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Topoisomerase II Cleavable Complex Formation within DNA Loop Domains

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ABSTRACT. The distribution of VM-26 (Teniposide)-stabilized cleavable complexes within DNA loops bound to the nuclear matrix was determined to provide further insights into the mode of DNA synthesis inhibition by VM-26. Covalent binding of [^{3}H]VM-26 was 9-fold greater per milligram of nuclear matrix protein compared with high salt-soluble nonmatrix protein of CEM cells. The ratio declined from 9-fold in CEM cells to 4-fold in drug-resistant VM-1/C2 cells, which have decreased nuclear matrix DNA topoisomerase IIα. VM-26 induced a concentration-dependent increase in the frequency of cleavable complex formation with actively replicating matrix DNA. At 25 μM VM-26, the frequency was 32 ± 2 (SEM) complexes per 10^6 bp of replicating matrix DNA compared with 13 ± 2 (SEM) complexes per 10^6 bp of nonreplicating DNA in the matrix fraction. VM-26 at concentrations as high as 25 μM stabilized less than 3 complexes per 10^6 bp in the various nonmatrix DNA domains, since the nonmatrix DNA comprises the DNA loop domains that are distal to the matrix-bound replication sites. A negligible frequency of cleavable complex formation was detected in both the matrix and nonmatrix DNA domains of drug-resistant VM-1/C2 cells. Compared with untreated control cells, VM-26 induced an accumulation of nascent DNA in the nuclear matrix fraction of CEM cells but decreased the amount of nascent DNA in the nonmatrix fraction. The extensive cleavable complex formation on matrix replicating DNA stalled most of the replication forks within 1 kb of the replication sites on the nuclear matrix. The results provide evidence that nascent DNA bound to the nuclear matrix is an important site of VM-26 cleavable complex formation, and that these complexes inhibit DNA synthesis by blocking the movement of nascent DNA away from replication sites on the nuclear matrix. BIOCHEM PHARMACOL **60**;1: 101–109, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. nuclear matrix; VM-26; Teniposide; topoisomerase II; DNA replication

Presently, the DNA topoisomerase II-active drugs are among the most frequently used anticancer agents. These drugs are referred to as "cleavable complex"-forming agents and include the epipodophyllotoxins (VP-16,† VM-26), anthracyclines (e.g. doxorubicin and daunorubicin), anthracenediones (mitoxantrone), and the aminoacridines (*m*-AMSA). Although much is known about the enzymatic mechanisms by which both the topoisomerase I- and II-active drugs interfere with enzyme activity [1–3], the critical biological consequences of drug treatment that lead to tumor cell death are less clear.

Neither the extent of inhibition of topoisomerase II activity nor the total amount of drug-induced cleavable complex formation appears to be related directly to the cytotoxic effects of the topoisomerase II drugs [4]. However, other studies have revealed some important determinants of the cytotoxic effects of the topoisomerase II-active drugs in proliferating cells. VM-26, *m*-AMSA, and mitoxantrone preferentially induce cleavable complex formation with topoisomerase II and newly replicated DNA compared with nonreplicating, bulk DNA [5, 6]. Arrest of DNA replication fork movement through the c-*myc* gene of CEM cells occurs specifically at sites of VM-26-induced cleavage of this gene [7]. In these cells, the cleavable complexes formed with nascent DNA contained only topoisomerase IIα, whereas those formed with nonreplicating DNA contained both the α and β topoisomerase II isozymes [8]. The extent of formation of topoisomerase IIα complexes with nascent DNA is related directly to the degree of VM-26-induced cytotoxicity [8]. Taken together, these results indicate that topoisomerase IIα complexes stabilized by VM-26 at DNA replication forks are critical to the development of cytotoxicity in CEM cells. In contrast, drug-induced cleavable complex formation with topoisomerase IIβ may be important in slowly proliferating tumors that have low activity of the α isozyme, since the expression of topoisomerase IIβ is not proliferation dependent [9, 10].

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†*Abbreviations*: VP-16 (Etoposide), 4′-demethylepipodophyllotoxin 9-[4,6-*O*-ethylidene-β-D-glucopyranoside]; VM-26 (Teniposide), 4′-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside); *m*-AMSA, *N*-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulfonamide; at-MDR, multidrug resistance associated with alterations in DNA topoisomerase II; FITC, fluorescein isothiocyanate; EMEM, Eagle's minimum essential medium; araC, 1-β-D-arabinofuranosylcytosine; and PMSF, phenylmethylsulfonyl fluoride.

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Several studies suggest that the topoisomerase II associated with the nuclear matrix has a major role in the formation of cleavable complexes at replication forks. The most basic level of DNA organization consists of the folding of the DNA into 50- to 100-kb loops, which are wound further into nucleosomes [11, 12]. Microscopic and biochemical studies have shown that the DNA loops are attached at their bases to the nuclear matrix [13–15]. The loops, in turn, form the basis for the regulation of the transcription and replication of these sequences as functional units or domains [16]. Current evidence is consistent with the concept that DNA replication takes place within adjacent clusters of DNA loops attached to the nuclear matrix, and that the DNA loops pass through the matrix-bound replication sites at the rate of replication fork movement [13, 17, 18]. The α isozyme of topoisomerase II is one of the most abundant proteins of the isolated nuclear matrix [15, 19]. It is thought to act at or near the matrix attachment regions to relieve the DNA supercoiling that accumulates during replication and transcription [20]. It is important to note that resistance of various cell lines to topoisomerase II-active drugs is associated specifically with a decreased amount and activity of nuclear matrix topoisomerase II [21, 22] and with a reduced ability of these drugs to inhibit nuclear matrix DNA synthesis [21, 23].

