or on the complementary strand containing 3' GGGAA. Nor did covalent adduct formation occur when topoisomerase was incubated with either of the two 18-mer oligonucleotides in single-stranded form. An RNA:DNA hybrid composed of radiolabeled CCCTT-containing RNA strand annealed to a complementary DNA nonscissile strand was inert in covalent complex formation. The same nonscissile strand when annealed to a labeled scissile DNA strand constituted a duplex that was readily cleaved. Although a trace level of label transfer was detected when topoisomerase was incubated with radioactive scissile DNA strand hybridized to a complementary RNA oligonucleotide, we observed a similar trace level of cleavage activity with scissile DNA strand in single-stranded form. Cleavage of the CCCTT-containing RNA strand (either as RNA:RNA hybrid or RNA:DNA hybrid) could not be induced by inclusion of magnesium over a concentration range of 0.5–10 mM (Fig. 4). Similarly, cleavage of the (CCCTT)DNA (GGGAA)RNA substrate was not enhanced by inclusion of either magnesium or manganese over the same concentration range (data not shown). These results indicate that vaccinia topoisomerase is unable to catalyze site-specific cleavage of RNA strands.

Effects of 2' Sugar Substitutions—Failure to cleave RNA, or to even to cleave a DNA:RNA hybrid, may reflect sensitivity to the global conformation of the helix (e.g. A-form versus B-form). Alternatively, the presence of a 2'OH group on individual sugars may interfere with specific protein-nucleic acid backbone interactions essential for covalent adduct formation. The latter possibility was tested using synthetic substrates containing 2'OMe substitutes at selected positions within the pentamer element. Initially, we examined the cleavage of substrates in which consecutive sugar residues were substituted in groups of two or three (e.g. CmCmCm or UmUm) on either the scissile or nonscissile strand. These sugar-substituted species were hybridized to unmodified complementary strands or to other sugar-substituted derivatives (Fig. 5). With respect to the nonscissile strand, O-methylation of the three consecutive G nucleotides virtually abrogated strand scission (lane 4), whereas methylation of the neighboring two A nucleotides had no effect whatsoever (lane 6). On the scissile strand, 2' O-methylation of the three C nucleotides had only a mild effect (lane 8), whereas sugar substitution of the two U nucleotides was sufficient to abolish the reaction completely (lane 14). Not surprisingly, the methylated strands that were ineffective when annealed to wild-type oligonucleotides remained so when hybridized with sugar-substituted strands (lanes 10, 16, and 18). It was noteworthy that the effect of combining of the CmCmCm scissile strand with the mAmA nonscissile strand was to eliminate cleavage (lane 12), even though neither substitution alone had such an effect. These data suggested that sugar-specific interactions within the pentamer motif play a significant role in site recognition and that the relative importance of individual sugars might vary with the context of the sugar-phosphate backbone on the opposite DNA strand.

This theme is underscored by the experiments presented in Tables IV and V, in which 18-mer oligonucleotides containing single 2'OMe sugars were tested for their ability to support covalent complex formation. The presence of a methylated sugar at any one of the five nucleotides of the 3'GGGAA element of the nonscissile strand had no appreciable affect on cleavage efficiency (Table IV, experiment 1). Within the CCCTT sequence of the scissile strand, single site methylations were also benign, with one exception. The 2'OMe group at position +2U was sufficient to reduce cleavage 9-fold (Table IV, experiment 2). That such an effect was limited to the +2 residue reinforces the findings of the base substitution analyses that the integrity of the +2 position is the most critical single determinant of topoisomerase specificity (5). Because the presence of an OMe group at the +1U nucleotide (i.e.

Figure captions:

**Fig. 3.** Vaccinia topoisomerase fails to cleave RNA strands. Cleavage reaction mixtures contained 800 fmol of topoisomerase (Enzyme +) and 32P-labeled substrate as indicated above the lanes. Reaction mixtures lacking enzyme (Enzyme −) were included as controls. The structure of the 18-bp RNA duplex is shown at the bottom. RNA strands (R) were 5' radiolabeled on either the scissile (top strand) or nonscissile (bottom strand) oligonucleotide. DNA strands (D) contained thymine bases in lieu of uracil. The identity of the 32P-labeled strand in each reaction is indicated by a circle around the R or D symbols above the lanes. Reaction mixtures contained either 500 fmol of 32P-labeled RNA (in single-stranded form or annealed to a complementary DNA or RNA strand) or 350 fmol of 32P-labeled DNA (as either single-stranded or annealed duplex). Reaction products were resolved by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the gel is shown. The position of the covalent protein-32P-nucleic acid adduct is indicated by the arrow at the left.

