The Topoisomerase II Inhibitor VM-26 Induces Marked Changes in Histone H1 Kinase Activity, Histones H1 and H3 Phosphorylation, and Chromosome Condensation in G2 Phase and Mitotic BHK Cells

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Abstract. We have examined the effects of topoisomerase inhibitors on the phosphorylation of histones in chromatin during the G2 and the M phases of the cell cycle. Throughout the G2 phase of BHK cells, addition of the topoisomerase II inhibitor VM-26 prevented histone H1 phosphorylation, accompanied by the inhibition of intracellular histone H1 kinase activity. However, VM-26 had no inhibitory effect on the activity of the kinase in vitro, suggesting an indirect influence on histone H1 kinase activity. Entry into mitosis was also prevented, as monitored by the absence of nuclear lamina depolymerization, chromosome condensation, and histone H3 phosphorylation. In contrast, the topoisomerase I inhibitor, camptothecin, inhibited histone H1 phosphorylation and entry into mitosis only when applied at early G2. In cells that were arrested in mitosis, VM-26 induced dephosphorylation of histones H1 and H3, DNA breaks, and partial chromosome decondensation. These changes in chromatin parameters probably reverse the process of chromosome condensation, unfolding condensed regions to permit the repair of strand breaks in the DNA that were induced by VM-26. The involvement of growth-associated histone H1 kinase in these processes raises the possibility that the cell detects breaks in the DNA through their effects on the state of DNA supercoiling in constrained domains or loops. It would appear that histone H1 kinase and topoisomerase II work coordinately in both chromosome condensation and decondensation, and that this process participates in the VM-26–induced G2 arrest of the cell.

When cells of higher eukaryotes enter mitosis, the nucleus undergoes major rearrangements which include condensation of interphase chromatin into metaphase chromosome, breakdown of the nuclear envelope, and the assembly of the mitotic spindle. An early event in this process is the G2 phase phosphorylation of histone H1 up to the hyperphosphorylated state at metaphase. Such a correlation of histone phosphorylation and progression through G2 was first observed in the highly synchronous slime mold, Physarum polycephalum, and led to the hypothesis that histone H1 phosphorylation could play a key role in the regulation of the cell cycle (4–6, 24). In the macroplasmodia of Physarum polycephalum there is a massive hyperphosphorylation of histone H1 through G2 to M phase with 22–24 phosphates/H1 molecule at metaphase (37). The physarum H1, however, has a flexible basic COOH-terminal domain of 200 residues compared to the 100 residues for mammalian H1. In mammalian cells, there are zero to one phosphate per histone H1 during the G1 phase of the cell cycle, and this increases by another two phosphates during S phase. The level of phosphorylation then rises through G2 up to the hyperphosphorylated state of four to six phosphates per histone H1 at metaphase (17–19, 21). This correlation between histone phosphorylation and chromosome condensation was also observed during the induction of premature chromosome condensation in sea urchin eggs and in mammalian cells (1, 27).

Advancement of mitosis by up to 1 h was reported when partially purified heterologous histone H1 kinase was added to Physarum polycephalum 3 h before the onset of mitosis (6, 24). This advancement is very similar to the induction of mitotic events by the maturation promoting factor (MPF)[1] which is active during late G2 (13, 27, 28, 35, 44). MPF has been identified with the growth-associated histone H1 kinase (GA kinase) and is a complex of the kinase $p34^{cdc2}$ and the p62 cyclin B (2, 8, 9, 11, 12, 28, 43). Microinjection of $p34^{cdc2}$ into mammalian cells was shown to induce chromatin condensation along with other events indicative of early phases of mitosis (30). Thus, the growth-associated histone H1 kinase has a key role in the initiation of mitosis. However,

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1. *Abbreviation used in this paper*: MPF, maturation promoting factor.

little is known about the mechanisms that govern chromosome condensation and its coordination with other mitotic events.

Another major participant in mitosis is topoisomerase II. This enzyme can relax supercoiled DNA and resolve catenated and knotted DNA by transiently breaking and rejoining both strands of DNA (51, 52). Topoisomerase II was shown to be essential for the untangling of sister chromatids and chromosome separation during anaphase in yeast cells (22, 23, 49, 50). In addition, in cstop2 mutant yeast cells, the chromosomes of the temperature-arrested cells are less compact than their normal counterparts and resemble prophase chromosomes in mammalian cells (49). This suggests that topoisomerase II is required for the final condensation of chromosomes, but not for the initial stages. In an in vitro assay system using *Xenopus* mitotic extracts and mammalian nuclei, Newport and Spann (39) showed that topoisomerase II inhibitors completely prevented condensation of chromosomes. This inhibition occurred before prophase, as evidenced by the absence of visible chromosomes when examined under light microscopy. Thus, the importance of topoisomerase II in chromosome condensation and mitosis is clear although the details of its activity are unknown. It would appear that at least two major factors are involved in the process of chromosome condensation (a) GA kinase which governs the condensation of chromatin loops through histone H1 phosphorylation; and (b) topoisomerase IIs which are located at the bases of loops and act on DNA topology. The interrelationships of these factors are not understood and are the subject of this study.

The activity of topoisomerase II has been studied with VM-26, a well-characterized inhibitor of the enzyme (32). VM-26 does not have any DNA binding properties, making it a better choice over other inhibitors that act by intercalating DNA, and could affect DNA topology in chromatin loops. VM-26 acts by stabilizing an intermediate in the topoisomerase reaction in which each monomer of the dimeric enzyme is covalently attached to the 5′ ends of the double-stranded break in the DNA. Topoisomerase II was shown to be the only protein associated with these DNA breaks (32).

An important property of VM-26 is its high specificity. Previous studies have shown that cells are sensitive to VM-26 mainly in the S and G2 phases (16, 26, 36, 42, 45, 52), while at Go when little topoisomerase II is present, VM-26 has no effect on the survival of the cells (46, 47). Furthermore, mutant cells that were isolated for resistance to VM-26 have altered topoisomerase II (48). A mutation in the gene coding for topoisomerase II was also reported in cells that are resistant to adriamycin, another topoisomerase II inhibitor (7). These lines of evidence point to a strong specificity of VM-26 to the enzyme.

