Effects of Base Mutations on Topoisomerase II DNA Cleavage Stimulated by *m*AMSA in Short DNA Oligomers†

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ABSTRACT: DNA cleavage by topoisomerase II in the absence or presence of *m*AMSA, and VM-26 was investigated in a series of oligonucleotides of 36 and 42 base pairs, which were derived from the DNA sequence of the major topoisomerase II cleavage site in the matrix-associated region of SV40 DNA. Topoisomerase II introduced strand cuts at several sites in the oligonucleotides, and the sequence selectivities of DNA cleavage with and without drugs were the same as in larger SV40 DNA fragments. A time course analysis showed that *m*AMSA specifically stimulated DNA cleavage at the 4263/4266 site, while DNA cleavage was specifically induced at the 4265/4268 site by the enzyme without drug or with VM-26. In agreement with recent findings on local nucleotide requirements in order for *m*AMSA to stimulate DNA cleavage, the 4263/4266 site had adenines at the two positions +1. This nucleotide requirement was challenged by mutating the bases 4263 and 4266 of the oligonucleotide representing the natural SV40 DNA sequence. New cleavage sites were not observed in the mutated oligonucleotides, and base mutations had an effect on DNA cleavage induced with and without the two drugs. This general effect was likely due to the sensitivity of topoisomerase II itself to the local DNA sequence. Nevertheless, effects of base mutations were more pronounced for *m*AMSA than for VM-26. Point mutations of either base 4263 or 4266, representing the two positions +1, reduced markedly the stimulative effect of DNA cleavage at the 4263/4266 site by *m*AMSA, and mutations of both bases completely abolised it. The effects of these base mutations on DNA cleavage at other sites varied depending on the site and the mutated base. In particular, a purine at position −2 of the 4265/4268 site increased cleavage at that site by the enzyme without drugs. A guanine at position +2 tended to favor topoisomerase II DNA cleavage with the studied drugs. A pyrimidine at position −1 of the 4259/4262 site allowed the stimulative effect of DNA cleavage by *m*AMSA at the site, while a purine did not. The results indicate that the dinucleotide cleaved by topoisomerase II is crucial for stimulation of DNA cleavage by *m*AMSA and that the in vitro DNA binding and cleavage by topoisomerase II is determined by nucleotides immediately surrounding the cleavage site.

DNA topoisomerase II is a nuclear enzyme required for DNA replication, RNA transcription, and other DNA metabolic processes (Cozzarelli, 1980; Gellert, 1981; Wang, 1985). This enzyme may be trapped by a strong denaturing agent in a complex wherein the DNA strands are broken and the two subunits of topoisomerase II are covalently linked to the two 5′ ends of the cleaved double helix (Cozzarelli, 1980; Gellert, 1981; Wang, 1985). Several anticancer drugs interfere with topoisomerase II functions and stabilize such enzyme–DNA complexes (Liu, 1989; Pommier & Kohn, 1989), by inhibiting the DNA religation step of the enzyme catalytic activity. This drug action on topoisomerase II is generally considered to be the molecular basis of drug antitumor activities (Liu, 1989; Pommier & Kohn, 1989; Zunino & Capranico, 1990).

The molecular mechanism of topoisomerase II trapping by drugs has not been definitively established yet, although a ternary complex DNA–drug–topoisomerase II is thought to form. Each drug is known to stimulate DNA cleavage at drug-specific DNA sites (Nelson et al., 1984; Tewey et al., 1984; Chen et al., 1984). A statistical analysis of 97 doxorubicin-specific DNA cleavage sites in SV40 DNA indicated an absolute requirement for adenines at positions −1 of the site (corresponding to the 3′ termini of the double-strand cut) in order for doxorubicin to stimulate DNA cleavage by mammalian topoisomerase II (Capranico et al., 1990a). Local nucleotide requirements were found also in the case of *m*AMSA and VM-26 (Pommier et al., 1991a). In particular, *m*AMSA required an adenine at position +1, and VM-26 required a cytosine at position −1. Therefore, the sequence specificities of the drug action may likely be due to specific interactions that can take place in the ternary complex among a drug molecule, the DNA bases immediately adjacent to the cleavage site, and amino acid residues of topoisomerase II.

The present investigation was undertaken in order to study the influence of the nucleotides at position +1 on the stimulation by *m*AMSA of DNA cleavage mediated by murine topoisomerase II. Oligonucleotides were designed corresponding to the major topoisomerase II cleavage site in the major matrix-associated region (MAR) of the SV40 genome (Pommier et al., 1990), and nucleotide mutations were introduced at positions +1 and +4 of the major *m*AMSA cleavage site present in the oligonucleotide. The results clearly indicate that the dinucleotide cleaved by topoisomerase II is crucial for *m*AMSA stimulation of DNA cleavage and are consistent with a previously proposed model (Capranico et al., 1990a).

