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Amsacrine-Promoted DNA Cleavage Site Determinants for the Two Human DNA Topoisomerase II Isoforms α and β

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ABSTRACT. Site-specific DNA cleavage by topoisomerase II (EC 5.99.1.3) is induced by many antitumour drugs. Although human cells express two genetically distinct topoisomerase II isoforms, thus far the role and determinants of drug-induced DNA cleavage have been examined only for α. Here we report the first high-resolution study of amsacrine (mAMSA) induced DNA breakage by human topoisomerase IIβ (overexpressed and purified from yeast) and a direct comparison with the recombinant α isoform. DNA cleavage in plasmid pBR322 and SV40 DNA was induced by α or β in the absence or presence of the antitumour agent mAMSA, and sites were mapped using sequencing gel methodology. Low-resolution studies indicated that recombinant human α promoted DNA breakage at sites akin to those of β, although some sites were only cleaved by one enzyme and different intensities were observed at some sites. However, statistical analysis of 70 drug-induced sites for β and 70 sites for α revealed that both isoforms share the same base preferences at 13 positions relative to the enzyme cleavage site, including a very strong preference for A at +1. The result for recombinant α isoform is in agreement with previous studies using α purified from human cell lines. Thus, α and β proteins apparently form similar ternary complexes with mAMSA and DNA. Previous studies have emphasized the importance of DNA topoisomerase II α; the results presented here demonstrate that β is an *in vitro* target with similar site determinants, strongly suggesting that β should also be considered a target of mAMSA *in vivo*. BIOCHEM PHARMACOL 52;11:1675–1685, 1996.

KEY WORDS. human DNA topoisomerase IIα; human DNA topoisomerase IIβ; amsacrine; DNA cleavage; pBR322; SV40 DNA

Type II DNA topoisomerases (EC 5.99.1.3) catalyse the interconversion of topological isomers of DNA by passing one DNA helix through a transient enzyme-bridged double-stranded DNA break in another. Different approaches have established that eukaryotic topoisomerase II is a major constituent of the metaphase chromosome scaffold, is essential for chromosome segregation and plays a role in releasing superhelical tension generated by transcription and replication [1–3]. Unlike lower eukaryotes such as *Drosophila melanogaster* and *Saccharomyces cerevisiae*, which appear to have a single form of topoisomerase II, human and other mammalian cells produce two genetically different isoforms, α and β, which have different patterns of temporal and spatial expression [4–11]. Topoisomerase IIα is expressed predominantly in tissues containing proliferating cells, and protein levels are highest during the G2/M phase of the cell cycle. By contrast, topoisomerase IIβ is expressed in a wide range of tissues, and protein levels remain relatively constant throughout the cell cycle [8, 9]. Immunofluorescence studies have reported the β isoform to be localised in the nucleolus, whereas the α isozyme is found throughout the nucleus [10, 11]. These observations suggest the two human isoforms are regulated independently and have different roles in the cell.

Topoisomerase II is the intracellular target for a number of structurally diverse antitumour agents including doxorubicin, epipodophyllotoxins and mAMSA.[‖] These agents interfere with DNA breakage–reunion by the enzyme, stabilising an enzyme-DNA intermediate in which the two DNA strands are broken and covalently attached at their 5′ termini, one to each subunit of the enzyme dimer. These "cleavable complexes" are the principal lesion by which

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‖ *Abbreviations:* mAMSA, amsacrine; PCR, polymerase chain reaction.

topoisomerase II inhibitors exert their cytotoxic effects. Transcription and replication processes convert these ternary complexes into double-strand DNA breaks, which can lead to illegitimate recombination, chromosome aberrations or apoptotic cell death [12–14]. Cells that express high levels of topoisomerase II are the most sensitive to these drugs, and thus proliferating tissues such as tumours and haematopoietic cells are targeted.

DNA breakage by topoisomerase II occurs at specific sites on DNA. Consensus cleavage rules have been proposed for enzymatic DNA breakage in the presence or absence of drugs. These studies have largely used native topoisomerase II purified from calf thymus, chicken erythrocytes or mouse leukaemic cells, and many were carried out before the existence of enzyme isoforms was generally recognised [15–17]. Work on DNA cleavage by human topoisomerase II has relied on enzyme from cell lines consisting entirely or predominantly of the α isoform [18, 19]. Difficulties in purifying human topoisomerase IIβ free of α and in quantity have precluded detailed studies of this isoform. Consequently, despite its obvious relevance for an understanding of drug action, little is known about the role and sequence specificity of DNA breakage by human topoisomerase IIβ.

We have engineered two yeast expression plasmids carrying cDNA sequences for human topoisomerase IIα or topoisomerase IIβ that allow purification of the individual human isoforms [20, 21]. We have used these homogeneous recombinant α and β proteins to analyse the sequence specificity of their DNA cleavage activity on pBR322 or SV40 DNA in the absence and presence of the anticancer drug mAMSA. Despite variation at a small number of sites, the results of statistical analysis of a large number of sites show that the β isoform cleaves DNA with the same base specificity as α and with similar efficiency: both isoforms share similar base preferences at 13 positions relative to the enzyme breakage site, including A at +1. This analysis of DNA cleavage by β confirms that β is efficiently inhibited by mAMSA and suggests that both isoforms could be intracellular drug targets.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents

mAMSA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). It was dissolved in dimethyl sulphoxide (DMSO) and stored at −20°C. Restriction enzymes were obtained from Northumbria Biologicals (Cramlington, Northumberland). [Gamma-^{32}P]ATP (3000 Ci/mmoi), [α-^{32}P]dCTP and [α-^{35}S]ATP were obtained from Amersham (Little Chalfont, Buckinghamshire). DE81 paper was obtained from Whatman (Maidstone, Kent). T4 polynucleotide kinase and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim (Lewes, Sussex). Recombinant human topoisomerases α and β were purified as described previously [20, 21]. Labelled DNA markers were prepared by incubating the "1-kb ladder" (Biorad, Hemel Hempstead, Hertfordshire) with Klenow fragment (New England Biolabs, Hitchin, Hertfordshire) and [α-^{32}P]dCTP. All other materials were purchased from commercial suppliers.

