# Chromosomes with Two Intact Axial Cores Are Induced by $G_2$ Checkpoint Override: Evidence That DNA Decatenation Is not Required to Template the Chromosome Structure

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*Abstract.* Here we report that DNA decatenation is not a physical requirement for the formation of mammalian chromosomes containing a two-armed chromosome scaffold. 2-aminopurine override of $G_2$ arrest imposed by VM-26 or ICRF-193, which inhibit topoisomerase II (topo II)–dependent DNA decatenation, results in the activation of p34[cdc2] kinase and entry into mitosis. After override of a VM-26–dependent checkpoint, morphologically normal compact chromosomes form with paired axial cores containing topo II and ScII. Despite its capacity to form chromosomes of normal appearance, the chromatin remains covalently complexed with topo II at continuous levels during $G_2$ arrest with VM-26. Override of an ICRF-193 block, which inhibits topo II–dependent decatenation at an earlier step than VM-26, also generates chromosomes with two distinct, but elongated, parallel arms containing topo II and ScII. These data demonstrate that DNA decatenation is required to pass a $G_2$ checkpoint, but not to restructure chromatin for chromosome formation. We propose that the chromosome core structure is templated during interphase, before DNA decatenation, and that condensation of the two-armed chromosome scaffold can therefore occur independently of the formation of two intact and separate DNA helices.

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$T$HE proper segregation of genetic material during mitosis in eukaryotes requires extensive condensation of DNA into discrete chromosomes, each containing two sister chromatids. Results from diverse experimental systems have supported the conclusion that DNA topoisomerase II (topo II)[1] plays an essential role both in chromosome formation and in the separation of sister chromatids. In $G_2$, topo II catalyzes the unravelling of the highly intertwined catenated sister chromatids that remain at the end of replication. This task is accomplished through a strand-passing activity, in which one double-stranded DNA segment passes through a transient double-strand break in another DNA duplex (Hsieh and Brutlag, 1980; Liu et al., 1980; for review see Roca, 1995). After the strand separation, topo II is required for the condensation of the chromosome (Newport and Spann, 1987; Adachi et al., 1991; Hirano and Mitchison, 1993). Finally, at the onset of anaphase, linkages that remain between the sister chromatids must be resolved by topo II–dependent DNA decatenation to allow sister chromatid segregation (Holm et al., 1985; Uemura et al., 1987; Downes et al., 1991; Shamu and Murray, 1992).

While the role of topo II in sister chromatid separation is clear, its role in chromosome condensation is not well understood. In mammalian cells, topo II localizes to an axial core (Earnshaw and Heck, 1985; Taagepera et al., 1993) or chromosome scaffold that maintains a chromosome-like morphology in the absence of DNA (Adolph et al., 1977; Earnshaw et al., 1985), suggesting a structural role for topo II in chromosome formation. DNA condensation has been accomplished in vitro by incubation of interphase nuclei with mitotic extracts (Newport and Spann, 1987; Adachi et al., 1991; Hirano and Mitchison, 1993), and topo II is absolutely required for the condensation of nuclei in these in vitro systems (Adachi et al., 1991; Hirano and Mitchison, 1993). However, it is unclear whether topo II–dependent decatenation of chromatin is a physical prerequisite for chromosome condensation or if it is only required as a structural component of the chromosome during condensation.

The problem can be rephrased as follows: How does the information content for condensation of an organized chromosome arise? It is possible that the decatenation of chromatin strands is an obligatory step before chromosome condensation and that this event allows the indepen-

1. *Abbreviations used in this paper*: 2-AP, 2-aminopurine; FIGE, field inversion gel electrophoresis; HU, hydroxyurea; PCC, premature chromosome condensation; SAR, scaffold attachment regions; topo II, topoisomerase II.

dent duplexes to form two chromatid arms, each with an axial core. Alternatively, it is possible that the information necessary to form the two chromatids with axial cores has been templated independently, and before, the decatenation event.

Here we have approached the problem of the interdependence of decatenation and chromatin condensation in intact mammalian cells. Mammalian cells contain a checkpoint that imposes $G_2$ arrest and blocks progression into mitosis if chromatin is not sufficiently decatenated (Downes et al., 1994). A drug block with any of several agents that interfere with topo II enzyme activity, such as VM-26 and ICRF-193, imposes a checkpoint on cell cycle progression in which the cell arrests in $G_2$ with undecatenated DNA. We have demonstrated, in a previous study, that when mammalian cells were arrested in $G_2$ with the topo II inhibitor VM-26, addition of the protein kinase inhibitor 2-aminopurine (2-AP) would override the drug block and induce mitosis (Andreassen and Margolis, 1992). Strikingly, we noted that the mitotic cells showed evidence of chromosome condensation after checkpoint override. The implication was that topo II–dependent decatenation of DNA in $G_2$ was not a physical prerequisite for the condensation of mitotic chromosomes. By contrast, Downes et al. (1994) found that complete chromosome condensation was blocked by ICRF-193 after checkpoint override, leading to the opposite conclusion that topo II–dependent decatenation of DNA is required for chromosome condensation.

In this report, we present a careful analysis of the formation of mitotic chromosomes during checkpoint override in mammalian cells after $G_2$ arrest with either of two topo II inhibitors, VM-26 or ICRF-193. Our data demonstrate that topo II–dependent decatenation is not required to remodel chromatin into a condensed chromosome with two axial cores. Further, the formation of two independent axial cores apparently does not require that intact and continuous DNA helices underlie them. Interestingly, while VM-26 permits the formation of fully condensed chromosomes, ICRF-193, which interacts with topo II in a different manner (Tanabe et al., 1991; Roca et al., 1994), results in formation of chromosomes with paired, but highly elongated, sister chromatids. It would thus appear that ICRF-193, but not VM-26, interferes specifically with a nonenzymatic topo II–dependent step leading to complete chromosome condensation.

Since the formation of chromosome cores is independent of decatenation, a template for the axial cores must exist independently of the intact DNA helices. This property requires that a template be formed before the $G_2$ decatenation step, perhaps during DNA replication. We propose that assembly of the axial core, independent of DNA decatenation, is required for the final steps of chromosome condensation, and we present a model in which the association of topo II with replicated DNA prepares a template for chromosome formation by assembly of the axial core.

## Materials and Methods

### Cell Culture and Synchronization

CHO and BHK cells were grown as monolayers in DME (GIBCO BRL, Paisley, UK) supplemented with 10% FBS (Biological Industries, Israel). Cells were maintained in a humid incubator at 37°C in a 5% $CO_2$ environment.

Nocodazole and hydroxyurea (HU) were obtained from Sigma Chemical Co. (St. Louis, MO). Stock solutions of 1 μg/ml nocodazole and 200 mM HU were prepared in DMSO and DME, respectively. VM-26 was obtained from Bristol-Myers Squibb (Princeton, NJ) and prepared as a 5 mM stock solution in DMSO. ICRF-193 was a gift from A.M. Creighton (St. Bartholomew's Hospital, London, UK), and was prepared as a stock solution in DMSO (70 mM). All stock solutions were kept frozen until used.

Synchronization of CHO cells just before $G_2$ was initiated by treatment with isoleucine-deficient DME containing 10% dialyzed FBS (GIBCO BRL). Subsequently, cells were treated with 2 mM HU in complete medium for 16 h and then released into drug-free medium for 5 h. Experimental protocols involving treatment with VM-26 commenced at this time.

### Chromosome Spreads

Cells were grown as subconfluent monolayers for a minimum of 24 h before any drug treatment. Cells were then treated with 2 mM HU, 10 μM ICRF, or VM-26 (0.20 μg/ml CHO; 0.02 μg/ml BHK) for 16 h. Where applicable, this was followed by 3 h of treatment with 10 mM 2-AP in the continued presence of the first drug. Treatment with nocodazole was at a concentration of 0.20 μg/ml for 3 h. To prepare chromosome spreads, cells were collected and swollen with 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 5 mM $MgCl_2$ for 5 min at room temperature (Earnshaw et al., 1985). Cells were then pelleted 2 min at 215 g and fixed for 30 min at room temperature in methanol/acetic acid (3:1). Spreads were then obtained by drying the cell suspension on coverslips at 30°C. The chromosome preparation was then washed with PBS, stained for 5 min with 0.5 μg/ml propidium iodide in PBS, washed again twice, and mounted for observation.

### Immunofluorescence Microscopy

For immunofluorescence microscopy, cells were grown on 12-mm-diam coverslips for a minimum of 24 h before drug treatment. For immunodetection of MPM-2 antigens (Davis et al., 1983), cells were fixed, permeabilized, and prepared with primary and FITC-conjugated affinity purified anti–mouse IgG secondary antibodies as previously described (Andreassen and Margolis, 1994). Counterstain was with 0.5 μg/ml propidium iodide in PBS.

For immunolocalization of topo II and ScII, cells were swollen by addition of 50 μl of 0.8% sodium citrate to each coverslip for 10 min at room temperature and were then fixed in 50% methanol/acetic acid (3:1) for 2 min, followed by 100% methanol/acetic acid (3:1) for 5 min as described by Saitoh et al. (1994). Chromosome spreads were obtained by drying at 30°C. Preparations were then rehydrated by three washes of 2 min each in TEEN (1 mM triethanolamine-HCl, pH 8.5, 0.2 mM EDTA, 25 mM NaCl containing 0.1% BSA and 0.5% Triton X-100 (Saitoh et al., 1994). Primary antibodies were diluted in TEEN containing 0.1% BSA and 0.5% Triton X-100 and were applied for 1 h at 37°C. Rabbit anti-human topo II (TopoGEN Inc., Columbus, OH) and rabbit anti-ScII antisera 966 and 1113 (kind gifts of N. Saitoh and W.C. Earnshaw, Johns Hopkins University, Baltimore, MD) were diluted 200-fold. Chromosomes were then washed three times, 5 min each, with $KB^+$ buffer (10 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.1% BSA, 0.1% Triton X-100) (Saitoh et al., 1994). FITC-conjugated affinity-purified goat anti–rabbit IgG antibodies (Cappel Laboratories, West Chester, PA) were applied at 2.5 μg/ml in $KB^+$ buffer for 30 min at 37°C. Chromosomes were then washed twice for 5 min each with $KB^+$, counterstained 5 min with 0.5 μg/ml propidium iodide in $KB^+$, and finally washed twice with $KB^+$.

Samples were observed using a microscope (model Optiphot II; Nikon Inc., Melville, NY) attached to a laser scanning confocal apparatus (model MRC-600, BioRad Microscience Division, Herts, England). Photographs were taken on TMAX-100 film with a film recorder (model CI-3000; Polaroid Corp., Cambridge, MA).

