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Drug Sensitivity and Sequence Specificity of Human Recombinant DNA Topoisomerases IIα (p170) and IIβ (p180)

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SUMMARY

Effective anticancer agents, such as epipodophyllotoxins and anthracyclines, exert their antitumor activity through stabilization of cleavable topoisomerase II/DNA complexes, which may result in DNA breakage on detergent addition. Two isozymes (α and β) of DNA topoisomerase II are present in human cells; however, their roles as drug targets have not been completely defined. We determined the *in vitro* isoenzyme sensitivities to VM-26 (teniposide) and 4-demethoxy-3′-deamino-3′-hydroxy-4′-*epi*-doxorubicin (an anthracycline analog) and established the sequence selectivity of isoenzyme-mediated DNA cleavage. Human topoisomerases IIα and IIβ were purified from yeast cells overexpressing the corresponding plasmid-borne cDNA. Enzyme sensitivities to drugs were measured by a DNA cleavage assay using ^{32}P-labeled simian virus 40 DNA fragments, and cleavage sites were mapped using agarose and sequencing gels. Both isozymes were sensitive to the studied poisons. They stimulated similar cleavage intensity patterns in agarose and sequencing gels; however, minor differences could be detected. The results showed that local base preferences for DNA cleavage without drugs were different at positions −2 and −1. On the other hand, sequence specificities of VM-26 and 4-demethoxy-3′-deamino-3′-hydroxy-4′-*epi*-doxorubicin were identical for both isozymes and corresponded to those of the native murine enzyme. The identical drug sequence specificities suggested that molecular interactions of the tested drugs in the ternary complex are likely similar between the two isozymes. The current findings indicate that both topoisomerase IIα and IIβ may be *in vivo* targets of antitumor poisons.

DNA topoisomerases are ubiquitous enzymes that regulate DNA topology (1, 2). Type II topoisomerases transiently break a DNA segment, catalyze the passage of a second duplex through the double-stranded cut, and then reseal the DNA break. Several functions have been proposed for eukaryotic DNA topoisomerase II: the enzyme is required at mitosis for chromosome condensation and chromatid disjunction, is likely involved in the removal of DNA supercoils during transcription and is possibly involved in the attachment of DNA loops to chromosomal scaffolds and the nuclear matrix of interphase cells (1, 2).

Effective anticancer agents, such as epipodophyllotoxins {e.g., VM-26 [teniposide; 4′-demethylepipodophyllotoxin-9-(4,6-*O*-thionylidine-β-D-glucopyranoside]} and anthracyclines (e.g., doxorubicin), are potent poisons of mammalian DNA topoisomerase II (3). It is well established that these drugs interfere with the enzyme breakage/reunion reaction, stabilizing a DNA/enzyme complex in which DNA strands are broken and covalently linked to protein subunits. Topoisomerase II generates a double-stranded cleavage with the two strand cuts staggered by 4 bp and the 5′-phosphoryl ends linked to tyrosine residues of the protein (Fig. 1). Drug action therefore results in DNA cleavage stimulation both in cultured tumor cells and in *in vitro* systems (3, 4). Antitumor drugs stimulate DNA cleavage in a sequence-selective manner, yielding drug-specific cleavage intensity patterns in sequencing gels (3, 4). The investigation of drug sequence selectivities disclosed that drug-specific nucleotides flanking the strand cut are required for drug stimulation of cleavage. For example, doxorubicin requires an adenine in at least one of the two 3′ termini (position −1) of a cleavage site (5), whereas VM-26 strongly prefers a cytosine at the same position (6, 7). Topoisomerase II poisons have been proposed to form ternary complexes by directly interacting with these nucleotides at the DNA/protein interface, resulting in inhibition of DNA religation and cleavage enhancement (4, 5, 8).

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ABBREVIATIONS: dh-EPI, 4-demethoxy-3′-deamino-3′-hydroxy-4′-*epi*-doxorubicin I; SV40, simian virus 40; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; bp, base pair(s).

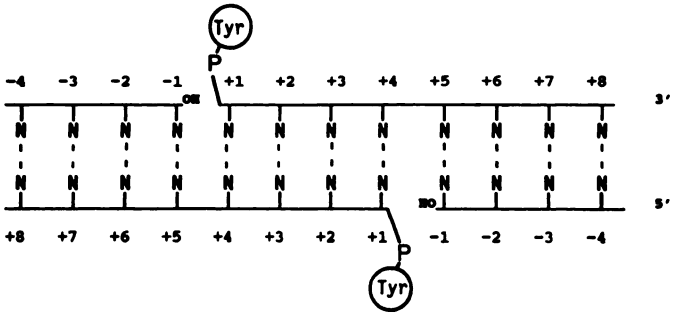

Fig. 1. Schematic diagram of DNA cleavage by topoisomerase II. The two strand cuts are 4-bp staggered and leave free hydroxy groups at 3′ ends. *Tyr*, tyrosine residue covalently linked to the 5′-phosphoryl end of DNA. The base numbering is also shown; note that −1 and +1 bases in one strand are the complementary nucleotides for the +5 and +4 bases of the other strand. *P*, phosphate group.

Two isoenzymes (α and β) of DNA topoisomerase II are present in human and murine cells: they have different molecular masses (170 and 180 kDa, respectively) and are encoded by distinct genes mapped on chromosomes 17 and 3, respectively, in the human genome (9–12). The human isoenzymes showed a high degree of homology with an overall 68% amino acid identity, which is higher in the amino-terminal portion and in the internal domain mediating DNA cleavage/reunion (10). The human enzymes have been overexpressed in a protease-deficient yeast strain, allowing purification of either isozyme in quantity (13, 14). Interestingly, the two isozymes have been shown to have a different pattern of gene expression during the cell cycle (15, 16) and in normal rodent tissues (17, 18); in addition, their phosphorylation states may be different during the cell cycle (19). It has also been reported that murine topoisomerases IIα was ~3-4-fold more sensitive to VM-26 than the β isoform in a catalytic assay and in a cleavage test using supercoiled plasmid pBR322 DNA (20). However, the authors could not compare the enzyme preparations at similar units of cleavage activity and did not report any high-resolution mapping of DNA cleavage sites produced by either isoform. Therefore, the specific roles of enzyme isoforms in the antitumor activity of anthracyclines, VM-26, and other topoisomerase II poisons remain to be fully established.

