Arrest of Replication Fork Progression at Sites of Topoisomerase II-Mediated DNA Cleavage in Human Leukemia CEM Cells Incubated with VM-26†

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ABSTRACT: Recent studies have shown that the anticancer drugs VM-26 and mitoxantrone stabilize preferentially the binding of topoisomerase IIα to replicating compared to nonreplicating DNA. To further understand the mechanisms by which cleavable complex-forming topoisomerase II inhibitors interfere with DNA replication, we examined the effects of VM-26 on this process in human leukemia CEM cells. Both the inhibition of DNA synthesis and cell survival were directly related to the total amount of drug-stabilized cleavable complexes formed in VM-26-treated cells. DNA chain elongation was also inhibited in a concentration-dependent fashion in these cells, which suggested that VM-26-stabilized cleavable complexes interfered with the movement of DNA replication forks. To test this hypothesis directly, we monitored replication fork progression at a specific site of VM-26-induced DNA cleavage. A topoisomerase II-mediated cleavage site was detected in the first exon of the *c-myc* gene in VM-26-treated cells. This cleavage site was downstream of a putative replication origin located in the 5′ flanking region of the gene. Replication forks, which moved through this region of the *c-myc* gene in the 5′ to 3′ direction, were specifically arrested at this site in VM-26-treated cells, but not in untreated or aphidicolin-treated cells. These studies provide the first direct evidence that a VM-26-stabilized topoisomerase II−DNA cleavable complex acts as a replication fork barrier at a specific genomic site in mammalian cells. Furthermore, the data support the hypothesis that the replication fork arrest induced by cleavable complex-forming topoisomerase II inhibitors leads to the generation of irreversible DNA damage and cytotoxicity in proliferating cells.

DNA topoisomerase II (topo II)[1] is involved in a number of essential nuclear processes, including DNA replication (Di Nardo et al., 1984a,b; Brill et al., 1987), transcription (Brill et al., 1987), recombination (Bae et al., 1988; Wang et al., 1990), and organization of chromatin loop domains (Cockerill & Garrard, 1986; Gasser & Laemmli, 1986; Gasser et al., 1986). The reaction catalyzed by topo II involves transient cleavage of the DNA, passage of an intact duplex through the break, and resealing of the broken DNA strands (Liu et al., 1983; Wang, 1985; Osheroff, 1986; Watt & Hickson, 1994). The result of the topo II action is a change of the DNA topology and degree of DNA supercoiling (Wang, 1985; Watt & Hickson, 1994). Cleavage of the DNA by topo II requires formation of a covalent intermediate in which the 5′ end of the broken DNA strand is linked to a tyrosine residue in the enzyme molecule (Liu et al., 1983; Wang, 1985; Zechiendrich et al., 1989). Some topo II inhibitors, such as the epipodophyllotoxins (VP-16 and VM-26) and the aminoacridines (*m*-AMSA), stabilize the transient complex (also known as cleavable complex) between topo II and the DNA (D'Arpa & Liu, 1989; Froelich-Ammon & Osheroff, 1995). This results in the inhibition of topo II catalytic activity and the formation of topo II-linked single- or double-stranded DNA breaks.

The cytotoxic effects of cleavable complex-forming topo II inhibitors are more closely related to the generation of topo II-mediated DNA damage rather than the inhibition of topo II activity (D'Arpa & Liu 1989). Cells exposed briefly to cytotoxic concentrations of these agents die despite the fact that most of the topo II−DNA complexes and topo II-mediated DNA strand breaks are repaired following removal of the inhibitor (Tewey et al., 1984; Rowe et al., 1985). Topo II−DNA cleavable complexes are thought to be only the initial lesions in a series of events that lead to cell death. A number of indirect observations suggest that ongoing DNA replication is required for the development of cytotoxic effects by topo II-active agents. Cleavable complex-forming topo II inhibitors are generally more effective in killing cells in the S phase of the cell cycle compared to cells either in G_1 or in G_2-M phase (Chow & Ross, 1987; Estey et al., 1987; Sullivan et al., 1987; D'Arpa et al., 1990; Del Bino &

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[1] Abbreviations: BrdU, bromodeoxyuridine; CsCl, cesium chloride; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; K-SDS, potassium−sodium dodecyl sulfate; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; RF, replication fork; SDS, sodium dodecyl sulfate; SSC, sodium chloride−sodium citrate buffer; topo II, DNA topoisomerase II; TCA, trichloroacetic acid; VP-16 (etoposide), 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); VM-26 (teniposide), 4′-demethylepipodopyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside).

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Darzynkiewicz, 1991). Topo II−DNA cleavable complexes are formed preferentially in replicating DNA following incubation of cells with either VM-26, VP-16, or *m*-AMSA (Nelson et al., 1986; Woynarowski et al., 1988). Furthermore, VM-26 stimulates preferentially the binding of the topo IIα isozyme to replicating DNA compared to nonreplicating DNA (Qiu et al., 1996). The formation of topo IIα−nascent DNA complexes correlates closely with the cytotoxic effects of VM-26 in CEM cells and is selectively impaired in VM-26-resistant CEM/VM-1 cells (Qiu et al., 1996). Consistent with the hypothesis that topo II−DNA complexes must interact with RFs to generate lethal damage is the observation that pretreatment of cells with the DNA polymerase inhibitor aphidicolin partially protects the cells from the cytotoxic effects of topo II-active drugs (Holm et al., 1989; D'Arpa & Liu, 1990). Additional information concerning the effects of cleavable complex-forming topo II inhibitors on DNA replication comes from studies of the SV40 virus in cell-free systems or intact cells. The cleavable complex-forming topo II inhibitors, VM-26 and VP-16, interfere with DNA chain elongation and induce breaks in replicating SV40 DNA (Snapka, 1986; Richter et al., 1987; Snapka et al., 1988; Richter & Strausfeld, 1988). In contrast, ICRF-193, a noncleavable complex-forming topo II inhibitor, blocks segregation of replicated SV40 DNA molecules by inhibiting topo II decatenating activity, which is required in the late stages of SV40 DNA replication (Ishimi et al., 1992).

Recent data suggest that a critical interaction between RFs and drug-stabilized cleavable complexes occurs at the level of the nuclear matrix. Topo IIα is a major component of the nuclear matrix of eukaryotic cells (Berrios et al., 1985; Earnshaw et al., 1985; Earnshaw & Heck, 1985), and DNA replication takes place in replication factories attached to the nuclear matrix (Nakayasu & Berezney, 1989; Hozak et al., 1993). VM-26 stabilizes cleavable complexes between matrix-associated DNA and topo IIα, which is the only topo II isozyme detected in the nuclear matrix of human leukemia CEM cells (Danks et al., 1994). Following cleavable complex formation, VM-26 and *m*-AMSA induce the dissociation of newly replicated DNA from the nuclear matrix of CEM cells, unlike other DNA synthesis inhibitors, such as araC and hydroxyurea (Fernandes et al., 1989). Furthermore, CEM/VM-1 cells have less topo II in the nuclear matrix and are resistant to the cytotoxic effects of topo II-active drugs (Fernandes et al., 1990).

