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SUBCELLULAR DISTRIBUTION OF THE α AND β TOPOISOMERASE II–DNA COMPLEXES STABILIZED BY VM-26

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Abstract—Studies were done to determine (a) the subcellular distribution of the α (170 kDa) and β (180 kDa) isozymes of topoisomerase II, and (b) the extent to which each isozyme forms complexes with DNA in tumor cells incubated with and without VM-26. Western blotting revealed that topoisomerase IIβ was highly unstable during cell fractionation. However, preincubation of human CEM leukemia cells with 5–100 μM VM-26 for 30 min protected the β isozyme from degradation by progressively increasing the amount of this isoform bound to DNA. The amount of topoisomerase IIβ detected in nuclei of CEM cells incubated for 30 min with 25 μM VM-26 was 7-fold greater than in nuclei from untreated control cells. VM-26 also had a protective effect on topoisomerase IIβ in HL-60 leukemia and WiDR colon carcinoma cells. In contrast, the intercalating agents mitoxantrone and m-AMSA did not protect topoisomerase IIβ from degradation during cell fractionation. The stabilization of topoisomerase IIβ by VM-26 allowed subsequent studies of the subcellular distribution of the topoisomerase II isozymes. Both isozymes were detected in the nonmatrix (high salt-soluble) fraction of nuclei from CEM cells, but only topoisomerase IIα was present in the nuclear matrix. VM-26 stabilized binding of the α and β topoisomerase II isoenzymes to nonmatrix DNA and topoisomerase IIα to matrix DNA. The differences observed in the subnuclear distribution and DNA binding pattern of the topoisomerase II isozymes support the hypotheses that each isozyme has a distinct cellular function, and that both the α and β isozymes are potential targets for VM-26 in intact cells. In addition, the results demonstrated that pretreatment of various cell lines with VM-26 is a useful way to stabilize topoisomerase IIβ during cell fractionation.

Key words: DNA topoisomerase II; teniposide; VM-26; nuclear matrix; m-AMSA; mitoxantrone

Chromosomal DNA is organized into loops and higher order structures that are maintained in a supercoiled state [1, 2]. Type II DNA topoisomerases are essential enzymes that alter the degree of DNA supercoiling by first cleaving the double-stranded DNA, passing a second double strand through the cleaved site, and then resealing the cleaved strands [3]. In eukaryotic cells topoisomerase II appears to be important in both DNA organization and replication. The enzyme is a component of the nuclear matrix [4–6], and topoisomerase II consensus cleavage site sequences are present near the attachment sites of DNA loops to the nuclear matrix [7, 8]. Inhibition of nuclear matrix topoisomerase II by either VM-26§ or m-AMSA results in the detachment of newly replicated DNA from the nuclear matrix [6]. Furthermore, topoisomerase II–DNA complexes are enriched on DNA replication forks compared with bulk DNA [9]. Following DNA replication, topoisomerase II catalyzes the decatenation of the intertwined DNA molecules [10, 11].

Two forms of mammalian topoisomerase II, with apparent molecular weights of 170 kDa (α) and 180 kDa (β), have been identified [12]. The two isozymes are encoded by different genes [13, 14] and differ in their catalytic properties [12]. It has been reported that the α isozyme is localized primarily in the nucleoplasm, while the β isozyme is present mainly in the nucleolus [15]. The roles of the individual isozymes in DNA organization and metabolism have not been established.

Certain anticancer agents in clinical use, such as VP-16, VM-26, and m-AMSA, increase DNA cleavage primarily by inhibiting religation of DNA cleaved by topoisomerase II [16, 17]. VM-26 inhibits both the 170 and 180 kDa isozymes, as measured in strand passing assays *in vitro* [12]. However, in intact cells the specific isozyme(s) inhibited by VM-26 has not been identified. In an attempt to address this question, we determined the extent to which each isozyme forms covalent enzyme–DNA complexes in whole cells, nuclei and subnuclear fractions of human CCRF-CEM leukemia cells incubated with and without VM-26.

MATERIALS AND METHODS

Chemicals, supplies, and cell culture conditions.

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§ Abbreviations: DTT, dithiothreitol; m-AMSA, 4′(9-acridinylamino)methanesulfon-*m*-anisidide; Me_2SO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; VM-26, teniposide, 4′-demethylepipodophyllotoxin 9-(4,6,*O*-2-thenylidene-β-D-glucopyranoside); and VP-16, etoposide, 4′-demethylepipodophyllotoxin 9-[4,6-*O*-ethylidene-β-D-glucopyranoside].

Sources for most chemicals and supplies have been reported previously [18–20]. Cycloheximide, hydroxyurea, cytosine arabinoside, pepstatin A, and aprotinin were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]L-Leucine (sp. act. 60 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, CA). [^{14}C]Thymidine (sp. act. 60 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). Descriptions of the cell lines and tissue culture conditions have been published [21–23]. HL-60 human promyelocytic leukemia cells were a gift of Dr. W. G. Harker, VA Medical Center, Salt Lake City, UT, and WiDR human colon adenocarcinoma cells were provided by Dr. F. Durr, Lederle Laboratories, Pearl River, NY. Protein concentrations were determined using the BioRad Dye Reagent as outlined by the manufacturer (BioRad Laboratories, South Richmond, CA). RNase-free DNase I was purchased from Worthington Biochemicals, Freehold, NJ. A monoclonal antibody to α tubulin was obtained from the Accurate Chemical Co., Westbury, NY. DNase I buffer consisted of 10 mM Tris–HCl (pH 7.8), 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$. Nuclei buffer consisted of 50 mM Tris–HCl (pH 7.9), 1 mM EDTA, 0.6 M NaCl, and a combination of six protease inhibitors consisting of benzamidine (1 mM), PMSF (1 mM), soybean trypsin inhibitor (10 μg/mL), leupeptin (50 μg/mL), aprotinin (2 μg/mL) and pepstatin (1 μg/mL). This combination of protease inhibitors is hereafter referred to as the protease inhibitor solution.

Isolation, salt extraction, and DNase I digestion of nuclei. CEM cells (8×10^7) were harvested and resuspended in fresh medium and serum at a density of 2×10^5 cells/mL. After 24 hr, the cells were incubated for 30–60 min with either Me_2SO/ethanol (1:1) at a final concentration of 0.1% (v/v), or with various concentrations of VM-26 in 0.1% Me_2SO/ethanol. The cells were harvested by centrifugation at 4°, and the pellets were washed with ice-cold medium without serum. The pellets were then resuspended at a density of 2×10^7 cells/mL in 10 mM Tris–HCl (pH 7) buffer that contained the protease inhibitor solution. The cells were allowed to swell on ice for 5 min and then were disrupted with 5 strokes in a Dounce homogenizer. The lysates from both control and VM-26-treated cells were layered over a solution of 45% (w/v) sucrose at 4°, and the nuclei were purified by centrifugation at 1900 *g* for 30 min at 4°.

In some experiments (see Fig. 3) the nuclear pellets were resuspended in nuclei buffer at 4° and extracted over a period of 30 min with 0.5 M NaCl. The extracts were then centrifuged at 48,000 *g* for 30 min at 4°. The pellets were resuspended in 200 μL of DNase I buffer and incubated for 20 min at 37° with 500 U of DNase I. At the end of this incubation the digests were centrifuged at 1000 *g* for 5 min at 4°. Aliquots of the 48,000 *g* and 1000 *g* supernatants were removed for measurement of protein concentration prior to western blotting of the proteins as described below.