Thus, as a first step to understanding putative isoform-specific effects relevant to antitumor drug activity, we studied the *in vitro* sensitivities of human topoisomerases IIα and IIβ to VM-26 and a potent doxorubicin derivative, dh-EPI (21), and determined the sequence selectivity of DNA cleavage with either isoform with and without drugs. The results showed that the human isoenzymes are sensitive to the test drugs to a similar but not identical extent. Slight differences in sequence selectivity of DNA cleavage may be observed without drugs; however, the studied drugs had identical base requirements with either isoenzyme. Our results show that both topoisomerase IIα and IIβ may be *in vivo* targets of antitumor poisons.

Materials and Methods

Drugs and other materials. VM-26 and dh-EPI were obtained from Bristol Italiana (Latina, Italy) and Pharmacia-Farmitalia (Milan, Italy), respectively. Drugs were freshly prepared in dimethylsulfoxide and then diluted in deionized water. SV40 DNA, T4 polynucleotide kinase, and acrylamide/bisacrylamide were purchased from GIBCO BRL (Basel, Switzerland). Agarose and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from FMC Bioproducts (Rockland, ME) and Amersham International (Milan, Italy), respectively. Restriction enzymes were purchased from New England Biolabs (Taunus, Germany).

Purification of native murine and recombinant human DNA topoisomerases IIα and IIβ. Human topoisomerase IIα and IIβ cDNAs, cloned in plasmids YEpWob6 and YEphTOP2β, respectively (13, 14), were expressed in *Saccharomyces cerevisiae* strain JEL1 (α *leu2 trp1 ura3-52 prb1-1122 pep4-37* Δ *his3::PGAL10-GAL4*) under the control of a galactose-inducible promoter and purified as previously described (13, 22). Briefly, the expression of the plasmid-borne human cDNA was activated by the addition of galactose (2%) to the medium for 3–16 hr. After cell harvesting and lysis, DNA was precipitated with polymin P; then, Celite (Fluka, Buchs, Switzerland) was added to protein mixture. The enzymes were eluted with 1 M KCl from Celite and precipitated with ammonium sulfate. Then, phosphocellulose column chromatography was performed, and the human isoforms were eluted with a KCl gradient. Topoisomerase-containing fractions were identified by SDS-polyacrylamide gel electrophoresis and DNA relaxation activity (13, 14). The purified isozymes were stored at −80° in 50 mM Tris·HCl, pH 7.7, 200 mM KCl, 10 mM EDTA, 10 mM EGTA, and 10% glycerol. The strand-passing activity of the recombinant isozymes was measured with a plasmid relaxation assay in L buffer (40 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 80 mM KCl, 0.5 mM dithiothreitol, 1 mM ATP, 15 μg/ml bovine serum albumin). One unit of topoisomerase II activity completely relaxed 0.2 μg of supercoiled pBR322 DNA at 37° in 30 min.

Native murine DNA topoisomerase II was purified from P388 leukemia cells as previously described (23). The purified protein, likely containing both murine topoisomerase II isoforms, was stored at −80° in 20 mM KH_2PO_4, pH 7.0, 50% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 1 mM β-mercaptoethanol. Its DNA strand-passing activity was determined in a P4 unknotting assay as previously described (23).

End-labeling of DNA fragments and DNA cleavage reaction. SV40 DNA was uniquely 5′-end labeled as described previously (5). Briefly, SV40 DNA was linearized with a restriction enzyme, 5′-end labeled with T4 kinase, and then digested with a second restriction enzyme. Labeled DNA fragments were purified by electroelution and ethanol precipitation (24). DNA fragments (15,000–20,000 cpm/sample) were reacted with 10–30 units of topoisomerase IIα or IIβ with or without drugs in L or H buffer (H buffer: 10 mM Tris·HCl, pH 6, 50 mM KCl, 10 mM $MgCl_2$, 1 mM ATP) for 20 min at 37°. VM-26 and dh-EPI were used at 50 and 10 μM, respectively. Reactions were stopped by the addition of 1% SDS and 0.3 mg/ml proteinase K and incubated at 42° for 45 min. Samples were then electrophoresed in a 1% agarose gel in 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8, and 0.1% SDS. For sequencing gels, after proteinase K treatment, DNA was ethanol precipitated; resuspended in 2.5 μl of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue; heated at 90° for 2 min; chilled on ice; and then loaded onto 8% denaturing polyacrylamide gels. Cleavage levels were determined by analyses of dried gels (PhosphorImager model 425, Molecular Dynamics, Sunnyvale, CA).

Statistical analyses of base sequences. The statistical tests have been described in detail previously (5, 6). Briefly, we used the χ^2 one-sample test to determine the deviation from the expected base distribution at each position of the aligned sequences and measurement of the probability (p) of the observed deviation from the expected base frequency. The opposite values of the logarithm of p, $-\log(p)$, are reported for each base at each position encompassing the cleavage site.

Results

To determine the sensitivities of human topoisomerases IIα and IIβ to antitumor poisons, DNA cleavage activities of the two isozymes with and without VM-26 or dh-EPI were studied in the SV40 genome with the use of agarose and sequencing gels. Cleavage reactions were carried out in H buffer, which increases the enzyme binding to DNA (24, 25), to map strong as well as weak cleavage sites. Preliminary experiments showed that cleavage sites were the same, although their intensities were lower, in L buffer (not shown), which is in agreement with a previous comparison of H and L buffers using the murine native topoisomerase II (24). SV40 DNA fragments were incubated with amounts of the two isozymes that yielded 1–2% cleavage of the full-length fragment at 37° in the absence of drugs. The protein amounts corresponded to 10–30 enzyme units as determined by a plasmid DNA relaxation assay (see Materials and Methods for details).