We have used VM-26 to inhibit topoisomerase II activity during the G2 and M phases of the cell cycle and monitored the effects on histone H1 kinase activity, on the levels of phosphorylation in histones H1 and H3, on chromosome condensation, and on nuclear envelope breakdown. Here we show that all these events are also affected by VM-26. We discuss how these changes might result either from the inactivation of topoisomerase II itself or from the DNA breaks induced by the action of VM-26 on topoisomerase II, and how these changes might participate in the cellular response to DNA damage.

Materials and Methods

Cell Synchronization

BHK cells were plated out at a density of 1×10^5 cells per 25-mm diameter culture dish for morphological studies, or at 3×10^6 cells per 100-mm diameter dish for phosphorylation experiments. A double block method was then applied for synchronization. The cells were first arrested in RPMI culture medium containing 0.25% calf serum for 48 h. They were then released by replacing the low serum medium with regular growth medium (containing 10% calf serum), and aphidicolin was added to a concentration of 1 μg/ml for 24 h to arrest the cells at early S phase. The cells were then released into S by washing off the aphidicolin and incubating with regular growth medium. After 6 h, colcemid was added to a concentration of 0.3 μg/ml to capture cells that enter metaphase.

Drug Treatment

VM-26 (Etoposide) was kindly provided by Dr. W. T. Bradner, Bristol Laboratories. Camptothecin, aphidicolin, and colcemid were purchased from Sigma Chemical Co. (St. Louis, MO). All drugs except for colcemid were dissolved in DMSO at 1,000× concentrations. Colcemid was dissolved in PBS as 1,000× stock.

Fluorescence Microscopy for Chromosome Morphology

Both attached and floating cells were recovered by trypsinization and fixed with 3.7% formaldehyde for 10 min on ice. The fixed cells were pelleted by centrifugation at 800 *g* for 5 min and resuspended in PBS containing 1 μg/ml of the fluorescent DNA stain Hoechst 33258 (3). After 2–5 min staining, the cells were pelleted again and resuspended in 10 μl Hanker Yates reagent containing 90% glycerol. The cells were mounted on glass slides and viewed through a Zeiss Standard microscope equipped with an epifluorescence unit. Photographs were taken on 35 mm Kodak Technical Pan #2415 film at 100 ASA. Chromosome condensation was assessed by counting the percentage of cells with mitotic chromosomes and by monitoring their morphology. Several hundred cells were counted in different fields on each slide. The slides were coded and scrambled so that mitotic cells can be scored without knowledge of their treatment or their position in the cell cycle.

Lamin Immunocytochemistry

Rabbit antiserum against BHK lamins A, B, and C (kindly provided by R. Hancock, Laval University Cancer Research Center, Hotel Dieu Hospital, Quebec) was used at a dilution of 1:1,000. Cells were grown on polylysine-coated coverslips and immunocytochemistry was performed as described by Earnshaw et al. (10), using TRITC-conjugated goat anti-rabbit IgG (Tago Inc., Burlingame, CA) as the secondary antibody. The cells were also counterstained with Hoechst 33258 for visualization of the chromosomes.

Histone Phosphorylation

To examine G2 phase-related histone phosphorylation, synchronized cells were released from aphidicolin block in normal culture medium for 4 h, after which the medium was replaced by phosphate-free medium containing 10% dialyzed calf serum. ^{32}P-orthophosphoric acid was then added to a concentration of 25 μCi/ml and the cells were incubated for the time periods specified in the figure legends. To extract histones, the medium was poured into a 50-ml polypropylene tube and centrifuged to collect any unattached cells. The cells that remained attached were harvested by trypsinization, and pooled with the pelleted cells from the medium. The cells were washed once with ice cold PBS before lysis in nuclei isolation buffer (250 mM sucrose, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1% (vol/vol) Triton X-100, 10 mM Tris-HCl, pH 8.0) containing 10 mM NaF, 50 mM iodoacetamide, 50 mM β-glycerophosphate, 50 mM $NaHSO_3$, and 2 mM PMSF. The nuclear pellets were collected by centrifugation at 10,000 *g* for 1 min at 4°C, and the supernatant was discarded. To extract total histones, the nuclear pellet was extracted twice with 0.4 N H_2SO_4 and the solubilized proteins were

precipitated with 20% TCA. This precipitate was then washed twice with acetone, dried under vacuum, and solubilized in acid-urea sample buffer (5% glacial acetic acid, 8 M urea, 5% glycerol). To isolate histone H1, the nuclear pellet was extracted twice with 5% PCA, and the solubilized proteins were precipitated and washed as described for the total histones. The very lysine-rich proteins which included histone H1 were then solubilized in acid-urea sample buffer containing 2.5 M urea instead of 8 M.

Gel Electrophoresis of Phosphorylated Histones

Phosphorylated histones H1 and H3 were resolved in 25 × 19 × 0.15-cm acid-urea gels (12% acrylamide, 0.08% bisacrylamide, 5% acetic acid, and 8 M urea), with 5% acetic acid as the running buffer. After an overnight preelectrophoresis, 40 μg protein was loaded onto each lane and electrophoresis was carried out for 36 h at 350 V at 4°.

To examine the degree of phosphorylation of histone H1, we used the acid-urea gel system described by Langan (31). The 25 × 19 × 0.075-cm gel was composed of 15% acrylamide, 0.1% bisacrylamide, 5% acetic acid, and 2.5 M urea. The running buffer was 5% acetic acid. After an overnight preelectrophoresis, 10 μg of the PCA extract was loaded onto each lane and electrophoresed for 48 h at 4°C at 350 V.

At the completion of electrophoresis, the gels were stained with Coomassie blue and dried before autoradiography using Kodak XAR 2 film to identify the phosphorylated species. The radioactivity of individual bands was quantitated with an Ambis radioactivity scanner or by scanning autoradiograms. Background readings were determined from adjacent areas of the gel.

