The EMBO Journal vol.12 no.5 pp.2085–2097, 1993

Mutational analysis of a type II topoisomerase cleavage site: distinct requirements for enzyme and inhibitors

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Communicated by C.Paoletti

We have analyzed the DNA sequence requirements for cleavage of a 30 bp oligonucleotide that contains a strong bacteriophage T4 type II topoisomerase site. A novel method was used to generate substrates with each of the four nucleotides at 10 positions surrounding the cleavage site, and mutant substrates were also prepared for the four internal positions of the staggered cleavage site. The substrates were tested for cleavage in the presence of several inhibitors that induce enzyme-mediated cleavage: four antitumor agents of different classes (an aminoacridine, a substituted anthraquinone, an ellipticine derivative and an epipodophyllotoxin) and one antibacterial quinolone. At eight nucleotide positions flanking the cleavage site, the same preferred bases were found regardless of which inhibitor was present. These preferred bases show dyad symmetry with respect to the cleavage site, indicating that both protomers of the topoisomerase homodimer interact with DNA in an analogous manner. In addition, we found that the preferred bases on the 5′ side of each cleaved phosphodiester bond are highly specific to the inhibitor used in the cleavage reaction. These results strongly suggest that the inhibitors interact directly with the DNA bases at the cleavage site, placing the inhibitor binding site precisely at the site of DNA cleavage.

Key words: antitumor agent/bacteriophage T4/inhibitor/oligonucleotide/type II topoisomerase

Introduction

The type II topoisomerases are important cellular enzymes which alter the topological state of DNA (for reviews see Maxwell and Gellert, 1986; Hsieh, 1990). All known type II topoisomerases introduce a transient break into one segment of DNA and pass a second duplex segment through the double-stranded break. Depending on the nature of the two DNA segments, this reaction mechanism can result in the introduction or removal of two superhelical twists, the knotting or unknotting of a circular substrate, or the catenation or decatenation of two circular DNAs. The physiologic roles of type II topoisomerases include introduction and removal of DNA supercoils (Liu and Wang, 1987; Drlica, 1990; Reece and Maxwell, 1991), segregation of DNA molecules during mitosis (Uemura *et al.*, 1987; Holm *et al.*, 1989; Downes *et al.*, 1991) and possibly attachment of DNA loops to eukaryotic chromosomal scaffolds (Berrios *et al.*, 1985; Earnshaw and Heck, 1985; Earnshaw *et al.*, 1985). The bacteriophage T4 type II topoisomerase, which is the focus of this study, is required for normal phage DNA replication and transcription (Yegian *et al.*, 1971; Mufti and Bernstein, 1974; McCarthy *et al.*, 1976; Mosig *et al.*, 1983) and may also be important for membrane–DNA association and for packaging DNA from the complex networks that are generated during the course of the infective cycle.

The type II topoisomerases are the targets of numerous chemotherapeutic agents, fostering ongoing clinical interest in these enzymes (for reviews see Ross *et al.*, 1988; D'Arpa and Liu, 1989; Liu, 1989). Specifically, bacterial DNA gyrase is inhibited by the quinolone antibacterial agents, and mammalian type II topoisomerases are inhibited by several classes of antitumor agents, including the acridines, anthracyclines, ellipticines and epipodophyllotoxins. Each of these inhibitors apparently acts by stabilizing an intermediate in the topoisomerase reaction cycle. This intermediate, termed the 'cleavage complex', consists of the topoisomerase covalently attached to the 5′ ends of a 4 bp staggered DNA break (Nelson *et al.*, 1984; Tewey *et al.*, 1984a,b; Zechiedrich *et al.*, 1989). Although the cleavage complex can be detected at a low level in the absence of inhibitor, the presence of an appropriate inhibitor greatly increases the amount of complex to the extent that nearly every enzyme molecule can be trapped in a cleavage complex. The simplest model to explain stabilization of the cleavage complex is that the inhibitors interfere with the religation step of the topoisomerase reaction cycle (Nelson *et al.*, 1984; Shen *et al.*, 1989b; Capranico *et al.*, 1990a; Robinson and Osheroff, 1990).

Although the topoisomerase cleavage complexes form at specific DNA sites, the determinants which define cleavage sites are not understood. Consensus sequences have been derived for bacterial DNA gyrase (Morrison and Cozzarelli, 1979; Lockshon and Morris, 1985) and eukaryotic type II topoisomerases (Sander and Hsieh, 1985; Spitzner and Muller, 1988; Capranico *et al.*, 1990a; Fossé *et al.*, 1991; Pommier *et al.*, 1991). In each of these studies, most significant nucleotide preferences were found within 5–10 nucleotides of the cleavage sites, and strong cleavage sites tended to match the consensus better than weak sites. However, the consensus sequences were only partially successful at predicting new cleavage sites; some cleavage sites did not match the consensus and some relatively good consensus matches were not cleaved by the enzyme (Sander and Hsieh, 1985; Spitzner and Muller, 1988). In addition, the consensus sequences determined in the studies cited above do not agree, although the lack of agreement could be caused by differences in the biological sources of the enzyme and/or the presence/absence of specific inhibitors. Finally, the consensus sequence approach has led to conflicting conclusions on whether the topoisomerase homodimer recognizes a dyad axis of symmetry (Sander

and Hsieh, 1985; Spitzner and Muller, 1988; Capranico *et al.*, 1990a; Fossé *et al.*, 1991; Pommier *et al.*, 1991).

The above discrepancies could be caused by problems of the consensus sequence approach, in particular, the selection of one of two DNA strands for analysis, the inclusion of weak cleavage sites and the possible merging of several classes of cleavage sites. A mutational analysis of a single topoisomerase cleavage site could avoid these problems and complement the consensus approach. Strand selection is not required for a mutational analysis, and a strong cleavage site could be selected for analysis. If there are several classes of cleavage sites, a mutational analysis should define the rules governing selection of one class rather than averaging information from different classes. Most importantly, a mutational analysis should accurately reveal both decreases and increases in the efficiency of cleavage caused by simple base substitutions in and around the cleavage site.

Topoisomerase–DNA interactions undoubtedly contribute to cleavage site selection because specific sites are cleaved by the enzyme in the absence of inhibitor (Brown *et al.*, 1979; Kirkegaard and Wang, 1981; Kreuzer and Alberts, 1984; Fisher *et al.*, 1986). Nonetheless, the inhibitors mentioned above appear to modulate cleavage site selection. The sites cleaved by both the mammalian and T4 topoisomerases are altered in a unique manner by each class of inhibitor (Ross *et al.*, 1984; Tewey *et al.*, 1984a,b; Rowe *et al.*, 1986; Capranico *et al.*, 1990a,b; Huff and Kreuzer, 1990). Therefore, the enzyme inhibitors may alter the rules which govern cleavage site selection or stabilize only a subclass of cleavage sites. Recent consensus sequence approaches strongly suggest that the bases immediately adjacent to the cleaved phosphodiester bonds are involved in the alteration of cleavage site specificity by inhibitors (Capranico *et al.*, 1990a; Fossé *et al.*, 1991; Pommier *et al.*, 1991; see Discussion).

Several models have been proposed in which the inhibitors bind near the active site of the enzyme, in the immediate vicinity of the two cleaved phosphodiester bonds (Liu, 1989; Shen *et al.*, 1989b; Capranico *et al.*, 1990a; Pommier *et al.*, 1991). Considering the inhibitor binding site, it is interesting that structurally diverse classes of inhibitors induce cleavage by a given type II topoisomerase. With both the mammalian and phage T4 enzymes, the relevant inhibitors include intercalators [e.g. 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA), mitoxantrone diacetate (mitoxantrone), 2-methyl-9-hydroxyellipticinium acetate (2-me-9-OH-E^+)] as well as non-intercalators [e.g. VP-16 (etoposide or 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside)] (Liu *et al.*, 1989; Huff and Kreuzer, 1990). Nonetheless, the diverse inhibitors probably bind to a common site, because mutant topoisomerases selected for resistance to one drug often show cross-resistance to other inhibitors (Sullivan *et al.*, 1989; Zwelling *et al.*, 1989; Huff and Kreuzer, 1990). A variety of results suggests that this common binding site consists of both protein and DNA. Most importantly, a radiolabeled quinolone forms a complex with DNA plus gyrase, but not with either component alone (Shen *et al.*, 1989a). In addition single point mutations in the T4 topoisomerase can alter DNA recognition and inhibitor sensitivity simultaneously, implying that DNA and inhibitor interact with common residues of the protein (Huff *et al.*, 1989; Huff and Kreuzer, 1990).

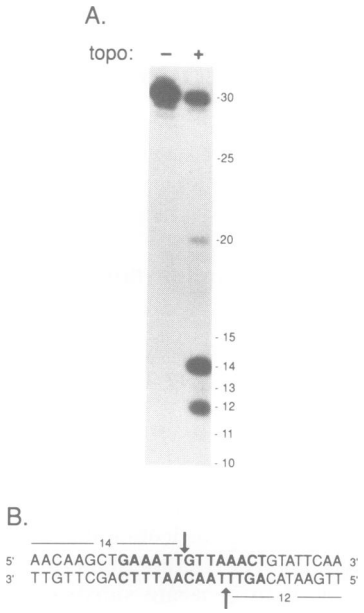

Fig. 1. Topoisomerase cleavage of the 30 bp substrate. (**A**) A 7 base primer was annealed to the 30 base oligonucleotide (bottom strand in B) containing the *rIIB* topoisomerase cleavage site sequence. The primer was extended by incubation with Klenow polymerase and cold nucleotide mix, and the full-length product was purified from a native 20% polyacrylamide gel. The purified duplex 30mer was then 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Approximately 0.3 pmol labeled duplex was incubated with (+) or without (–) 0.82 pmol topoisomerase in a 10 μl reaction containing *m*-AMSA (12.5 μM) and one-sixth of the products were then separated by electrophoresis through a denaturing 15% polyacrylamide gel. The cleavage products, which were visualized by autoradiography, had the expected size for cleavage at the *rIIB* site (14 and 12 bases; see Ripley *et al.*, 1988). A minor cleavage site resulted in faint bands of 20 and 6 bases. The size scale on the right was generated from the migration of sequencing reactions which were run on the same gel. (**B**) The sequence of the 30 bp oligonucleotide substrate is depicted. The principal cleavage site is denoted by arrows, and the expected cleavage products from each strand are indicated. The bold letters indicate the positions studied by mutational analysis in this report.