The results from the above studies suggest that cleavable complex-forming topoisomerase II-active drugs may preferentially stabilize the covalent binding of topoisomerase IIα to DNA replication forks on the nuclear matrix. This hypothesis was tested in the studies reported herein by examining the frequency of VM-26-induced cleavable complex formation within various nuclear matrix and nonmatrix DNA domains and then relating this effect to the movement of the replicating DNA within these domains.

MATERIALS AND METHODS

Materials

The parental human CCRF-CEM (CEM) cell line and the at-MDR CEM subline (CEM/VM-1) were obtained from Dr. William Beck of the University of Illinois. CEM/VM-1 cells were subcloned in agarose, and one clone, termed VM-1/C2, was used in the experiments described in this paper. VM-1/C2 cells were 63-fold resistant to VM-26 following a 48-hr continuous exposure to the drug. Immunoblotting revealed that the amount of nuclear matrix topoisomerase IIα in the resistant cells was 11% (range 10–12%) of that detected in CEM cells. All cell lines were propagated as previously described [21]. Nonradioactive VM-26 was provided by Rosemarie Schwindinger of Bristol-Myers Squibb. [4-^{14}C]Thymidine (specific radioactivity of 0.05 Ci/mmol) and [*methyl*-^{3}H]thymidine (specific activity of 64 Ci/mmol) was purchased from Moravek Biochemicals. Protease inhibitors were purchased from the Sigma Chemical Co. RNase-free pancreatic DNase I was obtained from Worthington Biochemicals. The sources of most other chemicals and supplies were as reported previously [19]. Protease inhibitor solution consisted of 1 mM PMSF, 1 mM benzamidine, 10 mg/mL of soybean trypsin inhibitor, 50 mg/mL of leupeptin, 1 mg/mL of pepstatin, and 2 mg/mL of aprotinin. High salt buffers consisted of either 1.5 or 3 M NaCl, 10 mM Tris–HCl (pH 7.0), and 0.2 mM $MgCl_2$ in protease inhibitor solution. Homogenization buffer consisted of 10 mM Tris–HCl (pH 7.0) and 2 mM $MgCl_2$ in protease inhibitor solution. Low salt buffer consisted of 10 mM NaCl, 10 mM Tris–HCl (pH 7.0), and 1 mM $MgCl_2$.

Measurement of Cell Viability, Apoptosis, and Necrosis

Quantitation of viable, apoptotic, and necrotic cells was done with annexin V and propidium iodide staining of the cells coupled with flow cytometric analysis [24]. The ApoNexin kit (Oncor, Inc.) was used for the assay according to the manufacturer's instructions. This assay is based on the different cellular alterations induced by the two types of cell death. During the early stages of apoptosis, phosphatidylserine is translocated to the external surface of the cell, and the exposed phosphatidylserine becomes available to bind FITC-labeled annexin V [25]. Early apoptotic cells remain impermeable to the DNA-binding agent propidium iodide. In contrast, loss of cell membrane integrity occurs during necrosis and the latter stages of apoptosis [26]. Thus, necrotic cells show propidium iodide fluorescence, while late-stage apoptotic or necrotic cells can also be stained with the larger label, FITC-annexin V. Viable cells do not stain significantly with either annexin V or propidium iodide.

Isolation of the Matrix and Nonmatrix Fractions of the Nucleus

The matrix and nonmatrix fractions of the nucleus were isolated as previously described [19]. Briefly, exponentially growing cells were resuspended in homogenization buffer at a density of 2×10^7 cells/mL. The cells were placed on ice for 5 min, and then the cell membranes were disrupted with 5 strokes in a Dounce homogenizer. Nuclei were purified by centrifugation at 1900 *g*, 4°, for 30 min through a 45% (w/v) sucrose gradient. The nuclear pellet was resuspended in 1 mL of low salt buffer containing the protease inhibitor solution, and then incubated with RNase-free DNase I (150 Kunitz units) for 20 min at 37°. An equal volume of 3 M high salt buffer was added over a period of 1 hr with gentle shaking, followed by a 30-min incubation on ice. The insoluble nuclear matrix and high salt-soluble nonmatrix fractions were separated by centrifugation at 7100 *g* for 15 min at 4°. The matrix pellet was washed at 4° with 4 mL of 1.5 M high salt buffer and then with 4 mL of low salt buffer.

Purification of [^{3}H]VM-26

[G-^{3}H]VM-26 (Teniposide) was purchased from the Amersham Co. The preparation was evaporated to dryness in a vacuum concentrator, reconstituted in 50 μL of 80% (v/v)

ethanol, and then purified by reversed phase HPLC as described by Strife *et al.* [27]. [^{3}H]VM-26 was identified by its retention time relative to authentic VM-26. Radiochemical purity was estimated to be at least 98%, following the purification by HPLC. The specific radioactivity of the purified [^{3}H]VM-26 was estimated to be 164 mCi/mmol, using a molar extinction coefficient of 21.6×10^3 that we previously determined for authentic VM-26.