**Fig. 4.** Covalent adduct formation in the presence of magnesium chloride. Cleavage reaction mixtures containing annealed nucleic acid substrates as indicated below the autoradiogram (with labeled scissile strand denoted by the asterisk) were supplemented with increasing concentrations of magnesium chloride as indicated above the lanes. Within each reaction series, the mixtures contained (from left to right) final magnesium concentrations of 0.5, 1.25, 5, and 10 mM. Reaction mixtures lacking enzyme were included as controls (lanes −E in each series).
We were labeled on the scissile strand. Reactions lacking enzyme contained 800 fmol of topoisomerase and approximately 250 fmol of 32P-labeled DNA substrate. The positions and sizes (in kDa) of prestained marker proteins are indicated at the left. The sequences of the 18-bp duplex substrates included in each pair of reaction mixtures are shown below (along with the relevant lane numbers). The locations of 2’OMe-substituted nucleotides are indicated by solid dots. The predicted site of adduct formation with the scissile DNA strand of the control substrate is denoted by the arrowhead. The extent of cleavage (expressed as the percent of input labeled DNA transferred covalently to protein) is indicated for each substrate directly below the lanes.

Fig. 5. Effect of 2’OMe sugar substitutions. Reaction mixtures contained 800 fmol of topoisomerase (even numbered lanes) and approximately 250 fmol of 32P-labeled DNA. All duplex substrates were labeled on the scissile strand. Reactions lacking enzyme (odd numbered lanes) were included as controls. Reaction products were resolved by SDS-polyacrylamide gel electrophoresis. An autoradiogram of the SDS gel is shown. The positions and sizes (in kDa) of prestained marker proteins are indicated at the left. The sequences of the 18-bp duplex substrates included in each pair of reaction mixtures are shown below (along with the relevant lane numbers). The locations of 2’OMe-substituted nucleotides are indicated by solid dots. The predicted site of adduct formation with the scissile DNA strand of the control substrate is denoted by the arrowhead. The extent of cleavage (expressed as the percent of input labeled DNA transferred covalently to protein) is indicated for each substrate directly below the lanes.

The predicted site of adduct formation with the scissile DNA strand of the control substrate is denoted by the arrowhead. The extent of cleavage (expressed as the percent of input labeled DNA transferred covalently to protein) is indicated for each substrate directly below the lanes.

Effect of single 2’OMe sugar substitutions on DNA cleavage

Cleavage reaction mixtures contained 800 fmol of topoisomerase and approximately 250 fmol of 32P-labeled DNA substrate. The structures of the substrates are shown. 2’OMe sugar-substituted positions are underlined.

| Expt. | Substrate                     | Cleavage (%) |
|-------|-------------------------------|--------------|
| 1     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 65.2         |
| 2     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 73.1         |
| 3     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 68.7         |
| 4     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 59.3         |
| 5     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 52.3         |
| 6     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 71.9         |
| 7     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 65.0         |
| 8     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 73.1         |
| 9     | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 68.4         |
| 10    | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 8.1          |
| 11    | CGTGTCGCCCTATATCCCTGGCAACGGGGAATAAAGGG | 58.2         |

The extent of cleavage (expressed as the percent of input labeled DNA transferred covalently to protein) is indicated for each substrate directly below the lanes.

Vaccinia DNA topoisomerase binds to and incises duplex DNA at specific sites. Although prior studies had shown that site selection is dictated by a pentapyrimidine sequence in the scissile strand (5’CCCTT, or close congeners thereof), the molecular interactions contributing to recognition of this motif had not been explored. The experiments presented above reveal that topoisomerase makes direct contact with guanine nucleotide bases of the pentamer motif complementary strand within the major groove of the DNA helix and that alteration of the binding surface by chemical modification is deleterious to the interaction. Additional contacts are made with guanine residues located upstream of the pentamer motif and with residues 3’ (downstream) of the site of strand scission.

