# Inhibition of DNA Topoisomerase II by ICRF-193 Induces Polyploidization by Uncoupling Chromosome Dynamics from Other Cell Cycle Events

**Ryoji Ishida,* Makoto Sato,*‡ Toshiharu Narita,*‡ Kazuhiko R. Utsumi,§ Takeharu Nishimoto,‖ Takashi Morita,¶ Hiroshi Nagata,** and Toshiwo Andoh***

*Laboratory of Biochemistry, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464; ‡Research Laboratory, Zenyaku Kogyo Co., Ltd., Nerima-ku, Tokyo 178; §Laboratory of Ultrastructure Research, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464; ‖Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812; ¶Department of Hygienic Chemistry, Meiji College of Pharmacy, Tanashi, Tokyo 188; **Central Research Laboratories, Yakult Honsha Co. Ltd., Kunitachi, Tokyo 186



*Abstract.* ICRF-193, a novel noncleavable, complex-stabilizing type topoisomerase (topo) II inhibitor, has been shown to target topo II in mammalian cells (Ishida, R., T. Miki, T. Narita, R. Yui, S. Sato, K. R. Utsumi, K. Tanabe, and T. Andoh. 1991. *Cancer Res.* 51:4909–4916). With the aim of elucidating the roles of topo II in mammalian cells, we examined the effects of ICRF-193 on the transition through the S phase, when the genome is replicated, and through the M phase, when the replicated genome is condensed and segregated. Replication of the genome did not appear to be affected by the drug because the scheduled synthesis of DNA and activation of cdc2 kinase followed by increase in mitotic index occurred normally, while VP-16, a cleavable, complex-stabilizing type topo II inhibitor, inhibited all these processes. In the M phase, however, late stages of chromosome condensation and segregation were clearly blocked by ICRF-193. Inhibition at the stage of compaction of 300-nm diameter chromatin fibers to 600-nm diameter chromatids was demonstrated using the drug during premature chromosome condensation (PCC) induced in tsBN2 baby hamster kidney cells in early S and G2 phases. In spite of interference with M phase chromosome dynamics, other mitotic events such as activation of cdc2 kinase, spindle apparatus reorganization and disassembly and reassembly of nuclear envelopes occurred, and the cells traversed an unusual M phase termed "absence of chromosome segregation" (ACS)-M phase. Cells then continued through further cell cycle rounds, becoming polyploid and losing viability. This effect of ICRF-193 on the cell cycle was shown to parallel that of inactivation of topo II on the cell cycle of the ts *top2* mutant yeast. The results strongly suggest that the essential roles of topo II are confined to the M phase, when the enzyme decatenates intertwined replicated chromosomes. In other phases of the cycle, including the S phase, topo II may thus play a complementary role with topo I in controlling the torsional strain accumulated in various genetic processes.

DNA topoisomerases (topo)[1] have been implicated in the maintenance of genetic processes such as replication, transcription, and recombination by controlling the higher order chromosomal structure through the cell cycle (for review, see Wang, 1985, 1987; Cozzarelli and Wang, 1990; Vosberg, 1985). Topo I and II catalyze topological changes of DNA by transiently introducing single- and double-strand breaks, respectively. While both topo I and II relax supercoiled DNA, topo II uniquely catalyzes knotting/unknotting and catenation/decatenation. In both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, species of yeast, topo I, and topo II play complementary roles in controlling the topological state of DNA during all the cell cycles, except metaphase, when topo II is essential for chromosome condensation and segregation (DiNardo et al., 1984; Uemura and Yanagida, 1984, 1986; Uemura et al., 1987; Holm et al., 1985).

In mammalian cells, however, the biological function of topo II has not been as clearly identified as in yeasts because of the lack of topo II mutants. For this purpose, specific topo

Address all correspondence to Ryoji Ishida, Department of Biochemistry, Aichi Cancer Center Institute, Chikusa-ku, Nagoya 464, Japan. Phone: 81-052-762-6111, ext. 8826; fax: 81-052-763-5233.

1. *Abbreviations used in this paper*: ACS, absence of chromosome segregation; CPT, camptothecin; DECC, 3-3′-dihexyloxacarbocyanine iodide; *m*-AMSA, 4-(9-acridinylamino)methanesulfon-*m*-anisidide; PCC, premature chromosome condensation; topo, topoisomerase; ts, temperature sensitive; VP-16, 4′-demethylepipodophyllotoxin-9-[4, 6-(0-ethylidene)-β-D-glycopyranoside]; VM-26, 4′-demethylepipodophyllotoxin-9-[4,6-(0-thenylidene)-β-D-glycopyranoside].

II inhibitors such as antitumor epipodophyllotoxins, 4′-demethylepipodophyllotoxin-9-[4,6-(0-ethylidene)-β-D-glycopyranoside] (VP-16) and 4′-demethylepipodophyllotoxin-9-[4,6-(0-thenylidene)-β-D-glycopyranoside](VM-26), have been applied to various biological systems. These agents induce DNA single- and double-strand breaks in mammalian cells via topo II–mediated cleavable complex formation. The treated cells are arrested in G2 of the cell cycle, as generally observed for proliferating eukaryotic cells exposed to a variety of DNA damaging agents (Krishan et al., 1975; Tobey, 1975). The G2 arrest is brought about by a cellular mechanism ensuring the order of cell cycle events, "check point control" (Hartwell and Weinert, 1989), which allows cells to repair damage before entering mitosis. The onset of mitosis is determined by activation of the master switch cdc2 protein kinase, which requires dephosphorylation and association with B-type cyclin (Nurse, 1990). Treatment of Chinese hamster cells in the G2 phase with an epipodophyllotoxin prevented their entering mitosis and also the cdc2 kinase activation, suggesting that inhibition of topo II through cleavable complex formation somehow interferes with the activation of the cdc2 kinase and the entry into mitosis (Lock and Ross, 1990; Lock, 1992; Roberge et al., 1990).

Biochemical studies in vitro with higher eukaryotic cells have suggested the involvement of topo II in condensation and segregation of chromosomes. Newport and Spann (1987) showed that in *Xenopus* mitotic extracts, novobiocin, and VM-26 prevented chromosome condensation. Downes et al. (1991) showed that in mitotic HeLa or PtK2 cells, VP-16 inhibited the segregation of metaphase chromosomes. Shamu and Murray (1992) used an elegant *Xenopus* in vitro system, in which all the mitotic events take place synchronously and sequentially, i.e., condensation and segregation of chromosomes followed by reassembly of nuclear envelope to form daughter nuclei. They demonstrated that VM-26 inhibited the chromosome segregation. While Sumner (1992) showed that not all topo II inhibitors block the passage of human lymphocytes through mitosis, the available data do lend support to the hypothesis that topo II plays an important role in chromosome dynamics in mitosis. However, this point remains controversial because of the problem of determining whether the inhibition of chromosome dynamics by these inhibitors results from a specific inhibition of topo II activity or a nonspecific effect of the drug-induced protein-linked DNA cleavage. Thus the arrest of metaphase progression by these inhibitors is in apparent conflict with observations in yeast, where inactivation of topo II activity in *top2* temperature-sensitive (ts) mutants at a nonpermissive temperature does not result in cessation of cell cycle progression, but allows cells to traverse mitosis, without chromosome segregation, to the next cell cycle, when DNA is replicated (Uemura and Yanagida, 1984, 1986; Uemura et al., 1987). It is relevant to refer here to the work done using cell-free extracts (Wood and Earnshaw, 1990; Adachi et al., 1991; Hirano and Mitchison, 1993), which obviated the complication caused by the use of drugs. They showed that chromosome condensation was prevented by immunodepletion of topo II from the extract, and the activity was restored by replenishment of purified topo II.

Bis(dioxopiperazines) were originally synthesized as potential intracellular chelating agents, and their biochemical and pharmacological properties, including antitumor activity, have been studied extensively over the past decades (for review, see Herman et al., 1982). The problem of inadequate bioavailability limiting clinical application (Hellman et al., 1969) has been approached by synthesis of various masked compounds showing improved bioavailability and higher antitumor activity than the parental compound ICRF-154 (Cai et al., 1989). MST-16, one of these compounds, has shown considerable therapeutic activity against a number of experimental tumors such as P388 and L1210 leukemias, B-16 melanoma, colon 26, etc. (Narita et al., 1990), and a cell cycle phase-specific mode of cytotoxicity (Narita et al., 1991), in line with the previous demonstration of toxic action specific for G2/M (Sharpe et al., 1970). We have previously reported that one of the potent derivatives of ICRF-154, ICRF-193, inhibits topo II in such a manner that, unlike VP-16, it does not stabilize the cleavable complex (Tanabe et al., 1991), but it does stabilize the enzyme in the form of a closed protein clamp by inhibiting its ATPase activity (Roca and Wang, 1992; Roca et al., 1994). We also showed that ICRF-193 in fact targets topo II in vivo, as shown by competition with the DNA cleavage and cytotoxicity caused by VP-16 (Ishida et al., 1991). In the present study, we show that the compound exhibits biological effects in mammalian cells analogous to those observed in *top2* ts mutants of yeast at nonpermissive temperature. Thus, in the presence of the drug, cells traverse many rounds of the cell cycle with their genome replicated but not segregated, resulting in polyploidization. Other cell cycle events associated with transition through S phase and M phase take place normally.