Isolation of nuclear matrices. To isolate nuclear matrices, nuclei from control and drug-treated CEM cells were resuspended in 4 mL of low salt buffer [10 mM Tris–HCl (pH 7.0) 1 mM $MgCl_2$, and 10 mM NaCl] containing the protease inhibitor solution. RNase-free DNase I (2560 U) was added and the samples were incubated for 30 min at 37°. Four milliliters of high salt buffer [3 M NaCl, 10 mM Tris–HCl (pH 7.0), and 0.2 mM $MgCl_2$] plus the protease inhibitor solution were then added over a period of 1 hr with gentle shaking. Following an additional 30-min incubation on ice, the samples were centrifuged at 7000 *g* for 15 min at 4°. The resultant nuclear matrix pellets were resuspended in 10 mL of 10 mM Tris–HCl (pH 7.0), 0.6 mM $MgCl_2$ and 1.5 M NaCl, centrifuged at 7000 *g*, 4°, and then washed an additional time with 10 mL of low salt buffer at 4°.

Separation of free and DNA-bound topoisomerase II by HPLC. Nuclear matrices prepared from untreated and VM-26-treated cells as described above were resuspended in 250 μL of 10 mM Tris–HCl (pH 8.0), 2 mM Na_2EDTA, and 2% SDS. The samples were heated for 5 min at 37° and then sonicated for 5 sec at the lowest setting of a Cole-Parmer 4710 ultrasonic homogenizer equipped with a microtip. The sonicates were injected onto a Beckman Spherogel TSK 4000 HPLC column that was eluted with 40 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 1 mM DTT at a flow rate of 1 mL/min. In separate experiments, molecular weight calibration curves were generated for this HPLC column using double-stranded DNA or protein standards. Fractions of 0.5 mL each were collected. Those fractions containing free and DNA-bound topoisomerase II, as determined by western blotting, were pooled. Magnesium chloride and sodium acetate were added (final concentrations of 10 and 300 mM, respectively) to the pooled fractions containing DNA-bound topoisomerase II, and the complexes were then precipitated overnight with 3 vol. of ethanol at −20°. The complexes were recovered by centrifugation for 60 min at 40,000 *g* at 4°. The pellets were resuspended in 100 μL of 50 mM Tris–HCl (pH 7.8), 1 mM DTT, 1 mM magnesium chloride and 0.1 mM calcium chloride. Each sample was then divided into two 40-μL aliquots, and either 50% glycerol or DNase I (750 U in 50% glycerol) was added to the aliquots. All aliquots were incubated for 20 min at 37°. The samples were stored at −100° for 2 days or less and analyzed by western blotting.

Reversal of VM-26–topoisomerase II–DNA complexes by heat. This technique was used only with whole cells, since rapid cell lysis destroys nuclei and nuclear matrices. Exponentially growing CEM cells were preincubated with [^{3}H]L-leucine (0.01 μCi/mL). After 24 hr, duplicate groups of 2×10^7 cells each were incubated for 30 min with either Me_2SO/ethanol (1:1) at a final concentration of 0.1% or with 12.5 or 25 μM VM-26 in 0.1% Me_2SO/ethanol. The cells were centrifuged at 4°, and one cell pellet from each group was preheated in the absence of medium for 5 min at 65° in order to dissociate any VM-26–topoisomerase II–DNA complexes [24]. These cell pellets were then lysed by resuspension in 1 mL of protease inhibitor solution containing 2% (w/v) SDS at 65°. The cell pellets in the second group were resuspended immediately in the protease

inhibitor solution containing 2% SDS at 65° and lysed without preheating. All the lysates were then vortexed at maximal intensity for 15 sec and then heated for 5 min at 65°. After cooling to room temperature for 10 min, a 10-μL aliquot of each sample was removed for liquid scintillation counting. This allowed a rapid estimation of the relative amount of protein (i.e. ^{3}H dpm) in each sample. Aliquots of the samples containing an equal amount of [^{3}H]protein were loaded on SDS–PAGE gels and analyzed by western blotting.

SDS–PAGE. Samples were equalized for protein, loaded onto a 5–15% polyacrylamide SDS gel, and then electrophoresed by the method of Laemmli [25]. Unless otherwise indicated, each lane contained 75 μg of protein. Qualitatively similar results were obtained when the amount of protein loaded in each gel lane varied from 25 to 125 μg.

Western blotting. Blots were incubated with antisera that recognize either the β isoform of DNA topoisomerase II or both the α and the β isoforms of the enzyme (MAC) [26]. The MAC antiserum was produced by Danks and Schmidt in rabbits immunized with a 72 kDa C-terminal fragment of the human topoisomerase II sequence of the α form of the enzyme. The plasmid producing this peptide was obtained from Dr. L. F. Liu (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ). The rabbit antiserum directed against a C-terminal peptide consisting of residues 1512–1530 of human topoisomerase IIβ was a gift of Dr. Fritz Boege of the University of Würzburg, Germany. After transfer of the proteins to nitrocellulose paper, nonspecific protein binding was blocked by incubating the paper at 37° for 2 hr in 3% (w/v) BSA, 5% (w/v) powdered milk, 0.2% (v/v) Tween 20, and 0.02% (w/v) sodium azide in 10 mM Tris–HCl (pH 7.5) and 140 mM NaCl. The MAC and topoisomerase IIβ antisera were diluted 1:2000 and 1:5000, respectively, with blocking solution prior to incubation of the nitrocellulose filter from 2 to 48 hr at room temperature. The blots were washed, incubated with alkaline phosphatase-linked goat anti-rabbit IgG [18], and then developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium according to the method of Harlow and Lane [27]. Control blots with preimmune serum diluted 1:1000 were negative. Quantitation of immunoblots was done with a Visage 110 laser scanner [20]. Briefly, the amount of immunoreactive topoisomerase II is the product of the band intensity and the band area, and is expressed in arbitrary units defined by internal computer standards. The products of the areas and band intensities calculated after reaction with antisera to topoisomerase II or a monoclonal antibody to α tubulin were linear with respect to the amounts of protein loaded on the gels. Statistical analysis was carried out using a two-tailed *t*-test.

RESULTS

Detection of topoisomerase II α and β in nuclei of cells incubated with and without VM-26. The initial studies were aimed at determining the extent to which VM-26 stabilized binding of the α and β isozymes of topoisomerase II to DNA. Nuclei isolated from untreated control and VM-26-treated cells were incubated with protease-free DNase I prior to electrophoretic separation of the proteins, since topoisomerase II bound to high molecular weight DNA did not migrate into 7.5% SDS–PAGE gels [28, 29]. Thus, incubation with DNase I allowed detection of free as well as DNA-bound enzyme. Topoisomerase IIα and a much smaller amount of topoisomerase IIβ were detected in nuclei of CEM cells not incubated with VM-26 (Fig. 1A). A 160 kDa immunoreactive band was also seen, and the possible origins of this protein are addressed in the Discussion. Incubation of CEM cells with 25 μM VM-26 for 30 min increased the amount of topoisomerase IIβ that was detected compared with the untreated control. In contrast, VM-26 had no apparent effect on the amount of either topoisomerase IIα or the 160 kDa protein.

This drug-induced increase in the amount of immunodetectable topoisomerase IIβ was unexpected. Since DNase I digestion of the samples prior to electrophoresis should permit detection of total nuclear topoisomerase II, we anticipated that the amount of topoisomerase IIβ would be equal in cells incubated with or without VM-26. The observed increase in detectable topoisomerase IIβ was maximal within 15 min after VM-26 treatment and was unaffected by 75 μM cycloheximide, which inhibited protein synthesis in CEM cells by greater than 98% (data not shown). These results suggested that the increase in topoisomerase IIβ did not occur as a result of *de novo* protein synthesis. The lack of a significant protective effect of VM-26 on topoisomerase IIα is consistent with the greater stability of the α compared with the β isozyme [12, 30].