5′ End Labeling of pBR322 and SV40 DNA Restriction Fragments

A 4.3-Kb *Hin*dIII–*Eco*RI fragment of pBR322 was uniquely 5′ end labelled with ^{32}P at its *Hin*dIII end as previously described [15] and the DNA was purified by using a Quiaquick spin column (Quiagen, Chatsworth, CA). Three regions of pBR322 were amplified by PCR using pairs of oligonucleotides corresponding to the following nucleotide positions in the plasmid: oligo 1 (4335–4349) and 2 (673–657); 3 (940–956) and 4 (1429–1413); 5 (1486–1502) and 6 (2931–2915). PCR conditions were 92°C for 1 min, 50°C for 2 min and 72°C for 3 min, repeated for 30 cycles. PCR products were gel purified and labelled using T4 kinase and 50 μCi [gamma-^{32}P]ATP. After ethanol precipitation, products were digested with appropriate restriction enzymes to generate uniquely end-labelled fragments, which were isolated following agarose gel electrophoresis in 1.5% agarose gels. Size markers for analysing cleavage of these fragments were generated by using the same oligonucleotides as primers for dideoxy sequencing reactions, with pBR322 as template. SV40 DNA fragments were uniquely 5′ end labelled and then purified by electroelution and ethanol precipitation as already described [17].

DNA Cleavage by Topoisomerase II Isoforms

One picomole of topoisomerase IIα or topoisomerase IIβ was incubated with the end-labelled 4.3-Kb *Hin*dIII–*Eco*RI fragment of pBR322 DNA (5000 cpm Cerenkov) in cleavage buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.5 mM EDTA, 30 μg/mL bovine serum albumin, 1 mM DTT, 100 mM KCl and 2.5% DMSO) in the presence or absence of m-AMSA (total volume 20 μl) at 37°C for 1 hr. SDS was added to 0.1% and proteinase K to 500 ng/mL; the samples were incubated for another hour at 37°C. Loading buffer (0.5% SDS, 25% glycerol, 0.1% bromophenol blue, 5 μl) was added, and the samples were electrophoresed in a 0.8% agarose gel for 5–16 hr at 1–5 V/cm. The gel was dried onto DE81 paper by capillary action and exposed to X-ray film for 3–5 days.

Cleavage of pBR322 fragments isolated by PCR were carried out similarly, except 6000–8000 cpm of radiolabelled DNA, 4 pmol of protein and mAMSA to a final concentration of 25.4 μM were used. Reactions were stopped by adding 0.1 vol sodium acetate, pH 5.2, 20 μg glycogen and 2 vol ethanol to precipitate the DNA. After 15 min at −80°C, DNA was pelleted by centrifugation and washed with 70% ethanol. DNA pellets were dissolved in 4 μl sequencing gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF), denatured for 2 min at 90°C and loaded onto a 6% denaturing polyacrylamide sequencing gel alongside appro-

priate ^{35}S-labelled DNA size markers (see below). Gels were run at 1100 V until the bromophenol blue (short run) or xylene cyanol dye (long run) reached the gel bottom, fixed in 10% acetic acid for 10 min and dried on to Whatman 3MM paper under *vacuo* at 80°C. Exposure to X-ray film was at −80°C for 3–4 days (to optimise ^{32}P exposure) and then at room temperature for 2–3 days (to optimise ^{35}S exposure) before development. Cleavage reactions and gels were performed at least three times to remove any ambiguities in alignment.

Topoisomerase II cleaves DNA to generate uncharged 3′ OH termini similar to the 3′ -H termini resulting for chain termination sequencing products. However, dideoxy sequencing products labelled by inclusion of [α-^{35}S] dATP derive from primers lacking the 5′ phosphate group present on the DNA substrates for topoisomerase II cleavage. To determine the effect of this phosphate group on the mobility of DNA, dideoxy sequencing reactions were also carried out by using primers 5′ labelled with ^{32}P but omitting the [α-^{35}S] dATP usually included in the labelling step. The two sets of sequencing products were electrophoresed alongside each other. The presence of the charged 5′ phosphate group increased the mobility of these sequencing products over the 5′ OH fragments as follows: mobility increased equivalent to 1.5 bp for 25–35-bp fragments, by 1 bp for 36–45-bp fragments and by 0.5 bp for 46–55-bp DNA fragments. These adjustments were used in fine mapping of DNA cleavage sites.

The 5′ ^{32}P-labelled SV40 DNA fragments were cleaved with topoisomerase II isoforms with or without mAMSA in 10 mM Tris-HCl, pH 6.0, 50 mM KCl, 10 mM $MgCl_2$, 1 mM ATP for 20 min at 37°C. Reactions were processed as described previously [17]. Cleavage sites were mapped by using A + G and C + T Maxam Gilbert sequencing products obtained for the parent fragment as size markers. The sequencing products have lost a G/A or C/T base at their 3′ ends and carry a 3′ phosphate group. Mobility differences with topoisomerase II cleaved products were allowed for as previously described [22].

Statistical Analysis of Cleavage Sites

Sites were classified as weak or strong (consistently and easily visible on the autorad), and the two categories were analysed separately and together. Each cleavage product was aligned with the sequencing marker of the same size, which corresponds to the base at position −1. Topoisomerase II makes a staggered cut on both strands to give a 4-bp 5′ overhang that is covalently linked to the enzyme. Three methods were used to choose the DNA strand at each cleavage site used for analysis. In method I, the ^{32}P-labelled strand was used [17, 23]. In method II, the sequences of both DNA strands at each site were analysed [18]. In method III, the "best strand" at each cleavage site was selected by an iterative process [16, 18, 24]. Briefly, the initially derived consensus was compared with both strands at each cleavage site, and the strand that matched best was then used to calculate a new consensus. This process was repeated until the consensus could not be improved. To derive each consensus, bases from −8 to +20 relative to each cleavage site were aligned, and the base frequencies analysed by chi-square and probability analysis, as described previously [17, 23], based on the overall composition of pBR322: f(C/G) = 0.269, f(A/T) = 0.231.