## Materials and Methods

### Drugs

The following drugs were gifts from companies: ICRF-193, Zenyaku Kogyo Co. Ltd. (Tokyo); VP-16, Bristol Meyers-Squibb (Brea, CA); camptothecin (CPT), Yakult Honsha Co. Ltd. (Tokyo); neocarzinostatin, Kayaku Antibiotics Research Co. Ltd. (Tokyo). The following drugs were purchased: TN-16 from Wako Pure Chemical Industries Ltd. (Osaka); 3,3′-dihexyloxacarbocyanine iodide (DECC) from Kodak Laboratory Chemicals (Rochester, NY); colcemid from GIBCO BRL (Gaithersburg, MD).

### Cell Culture

CHO strain AA8, HeLa S3, and tsBN2 cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum under a humidified atmosphere of 5% $CO_2$ in air. tsBN2 cells, a temperature-sensitive mutant of BHK21 cells, were cultured at 32.5°C (permissive temperature) or 41°C (nonpermissive temperature). Cell numbers were counted with a Coulter counter (Industrial D; Coulter Electronics Ltd., Luton Bedfordshire, United Kingdom). For clonogenic assay, cells were treated with the drugs indicated in Fig. 8 for 30 min, washed with Tris-buffered saline two times, and suspended by trypsinization. Cells were seeded at $3 \times 10^2$ cells/60-mm dish, cultured for 1 wk, fixed with 10% formalin in 0.9% NaCl, and stained with 0.1% crystal violet for counting colonies. Survival fractions of the drug-treated cells were expressed as percent of control cells treated with solvent alone.

### Cell Synchronization

Early S phase synchronization: HeLa S3 and CHO cells seeded at $1 \times 10^5$ cells/100-mm dish were cultured for 24 h in the presence of 1 mM thymidine, then for 9 h in the absence of thymidine, and then again for 16 h in the presence of 2 mM thymidine. Upon removal of thymidine, cells traverse synchronously from early S phase to G2 phase. tsBN2 cells seeded on the previous day at $1 \times 10^5$ cells/60-mm dish were cultured in isoleucine-deprived medium for 40 h, and transferred to and cultured in complete medium containing 2 mM hydroxyruea for 16 h.

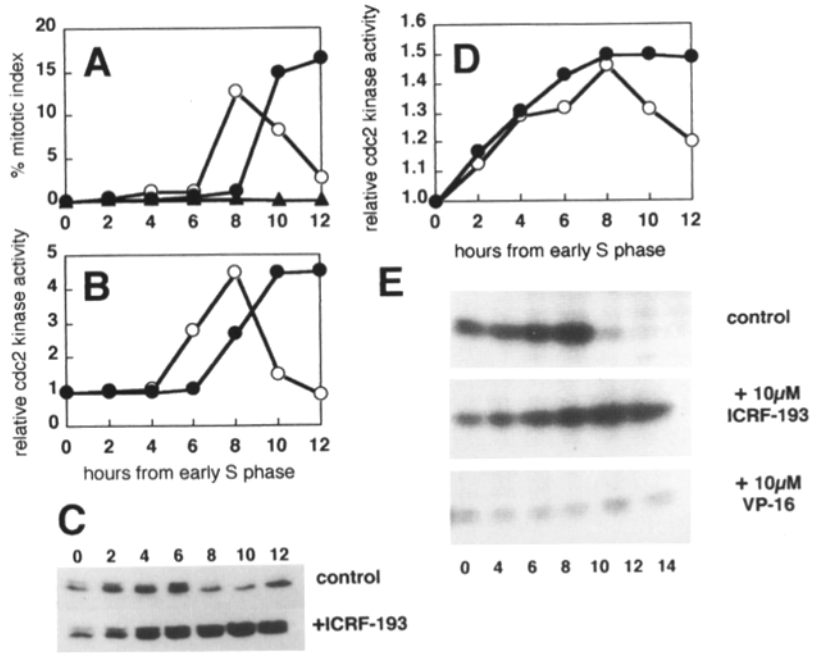


*Figure 1.* Lack of inhibition by ICRF-193 of cdc2 kinase activation and progression from early S phase to M phase. Cells were synchronized in early S phase by double thymidine treatment as described in the Materials and Methods. After release from the thymidine block, HeLa cells were cultured in the absence (○) or presence of 5 μM ICRF-193 (●) or 10 μM VP-16 (▲). (*A*) Percentages of mitotic cells were examined at the indicated times. (*B*) Cdc2 kinase activities in control (○) and ICRF-193–treated (●) HeLa cells, determined, as described in the Materials and Methods, using a specific oligopeptide S1 of 13 amino acid residues, which is the recognition sequence for cdc2 kinase, as a substrate (Yasuda et al., 1990). (*C*) Electrophoretic mobility of cdc2 kinase in control (*upper panel*) and ICRF-193–treated cells (*lower panel*) at the indicated times, examined by immunoblot analysis using polyclonal antibody against an oligopeptide of the carboxy terminus of human cdc2 kinase, as described previously (Yasuda et al., 1990). (*D*) Cdc2 kinase activities in control (○) and 5 μM ICRF-193–treated (●) CHO cells. (*E*) H1 kinase activities of control (*upper panel*), ICRF-193– (*middle panel*), and VP-16– (*lower panel*) treated HeLa cells were determined using histone H1 as a substrate. Reaction products were electrophoresed on SDS-PAGE, dried, and subjected to autoradiography.

***G2 Phase Synchronization.*** Isoleucine-starved tsBN2 cells, as described above, were transferred to and cultured in complete medium containing 1 μg/ml neocarzinostatin for 16 h.

***M Phase Synchronization.*** HeLa and CHO cells were seeded at 2 × $10^6$ cells/140-mm dish. On the next day, the medium was changed to fresh medium containing 0.5 mM 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione, an inhibitor of microtubule assembly (Hashimoto et al., 1972). After 4-h incubation, round mitotic cells were collected from monolayer culture by selective mechanical detachment, as described by Terashima and Tolmach (1963), and washed with Tris-buffered saline.

## *Mitotic Figures*

Cell samples were suspended in 1 ml of 75 mM KCl and stood at 0°C for 20 min. 1 ml of methanol-acetic acid (3:1) was slowly added to the suspension under constant mild agitation, and the cells were centrifuged down. After washing with 1 ml of the fixative, cells were resuspended in 30–100 μl of the fixative and dispensed onto glass slides. After drying, the samples were stained with Giemsa solution. Mitotic cells and premature chromosome condensation (PCC) cells were identified and counted under the microscope.

## *Immunofluorescence Microscopy*

Formalin-fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min and washed twice with PBS. For tubulin immunofluorescence, cells were attached to poly-L-lysine–coated coverslips and then incubated with tubulin-specific monoclonal antibody (Oncogene Science, Inc., Manhasset, NY) for 2 h at room temperature. After washing three times with PBS, the samples were further incubated with FITC-conjugated anti-mouse IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD), and stained with 1 μg/ml Hoechst 33258 in PBS. Coverslips were mounted in 50% glycerol solution with 2.5% 1,4-diazabicyclo-[2,2,2]octane as antibleaching agent. For membrane staining, HeLa cells were fixed with methanol-acetic acid (3:1) as described above in the "*Mitotic Figures*" section. Fixed cells were stained with 1 μg/ml of DECC for 5 min, and washed with PBS.

## *Assay of cdc2 Kinase Activity and Its Immunoblotting*

Frozen cells were suspended in hypotonic buffer (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM PMSF) at 0°C for 30 min. Nonidet P-40 was added to a final concentration of 0.1%, and the cells were vortexed and centrifuged. The precipitate was washed twice with the hypotonic buffer without NP-40, then extracted with 0.4 M NaCl for 30 min, the resultant samples being centrifuged at 105,000 *g* for 1 h. The supernatant was used as a nuclear fraction. Cdc2 kinase activity was assayed using histone H1 or S1 peptides of 13 amino acid residues, the specific recognition sequence of cdc2 kinase, as a substrate by the method of Yasuda et al. (1990). For immunoblotting, cell extracts were electrophoresed in 12.5% SDS-PAGE and transferred to Immobilon membranes (Millipore Corp., Bedford, MA). Filters were blocked with 5% skim milk and incubated with anti-cdc2 kinase (Yasuda et al., 1990). After washing with PBS, the filters were treated with $^{125}$I-protein A. Dried filters were exposed to x-ray film. To determine H1 histone phosphorylation, reaction products were electrophoresed in 12.5% SDS-PAGE, and the gels were dried and subjected to autoradiography.

# *Results*

## *ICRF-193 Does Not Inhibit Transition of Cells from S Phase to M Phase*

We have previously shown that when randomly growing cells are treated with ICRF-193, the cells are not arrested in the G2 phase, but they appear to continue further through cell cycle and they become polyploid (Ishida et al., 1991). This is unlike the inhibition observed with most other cleavable complex-stabilizing type topo II inhibitors.