The phage T4 topoisomerase used in this study is similar to the eukaryotic type II topoisomerase with respect to enzymatic activities and antitumor agent sensitivities (Kreuzer and Jongeneel, 1983; Huff and Kreuzer, 1990). In addition to the wild-type T4 enzyme, two *m*-AMSA-resistant topoisomerases have been purified and characterized: the *52*-A^R mutant enzyme with an altered gene *52* subunit (breaking–resealing) and the *39*-A^R mutant with an altered gene *39* subunit (ATPase) (Huff *et al.*, 1989, 1990). Both mutant enzymes show cross-resistance to other topoisomerase inhibitors. The *39*-A^R enzyme is particularly interesting because it appears to have a structural rearrangement in the inhibitor binding site. The *39*-A^R mutation caused differential effects on sensitivity to three members of the ellipticine family, implying that the mutation affects a region of the enzyme interacting with the ellipticine side groups (Huff and Kreuzer, 1990). Furthermore, the *39*-A^R enzyme is ultrasensitive to both the antitumor agent VP-16 and the bacterial DNA gyrase inhibitor oxolinic acid, suggesting that the altered binding site favors binding of certain inhibitors. Both mutant T4 enzymes show altered DNA cleavage site specificity and also display increased amounts of DNA cleavage in the absence of inhibitor.

In the present study we have analyzed the DNA sequence

requirements for T4 topoisomerase-induced cleavage of a particular site. The results indicate that the enzyme recognizes DNA sites by binding with dyad symmetry to the regions flanking the cleavage site. In addition, the results strongly support models in which the enzyme inhibitors bind near the nucleotide residues at the site of phosphodiester bond cleavage, thereby preventing religation of the cleaved phosphodiester bond.

Results

Cleavage of the wild-type 30 bp oligonucleotide and production of mutant oligonucleotides

We chose to analyze the interaction of the T4 topoisomerase with one particular cleavage site, an *m*-AMSA-induced site located in the T4 *rIIB* gene. This is the strongest *m*-AMSA-induced cleavage site within a region of ~1 kb (Ripley *et al.*, 1988). Duplex oligonucleotides containing this cleavage site were prepared and tested for *m*-AMSA-induced topoisomerase-dependent cleavage. Preliminary experiments demonstrated that a 57 bp and a 30 bp substrate, each with the strong cleavage site near the middle, were cleaved at the expected site with similar efficiencies. For example, cleavage of the 5′ ^{32}P-end-labeled 30 bp substrate by the T4 topoisomerase in the presence of *m*-AMSA yielded the expected 14 and 12 base products upon denaturing PAGE (Figure 1A). Therefore, the 30 bp substrate (Figure 1B) is sufficient for proper recognition by the T4 topoisomerase (for similar conclusions with eukaryotic type II topoisomerases, see Lee *et al.*, 1989; Lund *et al.*, 1990).

We analyzed the requirements at each of 14 nucleotide positions surrounding the cleavage site in the 30 bp substrate (bold positions in Figure 1B). The nomenclature used here refers to base positions relative to the two cleaved phosphodiester bonds. To illustrate any symmetry in the results, base preferences surrounding the top strand cleavage are indicated with respect to the top strand sequence (. . . −2,−1,+1,+2), and those surrounding the bottom strand cleavage with respect to the bottom strand sequence (+2′,+1′,−1′,−2′ . . .; see sequence at bottom of Figure 3). The detailed experimental procedure used for producing oligonucleotides containing each of the four possible bases at all positions with negative numbers will be described elsewhere (C.H.Freudenreich and K.N.Kreuzer, in preparation). Briefly, for each nucleotide position, a 30 base oligonucleotide was synthesized with 29 wild-type bases and a mixture of all four bases ('N') at the relevant position. For each position, a complementary primer was also synthesized; the primer hybridized to all bases between the 3′ end and the N nucleotide of the 30mer. Four separate extension reactions were then carried out, with different α-^{35}S-labeled deoxyribonucleotides in each to label only those template-primers with the complementary base at the N position. After labeling, a chase with an excess of all four cold deoxyribonucleotides yielded full-length duplex 30 bp products. Each substrate thus contained all four possible base pairs at the position of interest, but only one was labeled. For each set of four labeled substrates, one contained two (or more) identical bases in a row and thereby incorporated more labeled nucleotide than the other three. This and other factors that cause unequal labeling of the four substrates were negated by quantifying and then equalizing the specific activity of the four substrates to 30 000 c.p.m./pmol by adding cold extension products generated in parallel. The extension reactions generated many products that were incomplete at the 3′ end, most of which were removed by gel purification. Any remaining incomplete products would not generate labeled topoisomerase cleavage products as long as the label was 5′ to the cleavage site. For this reason, substrates with the N position to the left of the cleavage site were generated using a bottom-strand template and a top-strand primer, and those with the N to the right of the cleavage site using a top-strand template and a bottom-strand primer. Topoisomerase cleavage products from all of these labeled substrates would contain the labeled nucleotide on the strand that does not become covalently bound to the enzyme, simplying product analysis.

The method outlined above did not give acceptable results when the 'N' position was within the four-base staggered topoisomerase cleaved region, because the labeled strand became covalently linked to topoisomerase and contained the incomplete extensions. Therefore a more conventional approach was used for these four positions. For each, four different bottom strand oligonucleotides were synthesized with one of the four bases at the relevant position, and a 5′-end-labeled top strand primer was hybridized and

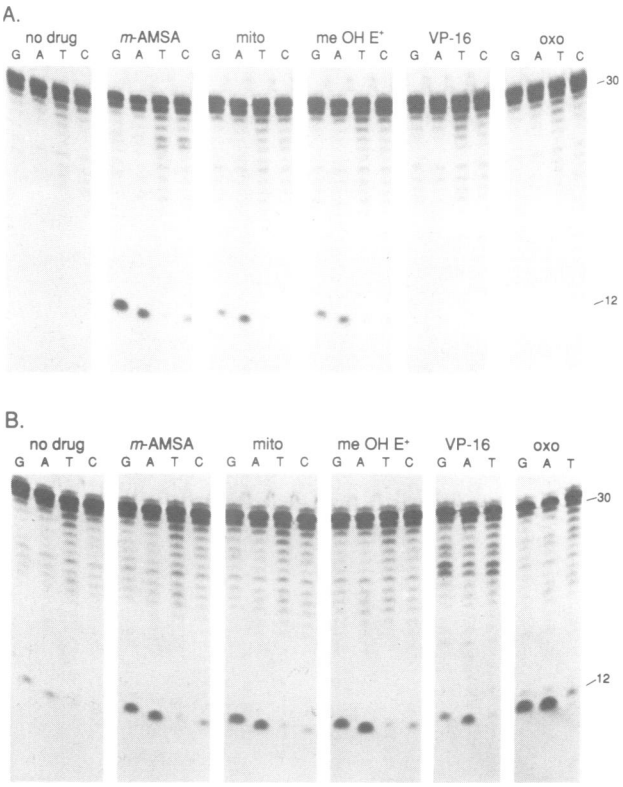

Fig. 2. Topoisomerase cleavage with nucleotide substitutions at the −3′ position. Each of the four specifically labeled double-stranded oligonucleotide substrates (radioactive G, A, T or C at position −3′; 2 pmol/10 μl reaction) were incubated with wild-type (**A**) or *39*-A^{R} (**B**) topoisomerase (3 pmol) in the presence of no drug, *m*-AMSA (12.5 μM), mitoxantrone (mito; 0.9 μM), 2-me-9-OH-E^{+} (me OH E^{+}; 7.5 μM), VP-16 (170 μM) or oxolinic acid (oxo; 1.9 mM). Cleavage products were separated on a denaturing polyacrylamide gel and visualized by autoradiography. The positions of the 30 base starting product and the expected 12 base cleavage product are indicated at the right. The ladder of bands in each lane is composed of incomplete extension products present in the untreated substrates. The C reactions for VP-16 and oxolinic acid in panel B were not done.

