

Site-specific DNA Cleavage by Vaccinia Virus DNA Topoisomerase I

ROLE OF NUCLEOTIDE SEQUENCE AND DNA SECONDARY STRUCTURE*

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Cleavage of linear duplex DNA by purified vaccinia virus DNA topoisomerase I occurs at a conserved sequence element (5'-(C/T)CCTT↓) in the incised DNA strand. Oligonucleotides spanning the high affinity cleavage site CCCTT at nucleotide 2457 in pUC19 DNA are cleaved efficiently *in vitro*, but only when hybridized to a complementary DNA molecule. As few as 6 nucleotides proximal to the cleavage site and 6 nucleotides downstream of the site are sufficient to support exclusive cleavage at the high affinity site (position +1). Single nucleotide substitutions within the consensus pentamer have deleterious effects on the equilibria of the topoisomerase binding and DNA cleavage reactions. The effects of base mismatch within the pentamer are more dramatic than are the effects of mutations that preserve base complementarity. Competition experiments indicate that topoisomerase binds preferentially to DNA sites containing the wild-type pentamer element. Single-stranded DNA containing the sequence CCCTT in the cleaved stand is a more effective competitor than is single-stranded DNA containing the complementary sequence in the noncleaved strand.

Type I DNA topoisomerases alter the topology of DNA by transiently breaking, passing, and rejoining single DNA strands (1, 2). The eukaryotic family of enzymes, including the nuclear type I enzyme and the topoisomerase I of vaccinia virus, catalyze this reaction via an intermediate consisting of DNA linked covalently to the topoisomerase through a 3'-phosphodiester bond to tyrosine (3–6). The intermediate, normally transient, can be trapped by the addition of a protein denaturant to the enzyme-DNA complex; this procedure results in strand cleavage at the site of covalent adduct formation (7).

The issue of whether topoisomerase I interacts with specific DNA sequences is of some interest insofar as the specificity of such interaction may have implications for topoisomerase action *in vivo*, be it in DNA replication, transcription, and/or recombination. Several studies (8–10) have demonstrated that cellular topoisomerase I cleaves defined DNA molecules frequently (*i.e.* at an average of one site every one to two turns of the DNA helix), but that the distribution of cleavage sites

at the nucleotide level is nonrandom. Recently, a 16-bp¹ element within the rDNA spacer of *Tetrahymena* was characterized as a high affinity site ($K_D = 10^{-10}$ M) for DNA binding and DNA cleavage and as a preferred site for DNA relaxation *in vitro* by cellular topoisomerase I (11–15). The location of this site in rDNA is remarkable given the involvement of topoisomerase I in ribosomal transcription *in vivo* (16–19). Cellular topoisomerase I has also been shown to cleave DNA preferentially at sites of DNA bending (20). Single-stranded DNAs are cleaved by cellular topoisomerase I (8, 10, 41, 46); however, the sites of cleavage are localized to regions of DNA with the potential for intramolecular base pairing (34), suggesting that DNA secondary structure is required for strand scission.

The vaccinia virus DNA topoisomerase I, a M_r 32,000 monomeric enzyme (21–23), is essential for replication of the virus in cultured cells (24); the exact role of the enzyme during the viral life cycle remains unclear. When expressed in a heterologous system *in vivo*, the vaccinia topoisomerase has been shown to promote illegitimate recombination (25). It is conceivable that the vaccinia topoisomerase acts as a recombinase; however, direct proof of such action is lacking (25). Site-specific recombination mediated by another type I DNA topoisomerase, λ-integrase, has been characterized extensively and shown to entail single-strand cleavage at a specific DNA sequence within *attP* and *attB* (26, 27, 45). Studies (28) of vaccinia virus DNA topoisomerase indicate that cleavage sites within a defined linear duplex DNA, pUC19, correlate with a conserved polypyrimidine motif (5'-(C/T)CCTT) at the site of strand scission and covalent adduct formation. Not all sites within pUC19 are cleaved equally well, however; and sites can be classified as higher or lower affinity based, for example, on their occupancy at limiting enzyme concentration. The features of the DNA substrate that contribute to the site specificity of the vaccinia enzyme are essentially unknown.

Clearly, pUC19 is not a suitable substrate for a more detailed analysis of topoisomerase binding and cleavage, containing as it does at least 12 cleavage sites and 17 copies of the consensus pentamer (C/T)CCTT (28, 29). I have therefore prepared for use as model substrates a series of complementary synthetic oligonucleotides whose sequences correspond to that of the DNA strands in the vicinity of the high affinity cleavage site at nucleotide 2457 of pUC19. The contributions of DNA length, DNA secondary structure, and DNA sequence to topoisomerase interaction with a single cleavage site have thereby been addressed as described herein.

EXPERIMENTAL PROCEDURES

Enzyme Purification—Vaccinia DNA topoisomerase was expressed in *Escherichia coli* and purified as described (30). The heparin-

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¹ The abbreviations used are: bp, base pair(s); SDS, sodium dodecyl sulfate.

agarose-enzyme preparation was used in this study unless stated otherwise. Vaccinia topoisomerase containing a Phe²⁷⁴ substitution at the active-site tyrosine residue was expressed in *E. coli* and purified by phosphocellulose column chromatography (6).

Oligonucleotides—Synthetic DNA oligonucleotides used in this study corresponded in sequence to pUC19 plasmid DNA (29) in the vicinity of the high affinity vaccinia topoisomerase cleavage site at position 2457 (Table I). Enrichment for full-length oligonucleotides was accomplished by preparative acrylamide gel electrophoresis of the crude deprotected synthesis products. Oligonucleotides were labeled at the 5'-end via enzymatic phosphorylation in the presence of [γ -³²P]ATP and T4 polynucleotide kinase. Labeled DNA was freed of protein and radioactive nucleotide by one of the following methods. (i) Kinase reaction products were extracted serially with phenol:chloroform:isoamyl alcohol (50:48:2) and chloroform:isoamyl alcohol (24:1) and then isolated by centrifugal gel filtration using a 1-ml Sephadex G-50 column. (ii) The kinase reaction products were electrophoresed through a nondenaturing 20% polyacrylamide gel. Full-sized labeled oligonucleotide was localized by autoradiographic exposure of the wet gel, and the labeled oligonucleotide was recovered from an excised gel slice by soaking the slice in 0.3 ml of H₂O for 4 h at room temperature.

Hybridization—Oligonucleotides were hybridized in a 0.25 M NaCl solution by heating to 65 °C for 2 min, followed by slow cooling to 25 °C at a rate of ~0.5 °C/min. Hybridization reactions in which both the top and bottom DNA strands were labeled or in which neither strand was labeled contained equimolar amounts of the complementary oligonucleotides. Hybridization reactions in which only the top (cleaved) strand was radioactively labeled included a slight molar excess of the unlabeled bottom-strand oligonucleotide (at a bottom:top strand molar ratio of 1.5–2.0). This was done to ensure complete hybridization of the labeled top-strand oligonucleotide.