In the present study, we examined more closely the effect of ICRF-193 on cell cycle transition. First, the transition from early S to M phases and the activation of cdc2 kinase were studied in HeLa and CHO cells synchronized in early S phase with a double thymidine block. After removal of the block, HeLa cells traversed through S, G2, and M phases during 12 h (Fig. 1 *A*; Ishida et al., 1991). Cdc2 kinase activity increased gradually and peaked at 8 h slightly before the M phase (Fig. 1 *B*). The kinase activity is modulated by de-

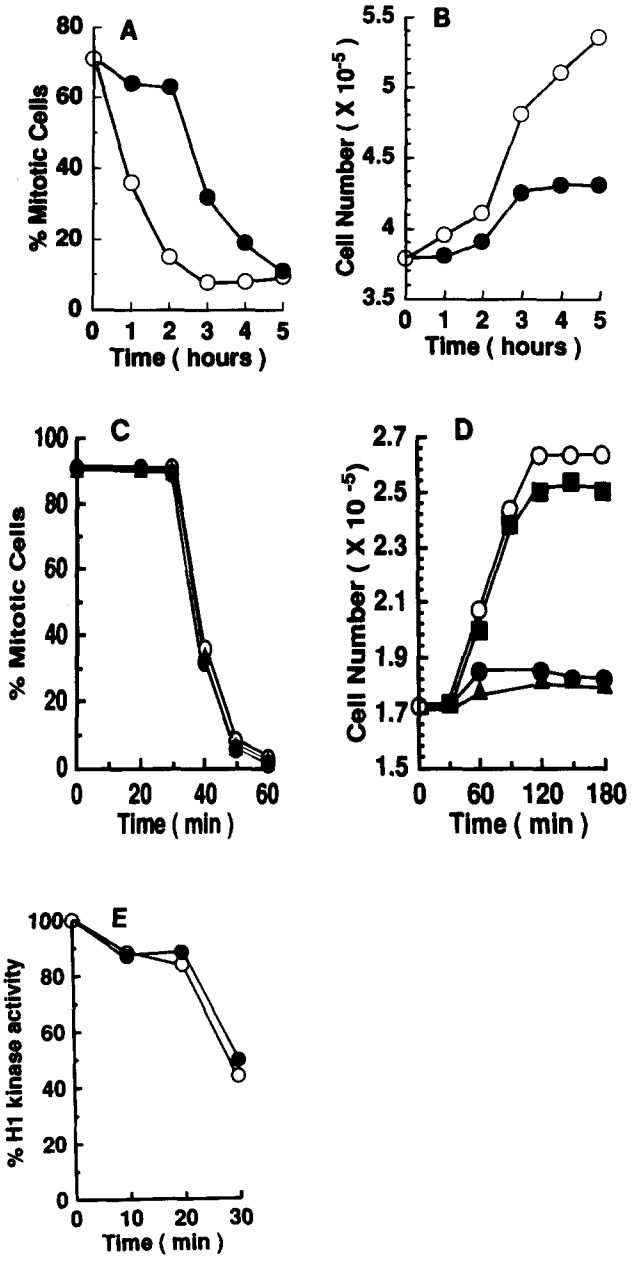


*Figure 2.* ICRF-193 inhibition of cell division. Mitotic cells were collected as described in the Materials and Methods, plated in fresh medium containing ICRF-193, VP-16, or CPT, and the percentages of mitotic cells, judged by nuclear envelope formation (*A* and *C*), total cell number (*B* and *D*), and cdc2 kinase activity (*E*) were determined at the times indicated. HeLa (*A* and *B*) and CHO cells (*C–E*) were treated with 5 μM ICRF-193 (●), 100 μM VP-16 (▲), 10 μM CPT (■), or were left untreated (○).

phosphorylation of the phosphorylated form, which can be detected by the mobility difference between the two forms on SDS polyacrylamide gel electrophoresis (Morla et al., 1989). As shown in Fig. 1, *C* and *E*, concomitant with increase in the kinase activity, dephosphorylation of the enzyme increased. Addition of ICRF-193 in the early S phase delayed the progression of HeLa cells to M phase and the increase of the kinase activity (Fig. 1, *A* and *B*): total amount of p34$^{cdc2}$ also increased; dephosphorylation thereof was delayed, and the phosphorylated form of the enzyme appeared to be maintained at a high level, even at 12 h after release from the thymidine block (Fig. 1, *B*, *C* and *E*).

In contrast, the epipodophyllotoxin VP-16 completely blocked the progression of cells to M phase and the increase in cdc2 kinase activity (Fig. 1, *A* and *E*), as reported earlier (Lock and Ross, 1990; Lock, 1992; Roberge et al., 1990). Unlike HeLa cells, a similar but undelayed rise in cdc2 kinase activity and transition to mitosis were observed in the drug-treated, synchronized CHO cells (Fig. 1 *D*), although the background activity level was high at 0 h after release from the double-thymidine block, due to poor synchronization.

### *ICRF-193 Inhibits Cell Division but Does not Affect the Traverse from M to G1 and S Phases*

Next, we examined the effect of ICRF-193 on cell cycle progression from M to G1 phases. Mitotic HeLa cells were collected using the microtubule inhibitor TN-16, and were released for traverse to the G1 phase in the presence of ICRF-193. In control culture, mitotic cells exited from mitosis and progressed into the G1 phase within 2 h, as revealed by reassembly of the nuclear envelope, and the cell number almost doubled (Fig. 2, *A* and *B*). ICRF-193 delayed the exit from M phase by 2 h, and the cell division was inhibited (Fig. 2, *A* and *B*). At 5 h, all the cells were in interphase, although the cell number did not increase. Essentially the same results were obtained with CHO cells, although the exit from metaphase was faster and the rate was not affected by ICRF-193 (Fig. 2, *C* and *D*). The cdc2 kinase activity decreased as the cells exited from mitosis in the presence or absence of the drug (Fig. 2 *E*).

Similar analyses were made to determine the effects of VP-16 and CPT, the latter being a cleavable, complex-stabilizing type topo I inhibitor on mitotic CHO cells. The results demonstrated that VP-16 inhibited cell division, as was the case with ICRF-193, whereas CPT affected neither exit from mitosis nor cell division (Fig. 2, *C* and *D*), confirming that topo II but not topo I is involved in chromosome segregation (Uemura and Yanagida, 1984, 1986; Holm et al., 1985).

### *ICRF-193 Inhibits Segregation and Retards Decondensation of Chromosomes*

Since ICRF-193 inhibits cell division, but does not affect the exit from the M phase, the question of how the drug influences the dynamic change of chromosomes and spindle formation in the M phase was addressed. As shown in Fig. 3, after release from metaphase, control untreated HeLa cells underwent mitosis through metaphase (Fig. 3 *D*), anaphase, and telophase (Fig. 3, *E–H*), and the chromosomes were separated. At 120 min, most of the cells had progressed to G1 phase. In contrast, however, in the presence of ICRF-193, cells in anaphase and telophase were seldom observed at any time after release from the M phase block (Fig. 3, *J*, *L*, and *N*), the result being consistent with the recent report of Clarke et al. (1993). Furthermore, the decondensation of unsegregated chromosomes was delayed, as it was not complete at 180 min, the cells having only slightly decondensed chromosomes like those of prophase, rarely seen in control cells because of the rapid progression through the phases.

