Human DNA Topoisomerase II: Evaluation of Enzyme Activity in Normal and Neoplastic Tissues†

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ABSTRACT: We have used both a quantitative filter binding assay and a decatenation assay to measure DNA topoisomerase II activity. The filter binding assay, which measures catenating activity, is able to detect topoisomerase II activity at 50–100-fold lower protein concentrations than the decatenation assay. Because of this remarkable sensitivity, we have been able to quantitate topoisomerase II activity in a variety of normal and neoplastic human tissues. The highest level of enzyme activity in normal tissues was found in the spleen and thymus. The highest level of enzyme activity in neoplasms was found in those that clinically behave in an aggressive manner and had a high proliferative status by flow cytometry. Surprisingly, these high topoisomerase II values in the neoplastic specimens are in the same range of values found in normal nonproliferating tissue. Since much previous data indicate that the enzyme is apparently a property of only proliferating cells, this finding might suggest that human tissues contain more than one form of the enzyme. The finding that 35–65% of the topoisomerase II activity in human tissues is resistant to teniposide suggests that more than one enzyme form exists.

DNA topoisomerases have been described from a variety of sources and may play important roles in replication, transcription, repair, and recombination (Wang, 1987; Vosberg, 1985). They have been divided into two types. The type I enzymes make transient single-stranded breaks in their DNA substrates while the type II enzymes make double-stranded breaks. In addition, type II enzymes require ATP. Mammalian type II topoisomerases have generated extensive interest since recent data have indicated that the enzyme may mediate the action of several clinically important anticancer drugs such as adriamycin, ellipticine, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA),[1] VP-16 (etopside), and VM-26 (teniposide) (Ross et al., 1988). These drugs apparently stabilize an intermediate in the topoisomerase II catalyzed reaction. This intermediate consists of a transient DNA double-strand break which is held together noncovalently by the enzyme, termed a "cleavable complex" (Rowe et al., 1986). In cells treated with these drugs, stabilization of the "cleavable complex" apparently occurs. By an unknown mechanism the stabilized "cleavable complex" is presumably lethal to the cell. This model has important clinical implications. It suggests that the clinical response to drugs like VP-16 and VM-26 is dependent on a cell's topoisomerase II level. In other words, the more topoisomerase II a cell has, the more cleavable complexes that can be formed in response to VP-16 or VM-26, and thus the greater chance of cell death.

The level of topoisomerase II in a cell has been thought to be dependent on the cell's proliferative status (Heck & Earnshaw, 1986). High levels of the enzyme appear to be present in cycling cells while noncycling cells have little, if any, enzyme. From these data, it might be postulated that human neoplasms with a high proliferative rate would contain high levels of topoisomerase II and thus be potential targets of therapy with topoisomerase II directed agents. Neoplasms with low topoisomerase II levels would be proportionately resistant. Indeed, it has recently been suggested that the poor response of patients with chronic lymphocytic leukemia (CLL) to adriamycin is directly related to the tumor's low topoisomerase II level (Potmesil et al., 1988). Also, the level of the enzyme in the nucleus appears to be related to the sensitivity of the cell to the topoisomerase II targeted drugs (Davies et al., 1988). These studies suggested to us that the measurement of topoisomerase II activity in nuclear extracts from tumor biopsy specimens might serve as a diagnostic test for responsiveness to chemotherapy. However, in spite of the extensive clinical interest in this enzyme, there is little information on the activity of topoisomerase II in human neoplasms and tissues. We have recently developed a quantitative and sensitive filter binding assay for topoisomerase II activity (Holden & Low, 1985). In this report, we describe the use of this assay to evaluate topoisomerase II activity in a variety of human neoplasms and normal tissue.

Materials and Methods

Chemicals, Resins, and Commercial Enzymes. The sources of these materials were as previously described (Holden & Low, 1985). In addition, teniposide (VM-26, NSC 122819) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. Phosphocellulose was obtained either from Whatman (P-11 cellulose phosphate) or from Bio-Rad (cellex P). Brain heart infusion was from Difco and Hemin (bovine type I); propidium iodide and ribonuclease A were from Sigma. Topoisomerase I was purified from human placenta by the same method as described for HeLa cells (Holden & Low, 1985; Miller et al., 1981). Purified recombinant T_4 topo-

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[1] Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; VP-16, etopside; VM-26, teniposide; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; RF, circular double-stranded replicative form; CLL, chronic lymphocytic leukemia; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; PEG, poly(ethylene glycol); SSC, 0.15 M NaCl–0.015 M trisodium citrate–$2H_2O$, pH 7.0; SDS, sodium dodecyl sulfate; PVA, poly(vinyl alcohol); kb, kilobase(s).

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isomerase II was a gift from Dr. W. Huang (University of Utah).

Nucleic Acids. The replicative form (RF) of M13 mp19 phage DNA, either ^3H labeled (10×10^3 cpm/μg) or unlabeled, was prepared by the lysozyme Triton method (Davis et al., 1980) or the alkaline lysis method (Maniatis et al., 1982). The latter procedure yields about 1 mg of supercoiled DNA from 2000 mL of a log-phase culture of phage-infected *Escherichia coli.* Relaxed M13 RF DNA was prepared by incubating the supercoiled molecule with purified human topoisomerase I as described (Holden & Low, 1985). Kinetoplast DNA from the mitochondria of *Crithidia fasiculata* (provided by R. Warters, University of Utah) was isolated by standard procedures (England, 1978). Crithidia were grown in the dark at room temperature in brain heart infusion supplemented with hemin (0.02 mg/mL). A ^{32}P-labeled DNA probe to the joining region of the heavy-chain immunoglobulin gene (J_H probe) was obtained from Oncor.