Covalent Binding of [^{3}H]VM-26 to Nuclear Matrix and Nonmatrix Protein

Exponentially growing CEM or CEM/VM-1 cells (2×10^7) were resuspended in fresh medium plus fetal bovine serum at a density of 2×10^7 cells/mL. Then the cells were incubated for 2 hr with either 2 μM [^{3}H]VM-26 (carrier-free) to measure total [^{3}H]VM-26 binding or with 2 μM [^{3}H]VM-26 plus a 90-fold excess of unlabeled VM-26 to measure nonspecific binding. The nuclear matrix and high salt-soluble nonmatrix fractions were isolated as described above. The samples were resuspended in a final concentration of 10% trichloroacetic acid (w/v) and then allowed to precipitate on ice for 30 min. Following centrifugation at 12,000 *g* for 5 min, the precipitates were washed with 1 mL of HPLC grade methanol. The pellets were resuspended partially in 1% SDS (w/v), and sufficient trichloroacetic acid was added to yield a final concentration of 10% trichloroacetic acid. The precipitates were washed with 5% trichloroacetic acid and solubilized by heating for 1 hr at 60° in 0.9 N NaOH. Sufficient HCl was added to reduce the pH to about 6.5 prior to liquid scintillation counting of the samples. Protein concentrations were determined by Coomassie blue staining. Specific [^{3}H]VM-26 binding was calculated as the difference between the total and the nonspecific binding. The specific binding was at least 5-fold greater than the nonspecific binding in the nuclear matrix samples.

K-SDS Precipitation of VM-26-Induced Protein–DNA Complexes

Exponentially growing CEM or VM-1/C2 cells were preincubated with 2 mM [^{14}C]thymidine (carrier-free) for 72 hr and then chased for 24 hr in radioactive-free EMEM plus serum. Groups of 2×10^7 cells were then incubated for 10 min with 11 nM [^{3}H]thymidine (carrier-free) followed by a 30-min incubation with either DMSO/ethanol (1:1) at a final concentration of 0.1% (v/v) or various concentrations of VM-26 in 0.1% DMSO/ethanol. The cells were harvested by centrifugation at 4°, and the pellets were washed with 10 mL of cold EMEM without serum. The K-SDS precipitation assay was carried out as described by Rowe *et al.* [28] with the following modifications: Nuclei, nuclear matrices, and the nuclear nonmatrix fractions were prepared as described above and then resuspended in 1 mL of SDS buffer consisting of 1.25% SDS (w/v), 80 mM NaCl, 2 mM EDTA, and 0.5 mg/mL of salmon sperm DNA. The average lengths of the nuclear, nuclear matrix, and nonmatrix DNAs were 2, 1, and 1 kb, respectively, and were estimated by comparing the migration distances of the sample DNAs in agarose gels to that of the *Hin*d III ladder from λDNA. Duplicate aliquots of the matrix and nonmatrix fractions were removed for determination of the total ^{3}H and ^{14}C incorporated into the DNA of these fractions. To dilute the salt concentration in the nonmatrix fractions, aliquots (100 μL) of the nonmatrix fractions were mixed with 800 μL of 10 mM Tris–HCl (pH 7.0), and then were diluted with an equal volume of 2.5% SDS, 4 mM EDTA, and 1.0% salmon sperm DNA. All samples were washed with 500 μL of the buffers described by Rowe *et al.* [28]. Duplicate aliquots of the solubilized K-SDS precipitates were also counted to determine the amounts of ^{3}H and ^{14}C bound in the protein–DNA complexes within the matrix and nonmatrix fractions. The numbers of cleavable complexes per DNA base pair were estimated based on a Poisson distribution and calculated according to the equation described by Woynarowski *et al.* [29]:

$$f = [- \ln (1 - BP)]/L$$

where f = the frequency of cleavable complexes per base pair, BP = (% $\text{bound}_{\text{VM-26}}$ − % $\text{bound}_{\text{control}}$), and L = length of the DNA fragment in base pairs.

RESULTS

Time Course of VM-26-Induced Apoptosis

Recent evidence suggests that the apoptotic degradation of DNA induced by metabolic stress and topoisomerase II-active drugs begins with the release of the 50- to 100-kb DNA loops from the nuclear matrix [30–32]. The free loops then are cleaved further to internucleosomal-size DNA fragments during the final stages of apoptosis [30, 31]. A goal of this study was to examine the formation of VM-26-stabilized topoisomerase II–DNA complexes within various nuclear matrix DNA domains. Thus, it was necessary to carry out experiments prior to the onset of apoptosis or necrosis.

VM-26-induced apoptosis was monitored over time by following the translocation of phosphatidylserine to the external surface of the cell that occurs during the early stages of apoptosis and prior to internucleosomal DNA ladder formation [25]. Neither a significant loss in cell viability nor a significant increase in early apoptotic cells was observed immediately after treatment of CEM cells for 30 min with either 1, 5, or 25 μM VM-26 (Fig. 1). Additional aliquots of cells were incubated for 30 min with VM-26 at the above concentrations, but then were washed with drug-free medium and analyzed 2, 4, and 6 hr later. No changes in cell viability were seen after 2 hr at any of the above drug concentrations compared with untreated cells. However, by 4 hr only 50 and 39% of the cells treated with 5 or 25 μM VM-26, respectively, were viable. The loss in cell viability was accompanied by a commensurate increase