### Flow Cytometric Analysis

Cells were collected by trypsinization, pooled with nonattached cells, and fixed 10 min with 90% methanol/PBS at −20°C. Fixed cells were then incubated with MPM-2 antibodies, washed, incubated with FITC-conjugated goat anti–mouse IgG secondary antibodies, and then incubated with propidium iodide in 4 mM sodium citrate containing RNase A as previously described (Andreassen and Margolis, 1994).

Data were collected using a FACScan® (Becton-Dickinson, San Jose, CA). For each sample, 10,000 events were collected. Aggregated cells were gated out and plots were prepared using LYSYS II software (Becton-Dickinson).

## Histone H1 Kinase Assay

CHO cells were grown as subconfluent monolayers for a minimum of 24 h before drug treatment. Cells were first treated for 16 h with 0.20 µg/ml VM-26 and were then either cultured with medium containing 0.20 µg/ml VM-26 plus 10 mM 2-AP or with VM-26 alone. At time points, the population was harvested by trypsinization, and cell extracts were prepared in lysis buffer (Andreassen and Margolis, 1994). Preclearing of proteins binding nonspecifically to Sepharose 4B–protein A beads, incubation with affinity-purified anti-p34[cdc2] antibody, incubation with protein A–Sepharose beads, washing of the resulting immune complex, and the kinase reaction were performed as described previously (Andreassen and Margolis, 1994). Samples were then subjected to SDS-PAGE on 12% polyacrylamide gels (19:1 ratio of acrylamide to bis-acrylamide), and autoradiographs were prepared by exposure to Hyperfilm-MP (Amersham Corp., Arlington Heights, IL).

## Analysis of Covalent DNA–Topoisomerase II Intermediates

Stabilization of the covalent DNA–topo II cleavage intermediate by VM-26 was measured using the In Vivo Link-Kit from TopoGEN Inc. Cells growing on 10-cm-diam culture dishes were synchronized just before $G_2$ as described above, and samples were prepared at time points after the introduction of 0.20 µg/ml VM-26. For the assay, cells were lysed in 10 mM Tris-HCl buffer at pH 7.5, containing 1 mM EDTA and 1% sarkosyl. These conditions extract noncovalently linked topo II from the DNA. Samples were then applied to a cesium chloride step gradient containing 0.20 µg/ml VM-26 and centrifuged to separate DNA from free protein (Trask and Muller, 1988). Fractions of 400 µl each were collected from the gradients, and 50 µl samples from each fraction were diluted with 100 µl of 25 mM sodium phosphate (pH 6.5) and applied to nitrocellulose using a Hybri-Dot manifold (GIBCO BRL). The nitrocellulose membrane was incubated 2 h with TBS-Tween (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% nonfat powdered milk and subsequently washed three times for 10 min each with TBS-Tween. Incubation with rabbit anti–human topo II antibody (TopoGEN Inc.) diluted 2,500-fold in TBS-Tween was carried out for 3 h at ambient temperature. After three washes with TBS-Tween, the dot blots were developed with enhanced chemiluminescence (Amersham Corp.). Levels of topo II covalent intermediates in the fractions were quantitated using a Vista-S6 scanner coupled to a Macintosh computer and NIH Image software to integrate the signals in the fractions containing DNA. The DNA peak was located and quantitated by staining with 10 µg/ml Hoechst 33258, followed by measurement of fluorescence on an Aminco-Bowman Series 2 luminescence spectrophotometer (SLM-Aminco, Rochester, NY), using an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

## Pulsed Field Gel Electrophoresis

Cells treated with 2-AP, or with VM-26 alone, were collected as whole cell populations by trypsinization. For those culture dishes treated with VM-26 plus 2-AP, mitotic cells were selectively detached from the culture dish at 3 h after 2-AP addition, resulting in a mitotic index of at least 85% in the harvested cells. Preparation of agarose plugs from cells and further treatment was according to Filipski et al. (1990). Cells were resuspended in 1.0 ml of nuclear buffer (2 mM $KH_2PO_4$, pH 6.4, 150 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$) and were then counted and resuspended at 3–5 × $10^7$ cells/ml in the same buffer depending on the experiment. An equal volume of molten 1.5% low–melting point agarose in nuclear buffer, also containing 0.4 mg/ml proteinase K, was then added to each sample. The mixture was injected into Tygon tubing (1/16 inch internal diameter) using a syringe with an 18-gauge needle and was solidified for 30 min on ice. Samples were then incubated in 10 mM Tris-HCl, pH 9.5, 10 mM NaCl, 25 mM EDTA, and 10% sarkosyl containing 33 µg/ml proteinase K for 18 h at 37°C, and then samples were washed thoroughly in 10 mM Tris-HCl, pH 8, and 1 mM EDTA at 4°C. For cell populations exposed to VM-26 either with or without 2-AP, all incubations and washes contained 0.20 µg/ml VM-26 to inhibit decatenation during processing.

Pulsed field gel electrophoresis was performed using a horizontal gel electrophoresis chamber (Pharmacia LKB Biotechnology, Piscataway, NJ)

and a power inverter (model PPI-200; MJ Research, Watertown, MA). Molecular weight standards were λ phage oligomeric DNA (50–700 kb) and *S. cerevisiae* chromosomal DNA (225–2,200 kb) supplied by BioRad Laboratories (Hercules, CA). Samples were sealed in place with LMP agarose in a 1% pulsed field grade agarose gel and were run at 8 V/cm with 0.5 × TBE. After 10 min, ramping began at 0.39 s in the forward direction and 0.13 s in the reverse direction, with 56 increments of 0.15 and 0.05 s, respectively. The total run time was 8 h. Chamber temperature was maintained at 23°C during the run, using a refrigerated circulating water bath (model DC3; HAAKE/Fisons Instruments, Valencia, CA). After completion of the run, photographs of gels were taken under UV light using Polaroid 667 film.

## Chromosome/Nuclear Isolation and Immunoblotting

Chromosome clusters were isolated from selectively detached mitotic cells, which were washed three times with PBS at 4°C. For this, cells were lysed and chromosomes were separated according to the technique of Saitoh and Laemmli (1994). Nuclei were similarly isolated after collection by trypsinization.

Samples were equalized on the basis of DNA content measured by absorbance at 260 nm. Electrophoresis on 8% polyacrylamide gels, transfer to nitrocellulose, and development with enhanced chemiluminescence (Amersham Corp.) were as previously described (Andreassen and Margolis, 1994). Anti-ScII antiserum 966 and anti–topo II antibodies (TopoGEN Inc.) were used at dilutions of 5,000- and 3,000-fold, respectively.

## Results

VM-26 inhibits topo II–dependent DNA strand passage activity, blocking the reaction at a step where the enzyme forms a covalent intermediate with a cleaved DNA double strand (Chen et al., 1984). This drug induced block results in $G_2$ arrest in mammalian cells (Misra and Roberts, 1975; Charron and Hancock, 1990). We have verified by flow cytometric analysis that 16 h of VM-26 treatment results in arrest of CHO cells in $G_2$ with 4N DNA content (Fig. 1 *A*, *left*). By contrast, untreated cycling CHO cells display a predominantly 2N DNA content.

MPM-2 antibodies recognize mitosis-specific phosphoepitopes (Davis et al., 1983). Using these antibodies, mitotic cells can be distinguished as the subset of cells that displays an elevated MPM-2 signal when analyzed by two-dimensional flow cytometry (Fig. 1 *A*, *right*). Among the cells with a 4N DNA content in an untreated cycling CHO population, a subset have an elevated MPM-2 signal. These represent the mitotic cells. Almost all cells after treatment for 16 h with 0.20 µg/ml (0.31 µM) VM-26 have a 4N DNA content. As expected of $G_2$-arrested cells, this population has a minimal MPM-2 signal. The VM-26–induced arrest in $G_2$ is solid, as CHO cells remain arrested in $G_2$ with 4N DNA content and exhibit a minimal MPM-2 signal through 24 h of treatment (data not shown). Cells arrested in $G_2$ by treatment for 16 h with VM-26 can be induced to enter mitosis by addition of 10 mM 2-AP in the continued presence of VM-26. While the cells maintain a 4N content of DNA, entry into mitosis is evident from the induction of an elevated MPM-2 signal, as measured by two-dimensional flow cytometry (Fig. 1 *A*, *right*).