Enzyme Assays. Topoisomerase II activity was followed by measuring the catenation of ^3H-labeled relaxed DNA with a filter binding assay as previously described (Holden & Low, 1985). Specific activity is expressed as the number of nanograms of relaxed DNA catenated per minute per nanogram of nuclear protein. The enzyme was also assayed by determining the extent of decatenation of kinetoplast DNA (Sullivan et al., 1986). For decatenation assays, reactions contained the following in a total of 20 μL: 50 mM Tris-HCl, pH 7.5, 85 mM KCl, 10 mM MgCl, 5 mM DTT, 5 mM EDTA, 30 μg/mL BSA, 1 mM ATP, and 250 ng of kinetoplast DNA. After incubation at 30 °C for 30–60 min, reactions were stopped by the addition of 2 μL of 10% SDS, 0.15 M EDTA, 0.2% bromophenol blue, and 50% glycerol. Samples were electrophoresed on 0.8% agarose gels in 40 mM Tris-HCl, pH 7.2, 20 mM sodium acetate, and 1 mM EDTA at 10 V for 15 h. The gels were stained with ethidium bromide, and the amount of monomer circle liberated from the kinetoplast network was estimated by a visual inspection of the gel. One unit of activity is the highest dilution of enzyme which decatenates all of the kinetoplast network. Topoisomerase I was assayed by following the relaxation of supercoiled phage DNA as peviously described (Holden & Low, 1985). All enzyme dilutions were performed in 50 mM Tris, pH 7.5, 200 mM KCl, 0.5 mM EDTA, 0.1 mg/mL BSA, 40% glycerol, 6% PEG, and 2 mM DTT.

Partial Purification of Topoisomerase II from Human Placenta. All purification procedures are performed at 4 °C unless otherwise stated. Fresh human placenta are obtained from the delivery room and immediately placed in buffer A [30 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.3 M sucrose, 0.2 mM EDTA, 2 mM DTT (or 15 mM β-mercaptoethanol), and 1 mM PMSF]. The umbilical cord and fetal membranes are removed, and the placenta is then washed several times in buffer A. The tissue is then placed through a meat grinder. This yields 200–250 g of crude placental tissue which is resuspended in 500 mL of buffer B [30 mM Tris-HCl (pH 7.5), 0.3 M sucrose, 4 mM $CaCl_2$, 2 mM DTT (or 15 mM β-mercaptoethanol), and 1 mM PMSF]. This is similar to a previously published method (Pedrini & Grossman, 1983). The crude placental tissue is then homogenized in 50-mL aliquots in a Kontes Scientific tissue grinder with a ground-glass tube and pestle. After homogenization, the suspension is filtered through cheesecloth and then centrifuged at 2000*g* for 15 min to pellet the nuclei. The nuclei are washed once with buffer C [10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 2 mM DTT, and 1 mM PMSF] and then resuspended in 250 mL of buffer C. After the suspension is stirred for 15 min, EDTA is added to a final concentration of 4 mM, and the suspension is stirred for an additional 15 min. At this time, the nuclei are lysed by the addition of 250 mL of buffer D [50 mM Tris-HCl (pH 7.5), 2 M NaCl, 2 mM DTT, and 1 mM PMSF]. The suspension is stirred for 15 min, and the lysing of the nuclei is monitored by phase-contrast microscopy and by noting the increased viscosity of the suspension. The DNA is then precipitated by the addition of 250 mL of buffer E [50 mM Tris-HCl (pH 7.5), 18% (w/v) poly(ethylene glycol) (PEG), 1 M NaCl, 2 mM DTT, and 1 mM PMSF]. The suspension is stirred for 30 min and then centrifuged at 20000*g* for 30 min. This procedure results in greater than 90% of the topoisomerase II activity being released into the supernatant. (Nuclear extracts prepared in this fashion can be frozen in liquid nitrogen and stored at −70 °C for at least a month without loss of activity.) To continue the purification, the supernatant which contains topoisomerase II activity is applied to a 30-mL hydroxylapatite column previously equilibrated with buffer E. After application of the sample, the column is washed with buffer F (0.2 M potassium phosphate, pH 7.0, 10% glycerol, 2 mM DTT, and 1 mM PMSF), and topoisomerase II is eluted by a potassium phosphate gradient from 0.2 to 0.7 M in buffer F.

Fractions containing the majority of activity are pooled, diluted 1:2 with 5 mM potassium phosphate, pH 7.0, 10% glycerol, 1 mM PMSF, and 2 mM DTT, and applied to a phosphocellulose column equilibrated with buffer F. Topoisomerase II is eluted with a linear potassium phosphate gradient from 0.2 to 0.7 M (pH 7.0) in buffer F. Fractions containing the peak of activity are pooled. This procedure is similar to the procedure used to purify topoisomerase II from HeLa cells (Miller et al., 1981). The pooled fractions are then brought to 40% saturation in ammonium sulfate, and after being stirred for 30 min, the solution is centrifuged at 8000*g* for 20 min. The supernatant is decanted and brought to 65% saturation in ammonium sulfate and, after being stirred for 20 min, is centrifuged at 8000*g* for 20 min. The pellet is resuspended in a small volume (0.5–1.0 mL) of buffer G (50 mM Hepes, pH 8.0, 500 mM KCl, 10% glycerol, 1 mM PMSF, and 2 mM DTT). The sample is stored at −70 °C. This represents a 300-fold purification of the enzyme with about an 8% yield.