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EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were either purchased from The Midland Certified Reagent Company (Midland, TX) or synthesized with a 380B DNA synthesizer (Applied Biosystems, Milan, Italy). T4 polynucleotide kinase and polyacrylamide/bis were purchased from GIBCO-BRL, Life Technologies (Basel, Switzerland). [γ-^{32}P]ATP was purchased from Amersham, Amity s.r.l., Milan, Italy. *m*AMSA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. VM-26 was obtained from Bristol Italiana (Latina, Italy). Drug stock solutions were made in dimethyl sulfoxide at 10 mM, and further dilutions were made in distilled water. DNA topoisomerase II was purified from murine leukemia P388 cell nuclei by published procedures (De Isabella et al., 1990; Minford et al., 1986) and was stored at −20 °C in 20 mM KH_2PO_4, pH 7.0, 50% glycerol, 0.5 mM PMSF, 0.1 mM EDTA, 1 mM β-mercaptoethanol. As previously described (De Isabella et al., 1990), topoisomerase II strand passing activity was determined with the P4 DNA unknotting assay in 20 mM Tris-HCl, pH 7.5, 80 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/mL bovine serum albumin. One unit of enzyme activity was defined as the minimum amount of protein that completely unknotted 0.2 μg of knotted P4 DNA at 37 °C in 30 min.

End-Labeling and Annealing of Oligonucleotides. Single-strand oligonucleotides were first purified by denaturing polyacrylamide gel electrophoresis and recovered by soaking gel slices in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8, 0.1% SDS and ethanol precipitation. Either all the upper or all the lower strands were ^{32}P-labeled concurrently, in order to obtain similar specific activities of the oligonucleotides being compared. Single-strand oligonucleotides were 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol) in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 1 mM spermidine for 30 min at 37 °C. After extraction with phenol–chloroform, the labeled strand was annealed with a 1.5-fold higher amount of unlabeled complementary strand in 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA. The mixture was heated at 65 °C for 5 min and slowly chilled at room temperature. After ethanol precipitation, the oligonucleotides were resuspended in distilled water and kept frozen at −20 °C.

DNA Cleavage Reaction. Topoisomerase II DNA cleavage reactions were performed in a volume of 20 μL in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, and 15 μg/mL bovine serum albumin for 10 min at 37 °C. Approximately 20 fmol of labeled oligonucleotides was incubated with 106 units of topoisomerase II (about 200 ng). Reactions were stopped by adding SDS, proteinase K, and EDTA (1%, 100 μg/mL, and 20 mM, respectively, as final concentrations) and incubated at 42 °C for an additional 30 min. After ethanol precipitation, the samples were resuspended in 2.5 μL of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue, heated at 95 °C for 2 min, chilled in ice, and then loaded into a 20% polyacrylamide sequencing gel (7 M urea, 89 mM Tris-HCl, pH 8, 89 mM boric acid, 2 mM EDTA). Gels were run at 70 W for 2 h, transferred to Whatmann 3MM paper sheets, dried, and autoradiographed with Amersham Hyperfilm MP. Topoisomerase II cleavage sites were located by comparison with purine sequencing lanes resulting from a Maxam–Gilbert reaction of the same oligonucleotides. As topoisomerase II-produced cleavage generates 3′-OH DNA termini instead of the 3′-P ends generated by the Maxam–Gilbert chemical reactions, a shft toward higher molecular weight (around 1.5 nucleotides) is expected for the topoisomerase II cleavage products (Tapper & Clayton, 1981). The quantification of the fractions of cleaved DNA was done by cutting out gel slices and counting with a MR 300 Automatic Liquid Scintillation System (Kontron).

Table I: DNA Sequences of the Studied Oligonucleotides[a]

oligomer[b]		nucleotide sequence
		4244 4263 4285
I	5′	GATTATAACTGTTATGCCTACTTATAAAGGTTACAGAATATT
(A/A)	3′	CTAATATTGACAATACGGATGAATATTTCCAATGTCTTATAA
		4266
		4247 4282
II	5′	TATAACTGTTATGCCTACTTATAAAGGTTACAGAAT
(A/A)	3′	ATTGACAATACGGATGAATATTTCCAATGTCTTATAAA
III	5′	TATAACTGTTATGCCTtCTTATAAAGGTTACAGAAT
(t/A)	3′	ATTGACAATACGGAaGAATATTTCCAATGTCTTATAAA
IV	5′	TATAACTGTTATGCCTcCTTATAAAGGTTACAGAAT
(c/A)	3′	ATTGACAATACGGAgGAATATTTCCAATGTCTTATAAA
V	5′	TATAACTGTTATGCCTgCTTATAAAGGTTACAGAAT
(g/A)	3′	ATTGACAATACGGAcGAATATTTCCAATGTCTTATAAA
VI	5′	TATAACTGTTATGCCTcCTgATAAAGGTTACAGAAT
(c/c)	3′	ATTGACAATACGGAgGAcTATTTCCAATGTCTTATAAA
VII	5′	TATAACTGTTATGCCTACTgATAAAGGTTACAGAAT
(A/c)	3′	ATTGACAATACGGATGAcTATTTCCAATGTCTTATAAA

[a] Bars and numbers at the ends of the oligonucleotides indicate the first and last bases of the upper strand (coding for the early message) and their genomic positions in SV40 DNA. Bars at the center indicate the two nucleotides covalently linked to topoisomerase II in the 4263/4266 cleavage site. Mutated bases are in italics and lowercase characters. [b] The studied oligomers are indicated with numbers (I–VII) and, in parentheses, with the two bases at positions 4263 and 4266, in the upper and lower strands, respectively.