These results are consistent with the hypothesis that drug-stabilized topo IIα−DNA cleavable complexes block the progression of RFs along the DNA template (Holm et al., 1989; D'Arpa et al., 1990; Fernandes & Catapano, 1991). The collision of RFs with drug-stabilized topo II−DNA complexes would promote the transformation of the reversible cleavable complexes into irreversible DNA strand breaks, which may be the major cytotoxic lesions induced by cleavable complex-forming topo II inhibitors. We have recently shown that the formation of cleavable complexes of topo IIα with nascent DNA is critical to the development of cytotoxicity by VM-26 (Qiu et al., 1996). In the present study, we examined the effects of VM-26-stabilized topo II−DNA cleavable complex formation on RF progression both globally and at a specific genomic site in order to further understand the cytotoxic mechanisms of the cleavable complex-forming topo II inhibitors. Our results showed that VM-26 inhibited RF progression in CEM cells and that RF arrest in the *c-myc* gene occurred specifically at a site of topo II-mediated DNA cleavage.

MATERIALS AND METHODS

Chemicals, Enzymes, and Other Reagents. Sources for most chemicals and supplies have been reported previously (Danks et al., 1994; Catapano et al., 1991). VM-26 and aphidicolin glycinate were obtained from Bristol Myers (Syracuse, NY) and the National Cancer Institute (Bethesda, MD), respectively. The ^{32}P-labeled dNTPs and NTPs were purchased from Dupont/NEN Radiochemicals (Boston, MA). The ^{14}C- and ^{3}H-labeled nucleosides were purchased from Moravek Biochemicals, Inc. (Brea, CA). Magnagraph nylon membranes for Southern and slot blotting were purchased from MSI (Westboro, MA). SSC (20×) buffer consisted of 3 M NaCl and 0.3 M sodium citrate (pH 7).

Cell Culture Conditions and Drug Incubation. Human leukemia CEM cells were grown at 37 °C under 95% air−5% CO_2 in E-MEM medium supplemented with 10% heat-inactivated fetal calf serum (Danks et al., 1994). VM-26 was dissolved in DMSO/ethanol (1:1). The cells were incubated with either 0.1% DMSO/ethanol or the indicated concentrations of VM-26 in 0.1% DMSO/ethanol for 30 min at 37 °C.

Probe Preparation. The pHSR-1 plasmid, which contained a 9.0 kb genomic insert of the *c-myc* gene, was obtained from the ATCC (Rockville, MD). To generate DNA probes, the pHSR-1 plasmid was digested separately with the restriction endonucleases *Pst*I and *Sac*I. The *Pst*I fragments of 1710 and 414 bp and the *Sac*I fragment of 606 bp were purified from agarose gels and labeled with [^{32}P]-dATP by random priming. To generate strand-specific RNA probes, the 367 bp *Xba*I/*Sac*I fragment at the pHSR-1 plasmid was subcloned into a pGEM vector (Promega). This vector contained the promoters for SP6 and T7 RNA polymerases in opposite orientation. Transcription with either SP6 or T7 RNA polymerase in the presence of [^{32}P]-CTP yielded radiolabeled probes complementary to either the leading or the lagging strand of the RF.

Potassium−SDS Precipitation of VM-26-Induced Covalent Protein−DNA Complexes. CEM cells were incubated with 0.1 μCi/mL [^{3}H]thymidine for 24 h prior to drug incubation. Control and VM-26-treated cells were washed at 4 °C, and two aliquots of 1×10^6 cells were taken from each sample. The cells in the first aliquot were lysed by addition of a buffer containing 1.25% SDS, and covalent protein−DNA complexes were precipitated as previously described (Rowe et al., 1986). The second aliquot from each sample was lysed in ice-cold 10% (w/v) TCA and used to determine the total amount of radioactivity incorporated into DNA. The results were expressed as the percentage of total DNA that was precipitated as protein−DNA complexes.

Measurement of DNA Synthesis Rates. CEM cells were incubated for 72 h (approximately 3 doubling times) with 0.01 μCi/mL [^{14}C]thymidine in order to uniformly label the DNA. After 24 h of growth in radiolabel-free medium, cultures of 2×10^6 cells were incubated with or without VM-26. Control and VM-26-treated cells were centrifuged at 4 °C, resuspended in drug-free medium, and incubated with 0.2 μCi/mL [^{3}H]thymidine for 30 min at 37 °C. The amount of radioactivity incorporated into DNA was measured as previously described (Catapano et al., 1991). The extent

of DNA synthesis inhibition was calculated as the ratios of [^{3}H]DNA/[^{14}C]DNA in drug-treated cells compared to the ratios in control cells.

Measurement of Cell Survival by the MTT Assay. Following drug incubation, aliquots of control and VM-26-treated cells were transferred to a 96 well microtiter plate. After 24 h, 100 μL of MTT solution was added to the wells, and the samples were processed as previously described (Qiu et al., 1996). The percentage of cell survival was determined as the ratio of the mean absorbance of wells containing drug-treated cells to the mean absorbance of wells containing control cells.

Alkaline Sucrose Density Gradient Centrifugation. CEM cells were prelabeled with 0.2 μCi/mL [^{14}C]thymidine for 72 h, chased in fresh medium for 24 h, and then incubated with or without VM-26. Aliquots of 1×10^6 cells were centrifuged at 4 °C, resuspended in drug-free medium, and incubated with 10 μCimL [^{3}H]thymidine for 10 min at 37 °C. Then, the cells were lysed on the top of 5−20% alkaline sucrose gradients, and the samples were centrifugated at 12 000 rpm for 16 h (Painter, 1978; Kaufmann et al., 1991). The amount of ^{3}H and ^{14}C acid-insoluble radioactivity in each gradient fraction was determined by liquid scintillation counting (Painter, 1978). The gradients were calibrated with λ DNA (48 kb) and an *Eco*RI restriction fragment of λ DNA (20 kb). The positions of these size standards are indicated at the top of the gradients.