Cleavage intensity patterns: comparison with native murine DNA topoisomerase II. Low-resolution mapping experiments consistently showed that in the absence of drugs, many cleavage products generated by topoisomerase IIα and IIβ were similar (Fig. 2). Nevertheless, minor differences in cleavage intensities were noted around the genomic position 4920, where topoisomerase IIβ produced a relatively higher cleavage intensity than topoisomerase IIα (Fig. 2). VM-26 (50 μM) and dh-EPI (10 μM) stimulated very similar cleavage intensity patterns with either isoform in agarose gels (Fig. 2 and results not shown). With the anthracycline dh-EPI, two SV40 DNA regions contained sites of strong cleavage stimulation with topoisomerase IIβ but not with topoisomerase IIα (Fig. 2). These regions were mapped around the 3750 and 3410 positions of the SV40 genome (Fig. 2). However, these differences were minor and could be the result of slightly higher cleavage activity of the β preparation in this case.

We also compared the human recombinant isoenzymes with the native murine topoisomerase II, and the results demonstrated that cleavage intensity patterns were very similar among the three enzymes in sequencing gels (Fig. 3). Weak cleavage sites stimulated by VM-26 were not readily observable for topoisomerase IIβ from 1700 to 1758 (Fig. 3), and this also could be the result of an overall minor cleavage level compared with the other isozyme. Nevertheless, other differences may be noted that are not explained by lower activity of one isozyme. Site 1684 was stimulated by VM-26

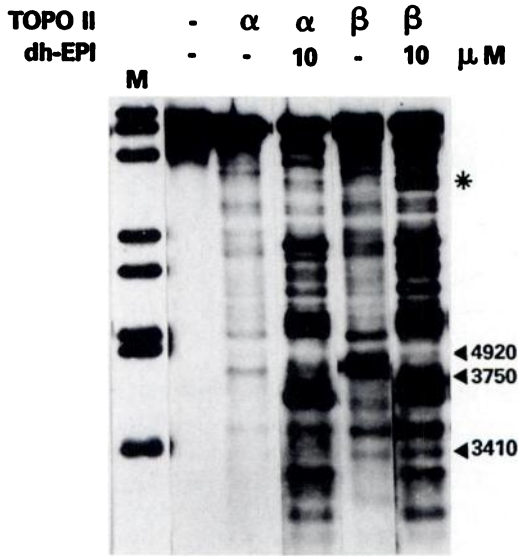

Fig. 2. DNA cleavage mediated by human topoisomerases IIα and IIβ in the SV40 genome: cleavage intensity patterns in agarose gels with and without dh-EPI. An SV40 DNA fragment was 5′-end ^{32}P-labeled at the *BclI* site and then incubated with topoisomerase IIα or IIβ and drug at 37° for 30 min. Reactions were stopped by the addition of SDS (1%) and proteinase K (0.3 mg/ml) and incubated for 1 hr at 42°. DNA was then electrophoresed in a 1.2% agarose gel. *Lane 1*, control DNA; *lane 2*, topoisomerase IIα without drug; *lane 3*, topoisomerase IIα with 10 μM dh-EPI; *lane 4*, topoisomerase IIβ without drug; *lane 5*, topoisomerase IIβ with 10 μM dh-EPI; *lane M*, molecular weight markers (fragment sizes from *bottom* to *top*: 224, 702, 1264, 1371, 1929, 2323, 3675, and 4324 bp). *Numbers and triangles*, sites of different cleavage intensities between the two isozymes. *, sites in the top portion of the gel cleaved more strongly by the β isoform.

