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DNA Topoisomerase II Is Required for Formation of Mitotic Chromosomes in Chinese Hamster Ovary Cells: Studies Using the Inhibitor 4′-Demethylepipodophyllotoxin 9-(4,6-*O*-Thenylidene-β-D-glucopyranoside)†

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Received April 4, 1990; Revised Manuscript Received June 14, 1990

ABSTRACT: To study the biochemical processes which DNA topoisomerase II carries out in mammalian cells, which have not been identified, we have examined the effects on chromosome replication in Chinese hamster ovary cells of an agent which traps molecules of topoisomerase II when they are covalently integrated into DNA during their reaction. This agent, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-thenylidene-β-D-glucopyranoside) (VM-26), targets this enzyme specifically according to a compelling body of evidence. Using synchronously growing cells, we found that VM-26 at a cytotoxic concentration (0.08 μM) did not affect DNA replication during the S phase. The formation of mitotic chromosomes was delayed by 4 h, and its rate was reduced thereafter, causing a delay in mitosis of >14 h in 65% of the cells; in some cells, the chromatin was aberrantly condensed, forming diffuse chromosomes or particles. Chromosome formation was completely inhibited at 0.32 μM VM-26. DNA fragments derived from topoisomerase II molecules covalently integrated in DNA and trapped by VM-26 were detected by FIGE analysis in the G2 period, but not during the S phase. The delay of chromosome formation appeared to be caused by two factors: first, a delay in the completion of DNA replication, because progress of some cells to mitosis after removal of VM-26 was prevented by aphidicolin, an inhibitor of DNA polymerases α and δ; and second, a delay of chromosome formation in cells which had apparently completed DNA replication. The observations reported here show that topoisomerase II carries out reactions which are essential for formation of mitotic chromosomes. They are compatible with a model in which topoisomerase II functions both during the completion of DNA replication and in a subsequent process which, by analogy with VM-26-sensitive steps in simian virus 40 DNA replication, may be the topological conversion of a series of replicated DNA loops or domains into two linear chromatid-length DNA molecules.

DNA topoisomerase II (Cozzarelli, 1980; Gellert, 1981; Wang, 1985; Vosberg, 1985; Maxwell & Gellert, 1986) carries out reactions which are essential for segregation of the chromosomes of simian virus 40 (SV40)[1] and of yeast. In SV40, its activity is required for the decatenation of progeny DNA molecules by passing one double-stranded daughter molecule across the second; except during replication of the terminal ≃250 bp region, which may occur simultaneously with decatenation (Varshavsky et al., 1983; Snapka et al., 1988), topoisomerase II is not required for DNA replication (Snapka, 1986; Richter et al., 1987; Yang et al., 1987; Richter & Strausfeld, 1988; Snapka et al., 1988) but can replace topoisomerase I in this process (Yang et al., 1987). In yeast, topoisomerase II is essential for the segregation of chromosomes at mitosis (DiNardo et al., 1984; Uemura & Yanagida, 1984, 1986; Uemura et al., 1987), but, as in SV40, it is not required for DNA replication although it can replace topoisomerase I in this process (Uemura & Yanagida, 1984, 1986). The prokaryotic homologue of topoisomerase II, DNA gyrase, is, in contrast, essential both for DNA replication and for chromosome segregation in *Escherichia coli* (Kreuzer & Cozzarelli, 1979; Snyder & Drlica, 1979; Steck & Drlica, 1984).

The biochemical processes which topoisomerase II carries out during the replication of the chromosomes of mammalian

† Supported by MRC of Canada Grant MA-9589, by the NCI of Canada, and by a scholarship to M.C. from the FRSQ (Québec).
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[1] Abbreviations: bp, base pair(s); CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; FIGE, field inversion gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; kbp, kilobase pair(s); LMP, low melting point; SV40, simian virus 40; VM-26, 4′-demethylepipodophyllotoxin 9-(4,6-*O*-thenylidene-β-D-glucopyranoside).

0006-2960/90/0429-9531$02.50/0

cells have not yet been identified. In the experiments described here, we employed an agent which specifically traps and inhibits topoisomerase II molecules covalently integrated into DNA during their reaction, to identify the period during chromosome replication in which the enzyme's activity is essential and the consequences which follow its trapping. For this purpose, we employed the epipodophyllotoxin VM-26, which targets topoisomerase II specifically according to a compelling body of evidence (Snapka, 1986; Richter et al., 1987; Yang et al., 1987; Richter & Strausfeld, 1988; Snapka et al., 1988; D'Arpa & Liu, 1989; Gupta & Ross, 1989; Liu, 1989; Hancock et al., 1990). The action of VM-26 differs from the classical types of enzyme inhibition, because it inhibits only enzyme molecules which are engaged in intermediate complexes with the DNA substrate; the term "poisoning" was introduced for this phenomenon in studies of DNA gyrase (Kreuzer & Cozzarelli, 1979), but in practice, VM-26 is termed an inhibitor of topoisomerase II (Minocha & Long, 1984; Richter et al., 1987; Drlica & Franco, 1988). Studies in vitro show that some enzyme molecules are trapped at low concentrations of VM-26 and that as the concentration is increased further enzyme molecules become integrated into DNA at the same and at other sites and are in turn trapped (Ross et al., 1984) and the reactions carried out by the enzyme are inhibited (Chen et al., 1984; Minocha & Long, 1984; Yang et al., 1987; Richter et al., 1987; Danks et al., 1988; Sullivan et al., 1989). Trapped enzyme molecules would be predicted to cause secondary effects such as interference with movement of polymerases along DNA.