The time course of override of VM-26–induced $G_2$ arrest by 2-AP was quantitated as the percentage of the whole cell population that entered mitosis, by immunofluorescence microscopic assay (Fig. 1 *B*). In this assay, we scored cells as mitotic if they displayed both an elevated MPM-2 signal and condensed chromatin. By immunofluorescence assay, cells arrested in $G_2$ after 16 h treatment
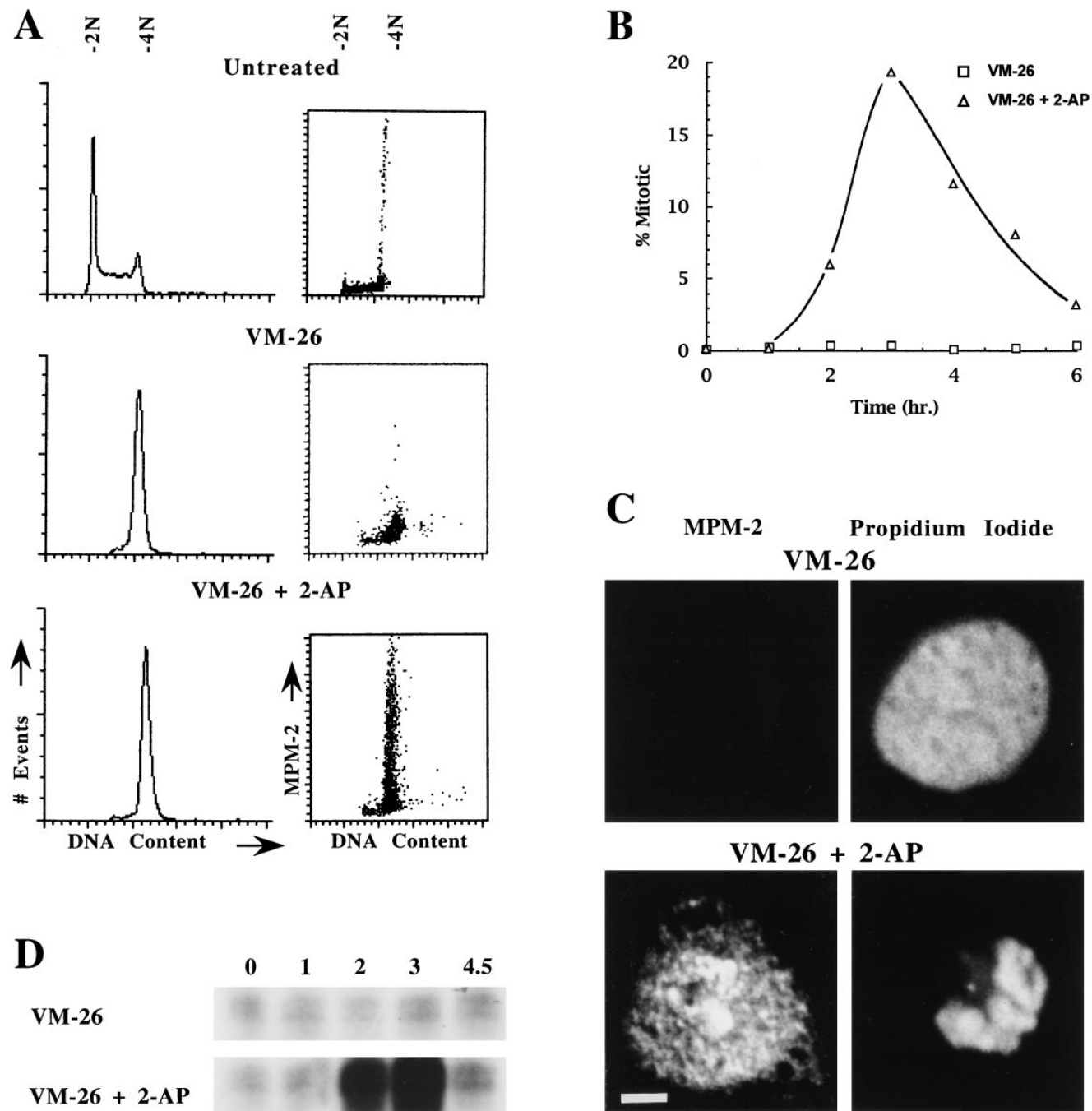
*Figure 1.* 2-AP overrides VM-26–induced $G_2$ arrest in CHO cells. (*A*) Histograms of DNA content (*left*) and two-dimensional dot plots of MPM-2, a mitosis-specific phosphoepitope, versus DNA content (*right*). In comparison to untreated controls, 16 h of treatment with 0.20 μg/ml VM-26 results in accumulation of cells in $G_2$ with 4N DNA content and low MPM-2 signal. Addition of 10 mM 2-AP during continuous exposure to VM-26 induces entry into mitosis, as indicated by the accumulation of cells with an elevated MPM-2 signal. (*B*) Quantitation of mitotic entry induced by 2-AP. Quantitation was performed by microscopy, counting cells that were both positive for MPM-2 and for chromatin condensation, as visualized with propidium iodide. Cells were synchronized in $G_2$ by 16 h of exposure to VM-26 and were subsequently exposed to both VM-26 and 2-AP or were continued in VM-26 alone. All points represent the average of three counts of at least 250 cells each. Standard deviation was <1.2% of the ordinate scale for each value. (*C*) Micrographs showing the MPM-2 immunofluorescence image of cells treated with VM-26 (*top*) or with both VM-26 and 2-AP (*bottom*). MPM-2 levels are low in interphase cells, which are identified by the presence of propidium iodide stained nuclei (*right*). In contrast, MPM-2 levels are elevated in mitotic cells, also containing condensed chromatin, after induction by 2-AP. (*D*) Assay of p34$^{cdc2}$ activity in cells arrested in $G_2$ with VM-26, and in VM-26–treated cells induced to enter mitosis with 2-AP. Cells were pretreated with VM-26 for 16 h before the time points of the assay. Bar, 5 μm.

with 0.20 μg/ml VM-26 display a low MPM-2 signal (Fig. 1, *B* and *C, top*). These cells also contain intact interphase nuclei, as determined by propidium iodide counterstain. After addition of 2-AP in the continued presence of VM-26, mitotic cells with high MPM-2 levels and condensed chromatin first become apparent within 2 h, and the number of mitotic cells peaks after 3 h of exposure to both VM-26 and 2-AP. A micrograph (Fig 1 *C, bottom*) displays a typical mitotic cell, containing both a high MPM-2 signal and condensed chromatin, that is scored positive in this assay. By contrast, a cell treated with VM-26 alone, as shown, displays low MPM-2 signal and has an intact interphase nucleus.

Activation of $p34^{cdc2}$ kinase is required to induce entry into mitosis in eukaryotes (Norbury and Nurse, 1992). As a further measure of the ability of 2-AP to induce mitotic entry after $G_2$ checkpoint override, we have assayed $p34^{cdc2}$ kinase activity at time points during exposure to either VM-26 alone or to VM-26 in combination with 2-AP (Fig. 1 *D*). Elevation of $p34^{cdc2}$ activity was detectable after VM-26 arrested cells were exposed to 2-AP for 2 h and peaked at 3 h after 2-AP exposure. This result correlates well with the time course of mitotic entry as demonstrated by quantitative analysis using immunofluorescence microscopy (Fig. 1 *B*). In contrast, $p34^{cdc2}$ activity was not elevated at any time point during continued exposure to VM-26 alone (Fig. 1 *D*).

It is evident, as we have previously reported (Andreassen and Margolis, 1992), that chromatin becomes condensed when cells enter mitosis after 2-AP–induced override of $G_2$ arrest (Fig. 1 *C, bottom*). To carefully examine the state of chromosome condensation and formation in these cells, we have prepared chromosome spreads from cells treated with both VM-26 and 2-AP (Fig. 2 *A*). Repeatedly, we have found that condensed chromosomes that are apparently normal in morphology are formed after 2-AP–induced override of $G_2$ arrest with VM-26. The level of condensation is equivalent to chromosomes in control cells arrested in mitosis with nocodazole. 2-AP–induced chromosomes resemble normal prophase chromosomes (Sumner, 1991) in that their chromatid arms remain largely unseparated. In contrast, chromosomes formed in the presence of nocodazole contain two well-separated chromatid arms. Chromatin condensation and chromosome formation results from 2-AP–induced override of the $G_2$ arrest, since only interphase nuclei are present in cells treated for an equivalent period of time with VM-26 alone.

The formation of intact chromosomes during checkpoint override with 2-AP is specific to $G_2$ arrest with VM-26. By comparison, formation of intact chromosomes does not occur in S-phase checkpoint override. It has previously been reported that premature progression to mitosis in the absence of completed DNA replication results in severe fragmentation of chromosomes (premature chromosome condensation [PCC]) (Johnson and Rao, 1970; Schlegel and Pardee, 1986). In accord with those results, we have also found that override of HU-induced S-phase arrest by 2-AP results in PCC (Fig. 2 *A*).

Nearly all mitotic cells contain condensed chromosomes after 2-AP–induced override of VM-26 arrest in $G_2$ (Fig. 2 *B*). In the chromosome spreads, cells with condensed chromatin were scored for the presence of normally condensed chromosomes, as well as for incompletely condensed (axially elongated) chromosomes and for chromatin exhibiting PCC. Reproducibly, 80% of such mitotic cells yield chromosome spreads that contain normal appearing chromosomes. We conclude that VM-26 inhibition of topo II enzyme activity does not prevent the formation of chromosomes during checkpoint override. The chromosomes that form in the presence of 2-AP are not perfectly intact, however. In comparison to chromosomes in nocodazole-treated cells, some degree of chromosome fragmentation in cells treated with VM-26 and 2-AP is detectable by a small increase (~10%) in the apparent number of chromosomes in spreads (data not shown).

At the maximum, 19.3% of the cells are in mitosis at 3 h treatment with 2-AP (Fig. 1 *B*). However, cumulatively, the majority of CHO cells finally override VM-26 arrest in the presence of 2-AP. We have previously reported that checkpoint override with 2-AP induces multinucleation as a result of failure of cells to segregate chromosomes in anaphase or to undergo cytokinesis (Andreassen and Margolis, 1991, 1994). After 6 h of treatment with VM-26 and 2-AP, >85% of cells are micronucleated, indicating that the majority of CHO cells have overridden $G_2$ arrest by this time point and have passed through mitosis.

To control for the possibility that a small amount of decatenation might continue in the presence of VM-26 and that the cumulative decatenation might permit chromosome condensation, we designed a protocol to test for chromosome formation after short exposures to VM-26 (Fig. 3 *A*). Cells were first synchronized at the onset of S-phase by growth in isoleucine-deficient medium for 24 h and then released into complete medium containing 2 mM HU. A histogram of DNA content shows that the cells are predominantly 2N in DNA content at the end of the HU block (Fig. 3 *A*).
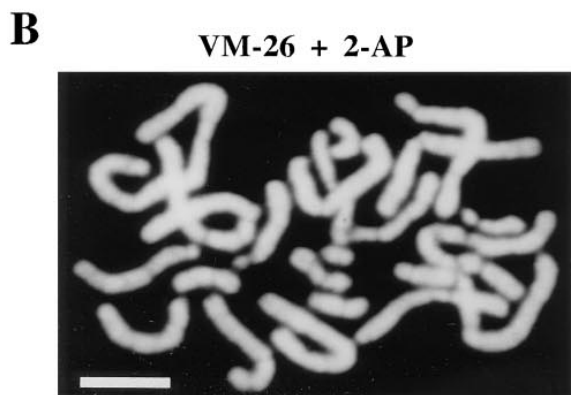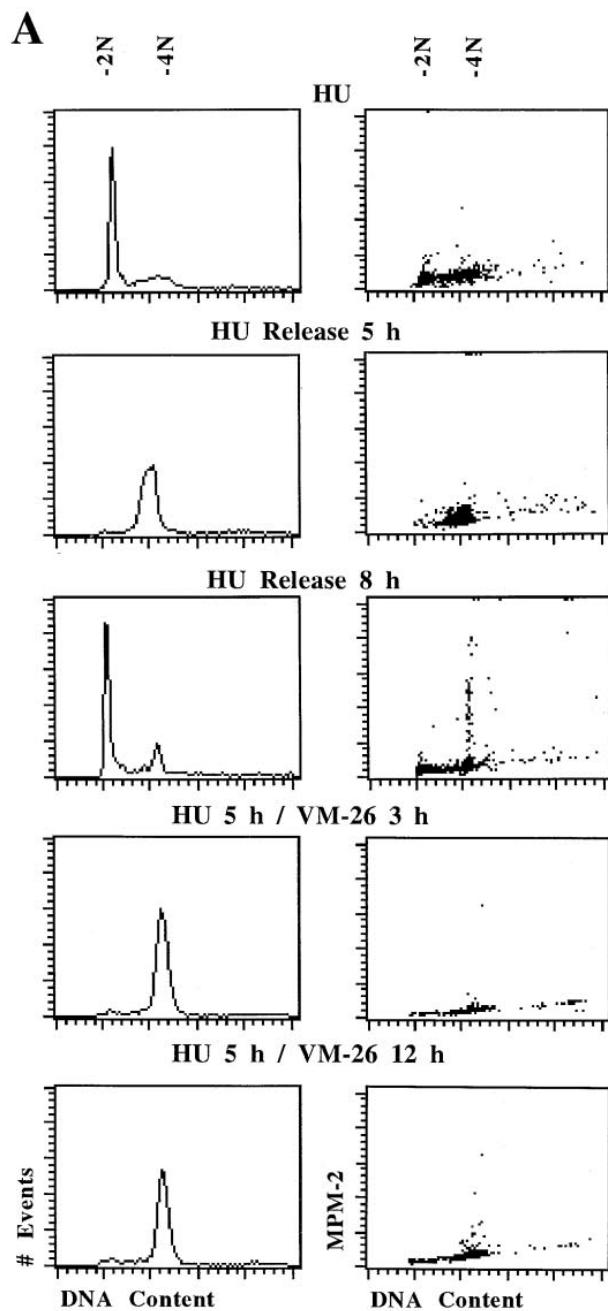
Cells were then released into drug-free medium. By 5 h after release from HU, the cells have largely progressed through S-phase and have nearly 4N DNA content. The absence of MPM-2 signal (Fig. 3 *A*) indicates that the population has not yet entered mitosis at this time. Cells exposed to VM-26 at 5 h after HU release uniformly exit S-phase within 3 h and have 4N DNA content. MPM-2 signal is low, indicating the cells are arrested in $G_2$ (Fig. 3 *A*). The arrest imposed by VM-26 is stable, as the synchronous population remains in $G_2$ after 12 h of exposure to VM-26 (Fig. 3 *A*). In contrast, the control population continues to cycle after release from HU and has largely progressed through mitosis by 8 h, as indicated by the predominant peak of cells with 2N DNA content (Fig. 3 *A*).