It was of interest to determine if incubation of other tumor cell lines with VM-26 also resulted in the detection of increased amounts of topoisomerase IIβ. HL-60 promyelocytic leukemic cells and WiDR colon carcinoma cells were incubated for 1 hr with 100 μM VM-26, and the nuclei were incubated with DNase I before SDS–PAGE. Western blotting revealed 2.1- and 8.6-fold increases in the β isozyme in nuclei of HL-60 and WiDR cells that were treated with VM-26 compared with the corresponding untreated controls (Fig. 1B). Also, in both cell lines VM-26 had no significant effect on the amounts of either topoisomerase IIα or the 160 kDa protein that were detected. The working hypothesis derived from the data in Fig. 1 is that topoisomerase IIβ is protected from breakdown when bound to DNA in the presence of VM-26 and, therefore, is detected in increased amounts in cells treated with this anticancer agent compared with control cells.

Concentration-dependent effect of VM-26 on the amount of immunodetectable topoisomerase IIα and topoisomerase IIβ. Figure 2A shows a western blot of nuclei from CEM cells incubated for 30 min with various concentrations of VM-26 from 0 to 100 μM. Quantitation of this blot is shown in Fig. 2B. All samples were treated with DNase I prior to separation of the proteins by SDS–PAGE. A concentration-dependent increase in the amount of the β isozyme was seen, which approached a plateau at about a 6-fold increase between 5 and 50 μM VM-26. In

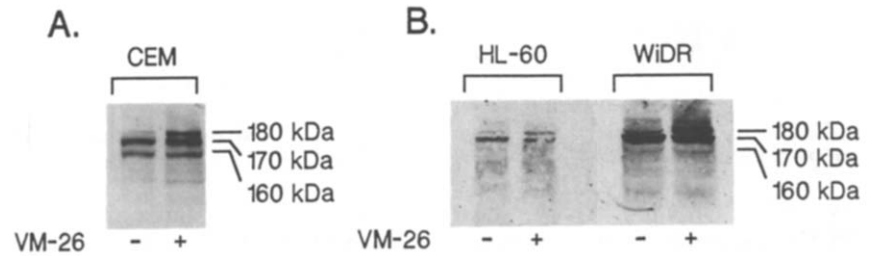

Fig. 1. Immunodetectable topoisomerase II isozymes in nuclei of CEM, HL-60, and WiDR cells. CEM cells were incubated for 30 min without and with 25 μM VM-26 (Fig. 1A). HL-60 cells and WiDR cells were incubated for 1 hr without or with 100 μM VM-26 (Fig. 1B). Nuclei were prepared and incubated with 500 U of DNase I for 20 min at 37° before electrophoresis. Each lane of the electrophoresis gel contained 75 μg of protein. Proteins were separated by SDS–PAGE and transferred to nitrocellulose paper. Western blotting was done with a polyclonal antiserum (MAC antiserum) that recognizes both the α and β isozymes of topoisomerase II.

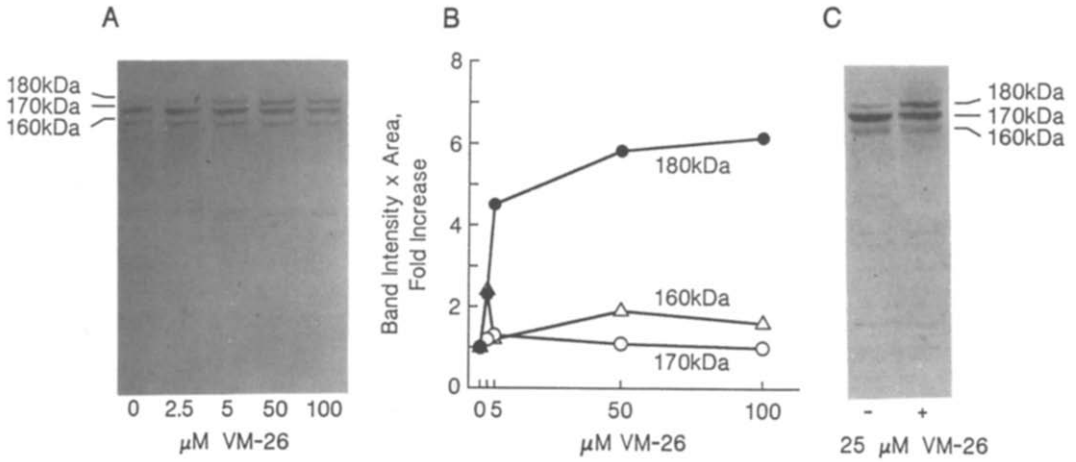

Fig. 2. Effects of various concentrations of VM-26 on the amounts of immunodetectable DNA topoisomerase II isozymes. CEM cells were incubated for 30 min in the absence or presence of the indicated concentrations of VM-26. Nuclei were isolated and all the preparations were incubated with DNase I before SDS–PAGE (Fig. 2A). Western blotting was done as described in the legend to Fig. 1. Figure 2B: quantitation of the results in Fig. 2A by laser densitometry. The results are expressed as the fold increase in arbitrary units (band intensity × area) as defined by internal computer standards. Figure 2C: effects of a single concentration of VM-26 (25 μM) on the amount of immunodetectable topoisomerase II. The experimental protocol was the same as in panel A.

contrast, no significant increases in the amounts of either topoisomerase IIα or the 160 kDa immunoreactive band was observed (1.2- and 1.8-fold changes at 50 μM VM-26, respectively). To evaluate the reproducibility of this VM-26-induced increase in topoisomerase IIβ as well as the minimal effect of VM-26 on the 170 kDa (α) and 160 kDa immunoreactive forms, the above experiment was carried out seven additional times using concentrations of either 0 or 25 μM VM-26. A representative western blot is shown in Fig. 2C. Based on the combined results of the eight determinations per group, the β isozyme was increased 7-fold ($P < 0.008$) in nuclei from cells that were incubated with VM-26 compared with the nuclei from cells that were not incubated with drug. In contrast, no significant differences were observed in the 170 and 160 kDa bands in preparations from drug-treated and untreated cells. The 180, 170 and 160 kDa bands represented 29 ± 4, 51 ± 6, and 20 ± 6% (mean ± SD), respectively, of the total immunoreactive protein in nuclei from cells incubated with 25 μM VM-26.