The observations described here provide information on the cellular processes which are affected by epipodophyllotoxins, which are successfully used in the chemotherapy of human tumors (Issell et al., 1984; D'Arpa & Liu, 1989; Gupta & Ross, 1989; Liu, 1989; Hancock et al., 1990) and on the functions of topoisomerase II in chromosome replication in mammalian cells.

Materials and Methods

Cell Cultures. CHO cells were grown in synchronous cultures in McCoy's 5a medium with 20 mM HEPES, pH 7.2, and 10% fetal calf serum. Mitotic cells of ≥95% purity were detached by shaking subconfluent monolayers on a horizontal platform, 2 h after shaking to dislodge debris and addition of colcemid (0.15 μM). Synchronous cultures were initiated by seeding 1×10^6 mitotic cells per 75 cm^2 culture flask (Stubblefield, 1968).

The inhibition of the colony-forming ability by VM-26 was determined by seeding single cells in medium containing different concentrations of VM-26 and counting stained colonies after 10 days. Determinations were made in triplicate. VM-26 and aphidicolin were stored at 1 mg/mL in DMSO at −20 °C.

DNA Replication. To measure the DNA content per cell by flow cytometry, cells were detached by trypsin, washed in cold PBS (100 mM NaCl/10 mM phosphate, pH 7.5), fixed in ethanol/PBS (1/1), digested with RNase (1 mg/mL, 15 min, 37 °C), and stained with propidium iodide (50 μg/mL in 0.1% sodium citrate) (Crissman & Steinkamp, 1973). The fluorescence signal from propidium iodide was measured in an Epics-V flow cytometer (Coulter) using $(1-2) \times 10^4$ cells. The percent of the cells with a G1 or G2/M content of DNA was calculated by assuming that the respective peaks were symmetrical, as described by Barlogie et al. (1976).

DNA replication was also measured by incorporation of [^{3}H]thymidine in medium containing [*methyl*-^{3}H]thymidine (110 Ci/mM, 0.5 μCi/mL) and unlabeled thymidine (2 μM). At the appropriate times, cells were detached from duplicate or triplicate cultures and washed as described above, deposited on glass fiber filters, and washed in 10% trichloroacetic acid and ethanol. Radioactivity was measured by liquid scintillation spectrometry.

FIGE Analysis of DNA Fragments. Cells detached by trypsin were washed in cold PBS, resuspended rapidly in melted agarose at 42 °C (1% LMP agarose in 100 mM EDTA, 10 mM Tris pH 7.5, and 20 mM NaCl), and immediately placed at 4 °C to gel the agarose. Blocks cut from the gel containing ≃10^6 cells were then incubated with sarkosyl and proteinase K (Tunnacliffe et al., 1987), washed, and sealed with LMP agarose in wells of a horizontal 1% agarose gel (10 × 5 × 0.5 cm). Electrophoresis was in TBE buffer (Tunnacliffe et al., 1987) circulated through a thermostat at 18 °C, at 100 V and ≃80 mA for 9 h with forward time ramped linearly from 0.9 to 10.5 s and reverse time from 0.3 to 3.5 s using a Bio-Rad Pulsewave 760 switcher. Markers were phage T2 DNA and oligomers of phage λ DNA. Gels were stained with ethidium bromide (1 μg/mL) and photographed on type 665 Polaroid film, and negatives were scanned in a Joyce-Loebl Chromoscan 3 recording densitometer at 626 nm to calculate peak areas using the integration function of the densitometer.

Visualization of Mitotic Chromosomes. Cells were trapped as they reached mitosis by colcemid (0.15 μM), added at 11 h. At different times thereafter, the cultures were fixed in acetic acid/methanol (1/3), washed in PBS, and mounted in 0.1 M glycine, pH 9.1, and 50% glycerol containing the DNA-specific fluorochrome Hoechst 33258 (1 μg/mL) (Latt & Wohlleb, 1975). The percent of the total cells which contained mitotic chromosomes was determined; >100 mitotic cells were counted at each time, and each experiment was carried out in duplicate or triplicate.