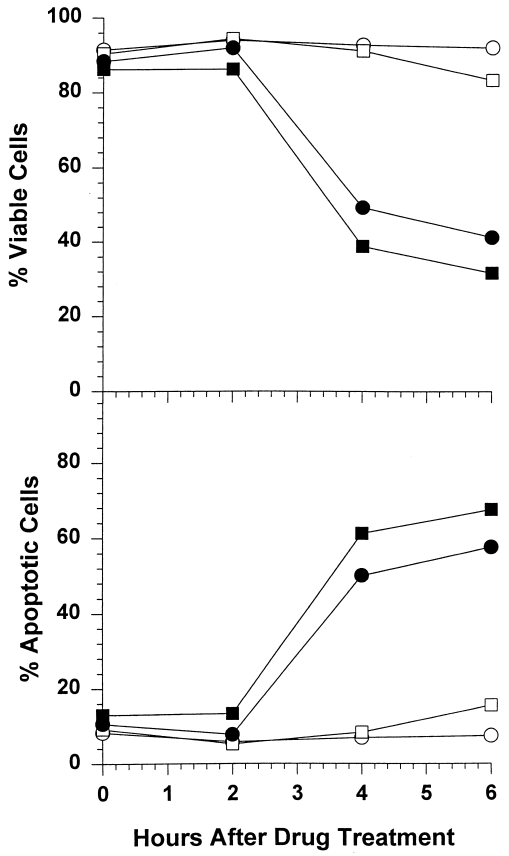

FIG. 1. Quantitation of viable and apoptotic cells following VM-26 treatment. CEM cells (2×10^5 cells/mL) were incubated with 0 (○), 1 μM (●), 5 μM (■), or 25 μM (ν) VM-26 for 30 min. Following VM-26 treatment, aliquots of cells from each group were resuspended in drug-free medium for either 2, 4, or 6 hr. Cell viability, apoptosis, and necrosis were monitored by counting 50,000 cells with a flow cytometer using FITC-annexin V and propidium iodide staining. A population of necrotic cells was not seen at any of the above VM-26 concentrations. Each point represents the mean of duplicate determinations, with the range of the individual values being less than the size of the symbol.

in the numbers of apoptotic cells. A significant population of necrotic cells was not seen even at the highest VM-26 concentration. Based on these results, all subsequent studies were carried out at either 30 min or 2 hr after drug treatment, when no significant apoptosis, necrosis, or loss in cell viability was detected.

Subnuclear Binding and Accumulation of [^{3}H]VM-26

The subnuclear distribution of [^{3}H]VM-26–protein complexes was determined in an initial attempt to identify important nuclear targets of the drug. This was accomplished by measuring covalent binding of [^{3}H]VM-26 that was resistant to trichloroacetic acid, methanol, and SDS extraction. Most of the [^{3}H]VM-26 was covalently bound to protein, since incubation of the nuclear matrix and nonmatrix samples for 2 hr at 60° with SDS–proteinase K solubilized at least 93% of the bound ^{3}H radioactivity.

TABLE 1. Binding of [^{3}H]VM-26 to nuclear matrix and nonmatrix protein of CEM and VM-1/C2 cells

	[^{3}H]VM-26 bound (pmol/mg protein)	
Cell line	Nuclear matrix	Nuclear nonmatrix
CEM	3.14 ± 0.17*	0.35 ± 0.12
VM-1/C2	1.48 ± 0.37*†	0.34 ± 0.52

Exponentially growing CEM or VM-1/C2 cells were incubated for 2 hr with 2 μM [^{3}H]VM-26. the nuclear matrix and nonmatrix fractions were isolated and sequentially extracted with 10% trichloroacetic acid (w/v), methanol, 1% SDS (w/v), and 10% trichloroacetic acid. The precipitates were solubilized by heating in 0.9 N NaOH, then were neutralized, and the [^{3}H]VM-26 was counted. Protein concentrations were determined by Coomassie blue straining. Values are means ± 1 SEM, N = 4.

* Significantly different ($P < 0.001$, two-tailed *t*-test) from the corresponding nuclear nonmatrix mean values.

† Significantly different ($P < 0.004$, two-tailed *t*-test) from the mean nuclear matrix value from CEM cells.

Incubation of the samples for 2 hr at 60° without SDS–proteinase K released less than 5% of the bound radioactivity. Table 1 shows that the covalent binding of [^{3}H]VM-26 was 9-fold greater per milligram of nuclear matrix protein of CEM cells than per milligram of high salt-soluble nonmatrix protein. The ratio decreased from 9-fold in CEM cells to about 4-fold in drug-resistant VM-1/C2 cells. The 2-fold decrease in binding of [^{3}H]VM-26 to matrix protein of VM-1/C2 cells was not the result of impaired nuclear uptake of [^{3}H]VM-26 in the drug-resistant cells. Total nuclear uptake of [^{3}H]VM-26 in CEM and CEM/VM-1/C2 cells averaged 79 ± 7 (SD) and 73 ± 8 (SD) pmol/10^7 cells, respectively.

Formation of VM-26-Stabilized Topoisomerase II–DNA Complexes at Various DNA Loop Domains

The above experiments did not address the question of whether the enhanced binding of [^{3}H]VM-26 to nuclear matrix protein represented increased drug binding to topoisomerase II–DNA complexes present in the nuclear matrix. This idea was explored by examining the VM-26-dependent formation of topoisomerase IIα cleavable complexes within several DNA loop domains.