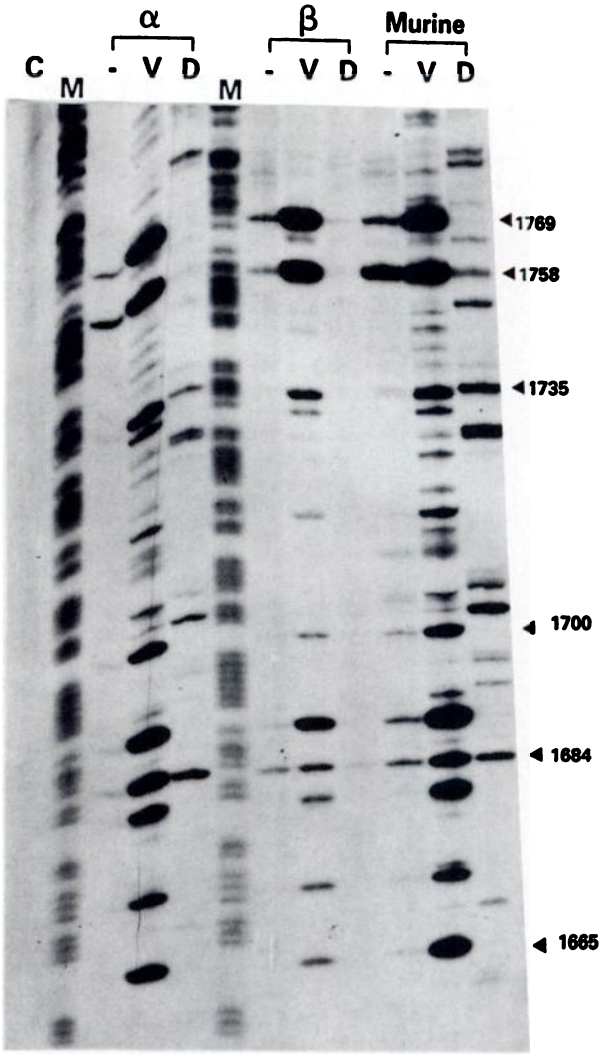

Fig. 3. Comparison of human recombinant topoisomerases IIα and IIβ with the murine native topoisomerase II. An SV40 DNA fragment was 5′-end ^{32}P-labeled at the *AccI* site, reacted with enzymes with or without drugs for 30 min at 37°, and then analyzed on a 8% polyacrylamide sequencing gel. *Lane 1*, control DNA; *lanes 2–4*, topoisomerase IIα; *lanes 5–7*, topoisomerase IIβ; *lanes 8–10*, murine native topoisomerase II; *lanes 2, 5, and 8*, no drugs; *lanes 3, 6, and 9*, 50 μM VM-26; *lanes 4, 7, and 10*, 10 μM dh-EPI; *lane M*, purine molecular weight markers.

more strongly in the case of the α isoform, whereas the drug was less effective in the case of the β isoform (Fig. 3).

High-resolution mapping of cleavage sites was performed in several regions of the SV40 genome: 1408–1607, 1670–1764, 1800–2000, 2630–2795, and 4610–4724 nucleotide positions in the coding strand of early mRNAs and 1653–1770, 1805–1900, 1928–2064, and 2760–2933 nucleotide positions in the coding strand of late mRNAs. In all of the studied DNA fragments, cleavage intensity patterns stimulated by VM-26 and dh-EPI were similar for both topoisomerases IIα and IIβ (Figs. 3 and 4 and not shown). Furthermore, regions of SV40 DNA were analyzed for cleavage sites in both strands to evaluate whether the cleavage was double stranded. No differences were observed between the two isoforms. Most of the sites of strong and intermediate intensities, at least in one strand, had corresponding sites on the other strand with a stagger of 4 bp, which is consistent with double-stranded cleavage. Thirty-eight of 41 weak sites did not have a 4-bp staggered cut on the other strand, suggesting that weak sites involved mainly single-stranded DNA cleavage.

In summary, a detailed comparison of cleavage intensity patterns between topoisomerases IIα and IIβ showed that most of the observed cleavage sites were shared in common and that the two isozymes produced similar relative levels of cleavage at each site. Nevertheless, some sites were indeed selected by one or the other isoenzyme, suggesting that subtle differences may exist in DNA site recognition by human topoisomerase II isoforms.

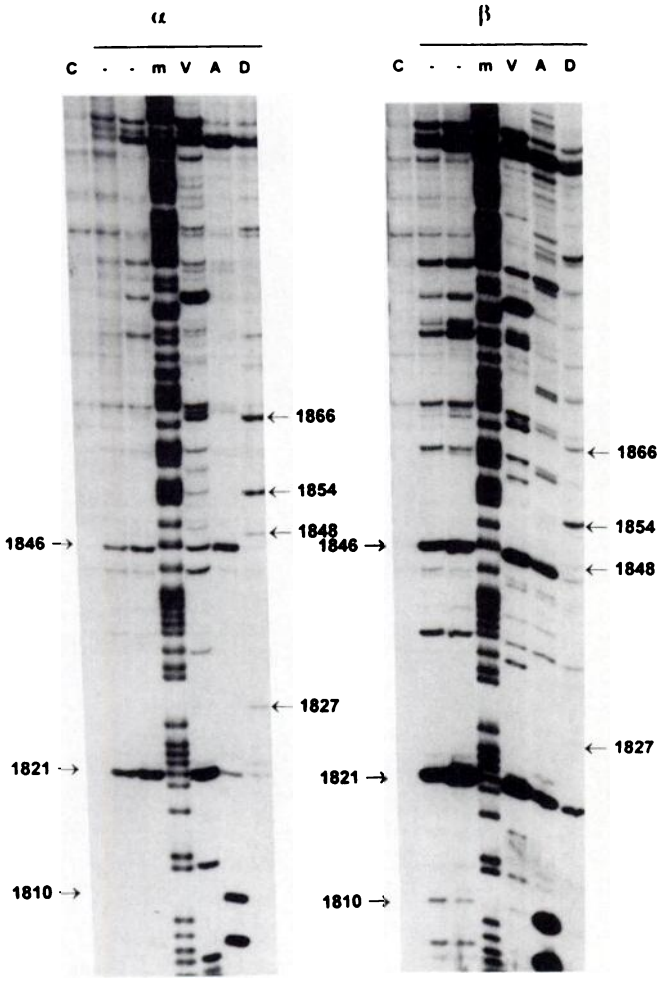

Fig. 4. Cleavage intensity patterns in sequencing gels. Lane C, control. *Lane* −, isoenzyme alone (*left and right lanes*, 1 and 3 units, respectively). *Lane m*, purine molecular markers; *lane V*, 50 μM VM-26; *lane A*, 50 μM amsacrine; *lane D*, 10 μM dh-EPI. See legend to Fig. 3 for additional details. *Arrows and numbers*, (*left of gel*) cleavage sites without drugs and (*right of gel*) dh-EPI-stimulated sites.

Human isozyme sensitivity to VM-26 and dh-EPI. We then determined the degree of drug stimulation of isozyme-mediated DNA cleavage by PhosphorImager analyses of agarose or sequencing gels. A cleavage level of each sample was calculated as the ratio of the radioactivity of all cleaved fragments to the total radioactivity in the lane and normalized relative to the corresponding control lane. Then, the drug stimulation factor was determined as the ratio of the cleavage level with the studied drug to the level without drugs. Under our conditions, the stimulation factors for dh-EPI (10 μM) were 4.9 and 3.0 for topoisomerases IIα and IIβ, respectively; for VM-26 (50 μM), the factors were 2.9 and 2.3, respectively (mean values of two or three determinations). Therefore, the data showed that the two drugs were as active or only slightly more active against the α isoform than the β isoform and that the anthracycline dh-EPI was more potent than VM-26.