Alkaline CsCl Density Gradient Centrifugation. CEM cells were labeled with 0.01 μCi/mL [^{14}C]thymidine for 72 h and chased for 24 h in fresh medium. The cells were then incubated with 25 μM BrdU for 1 h followed by a 30 min incubation with or without VM-26. Control experiments indicated that VM-26 did not affect BrdU incorporation into DNA under these conditions. After centrifugation at 4 °C, the cells were resuspended in drug-free medium, and pulse-labeled with 1.0 μCi/mL [^{3}H]deoxycytidine for 2 min at 37 °C. At the end of the pulse, nuclei were isolated from control and VM-26-treated cells (Catapano et al., 1991). The samples were incubated with RNase A (25 μg/mL) for 1 h at 37 °C followed by proteinase K (0.1 mg/mL) at 37 °C overnight. After extraction with phenol and chloroform, DNA was denaturated by addition of 0.15 M NaOH and mixed with a buffer consisting of 50 mM NaOH, 3 mM EDTA, and CsCl at a final density of 1.806 g/mL. Samples were centrifuged at 35 000 rpm in a 70.Ti Beckman rotor for 60 h to separate single-stranded BrdU−DNA from unreplicated DNA. Fractions of 0.45 mL were collected from the bottom of each gradient, and the amount of radioactivity was determined by liquid scintillation counting.

Analysis of Replication Fork Polarity. CEM cells were incubated with 10 μM fluorodeoxyuridine and 10 μM [^{3}H]-BrdU (0.025 μCi/mL) either in the presence or in the absence of 2 μM emetine. After 24 h, DNA was isolated from control and emetine-treated cells as previously described (Burhans et al., 1991). BrdU-labeled DNA was separated from unsubstituted DNA by centrifugation in alkaline CsCl gradients as described above. All the procedures involving BrdU-containing samples were carried out under subdued light. Equal amounts of BrdU−DNA (2−4 μg) were denatured by the addition of 0.1 volume of 3 M NaOH and incubation at 100 °C for 5 min. The samples were then neutralized with 1 volume of 6× SSC and applied to nylon membranes using a slot-blot apparatus. The blots were baked for 1 h at 80 °C and then were hybridized to ^{32}P-labeled strand-specific RNA probes. Hybridization and high-stringency washing were as described previously (Burhans et al., 1991).

In Vivo Mapping of Topo II-Mediated DNA Cleavage Sites. Control and VM-26-treated cells were centrifuged at 4 °C, washed in ice-cold drug-free medium, and then lysed in a buffer containing 1.25% SDS, 10 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.2 mg/mL proteinase K. DNA was purified from control and drug-treated samples as previously described (Pommier et al., 1992). Aliquots of 10 μg of DNA from control and drug-treated cells were digested with the restriction endonuclease *Bgl*II, electrophoresed in 1% agarose gels, and transferred to nylon membranes. The membranes were incubated at 50 °C in a buffer containing 0.1 M Tris-HCl (pH 7.5), 1 M NaCl, 30% formamide, 10% dextran sulfate, and 0.1 mg/mL salmon sperm DNA. After 2−4 h, the ^{32}P-labeled 414 bp *Pst*I probe [$(1-3) \times 10^6$ cpm/mL] was added to the buffer, and the hybridization was carried out at 50 °C overnight. Membranes were washed once at room temperature in 2× SSC for 15 min followed by two 30 min washes in 0.1× SSC and 0.1% SDS at 65 °C. Autoradiography was carried out at −70 °C for 2−4 days in the presence of an intensifying screen.

Centrifugal Elutriation. Exponentially growing CEM cells (5×10^8) were loaded in a standard elutriation chamber of a JE-6B Beckman rotor at a flow rate of 10 mL/min. Elutriation was performed at a rotor speed of 1500 rpm in a J2-MI Beckman centrifuge. The cells were collected in six fractions of 100 mL each by increasing the flow rate from 10 to 22 mL/min in increments of 2 mL/min. The cells number and mean cell volume of the elutriated cells were measured with a Coulter cell counter. Aliquots of elutriated cells were stained with propidium iodide and analyzed by flow cytometry to determine DNA content and cell cycle distribution (Catapano et al., 1987).

Analysis of Replication Fork Progression in Control and VM-26-Treated Cells. S-phase cells isolated by centrifugal elutriation (fractions 2 and 3 in Figure 7) were incubated with or without 50 μM VM-26 for 30 min. After centrifugation at 4 °C, the cells were resuspended in drug-free medium and incubated with 50 μM [^{3}H]BrdU (5 μCi/mL) for 15 min. The cells were centrifuged for 5 min at 4 °C. The cell pellets were washed in cold E-MEM medium without serum and then lysed in 10 mM Tris-HCl (pH 8), 0.1 M EDTA, and 0.5% SDS. DNA was purified and subjected to alkaline CsCl density gradient centrifugation as described above. Equal amounts of newly replicated DNA (2−4 μg) from control and VM-26-treated cells were blotted on nylon membranes. Duplicate filters containing identical samples were obtained in each experiment and hybridized with either the 1710 bp, the 606 bp, or the 414 bp probes. Prehybridization, hybridization, washing, and autoradiography were carried out as described for Southern blotting. To measure the amounts of newly replicated DNA in each sample, the blots were exposed to a phosphor screen (Molecular Dynamics) and analyzed with a phosphoimager. Quantitative data were obtained using the ImageQuant software provided with the phosphoimager.

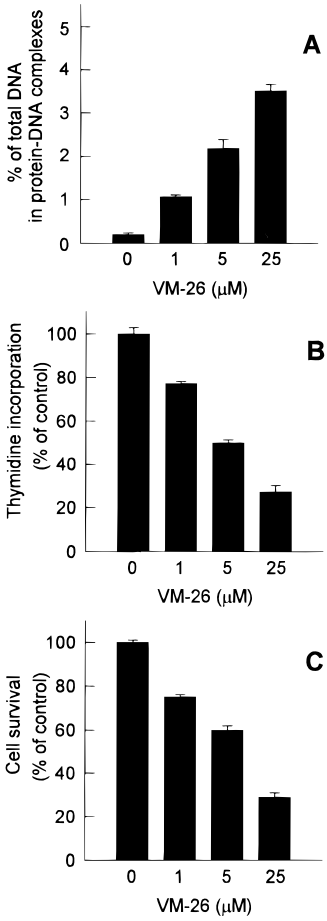

FIGURE 1: Effects of VM-26 on covalent protein−DNA complex formation, DNA synthesis, and cell survival. Panel A: Amounts of protein−DNA complexes precipitated by K-SDS in control and drug-treated cells. The results are expressed as a percentage of total [^{3}H]DNA precipitated as protein−DNA complexes. Panel B: Drug-induced inhibition of DNA synthesis. Control and VM-26-treated cells were pulse-labeled with [^{3}H]thymidine for 30 min in drug-free medium. The results are presented as the percentage of radiolabel incorporated into DNA of untreated control cells. Results in panels A and B are mean ± SD of four measurements. Panel C: Cell survival following a 30 min incubation with VM-26 and 24 h growth in drug-free medium. The number of viable cells was measured by the MTT assay, and the results are expressed as the percentage of cell survival compared to untreated control cells. Each point represents the mean ± SD of six determinations.