Effect of m-AMSA, mitoxantrone, cytosine arabinoside, hydroxyurea, and daunorubicin on the amounts of immunodetectable topoisomerase IIα and topoisomerase IIβ. To determine whether the increase in the β isozyme of topoisomerase II seen in the above experiments was unique to VM-26 or whether it could also be seen with other inhibitors of topoisomerase II, we incubated CEM cells for 1 hr with 0.5 to 20 μM mitoxantrone, 50 μM m-AMSA, or 5 μM daunorubicin. Mitoxantrone, m-AMSA, and daunorubicin at the above con-

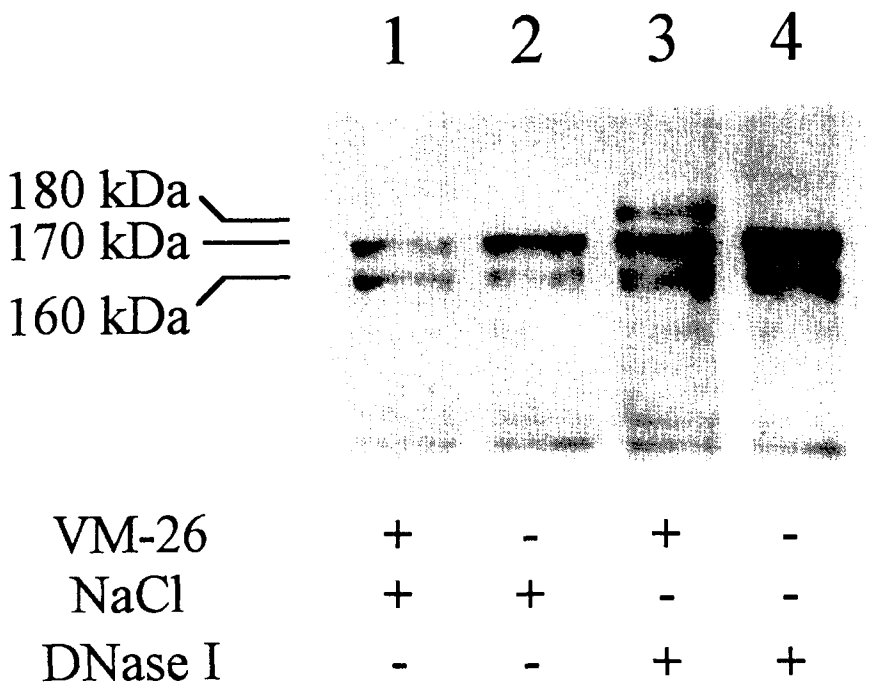

Fig. 3. Effects of salt extraction and DNase I digestion on the amounts of immunodetectable topoisomerase II isozymes. Nuclei isolated from CEM cells incubated with or without 25 μM VM-26 for 30 min were extracted with 0.5 M NaCl and centrifuged at 48,000 *g*. The pellets were then digested with DNase I and centrifuged at 1000 *g*. Salt extraction solubilized only about 2% of the total DNA, whereas 60% of the total DNA was solubilized after DNase I digestion. Aliquots of the 48,000 *g* and 1000 *g* supernatants were analyzed by western blotting as described in the legend to Fig. 1.

centrations increased the number of topoisomerase II–DNA complexes in CEM cells [19]. In addition, cytosine arabinoside and hydroxyurea, drugs that block DNA synthesis by mechanisms other than inhibition of topoisomerase II, were used as negative controls. CEM cells were incubated with either cytosine arabinoside (3 μM) or hydroxyurea (2.5 mM), both of which inhibited DNA synthesis greater than 90% compared with untreated cells ([6] and unpublished observations). Preparations of nuclei isolated from the untreated and these drug-treated cells were then analyzed by western blotting. No increase in topoisomerase IIα or β was seen with any of the above drugs (data not shown). These data indicate that the increase in topoisomerase IIβ induced by VM-26 (Figs. 1 and 2) did not result from inhibition of DNA synthesis *per se*. Furthermore, of the drugs used in the experiment, only VM-26 increased the detectability of topoisomerase IIβ on immunoblots.

Stabilization of topoisomerase IIα- and topoisomerase IIβ–DNA complexes by VM-26. The above results suggest that the topoisomerase IIβ detected in extracts of VM-26-treated cells was bound to DNA. A working hypothesis is that the topoisomerase IIβ bound to DNA in the presence of VM-26 is protected from degradation during subsequent preparation and analysis of the cell extracts. As one experimental approach to demonstrate that the immunodetectable topoisomerase IIβ was bound to DNA, CEM cells were preincubated for 72 hr in the presence of [^{14}C]thymidine to uniformly label the total cellular DNA. The cells were then incubated with and without VM-26 for 30 min, and the nuclei were extracted with 0.5 M NaCl. This salt extraction procedure solubilized only 1.4 and 2.2% of the [^{14}C]DNA from cells treated with and without VM-26, respectively. Figure 3 (lanes 1 and 2) shows that only the α isozyme and the 160 kDa species were detected on immunoblots of the 0.5 M NaCl extracts of nuclei obtained from VM-26-treated and untreated cells. In agreement with Fig. 1, VM-26 did not have a significant effect on the amounts of either topoisomerase IIα or the 160 kDa product. The pellets of the nuclear preparations were treated with DNase I, which solubilized 60% of the total [^{14}C]DNA, and the supernatants were analyzed by western blotting. Under these conditions, the β isozyme of topoisomerase II was readily detected in extracts from VM-26-treated cells (lane 3) but not from control cells (lane 4). Again, VM-26 had little effect on the amounts of both the α isozyme and the 160 kDa species (compare lanes 3 and 4). Thus, the β isozyme was detectable only in preparations from VM-26-treated cells that contained substantial amounts of solubilized DNA. Taken together, the results presented in Figs. 1–3 suggest that VM-26 selectively stabilized topoisomerase IIβ by increasing the amount of this isoform bound to DNA.

To directly evaluate binding of the topoisomerase II isozymes to DNA, CEM cells were incubated with either 0, 12.5 or 25 μM VM-26 for 30 min, and the

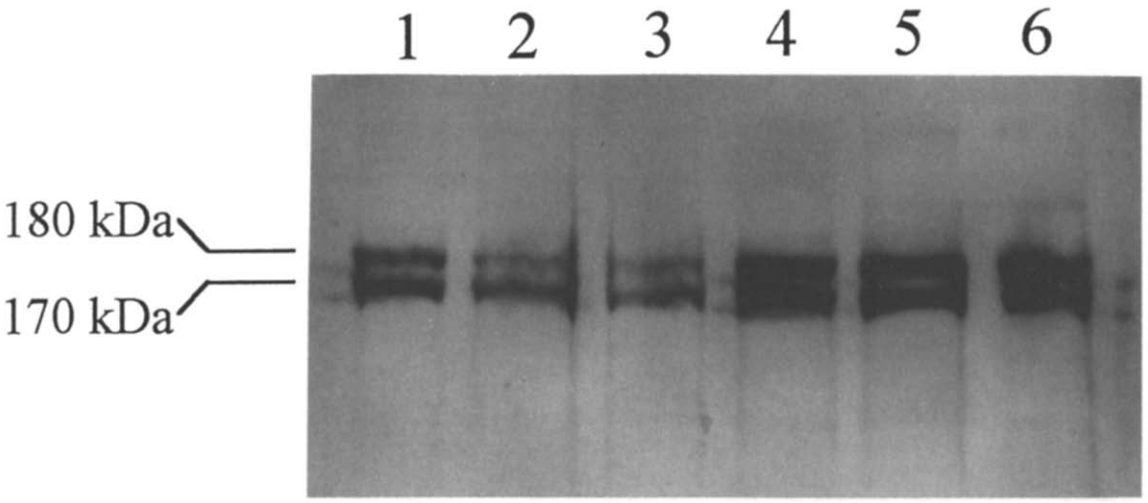

Fig. 4. Topoisomerase II isoforms bound to DNA in the presence of VM-26. CEM cells were incubated with or without various concentrations of VM-26 for 30 min. Some groups of cells were immediately lysed in hot SDS and electrophoresed, while other groups of cells were incubated for 5 min at 65° and then immediately lysed in hot SDS. The brief heat treatment dissociates VM-26-stabilized topoisomerase II complexes [24]. Western blotting was done as described in the legend to Fig. 1. Lane 1, untreated control; lane 2, 12.5 μM VM-26; lane 3, 25 μM VM-26; lane 4, untreated control → 65°; lane 5, 12.5 μM VM-26 → 65°; lane 6, 25 μM VM-26 → 65°.