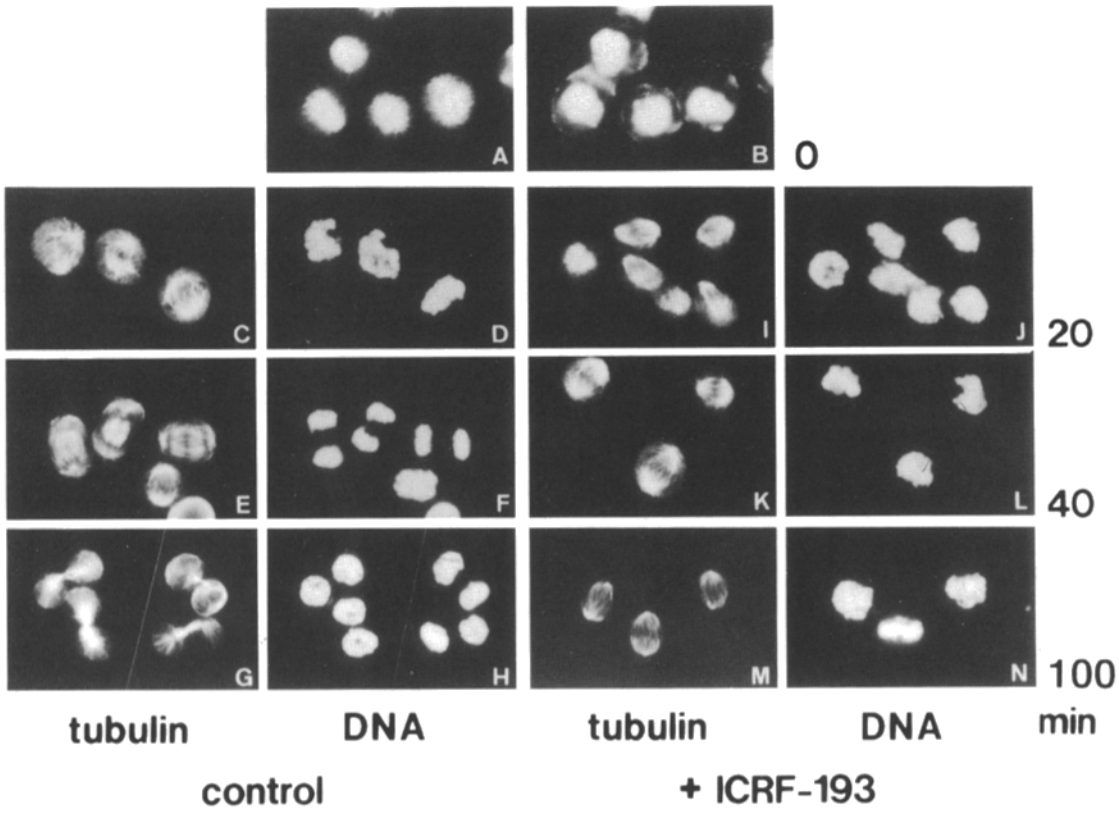


*Figure 3.* ICRF-193 inhibition of segregation and decondensation of chromosomes, but not spindle formation in mitotic HeLa cells. After removal of TN-16 as described in Fig. 2, cells were incubated in the presence (*I–N*) or in the absence (*C–H*) of 5 μM ICRF-193. At the indicated times, cells were fixed and stained sequentially with monoclonal antitubulin antibody (*A*, *C*, *E*, *G*, *I*, *K*, and *M*), and Hoechst 33258 (*B*, *D*, *F*, *H*, *J*, *L*, and *N*).

Since the failure of chromosome segregation in the presence of ICRF-193 may result from an inability to form spindles, we tested the effect of ICRF-193 on the formation of the spindle apparatus. After 20–60 min in the presence of the drug, spindles were formed and appeared to be connected with chromosomes (Fig. 3, *I*, *K*, and *M*). Thus, ICRF-193 does not appear to affect spindle dynamics.

ICRF-193 affected chromosome dynamics in CHO cells as in HeLa cells (Fig. 4), progression through mitosis being much the same, but at a faster rate. It is of particular interest to note that in telophase, in the presence of the drugs, spindle structures were transformed into midbodies at 40 min, as in untreated cells (Fig. 4, *E*, *F*, *K*, and *L*, *arrowheads*), showing cytokinesis taking place. The chromosomes, however, were not separated and appeared to be unequally separated to daughter cells (Fig. 4, *K* and *L*).

### *ICRF-193 Does Not Block Nuclear Envelope Reassembly*

As described above, cells enter an abnormal mitotic phase in the presence of ICRF-193. In normal cells, concomitant with the entry into M phase, nuclear envelope breaks down and disappears, and then upon exit from mitosis, the nuclear envelope reassembles to form the nucleus. This is illustrated in Fig. 5, mitotic HeLa cells, after release from metaphase, progressing into G1 phase during 75 min (Fig. 5, *B*, *D*, and *F*). Membrane vesicles first attach themselves to individual chromosomes and finally fuse to form the nuclear envelope (Fig. 5, *A*, *C*, and *E*). In the presence of ICRF-193, decondensation of chromosomes was delayed, and even after 120 min, the chromosomes did not completely decondense (Fig. 5, *H*, *J*, and *L*). However, nuclear envelopes form around the periphery of fused chromosomes (Fig. 5, *G*, *I*, and *K*).

### *ICRF-193 Does Not Inhibit Transition from Metaphase to the Next S Phase*

Cell cycle traverse from G1 to S phases was also examined by [$^3$H]thymidine incorporation monitoring the onset of DNA synthesis. Mitotic CHO cells were plated in the presence or absence of ICRF-193, VP-16, and CPT, and they were allowed to proceed to G1 and S phases. DNA synthesis was initiated as scheduled in the presence of ICRF-193 and CPT, whereas it was not in the presence of VP-16 (data not shown). Thus, the transition from G1 to S phases was not affected by ICRF-193 or CPT, but it was inhibited by VP-16. The results clearly show that ICRF-193 inhibits cell division but does not affect progression from M to G1 and further to the S phase, suggesting that topo I activity is sufficient for these transitions, and that topo II–mediated double-strand breakage induced by VP-16 blocks entry into the S phase.

### *ICRF-193 Partially Inhibits Premature Chromosome Condensation in S and G2 Phases*

In a previous paper (Ishida et al., 1991) we documented that

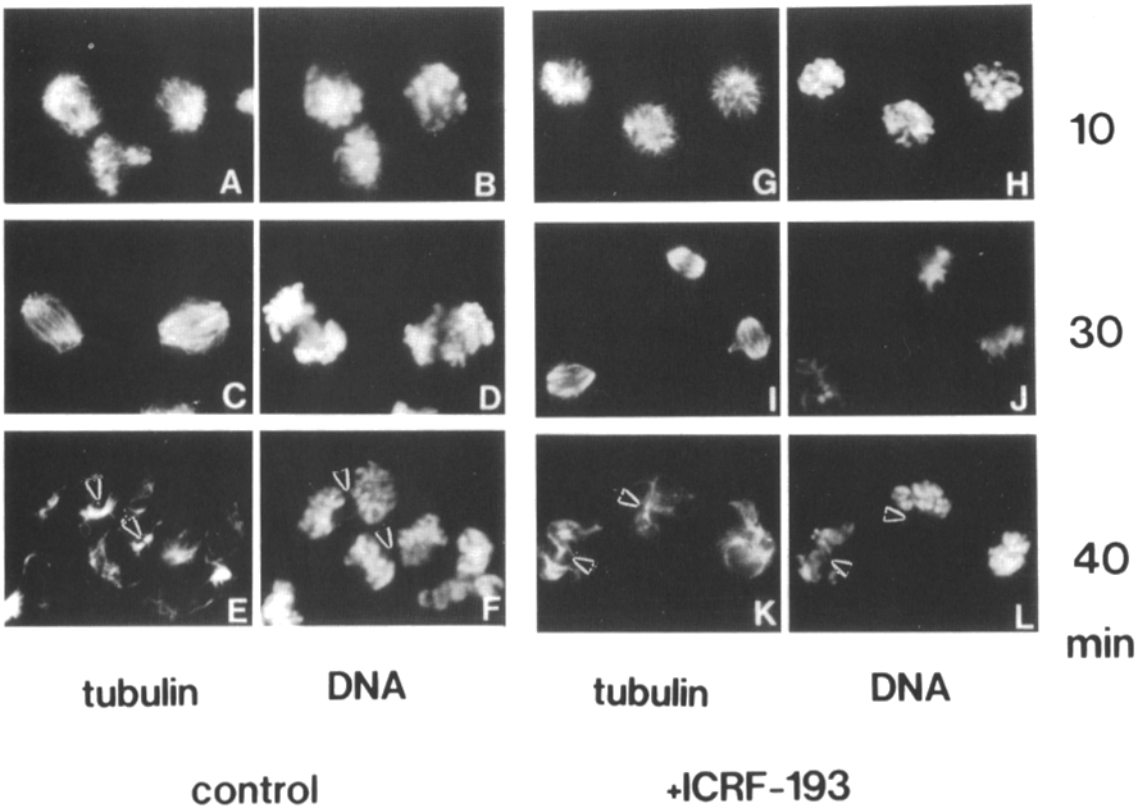


*Figure 4.* ICRF-193 inhibition of segregation of chromosomes in mitotic CHO cells. Mitotic cells collected as described in the Materials and Methods were incubated in the presence (*G–L*) or in the absence (*A–F*) of 5 μM ICRF-193. At the indicated time points, cells were fixed and stained with monoclonal antitubulin antibody (*A*, *C*, *E*, *G*, *I*, and *K*) and Hoechst 33258 (*B*, *D*, *F*, *H*, *J*, and *L*).

when RPMI8402 leukemic cells were treated with ICRF-193, the mitotic index did not increase, but abnormal mitotic figures were observed, e.g., early prophase-like figures and clumps of long entangled chromatid fibrils. It was suggested that the drug inhibits chromosome condensation at some early stage.