It must be noted that we compared the two isozymes at similar levels of DNA cleavage in the absence of drugs using the H buffer. These conditions favor enzyme binding to the DNA (24, 25), thereby increasing the DNA cleavage without drugs. Furthermore, at individual sites, the stimulation factor may be higher than that calculated taking into account all bands in the lanes. The calculated factor is an average value determined for all of the cleavage sites: for some sites, the drug may have no stimulatory effect or may even suppress cleavage (e.g., site 1846 in Fig. 4). At some sites, drug stimulation factors were higher than 20 (sites 1665 and 1700 for VM-26 in Fig. 3 and site 1854 for dh-EPI in Fig. 4). Nevertheless, the drug sensitivities of the two recombinant proteins were comparable at almost all sites. Marked isoform differences were noted in only a few instances, such as site 1684, which was strongly stimulated by VM-26 in the case of the α isoform and poorly stimulated by VM-26 in the case of the β isoform (Fig. 3).

Sequence selectivity with VM-26 or dh-EPI. Nucleotide sequences were aligned at the point of the observed cleaved phosphodiester bond with the bases immediately 5′ and 3′ to the cut corresponding to positions −1 and +1, respectively (Fig. 1). Note that the −1 and +1 bases of one strand are the complementary nucleotides to the +5 and +4 bases of the other strand, respectively (Fig. 1). The frequencies of cytosine, thymine, guanine, and adenine were calculated from positions −15 to +21 and compared with the expected base frequencies relative to the overall base composition of the SV40 genome. These values were used to calculate χ^2 values at each position (see Materials and Methods for details).

In the case of topoisomerase IIα, highly nonrandom base distributions were present at positions −1 and −3 with VM-26 and at position −1 with dh-EPI (Fig. 5). In the case of topoisomerase IIβ, biased base distributions were at positions −1, −3, +5, and +7 with VM-26 and positions −1, +4, and +5 with dh-EPI (Fig. 5). Interestingly, a more-pronounced symmetry is present in the χ^2 curves of the β isoform relative to those of the α isoform.