To examine cells by electron microscopy, they were fixed in glutaraldehyde (4% in 0.1 M sodium cacodylate, pH 7.4; 1 h) and OsO_4 (2%, 1 h), dehydrated through ethanol, immersed in 50% Epon 812/50% propylene oxide, and embedded in Epon 812. Sections stained with uranyl acetate and lead citrate were examined in a Jeol 1200 EX microscope operating at 60 kV.

Results

Effect of VM-26 on DNA Replication. VM-26 was employed at 0.08 μM unless indicated otherwise; this concentration was sufficient to prevent >98% of the cells from growing to form colonies (Figure 1). Traverse of the G1 phase and DNA replication during the S phase were not affected by VM-26, as shown by the kinetics of incorporation of [^{3}H]thymidine into DNA (Figure 2). This conclusion was confirmed by flow cytometric measurements which showed that during the S phase, the DNA content per cell increased at the same rate in the presence of VM-26 as in a control culture (Figure 3A,B).

Delay of Mitotic Chromosome Formation by VM-26. Flow cytometric studies showed that the progress of cells through mitosis was markedly delayed by VM-26 (Figure 3E). The data in Figure 3E show that ≃65% of the cells had not yet completed mitosis 14 h after the normal time (14 h, Figure 3C), calculated by considering that the division of one G2/M cell yields two G1 cells.

The delay in mitosis was found to be due to delay in the formation of mitotic chromosomes. In the presence of VM-26, the condensation of chromatin had not been initiated at 14 h as seen by electron microscopy, at which time the cells in a parallel control culture had divided; the chromatin remained

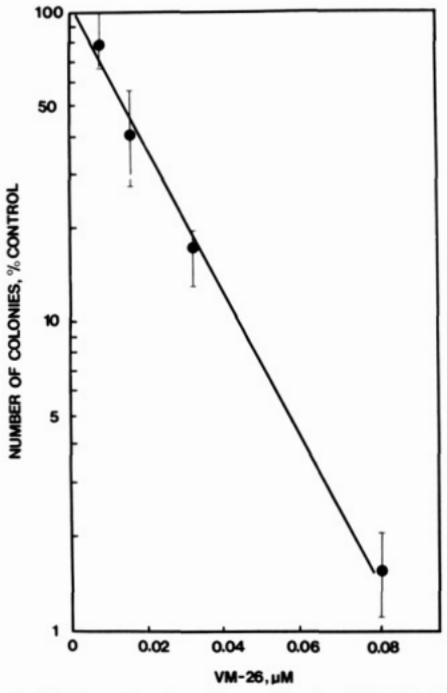

FIGURE 1: Inhibition of colony formation by VM-26. Single cells were seeded in medium containing the indicated concentration of VM-26, and colonies were stained and counted after 10 days. Data show the mean and range from three experiments.

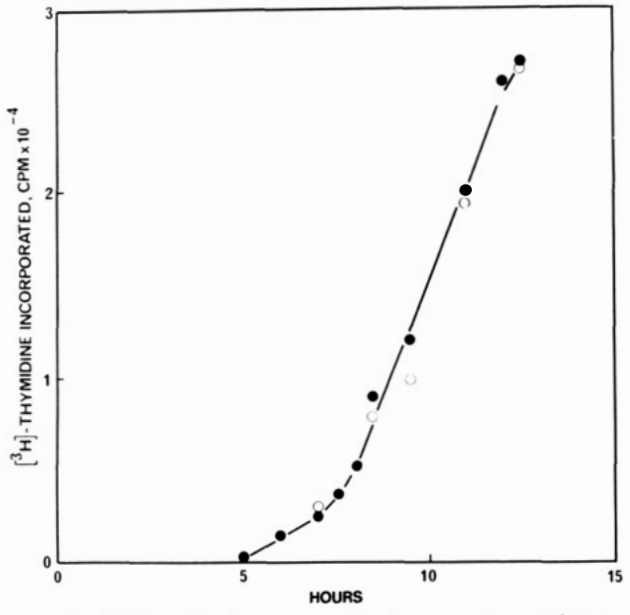

FIGURE 2: DNA replication measured by incorporation of [^{3}H]thymidine into DNA in the absence (●) or presence (○) of VM-26. Data are mean values of two or three independent experiments, each using two parallel cultures seeded from the same population of mitotic cells. The average variation of individual determinations from their mean was ±6.7% (n = 38).

dispersed homogeneously with no peripheral condensed regions which are normally formed progressively during the G2 period (Figure 4). The formation of chromosomes was delayed for 4 h in the presence of VM-26 at 0.08 μM, and thereafter they were formed at only 60% of the normal rate (Figure 5); these parameters were more severely affected at higher concentrations of VM-26 (Figure 5).