Table I. Quantification of topoisomerase cleavage products

WT		−6	−5	−4	−3	−2	−1	+1	+2	+2′	+1′	−1′	−2′	−3′	−4′
EXT	G	0	0	0	0	0	1.8	0.8	0	ND	0	0	0	0	0
	A	0	0	0	0	0	0	0	0	4.1	0	0	0	0	0
	T	0	0	0	0	0	0	0.7	1.3	0	1.6	0	0	0	0
	C	0	0	0	0	0	0.2	0	0	0	1.8	0	0	0	0
No drug	G	0	0	2.6	0	0	3.0	3.4	0	2.1	0	0	0	0	0
	A	0	0	3.4	0	0	0	0.2	0	4.2	0	0	0	0	0
	T	0	0	0.2	4.0	0	3.9	0.3	1.1	0.7	0.9	0	0	0	0
	C	0	0	2.4	0	0	1.1	0	0	2.0	0.3	0	0	0	0
m-AMSA	G	23	9.4	137	96	0	34	134	62	71	86	44	0.8	142	54
	A	68	98	91	66	4.0	109	200	61	70	150	104	1.2	86	15.8
	T	36	51	22	23	22	134	18.4	114	83	111	129	48	6.0	9.5
	C	58	22	27	31	0	33	43	40	91	73	35	0	18.5	6.4
MITO	G	3.4	0	35	38	0	30	35	33	17.8	29	0.4	0	31	1.3
	A	14.8	30	15.0	22	0	31	29	18.6	21	23	5.8	0	58	0.8
	T	9.6	11.9	1.8	10.6	6.8	45	4.5	24	19.6	39	57	8.0	4.2	0.8
	C	12.1	3.4	7.2	11.6	0	124	19.5	14.8	17.8	29	134	0	1.6	0
me OH E^+	G	8.0	0.4	40	44	0	18.2	61	81	43	30	2.5	0	34	22
	A	23	34	39	38	0.3	19.2	17.8	57	47	28	2.6	0	35	4.0
	T	10.2	11.1	2.8	11.2	4.7	90	8.3	55	32	39	92	14.7	6.7	5.0
	C	15.6	7.5	8.1	10.0	0	21	55	24	23	34	27	0	8.4	2.4
VP-16	G	0	0	5.4	0	0	11.0	0.8	0	3.3	0.4	0	0	0	0
	A	0	0	5.7	0	0	5.4	0	ND	5.1	0	8.3	0	0	0
	T	0	0	0.5	0.5	0	3.5	0.5	1.2	1.6	1.1	0	0	0	0
	C	0	0	4.1	0	0	6.4	0.8	0	1.6	0.2	9.8	0	0	0
OXO	G	0	0	1.2	0	0	12.0	0.3	0	4.6	6.0	0	0	0	0
	A	0	0	4.2	0	0	5.4	0	ND	2.6	0.2	2.3	0	0	0
	T	0	0	0	1.0	0	10.6	0	1.2	0	0	0	0	0	0
	C	0	0	1.3	0	0	2.4	0	0	0	1.6	0	0	0	0

39-A^R		−6	−5	−4	−3	−2	−1	+1	+2	+2′	+1′	−1′	−2′	−3′	−4′
No drug	G	0	0	3.8	0.8	0	4.0	2.4	3.7	1.8	4.6	11.2	0	11.6	0
	A	0	0	5.6	0.9	0	3.9	4.6	3.2	5.1	7.1	10.7	0	8.5	0
	T	0	0	0	0.1	0	1.9	0	2.6	1.6	6.0	13.0	0	0	0
	C	0	0	0.8	0.2	0	2.8	0	0.9	1.5	4.3	1.4	0	0	0
m-AMSA	G	3.4	0	21	19.6	0	6.5	12.5	6.0	9.9	21	32	0	35	3.1
	A	8.0	11.1	12.5	11.7	0	15.6	19.7	4.1	8.8	63	47	0	41	1.2
	T	4.3	3.4	0	7.2	2.0	8.9	2.4	6.6	9.3	25	37	3.2	1.6	0.8
	C	3.4	0.4	1.9	5.2	0	3.9	2.2	3.4	10.7	11.6	12.3	0	7.3	0
MITO	G	1.6	0	16.9	16.7	0	9.4	15.9	15.2	12.0	16.1	9.5	0	52	3.1
	A	7.1	11.2	9.7	14.4	0	12.9	22	6.3	9.9	17.7	21	0	50	1.6
	T	8.5	8.0	0	5.4	10.0	11.1	5.6	10.1	13.1	32	31	3.3	2.5	2.2
	C	7.7	2.0	3.3	9.0	0	8.5	18.7	5.5	13.6	25	15.0	0	5.2	0
me OH E^+	G	3.6	0	22	36	0	7.2	27	10.0	26	54	8.8	0	54	6.4
	A	14.9	16.8	17.4	33	0	10.2	15.2	7.4	16.3	32	19.5	0	80	1.9
	T	5.8	4.8	0	8.5	7.2	25	2.2	9.8	22	35	57	5.0	2.8	1.1
	C	4.6	0	1.5	5.6	0	7.3	22	6.0	27	42	14.8	0	6.6	0
VP-16	G	3.4	0	8.6	17.2	0	32	16.2	19.0	13.6	25	49	0	15.2	6.3
	A	4.5	8.0	5.4	7.2	0	33	24	6.0	11.6	18.3	85	0	32	0.4
	T	6.2	3.4	0	5.8	3.4	12.5	1.0	6.9	9.8	16.6	24	2.2	0.9	0
	C	4.8	1.5	2.8	4.4	0	35	9.7	3.5	12.7	21	87	0	ND	0
OXO	G	0.3	0	6.2	10.5	0	16.8	7.7	10.0	14.4	54	11.7	0	93	4.5
	A	7.3	10.3	5.6	11.4	0	26	5.9	13.9	23	50	35	0	120	0.1
	T	0	3.8	0	6.7	3.5	13.0	0	7.9	10.3	33	10.6	7.6	11.5	0
	C	1.0	1.8	2.8	2.8	0	2.7	3.0	4.6	6.6	19.4	1.5	0	ND	0

The amount of cleaved substrate (fmol) is shown for each position tested for both the wild-type (WT) and *39*-A^R enzymes in the presence of no drug, *m*-AMSA, mitoxantrone (MITO), 2-me-9-OH-E^+ (me OH E^+), VP-16 and oxolinic acid (OXO). In addition, the values for any background bands in the untreated substrates (EXT) are shown. All values were estimated by comparison with a standard curve (see Materials and methods). Note that the extension product background is not subtracted from the 'no-drug' values, and the 'no-drug' values are not subtracted from the inhibitor values. This table also contains additional data not shown in Figures 3 and 4: (i) cleavage in the presence of no drug, VP-16 and oxolinic acid for the wild-type enzyme (which was generally very low), and (ii) three additional positions (−6, −5 and −4′) which required altered reaction conditions; the fmol values at these positions may not be directly comparable with those at the other positions (see Materials and methods).

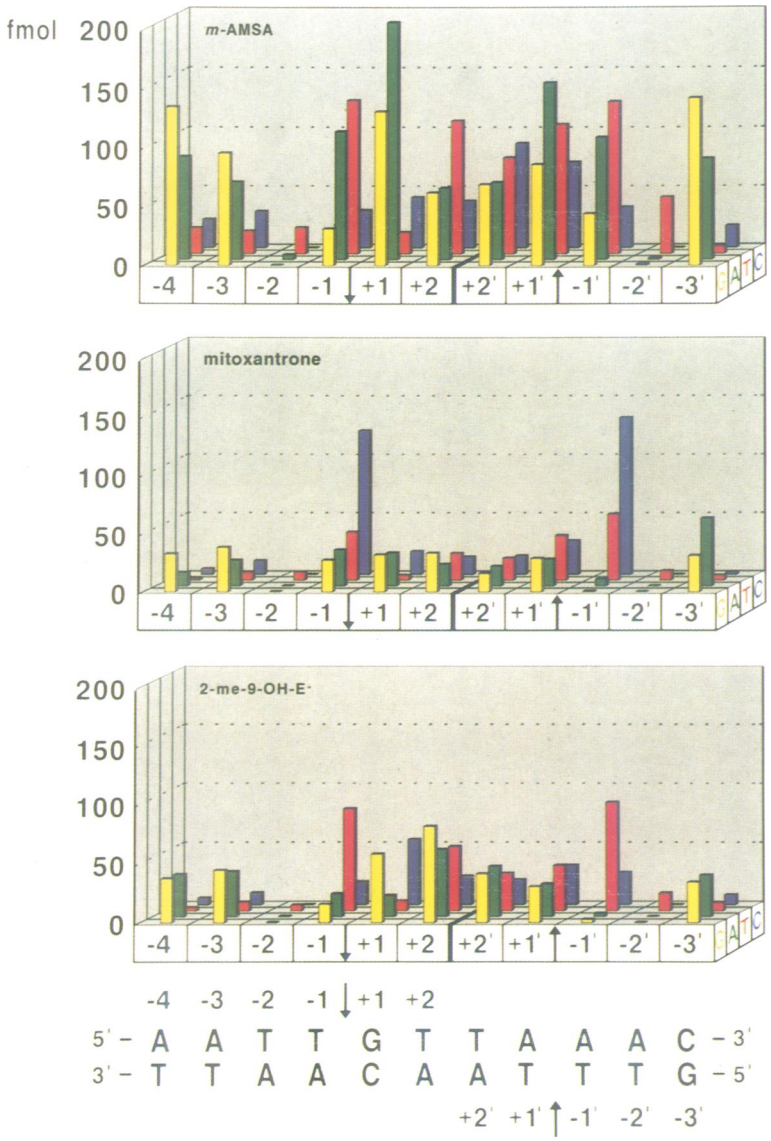

Fig. 3. Topoisomerase cleavage of mutant substrates by wild-type enzyme. These bar charts summarize the results of cleavage with the wild-type T4 topoisomerase in the presence of *m*-AMSA, mitoxantrone and 2-me-9-OH-E^+ for positions −4 to −3′. The cleavage site is indicated with arrows, and the center of symmetry with a bold line. The numbers at the bottom of each panel (*x* axis) correspond to the position tested, numbered with respect to the topoisomerase cleavage site. The reference strand for sequence numbers is the top strand to the left of the center of symmetry, but the bottom strand to the right of the center of symmetry (see sequence at bottom). The nucleotide tested at each position (G, A, T or C) is indicated on the *z* axis and is color coded. The height of each bar (the *y* axis) corresponds to the amount of cleavage product minus any no-drug product band (see Table I for uncorrected values). The wild-type sequence is indicated below, with cleavage site and reference numbering indicated.

extended with a mix of cold nucleotides. The specific activities of the products were adjusted to the standard level by altering the specific activity of the end-labeled primer.