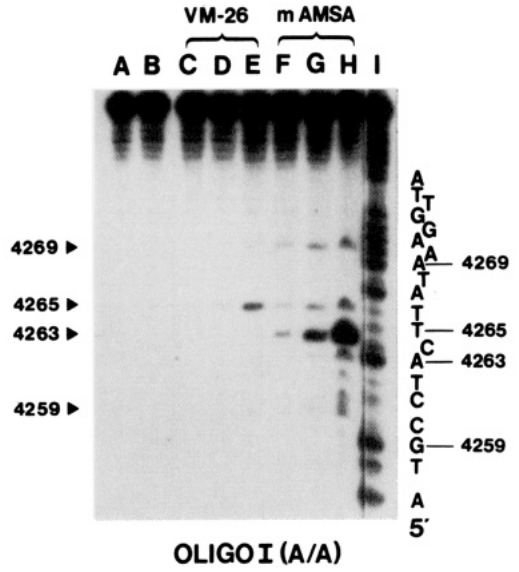

FIGURE 1: DNA cleavage produced by topoisomerase II in the oligomer I (A/A) with and without drugs. Cleavage reactions were for 10 min at 37 °C. Lanes: A, control; B, topoisomerase II without drug; C–E, +VM-26 at 5, 10, and 50 μM, respectively; F–H, +*m*AMSA at 5, 10, and 50 μM, respectively; I, purine molecular markers. Major cleavage sites are indicated on the left side. The nucleotide sequence is shown on the right side.

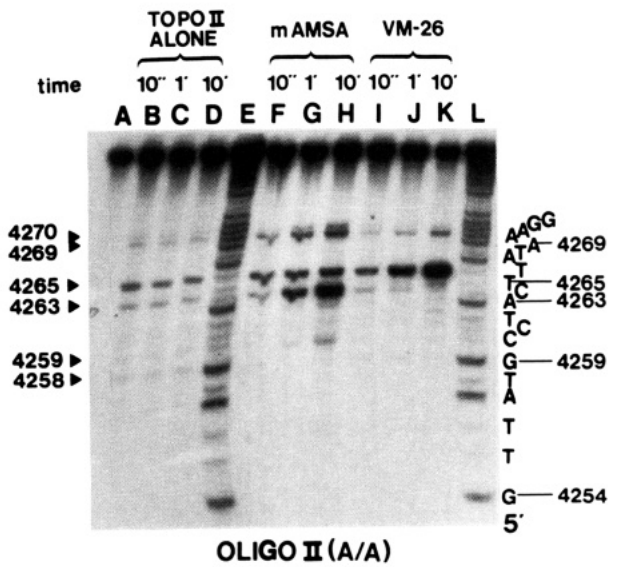

FIGURE 2: Time course of topoisomerase II DNA cleavage in the oligomer II (A/A). Cleavage reactions were for the indicated times at 37 °C without drug (lanes B–D), with 10 μM VM-26 (lanes F–H), or with 10 μM *m*AMSA (lanes I–K). Lanes: A, control; E and L, purine molecular markers. Major cleavage sites are indicated on the left side. The nucleotide sequence is shown on the right side.

RESULTS

Topoisomerase II-mediated DNA cleavage sites in the presence or absence of *m*AMSA or VM-26 were studied in 42– and 34–bp oligonucleotides (Table I). Oligonucleotide I (42 bp) corresponds to SV40 DNA from nucleotide 4244 to 4285, and oligonucleotide II (34 bp) from nucleotide 4249 to 4282 with additional terminal overhanging nucleotides (Table I). DNA cleavage was evaluated independently in the upper and lower strands and, for all the studied oligonucleotides, each DNA cleavage site on one strand was invariably paired with a corresponding site, staggered with the expected 5′ overhang of four bases, on the complementary strand. These results are consistent with the induction of double-strand cuts by topoisomerase II in this system. Thus, in the present paper, we indicate a cleavage site with the numbers of the two nucleotides covalently linked to topoisomerase II (corresponding to the +1 positions in the two strands; Table I).

The Sequence Specificities of DNA Cleavage by Topoisomerase II with and without mAMSA or VM-26 Are Conserved in Short Oligonucleotides. DNA cleavage mediated by topoisomerase II was observed at the same sites in oligonucleotides I and II as in larger SV40 DNA fragments (Figures 1 and 2). A good agreement was observed in the case of the *m*AMSA-stimulated cleavage sites (Table II). However, topoisomerase II without drug introduced strand cuts in oligonucleotides also at the 4258/4261, 4259/4262, and 4270/4273 sites, not observed in larger SV40 DNA fragments (Figure 2 and Table II). These last sites were probably too weak to be observed in larger DNA fragments (Pommier et al., 1991b). On the other hand, DNA cleavage without drug was not detectable at the 4248/4251 site, a strong cleavage site in larger SV40 DNA fragments (Pommier et al., 1991b), which is four bases distant from one terminus in oligonucleotide I (Table II and not shown). Moreover, the *m*AMSA-stimulated cleavage sites 4250/4253 and 4252/4255 observed in larger DNA fragments (Pommier et al., 1991b) were not detected in oligonucleotides (Table II). These sites were six and eight base pairs distant from one end of the oligomer. Thus, topoisomerase II preferentially produced strand cuts at the center of oligonucleotides, suggesting that flanking DNA sequences were required for appropriate DNA binding and/or cleavage by topoisomerase II.