RESULTS AND DISCUSSION

Cleavage of pBR322 and SV40 DNA by α and β Isoforms

MAJOR CLEAVAGE REGIONS IN pBR322. To compare the cleavage site specificities of human topoisomerase IIα and topoisomerase IIβ, cleavage reactions were carried out on a 4.3-kb *Hin*dIII–*Eco*RI fragment of plasmid pBR322. This fragment was 5′ labelled with ^{32}P at its *Hin*dIII end and was incubated with human topoisomerase IIα and topoisomerase IIβ isoforms in the presence or absence of mAMSA. DNA cleavage was induced by denaturation with SDS and, after proteinase K treatment to digest topoisomerase II, covalently linked to the 5′ ends of DNA breaks; cleavage products were examined by agarose gel electrophoresis (Fig. 1). Inclusion of mAMSA induced DNA breakage by both α and β (Fig 1: lanes 2–4, 6–8). Interestingly, the cleavage

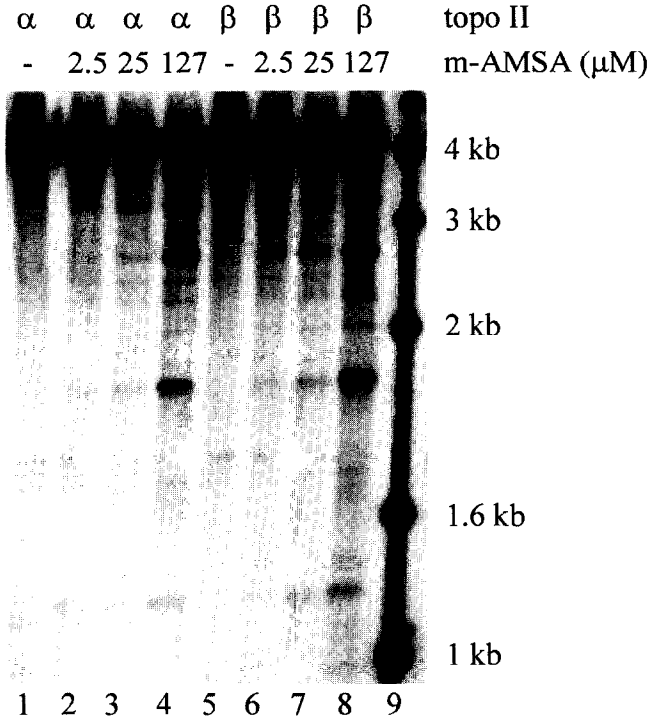

FIG. 1. DNA cleavage by topoisomerase IIα and topoisomerase IIβ in the presence of mAMSA. A *Hin*dIII–*Eco*RI pBR322 DNA fragment was 5′ end labelled at the *Hin*dIII site and incubated with 1 pmol of topoisomerase II and mAMSA at 37°C for 60 min. Cleavage was stabilised by the addition of 0.1% SDS and 0.5 mg/mL proteinase K followed by incubation at 37°C for 60 min. Loading buffer was added and samples electrophoresed on an 0.8% agarose gel as described in Experimental Procedures.

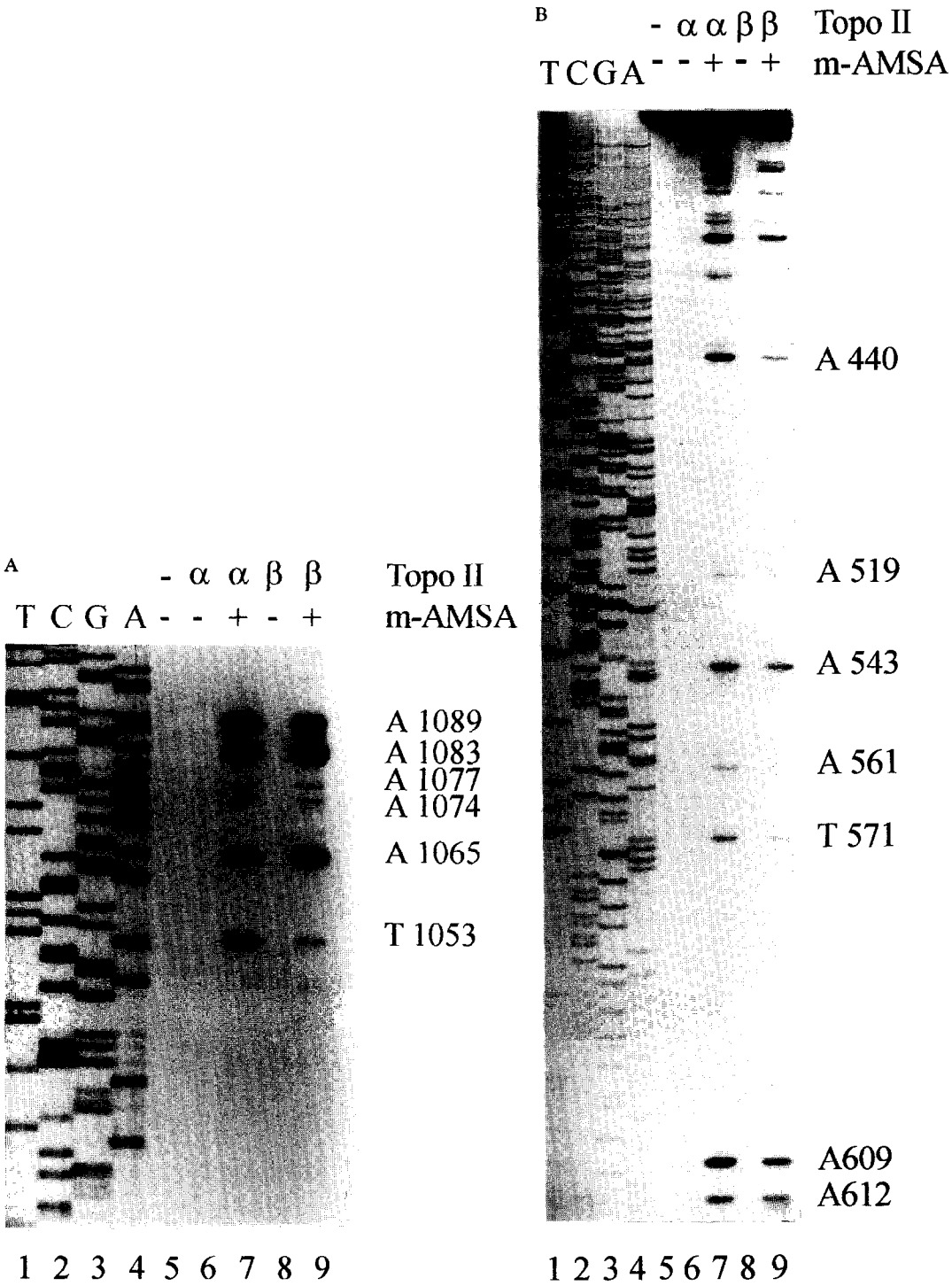

FIG. 2. Topoisomerase IIα and topoisomerase IIβ DNA cleavage intensity patterns stimulated by mAMSA. Lanes 1–4: T, C, G, A dideoxy sequencing reactions; lane 5: control DNA; lane 6: topoisomerase IIα; lane 7: topoisomerase IIα and mAMSA; lane 8: topoisomerase IIβ; lane 9: topoisomerase IIβ and mAMSA. Cleavage was performed on (A) an *Eag*I–*Sty*I fragment (940–1369 bp) of pBR322 DNA uniquely 5′ end labelled at the *Eag*I site and (B) a *Hin*dIII–*Sal*I fragment (30–673 bp) of pBR322 DNA uniquely 5′ end labelled at the *Sal*I site. Four picomoles of topoisomerase II were incubated with DNA and mAMSA to a final concentration of 25.4 μM for 60 min at 37°C. Reactions were then processed as described in Experimental Procedures and analysed by 6% polyacrylamide sequencing gels.