To demonstrate the ability of cells to form chromosomes after brief exposure to VM-26, we blocked cells in $G_2$ for 3 h with VM-26, starting 5 h after release from HU and then exposed them to 2-AP in the continuous presence of VM-26. After such brief exposures to VM-26, cells yield chromosome spreads of normal appearance (Fig. 3 *B*).

We also used such presynchronized cells to address the question of the stability of the VM-26 block. VM-26 inhibits DNA decatenation by stabilizing the covalent DNA–topo II cleavage intermediate (Chen et al., 1984). The covalent DNA–topo II complex can be isolated by lysis of blocked cells in the presence of denaturing detergent, fol-

**A**

**VM-26**  **VM-26 + 2-AP**  **VM-26 + 2-AP**

**Untreated Mitosis**  **Nocodazole**  **HU + 2-AP**



**B**
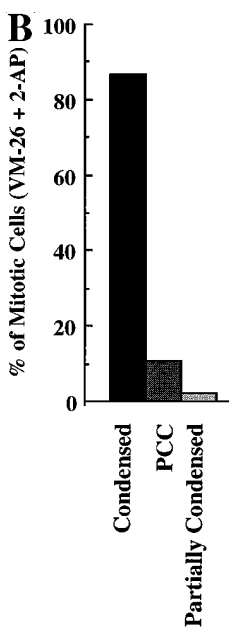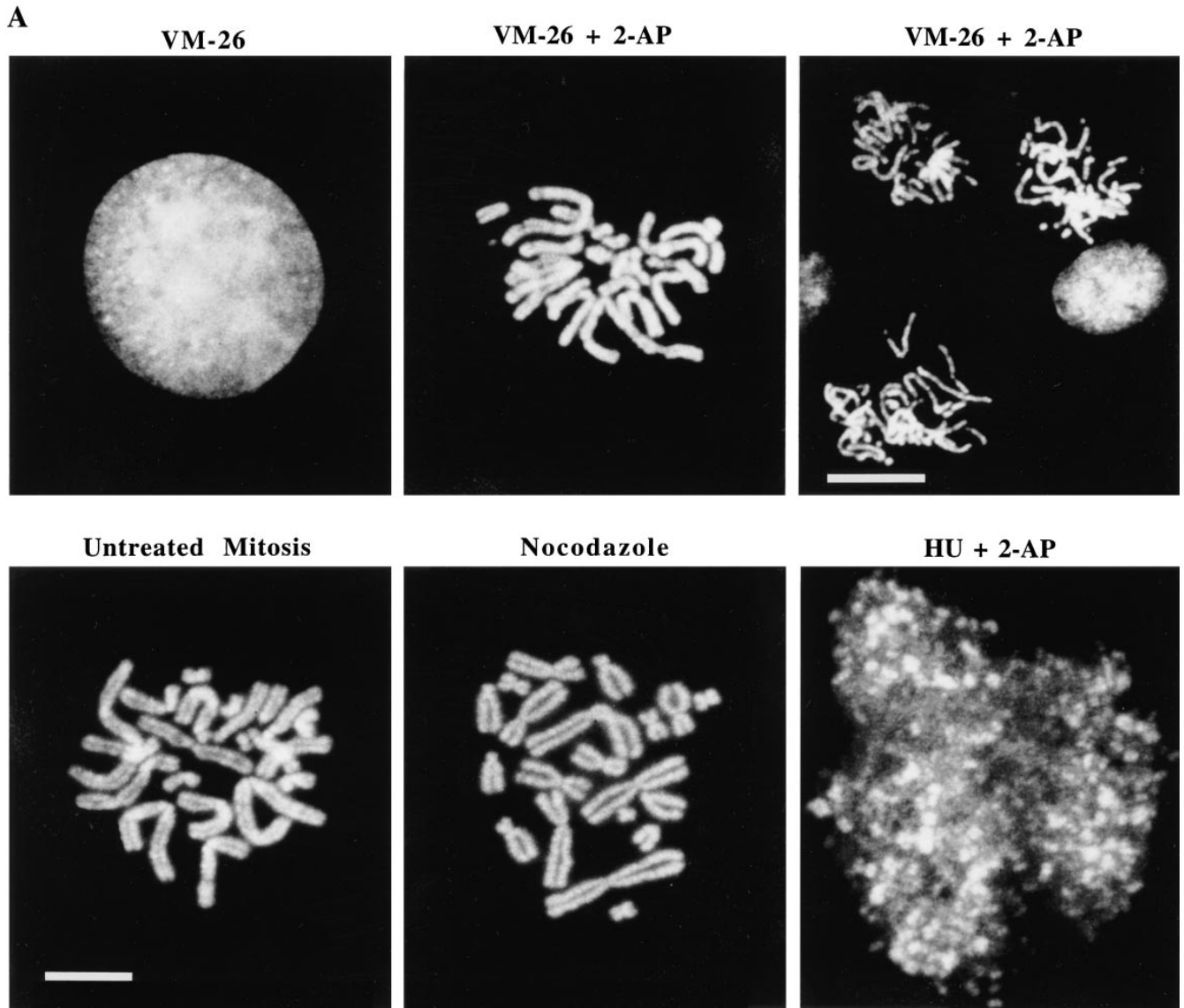
*Figure 2.* Condensed chromosomes form when CHO cells, blocked in G$_2$ with VM-26, are induced to enter mitosis with 2-AP. (*A*) Chromosome spreads of mitotic cells treated with VM-26 and 2-AP, and spreads from untreated mitotic cells, or from cells arrested in mitosis with 0.20 μg/ml nocodazole. For comparison, images are also shown of VM-26–treated G$_2$ nuclei and of chromosome fragmentation in spreads from cells blocked in S-phase with 2 mM HU and then advanced to mitosis by addition of 2-AP. (*B*) Quantitation of the percentage of VM-26–blocked cells, treated with 2-AP, that contain condensed chromosomes at mitosis. Counts were made from chromosome spreads prepared by a 16 h block in VM-26, followed by a 3-h treatment with both VM-26 and 2-AP. For comparison, the percentage of mitotic cells with incompletely condensed chromosomes or with fragmented chromosomes (*PCC*) were also quantitated. All data are derived from three counts each of 150 or more mitotic cells. Bars: (*lower left*) 10 μm (for all panels except upper right); (*upper right*) 20 μm.

A

HU

HU Release 5 h

HU Release 8 h

HU 5 h / VM-26 3 h

HU 5 h / VM-26 12 h

# Events

MPM-2

DNA Content

DNA Content

B

VM-26 + 2-AP

lowed by separation of the DNA on a CsCl gradient (Trask and Muller, 1988). Covalently associated topo II will migrate with the DNA fractions of the gradient. We have used this procedure to isolate the DNA–topo II complex from synchronous cells exposed to VM-26 for varying times in $G_2$ (Fig. 4). DNA isolated from untreated cells migrates to fractions 14–17, while DNA from cells treated for >9 h with VM-26 migrates predominantly in fractions 15–18 (Fig. 4 *A*). DNA is well separated from the protein fractions, which peak around fraction 5 (not shown).

We assayed for the presence of topo II in the DNA peak fractions (14–18) by immunoblotting procedures, using anti–topo II antibody as a probe (Fig. 4 *B*). Cells treated with VM-26 for 6, 9, or 12 h beginning 5 h after release from HU display strong signals for topo II conjugated with DNA, whereas untreated cells (Fig. 4 *B*) show no significant presence of topo II in these fractions. We scanned these immunoblots to quantitate the relative level of topo II present in the DNA fractions (Fig. 4 *C*). Importantly, we find that the level of topo II in the DNA fractions is essentially unchanged with time of exposure to VM-26. We conclude that VM-26 stably inhibits topo II activity in CHO cells under conditions that permit the formation of compact chromosomes after 2-AP induction. Further, we have found (data not shown) that cells still largely in S-phase after 3 h treatment with VM-26 form substantially fewer covalent intermediates (18% of maximum values) by this assay. Therefore, we conclude that the formation of covalent intermediates is specific to $G_2$ DNA decatenation and is not the result of random DNA damage.