mAMSA and VM-26 stimulated DNA cleavage mainly at the 4263/4266 and 4265/4268 sites, respectively, in oligonucleotide I in a dose-dependent manner (Figure 1). The sequence specificities of the two drugs were studied by adding the drug into the reaction mixture after an equilibrium had been reached between topoisomerase II and the labeled DNA (Figure 2). In a time-dependent manner, *m*AMSA switched the major cleavage from the 4265/4268 site to the 4263/4266 site and increased DNA cleavage also at other sites (4265/4268, 4269/4272, 4270/4273, and 4259/4262). Similar *m*AMSA-induced switches of close pairs of cleavage sites were also observed in larger SV40 DNA fragments, suggesting that, in the absence of drug, topoisomerase II DNA cleavage is in equilibrium with the religation and that, once the DNA is bound, the enzyme homodimer may move along the DNA duplex from site 4265/4268 to site 4263/4266 (Pommier et al., 1991b). In contrast, VM-26 stimulated DNA cleavage almost uniquely at the 4265/4268 site, and, to a much lesser extent, at site 4269/4272 (Figures 1 and 2). No differences of DNA cleavage patterns were observed between oligonucleotides I and II; thus, either one of them was used in the remaining part of this study.

Effects of Base Pair Mutations at the 4263 Position on Topoisomerase II DNA Cleavage at the 4263/4266 and 4265/4268 Sites. Topoisomerase II-mediated DNA cleavage was then studied in oligonucleotides III, IV, and V (Table I) carrying base pair mutations at position 4263 (Figure 3), which corresponds in the upper strand to position +1 of site 4263/4266 and to position –2 of site 4265/4268. In oligomers III (t/A), IV (c/A), and V (g/A), *m*AMSA and VM-26 still stimulated DNA cleavage at these sites (Figure 3). Topoisomerase II DNA cleavage with and without drug was overall reduced in oligomers III (t/A) and IV (c/A) as compared to oligomer V (g/A) (Figure 3). The fraction of cleaved DNA at the 4263/4266 site was lower in these mutated oligomers than in native oligomers I and II (A/A) (Table III). In the range of 10–50 μM *m*AMSA, from 2.1% to 17.7% of DNA

Table II: DNA Cleavage Sites in Oligonucleotides I and II and in the Corresponding SV40 DNA region[a]

Sites of DNA cleavage by topoisomerase II without drugs

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4244   4248            4265
   |   |          4263 |   4269
   |   |               | |   |
5′ GATTATAACTGTTATGCCTACTTATAAAGGTTACAGAATATT        in SV40 DNA

                       4265
                    4263|   4269
              4258 4259|  |   |4270
                   ||   | |   ||
5′ GATTATAACTGTTATGCCTACTTATAAAGGTTACAGAATATT        in oligonucleotides
                                                     I and II (A/A)
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Sites of DNA cleavage by topoisomerase II in the presence of mAMSA

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                       4263
   4244                |      4270
   |  4250 4252 4259   |4265 4269
   |     | |       |   | |   ||
5′ GATTATAACTGTTATGCCTACTTATAAAGGTTACAGAATATT        in SV40 DNA

                       4263
                       |4265 4269
                 4259  | |   |4270
                   |   | |   ||
5′ GATTATAACTGTTATGCCTACTTATAAAGGTTACAGAATATT        in oligonucleotides
                                                     I and II (A/A)
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[a] For clarity, only the upper strand is reported and topoisomerase II DNA cleavage sites are indicated with the number corresponding to the genomic position in SV40 DNA of the nucleotide covalently linked to topoisomerase II. The DNA cleavage sites in large SV40 DNA fragments are reported from Pommier et al. (1991b).

molecules were cleaved at the 4263/4266 site in oligomers I and II (A/A), while the fraction was reduced 4–10-fold in the mutated oligomers (Table III). On the other hand, VM-26-stimulated cleavage at the 4263/4266 site was reduced only 2–3-fold. Results were somewhat different in the case of the 4265/4268 site. DNA cleavage at that site was reduced 4–7.5-fold and 3.4-fold in the presence of *m*AMSA and VM-26, respectively, in oligomers III (t/A) and IV (C/A) as compared to oligomer I (A/A). However, the extent of DNA cleavage in the presence of either drug was essentially not decreased in the case of oligomer V (g/A) (Table III). In agreement with this observation, DNA cleavage by topoisomerase II without drugs was detectable only in the case of oligomers I (A/A) and V (g/A) at the same level (Table III). Thus, a specific reduction of the drug stimulation of DNA cleavage at the 4263/4266 site was observed in the oligomer V (g/A), and particularly for *m*AMSA, thus indicating that an adenine at position +1 is preferred over a guanine at *m*AMSA-stimulated cleavage sites.