Figure 2 is a schema that illustrates the strategy for radiolabeling various domains within DNA loops attached to the nuclear matrix. According to our hypothesis, VM-26-stabilized topoisomerase II cleavable complexes should be enriched in those regions of the DNA loops undergoing replication on the nuclear matrix. This hypothesis was tested by incubating CEM cells with [^{14}C]thymidine for 72 hr (about three doubling times) to uniformly label matrix, nonreplicating DNA (domain 1) and nonmatrix, nonreplicating DNA (domain 2). The cells then were incubated for 10 min with [^{3}H]thymidine to label both the actively replicating DNA on the nuclear matrix (domain 3) and the nascent DNA that had migrated away from the nuclear matrix during the 10-min labeling period as a result of replication fork movement (domain 4). Then the cells from each group were incubated with either no drug (control) or

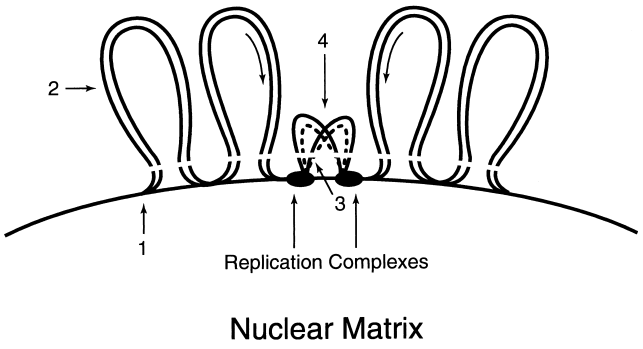

FIG. 2. Schema illustrating the strategy for radiolabeling various DNA loop domains. This schema shows DNA moving through fixed matrix-bound replication sets in a bidirectional fashion. For illustrative purposes, the DNA proximal and distal to the breaks in the DNA loops is termed nuclear matrix DNA and nonmatrix DNA, respectively. CEM cells were preincubated for 72 hr with [^{14}C]thymidine to label the nonreplicating matrix and nonreplicating nonmatrix DNA domains. Then the cells were incubated for 10 min with [^{3}H]thymidine to label both the actively replicating matrix DNA and the nascent DNA that had moved into the nonmatrix fraction during the 10-min incubation. The matrix and nonmatrix [^{3}H]DNA strands are shown as dashed lines. Domains: [1] matrix, nonreplicating [^{14}C]DNA; [2] nonmatrix, nonreplicating [^{14}C]DNA; [3] matrix, actively replicating [^{3}H]DNA; and [4] nascent [^{3}H]DNA that had migrated away from the nuclear matrix into the nonmatrix fraction.

with 5, 10, or 25 μM VM-26 for 30 min. Cleavable complex formation was also measured in whole nuclei from cells that were radiolabeled and incubated with VM-26 as described above.

The frequency of cleavable complex formation with newly replicated [^{3}H]DNA and nonreplicating [^{14}C]DNA in nuclei from cells treated with 25 μM VM-26 averaged 12 ± 2 (SEM) and 11 ± 2 (SEM) complexes per 1 x 10^{6} bp of DNA, respectively (Fig. 3A). A likely explanation for the lack of significant difference between the newly replicated and nonreplicating DNA is that the [^{3}H]DNA in whole nuclei is a mixture of DNA in domains 3 and 4, with domain 4 having a low frequency of cleavable complex formation (see below). VM-26 induced a concentration-dependent increase in the frequency of cleavable complex formation with actively replicating matrix [^{3}H]DNA (domain 3) (Fig. 3B). At 25 μM VM-26, the frequency was 32 ± 2 (SEM) complexes per 10^{6} bp, which was about 16-fold greater than that seen with either [^{3}H]DNA in nonmatrix domain 4 or [^{14}C]DNA in nonmatrix domain 2. Concentrations of VM-26 as high as 25 μM stabilized only minor amounts of cleavable complex formation in either of these nonmatrix DNA domains, since the DNA in the nonmatrix domains is distal to the matrix-bound replication sites. The frequency of cleavable complex formation was 13 ± 2 (SEM) complexes per 10^{6} bp with [^{14}C]DNA in the matrix fraction (domain 1). While some of this matrix [^{14}C]DNA was the parental DNA in the cleavable complexes, most of the matrix [^{14}C]DNA was present in DNA loops not undergoing replication during the drug treatment period (domain 1). When taken together, these results are consistent