Preparation of Nuclear Extracts from Human Tissues. Samples of normal and neoplastic human tissues are obtained fresh immediately following surgery from the Division of Surgical Pathology at the University of Utah. Only tissue not needed for pathologic diagnosis, and which would be subsequently discarded, is used. The use of human tissue for these experiments has been approved by the Institutional Review Board at the University of Utah. Normal specimens used must be grossly and microscopically unremarkable. Neoplastic specimens used must contain at least 95% tumor nuclei. To ensure that these criteria are met, frozen sections are performed on all specimens at the time of surgery and evaluated microscopically. Neoplastic specimens containing extensive inflammatory infiltrates or areas of necrosis are rejected. To prepare nuclear extracts from appropriate tissues, the samples are first finely minced with a scapel and then suspended in buffer B (5 mL/g of tissue). The suspension is homogenized in a ground-glass homogenizer with a tight-fitting pestel. After homogenization, the homogenate is filtered through cheesecloth, and the nuclei are isolated by centrifugation at 1000*g*. Nuclear extracts are then prepared as described above for human placenta. If the tissue cannot be processed into nuclear

extracts immediately, we have found that the tissue can be frozen in liquid nitrogen and then stored at −70 °C for at least a week without loss of enzyme activity.

The normal and neoplastic specimens analyzed were obtained from the following sources: specimen A is splenic tissue obtained from a 44-year-old man with Hodgkin's disease who underwent staging laparotomy. The spleen weighed 215 g and was grossly and microscopically normal. Specimen B represents a portion of normal thyroid tissue obtained from an 85-year-old man with a Hurthle cell carcinoma. The normal portion of thyroid was well separated from the tumor and was both grossly and microscopically unremarkable. Specimen C is a portion of normal thyroid tissue removed during exploratory surgery in a 17-year-old man. The patient had hyperparathyroidism secondary to end-stage renal disease. Specimen D represents tonsillar tissue obtained during routine tonsillectomy. Specimens E, F, and G all represent term placenta obtained at delivery during uncomplicated births. Specimen H represents a portion of small bowel in a 45-year-old man with Von Recklinghausen's disease. The bowel was removed because of small bowel obstruction secondary to mesenteric neurofibromas. A piece of bowel mucosa that grossly and microscopically was viable was dissected away from the external muscular layer and used for analysis. Specimen I is a portion of normal thymus obtained from the same patient as specimen C (see above). Specimen J is from a chest wall recurrence of breast carcinoma in a 78-year-old woman. Specimen K represents a portion of papillary thyroid carcinoma in a 25-year-old woman. Specimen L represents tissue from a parathyroid adenoma in a 48-year-old man. Specimen M is from a recurrent scalp leiomyosarcoma in an 80-year-old man. Specimen N is from the ovary of a 42-year-old woman which was diffusely infiltrated by a high-grade, non-Burkitts, malignant lymphoma. Specimen O represents tissue from an inguinal lymph node in a 42-year-old man. The node was also completely replaced by a high-grade, non-Burkitts, malignant lymphoma.

Immunoglobulin Gene Rearrangement. DNA was isolated from human tissue, digested with appropriate restriction enzymes, electrophoresed on 0.8% agarose gels, Southern transferred to nitrocellulose, and washed as described (Davis et al., 1986). The washed filters were baked at 80 °C for 2 h under vacuum. Filters were then prehybridized for 30 min at room temperature in heat-sealable bags containing 9.5 mL of 50% deionized formamide, 4 × SSC, 1 × Denhardt's solution, 1 mg of yeast tRNA, 0.2% SDS, and 20 mM sodium phosphate, pH 6.5. For hybridization, a ^{32}P probe to the joining region of the immunoglobulin heavy-chain gene (25 ng, 5 μCi) was added to 0.5 mL of prehybridization buffer, heated at 100 °C, for 2–3 min, and then added to the hybridization bags containing the filters. The joining region probe recognizes a 19-kb *Bam*HI restriction fragment and an 11-kb *Hin*dIII restriction fragment of the human immunoglobulin heavy-chain gene. Hybridization was performed overnight at 42 °C. The following day, the filters were washed to remove background radioactivity (Davis et al., 1986), and gene rearrangement was detected by autoradiography of the hybridized nitrocellulose. DNA isolated from autopsy liver was used to represent germ line configuration.

Heat Inactivation of Human Topoisomerase II. To determine the heat stability of topoisomerase II, a crude placental nuclear extract was diluted 10-fold into 50 mM Tris, pH 7.5, 200 mM KCl, 0.5 mM EDTA, 0.1 mg/mL BSA, 40% glycerol, 6% PEG, and 2 mM DTT. Aliquots (50 μL) were heated in a water bath at 50 °C for various times and then chilled in an ice/water bath. Remaining activity was assayed by the catenation assay. Where indicated, VM-26 was added to a concentration of 609 μM.

Cell Cycle Analysis by Flow Cytometry. Representative tissue samples showing greater than 95% tumor cell nuclei by frozen section were stored at −70 °C. Nuclei were isolated and stained for flow cytometry from the frozen tissue with "nuclear isolation medium" (Thornthwaite et al., 1989) except that propidium iodide (5 μg/mL) and ribonuclease A (1 mg/mL) were substituted for 4′,6-diamidino-2-phenylindole; 256-channel nuclear DNA content histograms were obtained with an EPICS C flow cytometer (Coulter) equipped with a 2-W argon laser tuned to excitation at 488 nm. Cell cycle analysis was performed by using the rectangular S-phase model (Baisch et al., 1975).

Protein Determination. The protein concentration of nuclear extracts was determined by the method of Bradford (1976) in which BSA served as a standard.

Photomicroscopy. Photomicrographs of neoplastic tissues were made with a Zeiss photomicroscope and Kodak Panatomic-X film. Magnification was 100×.