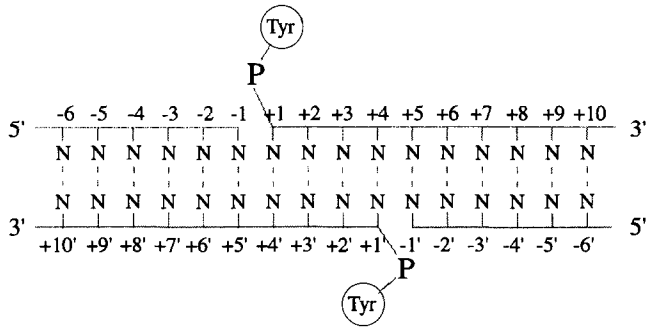

FIG. 3. Nucleotide positions at the site of DNA cleavage. The 5′ phosphate group of each cleaved strand becomes covalently linked to the active site tyrosine residue of a topoisomerase II monomer.

patterns for α and β were similar except for differences in intensity at some locations and a few sites that were cleaved only with α or β (Fig 1: compare lanes 4 and 8). Particularly intense DNA cleavage occurred at four locations in pBR322, which, from the migration of size markers, could be provisionally mapped to the vicinity of pBR322 nucleotide positions 3700, 3200, 2650 and 1850 (±50 bp). At this level of resolution, it was not clear whether these major cleavage sites were identical for α and β at the nucleotide level nor whether they represented individual sites or clusters of sites.

To resolve these questions, cleavage by α and β of pBR322 DNA fragments radiolabelled at a unique 5′ end was examined on high-resolution DNA sequencing gels. Regions of pBR322 encompassing cleavage sites identified in Fig. 1 and others were amplified by PCR using appropriate oligonucleotide primers (see Experimental Procedures). The PCR products were purified, 5′ end labelled with ^{32}P and cut with a restriction enzyme to generate a uniquely end-labelled fragment that could be used as a substrate in cleavage reactions. Figure 2A shows mAMSA-stimulated cleavage of an *Eag*I–*Sty*I fragment of pBR322 (940–1369 bp) by both α (Fig. 2A: compare lanes 6 and 7) and by β (Fig. 2A: lanes 8 and 9). By using a series of dideoxy sequencing products obtained for the same *Eag*I–*Sty*I fragment (Fig. 2A: lanes 1–4), it was possible to map these cleavage sites accurately at the nucleotide level (see

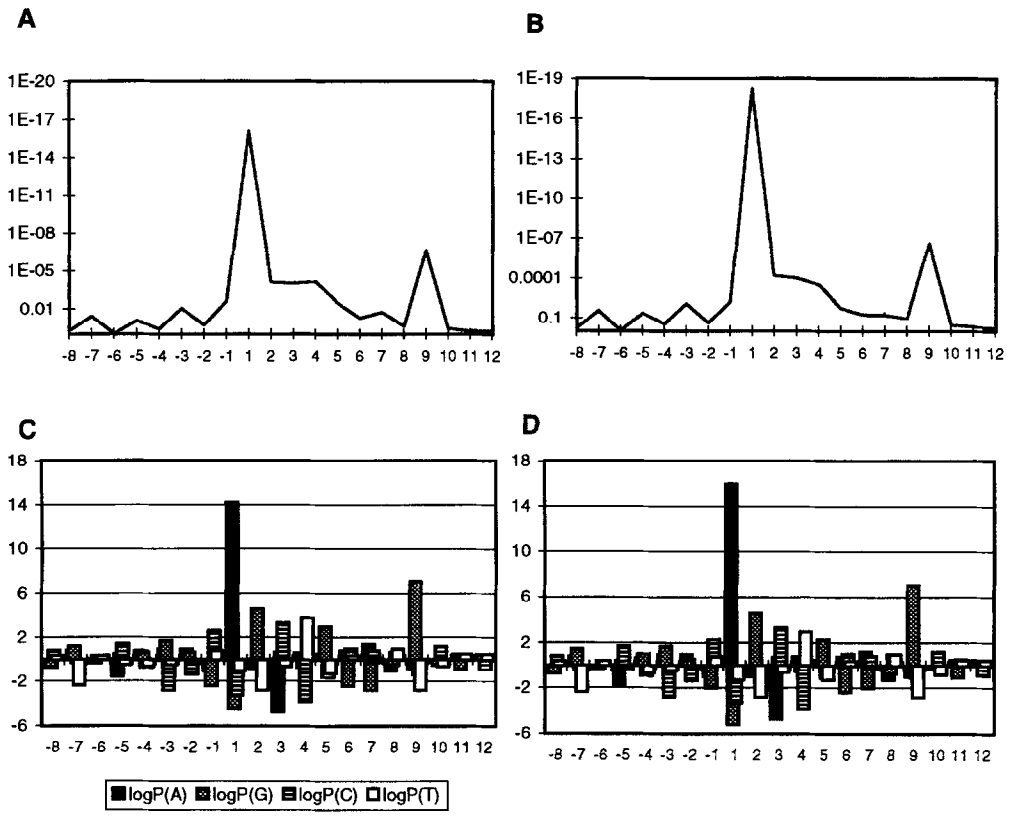

FIG. 4. Topoisomerase IIα and topoisomerase IIβ cleavage sites stimulated by mAMSA on pBR322 DNA. Analysis was done on the labelled DNA strand at each cleavage site for human α (A and C) and for human β (B and D). (A, B) Chi-square analysis of the base distribution at each position relative to the cleavage site vs. the overall base distribution in pBR322. The probability of each chi-square value (3 degrees of freedom) is plotted. (C, D) Log *P* for each base at each position relative to the cleavage site. *P* is the probability of obtaining the observed base frequency given the overall base composition of pBR322. Log*P* is displayed as positive where the base frequency is greater than expected and as negative where base frequency is lower than expected. Significance levels are log *P* = 1.3, *P* < 0.05; log *P* = 2, *P* < 0.01.