To confirm this, we turned to the tsBN2 mutant of BHK21 cells, which exhibit PCC at a nonpermissive temperature (Nishimoto et al., 1978). PCC mimics normal chromosome condensation, as evidenced by the activation of cdc2 kinase and association of mitosis-specific antigen recognized by monoclonal antibody MPM-2 with PCC chromosomes

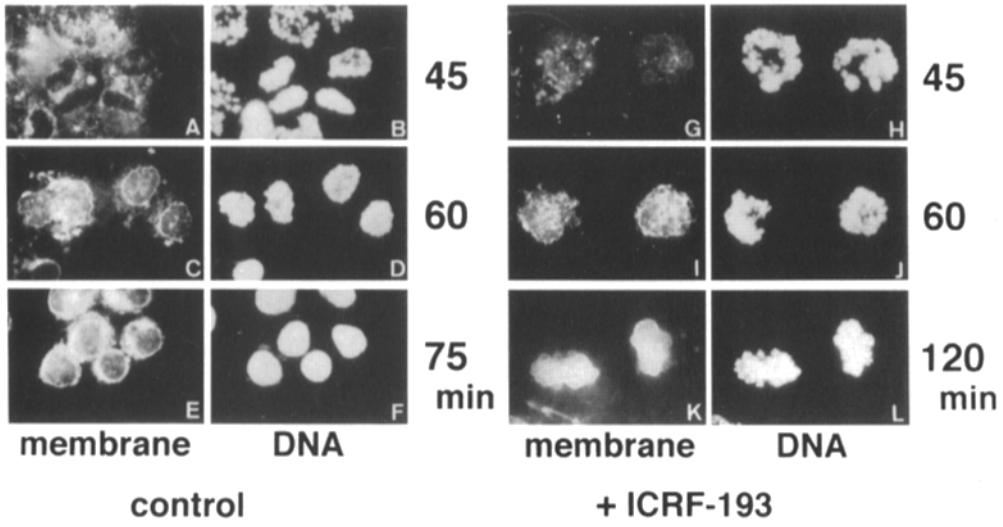


*Figure 5.* ICRF-193 does not block reassembly of nuclear envelopes. Mitotic HeLa cells were cultured in the presence (*G–L*) or in the absence (*A–F*) of 5 μM ICRF-193, as described for Fig. 3. At the indicated times, cells were double stained with the membrane-specific stain DECC (*A*, *C*, *E*, *G*, *I*, and *K*) and with Hoechst 33258 (*B*, *D*, *F*, *H*, *J*, and *L*).

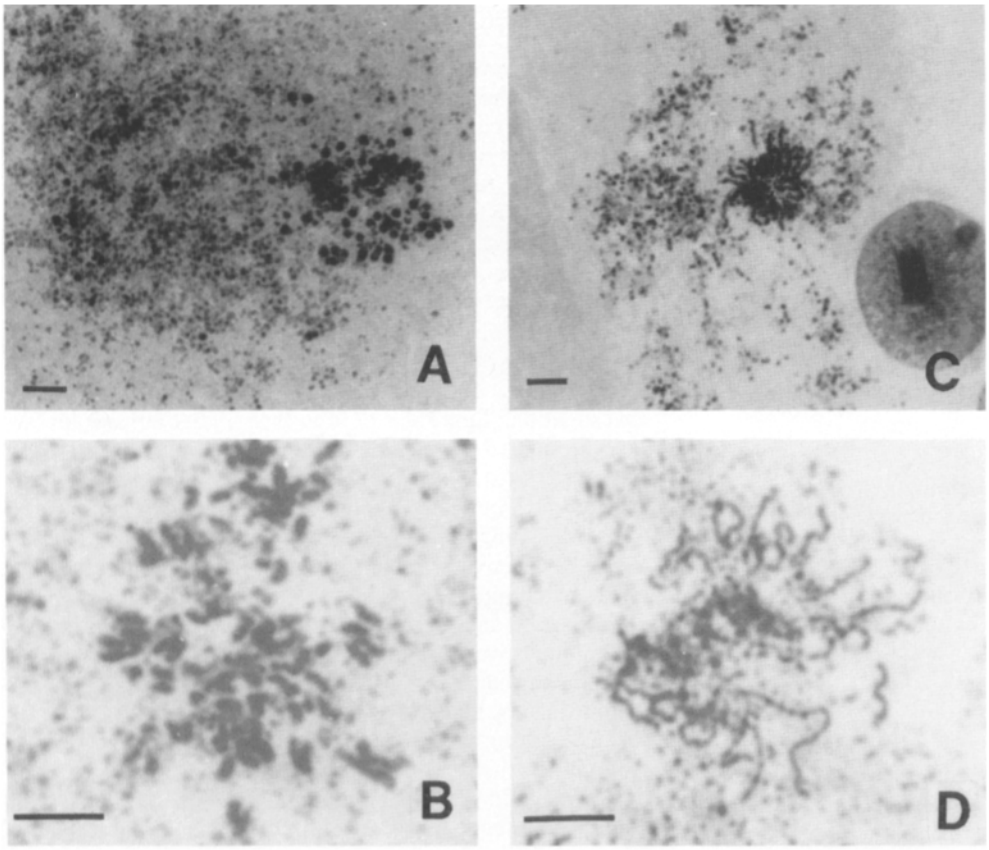


*Figure 6.* S-PCC figures in tsBN2 cells treated with ICRF-193. Cells were synchronized at early S phase by isoleucine deprivation followed by 2 mM hydroxyurea treatment (Nishimoto et al., 1981). Cells were shifted up to 41°C for 2 h in the presence of hydroxyurea. S-PCC preparations obtained from control (*A* and *B*) and ICRF-193-treated cells (*C* and *D*) were examined under the microscope and photographed. Bars, 5 μm.

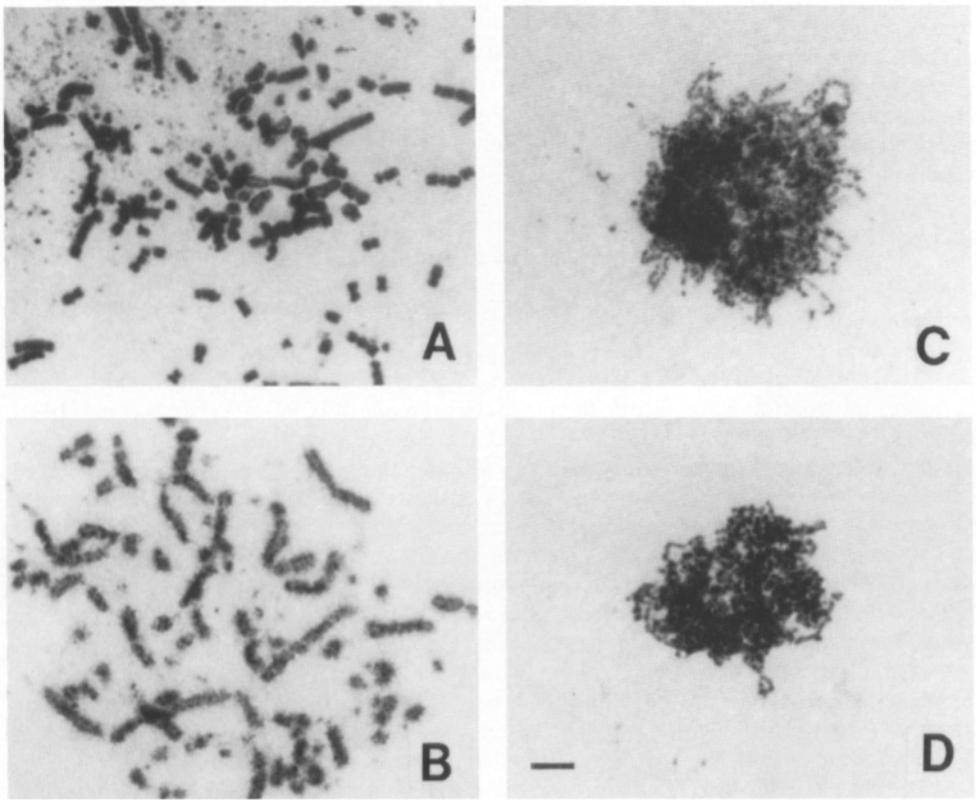


*Figure 7.* G2-PCC figures in tsBN2 cells treated with ICRF-193. tsBN2 cells were synchronized at G2 phase by isoleucine deprivation followed by neocarzinostatin treatment as described previously (Ishida et al., 1985). Cells were shifted up to 41°C for 2 h in the presence of neocarcinostatin. Control (*A* and *B*) and 10 μM ICRF-193-treated cells were examined for PCC. Bar, 5 μm; the magnification of all panels is the same.

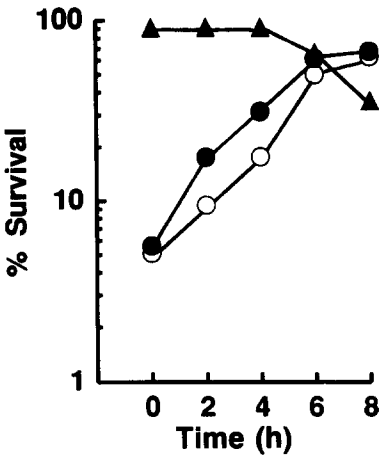


*Figure 8.* ICRF-193 kills mitotic cells. Mitotic CHO cells collected as described in the Materials and Methods were cultured in fresh medium. At the times indicated, aliquots were treated for 30 min with 2 μM (●) or 10 μM (○) ICRF-193, or 3 μM CPT (▲), washed with PBS, and plated for colony formation.

(Nishitani et al., 1991). To establish whether ICRF-193 inhibits the PCC in S or G2 phases, tsBN2 cells were first synchronized in early S phase by isoleucine deprivation followed by hydroxyurea treatment. Upon a temperature shift up to 41°C, PCC was induced after a lag of 1 h and reached 20% at 2 h and 32% at 4 h (data not shown). ICRF-193 did not appear to affect the induction of PCC. However, PCC figures considerably differed from those of normal PCC (Figs. 6 and 7). S-PCC figures comprised two distinct types of dot: highly condensed round or oval dots with a diameter of ~600 nm, and smaller and less dense dots with a diameter of 100–300 nm (Fig. 6, *A* and *B*). The dense dots increased and the light dots decreased as the cells progressed along the S phase, suggesting that the former are replicated chromatin segments and the latter are unreplicated segments. In the presence of ICRF-193, the chromosomes corresponding to the larger condensed dots became stretched with reduction of width to 300 nm while the other PCC dots were not apparently changed (Fig. 6, *C* and *D*).