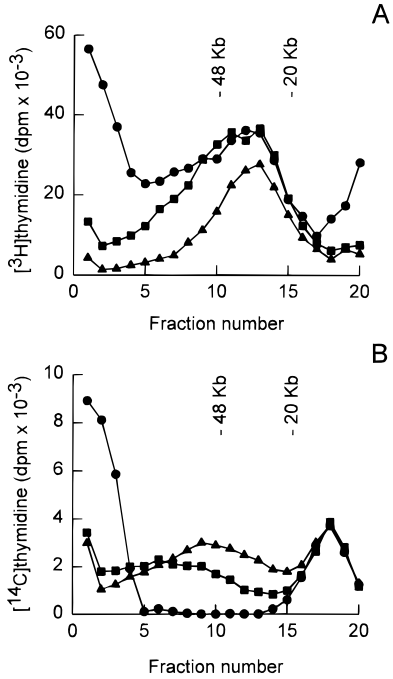

FIGURE 2: Velocity sedimentation analysis of DNA from control and VM-26-treated cells. CEM cells were preincubated with [^{14}C]-thymidine for 72 h to label total DNA. The cells were then incubated with either 0 (●), 5 (■), or 25 (▲) μM VM-26 for 30 min. Control and drug-treated cells were pulse-labeled with [^{3}H]thymidine for 10 min in drug-free medium to label newly replicated DNA. The cells were lysed on the top of alkaline sucrose gradients and centrifuged at 12 000 rpm for 16 h. Fractions were collected from the bottom of the centrifuge tubes. Panel A: ^{3}H-Labeled newly replicated DNA strand. Panel B: ^{14}C-Labeled parental DNA strand. λ DNA molecules of 20 and 48 kb served as standards, and their position is indicated at the top of the panels.

RESULTS

Formation of Topo II−DNA Cleavable Complexes and DNA Synthesis Inhibition in VM-26-Treated CEM Cells. Initial studies were carried out to determine the extent of topo II−DNA cleavable complex formation and DNA synthesis inhibition in CEM cells exposed for 30 min to 1−25 μM VM-26. This short incubation time was chosen, since longer incubation times may result in a generalized endonucleolytic DNA cleavage (Kaufmann, 1989). The K-SDS precipitation assay was used to measure the fraction of cellular DNA that was covalently bound to topo II as a result of the drug-induced stabilization of cleavable complexes (Rowe et al., 1986). As shown in Figure 1, panel A, untreated control cells contained only a small amount of protein−DNA complexes, whereas protein-bound DNA increased 5-, 10-, and 17-fold in cells incubated with 1, 5, and 25 μM VM-26, respectively. Incubation of CEM cells with these concentrations of VM-26 resulted also in a dose-dependent inhibition of DNA synthesis and cell survival (Figure 1, panels B and C). Taken together, these results showed that a 30 min incubation with 1−25 μM VM-26 induced topo II−DNA cleavable complex formation, DNA synthesis inhibition, and cytotoxicity in CEM cells.

Inhibition of DNA Chain Elongation in VM-26-Treated Cells. The cytotoxic effects of cleavable complex-forming topo II inhibitors have been attributed, at least in part, to the interaction of the cleavable complexes with the replication machinery (D'Arpa Liu, 1989; Fernandes & Catapano, 1991). However, little direct information is available concerning the actual mechanism by which drug-stabilized topo II−DNA complexes inhibit DNA replication in mammalian cells. In the following experiments, we examined the effects of VM-26 on the synthesis and size distribution of replicative intermediates by alkaline sucrose density gradient centrifugation. Following a 30 min incubation with or without VM-26, control and drug-treated cells were incubated for 10 min with [^{3}H]thymidine (Figure 2A). Under these conditions, the radiolabeled thymidine was incorporated into nascent DNA molecules at various levels of maturation (Painter, 1978; Kaufmann et al., 1991). Radioactivity in the lower part of the gradients (fractions 1−12) indicated incorporation of [^{3}H]thymidine into larger replicative intermediates that had initiated replication prior to the addition of the radiolabel and continued elongation during the 10 min of pulse-labeling. The radioactivity in the upper part of the gradients (fractions 14−20) corresponded to incorporation of [^{3}H]thymidine into smaller DNA fragments that initiated synthesis during the pulse with [^{3}H]thymidine. Fractions 18−20 contained very

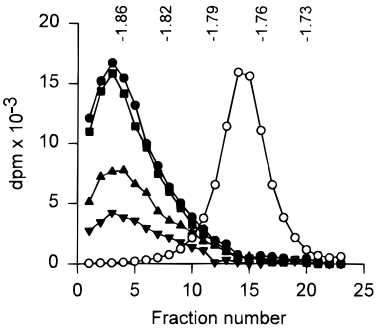

FIGURE 3: Analysis of DNA chain elongation in control and VM-26-treated cells. CEM cells were labeled for 72 h with [^{14}C]-thymidine to provide an internal standard of single-stranded parental DNA. The effect of VM-26 on DNA chain elongation was measured by incubating the cells for 1 h with 25 μM BrdU followed by the addition of the drug. After 30 min, the cells were washed at 4 °C, resuspended in drug-free medium, and pulse-labeled for 2 min with [^{3}H]deoxycytidine. Equal amounts of DNA purified from control and drug-treated cells were centrifuged to equilibrium in alkaline CsCl gradients. The amounts of ^{3}H and ^{14}C in each gradient fraction were determined by liquid scintillation counting. The ^{3}H counts in each gradient were normalized to the total amount of [^{14}C]DNA recovered in each sample. The density in grams per milliliter along the gradient is indicated at the top of the figure. Symbols: (○) ^{14}C-labeled parental DNA from control cells; (●) ^{3}H-labeled nascent DNA from control cells; (■) ^{3}H-labeled nascent DNA from cells treated with 1 μM VM-26; (▲) ^{3}H-labeled nascent DNA from cells treated with 5 μM VM-26; (▼) ^{3}H-labeled nascent DNA from cells treated with 25 μM VM-26.

small radiolabeled DNA fragments that likely represented Okazaki fragments (Okazaki et al., 1968) or partially degraded DNA. As shown in Figure 2A, a 30 min incubation of CEM cells with VM-26 resulted in decreased labeling of the largest DNA fragments, while the low molecular weight DNA was relatively unaffected. Thus, brief incubation of the cells with VM-26 induced accumulation of topo II−DNA complexes and inhibited preferentially DNA chain elongation compared to the initiation of new DNA chains.