The covalent DNA–topo II intermediate induced by VM-26 results in double-strand DNA breaks that can readily be detected by field inversion gel electrophoresis (FIGE) (Charron and Hancock, 1990; Roberge et al., 1990). We have used this technique as an additional assay of topo II inhibition by VM-26 (Fig. 5 *A*). Treatment with 0.20 μg/ml VM-26, the concentration used to bring about arrest of CHO cells in $G_2$, induces fragments of 200 kb and larger (Fig. 5 *A*). The size and degree of fragmentation is equivalent to that of samples treated with higher concentrations of VM-26, ranging to 2.0 μg/ml. The only difference apparent at higher concentrations of VM-26 is the generation of small fragments that result from random breakage in addition to the population of 200-kb fragments generated from DNA loops anchored by the chromosome scaffold (Charron and Hancock, 1990). To avoid problems arising from random breaks, we have used VM-26 at 0.20 μg/ml for

*Figure 3.* Condensed chromosomes form in CHO cells after 2-AP–induced override of short-term arrest with VM-26. (*A*) Histograms of DNA content (*left*) and two-dimensional dot plots of MPM-2 versus DNA content (*right*). Cells arrested with 2 mM HU were first presynchronized in $G_1$ by growth in isoleucine-deficient medium. Treatment with 0.20 μg/ml VM-26 beginning at 5 h after release from HU results in arrest at $G_2$ with 4N DNA content and low MPM-2 signal. (*B*) Condensed chromosomes form in synchronous cells treated with VM-26 for 3 h when they are induced to enter mitosis through checkpoint override by addition of 2-AP in the continued presence of VM-26 for 3 h. Cells were released from HU for 5 h and then treated with VM-26 just before arrival at $G_2$. Bar, 10 μm.

**A**

**B**

**CsCl Gradient Fractions**

**C**
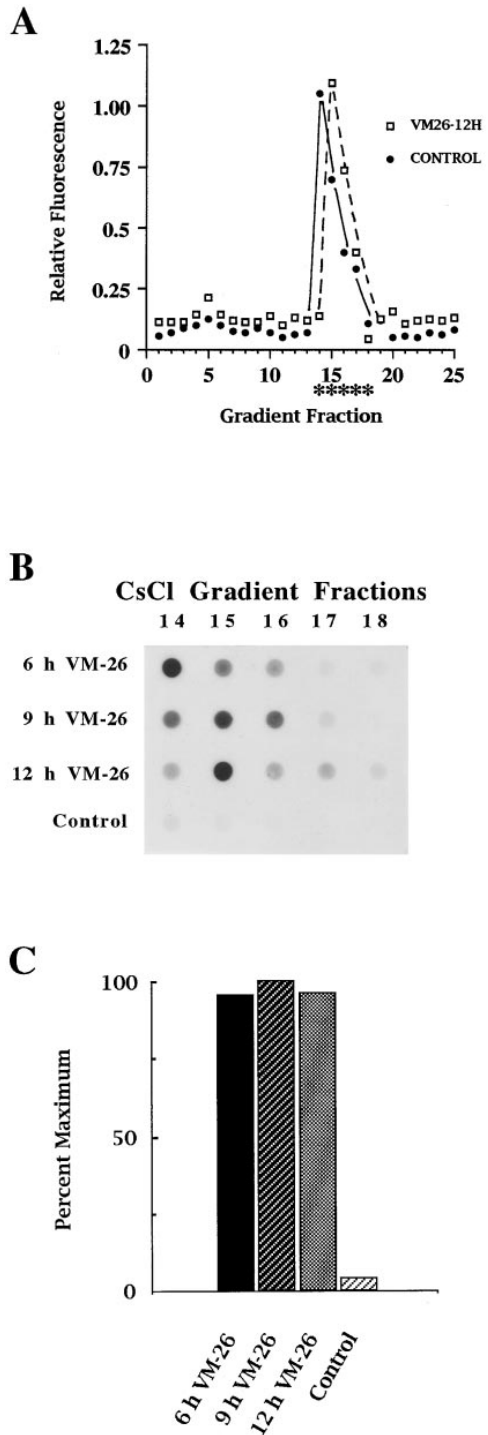
*Figure 4.* VM-26 treatment stably maintains DNA–topo II covalent complexes in G₂-arrested cells. The unchanging presence of covalent complexes indicates suppression of topo II activity. Covalent complexes of DNA and topo II were isolated on cesium chloride gradients after lysis of cells in the presence of sarkosyl. (*A*) The fractions containing DNA were identified by fluorescence measurement at an emission wavelength of 460 nm after Hoechst 33258 staining. DNA was always confined to fractions 14–18 in repeated experiments. The fractions shown in anti–topo II immunoblots are indicated with asterisks. (*B*) Topo II–DNA complexes were assayed by anti–topo II immunoblots of fractions containing DNA for cells exposed to 0.20 μg/ml VM-26 for 6, 9, or 12 h after release from presynchronization with isoleucine-

checkpoint override experiments. VM-26 at this concentration is sufficient to stably block decatenation, as shown by the constant levels of covalent intermediates generated at different times of drug exposure (Fig. 4 *C*).

We have also used FIGE to verify inhibition of topo II activity by VM-26 in CHO cells both during G₂ arrest and at mitosis after checkpoint override induced by 2-AP. (Mitotic cells were purified by mitotic shakeoff from the culture dish.) (Fig. 5 *B*). The degree of DNA cleavage and size of the fragments generated (∼200 kb) are equivalent during G₂ and in mitosis after checkpoint override. In contrast, fragmentation is minimally detectable in untreated CHO cells or CHO cells treated with 10 mM 2-AP alone (Fig. 5 *B*). We conclude that VM-26 suppresses DNA decatenation in G₂ and that decatenation remains blocked in mitosis after checkpoint override. From these results, it is evident that apparently normal chromosomes routinely form in VM-26 and 2-AP, although they contain fragmented DNA.

ICRF-193, another inhibitor of topo II activity, acts at a different step in the topo II reaction pathway, locking the enzyme in a "closed-clamp" conformation before DNA cleavage or strand passage (Tanabe et al., 1991; Roca et al., 1994). Exposure of CHO cells to 10 μM ICRF-193 for 16 h results in a cell population with 4N DNA content and low MPM-2 signal (Fig. 6 *A*). In certain cell types, ICRF-193 does not yield a G₂ block. Instead, these cells pass through mitosis without cytokinesis (Ishida et al., 1994). The DNA content profile observed in Fig. 6 *A* could be consistent with an effect of ICRF-193 on mitosis only, since in both cases 4N DNA content is expected. We demonstrate, however, that under the experimental conditions applied here, ICRF-193 induces an effective and complete G₂ block. Removal of ICRF-193 from the culture medium results in progression into mitosis with a 4N DNA content and a corresponding elevation in MPM-2 signal. If the cells had not blocked in G₂ but had instead passed through mitosis without cell division, they would have an 8N DNA content at the next mitosis after an intervening round of DNA replication. This is not observed. As observed with VM-26, 2-AP induces override of ICRF-193–dependent G₂ arrest. After addition of 2-AP, the blocked cells progress into mitosis, accompanied by an elevation in the MPM-2 signal in cells with 4N DNA content, as measured using two-dimensional flow cytometry.

The percentage of mitotic cells, scored positive by the presence of MPM-2 antigens and condensed chromatin, peaked at 19.5% of the total population 3 h after 2-AP was added in the continued presence of ICRF-193 (Fig. 6 *B*). The time course and amplitude of 2-AP–induced override of ICRF-193 arrest (Fig. 6 *B*) was similar to the time course observed in 2-AP–induced override of VM-26 arrest (Fig. 1 *B*). By contrast, cells in the continued presence of ICRF-193 alone remain arrested in interphase.

deficient medium and then HU. A control, representing cells subjected to synchronization but receiving no treatment with VM-26, is also shown. (*C*) The relative levels of topo II present in complexes with DNA were quantitated by scanning the dot blots in *b* and integrating and summing the signals displayed by all the fractions containing DNA.
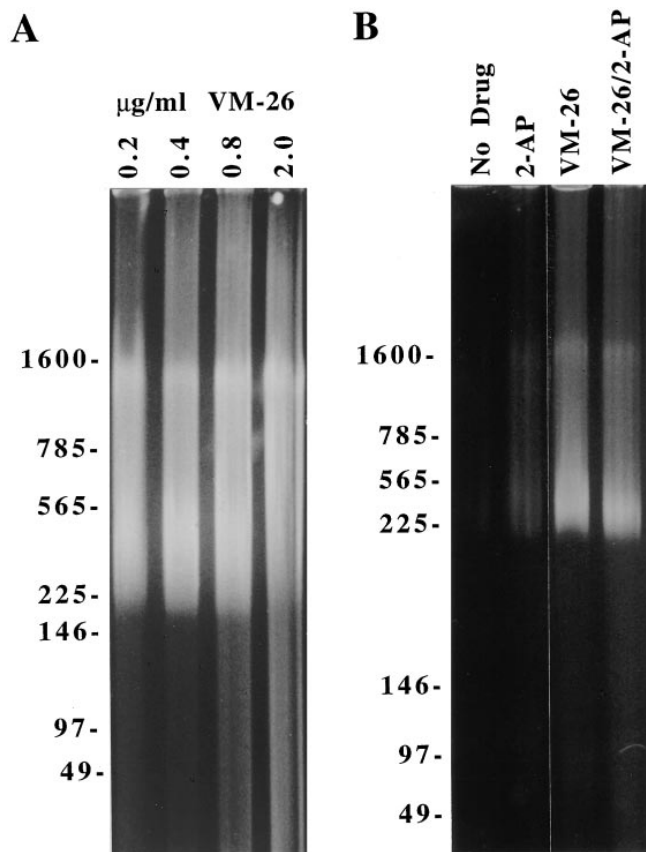
*Figure 5.* VM-26 inhibits topo II activity both during $G_2$ and at mitosis induced by checkpoint override with 2-AP. Inhibition of topo II activity was assayed by FIGE analysis of DNA fragments formed by VM-26 stabilization of topo II–DNA covalent intermediates. (*A*) The size and abundance of fragments is equivalent for cells treated for 16 h with 0.20, 0.40, 0.80, or 2.0 μg/ml VM-26 (0.31, 0.61, 1.22, and 3.1 μM VM-26, respectively). (*B*) The size and abundance of DNA fragments are equivalent, comparing $G_2$ cells, obtained by 16 h of exposure to 0.20 μg/ml VM-26, with mitotic cells collected by selective detachment after 16 h of treatment with VM-26 and then 3 h of exposure to VM-26 and 2-AP. By comparison, fragmentation is minimal in untreated control cells or in cells treated for 3 h with 2-AP alone.

ICRF-193 inhibits topoisomerase II at an earlier step than VM-26, before the initiation of DNA strand breaks (Tanabe et al., 1991; Roca et al., 1994). As expected from its mechanism, ICRF-193 does not induce significant levels of DNA strand breaks as measured by FIGE (data not shown). Chromosome spreads were prepared to examine chromosome formation in CHO cells after 2-AP–induced override of $G_2$ arrest with ICRF-193 (Fig. 7 *A*). Control cells that remain arrested in $G_2$ with ICRF-193 alone display normal interphase nuclei. In contrast, after induction of mitosis by addition of 2-AP in the continued presence of ICRF-193, the chromatin condenses into highly elongated fibers composed of two parallel arms (Fig. 7 *A*). Thus, ICRF-193 does not prevent the resolution of the chromatin into a pair of elongated sister chromatids.