TABLE 1. Consensus for mAMSA-stimulated cleavage by topoisomerase IIα and topoisomerase IIβ on pBR322 and SV40 DNA

	-8	-7	-6	-5	-4	-3	-2	-1	:	1	2	3	4	5	6	7	8	9	10	11	12
PBR322																					
β, all sites																					
Favoured	—	G	—	C	—	G	—	C	:	A	G	C	T	G	—	—	—	G	—	—	—
Disfavoured	—	t	—	a	—	c	c	g	:	gct	t	a	c	t	g	g	a	t	—	—	—
α, all sites	—	—	—	C	—	G	—	C	:	A	G	C	T	G	—	A	—	G	—	—	—
	—	t	—	a	—	c	c	g	:	gc	t	a	c	ct	g	g	—	ct	—	—	—
β, strong sites	—	—	—	C	—	—	—	C	:	A	G	C	—	—	—	A	—	G	—	—	—
	—	t	—	—	t	—	—	g	:	g	at	a	—	—	—	g	—	at	—	—	—
α, strong sites	—	—	—	C	—	—	—	C	:	A	G	C	—	—	—	A	—	G	—	—	—
	—	t	—	—	t	—	—	g	:	g	at	a	—	—	—	g	—	at	—	—	—
β, weak sites	—	—	—	—	—	G	—	—	:	A	G	C	T	G	—	C	—	G	C	—	—
	—	t	—	a	—	c	—	a	:	gc	t	a	c	—	g	—	—	t	—	—	—
α, weak sites	—	—	—	—	—	G	—	C	:	A	G	C	T	G	—	—	—	G	C	—	—
	—	t	—	—	—	c	—	a	:	gc	t	a	c	c	g	g	—	t	—	—	—
SV40 DNA																					
β, all sites	T	—	—	—	G	A	—	—	:	A	G	C	—	—	C	—	—	—	—	—	—
	—	—	—	—	t	—	c	—	:	ct	—	a	—	—	g	—	—	—	—	—	—
α, all sites	T	—	—	—	G	A	—	—	:	A	G	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	t	—	—	—	:	g	—	a	—	—	g	—	—	—	—	—	—

Analysis was done on sequence for the labelled DNA strand (mapped strand). In Tables 1–4, favoured bases are shown in upper-case letters and disfavoured bases are shown in lower-case letters. They are considered significant if log $P > 1.3$ ($P < 0.05$); bases with log $P > 2$ ($P < 0.01$) are shown in boldface type. The point of cleavage is indicated by a colon. Consensus sequences cleaved by topoisomerase IIα and topoisomerase IIβ on pBR322 are shown for three categories: all sites, strong sites and weak sites. Preferences for mAMSA-stimulated cleavage by topoisomerase IIα and topoisomerase IIβ on SV40 DNA are also shown.

Experimental Procedure). Six cleavage products were identified with topoisomerase II cleavage immediately 5′ to the indicated nucleotide bases in the pBR322 sequence on the labelled strand: sites A1089, A1083, A1077, A1074, A1065 and T1053 (Figs. 2a, 3). Of the six sites, a clear preference can be seen for A 3′ of the topoisomerase II break site. Each isoform cleaved the fragment at the same sites though the efficiency of enzymatic breakage differed, in particular at sites A1065 and T1053. Similar results were seen for a 644-bp Sal fragment of pBR322 (30–673 bp) (Fig. 2B): essentially the same spectrum of sites was cleaved by each isoform but with differing efficiencies, e.g., at A440.

Overall, cleavage by topoisomerase IIβ in the presence of mAMSA coincided with topoisomerase IIα at 68 of the 72 sites mapped, with levels of cleavage generally very similar for the two isoforms. The presence of ATP did not change the positions of cleavage and caused only slight increases in intensity (data not shown). In the absence of drug, the α and β isoforms cleaved identical sites, several of which were further stimulated by mAMSA, e.g. C102 and A86 (data not shown).

DRUG DETERMINANTS FOR SITE-SPECIFIC DNA BREAKAGE. Both human topoisomerase II proteins used in these studies were produced in yeast and then purified to homogeneity. In these recombinant α and β enzymes, the respective N-terminal 29 and 45 amino-acid residues of the 1531 and 1621 residue proteins were not present and were instead replaced by the first five residues of yeast topoisomerase II [20, 21]. Although unlikely from structural considerations of topoisomerase II, these recombinant proteins might have altered cleavage specificity when compared with native isoforms. To allow comparison between the recombinant isoforms and the native human α investigated previously [18, 19], a detailed statistical analysis of cleavage sites was performed to elucidate the sequence determinants for DNA breakage.

Statistical analysis of 70 topoisomerase IIβ cleavage sites on pBR322 was done by using a previously reported method to determine a consensus DNA sequence for cleavage in which the labelled DNA strand was used for all cleavage sites [17, 23]. Analysis was also done on the 70 topoisomerase IIα cleavage sites on pBR322, 68 of which are coincident with sites cleaved by the β isoform. The frequencies of C, T, G and A were calculated for positions −8 to +12 relative to the cleavage site. Chi-square analyses of the DNA strands analysed for α and β cleavage are shown in Fig. 4A and 4B, respectively. Base distribution was nonrandom at positions −7, −3, −1, +1, +2, +3, +4, +5 and +9 for both isoforms and at −5 for β and at +7 for α ($P < 0.05$). The strongest base selection was at position +1, immediately downstream of the cleavage site ($P < 1 \times 10^{-15}$). To determine the preferred bases at each position for topoisomerase IIα and topoisomerase IIβ, the probabilities of each of the observed base frequencies occurring by chance was calculated and their logarithms are plotted (Fig. 4C and 4D), respectively. Preferences for or against particular bases at several positions between −7 and +10 are revealed. The consensus sequences derived are shown in Table 1. The strongest selection is for A at +1: 69% of the cleavage sites sequenced had an A at +1.