DNA Cleavage Assay—Reaction mixtures (20 μ l) containing 50 mM Tris-HCl (pH 7.5), 5'-³²P-labeled oligonucleotide, and enzyme were incubated at 37 °C for 5 min. Cleavable complexes were disrupted by addition of SDS to 1%. Sample volume was adjusted to 40 μ l by addition of water. The mixtures were then digested with 15 μ g of proteinase K for 60 min at 37 °C. The samples were extracted serially with phenol:chloroform:isoamyl alcohol (50:48:2) and chloroform:isoamyl alcohol (24:1). An aliquot of the aqueous extract was adjusted to 50% formamide and then heated at 95 °C for 5 min. Samples were electrophoresed through a 20% polyacrylamide gel (acrylamide:bisacrylamide = 20:1) containing 7.5 M urea. Cleavage of the labeled DNA was detected by autoradiographic exposure of the gel.

DNA Binding Assay—Equilibrium parameters for DNA binding and cleavage were determined using a nitrocellulose filter binding assay essentially as described (28, 31). Binding reactions (20 μ l) contained 50 mM Tris-HCl (pH 7.5), 34 fmol of 5'-end-labeled oligonucleotide, and increasing amounts of purified topoisomerase (ranging from 20 fmol to 2 pmol, depending on which DNA substrate was being analyzed). After incubation for 5 min at 37 °C, two aliquots (9 μ l) were withdrawn. One was applied directly to nitrocellulose filters (25-mm diameter, 0.2- μ m pore size); the other was made 1% in SDS and then applied to filters that had (in either case) been wetted with 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. The filters were washed under vacuum with 5 ml of the same Tris:EDTA buffer, and the radioactivity retained on the filter was determined by liquid scintillation counting. The percent of input DNA bound to the filter was determined for each reaction. The data were corrected for the nonspecific binding of free DNA (generally ~8% for standard reactions and <1% for samples containing SDS). DNA retention in nondenatured samples (total DNA binding) represented the sum of covalent and noncovalent binding of protein to nucleic acid. DNA retention in SDS-treated samples reflected the extent of covalent binding of topoisomerase to DNA. The difference between these two values indicated the extent of noncovalent binding. The equilibrium dissociation constant for binding (K_D) was derived for different DNA substrates from the titration curve of total DNA binding versus enzyme concentration as described (31). The equilibrium constant for cleavage (K_{eq}) was taken as the ratio of covalently bound to noncovalently bound DNA as described previously (28).

RESULTS

Site-specific Cleavage of Duplex DNA Oligonucleotides by Vaccinia Topoisomerase—Vaccinia topoisomerase, in common with other eukaryotic type I enzymes, forms a cleavable

complex with duplex DNA in which the protein is linked covalently to a 3'-phosphate at the site of a topoisomerase-induced nick. Addition of SDS or alkali traps the normally transient cleavable complex and results in DNA strand cleavage. In studies of the cleavable complex, it is assumed generally that the sites of topoisomerase-dependent DNA breakage revealed by SDS are indeed sites of binding of topoisomerase to DNA and that the extent of cleavage at any given site is indicative of the instantaneous occupancy of that site by the enzyme; these points are assumed because it cannot be excluded that the SDS *per se* induces DNA cleavage at sites to which the topoisomerase is bound noncovalently (discussed in Ref. 44). These same assumptions apply to this study of covalent complex formation between vaccinia topoisomerase and DNA oligonucleotides; consequently, the term "cleavage" is taken to mean DNA breakage assayed after the addition of SDS.

A labeled duplex DNA spanning the pUC19 high affinity cleavage site at nucleotide 2457 was prepared by hybridizing complementary oligonucleotides JP1 (72-mer) and JP2 (73-mer), each of which had been 5'-³²P-end-labeled by enzymatic phosphorylation. The JP1/JP2 hybrid extended from 42 bp upstream of the high affinity site to 30 bp downstream of the known cleavage site (Table I). The labeled DNA was incubated with purified vaccinia topoisomerase under conditions previously shown to be favorable for covalent adduct formation (28). Trapping the bound enzyme by addition of detergent resulted in transfer of the end-labeled DNA to the protein (data not shown). The specificity of the cleavage reaction could therefore be assessed by electrophoretic analysis of the labeled reaction products after removal of attached protein by proteinase K. This procedure revealed the appearance of two classes of labeled products that depended on the inclusion of topoisomerase in the reaction (Fig. 1A, indicated by asterisks to the left). The more rapidly migrating class consisted of two discrete species that electrophoresed with apparent chain lengths of 43–44 nucleotides and was presumed to arise via topoisomerase cleavage of JP1 at the high affinity site associated with the CCCTT consensus motif (Table I, arrow). This site has been designated position +1 in the cleaved DNA strand. The apparent heterogeneity of cleavage products arising from a putative single cleavage event and the slightly retarded mobility of these species relative to the expected product of 42 nucleotides were most likely attributable to the covalent attachment of one or more amino acids to the 3'-end of the cleaved fragment (see below). The second cleavage product, migrating only slightly more rapidly than the input DNA oligonucleotides, likely arose via cleavage of JP2 at the TCCTT sequence near its 3'-end.

Oligonucleotides JP3 (60-mer) and JP4 (50-mer) extended from 30 and 20 nucleotides upstream of the high affinity cleavage site to 30 nucleotides downstream of the site, respectively. Both oligonucleotides, when hybridized to complementary strand JP2, were cleaved by vaccinia topoisomerase to yield a single (albeit heterogeneous) class of rapidly migrating products (Fig. 1B, indicated by asterisks on right). The product of JP3 cleavage migrated with an apparent size of 32 nucleotides (expected product for cleavage at position +1 was 30 nucleotides); the product of JP4 cleavage migrated as a 22-mer (expected product for cleavage at position +1 was 20 nucleotides). Oligonucleotide JP5 (54-mer), extending from +42 nucleotides upstream to -12 nucleotides downstream, was also, when hybridized to JP2, cleaved by topoisomerase, in this case yielding a product of 44 nucleotides (a 42-mer being anticipated; data not shown). Thus, it appeared from these initial experiments that: (i) purified vaccinia topoisom-