Preferences at position −3′ are enzyme specific

The four oligonucleotide substrates (referred to as G, A, T and C) differentially labeled at position −3′ (see sequence at bottom of Figure 3) will be used to illustrate the experimental procedure and presentation of results. Each cleavage reaction contained one of the four substrates (2 pmol) and 3 pmol of wild-type topoisomerase, either with or without one of the topoisomerase inhibitors. Following treatment with sodium dodecyl sulfate (SDS) and proteinase K, half of each reaction was subjected to denaturing gel electrophoresis and the cleavage products were visualized by autoradiography (Figure 2A). Cleavage in the presence of *m*-AMSA produced high levels of the expected 12-base band with the G (wild-type) and A substrates, whereas the T and C substrates supported only low levels of cleavage (Figure 2A, *m*-AMSA). Cleavage of each of the four substrates was strictly dependent on *m*-AMSA, because drug-independent cleavage could not be detected (Figure 2A, no drug).

In addition to the acridine *m*-AMSA, four other inhibitors of different drug classes were also tested for their effects on cleavage: mitoxantrone (a substituted anthraquinone), 2-me-9-OH-E^+ (an ellipticine derivative), VP-16 (an epipodophyllotoxin) and oxolinic acid (a quinolone). The concentrations of *m*-AMSA, mitoxantrone and 2-me-9-OH-E^+ were chosen to produce similar amounts of cleavage of pBR322 DNA (Huff and Kreuzer, 1990). VP-16 and oxolinic acid are poor inhibitors of the wild-type enzyme, and even relatively high concentrations of these two

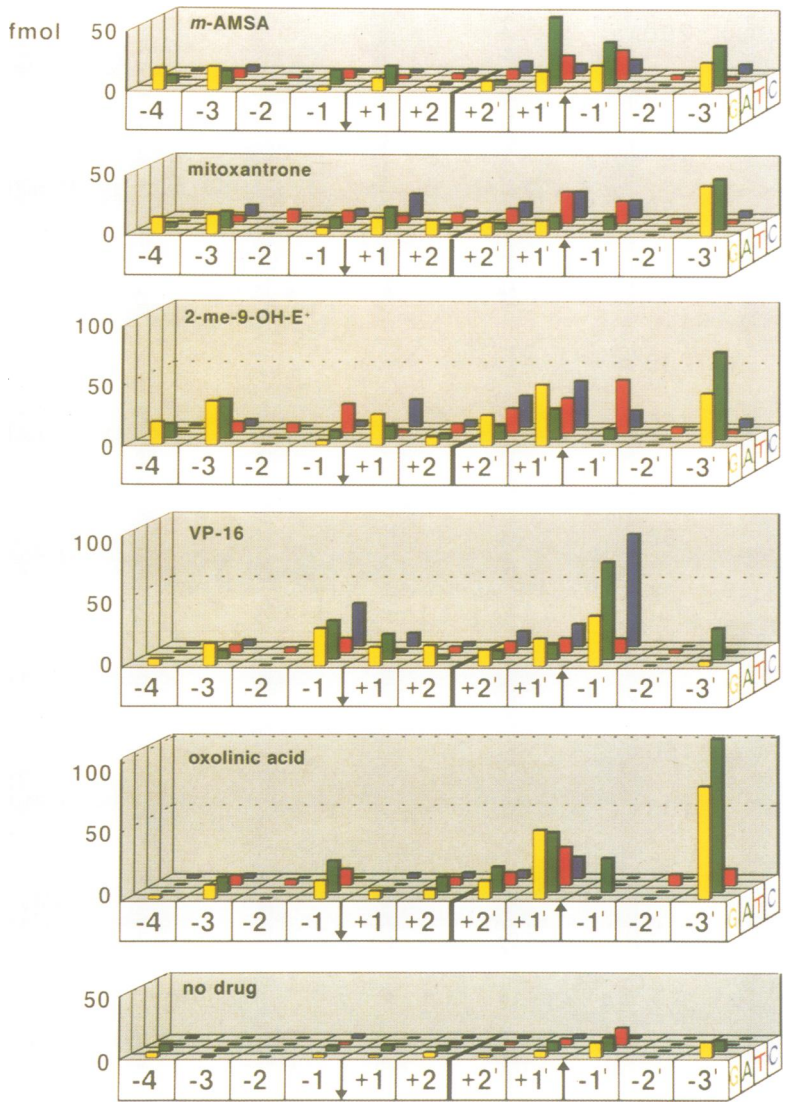

Fig. 4. Topoisomerase cleavage of the mutant substrates by the *39*-A^R enzyme. Inhibitor-induced cleavage is shown as in Figure 3, with additional results obtained in the presence of VP-16 and oxolinic acid. The 'no-drug' cleavage results have the extension-reaction products subtracted out, and the inhibitor panels have the 'no-drug' cleavage results subtracted out (see Table I for uncorrected values).

inhibitors induce much less cleavage of a pBR322 substrate (Huff and Kreuzer, 1990).

The base preferences for cleavage of the −3′ position substrates in the presence of mitoxantrone or 2-me-9-OH-E$^+$ were very similar to those in the presence of *m*-AMSA, with the G and A substrates being strongly preferred over the T and C substrates (Figure 2A). However, subtle changes in the order of preferred bases between the three inhibitors were detected (e.g. compare G and A lanes with *m*-AMSA and mitoxantrone). VP-16 and oxolinic acid induced no detectable cleavage of any of the four substrates (Figure 2A). Presumably, the lack of cleavage reflects the relative insensitivity of the wild-type T4 enzyme to these inhibitors, and may also indicate that the cleavage site being analyzed is not responsive to these two inhibitors (see below).

All of the experimental results in this study (including those in Figure 2A) were quantified by laser densitometry, using a standard curve to estimate the molar amount of cleavage product (Table I), and much of the data is summarized graphically in Figures 3 (wild-type enzyme) and 4 (*39*-A^R enzyme). In Figures 3 and 4, the amount of cleavage in the presence of each inhibitor represents only inhibitor-induced cleavage, with inhibitor-independent cleavage already subtracted, whereas Table I shows the uncorrected values. The quantification revealed that the base preferences at position −3′ are quite dramatic. For example, the G substrate was cleaved ~24-fold better than the T substrate in the presence of *m*-AMSA. The wild-type enzyme clearly has strong preferences for certain bases at the −3′ position, and these preferences are not affected much by the identity of the inhibitor used to induce cleavage. We therefore conclude that −3′ is an enzyme-specific position (i.e. base preferences imposed by the enzyme).

We also tested DNA cleavage by the *39*-A^R (*m*-AMSA resistant) mutant topoisomerase (Figure 2B). Compared with the wild-type T4 topoisomerase this enzyme is resistant to *m*-AMSA, mitoxantrone and 2-me-9-OH-E$^+$ but ultrasensitive to VP-16 and oxolinic acid. In addition, the *39*-A^R enzyme mediates an increased amount of cleavage in the absence of inhibitor (see Introduction). In the presence of *m*-AMSA, mitoxantrone or 2-me-9-OH-E$^+$, the −3′ position preferences of the *39*-A^R topoisomerase mirrored

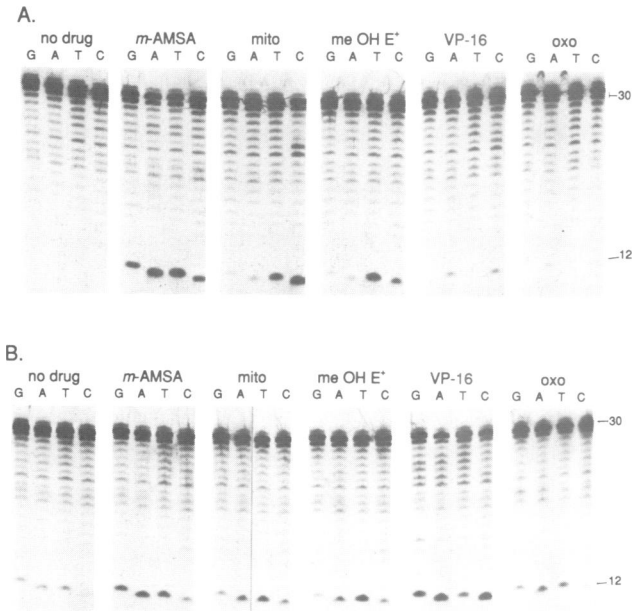

Fig. 5. Topoisomerase cleavage with nucleotide substitutions at the −1′ position. The four specifically labeled double-stranded oligonucleotide substrates (radioactive G, A, T or C at position −1′) were incubated with wild-type (**A**) or *39*-A^R (**B**) topoisomerase in the presence of no drug, *m*-AMSA, mitoxantrone (mito), 2-me-9-OH-E$^+$ (me OH E$^+$), VP-16 or oxolinic acid (oxo) as described in Figure 2. The positions of the 30 base starting product and the expected 12 base cleavage product are indicated at the right. The ladder of bands in each lane is composed of incomplete extension products which are also present in the untreated substrates.

those of the wild-type enzyme (G and A strongly preferred over T and C). *m*-AMSA-induced cleavage by the mutant enzyme was less extensive than that by the wild-type enzyme, in accord with the *m*-AMSA-resistant character of the mutant enzyme. Even more interesting, the mutant enzyme displayed the same strong preference for purines at −3′ in the presence of VP-16 or oxolinic acid, and even when no inhibitor was present (Figure 2B, Table I). These results with the *39*-A^R enzyme thereby strongly support the conclusion that the −3′ position is an enzyme-specific position.