Results

DNA Topoisomerase II Is Present in Term Human Placenta. We have used a filter binding assay to measure DNA catenation catalyzed by topoisomerase II (Holden & Low, 1985) and have found activity in crude nuclear extracts prepared from term human placenta (not shown). As little as 5–10 ng of placental nuclear extract results in measurable catenation activity. This is of the same order of magnitude as that seen in crude nuclear extracts of HeLa cells (Holden & Low, 1985). Placental nuclear extracts also contain decatenating activity (not shown). However, to observe this activity, the nuclear extracts must first be dialyzed to lower the salt concentration. The 1 M NaCl used in preparing the nuclear extracts inhibits the decatenating reaction. Also, decatenating activity is only seen with fairly large amounts of nuclear protein (600 ng). Others have also observed decatenating activity only with relatively large amounts of nuclear protein (Sullivan et al., 1986; Markovits et al., 1987). We have used mixing experiments to search for a possible inhibitor of the decatenating activity which might be present in crude nuclear extracts but have so far been unsuccessful. We also note that either 2 mM dithiothreitol or 15 mM β-mercaptoethanol must be present during the homogenization for optimal activity. Lower concentrations lead to poor catenation activity (less than 10% recovery) and undetectable decatenating activity (not shown). The effect of DTT or β-mercaptoethanol on topoisomerase II activity may be to prevent oxidation of the enzyme (Miller et al., 1981; Halligan et al., 1985) as well as to ensure the enzymes release from the nuclear scaffold (Gasser et al., 1986). A similar effect of mercaptoethanol on the extraction of chicken topoisomerase II from erythrocytes has recently been described (Muller et al., 1988). Both the filter binding assay to measure catenation and the agarose gel assay to measure decatenation can be used to follow the purification of the enzyme. Both activities copurify when crude placental nuclear extracts are chromatographed on a hydroxylapatite column (Figure 1) or subsequently on a phosphocellulose column (Figure 2).

Cantenation Is a Sensitive Measurement of DNA Topoisomerase II Activity. Studies with crude nuclear extracts of placenta suggested that the catenation assay is more sensitive than the decatenation assay. We quantitated this difference in sensitivity by measuring the catenating and decatenating activity of purified recombinant T_4 topoisomerase II. By

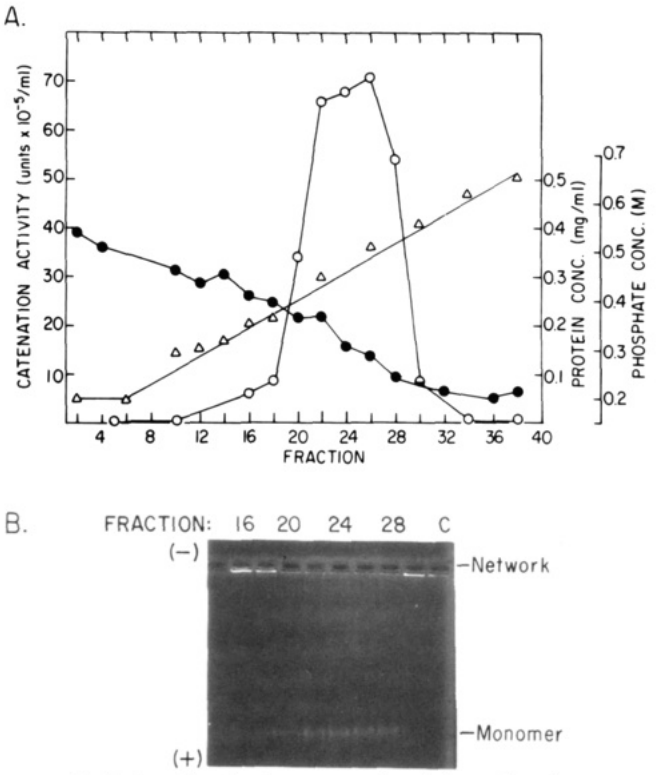

FIGURE 1: Hydroxylapatite chromatography of placental topoisomerase II activity. (A) Nuclear extract (700 mL) prepared from human placenta as described under Materials and Methods was applied to a 30-mL column (2.6 × 5.5 cm) of hydroxylapatite. Topoisomerase II activity (○) was eluted with a 400-mL phosphate gradient from 0.2 to 0.7 M and assayed by the catenation assay. The fractions had to be diluted 100-fold in order to measure catenation. The protein concentration (●) of each fraction was determined as described under Materials and Methods, and the phosphate concentration (△) was determined by conductivity measurements. Fraction size was 10 mL. The yield of catenating activity was 30%. (B) The indicated fractions from the hydroxylapatite column were assayed for decatenating activity as described under Materials and Methods. Fractions were assayed undiluted for 60 min. Lane C represents kinetoplast DNA without addition of enzyme.