TABLE I
Oligonucleotide substrates for topoisomerase I DNA cleavage

Cleaved (top) strand (5' → 3')	
JP1	AAAAGGAAGAGTATGAGTATTCAACATTCCTCGTGCGCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP3	ATGAGTATTCAACATTCCTCGTGCGCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP4	AACATTCCTCGTGCGCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP5	AAAAGGAAGAGTATGAGTATTCAACATTCCTCGTGCGCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP6	AACATTCCTCGTGCGCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP7	AACATTCCTCGTGCGCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP8	AACATTCCTCGTGCGCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP10	CCTGTGCGCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP11	GCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
JP16	AACATTCCTCGTGCGCCCTAATTCCTTTTGCGGCATTTGCCTTCCT
JP17	AACATTCCTCGTGCGCCATTATTCCTTTTGCGGCATTTGCCTTCCT
JP21	GCCCTTATTCCTTTTGCGGCATTTGCCTTCCT
Noncleaved (bottom) strand (3' → 5')	
JP2	TTTCCTCTCATTCACATAAGTTGAAAGGCACAGCGGGATAAGGGAAAAACGCCGTAAACCGGAAGGAG
JP9	TTGTAAGGCACAGCGGGATAAGGGAAAAAC
JP12	TTGTAAGGCACAGCGGGATAAGGG
JP13	TTGTAAGGCACAGCGGGATAAGGG
JP14	GCACAGCGGGATAAGGGAAAAAC
JP15	CGGGATAAGGGAAAAAC
JP18	TTGTAAGGCACAGCGGGATTAAGGG
JP19	TTGTAAGGCACAGCGGTATAAGGG
JP23	CGGGATAAGGG

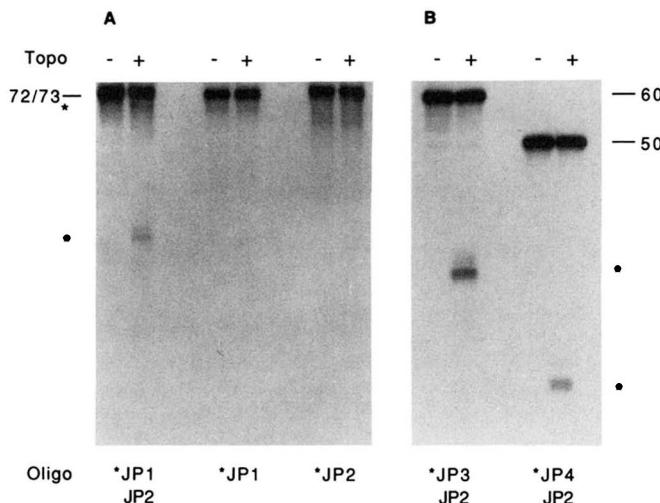


FIG. 1. Cleavage of DNA oligonucleotides by vaccinia DNA topoisomerase. Cleavage reactions contained 200 fmol of purified vaccinia topoisomerase (where indicated (+)) and 50 fmol of labeled DNA oligonucleotide. Control reactions from which topoisomerase was omitted are indicated (−). The oligonucleotide included in the reaction is indicated below each pair of lanes (refer to Table I for nucleotide sequence). Cleavage reaction products were analyzed by denaturing gel electrophoresis. Autoradiographic exposures of the gels are shown. The positions and sizes (in nucleotides) of the labeled DNA substrates are indicated to the left and right, as are the positions of the cleavage products (asterisks). A, in the case of the JP1/JP2 hybrid, both strands are 5'-end-labeled; B, in this experiment, only the top-strand oligonucleotide (either JP3 or JP4) of each hybrid is end-labeled.

erase could cleave duplex DNA oligonucleotides at the same site and with similar selectivity as the enzyme had displayed in previous studies of the cleavage of a much larger plasmid DNA; (ii) duplex structure, either partial or complete, was required for cleavage; and (iii) site-specific recognition, and not the distance from the end of the cleaved DNA strand, determined where the strand was cleaved. That the oligonucleotide cleavage reaction truly reflected topoisomerase (not nuclease) activity was confirmed by control experiments in

which no cleavage was observed with a purified mutant topoisomerase (Phe²⁷⁴) containing a Tyr → Phe substitution at the active site (data not shown).

DNA Strand Length as Determinant of Cleavage—Systematic deletion of DNA sequence information in the incised DNA strand was undertaken to address the minimal DNA size requirements for topoisomerase cleavage. First, a series of top-strand oligomers was prepared (JP6, JP7, and JP8) that deleted DNA sequence downstream of position +1 (Table I). JP6 (+20 → −12), when hybridized to JP2, was cleaved by topoisomerase to yield a discrete product of the expected size (Fig. 2A, lanes 1 and 2). The hybrid of JP7 (+20 → −6)/JP2 was cleaved by topoisomerase to give an identically sized species (Fig. 2A, lanes 3 and 4); however, the efficiency of the cleavage reaction was considerably higher for the latter substrate (Fig. 2A, compare lanes 2 and 4). In contrast, no cleavage was observed when the JP8 (+20 → +1)/JP2 hybrid was provided as substrate (Fig. 2A, lanes 5 and 6). Apparently, as few as 6 nucleotides downstream of the cleavage site on the cleaved DNA strand were sufficient to support strand scission. Significantly, in the absence of distal sequence information, there was no activation of alternative cleavage sites within the 20-mer JP8 fragment.

The requirements for upstream information in the cleaved DNA strand were investigated using a different set of deleted oligonucleotides (JP6 (+20 → −12), JP10 (+12 → −12), and JP12 (+6 → −12)), each of which was hybridized to complementary bottom-strand oligonucleotide JP9 (33-mer; +20 → −13). Reaction of topoisomerase with JP6/JP9 yielded a discrete class of products ~22 nucleotides long (Fig. 2B, lane 2). Cleavage of JP10/JP9 produced a prominent 14-mer and a less abundant 16-mer (Fig. 2B, lane 4). The sizes of these reaction products are, in both cases, consistent with cleavage at position +1, again taking into account the presence of one or more bound amino acids. Incubation of topoisomerase with JP12/JP9 produced two cleavage products whose electrophoretic mobility difference was more pronounced (Fig. 2B, lane 6). It was established, using 3'-end-labeled substrates, that heterogeneity of the bound peptide, and not the activation of novel cleavage sites by deletion of sequences from nucleotides

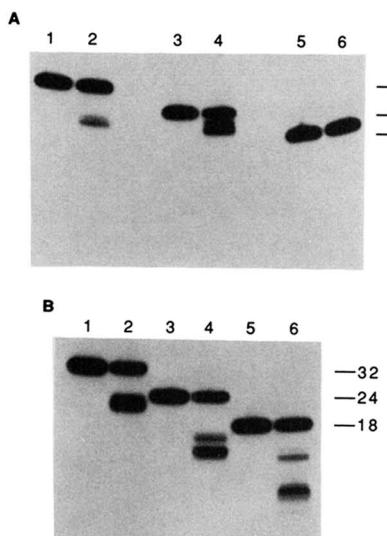


FIG. 2. Length of cleaved DNA strand affects cleavage efficiency. *A*, 3'-deletions of cleaved DNA strand. Cleavage reactions contained 90 fmol of duplex oligonucleotide, uniquely 5'-end-labeled on the top (cleaved) DNA strand (refer to Table I), and 400 fmol of vaccinia topoisomerase (lanes 2–5 and 7–10). Control reactions contained no enzyme (lanes 1, 3, and 5). The DNA substrates were JP6/JP2 (lanes 1 and 2), JP7/JP2 (lanes 3 and 4), and JP8/JP2 (lanes 5 and 6). The positions and sizes (in nucleotides) of the labeled DNA substrates are indicated to the right. *B*, 5'-deletions of cleaved DNA strand. Cleavage reactions contained 160 fmol of duplex oligonucleotide, uniquely 5'-end-labeled on the top (cleaved) DNA strand (refer to Table I), and 400 fmol of vaccinia topoisomerase (lanes 2, 4, and 6). Control reactions contained no enzyme (lanes 1, 3, and 5). The DNA substrates were JP6/JP9 (lanes 1 and 2), JP10/JP9 (lanes 3 and 4), and JP11/JP9 (lanes 5 and 6). The positions and sizes (in nucleotides) of the labeled DNA substrates are indicated to the right.