The chromatin was condensed in an abnormal manner in ≃5% of the mitotic cells after growth in the presence of VM-26 (0.08 μM). In some cells, the axis of the chromatids and their general form were visible, but the peripheral chromatin was not completely condensed (Figure 6A,C), and in others, the chromatin was condensed in the form of irregular particles (Figure 6B,D).

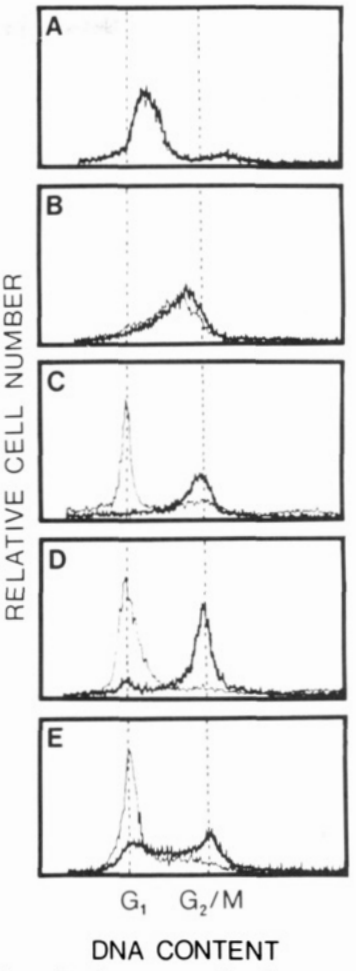

FIGURE 3: DNA replication measured by flow cytometric measurements of the DNA content per cell in the absence (light traces) or presence (heavy traces) of VM-26. Parallel cultures seeded from the same population of mitotic cells were harvested at (A) 6, (B) 10, (C) 14, (D) 18, and (E) 28 h. DNA replication is shown by an increase in the DNA content from the G1 to the G2 value, and mitosis by subsequent halving to the G1 value.

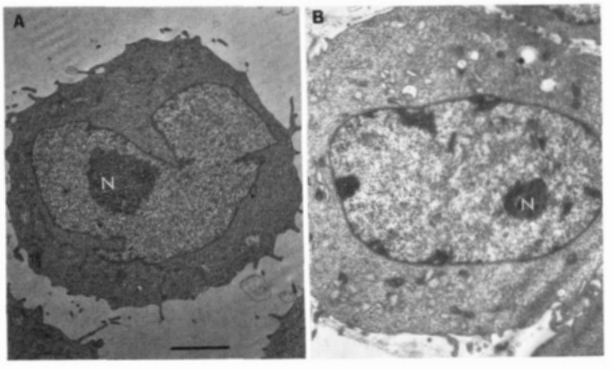

FIGURE 4: Electron microscope images of section cells after growth in the presence or absence of VM-26. Cells were harvested from parallel cultures seeded from the same population of mitotic cells. (A) A representative cell arrested by VM-26 at 14 h; (B) a representative G2 cell from a control culture with chromatin condensing at the nuclear periphery. N, nucleolus. Bar = 2 μm.

Topoisomerase II Covalently Integrated in DNA. Topoisomerase II molecules integrated into DNA during their reaction are dissociated by protein denaturants, forming double-strand breaks at the integration sites and releasing DNA fragments. These fragments have been studied by centrifu-

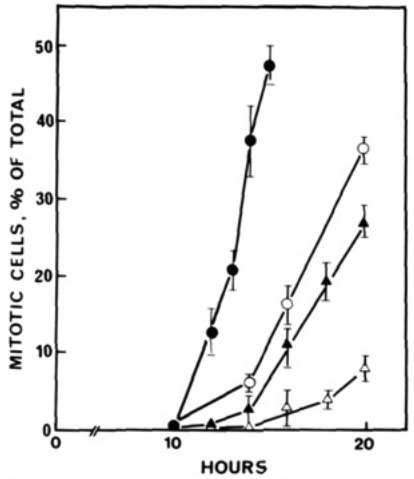

FIGURE 5: Time course of appearance of cells which have formed mitotic chromosomes in a control culture (●) and in cultures growing with VM-26 at 0.016 (○), 0.08 (▲), and 0.16 (△) μM. Parallel cultures were seeded from the same population of mitotic cells. Colcemid was added at 11 h to trap the accumulating mitotic cells (see Materials and Methods). Data show the mean and range from two or three independant experiments; >100 mitotic cells were counted at each time point.