Effects of Base Pair Mutations at the 4266 Position on Topoisomerase II DNA Cleavage at the 4263/4266 and 4265/4268 Sites. As the same enzyme–DNA complex might be stabilized either at 4263/4266 or at 4265/4268 sites, depending on the drug present (Figure 2), thus the general reduction of DNA cleavage in oligomers III (t/A) and IV (c/A) (Figure 3 and Table III) may suggest that DNA binding of topoisomerase II is reduced. Since these oligonucleotides have a stretch of seven pyrimidines (Table I), one possibility is that topoisomerase II does not bind to and/or cleave polypyrimidines. However, comparing the extent of cleaved DNA at the 4263/4266 site between oligomers III (t/A) and IV (c/A), it seemed that a cytosine at position +1 was less effective than a thymine in promoting the stimulation of DNA cleavage by *m*AMSA (Table III), in agreement with secondary base preferences observed in drug-stimulated cleavage sites in SV40 DNA (Pommier et al., 1991a). Thus, in order to test this hypothesis, topoisomerase II DNA cleavage was studied in oligomers VI (c/c) and VII (A/c), having two cytosines at the 4263 and 4266 positions or only one cytosine at the 4266 position, respectively (Table I). The results show that *m*AMSA- and VM-26-stimulated cleavage at 4263/4266 site was completely abolished in oligomer VI (c/c), while *m*AMSA

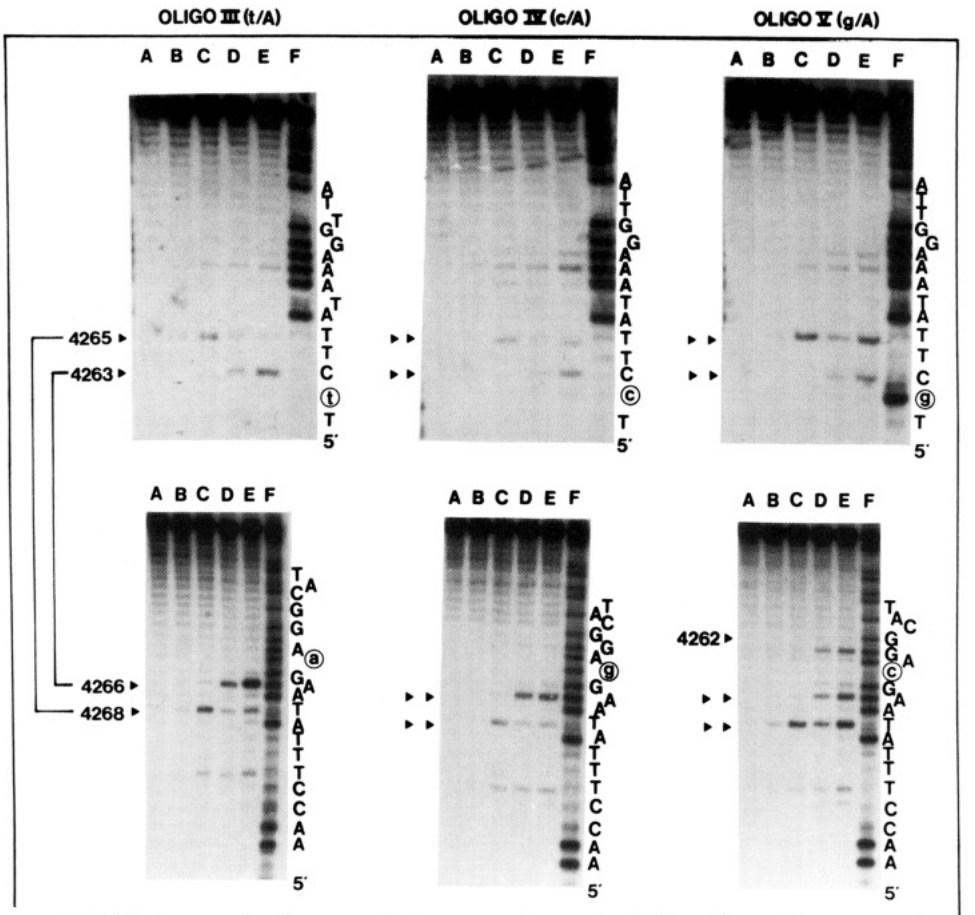

FIGURE 3: Topoisomerase II DNA cleavage in oligomers with base mutations at the 4263 position. Cleavage reactions were for 10 min at 37 °C. Lanes: A, control; B, topoisomerase II without drug; C, +10 μM VM-26; D and E, +10 and 50 μM *m*AMSA, respectively; F, purine molecular markers. Panels: upper, DNA cleavage in the upper strands; lower, DNA cleavage in the lower strands. Major cleavage sites are indicated on the left side of each gel by arrows and numbers. The nucleotide sequences are shown on the right sides of gels; mutated bases are circled lowercase characters.