The observed base-specific contacts outside the pentamer element occur at positions beyond the minimal region sufficient for site-specific cleavage of synthetic substrates (+6 to −2). No sequence conservation or strong nucleotide bias at positions outside the pentamer emerged from the mapping of topoisomerase cleavage sites within linear plasmid DNA (2). Yet, these sites could be classified as having higher or lower affinity for topoisomerase based on several criteria (2). (Higher affinity sites were cleaved at low enzyme concentration, were less sensitive to competition, and were most refractory to religation promoted by salt, divalent cations, and elevated temperature. Cleavage at lower affinity sites required higher enzyme concentration and was more sensitive to competition and induced religation.) This suggested that site affinity is influenced by the DNA sequence flanking the pentamer element. Studies of the effects of base changes within the pentamer element indicate that some alterations...
that abolish cleavage of a minimal substrate are actually tolerated by the vaccinia enzyme when they occur in the context of either a larger DNA or particular flanking sequences (5). Although flanking sequence effects on the vaccinia topoisomerase cleavage reaction have not been explored systematically, our findings that contact is made with nucleotide bases in the immediate flanking regions provide a rationale for the earlier observations and a framework for further studies. It is worth noting that the sequences of the synthetic DNA substrates used in the present study (and in earlier reports from this laboratory) are based closely on the sequence surrounding one of the high affinity sites mapped within plasmid DNA. Comparison of the protection and interference profiles of DNA substrates containing different flanking sequences may shed light on the issue.

Synthetic 18-bp RNA substrates containing a CCCUU motif are not cleaved by vaccinia topoisomerase under conditions permissive for the cleavage of DNA substrates of identical sequence. The observation that E. coli DNA topoisomerase I11 (a type I enzyme) can incise RNA strands (9) is therefore not applicable to this virus-encoded member of the eukaryotic family of type I enzymes. Control experiments using DNA substrates make clear that this is not caused by substitution of uracil for thymine within the pentamer element. Rather, our analysis of the cleavage of synthetic DNA duplexes containing 2'OMe sugars suggests that the inability of the vac-

**Table V**

| Expt. | Substrate | Cleavage | Expt. | Substrate | Cleavage |
|-------|-----------|----------|-------|-----------|----------|
|       |           | %        |       |           | %        |
| 1     | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 53.8 | 4     | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 68.6 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 40.4 |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 67.5 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 4.2  |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 35.0 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 2.1  |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 28.5 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 1.2  |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 0.4  |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 0.2  |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 5.1  |
| 2     | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 59.4 | 5     | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 80.4 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 56.2 |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 72.3 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 8.1  |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 75.5 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 15.0 |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 72.7 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 0.3  |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 7.2  |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 0.9  |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 49.2 |
| 3     | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 68.4 |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 70.7 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 59.2 |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 62.7 |
|       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 2.5  |       | CGTGTCGCCCTTATTCCC GCACAGGGGAAATAGGG | 43.0 |

Cleavage reaction mixtures contained 800 fmol of topoisomerase and approximately 250 fmol of 32P-labeled DNA substrate. The structures of the substrates are shown. 2'OMe sugar-substituted positions are underlined.
vaccinia topoisomerase to cleave either an RNA duplex or an RNA:DNA hybrid can be accounted for by the interfering effects of a 2′ sugar substituent at two or more sites within the pentamer motif. A key implication is that DNA topoisomerase makes specific contacts with the sugar moiety that are relevant to site recognition and/or catalytic activity. Interaction with the sugar at the +2T nucleotide appears to be the most critical, as judged by the effects of single sugar substitutions. Although we have not undertaken to assess the effects of 2′OMe sugar substitutions outside the pentamer motif, preliminary footprinting experiments with the chemical nuclease copper/phenanthroline show that the vaccinia topoisomerase protects the sugar-phosphate backbone of the scissile strand over a region extending from position +10 to −10.

We anticipate that the application of additional chemical footprinting methods (i.e. with different base and backbone specificities) will help define at higher resolution the spectrum of protein-nucleic acid interactions that contribute to binding and cleavage. Efforts to crystallize the covalent enzyme-DNA intermediate are being made concurrently.

REFERENCES

1. Champoux, J. J. (1990) in DNA Topology and Its Biological Effects (Cozzarelli, N. R., and Wang, J. C., eds) pp. 217-242, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Shuman, S., and Prescott, J. (1990) J. Biol. Chem. 265, 17929-17936
3. Shuman, S., Rea, K. M., and Morham, S. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9765-9769
4. Shuman, S. (1991) J. Biol. Chem. 266, 1796-1803
5. Shuman, S. (1991) J. Biol. Chem. 266, 11372-11379
6. Shuman, S., Golde, M., and Moss, B. (1988) J. Biol. Chem. 263, 16401-16407
7. Morham, S. G., and Shuman, S. (1992) J. Biol. Chem. 267, 15984-15992
8. Starr, D. R., and Hesley, D. K. (1991) Cell 67, 1231-1240
9. DiGate, R., and Marinas, K. J. (1992) J. Biol. Chem. 267, 20532-20535

2 J. Turner and S. Shuman, unpublished data.