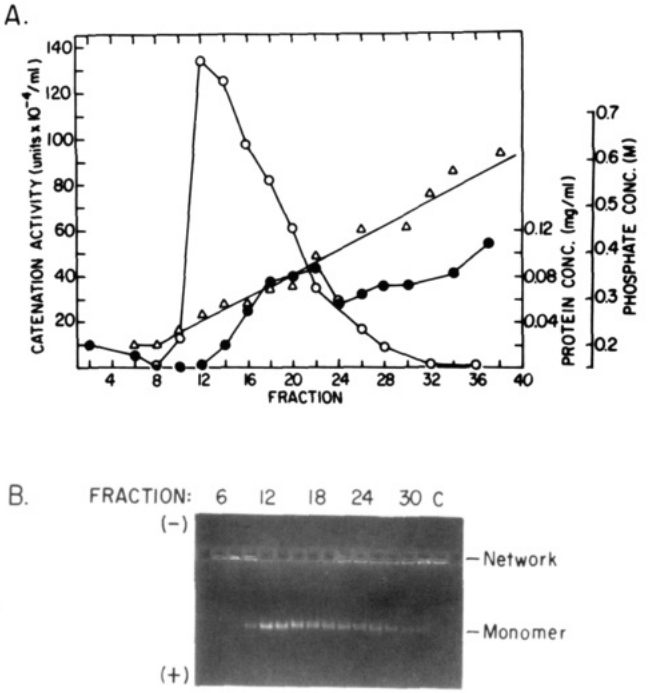

FIGURE 2: Phosphocellulose chromatography of placental topoisomerase II activity. (A) Fractions from hydroxylapatite chromatography containing the majority of topoisomerase II activity were pooled, diluted 2-fold, applied to a phosphocellulose column (1.7 × 4.0 cm), and eluted with a 50-mL 0.2–0.7 M phosphate gradient as described under Materials and Methods. Fractions were assayed for topoisomerase II by the catenation assay (○), for protein (●), and for phosphate concentration (△) by conductivity measurements. Fractions were 1.25 mL. The yield of catenating activity was 33%. (B) Fractions were assayed for decatenating activity for 60 min at 30 °C. Lane C contains kinetoplast DNA without the addition of enzyme.

defining a decatenation unit as that amount of activity which decatenates all (250 ng) of the kinetoplast DNA in 60 min at 30 °C, we find that only 1×10^{-2} decatenation unit is necessary to catenate all (500 ng) of the circular DNA in 10 min in the catenation assay (not shown). This suggests that the catenation assay is at least 2 orders of magnitude more sensitive than the decatenation assay.

DNA Topoisomerase Activity in Human Tissues. Because of its sensitivity, we have used the catenation assay to evaluate topoisomerase II activity levels in nuclear extracts prepared from various human tissues. These measurements have been correlated with measurements of topoisomerase I activity as well as with flow cytometric data (Table I). In normal tissues, the highest level of topoisomerase II activity was found in the spleen (specific activity about 2.4 units/ng) and thymus (specific activity 1.7 units/ng). Interestingly, the enzyme is present in several tissues which are terminally differentiated and probably have little capacity for further replication such as term placenta (specific activity about 0.5 unit/ng) and thyroid (specific activity about 0.5 unit/ng). Enzyme activity was also present in both benign and malignant neoplasms. The highest topoisomerase II activity was found in those neoplasms that generally are clinically aggressive (breast carcinoma, leiomyosarcoma, and lymphoma). These levels are, however, similar to those seen in normal spleen and thymus. Neoplasms such as papillary thyroid carcinoma and parathyroid adenoma, which generally behave in a clinically indolent fashion, show lower topoisomerase II activity. These lower levels are similar to those seen in normal thyroid, tonsil, placenta, and small intestine.

Photomicrographs of the neoplastic specimens evaluated are shown in Figure 3. These specimens contain at least 95% tumor nuclei which indicates that we are measuring topoisomerase II activity from the neoplastic cells and not from contaminating tissue. In the case of specimen N (lymphoma), we have been able to confirm this light microscopic impression by an independent method. We have shown that specimen N demonstrates a clonal rearrangement of the heavy-chain immunoglobulin gene (Figure 4). The rearranged gene is observed with both *Bam*HI and *Hin*dIII digestions, which suggests against a polymorphism, and the extremely small amount of germ line present argues that our sample is mostly comprised of pure tumor, in agreement with light microscopy. Suprisingly, this neoplasm did not contain highly elevated levels of topoisomerase II in spite of its apparent high proliferative status as indicated by the flow cytometric data (47% of the cells in S-G_2M). As expected, topoisomerase I activity remains fairly constant between different tissues although there is a suggestion of a somewhat higher level in specimen O (lymphoma). However, the topoisomerase I assay is only semiquantitative so we cannot be entirely sure whether this is a true elevated level or not.

Teniposide Inhibition of Catenation. Teniposide has been reported to be a potent inhibitor of topoisomerase II activity (Minocha & Long, 1984; Yang et al., 1987; Richter et al., 1987). To determine if there might be differences between teniposide inhibition of topoisomerase II activity from neo-