All the above observations have been made on CHO cells. We have also observed similar chromosome condensation in BHK cells on entry into mitosis after 2-AP–induced
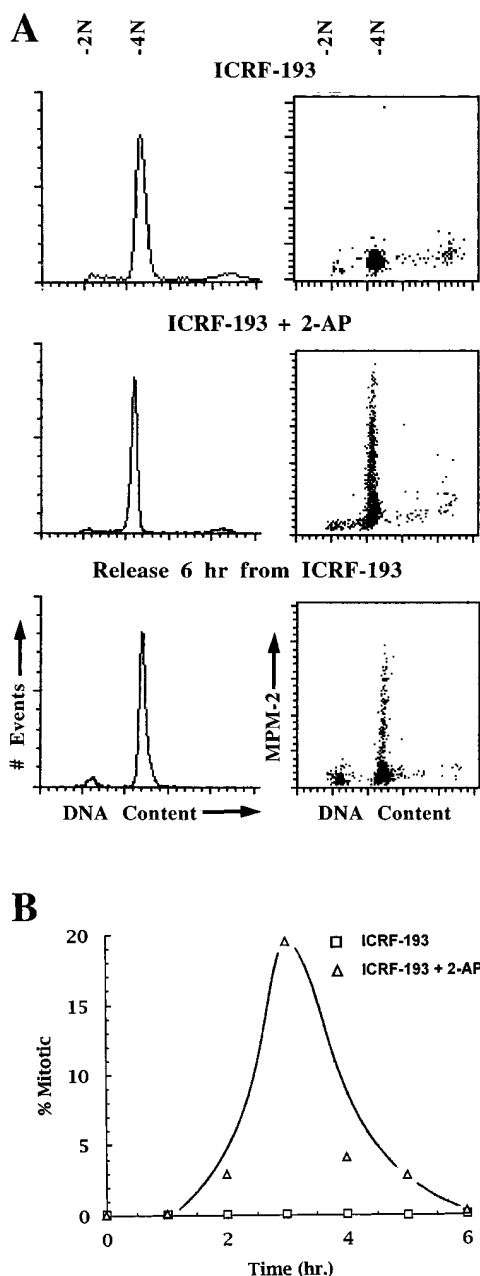


*Figure 6.* 2-AP overrides ICRF-193–induced $G_2$ arrest in CHO cells. (*A*) Histograms of DNA content (*left*) and two-dimensional dot plots of MPM-2 versus DNA content (*right*). Cells treated with ICRF-193 alone for 16 h contain 4N DNA. After 16 h of ICRF-193 treatment, cells enter mitosis when treated with both ICRF-193 and 10 mM 2-AP for 3 h, as indicated by elevated MPM-2 levels in cells with 4N DNA content. The result is equivalent to mitotic entry observed after removal of ICRF-193 for 6 h. (*B*) Quantitation of mitotic entry induced by 2-AP in ICRF-193–blocked $G_2$ cells. Mitotic cells were counted at the time points indicated by microscopic assay, scoring cells with both elevated MPM-2 levels and condensed chromatin, as detected by propidium iodide stain. Cells were arrested for 16 h in ICRF-193 before addition of 2-AP at the first time point. Each point represents the average of three counts of at least 250 cells each. Standard deviations were always <1.2% of the ordinate scale.
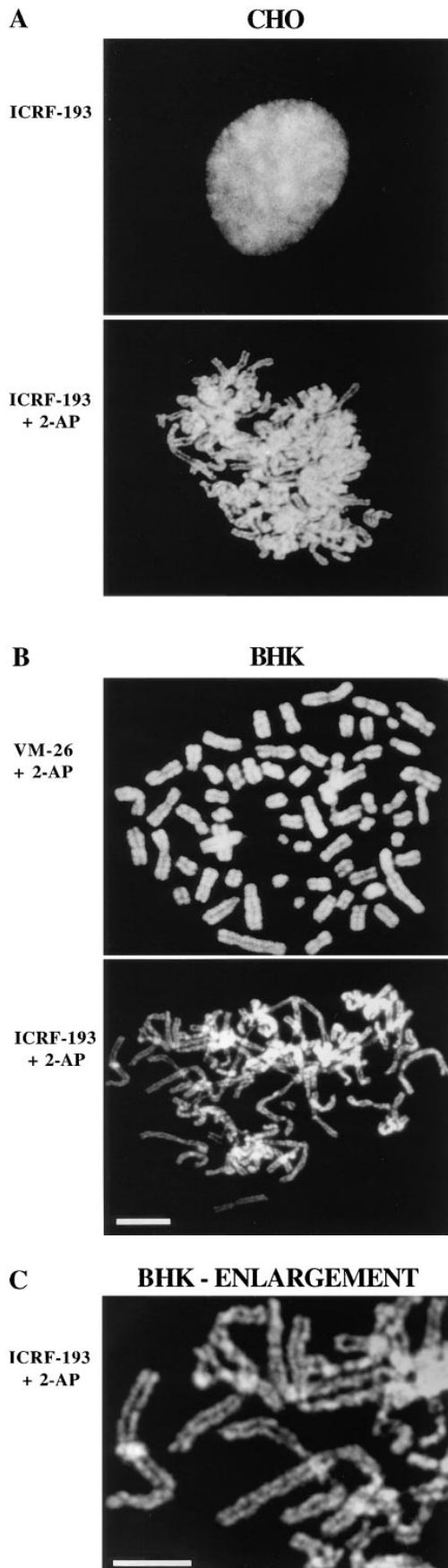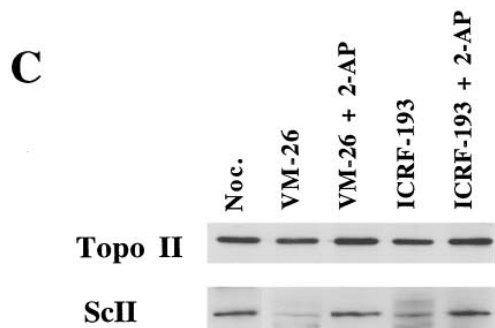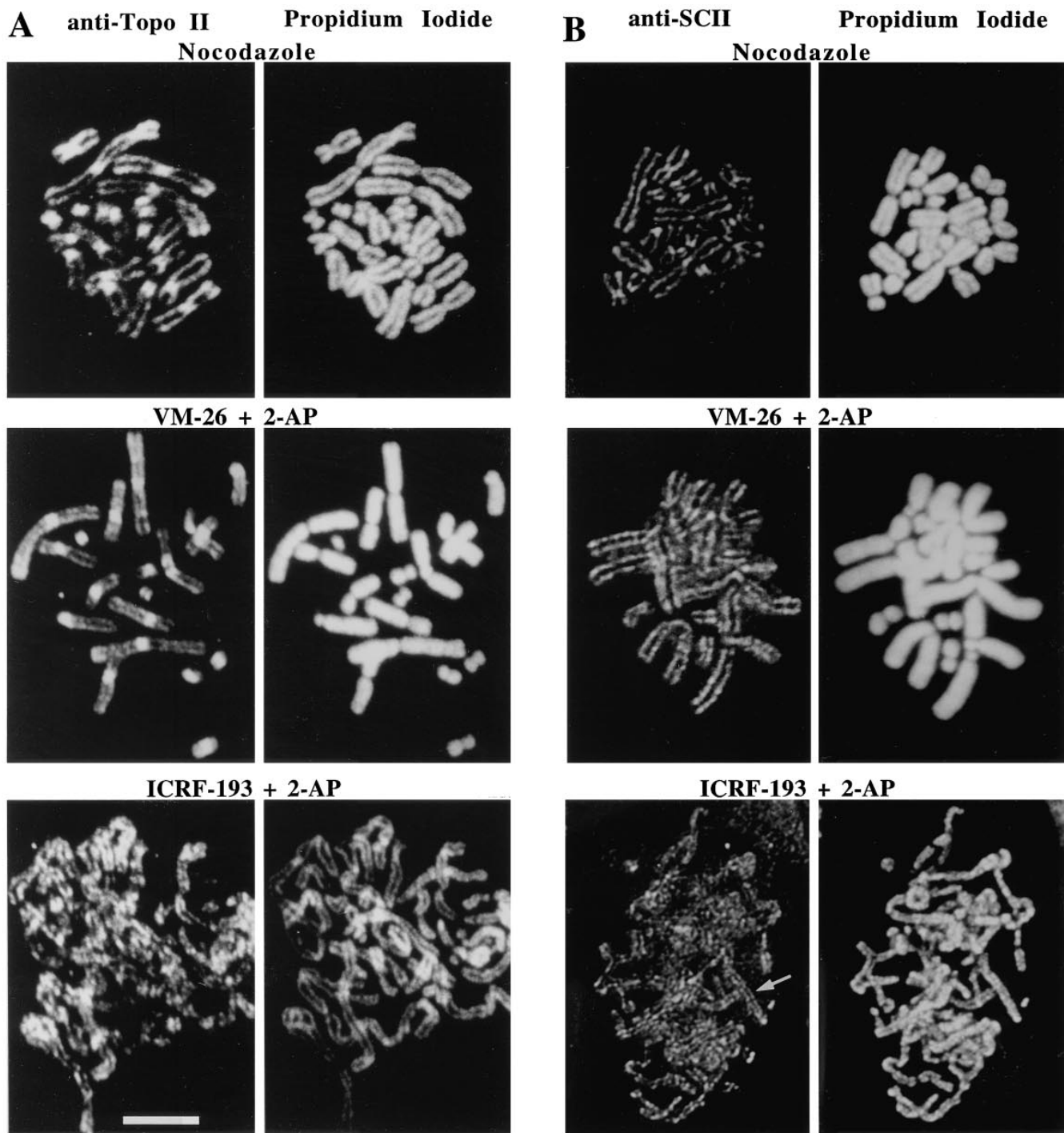
override of VM-26 or ICRF-193–dependent $G_2$ arrest (Fig. 7 *B*). As in CHO cells, condensed chromosomes form in the combined presence of VM-26 and 2-AP. In the combined presence of ICRF-193 and 2-AP, the chromosomes that form consist of two elongated parallel fibers. The formation of chromosomes with paired parallel fibers is strikingly evident in an enlarged image of the same cell (Fig. 7 *C*). In the two cell types observed, DNA decatenation is not required for chromosome formation, but the incomplete condensation of chromatin in ICRF-193 suggests that full chromosome condensation may require topo II to function in a nonenzymatic manner, since final condensation is inhibited by ICRF-193, but not by VM-26.