Assay of H1 Kinase Activity In Vitro

Crude nuclear extracts containing histone H1 kinase activity were prepared by a modification of the method described by Lake (29). BHK cells were harvested from monolayer cultures by trypsinization and washed in cold PBS supplemented with 0.1 mM PMSF. The cells were lysed with a Dounce homogenizer in 10 packed cell volumes (PCVs) of buffer A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM β-mercaptoethanol, 1.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5 mM β-glycerophosphate, 0.2 mM PMSF, 0.01% polyoxyethylene 23-lauryl ether [Brij-35], 0.25 M sucrose) supplemented with 0.5% Triton X-100. Nuclei were recovered by centrifugation at 1,000 *g* for 10 min, washed twice in buffer A, and pelleted after each wash by centrifugation. Histone H1 kinase activity was solubilized by gentle vortexing of nuclei suspended in one PCV of buffer B (20 mM Tris-HCl, pH 7.4, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 0.01% Brij-35, 10 mM β-glycerophosphate, 10% glycerol) containing 0.4 M NaCl and 0.1 mM EDTA. The nuclear homogenate was centrifuged at 20,000 *g* for 30 min and H1 kinase activity was precipitated from the resulting supernatant with the addition of solid ammonium sulfate to 40% saturation. The ammonium sulfate precipitate was collected by centrifugation at 20,000 *g* for 20 min and was resuspended in 0.5 PCV of buffer B and dialyzed against buffer B containing 5 mM EDTA for 2 h. The dialyzed sample was clarified by centrifugation at 20,000 *g* for 10 min.

Histone H1 kinase activity was assayed in a 10-μl reaction mixture containing 50 mM Tris-HCl, pH 7.4, 10 mM β-mercaptoethanol, 0.1 mM EGTA, 10 mM $MgCl_2$, 100 μM γ-^{32}P-ATP (1,000 cpm/pmole) extract, and 0.5 mg/ml HeLa histone H1 (generously provided by J. W. Breneman and P. Yau, University of California, Davis, California). Reactions were initiated by the addition of enzyme, incubated for 10–15 min at 30°, and terminated by the addition of H_3PO_4 to a final concentration of 150 mM. Incorporation of ^{32}P was quantitated by spotting 10-μl aliquots onto P81 phosphocellulose paper (Whatman Inc., Clifton, NJ). P81 paper was washed four times in 75 mM H_3PO_4, once in acetone, dried, and counted in Beta-Max liquid scintillation cocktail (ICN K&K Laboratories Inc., Plainview, NY) (15). Incorporation of ^{32}P specifically into histone H1 substrate was determined as the difference between reactions containing histone H1 substrate and control reactions in which no substrate was added. VM-26 and camptothecin were prepared by serial dilutions in DMSO, and then diluted 10-fold in 1× assay buffer to yield 10× stock solutions. H1 kinase activity was assayed in the presence of 1–150 μM VM-26 or camptothecin by the addition of 1/10 vol of the appropriate stock solution and further diluted 10× in the reaction mixture.

Pulse Field Gel Electrophoresis

Mitotic cells were embedded in 0.5% low-gelling agarose in cell culture medium. They were lysed and digested for 48 h at 37°C in 0.5 M EDTA, pH 7.4, 0.9% sarkosyl, 0.1% SDS, and 0.5 mg/ml proteinase K. Electrophoresis was in 1% agarose in 1× TBE at 200 V and 14° on a CHEF-DR II system (Bio-Rad Laboratories, Richmond, CA). Switching times were 70 s for 10 h followed by 120 s for 9 h. The gels were then stained with ethidium bromide.

Results

Cell Synchronization

BHK cells were synchronized using the double block method described above. Fig. 1 shows the flow cytometric profiles of the cells as they progress synchronously through S, G2, and into M phase after release from aphidicolin block at the G1/S boundary. The cells were accumulated at mitosis by colcemid, an inhibitor of microtubule formation. The extent of the G2 phase was calculated by subtracting the percentage of mitotic cells from the percentage of cells in G2 + M phase. G2 started at ~5 h after release from the aphidicolin block, peaked at ~7 h, and ended at ~8.5 h. Mitosis was observed to peak at 10–11 h.

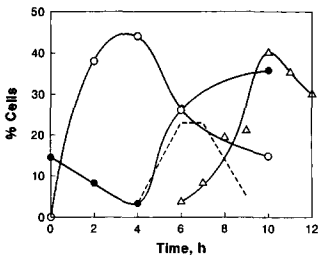

Figure 1. Cell synchronization. BHK cells were released from aphidicolin block at time 0 and colcemid was added at time 6. Their progress through S phase and G2+M phases was followed by collecting cells at different time points and analyzing their DNA content by flow cytometry. For the determination of M phase, additional time points were collected, the cells were fixed, their DNA stained with Hoechst 33258, and the mitotic cells were scored using fluorescent microscopy. The dashed line indicates the extent of the G2 phase; S phase cells are represented by open circles; G2+M phase cells are represented by filled circles; M phase cells are represented by open triangles.

VM-26 Blocks G2 and M Phase Phosphorylation of Histone H1

As cells progress through G2 into M, the level of phosphorylation on histone H1 increases from two to three phosphates per molecule at early G2 to four to six phosphates per molecule, depending on the subtype of histone, at metaphase. To determine the level of phosphorylation, synchronized BHK cells were allowed to progress through the cell cycle in the presence of inorganic ^{32}P-phosphate and colcemid to block the cells at metaphase. At different times, cells were collected, histone H1 was selectively extracted with 5% PCA, and analyzed by electrophoresis using an acid-urea gel system to resolve the different phosphorylated forms of H1 (31). As illustrated in Fig. 2 *A*, lanes *1–4*, the level of phosphorylation increased from a faster migrating, low phosphorylated form at early G2 (6 h) to slower migrating, highly phosphorylated forms at mitosis (12 h).

To examine the effect of VM-26, the drug was added to parallel cultures at 6, 7.5, or 9 h, corresponding to early, mid, and late G2, respectively. The cells were then collected at the 12-h time point, corresponding to the time of mitosis, and the levels of histone H1 phosphorylation determined. Fig. 2 *A*, lanes *5–7*, shows that in the presence of 5 μM VM-