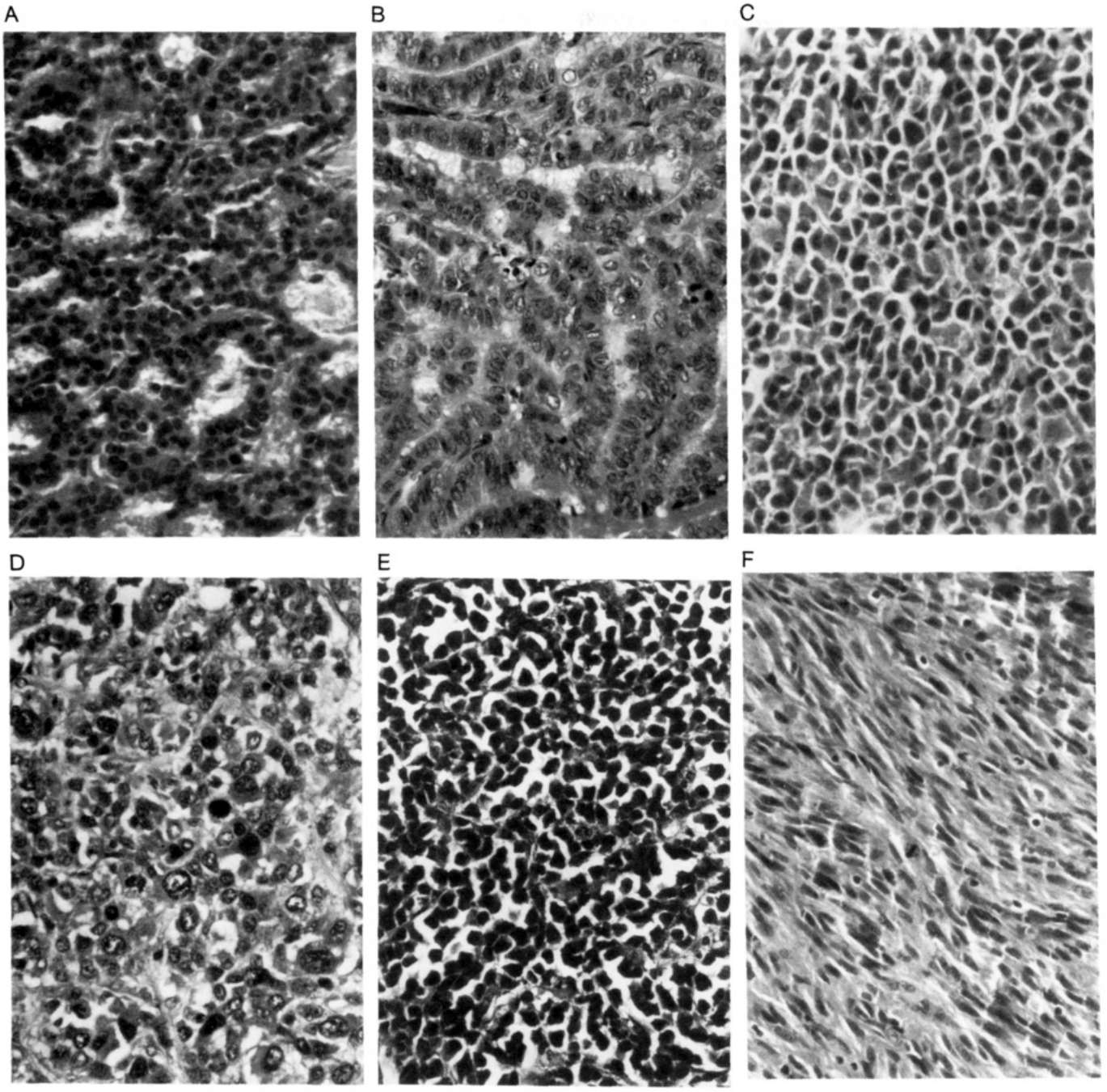

FIGURE 3: Photomicrographs of neoplastic specimens. Photomicrographs of neoplastic specimens were performed with a Zeiss photomicroscope as described under Materials and Methods; magnification is 50×. The photomicrographs are as follows: (A) parathyroid adenoma; (B) papillary thyroid carcinoma; (C) breast carcinoma; (D) high-grade lymphoma from an inguinal lymph node; (E) high-grade lymphoma from an ovary; (F) leiomyosarcoma.

plastic and normal tissues, we evaluated the ability of the drug to inhibit the catenating activity from specimen N (lymphoma), specimen D (tonsil), and specimen F (placenta) (Figure 5). All three show similar patterns of inhibition. There is an initial rapid decline of activity at around 10–20 μM followed by a plateau phase at around 50 μM teniposide where catenation is apparently resistant to inhibition. We have observed exactly the same pattern of inhibition in activity in specimen I (thymus) (not shown). In addition, decatenating activity present in the thymus gland is also quite resistant to the effects of teniposide (Figure 6). Extensive decatenating activity is present even at 609 μM teniposide.

In the above experiments, the reactions are terminated by the addition of SDS. Thus, it is possible that the inhibition observed is only an apparent inhibition caused by the generation of double-stranded DNA breaks formed in the presence of drug at the end of the reaction. Molecules with double-stranded breaks would be linearized and thus be released from the catenanes formed. However, the same inhibition is observed when the reactions are terminated with 500 mM NaCl for 15 min prior to the addition of SDS (not shown). This should be adequate to reverse any cleavable complexes at the end of the reaction (Liu et al., 1983) and suggests that the inhibition is due to a true inhibition of strand passing activity. These results are also in agreement with data indicating that the observation of cleavable complex formation requires several nanograms of purified enzyme (Tewey et al., 1984) and is generally not observed with catalytic amounts of nuclear extracts. Therefore, we would also not expect to see linear DNA in Figure 6.

Table I

specimen	tissue source[a]	Topo II act.[b] (units/ng)	Topo I act.[c] (units × 10^{-6}/mg)	% of cells in G_0G_1	S	G_2M[d]
		Normal Tissues				
A	spleen	2.4 ± 0.31	0.22	98	1	1
B	thyroid	0.72 ± 0.16	0.22			
C	thyroid	0.33 ± 0.10	0.18	97	1	2
D	tonsil	0.50 ± 0.07	0.15	88	9	3
E	placenta	0.34 ± 0.05	0.15	90	3.4	6.6
F	placenta	0.43 ± 0.07				
G	placenta	0.58 ± 0.03				
H	small intestine	0.51 ± 0.04	0.32	95	3	2
I	thymus	1.7 ± 0.17	0.53	85	5	6
		Neoplastic Tissues				
J	breast carcinoma	2.2 ± 0.36	0.35	73	18	9
K	thyroid carcinoma	0.75 ± 0.24	0.33	95	0	4
L	parathyroid adenoma	0.57 ± 0.07	0.16	97	0	3
M	leiomyosarcoma	1.6 ± 0.15	0.58	82	14	4
N	lymphoma	1.2 ± 0.30	0.36	53	39	8
O	lymphoma	1.9 ± 0.16	0.70	82	14	4