cell pellets were then immediately lysed in hot SDS and electrophoresed (Fig. 4). Samples containing the 180, 170 and 160 kDa species isolated from nuclei were run in adjacent gel lanes and served as molecular weight markers. If the β isoform of topoisomerase II is highly susceptible to degradation, then this more rapid preparation and analysis of the cell extracts may permit the detection of this isozyme in the absence of VM-26-induced stabilization. Consistent with this hypothesis, both the α and β isozymes of topoisomerase II were detected when whole untreated cells were rapidly lysed in hot SDS (Fig. 4, lane 1). Decreased amounts of both topoisomerase IIα and β were detected in preparations from drug-treated cells (lanes 2 and 3) compared with untreated controls (lane 1). Electrophoretic band depletion was observed because in the absence of DNase I treatment the drug-stabilized topoisomerase II–DNA complexes are too large to enter the 5–15% polyacrylamide gel [28, 29]. In contrast to these results were those obtained with intact untreated and drug-treated cells that were incubated for 5 min at 65° and then immediately lysed in hot SDS and electrophoresed (Fig. 4, lanes 4–6). The heat treatment dissociated any topoisomerase II–DNA complexes and permitted the migration of the topoisomerase II isozymes into the polyacrylamide gel. The amounts of both the α and β isozymes of topoisomerase II detected in cells incubated with either 12.5 or 25 μM VM-26 (lanes 5 and 6, respectively) were similar to, but not greater than, the amounts of each isozyme detected in untreated control cells (lane 4). VM-26 did not increase the amount of topoisomerase IIβ detected under these conditions, because the rapid cell lysis and immediate electrophoretic analysis prevented degradation of the β isozyme. It is concluded from the data presented in Figs. 3 and 4 that VM-26 stabilized binding of both the α and β isozymes to DNA. A similar study was carried out with various concentrations of either mitoxantrone (3, 6, and 12 μM) or m-AMSA (25 and 50 μM). Although mitoxantrone and m-AMSA enhanced the amount of both topoisomerase IIα- and topoisomerase IIβ–DNA complexes detected following rapid cell lysis in hot SDS (i.e. induced depletion of both the α and β isozyme bands on western blots, data not shown), neither drug was able to protect topoisomerase IIβ from degradation during isolation of nuclei and nuclear matrices (see above).

The results presented in Fig. 4 also appear to eliminate the possibility that the 180 kDa band identified as topoisomerase IIβ is not actually the product of the topoisomerase IIβ gene, but represents a complex of 170 kDa topoisomerase IIα and DNA that is stabilized by VM-26. The latter possibility is unlikely, because the 180 kDa band was detected in both untreated cells (lanes 1 and 4) and in VM-26-treated cells in which the topoisomerase II–DNA complexes were reversed by heating prior to electrophoresis (lanes 5 and 6). Furthermore, the VM-26-induced increase in topoisomerase IIβ did not occur at the expense of the α isozyme (Fig. 2). To confirm the identity of the 180 kDa band, additional groups of CEM cells were incubated with either 0, 12.5 or 25 μM VM-26 for 30 min, and the cell pellets were then immediately lysed in hot SDS (Fig. 5). Duplicate aliquots of each sample were electrophoresed in the same gel and blotted as described above. The blot was cut in half and one half was incubated with the MAC antiserum (lanes 1–3), while the other half was incubated with the topoisomerase IIβ-specific antiserum (lanes 4–6). Figure 5 shows that the 180 kDa protein revealed by

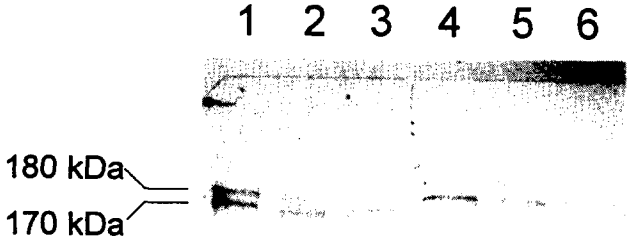

Fig. 5. Detection of DNA-bound topoisomerase II isozymes with the MAC and topoisomerase IIβ-specific antisera. CEM cells were incubated with various concentrations of VM-26 for 30 min, and the cell pellets were then immediately lysed in hot SDS as described in the legend to Fig. 4. Duplicate aliquots of each sample were electrophoresed in the same gel and blotted. The blot was cut in half, and one half was incubated with the MAC antiserum (lanes 1–3) while the other half was incubated with the topoisomerase IIβ-specific antiserum (lanes 4–6). Lanes 1 and 4, untreated control; lanes 2 and 5, 12.5 μM VM-26; lanes 3 and 6, 25 μM VM-26.

the MAC antiserum had the same electrophoretic mobility as the single protein identified by the topoisomerase IIβ-specific antiserum. In addition, depletion of both the 180 kDa and the 170 kDa bands was observed in samples from VM-26-treated cells (lanes 2 and 3, 5 and 6) compared with control cells (lanes 1 and 4). This confirms that VM-26 stabilized binding of both the α and β isozymes of topoisomerase II to DNA.

It should be noted that the 160 kDa protein is not evident in any of the lanes depicted in Figs. 4 and 5. This is consistent with the observations of others [31, 32] that the 160 kDa band represents a topoisomerase II degradation product, since rapid preparation of the cell extracts and inactivation of cellular proteases limited formation of this species.

Thus, topoisomerase IIβ can be detected in preparations from cells not incubated with VM-26 if the cells are rapidly processed to avoid degradation of the enzyme. However, recovery of topoisomerase IIβ following more extensive cell fractionation procedures, such as isolation of nuclei or nuclear matrices (see below), required pretreatment of the cells with VM-26.

Subnuclear distribution of the α and β isozymes of topoisomerase II. DNA topoisomerase II is a major nonhistone protein of the nuclear matrix, and current experimental evidence indicates that nuclear matrix topoisomerase II has a direct role in DNA organization, chromosome assembly, and possibly DNA replication [33]. Nuclear matrix topoisomerase II is also an important target for topoisomerase II-active agents [6, 20, 34]. Thus, information concerning the subnuclear distribution of the topoisomerase II isozymes may provide insights into the physiological roles of the individual isozymes as well as their importance as targets for topoisomerase II-active agents.

Since VM-26 stabilizes topoisomerase IIβ, this effect was exploited to investigate the distribution of the topoisomerase II isozymes in the matrix and nonmatrix (high salt-soluble) fractions of nuclei. Figure 6 represents a typical result obtained from five separate experiments and shows that topoisomerase IIα and the 160 kDa species are present in both the matrix and nonmatrix fractions of nuclei from untreated and VM-26-treated CEM cells. The striking observation was that the β isoform of topoisomerase II was detected in appreciable amounts only in the nonmatrix fraction of nuclei from cells incubated with VM-26. The amount of topoisomerase IIβ detected in the five experiments averaged 53 ± 10% (mean ± SD) of the amount of the α isozyme present in the nonmatrix fraction. The results indicate that the nuclear matrix isoform is topoisomerase IIα, while both the α and β isoforms are present in the nonmatrix fraction of the nucleus.