Velocity sedimentation analysis also provided the opportunity to examine the effects of VM-26 on the parental DNA strand, which was labeled with [^{14}C]thymidine. As shown in Figure 2B, the ^{14}C-labeled DNA banded mainly as high molecular weight DNA in untreated control cells (fractions 1−4). A shift of the sedimentation profile of [^{14}C]-DNA toward the low molecular weight region of the gradient was observed in cells treated with either 5 or 25 μM VM-26. This likely represented the result of topo II-mediated DNA cleavage in VM-26-treated cells. Thus, the effects of VM-26 on DNA replication may be a consequence of the formation of topo II-mediated strand breaks in parental DNA and inhibition of DNA chain elongation.

The results obtained with the velocity sedimentation analysis were confirmed with another assay which monitored selectively the addition of radiolabeled nucleotides to the ends of nascent DNA fragments (DNA chain elongation). CEM cells were prelabeled with [^{14}C]thymidine for 72 h, chased in fresh medium for 24 h, and then incubated in the presence of the density label BrdU. After a 1 h incubation with BrdU, the cells were treated with or without VM-26 for 30 min and then were pulse-labeled with [^{3}H]deoxycytidine for 2 min in drug-free medium. BrdU increased the density of the DNA and allowed subsequent separation of newly replicated BrdU−DNA from the parental [^{14}C]DNA strand in alkaline CsCl density gradients (Figure 3). When separated under alkaline conditions, the parental [^{14}C]DNA banded at a density between 1.79 and 1.76 g/mL. BrdU-labeled nascent DNA fragments that were elongated during the pulse-labeling with [^{3}H]deoxycytidine (i.e., the deoxycytidine added to the BrdU-labeled DNA) migrated in the CsCl gradients at a density greater than 1.82 g/mL, which was higher than that of parental DNA. As shown in Figure 3, DNA chain elongation was reduced to about 92, 49, and 26% of control in cells incubated with 1, 5, and 25 μM VM-26, respectively. These results provided additional evidence that VM-26 interfered with DNA chain elongation.

Organization of the c-myc Locus and Direction of DNA Replication in CEM Cells. If topo II−DNA cleavable complexes stabilized by VM-26 directly interfere with DNA chain elongation, then one would expect RF movement to be blocked specifically at sites of topo II-mediated DNA cleavage in VM-26-treated cells. The *c-myc* oncogene was chosen to test this concept because previous reports indicated that topo II-mediated DNA cleavage in this gene was stimulated by VM-26 both *in vitro* and *in vivo* (Riou et al., 1987, 1989; Pommier et al., 1992). Furthermore, the origin (Iguchi-Ariga et al., 1988; Leffak & James, 1989; McWhinney & Leffak, 1990; Vassilev & Johnson, 1990), direction (Leffak & James, 1989), and timing (Iqbal et al., 1987; Taljanidisz et al., 1989) of replication within the *c-myc* locus have been previously determined in various mammalian cell lines. Prior to testing our hypothesis in CEM cells, however, it was necessary to obtain similar information about both the organization of the *c-myc* gene and the direction of DNA replication within this locus in these cells. DNA was purified from untreated cells and digested with various endonucleases, and the restriction pattern was determined by Southern blot hybridization to *c-myc*-specific probes. The pattern of restriction fragments obtained in CEM cells was similar to that previously described in normal human cells (Saglio et al., 1986). Panel A of Figure 4 shows a restriction map of the *c-myc* locus in CEM cells based on the results of these studies. The absence of rearrangements in the gene facilitated the subsequent analysis of topo II-mediated DNA cleavage and RF progression.

Next, we analyzed the direction of replication fork movement within the *c-myc* locus. We used the imbalanced DNA synthesis approach to determine RF polarity (Handeli et al., 1989; Burhans et al., 1991; Kitsberg et al., 1993). This method is based on the ability of the protein synthesis inhibitor, emetine, to preferentially block lagging DNA strand synthesis in mammalian cells (Burhans et al., 1991; Kitsberg et al., 1993). Hence, the direction of DNA replication can be inferred from the unequal hybridization pattern of single-strand-specific probes to nascent DNA from control and emetine-treated cells. Samples containing equal amounts of nascent DNA isolated from either control or emetine-treated CEM cells were hybridized to strand-specific RNA probes (*plus* and *minus* probes in Figure 4, panel B). Nascent DNA from emetine-treated cells hybridized to the *plus* probe about 4-fold less than nascent DNA from control cells. In contrast, the *minus* probe hybridized to a similar extent to BrdU−DNA from both control and emetine-treated cells. Therefore, the DNA strand that was complementary to the *minus* probe represented the leading strand, and RFs moved in the 5′ to 3′ direction in this region of the *c-myc* gene. Although we did not directly identify the location of the *c-myc* replication origin in CEM cells, these results were consistent with the

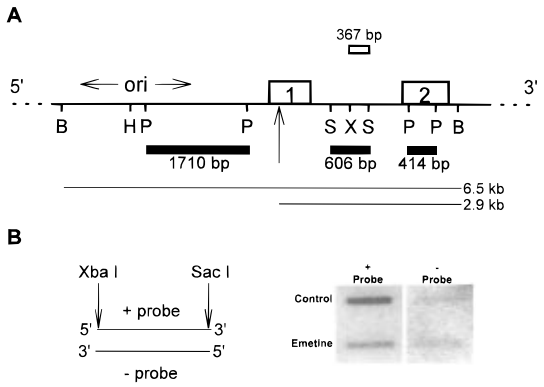

FIGURE 4: Organization of the *c-myc* gene and analysis of replication fork polarity in CEM cells. Panel A: Restriction map of the *c-myc* gene. Restriction endonuclease sites: B, *Bgl*II; H, *Hin*dIII; P, *Pst*I; S, *Sac*I; X, *Xba*I. *ori* is the putative origin of replication in the 5′ flanking region of the gene, and the *horizontal arrows* indicate the direction of replication forks originated from this site. The *vertical arrow* indicates the position of the major topo II-mediated DNA cleavage site identified by *in vivo* indirect end labeling. *Horizontal lines* represent the intact 6.5 kb *Bgl*II fragment and the shorter fragment of 2.9 kb generated in VM-26-treated cells. DNA probes, which correspond to sequences of the *c-myc* gene either upstream (1710 bp) or downstream (606 and 414 bp) of the topo II-mediated DNA cleavage site, are indicated by *solid bars*. The 367 bp *Xba*I/*Sac*I fragment, that was subcloned into a pGEM transcription vector, is indicated by an *open bar*. Panel B: Replication fork polarity in the *c-myc* gene. Duplicate slot blots, which contained equal amounts of BrdU−DNA from control and emetine-treated cells, were hybridized to the ^{32}P-labeled strand-specific RNA probes. The 5′ → 3′ strand of the *Xba*I/*Sac*I fragment is the *plus* (+) probe, and the complementary 3′ → 5′ strand is the *minus* (−) probe.

presence of an origin of replication in the 5′ flanking region of the *c-myc* gene and with RFs moving through the gene in the 5′ to 3′ direction as shown in Figure 4.