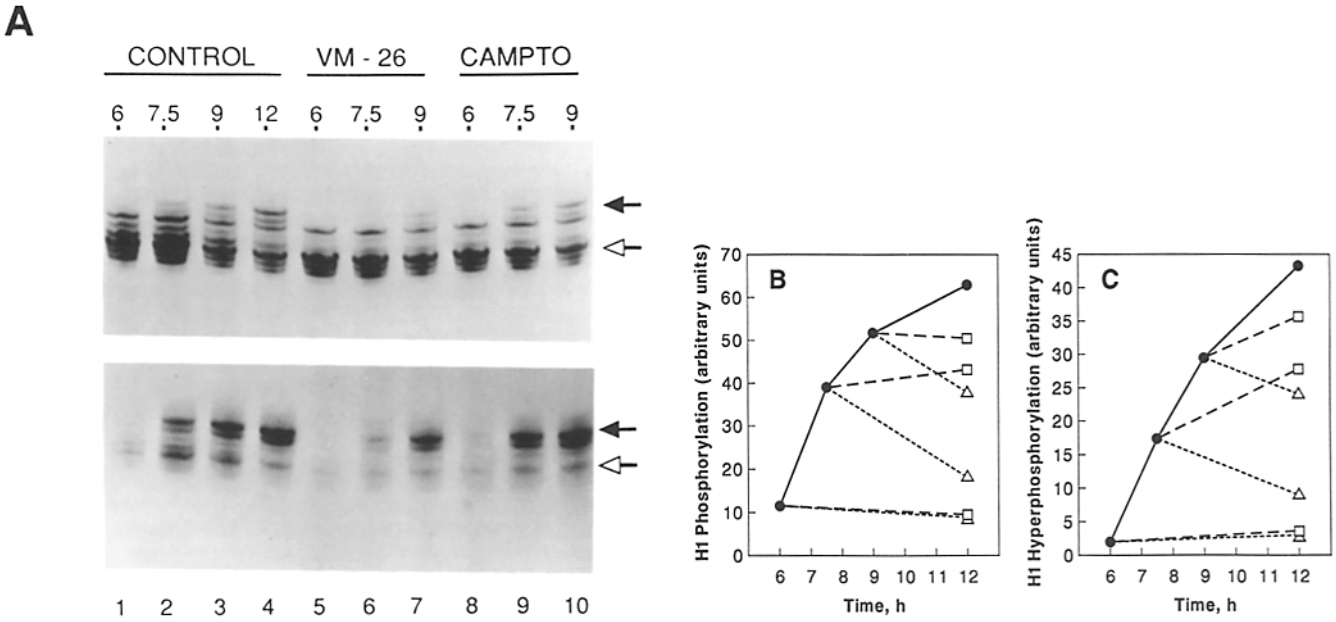

Figure 2. Effect of VM-26 and camptothecin on histone H1 phosphorylation. (*A*) $^{32}PO_4$ was added to synchronized cells 4.5 h after release from aphidicolin block. DMSO was added as a control to four cell aliquots at 6 h, and the cells were collected at 6, 7.5, 9, and 12 h (lanes *1–4*). At 6, 7.5, and 9 h, 5 μM VM-26 (lanes *5–7*) or 5 μM camptothecin (lanes *8–10*) was added to other aliquots and the cells were harvested at 12 h. Histone H1 was extracted and run on an acid–urea gel. The upper panel shows the Coomassie blue–stained gel and the lower panel is the corresponding autoradiogram. The open arrowhead shows the migration of low phosphorylated forms of histone H1, and the filled arrowhead shows the migration of the hyperphosphorylated forms. (*B*) The relative H1 phosphorylation of the samples in *A* was determined by quantitating the radioactivity associated with all H1 bands in each lane, then dividing the amount of protein determined from scans of the Coomassie blue–stained gel. (*filled circles*) Cells treated with DMSO; (*open triangles*) cells treated with VM-26; (*open squares*) cells treated with camptothecin. *C* is as *B* except that the relative H1 hyperphosphorylation is displayed, as determined from the radioactivity of the three slowest migrating radioactive bands in *A*.

26, H1 phosphorylation did not reach the high levels attained by control cells at mitosis (compare lanes *5*, *6*, and *7* with lane *4*). Fig. 2 *B* shows the quantitation of the total radioactivity associated with histone H1 and confirms that the level of phosphorylation did not increase further upon incubation with VM-26. In fact, Fig. 2 *B* reveals that the level of H1 phosphorylation actually decreased when the inhibitor was applied at 7.5 and at 9 h. Analysis of the most hyperphosphorylated (the slowest migrating) forms of histone H1 which are characteristic of the mitotic form of histone H1, shows that H1 hyperphosphorylation was blocked by VM-26 at the 6 h, and noticeably decreased when the inhibitor was added at 7.5 or 9 h (Fig. 2 *B*). These results indicate that in addition to the inhibition of histone H1 phosphorylation, VM-26 induces partial dephosphorylation.

*Table I. In Vitro Effects of VM-26 and Camptothecin on Histone H1 Kinase Activity**

Drug concentration	Relative H1 kinase activity
μM	%
VM-26	
1	89
3	95
5	89
30	90
50	94
150	101
camptothecin	
1	99
3	85
5	86
30	98
50	104
150	98

* Assay conditions were as described under Materials and Methods. Each activity value was determined at least three times. The mean values of the determinations are reported relative to control reactions in which no drug was added.

Table II. In Vivo Effects of VM-26 on Histone H1 Kinase Activity: Relative Activity of Histone H1 Kinase

Times after release from aphidicolin block	Control	VM-26
h		
6.0	1.0	0.5
7.5	1.5	0.6
9.0	1.8	1.5
11.0	2.1	–

Synchronized BHK cells were released from aphidicolin arrest and control samples were assayed for histone H1 kinase activity at the times indicated. For the VM-26–treated cells, 5 μM VM-26 were added to the cells at the times indicated after release from aphidicolin arrest, and the cells were harvested at the eleventh hour for assay of histone H1 kinase activity. Units of kinase activities are expressed relative to the control sample taken 6 h after release from aphidicolin block.

VM-26 Does Not Inhibit H1 Kinase In Vitro

This inhibition of histone H1 phosphorylation was unexpected and we investigated the possibility that VM-26 directly inhibits the activity of histone H1 kinase. The in vitro effect of VM-26 on the histone H1 kinase activity was measured using a crude nuclear extract. Histone H1 isolated from HeLa cells was used as the substrate for the GA kinase assay. Table I clearly shows that VM-26 has no inhibitory effect on H1 kinase activity up to 150 μM, a concentration 30-fold higher than that used for the above-mentioned experiments with living cells. Therefore, the observed inhibition of H1 phosphorylation is not due to a direct effect of the drug on the kinase, nor is it a result of a general inhibition of protein phosphorylation since histone H2A phosphorylation, which is cell cycle–independent, was minimally affected by VM-26 (see Fig. 5 *C*).