Topo II and ScII are the major proteins of the chromosome scaffold (Lewis and Laemmli, 1982). The presence of these proteins in the chromosome scaffold suggests that they play a role in the formation and organization of the chromosome structure (Earnshaw and Heck, 1985; Earnshaw et al., 1985; Saitoh et al., 1994). We have therefore examined chromosomes formed during override of $G_2$ arrest to determine the distribution of topo II and ScII (Fig. 8, *A* and *B*) and to quantitate their levels of association with chromatin under various conditions (Fig. 8 *C*). Confocal immunofluorescence microscopy (Fig. 8 *A*) demonstrates that topo II is localized to the two axial cores present within the arms of control chromosomes generated by mitotic arrest with nocodazole. Similarly, chromosomes formed in the presence of VM-26 and 2-AP display two axial cores containing topo II. This result is striking, as the two axial cores are present within chromosomes in which the chromatid arms remain unseparated. Topo II is similarly localized in unbroken double strands along the length of the two parallel fibers formed during mitosis induced in the presence of ICRF-193 and 2-AP.

We find that ScII displays the same pattern of distribution within paired axial cores in control chromosomes, and in chromosomes formed by $G_2$ checkpoint override in the presence of VM-26 and 2-AP (Fig. 8 *B*). Like topo II, ScII is evident throughout the length of the long parallel fibers generated after override of ICRF-193–dependent $G_2$ arrest. It is clear from these results that the association of topo II and ScII with paired axial cores within the chromosome does not require DNA decatenation as a prerequisite.

Immunoblots performed on chromosomes, isolated from mitotic cells generated by override of either VM-26 or ICRF-193 $G_2$ block, reveal that topo II and ScII are both



*Figure 7.* Partially condensed chromosomes containing two paired arms form at mitosis after 2-AP–induced override of ICRF-193 $G_2$ arrest. (*A*) Chromosome spreads of CHO cells treated with ICRF-193 for 16 h and then for 3 h with both ICRF-193 and 2-AP contain elongated chromosomes with two arms. In contrast, cells treated with ICRF-193 alone contain only nuclei with uncondensed chromatin. (*B*) Spreads of chromosomes formed by BHK cells arrested in $G_2$ with VM-26 or ICRF-193 induced to enter mitosis by addition of 2-AP. As in CHO cells, VM-26 does not inhibit full chromosome formation and ICRF-193 results in the formation of elongated chromosomes with well-resolved parallel arms. (*C*) Enlargement of chromosomes from same cell treated with ICRF-193 and 2-AP, shown in part *B*. Chromosomes are visualized by staining with propidium iodide. Bars: (*a* and *b*) 10 μm; (*c*) 5 μm.

# A anti-Topo II          Propidium Iodide
## Nocodazole



## VM-26 + 2-AP

## ICRF-193 + 2-AP

# B anti-SCII          Propidium Iodide
## Nocodazole

## VM-26 + 2-AP

## ICRF-193 + 2-AP

# C

|  | Noc. | VM-26 | VM-26 + 2-AP | ICRF-193 | ICRF-193 + 2-AP |
|---|---|---|---|---|---|
| Topo II | | | | | |
| ScII | | | | | |

*Figure 8.* Both topo II and ScII are present in paired axial cores of chromosomes formed by addition of 2-AP after $G_2$ arrest with either VM-26 or ICRF-193. (*A*) Topo II (*left*) was immunolocalized in chromosome spreads of mitotic cells treated with either nocodazole or both VM-26 and 2-AP, or both ICRF-193 and 2-AP. Chromosomes were visualized in images generated by counterstaining with propidium iodide (*right*). (*B*) ScII (*left*) was immunolocalized in chromosome spreads during mitotic arrest with nocodazole or after 2-AP–induced override of $G_2$ arrest with VM-26 or ICRF-193. Primary rabbit antibodies against topo II and ScII were detected with FITC-conjugated goat anti–rabbit secondary antibodies. (*C*) Immunoblots of isolated chromosomes and nuclei demonstrate that topo II is present at equivalent levels both during $G_2$ arrest with topo II inhibitors and in mitosis after 2-AP–induced checkpoint override of $G_2$-arrested cells. By contrast, ScII is present in chromatin only in mitosis either in normal mitotic cells or after 2-AP override of $G_2$ arrest induced by inhibitors of topo II. Bar, 10 μm.

present at levels comparable to control levels obtained with chromosomes isolated from nocodazole-blocked cells (Fig. 8 $C$). Topo II, but not ScII, is present in $G_2$ nuclei during arrest with either VM-26 or ICRF-193. The level of topo II in these nuclei is indistinguishable from the amount of topo II present in chromosomes isolated after override of $G_2$ arrest. Thus, during $G_2$, topo II is already present at levels required to participate structurally in the formation of the chromosome, but ScII is specifically recruited to the chromosome during mitotic onset. The presence, quantity, and distribution of both topo II and ScII in the chromosomes after checkpoint override indicates that the chromosomes formed during checkpoint override contain two independent axial cores. Further, it is apparent that, before DNA decatenation in $G_2$, topo II is already in a position to participate in formation of chromosomes with two axial cores.

## Discussion

Mammalian cells possess a $G_2$ checkpoint that requires topo II–dependent decatenation of sister DNA duplexes prior to entry into mitosis (Downes et al., 1994). Here we demonstrate that chromosomes with a two-armed axial core (chromosome scaffold) form in the absence of DNA decatenation after $G_2$ checkpoint override induced by the protein kinase inhibitor 2-AP. Thus, while topo II–dependent DNA decatenation is normally required to satisfy the $G_2$ checkpoint for progression into mitosis, its activity is not required to form the chromosome scaffold at mitosis. This suggests that topo II plays a noncatalytic structural role in chromosome condensation. The chromosome scaffold is present as an axial proteinaceous core within chromosomes in diverse systems (Adolph et al., 1977; Paulson and Laemmli, 1977; Klein et al., 1992; Hirano and Mitchison, 1994). We find that the two major scaffold proteins present in mammalian chromosomes, topo II and ScII (Lewis and Laemmli, 1982), are present in paired axial cores in chromosomes formed without DNA decatenation. Our results strongly suggest that the template for the chromosome structure has been put in place before the DNA decatenation event in $G_2$. We propose a model in which the scaffold is established by association of topo II with newly synthesized DNA as it is replicated during S-phase.

### Mitotic Entry Is Regulated by a Topo II–dependent Checkpoint in $G_2$

We have demonstrated, by flow cytometric analysis, that CHO cells arrest indefinitely in $G_2$ in the presence of the topo II inhibitors VM-26 or ICRF-193 but can be induced by the protein kinase inhibitor 2-AP to enter mitosis when topo II activity is suppressed. VM-26 inhibits topo II at an intermediate step, in which it is covalently linked with cleaved DNA (Chen et al., 1984). VM-26–induced $G_2$ arrest could therefore arise either through checkpoint response to DNA damage or failure of DNA decatenation (Lock and Ross, 1990). By contrast, ICRF-193 induces $G_2$ arrest without inducing DNA breaks (Ishida et al., 1991; Tanabe et al., 1991). Downes et al. (1994) observed a $G_2$ delay induced by ICRF-193, and on this basis they concluded that a DNA decatenation checkpoint exists in $G_2$.

As we show here, while topo II–dependent DNA decatenation is not physically required for chromosome condensation in mammalian cells, it is required both to pass the $G_2$ decatenation checkpoint and for chromosome segregation during mitosis. Since there appears to be no DNA decatenation checkpoint during mitosis (Uemura et al., 1987; Downes et al., 1991; Shamu and Murray, 1992), the $G_2$ checkpoint may serve to assure that decatenation is sufficient both to permit DNA condensation without strand breaks and to permit normal chromosome segregation in mitosis.

We find that CHO cells are stably arrested in $G_2$ with either VM-26 or ICRF-193 and that mitotic entry in their continued presence requires checkpoint override by 2-AP, accompanied by activation of p34[cdc2] kinase. Thus, topo II–dependent DNA decatenation in $G_2$ is a prerequisite for the activation of p34[cdc2] kinase and mitotic entry. Since chromosome formation normally occurs only upon entry into mitosis, inhibition of mitotic entry by the DNA decatenation checkpoint indefinitely inhibits chromosome formation.

The p34[cdc2] protein kinase is cyclically activated to induce mitosis during the eukaryotic cell cycle (Norbury and Nurse, 1992). We have previously shown that 2-AP exposure leads to the inactivation of p34[cdc2] kinase during override of mitotic arrest with taxol or nocodazole (Andreassen and Margolis, 1994). Here, we show that p34[cdc2] kinase, which is inactive during $G_2$ arrest with VM-26 or ICRF-193, is activated at mitosis after the induction of checkpoint override by 2-AP. Thus, taken together, these results show that 2-AP neither directly activates nor inactivates the p34[cdc2] kinase but instead acts to suppress checkpoint controls.

### DNA Decatenation Is Not Required for Chromosome Condensation

Our results demonstrate that DNA decatenation is not required for the formation of the two-armed chromosome scaffold (axial core) that is characteristic of paired sister chromatids at mitosis. We base this conclusion on our observation that two-armed axial cores form when cells are induced to enter mitosis by 2-AP treatment, while DNA decatenation is continuously blocked by either VM-26 or ICRF-193.

Previous studies have shown that topo II is required for chromosome condensation in *Xenopus* mitotic extracts in vitro (Newport and Spann, 1987; Adachi et al., 1991; Hirano and Mitchison, 1993). Such studies, however, have led to different conclusions as to the specific role that topo II might play in chromosome formation. In one study, after immunodepletion of topo II, addition of a high level of topo II was required for chromosome condensation, suggesting that topo II plays a structural role in chromosome formation (Adachi et al., 1991). Authors of another study, by contrast, concluded on the basis of VM-26 inhibition of chromosome condensation in mitotic extracts that chromosome condensation requires topo II enzymatic activity (Newport and Spann, 1987).

Because the above experiments were performed with exogenously added mitotic extracts containing active p34[cdc2] kinase, the involvement of topo II was independent of the

requirement to overcome a $G_2$ DNA decatenation checkpoint. Further, since such in vitro studies (Newport and Spann, 1987; Adachi et al., 1991; Hirano and Mitchison, 1993) used $G_1$ nuclei, they were unable to assess the role of topo II in the formation of two-armed chromosome morphology. In contrast to these previous studies, our analysis has uniquely permitted dissection of the role of topo II in checkpoint control and has permitted study of the capacity to form two-armed chromosomes in the absence of DNA decatenation.