+12 to +6 was responsible for these anomalous electrophoretic effects (see below).

Role of DNA Secondary Structure: Length of Complementary DNA Strand—The degree of strand complementarity required for topoisomerase cleavage of DNA was examined through deletion of DNA sequence information on the bottom (noncleaved) DNA strand (Fig. 3). In these experiments, a 5'-end-labeled top strand (either JP6 (+20 → -12) or JP7 (+20 → -6)) was reacted with topoisomerase in the absence of a complementary oligonucleotide or after hybridization with bottom-strand oligonucleotides of variable length. Both single-stranded oligonucleotides (JP6 and JP7) were themselves completely inert as topoisomerase cleavage substrates (Fig. 3A, compare lanes 1 and 2 with lanes 6 and 7), but were readily cleaved when provided with a complementary strand (e.g. Fig. 3A, lanes 3–5). The extent of JP6 cleavage increased significantly with deletion of nucleotides from positions -13 to -6 on the complementary strand (Fig. 3A, compare lanes 3 and 4). However, the deletion of all complementary sequences downstream of the cleavage site profoundly reduced the efficiency of the cleavage reaction (Fig. 3A, compare lanes 4 and 5). The cleavage of JP7 by topoisomerase was highly efficient and was not appreciably influenced by deletion of bottom-strand sequence from nucleotides -13 to -6 (Fig. 3A, lanes 8 and 9). Yet, when all downstream complementary sequence was deleted, cleavage was essentially abolished (Fig. 3A, compare lanes 9 and 10). Thus, although as few as 6 downstream complementary nucleotides allowed maximal DNA cleavage, the reaction was sustained, albeit poorly, by a duplex region spanning the CCCTT consensus motif and no other downstream nucleotides. Apparently, the use of such a minimal duplex substrate by the topoisomerase was acutely

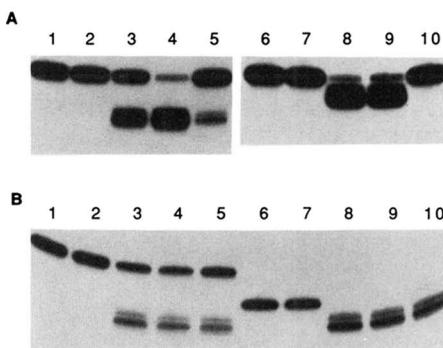


FIG. 3. Requirement for DNA secondary structure: length of noncleaved DNA strand affects cleavage efficiency. *A*, 3'-deletions of noncleaved DNA strand. Cleavage reactions contained 5'-end-labeled oligonucleotide and 400 fmol of vaccinia topoisomerase (lanes 2–5 and 7–10). Control reactions contained no enzyme (lanes 1 and 6). The DNA substrates were JP6 (160 fmol) (lanes 1 and 2), JP6/JP9 (160 fmol) (lane 3), JP6/JP12 (160 fmol) (lane 4), JP6/JP13 (160 fmol) (lane 5), JP7 (100 fmol) (lanes 6 and 7), JP7/JP9 (100 fmol) (lane 8), JP7/JP12 (100 fmol) (lane 9), and JP7/JP13 (100 fmol) (lane 10). Only JP6 and JP7 were radioactively labeled. *B*, 5'-deletions of noncleaved DNA strand. Cleavage reactions contained 5'-end-labeled oligonucleotide and 400 fmol of vaccinia topoisomerase (lanes 2–5 and 7–10). Control reactions contained no enzyme (lanes 1 and 6). The DNA substrates were JP6 (160 fmol) (lanes 1 and 2), JP6/JP9 (160 fmol) (lane 3), JP6/JP14 (160 fmol) (lane 4), JP6/JP15 (160 fmol) (lane 5), JP7 (100 fmol) (lanes 6 and 7), JP7/JP9 (100 fmol) (lane 8), JP7/JP14 (100 fmol) (lane 9), and JP7/JP15 (100 fmol) (lane 10). Only JP6 and JP7 were radioactively labeled.

dependent on the length of 3'-sequence present on the cleaved DNA strand (Fig. 3A, compare lanes 5 and 10).

The requirements for duplex structure upstream of the cleavage site were addressed using a different set of deleted bottom-strand oligonucleotides (JP9 (+20 → -13), JP14 (+12 → -13), and JP15 (+6 → -13)). Topoisomerase cleavage of either JP6 (Fig. 3B, lanes 1–5) or JP7 (Fig. 3B, lanes 6–10) at position +1 occurred equally well whether the upstream duplex region was 20, 12, or 6 nucleotides in length.

Cleavage of 3'-End-labeled Oligonucleotides—The heterogeneity and anomalous electrophoretic mobility of 5'-labeled peptide-linked cleavage products could be avoided by using substrates labeled uniquely at the 3'-end. Top-strand oligomers were hybridized to JP2 and extended by a single dGMP residue using the Klenow DNA polymerase and [α -³²P]dGTP. The 3'-radiolabeled 32-nucleotide top strand was electrophoretically purified and then hybridized to a complementary bottom strand for use in topoisomerase cleavage assays. Incubation of the [3'-³²P]-labeled JP6 (+20 → -13)/JP9 (+20 → -13) hybrid with topoisomerase resulted in the appearance of a single 13-nucleotide cleavage product (Fig. 4A, lane 2), as expected from strand scission at the T residue at position +1. An identically sized single fragment was produced when 3'-³²P-labeled JP6 was hybridized to JP12 (+20 → -6); however, the efficiency of the cleavage reaction was increased significantly (Fig. 4A, lane 4), just as had been seen in experiments using 5'-labeled substrates (compare to Fig. 3A, lanes 3 and 4). This effect could be caused by release of the distal fragment of the incised DNA strand from the DNA duplex by virtue of diminished capacity for base pairing with the bottom strand, thus creating, in essence, a "suicide substrate" for topoisomerase cleavage.