[a] Nuclear extracts were prepared from the indicated tissues as described under Materials and Methods. [b] Topo II activity is expressed as the number of nanograms of circular RF M-13 DNA catenated per minute per nanogram of nuclear protein. The results are expressed as the average ± the standard deviation of three to four separate assays. To assess the variability between similar tissues, samples of normal thyroid from two different patients and placentas from three different patients have been evaluated and included in the data. [c] Topo I activity was determined by measuring the relaxation of supercoiled M-13 RF DNA as described under Materials and Methods. One unit is that amount of enzyme which relaxes half (250 ng) of the supercoiled substrate in 15 min at 30 °C. These assays require a titration of enzyme to determine this point, and the titrations have been performed only once for the indicated extracts with the exception of specimen O. The titrations have been performed twice on this sample with identical results. [d] The proliferative fraction of the tissues was estimated by flow cytometry as described under Materials and Methods.

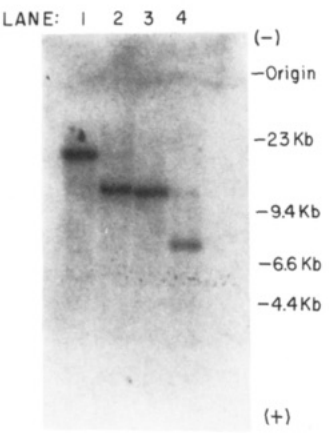

FIGURE 4: Immunoglobulin gene rearrangement of specimen N. Rearrangement of the heavy-chain immunoglobulin gene was detected by Southern blotting and hybridization to ^{32}P-labeled probe to the joining region of the heavy-chain immunoglobulin gene (JH probe) as described under Materials and Methods. Lane 1 represents hybridization to *Bam*HI-digested genomic DNA prepared from normal liver obtained at autopsy. Lane 2 represents hybridization to *Bam*HI genomic DNA isolated from specimen N. Lane 3 represents hybridization to *Hin*dIII-digested genomic DNA from autopsy liver. Lane 4 represents hybridization to *Hin*dIII-digested genomic DNA from specimen N.

Teniposide-Resistant Topoisomerase II Activity. The above results indicate that human tissues contain some topoisomerase II activity which is resistant to teniposide. This suggested that there may be more than one form of the enzyme and that these forms can be distinguishable by their interactions with the drug. If this is true, then these forms must be quite similar biochemically. They show a similar heat inactivation curve (Figure 7). Roughly 50% activity remains after heating at 50 °C for 10 min. Also the drug-resistant form purifies with known topoisomerase II through chromatography over both hydroxylapatite and phosphocellulose. Its chromatographic behavior on phosphocellulose is shown in Figure 8. Both teniposide-sensitive and teniposide-resistant topoisomerase II elute at identical salt concentrations.

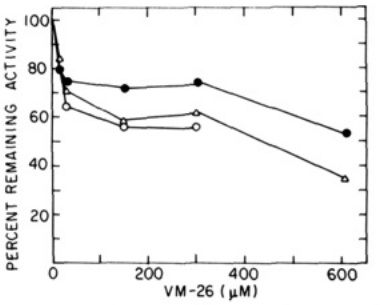

FIGURE 5: Teniposide inhibition of catenation. Nuclear extracts prepared from placenta (●), tonsil (△), and specimen N (○) were assayed for catenating activity as described under Materials and Methods in the presence of the indicated concentration of teniposide (VM-26). The drug was added prior to the addition of enzyme, and assays were performed for 10 min at 30 °C.

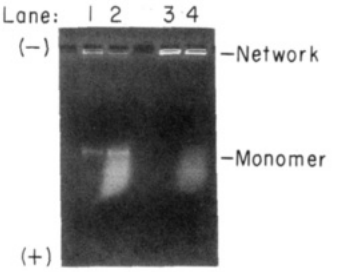

FIGURE 6: Decatenating activity of nuclear extracts from normal human thymus in the presence of teniposide. Nuclear extracts prepared from thymus were assayed for decatenating activity for 60 min at 30 °C as described under Materials and Methods. Lane 1 contains 900 ng of thymic nuclear protein. Lane 2 contains 900 ng of thymic nuclear protein and 609 μM teniposide. Lane 3 is a control without enzyme or drug. Lane 4 is a control containing drug but without the addition of enzyme.

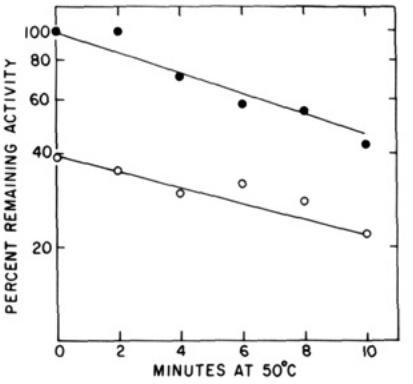

FIGURE 7: Heat inactivation of catenating activity from human placenta in the presence of teniposide. Nuclear extracts were prepared from human placenta as described under Materials and Methods. Extracts were diluted 10-fold and heated at 50 °C for the indicated times. After being cooled in an ice–water bath, the samples were assayed for remaining catenating activity either with (○) or without (●) the addition of 609 μM teniposide.