We have presented evidence that, in intact cells, topo II–dependent decatenating activity is not required for the formation of chromosomes with paired arms. ICRF-193 inhibits topo II before the initiation of DNA decatenation and does not induce DNA strand breaks (Ishida et al., 1991; Tanabe et al., 1991; Roca et al., 1994). We find that chromosomes with highly elongated parallel arms form in the presence of ICRF-193 and 2-AP. Recently, it has been demonstrated that antibodies to XCAP-C, a protein present in cores of chromosomes formed in *Xenopus* extracts in vitro, block a late step in chromosome condensation (Hirano and Mitchison, 1994), yielding chromosomes with elongated fibers. Since these results are similar to what we have observed in mammalian cells treated with ICRF-193 and 2-AP, we suggest that topo II plays a nonenzymatic role in completing a final step in chromosome condensation.

Perhaps the chromosome core itself undergoes a self-assembly step involved in final compaction of the chromosome (Hirano and Mitchison, 1994). Consistent with this possibility, topo II can selectively bind and aggregate scaffold attachment region (SAR) sites in vitro (Adachi et al., 1989). Further, topo II self-associates in a phosphorylation-dependent manner in vitro (Vassetzky et al., 1994). ScII, a major protein of the chromosome scaffold, forms a complex with topo II (Ma et al., 1993) and associates with chromatin only during mitosis (Saitoh et al., 1994). Thus, ScII might interact with topo II specifically to form the mature chromosome structure during mitosis. Importantly, both topo II and ScII are present in continuous, but elongated, chromosome cores in mitotic cells after treatment with ICRF-193 and 2-AP. It is possible that ICRF-193 disrupts the interaction of topo II with a chromosome core protein (perhaps ScII) that is necessary for final condensation of both the axial core and of the chromosome.

In contrast to results with ICRF-193 and 2-AP, which suggest that topo II–dependent decatenation might be required to obtain complete chromatin condensation, our results from cells treated with VM-26 and 2-AP indicate that the DNA decatenating activity of topo II is not required to completely condense chromosomes. VM-26 directly inhibits DNA decatenation by preventing topo II strand passage, inducing a covalent complex of enzyme and cleaved DNA (Chen et al., 1984; Osheroff, 1989). Despite the inhibition of decatenation, condensed chromosomes form in the continued presence of VM-26 after 2-AP–induced checkpoint override. Thus, the continued catenated state of duplex DNA strands does not prevent chromosome condensation, suggesting that chromosome structure is templated before, and independently of, DNA decatenation. Our results suggest that, while VM-26 directly inhibits DNA decatenation, it does not interfere with the non-enzymatic structural role of topo II, thereby permitting complete chromosome condensation.

We have verified by several criteria that condensed chromosomes form despite efficient inhibition of DNA decatenation by VM-26. Double strand breaks at topo II cleavage sites result in DNA fragments of relatively uniform size that can be resolved by FIGE. The size of such fragments is $\sim$200 kb at 0.20 µg/ml VM-26, corresponding roughly to the size of DNA loops anchored by topo II (Charron and Hancock, 1990). The SAR sites that bind topo II and anchor the DNA loops appear to be the essential target of topo II–dependent DNA decatenation during $G_2$ (Adachi et al., 1989; Strick and Laemmli, 1995). Use of higher VM-26 concentrations does not change the abundance of these loop fragments but does generate smaller fragments, which probably represent nonspecific DNA cleavage intermediates (Fig. 5; Ross et al., 1984). Newport and Spann (1987) demonstrated that 10 µM VM-26 inhibits chromosome condensation in *Xenopus* extracts. This high concentration of VM-26 may have induced the formation of nonspecific (non-SAR) covalent intermediates that interfered with chromosome condensation. Our FIGE results demonstrate that DNA decatenation is blocked by VM-26 in $G_2$ and remains blocked in mitosis after checkpoint override by 2-AP. Thus, FIGE demonstrates that DNA decatenation remains inhibited after checkpoint override and that chromosomes form with fragmented DNA in the presence of VM-26 and 2-AP. In this context, it is important to note that such chromosomes contain intact paired axial cores that must be functioning here to maintain the integrity of the chromosome structure.

We have also addressed the stability of the VM-26 block by quantitating the covalent DNA–topo II complex that forms in the presence of VM-26 (Fig. 4). The level of cleavage intermediates remains unchanged during 6 h of arrest in $G_2$. If inhibition of topo II–dependent DNA decatenation were incomplete, the level of topo II found in a covalent complex with DNA would have been expected to diminish. We have found, interestingly, that cells exposed to VM-26 in S-phase show minimal formation of topo II–DNA covalent intermediates (unpublished observations), indicating that this assay is an accurate measure of decatenation specific to $G_2$ at the drug concentration used.

## The Chromosome Structure Is Templated before Mitosis

We find that formation of the two-armed chromosome scaffold does not require DNA decatenation. Paired axial cores are present in cells that lack chromatid resolution after treatment with VM-26 and 2-AP, and in the partially condensed chromosomes that form after ICRF-193 and 2-AP treatment. These results suggest that the chromosome scaffold, and the structure of the chromosome itself, is templated before the DNA decatenation event in $G_2$. There is abundant evidence that the chromosome scaffold may determine the structure of the chromosome.

The fact that two axial cores are present in chromosomes that lack distinct chromosome arms in cells treated with VM-26 and 2-AP demonstrates that the paired axial cores can exist before chromosome arm separation. This finding suggests that the axial core actively creates and un-
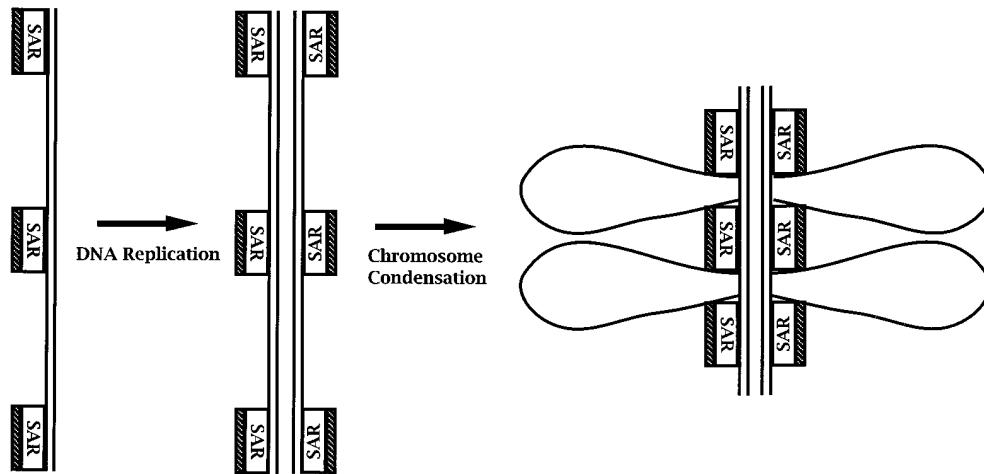
*Figure 9.* Model of the templating of the chromosome scaffold during DNA replication. In this model, we suggest that topo II (*stippled bar*) binds to new SAR sites as they are generated by DNA replication. At the termination of replication, this process yields sister DNA duplexes, each with independent scaffold elements that contain the information necessary to condense into sister chromatids independent of DNA decatenation.

derlies the chromosome morphology, in accord with results showing that the chromosome scaffold maintains the morphology of the chromosome when DNA is digested or histones are extracted (Adolph et al., 1977; Paulson and Laemmli, 1977; Earnshaw et al., 1985). Such a possibility is also supported by the recent demonstration that XCAP-C, which is a core protein in chromosomes assembled in vitro in *Xenopus* extracts, plays a role in both forming and maintaining chromosome organization. Antibody blocking of XCAP-C results in loss of chromosome organization and the appearance of elongated chromosome fibers (Hirano and Mitchison, 1994), similar to those created by combined ICRF-193 and 2-AP treatment. We have found that both topo II and ScII are present in highly elongated chromosome arms after treatment with ICRF-193 and 2-AP. This result demonstrates that an intermediate-stage axial core exists before final condensation of the chromosome, and also demonstrates that both topo II and ScII can be present in such intermediate fibers.

Recently Gimenez-Abian et al. (1995) have reported that Indian Muntjac chromatid cores separate during mitosis and that the separation is inhibited by ICRF-193. By contrast, our results show that topo II–dependent events required for separation of chromatid cores have occurred by $G_2$. It is possible that the requirements for topo II–dependent resolution of chromatid cores are different in Indian Muntjac and CHO cells. CHO cells arrest stably in $G_2$ in response to ICRF-193, whereas Indian muntjac cells arrest in mitosis (Gimenez-Abian et al., 1995). Alternatively, it is possible that the template for core separation has been completed at the molecular level during interphase but becomes manifest during mitosis. In this respect, it should be noted that Indian Muntjac chromatid cores do completely separate in a time-dependent manner in the continued presence of ICRF-193 (Gimenez-Abian et al., 1995).

We present a model (Fig. 9) in which topo II associates with the daughter DNA duplexes in a semiconservative manner during DNA replication. Topo II can bind DNA through SARs (Gasser and Laemmli, 1986; Adachi et al., 1989). We propose that topo II remains associated with SAR sites of the parental DNA strand and that topo II also associates with SAR sites that are generated on daughter DNA strands during DNA replication. In this

manner, the process of DNA replication yields independent templates for condensation of the sister DNA duplexes, such as those we have observed in cells treated with ICRF-193 and 2-AP. This model suggests that autonomous organization of the sister duplexes is templated by their independent associations with topo II and are therefore preorganized for chromosome condensation. By this model, final condensation of the chromosome would involve compaction of the topo II–associated axial cores, bringing with them condensed DNA loops that are anchored to topo II.

In support of this model, topo II binds to DNA through SAR sites both in interphase nuclei and mitotic chromosomes (Berrios et al., 1985; Mirkovitch et al., 1988), and topo II accumulates in the nuclei of proliferating cells at S-phase (Heck and Earnshaw, 1986). Furthermore, topo II is a component of the interphase nuclear matrix that supports DNA replication (Hozàk et al., 1993). Thus, topo II is positioned to associate with newly generated SAR sites at their site of synthesis. We have shown that the levels of DNA-associated topo II are equivalent in nuclei arrested in $G_2$ and in mitotic chromosomes. This result supports the possibility that the template for chromosome condensation is already in place in cells arrested in $G_2$ with inhibitors of topo II. In accord with Saitoh et al. (1994), we find that ScII only associates with mitotic chromosomes, and thus represents a mitotic factor that integrates with the topo II–based template to form the condensed and ordered chromosome.

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