5'-Deleted top-strand oligonucleotides were also 3'-end-labeled and tested for cleavage. 3'-³²P-Labeled JP10 (+12 → -13) and 3'-³²P-labeled JP11 (+6 → -13) were each cleaved by topoisomerase to yield a single labeled product of 13

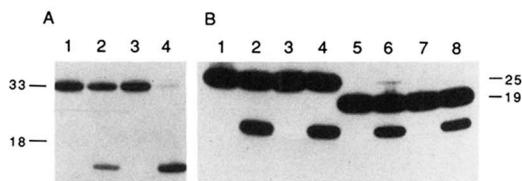


FIG. 4. Cleavage of 3'-end-labeled oligonucleotides. *A*, oligonucleotide JP6 was hybridized to JP2 and 3'-end-labeled with [α -³²P]dGTP using the Klenow DNA polymerase. Labeled oligonucleotide (33-mer) was gel-purified and hybridized to either JP9 or JP12. Cleavage reactions contained 50 fmol of 3'-end-labeled duplex oligonucleotide and 200 fmol of vaccinia topoisomerase (*lanes 2 and 4*). Control reactions contained no enzyme (*lanes 1 and 3*). The DNA substrates were JP6/JP9 (*lanes 1 and 2*) and JP6/JP12 (*lanes 3 and 4*). The positions and sizes (in nucleotides) of the labeled DNA substrate and an 18-mer marker oligomer are indicated to the *left*. *B*, oligonucleotides JP10 and JP11 were hybridized to JP2 and 3'-end-labeled with [α -³²P]dGTP. Purified labeled DNAs (25- and 19-mers) were hybridized to JP9 and JP14. Cleavage reactions contained 125 fmol of 3'-end-labeled duplex oligonucleotide and 400 fmol of vaccinia topoisomerase (*lanes 2, 4, 6, and 8*). Control reactions contained no enzyme (*lanes 1, 3, 5 and 7*). The DNA substrates were JP10/JP9 (*lanes 1 and 2*), JP10/JP14 (*lanes 3 and 4*), JP11/JP9 (*lanes 5 and 6*), and JP11/JP14 (*lanes 7 and 8*). The positions and sizes (in nucleotides) of the labeled DNA substrates are indicated to the *right*.

nucleotides (Fig. 4*B*). The efficiency of the reaction did not vary whether the complementary strand included sequence from nucleotides +20 to -13 (Fig. 4*B*, *lanes 2 and 6*) or sequence from nucleotides +12 to -13 (Fig. 4*B*, *lanes 4 and 8*). These results indicated that cleavage occurred exclusively at position +1, with as few as 6 nucleotides of upstream sequence information on the cleaved DNA strand. Other experiments established that topoisomerase cleaved a duplex 12-mer oligonucleotide substrate (JP21/JP23; see Table I) containing only sequences from +6 to -6 nucleotides (data not shown). Thus, the enzyme was active on a duplex DNA molecule having little more than one helical turn.

Effect of Point Mutations in CCCTT Motif on Topoisomerase Cleavage—26-Mer oligonucleotides (+20 → -6) were prepared that contained single nucleotide substitutions at position +3 (C → A) or +1 (T → A) in the 5'-CCCTT element of the cleaved DNA strand (JP16 and JP17; Table I). Mutated bottom-strand oligonucleotides included single nucleotide substitutions at complementary position +3 (G → T) or +1 (A → T) in the 3'-GGGAA sequence of the noncleaved strand (JP18 and JP19; Table I). Radiolabeled wild-type and mutant top strands were hybridized to wild-type and mutant bottom strands to provide heteroduplex and homoduplex mutant substrates for the topoisomerase cleavage reaction (Fig. 5). Neither the wild-type CCCTT substrate or the mutant CCATT and CCCTA substrates were cleaved by the enzyme in the absence of a complementary DNA strand (Fig. 5, *lanes 1, 5, and 9*) under conditions in which cleavage of the wild-type duplex substrate was nearly quantitative (Fig. 5, *lane 2*). C → A mutation at position +3 of the top strand completely abrogated cleavage in the context of a heteroduplex with the wild-type bottom strand (Fig. 5, *lane 10*). G → T mutation at position +3 of the bottom strand also had a profoundly suppressive effect on the cleavage reaction (Fig. 5, *lane 4*). Interestingly, the homoduplex mutant substrate at position +3, with mutations in both strands that serve to restore complete base pairing, was cleaved by the topoisomerase (Fig. 5, *lane 12*), although considerably less well than was wild-type DNA. Thus, the vaccinia enzyme appeared to be more sensitive to perturbations in DNA secondary structure than to changes in nucleotide sequence in the vicinity of the

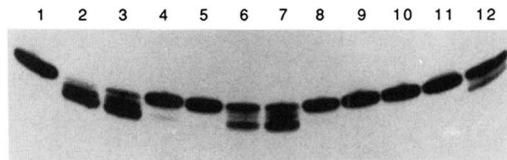


FIG. 5. Effect of DNA point mutations on topoisomerase cleavage: influence of DNA secondary structure and nucleotide sequence. Cleavage reactions contained 170 fmol of 5'-end-labeled oligonucleotide and 400 fmol of vaccinia topoisomerase. The DNA substrates were JP7 (*lane 1*), JP7/JP12 (*lane 2*), JP7/JP18 (*lane 3*), JP7/JP19 (*lane 4*), JP16 (*lane 5*), JP16/JP2 (*lane 6*), JP16/JP18 (*lane 7*), JP16/JP19 (*lane 8*), JP17 (*lane 9*), JP17/JP12 (*lane 10*), JP17/JP18 (*lane 11*), and JP17/JP19 (*lane 12*). Only the upper (cleaved) DNA strands were 5'-end-labeled. The sequences of the pentamer motifs for each DNA substrate are displayed in the box below the autoradiograph according to the sample lane that contains that substrate. The complete nucleotide sequences of the oligonucleotides are listed in Table I.

cleavage site. Mutations at position +1, *i.e.* the nucleotide to which the enzyme becomes covalently bound, adversely affected DNA cleavage, but considerably less so than did changes at position +3. Cleavage of the CCCTA mutant was suppressed partially whether the mutant was paired with a wild-type bottom strand or a compensatory mutant GGGAT strand (Fig. 5, *lanes 6 and 7*). Only slight inhibition of cleavage occurred when the cleaved T residue in the top strand was mispaired with a T residue in the bottom strand (Fig. 5, *lane 3*). Double heteroduplex mutants containing mispaired bases at both positions +3 and +1 were inert as substrates (Fig. 5, *lanes 8 and 11*).