VM-26 Inhibits H1 Kinase Activity In Vivo

Although VM-26 does not inhibit histone H1 kinase activity directly, it could have in vivo effects either by inhibiting the activation of the kinase or by promoting its inactivation. To examine this, synchronous cells were treated with VM-26 at 6, 7.5, and 9 h, harvested at the time corresponding to mitosis, and nuclear extracts containing the H1 kinase were prepared. Nuclear extracts were also prepared from untreated cells harvested at 6, 7.5, 9, and 11 h. The results of the measurements of the kinase activities are summarized in Table II. In the absence of VM-26, H1 kinase activity increased as the cell progressed through G2 and into M. When VM-26 was added to the cells, the kinase activity did not increase. Instead, a marked decrease in activity was noted. This result indicates that VM-26 affects the activation or inactivation of histone H1 kinase.

VM-26 Prevents Chromosome Condensation

If, as has been proposed (4–6), H1 hyperphosphorylation is essential for chromosome condensation and entry into mitosis, then, in the presence of VM-26, chromosome condensation should not occur because of the absence of histone H1 hyperphosphorylation. VM-26 was added to synchronized cells at 6, 7, 8, 9, 10, or 11 h after release from aphidicolin arrest, and chromosome condensation was determined morphologically at the time of mitosis (12 h). The condensed chromosomes were then stained with the fluorescent dye Hoechst 33258. The slides were scrambled so that the observer would not know the time points that were analyzed or whether the cells were treated with drugs. Several hundred cells were scored for each time point. Fig. 3 *A* shows that VM-26 completely prevented the formation of visibly condensed chromosomes when added at any time in the G2 phase. At the resolution afforded by light microscopy, the chromatin of VM-26–treated cells was indistinguishable from that of untreated G2 cells at the time when VM-26 was added (see Fig. 4). This inhibition of chromosome condensation was not observed when DMSO, the solvent for VM-26, was added to the cells (Fig. 3 *B*). This inhibition of chromosome condensation by VM-26 was concentration dependent,

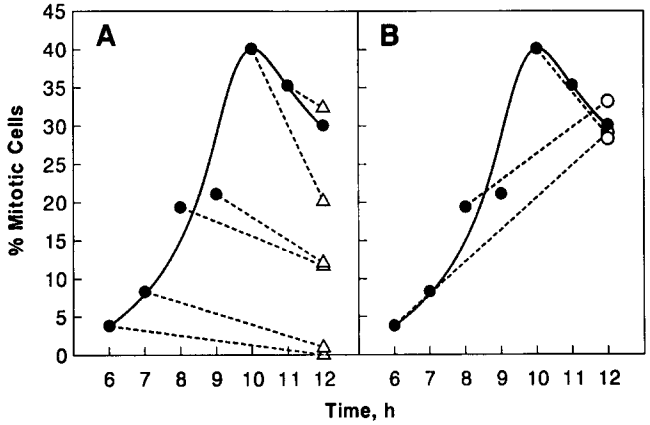

Figure 3. Effect of VM-26 on chromosome condensation. (*A*) Cells synchronized as in Fig. 1 were collected at 1-h intervals and the percentage of cells with visibly condensed chromosomes were determined (*closed circles*). At 6, 7, 8, 9, 10, and 11 h, 5 μM VM-26 was added and the percentage of mitotic cells was determined at 12 h (*open triangles*) (*B*) In a parallel run, control cells were treated with DMSO at 6, 8, and 10 h and collected at 12 h (*open circles*).

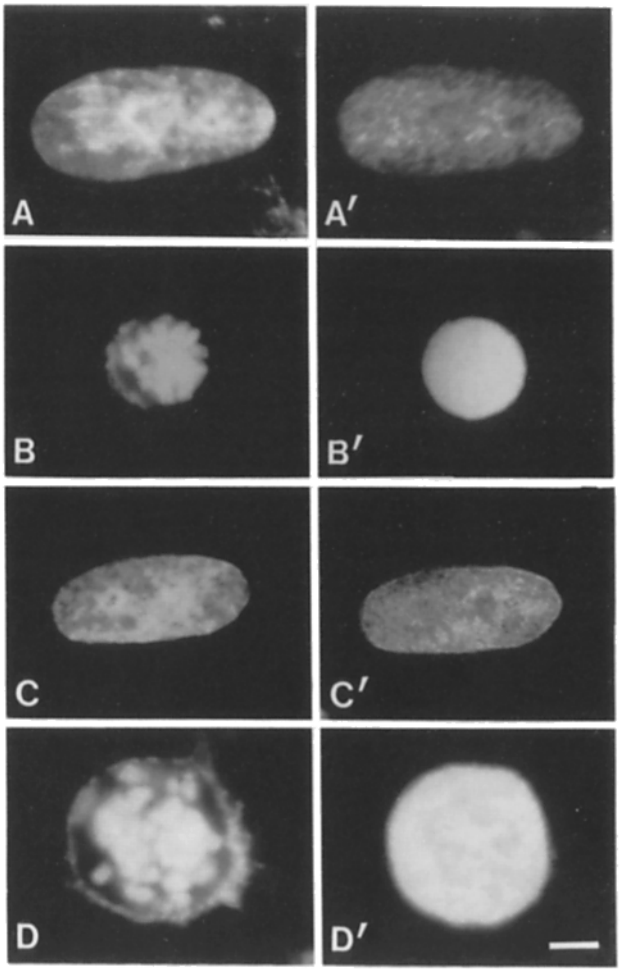

Figure 4. Effect of VM-26 and camptothecin on chromosome condensation and nuclear lamina depolymerization. DNA and lamins were visualized in the same cells by labeling with Hoechst 33258 (*A–D*) and lamin antibodies (*A′–D′*). DMSO (*A*, *A′*, *B*, *B′*), 5 μM VM-26 (*C*, *C′*), or 5 μM camptothecin (*D*, *D′*) were added at 9 h and the cells were harvested and fixed at 11 h. Control cells were fixed and stained at 9 h (*A*, *A′*) and at 11 h (*B*, *B′*). Bar, 5 μm.