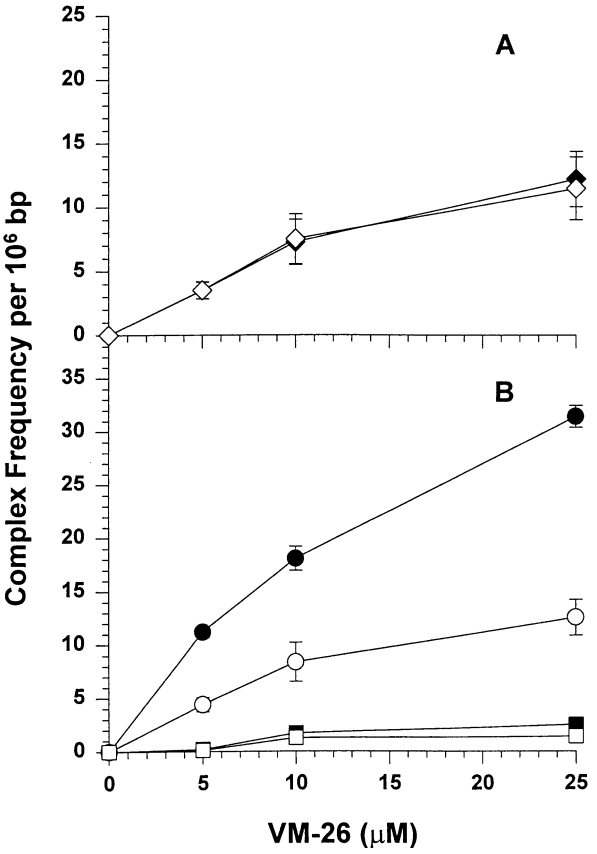

FIG. 3. Frequencies of VM-26-stabilized cleavable complexes at various DNA loop domains in CEM cells. Nonreplicating and newly replicated DNA in the nucleus (panel A) and in the nuclear matrix and nonmatrix fractions (panel B) were radiolabeled as described in the legend to Fig. 2. The cells were incubated for 30 min with the indicated concentrations of VM-26 after [^{14}C]thymidine labeling and prior to pulse-labeling with [^{3}H]thymidine. Cleavable complexes were quantitated using the K-SDS assay, and the frequencies of complex formation per DNA base pair were calculated as described in Materials and Methods. The average lengths of the nuclear, nuclear matrix, and nonmatrix DNAs were 2, 1, and 1 kb, respectively. Panel A: (△) nonreplicating [^{14}C]DNA; and (♦) newly replicated [^{3}H]DNA. Panel B: (○) matrix [^{14}C]DNA (domain 1); (□) nonmatrix [^{14}C]DNA (domain 2); (●) matrix [^{3}H]DNA (domain 3); and (■) nonmatrix [^{3}H]DNA (domain 4). Each point represents the mean of six determinations ± 1 SEM.

with the concept that VM-26 stabilizes cleavable complex formation preferentially with replicating DNA on the nuclear matrix (domain 3).

The above experiment was repeated using drug-resistant VM-1/C2 cells in which the amount of nuclear matrix topoisomerase IIα was 11% of that in CEM cells. Accordingly, a very low frequency of cleavable complex formation (less than 3 per 10^{6} bp) was observed in nuclei (Fig. 4A) as well as in nuclear matrix domains 1–4 of VM-1/C2 cells (Fig. 4B).

Effects of VM-26 on Nuclear Matrix-Bound DNA Replication

Since VM-26 preferentially stabilized cleavable complex formation with replicating DNA on the nuclear matrix,

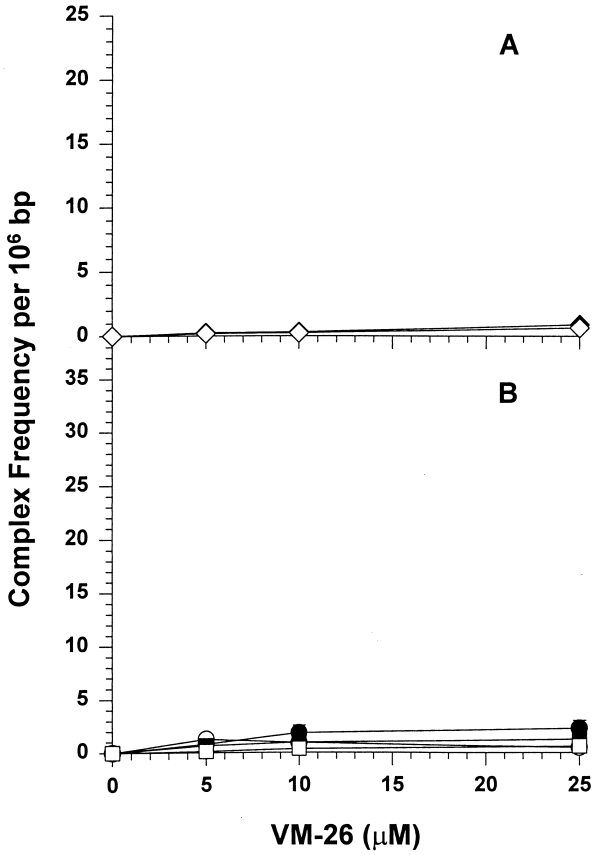

FIG. 4. Frequencies of VM-26-stabilized cleavable complexes at various DNA loop domains in VM-1/C2 cells. Nonreplicating and newly replicated DNA in the nucleus (panel A) and in the nuclear matrix and nonmatrix fractions (panel B) were radiolabeled as described in the legend to Fig. 2. The cells were incubated for 30 min with the indicated concentrations of VM-26 after [^{14}C]thymidine labeling and prior to pulse-labeling with [^{3}H]thymidine. Cleavable complexes were quantitated using the K-SDS assay, and the frequencies of complex formation per DNA base pair were calculated as described in Materials and Methods. Panel A: (△) nonreplicating [^{14}C]DNA; and (♦) newly replicated [^{3}H]DNA. Panel B: (○) matrix [^{14}C]DNA (domain 1); (□) nonmatrix [^{14}C]DNA (domain 2); (●) matrix [^{3}H]DNA (domain 3); and (■) nonmatrix [^{3}H]DNA (domain 4). Each point represents the mean of six determinations ± 1 SEM.