The core consensus sequences derived for the two isoforms are identical, C↓AGCTG being present in both, and bases +3C, +4T and +5G have dyadic symmetry with +1A,

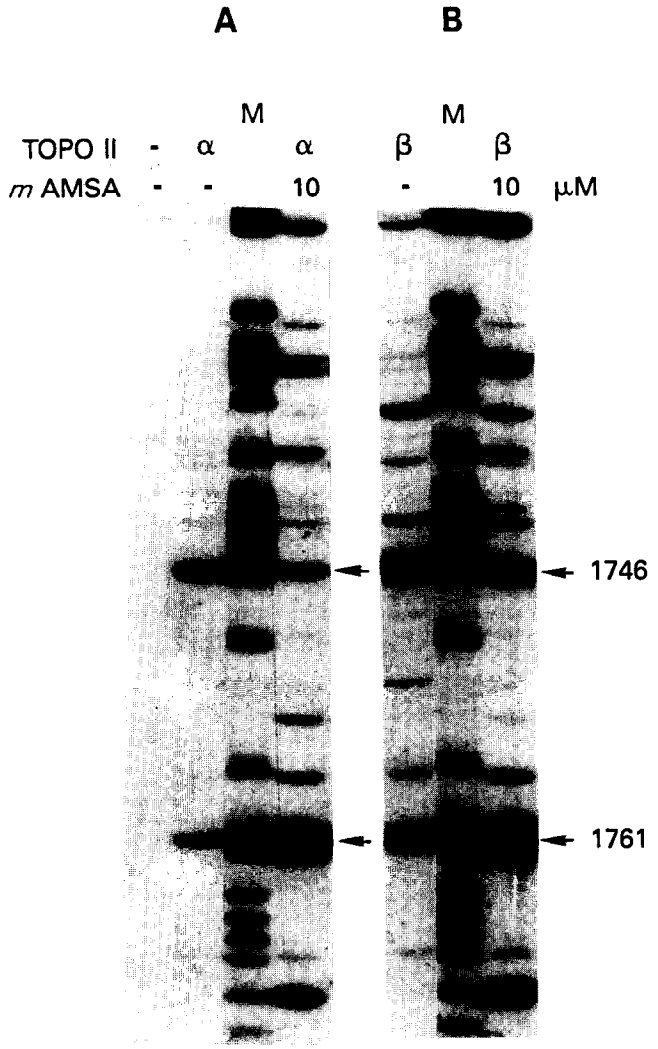

FIG. 5. Topoisomerase IIα (A) and topoisomerase IIβ (B) cleavage of SV40 DNA in the presence of 10 μM mAMSA. An *Eco*RI–*Hae*II SV40 DNA fragment was 5′-end-^{32}P labelled at the *Eco*RI site, then reacted with topoisomerase II with or without mAMSA for 20 min at 37°C and analysed on a 8% polyacrylamide sequencing gel. M, purine molecular weight markers.

+2G and −1C. Other base preferences at additional positions were revealed: +9G, −3G and −5C for both isoforms, +7A for α and −7G for β; interestingly, +9G and −5C also displayed dyadic symmetry. The consensus cleavage sequence of topoisomerase IIβ is thus virtually identical to that of human topoisomerase IIα.

To determine whether the efficiency of cleavage at a particular site is influenced by DNA sequence, sites cleaved by topoisomerase IIα and topoisomerase IIβ on pBR322 were classed as either weak or strong, and the two categories analysed independently. The strong sites usually coincide with double-strand breaks, whereas the weak sites are often only cleaved on one strand [17]. The consensuses for weak and strong sites (Table 1) were very similar but not identical. There were more weak than strong sites (42 and 26, respectively), which may account for the additional bases

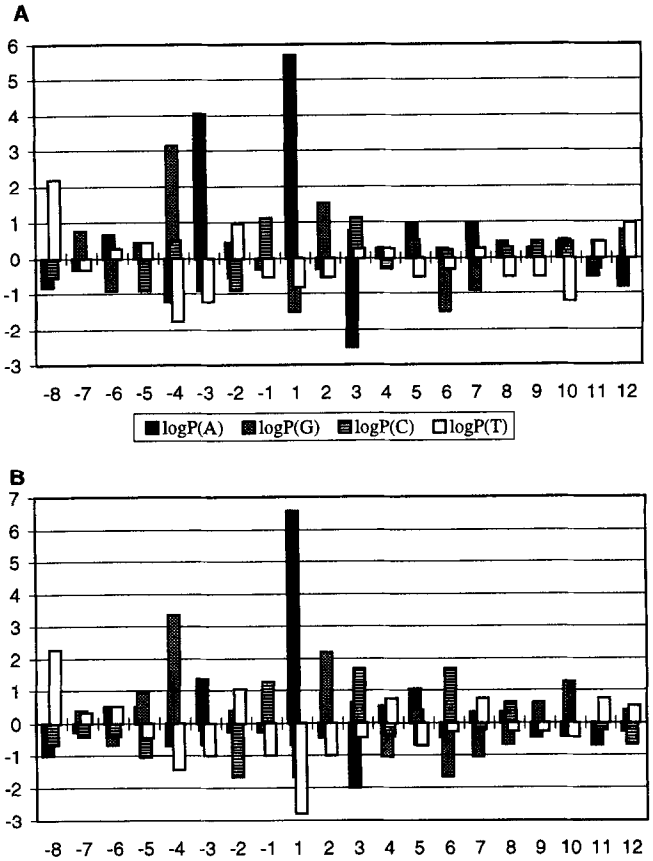

FIG. 6. Analysis of topoisomerase IIα and topoisomerase IIβ cleavage sites on SV40 DNA stimulated by mAMSA. (A) Topoisomerase IIα sites. (B) Topoisomerase β sites. Log *P* is plotted for each base at each the position relative to the cleavage site (see Fig. 4). Significance levels are log *P* = 1.3, *P* < 0.05; log *P* = 2, *P* < 0.01.

+4T, +5G and −3G in the weak consensus for both α and β and +7C in β; −5C and +7A were preferred in the strong but not the weak consensus, and their presence may increase the efficiency of cleavage at particular sites. The close similarity of the consensuses for strong and weak cleavage sites suggests that single-stranded breaks have similar sequence requirements for cleavage as the double stranded breaks.

Cleavage by topoisomerase IIα and topoisomerase IIβ was also observed at 18 sites on pBR322 in the absence of mAMSA, each of which was cleaved equally efficiently by both isoforms. Cleavage at nine of these sites was stimulated by mAMSA, but the intensities of the other sites were not altered by the drug. Thus, the two isoforms cleave very similar DNA sequences even in the absence of a strong cleavage-enhancing drug such as mAMSA. Statistical analysis was done, although the small number of sites meant only four positions showed significant base preferences (+5A, +7A, +8C, +11T). The striking difference for enzyme alone is the absence of very strong selection for +1A. This finding agrees with previous studies that have shown that the bases at positions −1 and +1 are strongly dependent on the presence and identity of any drug used [17, 23]