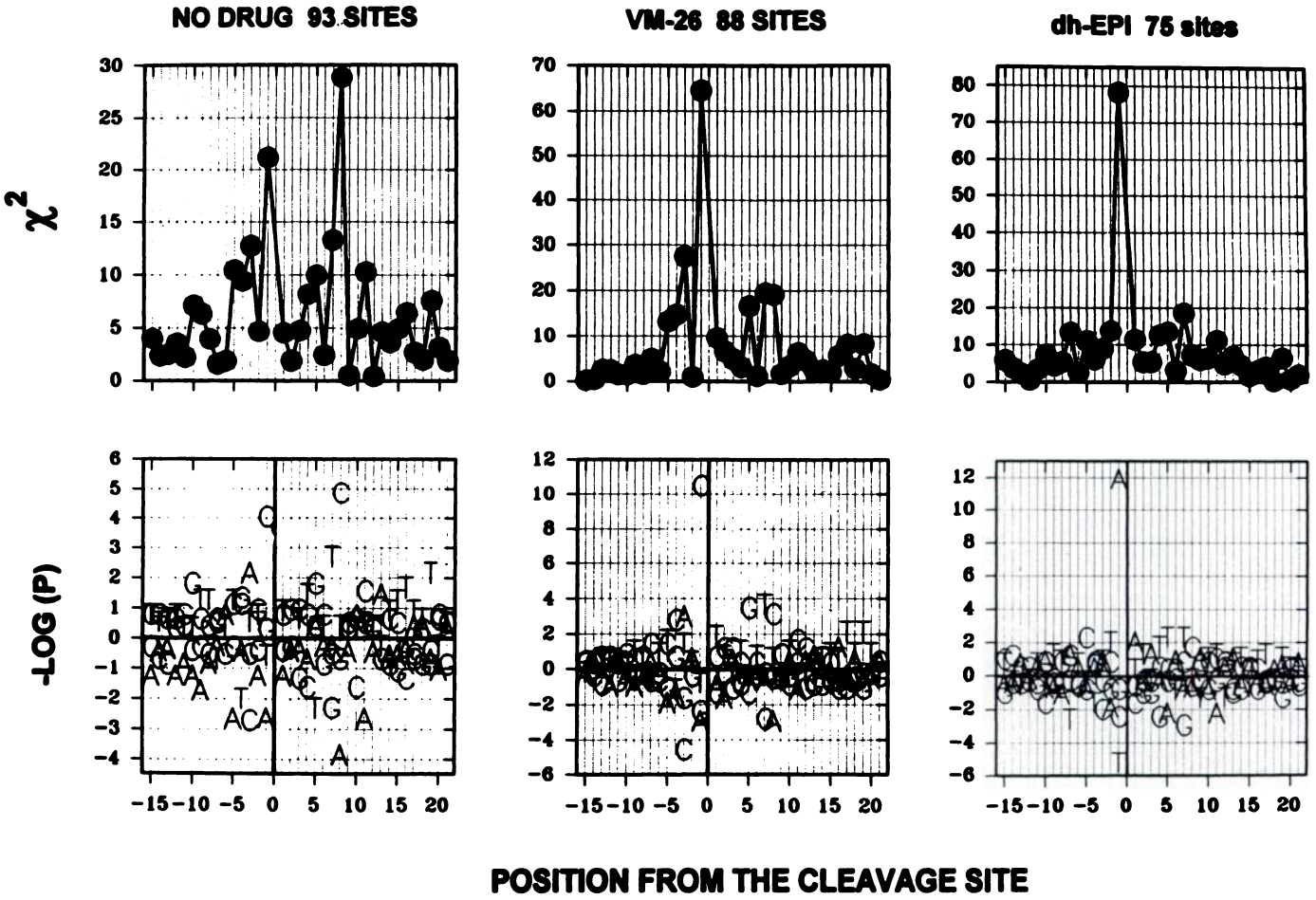

Fig. 5. The χ^2 and $-\log_{10}(p)$ plots for nucleotide sequences at cleavage sites generated by human recombinant topoisomerase IIα. Number of sites were generated in the SV40 genome (*left*) without drugs, or with (*middle*) VM-26 and (*right*) dh-EPI. For VM-26, a total of 245 sites were collected; reported results were obtained with only the 88 strongest sites. *Top*, χ^2 values indicate the deviation from the expected distribution of base frequencies (for $p = 0.05$ and 0.01, $\chi^2 = 5.99$ and 11.34, respectively). *Bottom*, probability (p) that deviation or higher value, as either excess (above the zero line) or deficiency (below the zero line) relative to the expected frequency of each base (A = T = 20.6%; G = C = 29.4%), is expressed as the opposite value of the $\log_{10}$ of p.

VM-26 strongly preferred a cytosine at the −1 position in the case of both isozymes with highly significant $-\log(p)$ values (10.43 and 5.58; see Figs. 5 and 6). Correspondingly, a guanine was preferred at the +5 position. The comparison with sequence consensuses published previously for native enzyme preparations showed a complete agreement not only for the −1 position but also for base preferences from −4 to +8 positions (Table 1).

dh-EPI showed a strong requirement for adenines at the −1 position for the α (11.84) and β (4.7) isoforms (Figs. 5 and 6). In the case of the β isoform, dh-EPI also showed stronger base preferences at other positions: thymines at +5, +4, and −2 positions (Fig. 6 and Table 1). Again, base preferences from −4 to +8 positions were in excellent agreement with previous statistical and mutational analyses of anthracycline-stimulated DNA cleavage sites of native murine topoisomerase II (5, 24).

Sequence selectivity without drugs. In the absence of drugs, highly nonrandom base distributions were present at positions +8, −1, +7, and −3 for the α isoform (Fig. 5) and at positions +7, −3, −2, and +5 for the β isoform (Fig. 6). Probability calculations of the observed nucleotide frequencies showed that at these positions, specific bases were preferred or excluded (Figs. 5 and 6). For topoisomerase IIα, highest $-\log(p)$ values were at the +8 position for cytosine preference (4.8) and adenine exclusion (3.9); in addition, a cytosine was preferred at position −1, a thymine at position +7, and an adenine at position −3 (Fig. 6 and Table 2). In the case of topoisomerase IIβ, the highest $-\log(p)$ value was for thymine preference (5.3) at position +7, where a guanine (3) was excluded (Fig. 6 and Table 2). A thymine and a guanine were preferred at positions +1 and −2, respectively.

Strikingly, differences between the two isozymes occurred at positions −1 and −2 (Table 2). The β isoform did not show any base preference or biased base distributions at position −1, whereas the α isoform preferred a cytosine at this position (Figs. 5 and 6 and Table 2). Relative to the −1 position, the α isoform resembled more closely than the β isoform the previously published sequence consensuses of native topoisomerases II from different species (Table 2). At all other positions, the base preferences of both human recombinant isozymes were in good agreement with those published previously for native topoisomerase II preparations (Table 2).