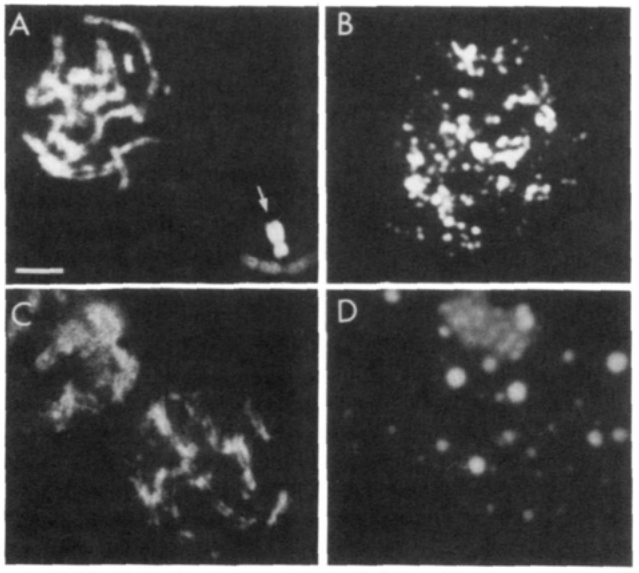

FIGURE 6: Defective condensation of chromatin observed in ≃5% of the mitotic cells at 20 h in the experiment shown in Figure 5 (VM-26 concentration, 0.08 μM). (A, C) Chromosomes in which the central chromatid axes are visible but the peripheal chromatin is incompletely condensed; the arrow indicates a chromosome formed normally. (B, D) Chromatin condensed into irregular particles. Bar = 2 μM.

gation in sucrose gradients (Loike & Horwitz, 1976; Marshall & Ralph, 1982), but this experimental approach has two limitations: because the quantity of DNA which can be analyzed is small, the cells must be labeled with [^{3}H]thymidine to a specific activity so high that cell cycle parameters are perturbed (Marshall & Ralph, 1982), and the interpretation of the fragment sizes which are obtained has been questioned (Kohn, 1979). To overcome these problems, we used FIGE analysis (Carle et al., 1986) to examine DNA fragments from cells harvested at different times during growth in the presence of VM-26. The cells were embedded in agarose and incubated with proteinase K and the strong cationic detergent sarkosyl (Tunnacliffe et al., 1987) to dissociate and digest topoisomerase II molecules integrated in DNA. To verify that trapped enzyme molecules were not released from DNA during these procedures, the FIGE pattern was examined after placing the

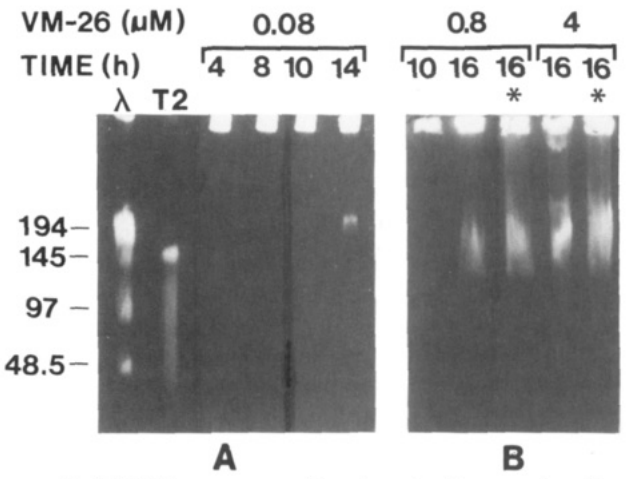

FIGURE 7: DNA fragments resulting from the denaturation of topoisomerase II molecules covalently integrated in DNA, analyzed by FIGE. Cells were grown in parallel cultures seed from the same population of mitotic cells, with VM-26 at the indicated concentration. (A) Cells harvested at different times during growth in the presence of 0.08 μM VM-26; (B) cells harvested at 10 h (S phase) or at 16 h (G2 phase) after growth in the presence of higher concentrations of VM-26 (0.8 or 4 μM). The samples marked with an asterisk in (B) were incubated for 1 h at room temperature before digestion with sarkosyl and proteinase K, to verify that topoisomerase II molecules were not released from DNA during these procedures; the level of DNA fragments was not detectably reduced. Length markers were phage T2 DNA and oligomers of phage λ DNA (length in kilobase pairs).

cells at room temperature for 1 h before incubation with sarkosyl and proteinase K; the amount of fragments was not detectably reduced (Figure 7B). Studies of DNA fragments from HeLa cells grown with VP-16 show that their level remains essentially constant at 37 °C for 30 min after removal of the drug (Loike & Hurwitz, 1976).

DNA fragments originating from trapped topoisomerase II molecules were present in cells harvested in the G2 period after growth with VM-26 at 0.08, 0.8, or 4 μM (Figure 7). They represented 12% of the total DNA at 0.08 μM VM-26 and 35% of the total at 0.8 μM VM-26, measured by densitometric scanning. Their length was ≃200–300 kbp at 0.08 μM VM-26, and was markedly more heterogeneous at higher concentrations (Figure 7B). DNA fragments were also present in cells exposed to VM-26 (0.08 μM) in the G2 period only (data not shown).