Topo II-Mediated DNA Cleavage in the c-myc Gene of VM-26-Treated CEM Cells. Drug-stabilized cleavable complexes consist of topo II molecules covalently bound to DNA (Liu et al., 1983; D'Arpa & Liu, 1989; Osheroff, 1989). Treatment of the cleavable complexes with a protein denaturant, such as SDS, results in inactivation of topo II, which remains linked to the 5′ ends of the topo II-concealed DNA breaks (Liu et al., 1983; Yang et al., 1985; D'Arpa & Liu, 1989). Then, incubation of the samples with proteinase K converts the topo II-concealed DNA breaks into actual DNA cleavage sites, which can be mapped by the indirect end-labeling method (Liu et al., 1983; Yang et al., 1985). Therefore, cells incubated for 30 min with or without VM-26 were lysed in a buffer containing SDS and proteinase K to determine the sites of topo II−DNA cleavable complex formation in the *c-myc* gene. DNA purified from these cell lysates was digested with *Bgl*II. This restriction enzyme generated a fragment of 6.5 kb that comprised the first and second exon and part of the 5′ flanking sequences of the *c-myc* gene (Figure 4, panel A). After gel electrophoresis and blotting to nylon membranes, the blots were hybridized to a 414 bp *Pst*I fragment that corresponded to the 3′ end of the *Bgl*II fragment. Figure 5 shows that samples from both control (lane 1) and VM-26-treated (lanes 2 and 3) cells exhibited the intact 6.5 kb *Bgl*II fragment. Samples from VM-26-treated cells (lanes 2 and 3) exhibited an additional band corresponding to a fragment of approximately 2.9 kb. This low molecular weight DNA fragment was the result of the VM-26-induced formation of topo II−DNA cleavable

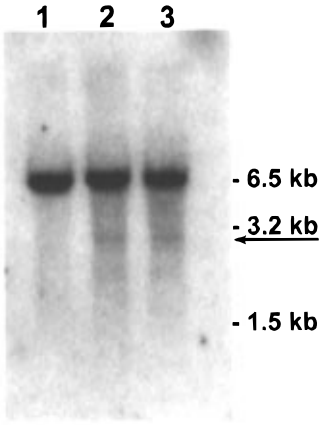

FIGURE 5: Topo II-mediated DNA cleavage in the *c-myc* gene of VM-26-treated cells. Exponentially growing CEM cells were incubated with either 0 (lane 1), 25 (lane 2), or 50 μM (lane 3) VM-26 for 30 min. DNA purified from control and drug-treated cells was digested with *Bgl*II, fractionated in a 1% agarose gel, and blotted to a nylon membrane. The blots were developed with the 414 bp *Pst*I probe, that corresponds to the 3′ end of the *Bgl*II fragment. Hybridization, high-stringency washing, and autoradiography were carried out as described under Materials and Methods. The position of DNA size markers (in kilobases) is shown to the right. The *arrow* indicates the 2.9 kb fragment present in VM-26-treated cells.

complexes and consequent DNA cleavage at a site approximately 2.9 kb from the 3′ end of the *Bgl*II fragment. As indicated in Figure 4, this corresponded to a region near the 5′ end of the first *c-myc* exon. Pommier et al. (1992) have previously shown that cleavage at this site occurred both *in vitro* and in cells with amplified *c-myc* following incubation with either VM-26 or *m*-AMSA.

Prior to addressing the question whether a VM-26-induced DNA cleavage can act as a replication fork barrier, it was necessary to demonstrate that VM-26-induced cleavage in the *c-myc* gene occurred in S phase cells. Asynchronous CEM cells were separated by centrifugal elutriation according to their position within the cell cycle. Figure 6A shows the cell cycle distribution of the cells in each of six elutriated fractions. Samples enriched with cells either in G_1 (elutriated fraction 1) or in S phase (elutriated fractions 2−5) were incubated with or without VM-26 for 30 min. Equal amounts of *Bgl*II-digested DNA from each treatment group were electrophoresed on an agarose gel, and the blot was hybridized to the 414 bp probe (Figure 6B). Untreated control cells either in G_1 or in S phase (lanes 1−3) and G_1 phase cells incubated with VM-26 (lane 4) exhibited only the intact 6.5 kb band. In agreement with Figure 5, samples from the S phase cells incubated with VM-26 (lanes 5−8) exhibited the additional 2.9 kb band, indicating the presence of a topo II-mediated DNA cleavage site in the first *c-myc* exon. These results showed that VM-26-stabilized cleavable complexes were present at this site in early and middle S phase cells and, therefore, could interfere with RF progression in the *c-myc* gene.

Replication Fork Progression Is Arrested at the Site of VM-26-Stabilized Topo II Cleavable Complex Formation in the c-myc Gene. To determine the effects of topo II−DNA cleavable complex formation on RF progression within the *c-myc* gene, early and middle S phase cells were isolated by centrifugal elutriation and incubated with or without 50 μM VM-26. This concentration of VM-26 reduced DNA synthesis to about 25% of untreated control cells. After 30 min,

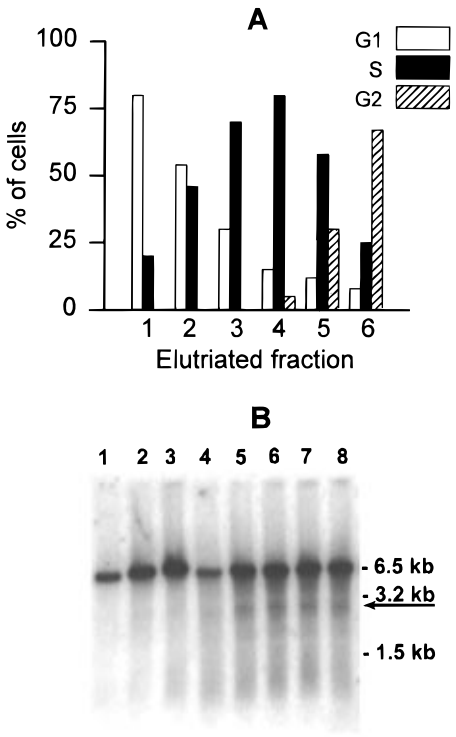

FIGURE 6: Induction of topo II-mediated DNA cleavage in S-phase cells. Panel A: CEM cells (5×10^8) were elutriated in six fractions by increasing the flow rate from 10 to 22 mL/min in increments of 2 mL/min. Cell cycle distribution of the cells in each fraction was determined by flow cytometry. Cell size of the elutriated cells was measured with a Coulter counter (Multisizer II). Mean cell volumes of the cells elutriated in fractions 1 and 6 were 1196 and 1920 μm^3, respectively. Panel B: Cells elutriated in fractions 1−5 were incubated with or without 50 μM VM-26. Aliquots of DNA (10 μg) were digested with *Bgl*II and analyzed by Southern blotting. The blots were hybridized to the 414 bp *Pst*I probe. Lanes 1−3, untreated control cells from elutriated fractions 1, 3, and 5, respectively; lanes 4−8, VM-26-treated cells from elutriated fractions 1−5, respectively. The position of DNA size markers (in kilobases) is shown to the right. The *arrow* indicates the 2.9 kb fragment present in VM-26-treated cells.