We have also examined the effect of ICRF-193 on induction of G2-PCC, using cells synchronized in the G2 phase by neocarzinostatin (Ebina et al., 1975; Ishida et al., 1985) after isoleucine deprivation. ICRF-193 again did not inhibit the induction of PCC itself. G2-PCC figures of control cells resembled those of normal mitotic cells (Fig. 7, *A* and *B*), whereas PCC figures of ICRF-193–treated cells were quite different and bizarrely shaped, i.e., much less condensed chromatids were highly entangled (Fig. 7, *C* and *D*). The same figures were earlier observed in randomly growing cells treated with the drug (Ishida et al., 1991). These observations clearly show that ICRF-193 does not inhibit entry into mitosis, but it does interfere with chromosome condensation toward the final step(s), i.e., resolution of the condensing chromatids followed by compaction thereof. In contrast to these observations, however, VP-16 at 100 μM inhibited entry into S-PCC by 90%, but CPT at an equitoxic dose of 2 μM had only marginal effects (data not shown).

### *ICRF-193 Kills Cells Most Effectively at Metaphase*

Finally, we examined the effects of ICRF-193 on cell survival. Metaphase-synchronized CHO cells were treated with ICRF-193 or camptothecin as a control for 30 min at 2-h intervals, and were plated for survival by colony formation. As shown in Fig. 8, cells appeared to be most sensitive to ICRF-193 in metaphase and least sensitive in the S phase, whereas cells were least sensitive to camptothecin in metaphase and most sensitive in the S phase, as previously described (Li et al., 1972; Horwitz et al., 1973).

## *Discussion*

Investigation of the roles of topo II in higher eukaryotes has been hampered by the lack of established mutants. In lower eukaryote yeast, however, studies using single *top2* and double *topltop2* mutants (DiNardo et al., 1984; Uemura and Yanagida, 1985, 1986; Uemura et al., 1987; Holm et al., 1985) have revealed a major role of topo II in the resolution of catenated replicated daughter DNAs, facilitating both the condensation and segregation of chromosomes. Thus, single *top2* mutants were found to show cell cycle–dependent arrest at metaphase at the nonpermissive temperature:nuclear division did not take place normally, but the cell plate was formed, cutting across the nucleus, presenting a "cut" phenotype. When the *top2* mutant was combined with a septum-defective, cold-sensitive mutation, *cdc11*, large cells with single nuclei containing several-fold higher levels of DNA, RNA, and protein accumulated at the nonpermissive temperature, strongly suggesting that the mutant cells traverse many rounds of cell cycle with their genome replicated but unsegregated (Uemura et al., 1987). The function of topo I is considered to be the maintenance of chromatin organization throughout the cell cycle, i.e., continuous removal of torsional strain accumulating in DNA molecules during genetic processes such as replication and transcription, and defects in topo I can be complemented with topo II. These observations point to the essential role of topo II in the cell cycle being the decatenation and/or unknotting of the catenated DNA necessary for chromosome dynamics in mitosis; upon inactivation of the enzyme, however, all the other cellular processes associated with cell cycle transition proceed normally, resulting in the accumulation of polyploid cells.

### *Induction of Polyploidy by ICRF-193*

In a previous paper, we showed that cells accumulate with a high DNA content, multilobed nuclei, and abnormal mitotic figures in the presence of ICRF-193 (Ishida et al., 1991, 1993; Andoh et al., 1993). In the present study, examination of how ICRF-193 induces polyploidy using synchronized cells demonstrated unusual mitoses with entry into G1 without cell divison, and continuation to S phase replication of DNA, resulting in the accumulation of cells with high DNA content and multilobed nuclei. Cells in mitosis have less condensed chromosomes that appear to be similar to those in prophase. These chromosomes were unable to segregate, and thus cells in anaphase and telophase were not observed.

From these results, we propose a model for the influence of ICRF-193 on cell cycle progression. In the presence of ICRF-193, cells continue to traverse the cell cycle and go through an unusual M phase, which we tentatively name the

"absence of chromosome segregation" (ACS-M) phase. In this ACS-M phase, late stage chromosome condensation and its segregation are inhibited, but decondensation of chromosomes does occur at reduced rates. Other mitotic events such as disassembly and assembly of nuclear envelopes, spindle formation, and cytokinesis, etc., triggered by activation of cdc2 kinase, proceed almost normally in CHO cells and are slightly delayed in HeLa cells. Thus, the other mitotic events are uncoupled from chromosome dynamics. These results differ in part from those obtained with the epipodophyllotoxins VP-16 or VM-26, where cell cycle progression was found to be inhibited and cells were arrested in G2 (Tobey et al., 1975; Krishan et al., 1975; Roberge et al., 1990). Since VP-16 and VM-26 cause DNA strand breaks in vivo as protein-concealed breakage or cleavable complexes (D'Arpa and Liu, 1989; Liu, 1989), G2 arrest is thought to be brought about by these rather than by inhibition of topo II activity per se (Lock and Ross, 1990; Lock, 1992). Our present observation that cells can traverse the cell cycle without cell division does not correlate with the effect of VP-16, but it agrees essentially with the behavior of *top2* ts mutants of yeast at nonpermissive temperature. This suggests that the functions of mammalian and yeast topo II in the cell cycle are essentially the same.

Polyploidization has been shown to be induced by various agents such as trichostatins (Yoshida and Beppu, 1988), K-252a, a staurosporine derivative (Usui et al., 1991), and spindle assembly inhibitors (Kung et al., 1990). However, unlike ICRF-193, trichostatins and K-252a, inhibitors of histone deacetylase and protein kinases, respectively, induce polyploidy, not by passage through mitosis, but rather by causing traverse from G2 directly to G1 without nuclear envelope breakdown. In the presence of inhibitors of microtubule assembly such as colcemid and nocodazole, CHO but not HeLa cells traverse into the next cell cycle without cell division (Kung et al., 1990). Even topoisomerase II inhibitors such as VM-26 and 4-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA) were found to induce polyploidization under certain conditions (Zucker et al., 1991). Thus, the induction of polyploidy by chemical agents is a widely observed phenomenon, but it differs mechanistically according to the drug and cell line. ICRF-193 is apparently unique in induction of polyploidy by allowing cells to traverse from a mitotic, prophase-like state to the G1 phase through the ACS-M phase.

### *Topoisomerase II is Required for Chromosome Condensation at a Late Stage, and for Segregation at Anaphase*

Topoisomerase II is localized in interphase nuclei and on metaphase chromosomes (Berrios et al., 1985; Earnshaw et al., 1985; Earnshaw and Heck, 1985; Gasser et al., 1986). In budding and fission yeasts, temperature-sensitive *top2* mutants have demonstrated that topo II is required for full condensation of chromosomes, as well as for the proper segregation of sister chromatids at mitosis (DiNardo et al., 1984; Uemura and Yanagida, 1986; Uemura et al., 1987; Holm et al., 1989). In higher eukaryotes, the enzyme has also been implicated in chromosome condensation and segregation (Earnshaw, 1988; Wood and Earnshaw, 1990; Wright and Schatten, 1990; Roberge et al., 1990; Adachi et al., 1991; Hirano and Mitchison, 1991; Downes et al., 1991; Shamu and Murray, 1992; Sumner, 1992; Buchenau et al., 1993). However, this point remains controversial, because many studies have used topoisomerase II inhibitors such as the epipodophyllotoxins VP-16 and VM-26, as well as *m*-AMSA, which stabilize enzyme-mediated cleavage of DNA, which itself may cause the arrest of chromosome condensation and segregation.

The problem was obviated, however, by approaches using immunological depletion of topo II from nuclear extracts, whereby chromosome condensation was prevented, and replenishment of the enzyme restored the activity (Wood and Earnshaw, 1990; Adachi et al., 1991; Hirano and Mitchison, 1993). In the present study, we applied the specific topo II inhibitor ICRF-193 to establish the role of the enzyme in chromosome dynamics. tsBN2 cells, when shifted up to a nonpermissive temperature, undergo PCC synchronously, associated with a series of mitotic events including activation of cdc2 kinase, and association of MPM-2 antigen with chromosomes (Nishitani et al., 1991). ICRF-193 added to this system did not inhibit PCC in S phase or G2 phase, but interestingly appeared to prevent the final stage of condensation, i.e., compaction of 300-nm fibrils to 600-nm chromatids (Figs. 6 and 7). In S-PCC, 300-nm fibrils of various lengths appeared, and in G2-PCC, more extensively tangled 300-nm fibrils were observed in the presence of the drug. However, how this step of folding is brought about remains unclear.