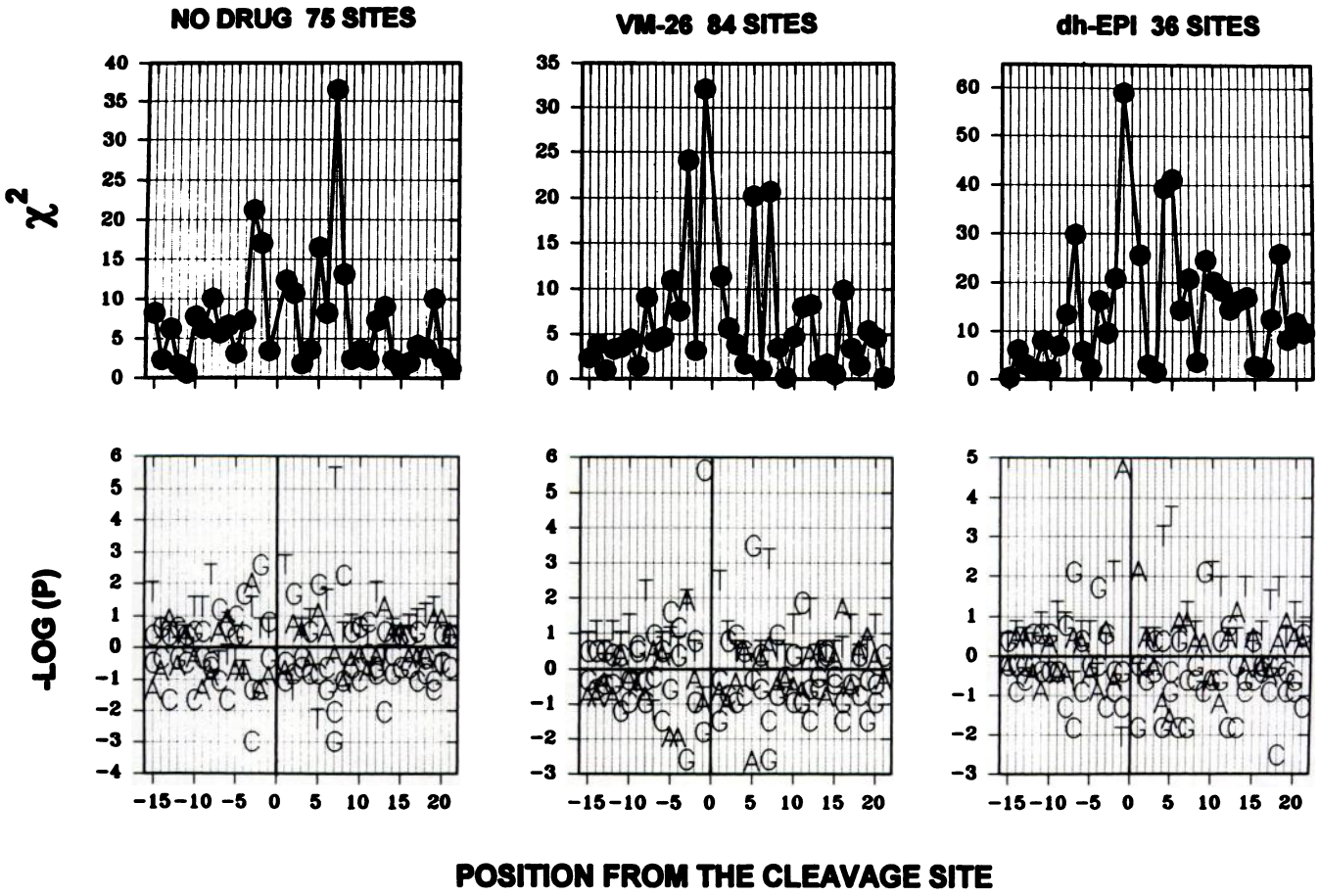

Fig. 6. The χ^2 and $-\log_{10}(p)$ plots for nucleotide sequences at cleavage sites generated by human recombinant topoisomerase IIβ. Number of sites collected in the SV40 genome (*left*) without drugs or with (*middle*) VM-26 and (*right*) dh-EPI. See legend to Fig. 3 for other details.

TABLE 1

Preferred base sequences at the DNA cleavage site for human recombinant isoenzymes and native murine, chicken, and human topoisomerases II in the presence of dh-EPI or VM-26

Enzyme	Nucleotide (cleavage between −1 and +1)														Reference
	−5	−4	−3	−2	−1	+1	+2	+3	+4	+5	+6	+7	+8	+9	
dh-EPI															
Human α	*C*	NP	*No C*	NP	**A, no T,** *no C*	NP	NP	NP	*No G*	*T, no A*	NP	**No G,** *T*	NP	NP	Current study
Human β	NP	NP	NP	*T*	**A,** *no T*	*A*	NP	NP	*T*	**T**	NP	NP	NP	*G*	Current study
Murine native	NP	NP	**No C,** *T*	**T**	**A, no T**	*No C*	NP	NP	*T*	**T, no A**	**A**	**No G, T**	NP	NP	5 and 24
VM-26															
Human α	NP	*C*	**No C,** *A*	NP	**C,** *no R*	NP	NP	NP	NP	**G**	NP	**T,** *no G*	**C, no A**	NP	Current study
Human β	NP	NP	*No G,* *no C*	NP	**C**	*T*	NP	NP	NP	**G,** *no A*	NP	**T,** *no G*	NP	NP	Current study
Human native	*C, no A*	*G, no T*	**A,** *no S*	NP	**C, no R**	NP	NP	NP	NP	**G,** *no T*	NP	**T,** *no G*	**C**	**G,** *no T*	31
Murine native	NP	**No T,** *G*	*No G, A*	NP	**C, no G**	NP	NP	NP	NP	**G, no T**	NP	*T*	*C*	NP	6

A, G, C, and T, the base was preferred; no A, no G, no C, and no T, a bias against the presence of that base; R, G or A; S, G or C; preferences in bold, $-\log(p)$ values were ≥3; preferences in italics, $-\log(p)$ values were 2–3; NP, no preference.

Discussion

To establish the role of individual human DNA topoisomerase II isoforms in cancer chemotherapy, we investigated the *in vitro* enzyme-mediated DNA-cleaving activity of two different topoisomerase II poisons: VM-26 and the potent doxorubicin analog dh-EPI. These compounds belong to two important classes of effective antitumor drugs and are used on a widespread basis in the treatment of human cancers (3, 4). VM-26 and dh-EPI were chosen because they are among

TABLE 2

Preferred base sequences at sites of DNA cleavage by human recombinant isoenzymes and native human, murine, and chicken topoisomerases II in the absence of drugs

Enzyme	Nucleotide (cleavage between −1 and +1)														Reference
	−5	−4	−3	−2	−1	+1	+2	+3	+4	+5	+6	+7	+8	+9	
Human α	*No A*	*No T*	*A, no C*	NP	**C,** *no A*	NP	NP	NP	NP	*No T*	NP	*T, no G*	**C, no A**	NP	Current study
Human β	NP	NP	**No C**	*G*	NP	*T*	NP	NP	NP	*No T*	NP	**T, no G**	*C*	NP	Current study
Human native	*C*	*No T, G*	**A**	NP	**No A,** *C*	NP	NP	NP	NP	NP	*No G*	*No G, T*	NP	NP	31
Murine native	*C*	**G**	*No G*	**No T,** *A*	**No A,** *C, T*	**A**	NP	NP	NP	**No T**	**T**	*A*	*C*	NP	5
Chicken native	C	G/A	T	G	T	A	G	C	T	A	C	A	C/T	G	30

A, G, C, and T, the base was preferred; no A, no G, no C, and no T, a bias against the presence of that base; preferences in bold, −log(*p*) values were ≥3; preferences in italics, −log(*p*) values were 2–3; NP, no preference.

the most active analogs within the respective chemical families, demethylepipodophyllotoxins and anthracyclines (3, 21). Our results demonstrate that human topoisomerase II isoforms are sensitive to the classic antitumor poisons VM-26 and dh-EPI to comparable extents. Furthermore, the sequence specificities of the tested drugs were identical for both isozymes. Previous studies on mammalian topoisomerase II with high-resolution DNA sequencing methods have used predominantly the α isoform or, more often, mixtures of α and β. We report the first study of the use of homogeneous α and β proteins, each known to be free of the other isoform, and compare DNA cleavage under identical conditions.