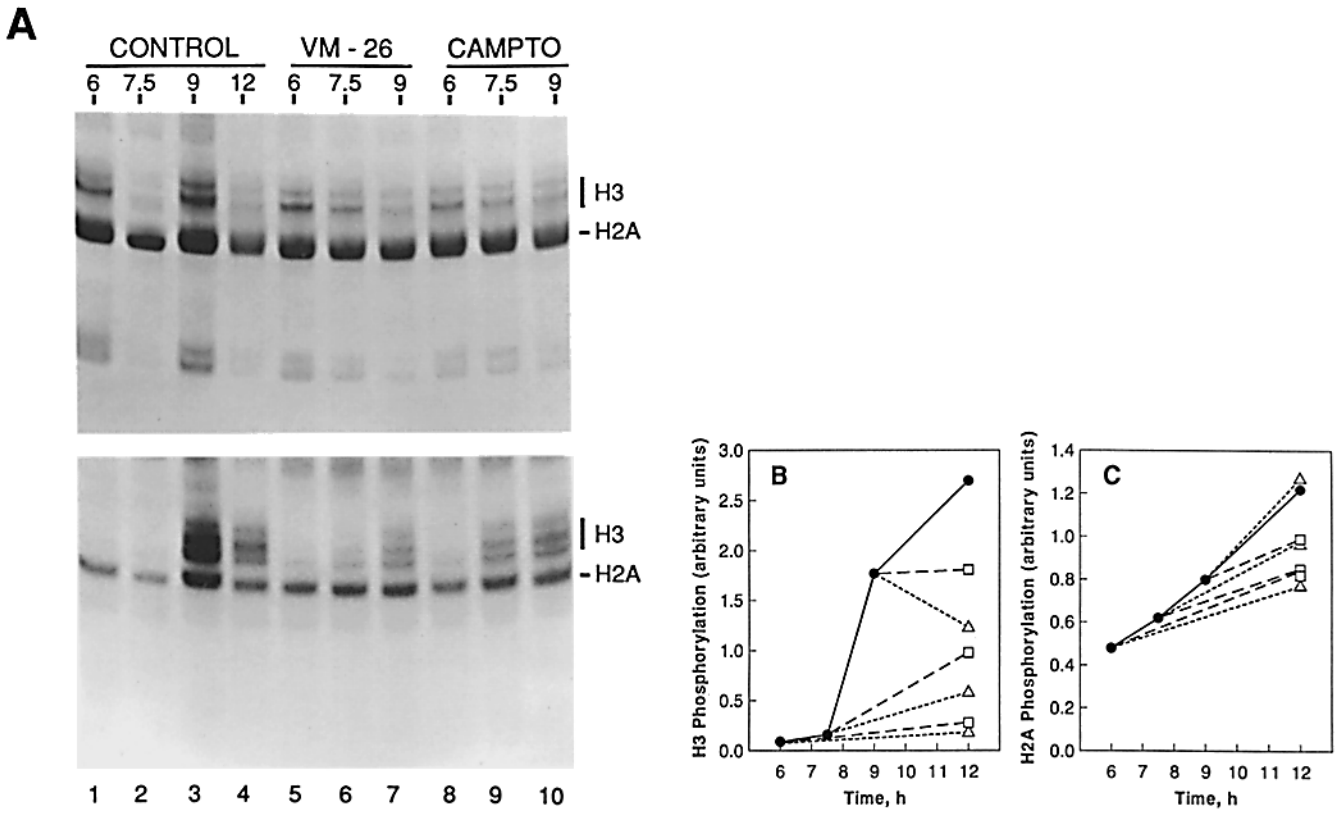

Figure 5. Effect of VM-26 and camptothecin on the phosphorylation of histone H3 and histone H2A. In the same experiment as in Fig. 2, aliquots of isolated nuclei were treated with sulfuric acid to extract core histones, and the proteins were separated in an acid–urea gel. The upper part of *A* shows the proteins stained with Coomassie blue and the lower part shows the corresponding autoradiogram. *B* shows the quantitation of histone H3 phosphorylation and *C* shows the quantitation of histone H2A phosphorylation; both are expressed relative to the amount of protein loaded as determined from the Coomassie blue–stained gel. (*filled circles*) Cells treated with DMSO; (*open triangles*) cells treated with VM-26; (*open squares*) cells treated with camptothecin.

with a 50% inhibition observed at a concentration of 1.5 μM (data not shown).

VM-26 Prevents Nuclear Lamina Disassembly and M Phase Phosphorylation of Histone H3

At mitosis, the onset of chromosome condensation is accompanied by phosphorylation of nuclear lamins, depolymerization of the nuclear lamina, and nuclear envelope breakdown at prometaphase. To determine if these events could occur independently of chromosome condensation and H1 phosphorylation and be unaffected by VM-26, the fate of the nuclear lamina was determined by immunofluorescence using antibodies raised against lamins A, B, and C from BHK cells. The cells were counterstained with Hoechst 33258 to monitor the degree of condensation of the chromosomes. During interphase, the nuclear lamina surrounds the nucleus and lamin immunofluorescence is localized at the nucleus. Upon entry into mitosis, the breakdown of the nuclear envelope is accompanied by depolymerization of lamins, and immunofluorescent staining shows dispersal of the lamins throughout the cytoplasm (Fig. 4). The addition of VM-26 in early, mid, or late G2 (6, 7.5, and 9 h, respectively) clearly prevented lamina depolymerization. At the time of mitosis, the cells that were blocked from activating their H1 kinase, phosphorylating histones, and chromosome condensation by the presence of VM-26 all had intact nuclear lamina.

Another mitosis-specific event is the phosphorylation of histone H3 at metaphase. As shown in Fig. 5, VM-26 also prevents this phosphorylation of histone H3. At the 9-h time point when a significant number of cells have already entered mitosis, a partial dephosphorylation of histone H3 can be observed. In the case of histone H2A which is phosphorylated through the cell cycle, VM-26 was found to slow down phosphorylation without the dephosphorylation that was seen with histones H1 and H3.

Therefore, the addition of VM-26 during G2 phase blocks at least four mitosis-related events: H1 phosphorylation, chromosome condensation, nuclear lamina depolymerization, and histone H3 phosphorylation.