TABLE 2. Comparison of the consensus sequences

	-8	-7	-6	-5	-4	-3	-2	-1	:	1	2	3	4	5	6	7	8	9	10	11	12
Recombinant	—	G	—	C	—	G	—	C	:	A	G	C	T	G	—	—	—	G	—	—	—
human β; pBR322	—	t	—	a	—	c	c	g	:	gct	t	a	c	t	g	g	a	t	—	—	—
Recombinant	—	—	—	C	—	G	—	C	:	A	G	C	T	G	—	A	—	G	—	—	—
human α; pBR322	—	t	—	a	—	c	c	g	:	gc	t	a	c	ct	g	g	—	ct	—	—	—
Native murine α	—	—	A	—	G	A	A	T	:	A	G	C	T	A	T	A	C	—	—	—	—
and β; SV40 [24]	—	—	c	—	—	g	c	ga	:	gct	t	a	—	t	g	g	g	—	—	—	c
Native human α	—	—	—	—	G	A	—	—	:	A	G	G	T	—	T	T	C	G	A	—	A
and β in 3:1 ratio; pBR322 [19]	—	—	—	a	t	cg	c	g	:	gct	a	a	—	—	g	g	—	—	t	—	—
Native human α [18]	—	—	A	C(T)	AG	T	AG	TC	:	A	C	G	T	AG	TC	A	TC	G(A)	T	—	—

Comparison of the consensus sequences for topoisomerase IIα and topoisomerase IIβ derived in this paper with those for (1) native murine topoisomerase II α and β [24], (2) a 3:1 mixture of native human topoisomerase II α and β [19] and (3) native human topoisomerase IIα [18]. Slight differences in the sequence preferences may be due to methodological differences among studies.

and these have been termed inhibitor-specific positions [19, 25].

To determine the effects of using a different DNA substrate, cleavage was also examined using labelled restriction fragments from SV40 DNA. For an *Eco*RI–*Hae*II SV40 DNA fragment 5′ ^{32}P end labelled at the *Eco*RI site, cleavage reactions were done at a lower pH and salt concentration to increase topoisomerase II DNA binding and cleavage (Fig. 5) [26]. In the absence of drug, cleavage was detected both for α and β at SV40 nucleotide positions 1746 and 1761, with several additional breakage sites seen for β. Inclusion of mAMSA stimulated cleavage at a range of other sites for both α and β. A similar spectrum of sites was cleaved by each isoform, although there were isoform-specific differences in the cleavage efficiency at some sites, as observed for pBR322. Of the 32 mAMSA-stimulated sites that were mapped on SV40 DNA, 17 were common to both isoforms, six were cleaved only by α and nine were cleaved only by β. Analyses of sites cleaved by α and β are shown in Fig. 6A and 6B, respectively, and the resultant cleavage consensus sequences are shown in Table 1. The two consensuses indicate the same base at five positions: both α and β favoured −8T, −4G, −3A, +1A and +2G and β also favoured +3C and +6C. These consensus sequences for topoisomerase IIα and topoisomerase IIβ on SV40 are very similar, with differences in the strengths of selection at some positions. This finding supports the conclusion drawn from pBR322 cleavage, that human β has *in vitro* DNA recognition and cleavage determinants similar to human α.

Comparison with the mAMSA consensus sequences reveals that selection for the bases at +1A and +2G is present in the consensuses for both isoforms on pBR322 and SV40. The very strong preference for an A at +1 for cleavage by topoisomerase IIα and topoisomerase IIβ in the presence of mAMSA on both pBR322 and SV40 DNA is therefore independent of DNA type or cleavage conditions. The absence of base preferences at several positions on SV40 when compared with the pBR322 consensuses is probably due to the difference in the number of sites analysed (25 for TOP2β on SV40 and 70 on pBR322) or possibly due to altered ionic conditions [26].

Comparison of Mammalian Topoisomerase II Cleavage Sequences

Consensus cleavages derived from sites cleaved by both topoisomerase isoforms on pBR322 and SV40 reveal that A at +1 on one or both strands is the strongest determinant for cleavage in the presence of mAMSA (Tables 1 and 2), representing an inhibitor-specific position, as has been observed previously for murine topoisomerase II (Table 2) [17]. We have compared the mAMSA cleavage consensus for both isoforms with the cleavage site consensuses previously derived for murine topo II (presumably a mixture of α and β) and for nonrecombinant human topoisomerase IIα [18] and a mixture of α and β [19] (Table 2). The bases preferred at +1A to +4T are identical to the murine consensus. When compared with the native human enzymes, +1A, +2G and +4T are conserved in all the consensuses;

TABLE 3. Cleavage site preferences determined by using three different statistical methods (I–III)

	-8	-7	-6	-5	-4	-3	-2	-1	:	1	2	3	4	5	6	7	8	9	10	11	12
β																					
Mapped strand	—	G	—	C	—	G	—	C	:	A	G	C	T	G	—	—	—	G	—	—	—
Both strands	—	G	—	C	G	G	—	C	:	A	G	C	T	G	—	C	C	G	—	C	—
Best strand	—	G	G	C	—	A	T	C	:	A	G	C	A	A	T	C	T	G	—	T	—
α																					
Mapped strand	—	—	—	C	—	G	—	C	:	A	G	C	T	G	—	A	—	G	—	—	—
Both strands	—	—	—	C	—	G	—	C	:	A	G	C	T	G	—	C	—	G	—	—	—
Best strand	—	G	G	C	—	A	—	C	:	A	G	C	A	A	T	C	T	G	—	T	—

TABLE 4. Alignment of cleavage site consensus sequences derived for recombinant human topoisomerase IIα and topoisomerase IIβ to compare the enzyme-specific base preferences for each isoform