Table III: Stimulation of Topoisomerase II-Mediated DNA Cleavage by *m*AMSA and VM-26 at Specific Sites in Oligonucleotides

		percentage of cleaved DNA[b]					
		site 4263/4266			site 4265/4268		
oligomer[a]	drug (μM)	upper strand	lower strand	relative[c] cleavage (%)	upper strand	lower strand	relative[c] cleavage (%)
I and II	none	0	<0.1		0.3	0.3	
(A/A)	*m*AMSA (10)	2.1	6.4	100	1.0	1.6	100
	*m*AMSA (50)	10.2	17.7	100	3.9	2.5	100
	VM-26 (10)	0.7	0.5	100	3.2	2.4	100
III	none	0	<0.1		<0.1	<0.1	
(t/A)	*m*AMSA (10)	1.2	1.0	26	0.4	0.3	27
	*m*AMSA (50)	1.8	3.4	19	0.5	0.3	13
	VM-26 (10)	0.4	0	33	1.0	0.6	29
IV	none	0	0		0	0	
(c/A)	*m*AMSA (10)	0.5	0.8	15	0.5	0.2	27
	*m*AMSA (50)	1.4	1.6	10	0.7	0.3	16
	VM-26 (10)	0.3	0.2	42	0.9	0.7	29
V	none	0	<0.1		0.3	0.2	
(g/A)	*m*AMSA (10)	1.1	0.9	24	1.3	1.0	88
	*m*AMSA (50)	2.4	0.9	12	3.8	1.1	88
	VM-26 (10)	0.4	0.2	46	3.6	0.9	80
VI	none	0	0		0.1	0	
(c/c)	*m*AMSA (10)	0	0	0	0.5	0.5	38
	*m*AMSA (50)	0	0	0	0.3	1.2	23
	VM-26 (10)	0	0	0	1.0	0.3	23
VII	none	<0.1	<0.1		0.1	0.1	
(A/c)	*m*AMSA (10)	0.3	0	4	2.1	1.4	135
	*m*AMSA (50)	0.6	0.4	3	4.7	3.7	131
	VM-26 (10)	0.3	0.2	42	4.9	1.9	121

[a] Oligonucleotide sequences are reported in Table I. [b] The percentage values shown are means of at least two independent experiments. [c] Calculated by dividing the mean of the percent cleavage on both strands in the studied oligomer by the percent cleavage mean in the nonmutated oligomers (A/A).

stimulation was specifically reduced at the same site in oligomer VII (A/c) (Figure 4). The influence of a cytosine at the 4266 position in the lower strand was even more marked than a cytosine at the 4263 position in the upper strand (Table