Other enzyme-specific positions

In addition to position −3′, seven other positions (−2 to −6, −2′ and −4′) were found to display enzyme-specific preferences (Figures 3 and 4; Table I). In each case, particular base preferences were consistently exhibited under all conditions tested, with similar results for the wild-type and *39*-A^R enzymes. This pattern of consistent base preference continued from the −2 and −2′ positions outward as far as was tested for both strands. At most positions, the preferred base substrate was cleaved at least several-fold more efficiently than the least favorable substrate. At certain positions, an unfavorable base severely reduced or eliminated cleavage, for example a T at position −3′ or any base other than T at positions −2 or −2′ (Figure 3). These results demonstrate that selection of a cleavage site by the T4 topoisomerase is highly dependent on specific bases surrounding the potential cleavage site, regardless of which inhibitor is present to induce DNA cleavage. Furthermore, in those cases where cleavage could be detected, the preferences of the *39*-A^R enzyme in the absence of inhibitor were similar to those detected in the presence of inhibitors.

Interestingly, these enzyme-specific positions showed a significant amount of symmetry (Figures 3 and 4; Table I). T was strongly preferred at positions −2 and −2′, G and A were preferred at positions −3 and −3′, and G was optimal at positions −4 and −4′. In addition, symmetrical positions usually showed exactly the same base-preference orders for the four substrates. Although many topoisomerase cleavage sites (including the wild-type *rIIB* site) do not have perfect symmetry, the two protomers of the dimer may nonetheless recognize DNA on opposite sides of the cleavage site in an equivalent manner.

Inhibitor-specific positions

Strikingly different results were obtained for the −1 and −1′ positions, which are directly adjacent to the cleaved phosphodiester bonds. At these two positions, every tested inhibitor resulted in unique preferences. The data for position −1′ are shown in Figure 5 (A, wild-type; B, *39*-A^R). In the case of the wild-type enzyme, the following preferences were observed for the three potent inhibitors: *m*-AMSA, T or A; mitoxantrone, C; and 2-me-9-OH-E$^+$, T. These inhibitor-specific preferences were mirrored at position −1, the symmetrical position with respect to the double-strand cleavage event (Figure 3). The difference in cleavage between the most and least preferred bases at these two positions was, in each case, ~4-fold or more; the most extreme preferences were detected at position −1′ for 2-me-9-OH-E$^+$ (37-fold) and mitoxantrone (>100-fold). We conclude that the position just outside the cleaved phosphodiester bond on each strand is a key determinant of inhibitor specificity.

Cleavage by the wild-type enzyme was very weak in the presence of VP-16 or oxolinic acid (Figure 5A). Nonetheless, some apparent preferences were detected, but these did not display symmetry at the two −1 positions (Table I). Due to the low level of cleavage, we are not confident in the VP-16- and oxolinic acid-induced preferences.

The base preferences observed at positions −1 and −1′ with the *39*-A^R topoisomerase were generally similar to those with the wild-type enzyme (Figures 3–5). For both enzymes, T and A were the preferred bases in the presence of *m*-AMSA and T was highly preferred in the presence of 2-me-9-OH-E$^+$. However, significant differences between the two enzymes were also detected. First, in the presence of *m*-AMSA, the base preference was T > A for wild-type but A > T for *39*-A^R. Second, in the presence of mitoxantrone, a C residue was highly preferred for the wild-type enzyme but suboptimal for the *39*-A^R enzyme. Therefore, the *39*-A^R mutation alters the cleavage site specificity of the T4 topoisomerase for these two positions (see Discussion). The *39*-A^R enzyme is ultrasensitive to VP-16 and oxolinic acid (Huff and Kreuzer, 1990), and accordingly mediated more cleavage than the wild-type enzyme in the presence of either inhibitor. At the −1 and −1′ positions, the mutant enzyme preferred G, A or C in the presence of VP-16 and A in the presence of oxolinic acid. These results demonstrate that VP-16 and oxolinic acid impose unique base preferences on the −1 and −1′ positions, as do the other inhibitors discussed above.

These data demonstrate that the identity of the inhibitor has a major influence on the amount of cleavage complex formed depending on which base is present 5′ to the cleaved phosphodiester bond. In spite of the large inhibitor-induced

preferences, it is also possible that the enzyme exerts preferences for this position. The wild-type enzyme supported a small amount of cleavage in the absence of drug with the −1T substrate (and somewhat less with the −1G and −1C substrates), but no cleavage was detected with any of the −1′ substrates (compare extension products with values in the absence of drug, Table I). With the *39*-A^R enzyme in the absence of drug, G, A and T appeared to be optimal at −1′, and very little preference was detected at −1. These data are consistent with the possibility that the two enzymes exert unique preferences for the −1 and −1′ positions in the absence of inhibitors, but the very low levels of detected cleavage preclude any firm conclusions. In any case, any enzyme-specific base preferences at the −1 and −1′ positions are overcome in the presence of inhibitors that induce cleavage.

Positions between the cleaved bonds

Unexpectedly, only relatively minor effects were detected with different bases at positions +2 and +2′, within the 4 bp staggered topoisomerase cleavage site (Figures 3 and 4). Therefore, the identities of the two innermost bases do not play a major role in determining cleavage site strength. The particular inhibitor that was present appeared to have some influence on base preference, but the alterations were subtle.

Both the enzyme and the inhibitors may exert preferences at positions +1 and +1′, although, again, the preferences are generally not very dramatic (Figures 3 and 4). With respect to inhibitor-specific effects, A was preferred with *m*-AMSA but somewhat unfavorable with 2-me-9-OH-E$^+$, and C was unfavorable only in the presence of *m*-AMSA. In spite of these differences, fairly similar patterns of +1 and +1′ base preferences were detected in the presence of different inhibitors, indicating that the enzyme exerts preferences regardless of the inhibitor present. Furthermore, the preferences of the *39*-A^R enzyme appeared to be similar to those of the wild-type. The most dramatic effect detected for all the interior bases was a disfavored T residue at position +1. T was disfavored regardless of the inhibitor present, with either the wild-type or mutant enzyme (Figures 3 and 4). Therefore, the aversion to T appears to be imposed by the enzyme and is not altered by the *39*-A^R mutation. Considering all of our data, the aversion to T at +1 deviates most strongly from symmetry: T is a perfectly adequate base at +1′. One possibility is that the run of five T residues (−2 to +2′) on the top strand of the +1T substrate is unfavorable for cleavage.

Alteration of both inhibitor-specific positions (−1 and −1′)

The inhibitor-specific preferences at positions −1 and −1′ suggest that the favored bases at each of these positions create good binding sites for that particular inhibitor (see Discussion). Assuming that this interpretation is correct, does the symmetry detected at these two positions imply that two good inhibitor binding sites are necessary for cleavage complex stabilization? For the wild-type topoisomerase in the presence of *m*-AMSA or 2-me-9-OH-E$^+$, the wild-type substrate with the preferred T at both positions supported more cleavage than seen with any of the substrates containing a T at only one of the two positions (Figure 3). In addition, for mitoxantrone, where C is the preferred base, substrates with a single C (at either position) were cleaved much more extensively than substrates that lacked the optimal C

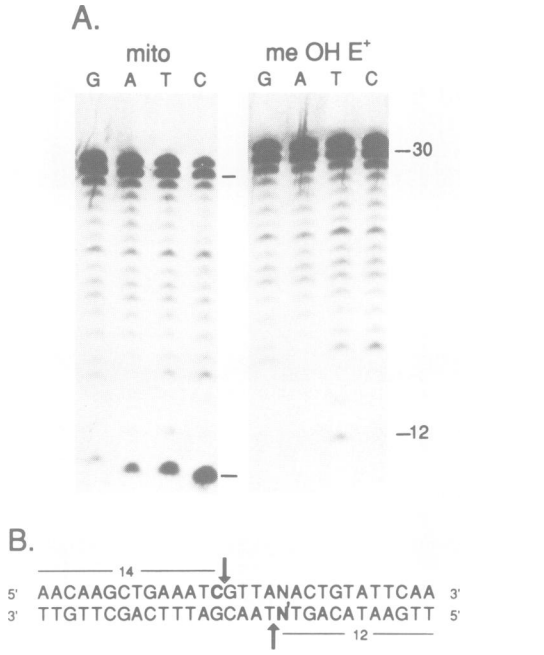

Fig. 6. Cleavage of the −1C, −1′N substrate by wild-type topoisomerase. A 30 base oligonucleotide template (top strand) was synthesized with a C residue at the −1 position and a mixture of all four bases (N) at position −1′. Duplex substrates were specifically labeled at the −1′ (N′) position in four separate reactions and prepared as described for other substrates with negative numbers (see Materials and methods). The sequence of the duplex substrate is shown at the bottom (**B**). Each differentially labeled substrate (G, A, T and C) was incubated with wild-type T4 topoisomerase in the presence of mitoxantrone (mito) or 2-me-9-OH-E$^+$ (me OH E$^+$) as described in the legend to Figure 2. Half of each reaction was analyzed on a denaturing 15% polyacrylamide gel, an autoradiograph of which is shown (1 week exposure; panel **A**). The positions of the 30 base starting product and the expected 12 base cleavage product are indicated at the right. Quantification of the cleavage products was performed as described in Materials and methods, with the estimated amounts of product being 6, 22, 58 and 147 fmol (G, A, T and C respectively) for mitoxantrone and 0, 0, 7.5 and 0 fmol (G, A, T and C respectively) for 2-me-9-OH-E$^+$.

residue at both positions (Figure 3). Thus, the data presented above suggest that one optimal inhibitor binding site is sufficient for cleavage, but that two optimal sites allow more efficient cleavage.

We attempted to compare systematically the cleavage efficiency with no, one or two optimal binding sites by generating an additional set of substrates which had a C at the −1 position and the four possible bases at position −1′ (−1C, −1′N; Figure 6B). The −1 and −1′ position substrates analyzed above (Figure 3) contained the wild-type T at the invariant position. Therefore, the 12 oligonucleotides together included substrates with no, one or two Cs at the critical positions, and also substrates with no, one or two Ts.