No DNA fragments were observed in cells harvested during the S phase at 0.08 μM VM-26 (Figure 7A), and densitometric scanning of the corresponding lanes of the gel detected no optical density above the background. DNA fragments were also not detectable in the S phase in cells growing in the presence of a 10-fold higher concentration of VM-26 (0.8 μM) (Figure 7B).

Evidence That VM-26 Inhibits Two Processes. Experiments using flow cytometry, in which VM-26 was added at a time when some cells had completed DNA replication and others had not, in order to examine separately its effects on these two classes of cells (a strategy explained under Discussion), suggested that delayed formation of chromosomes was caused by slowing of two processes.

To determine when DNA replication was completed, the progress of cells to mitosis was observed after addition of aphidicolin to inhibit DNA polymerases α and δ (Huberman, 1981; Dresler & Frattini, 1986) at a concentration (5 μg/mL) which reduced the incorporation of [^{3}H]thymidine into DNA by >97% within 10 min (data not shown); the remaining aphidicolin-resistant DNA synthesis has been shown in other

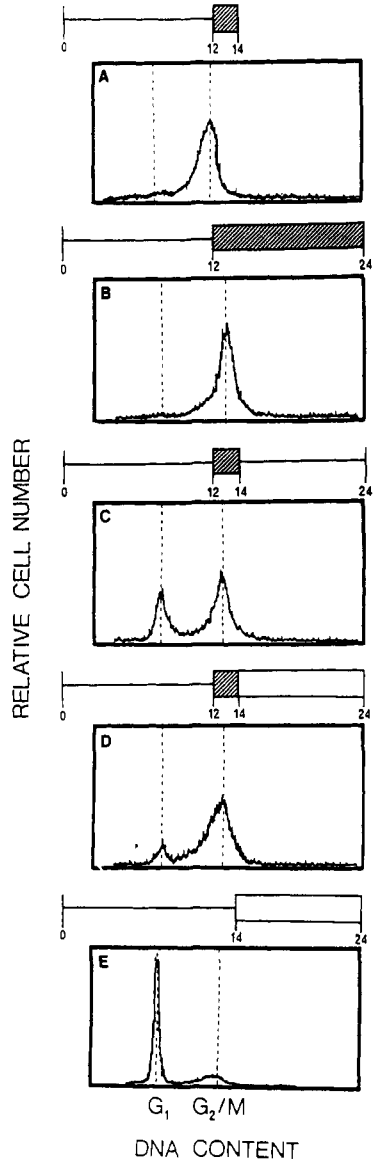

FIGURE 8: Inhibition by aphidicolin of the recovery of cells from VM-26 and their progression to mitosis, followed by cytometric measurements of the DNA content per cell. Five parallel cultures were seeded from the same population of mitotic cells, and VM-26 (0.16 μM) was added at 12 h to four cultures. Two of these were maintained in the presence of VM-26; these cells remained arrested with the G2/M content of DNA (A, B). The other two cultures were transferred at 14 h to VM-26-free medium without (C) or with (D) aphidicolin (5 μg/mL); aphidicolin reduced the number of cells which recovered and divided (G1 peaks) by 75%. Aphidicolin was also added at 14 h to the cultures which had not received VM-26, and did not prevent progression to mitosis (E). The cells were harvested for analysis at 14 h (A) or at 24 h (B–E). Hatched and open bars on the time scale show the periods of exposure to VM-26 and to aphidicolin.

mammalian cells to represent replication of mitochondrial DNA (Spadari et al., 1984). When aphidicolin was added at 12 h, 20% of the cells could proceed to mitosis, as shown by the G1 peak in Figure 9B. In contrast, when VM-26 was added at 12 h, all the cells remained arrested with a DNA content at the G2/M value for at least a further 12 h (Figure 8B). and microscopical examination showed that none formed mitotic chromosomes (data not shown). Both cells which had completed DNA replication at 12 h and those which had not were therefore arrested by VM-26.

We first examined the cells which had not completed DNA replication, as judged by the criterion that they could not recover and reach mitosis when aphidicolin was added upon removal of VM-26 at 14 h. In control cultures, aphidicolin

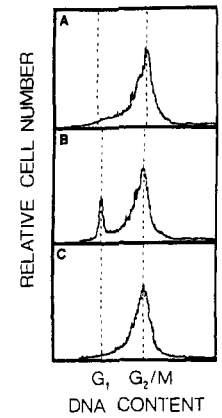

FIGURE 9: Cells which can reach mitosis in the presence of aphidicolin, added at 12 h, are prevented from progressing to mitosis by VM-26, as shown by flow cytometric measurements. Three parallel cultures were seeded from the same population of mitotic cells, and one was harvested for analysis at 12 h (A). At 12 h, aphidicolin (5 μg/mL) was added to the two other cultures (B, C) and VM-26 to one of them (C); these cells were harvested for analysis at 16 h.

showed no effect in these conditions: all the cells could progress to and complete mitosis (Figure 8E). The number of cells which could recover from VM-26 arrest was reduced by aphidicolin from 25% of the total cells (Figure 8C, G1 peak) to 6% (Figure 8D, G1 peak). The majority of these cells therefore appeared to have been arrested by VM-26 before completion of DNA replication.