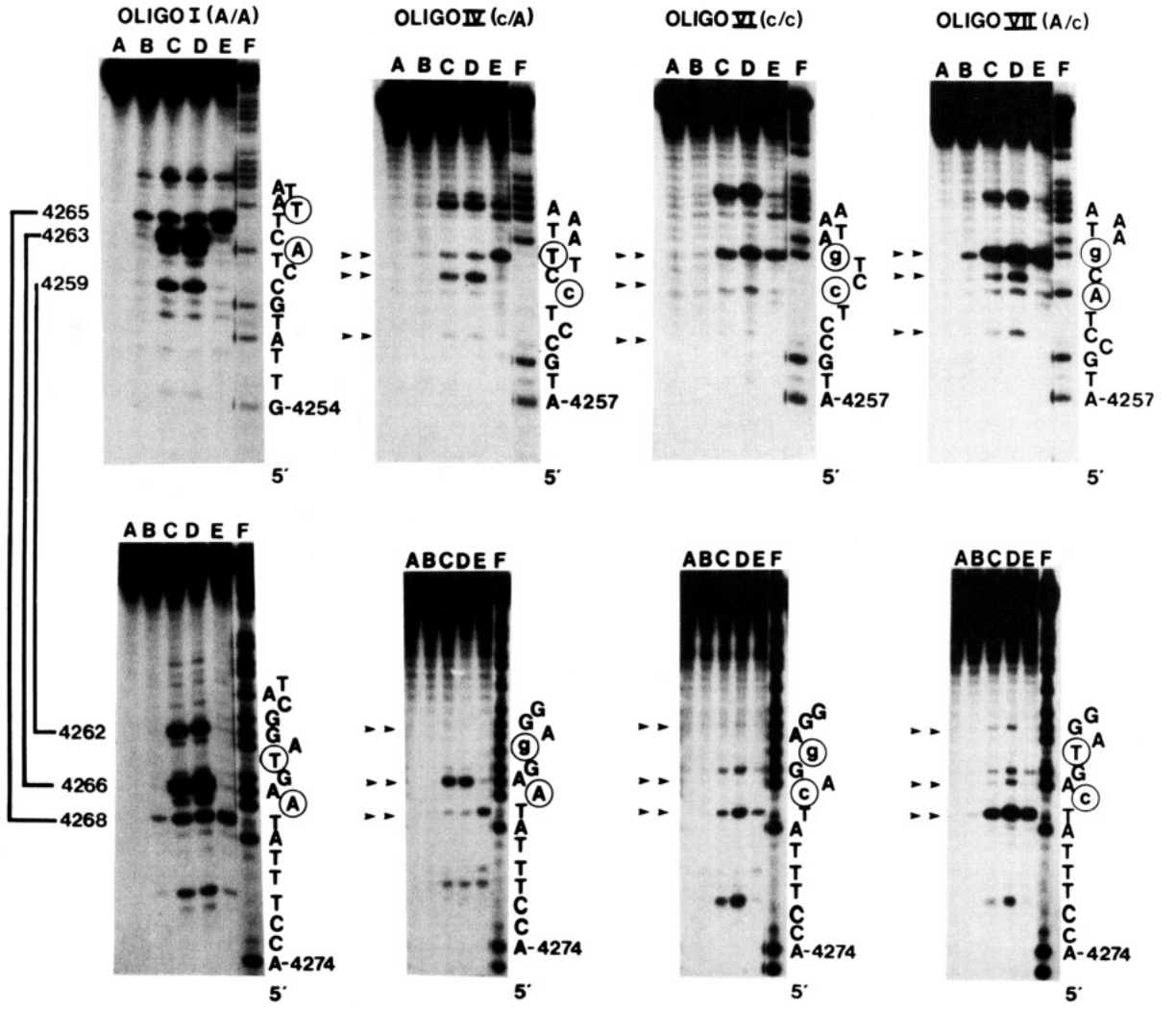

FIGURE 4: Topoisomerase II DNA cleavage in oligomers with base mutations at the 4263 and 4266 positions. Cleavage reactions were for 10 min at 37 °C. Lanes: A, control; B, topoisomerase II without drug; C and D, +10 and 50 μM *m*AMSA, respectively; E, +10 μM VM-26; F, purine molecular markers. Panels: upper, DNA cleavage in the upper strands; lower, DNA cleavage in the lower strands. Major cleavage sites are indicated on the left side of each gel by arrows and numbers. The nucleotide sequences are shown on the right sides of gels; mutated bases are lowercase characters.

III). Significantly, in the oligomer VII (A/c), the reduced cleavage was specific for the 4263/4266 site, as the cleavage at the 4265/4268 site was even increased as compared to the native oligomers I and II (A/A) (Table III). Moreover, *m*AMSA-stimulated DNA cleavage sites 4269/4272 and 4270/4273 was somewhat increased in oligomers VI (c/c) and VII (A/c) as compared to the oligomer I (A/A) (Figure 4), further indicating that the base mutations studied specifically reduced the cleavage at the 4263/4266 site. These findings strongly indicated that an adenine at position +1 enhances *m*AMSA-induced topoisomerase II DNA cleavage while a cytosine at the same position tends to suppress *m*AMSA-induced DNA cleavage.

Effects of Base Pair Mutations on Drug-Stimulated DNA Cleavage at the 4259/4262 and Other Sites. New *m*AMSA-stimulated DNA cleavage sites were not observed and the VM-26-specific pattern of DNA cleavage was unchanged in the mutated oligonucleotides. However, the *m*AMSA-specific pattern of DNA cleavage sites was somewhat altered, as the relative cleavage intensity at the 4259/4262 site was different among the studied oligonucleotides (Figures 1–4). *m*AMSA stimulated DNA cleavage at this site in the oligonucleotides I, II (A/A), V (g/A), and VII (A/c), while the cleavage was much lower, if any, in oligonucleotides III (t/A), IV (c/A), and VI (c/c) (Figures 1–4). Thus, DNA cleavage was stimulated by *m*AMSA only when a pyrimidine was present at the 4263 position in the lower strand. It has to be noted that an adenine is present at the +1 position of the lower strand at the 4259/4262 site, while a guanine is present at the corresponding position in the upper strand. The 4263 base in the lower strand corresponded to the −1 position for the 4259/4262 cleavage site, thus suggesting that both bases at the −1 and +1 positions may be crucial for stimulation of topoisomerase II DNA cleavage by *m*AMSA, at least in this case. However, this is not conclusive as topoisomerase II itself shows a preference for pyrimidines at position −1 (Capranico et al., 1990a).

VM-26- and mAMSA-stimulated DNA cleavage at site 4265/4268 was more intense in the oligonucleotide VII (A/c) (Table I) than in the other oligonucleotides (Figure 4). This increased cleavage at 4265 in the upper strand was paralleled with a similar increase of the cleavage at 4268 in the lower strand (Table III and Figure 4). The mutated base (a guanine for a thymine) corresponds to position +2 at the 4265/4268 site. This observation may suggest that a guanine at that position favored DNA binding and/or cleavage by topoisomerase II in oligomers, in agreement with a statistical analysis of cleavage sites in the SV40 genome (Pommier et al., 1991a).

DISCUSSION

Statistical analysis of several sets of topoisomerase II DNA cleavage sites induced by different antitumor drugs in SV40 DNA has led to the proposal of a molecular model of the ternary complex in which a drug molecule is stacked between the bases immediately adjacent to the cleaved phosphodiester bond (Capranico et al., 1990a; Pommier et al., 1991a; Capranico & Zunino, 1992). Consistently, the base mutation analysis presented in this report shows that a major determinant for *m*AMSA stimulation of DNA cleavage is constituted by these bases and agrees broadly with consensus sequences described for in vitro topoisomerase II DNA cleavage without drugs (Sander & Hsieh, 1985; Spitzner & Muller, 1988; Capranico et al., 1990a).