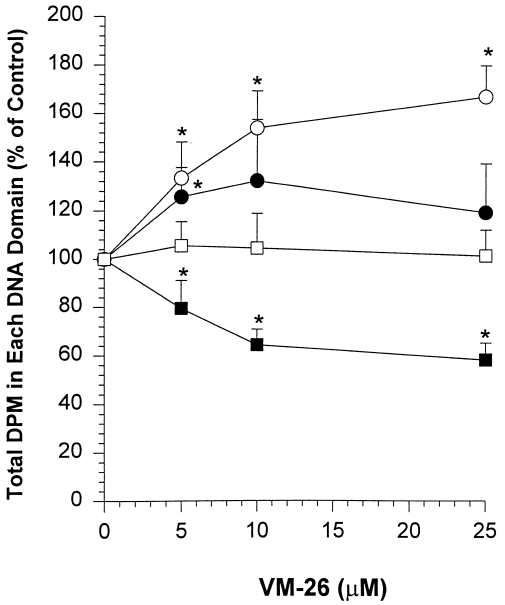

FIG. 5. VM-26-induced stalling of DNA replication forks on the nuclear matrix. Exponentially growing CEM cells were preincubated with 2 mM [^{14}C]thymidine (carrier-free) for 72 hr and then chased for 24 hr in radioactive-free EMEM plus serum. Then groups of 2 x 10^7 cells were incubated for 30 min with or without the indicated concentrations of VM-26 followed by a 10-min incubation with 11 nM [^{3}H]thymidine (carrier-free). The incorporation of ^{14}C and ^{3}H into nuclear matrix and nonmatrix DNA was determined as described previously [13]. Symbols: (○) matrix [^{14}C]DNA (domain 1); (□) nonmatrix [^{14}C]DNA (domain 2); (●) matrix [^{3}H]DNA (domain 3); and (■) nonmatrix [^{3}H]DNA (domain 4). Each point represents the mean of six determinations ± 1 SEM. Key: (*) $P < 0.02$ compared with the untreated control (two-tailed *t*-test).

further studies were done to evaluate the effects of VM-26 within the context of the nuclear matrix model of DNA replication (Fig. 2). In this model, newly replicated DNA moves away from the matrix-bound replication sites according to the rate of replication fork movement and then becomes incorporated into the nonmatrix (high salt-soluble) DNA [13, 17, 18].

CEM cells were incubated with [^{14}C]thymidine prior to VM-26 and with [^{3}H]thymidine after VM-26 to monitor drug effects on replication fork movement and DNA synthesis on the nuclear matrix, respectively. Under these conditions, the amount of [^{3}H]- or [^{14}C]DNA recovered from the nuclear matrix fraction at a specific time is determined by the rates of matrix binding of prelabeled [^{14}C]DNA or matrix synthesis of new [^{3}H]DNA minus the rates of migration of the [^{14}C]DNA or [^{3}H]DNA away from the nuclear matrix into the nonmatrix fraction. Analysis of the total ^{3}H and ^{14}C present in the nuclear matrix and nonmatrix fractions revealed that VM-26 induced an accumulation of both prelabeled [^{14}C]DNA and postlabeled [^{3}H]DNA in the nuclear matrix fraction (Fig. 5). These results support the concept that VM-26 inhibits the movement of the replication forks away from the matrix-bound replication sites to a greater extent than the synthesis of new [^{3}H]DNA on the nuclear matrix. This would occur if DNA continues to be synthesized on the nuclear matrix in the presence of VM-26, but further movement of the replication forks is blocked downstream where the forks encounter the VM-26-induced cleavable complexes or DNA damage. Equally important, the decreased amount of [^{3}H]DNA recovered from the nonmatrix fraction of VM-26-treated cells compared with that recovered from the control cells is also highly consistent with the VM-26-induced blockade of replication fork movement from the matrix to the nonmatrix fraction. In contrast, if VM-26 interfered with DNA synthesis to a greater degree than replication fork progression, then the amount of [^{3}H]DNA recovered in the matrix fraction of drug-treated cells would

have been less than that recovered in the matrix fraction from the untreated control cells. Similar amounts of [^{14}C]DNA were recovered from the nonmatrix fractions of VM-26 and untreated control cells, since the DNA in the nonmatrix fractions was labeled with ^{14}C prior to drug treatment (Fig. 5). As a negative control, CEM cells also were incubated with either 0, 0.1, 1, or 5 μM ara-C in place of VM-26, and the experiment was carried out as described above. Because ara-C interferes with DNA polymerization, it would be expected to decrease the amount of [^{3}H]DNA recovered from both the nuclear matrix and nonmatrix fractions rather than induce an accumulation of [^{3}H]DNA in the matrix fraction as observed in VM-26-treated cells. At 5 μM ara-C, the total amount of [^{3}H]DNA recovered from the matrix and nonmatrix fractions averaged 44 ± 2 and 47 ± 1% (SEM) of the untreated control, respectively. When taken together, the results indicate that VM-26 inhibits DNA synthesis by inducing replication forks to stall on the nuclear matrix.