Some evidence exists that nuclear matrix topoisomerase II is a structural protein [35–37], and it is possible that matrix topoisomerase II is not a target for VM-26. To examine the VM-26-induced binding of nuclear matrix topoisomerase II to DNA, we developed an HPLC gel filtration method for the separation of free and DNA-bound topoisomerase II present in the nuclear matrix. Prior to analysis of the nuclear subfractions, we generated molecular weight calibration curves for this HPLC column using protein or double-stranded DNA standards. The elution profiles of free and DNA-bound enzyme present in the samples were determined by western blotting of the fractions. Under denaturing conditions, free topoisomerase II eluted in a peak at 8.5 mL (fraction 17) corresponding to a molecular mass of about 170 kDa. DNA-bound topoisomerase II eluted in the void volume of the column (5.0 to 6.5 mL, fractions 10–13), which corresponded to a molecular mass of greater than 1000 kDa (data not shown). Following extensive digestion of the matrix samples with DNase I prior to HPLC, only free topoisomerase II was detected, and no topoisomerase II eluted in the void volume of the column (data not shown).

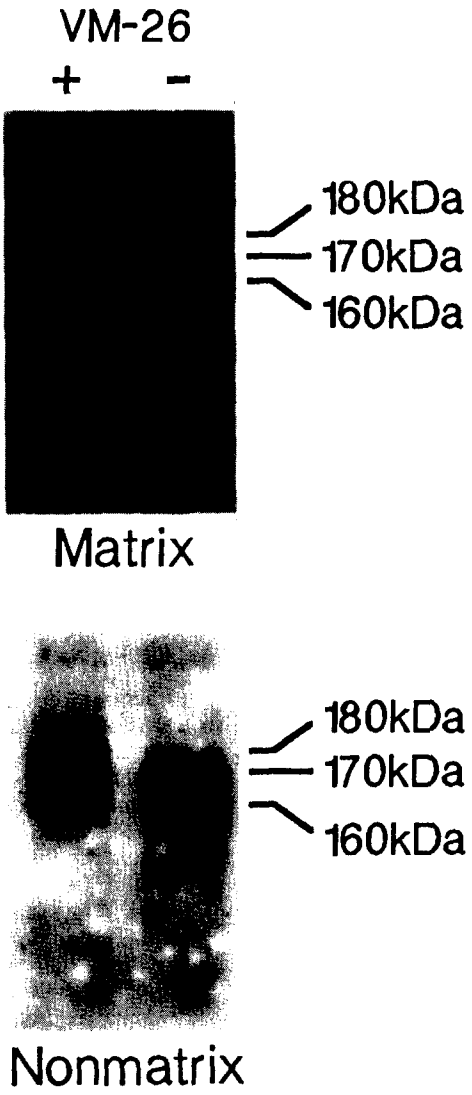

Fig. 6. Topoisomerase II isozymes in the matrix and nonmatrix fractions of nuclei from CEM cells. The cells were incubated for 1 hr in the presence or absence of 25 μM VM-26. Nuclear matrix and nonmatrix fractions were prepared from 4×10^7 control and VM-26-treated cells as described in Materials and Methods. Proteins were separated by SDS–PAGE and detected by western blotting as described in the legend to Fig. 1.

Figure 7 shows the results of one of five experiments in which HPLC and subsequent western analysis were used to identify the nuclear matrix isozyme bound to DNA. After separation of the free and DNA-bound topoisomerase II by HPLC, the pooled fractions representing the DNA-bound and free enzymes were extensively digested with DNase I to allow migration of the enzymes into the electrophoresis gel. By comparing the migration of the pooled HPLC sample of DNA-bound topoisomerase II from the nuclear matrix fraction (lanes 3 and 4) with that of known topoisomerase IIα and β (lanes 1 and 2), we could determine the particular isozyme(s) of topoisomerase II that was bound to nuclear matrix DNA. Lane 4 indicates that VM-26 stabilized binding of only the α isozyme and the 160 kDa species to nuclear matrix DNA. Lesser amounts of the α isozyme and the 160 kDa protein were bound to DNA in the absence of VM-26 (lane 3). No topoisomerase IIβ was detected in the nuclear matrix fraction of cells incubated with or without VM-26. Furthermore, western blotting of the pooled HPLC fractions of free nuclear matrix topoisomerase II from untreated cells revealed that the free enzyme was also topoisomerase IIα (lane 5). These results indicate that the α isozyme of topoisomerase II is the nuclear matrix target for VM-26 in CEM cells.

A similar HPLC analysis of the isozymes of topoisomerase II bound to nonmatrix DNA could not be carried out because of incomplete separation of these complexes from free topoisomerase II by the gel filtration column. The binding of topoisomerase II to nonmatrix DNA, which was degraded to a mixture of primarily mono- and oligonucleotides during the isolation of the nuclear matrix, did not increase the apparent molecular weight of the enzyme sufficiently to allow separation of the bound and free topoisomerase II. Therefore, this HPLC analysis could not provide information concerning the effect of VM-26 on the binding of the topoisomerase II isozymes to nonmatrix DNA. However, since VM-26 stabilizes binding of topoisomerase IIβ to DNA (Figs. 4 and 5) and the nuclear nonmatrix fraction contains most of the topoisomerase IIβ (Fig. 6), then it is likely that VM-26 stabilizes binding of this isozyme to nonmatrix DNA.

DISCUSSION

Anticancer drugs, such as VM-26 and m-AMSA, interfere with the activity of DNA topoisomerase II primarily by blocking religation of cleaved DNA [16, 17]. This increases the amount of a reaction intermediate, a covalent enzyme–DNA complex. While two isozymes of topoisomerase II have been identified, we are unaware of studies that have attempted to determine the extent to which each isozyme participates in DNA–complex formation in cells treated with inhibitors of topoisomerase II.

In the studies reported here, the identification of each topoisomerase II isozyme relies on its rate of migration in SDS gels and recognition by antibodies in immunoblots. The MAC antiserum was generated in one of our laboratories using a 72-kDa C-terminal fragment of topoisomerase IIα that was expressed in *Escherichia coli*. In earlier work involving the same cell line and the same antiserum, we detected only topoisomerase IIα[20]. The differences between this study and earlier studies include: the use of VM-26 to stabilize the β isozyme, rapid handling of samples, and the inclusion of six protease inhibitors in all preparations.

Our data indicate that topoisomerase IIβ, when bound to DNA, is protected from degradation. This is consistent with the observation that the β isozyme is a very unstable protein [12, 30]. This apparent protective effect was unique to VM-26 and was not seen with other topoisomerase II-active agents.