VM-26 Induces Partial Unfolding of Metaphase Chromosomes and DNA Strand Breaks in Colcemid-arrested Cells

In addition to preventing cells from entering mitosis, VM-26 also has a striking effect on the morphology of metaphase chromosomes in colcemid-arrested cells. Fig. 6 shows the appearance of chromosomes in cells that were treated with DMSO (Fig. 6 *A* and *E*) or VM-26 (Fig. 6, *C*, *D*, *G*, and *H*) as revealed by staining with Hoechst 33258. In untreated cells, the chromosomes displayed the typical X shape with brightly fluorescent and well-defined chromatids. DMSO alone did not have any visible effect on this morphology. In

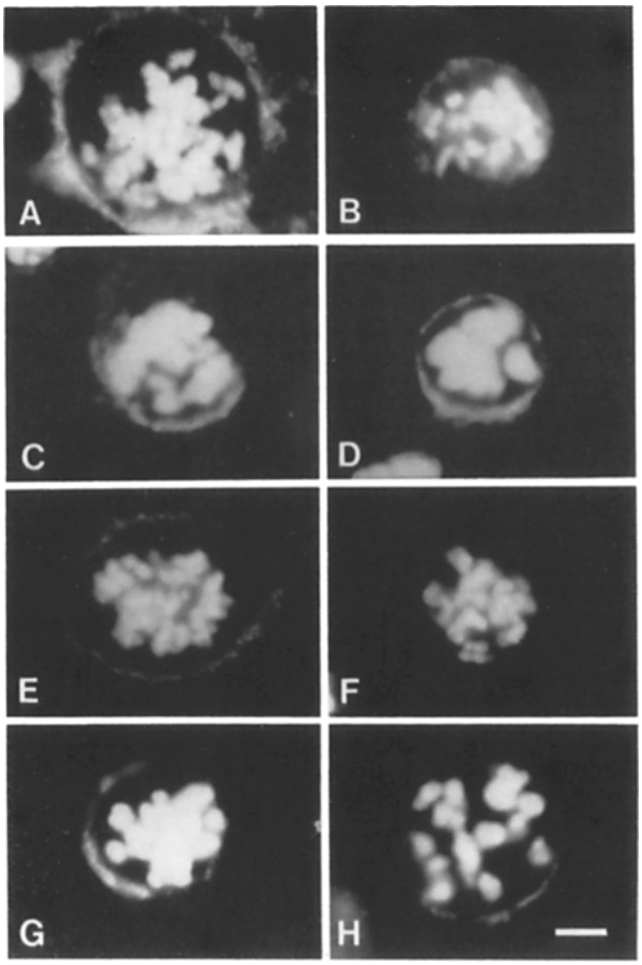

Figure 6. Effect of VM-26 and camptothecin on mitotic chromosome morphology. Cells were treated with DMSO (*A* and *E*), 5 μM camptothecin (*B* and *F*), or 5 μM VM-26 (*C, D, G, H*) for 2 h (*A–D*) or 3.5 h (*E–H*). The DNA was then stained with Hoechst 33258. Bar, 5 μm.

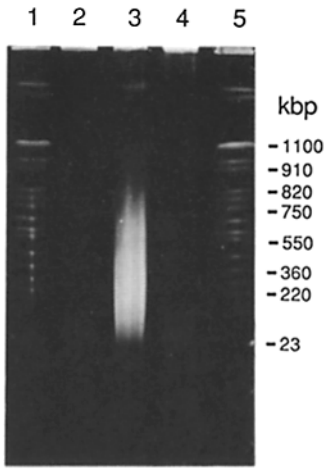

Figure 7. Induction of DNA strand breaks in metaphase chromosomes by VM-26. Mitotic cells (>95%) were collected by shake off of colcemid-arrested monolayers. DMSO (lane *2*), 5 μM VM-26 (lane *3*), or 5 μM camptothecin (lane *4*) was added for 2 h, after which the cells were embedded in agarose, lysed, and digested with proteinase K as described in Materials and Methods. (lanes *1* and *5*) *Saccharomyces cerevisiae* chromosomal DNA and Hind III digested lambda DNA. Agarose gel inserts in loading wells are shown at the top of the gel.

contrast, cells treated with VM-26 had chromosomes that appeared enlarged with more rounded and less fluorescent arms, and less defined edges. These chromosomes appeared partially decondensed. 1 h after the addition of VM-26, the chromosomes in several cells displayed such decondensed state. After 2 h, the chromosomes in every cell have adopted the partially unfolded conformation. With increasing times of exposure, the chromosomes become even more rounded and, in many cases, the X shape became unrecognizable, making it difficult to score them as mitotic cells. This could be the reason for the decrease in number of mitotic cells after treatment of VM-26 (Fig. 3).

In addition, double-stranded breaks in the DNA were detected with exposure to VM-26 (Fig. 7, lane *3*). Pulsed field gel electrophoresis showed a fragmentation of the DNA ranging from 20 to 800 kb, comparable with the 10–200 kb estimated for the sizes of DNA loops in mammalian chromosomes. As revealed in lanes *2* and *4*, DNA from untreated control (lane *2*) or the camptothecin-treated cells (lane *4*) showed no fragmentation.

The altered morphology of the chromosomes and inhibition of entry into mitosis by VM-26 was not a result of cell death because exposure of cells to 0.5–50 μM for up to 6 h did not cause any increase in the number of cells that took up trypan blue compared to untreated cells (data not shown).

A Topoisomerase I Inhibitor Blocks Histone H1 Phosphorylation and Entry into Mitosis Only in Early G1

A possible role of topoisomerase I on histone H1 phosphorylation was also investigated using the specific inhibitor camptothecin. Like VM-26, camptothecin inhibits topoisomerase I by stabilizing the enzyme–DNA complex containing a single strand DNA break (32). Fig. 2 shows the effects of 5 μM camptothecin on histone H1 phosphorylation when applied in early, mid, or late G2. Quantitation of histone phosphorylation in Fig. 2 *B* and H1 hyperphosphorylation in Fig. 2 *C* shows that camptothecin, like VM-26, completely inhibited H1 phosphorylation when applied in early G2. However, the effects later in G2 differ markedly from that of VM-26. At the 7.5- and 9-h time points, camptothecin partially inhibited H1 phosphorylation without inducing and dephosphorylation. Camptothecin had no effect on histone H1 kinase activity in vitro (Table I). Effects of camptothecin on the phosphorylation of histone H2A were small, comparable to that of VM-26 (Fig. 5).

Inhibition of chromosome condensation by camptothecin was seen only when applied at early G2 (Fig. 8). Similarly, the inhibitor prevented nuclear lamina depolymerization when applied in early G2. The effects on histone H3 phosphorylation were similar to that of H1 phosphorylation in that the phosphorylation was completely inhibited when the drug was added at early G2, and the level of H3 phosphorylation was reduced at late G2 (Fig. 5). Finally, camptothecin did not induce any unfolding of metaphase chromosomes (Fig. 6) nor did it induce any DNA strand breaks (Fig. 7). These results suggest that topoisomerase I may not be required at late G2 for chromosome condensation and entry into mitosis.