Cleavage of the C-C (−1, −1′) substrate in the presence of mitoxantrone was greater than that of the three substrates containing only one optimal C residue by 25-, 6.7- and 2.5-fold (C-G, C-A and C-T respectively; Figure 6, mitoxantrone). The C-T substrate in this set is equivalent to the −1C substrate used above, and that substrate was cleaved several-fold more efficiently than the three substrates with no optimal C residues (G-T, A-T and T-T; Figure 3). In addition, cleavage of the −1C, −1′N oligonucleotides in the presence of 2-me-9-OH-E$^+$ revealed that no cleavage occurred if neither position was a T, the preferred base for

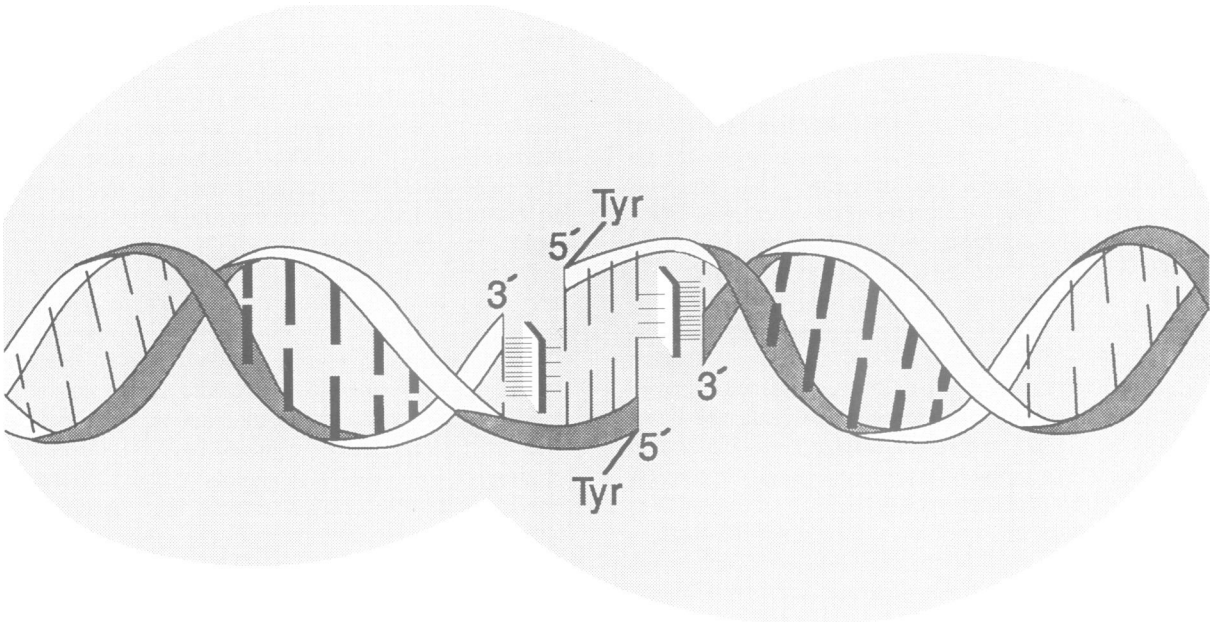

Fig. 7. Model of a topoisomerase cleavage complex trapped by an inhibitor. The topoisomerase recognizes a preferred cleavage site by specific interactions with the DNA helix, with the most important determinants being distal to the staggered cleavage sites. This model shows enzyme-specific bases in bold, assuming that symmetry extends to the −6 positions. DNA cleavage creates inhibitor binding sites at the internucleotide space of the cleaved phosphodiester bonds. The inhibitor (rectangular box) at each binding site interacts primarily with the base 5′ to the cleaved bond and secondarily with the base 3′ to the cleaved bond (illustrated by fine lines). The presence of the bound inhibitor blocks DNA religation and thereby stabilizes the cleavage complex.

this inhibitor (Figure 6, 2-me-9-OH-E$^+$). Substrates with a single T were cleaved with low efficiencies in the presence of the ellipticine derivative, while the −1T, −1′T substrates were cleaved effectively (Figures 3 and 6). These data strongly support models in which DNA cleavage requires inhibitor binding to at least one of two sites that are located very near the cleaved phosphodiester bonds, with maximal cleavage occurring when both sites can be readily occupied.

Discussion

In this study, we have analyzed the base preferences at 14 positions within and adjacent to a particular T4 topoisomerase cleavage site. Most of the 14 tested positions fell into one of three different categories, and the overall conclusions are summarized in Figure 7. First, at each of eight positions flanking the cleavage site (−6 to −2 and −2′ to −4′), the same preferences for certain bases were detected regardless of the tested inhibitor. We refer to these as enzyme-specific positions, and the preferred bases at these positions show strong symmetry with respect to the cleavage site. Second, the positions 5′ to the cleaved phosphodiester bond on each strand (−1 positions) are major determinants of which inhibitor causes effective cleavage, and these two positions are therefore referred to as inhibitor specific. Third, the two internal bases of the cleavage site had only minor influences on cleavage under any of the tested conditions. Finally, the positions just 3′ to the cleaved phosphodiester bond on each strand (+1 positions) did not fit any one category neatly, but appeared to have some importance both for enzyme and inhibitor recognition.

Recognition of potential cleavage sites: enzyme-specific positions

The results of this study reveal a general picture of how the T4 topoisomerase interacts with a particular cleavage site. The enzyme-specific positions outside the cleavage site apparently provide the major determinants for enzyme-mediated DNA site recognition. Bases at these positions may be in direct contact with the topoisomerase, or, in the context of surrounding bases, may generate local helix variations that are recognized by the enzyme. At least at position −3′, the enzyme and DNA are in very close contact because methylation of the −3′G interferes with cleavage (data not shown). Although we have identified eight important enzyme-specific positions flanking the cleavage site, positions further from the cleavage site may also play a role in site recognition. Consensus sequences determined with eukaryotic type II topoisomerases extend to position −6 (Sander and Hsieh, 1985) and −10 (Spitzner and Muller, 1988), with the most biased positions occurring within 7 bp of the cleavage site (Pommier *et al.*, 1991). Lee *et al.* (1989) showed that a 21 bp sequence contained the major determinants for recognition by the *Drosophila* type II topoisomerase, and also showed that the enzyme protects a region of ~25 bp (with the cleavage site in the middle) from nuclease action. Furthermore, Lund *et al.* (1990) showed that an 18 bp duplex oligonucleotide could be cleaved by eukaryotic topoisomerase, although slightly longer oligonucleotides were cleaved with much higher efficiencies (28 bp gave maximal cleavage). All these studies therefore indicate that recognition of DNA sites by the eukaryotic and T4 topoisomerase involves recognition of a 15−25 bp region, with the cleavage site near the center. There is no obvious relationship between the enzyme-specific preferences detected here for the T4 topoisomerase and the preferred bases at corresponding locations of the *Drosophila*, chicken or calf topoisomerase consensus sequences (Sander and Hsieh, 1985; Spitzner and Muller, 1988; Fossé *et al.*, 1991). However, the T4 topoisomerase preferences show some relationship with those determined for the mouse enzyme, in particular, a preferred G at positions −4 and

−4′ (Capranico *et al.*, 1990a; Pommier *et al.*, 1991). Therefore, it is possible that the phage and eukaryotic enzymes recognize some of the same enzyme-specific determinants.

The analysis of cleavage site substrates that differ from a known strong site by a single base pair has provided a wealth of information which was not obvious from consensus sequence analyses. The base preferences detected at many positions were quite dramatic, with single-base substitutions essentially abolishing cleavage at certain positions (e.g. −2, −2′ and −3′; see Figure 3). Thus, although consensus sequences do not indicate any absolute base requirements for topoisomerase cleavage, such requirements may exist at individual cleavage sites. Perhaps many sequences derived from different combinations of the more preferred bases constitute strong topoisomerase cleavage sites, explaining how enzymes with specific base preferences can nonetheless show degenerate consensus sequences.

A key finding that emerged from the mutational analysis was the symmetry of preferred bases with respect to the cleavage site. The type II topoisomerases are homodimeric enzymes which would be expected to interact with their DNA substrates in a symmetrical fashion. Considering the enzyme-specific positions of the cleavage site (−2 and −2′ outwards), two of the previous consensus sequences for the eukaryotic enzyme did not display a dyad axis of symmetry (Sander and Hsieh, 1985; Spitzner and Muller, 1988), but recent analyses of mammalian topoisomerase cleavage sites did show significant dyad symmetry (Capranico *et al.*, 1990a; Fossé *et al.*, 1991; Pommier *et al.*, 1991). Pommier *et al.* (1991) suggested that recognition occurs at one or the other, but not necessarily both, sides of the cleavage site. The mutational analysis presented here demonstrates that good sequence recognition on both sides of the cleavage site simultaneously is important for the T4 enzyme. This is most obvious from the strict requirement for a T at both −2 and −2′; mutation of either residue to G or C essentially abolished cleavage (Figure 3).

Assuming that symmetry extends to the −6 positions, we can summarize the enzyme-specific preferences in the −6 to −2 positions of each strand as 5′-A/C A G A/G T-3′. In addition, the T4 topoisomerase may prefer certain bases at the −1 positions and the +1 positions. The influence of inhibitors is dominant at the −1 positions, making it difficult to assess enzyme preferences there. The enzyme clearly disfavors T at +1 under all tested conditions, although the lack of symmetry suggests that this may be an unusual feature depending on the neighboring sequence of this particular site (see Results). The low level of cleavage in the absence of inhibitors has made it difficult to analyze directly the enzyme preferences in the absence of inhibitor. At three positions in the −6 to −2 regions, we detected significant cleavage by the *39*-A^R enzyme in the absence of inhibitor. In each case, the base preferences closely matched those detected in the presence of inhibitors. These results strongly support the conclusion that the −6 to −2 regions are recognized by the enzyme, regardless of the presence of inhibitor. A more complete analysis of base preferences in the absence of inhibitors would be quite useful to extend the current study, particularly with reference to the positions between −2 and −2′.