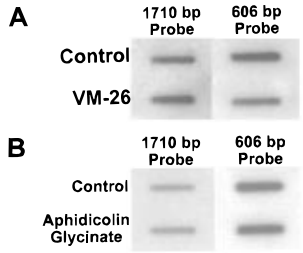

FIGURE 7: Effects of VM-26 and aphidicolin glycinate on replication fork progression in the *c-myc* gene. Panel A: S phase cells isolated by centrifugal elutriation (fractions 2 and 3 in Figure 6) were incubated with or without 50 μM VM-26 for 30 min and then pulse-labeled with 50 μM [^{3}H]BrdU for 15 min in drug-free medium. Newly replicated DNA was separated from parental DNA by centrifugation in alkaline CsCl density gradients. Equal amounts of newly replicated DNA from control and VM-26-treated cells were applied to nylon filters using a slot-blot apparatus. The duplicate filters were hybridized to either the 1710 bp or the 606 bp probe. Panel B: S phase cells were treated with 0.5 μM aphidicolin glycinate for 30 min and then pulse-labeled with BrdU as described above. Slot-blots containing equal amounts of newly replicated DNA from control and aphydicolin-treated cells were hybridized to either the 1710 bp or the 606 bp probe.

Table 1: Effects of VM-26 on the Relative Amounts of Newly Replicated DNA Upstream and Downstream of the Topo II-Mediated DNA Cleavage Site in the *c-myc* Gene[a]

probe	control[b]	VM-26[b]	VM-26/control[c]
group 1			
1710 bp	2280	2540	1.11 ± 0.05
606 bp	3560	1815	0.51 ± 0.05[d]
group 2			
1710 bp	1220	1135	0.93 ± 0.12
414 bp	2320	1100	0.47 ± 0.02[d]

[a] Blots from three separate experiments in each group were hybridized to either upstream (1710 bp) or downstream (606 or 414 bp) probes, exposed to a phosphor screen, and analyzed with a phosphoimager. [b] Mean of the hybridization signals in arbitrary units from three experiments. [c] Mean ± SD of the ratios of the hybridization signals from three experiments. [d] $p < 0.01$ compared to the mean ratios obtained with the 1710 bp probe.

the cells were pulse-labeled with [^{3}H]BrdU in drug-free medium for 15 min. The relative amounts of newly replicated DNA from control and VM-26-treated cells were determined by slot-blot hybridization to *c-myc*-specific probes that corresponded to sequences either upstream (1710 bp) or downstream (606 and 414 bp) of the topo II-mediated DNA cleavage site as shown in Figure 4. Two representative blots, which were hybridized with either the 1710 bp or the 606 bp probe, are shown in Figure 7 (panel A). Similar amounts of newly replicated DNA hybridized to the 1710 bp probe in samples from both control and VM-26-treated cells, since this probe hybridized upstream of the cleavage site. In contrast, the amount of newly replicated DNA that hybridized to the downstream 606 bp probe was lower in VM-26-treated cells than in control cells. Quantitative analysis with a phosphoimager of blots from three separate experiments showed that about 2-fold less newly replicated DNA was detected by the 606 bp probe in VM-26-treated cells compared to the untreated controls (Table 1). Similar results were obtained when blots from three independent experiments were hybridized to the 1710 bp and 414 bp probes (Table 1). Therefore, replication of the sequences upstream of the topo II−DNA cleavage site was not affected in VM-26-treated cells. In contrast, sequences downstream of the topo II−DNA cleavage site were underrepresented in newly replicated DNA from VM-26-treated cells compared to the untreated controls. These results demonstrated that topo II−DNA cleavable complexes formed at the site identified in the first *c-myc* exon arrested the progression of RFs moving through the gene.

The control experiment, which is shown in panel B of Figure 7, confirmed that the effects of VM-26 were not due to a general inhibition of DNA synthesis. S phase cells were incubated with the DNA polymerase inhibitor aphidicolin glycinate (Huberman, 1981). The concentration of aphidicolin glycinate (0.5 μM) that was used in this study inhibited DNA synthesis to 22% of control (a degree of inhibition similar to that induced by 50 μM VM-26). When samples containing equal amounts of newly replicated DNA from control and aphidicolin-treated cells were hybridized to either the 1710 bp or the 606 bp probe, similar hybridization signals were detected with both probes. Thus, unlike VM-26, aphidicolin glycinate did not induce a sequence-specific arrest of RF progression in this region of the *c-myc* gene.

DISCUSSION

Our analysis of DNA replication within the *c-myc* gene showed that the formation of a VM-26-stabilized topo II−

DNA cleavable complex in the first exon of the gene inhibited the progression of the replication forks. This is the first direct evidence that a VM-26-stabilized topo II−DNA cleavable complex can act as a replication fork barrier at a specific genomic site. The cleavable complex that blocked RF movement is likely to contain the topo IIα isozyme, since VM-26 was shown to stabilize the binding of only topo IIα to newly replicated DNA (Qiu et al., 1996).

An origin of replication has been identified approximately 2−3 kb upstream of the first *c-myc* exon in mammalian cells with a normal configuration of the gene (Iguchi-Ariga et al., 1988; Leffak & James, 1989; McWhinney & Leffak, 1990; Vassilev & Johnson, 1990). The region downstream of this putative origin of replication was also replicated in the 5′ to 3′ direction in CEM cells. Therefore, RFs, which originated from the putative initiation site in the 5′ flanking region and moved in the 5′ to 3′ direction, would encounter topo II−DNA cleavable complexes at the site identified in the first *c-myc* exon. Measurements of the relative amounts of *c-myc* sequences replicated upstream and downstream of the cleavage site identified in CEM cells revealed that RFs moving through the gene in the 5′ to 3′ direction were arrested within 500 bp or less from this site. These results demonstrated the physical correspondence of a site of RF arrest with a site of topo II-mediated DNA cleavage.