Uemura et al. (1987) elegantly demonstrated that topo II is required for chromosome segregation in anaphase using the double mutant *tstop2-csnda3*, in which spindle formation is cold sensitive. Our experimental conditions are in principle the same as for the yeast mutant: cells were arrested in metaphase by the microtubule inhibitor TN-16 (corresponding to cold-sensitive *nda3* at 20°C), followed by inhibition of topo II activity by ICRF-193 (corresponding to ts *top2* at 36°C). The results shown in Figs. 2–4 clearly demonstrate that mammalian topo II is also required for chromosome segregation. Clarke et al. (1993) recently reported similar ICRF-193 inhibition of chromosome segregation in HeLa and PtK2 rat kangaroo cells. However, in their study, they failed to observe any inhibition of chromosome condensation. This difference may have stemmed from the difference in the mode of treatment: they treated randomly growing cells for a short period of time, whereas we treated tsBN2 cells long enough before reaching PCC for topo II to become fully inactivated. Chen and Beck (1993) made a similar observation of inhibition of chromosome condensation and induction of polyploidization of human leukemic CEM/VM-1 cells exposed to merbarone, a noncleavable complex stabilizing type topo II inhibitor.

### *Extensive Catenation of the Genome Escapes Checkpoint Controls*

In eukaryotic cells, the initiation of mitosis is dependent on the completion of S phase, and this dependence is ensured by a surveillance mechanism, termed a checkpoint control (Hartwell and Weinert, 1989; Enoch and Nurse, 1990), which inhibits entry into the M phase until replication or the repair of damage to DNA has been completed. Premature M phase entry without completion of replication or repair of

DNA has been observed in some mutants of yeast, e.g., *rad9* in fission yeast (Hartwell and Weinert, 1989) and tsBN2 of baby hamster BHK21 cells (Nishimoto et al., 1978). Activation of cdc2 kinase by dephosphorylation has been found to play an important role in coupling the M and S phases (Nurse, 1990; Smythe and Newport, 1992).

A consistent finding is that cdc2 kinase is not activated in the presence of the epipodophyllotoxin VP-16, and cells were arrested in G2 phase (Lock and Ross, 1990; Lock, 1992; Roberge et al., 1990). In contrast, checkpoint control is not turned on, even if topoisomerase II function is inhibited by ICRF-193. However, the effects of ICRF-193 on activation of cdc2 kinase and on cell cycle transition differed slightly between the two cell lines HeLa and CHO, with delay of kinase increase only occurring in the former. The delayed activation of the kinase observed in HeLa cells appeared to parallel delayed dephosphorylation of $p34^{cdc2}$ and a significant increase in the total amount of $p34^{cdc2}$ (Fig. 1 *C*). This anomalous pattern may be ascribed to a behavior of cyclin B, a partner and the activator of $p34^{cdc2}$. Steinmann et al. (1991) reported that okadaic acid, a protein phosphatase inhibitor, induces premature chromosome condensation in rodent cells, but not in HeLa cells in S phase, with this cell-type specific difference being ascribed to the much increased accumulation of cyclin B only in the rodent case. Kung et al. (1990) showed that spindle assembly is more stringently coupled with certain karyokinetic events in HeLa than CHO cells in terms of cdc2 kinase activation by cyclin B. The difference in the response to ICRF-193 of the two cell lines used in the present study may thus be a reflection of this difference in cyclin B metabolism.

We recently reported that ICRF-193 does not inhibit chain elongation during SV-40 DNA replication in vitro, but that it blocks decatenation of replicated daughter molecules (Ishimi et al., 1992). If this same situation prevails in genomic DNA in vivo, it would appear that in the presence of ICRF-193, the extensively catenated genome escapes the cellular surveillance mechanism because the cells proceed to M phase. However, as we show in Fig. 8, entry into mitosis without active topo II is lethal, even though the cells are capable of traversing further rounds of G1 and S phases.

In conclusion, using a novel topo II inhibitor, ICRF-193, which inhibits only catalytic activity without forming cleavable complex, we could show that topo II plays an essential role as a decatenase in the resolution of replicated chromatin and of chromosomes in G2 and M phases. In other phases of the cell cycle, the enzyme may play a role complementary to topo I in transcription and replication. Of great interest is the fact that inactivation of the enzyme by the drug uncouples chromosome dynamics from the other cellular processes of the cell cycle, which normally proceed in coordination.

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*References*

Adachi, Y., M. Luke, and U. K. Laemmli. 1991. Chromosome assembly in vitro: topoisomerase II is required for condensation. *Cell.* 64:137–148.

Andoh, T., M. Sato, T. Narita, and R. Ishida 1993. Role of DNA topoisomerase II in chromosome dynamics in mammalian cells. *Biotechnol. Appl. Biochem.* 18:165–174.

Berrios, M., N. Osheroff, and P. A. Fisher. 1985. In situ localization of DNA topoisomerase II, a major polypeptide component of the *Drosophila* nuclear matrix fraction. *Proc. Natl. Acad. Sci. USA.* 82:4142–4146.

Buchenau, P., H. Saumweber, and D. J. Arndt-Jovin. 1993. Consequences of topoisomerase II inhibition in early embryogenesis of *Drosophila* revealed by in vivo confocal laser scanning microscopy. *J. Cell Sci.* 104:1175–1185.

Cai, J. C., H. L. Shu, F. C. Tang, T. Komatsu, T. Matsuno, T. Narita, S. Yaguchi, Y. Kide, and M. Takase. 1989. Synthesis and antitumor properties of *N*'-acyloxymethyl derivatives of bis(2,6-dioxopiperazines). *Chem. Pharm. Bull.* 37:2976–2983.

Chen, M., and W. T. Beck. 1993. Teniposide-resistant CEM cells, which express mutant DNA topoisomerase IIα, when treated with non-complex-stabilizing inhibiors of the enzyme, display no cross-resistance and reveal aberrant functions of the mutant enzyme. *Cancer Res.* 53:5946–5953.

Clarke, D. J., R. T. Johnson, and C. S. Downes. 1993. Topoisomerase II inhibition prevents anaphase chromatid segregation in mammalian cells independently of the generation of DNA strand breaks. *J. Cell Sci.* 105:563–569.

Cozzarelli, N. R., and J. C. Wang. 1990. DNA Topology and Its Biological Effects. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 480 pp.

D'Arpa, P., and L. F. Liu. 1989. Topoisomerase-targeting antitumor drugs. *Biochim. Biophys. Acta.* 989:163–177.

DiNardo, S., K. Voelkel, and R. Sternglanz. 1984. DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. *Proc. Natl. Acad. Sci. USA.* 81:2616–2620.

Downes, C. S., A. M. Mullinger, and R. T. Johnson. 1991. Inhibitors of DNA topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis. *Proc. Natl. Acad. Sci. USA.* 88:8895–8899.

Earnshaw, W. C., B. Halligan, C. A. Cooke, M. M. S. Heck, and L. F. Liu. 1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* 100:1706–1715.

Earnshaw, W. C., and M. M. S. Heck. 1985. Localization of topoisomerase II in mitotic chromosomes. *J. Cell Biol.* 100:1716–1725.

Earnshaw, W. C. 1988. Mitotic chromosome structure. *Bioessays.* 9:147–150.

Ebina, T., K. Ohtsuki, M. Seto, and N. Ishida. 1975. Specific G2 block in HeLa-S3 cells by neocarzinostatin. *Eur. J. Cancer.* 11:155–158.

Enoch, T., and P. Nurse. 1990. Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell.* 60: 665–673.

Gasser, S. M., T. Laroche, J. Falquet, E. Boy de La Tour, and U. K. Laemmli. 1986. Metaphase chromosome structure. Involvement of topoisomerase II. *J. Mol. Biol.* 188:613–629.

Hartwell, L. H., and T. A. Weinert. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science (Wash. DC).* 246:629–634.

Hashimoto, Y., H. Oshima, and H. Yuki. 1972. Metaphase-arresting action of carcinostatic tenuazonic acid. *Gann.* 63:79–85.

Hellmann, K., K. A. Newton, D. N. Whitmore, I. W. Hanham, and J. V. Bond. 1969. Preliminary clinical assessment of ICRF-159 in acute leukemia and lymphosarcoma. *Br. Med. J.* 1:822–824.

Herman, E. H., D. T. Witiak, K. Hellmann, and V. S. Waravdekar. 1982. Biological properties of ICRF-159 and related bis(dioxopiperazine) compounds. *In* Advances in Pharmacology and Chemotherapy. Vol. 19. S. Garattini, A. Goldin, F. Hawking, and I. J. Kopin, editors. Academic Press, New York. pp. 249–291.

Holm, C., T. Goto, J. C. Wang, and D. Bostein. 1985. DNA topoisomerase II is required at the time of mitosis in yeast. *Cell.* 41:553–563.

Horwitz, S. B., and M. S. Horwitz. 1973. Effects of camptothecin on the breakage and repair of DNA during the cell cycle. *Cancer Res.* 33:2834–2836.

Hirano, T., and T. J. Mitchison. 1991. Cell cycle control of higher-order chromatin assembly around naked DNA in vitro. *J. Cell Biol.* 115:1479–1489.

Hirano, T., and T. J. Mitchison. 1993. Topoisomerase II does not play a scaffolding role in the organization of mitotic chromosomes assembled in *Xenopus* egg extracts. *J. Cell Biol.* 120:601–612.