Antitumor poisons of topoisomerase II exert their pharmacological action through stabilization of the cleavable enzyme/DNA complex, which can result in DNA breakage on detergent addition rather than inhibition of enzyme catalytic activity (3, 4). Therefore, we were primarily interested in determining the enzyme sensitivity to drugs in a DNA cleavage assay as well as drug sequence-specific effects. Even though isoform differences in VM-26 and dh-EPI stimulation of cleavage varied depending on enzyme preparation and assay used, topoisomerase IIα was consistently slightly more sensitive than the β isoform. A comparison of cleavage activity is technically more difficult than a comparison of catalytic activity because in a cleavage assay, stoichiometric amounts of each enzyme must be used because one protein molecule is necessary for each cleavage event (1–3). When we compared isozyme amounts that gave comparable levels of cleaved DNA fragments without drugs (1–2%), topoisomerase IIα was ~1.6-fold more sensitive than topoisomerase IIβ to 10 μM dh-EPI. Enzyme sensitivities to VM-26 were equivalent. Previously, murine topoisomerase IIα was shown to be ~3-4-fold more sensitive to VM-26 than topoisomerase IIβ in catalytic and cleavage assays (20). Thus, the human isoenzymes might be more similar in their drug sensitivities than the murine isoenzymes. In the current study, we directly determined the drug stimulation factor as a ratio of cleavage level with drug to the level without drugs by using a radioactive DNA substrate, whereas the previous authors measured the conversion of supercoiled DNA to its linear form in the presence of VM-26 only (20). Thus, the discrepancy might be due to the different assays used (i.e., linear versus supercoiled DNA, and so on) or be related to species-specific characteristics of topoisomerase II. In any case, although the human β isoform is only slightly less sensitive than the α isoform to the studied poisons, we cannot rule out the possibility that compounds can be found that specifically stimulate DNA cleavage by one or the other isozyme.

Statistical analyses of nucleotide sequences at cleavage sites specifically stimulated by dh-EPI and VM-26 clearly showed that drug sequence requirements or preferences were identical for both isozymes as well as for those determined previously in the case of murine (5, 6, 24) and human native topoisomerases II (Table 1). VM-26 highly prefers a cytosine at the 3′ terminus of the strand cut, while dh-EPI requires an adenine at the same position (Table 1). Consistently, cleavage intensity patterns in sequencing gels were almost identical among human recombinant and murine native enzymes in the presence of drugs (Figs. 3 and 4). These results strongly suggest that molecular interactions of VM-26 and dh-EPI are similar in the ternary complex of both isozymes.

Thus, drug receptors in the DNA/enzyme complex may be very similar between the two proteins. This would be consistent with the high homology of amino acid sequences of the two human isoforms (10). Several conserved motifs have been found in eukaryotic topoisomerases II, and combined with sequence conservation and similar catalytic activity, the data suggest that type II topoisomerases may have common structural and mechanistic features (10). Evidence for structural similarities has been provided in the case of the human isozymes by the existence of very similar protein domains as determined through controlled proteolysis assays (14). Sequence analyses have also shown that the domains containing the active site and the protein regions that most likely contact the DNA double helices during strand passage (26) have the highest sequence homology (10). It is surprising, however, that the β isoform did not show any base preference at the −1 position, where base distributions are commonly very biased for the α isoform as well as for other topoisomerases II purified from several sources (5, 27–31). These findings might suggest that subtle differences exist between the human isozymes in contacting the DNA cleavage site.

The current results have important implications for the antitumor activity of topoisomerase II poisons. Our data strongly indicate that the β as well as the α isoforms should be considered targets of drugs *in vivo*. Mutant cells resistant to drugs may also provide some insights into the mechanism of action of cytotoxic compounds. A rough correlation exists between cellular topoisomerase II levels and cell sensitivity to topoisomerase II poisons (32). Cells with reduced enzyme amounts have been shown to be less sensitive to these antitumor agents than are cells with higher protein levels (33).

Data obtained from investigations in NIH-3T3 and *ras*-transformed NIH-3T3 cells indicated that cell sensitivity to drugs depended on the topoisomerase IIα content and the α/β ratio (15, 16). Indeed, several reports have supported the idea that an increased susceptibility of cancer cells may be due to a higher proportion of the α isoform (9, 15, 34).

Interestingly, cell lines selected for resistance to mitoxantrone or genistein were shown to have reduced amounts of topoisomerase IIβ (35, 36). However, further studies provided results showing that a truncated form of topoisomerase IIα, present in mitoxantrone-resistant HL-60 cells only, was found in the cytosol to a large extent, effectively reducing its nuclear content (37). The authors suggested that both a marked decrease of the β isoform and a reduced nuclear content of the α isoform may confer a growth advantage of the resistant cells in the presence of mitoxantrone (37). Furthermore, the intrinsic drug sensitivity of a panel of breast cancer cell lines was correlated to both α and β for mitoxantrone and, surprisingly, to β for VP-16 (a close analog of VM-26) (38). Therefore, drug resistance profiles in relation to topoisomerase II alterations have indicated that both the β and α isoforms can be important in the cytotoxic potency of antitumor poisons.

Whether the roles of individual human isoforms in the antitumor drug activity are markedly different remains to be fully established. Our current data demonstrate that were this the case, it would not be due to inherent differential drug sensitivities of the two isozymes, at least not for the studied agents. Instead, *in vivo* factors may markedly influence enzyme functions and drug activity. For example, the intranuclear localizations of the two isozymes have been proposed to be different, with the β isoform mainly localized at the nucleoli and the α isoform distributed diffusely in the nucleoplasm (39). In addition, the two isoforms are differentially regulated during the cell cycle. These properties may constitute important factors that markedly influence the tumor cell-killing activity of topoisomerase II poisons.

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