The wild-type *rIIB* sequence, which is cleaved efficiently in the presence of *m*-AMSA, matches the preferred base at most, but not all, enzyme-specific positions. Changing the wild-type A to G at position −3, −4 or −4′ moderately increased substrate cleavage, and the wild-type G at position −6 is the least favorable base. One question raised by this work is whether an 'ideal sequence' composed of the most preferred base at each position would be cleaved more efficiently than the wild-type substrate. Several additional questions are also raised by this work. First, do the rules deduced from this single site apply at all T4 topoisomerase cleavage sites, or does the enzyme recognize more than one class of sequence? We are currently attempting to use the preferences of Table I to predict the location and strength of topoisomerase cleavage sites in other DNA molecules. Secondly, do the rules of cleavage site recognition presented here also predict strong sites of enzyme activity? Thirdly, do strong inhibitor-independent cleavage sites fit the preferences deduced here, or do they consist of unique sequences that somehow stabilize the cleaved intermediate? We might expect that strong inhibitor-independent sites would contain the same optimal bases in the −6 to −2 flanks, but perhaps unique sequences at the sites of DNA cleavage.

The cleavage site preferences of the *39*-A^R enzyme were very similar to those of the wild-type enzyme, with no obvious differences in the −6 to −2 regions. However, one of the most interesting features of the mutant enzyme is that it was found to cleave pBR322 DNA with altered specificity compared with the wild-type enzyme (Huff *et al.*, 1989). The results presented here suggest that the alteration in cleavage site specificity may involve bases very near the cleavage sites, particularly the −1 and −1′ positions. Although these two positions are dominated by the effects of specific inhibitors, the wild-type and mutant enzymes displayed distinguishable preferences (compare Figures 3 and 4). The most striking difference was that the strong preference for C of the wild-type enzyme with mitoxantrone was abolished in the case of the *39*-A^R enzyme. Interestingly, the mutant enzyme strongly disfavored C at −1′ in the absence of inhibitor, suggesting that the mutational change could involve an aversion to C. In any case, a direct comparison of inhibitor-independent recognition of the −1 and −1′ positions by wild-type and mutant enzyme could clarify the change in site specificity. Huff and Kreuzer (1990) argued that the mutational alteration in the *39*-A^R enzyme was located very close to both the inhibitor binding site and the enzyme−DNA interface. This argument would be strongly supported if the mutational change in cleavage site specificity is predominantly at the base just 5′ to the cleavage site on each strand, because these two bases play a key role in the inhibitor binding site (see below).

Inhibitors interact with the nucleotide 5′ to the cleavage site

Several models have been proposed to explain how inhibitor binding could trap the cleavage complex with type II topoisomerases. Shen *et al.* (1989b) proposed that the region within the 4-base staggered cut produced by DNA gyrase becomes denatured in the course of the reaction cycle, and that this denatured region serves as the quinolone binding site. In this model, the inhibitor binding stabilizes the denatured region and prevents religation by the enzyme. A different model has been proposed for the mammalian type II topoisomerase by Capranico *et al.* (1990a) based on the finding that each of 97 doxorubicin-induced cleavage sites contained an A residue immediately 5′ to one or both cleaved

phosphodiester bonds (i.e. positions −1 and −1′). They suggested that doxorubicin intercalates between base-pairs at the cleavage site, with important stacking interactions between the intercalated doxorubicin and the 5′ A. Further consensus sequence analyses indicate strong biases for particular bases at the −1 positions or the +1 positions for *m*-AMSA, VM-26 and an ellipticine derivative (Fossé *et al.*, 1991; Pommier *et al.*, 1991).

This report provides the first direct test of the effect of base substitutions at the −1 and +1 positions of a particular cleavage site. Our results demonstrate that the base preferences at the positions just 5′ to the cleavage sites (−1, −1′) were highly specific for each tested inhibitor. The preferences for the positions just 3′ to the cleaved bonds (+1, +1′) also showed some degree of inhibitor specificity, but the differences were much more subtle than those at the −1 positions. None of the other positions surrounding the topoisomerase cleavage site exhibited notable inhibitor-specific behavior. The simplest explanation is that the inhibitors interact primarily with the −1 base-pairs, consistent with the intercalation/stacking model introduced above. The internucleotide space at the cleaved phosphodiester bond could be a favorable site for inhibitor insertion (see Figure 7). Binding might be promoted by a more flexible internucleotide distance between the −1 and +1 base-pairs when the phosphodiester bond is cleaved, perhaps explaining how inhibitors such as VP-16, which do not intercalate in naked DNA, nevertheless appear to induce cleavage complexes by the same mechanism as intercalators such as *m*-AMSA. When the inhibitor is bound, religation could be impeded by the difficulty of reducing this distance to allow interaction of the 5′-phosphate and 3′-OH groups (see Figure 7).

The intercalation model discussed above does not specify the precise chemical nature of the inhibitor-directed base specificities in formation of the cleavage complex. In the simplest case, the preferences for the −1 base-pairs could be identical to base preferences of the inhibitors in the absence of the topoisomerase. Alternatively, the presence of the covalently bound enzyme may constrain inhibitor binding and thereby influence the sequence preferences of the inhibitors. Unfortunately, we know of no reports that clearly define the sequence specificity of intercalation of any of the relevant inhibitors into DNA of heterogeneous sequence. Nonetheless, structural studies and molecular modeling of oligonucleotide−intercalator complexes suggest possible mechanisms for base specificities of intercalator binding. For example, ^{1}H-NMR analysis of mitoxantrone bound to the $[d(CpGpCpG)]_2$ duplex demonstrated close contact between the mitoxantrone side-chain methylene groups in the major groove and specific protons of the neighboring bases (Lown and Hanstock, 1985). Furthermore, the modeling studies of Chen *et al.* (1986) strongly suggest that mitoxantrone intercalates in a sequence-specific manner due to interactions of each mitoxantrone side chain with two backbone phosphates and with the N7 position of an adjacent purine (G preferentially; corresponding to the base paired with the −1 or −1′ position in our nomenclature). Substitution of a pyrimidine at that position results in loss of the purine N7 interaction and also prevents, by steric hindrance, the stabilizing side chain interactions with the backbone phosphates. The sequence preferences predicted by Chen *et al.* (1986) agree quite well with those reported here for mitoxantrone-induced cleavage complexes. In both cases, a C residue at the position just 5′ from the binding site (the −1 or −1′ position) is most highly preferred, while either purine residue in the same position is least favored. Further studies will be necessary to determine whether the sequence specificity of inhibitor-induced cleavage complex formation can be explained by simple inhibitor−DNA interactions such as those just described.

Some of the inhibitors tested in this study have also been used to generate consensus sequences for cleavage by eukaryotic type II topoisomerases. Our results for the inhibitor-specific positions agree reasonably well with those consensus sequences. First, Fossé *et al.* (1991) sequenced 25 strong pBR322 cleavage sites in the presence of an ellipticine derivative (2,11-dimethyl-5-ethyl-9-hydroxy-6H-pyrido[4,3-b]carbalzolium or EPC), and found that a T was always present at the −1 position on at least one cleavage strand. Likewise, we detected a strong preference for T at the −1 positions in the presence of 2-me-9-OH-E^+. Secondly, Pommier *et al.* (1991) found an increased incidence of T at the −1 positions of mouse topoisomerase cleavage sites induced by *m*-AMSA, and we found that either T or A led to efficient cleavage by the T4 enzyme. In the analysis of cleavage by mouse topoisomerase, the strongest preference was actually for A at position +1, leading to the hypothesis that *m*-AMSA stacks with the base-pair at position +1 rather than at −1. We also found A to be the preferred base at the +1 positions, suggesting that a TA dinucleotide is uniformly favored at the cleaved phosphodiester bond in the presence of *m*-AMSA. Thirdly, Pommier *et al.* (1991) found a bias for C at the −1 positions for cleavage by the mouse topoisomerase in the presence of VM-26. For the closely related VP-16, we also found that C is the most preferred base for cleavage by the *39*-A^R topoisomerase. However, we found that G and A also supported relatively good cleavage, whereas these two bases were least frequent at the −1 positions in the VM-26 sites. Overall, in spite of the large evolutionary distance between the T4 and mammalian topoisomerases, similar rules may govern the binding of inhibitors to the active site of the enzyme−DNA complex.

Analysis of substrates altered in both the −1 and −1′ positions demonstrated that maximal cleavage requires the preferred base at both positions (Figures 3 and 6). One possible explanation is that occupation of both putative inhibitor binding sites is necessary to stabilize the cleavage complex containing a double-strand break. This model may be correct in the case of the eukaryotic topoisomerase because many of the inhibitors induce cleavage complexes containing nicks, which could be promoted by inhibitor binding to only one site. However, in the case of the T4 enzyme, we did not detect nicking of the wild-type 30 bp substrate in the presence of *m*-AMSA or mitoxantrone (data not shown). This result suggests that religation of the two strands is tightly coupled in the reaction cycle of the T4 topoisomerase, with binding of an inhibitor molecule to either site blocking religation of both strands. Even if the binding of only one drug molecule is sufficient for stabilizing the double-stranded cleavage complex, a small dissociation constant at both inhibitor binding sites would increase the likelihood of at least one site being occupied at any given time.