Cleavage at the site in the first *c-myc* exon, as well as at other minor sites, was previously shown to occur in cells with amplified *c-myc* (Riou et al., 1989; Pommier et al., 1992). The *c-myc* gene is not amplified in CEM cells. This may explain the low intensity of the cleavage band observed in these cells and limit the detection of the minor cleavage sites which are observed in cells with amplified *c-myc* (Riou et al., 1989; Pommier et al., 1992). In addition, the low intensity of the cleavage band detected in CEM cells may be due to the shorter incubation time (30 min) used in this study compared to other studies (3−16 h; Riou et al., 1989; Aplan et al., 1996). Although long incubation times might generate stronger signals in a Southern blot, it would have been difficult to determine under these conditions whether the effects of VM-26 on RF progression were due to topo II-mediated DNA cleavage or endonucleolytic DNA fragmentation (Kaufmann, 1989; Barry et al., 1993). We observed also a temporal relationship between topo II-mediated cleavage and inhibition of DNA replication fork progression in the *c-myc* gene. Topo II-mediated DNA cleavage at the site identified in the first *c-myc* exon was more evident in cells in early and middle S phase than in G_1 phase cells. This may be due to either a higher topo IIα activity in S phase cells compared to G_1 cells or DNA topological changes which increase topo II-mediated cleavage at this site in S phase cells. In addition, drug-stabilized topo II−DNA complexes can be readily transformed in irreversible DNA breaks in S phase cells because of the presence of RFs (described below).

Although our results show that a barrier to RF progression is present within the *c-myc* gene in VM-26-treated cells, the actual mechanism by which topo II−DNA cleavable complexes block fork progression is still unclear. Both components of the VM-26-stabilized cleavable complexes (i.e., DNA strand breaks and covalent topo II−DNA complexes) may block the progression of RFs along the DNA template. As recently reported, topo II-concealed DNA cleavage can be converted into actual breaks by the action of a DNA helicase (Howard et al., 1994). Thus, DNA unwinding catalyzed by DNA helicase ahead of the RF may induce breaks in the DNA template at the sites of cleavable complex formation. Then, the replication complex would stop or run off the template at the cleavage site. Alternatively, topo II molecules covalently linked to DNA may act as a physical barrier to the movement of the replication complex along the DNA template. Evidence supporting both of these possibilities comes from other experimental systems. For example, cleavage of the DNA template by restriction endonucleases induces a runoff of the replication complex and arrest progression of DNA replication (James & Leffak, 1986; Leffak & James, 1989). In contrast, protein−DNA complexes formed by a protein encoded by the *tus* gene arrest DNA replication at specific termination sites (*Ter*) in the *E. coli* genome (Hill, 1992). Also, protein−DNA complexes or DNA secondary structures are thought to be responsible for RF arrest in the centromeric regions of yeast chromosomes (Greenfeder & Newlon, 1992) or in ribosomal RNA genes in yeast (Brewer et al., 1992) and humans (Little et al., 1993). Finally, it is possible that RF arrest induced in VM-26-treated cells can be an indirect consequence of the inhibition of topo II activity, which would lead to increased DNA supercoiling and prevent DNA unwinding ahead of the replication complex.

Taken together, the data allow speculation of the biological consequences of RF arrest at sites of VM-26-stabilized topo II−DNA cleavable complex formation. Inhibition of DNA chain elongation was the major effect of VM-26 on DNA replication in leukemia CEM cells. The formation of cleavable complexes of topo IIα with nascent DNA has recently been shown to be the critical element for the induction of cytotoxicity by VM-26 in these cells (Qiu et al., 1996). DNA strand separation ahead of a replication complex may be the driving force that converts the topo II-concealed DNA breaks to actual breaks, which are no longer held together by topo II molecules. Therefore, formation of irreversible damage is more likely to occur in the proximity of a RF, whereas the majority of cleavable complexes in bulk DNA are reversed upon removal of the drug. Furthermore, DNA breaks at stalled RFs may lead to dissociation of nascent DNA from replication sites. Studies from our laboratory indicated that VM-26 and *m*-AMSA induced the dissociation of newly replicated DNA from the nuclear matrix (Fernandes et al., 1989), which is the subnuclear site of DNA replication in eukaryotic cells (Hozak et al., 1993; Nakayasu & Berezney, 1989). This effect was seen with topo II-active agents, while other DNA synthesis inhibitors, such as araC and hydroxyurea, did not have a similar effect on the attachment of newly replicated DNA to the nuclear matrix (Fernandes et al., 1989). Furthermore, we have shown that the cytotoxic effects of VM-26 and other cleavable complex-forming topo II inhibitors are mediated mainly by the preferential stabilization of topo IIα complexes with newly replicated DNA (Qiu et al., 1996), which is enriched on the nuclear matrix (Danks et al., 1994). The preferential formation of cleavable complexes with replicating versus nonreplicating DNA may explain why VM-26 was quite effective in blocking replication through the *c-myc* gene while inducing a modest amount of cleavage in total *c-myc* DNA.

The induction of breaks at nuclear matrix-attached replication sites may be a very efficient cytotoxic mechanism in

proliferating tumor cells. Various phenomena, which have been described in cells treated with cleavable complex-forming topo II inhibitors, may be enhanced by the formation of DNA breaks at stalled RFs. Cleavage at the level of nuclear matrix-attached replication sites could (a) lead to dissociation of entire chromatin loops (Filipski et al., 1990; Walker et al., 1991; Gromova et al., 1995), (b) activate nucleases responsible for DNA fragmentation and apoptosis (Kaufmann, 1989; Walker et al., 1991; Barry et al., 1993), or (c) stimulate recombination events (Pommier et al., 1985; Bae et al., 1988; Sperry et al., 1989; Berger et al., 1991; Bodley et al, 1993; Han et al., 1993). In exponentially growing cells, multiple drug-induced breaks at RFs may disrupt the overall organization of matrix-attached chromatin loops. This would compromise many nuclear functions and, ultimately, activate a cell death program. DNA breaks at RFs are also potentially recombinogenic. RFs are clustered together in matrix-attached replication factories (Nakayasu & Berezney, 1989; Hozak et al., 1993), which places multiple potential recombination intermediates in close proximity. Illegitimate recombination may be responsible for the known mutagenic effects of cleavable complex-forming topo II inhibitors (Ratain et al., 1987; Broeker et al., 1996). Illegitimate recombination events may also result in deletions of large genomic segments (Berger et al., 1991; Shibuya et al., 1994), and the subsequent loss of essential genes may lead to cell death.

Our studies have identified a specific site where topo II−DNA cleavable complexes interact with RFs in the genome of VM-26-treated human leukemia cells. Future studies aimed at defining the events occurring at the stalled RFs may provide additional insights into the mechanisms of mutagenesis and cytotoxicity of this potent anticancer agent. In addition, drug-stabilized topo II−DNA cleavable complexes may represent a very useful model to analyze the biological consequences of RF arrest in eukaryotic cells.

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