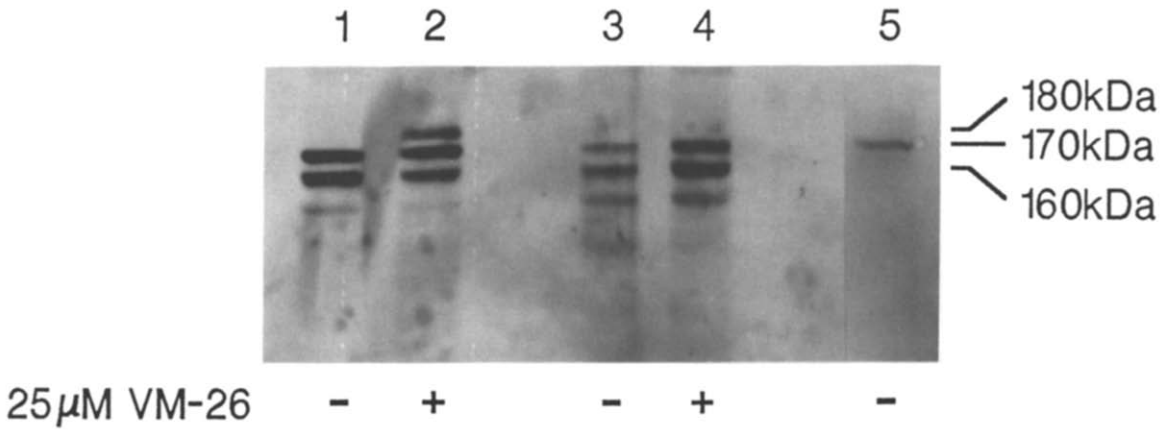

Fig. 7. Free and DNA-bound topoisomerase II in the nuclear matrix fraction of CEM cells. The cells were incubated with or without 25 μM VM-26 for 1 hr. Nuclear matrices prepared from untreated control and VM-26-treated cells were denaturated in SDS, sonicated, and then subjected to HPLC gel filtration as described in Materials and Methods. Fractions containing either free topoisomerase II or topoisomerase II bound to high molecular weight DNA were collected from the HPLC column, pooled, and digested with DNase I. Western blotting was done as described in the legend to Fig. 1. Lanes 1 and 2 are samples of nonmatrix proteins prepared from CEM cells incubated without and with VM-26, respectively. The nonmatrix samples contained detectable amounts of α (lane 1) or α and β (lane 2) topoisomerase II and were used to identify the isozyme present in the nuclear matrix samples. Lanes 3 and 4, topoisomerase IIα bound to nuclear matrix DNA isolated from cells incubated without and with VM-26, respectively. Lane 5, free topoisomerase IIα from CEM cells not incubated with VM-26.

Complexes of topoisomerase IIβ and DNA were also detected when the cells were incubated with either m-AMSA or mitoxantrone and rapidly lysed in hot SDS, but these intercalating agents did not protect the β isozyme from degradation during cell fractionation. One possibility to account for the specificity of the VM-26 effect on topoisomerase IIβ is that the stability or the three-dimensional structure of the topoisomerase IIβ–DNA complex induced by VM-26 is different from that induced by the intercalating agents. Robinson *et al.* [38] have shown that quinolone derivatives inhibit topoisomerase II by a different mechanism than m-AMSA and VM-26, yet all three drugs increase the number of covalent enzyme–DNA complexes. In addition, studies by Zwelling and coworkers [29, 39], using a mutant topoisomerase II from cells resistant to m-AMSA, show that the enzyme is sensitive to VP-16 but not m-AMSA. This suggests that the enzyme–DNA complexes formed in the presence of each drug are not identical. Thus, the stability or the three-dimensional conformation of the particular drug–topoisomerase IIβ–DNA ternary complex may greatly influence the degree to which the β isozyme is protected from degradation.

Considerable controversy surrounds the identity of the 160 kDa species. This species has been reported to be a degradation product of either topoisomerase IIα [31], or β [32], while others have suggested that it is a separate gene product [40]. The studies described herein were not designed to directly address this controversy and, thus, the identity of this immunoreactive protein remains an enigma. The 160 kDa protein was not seen on immunoblots when whole CEM cells were rapidly lysed in hot SDS and the lysates immediately electrophoresed. This result suggested that in CEM cells the 160 kDa species is a proteolysis product. In the experiments involving cell fractionation the amount of detectable topoisomerase β increased as a function of increasing VM-26 concentration from 0 to 50 μM. However, VM-26 did not affect significantly the amounts of either topoisomerase IIα or the 160 kDa protein that were recovered. This suggested that topoisomerase IIα was more likely the source of the 160 kDa product than topoisomerase IIβ. If topoisomerase IIβ was the source of the 160 kDa protein, then the amount of topoisomerase IIβ recovered would increase as a function of VM-26 concentration, while the amount of the 160 kDa protein would decrease. Our hypothesis based on these results is that some of the α isozyme is degraded to the 160 kDa product during cell fractionation, and this process is not affected by VM-26. This is consistent with the observation that the ratio of the amounts of the 170 and 160 kDa proteins remained constant in cells incubated without or with various concentrations of VM-26.

The differences that we observed in the subnuclear distribution of the α and β isozymes of DNA topoisomerase II are consistent with the concept that each isozyme has a different primary function in the cell. Drake *et al.* [12] previously speculated that topoisomerase IIα and β are involved in DNA replication and ribosomal RNA transcription, respectively. This speculation is supported by several lines of evidence. The expression of the α, but not the β, isozyme is thought to be cell proliferation dependent [12, 41]. DNA replication is thought to take place at fixed sites on the nuclear matrix

[2, 6, 42, 43], and the topoisomerase II-active drugs, VM-26 and m-AMSA, inhibit nuclear matrix DNA replication in CEM cells [6]. Thus, our finding that only topoisomerase IIα is associated with the nuclear matrix is consistent with the importance of the α isozyme in DNA replication and as a target for VM-26.

The localization of topoisomerase IIβ only in the nonmatrix fraction is in agreement with a proposed role of this isozyme in ribosomal RNA metabolism, which takes place in nucleoli [44]. Electron microscopic analysis and pulse labeling of CEM cells with [^{3}H]uridine revealed that most of the pre-ribosomal RNA was present in the nuclear nonmatrix fraction, which contained the nucleolar material ([6], unpublished data). Similarly, Zini *et al.* [45] recently reported that topoisomerase IIβ is present exclusively in nucleoli of K562 cells. In contrast, nuclear matrices of CEM cells are devoid of nucleoli and topoisomerase IIβ and contain only minor amounts of these RNAs ([6] and unpublished data). Additional studies will be needed to determine whether topoisomerase IIβ–DNA complexes stabilized by VM-26 interfere with ribosomal RNA metabolism and whether this effect contributes to drug-induced cytotoxicity.