The synthetic oligonucleotides studied were derived from the major SV40 MAR where preferential drug-induced DNA cleavage takes place (Capranico et al., 1990a; Pommier et al., 1991b). The observed agreement between cleavage sites found in large and short DNA fragments demonstrates that the local DNA sequence is sufficient to determine the localization of topoisomerase II DNA cleavage in in vitro systems. The results also show that DNA cleavage sites in oligonucleotides were not detected up to 9 base pairs from one end of the oligonucleotide; the site closest to one end was at 14 base pairs from it (Table II). Therefore, a minimal length from 9 to 14 base pairs of duplex DNA at each side seems to be required for topoisomerase II-mediated DNA cleavage in vitro. This observation is in agreement with the size of the DNA segment protected by topoisomerase II from nuclease digestion (25 base pairs) (Lee et al., 1989; Pommier et al., 1992). However, the design of oligonucleotides in which a strong cleavage site is moved progressively closer to the end may conclusively determine the minimum length that is required for cleavage.

The proposed model of the ternary complex predicts that the bases immediately flanking the cleaved bonds are essential for drug action. This has been tested in this work in the case of *m*AMSA which requires an adenine at position +1 for strong DNA cleavage stimulation. The importance of the identity of the +1 base for *m*AMSA action in oligonucleotides was evaluated in relation to the effects of the same base mutation on the cleavage stimulated by VM-26 at the same site and the cleavage stimulated by *m*AMSA at different sites. In some cases, the effects of the studied base mutations were not completely specific for *m*AMSA, and this was probably due to sequence-specific alterations affecting topoisomerase II itself. In particular, all the mutations studied had the effect of reducing the cleavage stimulation by both *m*AMSA and VM-26 at the 4263/4266 site. Nevertheless, the reduction was distinctively more pronounced for *m*AMSA (4–10-fold less) than VM-26 (2–3-fold less), in agreement with the hypothesis that an adenine at position +1 is required for *m*AMSA action on topoisomerase II. As an adenine at position +1 was also preferred for DNA cleavage in the absence of drugs (Capranico et al., 1990a), the observed reduction of VM-26-induced DNA cleavage at the 4263/4266 site may be due to a decreased ability of the enzyme to recognize this site.

Oligonucleotides V (g/A) and VII (A/c) were particularly significant as DNA cleavage was specifically reduced at site 4263/4266, while cleavage at site 4265/4268 was only slightly reduced or even enhanced. This further indicates that mutations of adenines at positions +1 had a specific effect on *m*AMSA stimulation of cleavage at the 4263/4266 site. Interestingly, DNA cleavage induced by either *m*AMSA or VM-26 was reduced to a similar extent at the 4265/4268 site in oligonucleotides III (t/A), IV (c/A), and VI (c/c), suggesting that the effect at this site was not drug specific. Moreover, cleavage seemed to be overall suppressed in these oligonucleotides in comparison with the native oligonucleotides I and II (A/A). As the mutation of the adenine 4263 to a pyrimidine (Y) produces a polypyrimidine stretch, 4260-CCTYCTT-4266, topoisomerase II binding to and/or cleavage of the DNA may be overall decreased in oligonucleotides with this sequence. This observation is consistent with previous findings showing that topoisomerase II DNA cleavage tends to occur in alternating purine/pyrimidine repeats (Spitzner et al., 1990).

Furthermore, the results of the present work show that mutations in one strand affected cleavage also at the opposite strand of a double-strand break. This is consistent with previous observations that preferred bases could be at only one of the two sites of a pair constitutive of a double-strand cleavage for drug-stimulated DNA cleavage in the SV40 genome (Capranico et al., 1990a; Pommier et al., 1991a). Altogether, these data suggest that the two subunits of topoisomerase II work in concert and that a drug molecule strongly interacting at one strand cut may be sufficient to trap the whole topoisomerase II homodimer.

DNA sequence is generally recognized to be an important determinant of the in vitro DNA cleavage by topoisomerase II. However, the intracellular interaction of other proteins with DNA may alter the frequency of DNA cleavage caused by topoisomerase II in living cells under the influence of *m*AMSA or other drugs (Yang et al., 1985; Richter & Ruff, 1991; Kas & Laemmli, 1992; Capranico et al., 1990b). Nevertheless, investigations on the in vitro sequence specificities of DNA cleavage stimulated by topoisomerase inhibitors have recently led to the idea of a common molecular model for the mechanisms of action of either topoisomerase I and II inhibitors (Capranico et al., 1990a; Pommier et al., 1991a; Kjeldsen et al., 1988; Jaxel et al., 1991; Capranico & Zunino, 1992). An approach based on the analysis of the effects of nucleotide mutations may provide additional and more direct evidence for the drug sequence specificity. Consistent with a common molecular model, mutations of the drug-required bases specificity reduce or even abolish the stimulation of topoisomerase DNA cleavage in either the case of *m*AMSA (present study) or the case of camptothecin (Jaxel et al., 1991).

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