We then examined the cells which had completed DNA replication at 12 h as judged by their ability to proceed to mitosis in the presence of aphidicolin, which represented 20% of the total population as shown by the peak of G1 cells in Figure 9B. These cells were prevented from reaching mitosis by VM-26, as shown by the absence of G1 cells in Figure 9c. The experiment shown in Figure 8 provided further data on this question: when VM-26 was removed at 14 h, 6% of the cells could proceed to mitosis in the presence of aphidicolin (Figure 8D, G1 peak) and therefore appeared to have been arrested by VM-26 after completion of DNA replication. These cells were, however, prevented from reaching mitosis by VM-26, as shown by the absence of G1 cells in Figure 8B.

Discussion

Effects of VM-26 on DNA Replication. DNA replication in CHO cells is not affected by VM-26 at a concentration which prevents >98% of the cells from growing to form colonies (Figures 2 and 3). Since this concentration of VM-26 is sufficient to inhibit topoisomerase II functions, as shown by the arrest of cells in the G2 phase (Figure 3), the enzyme does not appear to have essential functions in replication of chromosomal DNA. This situation is consistent with evidence that topoisomerase II is not required for DNA replication in other eukaryotic systems (DiNardo et al., 1984; Uemura & Yanagida, 1984, 1986; Snapka, 1986; Uemura et al., 1987; Richter et al., 1987; Yang et al., 1987; Richter & Strausfeld, 1988; Snapka et al., 1988), but contrasts with that in the prokaryote *Escherichia coli* where the homologue of topoisomerase II, DNA gyrase, is essential for DNA replication (Kreuzer & Cozzarelli, 1979; Snyder & Drlica, 1979), suggesting that this may be a fundamental difference between eukaryotic and prokaryotic cells.

The absence of an effect of VM-26 at a cytotoxic concentration on DNA replication suggests that few or no enzyme molecules are integrated in DNA during the period of DNA replication, because when these were trapped by VM-26 they would be predicted to prevent the progression of polymerases.

In support of this idea, DNA fragments derived from integrated enzyme molecules were not seen in the S phase in cells exposed to VM-26 (0.08 or 0.8 μM) (Figure 7). In studies of unsynchronized HeLa cells exposed to VP-16 (0.016 μM), DNA fragments were not detected by sucrose gradient analyses (Loike & Hurwitz, 1976). If topoisomerase II molecules are integrated in DNA during the S phase at sites spaced more widely than $\simeq$1000 kbp, the length of the largest DNA which enters these gels (Carle et al., 1986), they would, however, not have been detected.

Effects of VM-26 in the G2 Period. DNA fragments derived from topoisomerase II molecules trapped by VM-26 were only detected in the G2 period (Figure 7). The results of FIGE analyses are consistent with the idea that topoisomerase II molecules are not trapped at all possible sites at a low but cytotoxic concentration of VM-26 (0.08 μM) and that more are trapped at higher concentrations, as observed in vitro (Ross et al., 1984). The cells may be heterogeneous in their response at a low VM-26 concentration (0.08 μM), some containing frequent trapped enzyme molecules and others only a few, producing predominantly DNA fragments between 200 and 300 kbp in length (Figure 7A), while at higher VM-26 concentrations (0.8 or 4 μM) the fragments are much more heterogeneous in length (Figure 7B).

Taken together, these observations suggest that inhibition of processes in the G2 period by VM-26 is not caused by topoisomerase II molecules trapped in DNA during the S phase, but is due to interference with reactions which the enzyme carries out at the end of DNA replication or in the G2 period.

Topoisomerase II Functions in the Formation of Mitotic Chromosomes. The delay in progress of cells through mitosis by VM-26 (Figure 3E) reflects a delay in the formation of mitotic chromosomes (Figures 4 and 5). These processes are also delayed when VM-26 is present only after DNA replication is almost or completely terminated (Figure 8A,B).

The formation of chromosomes is the only process which we observe to be affected by VM-26 at a cytotoxic concentration; this is delayed for $\simeq$4 h, and its rate is reduced thereafter (Figure 5), with the result that division of 65% of the cells is delayed by >14 h (Figure 3). Chromosome formation is completely inhibited at 0.32 μM VM-26 (data not shown). The initiation of chromatin condensation to form chromosomes has not occurred at a time when control cells have divided (Figure 4); phosphorylation of histone H1 and phosphorylation of lamins (Matthews, 1977; Gerace & Blobel, 1980) are also delayed (data not shown), suggesting that the entire cascade of events which accompany chromosome formation is slowed. In some cells, the chromatin is incompletely or incorrectly condensed (Figure 6). We conclude that the only essential reactions which topoisomerase II carries out are concerned with the formation of mitotic chromosomes in the G2 period.