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REFERENCES

1. Paulson JR and Laemmli UK, The structure of histone-depleted metaphase chromosomes. *Cell* **12**: 817–828, 1977.
2. Vogelstein B, Pardoll DM and Coffey DS, Supercoiled loops and eucaryotic DNA replication. *Cell* **22**: 79–85, 1980.
3. Wang JC, DNA topoisomerases. *Annu Rev Biochem* **54**: 665–697, 1985.
4. Berrios M, Osheroff N and Fisher PA, *In situ* localization of DNA topoisomerase II, a major polypeptide component of the *Drosophila* nuclear matrix fraction. *Proc Natl Acad Sci USA* **82**: 4142–4146, 1985.
5. Earnshaw WC, Halligan B, Cooke CA, Heck MMS and Liu LF, Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J Cell Biol* **100**: 1706–1715, 1985.
6. Fernandes DJ, Smith-Nanni C, Paff MT and Neff TAM, Effects of antileukemia agents on nuclear matrix-bound DNA replication in CCRF-CEM leukemia cells. *Cancer Res* **48**: 1850–1855, 1988.
7. Gasser SM and Laemmli UK, The organization of chromatin loops: Characterization of a scaffold attachment site. *EMBO J* **5**: 511–518, 1986.
8. Cockerill PN and Garrard WT, Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell* **44**: 273–282, 1986.
9. Nelson WG, Liu LF and Coffey DS, Newly replicated DNA is associated with DNA topoisomerase II in cultured rat prostatic adenocarcinoma cells. *Nature* **322**: 187–189, 1986.
10. DiNardo S, Voelkel K and Sternglanz R, DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: Topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. *Proc Natl Acad Sci USA* **81**: 2616–2620, 1984.
11. Holm C, Goto T, Wang JC and Botstein D, DNA topoisomerase II is required at the time of mitosis in yeast. *Cell* **41**: 553–563, 1985.
12. Drake FH, Hofmann GA, Bartus HF, Mattern MR, Crooke ST and Mirabelli CK, Biochemical and pharmacological properties of p170 and p180 forms of topoisomerase II. *Biochemistry* **28**: 8154–8160, 1989.
13. Chung TDY, Drake FH, Tan KB, Per SR, Crooke ST and Mirabelli CK, Characterization and immunological identification of cDNA clones encoding two human DNA topoisomerase II isozymes. *Proc Natl Acad Sci USA* **86**: 9431–9435, 1989.
14. Tan KB, Dorman TE, Falls KM, Chung TDY, Mirabelli CK, Crooke ST and Mao J-i, Topoisomerase IIα and topoisomerase IIβ genes: Characterization and mapping to human chromosome 17 and 3, respectively. *Cancer Res* **52**: 231–234, 1992.
15. Zini N, Martelli AM, Sabatelli P, Santi S, Negri C, Astaldi-Ricotti GCB and Maraldi NM, The 180 kDa isoform of topoisomerase II is localized in the nucleolus and belongs to the structural elements of the nucleolar remnant. *Exp Cell Res* **200**: 460–466, 1992.
16. Osheroff N, Effect of antineoplastic agents on the DNA cleavage/religation reaction of eukaryotic topoisomerase II: Inhibition of DNA religation by etoposide. *Biochemistry* **28**: 6157–6160, 1989.
17. Robinson MJ and Osheroff N, Stabilization of the topoisomerase II–DNA cleavage complex by antineoplastic drugs: Inhibition of enzyme-mediated DNA religation by 4′-(9-acridinylamino)methane-sulfon-*m*-aminisidide. *Biochemistry* **29**: 2511–2515, 1990.
18. Danks MK, Schmidt CA, Cirtain MC, Suttle DP and Beck WT, Altered catalytic activity of and DNA cleavage by DNA topoisomerase II from human leukemic cells selected for resistance to VM-26. *Biochemistry* **27**: 8861–8869, 1988.
19. Danks MK, Schmidt CA, Deneka DA and Beck WT, Increased ATP requirement for activity of and complex formation by DNA topoisomerase II from human leukemic CCRF-CEM cells selected for resistance to teniposide. *Cancer Commun* **1**: 101–109, 1989.
20. Fernandes DJ, Danks MK and Beck WT, Decreased nuclear matrix DNA topoisomerase II in human leukemia cells resistant to VM-26 and *m*-AMSA. *Biochemistry* **29**: 4235–4241, 1990.
21. Danks MK, Yalowich JC and Beck WT, Atypical multiple drug resistance in a human leukemic cell line selected for resistance to teniposide (VM-26). *Cancer Res* **47**: 1297–1301, 1987.
22. Harker WG, Slade DL, Dalton WS, Meltzer PS and Trent JM, Multidrug resistance in mitoxantrone-selected HL-60 leukemia cells in the absence of P-glycoprotein overexpression. *Cancer Res* **49**: 4542–4549, 1989.
23. Wallace RE, Lindh D and Durr FE, Development of resistance and characteristics of a human colon carcinoma subline resistant to mitoxantrone *in vitro*. *Cancer Invest* **5**: 417–428, 1987.
24. Hsiang YH and Liu LF, Evidence for the reversibility of cellular DNA lesion induced by mammalian topoisomerase II poisons. *J Biol Chem* **264**: 9713–9715, 1989.
25. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
26. Friche E, Danks MK, Schmidt CA and Beck WT,

Decreased DNA topoisomerase II in daunorubicin-resistant Ehrlich ascites tumor cells. *Cancer Res* **51**: 4213–4218, 1991.
27. Harlow E and Lane D, *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988.
28. Hsiang YH and Liu LF, Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res* **48**: 1722–1726, 1988.
29. Zwelling LA, Hinds M, Chan D, Mayes J, Sie KL, Parker E, Silberman L, Radcliffe A, Beran M and Blick M, Characterization of an amsacrine-resistant line of human leukemia cells: Evidence for a drug-resistant form of topoisomerase II. *J Biol Chem* **264**: 16411–16420, 1989.
30. Woessner RD, Chung TDY, Hofmann GA, Mattern MR, Mirabelli CK, Drake FH and Johnson RK, Differences between normal and *ras*-transformed NIH-3T3 cells in expression of the 170 kD and 180 kD forms of topoisomerase II. *Cancer Res* **50**: 2901–2908, 1990.
31. Halligan BD, Edwards KA and Liu LF, Purification and characterization of a type II DNA topoisomerase from bovine calf thymus. *J Biol Chem* **260**: 2475–2482, 1985.
32. Negri C, Chiesa R, Cerino A, Bestagno M, Sala C, Zini N, Miraldi NM and Astaldi Ricotti GC, Monoclonal antibodies to human DNA topoisomerase I and the two isoforms of DNA topoisomerase II: 170- and 180-kDa isozymes. *Exp Cell Res* **200**: 452–459, 1992.
33. Fernandes DJ, Catapano CV and Townsend AJ, Topoisomerase-related mechanisms of drug resistance. In: *Drug Resistance in Oncology* (Ed. Teicher BA), pp. 479–498. Marcel Dekker, New York, 1993.
34. Fernandes DJ and Catapano CV, Nuclear matrix targets for anticancer agents. *Cancer Cells* **3**: 134–140, 1991.
35. Earnshaw WC and Heck MM, Localization of topoisomerase II in mitotic chromosomes. *J Cell Biol* **100**: 1716–1725, 1985.
36. Gasser SM, Laroche T, Falquet J, Boy de la Tour E and Laemmli UK, Metaphase chromosome structure: Involvement of topoisomerase II. *J Mol Biol* **188**: 613–629, 1986.
37. Adachi Y, Luke M and Laemmli UK, Chromosome assembly *in vitro*: Topoisomerase II is required for condensation. *Cell* **64**: 137–148, 1991.
38. Robinson MJ, Martin BA, Gootz TD, McGuirk PR, Moynihan M, Sutcliffe JA and Osheroff N, Effects of quinolone derivatives on eukaryotic topoisomerase II: A novel mechanism for enhancement of enzyme-mediated DNA cleavage. *J Biol Chem* **266**: 14585–14592, 1991.
39. Zwelling LA, Mayes J, Hinds M, Chan D, Altschuler E, Carroll B, Parker E, Deisseroth K, Radcliffe A, Seligman M, Li L and Farqhar D, Cross-resistance of an amsacrine-resistant human leukemia line to topoisomerase II reactive DNA intercalating agents. Evidence for two topoisomerase II directed drug actions. *Biochemistry* **30**: 4048–4055, 1991.
40. Harker WG, Slade DL, Drake FH and Parr RL, Mitoxantrone resistance in HL-60 leukemia cells: Reduced nuclear topoisomerase II catalytic activity and drug-induced DNA cleavage in association with reduced expression of the topoisomerase IIβ isoform. *Biochemistry* **30**: 9953–9961, 1991.
41. Woessner RD, Mattern MR, Mirabelli CK, Johnson RK and Drake FH, Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. *Cell Growth Diff* **2**: 209–214, 1991.
42. Pardoll DM, Vogelstein B and Coffey DS, A fixed site of DNA replication in eucaryotic cells. *Cell* **19**: 527–536, 1980.
43. Paff MT and Fernandes DJ, Synthesis and distribution of primer RNA in nuclei of CCRF-CEM leukemia cells. *Biochemistry* **29**: 3442–3450, 1990.
44. Scheer U and Benavente R, Functional and dynamic aspects of the mammalian nucleolus. *Bioessays* **12**: 14–21, 1990.
45. Zini N, Santi S, Ognibene A, Bavelloni A, Neri LN, Valmori A, Mariani E, Negri C, Astaldi-Ricotti B and Maraldi NM, Discrete localization of different topoisomerases in HeLa and K562 cell nuclei and subnuclear fractions. *Exp Cell Res* **210**: 336–348, 1994.