Differential Effects of Point Mutations on DNA Binding and Cleavage—Previous studies (28) had suggested that DNA cleavage by vaccinia topoisomerase was a two-step reaction in which noncovalent binding of enzyme to DNA preceded covalent complex formation. Therefore, the observed effects of point mutations on DNA cleavage (Fig. 5) could be due either to inhibition of enzyme binding to DNA or to inhibition of DNA cleavage by enzyme already bound, or both. To address this point, the equilibrium dissociation constant for DNA binding (K_D) and the equilibrium constant for the DNA cleavage reaction (K_{eq}) were determined for wild-type and mutant duplex DNA substrates using a nitrocellulose filter binding assay. The substrates were duplex 26-mers radiolabeled in the upper DNA strand containing the pentamer sequences indicated in Table II. The oligonucleotide containing the CCCTT consensus motif was bound by enzyme with a K_D of 4 nM. The same value (4 nM) was reported previously for vaccinia topoisomerase binding to pUC19 linear duplex DNA (28). Essentially all of the wild-type 26-mer DNA bound to protein was, in fact, bound covalently, as judged by the equivalent extent of filter binding in the presence and absence of protein denaturant. Therefore, the K_{eq} was estimated to be ≥ 50 . This value contrasted sharply with the K_{eq} of 0.16 estimated for topoisomerase cleavage of pUC19 linear duplex DNA (28); the implications of this disparity are considered under “Discussion.” Equilibrium parameters for the heteroduplex mutations at position +3 revealed a significant decrease in binding affinity (4–6-fold), but were more remarkable for the drastic decrease in the equilibrium constant of the

TABLE II

Effect of DNA sequence mutation and base mispairing on DNA binding and cleavage by topoisomerase I

Sequence	K_D (binding) nM	K_{eq} (cleavage)	K_{eq}/K_D M^{-1}
CCCTT GGGAA	4	≥ 50	$\geq 1.3 \times 10^{10}$
CCATT GGGAA	27	≤ 0.01	$\leq 4 \times 10^5$
CCCTT GGTAA	16	0.5	3×10^7
CCATT GGTAA	9	0.7	8×10^7
CCCTA GGGAA	7	6	9×10^8
CCCTA GGGAT	6	9	1.5×10^9

cleavage reaction. Creation of a properly base-paired homoduplex mutation at position +3 increased binding affinity, but did not restore equilibrium conditions favorable to cleavage. Heteroduplex and homoduplex mutations at position +1 had little effect on binding affinity, but did decrease the K_{eq} for cleavage by severalfold. The overall effect of a given mutation on the cleavage reaction was expressed quantitatively as the ratio of K_{eq} to K_D . Accordingly, it was clear that: (i) mutations at different sites within the CCCTT motif had effects on cleavage that differed in magnitude, i.e. in the range of a 1,000–10,000-fold depression of cleavage for mutations at position +3 versus a 10-fold depression for mutations at position +1; (ii) mutations had more prominent effects on cleavage than on binding; (iii) alterations of secondary structure had more severe effects than alterations of nucleotide sequence; and (iv) at least at position +3, mutation of the cleaved-strand sequence had more profound consequences than mutation of the bottom strand.

Inhibition of DNA Binding by Competitor DNAs: Role of Individual DNA Strands—The finding that the consensus region upstream of the cleavage site must be base-paired raised an intriguing question. Does vaccinia topoisomerase recognize the CCCTT binding motif in the cleaved DNA strand or the complementary sequence 3'-GGGAA in the noncleaved strand, or both? This was addressed by performing competition experiments using the nitrocellulose filter binding assay. Purified topoisomerase was incubated with radiolabeled duplex DNA (JP7/JP12 (+20 → -6)) in the presence of increasing amounts of unlabeled duplex JP7/JP12 and unlabeled upper-strand (JP7) or unlabeled lower-strand (JP12) oligonucleotides. Filter binding was effectively competed by unlabeled duplex DNA in a concentration-dependent manner (Fig. 6, left). Binding was also competed by single-stranded JP7, albeit to about half the extent as did duplex DNA. In contrast, the noncleaved strand JP12 had no discernible influence on the binding of topoisomerase to the duplex substrate (Fig. 6, left). These data suggested that the nucleotide sequence information responsible for cleavage specificity of the vaccinia topoisomerase resided primarily in the cleaved DNA strand.

Selective Binding of CCCTT-containing DNA in Presence of DNAs of Related Sequence—Purified topoisomerase did cleave mutant oligonucleotides, albeit inefficiently, when no alternative substrates were available to the enzyme (Fig. 5, lane 7). It was of interest therefore to determine whether the

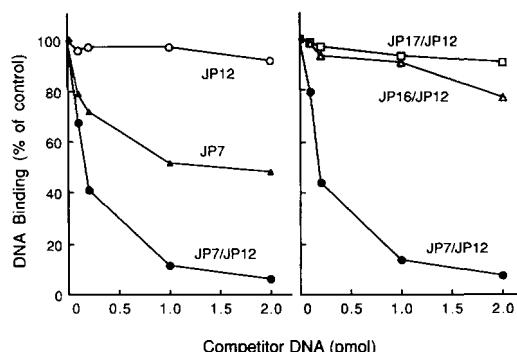


FIG. 6. Competition of topoisomerase-DNA complex formation by DNA oligonucleotides. Reaction mixtures contained 40 fmol of 5'-³²P-labeled JP7/JP12 duplex oligonucleotide, 200 pmol of vaccinia topoisomerase, and unlabeled competitor DNAs as indicated. DNA binding to protein was assayed by nitrocellulose filter binding as described under "Experimental Procedures." The absolute value of DNA binding for each sample (i.e. the percent of input DNA retained on the filter) is plotted as the extent of binding (percent) relative to control reactions (100%) containing no competitor DNA. The extent of binding in these control reactions was 85% (left) and 87% (right).

enzyme could discriminate between closely related sequences when present simultaneously. That this was indeed the case was shown by testing the ability of variant DNA sequences to compete for binding by enzyme of a CCCTT-containing 26-mer (Fig. 6, right). It was found, as before, that binding of radiolabeled wild-type oligomer was effectively competed by excess unlabeled DNA of the same sequence. In contrast, mutant oligonucleotides containing nucleotide substitutions at positions +3 and +1 were considerably less effective as competitors.

DISCUSSION

Topoisomerase Cleavage of Defined Oligonucleotides—Cleavage of linear duplex DNA by vaccinia type I topoisomerase occurs nonrandomly and infrequently. The finding of a conserved sequence element (5'-(C/T)CCCTT↓) at several sites of cleavage suggested that primary sequence may play a role in the process of DNA recognition and covalent catalysis (28). In this study, the issue was addressed through the use of defined DNA oligonucleotides containing a known high affinity cleavage site. Two features of the vaccinia virus type I DNA topoisomerase are underscored by this analysis, i.e. that the covalent interaction of enzyme with DNA is indeed sequence-specific and that covalent adduct formation requires DNA secondary structure.