In spite of the major role of the −1 and −1′ base-pairs in inhibitor specificity, it is clear that the enzyme also plays a role. First, closely related derivatives of the same drug

class have very different potencies, which, in at least some cases, have been attributed to drug–enzyme interactions (Zwelling *et al.*, 1982; Nelson *et al.*, 1984; Tewey *et al.*, 1984a; Ripley *et al.*, 1988; Baguley *et al.*, 1990; Huff and Kreuzer, 1990). Secondly, type II topoisomerases show unique spectra of inhibitor sensitivities, even though they presumably generate an identical reaction intermediate. Thirdly and most convincingly, simple mutational alterations can differentially affect sensitivity to various inhibitors, even within the same drug class (Sullivan *et al.*, 1989; Zwelling *et al.*, 1989; Huff and Kreuzer, 1990). Assuming that the inhibitors bind at the cleaved phosphodiester bonds, the structure of the enzyme may physically block the binding sites of certain inhibitors, or particular amino acid residues may interact productively with only certain inhibitors to stabilize the inhibitor–DNA interaction.

With both mammalian and T4 topoisomerases, cleavage site specificity changes in the presence of different classes of antitumor agents (Ross *et al.*, 1984; Tewey *et al.*, 1984a,b; Rowe *et al.*, 1986; Capranico *et al.*, 1990a,b; Huff and Kreuzer, 1990). One possibility that has been suggested repeatedly is that each inhibitor traps the topoisomerase at only a certain subset of sites. Our analysis of base preferences provides compelling support for this interpretation. The enzyme has specific base preferences, particularly in the regions flanking the cleavage site, and these preferences define potential cleavage sites. Overlaying these enzyme preferences, the identity of the bases immediately surrounding the cleavage sites determine whether a potential site will be stabilized by a certain inhibitor. Perhaps the most dramatic demonstration of this view is the conversion of the inhibitor specificity of the *rIIB* site. The wild-type cleavage site with which we began is a strong *m*-AMSA-inducible site, but a relatively poor site for mitoxantrone-induced cleavage. Substitution of either −1T or −1′T with a C residue increased mitoxantrone-inducible cleavage by ~3-fold (Figure 3), and provision of two optimal C residues caused a further 2.5-fold increase (Figure 6). At the same time, the T → C substitutions substantially reduced the efficiency of cleavage in the presence of *m*-AMSA or 2-me-9-OH-E$^+$.

In summary, the mutational analysis presented here has provided important new information about the sequence determinants involved in T4 type II topoisomerase cleavage site selection, as well as the location and nature of the binding site for common type II topoisomerase inhibitors. The results strongly support models in which the topoisomerase inhibitors bind precisely at the sites of phosphodiester bond cleavage, physically blocking DNA religation. Comparisons with studies of the mammalian topoisomerase suggest that the rules governing the specificity of inhibitor binding are very similar in distantly related type II topoisomerases.

Materials and methods

Enzymes and inhibitors

The wild-type and *39*-A^R T4 topoisomerases were purified essentially as described by Kreuzer and Jongeneel (1983) and Huff *et al.* (1990). *m*-AMSA and mitoxantrone diacetate were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. 2-me-9-OH-E$^+$ was generously given by Dr C.Paoletti (Institut Gustave Roussy, Villejuif, France). VP-16 was kindly provided by Bristol-Myers Pharmaceutical Co. (Wallingford, CT). Oxolinic acid was purchased from Sigma. 2-me-9-OH-E$^+$ was dissolved in water, oxolinic acid in 50 mM NaOH and all other inhibitors in 100% dimethylsulfoxide. Immediately prior to use, a 100× aliquot of the appropriate drug solution was diluted with water to 10× concentration, except in the case of oxolinic acid, which was dissolved at 10×. The final drugs concentrations were: *m*-AMSA, 5 μg/ml (12.5 μM); mitoxantrone, 0.5 μg/ml (0.9 μM); 2-me-9-OH-E$^+$, 2.5 μg/ml (7.5 μM); VP-16, 100 μg/ml (170 μM); oxolinic acid, 500 μg/ml (1.9 mM).

Preparation of oligonucleotide substrates

Oligonucleotides were synthesized by National Biosciences and resuspended in 1 × TE [10 mM Tris–HCl (pH 7.6), 1 mM Na_2EDTA]. For each tested position, a 30-base template oligonucleotide was synthesized with a mixture of all four bases (referred to as an 'N' base) at that position. A complementary primer oligonucleotide which hybridized up to the N base was annealed to the template at an equimolar ratio in 1 × Sequenase buffer [20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 25 mM NaCl] and allowed to cool slowly from 65°C to 4°C. The template-primer (110 pmol) was then subjected to extension reactions, which contained 25 pmol of one particular α-^{35}S-labeled deoxynucleoside triphosphate (New England Nuclear), 1 × Sequenase buffer, 5 mM dithiothreitol, BSA at 0.1 mg/ml and 2.4 U Sequenase (version 2.0; United States Biochemical). Incorporation of the labeled nucleotide was allowed for 5 min on ice, which resulted in efficient incorporation of the correct complementary nucleotide and very little misincorporation (C.H.Freudenreich and K.N.Kreuzer, in preparation). A mix of all four cold nucleotides was then added (2.5 mM final concentration each) and incubation was continued for 20 min at 14°C. The reaction was stopped by addition of Na_2EDTA (pH 8; 50 mM final concentration). The incorporated counts were measured by a DE81 filter-binding assay (Maniatis *et al.*, 1982), and cold extension products were added to produce a final specific activity of 30 000 c.p.m./pmol. The extension products were then purified from a native 20% polyacrylamide gel, and after elution and precipitation, the oligonucleotides were resuspended at 2 pmol/μl. Visualization of the purified products on a denaturing 15% polyacrylamide gel revealed some incomplete extension products. As described in the text, these do not interfere with the analysis. In rare instances, some incomplete extension products were the same size as topoisomerase cleavage products (see Table I for these background values). The extension products often went beyond the 30 base template by one or two bases, presumably because of non-templated addition commonly detected with polymerase lacking a 3′ → 5′ exonuclease (Clark *et al.*, 1987; Clark, 1988).

Because of poor annealing with very short primers, extension reactions for −5, −6 and −4′ positions were done at 4°C, and positions −5 and −6 were purified at a specific activity of only 20 000 c.p.m./pmol. Insufficient full-length product of position −5 and −4′ substrates was recovered because of poor extension; therefore, instead of the standard amount of substrate (2 pmol; see below), 1.5 and 0.67 pmol was used for each topoisomerase reaction respectively (the 2:3 ratio of substrate:enzyme was maintained). Longer exposures of these reaction products (−5, −6 and −4′) were done to compensate for the decreased radioactivity, so that the amount of cleavage product would be more comparable to that of the other positions (see below). These three positions are not included in Figures 3 and 4.

Substrates with N at each internal position (+1, +1′, +2 and +2′) were prepared as four separate substrates with different bases at the N position (13 substrates total). A 12 base primer was 5′ end-labeled using T4 polynucleotide kinase and [γ-^{35}S]ATP, and diluted with cold primer to a specific activity of 30 000 (incorporated) c.p.m./pmol. After annealing with the appropriate template, the primer was extended with a mixture of four cold nucleotides and gel purified, as described above.

Topoisomerase cleavage reactions

T4 topoisomerase cleavage reactions (10 μl) contained 2 pmol double-stranded 30mer (30 000 c.p.m./pmol), 3 pmol either wild-type or *39*-A^R T4 topoisomerase, 40 mM Tris–HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM Na_2EDTA, 0.5 mM ATP, BSA at 30 μg/ml, and the indicated inhibitor at the concentration listed above. Cleavage reactions were initiated by addition of topoisomerase, incubated at 30°C for 30 min and then terminated by the addition of 1 μl of 10% SDS. Covalently attached topoisomerase was removed by incubation with proteinase K (10 μg) at 30°C for 60 min. Reaction products were then purified using 0.5 ml Sephadex G-25 (Pharmacia) spin-columns and denatured by adding formamide loading buffer (formamide, 10 mM Na_2EDTA, xylene cyanol at 1 mg/ml, bromophenol blue at 1 mg/ml) and heating at 80°C for 2 min. Half of each reaction was loaded onto a denaturing 15% polyacrylamide gel, which was run at 53 W for 1.5 h in 1 × TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM Na_2EDTA). The gel was fixed for 15 min in 10% acetic acid/10% methanol, rinsed with water, transferred to Whatman 3MM paper, and dried under vacuum for 40 min using a Hoefer gel drier. Cleavage bands were visualized by autoradiography (one-week exposures), and the products were quantified

using a Helena densitometer. The areas under the peaks were determined by weighing cut-out peaks copied from the chart paper, and the amounts of product were estimated by comparison with a standard curve. The standard curve was generated in the following way: a set of typical topoisomerase cleavage reactions was separated on a denaturing 15% polyacrylamide gel alongside a lane with uncleaved substrate (1 pmol total oligonucleotide per lane). An autoradiograph of the gel (one-week exposure) was scanned by laser densitometry and the peaks were weighed as described above. In addition, the topoisomerase cleavage bands and the uncleaved substrate were cut out of the dried gel and subjected to liquid scintillation counting, providing a direct measure of the molar quantities of each cleavage band. The weights of the densitometric scans were then plotted against the fmol calculated from direct counting to provide one standard curve. Separately, the quantity of one particular topoisomerase cleavage band was determined using the standard curve just described; a linear dilution series of this reaction was then subjected to electrophoresis, and the cleavage bands were scanned and weighed as above, providing a second standard curve relating fmol to the weight of the cut-out bands. The two standard curves matched closely, and an average of the two was used for all quantification.

The wild-type sequence present in the substrates at different positions was cleaved at noticeably different levels, perhaps because of different unlabeled competitor substrates in the different reactions, or because of experimental error between experiments with different sets of substrates.

Acknowledgements

This work was supported by a grant from the Bristol-Myers Squibb Company. C.H.F. was supported by National Research Service Award 5 T32 GM07184. This work was done during the tenure of an Established Investigatorship from the American Heart Association to K.N.K.

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Received on October 21, 1992; revised on February 2, 1993