Discussion

We have shown that the topoisomerase II inhibitor, VM-26, when applied in the G2 phase, has a number of unexpected

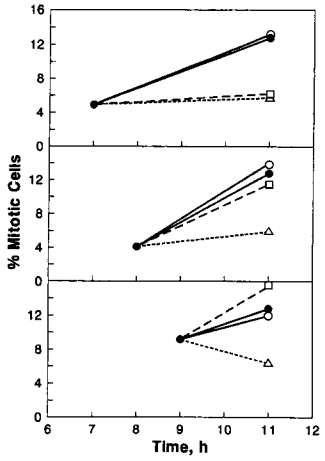

Figure 8. Effect of topoisomerase inhibitors on chromosome condensation. The inhibitors were added to a concentration of 5 μM to synchronized cells at 7 h (*top panel*), 8 h (*middle panel*), and 9 h (*bottom panel*), and the percentage of mitotic cells was determined at 11 h. (*closed circles*) No treatment; (*open circles*) DMSO treated; (*open triangles*) VM-26 treated; (*open squares*) camptothecin treated.

effects: it blocks H1 phosphorylation and inhibits H1 kinase activity by acting on factors involved in the control of its activity, it prevents chromosome condensation, and it blocks other mitotic events such as nuclear lamina disassembly and histone H3 phosphorylation. In addition, VM-26 may act to reverse some of these processes. This was most obvious in mitotic cells where it could be shown that chromosomes partially decondense and that H1 and H3 become partially dephosphorylated. It is possible that such a decondensation occurs in G2 phase cells, but without easy morphological identification of chromatin condensation, we could not make such a distinction.

Inhibition of H1 Kinase and of H1 Phosphorylation in G2 Cells

In cells that were exposed to VM-26 during the G2 phase, the activation of H1 kinase and the increase in H1 phosphorylation that would have occurred normally were completely blocked. Moreover, cells did not enter mitosis, as was determined morphologically by the absence of condensed chromosome and nuclear lamina depolymerization, and biochemically by the absence of hyperphosphorylated histone H1 and H3. However, an inherent limitation to the use of these drugs is that they do not allow the distinction between effects caused by inhibition of the enzyme per se and the effects due to the generation of DNA breaks. Studies done on topoisomerase II mutants in yeast show that cells can enter mitosis in the absence of any enzyme activity (50). This would suggest that the observed G2 block in our study may not be due to the inhibition of the topoisomerase II activity but to DNA breaks caused by the action of VM-26.

It is well documented that lesions in DNA induced by X-irradiation and other DNA-damaging agents arrest cells in the G2 phase (33, 34, 41). Recent studies showing that cells deficient in DNA repair are hypersensitive to VM-26 suggest that DNA breaks caused by the drug are recognized by cells as DNA lesions even though these breaks remain covalently bound to topoisomerase II (25). In yeast, the gene RAD9 was identified to control the cell cycle response to DNA damage, and a mutation in this gene permits cells to enter mitosis despite DNA damage (20, 53). It was suggested that RAD9 functions as a negative regulator by interacting with proteins such as MPF or cyclins that participate in the commitment to mitosis (53). The arrest of BHK cells in G2 by VM-26 could operate through such a mechanism. As MPF and H1 kinase were shown to be the same, it is of interest that VM-26 not only prevents the activation of the kinase, but promotes its deactivation. Hence, binding of a negative regulator to the H1 kinase would be a simple mechanism that would explain this dual effect.

In the temperature-sensitive tsBN2 cell line derived from the BHK, premature chromosome condensation (PCC) can be induced in the G2 phase by shifting the cells up to the restrictive temperature (40), and this can occur even when the cells are treated with DNA-damaging agents, resulting in condensed but fragmented chromosomes. A mutation in the RCC1 gene relieves the dependency of mitosis on intact DNA in these cells at the restrictive temperature. However, we have observed that shift-up of these cells to the restrictive temperature in the presence of VM-26 prevented PCC and histone H1 phosphorylation (Th'ng, J., M. Roberge, and E. M. Bradbury, manuscript in preparation). This raises the possibility that the G2 arrest caused by VM-26 is not dependent on a control mechanism for DNA repair. Obviously, more work will be necessary to clarify this point.

Chromosome Condensation and Histone Dephosphorylation in Metaphase-arrested Cells

A second effect of VM-26 on cells was observed when the drug was added to metaphase-arrested cells. When histones H1 and H3 were partially dephosphorylated, the chromosomes decondensed visibly, and double-stranded breaks occurred in the DNA. There appears to be a correlation between the level of phosphorylations on histones H1 and H3 and the degree of condensation of the chromosomes. These results suggest that either active topoisomerase II or intact DNA is necessary for the maintenance of the condensed state of metaphase chromosomes.

The level of histone H1 phosphorylation is determined by the net phosphorylating activity that results from the activities of the kinases and phosphatases. Partial or full inactivation of the kinases would result in a dephosphorylation of the substrate, as observed for H1 and H3. The effects of VM-26 are specific to the control of H1 and H3 kinases because histone H2A phosphorylation appears to be unaffected. These changes in histone phosphorylation and chromosome condensation probably constitute an intrinsic part of the cellular response to DNA damages such that the dephosphorylation of histones H1 and H3 would induce chromatin decondensation, thereby allowing the repair machinery access to the DNA. The partial decondensation was observed during mitosis when the chromosomes were visible under the light microscope. During G2, when the chromosomes have not sufficiently condensed to become visible under light microscopy, a similar decondensation of the chromatin can be expected because the histones were similarly dephosphorylated. These decondensation processes could be a determining factor in the inability of the cells to initiate mitosis when the DNA is damaged.

Camptothecin Does Not Block Entry into Mitosis in Mid and Late G2 Cells

Camptothecin, the topoisomerase I inhibitor employed in this study, prevented entry in mitosis only when applied in early G2. It would appear that topoisomerase I does not play a di-

rect role in the initiation of mitosis and in chromosome condensation. This is consistent with results obtained with topoisomerase I mutants in yeast showing that the cells could proceed normally through the cell cycle in the absence of a functional topoisomerase I (49). The inhibitory effects observed when camptothecin was applied in early G2 could be caused by interference with transcription of genes necessary for passage through G2 and subsequent entry into mitosis.

It is significant that although camptothecin slowed down phosphorylation of histones H1 and H3, no accompanying dephosphorylation was observed, nor was there any chromosome decondensation in mitotic cells. The implication is that these dephosphorylation events are specific responses to the inhibition of topoisomerase II or to DNA strand breaks.

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