Ishida, R., T. Takahashi, and T. Nishimoto. 1985. Chromosomes of G2 arrested cells are easily analyzed by use of tsBN2 mutation. *Cell Struct. Funct.* 10:417–420.

Ishida, R., T. Miki, T. Narita, R. Yui, S. Sato, K. R. Utsumi, K. Tanabe, and T. Andoh. 1991. Inhibition of intracellular topoisomerase II by antitumor bis(2,6-dioxopiperazine) derivatives: mode of cell growth inhibition distinct from that of cleavable complex-forming type inhibitors. *Cancer Res.* 51:4909–4916.

Ishida, R., K. Tanabe, T. Narita, M. Sato, R. Yui, K. R. Utsumi, and T. Andoh. 1993. Mechanism of action of noncleavable complex-forming type of topoisomerase II inhibitors, bis(2,6-dioxopipara-zine) derivatives. *In* Molecular Biology of DNA Topoisomerases and Its Application to Chemotherapy. T. Andoh, H. Ikeda, and M. Oguro, editors. CRC Press, Boca Raton, FL. pp. 207–214.

Ishimi, Y., R. Ishida, and T. Andoh. 1992. Effect of ICRF-193, a novel DNA

topoisomerase II inhibitor, on simian virus 40 DNA and chromosome replication in vitro. *Mol. Cell. Biol.* 12:4007–4014.
Krishan, A., K. Paika, and E. Frei III. 1975. Cytofluorometric studies on the action of podophyllotoxin and epipodophyllotoxins (VM-26, VP-16-213) on the cell cycle traverse of human lymphoblasts. *J. Cell Biol.* 66:521–530.
Kung, A. L., S. W. Sherwood, and R. T. Shimke. 1990. Cell line-specific differences in the control of cycle progression in the absence of mitosis. *Proc. Natl. Acad. Sci. USA.* 87:9553–9557.
Li, L. H., T. J. Fraser, E. T. Olin, and B. K. Bhuyan. 1972. Action of camptothecin on mammalian cells in culture. *Cancer Res.* 32:2643–2650.
Liu, L. F. 1989. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* 58:351–375.
Lock, R. B., and W. E. Ross. 1990. Inhibition of $p34^{cdc2}$ kinase activity by etoposide or irradiation as mechanism of G2 arrest in Chinese hamster ovary cells. *Cancer Res.* 50:3761–3766.
Lock, R. B. 1992. Inhibition of $p34^{cdc2}$ kinase activation, $p34^{cdc2}$ tyrosine dephosphorylation, and mitotic progression in Chinese hamster ovary cells exposed to etoposide. *Cancer Res.* 52:1817–1822.
Morla, A. O., G. Draetta, D. Beach, and J. Y. J. Wang. 1989. Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis. *Cell.* 58:193–203.
Narita, T., S. Yaguchi, T. Komatsu, M. Takase, A. Hoshino, M. Inaba, and S. Tsukagoshi. 1990. Antitumor activity of MST-16, a novel derivative of bis(2,6-dioxopiperazine), in murine models. *Cancer Chemother. Pharmacol.* 26:193–197.
Narita, T., Y. Koide, S. Yaguchi, S. Kimura, Y. Izumisawa, M. Takase, M. Inaba, and S. Tsukagoshi. 1991. Antitumor activities and schedule dependence of orally administered MST-16, a novel derivative of bis(2,6-dioxopiperazine). *Cancer Chemother. Pharmacol.* 28:235–240.
Newport, J., and T. Spann. 1987. Disassembly of the nucleus in mitotic extracts: membrane vesicularization, lamin disassembly, and chromosome condensation are independent processes. *Cell.* 48:219–230.
Nishimoto, T., E. Eilen, C. Basilico. 1978. Premature chromosome condensation in a tsDNA-mutant of BHK cells. *Cell.* 15:475–483.
Nishimoto, T., R. Ishida, K. Ajiro, S. Yamamoto, and T. Takahashi. 1981. The synthesis of protein(s) for chromosome condensation should be regulated by the post-transcriptional mechanism. *J. Cell Physiol.* 109:299–308.
Nishitani, H., M. Ohtsubo, K. Yamashita, H. Iida, J. Pines, H. Yasuda, Y. Shibata, T. Hunter, and T. Nishimoto. 1991. Loss of RCC1, a nuclear DNA-binding protein, uncouples the completion of DNA replication from the activation of cdc2 protein kinase and mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1555–1564.
Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature (Lond.).* 344:503–508.
Roberge, M., J. Th'ng, J. Hamaguchi, and E. M. Bradbury. 1990. The topoisomerase II inhibitor VM-26 induces marked changes in histone H1 kinase activity, histone H1 and H3 phosphorylation and chromosome condensation in G2 phase and mitotic BHK cells. *J. Cell Biol.* 111:1753–1762.
Roca, J., and J. C. Wang. 1992. The capture of a DNA double helix by an ATP-dependent protein clamp: a key step in DNA transport by type II DNA topoisomerases. *Cell.* 71:833–840.
Roca, J., R. Ishida, J. M. Berger, T. Andoh, and J. C. Wang. 1994. Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp. *Proc. Natl. Acad. Sci. USA.* 91:1781–1785.
Shamu, C. E., and A. W. Murray, 1992. Sister chromatid separation in frog egg extracts requires DNA topoisomerase II activity during anaphase. *J. Cell Biol.* 117:921–934.
Sharpe, H. B. A., E. O. Field, and K. Hellmann. 1970. Mode of action of the cytostatic agent "ICRF 159." *Nature (Lond.).* 226:524–526.
Smythe, C., and J. W. Newport. 1992. Coupling of mitosis to the completion of S-Phase in *Xenopus* occurs via modulation of the tyrosine kinase that phosphorylates $p34^{cdc2}$. *Cell.* 68:787–791.
Steinmann, K. E., G. S. Belinsky, D. Lee, and R. Schlegel. 1991. Chemically induced premature mitosis: differential response in rodent and human cells and the relationship to cyclin B synthesis and $p34^{cdc2}$/cyclin B comples formation. *Proc. Natl. Acad. Sci. USA.* 88:6843–6847.
Sumner, A. T. 1992. Inhibitors of topoisomerases do not block the passage of human lymphocyte chromosomes through mitosis. *J. Cell Sci.* 103:105–115.
Tanabe, K., Y. Ikegami, R. Ishida, and T. Andoh. 1991. Inhibition of topoisomerase II by antitumor agents bis(2,6-dioxopiperazine) derivatives. *Cancer Res.* 51:4903–4908.
Terashima, T., and L. J. Tolmach. 1963. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp. Cell Res.* 30:344–362.
Tobey, R. A. 1975. Different drugs arrest cells at a number of distinct stages in G2. *Nature (Lond.).* 254:245–247.
Uemura, T., and M. Yanagida. 1984. Isolation of type I and II DNA topoisomerase mutants from fission yeast: single and double mutants show different phenotypes in cell growth and chromatin organization. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1737–1744.
Uemura, T., and M. Yanagida. 1986. Mitotic spindle pulls but fails to separate chromosomes in type II DNA topoisomerase mutants: uncoordinated mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1003–1010.
Uemura, T., H. Ohkura, Y. Adachi, K. Morino, K. Shiozaki, and M. Yanagida. 1987. DNA topoisomerase II is required for condensation and separation of mitotic chromosomes in *S. pombe*. *Cell.* 50:917–925.
Usui, T., M. Yoshida, K. Abe, H. Osada, K. Isono, and T. Beppu. 1991. Uncoupled cell cycle without mitosis induced by a protein kinase inhibitor, K-252a. *J. Cell Biol.* 115:1275–1282.
Vosberg, H.-P. 1985. DNA topoisomerases: enzymes that control DNA conformation. *Curr. Topics Microbiol. Immunol.* 114:19–102.
Wang, J. C. 1985. DNA topoisomerases. *Annu. Rev. Biochem.* 54:665–697.
Wang, J. C. 1987. Recent studies of DNA topoisomerases. *Biochim. Biophys. Acta.* 909:1–9.
Wood, E. R., and W. C. Earnshaw. 1990. Mitotic chromatin condensation in vitro using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. *J. Cell Biol.* 111:2839–2850.
Wright, S. J., and G. Schatten. 1990. Teniposide, a topoisomerase II inhibitor, prevents chromosome condensation and separation but not decondensation in fertilized surf clam (*Spisula solidissima*) oocytes. *Devel. Biol.* 142: 224–232.
Yasuda, H., M. Kamijo, R. Honda, M. Nagahara, and Y. Ohba. 1990. The difference in murine cdc2 kinase activity between cytoplasmic and nuclear fractions during the cell cycle. *Biochem. Biophys. Res. Commun.* 172: 371–376.
Yoshida, M., and T. Beppu. 1988. Reversible arrest of proliferation of rat 3Y1 fibroblasts in both G1 and G2 phases by trichostatin A. *Exp. Cell Res.* 177:122–131.
Zucker, R. M., and K. H. Elstein. 1991. A new action for topoisomerase inhibitors. *Chem. Biol. Interact.* 79:31–40.