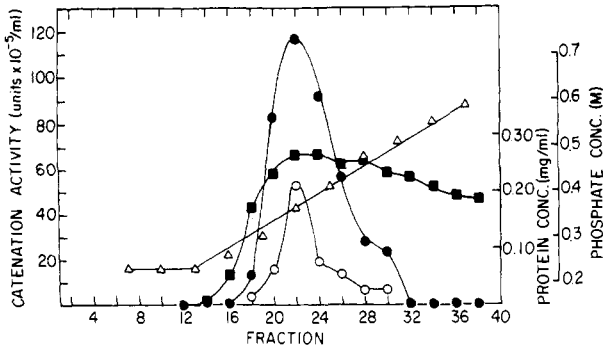

FIGURE 8: Phosphocellulose chromatography of teniposide-resistant catenating activity. Human topoisomerase II partially purified from human placenta by hydroxylapatite column chromatography was chromatographed on a phosphocellulose column as described under Materials and Methods. Fractions were assayed for catenating activity in the presence (○) or absence (●) of 609 μM teniposide. Fractions were also assayed for protein (■) and for phosphate (△) concentration by conductivity measurements. The yield of catenating activity was 27%.

Discussion

In this report, we have made use of a sensitive filter binding assay for topoisomerase II in order to investigate enzyme activity in a variety of human tissues. We believe this assay is accurately measuring topoisomerase II activity. We have shown this to be true in HeLa cells (Holden & Low, 1985), and the copurification of catenating activity and decatenating activity suggests that this is also true in human placenta. Furthermore, the activity we measure is ATP dependent and not affected by the topoisomerase I targeted drug camptothecin (Rolfson and Holden, unpublished data). A recent publication (Tsutsui et al., 1989) also indicates that topoisomerase II appears to be the sole enzyme in nuclei responsible for the catenation of circular DNA. The fact that catenation is a known function of type II topoisomerases and that catenating activity has been used to follow the purification of topoisomerase II from *Drosophila* (Shelton et al., 1983) leads us to believe that the activity we are measuring is catalyzed by DNA topoisomerase II.

The catenation assay appears at least 50–100 times more sensitive than the decatenation assay. This probably reflects the fact that theoretically only one strand passing event is necessary to link two circles. However, kinetoplast DNA is a complicated topological mixture (Marini et al., 1980; Ryan et al., 1988), and it is not clear how many strand passing events must occur before a single minicircle is released. The use of PVA in our assay as a condensing agent also means that the effect of endogenous nuclear factors such as spermidine and histone proteins which may stimulate catenation (Tse et al., 1984; Riou et al., 1985; Hsieh & Brutlag, 1980; Krasnow & Cozzarelli, 1982) becomes negligible. On the contrary, these factors, by stimulating catenation, may partially inhibit the decatenation assay which might also help to explain the low sensitivity of that measurement. The presence of such a factor which inhibits the decatenating activity in crude extracts of trypanosomes has recently been described (Douc-Rasy et al., 1986; Melendy & Ray, 1989).

Some of our results appear to be contradictory to previous published data. It has been suggested that topoisomerase II is present in proliferating (cycling) cells but drops to nearly undetectable amounts of resting or G_0 cells (Heck & Earnshaw, 1986; Hsiang et al., 1988; Nelson et al., 1987; Sullivan et al., 1986). Although it might be argued that tissues such as spleen, thymus, and tonsil are composed of lymphocytes which might not be in G_0, term placenta and thyroid tissue are certainly in a resting state with little capacity for further replication. Both of these tissues contain topoisomerase II activity measured either by our filter binding assay or by the more conventional decatenation assay. Some of the variation in activity we observed may be due to the source of tissue. These specimens were from patients of either sex and who differed greatly in age. Note that we observed a 2-fold difference in activity between two normal thyroid glands (Table I, specimens B and C). However, we were surprised not to find extremely high enzyme levels in specimen N. This was a lymphoma in which 47% of the cells were in S-G_2M phase (Table I).

To explain this contradiction, we postulate that human tissues may contain more than one form of topoisomerase II. Since most of the data indicating the proliferation dependence of the enzyme are based on immunological data, a second, antigenically distinct enzyme would go undetected. If this other enzyme is also active in catenation, we would detect it since we are using a sensitive functional assay.

Support for this hypothesis comes from the recent study of topoisomerase II in P-388 leukemia cells (Drake et al., 1987). These investigators observed two forms of topoisomerase II which differ in amino acid structure. One has a subunit molecular weight of 170000 and the other a subunit molecular weight of 180000. Significantly, antibodies prepared against the 170000 molecular weight protein do not inhibit the activity of the 180000 molecular weight form. In addition, other investigators have also indicated that topoisomerase II is present in nonproliferating tissues such as mature spermatids and chicken liver (Roca & Mezquita, 1989), rat brain (Tsutsui et al., 1986), confluent Chinese hamster cells (Schneider et al., 1988), and nonproliferating *Drosophila* tissues (Hadlaczky et al., 1988).

The prediction of more than one type II topoisomerase in human tissues must be reconciled with data indicating that the gene for the enzyme is present only in a single copy (Goto & Wang, 1984; Nolan et al., 1986; Tsai-Pflugfelder et al., 1988). Therefore, the second topoisomerase II must be a molecule derived from an RNA which underwent altered splicing or a molecule with extensive posttranslational modifications. Alternatively, it could be the product of a divergent gene. A new topoisomerase, similar to topoisomerase I but able to decatenate DNA, has recently been described in Chinese hamster cells (Kerrigan et al., 1988). Whether such an activity exists in human tissues is not yet known. Continued study of the activity present in proliferating and nonproliferating tissues should help resolve these questions and may indicate how topoisomerase II renders tumors susceptible to the topoisomerase II targeted drugs.

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Registry No. DNA topoisomerase, 80449-01-0; teniposide, 29767-20-2.

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