In duplex oligonucleotides, as in plasmid DNA, the vaccinia topoisomerase cleaves selectively at the 5'-CCCTT↓ motif. Deletion of DNA sequences around the 5'-CCCTT↓ consensus sequence indicates that as few as 6 nucleotides upstream of the cleavage site (GCCCTT) and 6 nucleotides downstream of the site are sufficient to support covalent complex formation. Thus, the topoisomerase can interact effectively with a DNA fragment containing little more than one duplex helical turn. A stringent requirement for DNA conformation, e.g. bending, wrapping, or looping, in the formation of the catalytic intermediate is thereby excluded. Studying the effects of additional incremental deletions in the 5'- and 3'-directions should reveal a true "minimal" substrate for cleavage. Prokaryotic type I topoisomerase has been shown to cleave single-stranded oligomers as small as 7–8 nucleotides in length, with cleavage occurring between nucleotides 4 and 5 from the 3'-end of the substrate (32). Wheat germ topoisomerase I can cleave a 22-bp duplex oligonucleotide containing 16 nucleo-

tides upstream and 6 nucleotides downstream of a site of strand scission (33). Footprinting of the *Tetrahymena* topoisomerase I at the hexadecameric rDNA cleavage element indicates that the enzyme protects both strands over a segment of 15–19 nucleotides within which the cleavage site is centrally located (14).

The requirement for DNA secondary structure in vaccinia topoisomerase-mediated cleavage of oligonucleotides is virtually total. This observation confirms those of Been and Champoux (34), derived from their studies of DNA cleavage by rat liver and wheat germ topoisomerases, that eukaryotic type I enzymes cleave single-stranded DNA at regions of intramolecular duplex structure and that they are inactive on regions of single-stranded DNA that are not base-paired. The vaccinia enzyme is exquisitely sensitive to even single base mismatches (at position +3) within the putative CCCTT cleavage motif. It is possible then that relaxation of negatively supercoiled plasmid DNA by the vaccinia enzyme occurs via selective interaction with fully duplex regions of the plasmid rather than with locally underwound segments (that are partially single-stranded in character). This is consistent with the ability of vaccinia topoisomerase (and all eukaryotic type I enzymes) to relax positively supercoiled substrates. Prokaryotic type I topoisomerase, on the other hand, forms its covalent intermediate with single-stranded DNA regions (35). Cleavage sites for prokaryotic topoisomerase I in negatively supercoiled DNA correlate with regions of single-strandedness, as demonstrated by S1 nuclease sensitivity (36). The interaction of vaccinia topoisomerase I with negatively supercoiled DNA has not yet been characterized.

Accuracy of Cleavage—If the (C/T)CCCTT consensus sequence represents a true recognition motif for the vaccinia topoisomerase, analogous to the canonical recognition sequences (typically 4-, 5-, or 6-bp elements) that distinguish individual type II restriction endonucleases, then it is expected that the topoisomerase should discriminate between substrates that contain the pentamer motif and substrates that differ in 1 base pair. Preliminary mutational analysis of the conserved motif suggests that this is the case, but that the specificity of the topoisomerase is not absolute. Changing a C/G → A/T at position +3 strongly suppresses cleavage, reducing the equilibrium constant of the reaction by >50-fold. In contrast, a T/A → A/T change at position +1 has only a mild effect on the reaction equilibrium. This is surprising at first because cleavage of plasmid DNA seems to occur only at T residues (28). The extent to which nonconsensus sequences are cleaved by topoisomerase (“star” activity) apparently varies with the location of the deviant base pair within the conserved motif. Position-dependent variations in star site activity have also been noted for the *EcoRI* endonuclease (37).

Several caveats arise in evaluating these experiments. First, the cleavage of “mutated” sequences presented to the enzyme in isolation does not imply that these sequences will be cleaved as well in the context of a more complex DNA molecule. Indeed, the mutant sequence at position +1 is not an effective competitor of the cleavage of a wild-type consensus element. Second, the cleavage reactions are performed under conditions (low ionic strength in the absence of a divalent cation) that favor cleavage at low affinity sites. Conceivably, as is the case with many restriction enzymes, the degree of topoisomerase star-like activity may vary considerably with reaction conditions. Third, a more exhaustive mutational analysis must be performed, under a variety of reaction conditions, before the sequence specificity of the cleavage reaction may be fully understood. Nonetheless, these data are consistent with selection of cleavage sites in complex DNA being governed by the

(C/T)CCCTT DNA sequence immediately upstream of the site.

Noncovalent binding of vaccinia topoisomerase to duplex DNA restriction fragments (at limiting concentrations of enzyme) correlates with the number and/or the relative affinity of cleavage sites contained therein; in large enzyme excess, duplex DNAs are bound nonselectively (28). Point mutations within the CCCTT motif reduce the affinity of the topoisomerase for defined duplex oligonucleotides containing a single binding site; however, the magnitude of the binding effect is far less than the effect on cleavage (see Table II). As with the cleavage reaction, substitutions at position +3 have a greater effect on binding than do changes at position +1. Apparently, vaccinia topoisomerase can bind DNA noncovalently (as assessed by filter binding) in a sequence-independent manner, albeit with reduced affinity. Again, certain caveats apply. (i) The effects of reaction conditions on nonspecific binding versus sequence-specific binding have not been evaluated; and (ii) the nitrocellulose filter binding assay does not reveal where on the DNA the enzyme is bound. Stevnser *et al.* (14) have shown, using a nuclease protection assay, that a single T → A mutation in the rDNA motif abolishes noncovalent binding of *Tetrahymena* topoisomerase I to the region of the cleavage site.

As discussed above, secondary structure in the CCCTT motif is more critical to the cleavage reaction than is primary sequence insofar as single base mismatches are more inhibitory than are base-paired mutations (at either position +3 or +1). This is the case even when the noncleaved strand is mutated and the cleaved strand sequence is unperturbed. It is noteworthy that the converse is true for the *EcoRI* endonuclease; the restriction enzyme is better able to bind and cleave heteroduplex recognition sequences containing an altered base in one strand than homoduplex substrates with a base-paired mutation in both strands (37).

Implications for Recombination—The equilibrium constant for the cleavage of linear plasmid DNA by vaccinia topoisomerase is estimated to be 0.16, indicating that noncovalent interaction with DNA predominates over covalent adduct formation (28). It is evident, however, that the efficiency of the cleavage reaction can be influenced by the length of the DNA strand. Progressive shortening of the substrate 5' to the cleavage site has no qualitative effect on the cleavage reaction; however, deletion of DNA 3' to the cleavage site (*e.g.* from 12 to 6 nucleotides) increases significantly the extent of strand scission, such that cleavage becomes virtually quantitative (see Figs. 2 and 3 and Table II). This is observed whether the deletion is in the cleaved strand or the noncleaved strand, suggesting that the degree of the base pairing 3' to the cleavage site is the key variable. This effect is probably caused by instability of the short duplex sequence 3' to the cleavage site, which, upon dissociation of the distal segment of the cleaved strand, generates a suicide intermediate with no readily available acceptor for religation. Apparently, the vaccinia enzymes does not “hold on” to the DNA 3' to the point of scission of the suicide substrate once the covalent intermediate has formed. Are such intermediates recombinogenic?