DISCUSSION

Previous studies have shown that various topoisomerase II-active drugs preferentially stabilize cleavable complex formation with topoisomerase II and newly replicated DNA compared with nonreplicating, bulk DNA [5, 6, 8]. These complexes, or the DNA damage induced by the complexes, block DNA replication fork movement [7]. The results reported herein provide further insights into the subnuclear effects of VM-26 that result in the inhibition of replication fork movement. This study directly shows for the first time that VM-26-induced cleavable complexes form at the highest frequency on the actively replicating DNA that is bound to the nuclear matrix. Matrix-bound DNA loops that did not contain actively replicating DNA and nonmatrix regions of replicating DNA loops were considerably less enriched in cleavable complexes. These results may explain why VM-26 was very effective in blocking replication through the c-*myc* gene while inducing only a modest amount of cleavage in total c-*myc* DNA [7]. The preferential formation of VM-26-induced cleavable complexes at or near matrix-bound DNA replication forks appears to be related to several factors. DNA topoisomerase IIα is a major protein of the nuclear matrix of CEM and other cells [15, 19]. Also, the movement of replication forks creates single-stranded regions in matrix-bound DNA that are nucleosome-free [33]. Replicating and transcriptionally active DNA in an open conformation is likely more accessible to topoisomerase II and the topoisomerase II-active drugs than bulk DNA [34, 35].

Our work further supports the nuclear matrix model of DNA replication and provides evidence that the nascent DNA bound to the nuclear matrix is an important site of action of VM-26 stabilized cleavable complexes. When present in high concentrations on replicating matrix DNA, the cleavable complexes or the resultant DNA damage interfered with replication fork movement away from the replication sites on the nuclear matrix to a greater extent than the synthesis of new DNA. Since the average length of nuclear matrix DNA was about 1 kb in CEM cells, replication fork movement was blocked within this distance from the matrix-bound replication sites. Several lines of evidence indicate that the inhibition of replication fork movement results from VM-26-induced DNA damage ahead of the replication fork and not from direct drug effects on the enzymes involved in DNA replication. VM-26 at concentrations as high as 50 μM did not inhibit purified DNA primase or DNA polymerase α activities on poly(dT) or activated calf-thymus DNA templates, respectively (data not shown). Also, in CEM cells incubated with VM-26, replication fork movement stops at specific VM-26-induced cleavage sites ahead of the replication fork [7]. Others have reported that *m*-AMSA inhibits replicon initiation [36]. Their results may not be in conflict with the results reported herein. In their study, the effect of *m*-AMSA on the synthesis of DNA intermediates of about 50 kb and higher was monitored using sucrose density gradient centrifugation. The 50-kb intermediates are much larger than the nuclear matrix DNA of CEM cells (about 1 kb). Thus, inhibition of the synthesis of 50-kb DNA also reflects some inhibition of replication fork movement at sites downstream of the replication origins on the nuclear matrix.

In addition to the preferential formation of cleavable complexes at matrix-bound replication forks, it is likely that these complexes are more potent inducers of cytotoxicity than cleavable complexes formed with nonreplicating DNA away from the nuclear matrix. Replication forks are located at the bases of the matrix-bound DNA loops [16, 18]. Indirect evidence suggests that VM-26 and *m*-AMSA rapidly induce cleavage at replication forks and the detachment of the replicating DNA loops from the nuclear matrix [17]. Thus, the substantial enrichment of cleavable complexes that we observed on replicating matrix DNA may be an underestimate of the actual frequency of cleavable complex formation within this DNA domain.

Some recent evidence suggests that apoptotic degradation of DNA begins with the release of 50- to 100-kb DNA loops from the nuclear matrix [30, 31]. However, it is important to emphasize that the metabolic fates of the VM-26-stabilized cleavable complexes formed with either replicating matrix DNA or nonmatrix DNA are unknown. Some cleavable complexes may be reversed without inducing DNA loop release or apoptosis. Further work will need to address the efficacy of VM-26 in inducing cleavage within various nuclear matrix versus nonmatrix DNA domains as well as the relative potencies of the cleavages within these individual domains in generating apoptotic signals.

This study of the interactions of VM-26 with the nuclear matrix sheds light on possible alternative targets of the drug. There was a negligible frequency of cleavable complex formation in the nuclear matrix fraction of VM-1/C2 cells. However, covalent binding of highly purified [^{3}H]VM-26

was only 2-fold lower to nuclear matrix protein of VM-1/C2 cells than CEM cells. The extent to which the [^{3}H]VM-26 was bound to topoisomerase II in cleavable complexes versus other nuclear matrix proteins could not be determined with this assay. It has been reported that oxidative metabolism of VP-16 and VM-26 generates reactive intermediates that covalently bind to DNA or protein [37–39]. Thus, it was possible that some of the highly reactive metabolites of [^{3}H]VM-26 bound to alkylation sites on nuclear matrix proteins other than topoisomerase II. Nuclear matrix proteins have important roles in chromatin organization, replication, and RNA processing [16]. Therefore, the alkylation of matrix proteins by VM-26 metabolites may add to the cytotoxicity that results from the interaction of the parent drug with DNA topoisomerase II. Nevertheless, the decreased binding of VM-26 to nuclear matrix protein of VM-1/C2 cells does not appear to be the primary mechanism of VM-26 resistance, since VM-1/C2 cells were 63-fold resistant to VM-26 whereas binding of VM-26 was only 2-fold less to matrix protein of VM-1/C2 cells compared with CEM cells.

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