Evidence for Functions of Topoisomerase II both during and after the Completion of DNA Replication. The observation that aphidicolin prevents the progress to mitosis of the majority of the cells which recover after removal of VM-26 at 14 h (Figure 8) suggests that further DNA synthesis must occur in these cells before mitosis. This finding is compatible with the conclusion that VM-26 slows or arrests DNA replication at a late stage, but other experimental approaches to examine late intermediates in DNA replication are necessary to confirm this hypothesis.

Cells which have completed DNA replication, as judged by the criterion that they can divide in the presence of aphidicolin, are also unable to progress to mitosis in the presence of VM-26 (Figure 9). Similar studies using unsynchronized cells confirmed this conclusion (data not shown). This effect of VM-26 is masked by its prior effect on completion of DNA replication when it is present during the entire cell cycle, a situation which led us to choose this experimental strategy. Further, aphidicolin does not affect 25% of the cells which recover after the removal of VM-26 (Figure 8). The interpretation of experiments in which aphidicolin shows no effect is, however, subject to the caveat that complete arrest of all nuclear DNA replication cannot be rigorously proved, so that although these results suggest that trapping of topoisomerase II prevents progress to mitosis after DNA replication is completed, they do not provide unequivocal support for this hypothesis.

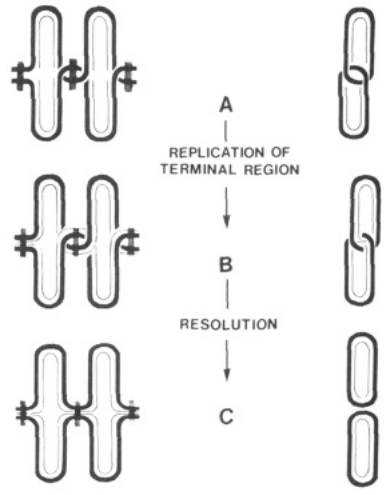

FIGURE 10: Stages of terminal replication and segregation of adjacent 100–200 kbp long loops or domains of chromosomal DNA (left) compared with the topologically analogous processes in the $\simeq$5 kbp long closed circular DNA of SV40 (right). Parental and new DNA strands are heavy and light, respectively. The rectangles (left) represent sites where chromoosmal DNA is attached and topologically constrained within the nucleus; these are shown in the termination region, but other locations are possible. The catenation linking number when replication is complete is underrepresented for clarity; it is up to 25 in SV40 (Varshavsky et al., 1983; Snapka et al., 1988).

Topological Aspects of Processes Requiring Topoisomerase II. To further interpret our observations, we compared the effects of VM-26 on chromosome replication in CHO cells with those on the replication of SV40 DNA (Snapka et al., 1988). The DNA molecules in cellular chromosomes are of the order of 10^5 kbp in length (Kavenoff et al., 1974) but are divided into independent topologically constrained domains or loops (Benyajati & Worcel, 1976) which are $\simeq$140 kbp long in CHO cells (Hartwig, 1978). The replication of each loop shows topological analogies with that of a closed circular DNA such as SV40 (Hancock, 1982), as illustrated in Figure 10.

Our observations are consistent with a requirement for topoisomerase II during the last stage of chromosomal DNA replication (Figure 10A,B). Topoisomerase II is required during replication of the terminal $\simeq$250 bp region of SV40 DNA; the molecular basis for this requirement has not been elucidated, but it may arise because replication of this region occurs simultaneously with its progressive decatenation (Varshavsky et al., 1983; Snapka et al., 1988).

Topoisomerase II is required in a second process in SV40 replication, the decatenation of completed progeny molecules (Snapka, 1986; Richter et al., 1987; Yang et al., 1987; Richter & Strausfeld, 1988; Snapka et al., 1988). In cellular DNA, the topological analogy of this process is the conversion of the series of replicated DNA domains or loops into two long linear molecules which form the two chromatids of a mitotic chro-

mosome (Figure 10B,C). Our evidence that VM-26 specifically delays the formation of mitotic chromosomes, and causes abnormal chromatin condensation in some cells, is consistent with a model in which the essential reaction of topoisomerase II is to carry out this double-strand passing process, whose arrest by VM-26 prevents the folding of the chromatin to form mitotic chromosomes.

ACKNOWLEDGMENTS

We thank R. Ralph for introducing us to this subject, J. Aubin and J. Gauthier for help with flow cytometry, E. Beaulieu for developing FIGE methods, R. Pankov and N. Pépin for help with electron microscopy, J. Chafouleas for discussions, and Bristol-Myers for a gift of VM-26.

Registry No. VM-26, 29767-20-2; DNA topoisomerase, 80449-01-0.

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