Suicide cleavage substrates have been described for another type I topoisomerase, λ -integrase. Creation of a medial nick in the overlap region of *attB* generates, upon strand scission, an unstable 3-bp duplex segment 3' to the cleavage site. Dissociation of the distal fragment results in significantly increased yield of covalent intermediate compared to conventional cleavage substrates (38). Through the use of specialized substrates that stabilize the covalent intermediate, Nash and Robertson (39) and Nunes-Duby *et al.* (40) have shown that integrase protein can join the covalently bound strand indis-

criminate to a receptor DNA. Although the ability to catalyze heterologous strand transfer has not been demonstrated for the vaccinia topoisomerase, as it has for λ -integrase and for the cellular type I topoisomerase (33, 41), the finding that the vaccinia enzyme can promote excisive recombination of λ -prophage *in vivo* suggests that such a reaction is likely (25). Champoux and co-workers (42, 43) have argued persuasively that eukaryotic topoisomerase I plays a role in illegitimate recombination. Given the sequence specificity of the vaccinia enzyme, it should be feasible to design model substrates for topoisomerase-catalyzed recombination *in vitro*. The relevance of the sequence specificity of the cleavage reaction to an *in vivo* model of topoisomerase-mediated illegitimate recombination will require sequencing of the recombination junctions of isolated phage excisants (25), as has been done for the products of SV40 excision (42).

REFERENCES

1. Wang, J. C. (1971) *J. Mol. Biol.* **55**, 523-533
2. Champoux, J. J., and Dulbecco, R. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 143-146
3. Champoux, J. J. (1981) *J. Biol. Chem.* **256**, 4805-4809
4. Lynn, R. M., Bjornsti, M., Caron, P. R., and Wang, J. C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 3559-3563
5. Eng, W., Pandit, S. D., and Sternglanz, R. (1989) *J. Biol. Chem.* **264**, 13373-13376
6. Shuman, S., Kane, E. M., and Morham, S. G. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 9793-9797
7. Champoux, J. J. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 3800-3804
8. Edwards, K. A., Halligan, B. D., Davis, J. L., Nivera, N. L., and Liu, L. F. (1982) *Nucleic Acids Res.* **10**, 2565-2576
9. Been, M. D., Burgess, R. R., and Champoux, J. J. (1984) *Nucleic Acids Res.* **12**, 3097-3114
10. Been, M. D., Burgess, R. R., and Champoux, J. J. (1984) *Biochim. Biophys. Acta* **782**, 304-312
11. Bonven, B. J., Gocke, E., and Westergaard, O. (1985) *Cell* **41**, 541-551
12. Christiansen, K., Bonven, B. J., and Westergaard, O. (1987) *J. Mol. Biol.* **193**, 517-525
13. Thomsen, B., Mollerup, S., Bonven, B. J., Frank, R., Blocker, H., Nielsen, O. F., and Westergaard, O. (1987) *EMBO J.* **6**, 1817-1823
14. Stevnsner, T., Mortensen, U. H., Westergaard, O., and Bonven, B. J. (1989) *J. Biol. Chem.* **264**, 10110-10113
15. Busk, H., Thomsen, B., Bonven, B. J., Nielsen, O. F., and Westergaard, O. (1987) *Nature* **327**, 638-640
16. Brill, S. J., DiNardo, S., Voelkel-Meiman, K., and Sternglanz, R. (1987) *Nature* **326**, 414-416
17. Fleischmann, G., Pflugfelder, G., Steiner, E. K., Javaherian, K., Howard, G. C., Wang, J. C., and Elgin, S. C. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 6958-6962
18. Egyhazi, E., and Durban, E. (1987) *Mol. Cell. Biol.* **7**, 4308-4316
19. Zhang, H., Wang, J. C., and Liu, L. F. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1060-1064
20. Caserta, M., Amader, A., DiMauro, E., and Camilloni, G. (1989) *Nucleic Acids Res.* **17**, 8463-8474
21. Bauer, W. R., Ressner, E. C., Kates, J., and Patzke, J. V. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 1841-1845
22. Shaffer, R., and Traktman, P. (1987) *J. Biol. Chem.* **262**, 9309-9315
23. Shuman, S., and Moss, B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7478-7482
24. Shuman, S., Golder, M., and Moss, B. (1989) *Virology* **170**, 302-306
25. Shuman, S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 3489-3493
26. Kikuchi, Y., and Nash, H. A. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 3760-3764
27. Landy, A. (1989) *Annu. Rev. Biochem.* **58**, 913-949
28. Shuman, S., and Prescott, J. (1990) *J. Biol. Chem.* **265**, 17826-17836
29. Yanisch-Perron, C., Viera, J., and Messing, J. (1985) *Gene (Amst.)* **33**, 103-109
30. Shuman, S., Golder, M., and Moss, B. (1988) *J. Biol. Chem.* **263**, 16401-16407
31. Riggs, A. D., Suzuki, H., and Bourgeois, S. (1970) *J. Mol. Biol.* **48**, 67-83
32. Tse-Dinh, Y. C., McCarron, B. H. G., Arentzen, R., and Chowdry, V. (1983) *Nucleic Acids Res.* **11**, 8691-8701
33. Champoux, J. J., McCoubrey, W. K., and Been, M. D. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49**, 435-442
34. Been, M. D., and Champoux, J. J. (1984) *J. Mol. Biol.* **180**, 515-531
35. Depew, R. E., Liu, L. F., and Wang, J. C. (1978) *J. Biol. Chem.* **253**, 511-518
36. Shisido, K., Noguchi, N., and Ando, T. (1983) *Biochim. Biophys. Acta* **740**, 108-117
37. Thieling, V., Alves, J., Fliess, A., Maass, G., and Pingoud, A. (1990) *Biochemistry* **29**, 4682-4691
38. Pargellis, C. A., Nunes-Duby, S. E., Moitoso de Vargas, L., and Landy, A. (1988) *J. Biol. Chem.* **263**, 7678-7685
39. Nash, H. A., and Robertson, C. A. (1989) *EMBO J.* **8**, 3523-3533
40. Nunes-Duby, S. E., Matsumoto, L., and Landy, A. (1989) *Cell* **59**, 197-206
41. Halligan, B. D., Davis, J. L., Edwards, K. A., and Liu, L. F. (1982) *J. Biol. Chem.* **257**, 3995-4000
42. Bullock, P., Champoux, J. J., and Botchan, M. (1985) *Science* **230**, 954-958
43. Champoux, J. J., and Bullock, P. A. (1988) in *Genetic Recombination* (Kucherlapati, R., and Smith, G. R., eds) pp. 655-666, American Society for Microbiology, Washington, D. C.
44. 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
45. Craig, N. L., and Nash, H. A. (1983) *Cell* **35**, 795-803
46. Been, M. D., and Champoux, J. J. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 2883-2887