	Enzyme-specified bases							Drug-specified bases						Enzyme-specified bases						
	-8	-7	-6	-5	-4	-3	-2	-1	:	1	2	3	4	5	6	7	8	9	10	11	12
β; m-AMSA;	—	G	—	C	—	G	—	C	:	A	G	C	T	G	—	—	—	G	—	—	—
pBR322	—	t	—	a	—	c	c	g	:	gct	t	a	c	t	g	g	a	t	—	—	—
β; m-AMSA;	T	—	—	—	G	A	—	—	:	A	G	C	—	—	C	—	—	—	—	—	—
SV40	—	—	—	—	t	—	c	—	:	ct	—	a	—	—	g	—	—	—	—	—	—
β; no drug;	—	—	—	—	—	—	G	—	:	T	—	—	—	—	—	T	C	—	—	—	—
SV40	—	—	—	—	—	c	—	—	:	—	—	—	—	t	—	g	—	—	—	—	—
β; VM26;	T	—	—	C	—	AT	—	C	:	T	—	—	—	G	—	T	—	—	—	C	T
SV40	—	—	—	a	a	gc	—	g	:	g	—	—	—	a	—	gc	—	—	—	—	c
β; dh-EPI;	—	G	—	—	G	—	T	A	:	A	—	—	T	T	—	—	—	G	T	T	—
SV40	c	c	—	—	—	c	—	tc	:	g	—	—	g	a	c	g	—	—	—	—	c
α; m-AMSA;	—	—	—	C	—	G	—	C	:	A	G	C	T	G	—	A	—	G	—	—	—
pBR322	—	t	—	a	—	c	c	g	:	gc	t	a	c	ct	g	g	—	ct	—	—	—
α; m-AMSA;	T	—	—	—	G	A	—	—	:	A	G	—	—	—	—	—	—	—	—	—	—
SV40	—	—	—	—	t	—	—	—	:	g	—	a	—	—	g	—	—	—	—	—	—
α; no drug;	—	—	—	—	—	A	—	C	:	—	—	—	—	—	—	T	C	—	—	—	—
SV40	—	—	—	a	t	c	—	a	:	—	—	—	—	t	—	g	a	—	—	—	—
α; VM26;	—	C	—	TC	C	AT	—	C	:	T	—	—	—	G	—	T	C	—	—	C	—
SV40	—	—	—	a	a	cg	—	ag	:	g	a	—	—	c	—	g	a	—	—	—	—
α; dh-EPI;	—	—	—	C	—	—	T	A	:	A	—	—	T	T	—	T	C	—	—	—	—
SV40	—	t	—	—	t	c	a	tc	:	c	—	—	g	a	—	g	—	t	—	a	t

however, position +3 was degenerate, being either a G or C. Our results show that recombinant human topoisomerase IIα and topoisomerase IIβ behave in a manner similar to native topoisomerase II. Each isoform interacts with mAMSA in a similar manner, both favouring cleavage with +1A. This key drug-specific position is also independent of the statistical method of analysis used. The statistical method used to derive α and β consensus and those for the murine and human α and β mixtures were identical [17, 19], whereas the studies of native human α used a different method [18]. We also analysed our cleavage sequence data by two additional previously reported methods (Table 3) [18]. In method II, both DNA strands at each cleavage site were included in the analysis, thus avoiding the arbitrary use of only the ^{32}P-labelled strand. In method III, the DNA strand that best fits the final consensus was selected at each site by an iterative procedure. No matter which method of analysis was used, an A at +1 was always the most highly significant base selection. Seven other bases within the consensus (−7G, −5C, −1C, +1A, +2G, +3C, +9G) were also independent of the method used.

```
Human topo IIα  PGQRKVLFTCFKRNDKREVKVAQLAGSVAEMSSYHHGEMSLMMT
Human topo IIβ  PGQRKVLFTCFKRNDKREVKVAQLAGSVAEMSAYHHGEQALMMT
Yeast topo II   PGQRKVLYGCFKKNLKSELKVAQLAPYVSECTAYHHGEQSLAQT
                ********..***.* *.*.****** *.* ..***** .*  *

Human topo IIα  IINLAQNFVGSNNLNLLQPIGQFGTRLHGGKDSASPRYIFTMLSS
Human topo IIβ  IVNLAQNFVGSNNINLLQPIGQFGTRLHGGKDAASPRYIFTMLST
Yeast topo II   IIGLAQNFVGSNNIYLLLPNGAFGTRATGGKDAAAARYIYTELNK
                *..**********. ** * *.****  ****.*..***.* *..
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FIG. 7. Clustal alignment of the CAP-like regions of human topoisomerase IIα (amino acids 724–812), human topoisomerase IIβ (740–828) and yeast topoisomerase II (702–790) performed by using PC/GENE software. Identical amino acids are indicated by a star and a well-conserved position is indicated by a period.

The length of DNA to which sequence specific contacts are made appears to be a minimum of 20 bases (positions −8 to +12). The cleavage consensus may be composed of two domains [18, 19, 25] one that is inhibitor specific (−1 to +5) and includes the bases immediately flanking the cleaved phosphodiester bond (positions −1 and +1) and a second domain that is enzyme specific (−2 to −6 and +6 to +10). Our data is consistent with the DNA consensus sequences containing two domains [19, 25]. The mAMSA drug-specified domain was the same for the α and β isoforms as demonstrated by an A at the +1 position. To assess whether there were any isoform-specific differences in the enzyme-specified base preferences, we compared our cleavage consensuses with the only other consensuses for α and β isoforms (Table 4), which are on SV40 DNA without drug and with VM26 or dh-EPI [27]. Both isoforms show preferences for and against particular bases at several positions: −3 A no c, −5 C no a, +6 no g, +7 T no g, +8 C no a and +9 G no t. There were no obvious isoform-specific differences in the enzyme-specified positions, suggesting both human isoforms interact with DNA in a very similar manner, both in the presence and absence of drug and therefore that the region within the enzyme responsible for DNA recognition is conserved between the two isoforms. The two human isoforms share 68% amino acid identity [7] and have a similar domain structure to each other and to the yeast topoisomerase II [21]. The crystal structure for the central DNA breakage and rejoining domain of yeast topoisomerase II has been solved [28]. This beautiful structure reveals a CAP-like domain that could be involved in DNA

interaction. Alignment of this region from yeast topoisomerase II with α and β is shown in Fig. 7. Alpha and β show 66% and 69% identity, respectively, with the yeast enzyme in this region, whereas α and β are 92% identical in this region, providing one possible explanation for their similar DNA cleavage consensus sequences.

Relevance for mAMSA Action In Vivo

mAMSA is highly cytotoxic to human tumour cell lines [15]. This toxicity arises from the formation of a cleavable complex of topoisomerase II on DNA and the subsequent cellular response to this form of DNA damage. Clearly, whether α or β isoforms (or both) are killing targets for the drug *in vivo* will depend on the nature and levels of their respective cleavable complexes and how these lesions are processed. The observations reported here indicate that α and β do not differ markedly in either the efficiency or specificity of cleavable complex formation *in vitro*. The data suggest that if α and β do confer different drug sensitivities *in vivo* it must arise from factors other than intrinsic drug interactions with the target, such as the levels of cleavable complex formation, the intracellular α and β levels, target accessibility or alternatively through differential cellular responses to α- and β-induced DNA damage, about which little is known. Despite strong evidence implicating both isoforms in drug resistance [29–32], previous studies have tended to emphasize the importance of α. The results presented in this paper show that α and β respond similarly to drugs *in vitro*, suggesting that β should be considered as a potential drug target *in vivo*. Further studies are underway to examine the roles of α and β in drug-induced DNA cleavage and cytotoxicity *in vivo*.

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